

# Characterization of natural occurring *Pneumocystis carinii* pneumonia in pigs by histopathology, electron microscopy, *in situ* hybridization and PCR amplification

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**Summary.** Macroscopic, histologic, ultrastructural, microbiologic, *in situ* hybridization (ISH) and PCR detection results in three 8-week-old pigs naturally infected with *Pneumocystis carinii* (PC) are described. All animals had a nonsuppurative interstitial pneumonia and intra-alveolar *Pneumocystis* organisms with foamy eosinophilic and PAS positive appearance. Ultrastructurally, PC trophozoites and cysts were observed in pigs No. 2 and No. 3, with the former being much more numerous. PC organisms were located on the alveolar surface or within the alveolar septa. Trophozoites had numerous filopodia and were thin-walled. Cysts had no or few filopodia, were thick-walled and contained intracystic bodies. Using non-isotopic ISH on formalin-fixed, paraffin-embedded lung tissue sections, PC DNA from pigs No. 2 and No. 3 hybridized with a probe specific for PC ribosomal RNA (rRNA). Using primers specific for mitochondrial rRNA gene (pAZ102-E/pAZ102-H), and for the internal transcriber spacers of ribosomal gene of PC, PCR methods amplified a product in the lung of pigs No. 2 and No. 3 using either frozen or formalin-fixed and paraffin-embedded lung tissue. DNA from Pig No. 1 samples did not amplify with any primer. This is the first time that molecular biology techniques (*in situ* hybridization and PCR) have been applied to the study of porcine pneumocystosis.

**Key words:** *Pneumocystis carinii*, Pneumonia, Electron microscope, ISH, PCR

## Introduction

*Pneumocystis carinii* (PC) is an unicellular eukaryote that inhabits the lungs of a wide variety of homeothermic terrestrial vertebrates (Armstrong and

Cushion, 1994). The taxonomy of PC is controversial (Sukura, 1995). Some authors classify this organism as a protozoan because of its morphologic similarities to protozoa and susceptibility to some antiprotozoan drugs. Other authors classify PC as a member of the fungi considering that its ribosomal RNA has a high homology to the group of fungi Rhizopoda/Myxomycota/Zygomycota group (Watanabe et al., 1989). PC emerged as a leading cause of opportunistic infection and mortality in HIV-positive patients in the 1980s (Walzer, 1993).

Two major life stages have been found in the lungs of infected animals: trophozoites and cysts (Ruffolo, 1994; Sukura, 1995). Trophozoites are irregular, 1-5  $\mu$ m in diameter, thin-walled and with a variable number of filopodia (tubular expansions of the cell membrane and cell wall) (Bedrossian, 1989). The cytoplasm of trophozoites contains mitochondria, rough endoplasmic reticulum, ribosomes, glycogen particles, lipid droplets and dense round bodies. The cyst stage is round, about 5  $\mu$ m in diameter, thick-walled, usually smooth without filopodia, and contains intracystic bodies with morphological features similar to free trophozoites. The cyst wall has an outer dense layer containing chitin, a middle electron-lucent layer containing glucan (Matsumoto et al., 1989) and a cell membrane.

Lesions produced by PC occur primarily in the lungs. Disseminated infections in immunocompromised human beings have been reported (Travis, 1994). The classic histopathologic finding is a prominent eosinophilic, PAS-positive foamy intra-alveolar material accompanied by a mild, interstitial pneumonia and proliferation of type II pneumocytes (Travis, 1994). The alveolar exudate consists of different stages of PC with the cyst wall stained by Gomori methenamine silver (GMS) or toluidine blue O stains (Holten-Andersen and Kolmos, 1989). A broad spectrum of pulmonary changes can be seen in human beings and animals with chronic infections. These changes can be related to progression of the disease while others are due to associated pathogens (Kucera et al., 1968; Morin et al., 1990;

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Kondo et al., 1993; Koziel et al., 1993; Travis, 1994).

*P. carinii* pneumonia in domestic animals has been described in foals (Ainsworth et al., 1993); goats (McConnell et al., 1971); dogs and cats (Settnes and Hasselager, 1984; Sukura et al., 1996). There are several reports of PC pneumonia or detection of this organism in pigs (Kucera et al., 1968; Seibold and Munnell, 1977; Fujita et al., 1989; Bille-Hansen et al., 1990; Kondo et al., 1993).

In this study we compare the macroscopic, histopathologic, electron microscopic and microbiologic features of a natural PC infection of three pigs with those described in the literature. In addition, this is the first report describing molecular biology techniques (*in situ* hybridization and PCR) for the study and diagnosis of porcine pneumocystosis.

## Materials and methods

### Animals, histopathology and electron microscopy

Tissues were collected from one male (Pig No. 1) and two female (Pigs No. 2 and 3) 8-week-old pigs (pigs No. 1 and No. 2 were from the same farm), fixed in 10% neutral buffered formalin and processed routinely for histopathology. Six-micron lung sections were stained with HE, Giemsa, PAS and Gomori's methenamine silver (GMS) stains. Formalin-fixed lungs from pigs No. 2 and No. 3 were postfixed in 1%  $\text{OSO}_4$ , processed and embedded in a mixture of Epon-Araldite, stained with uranyl acetate and lead citrate and examined with a Philips 301 transmission electron microscope at 60 Kv.

