Journal of Visualized Experiments

Use of the Invertebrate Galleria Mellonella as an Infection Model to Study the Mycobacterium Tuberculosis Complex

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59703R1
Full Title:	Use of the Invertebrate Galleria Mellonella as an Infection Model to Study the Mycobacterium Tuberculosis Complex
Keywords:	Galleria mellonella, mycobacteria, tuberculosis, Mycobacterium bovis BCG lux, infection model, host-pathogen interactions, Mycobacterium tuberculosis complex, hemocytes
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Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	London, UK

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14th January 2019

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FAO, Dr Jaydev Upponi (Science Editor)
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Dear Dr Upponi,

Further to our recent telephone and email correspondence, please find submission of the manuscript as requested titled "**Methods for the use of the invertebrate, *Galleria mellonella,* as an infection model to study the *Mycobacterium tuberculosis* complex".** We would be grateful if you would consider our manuscript for publication in the Journal of Visual Experiments.

Tuberculosis remains one of the most important human diseases worldwide. Treatment of the disease has become increasing challenging due to drug-resistant strains of *Mycobacterium tuberculosis*. There is therefore an urgent need for a greater understanding of host-*Mycobacterium tuberculosis* interactions, the development of new, anti-mycobacterial agents to target drug-resistant tuberculosis (TB) and shorten treatment regimens, and also a need for novel drug screening models that reduce and/or replace the currently used conventional animal models, all of which have limitations.

The larva of the insect, *Galleria mellonella*, has been increasingly used as a surrogate organism to study host-pathogen interactions in a range of bacterial pathogens, and as a rapid model to

screen novel antimicrobial drug candidates. In a recent study, (Li *et al*., Virulence 9:1126-37, 2018) we evaluated and established *G. mellonella* as a suitable novel infection model for the *M. tuberculosis* complex demonstrating a dose-response for *G. mellonella* survival infected with different inocula of bioluminescent, *Mycobacterium bovis* BCG *lux*, and demonstrated suppression of mycobacterial luminescence over 14 days. Through histopathological staining and transmission electron microscopy we further demonstrated that the *G. mellonella* - mycobacteria infection model can be used to study mycobacterial pathogenesis, particularly in the context of granuloma formation.

Here we describe the methods in detail for the use of *G.mellonella* larvae combined with bioluminescent mycobacteria as an infection model. *G. mellonella* has the potential to be used as a low-cost, reproducible and high-throughput, model to understand host-pathogen interactions in mycobacterial infection, and be used as a pre-screening model to assess the toxicity and activity of antimycobacterial drugs. Moreover *G. mellonella* has the capacity to significantly reduce and replace the use of animal models in TB research.

We believe these methods will be of interest to the specific community of tuberculosis, microbiologists, and infectious diseases researchers, as well as to researchers involved in drug development and discovery.

The final manuscript has been approved by all authors. The authors declare there are no conflicts of interest.

On behalf of my co-authors, I would like to thank you again for your time and kind consideration in reviewing our submission.

Yours sincerely,

S. M. Newton

TITLE:

Use of the Invertebrate *Galleria Mellonella* as an Infection Model to Study the *Mycobacterium Tuberculosis* Complex

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KEYWORDS:

Galleria mellonella, mycobacteria, tuberculosis, *Mycobacterium bovis* BCG *lux*, infection model, host-pathogen interactions, *Mycobacterium tuberculosis* complex, hemocytes

SUMMARY:

Galleria mellonella was recently established as a reproducible, cheap, and ethically acceptable infection model for the *Mycobacterium tuberculosis* complex. Here we describe and demonstrate the steps taken to establish successful infection of *G. mellonella* with bioluminescent *Mycobacterium bovis* BCG *lux.*

ABSTRACT:

Tuberculosis is the leading global cause of infectious disease mortality and roughly a quarter of the world's population is believed to be infected with *Mycobacterium tuberculosis*. Despite decades of research, many of the mechanisms behind the success of *M. tuberculosis* as a pathogenic organism remain to be investigated, and the development of safer, more effective antimycobacterial drugs are urgently needed to tackle the rise and spread of drug resistant tuberculosis. However, the progression of tuberculosis research is bottlenecked by traditional

mammalian infection models that are expensive, time consuming, and ethically challenging. Previously we established the larvae of the insect *Galleria mellonella* (greater wax moth) as a novel, reproducible, low cost, high-throughput and ethically acceptable infection model for members of the *M. tuberculosis* complex. Here we describe the maintenance, preparation, and infection of *G. mellonella* with bioluminescent *Mycobacterium bovis* BCG *lux.* Using this infection model, mycobacterial dose dependent virulence can be observed, and a rapid readout of in vivo mycobacterial burden using bioluminescence measurements is easily achievable and reproducible. Although limitations exist, such as the lack of a fully annotated genome for transcriptomic analysis, ontological analysis against genetically similar insects can be carried out. As a low cost, rapid, and ethically acceptable model for tuberculosis, *G. mellonella* can be used as a pre-screen to determine drug efficacy and toxicity, and to determine comparative mycobacterial virulence prior to the use of conventional mammalian models. The use of the *G. mellonella*-mycobacteria model will lead to a reduction in the substantial number of animals currently used in tuberculosis research.

INTRODUCTION:

Tuberculosis (TB) is a major threat to global public health with 9 million new cases per year and 1.5 million deaths[1]. In addition, it is estimated that one quarter of the world's population is infected with the causative agent of the disease, *Mycobacterium tuberculosis* (*Mtb*). Amongst the infected population, 5−10% will develop active TB disease over their lifetime. Furthermore, the emergence and spread of multi-drug resistant (MDR) and extensively-drug (XDR) resistant *Mtb* poses a serious threat to disease control, with 123 countries reporting at least one XDR case[1]. Treatment of TB requires a cocktail of at least four anti-mycobacterial drugs, of which isoniazid and rifampicin are prescribed for a minimum duration of six months; treatment is often associated with complex side effects and toxicities. Protection from the only licensed vaccine against TB, *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), is variable[2]. An incomplete understanding of the pathogenesis of TB significantly hampers the development of new therapeutic and vaccination strategies.

For decades animal infection models have been vital for TB research to understand the basic pathogenesis and host response to infection, and to evaluate novel anti-mycobacterial agents, immuno-therapeutics and new vaccine candidates[3,4]. However, research using animal infection models of TB is notoriously difficult as the pathogenesis and progression of TB infection are complex, and there is no single animal model that mimics the full spectrum and important features of the disease[5,6]. Furthermore, animal experiments are expensive, time consuming to undertake and require full ethical justification. Nevertheless, animal infection models of TB have been described in non-human primates (e.g., macaques), guinea pigs, rabbits, cattle, pigs, mice and zebrafish, with each having their limitations[3,4]. The murine model is the most commonly used model due to cost, availability of inbred lines, reproducibility of infection and abundance of immunological reagents. However, they do not typically form granulomas associated with areas of hypoxia that are characteristic of latent tuberculosis infection (LTBI)[6]. Guinea pigs are highly susceptible to *Mtb* infection, with pathology and early granuloma formation similar to those in humans, and are widely used in vaccine testing; yet the lack of immunological reagents hampers their use as an infection model[7]. Zebrafish are suitable for large-scale screening in early-stage

preclinical studies due to their small size, rapid reproduction and advanced genetic tools, but are anatomically and physiologically different to humans and are only susceptible to *Mycobacterium marinum* infection[3]. The animal models most closely resembling human *Mtb* infection are non-human primates (e.g., the macaque), but they are expensive and have significant ethical and practical considerations which considerably limits their use[8].

