IN UTERO TRANSMISSION OF Pneumocystis carinii sp. f. oryctolagi

CERE N.*, DROUET-VIARD F.*, DEI-CAS E. **,***, CHANTELOUP N.* & COUDERT P.*

Summary:
Although vertical transmission of Pneumocystis in human or animal hosts has often been suspected, no evidence demonstrating this infection route has been furnished until now. This widespread parasite is constantly found in the lungs of rabbits, which spontaneously develop a benign pneumocystosis at weaning. However, the infection source, the method of entry of Pneumocystis organisms into the rabbit and when this mammal is infected, remain to be known. As a few parasites have been microscopically observed and detected by PCR in the lungs of rabbits at birth, in utero Pneumocystis infection has been hypothesized. The presence of Pneumocystis was therefore carefully assessed in 16 pregnant does, their embryos and fetuses by using several detection methods. Pneumocystis was detected by PCR in maternal blood, embryos, amniotic fluid and fetuses. The parasite was also revealed histologically and by immunofluorescence in fetal and maternal lungs and in placentas. The results suggest that vertical transmission of P. carinii sp. f. oryctolagi occurs as early as at the 10th day of pregnancy.

KEY WORDS: Pneumocystis carinii sp. f. oryctolagi, transplacental transmission, in utero infection, rabbit. Pneumocystis carinii pneumonia.

INTRODUCTION

Pneumocystis carinii is an opportunistic agent primarily found in the lungs of various mammals. This parasite causes severe pneumonia in immunocompromised hosts. It can be transmitted by the airborne route (Hughes et al., 1987; Soulez et al., 1991) but other modes of transmission cannot be totally excluded (Hughes et al., 1987). Thus, although no definitive proof has been furnished (Hughes et al., 1995), vertical transmission of P. carinii has been suspected for a long time in rats (Pifer et al., 1984) and humans (Bazaz et al., 1970; Mortier et al., 1995). In contrast, the parasite is not transmitted through the placenta in SCID mice (Ito et al., 1991). The rabbit is an interesting model to investigate vertical transmission of Pneumocystis. We have reported that almost all untreated (i.e. not submitted to immunosuppressive drugs) young rabbits are spontaneously and heavily infected by P. carinii at weaning (28-day-old rabbits) (Soulez et al., 1989; Dei-Cas et al., 1990a) but we do not know when, nor how P. carinii infects them. Most rabbits recover spontaneously from this spontaneous P. carinii pneumonia (PCP) within 2-4 weeks (Soulez et al., 1989).

We have previously reported P. carinii infections in 7-day-old rabbits (Dei-Cas et al., 1990b). The aim of the present work was to determine when the first contamination of this mammal with the parasite occurs. The presence of Pneumocystis was carefully assessed in newborn rabbits, pregnant does, their embryos and fetuses by using several detection methods. Considerable evidence suggesting that in utero transmission of Pneumocystis occurs in rabbits was found and is reported here.
MATERIALS AND METHODS

Animals, experiments and sampling procedures

Thirteen experiments were developed using hybrid California/New Zealand white rabbits purchased from a commercial supplier. Eight newborn rabbits, 16 pregnant does and 67 fetuses or blastocysts were used. Two females were at the 26th day of pregnancy and bore 28 fetuses. Four females were at the 15th day of pregnancy and bore 20 fetuses. Four females were at the 10th day of pregnancy and bore 12 fetuses. Six females were at the 5th day of pregnancy and bore 29 embryos (blastocystic stage) pooled in 7 sets.

Newborn rabbits were sacrificed at birth, their lungs were removed under aseptic conditions. The presence of *Pneumocystis* was assessed in all lungs by microscopy (on smear impression and histologic sections) and PCR methods (see below).

