**Solenopsis invicta** virus-1 tissue tropism and intra-colony infection rate in the red imported fire ant: A quantitative PCR-based study

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**Abstract**

Quantitative real-time PCR was employed to measure the *Solenopsis invicta* virus 1 (SINV-1) load in tissues, individuals, and among colonies of the red imported fire ant, *S. invicta* Buren. Among tissues examined from SINV-1-infected adults and larvae, the alimentary canal (specifically the midgut) consistently had the highest number of SINV-1 genome copies (91.1 and 99.9%, respectively). Negative staining of a supernatant of the gut homogenate demonstrated the presence of spherical virus particles with a diameter of 30–35 nm, consistent with SINV-1. The number of SINV-1 genome copies in infected larvae and workers from the same queenright colonies were similar to each other. In other words, the infection rate was consistent among both developmental stages. No significant differences were observed in SINV-1 genome copy number among infected colonies sampled during the winter and summer. Although the SINV-1 infection rate of summer-collected mounds was previously shown to be six-times higher than winter-collected mounds, the intra-colony infection rate appears to be unaffected by season. Perhaps less inter-mound interaction during the winter months among *S. invicta* restricts spread of the virus. A positive correlation between intra-colony infection rate and mean SINV-1 genome copy number per ant was also observed. Based on these results, it is likely that SINV-1 replicates in gut epithelia of *S. invicta* and virus is shed into the gut lumen where it may be transmitted to nestmates by trophallaxis.

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**Keywords:** *Solenopsis invicta* virus 1; Tropism; Quantitative real-time PCR

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1. Introduction

*Solenopsis invicta* virus 1 (SINV-1) is the first virus isolated and characterized from the red imported fire ant, *S. invicta* Buren (Valles et al., 2004). SINV-1 possesses a dicistronic RNA genome (8026 nts and poly (A) tail), encoding structural (3' proximal ORF) and non-structural (5' proximal ORF) polyproteins. Multiple alignments of amino acid sequences of conserved regions of these polyproteins with those of corresponding regions of characterized insect-infecting RNA viruses revealed that the SINV-1 sequences were homologous to those of dicistroviruses such as cricket paralysis virus (CrPV), acute bee paralysis virus (ABPV), Kashmir Bee Virus (KBV), and black queen cell virus (BQCV).

SINV-1 RNA sequence was initially identified from an expression library constructed from a monogynous *S. invicta* colony that did not exhibit any apparent symptoms of viral infection. SINV-1-infected ants are typically found in field colonies exhibiting no disease symptoms. However, in many instances, after being brought into the laboratory, SINV-1-positive colonies exhibited brood death, which sometimes resulted in colony death. Based on previous research on homologous single stranded-RNA (ssRNA) viruses infecting honeybees and other insects, we hypothesized that SINV-1 fits the current paradigm of being an asymptomatic, unapparent infection that begins to cause animal death after exposure to certain stressors. Colony collapse caused by similar ssRNA viruses in honeybees has been shown to be induced by environmental...
factors (Tentcheva et al., 2004), virus titer (Bowen-Walker et al., 1999; Chen et al., 2005b), malnutrition (Chen et al., 2005b), and associations with microsporidial or bacterial pathogens (Bailey, 1967; Brodsgaard et al., 2000; Yang and Cox-Foster, 2005) or exposure to Varroa mites (Martin, 1998; Shen et al., 2005; Yang and Cox-Foster, 2005).

In an effort to continue characterization of SINV-1 biology and advance our understanding of the behavior of SINV-1 within individual ants and colonies, we conducted experiments to examine the tissue tropism of the virus in S. invicta adults and larvae. In addition, experiments were conducted to determine the intra- and inter-colony SINV-1 infection rate in S. invicta. Although completely descriptive, these studies are essential to develop an understanding of SINV-1 in its host, S. invicta.