### *In situ* hybridization

The *in situ* hybridization technique (ISH) to demonstrate *Pneumocystis carinii* has been described elsewhere (Montone, 1994). Briefly, five-micron sections were deparaffinized, rehydrated and digested with a solution (2.5 mg/ml) of pepsin at 105 °C for 3.0 minutes. The biotin-labeled oligonucleotide probe, consisting of 22 base sequences (5'-TCTCTGAGGTA TGGCCGTA ACT-3') complementary to the first 22 nucleic acid sequences of PC 5S rRNA, was diluted to 200 ng/ml in a non-formamide based cocktail. The probe solution was applied to the slides and the tissues were heated at 105 °C for 2 min. to denature any secondary rRNA structures. The tissue target and the probe hybridized for 10 min at 40 °C. Streptavidin-peroxidase was the detection system with diaminobenzidine as the chromogen. Sections were counterstained with hematoxylin. Negative controls included sections containing a variety of bacteria, fungal or protozoal organisms including *Aspergillus flavus* and *Toxoplasma gondii*. Another negative control was substitution of the specific PC probe by an unrelated probe targeting the 5S rRNA of *Aspergillus* nucleic acids 1-22. Human lung naturally infected with PC was used as positive control.

### Polymerase chain reaction

The polymerase chain reaction (PCR) was performed according to established protocols using frozen or formalin-fixed, paraffin-embedded lung (Wakefield et al., 1990; Lee et al., 1993; Lu et al., 1994). Briefly, 50  $\mu\text{m}$ -thick paraffin sections were deparaffinized or thawed, homogenized, and centrifuged. Pellets were washed with PBS-EDTA buffer several times. The pellet was resuspended and digested with proteinase K diluted in digestion buffer and incubated at 55 °C for 45 min. The mixture was extracted with phenol and chloroform. The DNA present in the aqueous phase was precipitated with ethanol which was then removed by vacuum drying. The DNA was dissolved in 50  $\mu\text{l}$  of Tris-EDTA buffer (PCR solution). The oligonucleotide primers used for both paraffin and frozen samples were pAZ102-E (5'-GATGGCTGTTTC CAAGCCCA-3') and pAZ102-H (5'-GTGTACGTTGC AAAGTACTC-3'), that target the mitochondrial rRNA coding region in rat- and human-derived PC DNA. Amplification with these primers results in a fragment of approximately 350 base pairs (bp). In addition, a nested PCR with primers amplifying the internal transcribed spacer (ITS) was used. The nested PCR was performed using primers 1724 F (5'-AAGTTGGTCAAATTTGGT C-3') and ITS2R (5'-CTCGGACGAGGATCCTCGCC-3') for the first step and primers ITS1F (5'-CGTAGG TGAACCTGCGGAAGGATC-3') and ITS2R1 (5'-GTTACGCGGGTGATCCTGCCTG-3') for the second step (nested) (Lu et al., 1995). Amplification of a diagnostic fragment of 550 bp is specifically obtained with the ITS nested PCR for *P. carinii*.

PCR with pAZ primers was performed in a 100  $\mu\text{l}$  mixture containing template DNA, PCR buffer, 20 pmol of PCR primers, 0.2 mM of the four deoxynucleoside triphosphates, and 2 U of *TaqI* DNA polymerase. PCR was performed in two stages. The initial stage was 35 cycles, with each cycle consisting of 1.5 min at 94 °C, 1.5 min at 55 °C, and 3 min at 72 °C. The final stage was extension at 72 °C for 10 min. The PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide to determine the sizes of the amplified products. For nested ITS PCR the same conditions for the pAZ primers were used, but the annealing temperature was 50 °C for the first PCR reaction and 65 °C for the second nested PCR reaction (Lee et al., 1993; Lu et al., 1994). Rat lung experimentally infected with PC was used as a positive control. Non-infected rat lung and liver from all three pigs were used as negative controls.

### Bacteriological and virological examination

Lung, spleen, liver and small intestine were cultured for bacterial pathogens. Samples of spleen, liver and lung were tested by fluorescent antibody and virus isolation for pseudorabies virus, and lung was tested by both methods for influenza virus, and porcine



reproductive and respiratory syndrome (PRRS) virus.

## Results

### Gross lesions

All pigs were emaciated. Pig No. 1 had a rough hair coat with areas of hyperkeratosis on its dorsum and tail. The pericardial sac and the epicardium were almost completely adhered to one another. The lungs were diffusely congested with consolidation of the left middle lobe. Tracheobronchial and cervical lymph nodes were enlarged. Pig No. 2 had also a rough hair coat with areas of alopecia and hyperkeratosis along the head and spine. An umbilical abscess was noted. There was mild fibrinous peritonitis. The lungs did not collapse, were palpably firm and mottled red. The cranioventral lobes were consolidated. Pig No. 3 lung had cranio-ventral atelectatic areas with lobular pattern.

### Histopathology

An interstitial pneumonia associated with organisms consistent with PC was present in all pigs examined although organisms and the intensity of the lesions were much more extensive and severe in pigs No. 2 and No. 3. The alveolar septa were congested and thickened with a mild to moderate number of lymphocytes, plasma cells, macrophages and fewer neutrophils. The alveolar lumen, and occasionally the alveolar ducts and respiratory bronchioles contained edema fluid, fibrin, and a foamy eosinophilic material (Fig. 1) that was strongly PAS positive and consisted of small round bodies (Fig. 2). Most of these bodies had single central basophilic dots, and were considered PC trophozoites. Some bodies had a thicker cell wall and multiple (up to 8) basophilic dots (Fig. 3) and were considered PC cysts containing intracystic bodies. A GMS stain stained the cyst wall in all pigs (Fig. 4). Pigs No. 2 and No. 3 had also a bacterial bronchopneumonia affecting the cranial lobes with numerous neutrophils and fewer macrophages in the small airways and alveoli. In addition, pig No. 3 had peribronchiolar and perivascular lymphoplasmacytic aggregates. Necropsies of 6 other 1-month-old pigs from the farm where pigs No. 1 and No. 2 were housed did not reveal PC but multiple bacterial pathogens were isolated.

