DELIVERY SYSTEM USING SODIUM ALGINATE VIRUS LOADED PELLETS TO RED IMPORTED FIRE ANTS (*SOLENOPSIS INVICTA*, HYMENOPTERA: FORMICIDAE)

DANIELLE M. TUFTS[1,3], KYLE SPENCER[1], WAYNE B. HUNTER[2] AND BLAKE BEXTINE[1]
[1]University of Texas—Tyler, Biology Department, 3900 University Blvd., Tyler, TX 75799

[2]ARS, USDA, U.S. Horticultural Research Laboratory, 2001 South Rock Road, Fort Pierce, FL 34945

[3]Current Affiliation: School of Biological Sciences, University of Nebraska-Lincoln, 323 Manter Hall, Lincoln, NE 68588

ABSTRACT

Microencapsulation as a delivery mechanism of SINV-1 and other molecules such as dsRNA, offers an approach to *Solenopsis invicta* Buren management that is target specific and fits current approaches to baiting ants with toxins and/or RNA-interference. The delivery method presented here targets ground dwelling, foraging ants with an ant-infecting virus which is specific to the genus, *Solenopsis*. Endemic ant-infecting viruses, like *S. invicta* viruses (SINV-1, SINV-2, and SINV-3) are being evaluated for efficacy in *S. invicta* population suppression. In this study, SINV-1 (TX5 strain) was extracted from *S. invicta* colonies and microencapsulated in sodium alginate pellets. Pellets containing extracted whole virions were offered to confirmed non-infected *S. invicta* colonies. Colonies were sampled every 5 d and tested by reverse transcription polymerase chain reaction (RT-PCR) for presence of viral RNA. The longevity of control and viral pellets were also evaluated. Within 30 d, post-feeding of virus, 35% of *S. invicta* colonies acquired SINV-1 infection ($P = 0.03$). Thus, microencapsulation as a delivery mechanism was successful to deliver SINV-1 to *S. invicta* colonies. Future incorporation of this economically affordable method can be implemented to deliver biological agents for specific ant species and to augment current approaches that bait ants. While a virus was used to demonstrate delivery, an adequate and affordable virus production system still needs to be developed before a viral strategy can be adopted as a tool for biological control of fire ants.

Key Words: ant management, anti-infecting virus, *Solenopsis invicta* virus (SINV), microencapsulation, mortality, biological control

RESUMEN

Microencapsulación como un mecanismo para entregar el virus SINV-1 y otras moléculas como dsARN, ofrece una aproximación al manejo de *Solenopsis invicta* Buren que es un objetivo específico y se ajusta a los enfoques actuales de cebos para hormigas tóxicos y / o interferencia de ARN. El método de entrega presentado aqui se enfoca sobre hormigas que hábitan el suelo o que forrajean y utiliza un virus que infecta específicamente a hormigas del género *Solenopsis*. Los virus endémicos que infectan las hormigas, como los virus de *S. invicta* (SINV-1, SINV-2 y SINV-3) estan siendo evaluados para su eficacia en suprimir poblaciones de *S. invicta*. En este estudio, el virus SINV-1 (cepa TX5) fue extraido de colonias de *S. invicta* y microencapsulado en granulos de alginato de sodio. Se ofrecieron granulos con los virus encapsulados a las colonias de *S. invicta* que fueron confirmadas de no estan infectadas. Las colonias fueron examinadas cada cinco dias y probadas usando la reacción reversa de la transcripción de la cadena polimerasa (RT-RCP) para la presencia de ARN viral. La longevidad de los granulos virales y su control fueron evaluados. En un período 30 días, posterior a la ingestión de la virus, el 35% de las colonias de *S. invicta* adquireron una infección de SINV (P = 0,03). Por lo tanto, la microencapsulación como un mecanismo para entregar el SINV-1 a las colonias de *S. invicta* fue exitosa. La incorporación de este método economicamente asequible puede ser implimentado en el futuro para entregar agentes biológicos para especies específicas de hormigas y para aumentar los métodos actuales que usan cebo para controlar las hormigas. Aunque el virus fue usado para demonstrar la entrega, todavia se necesita desarollar un sistema de producción adecuada y asequible antes que una estrategia viral pueda ser adoptada como una herramienta de control biológico para las hormigas de fuego.

