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ABSTRACT

Pneumocystis pneumonia is a severe lung infection that occurs primarily in largely immunocompromised patients. Few treatment options exist and the mortality remains substantial. To develop new strategies in the fields of diagnosis and treatment, it appears critical to improve the scientific knowledge about the biology of the *Pneumocystis* agent and the course of the disease. In the absence of *in vitro* continuous culture system, *in vivo* animal studies represent a crucial cornerstone for addressing *Pneumocystis* pneumonia in laboratories. These models constitute an essential complement to clinical studies. Here, we provide an overview of the animal models of *Pneumocystis* pneumonia that were reported in the literature over the last 60 years. It summarizes the various technical parameters to consider in the preparation of the model and the interpretation of the limits / results of such studies. Overall, this review highlights the great heterogeneity of the variables studied: the choice of the host species and its genetics, the different immunosuppressive regimens to render animal susceptible, the experimental challenge, and the different validation methods of the model.

Keywords: *Pneumocystis* pneumonia, animal model, *Pneumocystis* spp., *in vivo*, infectious challenge.

INTRODUCTION

Pneumocystis pneumonia is a lung infection involving *Pneumocystis jirovecii*, an ubiquitous fungus with opportunistic behavior (1). First described in malnourished children during and after World War II (2), fatal *Pneumocystis* pneumonia was one of the first signals of the AIDS epidemic in the United States in the early 1980s (3). The advent of antiretroviral drugs has resulted in a significant decrease in the incidence of *Pneumocystis* pneumonia in Human Immunodeficiency Virus (HIV)-positive patients. Today, in regions where HIV testing and treatment are available without restrictions, *Pneumocystis* pneumonia primarily occurs in subjects undergoing non-viral sources of immunosuppression. This includes pathological conditions responsible for the decrease of blood leucocytes such as hematological malignancies, auto-immune diseases, and drug-induced immunosuppression such as corticosteroids, TNF-*alpha* inhibitors, alkylating agents (4, 5). Actually, *Pneumocystis* pneumonia occurs mainly when risk factors are cumulative, *i.e.* immunosuppressive therapeutic associated to a fragile medical condition. Altogether, *Pneumocystis* pneumonia affects each year more than 500,000 patients worldwide. After *Candida* spp, *P. jirovecii* is the second most common fungal agent among invasive fungal infections (6). *Pneumocystis* pneumonia mortality is significant and has been estimated at 10-20% in HIV-positive patients and 20-40% in HIV-negative patients (7, 8).

Two main forms co-exist during *P. jirovecii* life cycle: the asci and the trophic forms that are differentially involved. The transmission, which is human-to-human airborne, is ensured by the asci form, the only form capable of living transiently in the external environment (9, 10). Next, the trophic forms thrive at the surface of type I-pneumocytes in the pulmonary alveoli. This leads to the generation of a local inflammation, while the infection remains extracellular and never becomes invasive in tissues (11, 12). Clinically non-specific, *Pneumocystis* pneumonia can manifest with fever associated with non-productive cough and chest pain (1, 13, 14). Chest scans usually show bilateral interstitial alveolar syndrome revealed by ground-

glass findings, that are non-pathognomonic of *Pneumocystis* pneumonia (15). The specific biological diagnosis relies on microbiological identification of *P. jirovecii* in pulmonary secretions / lung tissues by microscopic examination and qPCR. Likewise, it can be indirectly suggested by measuring (1,3)-β-D-glucan, a polysaccharide component of the cell wall of *P. jirovecii* and other fungi, in the serum of patients (16, 17).

Despite some advance in the scientific knowledge, *Pneumocystis* pneumonia still contains many unknowns. The cycle of *Pneumocystis* is not fully elucidated yet, thus preventing from dispensing clear prevention guidelines. Concerning the pathophysiology, there is a critical need to investigate all the immune mechanisms integrated in the host response. Therefore, experimental models are essential to complete clinical studies. Although theoretically easier and sparing animal lives, *in vitro* models are unable to mimic the complexity of host-fungus interactions. Importantly, there is no *in vitro* continuous culture system for *Pneumocystis* spp., despite long research in this area (18, 19). Animal models can circumvent these limitations (20–22). Therefore, various animal models of *Pneumocystis* pneumonia have been developed in attempt to address pathogenesis, virulence, immune response, diagnosis or therapy concerns. However, a single model cannot answer all the aforementioned questions, which explains in part the great multiplicity of supports that have been developed so far. This variability can hind the scientific comparisons, and each mammal species has its own *Pneumocystis* species (*e.g. P. murina* for the mouse or *P. carini* for the rat). Of all the animal model variables, the investigator has to question at the pivotal experimental parameters and major technical features that are assumed to likely influence the results according to the question asked.