The insect larva of the greater wax moth or honeycomb moth*, Galleria mellonella,* have become increasingly popular as an infection model for a variety of bacterial and fungal pathogens[9], and as a screen for novel antimicrobial drug candidates[10]. *G. mellonella* is a successful invertebrate model due to its sophisticated innate immune system (comprised of cellular and humoral defenses) that shares a high degree of structural and functional similarity to that of vertebrates[11]. This includes immune mechanisms such as the phagocytosis of pathogens by hemocytes (functionally similar to mammalian macrophage and neutrophils)[12,13], the production and circulation of anti-microbial peptides (AMPs) and complement-like proteins within the hemolymph (analogous to mammalian blood) of *G. mellonella*[11]. Other advantages[9,14,15] of *G. mellonella* larvae as a model include 1) their large size (20−30 mm) which allows for easy manipulation and infection, as well as the collection of tissue and hemolymph for analyses, 2) easy maintenance at 37 °C, compatible for studying human pathogens, 3) precise infection by injection without the need for anesthesia, 4) efficacy of antimicrobial agents can be assessed utilizing less drug for evaluation, 5) lack of ethical constraints compared to the use of mammals, 6) large group sizes can be used compared to animal models allowing greater reproducibility, and 7) shorter times for infection experiments are required.

In a recent study, we demonstrated that *G. mellonella* can be used as a novel infection model for studying the pathogenesis of infection by bioluminescent *M. bovis* BCG *lux*, a genetically modified version of the vaccine strain and member of the *Mtb* complex (MTBC)[16]. While *G. mellonella* has previously been used as an infection model for non-tuberculous mycobacteria (NTM), mainly *M. marinum* and *Mycobacterium abscessus*[17,18], studies using MTBC are limited to that of Li et al.[16]. Bioluminescent non-pathogenic mycobacterial strains, which can be used at containment level (CL) 2 as a surrogate for *Mtb,* offer the advantages of safety and practicality over pathogenic mycobacteria. Following infection with BCG *lux*, larvae begin to develop early granuloma-like structures, which could provide valuable insight into the role of innate immunity in the establishment of TB infection[16]. In addition, this simple invertebrate infection model has the potential to provide a rapid, low-cost, and reliable evaluation of TB pathogenesis incorporating controlled challenge and multiple replicates for reproducibility. Furthermore, the model has the potential to be used to screen novel anti-TB drug and vaccine candidates in early development, reducing the overall number of animals in experimentation. The ability to measure changes in host and pathogen structure, transcriptome and proteome to determine drug targets and assess mechanisms of action of novel drugs and therapeutic vaccines, are also advantageous.

Here we describe the experimental protocols for the preparation of a bioluminescent *M. bovis* BCG *lux* inoculum and *G. mellonella* larvae for mycobacterial infection, as well as the determination of both larval and mycobacterial survival in response to infection.

PROTOCOL:

NOTE: All work described below are to be carried out in a CL2 laboratory within a class 2 microbiological safety cabinet (MSC) following local health and safety guidelines.

1. Preparation *of M. bovis* BCG *lux* for infection

1.1. Defrost a frozen 1.2 mL glycerol (15%) stock of *M. bovis* BCG *lux*, the Montreal vaccine strain transformed with the shuttle plasmid pSMT1 carrying the *luxAB* genes from *Vibrio harveyi* encoding the luciferase enzyme[19].

1.2. Inoculate 15 mL of Middlebrook 7H9 broth containing 0.2% glycerol, 10% albumin, dextrose, catalase (ADC) enrichment and 50 µg/mL hygromycin with a defrosted 1.2 mL aliquot of BCG *lux*, in a labelled 250 mL Erlenmeyer flask.

1.3. Place the flask in a sealed biosafety container and incubate at 37 °C in an orbital shaker incubator at 220 rpm for 72 h (or until the culture reaches the mid-log phase of growth).

1.4. Check the growth of BCG *lux* culture by preparing 1:10 dilutions of the culture in luminometer tubes using phosphate buffered saline (PBS, pH 7.4, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) in duplicate. Vortex, and load the luminometer tubes into the luminometer and measure the bioluminescence (relative light unit [RLU]/mL) using n-decyl aldehyde as the substrate (1% v/v in absolute ethanol)[20].

NOTE: The ratio of RLU/colony forming units (CFU) was previously determined to be 3:1 when BCG *lux* was grown in vitro in Middlebrook 7H9 broth[20].

1.5. Centrifuge the culture at 2175 x *g* for 10 min at room temperature to pellet the cells and discard the supernatant into an appropriate disinfectant with known mycobactericidal activity. Dispose all culture waste with disinfectants appropriate for mycobacteria following local guidelines.

1.6. Wash the cell pellet twice in PBS containing 0.05% polysorbate 80 (PBS-T) to prevent bacterial clumping.

1.7. Following the final wash, decant waste supernatant, resuspend the mycobacterial cell pellet in PBS-T and dilute the mycobacterial suspension to the desired cell density using RLU measurements.

1.8. Prepare ten-fold serial dilutions of the inoculum in 24-well plates using PBS-T. Plate out 10 µL onto Middlebrook 7H11 agar plates (0.5% glycerol, 50 µg/mL hygromycin, 10% oleic acid, albumin, dextrose, catalase [OADC]) in duplicate to enumerate inoculum CFU counts.

2. Preparation of *G. mellonella* larvae

2.1. Purchase last instar larvae from appropriate sources and maintain the larvae in the dark at 18 °C upon arrival and use within 1 week of purchase. Alternatively, larvae can be self-reared following protocol by Jojão et al.[21]. For purchased larvae, discard any dead, discolored or pupating larvae prior to storage.

NOTE: Pupating larvae are morphologically distinguishable to the last instar larvae.

2.2. Identify and select healthy larvae for experimentation, based on uniform cream color with little to no discoloration (melanization), size (2−3 cm in length), weight (approximately 250 mg), displaying a high level of motility, and possessing the ability to right themselves when turned over.

2.3. Carefully count the healthy larvae (minimum of 20−30 larvae per group) into a Petri dish (94/15 mm) lined with a layer of filter paper (94/15 mm) using blunt-end tweezers to minimize contamination and store at room temperature in the dark until use.

3. Infecting *G. mellonella* with BCG *lux*

3.1. Minimize the clutter within the MSC to reduce the risk of contamination and needle stick injury.

3.2. Prepare the injection platform by taping a circular filter paper (94 mm) to a flat raised surface. Soft or hard surfaces can be used and is entirely dependent on user preference, e.g., pipette box lids or a nylon scouring sponge.

3.3. Sterilize a 25 µL microsyringe (25 G) by aspirating 3 volumes of 70% ethanol and further rinse with 3 volumes of sterile PBS-T.

