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Pneumocystis Infection in Goat Kids in Uganda

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Abstract

Pneumocystis pneumonia was identified in two female (cases 1 and 2) and a male (case 3) Boer cross goat kids aged 3 months on a farm in Uganda. This was fatal in cases 1 and 2, with symptoms of coughing. Severe infestation with *Haemonchus contortus* was found in the euthanized case 3. Histology revealed numerous *Pneumocystis* organisms in cases 1 and 2, but fewer in case 3. Although macrophages reactive to the organisms were observed in all cases, the lung lesions were not infiltrated with lymphocytes in cases 1 and 2. Polymerase chain reaction analysis for *Pneumocystis* DNA identified five genetic clones in case 1. This diversity may be due to repeated infection from other goats, and may also be associated with selective pressure from the innate immune system activated by mild *Haemonchus* infestation in young kids.

Discipline: Animal health
Additional key words: Africa, genetic variation, *Haemonchus contortus*

Introduction

Pneumocystis pneumonia is an opportunistic infection in immunosuppressed individuals. The host range is wide and includes humans and other mammals such as rabbits, dogs, cats, swine and horses. However, *Pneumocystis* organisms from different host species have very different DNA sequences, and cross infection between different host species is not possible (Kovacs & Masur 2009, Wazir & Ansari 2004). The organism causing the disease in humans is *Pneumocystis jirovecii* (Wazir & Ansari 2004). Although it may occur as an epidemic in premature or malnourished children (Wazir & Ansari), *Pneumocystis* pneumonia is mainly detected in patients with human immunodeficiency virus (HIV) infection, cancer or transplants (Kovacs & Masur 2009). The finding that different strains are present in immunocompetent infants and HIV-infected adults suggests independent transmission cycles (Beard 2005). Adult patients infected with multiple strains of *P. jirovecii* are not rare (Kovacs & Masur 2009), and this organism can evolve under positive selective pressure from sulfa drugs (Lane et al. 1997).

Pneumocystosis is also found in young swine, and can occur concurrently with certain viral or bacterial infections (Kondo et al. 2000). Although reports on *Pneumocystis* infection are extremely rare in ruminants, molecular phylogenetic analyses demonstrated a goat-specific strain from a 3-year-old doe suffering from heavy paratuberculosis (Sakakibara et al. 2013). In this paper, we describe the histology of pneumocystosis in three goat kids, with five different clones detected in one of the cases.

Materials and methods

1. Animals and gross pathology

We examined two female (cases 1 and 2) and a male (case 3) Boer cross goat kids aged 3 months, which were raised on a farm in the Mpigi district of Uganda. New animals had been introduced to this farm from other farms more than eight times. Although parasitic disorders occurred quite often on the farm, occasional anthelmintic administration appeared relatively ineffective. Because approximately 40 kids had suffered from coughing and then died, formalin-fixed tissues from two of them (cases 1 and 2) were submitted to the National Animal Disease Diagnosis

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Center. To ascertain the cause of death, another kid with anemia and slight nasal discharge (case 3) was euthanized and necropsied. Numerous *Haemonchus contortus* were detected in the abomasum of this animal, while the lungs were pale pink and slightly edematous.

2. Histological and immunohistochemical examinations

Formalin-fixed tissue samples were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (HE) and Giemsa. Selected sections were dewaxed and labeled by the streptavidin-biotin-peroxidase complex (SAB) method. The primary reagents employed were mouse monoclonal antibodies to *Pneumocystis* (1:20; product number 0921, ViroStat, Portland, ME, USA) and CD68 (1:50; Dako A/S, Glostrup, Denmark), and rabbit polyclonal antibodies to CD3 (1:50; Dako A/S) and CD20 (prediluted; Spring Bioscience, Pleasanton, CA, USA). Subsequent procedures were carried out using an immunoperoxidase labeling system (Nichirei, Tokyo, Japan). After visualization with diaminobenzidine (DAB) in the presence of nickel and cobalt ions (Adams 1981), some sections stained for *Pneumocystis* antigen were successively treated for CD68 staining, whereupon the positive sites were visualized with DAB.

