


RESEARCH PAPER/REPORT



The *Galleria mellonella* larvae as an *in vivo* model for evaluation of *Shigella* virulence

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ABSTRACT

Shigella spp. causing bacterial diarrhea and dysentery are human enteroinvasive bacterial pathogens that are orally transmitted through contaminated food and water and cause bacillary dysentery. Although natural *Shigella* infections are restricted to humans and primates, several smaller animal models are used to analyze individual steps in pathogenesis. No animal model fully duplicates the human response and sustaining the models requires expensive animals, costly maintenance of animal facilities, veterinary services and approved animal protocols. This study proposes the development of the caterpillar larvae of *Galleria mellonella* as a simple, inexpensive, informative, and rapid *in-vivo* model for evaluating virulence and the interaction of *Shigella* with cells of the insect innate immunity. Virulent *Shigella* injected through the forelegs causes larvae death. The mortality rates were dependent on the *Shigella* strain, the infectious dose, and the presence of the virulence plasmid. Wild-type *S. flexneri* 2a, persisted and replicated within the larvae, resulting in haemocyte cell death, whereas plasmid-cured mutants were rapidly cleared. Histology of the infected larvae in conjunction with fluorescence, immunofluorescence, and transmission electron microscopy indicate that *S. flexneri* reside within a vacuole of the insect haemocytes that ultrastructurally resembles vacuoles described in studies with mouse and human macrophage cell lines. Some of these bacteria-laden vacuoles had double-membranes characteristic of autophagosomes. These results suggest that *G. mellonella* larvae can be used as an easy-to-use animal model to understand *Shigella* pathogenesis that requires none of the time and labor-consuming procedures typical of other systems.

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Introduction

Shigella spp. are human-adapted pathogens that are transmitted orally through contaminated food and water and cause an acute inflammation of the distal colon and rectum. The symptoms of infection include moderate to severe diarrhea, dysentery, fever, abdominal cramps, tenesmus, nausea and various other gastrointestinal and constitutional symptoms. As low as 10–100 virulent *Shigella* can cause disease in some humans. Within 10–14 hours of infection, the level of serum TNF- α , a proinflammatory cytokine rises and the strain can be detected in diarrheal stools with either symptoms of fever, diarrhea and/or dysentery appearing in the

next few hours.¹ Primates such as rhesus monkeys are also susceptible to oral *Shigella* infections and become very sick, sometimes resulting in death.^{2,3} However, the multiplicity of infections in such animals is much higher, in the range of 10⁹ to 10¹⁰ CFU.

Shigella is an enteroinvasive gram negative pathogen with a genome backbone very similar to *E. coli*. There are 4 major *Shigella* serogroups, *S. flexneri*, *S. sonnei*, *S. dysenteriae* and *S. boydii* and within each group, there are multiple serotypes and subtypes. Only *S. sonnei* has one serotype. *Shigella* virulence is an acquired trait and is due to the presence of a large mosaic 180–220 kb plasmid, also known as the invasion plasmid or the

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virulence plasmid (VP), composed of multiple virulence associated genes and a type III secretion system (T3SS). During pathogenesis, the presence of the VP allows the bacteria to initially be rescued from phagocytic macrophages and then to invade epithelial cells and subsequently spread from one cell to another.⁴⁻⁸ These 2 essential steps in pathogenesis, invasion and spread, can be assayed in the laboratory using cultured cells. Plasmid-cured strains or strains lacking critical regions of the VP are noninvasive, avirulent and unable to cause disease. *Shigella* mutants that are invasive but lack the ability to spread intercellularly are significantly attenuated in virulence and are being evaluated as live attenuated vaccine candidates. Since humans and primates alone are naturally susceptible to infection, several small animal models have been developed to test different facets of *Shigella* pathogenesis and assist in vaccine development.⁹⁻¹⁶

Rhesus macaques and more recently *Aotus nancymae* monkeys have been used to study oral *Shigella* infections.^{2,9,17,18} However, the monkey models are very expensive, technically challenging and difficult to sustain. Alternately, several small animal models are being used to understand specific steps in pathogenesis. For example, the Sereny keratoconjunctivitis reaction in guinea pig eyes is a measure of virulence of a *Shigella* strain and requires both bacterial invasion and intercellular spread of the bacteria.¹⁶ Sereny negative strains are significantly attenuated in virulence properties.¹⁹ The mice intranasal model is used to probe the inflammatory potential of a *Shigella* strain and has been previously used to evaluate the endotoxicity of LPS mutants.²⁰ Oral feeding of *Shigella* to 3-day old gnotobiotic piglets results in diarrhea and has been used to demonstrate the enterotoxicity of some *Shigella* strains although the animals become quickly resistant to the bacteria.¹⁰ However, most small animal models do not simulate natural infections in humans. Additionally, monkeys, mice, guinea pigs and gnotobiotic piglets are hard to maintain, expensive and labor-intensive to work with and require regulatory approval and adequate veterinary resources. Thus, most of the small animal models have some drawbacks including practicality, ethical considerations, inter-laboratory reproducibility, ease of use, specialized training, and relevance to shigellosis. Due to these limitations, alternative *in vivo* infection model systems

are being described that are easier and cheaper to house and maintain without the need for specific approvals.²¹⁻²³ This study focuses on insect model organisms that are attractive as they are relatively cheap, readily available, and present limited ethical issues. Like other animal models they typically shed light on relevant aspects of pathogenesis. It is believed that the epithelial cells of the insect larval midgut have similarities to the intestinal cells of the mammalian digestive system. The human and insect innate immune systems demonstrate several similarities which in-turn enable testing hypothesis across kingdoms.²⁴ Most insect species contain specialized cells known as haemocytes that phagocytose pathogens and form aggregates which encapsulate and neutralize foreign microorganisms.²⁵ Moreover, activated haemocytes can trigger a phenoloxidase (PO) melanization cascade.²⁴ As a result of bacterial infection haemocyte-mediated responses are complemented by the production and secretion of antimicrobial peptides (AMPs) by the insect fat body, an organ similar to the mammalian liver, activation of specific signaling pathways, and generation of reactive oxygen species.²⁵

