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Pneumocystis carinii Pneumonia in Thoroughbred Foals: Identification of a Genetically Distinct Organism by DNA Amplification

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Genetically distinct forms of *Pneumocystis carinii* infect several mammalian hosts. We report the amplification of *P. carinii* DNA from samples of two infected thoroughbred foal lungs by using primers designed from the sequence of a *P. carinii* mitochondrial rRNA gene; these primers also prime the amplification of *P. carinii* DNA from other hosts. The nucleotide sequence of part of the mitochondrial rRNA gene amplified from *P. carinii* infecting one of the foals was determined and found to be distinct from that of published rat-, rabbit-, ferret-, and human-derived *P. carinii* sequences.

Pneumocystis carinii is an opportunistic fungal pathogen of humans that causes pneumonia in the immunosuppressed, such as AIDS sufferers and patients receiving immunosuppressive therapy (1, 8, 19, 22). Furthermore, *P. carinii* infection has been reported in animals with immune deficiencies or malnourishment, including foals (16, 17), piglets (4), and dogs (9). The infection may also be experimentally induced in laboratory animals by steroid treatment (10).

P. carinii pneumonia was recently retrospectively diagnosed in four English thoroughbred foals by use of histochemical and immunocytochemical staining (25). The 6- to 12-week-old foals died between 1969 and 1991 after short periods of diffuse interstitial pneumonia. Staining of sections of lung tissue with Grocott's methenamine silver and immunostaining with a mouse monoclonal antibody to *P. carinii* of human origin led to a diagnosis of *P. carinii* pneumonia. One of these foals was a 6-week-old male thoroughbred, which presented with a sudden onset of severe dyspnea and pyrexia and died after 2 days in 1991. Formalin-fixed lung samples were available from this foal. A more recent case (1992) involved a 10-week-old female thoroughbred, which had scour for 12 h, followed by dyspnea and death. Frozen and formalin-fixed lung samples were available from this foal. No underlying immune defect which would have predisposed these animals to *P. carinii* infection was determined, although no appropriate tests were performed at or before the postmortem examination.

In this study, we examined the 1991 and 1992 cases of *P. carinii* infection in foals by using a DNA amplification technique which is highly sensitive and specific for the diagnosis of *P. carinii* infection in AIDS patients and individuals undergoing immunosuppressive treatment for organ transplantation (15, 24). With this technique, it is possible to detect *P. carinii* to a lower limit of one or two organisms (18). The amplification primers used were derived from the sequence of the large subunit of mitochondrial rRNA from *P. carinii* and will amplify *P. carinii* DNA isolated from the lungs of steroid-treated animals (rats, rabbits, and ferrets) that have acquired the infection. DNA sequencing of the amplification products has shown that the *P. carinii* organisms infecting different mammalian hosts are genetically distinct at this locus (21, 23). Similarly, the β-tubulin genes from rat- and human-derived *P. carinii* are only 81% identical at the DNA level, although they are completely conserved at the amino acid level (7). Studies with monoclonal antibodies have also highlighted differences between human- and rat-derived *P. carinii* (13), and experiments with convalescent-phase antisera have shown ferret-, rat-, and mouse-derived *P. carinii* to be antigenically different (2). Here we report the amplification of equine-derived *P. carinii* DNA from fresh and formalin-fixed material by using the primers described above. We compare the DNA sequence of the amplification product with the DNA sequences of this product from human-, rat-, rabbit-, ferret-, and mouse-derived *P. carinii* and show that the organisms infecting these different hosts are genetically distinct at this locus.

