

# ***Pneumocystis Murina* Infection in Immunodeficient Mice in a Closed Barrier Unit: a Case Report**

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## **Summary**

*Pneumocystis* is an important pathogen in immunocompromised individuals. In colonies of immunodeficient mice, *P. murina* can cause wasting disease and make the breeding and maintenance of immunodeficient animals difficult, unless they are continuously treated with sulfadiazin/trimethoprim. At University of Aarhus immunodeficient and immunocompetent mice were co-housed in a barrier unit. The facility was closed for entrance of animals (except for embryos for embryo transfer) and the entrance for personnel was highly restricted. The breeding performance of immunodeficient animals was comparable to that of the immunocompetent mice for a period of more than 3 years, until wasting disease and decreased litter size specifically in the breeding colony of immunodeficient mice occurred. Clinical symptoms of affected mice included laboured breathing, hunched up position, unwillingness to move, and ruffled coat. *Pneumocystis* infection was confirmed by histological examination and PCR. The partial sequence of the mitochondrial large subunit rRNA gene obtained (GenBank accession no AF548626) displayed 99 % identity to that of *Pneumocystis murina* (formerly *Pneumocystis carinii* f.sp.*muris*) found in laboratory mice. The immunodeficient animals were removed from the barrier and treated with sulfadiazin/trimethoprim in a separate unit. After the removal of immunodeficient animals, *Pneumocystis* could not be detected by PCR in the remaining animals. Our data add to the growing evidence that immunocompetent animals harboring *Pneumocystis* as a subclinical infection may be reservoirs for this organism. Still it remains to be determined how the infection was introduced and whether a latent infection can persist or the outbreak was caused by leakage in the barrier.

## **Introduction**

*Pneumocystis* pneumonia is considered one of the most serious fungal respiratory infections in immunocompromised patients and animals. Molecular comparisons of various gene sequences clearly demonstrated that the single name *P. carinii* corresponds in fact to a complex group of eukaryotic organisms, which should be assigned to the kingdom Fungi (Edman et al., 1988; Stringer, 1996; Aliouat-Denis et al., 2008; Li et al., 2008 ). Thus, several

*Pneumocystis* species can be distinguished, each of them residing in a specific mammalian host (Wakefield, 2002; Aliouat-Denis et al., 2008). *P. murina* is the species found in laboratory mice (Keely et al., 2004). The concept of host specificity has been further supported by the failure of most cross-infection experiments (Aliouat et al., 1994; Gigliotti et al., 2003). However, Sethi (Sethi, 1992) described the possibility for human-derived *P. carinii* to develop in SCID mice, and one study suggested transient colonization of owl monkeys (*Aotus nancymai*) by human-derived *P. carinii* (Beard et al., 1999).

Because a continuous *in vitro* propagation system for *Pneumocystis* is still lacking, the basic biology of this fungus as well as the epidemiology of *Pneumocystis* pneumonia remain poorly understood (Cailliez et al., 1996; Stringer, 1996). Airborne

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transmission appears likely. It has been hypothesized that the main source of *P. jiroveci*, the species found in humans (Stringer *et al.*, 2002), is patients with *P. jiroveci* pneumonia (Schmoltdt *et al.* 2008). Another source of transmission may occur through maternal-infant exposure (Cere *et al.*, 1997). *P. murina* causes disease in laboratory mice (Keely *et al.*, 2004), and several factors in the immune system have been implicated in controlling and clearing infection with *P. murina* (e.g. Hernandez-Novoa *et al.*, 2008; Atochina-Vasserman *et al.*, 2009; Linke *et al.*, 2009; Nelson *et al.*, 2009). In this report we describe the outbreak of *Pneumocystis* pneumonia in a colony of immunodeficient animals co-housed in a closed barrier together with immunocompetent mice.