3. Polymerase chain reaction (PCR) assay

Total DNA was extracted from paraffin-embedded lung sections via deparaffinization by xylene, proteinase K digestion and phenol-chloroform-isoamyl alcohol extraction. Standard *Pneumocystis* primers for the 5' end of the large subunit of the mitochondrial ribosomal RNA gene (mtLSU rRNA; primers pAZ102-E and pAZ102-H) and ExTaq DNA polymerase (Takara, Tokyo, Japan) were used for the analyses (Wakefield et al. 1997). The amplicons were separated by 2.0% agarose gel electrophoresis, purified using QIAquick Gel Extraction Kits (Qiagen, Hilden, Germany) and ligated into pCR2.1-TOPO vectors, followed by transformation into One Shot TOP10 competent cells (TOPO TA Cloning; Invitrogen, Carlsbad, CA, USA). The bacteria were selectively grown on Luria Broth agar plates containing 50μg/ml kanamycin, and several clones were selected. The plasmids were extracted from the bacterial clones using QIAprep Spin Miniprep Kits (Qiagen), and their inserts were sequenced with M13 universal primers using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and ABI 3130 Genetic Analyzer (Applied Biosystems). Similarity searches were performed using BLAST (http://blast.ddbj.nig. ac. jp/top-j.html) and FASTA (http://fasta.genome.jp) of the National Center for Biotechnology Information.

Results

1. Histological findings

In cases 1 and 2, there were many masses of foamy eosinophilic material comprising cysts with spores within alveolar and bronchiolar spaces (Fig. 1). The cysts stained positively with anti-*Pneumocystis* antibody (Figs. 2, 3), while the alveolar septa were thickened with inflammatory cells, consisting mainly of CD68-positive macrophages, with few CD3- or CD20-positive cells present (Fig. 4). Although macrophages were similarly observed in case 3, fungi were far less numerous. CD3-positive cells were present in abundance in the septa (Fig. 5), as were CD20-positive cells in peribronchial areas. Intra-alveolar macrophages adherent to fungal colonies (Fig. 6) or phagocytosing some cysts (Fig. 7) were detected in all cases, and intra-vascular neutrophilia was marked in the lungs and other organs examined.

2. PCR analysis findings

Pneumocystis-specific DNA in the mtLSU rRNA region (363-365 base pairs) could be amplified from the lung tissue sample of case 1. The other two cases were negative by PCR, presumably due to the prolonged formalin fixation time. Nucleotide sequencing of the amplified DNA was accomplished by the cloning and sequencing method, and five different sequences (clones #1-5) were determined. Pair-wise comparisons showed a 0.6-8.0% (average 6.3%) sequence divergence among the five clones (Table 1). Relative to other host species, there was an 8.5-11.7% (average 9.9%) sequence divergence from the *Pneumocystis* from *Capra hircus*, 9.4-12.8% (average 11.0%) divergence from *Sus scrofa domesticus*, 13.7-16.2% (average 15.0%) divergence from *Oryctolagus cunicullus*, 14.4-17.3% (average 15.8%) divergence from *Saguinus midas*, and 17.3-19.9% (average 19.0%) divergence from *P. jirovecii* from *Homo sapiens* (Table 1). These results indicate that the *Pneumocystis* species isolated in this study are most closely related to the *Pneumocystis* from a goat in Japan and most distantly related to the *P. jirovecii* from humans.

Discussion

There is substantial evidence that many human patients with *Pneumocystis* pneumonia are infected with multiple strains of human *Pneumocystis* (Nahimana et al. 2000). Multiple episodes of *Pneumocystis* pneumonia are only common among patients with HIV, and are sometimes due to reinfection rather than relapse (Kovacs & Masur 2009). In the current study, multiple genetic clones were detected in one of three goat kids. Considering the environment of the farm in which many goats graze and new animals have been introduced from other farms multiple times, this

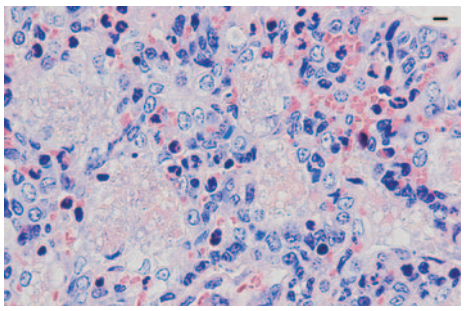

Fig. 1. Case 1
Because of thickening of alveolar septa, the alveolar spaces are narrowed and filled with foamy material. Giemsa. Bar = 5 µm.