Blood of pregnant does was collected from the central ear vein on EDTA before euthanasia. Blood was not collected by cardiac puncture in order to avoid potential contamination with *Pneumocystis* from lung. The buffy coat was tested for *Pneumocystis* by using microscopy and PCR methods. Hysterectomy was performed in aseptic conditions. The external surface of the uterus was carefully disinfected with quaternary ammonium salts before dissection in order to avoid enteric microbial contamination. All the fetuses were collected aseptically under a laminar air flow hood. Lungs, liver, spleen, fetal side of placenta, amniotic fluid and maternal side of placenta were collected from the six females (5th day of pregnancy) and the uterine washing fluid using only TBO. Parasite extraction was performed as described by Aliouat et al. (Aliouat et al., 1993). Lungs, liver, spleen, fetal side of placenta, amniotic fluid and maternal lungs were collected, frozen and fixed as described by Drouet-Viard et al. (Douet-Viard et al., 1994). *Pneumocystis* organisms were detected by means of an immunofluorescence assay (IFA) using a monoclonal antibody anti-rabbit-derived *Pneumocystis* (Mab 1H1, INSERM U42, Lille, France). A goat anti-mouse IgG coupled to fluorescein isothiocyanate (GAM FITC, Nordic, Netherlands) was used to label Mab 1H1. The sections were counterstained with Evans blue.

Detection of *Pneumocystis* in rabbit tissues by immunofluorescence

Immunofluorescence detection of *Pneumocystis* was carried out on 2 females and their fetuses at the 26th day of pregnancy. Samples of maternal lungs and fetal lungs, as well as of maternal and fetal sides of placentas were collected, frozen and fixed as described by Drouet-Viard et al. (Douet-Viard et al., 1994). *Pneumocystis* organisms were detected by means of an immunofluorescence assay (IFA) using a monoclonal antibody anti-rabbit-derived *Pneumocystis* (Mab 1H1, INSERM U42, Lille, France). A goat anti-mouse IgG coupled to fluorescein isothiocyanate (GAM FITC, Nordic, Netherlands) was used to label Mab 1H1. The sections were counterstained with Evans blue.

Detection of *Pneumocystis carinii* by PCR

Tissues were homogenized with a hand Potter homogenizer. The resulting homogenate was poured through gauze, centrifuged at 3,000 g for 10 min, and the resulting pellet was washed with PBS. Red blood cells were lysed using the buffered hemolytic solution. Template DNA was prepared using an adapted protocol of Maniatis et al. (Maniatis et al., 1981). Each sample was treated with proteinase K (0.2 mg/ml) (Boehringer Mannheim, France) in Sodium (0.1 M) Tris HCl (10 mM) EDTA (1 mM) buffer (pH 8) in the presence of 1 % SDS. DNA was purified by phenol-chloroform extraction and ethanol precipitation (Sambrook et al., 1989). Fifty nanograms were processed in 20 μl amplification buffer containing 5 mM MgCl2, 0.02 mM dextynucleoside triphosphate, 3.5 μl reaction buffer (750 mM Tris HCl pH 9 at 25 °C, 200 mM (NH4)2SO4 and 0.1 % Tween 20), 0.02 U/μl Goldstar DNA polymerase (Eurogentec, 4102 Seraing, Belgium) and 0.05 mM of each primer. The *Pneumocystis*-specific primers used were pAZ102-E and pAZ102-H complementary to sequences of the gene coding for the large subunit of the mitochondrial ribosomal RNA from the parasite (Wakefield et al., 1990). Reaction temperatures were 92 °C for 40 s, 51 °C for 20 s and 72 °C for 20 s;
35 cycles were repeated in both amplification steps. PCR was performed in a MJ Research thermal cycler. Negative controls were water, rabbit plasma, rabbit brain and rabbit sperm. The amplification products were visualized by ethidium bromide staining (0.5 μg/ml) after electrophoresis on a 2% agarose gel.

PCR FRAGMENT SEQUENCING
AND SEQUENCE COMPARISONS

PCR amplified fragment sequences were determined by the dideoxy chain termination technique (Sanger et al., 1980) and subsequently loaded on a fluorescent 373A automated DNA sequencer (Applied Biosystems). Sequencing data were analysed using the FASTA program (Pearson et al., 1988) of the Genetics Computer Groups (GCG) package.