### Materials and Methods

At the Faculty of Health Sciences, University of Aarhus, a barrier facility is managed with double door pass-through, floor loading bulk autoclave, two ventilated entry and exit vestibules with interlocking doors for personnel. A pass-through dip tank filled with hypochlorite solution was used for introduction of small devices such as test tubes etc. All mice in the barrier were housed in rooms with controlled environment ( $21 \pm 2^\circ\text{C}$ ,  $55 \pm 5\%$  relative humidity, 12 h light/ dark cycle, 12- 16 changes of HEPA filtered air per hour). Personnel entering the barrier facility were required to shower and change to sterile clothing, cap, surgical mask, and gloves prior to entering the barrier. Entrance to the room was only for allowed personnel, who had not had any other animal contact within the last 24 hours. The animals were kept in autoclaved Macrolon® cages (one male and three female mice or 10 weaned offspring per type III cage), with autoclaved water and food (Altromin 1314/1324). Diet and water in Macrolon® bottles were provided *ad libitum*. Routine microbiological monitoring according to FELASA recommendations (Rehbinder *et al.*, 1996) did not reveal any evidence of infection with common murine pathogens.

The barrier was reestablished 6 years before the out-

break by disinfection and resupplying with breeding animals from a commercial breeder. However, after 3 years the barrier was closed to further import of live animals, and all new genetic material was only introduced by embryo transfer. Many different strains were introduced to the barrier facility by embryo transfer. Embryos from strains to be 'imported' were flushed from the oviducts from newly mated female mice. This procedure was performed outside the barrier in a sterile hood and the embryos were transported in a sterile test tube to the dip tank, where the test tube was passed into the barrier. In the barrier facility the embryos were flushed 10 times in sterile medium before surgical transplantation to pseudopregnant fostermothers. Among imported strains were immunodeficient mice: RAG-2 knock-out mice (Shinkai *et al.*, 1992) and mice lacking all conventional MHC class II genes (Madsen *et al.*, 1999). Among those animals, approximately 15 % (21/140) of the animals exhibiting symptoms before treatment were selected and were examined macroscopically. Samples from the lungs were frozen for PCR analysis from 10 animals. After the removal of the immunodeficient animals from the colony, lungs from animals in the routine health monitoring programme were examined by PCR analysis.

For histology, representative samples of heart, lungs, spleen, liver, kidneys, stomach, duodenum and colon were collected from three animals. Tissues were fixed in 4 % neutral buffered formalin, routinely processed, embedded in paraffin, sliced into 4- $\mu\text{m}$  sections and stained with hematoxylin and eosin or with Gomori's methenamine silver staining technique.

### Amplification and sequencing of *Pneumocystis* mitochondrial rRNA sequences.

Lung tissue was minced with a scalpel and an equal volume of water was added. The sample was heated for 5 min in a boiling water bath and 20  $\mu\text{l}$  was used for 35 cycles of PCR in a 25  $\mu\text{l}$  reaction volume using primers pAZ102-E and pAZ102-H and conditions as described (Wakefield *et al.*, 1990). These primers specifically amplify a fragment of the gene

encoding the large subunit of mitochondrial rRNA in *Pneumocystis*. The resulting amplicons were analyzed by agarose gel electrophoresis and the presence of a band corresponding to 364 bp indicated the presence of DNA from *Pneumocystis* sp. For sequencing we used the same primers as for the PCR.

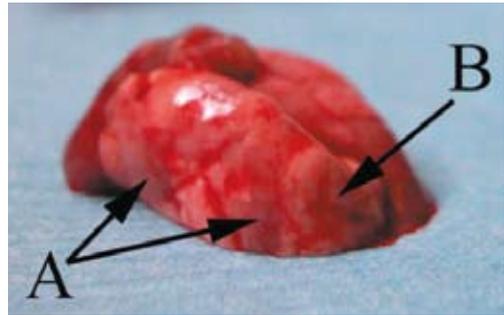
### Results

The breeding of immunodeficient animals was comparable to that of the immunocompetent mice (C57BL/6J, DBA/2J, and B6D2F1 hybrids between these two strains) for a period of more than 3 years, until disease and decreased litter size occurred specifically in the breeding colony of immunodeficient mice. Clinical manifestations of affected mice included laboured breathing, hunched up position, unwillingness to move, and ruffled coat (Fig. 1).

