

Expanding the use of alternative models to investigate novel aspects of immunity to microbial pathogens

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Keywords: *Galleria mellonella*, offspring immunity, microbial virulence, alternative hosts, innate immunity

In the present issue of *Virulence*, an article entitled “The maternal transfer of bacteria can mediate trans-generational immune priming in insects”¹ describes an elegant study that illustrates the use of the lepidopteran *Galleria mellonella* to investigate a specific aspect of immunity to microbes. The authors show that exposure of mothers to bacteria results in enhanced immunity in the offspring. Furthermore, they have demonstrated that bacteria ingested by female larvae are found in the eggs, suggesting that enhanced immunity of the offspring is a consequence of direct exposure of the eggs to bacteria. This is a relevant and novel study for several reasons. The authors provide a mechanism for an important aspect of insect immunity, which is that direct transfer of bacterial fragments from the mother to the eggs primes their immune response. But in addition, this study opens the scope on the use of non-conventional models and illustrates how they can be used to investigate aspects of immunity against pathogenic microorganisms.

Classically, mammals have been used to study microbial pathogenesis (rodents such as mice and rats) and the immune response elicited by the host. These models have been a useful tool for centuries, and the development of their genetic manipulation offers new alternatives to investigate the role of specific factors of the immune system in the defense against pathogens. However, animal experimentation is associated with important bioethical problems, mainly due to the pain and suffering inflicted to the animals. For this reason, animal experimentation is nowadays regulated by authorities and bioethical committees. Furthermore, to reduce these bioethical problems, there is a strong trend to apply the “3 Rs” rule in experiments that involve animal use, which are: **reduce** the number of animals used in the laboratory; **refine** the protocols to increase animal comfort and reduce pain; and **replace** animals for other models that do not have bioethical problems associated. In this context, there has been an increasing interest in the scientific community to implement other systems that could be used as an alternative to protected animals, with special emphasis on animals with poorly developed neural systems in which the feeling of pain is almost absent. For this reason, “non-conventional” hosts are being used

to investigate microbial pathogenesis, including both invertebrates and vertebrates. These organisms have been proven to be very useful to investigate specific virulence traits of the pathogen and their role in infection. But although they are not closely related to higher vertebrates from an evolutionary point of view, they share important aspects in their response to microbes, in particular, in their innate immunity. So these models can also provide information about the immune response elicited against microbial pathogens.

Among vertebrates, two alternative different models are being used as infection models: zebra fish embryos and embryonated chicken eggs. In both cases, and to reduce the bioethical issues associated with the use of adult individuals, infections are performed in the embryonated stage of development. These models present the advantage that they have a closer immunity to mammals than invertebrates. Zebra fish (*Danio rerio*) is used as model host during the first seven days after eggs deposition, and infections can be performed by microinjection in different areas.² The zebra fish has both innate and acquired immunity, although this last one is not developed until day 30 of development, so the zebra fish embryo infection model is of particular interest to investigate virulence of pathogens controlled mainly by innate immunity. One advantage of this model is that the anatomy of the embryos is easily visible under the microscopy due to their transparency. Embryonated chicken eggs offer also an alternative to investigate microbial pathogenesis, and as it occurs with the zebra fish, the immunity of the eggs is similar to that of higher mammals. Infections are performed by injection of the pathogen in chorioallantoic membrane or directly in the embryos of eggs. The use of zebra fish and embryonated chicken eggs is limited in many cases because they require specific facilities to host and maintain the animals, and also due to the expertise required to handle them. Despite these limitations, these models have been used to investigate the virulence of fungal, bacterial, and viral pathogens.^{3–15}

Invertebrate animals are also extensively used as models to study immunity and microbial virulence. There are three main alternative hosts that have been widely utilized: amoebas, insects, and nematodes. Amoebas are environmental predators, and for

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Submitted: 03/28/2014; Accepted: 04/03/2014; Published Online: 04/09/2014

<http://dx.doi.org/10.4161/viru.28775>

Comment on: Freitag D, Schmidtberg H, Dickel F, Lochnit G, Vogel H, Vilcinskas A. The maternal transfer of bacteria can mediate trans-generational immune priming in insects. *Virulence* 2014; 5:547–54; PMID: 24603099; <http://dx.doi.org/10.4161/viru.28367>

this reason, they are considered an optimal model to investigate phagocytic activity.¹⁶ This is of particular interest for the study of facultative intracellular pathogens, since some of them can also survive inside amoebas and the mechanisms that result in intracellular pathogenesis seem to be conserved from amoebas to mammalian phagocytic cells. In addition, survival of microbial pathogens inside amoebas drives selection of microbes resistant to killing, which has important implications to understand the acquisition of virulence traits that are also used to cause disease in more complex organisms.¹⁶ This survival is also relevant for the infection cycle of some bacteria, which are phagocytosed in the environment by amoebas, and use them as vehicle to infect humans.^{17,18}