### In situ hybridization

Lung from pig No. 1 was negative when tested with a probe for PC rRNA but lung from pigs No. 2 and No. 3 gave an intense granular brown reaction (Fig. 5) that was localized in the areas where PC organisms were observed by histopathology and electron microscopy. Most of the reaction appeared to be related to trophozoites. There was no background staining. Positive and negative controls performed as expected.

### Electron microscopy

The lungs from pigs No. 2 and No. 3 were examined. The alveolar surfaces were covered by single or multiple layers of irregular shaped bodies, 3-5  $\mu\text{m}$  in length, with a 20-25 nm cell wall (Fig. 6). The morphology of these organisms was consistent with trophozoites of PC. Variable numbers of filopodia, less than 100 nm in diameter, were arising from the surface of the trophozoites. Cross section of these expansions revealed that they had a central hollow core surrounded by spikes (Fig. 7). Tubular expansions from adjacent trophic forms were intermingled or very close to the surface of alveolar cells but no direct contact was observed. Type I pneumocytes had cytoplasmic projections that partially surrounded trophozoites. Other structures, identified as PC cysts, were 3-5  $\mu\text{m}$  in diameter, round to ovoid, and were intermingled with trophic forms (Fig. 8). The wall of the cysts was 80-90 nm in thickness, with a smooth surface and had three identifiable layers: an outer dense layer, a middle and thicker electron-lucent layer, and an inner cell membrane. Most of the cysts had one or more (up to 6) intracystic bodies. Some cysts were collapsed, crescent-shaped, and empty. The ratio of trophozoites:cysts was usually higher than 100:1. Both trophozoites and cysts were seen in the interstitium.

### Polymerase chain reaction

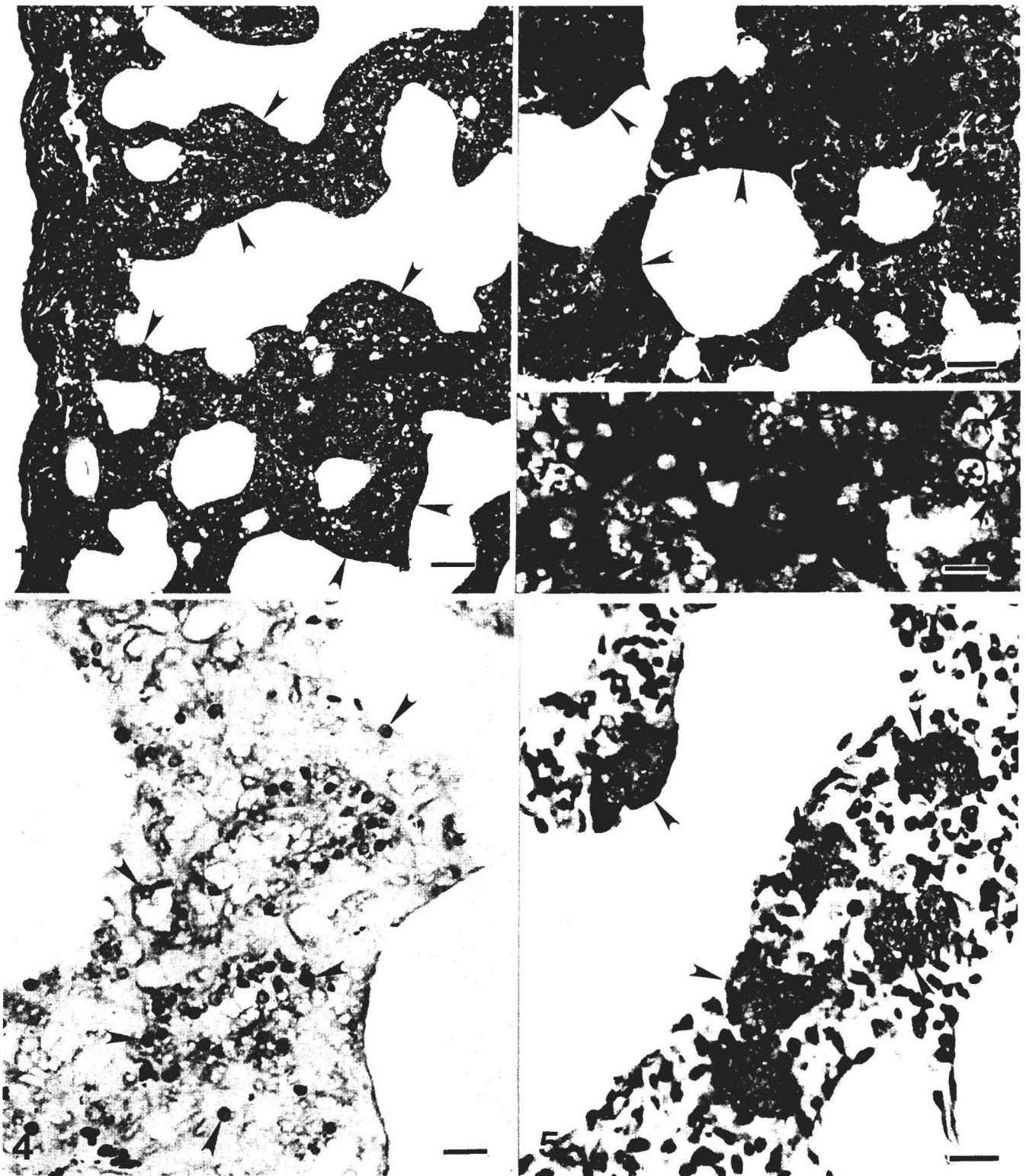
There were no PCR-amplified products when PCR with pAZ primers and nested ITS PCR were performed on frozen or paraffin-embedded lung tissue of pig No. 1. Lung samples (paraffin-embedded and frozen) from pigs No. 2 and 3 were amplified by both types of PCR. The molecular size of the amplified products for PCR with pAZ primers and for nested ITS PCR was approximately 350 bp and 550 bp respectively (Fig. 9). Positive and negative controls performed as expected.