Here, we conducted an extensive literature review of published reports related to animal models of *Pneumocystis* pneumonia using a search strategy in the PubMed database for articles published up to December 2020, based on MeSH terms. Our electronic request about animal models of *Pneumocystis* pneumonia retrieved 1,444 publications. Experimental animal

studies were included when they met the following inclusion criteria: (a) the article was accessible and written in English; (b) the study was an original article, (c) the animal model was *not* exclusively used to produce *Pneumocystis* organisms for an *in vitro* study; (d) the study was *not* a *post hoc* analysis in laboratory or wild animals. After thorough reviewing, a total of 341 articles, corresponding to 749 distinct animal models, were finally retained for complete analysis (**Figure 1**). Initially, articles were mostly dedicated to the description of the implementation of animal models and to preclinical therapeutic studies (**Figure 2**). Then, at the beginning of the 2000s, pathophysiology studies became by far the largest area of experimentation. We now propose to the reader a progressive and in-depth review of the elements that we consider essentials in the design of an animal model of *Pneumocystis* pneumonia, *i.e.* the host species, the parameters inducing susceptibility to *Pneumocystis* pneumonia, , the implementation of the experimental infection (route of inoculation, fungal inoculum) and and the biological parameters to follow up to assert correct implementation of the disease the validation methods of the model.

GENERAL DESCRIPTION OF THE VARIOUS ANIMAL MODELS: HOST SPECIES AND STRAINS, SEX, WEIGHT AND AGE

The choice of the host species is critical to reproduce as faithfully as possible the pathology that develops in Humans, but also to ensure the best reproducibility. Indeed, as in Humans, animals need to be carriers of *Pneumocystis* and transmit it to their congeners by air. Also, as in Humans, depending on their immune status, they must be able to eliminate the fungus naturally without developing a disease if they are immunocompetent, or on the contrary, in case of immunosuppression. Overall so far, more than ten animal species have been used as host models to *in vivo* study *Pneumocystis* pneumonia (**Table I**). Not surprisingly, rodents, were extensively exploited (95.9%) compared to other orders of mammals. Mice were used in 74.8% of the selected studies, compared to 20.8% and 0.3% for rats and other rodents (*e.g.* Guinea pigs and hamsters), respectively. The mouse model was widely used for its well characterized physiology, as well as biochemical and genetic homologies with Humans (23), but also for the dedicated toolbox that has been developed. Rabbits were used in 1.3% of the studies. Nonetheless, rabbits usually display lower fungal loads than other animals, and few tools and products are adapted to rabbit's biology. In addition, they are more expensive and difficult to handle than rats and mice. In 1.3% of the models, non-human primates (NHP) were used, from two species belonging to the family of Cercopithecidae (26–33). The latter, thanks to their physiological similarities and evolutionary conservation with Humans, represented privileged models for studying *Pneumocystis* pneumonia in a viral immunodeficiency background. Nevertheless, even if Humans and NHP are closely related, it should be kept in mind that, in normal conditions, each is contaminated by its own species, *P. jirovecii* for Humans and *P. macacae* for macaques. Other mammals were rarely used such as ferrets (34–36), pigs (37–39), cats (37) and dogs (37). Lastly, two arthropod-based studies, with *Drosophila melanogaster* and *Galleria mellonella,* assessed the non-susceptibility of

non-mammalian species to *Pneumocystis* pneumonia (40, 41). Depending on the purpose/issue of the study, some animals were used more frequently than others (**Figure 3**). Rabbits have most commonly been used to study the *Pneumocystis* agent and its transmission. Indeed, spontaneous P*neumocystis* pneumonia is described in the absence of induced immunosuppression at the time of weaning, thus naturally facilitating its study (24, 25). Mice and rats have also been used to study the transmission of *Pneumocystis* between the same or different host species. Mice have been are mostly used to study host-pathogen interactions and host immune response. Non-human primates have been little used, in part for ethical restrictions; but they have the great advantage to reproduce the development of *Pneumocystis* pneumonia in a context of virus-induced immunodepression. Finally, rats have been the preferred species for pre-clinical therapy studies (prophylactic, immunization, curative). The relative benefits and limitations of these models for the study of *Pneumocystis* pneumonia are summarized in the **Table II**.