The red imported fire ant (*Solenopsis invicta* Buren) invaded North America in the 1930s and since then has become a serious threat to humans and devastated many endemic wildlife species

(Taber 2000). *Solenopsis invicta* colonies were quickly established in the USA because of their aggressive behavior, large colony size, and lack of natural predators (Taber 2000). Therefore, a safe and species specific method for reducing ant colony size and population density of *S. invicta* is desperately needed.

A positive, single-stranded RNA virus in the Family *Dicistroviridae* was reported to only infect ants in the S*olenopsis* genus (Valles et al. 2007). The *S. invicta* viruses (SINV-1, SINV-2, and SINV-3) and genotypes (SINV-1A and SINV-1 TX5) infect all stages of development and caste members (Valles et al. 2004; Valles & Strong 2005; Valles et al. 2007; Valles & Hashimoto 2009; Tufts et al. 2010). Rapid replication of SINV-1 (TX5) can quickly produce an infected fire ant colony. Although acute mortality observed within field populations of ants under natural viral infection has not been significant, when the entire colony becomes infected under controlled conditions the colony dies (Valles et al. 2004). Other examples of insects which became infected with single stranded RNA (ssRNA) viruses related to SINV-1 also showed increased mortality and colony collapse, e.g., leafhoppers (Hunter et al. 2006; Hunnicutt et al. 2006) and honey bees (*Apis mellifera* L.) (Cox-Foster et al. 2007). These studies suggest that the effect of high virus titers or multiple virus infections may be required to cause colony collapse. A virus closely related to SINV-1, the Israeli acute paralysis virus (IAPV), infects honey bees in approximately 90% of apiaries (Johnson 2010). However, only in conjunction with other pathogens (i.e. other viruses or parasites) did it produce colony collapse (Cox-Foster et al. 2007; VanEngelsdorp et al. 2009).

Fire ants, which are also hymenopterans, are under increased stress from the implementation of several biological control agents, e.g., phorid flies (*Pseudacteon* spp.; Graham et al. 2003), fungi (*Beauveria bassiana*; Baird et al. 2007), and microsporidian protozoan (*Thelohania solenopsae*; Oi & Williams 2002; Oi & Valles 2008). These natural enemies continue to be investigated with limited success with respect to their effective use in reducing fire ant populations; therefore, the addition of multiple viral pathogens may increase the efficacy of these currently used approaches. Costly, chemical control programs have been successful, but often displace fire ant colonies to surrounding regions, with re-colonization occurring as the chemicals break down and fire ant populations increase.

The entomopathogenic fungus, *B. bassiana*, was microencapsulated in sodium alginate pellets and tested as a potential biological control method with success (Bextine & Thorvilson 2002). *Beauveria bassiana* was delivered to *S. invicta* colonies by workers that had fed on pellets. The objective of this study was to develop an effective delivery method that would transfer viral infection to *S. invicta* colonies. Thus, SINV-1 (TX5) was encapsulated in pellets and the delivery method assessed. While not tested directly, the potential of this strategy is to provide the means to infect the majority of ants with a high titer of virus, consequently inducing colony collapse.

Materials and Methods

Colony Collection and Viral Detection

Solenopsis invicta colonies were collected during May and Aug 2008 from Smith, Cherokee, Hunt, and Gregg Counties, Texas. A total of 58 colonies were collected from Smith County, 15 colonies from Cherokee County, 9 colonies from Hunt County, and 7 colonies from Gregg County; all colonies were of the polygyne phenotype. To rigorously evaluate variability of acceptance of these pellets by fire ants, ant colonies were collected from widely disparate locations to increase the probability of including behavioral and genetic variations in the ant colonies tested. All colonies collected were tested for the presence of the *Solenopsis invicta* virus - 1 (SINV-1) by reverse transcription polymerase chain reaction (RT-PCR) and gel electrophoresis. RNA was extracted from entire *S. invicta* colonies (workers, queens, and brood) using TRIzol® reagent (Invitrogen, Carlsbad, CA) following manufacturer's protocol. Samples were then tested for virus using a SuperScript One-Step Reverse Transcriptase PCR (RT-PCR) kit (Invitrogen, Carlsbad, CA). RT-PCR was completed using a specific primer set (p62 and p63; Valles & Strong 2005) for a short segment (326 bp) of SINV-1 and performed in duplicate (Tufts et al. 2010). Purified, active virus was extracted from colonies which tested positive for SINV-1 using a modified protocol by Hunter, USDA, ARS (Tufts et al. 2010).