3.4. Aspirate 10 µL of BCG *lux* inoculum (prepared in section 1) or PBS-T into the sterilized 25 µL microsyringe. Use a separate syringe for PBS-T negative control.

NOTE: Resuspend the BCG *lux* inoculum following 10 injections to ensure uniform cell suspension.

3.5. Use tweezers to pick up one larva and place onto the injection platform.

3.6. On the platform, flip the larva on to its back and immobilize by securing the head and tail with the tweezers. Locate the last left proleg counting down from the head of the larva and carefully insert the tip of the needle (5−6 mm) at a 10−20° angle to the horizontal plane.

NOTE: Short and narrow tweezers allow for easy immobilization with minimum larval stress. Pay attention not to over penetrate which may puncture the gut and cause non-BCG *lux* specific melanization or death.

3.7. Count the infected larvae into a Petri dish lined with a layer of filter paper, a single 90 mm Petri dish can accommodate up to 30 larvae.

3.8. Store the Petri dish containing the larvae in a vented or non-sealed dark box inside an incubator at 37 °C with 5% CO_2.

4. Monitoring the survival of *G. mellonella* following infection

4.1. Over the time course, monitor the survival of the larvae every 24 h. Larvae are considered dead when they fail to move in response to touch.

5. Measuring the in vivo burden of BCG *lux* in *G. mellonella*

5.1. At each time point, randomly select five infected larvae previously prepared in section 3 and gently sterilize the larval surfaces using a cotton bud swabs soaked in 70% ethanol.

NOTE: This step is important when plating larval homogenate for mycobacterial CFU enumeration as non-sterilized larvae can lead to contamination.

5.2. Place the larvae individually into 2 mL lysing matrix tubes containing 800 µL of sterile PBS.

5.3. Homogenize the larvae using a homogenizer for 60 s at 6.0 m/s.

5.4. Centrifuge the lysing tubes at 3500 x *g* for 5 s to remove homogenate from the lids, and carefully decant the homogenate into sterile luminometer tubes individually.

NOTE: For CFU enumeration (step 5.7), ensure to reserve 100 µL of homogenate in a sterile 1.5 mL reaction tube.

5.5. Recover any remaining homogenate by washing the lysing matrix tubes with 1 mL of PBS-T and pipette into the corresponding luminometer tubes.

5.6. Vortex the luminometer tubes and measure the bioluminescence of the homogenates previously described in step 1.4.

5.7. Prepare ten-fold serial dilutions of the homogenate in 24-well culture plates using PBS-T. Plate out 10 µL of the dilution onto Middlebrook 7H11 agar plates containing 0.5% glycerol, 50 µg/mL hygromycin, 10% OADC and 20 µg/mL piperacillin, to determine the RLU/CFU ratio of BCG *lux* following in vivo infection.

NOTE: Piperacillin eliminates native *G. mellonella* microbiota with minimal growth inhibition of BCG *lux*[16].

6. Statistical analysis

6.1. Plot the Kaplan-Meier survival curve using data collected and carry out the Mantel-Cox (Log-rank) test to determine the significance of the result, where *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

REPRESENTATIVE RESULTS:

Here we present representative data that can be obtained using the *G. mellonella* – BCG *lux* infection model and highlight the benefits of *G. mellonella* as an infection model for members of the MTBC (**Figure 1**). Experimental procedures with key technical points are outlined in **Figure 2**.

[Place Figure 1 and Figure 2 here]

BCG *lux* dose dependent virulence was observed in *G. mellonella* larvae over a 96 h incubation period (**Figure 3**), and the lethal dose required for 50% larval mortality (LD_{50}) was determined to be 1 x 10^7 CFU. The survival distribution reflecting the virulence of the BCG *lux* doses tested was significantly different ($p < 0.0001$). Control groups injected with a 10 µL dose of PBS-T or those simply pricked simulating needle injuries, did not affect larval health or lead to an increase in mortality as determined by observational checks on motility and melanization at different time points.

[Place Figure 3 here]

All larvae infected with BCG *lux*, displayed physiological changes over time and for larvae infected with a 2 x 10^7 CFU dose of BCG *lux*, melanization of the larval dorsal line was observed from 48 h post infection (pi), and systematic melanization was observed from 96 h pi (**Figure 4**). Furthermore, the motility of the larvae reduced with the severity of melanization and the ability of larvae to pupate was lost upon infection in comparison with uninfected controls.

[Place Figure 4 here]

Survival of BCG *lux* within the *G. mellonella* larvae was determined over 2 weeks through bioluminescence measurement of larval homogenates. Infection with a 1 x 10^7 CFU dose of BCG *lux* resulted in an initial decline of BCG *lux* bioluminescence from 0−72 h pi. However, from 72−144 h pi, the bioluminescence of BCG *lux* plateaued, indicating the establishment of persistent infection (**Figure 5**).

[Place Figure 5 here]

As the rapid and reproducible quantification of in vivo mycobacterial growth in these studies was determined by the measurement of bioluminescence, the ratio of RLU and CFU should also be determined in vivo. In our particular infection system, the in vivo ratio of RLU and CFU ranged from 2:1–5:1, with an average of 4:1 over the 168-h time course (**Figure 6**).

[Place Figure 6 here]

FIGURE LEGENDS:

Figure 1: The benefits of *G. mellonella* as an infection model. This figure has been adapted from Kavanaugh and Sheehan[22].

Figure 2: Outline of experimental procedures. (**A**) Maintenance and preparation of *G. mellonella* for infection with BCG *lux*. (**B**) Preparation of BCG *lux* culture and inoculum for infection. (**C**) Infection of *G. mellonella* with BCG *lux*. (**D**) Measurement of virulence and in vivo burden of BCG *lux* in *G. mellonella* larvae.

Figure 3: Kaplan-Meir survival curve of *G. mellonella* in response to varying inocula of *M. bovis* BCG *lux*. Healthy larvae (n ≥ 10 per group), were infected with varying doses of BCG *lux*. Larvae were incubated at 37 °C and monitored for survival every 24 h for up to 96 h. The uninfected group was injected with PBS, and a pricked group (insertion of needle only) demonstrated the effect of needle injury on larval health. The means of two independent experiments are shown, with 95% confidence interval, represented as dotted lines in corresponding color to the inoculum. This figure has been adapted from Li et al.[16].

Figure 4: Melanization of *G. mellonella* in response to infection with *M. bovis* BCG *lux*. Healthy larvae at 0 h were infected with a 2 x 10^7 CFU dose of BCG *lux*. At 48 h and 96 h post infection, melanization along the larval dorsal line and systematic melanization, respectively, was observed.

Figure 5: In vivo burden of *M. bovis* BCG *lux* in *G. mellonella*, quantified using bioluminescence (relative light unit, RLU/mL) over a two week time course. Healthy larvae (n = 30) were infected with 1 x 10^7 CFU dose of BCG *lux*. In vivo burden was quantified by homogenizing five larvae at each time point (0, 24, 48, 72, 96, 168, 336 h), and measuring the bioluminescence of the homogenate. The means of three independent experiments are shown, and the error bars represent the standard deviation of the mean. This figure has been reprinted from Li et al.[16].

