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***Galleria mellonella* as a model host to study infection by the *Francisella tularensis* live vaccine strain**

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Abstract

We used the killing of *Galleria mellonella* (Lepidoptera: Pyralidae; the greater wax moth) caterpillar by the live vaccine strain (LVS) of *Francisella tularensis* to develop an invertebrate host system that can be used to study *F. tularensis* infection and the *in vivo* effects of antibacterial compounds on *F. tularensis* LVS. After injection into the insect hemocoel, *F. tularensis* LVS, killed caterpillars despite the association of LVS with hemocytes. The rate of killing depended on the number of bacteria injected. Antibiotic therapy with ciprofloxacin, levofloxacin or streptomycin administered before or after inoculation prolonged survival and decreased the tissue burden of *F. tularensis* in the hemocoel. Delayed drug treatment reduced the efficacy of antibacterials and especially streptomycin. The *G. mellonella* – *F. tularensis* LVS system may facilitate the *in vivo* study of *F. tularensis*, efficacy with antibacterial agents.

Keywords

Francisella tularensis; *Galleria mellonella*; tularemia; phagocytosis

1. Introduction

Francisella tularensis, the cause of tularemia, is one of the most infectious bacterial pathogens known. Two subspecies of major clinical importance have been identified, type A (subspecies *tularensis*) and type B (subspecies *holarctica*); type A is more virulent in humans [1]. Infection with *F. tularensis* subspecies *tularensis* (Type A) if not treated is associated with a high mortality rate (80% mortality for the pneumonic form and 10% for the ulceroglandular form) [2]. Due to the various routes of transmission and dissemination and the high virulence, *F. tularensis* is considered a category A bioterrorism agent by the Center for Disease Control and Prevention [3,4].

The availability of genome sequence data for *F. tularensis*, new genetic tools and animal models, as well as studies in cell lines have aided in the study of this organism [5]. Mice have been used as a model system to study *F. tularensis*, while studies have shown that this pathogenic bacteria is also able to survive within amoebae [6] and is able to survive and grow

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within macrophages [7,8]. An attenuated live vaccine strain (LVS) has been developed from *F. tularensis* subspecies *holarctica* [3]. The LVS confers some protection against tularemia infection in humans and in a number of animal models [9]; however, the basis of attenuation of LVS strain is not known. The vaccine is no longer available in the United States and its future is undetermined [3].

We propose to use the greater wax moth *Galleria mellonella* as a model host for *F. tularensis* LVS. A number of human pathogens have been studied in the *G. mellonella* pathosystem [10-16]. For example, the gram-negative bacterium *Pseudomonas aeruginosa* has been extensively studied in this system. Strain PA14, a human clinical isolate of *P. aeruginosa*, is pathogenic in mice and *G. mellonella*. Analysis of 32 different PA14 mutants in mice and *G. mellonella* showed a positive correlation in virulence patterns [11]. Pathogen defense is facilitated by the hemolymph system. *G. mellonella* is an attractive alternative host that offers the benefit of being able to maintained at a temperature range of 15 °C to 37 °C. It can be inoculated with a pathogen by means of injection [10] or by topical application [17].

We used the host-pathogen interaction between the *G. mellonella* larvae and the LVS of *F. tularensis* to develop an invertebrate host model to further study LVS. We are particularly interested in developing this model to study antibacterial activity to LVS as a model system for further exploration of antibacterial agents to *F. tularensis* virulent strains. We found that *F. tularensis* LVS killed *G. mellonella* larvae and killing of caterpillars depended on the number of bacteria in the inoculum and the incubation temperature following inoculation. The *G. mellonella* host response to *F. tularensis* involved melanization and association of bacteria with hemocytes. Antimicrobial therapy with streptomycin, levofloxacin or ciprofloxacin, but not ampicillin, administered before or after inoculation was effective in prolonging survival of caterpillars. However, in the case of streptomycin treatment, a delay in therapy by 24 hours neutralized the efficacy of this agent in *G. mellonella* survival.