The importance of an informed choice for the animals concerns not only the species, but also the strain. Focusing on mouse models, studies using inbred strains predominated: BALB/c and C57BL/6 were the more reported before C3H/HeN. Attention should be paid to the selection of strains, as highlighted in a study conducted by Swain *et al*, in which BALB/c and C57BL/6 mice have been shown to develop a specific early immune reaction after inoculation of *P. murina* (42). Strains also appeared to show a different permissiveness to *Pneumocystis* infection with variable lung burdens as shown by Tisdale *et al.* (43). Considering all animal models other than mice, outbred animals were used more frequently than inbred ones. For studies with outbred rats, Sprague-Dawley represented 64.1% of the rat models, while Wistar strain was associated with 14.7% reports. The data on susceptibility in different rat strains do not seem to be unanimous. Whereas Boylan *et al.* evaluated that Sprague-Dawley, Fisher 344, and Lewis rats immunosuppressed by steroid developed the same heavy infection six weeks after the inoculation, Hong *et al.* showed that Wistar rats developed an earlier and severer infection than Fisher and Sprague-Dawley rats under steroid immunosuppression (37, 44).

The sex of the animal chosen is also important although, in the majority of models (64.6%), it was not specified. When reported, they were females in 48.3%, males in 37.4%, and both genders in 14.3%. In a study comparing the progression of *Pneumocystis* pneumonia in male and female, Tisdale *et al. s*howed that females of three distinct mouse strains had higher fungal burdens as compared to males after six weeks of infection (43). This contrasts with what is usually observed in Humans where men are the most affected by *Pneumocystis* pneumonia (45, 46). Concerning the weight of animals used, when informed (14.2%), it was quite homogeneous and standard with 21.0 ± 4.5g and 189.4 ± 48.4g, for mice and rats respectively. In models of *Pneumocystis* pneumonia, weight loss is rarely reported and appears as a poor and irrelevant indicator of disease. Moreover, in human medicine, there are very few data on the importance of the initial weight of patients suffering from *Pneumocystis* pneumonia, only a few cases reported in a context of nutritional deprivation (47, 48). In contrast, the choice of life stage of the animals may be an important element, especially considering that the immune system is not fully developed during the first weeks of life and strongly evolves throughout aging (49). Indeed, studies have compared the different life stages of mice in relation to the immune response: neonates showed a delay in the onset of the immune response due to an inadequate lung environment coupled with an inherent inability to develop a robust innate immune response to infection and an inexperienced adaptive immune system (50–52).

SELECTION OF THE REGIMEN INDUCING SUSCEPTIBILITY TO *PNEUMOCYSTIS* PNEUMONIA

In the great majority of cases, tools to render animals susceptible to *Pneumocystis* pneumonia are an essential element to consider. Indeed, patients susceptible to *Pneumocystis* infection have the particularity of presenting pre-existing underlying conditions. Therefore, usage of a regimen inducing susceptibility to *Pneumocystis* pneumonia was reported in 663 animal models, *i.e.,* 88.5% of those described. The advantages and disadvantages of the principal strategies to render animal susceptible to *Pneumocystis* pneumonia are summarized in **Table III.**

Based on analogy with other models of fungal infections of the respiratory tract *(e.g.* aspergillosis), anti-cancerous drugs like alkylating substances, and more specifically cyclophosphamide, were used to induce adequate immunocompromised conditions (53, 54). However, alkylating agents primarily target neutrophils, that are less involved in the response to *Pneumocystis* than T-lymphocytes and macrophages. The latter are rather targeted by steroids, recognized as major risk factor for the development of *Pneumocystis* pneumonia (55–57). They have been largely used to induce immunosuppression in animal models of *Pneumocystis* pneumonia (30.8% of the animal models) (58–64). Dexamethasone administered in drinking water at a concentration of 1 to 4 mg/L was the most commonly used (57.8% of steroids models), ahead of injectable cortisone acetate (23.9% of steroids models) and injectable methylprednisolone (15.2% of steroids models), both administered subcutaneously. Dexamethasone has the advantage of a longer duration of action, but also a higher anti-inflammatory potency than cortisone and methylprednisolone. Oral administration is convenient, relatively safe, economical and compatible with refinement of experimental procedures, although it does not possess the highest bioavailability compared to parenteral routes of administration (65). In most models, steroid-dependent immunosuppression started