Figure 6: Determining the in vivo RLU/CFU ratio of *M. bovis* BCG *lux* in *G. mellonella*. Healthy larvae (n = 30) were infected with 1 x 10^7 CFU dose of BCG *lux*. At each time point (0, 24, 96 and 168 h), four infected/control (PBS-T) larvae were homogenized, and the homogenates were measured for bioluminescence and plated out onto 7H11 agar to enumerate CFU counts. The means of two independent experiments are shown and the error bars represent the standard deviation of the mean.

DISCUSSION:

The use of *G. mellonella* as an infection model has been established for a number of bacterial and fungal pathogens for the study of virulence, host-pathogen interaction, and as a screen for novel therapeutics[10,22]. The following discussion is based on the experimental procedure in the use of *G. mellonella* as an infection model for the MTBC.

The health of the naïve larvae prior to experimentation can have a considerable impact on the

outcome of the experiment. Therefore, it is vital that any discolored and/or injured larvae are removed upon arrival and are not used for any experimentation. If a large number of larvae are found dead within the same container upon arrival, it is advisable to discard the batch as pre-existing infections may be the cause of death. When possible perform experiments using the larvae as close to the day of purchase/arrival. Before use ensure to store the larvae at 18 °C to prevent pupation and to maximize the number of larvae available for experimentation. Healthy larvae can be used for up to 7 days following delivery/purchase. Following infection, ensure to remove any dead larvae from the Petri dish as for reasons yet unknown, the presence of dead larvae appears to increase the rate of mortality in the sample population. For users of self-reared larvae, it is important to be aware of biological variability in comparison to purchased larvae, as variance in diet and growth conditions can have an impact on larval immunity[21,23]. Inter-experimental variabilities can be limited by keeping the source, if purchased, or the feed and growth conditions of the reared larvae consistent between experimentation. In all experiments, the inclusion of 'blank' and 'pricked' negative controls are essential; the blank control is an indicator of contamination or toxicity of the suspension matrix (PBS-T or media broth), and the pricked control mimics the effect of the needle injury on larval health. Furthermore, these controls normalize any biological variation between batches of larvae, ensuring reproducibility and accuracy between experiments.

For injection of *G. mellonella* larvae, the use of a 25 G needle is recommended as larger gauge needles can cause excessive bleeding and sharper smaller gauge needles can easily puncture the gut of the larvae, leading to larvae mortality and false positive results. Conventional methods of needle injection typically immobilize the larvae by hand, which increases the risk of needle injury. By using tweezers to immobilize the larvae, the risk of needle stick injury is significantly reduced as the hand is not in close proximity to the needle at any point during infection. Alternatively, larvae could be immobilized by cooling. However, cold shock at 12 °C for 15 min prior to infection has been documented to enhance the innate immune response to infection. Therefore, the analysis of the results obtained via cooling should carefully consider its impact[24]. Using our technique, medium throughput of injection (2−3 larvae per minute) can be achieved with user practice; this is comparable to the speed of conventional injection from our experience (3−4 larvae per min). Furthermore, our method can be adapted for higher throughput injection by utilizing a pedal operated injection platform comprised of an infusion pump, with a disposable syringe, connected to a 25 G butterfly cannula.

The preparation of BCG *lux* inoculum is based on the rapid estimation of CFU using RLU of the mycobacterial culture[20]. In our experience with broth culture, the ratio of RLU to CFU is 3:1[20], contrasting with BCG *lux* growing in *G. mellonella* where the RLU to CFU ratio is 5:1[20]. Mycobacterial cell aggregation or 'clumping' commonly seen in dense cultures can have an impact on the RLU measurements, as clumping can result in unreliable bioluminescence measurements. As such, the use of clumped mycobacterial culture for inoculum preparation is not recommended. However, the addition of polysorbate 80 to the growth media minimizes cell clumping without altering the growth of BCG *lux*.[16] Any minor cell clumping can be resolved by washing the culture with PBS-T and is vital for preparing accurate inoculum using RLU as the readout. Furthermore, the PBS-T wash is vital for removing any extracellular virulence factor

secreted during growth. Mycobacterial strain and passage number should also be taken into consideration, as this can impact the severity of mycobacterial aggregation[25]. In all cases, the inoculum should be enumerated by CFU on 7H11 agar plates to ensure that the correct CFU was prepared by bioluminescence measurement. Additionally, the RLU/CFU ratio in vivo should be determined with new bioluminescent reporter or stocks, as the ratio will likely vary.

G. mellonella holds several advantages over conventional models of TB infection, including cheaper acquisition and maintenance costs, ease of infection and research throughput, especially in combination with bioluminescent strains[16]. The lack of ethical constraints allows for greater sample size (minimum of 20−30 larvae per group) in comparison to mammalian model, giving greater confidence and reliability in the results obtained[26,27]. However, there are a number of limitations in the use of *G. mellonella* as an infection model. As an invertebrate, they naturally lack adaptive immunity making them unsuitable for antigenicity or immunological studies[10]. Cellular innate immune responses of *G. mellonella* are comprised of a number of hemocyte types, and plasmatocytes and granulocytes have been reported to function similarly to mammalian phagocytes (neutrophils and macrophages)[12]. However, the role and mechanisms of these cell types remain under characterized, and direct comparative studies between mammalian and insect phagocytes are yet to be carried out. Furthermore, the lack of an annotated *G. mellonella* genome hinders the analysis of the host response to infection, and this is currently reliant on gene ontology analysis against transcriptomic libraries of other invertebrates, such as *Drosophila melanogaster* and *Bombyx mori*[28].

As a TB infection model, *G. mellonella* holds a promising future with its ability to develop granuloma-like structures in response to BCG *lux* infection[16], which are vital pathophysiological hallmarks of TB infection and are a key feature in the development of LTBI. Future work will aim to characterize this model with a particular interest in the formation of granuloma-like structures using reference, clinical and mutant *Mtb* isolates under CL3 conditions. Additionally, we anticipate that it may also be useful for novel anti-mycobacterial agent screening, as a similar *G. mellonella* model was used for NTMs[18], but this remains to be determined. The adoption of this model has the ability to significantly reduce the number of animals used within the TB research community, while simultaneously accelerating in vivo TB research output under ethically more acceptable conditions.