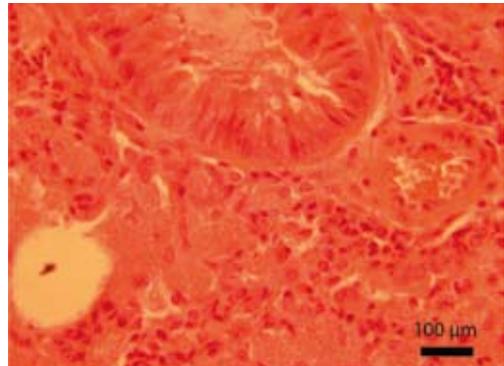


**Figure 1.** Clinical symptoms of affected mice included laboured breathing, hunched up position, unwillingness to move, and ruffled coat.

At the macroscopic examination the lungs did not collapse when the thorax was opened. The lungs were extensively consolidated and red-greyish in colour, only the caudal parts of the diaphragmatic lobes still being aerated (Fig. 2). Histological examination using routine staining (H&E) showed thickening and epithelialization of alveolar septa, and infiltration by lymphocytes and plasma cells (Fig. 3). The alveolar ducts and alveoli were distended by an eosinophilic, amorphous and finely foamy proteinaceous material. Gomori's methenamine silver staining technique demonstrated cystic or crescent-shaped structures (Fig. 4 & 5), consistent

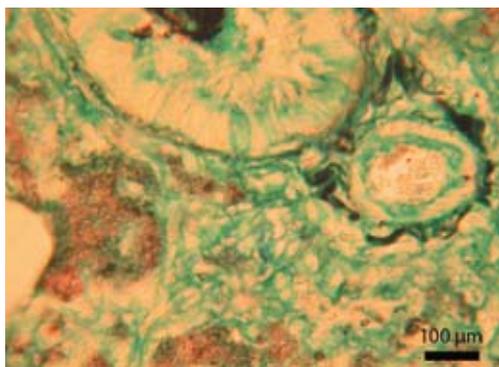


**Figure 2.** At the macroscopic examination the lungs did not collapse when the thorax was opened. The lungs were extensively consolidated and red-greyish in colour (A arrows), only the caudal parts of the diaphragmatic lobes still being aerated (B).

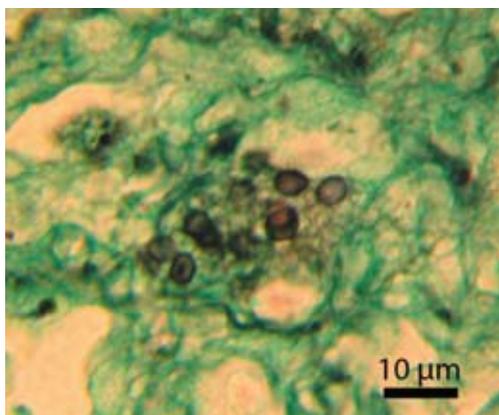


**Figure 3.** Histology using routine staining (H&E) showed thickening and epithelialization of alveolar septa, and infiltration by lymphocytes and plasma cells. The alveolar ducts and alveoli were distended by an eosinophilic, amorphous and finely foamy proteinaceous material.

with *Pneumocystis* infection. Lung tissue was used in a PCR that specifically detects a fragment of the gene encoding the large subunit of mitochondrial rRNA in *Pneumocystis* sp. The diseased mice were all positive for *Pneumocystis* sp. DNA as revealed by PCR, whereas only a few (3/10) of the healthy ones tested were positive. The obtained sequence (GeneBank accession nr AF548626) showed 99 %



**Figure 4.** The amorphous and finely foamy proteinaceous material showed specific staining by the Gomori's methenamine silver staining technique.



