International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 13-08-2021, Accepted: 27-08-2021, Published: 13-09-2021
Volume 6, Issue 5, 2021, Page No. 42-47

Greater wax moth of *Galleria mellonella* (Lepidoptera: Pyralidae) as a model host for human pathogen in *Escherichia coli*

Aysha A[1], Chitra P[2]
[1] MSc, Student, Avinashilingam Institute for Home science and Higher Education for Women, Coimbatore, Tamil Nadu, India
[2] Assistant Professor, Avinashilingam Institute for Home science and Higher Education for Women, Coimbatore, Tamil Nadu, India

Abstract
Larvae of *Galleria mellonella (*greater wax moth or honeycomb moth) are a widely used model for studying virulence factors and pathogenesis of many prominent bacterial and fungal human pathogens, the efficacy of new antimicrobial compounds, and immune responses. *G. mellonella* larvae are inexpensive easily obtained and are simple to use as they don't require special lab equipment. The immune system of *G. mellonella* larvae is structurally and functionally analogous to the innate immune response of mammals, which makes this model suitable for study of virulence and pathogenesis of human pathogens. The present research is undertaken to assess the suitability of *G. mellonella* as an alternative model for studying the virulence and pathogenicity of Human pathogen *E. coli* and also the cellular and humoral immunity of *E. coli* to *G. mellonella.* Mortality and Survival record of the larvae challenged with 10 µl of10^5 to10^7 CFU/Ml and with 0.1% peptone water as control were observed over 72 hrs and dose responses data obtained. Phenoloxidase enzyme activity was investigated as a marker for immune response. Results demonstrate that *E. coli* is pathogenic towards *G. mellonella* Infection with *E. coli* strains activated the melanisation pathway resulting in melanin deposition. The findings suggest *G. mellonella* might be a useful *in vivo* model of infection and convenient as a prescreening assay for virulence of *E. coli.*

Keywords: *Galleria mellonella, Escherichia coli, Phenoloxidase*, humoral immunity

Introduction

Galleria mellonella is also called greater wax moth or honeycomb moth; it includes the order Lepidoptera and family Pyralidae. The greater wax moth is notable for its parasitization of honeybees and their hives. *G. mellonella* is a typical holometabolous insect, that is, it goes through four developmental stages in its life cycle, namely, the egg, larva, pupa and adult. *G. mellonella* larvae have considerable benefits when compared to traditional mammalian models: they are easily inexpensive. Additional advantages are that ethical approval is not required for their use, and their short life cycle (7–8 weeks) makes them ideal for large-scale studies. Large size of moth larvae (12-20 mm) enables simple manipulation and encourages the processing of tissue and haemolymph for examination. The popularity of this model organism is driven by the low cost of purchase and the reduced ethical concerns for the experimental manipulation of insects. This allows the challenge of a large number of larvae in a single experiment, which can improve the statistical power of an assay. Importantly, *G. mellonella* can be maintained at mammalian body temperature and the outcomes of infection can reproduce that of mammalian animal models. This is likely due to similarities in the innate immune response to pathogens mediated by elements of cellular and humoral immunity between insects and mammals.

The *G. mellonella* immune system shares a high degree of structural and functional similarity to the vertebrate innate immune system. Because of these similarities, their larvae have been employed to study the virulence of bacterial and fungal pathogens of mammals. The immune response of *G. mellonella* is innate (first-line defense), and it consists of three parts: physical as well as chemical barriers that protect the insect from the external environment, the humoral response, and the cellular response. The cellular response is mediated by haemocytes (blood cells of *G. mellonella*). There are at least six types of haemocytes in insects and these show in distinguish ability's to mammalian neutrophils in terms of their ability to phagocytosis and kill pathogens by the production of superoxide. The functions of these hemocytes include storage of various types of substances, as well as providing a defense of the organism, based on coagulation mechanisms, phagocytosis, nodulation, and melanization. The humoral response is mediated by different types of soluble molecules secreted against pathogens. This response mainly comprises antimicrobial peptides, lytic enzymes (lysozyme), and melanin.

Materials and Methods

Collection and culture of *Galleria mellonella:*

The egg mass and larvae of *G. mellonella were* collected from Marutham Honey Bee farm at Nallampalayam area of Coimbatore and brought to the laboratory. The collected egg mass were isolated and kept in a separate plastic containers. The collected larvae were isolated according to the size and weight and placed in separate containers, fed with artificial diet. The culture was maintained at 27±2º C and 42 Rh.