ACKNOWLEDGMENTS:

This project was supported by grants from the Biotechnology and Biological Science Research Center (BBSRC), awarded to PRL and YL (BB/P001262/1), and the Center for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), awarded to PRL, SMN, BDR, and YL (NC/R001596/1).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

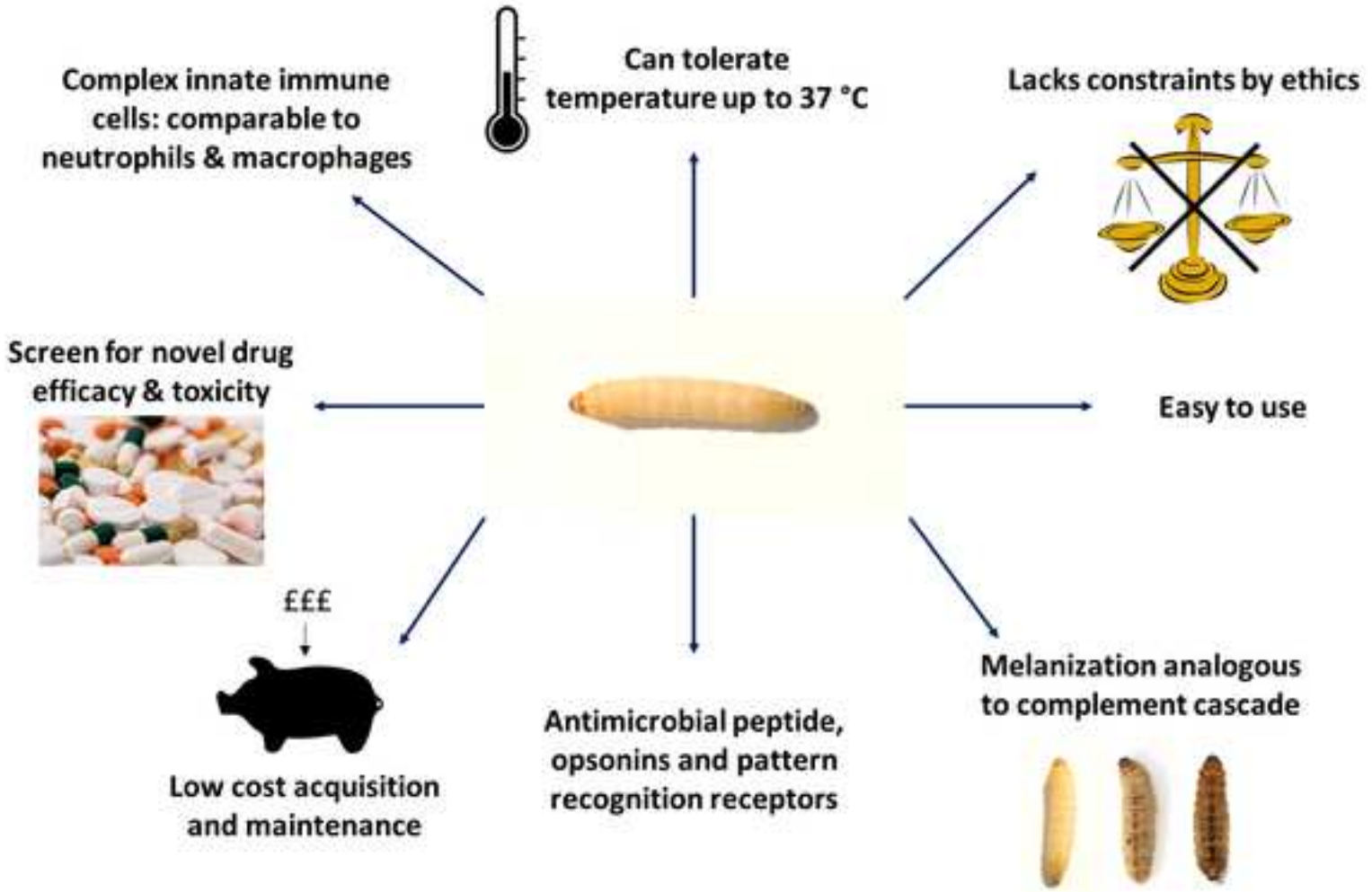

Figure 2

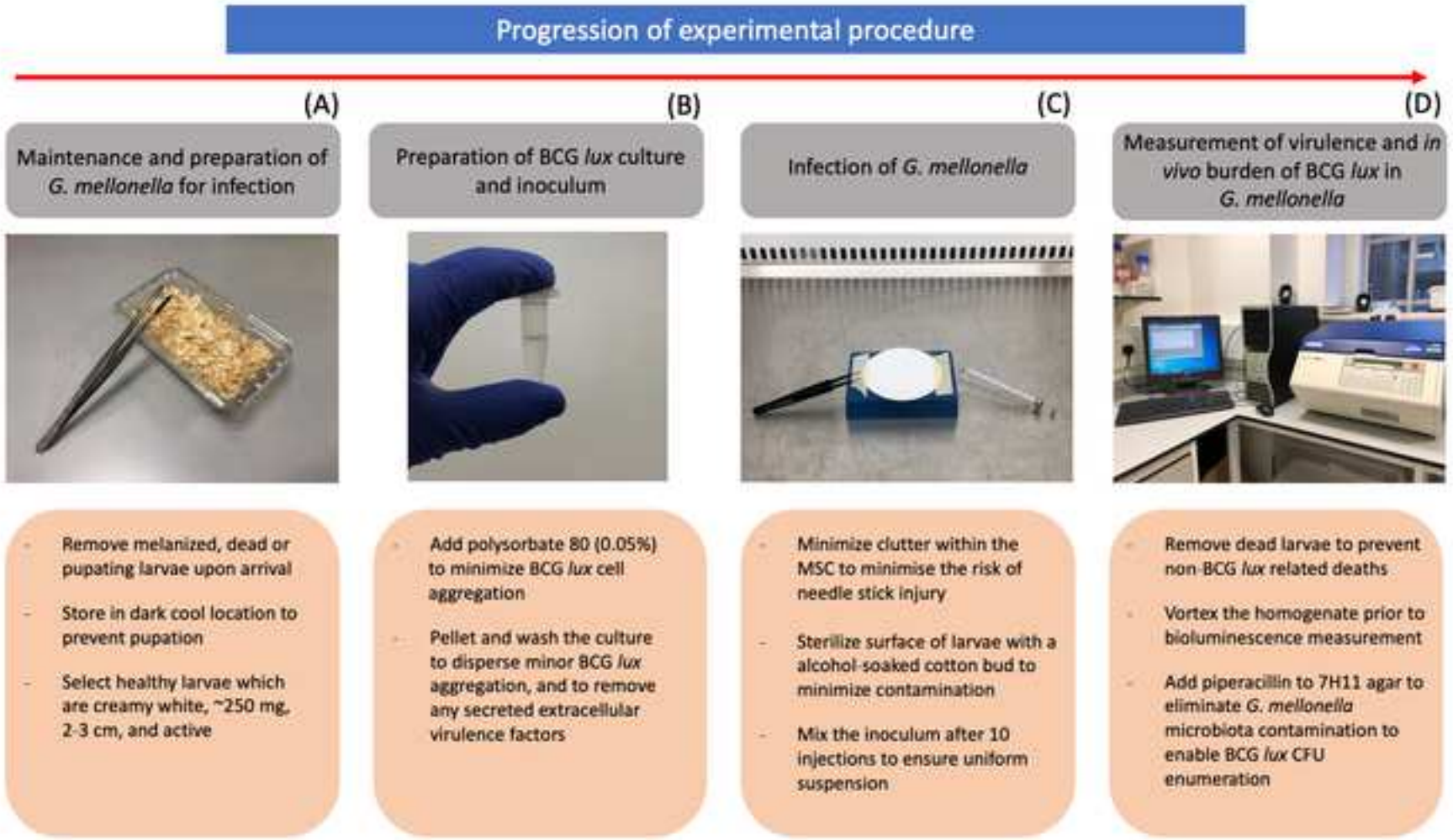

Figure 3

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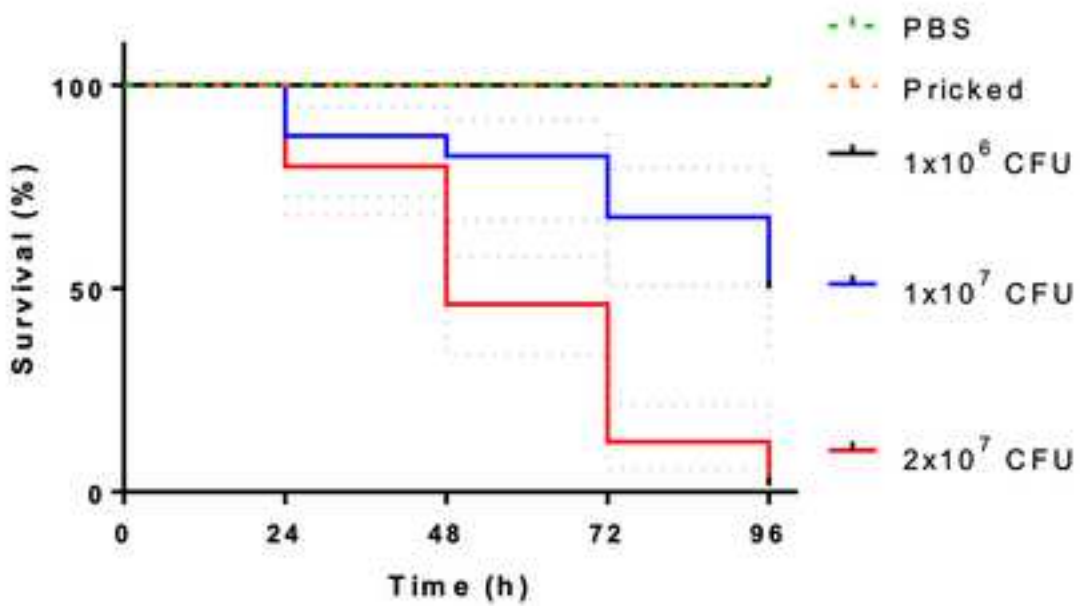

Figure 4

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Figure 5

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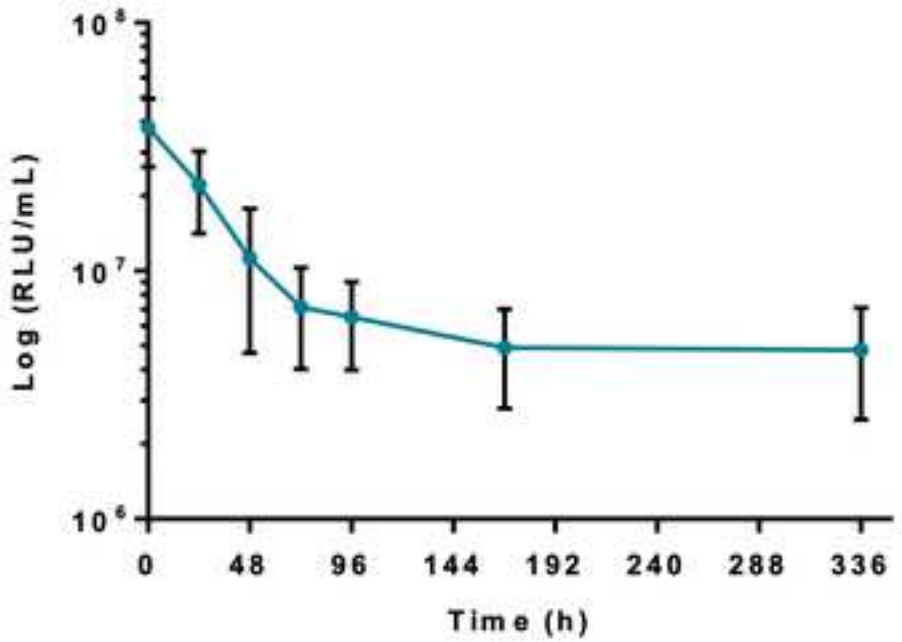

Figure 6

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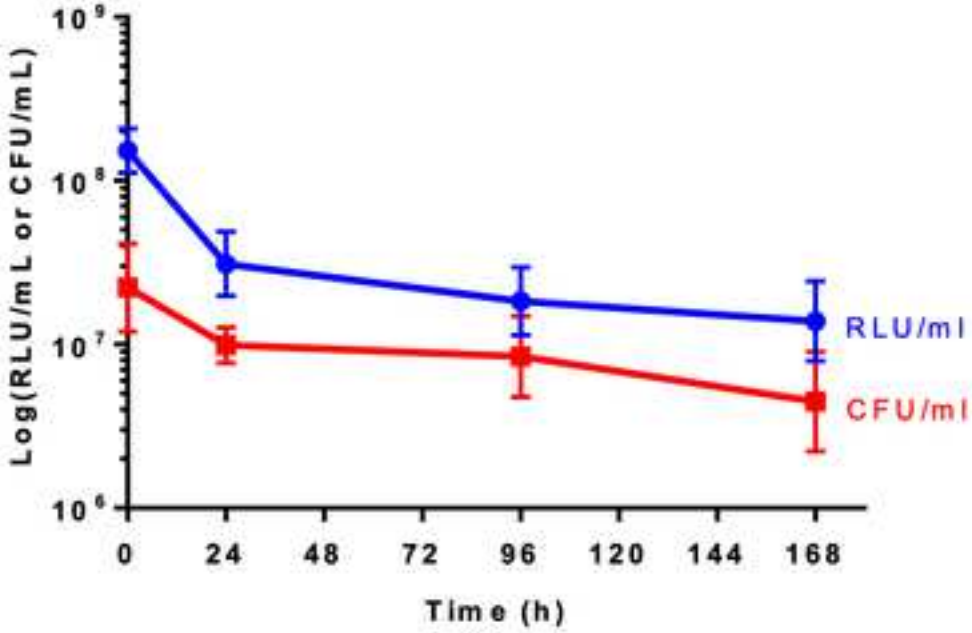

Name of Material/ Equipment	Company		Catalog Number
1.5ml reaction tube (Eppendorf)	Eppendorf		22431021
20, 200 and 1000 µl pipette and filtered tips	Any supplier		n/a
24 well culture plate	Greiner		662160
25 ml pipettes and pipette boy	Any supplier		n/a