2. Materials and methods

2.1. SINV-1-infected fire ants

SINV-1-infected S. invicta mounds were identified in Gainesville, Florida, by one-step, RT-PCR (Invitrogen, Carlsbad, CA) with genotype-specific oligonucleotide primers (Valles and Strong, 2005). RT-PCR was conducted in a PTC 100 thermal cycler (MJ Research, Waltham, MA) under the following optimized temperature regime: 1 cycle at 45 °C for 30 min, 1 cycle at 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 54 °C for 15 s, 68 °C for 30 s, followed by a final elongation step of 68 °C for 5 min. SINV-1-positive nests were excavated by shovel and ants were removed from the soil using the floating technique described previously (Jouvenez et al., 1977). The ants were then maintained in rearing trays.

2.2. Dissection and RNA extraction from body parts and tissues

Ice-chilled fire ant workers from a SINV-1-positive colony were placed on a glass slide, and head, thorax, and abdomen were separated with a surgical blade under a Leica MS5 dissecting microscope. The abdomen was immersed in a drop of 10 mM Tris–HCl, pH 8.0, and tissues were isolated and removed with micro-dissecting forceps in the following order: crop, poison sac, midgut, hindgut, and the abdominal carcass, which included malpighian tubules, Dufour’s gland, ovary, rectum, fat body, muscle, and cuticle. Larvae were dissected and separated into alimentary canal, malpighian tubules, and the remaining carcass. All alimentary canal components were opened longitudinally and contents removed. The alimentary canal tissues were subsequently rinsed with 10 mM Tris–HCl, pH 8.0. Body parts and tissues were pooled from 10 workers or larvae in a 1.5 ml microcentrifuge tube that contained 250 μl of Trizol reagent (Invitrogen). The body parts or tissues were then homogenized with a plastic pestle and vortexed after addition of 100 μl of chloroform. The mixture was centrifuged at 20,817 g for 5 min at room temperature. The supernatant was transferred to a clean 1.5 ml microcentrifuge tube, mixed with 250 μl of isopropanol and 0.5 μl of mussel glycogen as carrier (20 mg/ml; Sigma, MO), and held at −20 °C for 30 min. RNA was pelleted by centrifugation at 20,817 g for 15 min at 4 °C and the pellet was rinsed with cold 70% ethanol, dried, and re-suspended in 20 μl of diethylpyrocarbonate-treated, double-distilled water (DEPC water). The same procedure was used to extract total RNA from individual workers and larvae. RNA concentration was measured spectrophotometrically.

2.3. First-strand cDNA synthesis and quantitative PCR

cDNA was synthesized from the SINV-1 genome region corresponding to the RNA-dependent RNA polymerase (RdRp) with total RNA isolated from individual ants, body parts, or dissected tissues using SuperScript III Reverse Transcriptase (SsRT; Invitrogen) and a gene-specific primer (p523 [SINV-1 genome position 3925–3955, 5’-CCTCATTTGAAGATAATCCTCTCTTGGAGAAA] (Valles et al., 2004; Hashimoto et al., 2007). In a 0.5 ml PCR tube, 2 μl of the RdRp-specific primer (1 μM), 1 μl of a dNTP mix (10 mM), and 10 μl of total RNA (100 ng) were mixed and heated to 65 °C for 5 min in a thermal cycler, followed by incubation on ice for 1 min. Then, 4 μl of first-strand buffer (250 mM Tris–HCl, pH 8.3; 375 mM KCl, 15 mM MgCl2), 2.5 μl of DEPC water, and 0.25 μl of SsRT (200 U/μl) were added. The mixture was incubated at 55 °C for 30 min, followed by inactivation of SsRT by heating to 70 °C for 15 min. Dithiothreitol (DTT) was omitted because it has been shown to interfere with QPCR reactions using SYBR Green (Lekanne Deprez et al., 2002; Hashimoto et al., 2007).