The caterpillar larva (also called wax worms) of the *Lepidopteran* greater wax moth (*Galleria mellonella*) has become a widely adopted insect model to study several human pathogens including *Listeria* spp,²⁶ *Streptococcus*,²⁷ *Legionella*,²⁸ *Campylobacter*,²⁹ *Yersinia*,^{30,31} *Pseudomonas*,³²⁻³⁴ *Enterococcus*,³⁵ *Staphylococcus*,³⁶ *Candida*,^{37,38} *Aspergillus*³⁹⁻⁴¹ and other bacterial and fungal pathogens.⁴²⁻⁴⁵ The life cycle of this organism comprises a larval stage that transforms within 6–8 weeks into pupae and finally into moth. The larvae are fairly large in size, the last instar larva is 2 cm long and 250 mg in weight and can be reared at various temperatures (20–30°C). *G. mellonella* larvae tolerate prolonged incubation at elevated temperatures (up to 42°C) and do not require anesthesia. The size of the larvae allows for easy manipulation and injection. After introduction of microbial pathogens the larvae is conveniently monitored for survival and larvae death is accompanied by unresponsiveness to physical stimuli and strong melanization. However, intrinsic differences between bacterial species in mechanisms of colonization, invasion, and pathogenicity means that it is necessary to examine the suitability of this model for the study of *Shigella* spp. In this study, the *G. mellonella* larvae is evaluated as an *in-vivo* model for studying *Shigella* infections. Its utility as a

quick and inexpensive tool to evaluate virulence and compare isolates, species, and mutant *Shigella* strains is demonstrated.

Materials and methods

Bacterial strains and preparation for larval infections

The bacterial strains used in this study are listed in Table 1 and came from the WRAIR collection. Bacterial strains were routinely grown on Tryptic Soy Agar (TSA) plates containing 0.05% Congo Red (w/v) and in Tryptic Soy Broth (TSB) medium. A Congo Red positive colony usually correlates with a virulent *Shigella*. A Form I Moseley strain represents an invasion-plasmid containing *S. sonnei* isolate whereas a Form II isolate refers to a plasmid-cured strain. Where appropriate, carbenicillin (50 µg mL⁻¹) or chloramphenicol (35 µg mL⁻¹) was added to the growth media. *Shigella* strains were cultured on TSA plates for 16 hours before inoculation in TSB. After overnight growth in TSB, mid-log phase cultures were derived by inoculating fresh TSB medium with an aliquot of the overnight growth at a 1:100 dilution. Unless specified differently bacteria growth was done at 37°C. The culture at mid-log phase of growth (O.D. = 0.4, ~2 hours) was harvested by centrifugation (5,000 X g, 5 minutes), washed and diluted in Dulbecco's phosphate-buffered saline (D-PBS) to the desired optical density at 600 nm (OD₆₀₀). The overnight growth culture represents the stationary growth phase. 1 O.D. unit was estimated to correspond to 5×10⁸ CFU/ml and the actual concentration was verified by serial dilutions and plating.

Table 1. List of bacterial isolates and plasmids used in the *G. mellonella* infection assays.

Strain	Description
2457T	Virulence plasmid-containing <i>S. flexneri</i> 2a
BS103	Virulence plasmid-cured <i>S. flexneri</i> 2a 2457T
2457TΔipaB	ΔipaB <i>S. flexneri</i> 2a 2457T
2457TΔipaB::ipaB	ΔipaB <i>S. flexneri</i> 2a+IpaB clone
Moseley form II	Virulence plasmid-containing <i>S. sonnei</i>
1617	Virulence plasmid-cured <i>S. sonnei</i> Moseley
M90T-W	Wild type virulent <i>S. dysenteriae</i> 1
M90T-A3	Wild type <i>S. flexneri</i> 5
M90T 55	M90T-W with a 65 kb deletion which includes ΔipaBCDA and ipaR
2457T-GFP	Virulence plasmid-cured M90T-W containing pGFPuv
plasmid	Description
pGFPuv	pUC18 with GFP gene (ClonTech)
IpaB clone	pUC18 encoding full length IpaB

G. mellonella larvae and infections

Final instar *G. mellonella* larvae were obtained from Vanderhorst Wholesale Inc. (St. Marys, OH) and stored in darkness at room temperature. Only healthy-looking caterpillars with no melanization were used in the experiments. Randomly assigned sets of 10 *G. mellonella* larvae were contained in a petri dish and injected with 5 µl of bacterial suspension using a modified Hamilton syringe with a 31 gauge 8 mm long needle. The plates containing the caterpillars were incubated at 37°C in the dark unless specified differently. All injections were administered via the last left pro-leg into the haemocoel. As controls, equal numbers of larvae kept in a second Petri dish were injected with PBS alone while a third group of larvae with no treatment were included in every experiment. Larvae were individually examined for pigmentation, and the number of live larvae was recorded at designated intervals. Larvae that did not show any movement in response to touch were considered dead. Assays proceeded for 48–72 hours and monitored twice a day. At least 3 independent replicates of each experiment were performed.

Assessment of bacterial proliferation in *G. mellonella*

At designated time post-infection (p.i.), 4 live infected larvae were pooled and killed. After homogenization in PBS+0.1% Triton X100 and a 10 minute incubation to allow for release of the bacteria, the sample was serially diluted in PBS and plated. Plates were incubated at 37°C for 16 hours and the number of CFU was recorded.

Plasmids and standard molecular biology techniques

Construction of *S. flexneri ipaB* mutant: An *ipaB* deletion of the wild-type *S. flexneri* strain 2457T was made by the lambda-red recombineering method.^{46,47} The chloramphenicol cassette/linear DNA was PCR amplified from the pKD3 plasmid with the primers ipaB KO for and ipaBC KO rev (CATTCTCCTTATTTG-TATCAAGCAGTAGTTTGTTCG-AAAATTGCTTT TGC GTGTAGGCTGGAGCTGCTTCG and AAAGC ACAATCATACTTGGACGCAATT-CAGGATATCA AGGAGTAATTATTCATATGAATATCCTCCTTAGT). The *ipaB* gene was cloned by PCR amplification into pUC18 (IpaB clone). The plasmid was transformed

into 2457TΔipaB and the expression of IpaB was evaluated by Western blot analysis using monoclonal anti-IpaB antibodies. Transformation of *Shigella* strains with pGFPuv (Clontech) was done according to standard molecular biology method and GFP expression was checked spectroscopically.

Haemocytes quantification

Haemolymph was extracted at $t = 0$ and $t = 24$ hours p.i. Trypan blue (0.02% [vol/vol] in PBS) was added to the cells in the hemolymph and incubated at room temperature for 10 min. Viable cells were enumerated using a hemocytometer (TC-10, Bio-rad), and each sample was analyzed in triplicate. The average of 3 independent experiments are reported.

