frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 04 February 2015
doi: 10.3389/fmicb.2015.00038

Animals devoid of pulmonary system as infection models in the study of lung bacterial pathogens

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Biological disease models can be difficult and costly to develop and use on a routine basis. Particularly, *in vivo* lung infection models performed to study lung pathologies use to be laborious, demand a great time and commonly are associated with ethical issues. When infections in experimental animals are used, they need to be refined, defined, and validated for their intended purpose. Therefore, alternative and easy to handle models of experimental infections are still needed to test the virulence of bacterial lung pathogens. Because non-mammalian models have less ethical and cost constraints as a subjects for experimentation, in some cases would be appropriated to include these models as valuable tools to explore host–pathogen interactions. Numerous scientific data have been argued to the more extensive use of several kinds of alternative models, such as, the vertebrate zebrafish (*Danio rerio*), and non-vertebrate insects and nematodes (e.g., *Caenorhabditis elegans*) in the study of diverse infectious agents that affect humans. Here, we review the use of these vertebrate and non-vertebrate models in the study of bacterial agents, which are considered the principal causes of lung injury. Curiously none of these animals have a respiratory system as in air-breathing vertebrates, where respiration takes place in lungs. Despite this fact, with the present review we sought to provide elements in favor of the use of these alternative animal models of infection to reveal the molecular signatures of host–pathogen interactions.

Keywords: alternative model, pneumonia, zebrafish, *C. elegans*, *Drosophila melanogaster*, *Galleria mellonella*, tuberculosis, cystic fibrosis

INTRODUCTION: A GOOD ANIMAL MODEL

To study the pathology, host immune response and the complex interactions between host and pathogen, the use of animal models have been invaluable, but for many years, it have presented strong public, scientific concerns, as well as philosophical contradictions (Lipscomb et al., 2010). From the great contributions of Luis Pasteur and Robert Koch in the use of animal models to decipher the causal agents of several diseases, including *Bacillus anthracis*, *Mycobacterium tuberculosis* or the rabies virus, concepts related to the animal use and handle and bioethics have arise (Baumans, 2004). Russell and Burch (1959) developed the "Three Rs" principle (Replacement, Reduction, and Refinement), although their work remained largely ignored well into the 1970s (Balls and Halder, 2002). At this time, rodents were considered despicable animals and consequently they were treated without consideration, wide spreading its use as research model (Damy et al., 2010). In 1999, the Declaration of Bologna reaffirmed that "humane science is a prerequisite for good science, and is best achieved in relation to laboratory animal procedures by the vigorous promotion and application of the Three Rs" (Balls, 2009). In 2002, the genome sequence of mouse was completed (first mammal after humans; Watkins-Chow and Pavan, 2008). This fact largely contributed to the great use of mouse as animal model. However, and taken into account the principle of Replacement, several alternative models far different from the mammals classic ones, have been developed in the last years (Hendriksen, 2002).

A perfect animal model would be a model that satisfied not only scientific, but also ethical criteria (Zak and O'Reilly, 1993). Among the most controversial experimental animal models from the point of view of ethics, lung infections induced by bacteria are considered by far the most aggressive. For instance, several pathogens are able to kill non-human mammals due to lung infections. For this reason, these models need to be optimized to better reproduce human infections and acute lung damages. In addition, when an animal model is used in preclinical research, we have to consider that not all results will be successfully extrapolated from animal studies to humans (Fuchs et al., 2009; Evans et al., 2010). Some important anatomical, physiological, genetic, and molecular differences are clearly present between species.

The pathogenesis of infection is a result of the balance of the host–pathogen interaction (Mason et al., 1995). The pathogen and host genetic backgrounds are a relevant determinant of this outcome, as continuously several genes are activated or repressed depending on environmental changes during experiments. For many years it was thought that mammalian models of infection were the unique choice to study host–pathogen interactions as well as for pre-clinical evaluation of vaccines and drugs before their use in humans (Means and Aballay, 2011). However, there

are presently numerous scientific data that have been argued to the more extensive use of several kinds of alternative infection models, such as, small vertebrates, insects, and nematodes (Kurz and Ewbank, 2007). The present review summarizes, compare and discuss the published experience in classical animal models, such as mice, and alternative animal models, particularly the zebrafish (*Danio rerio*), *Caenorhabditis elegans* and the insects *Drosophila melanogaster* and *Galleria mellonella* (the greater wax moth) in the study of bacterial agents which are considered the principal causes of lung injury.

ANIMAL MODELS TO STUDY BACTERIAL LUNG PATHOGENS, SAFETY AND ETHICAL ISSUES AND THE NEED OF ALTERNATIVE METHODS

OVERVIEW OF MAMMALIAN MODELS FOR RESEARCH ON PULMONARY INFECTION

The respiratory system of most non-human mammals mimics in general a lung environment in humans, in terms of chemical and physical conditions and spatial structures. In addition, the pulmonary defenses to respiratory infections in non-human mammals are somehow similar to humans. Hence, most of the pneumonia animal studies are carry out in mammals to facilitate some types of investigation (Mizgerd and Skerrett, 2008). However, extrapolation of results to humans is not straightforward owing to significant anatomical and physiological differences between species. The different mammals do not appear to present similar mucociliary clearance and alveolar macrophage morphometry (Fernandes and Vanbever, 2009).

To produce experimental pulmonary infection, mammalians offer a wide diversity of inoculation routes [reviewed and compared in Bakker-Woudenberg (2003)], being intranasal (i.n.) and intratracheal (i.t.) inoculations the infection routes seem to be the most "naturally acquired." The intratracheal model of infection requires a complex and invasive technique for disease induction but offers the advantage of allowing almost total delivery of the bacterial inoculum to the lungs. The model of infection through intranasal aspiration is the most commonly used, as it is fast and easier to perform without invasive surgical procedures and also because it mimics the natural route of infection in humans. When the inoculum is administered intranasally it could be applied as an aerosol (passive inhalation) or as nasal droplets. The intranasal aerosol model instead requires an exposure chamber with a nebulizer, and permits the simultaneous infection of several mice (Mizgerd and Skerrett, 2008). However, aerosol studies carry the greatest potential risk of infection with airborne pathogens (Chen et al., 2011), the inhaled dose varies considerably and the equipment to perform the infection is not always available in research laboratories. Most of these routes may require anesthetize animals and sometimes post-administration of pain relief drugs.

Rodents, more than other animals, are commonly used to study pneumonia. Mice are the most used in infection experiments and offer many advantages, including that they are relatively inexpensive, easy to maintain, easy to handle and their genome can be manipulate (Denny, 2005; Leung et al., 2013). Despite of this, surgical and invasive techniques are required to reproduce some acute or chronic lung diseases in mice. However, the use of general anesthesia with their side effects in host is considered as a great disadvantage of these techniques (Mizgerd and Skerrett, 2008). Guinea pigs, rats and hamsters apart of mice, are some of the other animals employed as models of lung infections (Bakker-Woudenberg, 2003).

There are well-documented relevant differences relative to lung infection between mice and humans. Differences in the structural anatomy, cellular composition of the tracheobronchial epithelium, local phagocytic and chemical defenses, inflammatory or immune response, need to be analyzed at the moment of interpreting the experimental results. Mice are different in terms of their lower complexity of the airway branches and a relatively large caliber of the airway lumen (Irvin and Bates, 2003). Furthermore, they have different cellular expression and ligand binding for selected Toll like receptors (TLRs; Mizgerd and Skerrett, 2008; Apt and Kramnik, 2009). In contrast with humans, this species has a well-developed broncus associated lymphoid tissues (BALTs) system, may be due to their life habits. In healthy humans, this system is practically absent (Hyde et al., 2009). However, the availability of immunological reactives as well as several mice transgenic lines has made their use almost indispensable in the majority of infection studies.