Development of Sodium Alginate Pellets

The virus extract was microencapsulated using a 1% sodium alginate suspension (Bextine & Thorvilson 2002). The solution was prepared by dissolving 2.5 g sodium alginate (Spectrum, Gardena, CA) and 2 g corn meal (Quaker Oats Co., Chicago, IL) in a solution of 10 mL 95% ethanol and 8.5 ml purified virus extract, the mix was then brought to a final volume of 100 mL using autoclaved, nano-pure filtered water. The gelatinous solution was mixed vigorously for 10 min and then slowly dripped into an aqueous solution of 0.25 M calcium gluconate with a sterile 10 mL disposable pipette. After 5 min, the pellets were strained out of the gluconate solution by a sieve and allowed to dry at 22°C on 2 sheets of wax paper for 24 h. Control pellets were produced in the same manner, substituting autoclaved, nano-pure

filtered water for viral extract. Pellets were observed to shrink substantially from their original size over the following 24 h and were stored in airtight plastic vials at 22°C, in the dark. Dried pellets had an average weight of 5.7 mg and an average diameter of 2 mm (n = 20).

Introduction of Pellets to Laboratory Colonies

All colonies were tested by RT-PCR for the presence or absence of SINV, protocols as stated above. A sample of 20 worker ants from each colony was tested for SINV presence, for a total of 1780 ants. Ten of the established laboratory colonies which were negative for SINV infection were randomly chosen; 5 with brood present and 5 colonies without brood. All 10 treated colonies were offered 5 virus pellets. An additional 5 colonies, which also tested SINV negative, were chosen and offered 5 control pellets. All pellets (control and treated) were coated with 200 µL of Vienna sausage liquid (Libby's, Chicago, IL) and placed approximately 16 cm away from the brood box (14 cm × 10 cm × 4 cm). A circle drawn around the pellets on the underside of the ant's observation tray (57.5 cm × 41 cm × 14.5 cm) delineated pellet movement. Throughout the experimental trial period (30 d) 2 samples of 10 ants each (a combination of foragers and workers) were collected every 5 d from each colony (control and treated), resulting in a total of 120 ants tested per colony. RNA was extracted and analyzed immediately for the presence or absence of virus using specific primer sets (p62 and p63; Valles & Strong 2005). Chi-square analysis of SINV positive ants between those with or without brood was performed with pooled data from the experimental groups.

The experiment was replicated with pellets (control and treated) that were stored in dark, air tight containers at room temperature (22°C) for 12 mo. Fifteen new colonies were collected, screened for SINV presence, and established as before. The experimental period for the aged pellets was 35 d. At the conclusion of the second replicated experiment, queens, current brood, and males from 2 infected colonies were sacrificed and tested for the presence of virus.

Results

Of the 89 colonies collected only 26 were found to be positive for SINV, 13 from Smith County, 9 from Cherokee County, 2 from Hunt County, and 2 from Gregg County. Overall ant colonies within 2 counties in Texas (Smith and Cherokee, Co) showed a higher incidence of SINV-1 in Cherokee County with 9 of 15 colonies testing positive for SINV (60%), while Smith County had 13 of 58 colonies test positive (22.4%). Sample sizes for Hunt and Gregg, Co were not large enough to make statistical inferences.