Fixation and staining of *G. mellonella*

G. mellonella larvae were fixed in 4% Paraformaldehyde for 3–4 weeks at room temperature, processed on Sakura Tissue-Tek VIP processor, embedded, sectioned and stained with hematoxylin and eosin (H&E) by Sakura Tissue-Tek Prisma stainer.

Transmission electron microscopy (TEM)

Haemolymph was extracted from infected *G. mellonella* larvae. Cells were spun down, washed once with PBS, and fixed in 1% formaldehyde and then 4% glutaraldehyde buffered with sodium phosphate pH 7.2. Samples were post-fixed with 2% OsO₄ and then post stained with 2% uranyl acetate for TEM. The material was sectioned on Leica microtome and viewed on 1400 Jeol electron microscope.

Fluorescence and confocal microscopy

Bacterial cells were transformed with pGFPuv to constitutively express GFP (2457T-GFP). Haemolymph was extracted from *G. mellonella* larvae infected with 2457T-GFP at the designated time points. Haemocytes were spun down, washed once with PBS, mounted on glass coverslips and viewed directly using a fluorescence microscope (Zeiss, AxioObserver.Z1). For confocal microscopy *G. mellonella* larvae were fixed and sectioned as written above, labeled with monoclonal anti-*S.flexneri* antibody followed by Alexa-fluor 594 conjugated secondary antibody (goat anti-mouse antibody) and detected by Olympus Fluoview FV1200.

Statistical analysis and LD₅₀ calculations

Individual statistical analysis of data are indicated either in the text or in the Figure legends. The Spearman-Kärber method (Haas 1989) was used to estimate the Lethal Dose (LD) causing 50% mortality (LD₅₀) of the infected *G. mellonella* larvae.

Results

Galleria mellonella larvae are susceptible to *Shigella* spp. infection

G. mellonella larvae were injected with representative virulent strains of 3 major *Shigella* species. The larvae survival was monitored over time following injection of the bacterial suspension as shown in a representative experiment (Fig. 1, panel A). *S. flexneri* 2a strain 2457T, *S. sonnei* strain Moseley, and *S. dysenteriae* 1 strain 1617 caused time-dependent larval death that was assayed by touch-induced lack of larval movement. In most cases, larval mortality was accompanied by melanization of the larvae (Fig. 1, panel B). Compared to the other 2 species of *Shigella*, strain 1617 induced higher mortality at the same bacterial inoculum dose indicating an increased virulence of this strain in this model (Fig. 1, panel A). No mortality was observed in the control PBS-injected larvae. An avirulent *S. flexneri* 2a strain BS103, under the same conditions behaved like PBS (Fig. 1, panel B). Oral force feeding of larvae with 2457T did not show any specific phenotype (data not shown). These results demonstrate that, with the limited number of strains studied here, *G. mellonella* larvae succumb to *Shigella* infection and appear to show strain-specific differences.

Shigella-induced larval mortality is dose dependent and requires the presence of the *Shigella* virulence plasmid

Infection with increasing doses of 2457T (and Moseley Form I, data not shown) resulted in increased rates of larval mortality indicating that larval death was dose dependent (Fig. 2, panel A). Infection with 2×10^6 CFU of 2457T resulted in 100% mortality of the larvae within 24–30 hours while mortality was reduced to 10% in larvae injected with 1.8×10^4 CFU of 2457T, even with increased time of infection to more than 40 hours (Fig. 2, panel A). VP-cured strains BS103 (and Moseley Form II, data not shown) caused lower rates of mortality, demonstrating the role of the invasion plasmid in causing early larval death (Fig. 2,



Figure 1. *Shigella* spp. infection of *G. mellonella* induces strain dependent lethality. Panel A, Log phase cultures of *S. flexneri* 2a strain 2457T (—■—), *S. sonnei* strain Moseley (—▲—), and *S. dysenteriae* 1 strain 1617 (●—▼—●) were harvested and 7×10^5 CFU bacteria were injected into the larva. Each group contained 10 larvae. PBS mock injection was used as a negative control (---●---). Survival was monitored over 54 hours p.i. Panel B, photographic images of the *G. mellonella* larvae injected with 7×10^5 CFU/larvae of 2457T, BS103 or PBS at 24 hours p.i., showing melanization of the larvae by injection of 2457T.

panels A-B). A similar profile was seen when *G. mellonella* was injected with *S. flexneri* 5a strain M90T-W, and an isogenic VP-cured strain M90T-55. M90T-A3, that lacks a portion of the VP, is less virulent than M90T-W (data not shown). These differences between plasmid-containing and plasmid-cured strains were reflected in the LD_{50} values for larval death (Table 2).

Shigella IpaB is required for *G. mellonella* larvae mortality

IpaB is a T3SS effector protein that is present on the VP and is critical for epithelial cell invasion and release of the bacteria from endocytic vacuoles. IpaB mutants are noninvasive and hence avirulent. *G. mellonella* were

injected with different doses of 2457T, 2457T Δ ipaB, 2457T Δ ipaB::ipaB and BS103 (Fig. 2, panels A-D). Larvae injected with 2457T Δ ipaB exhibited very low rates of mortality (Fig. 2, panel C). 2457T at a dose of 1.4×10^6 CFU resulted in 100% mortality within 24–30 hours of infection. However, only 10% of the larvae died when infected with a similar dose of a 2457T Δ ipaB mutant over 48 hours of infection (Fig. 2, panel C). Complementation of the Δ ipaB mutant with an ipaB gene (2457T Δ ipaB::ipaB) restored larval mortality rates indicating that ipaB-mediated virulence is required for larval death (Fig. 2, panel D). The significance of the differences in the survival rates, performed using the Log-Rank test, and the p-values derived for larvae infected with 2457T, 2457T Δ ipaB and 2457T Δ ipaB::ipaB at 1.2×10^6 CFU,