QPCR was performed on an ABI PRISM 7000 Sequence Detection System interfaced to the ABI prism 7000 SDS software (Applied Biosystems, Foster City, CA) in a 25 μl reaction volume (Hashimoto et al., 2007). The reaction contained 12.5 μl of SYBR Green SuperMix (with UDG and ROX, Invitrogen), 0.4 μl each of 10 μM RdRp-specific primers (p517; SINV-1 genome position 3332–3364, 5’-CAATAGGCACCAACGTATATAGTAGAGATTGGA and p519; SINV-1 genome position 3559–3585, 5’-GGAATGGGTCATCATATAGAATAGGCAACCAACGTATATAGTA GAGATTGGA and p519; SINV-1 genome position 3559–3585, 5’-GGAATGGGTCATCATATAGAAGA ATTG), 3 mM MgCl2, 1 μl of the cDNA synthesis reaction, and 10.7 μl of DEPC-water. QPCR conditions consisted of one cycle at 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 56 °C for 15 s, 72 °C for 1 min. The non-template control for QPCR included a mock-cDNA synthesis reaction that was carried out without RNA template. A standard curve was constructed from a plasmid clone of the RdRp region using a copy number range of 5 × 103 copies per QPCR. Reaction efficiency was determined by regressing C_T values against the template copy number (log) and calculated according to the formula \[E = (10^{\frac{1}{Slope}}) - 1\] (Klein et al., 1999). After QPCR, amplification fidelity was confirmed by determining the melting temperature with the
dissociation protocol and visually by examination of amplicons after separation on an agarose gel.

QPCR was conducted on RNA samples from workers (n = 10) and late-stage larvae (n = 14) comprised of body parts or tissues pooled from 10 individuals from 4 SINV-1-infected colonies. Larvae were staged by the method of Petralia and Vinson (1979). In later experiments, the alimentary canal of workers was further dissected to midgut and hindgut and four sets of RNA samples comprised of pools of ten worker midguts or hindguts from two SINV-1-positive colonies were analyzed by QPCR. These colonies were collected in November and December 2006. The tissue data were analyzed by analysis of variance (ANOVA) and Scheffe’s multiple comparison procedure in instances where the ANOVA results were significant (p < 0.05). Student’s t-test was used to compare SINV-1 genome number in the midgut and hindgut of workers.

2.4. Seasonal infection rate

Valles et al. (2007) had shown previously that the SINV-1 infection exhibited a seasonal pattern and the inter-colony infection rate was most prevalent among colonies during the spring and summer in S. invicta in Florida. Indeed, the prevalence was correlated with temperature—a characteristic shown also in other arthropod-infecting single-stranded RNA viruses (Tentcheva et al., 2004). In an effort to further understand the seasonal prevalence of SINV-1 in S. invicta, experiments were conducted to compare the SINV-1 infection rate in summer-collected and winter-collected S. invicta. The genome copy number was determined as well as the intra-colony and inter-colony infection rates among these two groups. QPCR was conducted on RNA samples from workers and larvae of four winter-collected (November-December 2006) colonies (colony-10, 10 workers and 15 larvae; colony-16, 3 workers and 15 larvae; colony-20, 20 workers and 15 larvae; and colony-30, 10 workers and 15 larvae) and from workers of three summer-collected (May-June 2006) colonies (colonies-V5, -V12, and -3, 15 workers from each colony). The intra-colony infection rate was determined by QPCR evaluation of individual ants. Correlation analysis using the Spearman correlation procedure was conducted between intra-colony infection rate and mean RdRp copy per ant using Proc Corr (SAS, 1988).