### Microbiology and virology

Fluorescent antibody test and virus isolation were negative for pseudorabies, PRRS, and swine influenza in all pigs. *Haemophilus parasuis* was isolated from lung of pigs No. 1 and No. 3 and *Streptococcus suis* type II was isolated from lung of pig No. 2. *Actinomyces pyogenes* and *Pasteurella multocida* were isolated from the umbilical abscess. *Bordetella bronchiseptica* was also isolated from lung of pig No. 3.

## Discussion

This report describes macroscopic changes, microscopic lesions, ultrastructural changes, *in situ* hybridization and PCR studies in three pigs with pulmonary pneumocystosis. Microscopic changes in the lungs were consistent with a diagnosis of acute to subacute interstitial pneumonia due to PC and



**Fig. 1.** Pig No. 2. Lung. Interstitial pneumonia. Eosinophilic foamy exudate in alveoli (arrowheads) and interstitium (circles) containing *Pneumocystis* organisms. HE stain. Bar: 80  $\mu$ m.

**Fig. 2.** Pig No. 3. Lung. Interstitial pneumonia. The alveolar septa are thickened with lymphocytes, plasma cells and foamy macrophages interspersed between *Pneumocystis* organisms. The foamy exudate is strongly PAS positive (arrowheads). PAS stain. Bar: 40  $\mu$ m.

**Fig. 3.** Pig No. 2. Lung. Trophozoites (arrowheads) and cysts (arrows) containing intracystic bodies are admixed. HE stain. Bar: 8  $\mu$ m.

**Fig. 4.** Pig No. 2. Lung. The wall of the cysts (arrowheads) is strongly stained with Gomori's methenamine silver stain. GMS stain. Bar: 20  $\mu$ m.

**Fig. 5.** Pig No. 3. Lung. The foamy exudate has a brown strong reaction (arrowheads) for *P. carinii* ribosomal RNA by *in situ* hybridization. Streptavidin-peroxidase. Bar: 20  $\mu$ m.