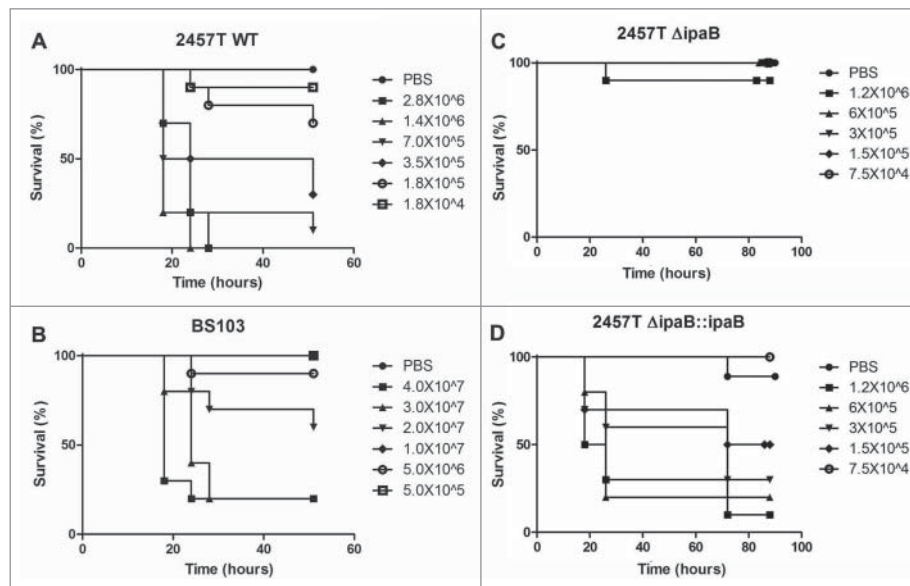


Figure 2. Dose, time and IpaB dependence of *G. mellonella* larvae death after infection with *Shigella* spp. *G. mellonella* larvae were injected with different concentrations of panel A, 2457T, panel B, BS103, panel C, 2457T $\Delta ipaB$ and panel D, 2457T $\Delta ipaB :: ipaB$. Larval death was monitored over time and scored by lack of movement after a touch. Significance was determined by the Log Rank test.

6×10^5 CFU, 3×10^5 CFU and 1.5×10^5 CFU were $p < 0.0001$, 0.0001 , 0.004 and 0.026 , respectively. *S. sonnei ipaB* mutants were also attenuated in this infection model (data not shown).

Effect of bacterial growth temperature and growth phase on *Shigella* virulence in *G. mellonella*

G. mellonella larvae have the advantage of being able to grow at a broad range of temperatures. And as with many pathogens, *Shigella* virulence is temperature regulated. 2457T grown at 37°C or 30°C in TSB medium was used to infect *G. mellonella*. After infection, the larvae were incubated at the same temperatures as the bacterial growth, at either 37°C or 30°C . The larval mortality rates infected with 1×10^6 CFU of 2457T are shown in Figure 3. Infection with 2457T that was grown at 30°C did not cause larval death at 24 hours p.i. even at doses of more than 4 times the LD_{50} of the strain at 37°C (data not shown). At 72 hours p.i. the

larval mortality at 30°C did not exceed 20% indicating that bacterial growth temperatures determined the outcome of the infection (Fig. 3).

Larval mortality was also dependent upon the growth phase of the bacteria (Fig 3). Overnight, stationary phase bacterial cells induced much lower mortality than bacterial cells grown to log phase ($\text{OD}_{600} = 0.4\text{--}0.6$) (Fig 3).

Table 2. LD_{50} of *Shigella* strains in the wax worm.

Bacterial Strain	LD_{50}
<i>S. flexneri</i> 2a 2457T	$5.1 \pm 1.4 \times 10^5$
<i>S. flexneri</i> 2a BS103	$2.3 \pm 0.7 \times 10^7$
<i>S. flexneri</i> strain M90T-W	$9.7 \pm 2.0 \times 10^5$
<i>S. flexneri</i> strain M90T- A3	$8.4 \pm 1.6 \times 10^6$
<i>S. flexneri</i> strain M90T 55	$3.0 \pm 0.7 \times 10^7$
<i>S. sonnei</i> strain Moseley form I (MI)	$1.7 \pm 0.5 \times 10^5$
<i>S. sonnei</i> strain Moseley form II (MII)	$>5.5 \pm 1.4 \times 10^7$
<i>S. dysenteriae</i> 1617	$2.1 \pm 0.4 \times 10^5$

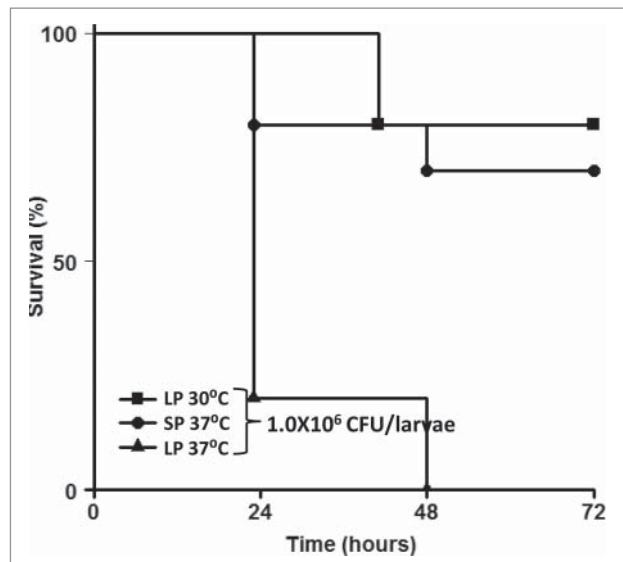


Figure 3. *G. mellonella* larval death is dependent on bacterial growth temperature and bacterial growth phase. *G. mellonella* larvae were injected with log (LP) and stationary phase (SP) 2457T that were grown at 37°C and 30°C . After injection, the larvae were incubated at either 37°C or 30°C , and death was monitored over time.

Virulent *Shigella replicates within G. mellonella and induces haemocyte cell-death*

To determine the fate of the bacteria within the wax worm larvae, *Shigella*-infected larvae were killed and aliquots of PBS/TritonX-100 larval lysates were plated out on TSA plates. The number of 2457T bacteria recovered from the lysates increased with time by more than one log indicating that the VP-containing strain replicates within *G. mellonella* while at the same time resulting in larval death (Fig. 4, panels A-B). In contrast, the number of BS103 recovered post infection reduced by more than a log indicating that the VP-cured noninvasive strain does not replicate and persist and is cleared from the larvae (Fig. 4, panel B). At the same time enumeration of larval haemocyte counts indicated that infection of *G. mellonella* with 2457T resulted in the drastic reduction of haemocyte counts in contrast to larvae infected with BS103 where the haemocyte counts remained almost unchanged (Fig. 4, panel C).