Mice are resistant (not susceptible) too many human pathogens (Lyons et al., 2004; Di Pietrantonio and Schurr, 2005; Aziz et al., 2007). Consequently, in addition to rodents, several other mammalian animal models have been explored in respiratory diseases studies: cattles, goats, sheeps, pigs, dogs, and non-human primates. The fact that non-human mammalian species are more phylogenetically related to humans justifies at least in part the use of these species by researchers. However, none of them completely reproduces all the aspects of lung diseases in humans. The most clinically relevant model has been the primate infection models, due to the high genetic and structural similarity with humans (Bem et al., 2011; Kaushal et al., 2012). Nonetheless, the cost, time, logistic and ethical considerations and the risk of zoonoses of this model mean that it can only be used in a limited fashion.

Despite the simplicity of their immune system, and their evolutionary distance to human, some non-mammalian models using small animals (fishes, nematodes, and insects) are characterized by their short generation time which redundance in the low cost of experiments. Curiously none of these animals have a respiratory system as in air-breathing vertebrates, where respiration takes place in lungs. However, such models have allowed successful screening for virulence genes in the most common bacterial lung pathogens (**Table 1**).

ZEBRAFISH (*Danio rerio*) AS AN ALTERNATIVE VERTEBRATE MODEL FOR STUDYING LUNG INFECTION AGENTS

In adult fish, respiration takes place mainly through the gills. In embryo zebrafish gill development begins by 3 days post-fertilization, in the meantime cutaneous respiration accounts for nearly all gas exchange (Rombough, 2007). Naturally, zebrafish get infected by pathogens through the digestive route, the damaged fish surface or through the gills (Cantas et al., 2012). Over the past decade, several bacteria and viruses have been studied in their ability to infect zebrafish (Martin and Renshaw, 2009).

Table 1 | Most significant contributions of alternative animal models for the study of some relevant lung pathogens.

Lung pathogen	Alternative model	Relevant contribution to pulmonary infection in mammals	Reference
Streptococcus pneumoniae	Zebrafish embryos	Pneumococci evade immune clearance by interfering with phagocytic functions.	Rounioja et al. (2012)
	Adult zebrafish	Virulence attenuated mutants are defective in polysaccharide capsule, autolysin, or pneumolysin.	Saralahti et al. (2014)
	Galleria mellonella larvae	Non-capsulated and pneumolysin defective strains were less virulent than their respective wild types. The role of antimicrobial peptide activity and resistance has been addressed.	Evans and Rozen (2012)
Staphylococcus aureus	Zebrafish embryos	Role of Macrophages in the ingestion of *S. aureus* during *in vivo* infections.	Prajsnar et al. (2008)
	Caenorhabditis elegans	Several genes encoding virulence factors, including biofilm-related, identified in *S. aureus* were relevant for mammalian pathogenesis.	Sifri et al. (2003, 2006), Begun et al. (2005, 2007)
	G. mellonella larvae	Both bacterial glycolysis and gluconeogenesis have important roles in virulence.	Purves et al. (2010)
Mycobacterium tuberculosis	Zebrafish infected with *Mycobacterium marinum*	Infection with *M. marinum* induces host proteases in epithelial cells and enhances recruitment of macrophages in order to promote granuloma formation to facilitate bacterial dissemination.	Volkman et al. (2010)
	Zebrafish infected with *M. marinum*	The importance of Th2-type response in controlling mycobacterial infection.	Hammarén et al. (2014)
	Drosophila melanogaster infected with *M. marinum*	Model to reveal the relationship between phagocytes and bacteria.	Dionne et al. (2003)
Pseudomonas aeruginosa	*C. elegans* (Slow killing)	Positive correlation between *P. aeruginosa* genes required to kill *C. elegans* and those for pathogenesis in mammals.	Tan et al. (1999a)

(Continued)

Table 1 | Continued

Lung pathogen	Alternative model	Relevant contribution to pulmonary infection in mammals	Reference
	C. elegans (Fast killing)	The diversity of toxic molecules produced and released by *P. aeruginosa* facilitates its pathogenicity and contributes to impair lung function in CF.	Cezairliyan et al. (2013)
	Zebrafish embryos	The T3SS, biofilm formation and quorum-sensing systems are involved in virulence, and these systems correlate with increased *P. aeruginosa* virulence in murine models and in humans.	Clatworthy et al. (2009)
	Zebrafish embryos	Helped to support a connection between the cystic fibrosis transmembrane conductance regulator (CFTR) and the innate immune response.	Phennicie et al. (2010)
	D. melanogaster	The model allowed *in vivo* study of *P. aeruginosa* biofilm infections.	Mulcahy et al. (2011)
	G. mellonella larvae	Identification of mammalian virulence factors of *P. aeruginosa*, particularly biofilm-related genes.	Jander et al. (2000)
Burkholderia pseudomallei	*C. elegans*	Disruption of calcium signal transduction, a second messenger in the epithelial response to bacteria, as mechanism for nematode neuromuscular intoxication caused by *Burkholderia spp.*	O'Quinn et al. (2001)
	C. elegans	Identification of virulence factors further validated in an intranasal infection model in BALB/c mice.	Gan et al. (2002)
	D. melanogaster infected with *Burkholderia thailandensis*	Potential model host to study the role of innate immunity in melioidosis.	Pilátová and Dionne (2012)
Stenotrophomonas maltophilia	Zebrafish adults	Abundance of protein Ax21, a quorum-sensing factor, proved correlation to mortality in the zebrafish infection model. This protein triggers innate immunity in both plants and animals.	Ferrer-Navarro et al. (2013)
	C. elegans (Slow killing)	Diffusible signal factor (DSF) that controls cell–cell communication, is involved in virulence, biofilm formation, and motilities.	Huedo et al. (2014)
	G. mellonella larvae	The model confirms protease StmPr1 as relevant virulence factor of *S. maltophilia*.	Nicoletti et al. (2011)

A major advantage for its use has been that during the first days after fertilization (≈48 h until hatching) the embryos look transparent and until 3 weeks the larvae are quite translucent (Ali et al., 2011). Therefore, it is possible to follow in real time the progression of infected living embryos, using fluorescent techniques (Redd et al., 2006). However, adult fishes are gaining recognition as a model for bacterial infections because they possess a fully developed adaptive immune system (Meijer and Spaink, 2011).

The second main advantage of this model is the great possibilities that it offers for genomic and large-scale mutant analysis. Zebrafish genome is already available (Ramachandran et al., 2010) and quite well-annotated (ZF version 9; http://www.ensembl.org/Danio_rerio/Info/Index). More than 26,000 genes encoding proteins have been sequenced and annotated, showing high conservation between innate and adaptative related genes with the respective orthologs in humans (Meeker and Trede, 2008; Cui et al., 2011; Rauta et al., 2012).

Most of the mammalian immune system components and molecules have been identified in zebrafish or in other teleost species (Mitra et al., 2010; Alejo and Tafalla, 2011; Xu et al., 2012; Page et al., 2013), including a population of antigen-presenting cells very similar to the mammalian dendritic cells (Lugo-Villarino et al., 2010). Innate immunity is functional, with macrophages and neutrophils that are active at 48 h post-fertilization. These species have an active complement system which can be started /initiated by the same ways presents in mammals (Holland and Lambris, 2004; Sunyer et al., 2005). The adaptive immune system also consists of T cells and B cells although the main site for antigen presentation and T cell maturation is the spleen. Furthermore, multiple waves of hematopoiesis in zebrafish occur at distinct anatomical sites analogous to mammalian hematopoiesis (Kanther and Rawls, 2010).