DNA was isolated from frozen or fixed samples of lungs from two foals with *P. carinii* infection and also from a frozen *P. carinii*-infected severe combined immunodeficiency (SCID) mouse lung. To isolate DNA from frozen material, 3-cm^3 lung samples were defrosted and minced finely. DNA was extracted by digestion with proteinase K at a final concentration of 500 $\mu g\ ml^{-1}$ (in the presence of 10 mM EDTA–1% sodium dodecyl sulfate at 50°C with shaking for 16 h), phenol-chloroform extraction, and ethanol precipitation. For DNA isolation from formalin-fixed material, 7-μm-thick paraffin-embedded sections were dewaxed with 500 μl of xylene, vortexed, and pelleted in a microcentrifuge for 5 min. This step was repeated twice. The pellet was washed twice with ethanol, dried, and resuspended in 200 μl of sterile distilled water. Proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation were carried out as for frozen material.

Two pairs of oligonucleotide primers were used for DNA

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              1                                                  50
   Pc-foal    GATATAGCTG GTTTTCTGCG AAATTTGTTT TAGCAAATTG TTTATTAT..
    Pc-rat    GATATAGCTG GTTTTCTGCG AAATCTATTT TGGTAGATGA CTTGTTAT..
 Pc-rabbit    GATATAGCTG GTTTTCTGCG AAATTTGTAT TAGCAAAGTG TTTATGTA..
 Pc-ferret    GATATAGCTG GTTTTCTGCG AAATTTGTTT TAGCAAGTTG TTTATTAG..
  Pc-human    GATATAGCTG GTTTTCTGCG AAAATTGTTT TGGCAAATTG TTTATTCCTC
  Pc-mouse    GATATAGCTG GTTTTCTGCG AAATCTATTT TAGTAGATGA TTTATTAT..

              51                                                100
   Pc-foal    ..ATTATTAG TAGGTATAGC ACTGAATATC TATTCTTAAT AGAATAAA.A
    Pc-rat    .TATTTGTAG TGGGTATAGC ACTGAATATC TAACTTATGT TAGA.....A
 Pc-rabbit    .ATAAATTAG TAGGTATAGC ACTGAATATT CATTTAG... .........A
 Pc-ferret    .AATTATTAG TAGGTATAGC ACTGAATATC TAAATAATTA GG.......A
  Pc-human    TAAAAAATAG TAGGTATAGC ACTGAATATC TCG....... .........A
  Pc-mouse    ..TTTATTAG TGGGTATAGC ACTGAATATC TGTAATTTGA ATTACAAGAA

              101                                               150
   Pc-foal    GGGAGTATTA TTAATACTTA TCTTGGATAT TTAATCTCAA AATGACT.AA
    Pc-rat    GGGAGTATGA AGG.TACTTA CTTTGGATAT TTAATCTCAG AATAGCT.AT
 Pc-rabbit    GGGAGTACGT AAG.TATTTA TCTCGGATAT TTAATCTCAG AATGACT.AA
 Pc-ferret    GGGAATATTA AAGATATTT. TCTTGGATAT TTAATCTCAG AATGACTTAA
  Pc-human    GGGAGTATGA AAA.TATTTA TCTCAGATAT TTAATCTCAA AATAACT.AT
  Pc-mouse    GGGAGTATGA AAG.TACTTA CTTTGGATAT TTAATCTCAG AATAACT.AA

              151                                               200
   Pc-foal    TAAGT....A AGATAAATA. ATCAGACTTT TTGCGATAAG GTAGAAAGTC
    Pc-rat    TAATAT...A TGATGAGTT. ATCAGACTTC TTGCGATAAG GTGAGGAGTC
 Pc-rabbit    TTTAT....A AGATAAATCG ATCGGACTTT TTGCGAGAAG GTGGAAAGTC
 Pc-ferret    TAATA....G TGGTAAATA. ATGGGACTTC TTGCGATAAG GTGGGAAGTC
  Pc-human    TTCTT....A AAATAAATA. ATCAGACTAT GTGCGATAAG GTAGATAGTC
  Pc-mouse    TAAGAAGATA AGATAAATT. CTCAGACTTT TTGCGATAAG GTGAAAAGTC

