A Diagnostic Method for *Pneumocystis carinii* a Causative Agent of Pneumonia in Immunodeficient Rats

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Abstract: Immunodeficient animals are in demand in current biomedical research, and they contribute to medical progress. In *Pneumocystis* infections, a specific histological diagnostic tool may be immunochemistry (IC). However, it was recently reported that the antibody (3F6) was not suitable for detecting *Pneumocystis* in rats. We purchased another antibody [PNC007] from a commercial source for IC. We could detect positive signals at identical locations with IC and Toluidine blue O in lungs of infected rats. These results corresponded to the results obtained with PCR. We should study the relationship between unexpected positive signals seen in IC and trophic forms in lungs of infected rats. We could clinically diagnose pneumonia caused by *Pneumocystis carinii* with the diagnostic method we developed.

Key words: immunochemistry, immunodeficient rat, *Pneumocystis carinii*

Though genetically immunodeficient animals are in great demand in biomedical research, they tend to suffer severe infections from opportunistic pathogens.

*Pneumocystis* spp. organisms are able to dwell and replicate in the lungs, especially in naïve immunocompetent hosts, like young mammals and immunodeficient animals, and they can be transmitted by airborne routes as long as their host remain infected [1]. Healthy hosts not showing the onset of pneumonia could behave as a sort of dynamic reservoir for *Pneumocystis* species [1, 2].

*Pneumocystis carinii* were mistaken as protozoans [1, 3] for many years and *Pneumocystis carinii* was finally classified as a fungus by sequencing of the gene encoding of the nuclear small subunit ribosome RNA (rRNA) [6] and by direct sequencing of rRNA [17] in the late 1980s. In the late 1990s, new evidence was provided showing that *Pneumocystis* spp. strains that adapted to mammal species had been genetically isolated from each other for a very long time [13, 14]. The human form of the organism was determined as *Pneumocystis jirovecii*, according to the requirements of the International Code of Botanical Nomenclature (ICBN) in 1999 [8]. In rats (*Rattus norvegicus*), two species of *Pneumocystis*,
Pneumocystis carinii and Pneumocystis wakefieldiae, had been reported but there is a confusion about the description of Pneumocystis even in scientific reports; for example, many authors still cite Pneumocystis carinii, which was derived from the human specimen [5, 7, 16].

The current practical methods of detecting Pneumocystis spp. are PCR and cystic form staining in lung smears or sections with Grocott’s methenamine-silver nitrate (GMS) or toluidine blue O (TBO) [1, 9]. These staining methods are directed at common fungal polysaccharide moieties of the cystic wall and leave the trophic forms unstained [9]. An immunochemical method has been reported to detect all the developmental stages of Pneumocystis in foals, pigs and rats [4, 9], and it is thought to be a more sensitive method than GMS and TBO. There are several reports describing immunochemical staining methods with non-commercially available antibodies generated from immunized animals with purified antigen [4, 12]. Commercially available antibodies seem to be more practical than antibodies generated in the laboratory, because it is unnecessary to conduct specificity and sensitivity tests of commercial antibodies to Pneumocystis carinii. An immunochemical method might have potential for improving the ability to diagnose pneumonia caused by Pneumocystis spp. There are a few reports about common diagnostic methods for rats which used commercially available primary antibodies [10, 18]. Kobayashi et al. [10] reported that Pneumocystis spp. was more weakly stained with the monoclonal antibody (3F6), which is commonly used in the medical field, in the rat lung than in the human lung. The technical instructions for the most of commercially available antibodies (3F6) indicate that the antibody (3F6) was raised against Pneumocystis carinii isolated from a human lung. Therefore, the antibody (3F6) might cause pseudo negative diagnosis in rats.

The PCR method has proved the existence of a specific DNA nucleotide, but not a host defensive reaction to Pneumocystis carinii. There are no effective methods which can detect both Pneumocystis spp.’s pathogenicity and its existence at the same time, except for histopathological methods.

To solve these problems, we developed a new diagnostic method. The new method is a combination of histopathological methods, including fluorescence immunochemistry (IC) with mouse monoclonal antibody [PNC007] to Pneumocystis carinii, HE staining, and DNA amplification.

For the development of the detection method, a F344/NJcl-rnu/rnu female rat aged four weeks was purchased from CLEA Japan, Inc. (Tokyo, Japan). Also, a SPF CrI:WI (Wistar) retired rat was purchased from Charles River, Inc. (Kanagawa, Japan). For the clinical application, six F344/NJcl-rnu/rnu female sentinel rats aged six weeks were purchased from CLEA Japan, Inc. were observed. Vendor reports indicated that the F344/NJcl-rnu/rnu female rats were free from common viral, parasitic, mycoplasmal, and bacterial pathogens including Pneumocystis carinii, in accord with the recommendation of the Japanese Association of Laboratory Animal Facilities of National University Corporations. Vendor reports (http://www.crj.co.jp/service/quality/list.cgi, accessed November 10, 2009) also indicated that CrI:WI (Wistar) rats were free from common viral, parasitic, mycoplasmal and bacterial pathogens, and that the rats showed no significant lesions consistent with an active infectious disease in gross pathology.