Ants quickly accepted the pellets and within 24 h of pellet introduction, some colonies had moved most of the pellets into the brood box. By d 15 all colonies including control colonies (100%) had moved all 5 pellets into their brood boxes. By d 5, post-feeding one set of forager/worker ants (20 individuals) tested positive for SINV. By d 10, post-feeding 2 additional colonies (40 individuals) tested positive, and by d 15 post-feeding, forager/worker groups in 4 of the 10 colonies (80 individuals) had tested positive for SINV (Fig. 1). Additionally, none of the control colonies tested positive for SINV infection, during or after the experiment. After 30 d the experiment was terminated, no additional colonies tested positive for SINV after d 15, resulting in a 40% infection rate of foragers/workers tested for the 10 experimental colonies. The presence or absence of

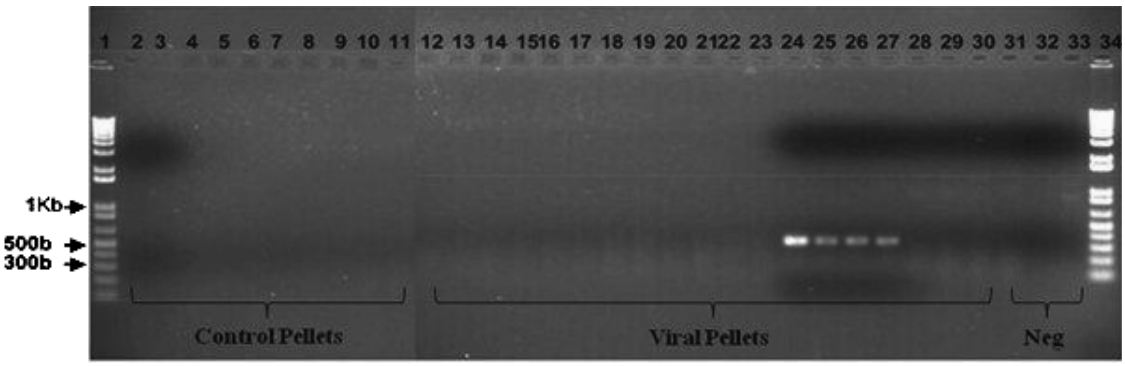

Fig. 1. Gel electrophoresis of the RT-PCR from d 15 post-feeding. This gel illustrates 2 samples as duplicates in neighboring lanes, thus displaying 2 of the 4 colonies that were SINV-1 positive over the duration of the testing period (30 d). Each sample depicts 10 forager/worker ants that were offered 2 d old pellets. A 1Kb ladder (TrackIt™ 1Kb Plus DNA Ladder, Invitrogen, Cat. no. 10488-085) (lanes 1 and 34), ants given control pellets for 5 colonies (lanes 2-11), ants given viral pellets for 10 colonies (lanes 12-31), lanes 24-27 illustrate positive detection of virus for two colonies (~300bp), and negative controls for the RT-PCR (lanes 32-33). Multiple gels are shown.

brood in a colony did not appear to have an effect on colony infection or detection of SINV.

For the experiment with aged pellets, it was found that 30% of tested foragers/workers from the colonies tested positive for SINV 5 d post-feeding. No additional colonies tested positive for SINV over the duration of the trial (Fig. 2) and no control colonies were found to be SINV positive. Upon termination of the second experiment, queens, remaining brood, and males did not test positive for the presence of virus. Based on Chi-square analysis, a significant difference was observed between the virus exposed and unexposed colonies ($\chi^2 = 4.565$, $df = 1$, $P = 0.03$).