              201       219
   Pc-foal    AAAAGGGAAA CAGCCCAGA
    Pc-rat    GAGAGGGAAA CAGCCCAGA
 Pc-rabbit    AAGAGGGAAA CAGCCCAGA
 Pc-ferret    AAGAGGGGAA CAGCCCAGA
  Pc-human    GAAAGGGAAA CAGCCCAGA
  Pc-mouse    GAAAGGGAAA CAGCCCAGA
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FIG. 1. DNA sequence of a portion of the pAZ102-H–pAZ102-E amplification product from foal-, rat-, rabbit-, ferret-, human-, and mouse-derived *P. carinii*. Dots indicate gaps to allow alignment of the sequences according to secondary structure.

amplification. For DNA extracted from frozen material, we used the previously described primers pAZ102-H and pAZ102-E (24), which prime the amplification of a product of approximately 350 bp, depending on the host of origin of the *P. carinii* DNA. For DNA extracted from fixed material, we used primers pAZ102-E and pAZ102-L2, which prime the amplification of a smaller product, of 120 bp. The primers were used in amplification reactions at a final concentration of 1 μM with 0.025 U of *Taq* DNA polymerase (Boehringer Mannheim) per μl, buffer (Boehringer) supplemented with $MgCl_2$ to a final concentration of 3 mM, and 400 μM deoxynucleoside triphosphates (Boehringer). Amplification conditions for pAZ102-H and pAZ102-E were 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 2 min for 40 cycles; those for pAZ102-E and pAZ102-L2 were the same, except for an annealing step of 50°C for 1.5 min. Amplification products from reactions primed with pAZ102-H and pAZ102-E were electrophoresed in 1.5% agarose gels, and those from reactions primed with pAZ102-E and pAZ102-L2 were electrophoresed in 3% GTG Nusieve–1% SeaPlaque agarose gels (Flowgen Instruments Ltd.). Southern blots of the gels were hybridized with an equine *P. carinii*-specific internal oligonucleotide, pAZ102-T (5′ TAG GTA ATT ATT TTA TTA CAA TC 3′), end labelled with [^{32}P]dATP (Amersham International). Hybond N filters (Amersham) were washed at melting temperature − 5°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

The DNA sequences of the amplification products were determined by performing the amplification reactions with one biotinylated and one unmodified oligonucleotide primer. The products were purified with streptavidin-coated Dynabeads (Dynal) by magnetic separation and sequenced by the dideoxy chain termination method (3, 12, 20).

When DNA extracted from frozen samples of lungs from the foal which died in 1992 (foal A) was amplified with the primers pAZ102-H and pAZ102-E, it produced a product of 350 bp (data not shown). The sequence of the amplification product was determined and aligned with the DNA sequences of the same region amplified from other mammalian hosts (Fig. 1). Over 219 bp of the amplification product from equine *P. carinii*, there was 78% sequence identity with rat-derived *P. carinii*, 84% sequence identity with rabbit- and SCID mouse-derived *P. carinii*, and 85% sequence identity with ferret- and human-derived *P. carinii* (University of Wisconsin Genetics Computer Group Sequence Analysis Software Package, Distances Program [6]). These data confirm the diagnosis of *P. carinii* infection in this foal and show that equine-derived *P. carinii* DNA is distinct at this locus from *P. carinii* DNA from other mammalian hosts.

Amplification of DNA from fixed material is problematic because of cross-linking of DNA by formalin. We were unable to amplify DNA from fixed material with pAZ102-H and pAZ102-E but successfully amplified DNA from fixed material with pAZ102-L2 and pAZ102-E for the foals which died in 1992 (foal A) and in 1991 (foal B) (Fig. 2). The amplification product was smaller (120 bp) than that obtained with pAZ102-H and pAZ102-E, and to establish that the PCR product was specific for equine-derived *P. carinii*, we used an internal oligonucleotide probe, pAZ102-T, which was designed from the sequence of the larger, pAZ102-H–