RESULTS

PNEUMOCYSTIS IN NEWBORN RABBITS

A few Pneumocystis organisms were microscopically detected in all 2 to 4-hour-old rabbits in lung extracts smears stained with BTO and in all histological sections (Fig. 1e). A Pneumocystis specific band of 346 bp (Wakefield et al., 1990) was amplified by PCR (Fig. 2).

<table>
<thead>
<tr>
<th>Day of pregnancy</th>
<th>Host</th>
<th>Detection (1)</th>
<th>Lung</th>
<th>Blood</th>
<th>Placenta</th>
<th>Amniotic fluid</th>
<th>Whole body (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Blastocysts (3) (n = 29)</td>
<td>PCR</td>
<td>0/6</td>
<td>4</td>
<td>0/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(from 6 pregnant rabbits)</td>
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</tr>
<tr>
<td>10</td>
<td>Fetuses (n = 12)</td>
<td>PCR</td>
<td>0/12</td>
<td>0/12</td>
<td>12/12</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(from 4 pregnant rabbits)</td>
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</tr>
<tr>
<td>15</td>
<td>Fetuses (n = 20)</td>
<td>PCR</td>
<td>20/20</td>
<td>6/10</td>
<td>20/20</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(from 4 pregnant rabbits)</td>
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</tr>
<tr>
<td>26</td>
<td>Fetuses (n = 28)</td>
<td>TBO</td>
<td>6/6</td>
<td>6/6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(from 2 pregnant rabbits)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Mab</td>
<td>6/6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td></td>
<td>PCR</td>
<td>28/28</td>
<td>ND</td>
<td>28/28</td>
<td>6/6</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

(1) Detection methods were the following: toluidine blue O (TBO), fluorescent specific monoclonal antibody staining (Mab) or PCR assay (see Materials and Methods).
(2) Whole body = PCR was carried out from a total DNA extract of each fetus.
(3) The 29 blastocysts were divided in 7 sets of 4 to 5 pooled blastocysts.
(4) Uterine washing fluid to collect blastocysts.
ND = Not done.

Table 1. = Pneumocystis carinii in tissues of embryos or fetuses. Number of positive embryos or fetuses/number tested.
Fig. 1. – *Pneumocystis in utero* transmission in rabbits. a) Cystic forms of the parasite in the lung of a pregnant rabbit (26th day of pregnancy); b), c) and d) *Pneumocystis* cystic forms (arrowheads) in fetal lungs (26th day of pregnancy); e) *Pneumocystis* cystic forms in the lung of a newborn rabbit (arrowhead). a) and b) IFA without a) or with b) Evans blue counterstaining. c), d) and e) histological sections stained with toluidine blue O. Bar = 10 μm.
Pneumocystis organisms were revealed in fetal (Fig. 1b, c, d) lungs and at the fetal side of placetas using light microscopy or IFA.

The Pneumocystis-specific band of 346 bp was amplified in all samples of tissues and fluids of all pregnant females. Maternal serum and plasma were negative but the buffy coats were positive. Rabbit sperm and rabbit brains used as controls were negative. These were the only samples (with plasma and serum of pregnant females) of rabbit origin which were found free of Pneumocystis by using PCR. At the 15th day of pregnancy, PCR assay revealed that all tissues were positive including placentas and amniotic fluid (6/10). At the 10th day of pregnancy, PCR assay revealed Pneumocystis in the whole body but not in amniotic fluid. In contrast, PCR assay did not reveal Pneumocystis in the blastocysts and uterine washing fluid from the 5th day of pregnancy (3 days before nidation).

SEQUENCING OF THE AMPLIFIED PRODUCTS

The sequence of the amplified fragments were 98% identical to the published rabbit-derived Pneumocystis homologous fragment (Peters et al., 1994) over 219 bp.