Renshaw et al. (2007) established a model of inflammation, injuring to the zebrafish tailfin and inducing a characteristic neutrophilic inflammatory response, which resolves with a similar kinetics as in mammals. These authors defined a new model for *in vivo* study of inflammation resolution and their link with apoptosis (Renshaw et al., 2007). By other way, Benyumov et al. (2012) developed an *in vivo* zebrafish model to test phenotypic differences between human fibroblasts that participate in physiological and pathological process.

Additionally, zebrafish has been considered as replacement method for animal experiments because they present some characteristics such as high rate of fecundity, small size, easy maintenance, fast development and less stringent regulatory and ethical considerations since it has been considered that fish embryos in early developmental stages do not experiment pain, suffering, or distress. Although the ethical constraints become apparent, one study suggests that experiments with zebrafish should be subject to regulation from 5 days post-fertilization onward (Strähle et al., 2012) since is between days 5 and 6 when larvae start to feed. Thus, an animal protocol should be required to infect zebrafish older than the time the animals become free feeding. On the other hand, some recent studies say that common anesthetics are not the most "human" or humanize option for zebrafish euthanasia and could cause animal suffering (Cressey, 2014), adding evidences that it is necessary to minimize distress or death.

As in preclinical researches with mammalian models, adult animals should be allocated for several days under special conditions before beginning the experimental procedures, in order to reach their adaptation to new laboratory conditions and to recover from stress (**Figure 1**). When zebrafish embryos are used, this time is relatively short because they develop very rapidly. To reach a pathogenic dose, as for traditional infection in mice models, zebrafish infection involves a single dose of bacteria requiring an initial population of pathogens able to proliferate avoiding, long enough, the detection by immune cells. However, when adult fish are less susceptible hosts for bacterial infection, higher amount of live bacteria are required when compared to infection in mice. For instance, intraperitoneal infection of zebrafish with the

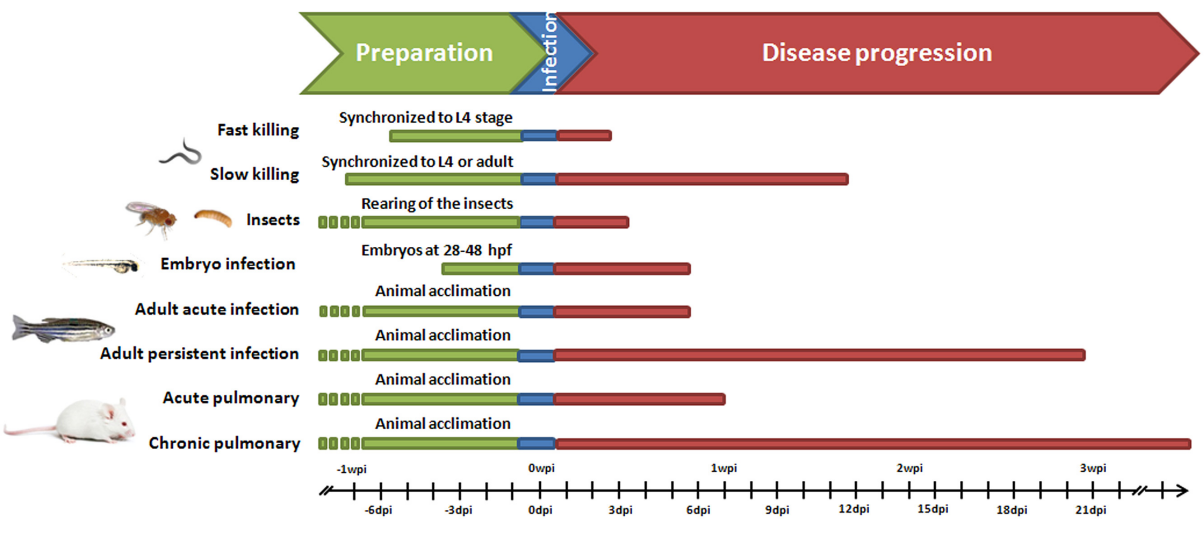

FIGURE 1 | Time course of implementation of mice and alternative models for bacterial infections. Persistent infection in adult zebrafish refers to a latent disease *Mycobacterium marinum* model (Parikka et al., 2012).

Pseudomonas aeruginosa PAO1 strain or with *Stenotrophomonas maltophilia* clinical isolates reported median lethal dose (LD_{50}) values of approximately 5×10^7 cfu/dose or 5×10^8 cfu/dose respectively (Ferrer-Navarro et al., 2013; Huedo et al., 2014; Ruyra et al., 2014). Infection models in zebrafish usually are conducted within the time frame of days rather than weeks to months (**Figure 1**), but in the adult persistent mycobacterial infection model a timeline of progression of infection could reach to weeks post-infection (Cronan and Tobin, 2014; Hammarén et al., 2014). Also in zebrafish embryos and larvae, *P. aeruginosa* requires higher dose of pathogens to establish a virulent infection because many of these pathogens are killed by macrophages and neutrophils (Brannon et al., 2009).

Caenorhabditis elegans AS A NON-VERTEBRATE MODEL FOR STUDYING OF LUNG BACTERIAL AGENTS

In 1963 Sydney Brenner proposed the nematode *C. elegans* as an experimental organism for pursuing research in developmental biology and neurology (Ankeny, 2001). *C. elegans* has fewer than 1000 body cells when completely grown. *C. elegans* possesses remarkable advantages that make it an ideal model, for example, low cost, simple growth conditions, and a short generation time with an invariant cell lineage. By other way, at present, there are many molecular and genetic methods for its manipulation (Kurz and Ewbank, 2000). The genome of *C. elegans* was completely sequenced at the end of 1998 (Schulenburg and Ewbank, 2007). In comparison with mice, is obvious that culture and maintenance of *C. elegans* is far simpler and cheaper. Besides, ethical regulations are practically absent for experimentation with worms. Regarding to cell cultures, *C. elegans* contaminated stocks are easily identified and cleaned better than mammalian contaminated cells (Hulme and Whitesides, 2011).

Fascinatingly, this worm shares similarity to mammalian immune system, particularly signaling cascades in innate immunity in response to pathogen invasion (Alper et al., 2008). Because nematodes consume microorganisms as their food source, there is presumably selection pressure to evolve and maintain immune defense mechanisms (Kim et al., 2002; Alper et al., 2010; Shivers et al., 2010). While the human lung and the nematode intestine clearly differ in several anatomical, cellular and biochemical characteristics, in the nematode intestine it is possible to identify pathogen-specific virulence factors that interact with epithelial surfaces. To reinforce the wide field of applications of such non-vertebrate models, the study carried out by Green et al. (2009) describe, despite of the absence of lungs in the nematode, a practical model to evaluate the impact of the cigarette smoke exposure on innate immunity. In this study, *C. elegans* responded to nicotine, which is one of the main components of cigarette smoke, converting nicotine to cotinine, in a similar manner to mammals and opening the path to demonstrate that the animals are absorbing the smoke. In this model, *C. elegans* was subjected to whole cigarette smoke exposure, overcoming several aspects impossible to elucidate using human epithelial cell lines, showing down-regulation in many genes in response to the smoke, mainly several host defense genes (Green et al., 2009).

Additional advantage of using nematodes as animal model of bacterial infection lies in reduced experimental time without the need of animal acclimation before infection, and time-kill curves taking only few hours (fast killing) or days (slow killing) post-infection depending on the killing mechanism (**Figure 1**). However, nematodes need to be synchronized about 1 week prior to infection to reduce the variation in results associated to differences of age. There are two methods to synchronize worms: egg preparation via bleaching and egg lay (Sulston and Hodgkin, 1988). The first method produces more progeny than the latter; however, egg lay can generate a better synchronized population. Scientists take also advantage of temperature-sensitive sterile *C. elegans* mutants (e.g., the CF512 strain) to avoid production of a brood that would complicate the scoring of death of the infected worms. These animals do not produce progeny at working temperature (25°C), thus simplifying the procedure. However, these strains appear to be slightly more resistant to bacterial infections (Feinbaum et al., 2012).