Fluorescence microscopy of infected larvae

G. mellonella larvae were infected with 2457T carrying a constitutive GFP-expressing plasmid. The introduction

of the plasmid had no effect on bacterial growth inside the larvae or the LD₅₀ values (data not shown). The hemolymph was extracted and observed under fluorescence microscopy. Soon after injection, GFP-tagged bacterial cells were observed in close association with haemocytes. At 2 hours post infection many of the haemocytes contained fluorescent 2457T could be seen (Fig. 5, panels A-F). Within a few hours the number of bacterial cells within the haemocytes increased, the percentage of haemocytes containing bacterial cells also increased while the number of free floating bacterial cells diminished significantly (data not shown). At 24 hours post infection very few haemocytes were seen and those that remained contained multiple GFP-tagged bacteria.

S. flexneri interactions with *G. mellonella*

Whole body sections of an infected *G. mellonella* larvae were analyzed using H&E staining accompanied by immune-staining confocal microscopy. At 2 hours post infection with 2457T, H&E stained cross sections of larvae revealed clusters of haemocytes exhibiting phagocytosis of bacteria (finely stippled blue dots throughout section) with small area of melanization around haemocyte aggregates (Fig. 6, panel A). At

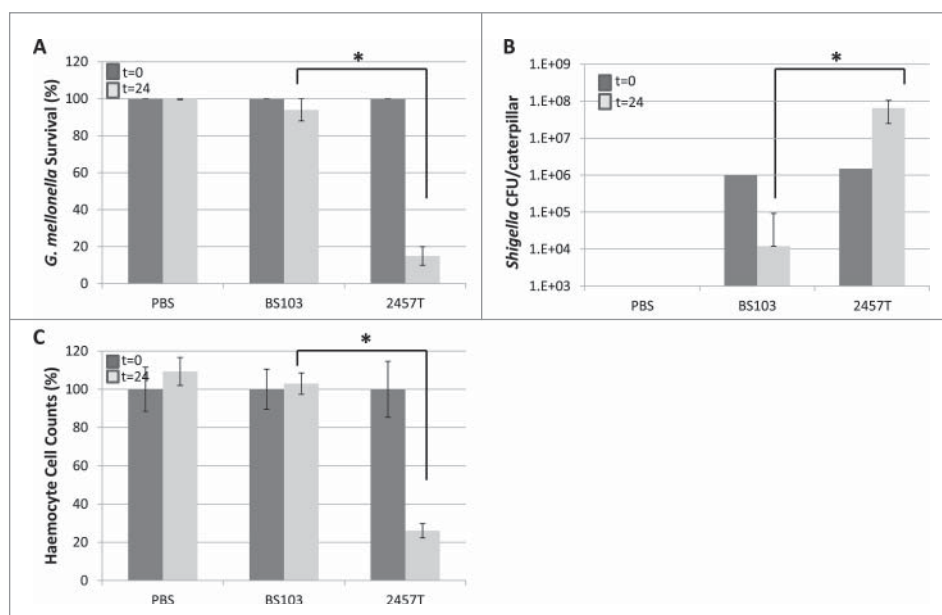


Figure 4. Bacterial replication and haemocyte depletion after *Shigella* infection in *Galleria* larva. *G. mellonella* larvae were infected with PBS, 1.0×10^6 CFU of 2457T and 1.0×10^6 CFU of BS103. After 24 hours, larvae survival rate, bacterial load in larvae and haemocyte counts were recorded. 4 live larvae at $t = 0$ and $t = 24$ were killed for these assays. Panel A represents percent survival of larvae at $t = 0$ and $t = 24$. Panel B represents changes in bacterial load in whole body at the 2 time points. Panel C represents per cent haemocyte counts at the 2 time points. These experiments were repeated 4 times. Each error bar represents one standard deviation from the mean. Differences between wild-type and BS103 were assessed for statistical significance by using a 2-sided t-test and assuming unequal variances. * $p < 0.05$. Dark-filled bars $t = 0$, light gray bars, $t = 24$.

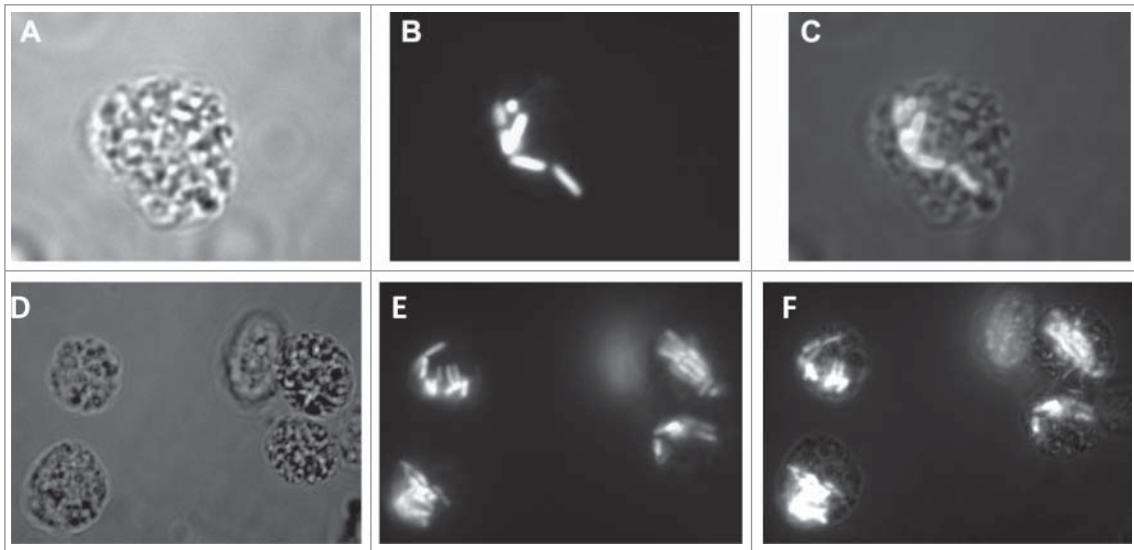


Figure 5. *S. flexneri* replicates within *G. mellonella* haemocytes. 2457T was tagged with a plasmid encoding GFP and used to infect larvae of *G. mellonella* at a dose of 1×10^6 CFU/larvae. The larvae were killed 2 hours post infection. Samples of the larval hemolymph were observed using panel A, bright field and panel B, fluorescent microscopy. The merged image in panel C shows GFP-tagged bacteria within the haemocytes. Panels D, E, and F represent a different field where multiple haemocytes can be seen with GFP-laden bacteria. Panel F represents the merged image of panels D and E.