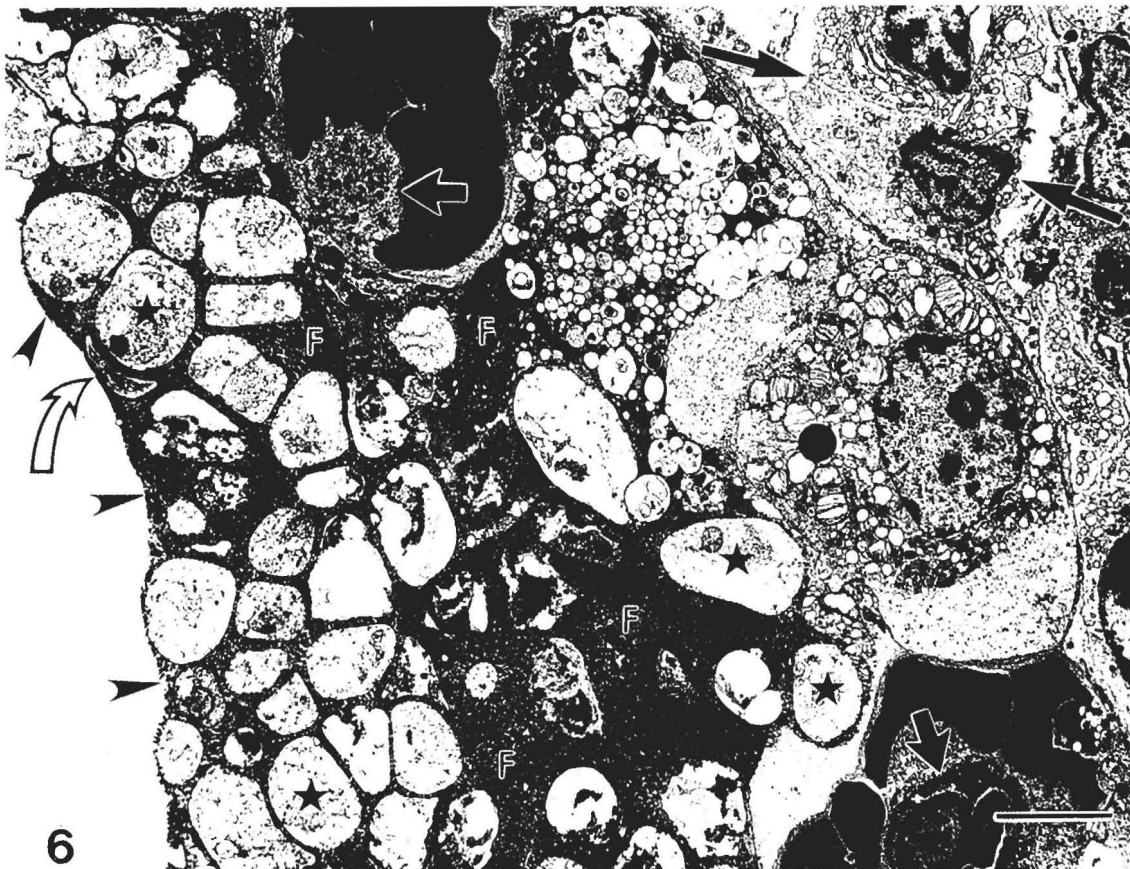
*Porcine pneumocystosis*

concurrent bacterial pneumonia (Travis, 1994). Swine pneumocystosis is more common between 6 and 11 weeks of age (Kucera et al., 1968; Seibold and Munnell, 1977; Bille-Hansen et al., 1990; Kondo et al., 1993). The interstitial pneumonia is initially multifocal but eventually becomes diffuse. Although microorganisms capable of producing pneumonias were isolated from all pigs, the most likely cause of the pulmonary interstitial lesions was PC. The lesions found in the cranial lobes of pig No. 2 were most likely produced by *Streptococcus suis* type II and the cuffing pneumonia in pig No. 3 by *Mycoplasma* sp. although there was no isolation of this organism. Bacterial or viral infections have been reported in cases of swine PC pneumonia (Kucera et al., 1968; Seibold and Munnell, 1977; Morin et al., 1990; Kondo et al., 1993).

Incomplete descriptions of the ultrastructure of swine PC are available in the literature (Seibold and Munnell, 1977; Kondo et al., 1993). PC from pigs No. 2 and No. 3 were morphologically indistinguishable from other PC by electron microscopy (Bedrossian, 1989). We detected two different stages, the more numerous trophozoite, usually with filopodia, and the cyst stage. It has been hypothesized that filopodia are involved in the reproduction of trophozoites, transfer of nutrients and/or the attachment to alveolar cells (Bedrossian, 1989). None of these hypotheses has been proven convincingly.

We observed that trophozoites were in close contact with pneumocytes with the epithelial cells "embracing" trophozoites by means of cytoplasmic projections. This contact required the smooth portion of the trophozoite cell wall to undulate and conform to the irregularities of the pneumocytes, but no filopodia were observed in the region of contact (Bedrossian, 1989; Pottratz and Martin, 1994). Close contact between epithelial alveolar cells and cysts was not seen. Receptors in the alveolar epithelium and macrophages for vitronectin, fibronectin, mannose, surfactant protein D or the Fc fraction of immunoglobulins in macrophages mediate the attachment of PC (Pottratz and Martin, 1994; Limper, 1995). PC contains a major mannose-rich surface antigen complex termed glycoprotein A (Pottratz and Martin, 1994; Limper, 1995). It has been suggested that the susceptibility of HIV-infected patients to PC is related to impairment of the alveolar macrophage mannose receptor by HIV (Koziel et al., 1993). PC not only attaches to macrophages, but when this binding is through mannose (glycoprotein A) or immunoglobulins (Fc receptor in macrophages), it activates macrophages and phagocytosis of PC (Pottratz and Martin, 1994).

Ribosomal RNA are abundant RNA sequences found within all eukaryotic and prokaryotic cell types. These sequences are conserved, and they are widely utilized for the phylogenetic classification of infectious



**Fig. 6.** Pig No. 2. Lung. The alveolar surface (arrowheads) is covered with several layers of *P. carinii* organisms, most of them trophozoites (stars). There is also a collapsed cyst (open arrow). The interstitial infiltrate contains plasma cells (long arrows). Capillaries are dilated with erythrocytes and neutrophils (short arrows). There are numerous filopodia (F) between trophozoites. Pneumocyte type II (circle). Uranyl acetate and lead citrate stain. Bar: 470 nm.



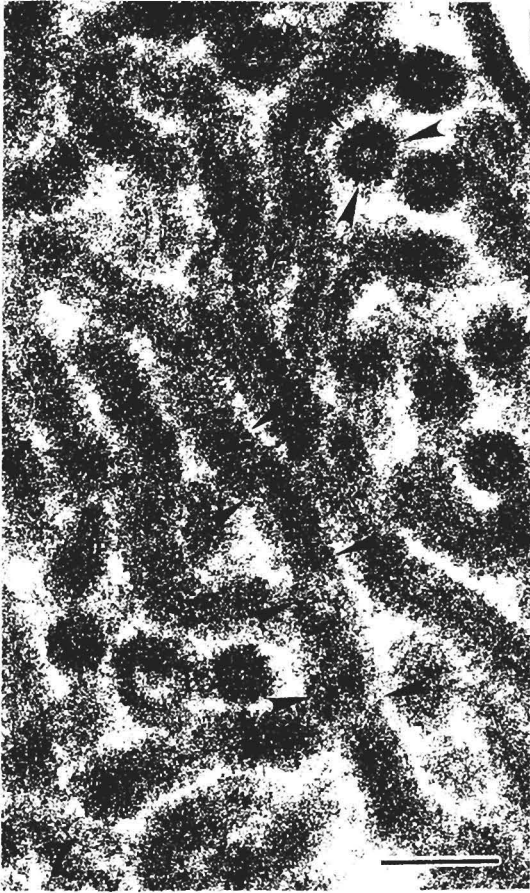


Fig. 7. Pig No. 2. Lung. Detail of the filopodia showing on cross and longitudinal sections a central hollow structure surrounded by spikes (arrowheads). Uranyl acetate and lead citrate stain. Bar: 26 nm.

organisms (Olsen and Woese, 1993). Using non isotopic *in situ* hybridization (ISH) we demonstrated the presence of rRNA specific for PC in the foamy alveolar exudate. This technique proved to be simple and quick to perform (Montone, 1994). Other advantage of this technique was an intense and specific reaction in paraffin-embedded tissues. The intensity of the reaction using paraffin embedded lung sections of pig No. 2 was comparable to the human control. The synthesis of oligonucleotides for different areas of the PC genome might be appropriate for retrospective epidemiological studies and classification of PC isolates.