More than 40 human pathogens, or their close relatives, are known to cause disease in *C. elegans* (Sifri et al., 2005). *P. aeruginosa* was the first broad host-range pathogen, able to kill *C. elegans* (Tan et al., 1999a,b). *C. elegans* infection process has the advantage to closely resemble chronic infection, because the host is usually exposed to maximal dose of pathogen. On the other hand, among killing mechanisms, slow killing involves a process similar to those related to an infection process. At present, there are five distinctive mechanisms of worm killing identified: infection with intestinal colonization, persistent infection, invasion, biofilm formation, and toxin-mediated killing (Smith et al., 2002; Beale et al., 2006; Dunbar et al., 2012). Obviously, in the *C. elegans* model will be possible to study only those human diseases caused by pathogens able to infect the nematodes. In addition to the bacterial species, the killing mechanism is in most cases dependant to the way the bacterium is prepared prior to infection. For instance, depending on the composition of the agar medium where the bacterium is grown, the rate of *P. aeruginosa*-mediated killing of *C. elegans* will be different (Tan et al., 1999a). If the *P. aeruginosa* tested strain is grown on minimal medium, the killing will occur in the course of several days; by the contrary, if a high-osmolarity medium is used, the killing will occur in the course of several hours.

INSECT AS MODELS OF INFECTION

In opposite to mammals, the respiration process in insects occurs by a network of tubes called tracheae and tracheoles. Despite this, insects have recently been shown to be a valuable alternative to animal models for bacterial pathogenesis studies. This is mainly due to the fact that insects have a relatively advanced system of antimicrobial defenses. Like mammals, insects possess a complex innate immune system and display evolutionary conservation of signaling cascades (Lemaitre and Hoffmann, 2007). In addition, as with other non-vertebrate models, the advantages are the low cost of maintenance and no ethical concerns. There are multiple genetic and molecular tools available. By other way, these models have a precise endpoint. The low cost of maintenance and the rapid development, allow their use in high animal number for proper statistical analysis of results. Among these models, the common fruit fly *D. melanogaster* and the larvae of *G. mellonella*, have been

shown to be relevant for several fungal and bacterial mammalian pathogens (Limmer et al., 2011; Sprynski et al., 2014).

Most insects have a very rapid life cycle, which consists of four clearly defined stages: the embryo, the larva, the pupa, and the adult. In addition, insect rearing is easy and relatively cheap (Ramarao et al., 2012). For larva, drugs can be administered directly injecting the organism or mixing with media (solid or liquid with 2% yeast paste). For adults, drugs may be delivered as aerosol, mixed with food, injected or applied directly to the nerve cord. In the injection method, a needle or a nanoinjector preloaded with pathogen culture is used to prick the body cavity (insect hemocoel). Injection requires anesthetization, which is usually done with carbon dioxide, and requires the transfer of insects into vials containing food, where the worms incubated at 25–30°C and their survival is evaluated (Igboin et al., 2012). For ingestion, it is common to introduce the insects into small laboratory tubes containing filter disks embedded with media containing pathogens of interest.

Galleria mellonella larvae are cost effective, widely available and the results can be obtained within 2 or 3 days (**Figure 1**). There are three main ways in which *Galleria* fight bacterial infections: circulating phagocytic hemocytes that patrol the hemolymph; proteolytic cascades that can be quickly triggered, activating the melanization response and inducing antimicrobial immune effectors such as lysozymes, as well as antimicrobial peptides which can be rapidly synthesized by the fat body (Yuen and Ausubel, 2014). An added benefit of using *Galleria* for pathogenesis studies is that infections can be carried out at 37°C or higher, as *Galleria* tolerates relatively high temperatures, unlike the Zebrafish, *D. melanogaster* and *C. elegans* (maximum 25–28°C; Glavis-Bloom et al., 2012). The larger size of the *Galleria* larva, compared to other invertebrate models, also allows it to be infected with larger and more controlled doses of the pathogen without significantly traumatizing the insect. Some disadvantages of the *G. mellonella* model rely in the fact that genetic methods for generating recombinant organisms and to sequence them are not completely available. However, this model could be improved in the next years and hopefully, could be used for more pathogens, for which no alternative models of infection exist.

FROM CLASSIC TO ALTERNATIVE MODELS IN STUDYING RELEVANT BACTERIAL LUNG PATHOGENS

The most common causes of bacterial lung infections in normal human hosts include *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*, and the recent increase of *M. tuberculosis*. Pneumonia is classified according the source of infection into community-acquired pneumonia (CAP), hospital-acquired or nosocomial, aspiration of foreign material and immunocompromised host (Woodhead, 2013). In basis of their presentation, pathogens have been classified into "typical" and "atypical." Typical organisms in CAP include *S. pneumoniae*, *H. influenzae*, *S. aureus*, *Moraxella catarrhalis,* and *P. aeruginosa* (Musher and Thorner, 2014). Atypical organisms include *Legionella* species, *Mycoplasma pneumoniae, Burkholderia spp.*, *Chlamydia spp.*, *Chlamydophila spp.*, *Coxiella burnetii* and viruses (Jones, 2010). In almost all epidemiological studies of hospital-acquired pneumonia (Jones, 2010; Barbier et al., 2013; Polverino et al., 2013; Erdem et al., 2014), a consistent six organism groups (*S. aureus*, *P. aeruginosa*, *Klebsiella* species, *Escherichia coli*, *Acinetobacter* species, and *Enterobacter* species) caused ∼80% of episodes, with lower prevalences of *Serratia* species, *S. maltophilia*, and community-acquired pathogens, such as pneumococci and *H. influenzae*. In compromised hosts, the bacterial causes of pneumonia are much broader, including species not usually considered of high virulence in humans. For instance, *Mycobacterium sp.*, *Burkholderia spp.*, *P. aeruginosa,* and *S. aureus* are the most important infectious agents in cystic fibrosis (CF) patients (Coutinho et al., 2008).

For the most frequent bacteria causing pneumonia, scientists have developed animal models of infection, mainly using mice (**Figure 2** and **Table 1**). However, the introduction of alternative non-mammalian models is still at its beginning and obviously for host-permissive pathogens the contribution would be higher. For instance, despite several mouse *M. tuberculosis* lung infection models are utilized, and *Mycobacterium marinum* infection of fishes results in chronic granulomatous diseases similar to mycobacterioses in mammals (Cosma et al., 2006b), *C. elegans*, a well-established model host, is resistant to mycobacterial infection (Couillault and Ewbank, 2002). The most extreme example is that of pathogens for which there are no or very few alternative models of infection, such as *H. influenzae*, *M. catarrhalis,* and *M. pneumoniae* (**Figure 2**). On the other side as will be discussed later, *P. aeruginosa*, a versatile and ubiquitous bacterium, is capable to survival and colonize various living host organisms facilitating the development of infection models spanning from nematodes to small vertebrates (**Figure 2**). Here, we discuss the models that have been developed for studying most common human lung pathogens by comparing the mouse model with alternative ones in zebrafish, nematodes, and insects.

ANIMAL MODELS OF PNEUMOCOCCAL INFECTION

Streptococcus pneumoniae is considered the most common bacterial agent in CAP and a great number of animal models of pneumococcal diseases are available. Some review articles from the past decade have specifically mentioned the value of animal models to test pneumococcal vaccines (Briles et al., 2000; Adamou et al., 2001; Bogaert et al., 2004; Kadioglu and Andrew, 2004; Morsczeck et al., 2008) and the use of murine models of pneumonia to evaluate protein-mediated antimicrobial responses (Srivastava et al., 2007), the mouse genetic susceptibility to pneumococcal disease (Proft and Fraser, 2003) and for investigating the pathophysiology of bacterial meningitis (Teles et al., 1997; Gerber et al., 2001; Blair et al., 2005; Endo et al., 2012).