one to two weeks prior experimental challenge, in order to reproduce a suitable condition for the development of *Pneumocystis* pneumonia (66), and was continuously pursued until the infection had been established (67, 68). Other immunosuppressive drugs were alternatively used in rare models: dichloromethylene diphosphonate-containing liposomes or clodronate-liposomes for the specific depletion of macrophages (69, 70, 64), or more broad-spectrum medicines such as calcineurin inhibitors, tacrolimus and ciclosporin (71), mTOR inhibitor, sirolimus (72), or inhibitor of inosine-5'-monophosphate dehydrogenase, mycophenolate mofetil (72).

Considering that $CD4^+$ T-lymphocytes count is a reliable predictor of opportunistic *Pneumocystis* pneumonia during HIV infection (66), a more specific treatment of this lineage has also been tested. Depleting monoclonal antibodies (mAbs) targeting $CD4^+$ T-lymphocytes (clone GK1.5) were widely used (81.7% of these models based on immunotherapy) alone or in combination with other T-cell depleting mAbs such as anti-CD8 (clone 2.43) or anti-Thy1.2 (clone 30H12) mAbs in mice. Some other antibodies were given, such as anti-CD20 mAb (clone 5D2 or 18B12) allowing B cell depletion (73, 74). mAbs could be administered either once, before or just after the experimental infection, or several times throughout the course of infection. Immunotherapy was most often administered by intraperitoneal injection and almost exclusively in mice. Unfortunately, the risk of hypersensitivity reaction or cytokine release-associated acute reactions, and the multiplication of parenteral injections constitute major drawbacks (75, 76).

Genetically modified mice also offer interesting advantages for the development of *Pneumocystis* pneumonia and have been widely used (56.4% of the studies in mouse models). They can be grossly divided into two groups: 1) models displaying a general immunodeficiency such as SCID or $RAG1^{-/-}$ mice that lack functional T-cells and/or B-cells, or 2) more refined models that target a specific gene implicated in the host response. The first ones aforementioned were primarily used to study *Pneumocystis* biology including its life

cycle and efficiency of anti-*Pneumocystis* curative drugs. In 1993, the study by Chen *et al*. used CB17/scid (SCID) mice to support the concept that *Pneumocystis* pneumonia develops in immunocompromised patients because of recent exposure to an exogenous source and not necessarily because of reactivation of latent infection (77). The second ones were exploited to study and identify cellular and molecular entities involved in the innate and adaptive anti-*Pneumocystis* immune responses. For example, the involvement of the surfactant proteins A and D in fighting against *Pneumocystis* was highlighted by the generation of deficient mice that were knocked out for the relative encoding genes (78–84). Later in 2018, Elsegeiny *et al*. used several mouse models to recapitulate human primary immune disorders, enabling them to understand which types of CD4 T-cells were involved or relevant to mediate the clearance of *Pneumocystis* (22). However, care should be taken when interpreting outcomes in these models because of redundancy in the immune system and/or compensatory hyperactivity that can lead to confounding effects (85). In addition, scientists have to keep in mind that the use of such genetically modified or defined mice under standardized environmental conditions may influence host immunity and inflammation (86). While the generation of such mice still remains complicated, expensive and time consuming, they represent very useful biological tools for studying the host immune response to *Pneumocystis*.

Alternative immunosuppression procedures have also been implemented. This was the case for the majority of *Pneumocystis* pneumonia models in NHP. In order to reproduce as closely as possible the immunosuppression that affects AIDS patients, NHP were infected intravenously with Simian Immunodeficiency Virus (SIV) (26–32).

To enhance the magnitude of *Pneumocystis* infection, low protein diet was used in 7.9% of the models (87). This particular diet, harmful to longevity/metabolic health, was set up to reproduce the malnutrition status observed in some patients suffering from *Pneumocystis* pneumonia. However, it was quite expensive and barely used after the 2000s.