The PCR technique proved to be a reliable and specific method to detect different components of swine PC genome. Primers specific for PCR amplified the genome of swine PC in pigs No. 2 and 3, either using frozen or formalin-fixed and paraffin-embedded lung tissue. PCR is being used frequently in the diagnosis of human PC pneumonia as well as for epidemiologic studies (Wakefield et al., 1990; Lee et al., 1993; Lu et al., 1994). We used different primers for mitochondrial rRNA, and for the ITS regions. Amplification of ITS

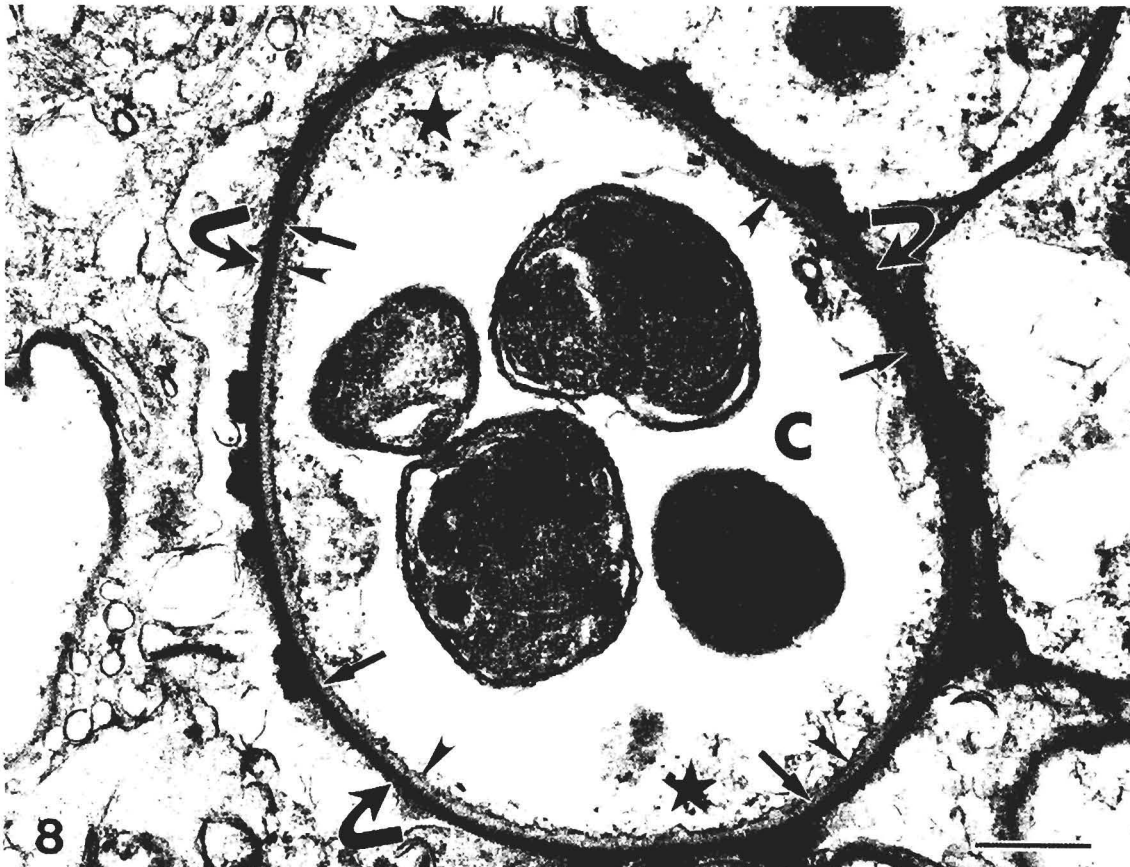
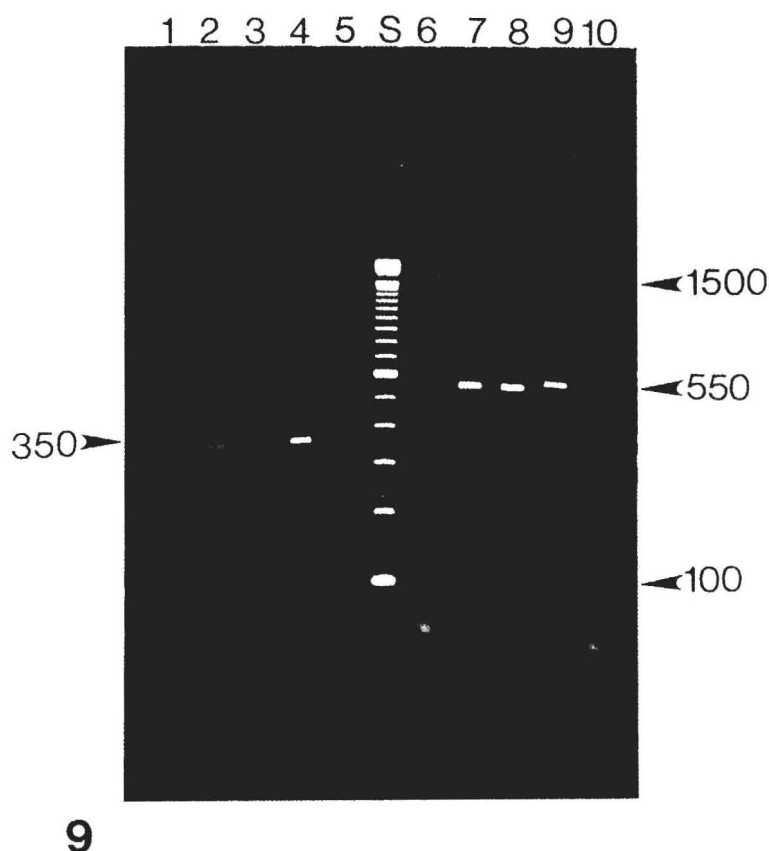