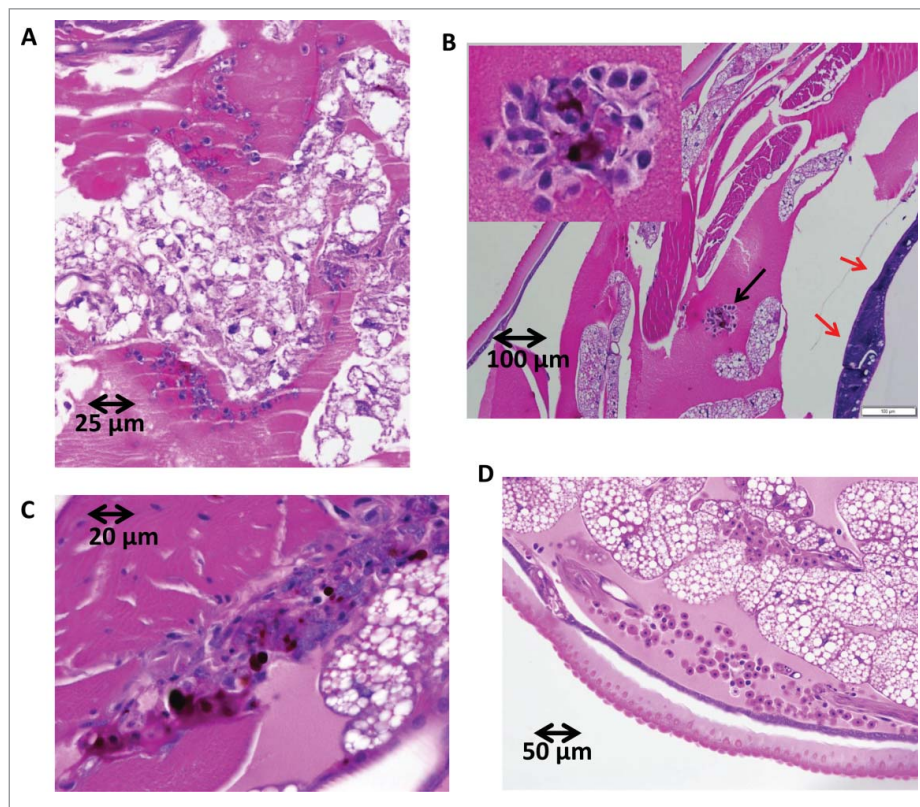


Figure 6. H&E-stained sections of *G. mellonella* after *Shigella* infection. *G. mellonella* were infected with 1×10^6 CFU/larvae of 2457T. After 2 hours and 20 hours of growth at 37°C the larva were fixed and stained. Panel A, 2 hours p.i. Haemocytes slightly more clustered than observed in uninfected hemolymph (panel D). Panels B and C, 20 hours p.i. Haemocytes form nodules with evidence of melanization (black arrow in panel B and inset in panel B) and a load of bacteria around tubular organelle (red arrows in panel B). Panel D, PBS injected sample. No bacteria are observed in haemocoel and no obvious aggregates of haemocytes or melanization in uninfected samples.

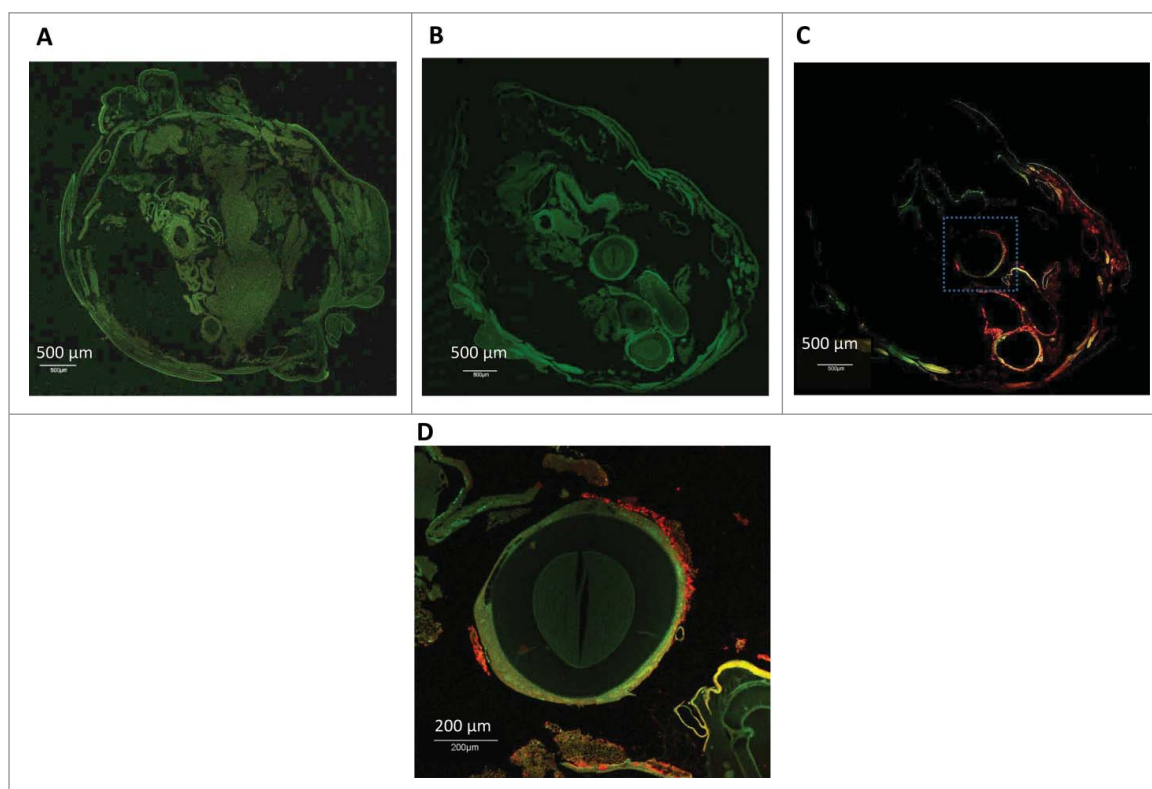


Figure 7. Immunofluorescence microscopy of 2457T-infected *G. mellonella*. *G. mellonella* larvae were infected with 1.0×10^6 CFU of 2457T/larvae. After 20 hours the larvae were fixed and stained using monoclonal anti-*S. flexneri* LPS antibody as primary antibody and Alexa-fluor 594 conjugated secondary antibody. Panel A, autofluorescence of uninfected *G. mellonella* section, panel B, autofluorescence of infected larvae section, panel C, immunofluorescence of anti *S. flexneri* specific antibodies and panel D, higher-magnification view of the square marked region in panel C showing red-labeled bacteria clustering around tubular organs.

