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## **Pneumocystis pneumonia: pitfalls and hindrances to establish a reliable animal model**

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### ► **To cite this version:**

Adélaïde Chesnay, Christophe Paget, Nathalie Heuzé-Vourc'h, Thomas Baranek, Guillaume Desoubieux. Pneumocystis pneumonia: pitfalls and hindrances to establish a reliable animal model. Journal of Fungi, 2022, 8 (2), pp.129. 10.3390/jof8020129 . hal-03687287

**HAL Id: hal-03687287**

**<https://univ-tours.hal.science/hal-03687287>**

Submitted on 3 Jun 2022

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1 ***Pneumocystis pneumonia: pitfalls and hindrances to***  
2 ***establish a reliable animal model***

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15  
16  
17 *Word count for the Abstract: 174*

18 *Word count for the body of the text: 4916*

19 *Number of references: 120*

20 *Number of figures: 4*

21 *Number of tables: 3*

22 *Conflict of interest: none*

23 *Funding source: none*

24

25

26 **ABSTRACT**

27

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

41

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

44

## 45 INTRODUCTION

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

63 Two main forms co-exist during *P. jirovecii* life cycle: the asexual and the trophic forms that are  
64 differentially involved. The transmission, which is human-to-human airborne, is ensured by  
65 the asexual form, the only form capable of living transiently in the external environment (9, 10).  
66 Next, the trophic forms thrive at the surface of type I-pneumocytes in the pulmonary alveoli.  
67 This leads to the generation of a local inflammation, while the infection remains extracellular  
68 and never becomes invasive in tissues (11, 12). Clinically non-specific, *Pneumocystis*  
69 pneumonia can manifest with fever associated with non-productive cough and chest pain (1,  
70 13, 14). Chest scans usually show bilateral interstitial alveolar syndrome revealed by ground-

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

76 Despite some advance in the scientific knowledge, *Pneumocystis* pneumonia still contains  
77 many unknowns. The cycle of *Pneumocystis* is not fully elucidated yet, thus preventing from  
78 dispensing clear prevention guidelines. Concerning the pathophysiology, there is a critical  
79 need to investigate all the immune mechanisms integrated in the host response. Therefore,  
80 experimental models are essential to complete clinical studies. Although theoretically easier  
81 and sparing animal lives, *in vitro* models are unable to mimic the complexity of host-fungus  
82 interactions. Importantly, there is no *in vitro* continuous culture system for *Pneumocystis* spp.,  
83 despite long research in this area (18, 19). Animal models can circumvent these limitations  
84 (20–22). Therefore, various animal models of *Pneumocystis* pneumonia have been developed  
85 in attempt to address pathogenesis, virulence, immune response, diagnosis or therapy  
86 concerns. However, a single model cannot answer all the aforementioned questions, which  
87 explains in part the great multiplicity of supports that have been developed so far. This  
88 variability can hind the scientific comparisons, and each mammal species has its own  
89 *Pneumocystis* species (e.g. *P. murina* for the mouse or *P. carini* for the rat). Of all the animal  
90 model variables, the investigator has to question at the pivotal experimental parameters and  
91 major technical features that are assumed to likely influence the results according to the  
92 question asked.

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

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

111

112

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

115 The choice of the host species is critical to reproduce as faithfully as possible the pathology  
116 that develops in Humans, but also to ensure the best reproducibility. Indeed, as in Humans,  
117 animals need to be carriers of *Pneumocystis* and transmit it to their congeners by air. Also, as  
118 in Humans, depending on their immune status, they must be able to eliminate the fungus  
119 naturally without developing a disease if they are immunocompetent, or on the contrary, in  
120 case of immunosuppression. Overall so far, more than ten animal species have been used as  
121 host models to *in vivo* study *Pneumocystis* pneumonia (**Table I**). Not surprisingly, rodents,  
122 were extensively exploited (95.9%) compared to other orders of mammals. Mice were used in  
123 74.8% of the selected studies, compared to 20.8% and 0.3% for rats and other rodents (*e.g.*  
124 Guinea pigs and hamsters), respectively. The mouse model was widely used for its well  
125 characterized physiology, as well as biochemical and genetic homologies with Humans (23),  
126 but also for the dedicated toolbox that has been developed. Rabbits were used in 1.3% of the  
127 studies. Nonetheless, rabbits usually display lower fungal loads than other animals, and few  
128 tools and products are adapted to rabbit's biology. In addition, they are more expensive and  
129 difficult to handle than rats and mice. In 1.3% of the models, non-human primates (NHP)  
130 were used, from two species belonging to the family of Cercopithecidae (26–33). The latter,  
131 thanks to their physiological similarities and evolutionary conservation with Humans,  
132 represented privileged models for studying *Pneumocystis* pneumonia in a viral  
133 immunodeficiency background. Nevertheless, even if Humans and NHP are closely related, it  
134 should be kept in mind that, in normal conditions, each is contaminated by its own species,  
135 *P. jirovecii* for Humans and *P. macacae* for macaques. Other mammals were rarely used such  
136 as ferrets (34–36), pigs (37–39), cats (37) and dogs (37). Lastly, two arthropod-based studies,  
137 with *Drosophila melanogaster* and *Galleria mellonella*, assessed the non-susceptibility of