2. Materials and Methods

2.1. Preparation of inoculum

The LVS of *F. tularensis* was used in all experiments. Bacterial cultures of LVS were maintained on Cysteine Heart Agar with 2% hemoglobin (CHA-H) and as frozen stocks. Frozen stocks of LVS were maintained on Brain Heart Infusion with 10% glycerol (BHI-G) at -80 °C. The concentration of LVS in the frozen stocks was 3×10^8 Colony Forming Units (cfu)/ml, established by reading the Optical Density (OD) on a spectrophotometer and confirmed by plating serial dilutions on CHA-H plates and counting the cfu 72-96 hours later. For the heat-killing assay, LVS were exposed to 65 °C for 90 minutes. Heat killed LVS cells were washed in Phosphate Buffered Saline (PBS), resuspended and diluted to the appropriate concentration. For experiments utilizing heat-killed bacteria, the same concentration of the heat killed *F. tularensis* LVS was plated on CHA-H plates, to confirm the absence of colonies.

2.2. *G. mellonella* injection

G. mellonella caterpillars in the final larval stage (Vanderhorst Wholesale, St. Marys, Ohio) were stored in the dark. Caterpillars 0.30-0.35 g in weight were employed in all assays. A 10- μ l Hamilton syringe was used to inject 10- μ l aliquots of the inoculum into the hemocoel of each caterpillar via the last left proleg. Before injection, the area was cleaned using an alcohol swab. After injection, caterpillars were incubated in plastic containers. Sixteen randomly chosen caterpillars of the required weight were used per group and the number of dead caterpillars was scored daily. Caterpillars were considered dead when they displayed no movement in response to touch. For all the killing assays, we plotted killing curves and analyzed

differences in survival with the Kaplan-Meier method using STATA 6 statistical software (Stata). A *P* value of less than 0.05 was considered significant.

The following control groups were used in all experiments. The first group included caterpillars that received doses of PBS. This group was used to assess the mortality related to trauma. A second group of larvae received no injection. In the studies evaluating antimicrobial efficacy, a third control group received the bacterial inoculum, but instead of antibacterial agents, received injection(s) with PBS. This control group was used as a control in the evaluation of the efficacy of the antimicrobial agents.

2.3. Administration of antimicrobials

A 10- μ l Hamilton syringe was used to inject aliquots of ciprofloxacin 20 mg/kg, levofloxacin 15 mg/kg, streptomycin 15 mg/kg, or ampicillin 150 mg/kg. For experiments that required multiple injections, a different proleg was used for each injection, starting from the left last proleg and rotating left to right and moving proximally (i.e., injecting through the left last proleg, right last proleg, etc., as needed).

2.4. Tissue-burden culture studies

For the evaluation of the tissue burden of *F. tularensis* in caterpillars over time, the hemolymph from five caterpillars per group was collected into 1.5 ml Eppendorf tubes and serial dilutions were plated on CHA-H agar plates with 7.5 mg of colistin, 2.5 mg of amphotericin, 0.5 mg of lincomycin, 4 mg of trimethoprim, and 10 mg of ampicillin per liter [18]. Four groups (with 5 larvae each) were used for each condition. Plates were incubated at 37 °C for at least 72 hours before colonies were counted. Plotting was performed using Microsoft Excel, while the software program STATA 6 (Stata) was used for the statistical analysis of the cfu of *G. mellonella* in the hemocoel (Mann-Whitney and Kruskal-Wallis tests). A *P* value of less than 0.05 was considered significant.

2.5. Microscopy

Caterpillars after injection were maintained at 37 °C and 48 hours after inoculation, we collected their hemolymph into 1.5 ml Eppendorf tubes and fixed using 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, Pennsylvania). Following fixation, the hemolymph was centrifuged and the supernatant was discarded. The cells were diluted in Grace's insect medium (Invitrogen, Carlsbad, California) and stained with mouse monoclonal antibody to *F. tularensis* lipopolysaccharide (LPS) as primary antibody (Abcam, Cambridge, Massachusetts), followed by staining with secondary monoclonal IgG2a Alexa Fluor 488 goat anti-mouse (Molecular Probes, Eugene, Oregon). Light and fluorescence microscopy were performed using an Olympus BX51 microscope and Vectashield mounting medium (Vector Laboratories, Youngstown, Ohio).

3. Results

3.1. Killing of *G. mellonella* by *F. tularensis* LVS

As shown in Fig. 1, inoculation of *G. mellonella* caterpillars with *F. tularensis* LVS at 37°C resulted in killing of the caterpillars, and killing depended on the number of bacteria injected. For example, after injection of 3×10^5 cfu/larva, the median time to death was 3 days, while after injection of 3×10^3 cfu/larva, the median time to death was >10 days. Injection of 3×10^5 resulted in significantly faster killing compared to the control group of caterpillars that received sham injections with PBS ($P < 0.0001$) and to the groups that received 3×10^3 and 3×10^4 cfus/larva ($P < 0.0001$ for both). Moreover, injection of 3×10^4 resulted in significantly faster killing compared to the group that received 3×10^3 cfu/larva ($P = 0.0017$). Of note, non-pathogenic

microbial species do not kill *G. mellonella*. For example, killing of caterpillars by inocula of *Escherichia coli* (strain *E. coli* OP50), *Saccharomyces cerevisiae* or non-pathogenic strains of *A. fumigatus* as high as 1×10^7 cfu/larva was not different previously than a control group that received PBS [10].