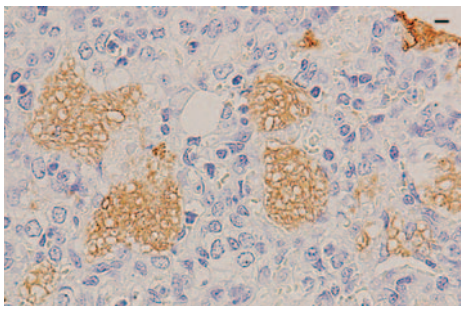

Fig. 2. Case 1
As in Fig. 1, intra-alveolar foamy material positive for *Pneumocystis* antigen is visible. SAB. Bar = 5 µm.

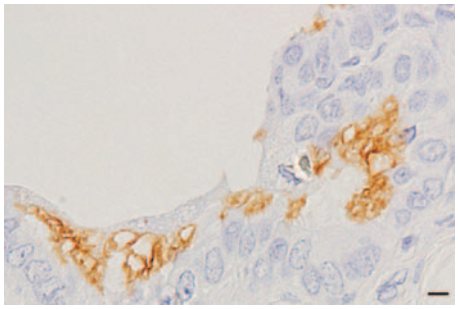

Fig. 3. Case 1
Pneumocystis antigen-positive organisms are observed on the surface of bronchiolar epithelial cells. SAB. Bar = 5 µm.

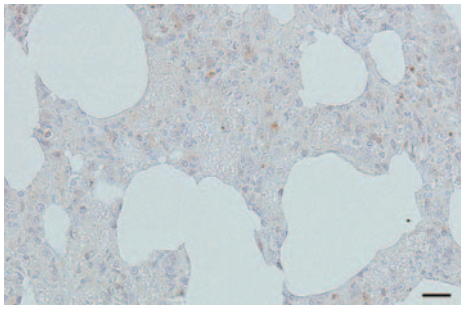

Fig. 4. Case 1
CD3-positive cells are almost absent in alveolar septa. SAB. Bar = 20 µm.

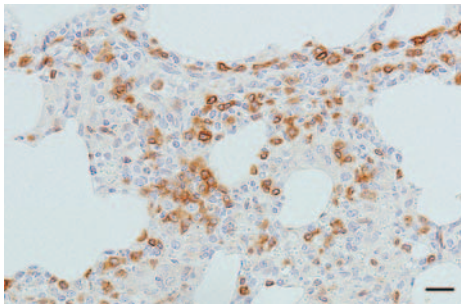

Fig. 5 Case 3
Some CD3-positive cells are present in alveolar septa. SAB. Bar = 20 µm.

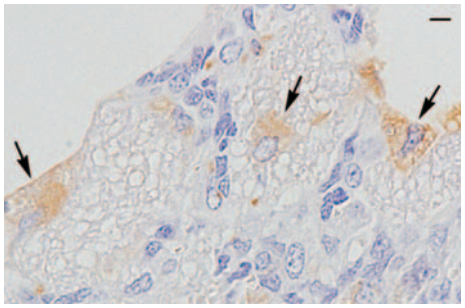

Fig. 6 Case 1
CD68-positive macrophages (arrows) are visible on or within *Pneumocystis* masses. SAB. Bar = 5 µm.

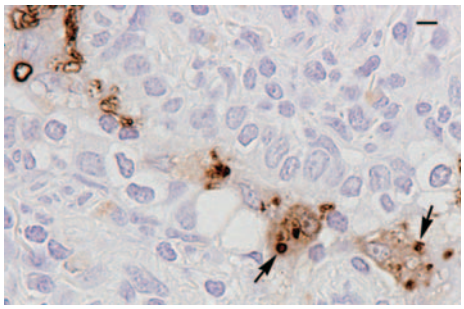

Fig. 7 Case 3
Pneumocystis antigen is visible as dark dots (arrows) in the cytoplasm of CD68-positive macrophages. SAB. Bar = 5 µm.