DISCUSSION

Nonimmunodepressed young weanling rabbits from conventional breeders are spontaneously and heavily infected with Pneumocystis (Soulez et al., 1989; Dei-Cas et al., 1990a). This natural infection has been used as an experimental model of P. carinii pneumonia (PCP) (Goyot et al., 1984; Dei-Cas et al., 1990a; Dei-Cas et al., 1994; Akono & Pal luault, 1994; Mazars et al., 1995). This model presents at least two advantages. First, the infection occurs in the absence of drug-induced immunodepression (Soulez et al., 1989; Dei-Cas et al., 1990a). The rabbit can therefore be used to investigate host-parasite interactions in a nonimmunodepressed natural host, especially primary Pneumocystis infection. Second, antigenic (Goyot et al., 1984; Soulez et al., 1988) and genomic (Dei-Cas et al., 1994; Mazars et al., 1995) data suggest that rabbit-derived Pneumocystis (P. carinii sp. f. oryctolagi) strains are more related to human Pneumocystis (P. carinii sp. bominis) than those of mice or rats.

In this work, PCR allowed more accurate detection of Pneumocystis than conventional staining or IFA methods. It is well known that PCR is an extremely sensitive technique and unfortunately contamination in PCR experiments is a frequent occurrence. Nevertheless, we never observed any positive PCR in the 5 negative controls in any of the 13 successive experiments which were performed to obtain these results. We specially emphasize the importance of the negative brain control which was treated with exactly the same material (hand potter homogenizer, surgical tools,..) as the other solid tissues (lungs, whole body,..).

This observation that newborns were already infected with Pneumocystis at birth is sufficient to assert that in utero transmission occurred. Thus, 16 pregnant does, their 60 fetuses and 29 blastocysts were examined to investigate vertical Pneumocystis transmission. Four days before birth, Pneumocystis was detected by PCR in all organs tested from mothers and fetuses. The fact that PCR revealed Pneumocystis in the buffy coat of blood samples from pregnant mothers suggested that parasites could reach the fetuses by the hematogenous route. Unfortunately we were not able to identify microscopically the parasite in buffy coat samples. Moreover, the lack of detection of Pneumocystis in blastocysts and its presence in fetuses suggests that the placenta is necessary to fetal infection. Steven (Steven, 1975) has shown that the endothelium of maternal capillaries in rabbits disappears on the 10th day of pregnancy and that the placenta is hemochorial until the 17th day. Thereafter, from the 17th day until birth, the placenta is hemoendothelial. Thus, placenta permeability increases with the stage of pregnancy. It was found in the present study that fetuses were already infected at 10 days of pregnancy, corresponding to the hemochorial stage of placentation. The placental barrier is then relatively permeable and parasites circulating in the maternal blood could reach the fetus. Another route might be the amniotic fluid, where Pneumocystis was detected by PCR. Nevertheless, it was difficult to sample the amniotic fluid from 15- and 26-old day fetuses; most of these samples were slightly contaminated with blood and therefore these results should be considered with caution.

As humans also have a hemochorial type of placenta, in utero transmission of Pneumocystis might also occur (Hughes, 1987). However, no definitive proof of vertical transmission of Pneumocystis either in humans or in rats has been found. Ito et al. (Ito et al., 1991) did not find evidence of transplacental infection with Pneumocystis in SCID mice.

In summary, clear evidence of in utero transmission of Pneumocystis in rabbits is presented in this work. In utero transmission might be a supplementary route for Pneumocystis, at least in rabbits, besides the airborne route already shown in rats (Hughes, 1987) and in mice (Soulez et al., 1991). The mechanism of transmission of Pneumocystis from the mother to the fetus remains to be elucidated. Likewise, it has to be determined whether the transplacental transmission of the Pneumocystis organisms infecting the fetus in utero is the origin of the spontaneous pneumocystosis observed in rabbits at weaning (Soulez et al., 1989; Dei-Cas et al., 1990a).
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