**Figure 5.** A higher magnification of the Gomori's methenamine silver stained areas showed cystic or crescent-shaped structures, consistent with *Pneumocystis* infection.

identity to that of large subunit mitochondrial rRNA of *P. carinii* found in laboratory mice (GeneBank accession nr AF257179) which presumably is identical to *P. murina* (Keely *et al.* 2004).

Treatment of the colony was initiated shortly after the verification of the diagnosis using sulfadiazin/trimethoprim (Tribrissen® vet Forte) in the drinking water (4 mg Sulfametoxazol and 0.8 mg trimethoprim / ml, corresponding 600 mg Sulfametoxazol /

120 mg trimethoprim /kg/day). After the start of the treatment no new cases of disease occurred and the breeding capacity of the immunodeficient animals was restored. Only immunodeficient animals or animals supposed to deliver immunodeficient offspring were treated. The immunodeficient animals were moved to another animal facility outside the barrier and here all mice in the colony were treated for practical reasons. Examination by PCR of the immunocompetent animals in the barrier after removal of the infected immunodeficient animals revealed only few positive animals after two months (2 positive / 88 tested) and later no positive animals were found, indicating elimination of the agent from the colony.

#### Discussion

It was thought for many years that *Pneumocystis* pneumonia resulted from reactivation of a latent infection. There is now an increasing body of data to support the idea that in some instances *Pneumocystis* infection is acquired *de novo*. It has been shown that *Pneumocystis* organisms were eliminated from the lungs after pneumonia, and that the persistence of organisms was limited in rats and mice (Chen *et al.*, 1993; Vargas *et al.*, 1995). This is in concordance with the finding in our case where PCR failed to detect *Pneumocystis* two months after the removal of the immunodeficient mice from the colony. Similar results have been seen in humans (Millard and Heryet, 1988; Peters *et al.*, 1992; Oz and Hughes, 2000), where the main source of *P. jiroveci* is other patients with *P. jiroveci* pneumonia (Schmoldt *et al.* 2008). It is therefore likely that a temporary sub-clinical infection in immunocompetent mice or the clinical infection in the immunodeficient mice have been the source from where the infection spread after introduction into the colony. Furthermore as *Pneumocystis* were isolated from mice and host specificity has been reported, the origin of the infection is most likely to be murine. This was confirmed by sequencing a fragment of the mitochondrial large subunit rRNA gene which showed high homology to *P. murina* found in mice. It is unlikely that the

immunocompetent animals had housed a latent infection of *Pneumocystis* for more than 3 years, as clinical manifestations should have occurred at an earlier time point. Speculation on how the agent was introduced into the barrier facility included the possibility that personnel were carriers of the agent as no live animals were introduced into the colony. Transmission with fertilised embryos seems very unlikely. Contaminated equipment may also be a possibility.

Interestingly, *Pneumocystis* DNA has been detected in samples of airborne fungal spores (Wakefield, 2002). This suggests that *Pneumocystis*, as one part of the life-cycle, produces stable spores that can be disseminated into the environment. Such spores are not necessarily capable of propagation. Indeed, it is not yet known whether *Pneumocystis* is an obligate fungal 'parasite', which can only propagate within its specific host, or whether it is also capable of reproducing in an environmental niche (Wakefield, 2002).

### Conclusion

Based on the observed data from the colony, it is most likely that *Pneumocystis* was introduced into the colony by a leakage in the barrier. However, knowledge about the presence and persistence of this fungus in the environment is sparse. The introduction *de novo* of *Pneumocystis* in apparently closed barrier shows that it might serve as an indicator of leakage in the barrier and co-housing of immunodeficient animals in a barrier could serve as a control system to catch leakage in the barrier system.

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