Nematodes, in particular *Caenorhabditis elegans*, can be used as model hosts for infections.¹⁹ Immunity of *C. elegans* is based on three major responses: avoidance behavior, which relies on chemosensory neurons that sense pathogens and induce escape, physical barriers (cuticle and the pharyngeal grinder), and innate immunity. This last response depends on pattern recognition receptors (scavenger receptors, c-lectins, FSHR, and TOLL), which regulate different signaling pathways (mainly MAPK, unfolded protein response, DAF, and TGF- β). As a consequence, antimicrobial responses (such as antimicrobial peptides, caenopores, lysozymes, and reactive oxygen species, ROS) and autophagy are induced. Remarkably, *C. elegans* does not have phagocytic cells.²⁰ The main advantage of this model is the availability of genetic tools. KO collections are available, which makes this worm suitable to investigate the role of specific elements of the host in the response to pathogens. Moreover, due to their small size and the possibility to perform assays in microdilution plates, *C. elegans* offers an excellent model to perform large screenings of antimicrobial compounds.²¹ However, this model exhibits also several limitations. Infection is performed by placing the worms on agar plates with a layer of the microorganism, so it is difficult to estimate the amount of inoculum used in each experiment. In addition, the worms do not tolerate high temperatures, so it is not an optimal model to analyze host–pathogen interaction at 37 °C.

Among insects, there are two species largely used as model hosts to study microbial virulence, *Drosophila melanogaster* and *Galleria mellonella*.²² *Drosophila melanogaster* is a fly that has been used in research for decades. Its immunity depends mainly on physical barriers, and on both cellular (hemocytes), and humoral (Toll and Imd pathways) responses, that induce the production of antimicrobial peptides and ROS.²³ Investigation with *Drosophila melanogaster* has elucidated some of the main elements of the immunity against pathogenic microorganisms, such as the Toll receptors, which were identified for the increased susceptibility of KO flies lacking this receptor to *Aspergillus fumigatus*,²⁴ a discovery that was awarded the Nobel Prize of Physiology or Medicine in 2011. Pathogens can be introduced in the flies aerosolized, by microinjection, or administered in the food. The development of genetics and possibility to obtain knockout strains make *D. melanogaster* also a suitable model to investigate the role of host elements in the response against microbial pathogens.²⁵

The other insect that is currently widely used to investigate microbial virulence is the lepidopteran *Galleria mellonella*.^{26–28} The life cycle of this organism comprises a larval stage (size around 1–3 cm) that transform into pupae and finally into moth. The size of the larvae makes easy their manipulation and injection. Survival monitoring is also very convenient because when they die, they become unresponsive to physical stimuli and acquire a dark color due to strong melanization. In addition, it is possible to easily administer accurate doses of antimicrobial compounds to test toxicity and in vivo efficacy. Immune response of this insect is mainly based on the presence of hemocytes with phagocytic activity, on antimicrobial peptides and on the induction of melanization. Furthermore, different routes of infection can be performed, such as direct injections in the hemocoel, or by ingestion after placing the pathogen in the food. *Galleria mellonella* is becoming a reference model to investigate microbial pathogenesis, such as the role of virulence factors in disease and efficacy of antimicrobial compounds. But this model can be used to investigate more complex aspects of immunity and virulence. Dr Vilcinskas' group has elegantly demonstrated that this lepidopteran can be used to investigate specific features of microbial disease, such as brain infection caused by *Listeria monocytogenes*²⁹ (comment in ref. 2). The article by Freitak on maternal transfer of immunity to the offspring¹ illustrates another example of the versatility of non-mammalian models to investigate relevant aspects of immunity, and applies to different fields, from entomology to immunity. Furthermore, this work opens new perspectives and research lines (such as the investigation of the susceptibility to infection of worms derived from mothers exposed to pathogens), a matter that could be addressed using other models, such as *C. elegans* or *D. melanogaster*. But furthermore, this is an exciting article from an intellectual point of view, because it suggests a mechanism of natural selection of microbe-resistant worms through evolution not based on the acquisition of specific genes or mutations.

Finally, we would like to stress that at the moment, despite the bioethical issues associated with animal experimentation, full replacement of classical models does not seem to be an option, since there is still a need to validate the use of non-conventional hosts to fully understand how much information obtained with them correlates with the results observed in more complex organisms. But the use of “non-conventional” models to investigate immunity to microbes is an emerging field, and the number of articles in which virulence and immunity is assessed in these models and not in “classical” animals, such as rodents, is increasing. For this reason, we believe that this type of host should be designated in the future as “alternative” models as opposed to the term “non-conventional”.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

O.Z. is funded by grant SAF-2011-25140 from the Spanish Ministry for Economics and Competitiveness. N.T.-C. is supported by a FPI fellowship (reference BES-2012-051837).

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