The larvae were maintained in a large container at room temperature on an artificial diet. Aeration was provided by making small holes on the lid. Once in a week larvae was transferred in to fresh diet. Some of the larvae were kept as

such in a container for pupation. During adult stage, they were placed on a container having wax coated butter paper, in which the female laid eggs. The eggs were transferred carefully using sterile blade. Then washed with sterile distilled water and 0.01% formalin and then transferred to fresh diet. The eggs hatch within 3-4 days. The greater wax moth larvae of *Galleria mellonella* were reared on artificial diet in the laboratory.

Fig 1: Separation of Eggs and larva of *Galleria mellonella*

Fig 2: Rearing of Galleria mellonella in the laboratory

Bacterial strains and preparation of inoculum

E. coli was sourced from our culture collection and isolates selected based on toxin profiles. Cultures were stored in 30% glycerol (Thermo Fisher Scientific, United Kingdom), 70% brain heart infusion (BHI) media at -80°C and cultured on BHI agar and incubated at 37°C for 5 h. Bacterial inoculum were adjusted by absorbance (OD_{620}) and CFU/mL determined by traditional serial dilution and plating on TSC agar. Cells in adjusted cultures were pelleted by centrifuging at 3170 ×*g* at room temperature for 2 min and washed once with 0.1% peptone water. Dilutions were drawn into a 1 mL syringe fitted with a 30 g sterile needle.

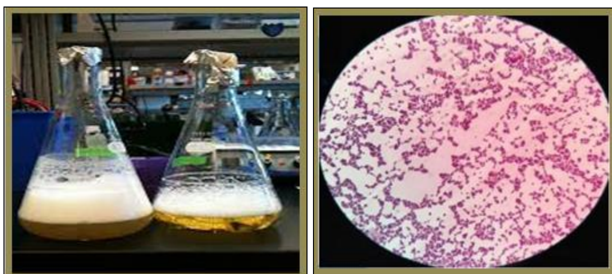

Plate 3: Culture of Escherichia coli

Galleria mellonella challenge assay

Larvae were stored in wood shavings, at room temperature, in the dark. To avoid sampling biases, larvae with any signs of melanisation or deformity were rejected. Larvae were weighed and only larvae meeting the criteria of 250 ± 50 mg were utilized. Prior to all injections, larvae were immobilized by standing the 24 micro-well plate on ice for 15 min (Sammy Kay *et al,* 2019).

Plate 4: Challenge assay of *Galleria mellonela* before introduced bacterial inoculum

Pathogencity screening

E.coli was prepared for inoculation. Groups of 10 larvae were each challenged with 10 µl of10^5 to10^7 CFU/Ml and 10 larvae injected with 0.1% peptone water as controls. Larvae were placed in Petri-dishes lined with greaseproof paper to allow for greater contrast in images. All groups were incubated aerobically at 37°C and survival recorded at 72 h. The experiments were repeated in triplicate and an average survival result recorded (Sammy Kay *et al,* 2019).

Dose dependent challenge

Final instar *G. mellonella* larvae were obtained from the culture. Only healthy looking caterpillar with no melanization was used in the experiments. In dose dependent challenge studies, larvae were prepared by placing one per well in 24 micro-well plastic plates. All larvae were treated identically to ensure injection and isolate continuity throughout. *E.coli* were investigated by preparing a dilution series of isolate and larvae were injected with a dilution of10 µL of washed cultures ranging from approximately 10^5 to 10^7 CFU/mL. in addition, 24 larvae were injected with 10 µL of 0.1% peptone water as negative control. All six plates for each isolate were recorded simultaneously with the image recording system described previously. The larvae were incubated for 72 h at 37°C. Images were taken every 10 min for 72 h. Melanisation scores and mortality were recorded at 0, 24, 48, 72 and 96 h post injection. All experiments were repeated in triplicate and average scores recorded (Sammy Kay *et al,* 2019).

Results and Disscussion

A variety of different insects species have been employed to study microbe host interactions and include *Drosophila melanogaster*, *Galleria mellonella, Bombyx mori* and *Manduca sexta.* A wide range of microorgansims have been studied in insects including many bacterial (Dunphy *et al.,* 1986; Morton *et al.,* 1987; Bergin *et al.,* 2005) [4] and fungal pathogens (Mylonakis *et al.,* 2005; Reeves *et al.,* 2004; Cotter *et al.,* 2000) [2, 18, 10]. In this study the human pathogen *E. coli* was introduced in to the 5th instar larvae of *G. mellonella* in different inoculum levels.