Since the models are mostly immunocompromised, it is important to use antibiotic prophylactic strategy to prevent from the occurrence of opportunistic bacterial infection, that would occur more quickly than the *Pneumocystis* pneumonia. Antibiotics were used in 23.0% of the models. The molecules used belonged to a broad spectrum of antibiotic families. Cyclins were the most widely used, in 70.9% of the models using antibiotics. Tetracycline was administered in drinking water at a concentration between 0.5 and 1 mg/mL, and doxycycline, by far less used, was administrated by subcutaneous injection. Beta-lactamins were used in 26.2% of the models, along with ampicillin, cephadrin, penicillin G, amoxicillin with or without clavulanic acid. They were mostly administered in drinking water. Other antibiotics were less used, such as quinolones with ciprofloxacin (69), aminosides with streptomycin and gentamicin (88, 89), or sulfamides with sulfadiazine (90). Anecdotally, 2.4% of the models used polyenes, nystatin or amphotericin B to prevent for other fungal diseases. The antibiotic prophylaxis strategy based on the use of cyclins, especially tetracycline, which is widely used, inexpensive and easily administered in drinking water, is to be preferred. Concerning the use of antibiotic prophylactic strategy, the parallel with what can be observed in human medicine is complicated to establish. Indeed, while most cases of *Pneumocystis* pneumonia occur in immunocompromised patients, little or no retrospective data are available on the use of antibiotics concomitant with the development and/or diagnosis of *Pneumocystis* pneumonia. Such information could be of interest in assessing the impact of such treatment might have on the pathophysiology of the disease.

IMPLEMENTATION OF THE EXPERIMENTAL INFECTION

Setting up a relevant animal model of fungal infection first requires considering the route of infection. Three main methods of experimental challenge have been proposed in the literature for generating *Pneumocystis* pneumonia.

A first passive strategy was based on the presumed latency of *Pneumocystis* within the lung alveoli and its subsequent reactivation following the induction of immunosuppression. This strategy was adopted in 20.7% of the models, especially in the pioneer reports. With respect to the recent evidences rather in favor of a *de novo* infection, this protocol seemed clearly inadequate and moreover insufficient to ensure a methodologically strict and reproducible study. Indeed, in most of these ancient reports, animals were kept under unspecified exposure conditions, and the occurrence of *Pneumocystis* pneumonia was quite random and most likely due to the transmission of *Pneumocystis* organisms by the other animals housed in the same facilities. Nowadays, one acknowledges that it is essential to use animals with the SOPF (Specific and Opportunistic Pathogen Free) certification in housing conditions such as microisolator/filtered cages that eliminate the risk of transmission from other animals.

A second passive strategy, used in 17.0% of the studied models, was implemented by co-housing healthy animals with *Pneumocystis*-pre-infected seeder mate-fellows. Indeed, the airborne route was clearly established in the early 1980's in germ-free immunocompromised rats that had been exposed to potential sources of *Pneumocystis carinii* (*i.e.* natural *Pneumocystis* species in rats) (91). In isolators, animals exposed to filtered sterile air and unsterile water and food did not acquire *P. carinii*, while rats exposed in open cages to room air but maintained on sterile diet acquired the infection. Thus thanks to this model, it has been demonstrated that *Pneumocystis* was naturally acquired by horizontal transmission as an airborne organism in a *de novo* infection (67, 91, 92). In the same vein, healthy immunocompromised animals were co-housed with fellows of the same species infected with *Pneumocystis*, for a time varying from one day to several weeks (93–98). It appeared that

inoculum or dose effect determined the rate at infection progression (99). Although this kind of strategy replicates the natural transmission of *Pneumocystis* in mammals, it could lack control and reproducibility.