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

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



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

183 **SELECTION OF THE REGIMEN INDUCING SUSCEPTIBILITY TO**  
184 ***PNEUMOCYSTIS* PNEUMONIA**

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

192 Based on analogy with other models of fungal infections of the respiratory tract (*e.g.*  
193 aspergillosis), anti-cancerous drugs like alkylating substances, and more specifically  
194 cyclophosphamide, were used to induce adequate immunocompromised conditions (53, 54).  
195 However, alkylating agents primarily target neutrophils, that are less involved in the response  
196 to *Pneumocystis* than T-lymphocytes and macrophages. The latter are rather targeted by  
197 steroids, recognized as major risk factor for the development of *Pneumocystis* pneumonia  
198 (55–57). They have been largely used to induce immunosuppression in animal models of  
199 *Pneumocystis* pneumonia (30.8% of the animal models) (58–64). Dexamethasone  
200 administered in drinking water at a concentration of 1 to 4 mg/L was the most commonly used  
201 (57.8% of steroids models), ahead of injectable cortisone acetate (23.9% of steroids models)  
202 and injectable methylprednisolone (15.2% of steroids models), both administered  
203 subcutaneously. Dexamethasone has the advantage of a longer duration of action, but also a  
204 higher anti-inflammatory potency than cortisone and methylprednisolone. Oral administration  
205 is convenient, relatively safe, economical and compatible with refinement of experimental  
206 procedures, although it does not possess the highest bioavailability compared to parenteral  
207 routes of administration (65). In most models, steroid-dependent immunosuppression started

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

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

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

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

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

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

259 Since the models are mostly immunocompromised, it is important to use antibiotic  
260 prophylactic strategy to prevent from the occurrence of opportunistic bacterial infection, that  
261 would occur more quickly than the *Pneumocystis pneumonia*. Antibiotics were used in 23.0%  
262 of the models. The molecules used belonged to a broad spectrum of antibiotic  
263 families. Cyclins were the most widely used, in 70.9% of the models using antibiotics.  
264 Tetracycline was administered in drinking water at a concentration between 0.5 and 1 mg/mL,  
265 and doxycycline, by far less used, was administrated by subcutaneous injection. Beta-  
266 lactamins were used in 26.2% of the models, along with ampicillin, cephadrin, penicillin G,  
267 amoxicillin with or without clavulanic acid. They were mostly administered in drinking  
268 water. Other antibiotics were less used, such as quinolones with ciprofloxacin (69),  
269 aminosides with streptomycin and gentamicin (88, 89), or sulfamides with sulfadiazine  
270 (90). Anecdotally, 2.4% of the models used polyenes, nystatin or amphotericin B to prevent  
271 for other fungal diseases. The antibiotic prophylaxis strategy based on the use of cyclins,  
272 especially tetracycline, which is widely used, inexpensive and easily administered in drinking  
273 water, is to be preferred. Concerning the use of antibiotic prophylactic strategy, the parallel  
274 with what can be observed in human medicine is complicated to establish. Indeed, while most  
275 cases of *Pneumocystis pneumonia* occur in immunocompromised patients, little or no  
276 retrospective data are available on the use of antibiotics concomitant with the development  
277 and/or diagnosis of *Pneumocystis pneumonia*. Such information could be of interest in  
278 assessing the impact of such treatment might have on the pathophysiology of the disease.  
279

## 280 **IMPLEMENTATION OF THE EXPERIMENTAL INFECTION**

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

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

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

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

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

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

333 Other concerns raised from the variability of the composition and the size of the *Pneumocystis*  
334 inoculum. Because, so far, *in vitro* production of *Pneumocystis* has not been successful,  
335 *Pneumocystis* were extracted, and mostly purified, from fresh or frozen pulmonary grindings  
336 of previously-infected animals. Extraction could be based on different methods, such as  
337 stomacher blending, ultrasonication or magnetic stirring (10, 105–107). Because  
338 *Pneumocystis* organisms can only be partially purified, inoculum will contained immune  
339 cells, cytokines, or other immune stimulators that may affect the host's pulmonary immune  
340 response. Thus, a control with lungs from healthy animals having undergone the same  
341 purification process seems essential. In some rare publications, the animal received  
342 *Pneumocystis* asci from another animal species (40, 41, 108, 109). Although Walzer *et al.*  
343 initially showed that the sporadic transmission of *Pneumocystis* was possible between rats and  
344 mice (108), the opposite was subsequently demonstrated and definitively admitted (109).  
345 Furthermore, there was a great diversity in the way to count the number of *Pneumocystis*  
346 organisms in order to prepare the infectious suspension for the experimental challenge. When  
347 some counted only the asci through microscopic observation, others counted in addition the  
348 trophic forms (80, 110–112). Noteworthy, counting of trophic forms is a tedious task and  
349 requires a great deal of experience on the part of the microscopist, and taking trophic forms in  
350 account is also quite sensitive, since they were shown as insufficient to induce *Pneumocystis*  
351 pneumonia (9, 10, 111, 113). In a concern of homogeneity and scientific relevance, it seems  
352 more appropriate to consider and count only the asci for the inoculum. Large variations in the  
353 inoculum size, defined by the prior numbering of *Pneumocystis* forms, were observed, from  
354  $1.0 \times 10^4$  to  $1.0 \times 10^8$  *Pneumocystis* forms, with an average around  $1.0 \times 10^6$  to  $1.0 \times 10^7$   
355 *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

358 **VALIDATION OF THE MODEL AND OUTCOME PARAMETERS TO**  
359 **FOLLOW UP**

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

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

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

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

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

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

396

397 **CONCLUSION**

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

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

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

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

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

441

442

443

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