Discussion

We have successfully demonstrated an inexpensive method to produce pellets containing ant-infecting virus for delivery to fire ants. Pellets with attractive flavor and virus were produced by a simple method which provides potential application for increasing the efficacy of currently used biological control agents of *S. invicta*. The virus containing pellets provide substantial evidence that this method may have potential, after further refinement, as an effective tool to introduce SINV and other viruses to fire ants in the field. The longevity of these viral pellets was shown to be at least 1 year when storing pellets in dark, airtight plastic containers, at room temp (22°C). Delivery of virus to multiple colonies for both the freshly prepared and extendedly stored pellets (2 d and 12 mo, respectively) was successful. A crude virus preparation was used to produce these pellets, therefore a precise virus titer was not obtained, calculations based on nanodrop readings showed a 139.5 ng of protein/µL of inoculum was used. Even though a total of 600 ants were sampled in virus exposed colonies, detection of SINV-1 was sporadic when sampling workers that may or may not have yet been exposed to the virus through food sharing, i.e., trophallaxis. A plausible explanation for this anomaly is that SINV-1 was only being ingested and not transferred at 100% success from queen to offspring or worker to worker. However, trials were terminated after 30 d and infections may have persisted. Long term trials are needed but were beyond the current scope of this trial. External contamination is not a likely candidate for explaining infection because colonies that tested positive for SINV-1 remained positive throughout the experimental period. If external contamination were contributing to the detection of infection we would expect more colonies to have tested positive randomly or only during a single sampling time. Queens, males, and brood tested negative for the presence of virus; increasing the likelihood that SINV was not being transferred by contact between individuals but by ingestion alone. An individual may have become infected with the virus and died, but due to rapid mortality under laboratory conditions the virus may not have spread to nest mates. Conversely, the experiment clearly demonstrated that delivery of virus to foragers in a colony using this pellet formulation method was possible.

Future studies will evaluate combinations of microencapsulated virus with the fungus, *Beauveria bassiana*, previously shown to also reduce *S. invicta* colonies (Bextine & Thorvilson 2002) and various chemical control agents (e.g. Amdro (Tetrahydro-5,5-dimethyl-2(1H)-pyrimidinone [3-[4(trifluoromethyl)phenyl]-1-[2-[4-(trifluoromethyl)phenyl]ethyenyl]-2-propenylidene]hydrazone), and Over-and-Out (5-amino-1-(2,6-dichloro-4-(trifluoromethyl) phenyl)-4-((1,R,S)-(trifluromethyl) sulfinyl)-1-H-pyrazole-3-carbonitrile)). Given that SINV-1 is taxonomically related to IAPV, adding additional immune stressors to *S. invicta* colonies may be able to induce a 'colony collapse' effect, thus effectively decreasing populations of *S. invicta*.

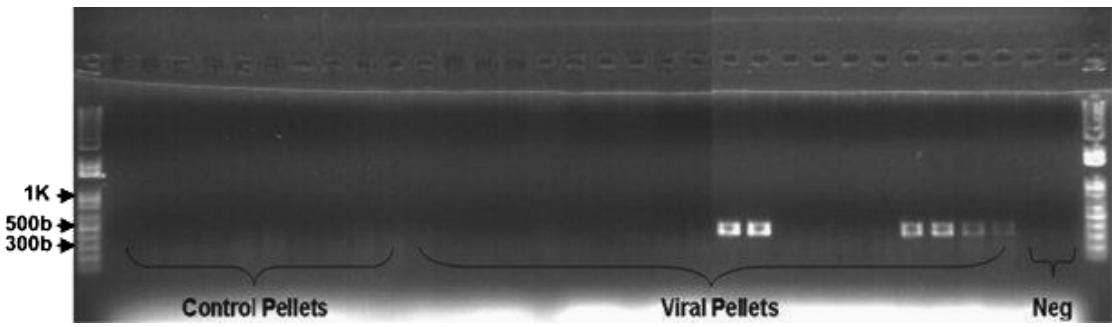

Fig. 2. Gel electrophoresis of the RT-PCR from d 5 presented in duplicate, illustrating 3 different colonies with positive bands for viral infection. Each sample depicts 10 forager/worker ants that were offered 12-month-old pellets. A 1Kb ladder (TrackIt™ 1Kb Plus DNA Ladder, Invitrogen, Cat. no. 10488-085) (lanes 1 and 34), ants given control pellets for 5 colonies (lanes 2-11), ants given viral pellets for 10 colonies (lanes 12-31), lanes 22-23 and 28-31 confirm positive detection of virus for 3 colonies (~300bp), and negative controls for the RT-PCR (lanes 32-33). Multiple gels are shown.