3 compartment Petri dish (94/15mm)	Greiner		637102
Centrifuge	Any supplier		n/a
Class II saftey cabinet	Any supplier		n/a
Erlenmeyer flask with vented cap (250 ml)	Corning		CLS40183
Ethanol (>99.7%)	VWR		208221.321
Galleria mellonella (250 per pk)	Livefood Direct UK		W250
Glycerol	Sigma-Aldrich		G5150
Homogeniser (FastPrep-24 5G)	MP Biomedicals		116005500
Hygromycin B	Corning		30-240CR
Luminometer (Autolumat LB 953)	Berthold		34622
Luminometer tubes	Corning		352054
Lysing matrix (S, 2.0ml)	MP Biomedicals		116925500
Micro syringe (25 µl, 25 ga)	SGE		3000
Microcentrifuge	Any supplier		n/a
Middlebrook 7H11 agar	BD Bioscience		283810
Middlebrook 7H9 broth	BD Bioscience		271310
Middlebrook ADC enrichment	BD Bioscience		212352
Middlebrook OADC enrichment	BD Bioscience		212240
Mycobacterium bovis BCG *lux*	Various	n/a	
n-decyl aldehyde	Sigma-Aldrich		D7384-100G
Orbital shaking incubator	Any supplier		n/a
Phosphate buffered saline	Sigma-Aldrich		P4417-100TAB
Polysorbate 80 (Tween-80)	Sigma-Aldrich		P8074-500ml
Small box	Any supplier		n/a
Tweezer	Any supplier		n/a