On the other hand, as there are several evidences about the positive correlation between infection with *Streptococcus iniae* and *Streptococcus pyogenes* in zebrafish and mammalian models, there is simple to assume the use of zebrafish to evaluate host–pathogen interactions during pneumococcal infection (Borst et al., 2013). In a recent work, Rounioja et al. (2012) reported the use of zebrafish embryo to evaluate the response of the host immune system against challenge with pneumococci. They also reported that this response is dependent on whether the pneumococci could evade clearance by interfering with host phagocytic

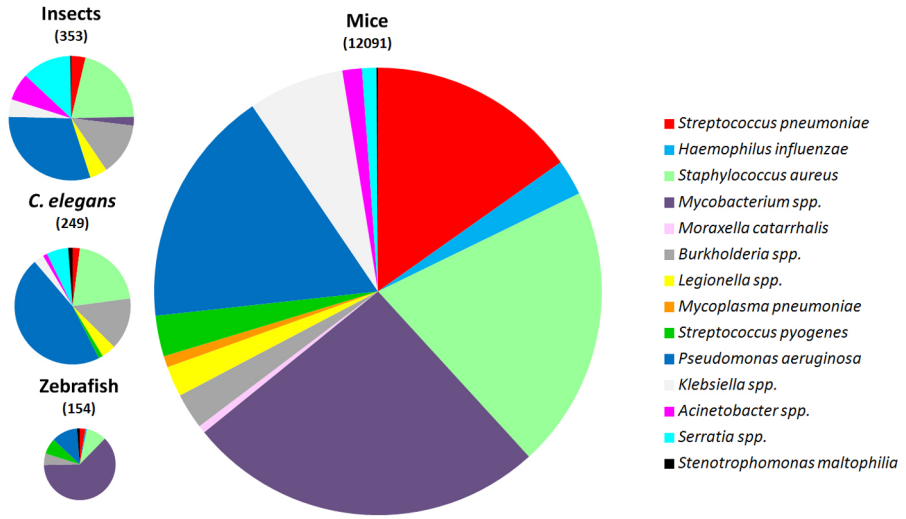

FIGURE 2 | Comparative number of publications for different lung pathogens employing mice, *Caenorhabditis elegans*, zebrafish, and insects as animal models of infection. The number of publications is shown between parentheses. A systematic search was conducted using PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Literature searches were conducted to identify studies published until 1 November, 2014.

function. Moreover, pneumococcal mutants defective in important virulence factors were attenuated in this *in vivo* model system (Rounioja et al., 2012). The adult zebrafish model can be also used to investigate pneumococcal diseases (Saralahti et al., 2014). The authors showed that *S. pneumoniae* mutants defective in polysaccharide capsule are also attenuated and that elimination of pneumococci depends mainly on host innate immune responses.

To our knowledge, little is the information available up to day related to the use of *C. elegans* to study pathogenesis of *Streptococcus*. Garsin et al. (2001, 2003), in two separate works, demonstrated the suitability of using *C. elegans* as a model host for Gram-positive infection, including *Enterococcus faecalis*, *S. aureus*, and *S. pneumoniae*. Jansen et al. (2002) demonstrated that *S. pyogenes* kills *C. elegans*, both on solid and in liquid medium, mediated by the hydrogen peroxide production (Jansen et al., 2002; Bolm et al., 2004).

The potential application of the larvae of *G. mellonella* as an informative infection model for *S. pneumoniae* has been also studied (Evans and Rozen, 2012) since strains differing in known virulence factors could be distinguished in this host. Strains lacking capsule or pneumolysin showed less virulence than their respective wild types. Particularly, pneumolysin plays a role in damaging human lung epithelium, allowing the establishment of infection (Rayner et al., 1995).

ANIMAL MODELS OF STAPHYLOCOCCAL INFECTION

As nasal colonization is a main requisite for the establishment of *S. aureus* infection in humans (Baur et al., 2014), mice infected by using this route is a useful model for characterizing early host responses. However, this model has failed in mimic the whole natural route of infection, resulting in self-limited disease (Alami et al., 1968; Bartell et al., 1968; Anatoliĭ et al., 1971; Ansfield et al., 1977; Bragonzi et al., 2004; Bloemendaal et al., 2011). The Bolus infection models, where mice are challenged by i.t. and i.n. inoculation have been more successful in producing intrapulmonary infection and host mortality (Jakab, 1976; Hu et al., 2006; Rajagopalan et al., 2006; Huzella et al., 2009; Fernandez et al., 2011; Park et al., 2011). Sawai et al. (1997) described a murine model of acute staphylococcal pneumonia inoculating mice by intravenous (i.v.) injection of a suspension containing bacteria enmeshed in agar-beads. This model allows the bacteria to remain in the lung for several weeks, and it is reproducible and simple (Sawai et al., 1997).

Li and Hu (2012) developed a zebrafish embryo infection model with *S. aureus* at 36 h post-fertilization. These researches inoculated the bacteria directly into the pericardial cavity, eye, and yolk body. By using GFP-expressing *S. aureus* and transgenic zebrafish lines along with multicolored confocal fluorescence methods, they could analyze different phases of bacterial infection. As important conclusion from this work, the dynamic of infection clearly depends on the bacterial entry routes (Li and Hu, 2012). Prajsnar et al. (2008), using a similar model, identified staphylococcal virulence genes, whose respective mutants are attenuated in zebrafish. The virulence factors include a peroxide regulon repressor, a protein involved in starvation survival and a response regulator involved in controlling exoproteins production. They also demonstrated that in zebrafish embryos, macrophages phagocytize *S. aureus* during *in vivo* infections. Accordingly to Kubica et al. (2008), these immune cells act as a reservoir during infection. This model in both zebrafish embryos and adults also allowed rapid screening of mutants for those strains with attenuated pathogenicity, identifying relevant factors of pathogen virulence and host immunity (Lin et al., 2007; Li and Hu, 2012; Lü et al., 2012).

On the other hand, several studies used the *C. elegans* model to assess virulence levels between some different methicillin-resistant *S. aureus* strains, demonstrating the suitability of this

model for studying the virulence and pathogenicity of *S. aureus* strains (Wu et al., 2010, 2012, 2013). In addition, several *S. aureus* virulence determinants recognized as important in mammalian pathogenesis are also identified as relevant for full pathogenicity in nematodes, including *agr* (a quorum-sensing global virulence regulatory system), *sarA* (global virulence regulator), the alternative sigma factor B, alpha-hemolysin, and V8 serine protease (Sifri et al., 2003, 2006). However, Polakowska et al. (2012) showed that there is no substantial variation in virulence among different staphylococcal strains using this experimental model, questioning the usefulness of it.

In another work, JebaMercy et al. (2011) performed solid and liquid assays for the infection of *C. elegans* with *S. aureus*, demonstrating that *S. aureus* took ∼90 h for the complete killing of *C. elegans* and thereby postulating that colonization with live bacteria was necessary for worm killing. Using an interactive genetic approach, Begun et al. (2007) established a novel *in vivo* experimental model to explain the interaction between the bacteria biofilm matrix and components of the innate immune system. This study demonstrates the ancient conserved function against predation linked to the protective activity of biofilms. In another work based on *C. elegans* infections, the same authors identified staphylococcal genes relevant for mammalian pathogenesis, including the product of the *nag*D gene, which was not previously described as a virulence factor (Begun et al., 2005).

