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Comparison of Assays for the Determination of Entomogenous Nematode Infectivity

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Abstract: Injection, contact, and soil assays were used to compare infectivity of *Heterorhabditis bacteriophora* strain HP88 and *Steinernema carpocapsae* strain All to final instar *Galleria mellonella* larvae. Under comparable assay conditions, *H. bacteriophora* produced less *Galleria* mortality and showed greater within-assay variability in infectivity than *S. carpocapsae*. Injection of individual *S. carpocapsae or H. bacteriophora* infective juveniles into *Galleria* indicated that a comparatively greater percentage of *S. carpocapsae* was capable of initiating infection. In addition to nematode species, other major components of variability in assay estimations of nematode infectivity were number of nematodes used in the assay, assay type, date of the assay, and possibly, *Galleria* age.

Key words: Bioassay, infectivity assay, injection, nematode, *Galleria mellonella, Heterorhabditis bacteriophora, Steinernema carpocapsae.*

During the infective juvenile (IJ) stage, species of *Steinernema* and *Heterorhabditis* nematodes actively seek out and invade susceptible insect hosts (10). Infective juveniles contain symbiotic *Xenorhabdus* bacteria, which are released into the hemocoel of the insect and kill it within 48 to 72 hours after infection. The infectivity of nematodes may be influenced by several biological parameters. These include nematode behavior, activity level, host finding ability, and ability to penetrate the host (4,6,8). Other possible influences on the infectivity of the nematode-bacterial complex are the number of bacteria per IJ (5), the virulence of the bacteria (8), and the percentage of IJ carrying bacteria (1). The biological parameters of infectivity act in concert with environmental influences such as temperature (3) and soil composition (7). Determination of nematode infectivity in the laboratory is important in host range evaluation, interstrain comparisons, and quality control monitoring of laboratory-reared material. This investigation reports on the comparative infectivity of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* using three types of assays.

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MATERIALS AND METHODS

Nematode species *Heterorhabditis bacteriophora* strain HP88 (Biosys, Palo Alto, CA) and *Steinernema carpocapsae* strain All (Dr. H. Kaya, University of California, Davis) were propagated on *Galleria mellonella* larvae (12). Infective juveniles aged 1–7 days, at a density of 10,000 IJ/ml, were stored at 6–8 C (All) or 10–12 C (HP88) in 125 ml sterile distilled water within 650-ml, 150-cm^2, loosely capped tissue culture flasks. Survivorship of IJ was >90% during the 5-week study. (Final instar *G. mellonella* larvae used for testing were obtained from Sunfish Bait, Webster, WI.)

Assays: Weekly samples of IJ were tested during weeks 0–5 for infectivity to larvae of *G. mellonella* using the one-on-one, five-on-one, and tube soil assays. Two additional assays, the injection assay and the tray soil test, were conducted on different days during weeks 0–5 using IJ sampled from the same storage containers. Appropriate control *Galleria,* unexposed to IJ, were used for each assay-sample date. All assays were maintained at L:D 24:0, 22 C, and 40–60% RH. Assays were scored for *Galleria* mortality at 4 days after initiation. Only larvae showing the characteristic color change associated with *Xenorhabdus* growth (*X. nematophilus* = tan; *X. luminescens* = brick red) were judged as dead due to IJ infection. The percentage of mortality for each assay date was corrected

for control mortality using Abbott's formula.

Contact assays: The general procedure for one-on-one and five-on-one assays was outlined by Miller (9). The test arena was a 24-well Falcon #3047 tissue culture plate. Each well received one Bacto 1.3-cm-d concentration disk, 75 µl of water to maintain cell humidity, one final instar *Galleria* larva, and either one or five IJ. The IJ were pipetted individually using a 100 µl glass pipet with a heat-tapered tip. Controls contained larvae not exposed to IJ. The wells were sealed with Mylar film ventilated with four holes introduced by a 23-gauge hypodermic needle. Sample size was 24 larvae, with four replications per sample date.

Soil assays: The test arena was a 50-ml Corning #25339 centrifuge tube. One final instar *Galleria* larva was placed in the bottom of each tube and covered with 25 ml of sandy soil (80% medium sand, 20% nonsterile soil). The soil was moistened with 3 ml of water, and 10 IJ were individually pipetted onto the top soil surface using a pipet of the type described in the previous assay. Control tubes received 3 ml water but no nematodes. Tubes were loosely capped and kept upright during the experiment. Sample size was 24 tubes, with four replications per sample date.

In tray soil tests, the test arena was a 14-well plastic ice cube tray, 27 × 11 × 4 cm, well capacity 25 ml. One final instar *Galleria* larva was placed in the bottom of each well and covered with 25 ml of the previously described sand:soil mix. The soil was moistened with 3 ml water, and 5 IJ were individually pipetted onto the soil surface. The wells were covered with Mylar perforated with four ventilation holes. Control wells received water but no nematodes. Sample size was 14 wells, with two replications per sample date.