Winterm (V1.08)	Berthold	n/a	
Petri dish (94/15mm)	Greiner		633181
Filter paper (94mm)	Any supplier	n/a	

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Title of Article: Methods for the use of the invertebrate, Galleria mellonella, as an infection model to study the Mycobacterium tuberculosis complex

Author(s): Masanori Asai, Yanwen Li, Jasmeet Singh Khara, Camilla Gladstone, Brian Robertson, Paul Langford, Sandra Newton

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27th Febraury 2019

Sandra Newton BSc PhD FHEA
Senior Research Fellow

FAO Dr Xiaoyan Cao,
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Dear Dr Xiayan Cao,

Re: Manuscript No. JoVE59703, titled 'Methods for the use of the invertebrate, *Galleria mellonella*, as am infection model to study Mycobacterium tuberculosis complex.'

The authors and I thank the reviewers of our above-referenced manuscript for their helpful comments and suggestions.

We have considered all of their remarks and incorporated respective changes in the revised manuscript with line numbers. Please note line numbers stated below correspond to those in the 'cleaned' revised manuscript. Our specific responses are provided on the following pages.

We believe that we have addressed all of their concerns and hope that the manuscript is now acceptable for publication.

On behalf of my co-authors, I would like to thank you again for your time and kind consideration.

Yours sincerely,

S. M. Newton

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Changes to be made by the author(s) regarding the manuscript:

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Response: Figure 5 has been published before in Li *et al*. Virulence 9 (1) 2018. However this article has been published with a CC BY licence (open access) which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. The journal ‘Virulence’ confirmed that we can use the figure in another article. In this manuscript we have cited the figure in the figure legend. A copy of the email correspondence with Virulence is enclosed.

3. Please revise lines 80-82 to avoid previously published text.

Response: L80-84 (previously L80-82) has been changed to avoid previously published text.

4. Please abbreviate liters to L (L, mL, µL) to avoid confusion.

Response: Done

5. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

Response: Done

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Response: Apologies for our oversight. As requested, we have now changed any commercial language, and protocols have been adjusted to the imperative tense.

8. 2.3: How many healthy larvae are needed? How large is the petri dish?
9. 3.1: Please specify the size of the filter paper.

Response: We recommend minimum of 20-30 larvae per group – also typically used with other pathogens and the size of the Petri dish and filter paper used were 94/15 mm in diameter. This information has been added to L187-188.

10. Figure 1 and Figure 2: Please use American English (melanization instead of melanisation, minimize instead of minimize, sterilize instead of sterilise, etc.).

Response: As requested, American English has now been used throughout the manuscript.

11. Figure 2: Please replace commercial language (Tween-80) with a generic term.

Response: Tween-80 has been changed to “polysorbate 80” throughout the manuscript.

12. Figure 5 and Figure 6: Please abbreviate liters to L (L, mL, µL) to avoid confusion.

Response: Throughout the manuscript, all liters (as l) have been abbreviated to L

13. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

Response: Done as requested. In addition, we also included the relevant information for Petri-dishes, and software used for the luminometer, which had been inadvertently omitted.

14. References: Please do not abbreviate journal titles.

Response: The journal titles are now in their full format.

Reviewer #1:

(1) I doubt that injecting 10^7 bacteria into the larva really is a model for "pathogenesis" - the pathogenesis of mycobacteria is mainly intracellular, and i doubt injecting that many bacteria allows for most of them to be phagocytized by specialized cells. i do think the model is valid, especially for drug testing (less so for pathogenesis), but this point should be discussed (in the discussion...)

Response: We agree with the reviewer that this model is likely to be suitable for the study of drug efficacy and toxicity. In our original paper (Li *et al*., 2018 Virulence 9:1126-1137), we focused on pathogenesis to get a handle on the strengths and weaknesses of the model. Experiments are in progress to establish whether it can also be used for drug efficacy, but those studies will not be complete for some time. Antimicrobial efficacy has been evaluated in the case of non-tuberculous bacteria (Entwistle and Coote, 2018). In view of the referee’s

comment, we have now discussed the point raised in the text (L419-421) and cited the reference (Number 18).

(2) A highly similar paper was published recently, i believe it was on M. abscessus (a luminescent mutant). i think it was Meir et al, in 2018. this paper should probably be cited.

Response: We thank the reviewer for bringing this paper to our attention, we have now included Mair *et al.* 2018 (Reference 17) in a sentence to highlight pre-existing models for the study of non-tuberculous mycobacteria.

Reviewer #2:

(1) The method has some omissions –

a) the authors must include statistical methods used to assess the significance of larval survival data eg. log-rank test, Kaplan-Meier plots;

b) the authors mention use of the model as a tool to identify novel treatments for M. tuberculosis in their Introduction yet they do not include methodology on how drugs are administered and these tests performed eg. dose response of antibiotics, toxicity etc. For the method to be complete these should be included.

c) Also, why pitch the methodology at just M. bovis? The methodology they describe is equally applicable to the study of virulence and drug sensitivity of scores of infective bacteria? It is generic.

Response 1a: We have now altered Figure 3 including 95% CI to provide more information on the survival of the larvae overtime. Furthermore, as suggested we have carried out log-rank test and report a significance of $P < 0.0001$ between the all groups. Statistical testing has been added as Section 6 (L260-264) and significance has been reported in L276.

Response 1b: While we agree that such description would be ideal, the use of this model as a drug efficacy/toxicity is work in progress (L419-421), and as such was not included in this manuscript. See also response to Reviewer #1 point (1).

Response 1c: We do agree that *G. mellonella* is suitable for other bacterial (and fungal) pathogens, and this is referred to in L343-345. Our paper (Li et al. 2018) was the first description of the use of *G. mellonella* as a surrogate model for the *Mycobacterium tuberculosis* complex, and has received substantial interest. Working with mycobacteria is a challenge in its own right, and we do not feel a generic paper would do justice to those aspects. We felt that JOVE was a natural fit – as the other reviewers' comments substantiate - to enable those in the field of mycobacteria to use this method. We anticipate that publication in JOVE will be of great benefit to the research field and maximize uptake of the model within the growing mycobacterial community.

(2) line 144 - Section 1.4 is not clear. The second sentence should start the section and the NOTE should be included in the paragraph as it is integral to the effective use of the technique.

Response: This section has been amended as requested (L150-156).

(3) line 187 - MSC – define

Response: MSC was previously defined as microbiology safety cabinet in L130 (in the original manuscript) and in L135 in the current version.