20 hours post infection no individual haemocytes were seen and those that were observed were present in nodules, some attached to organ structures with evidence of melanization (Fig. 6, panels B-C). Tubular organs became surrounded by a load of bacteria (Fig. 6, panel B). In contrast, cross sections of H&E-stained uninfected larva looked healthy with individually distributed haemocytes showing no noticeable aggregates or melanization (Fig. 6, panel D). To confirm that the bacteria observed in the fixed sections of the infected *G. mellonella* were *S. flexneri* 2a, sections were stained with an 2457T-specific LPS antibody. Confocal microscopy of cross sections of the worm indicated that some 2457T bacteria localized in the proximity of the tissue surrounding tubular organs (Fig. 7, panels A-D).

Transmission electron microscopy (TEM).

Haemocytes at different times post infection were extracted and analyzed using TEM (Fig. 8, panels A-H). The electron micrograph demonstrates internalization of

the 2457T strain within a distinct vacuole lying within the cytoplasm of a granular haemocyte (Figs. 8, panels A-F). The cytoplasm shows numerous mitochondria as well as Golgi and rough endoplasmic reticulum (RER) studded with distinct ribosomes (Fig. 8, panels A-F). Several darker and less darker vesicular bodies are seen which in previous publications have been referred to as dense granules and structured granules.²⁵ The structured granules appear to have “microtubular” structures within the organelle. At 8 hours after infection with 2457T, the haemocyte contains multiple bacteria within enclosed vacuoles. In some cases the bacterium is seen replicating within the vacuole (Fig. 8, panel C, F). Most of the bacteria-laden vacuoles are encased within a distinct double membrane characteristic of autophagosomes that appears in some cases to have a fibrillar edge (see thin arrow in Fig. 8, enlarged panels D-F). While some vacuoles within the infected haemocyte are clearly empty or have faint granular material, other darker vesicles present within individual haemocytes appear to contain multiple smaller bodies or organelles. Several of the vacuoles have whorls of filaments forming concentric rings

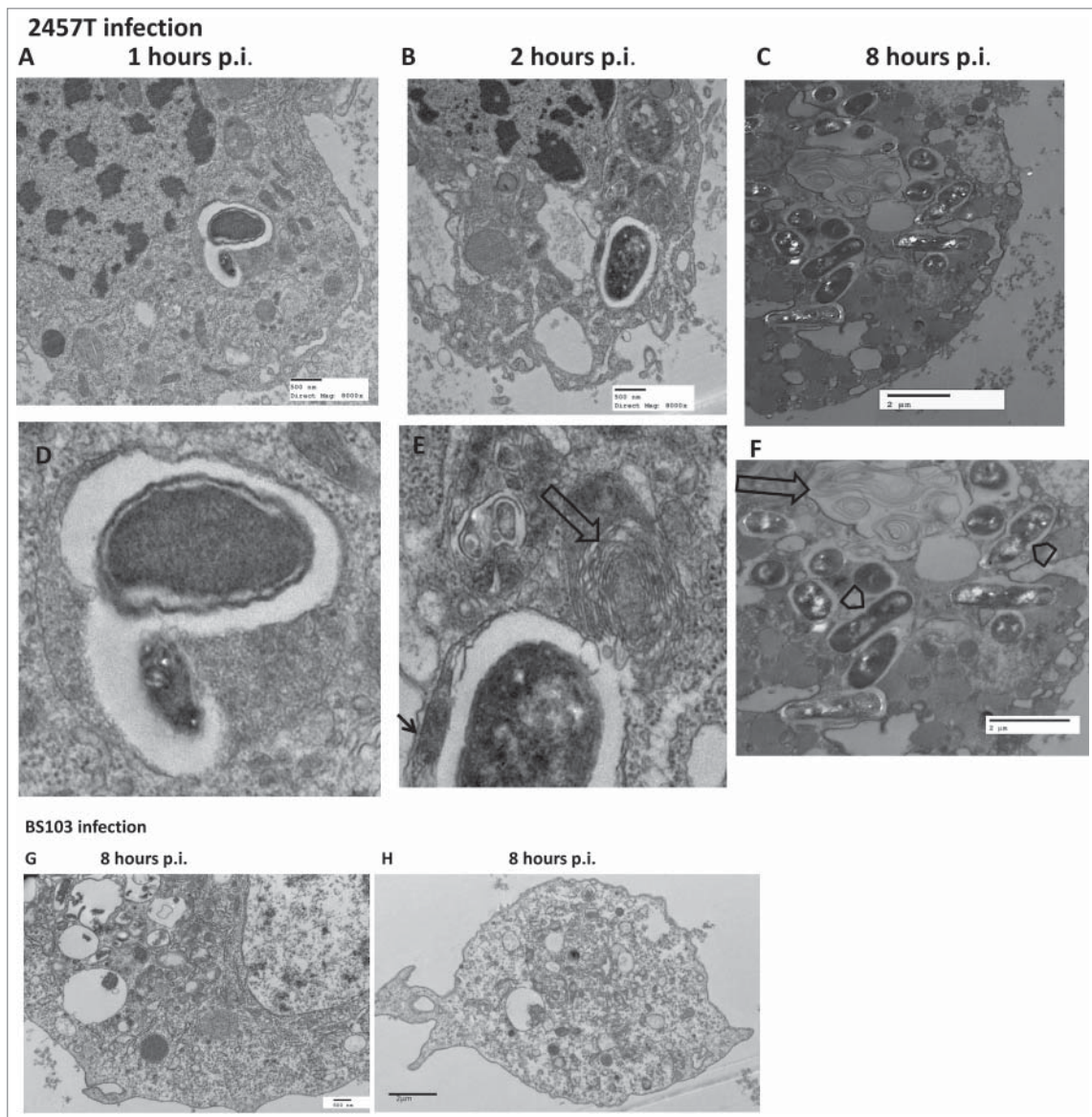


Figure 8. TEM of *G. mellonella* larval haemocytes after *Shigella* infection. Haemocytes of *G. mellonella* infected with 1.0×10^6 CFU/larva, were fixed, sectioned and observed under the TEM. Panel A-F, sections of *G. mellonella* larval haemocytes infected with *S. flexneri* 2a 2457T. The lower panels D-F represent higher magnification images of bacteria-containing regions in the corresponding upper panels A-C (wide arrows-structures appearing as onion rings, arrow heads-replicating bacteria, thin arrow-fibrillar edges). Panels G-H, larval haemocytes infected with BS103.