E. coli inoculation of *Galleria mellonella* larvae

The groups of *G. mellonella* larvae injected different inoculum levels (10^5, 10^6 and 10^7 CFU/ml of *E. coli* suspension. Larvae of *G. mellonella* are easy to inoculate via injection into the haemocoel through the last left pro-leg. The base of the pro-leg can be opened by applied gentle pressure to the sides of the leg and this aperture will re-seal after removal of the syringe needle without leave a scar. Larvae can be stored at 15˚C prior to use and, once inoculated, may be maintained at temperatures up to 37˚C as long as appropriate controls are implemented to quantify the

effect of temperature on survival. Larvae with any signs of melanisation or deformity were rejected. Larvae were weighed and only larvae meeting the criteria of 250 ± 50 mg were utilized. Prior to all injections, larvae were immobilized by standing the 24 micro-well plate on ice for 15 min.

Pathogenesity screening

Groups of 10 larvae were injected with 10 µl of $10^5$to 10^7 CFU/mL and 10 larvae injected with 0.1% peptone water as controls. All groups were incubated aerobically at 37°C and survival recorded at 72 h. Injection with 10µL of 10^7CFU/ml *E. coli* bacteria cause visible disease to the larvae within 6-12 h. melanisation occurs at the site of injection and other parts of body. No melanisation occurs in 0.1% peptone control. Control shows high survival rate compared to the infected *E. coli* strains in larvae of *G. mellonella* isolates of high virulence could be detected by a decrease in circulating haemocyte density and an increase in bacterial load whereas isolates of low virulence were detectable by a high haemocyte density and a low bacterial load (Bergin *et al.,* 2003). Changes in the expression of selected anti-microbial peptides can be used as an indicator of the immune response to infection and assist in differentiating pathogenic from non-genic infections (Bergin *et al.,* 2006) [3]. Irrespective of which endpoint is used (larval death, Bacterial load, haemocyte density, anti-microbial peptide expression, Proteomic profile) results can be obtained within 12 h. While *G. mellonella* larvae offer many advantages as an alternative model for studying bacterial pathogens of humans a number of points should be borne in mind regarding the appropriate of their use.

Plate 5: Living (A) and dead (B) *G. mellonella* larvae that had been infected with *E. coli*

Dose Dependent Challenge

In dose dependent challenge larvae were injected with different concentration of *E.coli* bacteria such as10^5, 10^6 and 10^7CFU/Ml. In addition, larvae were injected with 10 µL of 0.1% peptone water as negative controls. After 6hrs of incubation larvae exhibits nodulation and blackening of the cuticle. The rate of development of infection appears dependent on inoculums size as melanization increases rapidly with increasing inoculum dose. Infected group demonstrates melanization at 1-2 hrs post incubation compared to no melanization in 0.1% peptone control. Infection with lower dilutions did not cause significant melanization compared to higher dilutions.

Inoculated larvae were start changed to turn black color within two hours post infection of *E. coli.* It may occurred depending upon the virulence of the pathogen *(E. coli).* This is due to the onset of melanisation an insect immune response resulted from the cleavage of prophenoloxidase to active phenoloxidase designed to limit the growth of the *E. coli* within the haemocoel (Kavanagh and Reeves, 2004) [18]. Dead larvae were appeared black in color. However it is worth noting that some pathogens (*E. coli*) may lead to the appearance of arid/brown pigmentation in the insect cuticle. While larval death is often used as the end point in an experiment other parameters may also be employed particularly when dealing with a relatively ‘weak ‘pathogen or mutant with attenuated virulence which may not actually kill the test larvae. Fluctuations if Bacterial culture load and haemocyte density have been used as accurate indicators of bacterial virulence in larvae (Bergin *et al.,* 2003) as have changes in the expression of anti-microbial peptides (Bergin *et al.,* 2006) [3].