We also evaluated killing of *G. mellonella* at 30 °C. Similar to our findings at 37 °C, inoculation of *G. mellonella* with *F. tularensis* LVS resulted in killing of the caterpillars and killing depended on the number of bacterial cells injected. Again, in both cases, killing was significantly faster compared to a control group of caterpillars that received placebo injections with PBS ($P < 0.0001$; Fig. 2). For example, injection of 3×10^5 cfu/larva did not result in killing of all larvae at 30 °C, while it resulted in 100% mortality at 37 °C. Killing appeared to be more effective at 37 °C, a more effective growth temperature for *F. tularensis*.

3.2. Hemocytes are part of the *G. mellonella* response to *F. tularensis*

We found that the hemocytes are part of the *G. mellonella* response to *F. tularensis* and, by day 2 after inoculation, more than 90% of hemocytes are associated with *F. tularensis* bacteria (Fig. 3). Of note is that “nodulation” (encapsulation of large invading pathogens by layers of hemocytes) is part of the *G. mellonella* response to infection by large pathogens, such as fungi or parasites [10]. We did not find nodulation to be part of the insect response to *F. tularensis* and even when hemocytes adhered to each other, bacteria were bound to or phagocytosed by individual hemocytes and not placed between hemocytes. Melanization is also part of the insect reaction to infection [19] and over the progress of the *F. tularensis* infection, caterpillars demonstrated obvious signs of progressive melanization (Fig. 4).

3.3. Study of antimicrobial agents in the *G. mellonella* - *F. tularensis* system

In order to evaluate if the *G. mellonella* - *F. tularensis* system could be used to study the effect of antibacterial agents, we investigated the role of antibacterials by administering single doses of streptomycin (15 mg/kg), ciprofloxacin (20 mg/kg), levofloxacin (15 mg/kg), or ampicillin (150 mg/kg), two hours after the inoculation of caterpillars with 3×10^6 cfu/larva of *F. tularensis* LVS. Of note is that streptomycin is the most widely used treatment for management of severe human tularemia, while ciprofloxacin is an alternative treatment that is used in treatment or post-exposure prophylaxis [20].

Interestingly, streptomycin, ciprofloxacin and levofloxacin prolonged the survival of *G. mellonella* caterpillars at 37 °C compared to an infected control group that received ampicillin, an antibacterial that has no efficacy against LVS (Fig. 5; P values < 0.0001 for all groups compared to controls and to the group received ampicillin). The doses of antibiotics used in these experiments were similar to the widely accepted doses of the same antibiotics in humans. The difference between ciprofloxacin, levofloxacin and streptomycin was not statistically significant.

We also evaluated the efficacy of ciprofloxacin (20 mg/kg), levofloxacin (15mg/kg) or streptomycin (15 mg/kg) given one hour before inoculation with *F. tularensis* (3×10^6 cfu/larva) (“prophylaxis” group). Again ciprofloxacin, levofloxacin and streptomycin were effective in prolonging the life span of caterpillars compared to a control group that received ampicillin 150 mg/kg ($P < 0.0001$; Fig. 6A). In order to rule out any killing of larvae by ciprofloxacin, levofloxacin or streptomycin, we administered the same doses of these antibiotics alone to control groups of caterpillars. There were no deaths in these groups (data not shown).

For the evaluation of the effectiveness of ciprofloxacin, levofloxacin and streptomycin in the reduction of the bacterial burden in the hemolymph, we administered the above noted doses of antibiotics after infection with 3×10^4 cfu/larva; another group received PBS after infection.

Ciprofloxacin, levofloxacin and streptomycin resulted in statistically significant reductions in bacterial counts compared to ampicillin and controls ($P=0.02$). There were no statistically significant differences between the groups that received ciprofloxacin, levofloxacin or streptomycin (Fig. 6B).