Animal husbandry procedures were performed according to the guidelines for laboratory animals and the standard operating procedures of the Institute of Experimental Animal Sciences, Osaka University Graduate School of Medicine. In summary, rats were housed in open TPX cages (KN-601-T, Natsume Seisakusyo Co., Ltd., Tokyo, Japan) using pulp bedding (ALPHA-dri: Shepherd Specialty Papers, Watertown, TN, USA). The cages with bedding were autoclaved. Rats were given water processed by a reverse osmosis system (Model RO-8600, Edstrom Industries, Inc., Waterford, WI, USA) ad libitum and were fed a commercial laboratory diet (MF: Oriental Yeast Co., Tokyo, Japan). Cages were changed once or twice a week. A 12:12-h light:dark cycle was used with a room temperature of 21.5 to 24.5°C and a humidity range of 30–60%.

We prepared a rat with Pneumocystis carinii infection. A F344/NJcl-rnu/rnu female rat aged 4 weeks was exposed to the environment, which may spontaneously introduce infection with Pneumocystis carinii, for a long time. The rat was held with immunocompetent animals in a holding room that had not been sterilized with form-
aldehyde fumigation for ten years. It was housed for six months in open TPX cages.

At seven months old, the F344/NJcl-rnu/rnu rat showed signs of coughing. It was euthanized by CO₂ inhalation. The lung was then collected for PCR and histopathological analysis. Briefly, the right lung specimen was frozen at −80°C immediately after the autopsy. The left lung specimen was dissected, fixed in 10% neutral buffered formalin and embedded in paraffin. A Crlj:WI (Wistar) rat was euthanized by CO₂ inhalation on the day of purchase. The lung was collected and frozen at −80°C for PCR analysis as a negative control.

For the clinical application, we used two different types of holding rooms in terms of sanitization. One room had been sterilized with formaldehyde fumigation one year before the commencement of the study. Three F344/NJcl-rnu/rnu rats were housed in a TPX cage that was held in the “sterilized room”. The other three rats were housed in a TPX cage that was held in a “non-sterilized room” that had not been sterilized for 16 years. At the time of changing cages, we observed the health conditions of the rats. The three rats held in the sterilized room and the other three rats held in the non-sterilized room were kept for 14 weeks. We took samples of fecal and dirty bedding substrates with a sterilized spoon from the cages of other immunocompetent rats and deposited them in the sentinel cages once a month.

We applied the detection method to clinical diagnostic procedures. The clinical examination was performed when the rats were twenty weeks old. The six rats held in the two different types of holding room for microbiological monitoring were euthanized in a carbon dioxide chamber. The lungs were dissected and examined with PCR analysis and histological observations with IC, TBO, and HE staining.

The serial sections of the embedded lung were deparaffinized in xylene to study the histology. The hydrated serial sections were permeabilized with diluted PBS pH 7.2, 10× (GIBCO, Life Technologies Japan Ltd., Tokyo, Japan) followed by heat-induced retrieval with diluted Target Retrieval solution, pH 9, 10× (Dako Japan Inc., Tokyo, Japan) according to the manufacturer’s instructions. The slides were incubated with two primary antibodies; mouse monoclonal [PNC007] to Pneumocystis carinii (1:100, Abcam, Cambridge, England) and mouse monoclonal [80] to pan Keratin (1:10, Abcam) for a negative control, and then incubated with a secondary antibody, Alexa Fluor 488 goat anti-mouse IgG1γ1 (1:400; Invitrogen, Life Technologies Japan Ltd.). The slides were observed with either a confocal laser scanning microscope, TCS SL (Leica, Solms, Germany), or an imaging system with a fluorescence microscope, Axioplan2 IPlan Spectrum (Carl Zeiss, Oberkochen, Germany).

The left lung serial sections were deparaffinized in xylene and stained with HE and TBO [19].

We made a smear preparation from frozen lung tissue and stained it with TBO [19]. The slides were fixed with cold acetone and stained by IC.