In order to control these points, a third experimental infection strategy was developed through the direct inoculation of *Pneumocystis* organisms into the animals' respiratory tract. Various modes of administration have been developed. Most of the time, animals were sedated or anesthetized prior delivery, in order to minimize struggling and sneezing. The anesthesia procedure and the operator skills were critical steps to achieve a robust and reliable infection (100). Inoculation of *Pneumocystis* organisms could be achieved by intranasal, oropharyngeal, intratracheal instillation or by transtracheal deposition. The intranasal instillation, consisting in the deposition of droplets of a *Pneumocystis* suspension close to the nostrils, appears as the softest methods (easiest and the least invasive technique). At the opposite, the transtracheal alternative requires to expose the trachea surgically to a direct injection of organisms inside. Intratracheal delivery of *Pneumocystis via* blunted - needle or feeding cannula allows refining of the procedure by getting rid of the surgical incision. Overall, the direct inoculation strategy was the most common method used in mouse models, with a majority of administration based on intratracheal instillation (**Table I**). The frequency of *Pneumocystis* inoculation was generally based on a single administration, except for some specific studies that completed two or three successive inoculations, separated by two to twenty days (50, 101–104). Garvy *et al.* performed several inoculations to induce immunization (50), whereas Vuk *et al.* used a second inoculation to be certain that mice strains used, known to exhibit low levels of *Pneumocystis* infection according to them, were sufficiently exposed to *P. murina* organisms (104). None of the studies compared multiple inoculations *vs.* a single one; thus, it is difficult to appreciate whether this resulted in a greater infection. However, the time until the onset of *Pneumocystis* pneumonia was similar whatever

the number of inoculations used. The advantages and disadvantages of each strategy to implement *Pneumocystis* pneumonia are summarized in **Table III.**

Other concerns raised from the variability of the composition and the size of the *Pneumocystis* inoculum. Because, so far, *in vitro* production of *Pneumocystis* has not been successful, *Pneumocystis* were extracted, and mostly purified, from fresh or frozen pulmonary grindings of previously-infected animals. Extraction could be based on different methods, such as stomacher blending, ultrasonication or magnetic stirring (10, 105–107). Because *Pneumocystis* organisms can only be partially purified, inoculum will contained immune cells, cytokines, or other immune stimulators that may affect the host's pulmonary immune response. Thus, a control with lungs from healthy animals having undergone the same purification process seems essential.. In some rare publications, the animal received *Pneumocystis* asci from another animal species (40, 41, 108, 109). Although Walzer *et al.* initially showed that the sporadic transmission of *Pneumocystis* was possible between rats and mice (108), the opposite was subsequently demonstrated and definitively admitted (109). Furthermore, there was a great diversity in the way to count the number of *Pneumocystis* organisms in order to prepare the infectious suspension for the experimental challenge. When some counted only the asci through microscopic observation, others counted in addition the trophic forms (80, 110–112). Noteworthy, counting of trophic forms is a tedious task and requires a great deal of experience on the part of the microscopist, and taking trophic forms in account is also quite sensitive, since they were shown as insufficient to induce *Pneumocystis* pneumonia (9, 10, 111, 113). In a concern of homogeneity and scientific relevance, it seems more appropriate to consider and count only the asci for the inoculum. Large variations in the inoculum size, defined by the prior numbering of *Pneumocystis* forms, were observed, from $1.0x10^4$ to $1.0x10^8$ *Pneumocystis* forms, with an average around $1.0x10^6$ to $1.0x10^7$ *Pneumocystis* organisms. Thereafter, the experimentalist should be aware that the

356 establishment of the clinical *Pneumocystis* pneumonia is a long process requiring four to
357 seven weeks after the inoculation

VALIDATION OF THE MODEL AND OUTCOME PARAMETERS TO FOLLOW UP

In all the infectious animal models, it is essential to verify the effective infection or colonization and quantify the microorganism load. Since the clinical and radiological signatures of *Pneumocystis* are not specific, the use of histological / biological techniques was almost systematic, although none of these methods provided actual information about the viability of the fungal elements. Overall, 98.4% of the articles reported at least one histological or biological test (including microscopic approaches) to confirm that the experimental infection has been correctly implemented in the exposed animals or to assess the fungal burden. However, most of the models exploited only one technique (78.6%).

Microscopic observations of pulmonary secretions/lung sections/lung grindings slides, longtime considered as the reference standard to prove *Pneumocystis* pneumonia or colonization, have been largely described, in 81.8% of all models. These direct methods used different types of staining like Diff quick, Giemsa, Grocott methanamine silver nitrate (GMS), and toluidine blue O or calcofluor-blue brightener to demonstrate the presence of discoid *Pneumocystis* asci and/or ascospores and/or trophic forms. Microscopic approaches require substantial microscopic expertise, but they seem essential because they allow to distinguish quickly the asci forms, while being easy-to-implement and inexpensive methods.