ACKNOWLEDGMENTS

We thank our anonymous reviews for constructive criticism and Christopher Powell for assistance in the laboratory. Funding was provided by a University of Texas at Tyler research grant. The mention or use of products within does not imply nor guarantee an endorsement by the USDA, ARS, to the exclusion of other similar, suitable products.

REFERENCES CITED

BAIRD, R., WOOLFOLK, S., AND WATSON, C. E. 2007. Survey of Bacterial and Fungal Associates of Black/Hybrid Imported Fire Ants from Mounds in Mississippi. Southeast Nat. 6(4): 615-632.

BEXTINE, B. R., AND THORVILSON, H. G. 2002. Field applications of bait-formulated *Beauveria bassiana* alginate pellets for biological control of the red imported fire ant (Hymenoptera: Formicidae). Environ Entomol 31(4): 746-752.

COX-FOSTER, D. L., et al. (21) 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318: 283-287.

GRAHAM, L. C., PORTER, S. D., PEREIRA, R. M., DOROUGH, H. D., AND KELLEY, A. T. 2003. Field releases of the decapitating fly *Pseudacteon curvatus* (Diptera: Phoridae) for control of imported fire ants (Hymenoptera: Formicidae) in Alabama, Florida, and Tennessee. Florida Entomol. 86(3): 334-339.

HUNTER, W. B., KATSAR, C. S., AND CHAPARRO, J. X. 2006. Nucleotide sequence of 3' -end of *Homalodisca coagulata Virus-1.* A new leafhopper-infecting virus from the glassy-winged sharpshooter. J. Insect Sci. 6.28 (Online: insectscience.org/6.28/).

HUNNICUTT, L. E., HUNTER, W. B., CAVE, R. D., POWELL, C. A., AND MOZORUK, J. J. 2006. Complete genome sequence and molecular characterization of *Homalodisca coagulata virus-1*, a novel virus discovered in the glassy-winged sharpshooter (Hemiptera: Cicadellidae). Virology 350: 67-78.

JOHNSON, R. 2010. Honey Bee Colony Collapse Disorder. Congressional Research Service Report for Congress (www.crs.gov).

OI, D. H., AND WILLIAMS, D. F. 2002. Impact of *Thelohania solanapsae* (Microsporidia: Thelohaniidae) on polygyne colonies of red imported fire ants (Hymenoptera: Formicidae). J. Econ. Entomol. 87: 623-630.

OI, D. H., AND VALLES, S. M. 2008. Fire ant control with entomopathogens in the USA, pp. 237-257 *In* H. M. T. Hokkanen, A. E. Hajek, T. R. Glare, and M. O'Callaghan [eds.], Use of Microbes for Control and Eradication of Invasive Arthropods. Springer Netherlands (doi: 10.1007/978-1-4020-8560-4).

TABER, S. W. 2000. Fire Ants. Texas A&M University Press, College Station.

TUFTS, D. M., HUNTER, W. B., AND BEXTINE, B. 2010. Discovery and effects of the *Solenopsis invicta* virus (SINV-TX5) on red imported fire ant populations. J. Invertebr. Pathol. 104: 180-185.

VALLES, S. M., et al. (7) 2004. A picorna-like virus from the red imported fire ant, *Solenopsis invicta*: initial discovery, genome sequence, and characterization. J. Virol. 328: 151-157.

VALLES, S. M., AND STRONG, C. A. 2005. *Solenopsis invicta* virus-1A (SINV-1A): Distinct species or genotype of SINV-1? J. Invertebr. Pathol. 88: 232-237.

VALLES, S. M., et al. (20) 2007. Phenology, distribution, and host specificity in *Solenopsis invicta* virus-1. J. Invertebr. Pathol. 96: 18-27.

VALLES, S. M., AND HASHIMOTO, Y. 2009. Isolation and characterization of *Solenopsis invicta* virus 3, a new positive-strand RNA virus infecting the red imported fire ant, *Solenopsis invicta*. Virology 388: 354-361.

VANENGELSDORP, D., et al. (12) 2009. Colony collapse disorder: A descriptive study. PLoS One 4(8): e6481. Doi:10.1371/journal.pone.0006481.