The total DNA of the frozen lungs was isolated using DNeasy Blood and Tissue Kit (QIAGEN K. K., Tokyo, Japan) according to the manufacturer’s instructions. PCR was performed in a reaction mix from TaKaRa Taq (Mg²⁺ free Buffer, TaKaRa, Kyoto, Japan) containing 50 mM KCl, 10 mM TRIS (pH 8.3), 3.5 mM MgCl₂, 0.25 μM oligonucleotide primers and 0.02 units/μl Taq polymerase. The primers used were RC1: 5’-TTTTGGTAGATGACTTGTTATT-3’ and RC2: 5’-AGTCTGACTAACCATCATATAT-3’ targeting the mitochondrial large subunit rRNA of Pneumocystis carinii (137 bp), and RR1: 5’-GTAGATAGCTTAATAAGGATG-3’ and RR2: 5’-TTCTTGACTGTCTATGAAGT-3’ targeting the mitochondrial large subunit rRNA of Pneumocystis wakefieldiae (251 bp) [15]. Thirty-five cycles of amplification were performed as previously reported [15]. PCR products were electrophoresed on a 3.0% agarose gel and visualized by staining with ethidium bromide.

PCR products amplified by RC1 and RC2 were purified using QIA quick PCR Purification Kit (QIAGEN K. K.) according to the manufacturer’s instruction. Sequence analysis of the PCR products was performed in both directions with a 3100 Genetic Analyzer at Osaka University Medical School, Center for Medical Research and Education using the Big Dye Terminator Cycle Sequencing kit ver1.1 (Applied Biosystems, Life Technologies Japan) according to the manufacturer’s instructions. The sequences used for comparison were previously described [15] and were downloaded from the GenBank database (http://www.ncbi.nlm.nih.gov/Gen-

The lung of the F344/NJcl-rnu/rnu rat, which had begun showing signs of coughing, showed severe pneumonia under macro observation. The microscopic observation of the smear specimens and serial histological paraffin sections stained by IC revealed oval shaped or circular objects 5–10 µm in size similar to the specimens or sections which were stained with TBO (Fig. 1A–D). The shapes and sizes of objects stained with TBO were in agreement with those described for *Pneumocystis* spp. [1, 3, 19]. We did not find any pseudo positive signals in smear specimens. There were unexpected positive signals in IC and cystic forms were not detected at identical locations with TBO. Also, no staining was observed in IC at the location when we used PBS and monoclonal antibody to pan Keratin as the primary antibody for negative controls.

PCR analysis with the primer pair RC1 and RC2 of the lung of the F344/NJcl-rnu/rnu rat prepared with natural *Pneumocystis* infection showed a band located at 137 bp, which was expected and was a positive indication of the existence of *Pneumocystis carinii* (Fig. 2). The DNA sequence obtained from the PCR products with RC1 and RC2 primers matched (97.1%) a sequence previously reported [15] as a large subunit rRNA of *Pneumocystis carinii* which was deposited in the GenBank database (accession no. S42914). The products expected for *Pneumocystis wakefieldiae* (251 bp) were not detected. No amplification products were found for the Crlj:WI (Wistar) rat used as a negative control.

The method developed to diagnose *Pneumocystis carinii* in rats was clinically applied to sentinel rats. Serial sections of the lungs of the six rats stained with HE, TBO, and by IC were observed (Fig. 3). In all six lungs, we could observe histopathological lesions, such as perivascular or peribronchiolar lymphocytic or neutro-
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Philic inflammation. Foamy macrophages were observed in only one specimen from the non-sterilized room (Fig. 3A). We found circle or oval shaped purple organizations on the sections stained with TBO in three rats from the non-sterilized room (Fig. 3B), though the staining results of two out of three specimens were not very clear. Then serial sections stained by IC were observed and the oval shaped or circular objects 5–10 \( \mu m \) in size were detected on the sections of all three lung specimens from the non-sterilized room (Fig. 3C). We did not find any specifically shaped objects on the serial sections stained with TBO and by IC in the three rats from the sterilized room. Samples from lungs of rats were PCR amplified using two pairs of primers, RC1 and RC2 or RR1 and RR2. Amplification products produced by the RC1 and RC2 primers were obtained from all of the lungs of the three rats kept in the non-sterilized room, but no products were observed in any of the lungs of the three rats kept in the sterilized room. There were no amplification products produced by the RR1 and RR2 primers for any of the specimens.

We applied a commercially available primary antibody [PNC007] to *Pneumocystis carinii* in IC. The target of this antibody was *Pneumocystis carinii* isolates as per the instructions on the product, and the antibody was reported to stain *Pneumocystis* spp. in the lungs of humans before the taxonomy dispute [5, 16]. The signals of IC were consistent with the signals of TBO on the serial sections. We could identify circle or oval shaped forms specific to *Pneumocystis* with the antibody [PNC007] in IC, as was previously reported for other antibodies [4, 11, 18].