Larvae of the greater wax moth also have provide insights into the pathogenesis of *S. aureus*, principally as a suitable host for testing the *in vivo* efficacy of antimicrobial agents (Gao et al., 2010; Desbois and Coote, 2011; Gibreel and Upton, 2013; Apolónio et al., 2014). In addition, using this models authors have demonstrated for the first time that both glycolysis and gluconeogenesis have important roles in virulence (Purves et al., 2010). Their results showed that two glyceraldehyde-3-phosphate dehydrogenase (GAPDH) homologs (GapA and GapB) are required for the virulent phenotype of *S. aureus* in this model. In *S. pyogenes* surface-associated GAPDH was associated with antiphagocytic properties and host cell adherence (Boël et al., 2005).

ANIMAL MODELS FOR MYCOBACTERIAL PATHOGENESIS

Due to the complex interaction between the pathogen and the host, it has been very difficult to find out an ideal model to study mycobacterial pathogenesis. Some mice strains can be easily infected via aerosol with a low dose of *M. tuberculosis*, multiplying in the lungs and subsequently spreading to liver and spleen. The infection is controlled but not eliminated, by cell-mediated immunity, mainly T cell responses, and the infection is well-tolerated for more than 1 year (Beamer and Turner, 2005; Aguilar et al., 2007, 2010; Andreevskaia et al., 2007). Hence, the mouse model has been largely a suitable infection model (Orme, 2005b; Cooper, 2014). Surprisingly, there is still no ideal and validated model of experimental tuberculosis disease (Vilaplana and Cardona, 2014), and the mechanisms leading to latency and reactivation of are still unclear (Parikka et al., 2012).

BALB/C mice infection models by i.t. injection using a high dose of bacilli (Hernández-Pando et al., 1996) is one of the most employed models. This model has greatly contributed to elucidate the role of antibodies in the protection against mycobacterial infections (López et al., 2009, 2010; Alvarez et al., 2013), and to the screening and validation of new vaccine candidates (Castillo-Rodal et al., 2006; López et al., 2006). Alternatively, C57BL mice have been infected i.n. via aerosol with a low dose of *M. tuberculosis*, which produces a well-tolerated infection dominated by Th1 response (Kelly et al., 1996; Cardona et al., 2003; Aldwell et al., 2005; Saini et al., 2012). For this reason, this is actually a model of slow progressive disease, and the animal death is produced by excessive inflammation or immunopathology (Mustafa et al., 1999a,b, 2000). The model of latent tuberculosis has been also partially reproduced experimentally in mice (Phyu et al., 1998; Shi et al., 2011; Zhang et al., 2011) which are injected intratracheally with relatively low numbers of the virulent strain H37Rv (Hernandez-Pando et al., 1998). After that, low and stable bacillary counts with few granulomas appear and the mice continue gaining weight and appear healthy for more than 2 years.

Although mice are the most employed animal model for studying human TB, it has important drawbacks. Due to the fact that *M. tuberculosis* is not a natural pathogen of mice, the pathological development of TB will be clearly different from that in human. As relevant characteristic, we can mention the absence of granulomas formation in lungs from mice (Grumbach et al., 1967; Karakousis et al., 2004; Mollenkopf et al., 2004; Orme, 2005a,b; Hunter et al., 2007; Dharmadhikari and Nardell, 2008; Young, 2009; Apt, 2011). The immune response elicited in mice after mycobacterial infection is able to control bacillary load even without causing marked lesions (Vilaplana and Cardona, 2014). Therefore, mice are generally resistant to TB infection when compared with other rodents, and even humans, as evidenced by their ability to tolerate relatively large bacterial numbers within their lungs without signs of disease (Be et al., 2008; Dharmadhikari and Nardell, 2008; De Steenwinkel et al., 2009). Also, ethically, these models in conjunct appear to be more aggressive to the mice.

Zebrafish and fruit fly are emerging as alternative models and have provided new insights into the pathogenesis of the tuberculosis disease. By the contrary, *C. elegans*, to our knowledge, seems to be not a feasible model for infection with *M. tuberculosis*. The zebrafish has been a key model in our understanding of mycobacterial infection. Studies on this model employ a fish pathogen, *M. marinum*, a close relative to *M. tuberculosis* (Tobin and Ramakrishnan, 2008). This bacterium is a natural pathogen of fish and amphibian (van der Sar et al., 2004a,b). The *M. marinum* infection produced in these hosts is quite similar to those produced in humans, mainly in the granuloma formation (Prouty et al., 2003; Berg and Ramakrishnan, 2012). Low doses (<100 bacteria) of *M. marinum* lead to a chronic infection in adult zebrafish (Parikka et al., 2012), while higher doses cause a fatal acute infection (Cosma et al., 2006b). Besides, *M. marinum* grows faster than *M. tuberculosis*, it can be easily manipulated and requires only common laboratory precautions (biosafety level 2). The zebrafish/*M. marinum* infection model changed the old conception that granuloma formation requires lymphocytes and by the contrary, postulated that the granuloma actually functions as a bacterial tool for disseminating the disease.

Experiments conducted by Ramakrishnan and colleagues using the zebrafish/*M. marinum* infection model (Davis et al., 2002;

Davis and Ramakrishnan, 2009; Ramakrishnan, 2013), demonstrated for the first time new mechanisms of bacterial dissemination: the bacterial transfer between two macrophages through membrane tethers and re-phagocytosis of bacteria associated with dead macrophages in tissues. They also observed that, as early as 72 h post-injection, the extravasated infected macrophages began to form granuloma-like aggregates in the tissues, establishing that there is not needed the participation of adaptive immunity to initiate granuloma formation (Davis et al., 2002). Using this model, and taking advantages of the powerful live imaging of the zebrafish model, it was determined that an efficient bacterial expansion depends on the mycobacterial region of deletion 1 (RD1) locus. The researchers also demonstrated that the bacterial protein ESAT6 elicits the expression of metalloproteinase 9 (MMP9) in the host, and both proteins act together for granuloma formation (Pozos et al., 2004; Volkman et al., 2004; Cosma et al., 2006a; Berg and Ramakrishnan, 2012; Takaki et al., 2013; Cambier et al., 2014). These findings showed that one protein from the host and one from the bacteria, constitute a virulence axis which evade the host's early immune responses and lead to mycobacterial dissemination. This study is, from our point of view, the most important evidence of how the zebrafish model can be used to validate and to re-postulate host–pathogen interactions during mycobacterial infection.

On the other hand, Swaim et al. (2006) reported that lymphocytes play the same critical role in controlling mycobacterial infection in fishes and mammals by the use of a defective zebrafish mutant in the *rag1* gene. They also demonstrated that bacteria defective in RD1 region are also attenuated in zebrafish. In addition, the zebrafish/*M. marinum* model has proven to be useful for studying the latency, dormancy, and reactivation of latent or subclinical tuberculosis (Parikka et al., 2012). This group has recently studied using this model the T cell responses in mycobacterial infection and they have found associations between the disease severity (bacterial load) and the type and magnitude of T cell responses, particularly an adequate Th2-type response (Hammarén et al., 2014). This infection model also helped to demonstrate that mycobacterial antigens Ag85B, CFP-10, and ESAT-6 protect adult zebrafish from mycobacterial infection (Oksanen et al., 2013), paving a new way in tuberculosis vaccine research.

Mycobacterium marinum also causes a lethal infection in the fly *D. melanogaster* characterized by a widespread tissue damage, even at significant low bacterial doses (Dionne et al., 2003). These initial stages of the infection were very similar to the early stages in frogs and fishes infected with *M. marinum* (Pozos et al., 2004). This model may be valuable in testing the activity of new antimycobacterial agents (Oh et al., 2013).

ANIMAL MODELS TO STUDY THE VIRULENCE FACTORS OF *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic human pathogen that can also infect several diverse organisms, such as plants, nematodes, insects, and mammals. Thus, we counted on good mammalian and non-mammalian models for studying virulence factors of *P. aeruginosa*. In humans, *P. aeruginosa* is widely associated with nosocomial infections in CF patients (Moreau-Marquis et al., 2008) and other immunocompromised individuals, and the resolution of the infections is hampered due to the formation of drug resistance biofilms. Until the moment, a difficult exists on studying biofilms formation in the context of animal and human lungs. However, several non-mammalian models have provided compelling data regarding *P. aeruginosa* biofilm formation (reviewed in Lebeaux et al., 2013).