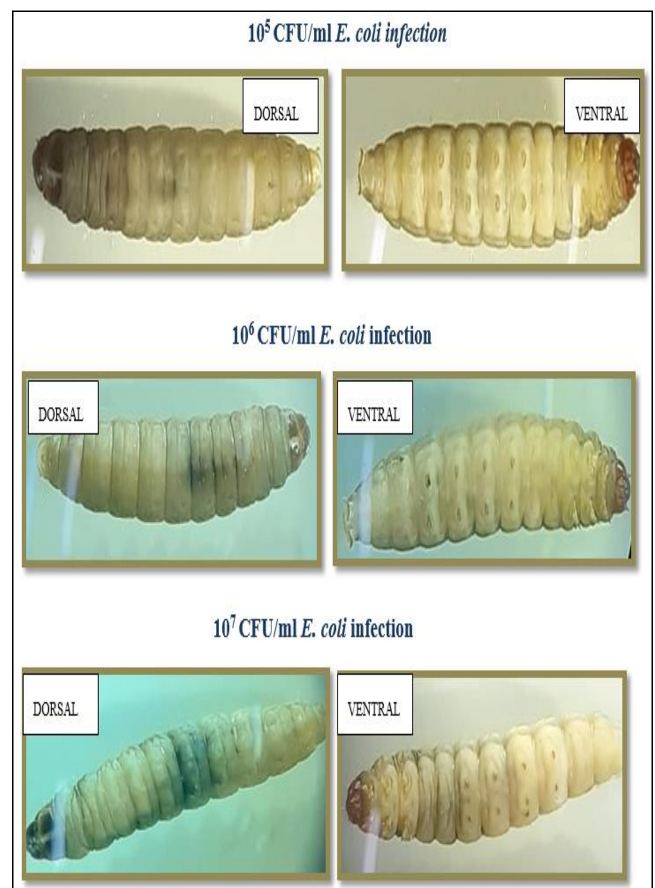

Plate 6: Melanisation occurs dorsal and ventral side of *G. mellonella* larvae, one hour post infection with *E. coli* 10^5 cfu/ml *E. coli infection*

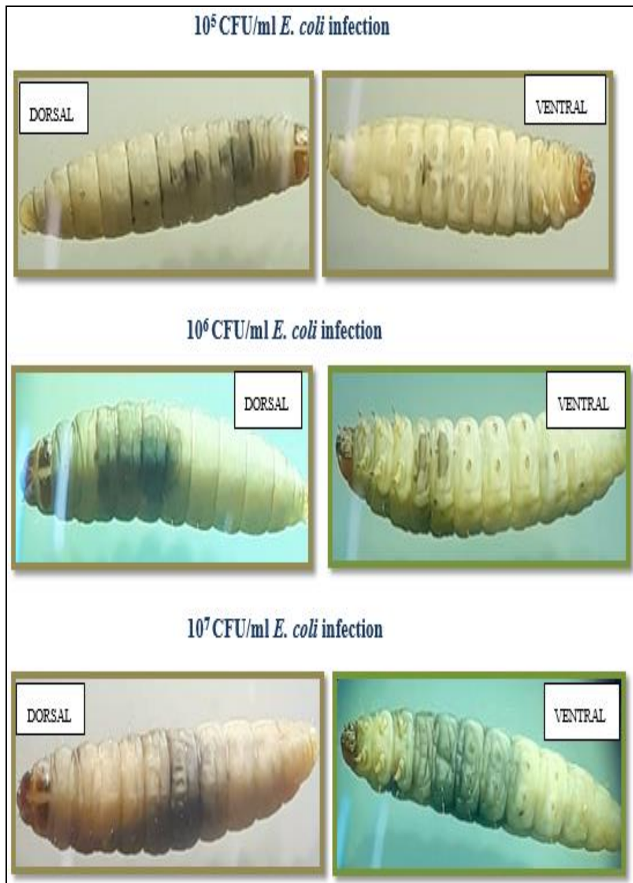

Plate 7: Melanisation occurs dorsal and ventral side of *g. Mellonella* larvae, two hours post infection with E. *coli*