We further evaluated the efficacy of streptomycin (15mg/kg), ciprofloxacin (20mg/kg) and levofloxacin (15mg/kg) when administered for two doses at a delayed time point from the time of inoculation. *G. mellonella* infected with LVS were treated at 6 hours post infection followed by an additional drug dose at 24 hours. Treatment with streptomycin, ciprofloxacin and levofloxacin all significantly increased survival when drugs were administered 6 hours and 24 hours after infection with LVS compared to control groups ($P=0.0075$ for streptomycin and $P<0.0001$ for ciprofloxacin and levofloxacin respectively).

In another delayed drug dose assay, we delivered a dose of the antibacterial agent at 24 hours post infection, followed by an additional dose at 48 hours post infection. Treatment with ciprofloxacin and levofloxacin remained significant compared to treatment with PBS at 24 and 48 hours with $P<0.0001$ and $P=0.0001$ respectively. However, streptomycin was not significantly better compared to LVS infected *G. mellonella* that received placebo treatment ($P=0.1071$).

4. Discussion

In this report, we describe the use of the *G. mellonella* larvae and the LVS of *F. tularensis* to develop an invertebrate host system for the study of tularemia and the efficacy of antibacterial agents. We found that the LVS of *F. tularensis* killed *G. mellonella* larvae. Killing of larvae depended on the number of bacteria in the inoculum and the incubation temperature following inoculation. The *G. mellonella* host response to *F. tularensis* infection involved melanization and phagocytosis. Antimicrobial therapy with ciprofloxacin, levofloxacin or streptomycin administered before or after inoculation prolonged survival of larvae.

G. mellonella (the greater wax moth) is a unique invertebrate host model because it can survive at mammalian temperatures and can be used for the study of microbial pathogenesis, including phagocytosis. The host response of *G. mellonella* to infection consists of structural and passive barriers, as well as cellular and humoral responses that are mediated by hemocytes within the hemolymph. Different types of hemocytes have been identified in *G. mellonella* and the insect response includes phagocytosis, “nodulation” (encapsulation of large invading pathogens by layers of hemocytes), and melanization [21].

In addition to *in vivo* defense responses, the use of drug treatment for LVS can be evaluated in the *G. mellonella* model system. Drug treatment with ciprofloxacin or levofloxacin significantly prolongs survival of LVS infected larvae under each condition. Streptomycin significantly prolonged survival of *G. mellonella* larvae when administered at 2 or 6 hours after infection. However, when streptomycin treatment was delayed to 24 hours post infection, there was no significant difference compared to *G. mellonella* infected with LVS that received PBS treatment. Delayed drug treatment has also been shown to decrease survival in a mouse model system infected with *F. tularensis* [22].

The aminoglycosides streptomycin and gentamicin are bactericidal against *F. tularensis* and are currently the drugs of choice for the treatment of tularemia infection in humans [20]. Fluoroquinolones have good bactericidal activity against *F. tularensis* *in vitro* [23]. In contrast, beta-lactams, macrolides, lincosamides, and co-trimoxazole are not reliable for treatment of tularemia. Our results with antibacterials demonstrated efficacy in the *G. mellonella* system that was similar to results from clinical trials [3,23].

Important limitations of the *G. mellonella* model should be considered. The genome of this organism has not been sequenced; this means that *G. mellonella* has limited genetic tractability compared to other invertebrate models such as *Caenorhabditis elegans* and *Drosophila melanogaster* [10]. Also, administration of inocula by injection makes high throughput screening difficult. Finally, the *G. mellonella*-*F. tularensis* LVS model requires the inoculation of larvae with a significant number of bacteria. This suggests that the hemocytes are able to clear a smaller number of *F. tularensis* LVS bacteria. It is anticipated that virulent bacterial strains such as *F. tularensis* Schu 4 will be able to kill *G. mellonella* larvae at significantly lower inocula than the LVS strain.

In conclusion, *G. mellonella* is a facile model that complements more cumbersome and expensive mammalian models and may minimize mammalian suffering for the study of *F. tularensis* pathogenesis. This host model provides a novel approach to the study of *F. tularensis* host-pathogen interactions at both mammalian and lower temperatures, study of the interaction of *F. tularensis* with insects, and evaluation of drug sensitivity profiles *in vivo*. Future studies are needed to investigate the use strains such as *F. tularensis* Schu 4, in order to establish the usefulness of this model in the study of virulent strains.

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Abbreviations

BHI-G, Brain Heart Infusion with 10% Glycerol; CFU, Colony Forming Units; CHA-H, Cysteine Heart Agar with 2% Hemoglobin; LVS, Live Vaccine Strain; PBS, phosphate-buffered saline.