2.5. Electron microscopy

Samples from ants testing positive for SINV-1 by QPCR were further examined by transmission electron microscopy in an effort to identify corresponding virus particles. Midguts were dissected from worker ants from SINV-1-infected and -uninfected colonies. The individual midgut (with gut contents in place) was transferred to a 1.5 ml microcentrifuge tube containing 50 μl of DEPC-water and was homogenized with a plastic pestle. The homogenate was vortexed and centrifuged at 2300g for 1 min at room temperature. Two sets of 20 μl of the supernatant were prepared in new 1.5 ml microcentrifuge tubes, and one set was processed for total RNA extraction, followed by cDNA synthesis and QPCR as described previously. Two SINV-1-positive and -negative samples were submitted to the Interdisciplinary Center for Biotechnology Research (University of Florida) for examination by electron microscopy. An aliquot of supernatant of homogenate prepared from worker ant gut was negatively stained with aqueous 2% phosphotungstate acetate, pH 7.0, on a formvar film coated-grid and photographed with a Hitachi H-7000 transmission electron microscope (Tokyo, Japan).

3. Results and discussion

3.1. Tissue tropism of SINV-1

Based on QPCR results, SINV-1 genome was most prevalent in the alimentary canals of S. invicta workers (91.6 ± 4.7%) and larvae (99.9 ± 0.1%) (Table 1). Among workers, the SINV-1 genome was detected in all of the other tissues examined—albeit at an insignificant level most likely the result of cross contamination during the dissection process. When the alimentary canals of workers were further separated into midgut and hindgut, the majority of the SINV-1 genome was detected in the midgut (99.4 ± 0.9%). SINV-1 specificity for the midgut of S. invicta is consistent with a number of other insect-infecting single-stranded RNA viruses. Rhopalosiphum padi virus

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Tissue/ body parts</th>
<th>SINV-1 presence by RT-PCR</th>
<th>RdRp distribution by QPCR (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>Head</td>
<td>–</td>
<td>1.6 ± 1.4 A</td>
</tr>
<tr>
<td></td>
<td>Thorax</td>
<td>–</td>
<td>1.3 ± 2.3 A</td>
</tr>
<tr>
<td></td>
<td>Crop</td>
<td>–</td>
<td>0.0 ± 0.1 A</td>
</tr>
<tr>
<td></td>
<td>Poison sac</td>
<td>–</td>
<td>0.1 ± 0.2 A</td>
</tr>
<tr>
<td></td>
<td>Abdominal carcass</td>
<td>–</td>
<td>5.4 ± 5.4 A</td>
</tr>
<tr>
<td>Alimentary canal</td>
<td>+</td>
<td>91.6 ± 4.7 B</td>
<td></td>
</tr>
<tr>
<td>Hindgut</td>
<td>+</td>
<td>0.6 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Midgut (tissue and contents)</td>
<td>+</td>
<td>99.4 ± 0.9*</td>
<td></td>
</tr>
<tr>
<td>Midgut contents</td>
<td>+</td>
<td>84.6 ± 20.6</td>
<td></td>
</tr>
<tr>
<td>Midgut tissue</td>
<td>+</td>
<td>15.3 ± 20.6</td>
<td></td>
</tr>
<tr>
<td>Larvae</td>
<td>Malpighian tubules</td>
<td>–</td>
<td>0.0 ± 0.0 B</td>
</tr>
<tr>
<td>Carcass</td>
<td>–</td>
<td>0.1 ± 0.1 B</td>
<td></td>
</tr>
<tr>
<td>Alimentary canal</td>
<td>+</td>
<td>99.9 ± 0.1 A</td>
<td></td>
</tr>
<tr>
<td>Alimentary canal</td>
<td>+</td>
<td>99.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Alimentary canal</td>
<td>+</td>
<td>0.1 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