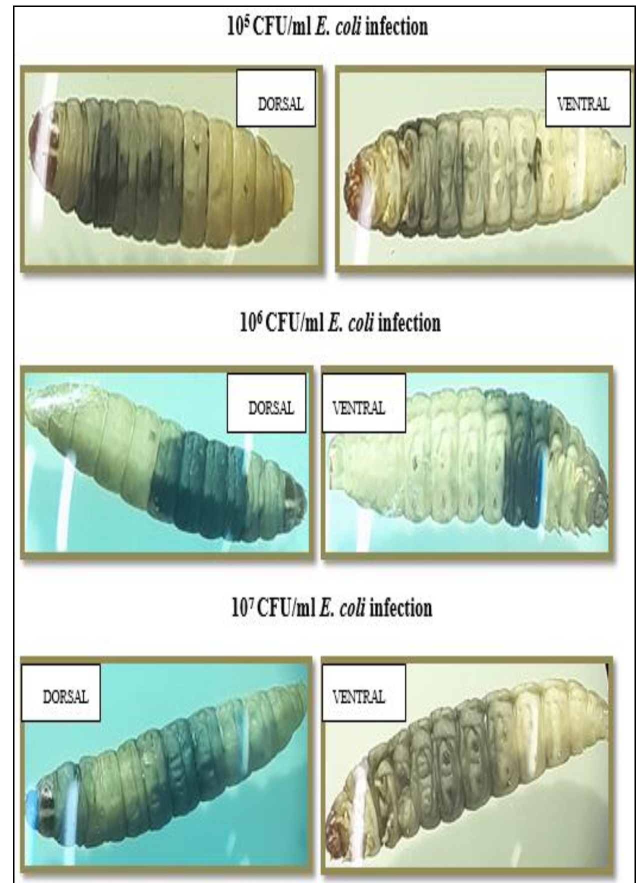

Plate 9: Melanisation occurs dorsal and ventral side of *G. mellonella* larvae, four hours post infection with *E. coli*

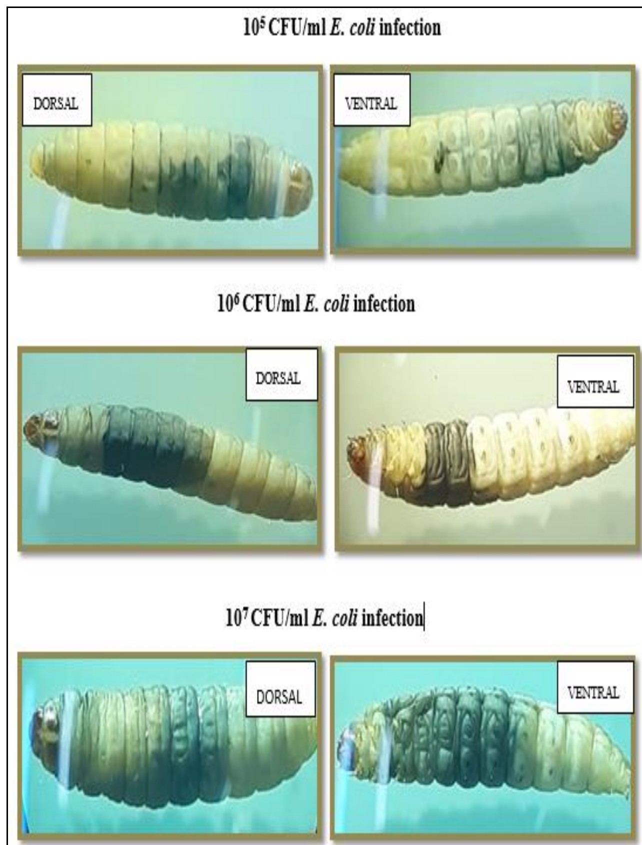

Plate 8: Melanisation occurs dorsal and ventral side of *G. mellonella* larvae, three hours post infection with *E. coli*