Injection assay: A modification of the injection techniques developed by Poinar and Thomas (11) and Akhurst and Bedding (2) was used. Final instar *Galleria* larvae were surface sterilized in 0.04% sodium hypochlorite for 60 seconds and then rinsed for 60 seconds in sterile distilled water. Infective juveniles were surface sterilized for 3 hours in a solution of 0.1% merthiolate plus 5,000 units/ml streptomycin, with constant slow agitation provided by a magnetic stir bar. Surface sterilized IJ were rinsed three times in sterile distilled water. A Hamilton 50-µl syringe was used to pick up individual IJ in 4 µl of sterile water. Nematodes were injected into the larval hemocoel via the pleural area of the 6th abdominal segment. Control larvae were injected with 4 µl of sterile distilled water. Injected larvae were maintained within individual wells of 24-well tissue culture plates using the one-on-one assay protocol. To minimize the possibility of bacterial cross contamination, the injection needle and inner syringe barrel were cleaned with 70% ethanol before and after injections of each nematode species.

Statistical analysis: We used an analysis of variance (GLM procedure, SAS Institute, Cary, NC) to determine the significance of assay type and sample date on the estimation of infectivity for HP88 and SC-All derived from storage. The experimental design was a randomized complete block with sample dates (weeks) considered as blocks. The two nematode species were evaluated separately. Because the injection and tray soil assays were performed on different sampling dates within the 5-week period, these data were not included in the main statistical analysis of variance. To examine relative effectiveness and variability among all assays, the mean, standard deviation, and coefficient of variation of corrected *Galleria* mortality, among sample dates, were calculated for the one-on-one, five-on-one, and tube assays and were compared with data from the injection and tray assays.

Results and Discussion

Control mortality of *Galleria* did not exceed 10% for any of the assays. Estimates of nematode infectivity, within species, were significantly affected by variation as-

TABLE 1. Sources of variation in mortality of final instar *Galleria mellonella* larvae exposed to infective juveniles of *Heterorhabditis bacteriophora* (HP88) or *Steinernema carpocapsae* (All) using three assays.

Species-strain	Source of variation†	df	Sum of squares	*F*	*P*
HP88	Sample week	5	5,784.9	6.06	0.0002
HP88	Assay	2	37,260.2	97.60	0.0000
HP88	Sample week · assay	10	2,965.6	1.55	0.1480
HP88	Error	51	9,734.8	—	—
SC-All	Sample week	5	1,314.0	2.47	0.046
SC-All	Assay	2	16,946.0	79.52	0.0000
SC-All	Sample week · assay	10	1,840.1	1.73	0.102
SC-All	Error	48	5,114.6	—	—

† Sample week · assay = interaction effect.

sociated with assay type and sample week (Table 1). Significant variability among assays was not surprising, given the distinctiveness of the assays and the different number (1–10) of IJ used to challenge individual *Galleria* larvae. No time trend was evident in infectivity because infectivity varied randomly over time (all $P > 0.05$). One possible source of the significant between-week variation was age of *Galleria* larvae used in the assays. *Galleria* larvae were stored at 10 C in sawdust for 1 to 4 weeks prior to use. Therefore, larval age at the time of the assay ranged from approximately 1 to 5 weeks. Variation in the general level of activity, host-seeking ability, and number of *Xenorhabdus* cells in the gut of nematodes sampled for the assays may have also influenced variability in *Galleria* mortality.

Galleria larval mortality in the injection assay estimated the maximum potential infectivity in the nematode strains. In direct comparison of injection, contact, and soil assays, HP88 was less infective to *Galleria* larvae than SC-All (Table 2). The magnitude of the difference between *Galleria* mortality produced by the injection versus the one-on-one assays suggests that SC-All may be superior to HP88 in locating and or invading *Galleria* larvae within small test arenas, although this was not directly tested (Table 2). The one-on-one contact assay had the highest coefficient of variability, for both species, of all assays studied (Table 2). HP88 infectivity was particularly variable in the one-on-one assay.

In general, nematode species and the number of IJ significantly influenced infectivity and subsequent mortality of *Galleria* larvae. For *H. bacteriophora* (HP88), assays using a larger number of IJ and a soil substrate separating IJ from the host were more consistent measures of *Galleria* mor-

TABLE 2. Corrected mortality (mean and standard deviation) of *Galleria* larvae in five assays used to estimate infectivity of *Heterorhabditis bacteriophora* (HP88) and *Steinernema carpocapsae* (All).

Species-strain	Assay	n†	Mean CM‡	SD	CV (%)§
HP88	One on one	6	11.4	12.0	105.3
HP88	Injection	7	50.0	11.8	23.6
HP88	Five on one	6	45.0	16.7	37.1
HP88	Tube	6	68.7	20.5	29.8
SC-All	One on one	6	61.2	18.2	29.7
SC-All	Injection	8	71.0	13.7	19.3
SC-All	Five on one	6	94.5	7.6	8.0
SC-All	Tray	5	76.8	11.7	15.2
SC-All	Tube	6	96.9	3.5	3.6

† replications.
‡ CM = corrected mortality.
§ CV = coefficient of variation (SD × 100) ÷ CM.

tality than the direct contact assays. With *S. carpocapsae* (SC-All), *Galleria* mortality in small arenas was more consistent, indicating that direct-contact tests such as the one-on-one and five-on-one may be useful baseline indicators of *S. carpocapsae* infectivity.

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