a Mouth to posterior of midgut including gut contents.
b Means with different letters (within a group demarcated by horizontal lines) are significantly different by Scheffe’s multiple comparison procedure or with * by Student’s t-test (p < 0.05).
(Gildow and D’Arcy, 1990), *Plautia stali* intestine virus (Nakashima et al., 1998), and *Acyrthosiphon pisum* virus (van den Heuvel et al., 1997) were found primarily restricted to the gut and gut contents. The distribution profile of the SINV-1 genome among the different body parts and tissues was consistent between colonies. Presence of the SINV-1 genome in the alimentary canal of both workers and larvae indicated that *S. invicta* gut epithelia were susceptible to SINV-1 infection. Additionally, we detected considerable SINV-1 genome in the alimentary canal contents which could also explain detection in the head and thorax (by way of the esophagus) and may even serve as a means of dissemination throughout the colony. The presence of SINV-1 in the gut was confirmed by electron microscopy. When a homogenate of the gut/gut contents from a SINV-1-infected worker ant was negatively stained, virus-like particles with a diameter of 30–35 nm were detected in high abundance (Fig. 1). No corresponding particles were observed from ant samples testing negative for SINV-1 by QPCR.

### 3.2. SINV-1 distribution among larvae and workers

To compare the distribution of SINV-1 genome between SINV-1-infected larvae and workers among different colonies, ants were collected directly from the field and analyzed without laboratory rearing by QPCR for the RdRp gene (Fig. 2). An interesting pattern was noted with regard to the SINV-1 infection rate. The SINV-1 genome copy number was similar among larvae and workers taken from the same infected colony. Despite different levels of infection among colonies, workers and larvae from the same colony nearly always exhibited similar levels of SINV-1 genome. Student’s *t*-test showed that three of four colonies exhibited no significant difference of SINV-1 genome copy numbers between larvae and workers. This result is unique and may be associated with pathology or transmission of the virus throughout the colony structure. Chen et al. (2005a) postulated that virus titer was a key factor in the appearance of wing deformity in adult worker honeybees infected with deformed wing virus. They hypothesized that a critical threshold of viral titer must be met before the onset of symptoms appeared. Typically, SINV-1 infections are asymptomatic in *S. invicta*. Perhaps the SINV-1 genome copy number differences observed between colonies indicate different stages of a developing infection.

### 3.3. SINV-1 genome copy distribution in winter- and summer-collected workers of *S. invicta*

A previous study of SINV-1 infection in *S. invicta* in Florida revealed that virus prevalence (inter-colony) was higher among summer mounds compared with those sampled during the winter (Valles et al., 2007). A strong correlation between temperature and SINV-1 mound infection rate was also observed. To further understand the seasonal prevalence of SINV-1 infection in individual colonies, the SINV-1 intra-colony infection rate was quantified and compared from workers from winter-collected colonies and those from summer-collected colonies. Although the inter-colony infection rate was significantly higher in summer, among SINV-1-infected colonies the intra-colony infection rate and genome copy number were not significantly different (Table 2). In other words, despite a lower inter-colony incidence of SINV-1 among fire ant mounds during the winter, colonies found to be infected during the winter exhibited the same intra-colony infection rate and genome copy number as summer colonies. The variation was also quite high among summer- and winter-collected colonies. In summer-collected colonies, the intra-colony infection rate ranged from 33 to...
100% with genome copy number from $5 \times 10^3$ to $5 \times 10^8$. Winter colonies exhibited an intra-colony infection rate of 80–100% and genome copy number in the range of $1 \times 10^5$ to $2 \times 10^8$. Thus, seasonal changes do not appear to influence the SINV-1 intra-colony infection rate. Perhaps, during colder periods, less inter-colony interaction among S. invicta restricts spread of the virus.

3.4. Relationship between SINV-1 genome copy numbers in individual workers with SINV-1 infection rate in colony

We noticed a pattern between the intra-colony infection rate and mean number of genome copies per ant taken from the same colony (Table 2). Indeed, a significant correlation ($y = 0.061x + 1.7417$, $r = 0.86$, $p = 0.03$) was observed between the SINV-1 genome copy number of individual ants and the corresponding intra-colony infection rate (Fig. 3). These data may suggest the development of the infection through time. Perhaps as the virus number increases, it is more easily disseminated among nestmates resulting in an increased intra-colony infection rate.