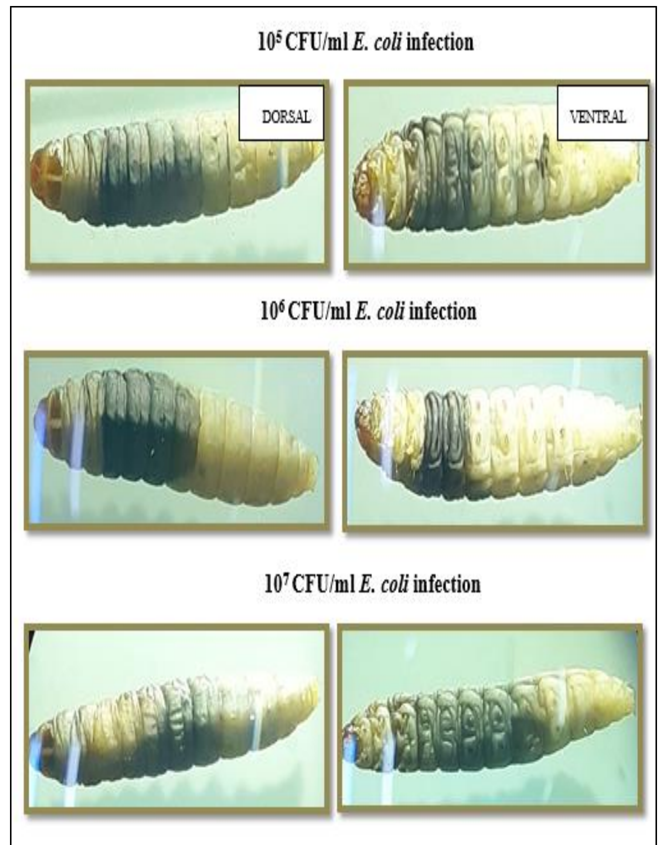

Plate 10: Melanisation occurs dorsal and ventral side of *G. mellonella* larvae, six hours post infection with *E. coli*

Conclusion

Over recent years, *G. mellonella* has become increasingly popular as a surrogate host to study infectious diseases, as well as a screening platform for antibiotics. The use of this larval model has provided several advantages over the use of the murine model, including the ability to test many bacterial strains in a limited period at low cost with easy management, these characteristics make *G. mellonella* an ideal model for the *in vivo* evaluation of new strategies, the efficacy of novel drugs and the characterization of host pathogen interaction. In this review, the cellular and humoral aspects of the human immune responses of *G. mellonella* larvae when challenged with human pathogen *E. coli* have been described. Cellular responses and some humoral responses such as antimicrobial peptides, lytic enzymes, melanization and opsonins are well defined based on the number of species studied. These factors could be important for a better understanding of *G. mellonella* as a model organism to study host-pathogen interactions and the treatment of infections.

References

1. Altincicek B, Linder M, Linder D, Preissner KT, Vilcinskas A. Microbial metalloproteinases mediate sensing of invading pathogens and activate innate immune responses in the lepidopteran model host Galleria mellonella. Infect Immun,2007:75:175-83.
2. Aperis G, Fuchs BB, Anderson CA, Warner JE, Calderwood SB, Mylonakis E. *Galleria mellonella* as a model host to study infection by the *Francisella tularensis* live vaccine strain. Microbes Infect,2007:9:729-34.
3. Bergin D. Pre-exposure to yeast protects larvae of Galleria mellonella from a subsequent lethal infection by Candida albicans and is mediated by the increased expression of antimicrobial peptides. Microb. Infect,2006:8:2105-2112.
4. Bergin D. Superoxide production in haemocytes of *Galleria mellonella* identification of proteins homologous to the NADPH oxidase complex of human neutrophils. Infect. Immun,2005:73:4161-4173.
5. Binder U, Maurer E, Lass-Flörl C. *Galleria mellonella:* An invertebrate model to study pathogenicity incorrectly defined fungal species. Fungal Biol,2016:120:288-295.
6. Boman HG, Hultmark D. Cell-free immunity in insects. Annu. Rev. Microbiol,1987:41:103-126.
7. Browne N, Heelan M, Kavanagh K. An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes. Virulence,2013:4:597-603.
8. Coleman JJ, Muhammed M, Kasperkovitz PV, Vyas JM, Mylonakis E. Fusarium pathogenesis investigated using *Galleria mellonella as* a heterologous host. Fungal Biol,2011:115:1279-1289.
9. Cook SM, McArthur JD. Developing *Galleria mellonella* as a model host for human pathogens. Virulence,2013:4:350-353.
10. Cotter G. Development of an insect model for the *in vivo* pathogenicity testing of yeasts. FEMS Immunol. Med. Microbiol,2000:27:163e169.
11. Eleftherianos I, Revenis C. Role and importance of phenoloxidase in insect hemostasis. J Innate Immun,2011:3:28-33.
12. Fuchs BB, O'Brien E, Khoury JB, Mylonakis E. Methods for using *Galleria mellonella* as a model host to study fungal pathogenesis. Virulence,2010:1:475-482.
13. Gillespie JP, Kanost MR, Trenczek T. Biological mediators of insect immunity. Annu. Rev. Entomol,1997:42:611-643.
14. Götz P. Encapsulation in arthropods. Immunity Invertebr, 1986, 153-170.
15. Grizanova EV, Semenova AD, Komarov DA, Chertkova EA, Slepneva IA, Dubovskiy IM. Maintenance of redox balance by antioxidants in hemolymph of the greater wax moth Galleria mellonella larvae during encapsulation response. Arch. Insect Biochem. Physiol,2018:98:21460.
16. Jiravanichpaisal P, Lee BL, Söderhäll K. Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. Immunobiology,2006:211:213-236.
17. Joyce SA, Gahan CG. Molecular pathogenesis of Listeria monocytogenes in the alternative model host Galleria mellonella. Microbiology,2010:156:3456-3468.
18. Kavanagh K, Reeves EP. Exploiting the potential of Insects for *in vivo* pathogenicity testing of microbial pathogens. FEMS Microbiol. Rev,2004:28:101-112.
19. Konkel ME, Tilly K. Temperature-regulated expression of bacterial virulence genes. Microbes Infect,2000:2:157-66.
20. Lavine MD, Strand MR. Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol,2002:32:1295-1309.
21. Li D, Scherfer C, Korayem AM, Zhao Z, Schmidt O, Theopold U. Insect hemolymph clotting: Evidence for interaction between the coagulation system and the prophenoloxidase activating cascade. Insect Biochem. Mol. Biol,2002:32:919-928.
22. Lionakis MS. Drosophila and Galleria insect model hosts: new tools for the study of fungal virulence, pharmacology and immunology. Virulence,2011:2:521-527.
23. Lu A, Zhang Q, Zhang J, Yang B, Wu K, Xie W *et al*. Insect prophenoloxidase: The view beyond immunity. Front. Physiol,2014:5:252.
24. Mowlds P, Kavanagh K. Effect of pre-incubation temperature on susceptibility of *Galleria mellonella* to infection by *Candida albicans.* Mycopathologia,2008:165:5-12.
25. Mylonakis E. *Galleria mellonella* and the study of fungal pathogenesis: making the case for another genetically tractable model host. Mycopathologia,2008:165:1-3.
26. Park SY, Kim CH, Jeong WH, Lee JH, Seo SJ, Han YS *et al*. Effects of two hemolymph proteins on humoral defense reactions in the wax moth, *Galleria mellonella.* Dev. Comp. Immunol,2005:29:43-51.
27. Pech LL, Strand MR. Granular cells are required for encapsulation of foreign targets by insect haemocytes. J. Cell Sci,1996:109:2053-2060.
28. Ramarao N, Nielsen-Leroux C, Lereclus D. The insect Galleria mellonella as a powerful infection model to investigate bacterial pathogenesis. J. Vis. Exp,2012:70:e4392.
29. Ratcliffe NA. Invertebrate immunity–a primer for the