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Fig 1. Killing of *G. mellonella* caterpillars by *F. tularensis* LVS depends on the number of bacteria inoculated

Kaplan-Meier plots of *G. mellonella* survival after injection of different inocula of *F. tularensis* LVS. Injection with 3×10^5 cfu/larva resulted in significantly higher death rate, compared to injection with 3×10^4 cfu/larva ($P < 0.0001$), or 3×10^3 cfu/larva ($P < 0.0001$). Injection with 3×10^4 cfu/larva resulted in significantly higher death rate compared to injection with 3×10^3 cfu/larva ($P = 0.0017$). There was no killing of caterpillars that received heat killed bacterial cells of the same strain (3×10^5 cells/larva). The caterpillars were maintained at 37°C .

Fig 2. Killing of *G. mellonella* caterpillars by *F. tularensis* LVS depends on the temperature after inoculation

Kaplan-Meier plots of *G. mellonella* survival after injection of different inocula of *F. tularensis* LVS. The caterpillars were maintained at 30°C. Injection with 3×10^6 cfu/larva resulted in significantly higher death rate compared to controls and to injection with 3×10^5 cfu/larvae. However, the killing rate at 30°C was slower than at 37°C (Figure 1), and at 30°C injection with 3×10^5 cfu/larva was associated with significant survival (50% on Day 15).

Fig 3. Association of *F. tularensis* LVS cells with *G. mellonella* hemocytes

Hemocytes from caterpillars infected with *F. tularensis* LVS. After injection, caterpillars were maintained at 37°C and 48 hours after inoculation we collected their hemolymph. Following fixation, cells were diluted in Grace's insect medium and stained with mouse monoclonal antibody to *F. tularensis* lipopolysaccharide (LPS), as described under Materials and Methods.

Left panel: Arrows point to FITC-labeled *F. tularensis* bacteria associated with a *G. mellonella* hemocyte. **Right panel:** Group of *G. mellonella* hemocytes with FITC-labeled associated *F. tularensis* bacteria.

Fig 4. Melanization is a part of the insect response to infection by *F. tularensis* LVS

Over the progress of the *F. tularensis* infection, caterpillars demonstrated obvious signs of progressive melanization. From right to left: *G. mellonella* at different time points after challenge with an inoculum of the LVS of *F. tularensis* 2×10^6 cfu/larva. Infected larvae show progressively increasing melanization. From right to left larvae are at 0, 12, 24 (three larvae) and 48 hours (the last three larvae) after inoculation. Petri dish diameter is 8.5 cm.

Fig 5. Antibacterial agents prolong the survival of *G. mellonella* caterpillars after challenge with *F. tularensis* LVS

We examined the role of the most commonly used agents for *F. tularensis* infection by administering single doses of ciprofloxacin (CIP) 20 mg/kg, levofloxacin (LEV) 15 mg/kg, streptomycin (STR) 15 mg/kg or ampicillin (AMP) 150 mg/kg after the inoculation of caterpillars with 3×10^6 cfu of *F. tularensis* LVS per larva. A control group received the *F. tularensis* inoculum and PBS instead of antibacterials. Treatment with ciprofloxacin, levofloxacin or streptomycin prolonged the survival of *G. mellonella* caterpillars ($P < 0.0001$ compared to control). However, ampicillin was not effective against *F. tularensis* LVS. The caterpillars were maintained at 37°C.

Figure 6. Antibacterial agents before challenge with *F. tularensis* LVS prolong the survival of *G. mellonella* caterpillars

A. We examined the role of the most commonly used agents for *F. tularensis* infection by administering a single dose of ciprofloxacin (CIP) 20 mg/kg, levofloxacin (LEV) 15 mg/kg, or streptomycin (STR) 15 mg/kg, one hour before infection with *F. tularensis* LVS 3×10^6 cfu/larva. A control group received *F. tularensis* LVS and PBS instead of these drugs. Treatment with CIP, LEV or STR prolonged the survival of *G. mellonella* caterpillars ($P < 0.001$ compared to control). Ampicillin (AMP) 150mg/kg given 1 hour before the infection with *F. tularensis* LVS 3×10^6 cfu/larva did not protect *G. mellonella* caterpillars. **B.** We also examined the bacterial burden in the hemolymph after 48 hours using a single dose of ampicillin,

ciprofloxacin, levofloxacin or streptomycin. A control group received treatment with PBS. CFU were counted per five larvae. The caterpillars were maintained at 37°C.