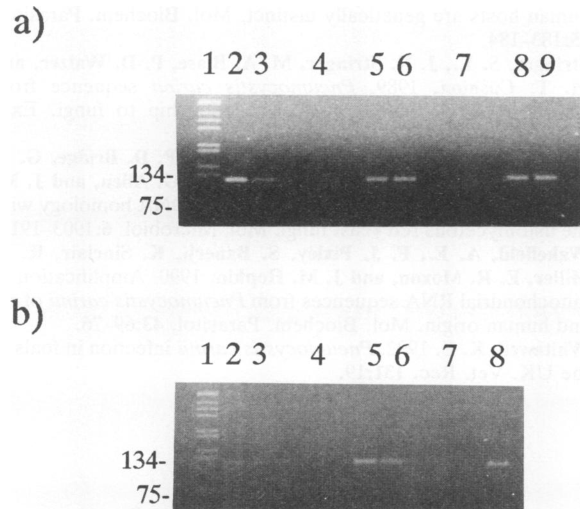

FIG. 2. Agarose gel electrophoresis (3% Nusieve–1% SeaPlaque agarose) of PCR products from amplifications with primers pAZ102-L2 and pAZ102-E and DNA extracted from frozen material (a) and fixed material (b). (a) Lanes: 1, 1-kb DNA ladder (GIBCO BRL) (sizes are in base pairs); 2, rat-derived *P. carinii* (1/5 dilution); 3, rat-derived *P. carinii* (1/25 dilution); 4, no-template-DNA control (NT); 5, human-derived *P. carinii* (1/5 dilution); 6, human-derived *P. carinii* (1/25 dilution); 7, NT; 8, foal A-derived *P. carinii* (1/5 dilution); 9, foal A-derived *P. carinii* (1/25 dilution). (b) Lanes: 1, 1-kb ladder; 2, foal B-derived *P. carinii* (1/5 dilution); 3, foal B-derived *P. carinii* (1/25 dilution); 4, NT; 5, foal A-derived *P. carinii* (1/5 dilution); 6, foal A-derived *P. carinii* (1/25 dilution); 7, NT; 8, rat-derived *P. carinii*.

pAZ102-E product as an equine *P. carinii*-specific probe when used under high-stringency conditions. The amplification products from foal A and foal B both hybridized with this probe (data not shown), confirming that they originated from the amplification of equine-derived *P. carinii* DNA.

As *P. carinii* cannot be cultured in vitro, routine diagnosis is based on microscopic observation of stained organisms in lung samples. The diagnostic technique based on DNA amplification, which we have previously reported for use in humans (24), has also proved to be sensitive and specific for the diagnosis of *P. carinii* infection in foals and may be of use for the prompt diagnosis of equine *P. carinii* pneumonia. The primers on which this technique is based amplify DNA of *P. carinii* from all mammalian hosts tested to date. The primers are designed to conserved regions of a mitochondrial rRNA gene, and the intervening sequence spans a variable region. Hence, these primers can be used for the amplification of *P. carinii* DNA from different mammalian hosts under high-stringency conditions (melting temperature − 3°C), but sequencing of the products reveals significant genetic differences among *P. carinii* organisms from the various hosts. This study shows that *P. carinii* infecting foals is genetically distinct from that infecting other hosts.

Lee et al. (14) have reported genetic variations among *P. carinii* strains infecting several different human hosts by using the same amplification primers as those used in this study. Moreover, two genetic variants of *P. carinii* coinfecting laboratory rats have been reported, and it may be appropriate to consider these variants separate species (5). Hence, it should be noted that the DNA sequence of equine-derived *P. carinii* reported here may be from one *Pneumocystis* species capable of infecting foals and that there may be others. This study shows that equine *P. carinii* is genetically distinct from isolates from other hosts and supports the views of Hughes and Gigliotti (11) and Cushion et al. (5) that the nomenclature of *P. carinii* should be reviewed.

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