![Fig. 2. PCR amplification of three samples using the primers for *Pneumocystis carinii* (P.c.), and *Pneumocystis wakefieldiae* (P.w.). Lanes are marked as to the sample on the top and bottom of the gel. C indicates primers for P.c. and W indicates primers for P.w.; Lanes 2, 3: A coughing F344/NJcl-rnu/rnu rat, which had severe pneumonia; Lanes 5, 6: A specific pathogen free CrIj:WI(Wistar) rat; Lanes 8, 9: diluted water as negative control; Lanes 1, 10: 100 bp molecular weight marker; Lanes 4, 7: Blank.](image)

![Fig. 3. Micrographs of the clinical application for sentinel F344/NJcl-rnu/rnu rat lungs in the non-sterilized room. Serial sections of the rat lungs (A–C) were stained with HE (A), TBO (B), and by IC (C). (A) Foamy macrophages were observed (arrow). (B) Cystic forms were stained with TBO (arrow). (C) Expected organizations were stained by IC (arrow). Scale bar, 20 \( \mu m \).](image)
There were unexpected positive signals with IC on the paraffin sections in spite of no signals being found on the negative control. Sukura [18] reported the IC staining results of trophic forms and cystic forms in rats. The IC staining results reported by Sukura resembled the unexpected positive signals seen in this study. Further study is required to clarify the relationship between trophic forms and the unexpected positive signals that we observed.

An effective method for detecting *Pneumocystis carinii* is PCR. We could identify specific PCR products with *Pneumocystis carinii* as previously reported [15]. PCR is an effective method, but it is difficult to detect the causative agent of pneumonia with only the PCR method because it is too sensitive for property. The PCR method can detect the existence of *Pneumocystis carinii* but not the causative agent in pathological lesions of pneumonia. In the case of suspected pneumonia related to *Pneumocystis* spp., we should diagnose using the results of the combination of PCR and histology, including immunohistochemistry.

We tried to examine the presence of *Pneumocystis wakefieldiae* as well as *Pneumocystis carinii* with the PCR method. *Pneumocystis wakefieldiae* was the second species of *Pneumocystis* formally described in laboratory rats. The differences between *Pneumocystis carinii* and *Pneumocystis wakefieldiae* are too profound to reconcile with the view that they belong to the same species [4]. We tried to identify the two types of *Pneumocystis* in pneumonia in rats using the PCR method, but we could not detect *Pneumocystis wakefieldiae* in this study.

We thought that we could clinically diagnose the infection of *Pneumocystis carinii*, which is a known causative agent of lung lesions in immunodeficient rats, using a combination of IC with the commercially available antibody [PNC007], TBO and PCR, even though it was said that the proof of a novel causative agent for infection is established by the separation and confirmation of the infectivity. The method commonly used for detecting *Pneumocystis* is a combination of cystic form staining with TBO or GMS in lung smears or paraffin sections and PCR [1, 9]. Such staining methods may not be used for a diagnosis because they are specific to not only *Pneumocystis* spp. but also common fungal polysaccharide moieties of the cystic wall and they leave the trophic form unstained [9]. We could obtain clear staining results in IC with two specimens from the non-sterilized room although their TBO staining results were not very clear. Also, the PCR method proves only the existence of the specific DNA nucleotide. If we prepare the serial sections once, we can prove the existence by IC and the pathogenicity with HE. The method proposed in this study, a combination of IC, traditional staining and PCR, could prove precisely both the pathogenicity and existence of *Pneumocystis carinii*.

We found different results for infection with *Pneumocystis carinii* between two different types of holding room. In the non-sterilized room, we detected infection of *Pneumocystis carinii* in immunodeficient rats. Immunocompetent animals are not usually infected with opportunistic pathogens. On the other hand, non-regular sterilization procedures of animal holding rooms might result in accumulation of opportunistic pathogens and specific pathogens creating a reservoir of *Pneumocystis carinii*. Histological lesions in the lungs of the three rats kept in the sterilized room were observed, but *Pneumocystis carinii* was not detected by any of the methods applied in this study. The lung lesions of the three rats might have been caused by other opportunistic pathogens because of the rat’s immunodeficient property.

We strongly suggest that *Pneumocystis carinii* infection should be diagnosed clinically with not only PCR and traditional stainings, but also IC staining for confirmation of the pathogenicity of *Pneumocystis carinii* at a specific lesion observed in HE staining. The order of the combination of methods proposed is PCR, HE and then IC, for the routine monitoring, and HE, IC and then PCR for the more precise detection. In future, we need to search for more easily purchased antibodies for the detection of *Pneumocystis carinii* in rats.

**References**