Fig. 8. Pig No. 2. Lung. The cell wall of a cyst has three defined layers; an outer electron-dense layer (curved arrows), a middle electron-lucent layer (arrows) and the inner cell membrane (arrowheads). The cyst contains a rudimentary cytoplasm (star). There are four intracystic bodies (I). Uranyl acetate and lead citrate stain. Bar: 61 nm



**Fig. 9.** Ethidium bromide stained agarose gel analysis of PCR-amplified products using primers pAZ102-E/pAZ102-H (lanes 1-5) and nested ITS PCR (lanes 6-10). **Lanes 1 and 6:** sample from pig No. 1 which was negative in both types of PCR. **Lanes 2 and 3:** amplified products obtained with pAZ primers of pigs No. 2 and 3 respectively. **Lanes 7 and 8:** amplified products obtained by nested ITS PCR of pigs No. 2 and 3 respectively. **Lanes 4 and 9:** positive control (lung sample from a rat infected with *P. carinii*) for PCR with pAZ primers and nested ITS PCR. **Lanes 5 and 10:** negative PCR controls (lung sample of uninfected rat). **Lane S:** contains the 100 base pair ladder standard. Molecular sizes (in base pairs) are shown on the right and left sides of the figure.

regions is being used in human beings and laboratory animals for typing of PC strains (Lu et al., 1994) and has been considered by some authors one of the best PCR methods to detect human PC (Lu et al., 1995). Due to the lack of other studies in swine we could not determine whether this approach will be suitable in the porcine species. Although PCR has been described to detect PC in infected horses and dogs, the primers used did not include those for ITS regions (Peters et al., 1994; Sukura et al., 1996).

Samples from pig No. 1 did not hybridize with the specific probe for PC and did not amplify the PC specific DNA band by PCR using the same conditions and primers as those for pigs No. 2 and No. 3. Furthermore, samples from all pigs were processed in an identical way. These results are puzzling and a definitive explanation is lacking. One possibility could be that due to the multifocal nature of the foamy exudate in pig No. 1, sampling may have influenced the detection of PC when using frozen sections. However, both ISH and PCR techniques were also done using paraffin sections with organisms resembling PC, as demonstrated by HE and GMS stains, although much less abundant than in the other two pigs. Another possibility entertained was the existence of different strains of PC in swine, as it has been described in other species (Gigliotti et al., 1993), but pigs No. 1 and No. 2 were housed in the same barn

which makes it more likely that both animals were infected with the same strain of PC. Furthermore, pAZ primers were designed on conserved regions of PC and amplify rat, ferret, human and other PC strains which makes unlikely that PCR negative result in pig No. 1 was due to the presence of a different strain. Sensitivity of both ISH and PCR may be an issue in this case. It has been shown that in some cases ISH using digoxigenin labeled probes is more sensitive than when biotin labelled probes are used (McQuaid et al., 1995). In our case we used only biotin labelled probes.

We conclude that in addition to the "classic" morphological methods to study and diagnose pneumocystosis in the porcine species, molecular biology techniques can be successfully used in porcine PC infections. Future research using molecular biology techniques will include epidemiologic studies in the swine population as well as the complete sequencing of the amplified porcine PC products and comparison with those from other species.