(4) line 194 - SGE and 25 Ga – define

Response: We have removed SGE as it is a commercial name, and Ga has now been defined as gauge in L200.

(5) line 203 - use of tweezers to pick up larvae seems unnecessary - it is much easier and quicker to gently handle larvae using gloved hands. Tweezers could pinch and stress the larvae? This also removes the need for a dedicated platform as described in section 3.5.

Response: The reason for using the tweezer/platform was to securely immobilize the larvae while minimizing the risk of needle stick injury and is part of a standard protocol agreed with our safety department. Such concerns are addressed in the discussion (L370-373).

(6) line 260 - use of piperacillin to eliminate larval flora requires a reference source.

Response: The reference for piperacillin usage has now been added to the main text (Reference 16 = Li *et al*., 2018 Virulence 9:1126-1137), L257-258.

(7) Figure 3 - present the 2 control treatment lines so that they can be seen.

Response: Figure 3 has now been modified to show both controls.

(8) Figure 5 should be removed as a similar dataset is shown in Figure 6. There is no need to show this twice.

Response: We thank the reviewer for the suggestion. However, while these figures are indeed similar (but not the same), the purpose of Figure 6 is to demonstrate the ratio between CFU and RLU which you can NOT obtain from that presented in Figure 5. NB: The reviewers of Li et al. (2018) were concerned that the ration of CFU to RLU of mycobacteria in *G. mellonella* and broth would differ. Therefore, presenting data in such a way that others could calculate the ratio is likely to be of high interest to those using the method.

Reviewer #3:

(1) Ln 67 Only 2 drugs required for 6 months

Response: We referred to the treatment as cocktail of drugs for 6 months in a broad sense, total cumulative treatment period 2 month of HRZE followed by 4 months of HR. L67-69 have been amended to include specificity of treatment.

(2) Ln 168 - Why do CFU when RLU correlates - Is there a difference sometimes?

Response: The need to correlate CFU are described in L384-385. We have modified the text to point out that the BCG *lux* RLU to CFU ratio is typically 3:1 in broth and 4:1 in *G. mellonella* (see also point 8 of Reviewer #2).

(3) Fig 2 & Ln 260 -the term "flora" is no longer appropriate and should be replaced with microbiota.

Response: The term flora has now been replaced with microbiota in Figure 2 and L258

Reviewer #4:

(1) authors consider the use of moth larvae that are purchased. Although, in most cases in the current literature, the larvae are obtained in the same lab that grows pathogens or labs of research institutions (that are donated, not necessarily purchased). It is necessary to consider this experimental context in item 2.1 and other parts, such as discussion (L345-346).

Response: We thank the reviewer for the comment and we agree to the importance of such consideration. Changes have been added to reflect non-purchased larvae in our manuscript (L177-178, 357-361).

(2) Reference about hemocytes of Galleria needs to be updated. Ref #12 is not specific to the studied model and other references are more appropriate. For example, refer to Differential cellular immune response of Galleria mellonella to Actinobacillus pleuropneumoniae. Cell and Tissue Research, 2017, 370(1).DOI: 10.1007/s00441-017-2653-5

Response: The new reference has been added to the manuscript in the text (L101) + reference section (13).

(3) L146: pH and concentration of PBS?

Response: The pH of the PBS is 7.4 and the concentrations are as follows: 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride. This information has been added to the manuscript (L151-152).

(4) L175: how pupating individuals are identified?

Response: Pupating larvae are identified by clear morphological differences and non-trained individual should easily be able to distinguish them from the general populations. This point has been added to the manuscript L179-180

(5) L197: …into the same 25uL…?

Response: BCG *lux* inoculum was aspirated into the sterilized 25 µL micro syringe and section 3.4 has been changed to clarify this point (L203)

(6) L228: …select five infected larvae…

Response: Section 5.1 has been clarified: as “five infected larvae previously prepared in section 3.” (L232)

(7) L272: Figure 3

Response|: We are not quite sure what the reviewer is referring to, but Figure 3 has been amended to improve distinction between the control groups, and the 95% confidence interval has been added to provide greater detail into the survival of the larvae.

(8) L341: Confuse. Did you mean: the health of the naïve larvae? Prior to the experiments?

Response|: For clarity we have added the word naïve as has been suggested (L348)

(9) L358: why not cold the larvae for immobilization? This procedure is not considered in the discussion.

Response: We have included a discussion on cold immobilization of larvae in L373-376.

From: authorqueries@tandf.co.uk [mailto:authorqueries@tandf.co.uk]
Sent: 05 December 2018 16:08
To: Newton, Sandra M <s.newton@imperial.ac.uk>
Subject: Copyright or permissions [ref:_00D0Y35Iji._5001nrZ5Yk:ref]

Dear Sandra Newton,

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I hope this helps and if you have further questions or concerns, please let me know.

Best wishes,

Shirmae Ampo
On behalf of the Taylor & Francis Journals Helpdesk

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Sent on: 03/12/2018

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Manuscript ID: KVIR 1491255 DOI 10.1080/21505594.2018.1491255
Message:
Dear Sir/Madam,

I would like to request permission to use a figure that I recently published in the above manuscript in the Virulence journal.
The Figure is no 2. (Recovery of M. bovis BCG lux). I would like permission to use this figure in a technical paper that I am writing for the Journal of Visual Experiments (J Vis Exp). Please let me know if this is possible.

Best wishes

Sandra Newton

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jove

Xiaoyan Cao <xiaoyan.cao@jove.com>

RE: Response Requested: JoVE Submission JoVE59703R1 - [EMID:3d43a33d6c2ce6eb]

1 message

Newton, Sandra M <s.newton@imperial.ac.uk> Wed, Feb 27, 2019 at 4:45 AM
To: Xiaoyan Cao <xiaoyan.cao@jove.com>

Dear Xiaoyan,

The Kavanaugh and Sheenan paper 2018 was published in the Journal of Fungi, link attached here https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6162640/#!po=55.7018

The article states that it is published with a CC BY license (https://creativecommons.org/licenses/by/4.0/). According to the link here for a CC BY license, figures from an article can be used and adapted as long as the original source is given appropriate credit. We have clearly referenced and stated in the JOVE manuscript that the figure has been adapted from the Kavanaugh and Sheenan paper 2018.

Please can you confirm that this is acceptable?

Many thanks

Sandra

From: em.jove.187f8.618b2b.d80b1597@editorialmanager.com [mailto:em.jove.187f8.618b2b.d80b1597@editorialmanager.com] **On Behalf Of** Xiaoyan Cao
Sent: 26 February 2019 22:09
To: Newton, Sandra M <s.newton@imperial.ac.uk>
Subject: Response Requested: JoVE Submission JoVE59703R1 - [EMID:3d43a33d6c2ce6eb]

Dear Dr. Newton,

Regarding your JoVE submission JoVE59703R1 Methods for the use of the invertebrate, Galleria mellonella, as an infection model to study the Mycobacterium tuberculosis complex, please obtain explicit copyright permission to reuse the figure from Kavanaugh and Sheehan (2018). Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please email me this information at your earliest convenience.

Please feel free to email me with any questions or concerns about your manuscript.

Best Regards,

Xiaoyan Cao, Ph.D.
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