Various methods have been employed to detect single-stranded RNA viruses in arthropods including in situ hybridization (Deformed wing virus [DWV], Fievet et al., 2006), immunohistochemistry (Infectious flacherie virus [IFV], Inoue and Ayuzawa, 1972; DWV, Fievet et al., 2006), immunodiffusion (Chronic bee paralysis virus [CBPV], Ribière et al., 2000), ELISA (Himetobi P virus [HPV], Suzuki et al., 1993; ABPV, Allen et al., 1986; BQCV, CBPV, Sacbrood virus [SBV], Kashmir bee virus [KBV], Anderson, 1984), and RT-PCR (ABPV, Bakonyi et al., 2002; ABPV, BQCV, Benjeddou et al., 2001; KBV, ABPV, Evans, 2001; SBV, Grabenstiner et al., 2001; KBV, Hung et al., 2000; SINV-1, Valles and Strong, 2005). Recently, QPCR protocols have been developed providing a powerful tool to aid identification of tissue specificity, or tropism, and in measuring virus replication levels in different life stages of insect hosts (DWV, Chen et al., 2005a; KBV, ABPV, CBPV, DWV, BQCV, SBV, Chantawannakul et al., 2006; Kakugo virus [KV], Fujiyuki et al., 2004). A QPCR study by Hashimoto et al. (2007) on the SINV-1 infection level in fire ants showed that SINV-1 infection peaked in late stage larvae, decreased in pupae, and peaked again in workers. In the present work, we showed that SINV-1 genome copy was present abundantly in the alimentary canal of both workers and larvae and virus was also found overwhelmingly in midgut contents. These results lead us to hypothesize that SINV-1 replicates in gut epithelia of S. invicta and virus is shed into the gut lumen where it is likely disseminated to nestmates by trophallaxis and associated colony behaviors (e.g., grooming). Similar tissue tropism and route of transmission have been reported and proposed for Rhopalosiphum padi virus (Gildow and D’Arcy, 1990), Plautia stali intestine virus (Nakashima et al., 1998), and Acyrthosiphon pisum virus (van den Heuvel et al., 1997). Transmission studies are underway to follow and quantify the progression of SINV-1 infection throughout the fire ant colony structure in an effort to test our hypothetical mode of SINV-1 dissemination in fire ants.

Table 2

<table>
<thead>
<tr>
<th>Season</th>
<th>RdRp copy/infected ant*</th>
<th>Colony infection rate (%) (n)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SE</td>
<td>Intra-</td>
</tr>
<tr>
<td>Winter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colony</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>757,508</td>
<td>374,250</td>
</tr>
<tr>
<td>20</td>
<td>242,586,870</td>
<td>172,944,380</td>
</tr>
<tr>
<td>30</td>
<td>100,773</td>
<td>61,915</td>
</tr>
<tr>
<td>Overall mean</td>
<td>81,148,384</td>
<td>57,793,515</td>
</tr>
<tr>
<td>Summer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colony</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V5</td>
<td>5816</td>
<td>1025</td>
</tr>
<tr>
<td>V12</td>
<td>302,179</td>
<td>282,216</td>
</tr>
<tr>
<td>3</td>
<td>573,685,767</td>
<td>210,983,580</td>
</tr>
<tr>
<td>Overall mean</td>
<td>191,331,254</td>
<td>70,422,274</td>
</tr>
</tbody>
</table>

* No significant difference between winter and summer overall mean ($p = 0.62$) or intra-colony infection rate ($p = 0.45$) by Student’s t-test.
\(^b\) Data taken from Valles et al. (2007).

\(^*\) Inter-colony infection rate is significantly ($p < 0.05$) greater in summer-collected colonies by Student’s t-test.

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Fig. 3. Relationship between intra-colony infection rate and mean SINV-1 genome copy number per worker among winter- and summer-collected colonies.
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