(“onion rings”?) (Fig 8, panels B-C, E-F). In some cases these vacuoles with onion rings along with the structured granules appear to lie in close association with the bacteria-laden vacuoles. No intact bacteria was seen in the haemocytes of BS103 infected larva (Fig. 8B, panels G-H). In some of these BS103-infected haemocytes there were vacuoles that appear to contain what looks like disintegrating bacteria. However, further analysis of the material within the BS103-infected haemocytes is needed (Fig. 8, panels G-H). Unlike the haemocyte vacuoles present within the 2457T-infected larvae, haemocytes from BS103-infected larvae looked intact after 8 hours p.i., and

did not contain autophagosomal-like structures or vacuoles with “onion rings” (Fig. 8, panels G-H).

Discussion

In this study the larvae of the greater wax moth *G. mellonella* served as a non-vertebrate animal host model for *Shigella* infections. *G. mellonella* was chosen because it has several advantages over other non-vertebrate infection systems such as *Drosophila melanogaster* or *Caenorhabditis elegans*. The larvae are quite large in size, commercially available, can be

easily stored at room temperature and do not need to be propagated in the laboratory.⁴³ *G. mellonella* larvae can be incubated at 37°C, a temperature that is necessary for virulence of many pathogens such as *Shigella*.

The larval death was dose dependent, strain dependent, and required IpaB. VP-cured *Shigella* required 2–3 log higher doses to cause larval death that occurred over a longer period of time. Virulent *Shigella* replicates within the larvae and results in haemocyte cell death, melanization and ultimately larval death within 48–72 hours. The bacteria-laden vacuoles within 2457T-infected larval haemocytes appear to be encased within a double-membraned structure that resembles autophagosomes, reminiscent of what has been recently described in *Shigella*-infected zebra fish.²¹ Some of the vacuoles containing individual or replicating bacteria resemble endocytic vacuoles described previously in *Shigella*-infected mouse and human macrophages and in rabbit PMNs. At 8 hours after infection with 2457T the haemocytes appear necrotic concomitant with an increase in the number of bacteria. At 24 hours of infection haemocyte counts reduced drastically. Molecular and biochemical analysis is required to understand the mechanism of *Shigella*-mediated haemocyte cell death although a recent report describes apoptosis of *Galleria* haemocytes to occur upon *Shigella* infection.⁴⁸ Avirulent strains were not recovered from the haemocytes at later times of infection and were not seen as intact bacteria within the haemocyte vacuoles. Unlike 2457T-infected haemocytes, BS103 infected haemocytes larvae showed large vacuoles with disintegrating bacteria. It is not clear whether BS103 bacteria are taken up and immediately destroyed within the phagocytic vacuole similar to what has been described with *Galleria* larvae infected with *Legionella pneumophila*. In this system wild type strains replicated within the larvae while type IV secretion system mutants were rapidly cleared.^{49–51}

Unlike *C. elegans*, *G. mellonella* has a relatively complex innate immune response, with homology to mammalian innate immunity.⁵² Circulating granular haemocytes that function as mammalian macrophages as well as plasmatocytes that can adhere to and encapsulate foreign bodies are the main drivers of the larval humoral and cellular defense responses.^{24,25,52–58} Bacterial infections can be cleared by haemocyte-mediated aggregation and nodulation, or by phagocytosis of the bacterial strain, resulting in production and release of antimicrobial peptides, haemocyte cell

death and in some cases melanization of the larval body.^{27,28,37,59–66} Some of the physical features such as phagocytosis of the bacterial strain, haemocyte-aggregation, nodulation, and melanization were also observed in *Shigella*-infected larvae; however future work will determine whether specific AMPs are increased after infection.

G. mellonella larvae has been widely adopted as a simple and inexpensive model for assessing host-pathogen interactions that includes bacterial, plant and fungal pathogens.^{67,68} Different *Shigella* strains, such as *S. flexneri* 2a strain 2457T, *S. flexneri* 5 strain M90T, *S. sonnei* strain Moseley and *S. dysenteriae* 1 strain 1617 as well as mutants of these strains showed different kinetics of survival and larval mortality suggesting that this model is useful in discriminating between virulent as well as avirulent strains. Similar pattern of discrimination between strains of the same species in *Galleria* has been seen with other organisms such as *Yersinia*, *Campylobacter* and *H. pylori*.^{29,30,31,69,70} For example, OxyR and PhoP mutants of *Y. pestis* are less virulent in *Galleria*, and *H. pylori* VacA, CagA, CagE, cag PAI, or urease mutants, were less virulent than the respective parental strain.⁷¹

Like mammalian cells, *Galleria* can recognize microbe-associated molecular patterns by receptors such as Toll and peptidoglycan recognition proteins.^{72,73} *Galleria* larvae also exhibit apoptosis and autophagy as cellular processes that, like in mammalian cells, could potentially be activated during microbial infection.^{74,75} Thus, bacterial factors that enable survival in this insect host are likely to be directly relevant to human infection.^{75,76} More recently, a cDNA encoding a caspase 1 has been cloned and sequenced from the midgut of *G. mellonella* larvae and shown to be similar to human *ced-3* and proteins homologous to human NADPH oxidase system have also been shown to exist in *G. mellonella* larvae.^{77,78} Thus, it appears that mechanisms used by mammalian neutrophils and macrophages including an oxidative burst, production of lysozyme and numerous antimicrobial peptides that kill ingested microbes are also in play in *Galleria* haemocytes. Future studies will be directed toward which of these defense mechanisms are utilised during *Shigella* infections.

Obviously there are differences since *Galleria* does not have an adaptive immune system and lacks antibodies and *Galleria* innate immune system lacks natural killer cells, dendritic cells and cytokines that

play a crucial role in the human immune response.⁶⁷ Recently, a comprehensive transcriptome of *G. mellonella* genes that participate in its immune response has been identified and will help to further studies in this host.⁶⁶ This transcriptome will be useful since *Galleria* does not have a fully sequenced genome. The response of *Galleria* larvae to virulent strains of *Shigella* was sensitive to both temperature and growth phase of the bacteria before infection, 2 factors that also influence virulence in mammalian models.⁷⁹ However, it is known that environmental factors such as heat, nutrient deprivation and other kinds of stress also influence the cellular and humoral immune response of the larvae.^{54,80-83} suggesting that careful interpretation of data arising from effect of specific environmental factors is needed.⁸⁴

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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