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## References

- Ainsworth D.M., Weldon A.D., Beck K.A. and Rowland P.H. (1993). Recognition of *Pneumocystis carinii* in foals with respiratory distress. *Equine Vet. J.* 25, 103-108.
- Armstrong M.Y.K. and Cushion M.T. (1994) Animal models. In: *Pneumocystis carinii* pneumonia. 2nd ed. Walzer P.D. (ed). Marcel Dekker. New York. pp 181-222.
- Bedrossian C.W.M. (1989). Ultrastructure of *Pneumocystis carinii*: a review of internal and surface characteristics. *Semin. Diagn. Pathol.* 6, 212-237.
- Bille-Hansen V., Jorsal S.E., Henriksen S.A. and Settnes O.P. (1990). *Pneumocystis carinii* pneumonia in Danish piglets. *Vet. Rec.* 127, 407-408.
- Fujita M., Furuta T., Nakajima T., Kurita F., Kaneuchi C., Ueda K. and Ogata M. (1989). Prevalence of *Pneumocystis carinii* in slaughtered pigs. *Jpn. J. Vet. Sci.* 51, 200-202.
- Gigliotti F., Haidaris P.J., Haidaris C.G., Wright T.W. and Van der Meid K.R. (1993). Further evidence of host species-specific variation in antigens of *Pneumocystis carinii* using the polymerase chain reaction. *J. Infec. Dis.* 168, 191-194.
- Holten-Andersen W. and Kolmos H.J. (1989). Comparison of methenamine silver nitrate and Giemsa stain for detection of *Pneumocystis carinii* in bronchoalveolar lavage specimens from HIV infected patients. *Acta Pathol. Microbiol. Immunol. Scand.* 97, 745-747.
- Kondo H., Taguchi M., Abe N., Nogami Y., Yoshioka H. and Ito M. (1993). Pathological changes in epidemic porcine *Pneumocystis carinii* pneumonia. *J. Comp. Pathol.* 108, 261-268.
- Koziel H., Kruskal B.A., Ezekowitz R.A.B. and Rose R.M. (1993). HIV impairs alveolar macrophage mannose receptor function against *Pneumocystis carinii*. *Chest* 103, 111S-112S.
- Kucera K., Slesinger L. and Kadlec A. (1968). Pneumocystosis in pigs. *Folia Parasitol. (Praha)* 15, 75-78.
- Lee C-H., Lu J-J., Bartlett M.S., Durkin M.M., Liu T-H., Wang J., Jiang B. and Smith J.W. (1993). Nucleotide sequence variation in *Pneumocystis carinii* strains that infect humans. *J. Clin. Microbiol.* 31, 754-757.
- Limper A.H. (1995). Adhesive glycoproteins in the pathogenesis of *Pneumocystis carinii* pneumonia: host defense or microbial offense? *J. Lab. Clin. Med.* 125, 12-13.
- Lu J-J., Bartlett M.S., Shaw M.M., Queener S.F., Smith J.W., Ortiz-Rivera M., Leibowitz M.J. and Lee C-H. (1994). Typing of *Pneumocystis carinii* strains that infect humans based on nucleotide sequence variations of internal transcribed spacers of rRNA genes. *J. Clin. Microbiol.* 32, 2904-2912.
- Lu J-J., Chen C-H., Bartlett M.S., Smith J.W. and Lee C-H. (1995). Comparison of six different PCR methods for detection of *Pneumocystis carinii*. *J. Clin. Microbiol.* 33, 2785-2788.
- Matsumoto Y., Matsuda S. and Tegoshi T. (1989). Yeast glucan in the cyst wall of *Pneumocystis carinii*. *J. Protozool.* 38, 21S-22S.
- McConnell E.E., Basson P.A. and Pienaar J.G. (1971). Pneumocystosis in a domestic goat. *Onderstepoort J. Vet. Res.* 38, 117-126.
- McQuaid S., McMahon J. and Allan G.M. (1995). A comparison of digoxigenin and biotin labelled DNA and RNA probes for *in situ* hybridization. *Biotech. Histochem.* 70, 147-154.
- Montone K.T. (1994). *In situ* hybridization for ribosomal RNA sequences: a rapid sensitive method for diagnosis of infectious pathogens in anatomic pathology substrates. *Acta Histochem. Cytochem.* 27, 601-606.
- Morin M., Girard C., ElAzhary Y., Fajardo R., Drolet R. and Lagacé A. (1990). Severe proliferative and necrotizing pneumonia in pigs: a newly recognized disease. *Can. Vet. J.* 31, 837-839.
- Olsen G.J. and Woese C.R. (1993). Ribosomal RNA: a key to phylogeny. *FASEB J.* 7, 113-123.
- Peters S.E., Wakefield A.E., Whitwell K.E. and Hopkin J.M. (1994). *Pneumocystis carinii* pneumonia in thoroughbred foals: identification of a genetically distinct organism by DNA amplification. *J. Clin. Microbiol.* 32, 213-216.
- Pottratz S.T. and Martin II W.J. (1994). Mechanisms of *Pneumocystis carinii* attachment to lung cells. In: *Pneumocystis carinii* pneumonia. 2nd ed. Walzer P.D. (ed). Marcel Dekker. New York. pp 237-250.
- Ruffolo J.J. (1994). *Pneumocystis carinii* cell structure. In: *Pneumocystis carinii* pneumonia. 2nd ed. Walzer P.D. (ed). Marcel Dekker. New York. pp 25-43.
- Seibold H.R. and Munnell J.F. (1977). *Pneumocystis carinii* in a pig. *Vet. Pathol.* 14, 89-91.
- Settnes O.P. and Hasselager E. (1984). Occurrence of *Pneumocystis carinii* Delanoë & Delanoë, 1912 in dogs and cats in Denmark. *Nord. Vet. Med.* 36, 179-181.
- Sukura A. (1995). *Pneumocystis carinii* in a rat model. Academic Dissertation. College of Veterinary Medicine. Helsinki. Finland.
- Sukura A., Saari S., Järvinen A-K., Olsson M., Kärkkäinen M. and Ilvesniemi T. (1996). *Pneumocystis carinii* pneumonia in dogs: a diagnostic challenge. *J. Vet. Diagn. Invest.* 8, 124-130.
- Travis W.D. (1994). Pathological features. In: *Pneumocystis carinii* pneumonia. 2nd ed. Walzer P.D. (ed). Marcel Dekker. New York. pp 155-180.
- Wakefield A.E., Pixley F.J., Banerji S., Sinclair K., Miller R.F., Moxon E.R. and Hopkin J.M. (1990). Detection of *Pneumocystis carinii* with DNA amplification. *Lancet* 336, 451-453.
- Walzer P.D. (1993). *Pneumocystis carinii*: recent advances in basic biology and their clinical application. *AIDS* 7, 1293-1305.
- Watanabe J., Hori H., Tanabe K. and Nakamura Y. (1989). Phylogenetic association of *Pneumocystis carinii* with the "Rhizopoda/Myxomycota/Zygomycota group" indicated by comparison of 5S ribosomal RNA sequences. *Mol. Biochem. Parasitol.* 32, 163-168.

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