Table 1. Matrix of mtLSU rRNA sequence divergence of *Pneumocystis* derived from various mammalian hosts

Source of rRNA	% divergence from				
	#1	#2	#3	#4	#5
Clone #1					
Clone #2	7.4				
Clone #3	7.1	6.5			
Clone #4	0.6	8.0	7.8		
Clone #5	6.8	5.9	5.6	7.4	
Goat (*Capra hircus*)	8.5	11.7	9.9	9.2	10.4
Pig (*Sus scrofa domesticus*)	9.4	12.8	11.8	9.9	11.3
Rabbit (*Oryctolagus cuniculus*)	13.7	16.2	15.7	14.2	15.2
Red-handed tamarin (*Saguinus midas*)	14.4	15.7	16.3	15.1	17.3
Human (*Homo sapiens*)	19.5	19.2	19.9	19.2	17.3

The DDBJ accession numbers of Pneumocystis species used in this study are as follows: AB602435 (Pneumocystis from *Capra hircus*); S42915 (from *Oryctolagus cuniculus*); AF362455 (from *Saguinus midas*); FJ357851 (*P. jirovecii* from *Homo sapiens*)

genetic diversity implies that the kid may have been repeatedly infected by other goats. In contrast, a single strain was identified in a 3-year-old goat with paratuberculosis (Sakakibara et al. 2013), which was one of nine goats raised on a farm where no other goats were present in neighboring areas (unpublished data). Activation of fungi having infected this animal soon after birth is the most likely event under such circumstances (Kovacs & Masur 2009).

Another factor may also be responsible for the genetic variability of *Pneumocystis* in the Uganda case. Sulfa drugs are associated with the evolution of *P. jirovecii* in HIV-infected adults (Beard et al. 2005, Lane et al. 1997). Since natural selection exerted by the innate immune system is thought to generate hypervirulent bacterial variants in streptococcal infections (Walker et al. 2007), it is probable that not only sulfa drugs but also host immune responses impose selective pressure on *Pneumocystis* organisms. Immunocompetent human infants (Beard et al. 2005), suckling piglets (Kondo et al. 2000) and newborn rabbits (Dei-Cas et al. 1998) are considered to serve as reservoirs for *Pneumocystis* organisms. The infection subsequently clears as the immune response matures (Beard et al. 2005), and an elevation of antibody titers to the organisms in pigs after weaning (Kondo et al. 2000) suggests the involvement of the acquired immune system. Healthy young goat kids are presumably also a reservoir, but in the present farm and surrounding areas, many goats were infested with *Haemonchus contortus*, capable of causing anemia, weakness and weight loss (Kaufmann 1996). The parasites are not usually fatal to most goats in such *Haemonchus* endemic areas. However, activation of the innate immune system caused by mild infestation in some young kids may induce alteration of host-fungal interactions, which can lead to infection with multiple genetic clones of *Pneumocystis* in these areas.

As in the close relationship between paratuberculosis and pneumocystosis in a doe (Sakakibara et al. 2013), the opportunistic infection in the lungs can be linked to the severe haemonchosis in case 3 described here. The fact that *Pneumocystis* organisms were more abundant in the fatal cases suggests that the two kids were also infested with parasites and were anemic, malnourished, and in an immunocompromised status. This view was supported by further histological findings. *Pneumocystis* organisms were phagocytosed by macrophages, which suggests that macrophages play an important role in the host defense against *Pneumocystis* (Martin & Pasula 2000). However, their number was too small to exclude or suppress the fungi in cases 1 and 2, relative to the abundance of fungal colonies. Additionally, CD3-positive cells were sparse in lung tissues; CD4-positive helper cells are known to be important in preventing progression to pneumocystosis in HIV-infected patients (Masur et al. 1989). In contrast, numerous CD3- or CD20-positive lymphocytes were observed in case 3, and despite the small size and number of *Pneumocystis* colonies, there were similar numbers of macrophages as in cases 1 and 2. These findings suggest that immune competence was retained to some degree in case 3, while in the other kids, serious immune dysfunction suggested the existence of more severe haemonchosis.

Except for two caprine cases (McConnell et al. 1971, Sakakibara et al. 2013), there is essentially no available information on *Pneumocystis* in ruminants, which has left the impression that these animals are rarely infected with this pathogen. However, taking into account the occurrence of caprine *Pneumocystis* infection in Africa and Japan (McConnell et al. 1971, Sakakibara et al. 2013), the infec-

tion may, in fact, not be so rare in immunocompromised goats.

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