Acute and chronic models of lung *P. aeruginosa* infection have been developed using several mammalian species (rats, guinea pigs, hamsters, mice, sheep, rabbits, and baboons; Seidenfeld et al., 1986; Starke et al., 1987; Pier et al., 1990; Collins et al., 1991; Iwata and Sato, 1991; Hart et al., 1993; Terashima et al., 1995; Bakker-Woudenberg et al., 2002; Luna et al., 2007, 2009; Kurahashi et al., 2009; Rodríguez-Rojas et al., 2009; Collie et al., 2013). The chronic infection model has been extensively used and characterized, showing certain similarities with human pathology due to the persistence of the inoculum and the resultant lung pathology (van Heeckeren and Schluchter, 2002). Depending on the route, dose administered, and the frequency of dosing, acute lung infection with either rapid clearance of the bacteria or acute sepsis and death could take place (George et al., 1991). Using this model, it has been shown that *P. aeruginosa* must express several key virulence factors (Balloy et al., 2007).

A literature survey about acute vs. chronic *P. aeruginosa* lung infections clearly shows that to induce an infection for more than 1 month, it is necessary to use an immobilizing agent such as agar, agarose, or seaweed alginate together with the bacterial suspension (Iwata and Sato, 1991; Hart et al., 1993; McMorran et al., 2001; Moser et al., 2002). The initial agar-beads model of chronic pulmonary infection with *P. aeruginosa* was modified for its use in mice by Starke et al. (1987) and has been widely used to study CF lung disease, bacterial pathogenesis (Gosselin et al., 1995, 1998; Morissette et al., 1995; Stevenson et al., 1995; Moser et al., 1997; Sapru et al., 1999; Tam et al., 1999; McMorran et al., 2001), and for the evaluation of new therapies (Pier et al., 1990; Staczek et al., 1998; Chmiel et al., 1999; Wilmott et al., 2000) and virulence factors (Rodríguez-Rojas et al., 2009). Researchers have also developed a mouse model with a lung pathology similar to human CF. One group generated mice that absorb excess sodium in the airways (Mall et al., 2004; Mall, 2008) and these animals developed airway obstruction with dehydrated mucus. This CF model promises to answer important questions about the cause of the inflammation that leads to lung damage and failure in CF (Mall et al., 2004; Mall, 2008). Both acute and chronic models require extensive use of animals and labor-exhausting techniques to prepare and immobilizing bacteria in agar, as well as good surgery skills.

Brannon et al. (2009) developed a zebrafish embryo infection model for the study of systemic *P. aeruginosa* infection, and for evaluating the virulence of a type 3 secretion system (T3SS) mutant. By fluorescence microscopy it was possible to follow in real time *P. aeruginosa* infection in transgenic zebrafish with fluorescently labeled neutrophils and macrophages (Hall et al., 2007). Clatworthy et al. (2009) demonstrated that lethal infection requires quorum-sensing and the T3SS for full virulence in late-stage zebrafish embryos infected with *P. aeruginosa*. Curiously, T3SS expression has been associated also to increased risk

of *P. aeruginosa* infection in hospitalized patients (Ledizet et al., 2012) and it is also associated with initial infections in patients with CF (Jain et al., 2008). They demonstrated that the infection outcome could be influenced on the pathogen side, by both the inoculum size and the presence of known virulence determinants (*las*R, *mvf*R, and *psc*D) and on the host side by developmental stage and the modulation of the immune system (Clatworthy et al., 2009). They also showed that the infection process can be modified through the use of morpholinos or antibiotics, which were used to shift immune cell numbers or rescue embryos from lethal challenge respectively.

In one study performed by Phennicie et al. (2010) in zebrafish, the role of the cystic fibrosis transmembrane conductance regulator (CFTR) in the innate immune response to acute infection with *P. aeruginosa* was evaluated. The authors found that the *P. aeruginosa* bacterial load was significantly higher in *cftr* morphants (knockdown of the zebrafish ortholog to human *cftr*) than in control embryos, according with similar studies performed with mice and human bronchial epithelial cells.

The *P. aeruginosa*-zebrafish infection model has allowed conducting chemical screens for small molecules or antimicrobial compounds. For instance, the treatment of infected embryos with front line antipseudomonal agents could save zebrafish embryos from a lethal *P. aeruginosa* challenge (Clatworthy et al., 2009). More recently, Ruyra et al. (2014) reported the use of immunostimulant-loaded nanoliposomes to protect adult fishes against bacterial or viral infections. In a model of adult zebrafish infection developed by these researchers, nanoliposomes protected zebrafish against otherwise lethal bacterial (*P. aeruginosa* PAO1) and viral (SprinViraemia of Carp Virus) infections.

Tan et al. (1999a, 1999b) conducted several studies (Tan and Ausubel, 2000; Tan, 2002) showing for the first time the use of *C. elegans* in the study of *P. aeruginosa* pathogenesis. They demonstrated that accumulation of *P. aeruginosa* cells in intestines is crucial to explain the killing mechanism. In addition, as other authors have demonstrated, they showed that bacterial genes required for this killing were also described in mammalian or plant hosts pathogenesis. Feinbaum et al. (2012) screened mutants with reduced ability to kill *C. elegans* using a mutant library representing ∼80% of the non-essential *P. aeruginosa* PA14 genes. They described a set of 180 *P. aeruginosa* genes necessary for normal levels of virulence. The principal contributors to *P. aeruginosa* virulence in the *C. elegans* infection model were genes that play key roles in survival of *P. aeruginosa* within the host intestine, particularly regulatory genes that are involved in quorum-sensing (Feinbaum et al., 2012).

Insects have been also surrogate model systems for identifying mammalian virulence factors of *P. aeruginosa*. Previous studies showed that this bacterium is a virulent pathogen of fruit flies (Boman et al., 1972). Using the *D. melanogaster* as model host, D'Argenio et al. (2001) have identified mutants of *P. aeruginosa* with reduced virulence. Among these mutants, the *pil-chp* signal transduction system is particularly relevant also in mammals and is involved in type IV pilus synthesis and biofilm formation (Kato et al., 2008). The *D. melanogaster* model allowed *in vivo* study of *P. aeruginosa* biofilm infections by oral administration (Mulcahy et al., 2011). By the other hand, several studies summarized by Jander et al. (2000) point out similarities between virulence of *P. aeruginosa* mutants in mice and *G. mellonella*. This infection model helped to demonstrate that human anti-microbial peptides that inhibited the initial steps in biofilm formation could be used in the development of new therapies for *P. aeruginosa* infection (Dean et al., 2011).

ALTERNATIVE MODELS FOR TWO LESS COMMON BACTERIAL LUNG PATHOGENS

Previous works suggest a limited invasiveness of *Stenotrophomonas maltophilia* in mice, as indicated by a transient and minimal presence of the bacteria in animal organs after infection. *S. maltophilia* CF strains were shown to cause no mortality in a neonatal mouse model of respiratory tract infection (Waters et al., 2007). Despite this lack of strong invasiveness, mouse models of *S. maltophilia* infection have been useful answering questions about immune response against this pathogen (Brooke, 2012). Additionally, a model of acute respiratory infection in DBA/2 mice inoculated with aerosolized *S. maltophilia* has allowed the study of lung pathology and the mechanisms of infection resolution (Di Bonaventura et al., 2010). However, in this model, most of the animals were able to control the infection in a short time period, even at high doses of virulent inoculums, being the animal weight the best criterion to evaluate the virulence of tested strains (Di Bonaventura et al., 2010; Pompilio et al., 2011). One study also showed bacterial colonization in rat lungs after 7 days post-infection (McKay et al., 2003).