non-specialist. Immunol. Lett,1985:10:253-270.
30. Renwick J. Translocation of proteins homologous to human neutrophil p47phox and p67phox to the cell membrane in activated hemocytes of *Galleria mellonella*. Dev. Comp. Immunol,2007:31:347-359.
31. Rock FL. A family of human receptors structurally related to Drosophila. Dev. Biol,1998:95:588-593.
32. Satyavathi VV, Minz A, Nagaraju J. Nodulation: an unexplored cellular defense mechanism in insects. Cell Signal,2014:26:1753-1763.
33. Schmit AR, Ratcliffe NA. The encapsulation of foreign tissue implants in *Galleria mellonella* larvae. J. Insect Physiol,1977:23:175-184.
34. Scoble M. Classification of Lepidoptera. Oxford university press, 1995.
35. Slater JL, Gregson L, Denning DW, Warn PA. Pathogenicity of *Aspergillus fumigatus* mutants assessed in *Galleria mellonella* matches that in mice. Med. Mycol,2011:49:S107-S113.
36. Soderhall K, Cerenius L. Role of the prophenoloxidase activating system in invertebrate immunity. Curr Opin Immunol,1998:10:23-8.
37. Strand MR. The insect cellular immune response. Insect Sci,2008:15:1-14.
38. Tang H. Regulation and function of the melanization reaction in Drosophila. Fly,2009:3:105-111.
39. Thomas RJ, Hamblin KA, Armstrong SJ, Muller CM, Bokori-Brown M, Goldman S *et al*. *Galleria mellonella* as a model system to test the pharmacokinetics and efficacy of antibiotics against Burkholderia pseudomallei. Int J Antimicrob Agents,2013:41:330-6.
40. Tojo S, Naganuma F, Arakawa1 K, Yokoo1 S. Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, *Galleria mellonella.* J. Insect Physiol,2000:46:1129-1135.
41. Tsai CJ, Loh JM, Proft T. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. Virulence,2016:7:214-229.