Methods based on molecular biology like nucleic acid amplification by qPCR or fluorescence *in situ* hybridization (FISH) are more sensitive techniques. They are more refined to determine the fungal load (asci and trophic forms included), and could be used in various kinds of samples (lung tissues, bronchial-alveolar lavage fluids (BALF), or oral swab samples). They were widely used, in 31.8% of the models, with the following targets: mitochondrial large subunit (mtLSU) rRNA gene, 5.8 S rRNA gene or dihydrofolate reductase (DHFR) gene. As for other molecular biology methods, qPCR requires specialized

costly equipment and reagents, which are now available in a large number of laboratories. It should be noted that the primers used for *Pneumocystis jirovecii* usually do not overlap with those of other *Pneumocystis* spp., like *P. jirovecii.*

Other tools were used, such as detection of anti-*Pneumocystis* antibodies, performed in 6.9% of the models, or the blood / BALF detection of (1,3)-β-D-glucan (17, 112, 114–116). The serology, never used alone, was attended by huge difficulties involving potential false-negative test results and is questionable with regard to the production of antibodies in immunocompromised animals. In Humans, its use is restricted to epidemiological questions (117). Detection of (1,3)-β-D-glucan is not specific to *Pneumocystis* pneumonia and is quite costly.

In general, and whatever the type of study, to assess *Pneumocystis* presence, identify its forms, and ensure the most accurate quantification possible, the combination of a microscopic and a molecular biology technique appears the most suitable.

CONCLUSION

Pneumocystis pneumonia is a severe respiratory disease that occurs especially in immunocompromised patients. Worldwide, the number of deaths due to *Pneumocystis* spp. is estimated at almost 250,000 (Gaffi data, 2017). In absence of models of continuous *in vitro* culture, *in vivo* animal studies represent a crucial cornerstone for the study of *Pneumocystis* pneumonia. However, it is important to keep in mind that *Pneumocystis* species are host-specific (35); they have progressively diverged several tens of millions years ago and co-evolved with their hosts, thus defining their host obligate nature (118, 119). Therefore, these models are imperfect, and we can wonder about the extrapolation of the results obtained with models using microorganisms genetically different from those infecting Humans.

Ethical considerations are important when planning the use of an animal model and should be governed by the "3 R's" rule: Replacement, Reduction, and Refinement (120). Animal experiments should be designed in such a way that they allow statistically significant results with the smallest possible number of animals, while being robust and reproducible. In such a manner, the choice of the animal species and strains to study *Pneumocystis* pneumonia is decisive. As seen previously, the mouse seems to be the most suitable species. Refinement in animal models of *Pneumocystis* pneumonia can be achieved by choosing a mean of immunosuppression that avoids parenteral administration (same comment for the choice of antibiotics prophylaxis), and by using parameters other than the overall mortality to assess the disease progression.

Studying articles published for the last 60 years has enabled us to establish a wide range of criteria and factors to be considered for implementing an animal model to address *Pneumocystis* pneumonia. It requires to make choices to best answer the question posed and includes many elements such as permissiveness to infection, homology, analogy, and fidelity with Humans, reproducibility, ease of handling, safety, and of course cost. Thus, if one wonders about the cycle of *Pneumocystis*, it seems more relevant to replicate the natural

transmission of *Pneumocystis* in mammals by using co-housing of healthy animals with infected fellows, whether rodents with which we have the most experience, or NHP, whose *Pneumocystis* species is the closest phylogenetically to that of Humans. In contrast, in pre-clinical therapeutic studies that require rigorous design to obtain homogenous population, a model with an implementation of the infection by direct inoculation of *Pneumocystis* organisms allows necessary reproducibility and high control. For studies focusing on the understanding of the pathophysiology and particularly the host immune response, several types of models can be suggested. The first ones use refined genetically modified mice with a very specific immunodeficiency to study its specific involvement in the host response. The other ones study the immune response more generally, using models displaying general immunodeficiency such as genetically modified SCID or RAG1-/- mice, or animals immunosuppressed by the use of corticosteroids, the major iatrogenic risk factor of *Pneumocystis* pneumonia in Humans (55–57).

Finally according to our experience, we can propose a relevant example of an animal model to study the immune response that uses genetically modified or not and steroid immunosuppressed rodents (**Figure 4**), challenged by intranasal inoculation of *Pneumocystis murina* and validated by a microscopic and a molecular biology technique. However, the scientific debate is not close to be shut.

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