S. maltophilia has also been isolated from channel catfish (*Ictalurus punctatus*) with infectious intussusception syndrome (Geng et al., 2010), suggesting that the use of fish as a model to evaluate the pathogenicity and susceptibility of *S. maltophilia* to available antimicrobial agents is adequate. Recently, a model of intraperitoneal infection in zebrafish confirms the attenuation of a *S. maltophilia* collection strains when compared with recent clinical isolates (Ferrer-Navarro et al., 2013), paving the way for new approaches to gain relevant information on pathogenesis of this bacterium. An infection model using *C. elegans* has been proposed for routine screening of *S. maltophila* isolates for pathogenesis (Thomas et al., 2013). In this work the *in vivo* killing efficiency was evaluated by four different methods: classical fast killing assay, filter-based fast killing assay, slow killing assay and virulence assay using heat inactivated bacteria. Moreover, virulence regulation in *S. maltophilia* mediated by a quorum-sensing system has been recently studied in the *C. elegans* and zebrafish infection models (Huedo et al., 2014). In that work, it has been demonstrated that *S. maltophilia* inoculated by intraperitoneal route in zebrafish is characterized by rapid body dissemination. By the other hand, one study using the insect *G. mellonella* suggests the proteolysis as a possible pathogenic mechanism in *S. maltophilia* isolates from CF infections (Nicoletti et al., 2011).

On the other hand, respiratory pathogens like *Burkholderia pseudomallei* has the same type of tropism in mice than that observed in humans, regardless of its acute or chronic output (Stundick et al., 2013). Modeling of experimental melioidosis has been conducted in numerous biologically relevant models including mammalian and invertebrate hosts (reviewed in Warawa, 2010). Non-mammalian models have been explored since the

mechanisms of *Burkholderia* virulence may be conserved during evolution from worms to mammals. *Drosophila* and *G. mellonella* have also shown to be useful alternative infection models for *Burkholderia spp.* (Castonguay-Vanier et al., 2010; Wand et al., 2011; Pilátová and Dionne, 2012). Particularly, *Burkholderia thailandensis* is highly virulent in the fruit fly (Pilátová and Dionne, 2012), a closely related organism to *B. pseudomallei* known to be avirulent in humans, thus being a useful model for mammalian melioidosis.

Data shown by O'Quinn et al. (2001) suggest that the disease phenotype observed in nematode after exposure to *B. pseudomallei* may be also valuable for investigating the pathogenesis of these bacteria. *Burkholderia* species are able to cause 'disease-like' symptoms and kill the nematode *C. elegans* either by infection or intoxication (O'Quinn et al., 2001; Darby, 2005) or suppressing worm immunity by specific degradation of a GATA transcription factor (Lee et al., 2013). The study of O'Quinn et al. (2001) suggests that the neuromuscular intoxication caused by *B. pseudomallei* is related to a signal transduction mechanism involving calcium. It is well-known that bacterial toxins can increase the content of free calcium (Ca^{2+}) in the cytosol of the host (TranVan Nhieu et al., 2004). In this sense, calcium acts as a second messenger in several physiological processes and immune mechanisms. The common respiratory bacterial pathogens, *P. aeruginosa* and *S. aureus*, activate Ca^{2+} fluxes after contact with y epithelial cells from the respiratory tract, activating proinflammatory signaling events (Ratner et al., 2001). The Ca^{2+} fluxes mediate the expression of proinflammatory cytokines and chemokines necessary to recruit leukocytes to the lung and also to initiate modifications in the epithelial junctions to facilitate leukocyte transmigration into the airway lumen (Chun and Prince, 2009). The *C. elegans* system has been used to screen for new virulence factors in *B. pseudomallei*, and selected attenuated bacterial mutants were further evaluated in an intranasal infection model in BALB/c mice (Gan et al., 2002). The results in mice validate positively the use and clinical relevance of *C. elegans* as an alternative model in the screening of virulence factors in *B. pseudomallei*.

CONCLUDING REMARKS

The nematode was the first invertebrate alternative model described, followed by the larvae and adult fruit fly models (*Drosophila sp.*) and more recently, the wax moth larvae (*G. mellonella*) model. Non-mammalian vertebrates, as fish and amphibians, which are able to mount an adaptive immune response, are now available as excellent tools. These kinds of models are usually criticized as being too distant from human. Several limitations such as their reduced complexity and the simplicity of their immune system, differences in temperature, target organs, or particular receptors have impaired the use of these models. However, these have been mentioned also for the murine models of other diseases. No model is perfect, each one has its specific strengths and weaknesses, but the most important thing is to combine the information gained from one to other, taking advantages of the incredible genomics and bioinformatics tools on-hand, before to extrapolate to humans beings. The belief that vertebrates are necessary the best available models in biomedical research was called the high-fidelity fallacy (Stevens, 1992; Russell, 1995). To avoid some of the limitations of these models, researchers begin to study infection and immunity in non-mammalian models. Since our knowledge of the immune system and their evolutionary conservation has increased, the usefulness of alternative models different for mammals has been accepted more. Although could seems irrational to study lung diseases in animals who do not have lungs, several evidences support the benefits that these studies, if carried properly, may to bring in the elucidation of human lung pathology diseases (**Table 1**). Thus, these non-mammalian organisms have been successfully employed to elucidate conserved and universal immune mechanisms. In addition, the small size of the most used non-mammalian organisms enables to perform high throughput and automated studies (Letamendia et al., 2012). Most of these model organisms have their genome completely sequenced, offering the possibility to do genetic studies both on the bacteria and the host. And what we consider one of the most practical advantages, the alternative models described here provide a way to easily bypass the ethical limitations of some types of studies in higher animal models.

What we do recommend to the scientific community facing the design of experimental infection with bacterial pathogens? Firstly, we have to consider several aspects related to the immunopathology of the diseases that we want to reproduce in an animal model. The relationship between the host and the guest in terms of molecular interactions is crucial to determine which type of response we will observe and consequently, to plan strategies for measuring it. But, a question arises. Which, among the methods that we will employ, are better in terms of cause less injury or damage to the animal? Is this response only measurable in vertebrate animal models? There are now sufficient evidences about the similarities of non-vertebrates immune systems and mammals. By other way, it has been demonstrated that the handling and maintenance of non-mammals organisms is easier and shaper. It is common to think in mice immediately when we are planning an *in vivo* experimental infection. Certainly, it is the most employed animal model in biomedical research. However, we could be aware about ethical restrictions that the use of large amount of animals and certain experimental procedures could implicate for the investigation. So, we modestly recommend being in mind the possibility of considering the use of no vertebrate animal models, as worms or insect as a first screening when it is reasonable. For the latter stages of the research we may to use other mammalian models. However, for certain lung diseases, conclusive points will arise more properly from the conjunction of one or more experimental studies carrying on in different species.

AUTHOR CONTRIBUTIONS

Yamilé L. Hernández and Daniel Yero authored first draft of manuscript with academic input and expertise provided by Isidre Gibert and Juan M. Pinos-Rodríguez. All authors were involved in reviewing manuscript and have approved the final version.

ACKNOWLEDGMENTS

The authors would like to acknowledge the significant review and the many valuable suggestions made by Nerea Roher, head of the group of Evolutive Immunology at IBB (Institut de Biotecnologia i de Biomedicina) and professor at the Departament de Biologia

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 November 2014; accepted: 12 January 2015; published online: 04 February 2015.

Citation: López Hernández Y, Yero D, Pinos-Rodríguez JM and Gibert I (2015) Animals devoid of pulmonary system as infection models in the study of lung bacterial pathogens. Front. Microbiol. ***6****:38. doi: 10.3389/fmicb.2015.00038*

This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology.