

STUDY ON PLACENTAL TRANSMISSION OF *PNEUMOCYSTIS CARINII* IN RABBITS

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Abstract - Eighty to 100% of rabbits at weaning are spontaneously and heavily infected with *Pneumocystis* (*P. carinii* sp.f. *oryctolagi*). Lung and other tissues have been found infected with *Pneumocystis* after microscopic examination, immunofluorescence or PCR. However; the infection source, the method of entry of *Pneumocystis* organisms into the rabbit and when they become infected remain to be known. The parasite rate was highest at weaning. As a few parasites were microscopically observed at birth and detected by PCR, in utero *Pneumocystis* infection was hypothesised. Thus, four pregnant does and their foetuses or embryos were examined. *Pneumocystis* was detected by PCR in maternal blood, foetuses, embryos and amniotic fluid. It was also revealed histologically and immunofluorescence in foetal and maternal lungs and placentas. Results suggest that transplacental transmission of *Pneumocystis* occurs as early as at the 15th of pregnancy.

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 but other modes of transmission cannot be totally excluded [1]. The results published by ITO *et al.* [2] suggest that *P. carinii* cannot be transmitted transplacentally in mice. On the other hand, the vertical transmission of *P. carinii* has been suggested in rats [3] and humans [4] but no definitive proofs were furnished [5].

It was reported that 80 - 100 % of untreated (not submitted to immunosuppressing drugs) young rabbits at weaning are spontaneously and heavily infected with *Pneumocystis* [6]. Most animals recover spontaneously within 2-4 weeks. The infection source, the method of entry of *Pneumocystis* infectious organisms into the rabbit and when they become infected remain to be known. The untreated rabbit has been used as an experimental model of *Pneumocystis carinii* pneumonia (PCP) [7, 8, 9, 10, 11]. It presents at least two advantages: the infection occurs in the absence of drug induced immunodepression [6, 7] and antigenic [11, 12] and genomic [8, 10] data suggest that rabbit-derived *Pneumocystis* (*P. carinii* sp.f. *oryctolagi*) strains are more related to human *Pneumocystis* (*P. carinii* sp.f. *hominis*) than those of mice or rats. Moreover, the rabbit can be used as an animal model to investigate the *Pneumocystis* primary infection as well as the host-parasite relationships in a nonimmunodepressed natural host. The aim of the present study was to determine the time of first contamination of rabbits with this parasite. Important evidences suggesting that in utero transmission of *Pneumocystis* occurs in rabbits are here reported.

MATERIALS AND METHODS

Animals

In order to determine when the primary infection with *Pneumocystis* takes place in rabbits, 2 experiments were carried out. In the experiment 1, 8 New Zealand white rabbits (INRA 1077 strain) were used. They came from the specific pathogen free (SPF) breeding colony of the Station de Pathologie Aviaire et de Parasitologie, INRA, (Tours, France). They were free of coccidia, oxyurids, *Pasteurella*, *Clostridium spiroforme* and pathogenic *Escherichia coli* [13]. In the experiment 2 (Table I), 4 pregnant does purchased from a commercial supplier were used. Two females were at the 26th day of pregnancy and both 14 foetuses. One female was at

the 15th day of pregnancy and bore 3 foetuses. Another female was at the 5th day of pregnancy and bore 5 embryos (blastocystic stage).

Experiments and sampling procedure

Experiment 1 - The 8 New Zealand white rabbits were sacrificed, their lungs were removed under aseptic conditions, washed and finely minced with scissors in phosphate buffered saline (PBS). Lungs were used to test the presence of *Pneumocystis* at birth. *Pneumocystis* cystic forms were detected and counted on toluidine blue O stained microscopical smears. PCR for *Pneumocystis* (see below) was performed on all the lungs.

Experiment 2 - Before euthanasia of the pregnant does, blood was collected from the central ear vein on EDTA. Blood was not collected by cardiac puncture in order to avoid contamination with *Pneumocystis* from lung. The buffy coat was tested for *Pneumocystis* by using microscopic and PCR methods. Hysterectomy of each female was performed in aseptic conditions. Moreover, the external surface of the uterus was disinfected with AGRIBAC 80 POA solution (benzyl dimethyl lauryl ammonium bromide, Lab. SICCA Hygiène, Colombes, France) before dissection, in order to avoid enteric microbial contamination. All the foetuses were collected aseptically under a laminar air flow hood. In all the 26-day-old foetuses, lungs, liver, spleen, foetal side of placenta, amniotic fluid and maternal side of placenta were collected and PCR testing was performed on samples of each organ to detect *Pneumocystis*. In addition, samples of lungs and placenta of 6 foetuses and samples of lung of the 2 pregnant does were frozen (-80°C) and sections (5µm) were made in order to detect *Pneumocystis* by immunofluorescence and toluidine blue O staining (TBO) [14]. In the three 15-day-old foetuses (7 days after nidation) from the third female, whole bodies, amniotic fluid and placentas were tested for *Pneumocystis* using only PCR. Likewise, the blastocystic embryos from the fourth female (5th day of pregnancy), as well as the uterine washing fluid were tested for *Pneumocystis* using only PCR.

Light microscopic detection and quantification of *Pneumocystis* organisms

Lung samples of infected suckling rabbits (from birth) were removed aseptically and assessment of the level of *Pneumocystis* infection was carried out on lung impression smears stained with TBO. Lungs were cut into 1mm³ pieces in sterile Dulbecco minimum essential medium (DMEM) (F0455-Sigma, France) and homogenised with a hand Potter homogeniser (A14.197.31, OSI, France). The homogenate was poured through gauze and centrifuged at 3,000g for 10 min (4°C). The pellet was resuspended in buffered haemolytic solution (NH₄Cl at 150 mM and NaHCO₃; 10:1), incubated for 10 min (4°C) and centrifuged. The pellet was resuspended in DMEM and organisms were purified as previously described by SETTNER [15] but using a Percoll gradient density of 1.026. Parasite counts were performed on TBO stained smears as previously described [16].

Detection of *Pneumocystis* in rabbit tissues using a monoclonal antibody

The immunofluorescence detection of *Pneumocystis* was carried out on the 2 females at 26th days of pregnancy and their foetuses. Samples of foetal lung and maternal lung, as well as maternal or foetal side of placenta were collected, frozen immediately on dry ice and stored at - 80°C. Frozen sections of tissues (5µm) were collected on gelatine coated slides and fixed in a volume-matched ethanol-acetone mixture as described by DROUET-VIARD *et al.* [17]. *Pneumocystis* forms 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

Purification of total mitochondrial DNA - Purification of mitochondrial DNA (mtDNA) was performed following modified published methods [18, 19]. The entire procedure was carried out at 4°C. Tissues were homogenised with a hand Potter homogeniser. The resulting homogenate was poured through gauze, centrifuged at 3,000g for 10 min (4°C), and the resulting pellet was washed with PBS. Red blood cells were lysed using the buffered haemolytic solution. The preparation was centrifuged again and the pellet was washed 3 times with PBS and then homogenised with a buffer containing of 0.25M sucrose, 130mM KCl, 30mM Tris HCl pH 7.5, 3mM Magnesium acetate and 1mM Dithiothreitol. The preparation was incubated on ice for 15 min and then centrifuged at 650g for 10 min (4°C); the supernatant, which contained un-pelleted mitochondria, was centrifuged in the same conditions. The supernatant was pelleted in Corex tubes (30ml) at 11,000g for 20 min;

the pellet was resuspended in 1.5 ml in TE buffer (10 mM Tris HCl pH 8, 1 mM EDTA) and the preparation was incubated on ice for 10 min and centrifuged at 4°C for 20 min at 11,000g. The pellet was resuspended in a fresh homogenization buffer consisting of 50mM glucose, 20mM Tris HCl pH 8, 10mM EDTA and 20mg/ml lysozyme. The preparation was incubated on ice for 20 min and then homogenised with 2ml of 0.2M NaOH/1% SDS; the solution was stored on ice for 5 min. 3M sodium acetate (1.5 ml) was added to the solution to neutralise the sample. The homogenate was mixed gently and kept on ice for one hour and then centrifuged at 11000g for 20 min at 4°C. The mtDNA in the supernatant was extracted by the phenol:chloroform method [14], then precipitated by ethanol and dried. The pellet of mtDNA was dissolved in TE buffer and its concentration was measured (absorbance at 260nm with a spectrophotometer). The solution was stored at 4°C until use.

DNA amplification - After the purification of mitochondrial DNA, 5µl of the mtDNA sample (equivalent to 50ng) were processed in 20µl amplification buffer containing 5mM MgCl₂, 0.02mM deoxynucleoside triphosphate, 3.5µl reaction buffer (750mM Tris HCl pH 9 at 25°C, 200mM (NH₄)₂SO₄ and 0.1% Tween 20), 0.02U/µl of Goldstar DNA polymerase (Eurogentec, 4102 Seraing Belgium) and 0.05mM of each primer. The 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 *Pneumocystis* [21]. Reaction temperatures were 92°C for 60 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. Controls were water, plasma and rabbit sperm. These controls were always negative after 35 cycles. The amplification products were visualised by ethidium bromide staining (0.5µg/ml) after electrophoresis on a 2 % agarose gel.

Mitochondrial DNA Sequencing and sequence comparisons - Mitochondrial DNA sequences in rats (DNA from rat-derived *Pneumocystis*, INSERM U42) and in rabbits were determined by the dideoxy chain termination technique [22] and subsequently loaded on a fluorescent 373A automated DNA sequencer (Applied Biosystems). Sequencing data were analysed using the FASTA program [23] of the Genetics Computer Groups (GCG) package.

RESULTS

Experiment 1

By using microscopic or PCR detection methods, all rabbits were found to be spontaneously infected with *Pneumocystis* at birth. This suckling rabbits were little extensively infected by *Pneumocystis*.

Experiment 2 (Table 1)

In all samples of tissues and fluid of 26-day pregnancy females and their foetuses (lung and blood of mothers, maternal side of placenta, foetal side of placenta, foetal lung, spleen or liver and amniotic fluid), the fragment of the mitochondrial gene encoding the large subunit of the ribosomal RNA of *Pneumocystis* was amplified (Figure 1). In the blood, maternal sera and plasma were negative but the buffy coat was positive. As expected, in positive maternal or foetal samples, a *Pneumocystis* specific band of 346 pb was amplified. PCR did amplify *Pneumocystis* DNA in all infected samples, and no product was amplified from the negative controls (Figure 1). In the same way, *Pneumocystis* organisms were revealed in maternal or foetal lungs and in the foetal side of the placenta by using light microscopy or IFA. In contrast, *Pneumocystis* was undetectable in buffy coat using light microscopic examination or IFA assay.

With regard to the 15-day pregnancy female, the PCR assay revealed *Pneumocystis* in its 3 foetuses. The parasite was detected also by light microscopy and IFA in the lung of this female.

In contrast, PCR assay did not revealed *Pneumocystis* in the blastocysts and uterine washing fluid from the 5-day pregnancy female, though *Pneumocystis* organisms were detected using light microscopy, IFA or PCR in the maternal lungs. Moreover, the PCR assay detected the parasite in the blood of this female.

The amplified products from rabbit-derived *Pneumocystis* were sequenced and compared to the homologous sequences of rat-derived *Pneumocystis* (Figure 2). The rabbit-derived *Pneumocystis* amplified product showed 56 mutations (the similarity coefficient was about 76,4%).

Table 1: Pneumocystis in tissues of 4 pregnant does and their embryos or foetuses.

Host	Day of pregnancy	Detection*	<i>P. carinii</i> in:				
			Lung	Blood	Placenta	Amniotic fluid	Whole body**
Pregnant rabbit No1	5	TBO	+	ND			
		Mab	+	ND			
		PCR	+	+			
Blastocysts (n= 5)***	5	TBO					ND
		Mab					ND
		PCR					- (5/5)
Pregnant rabbit No2	15	TBO	+	ND	ND		
		Mab	+	ND	ND		
		PCR	+	+	+ (3/3)		
Fetuses (n= 3)	15	TBO			ND	ND	ND
		Mab			ND	ND	ND
		PCR			+ (3/3)	+ (3/3)	+ (3/3)
Pregnant rabbit No3	26	TBO	ND	ND	ND		
		Mab	+	ND	+ (3/3)		
		PCR	+	+	+ (14/14)		
Fetuses (n= 14)	26	TBO	+ (3/3)	ND	ND	ND	ND
		Mab	+ (3/3)	ND	+ (3/3)	ND	ND
		PCR	+ (14/14)	ND	+ (14/14)	+ (3/3)	ND
Pregnant rabbit No4	26	TBO	ND	ND	ND		
		Mab	+	ND	+ (3/3)		
		PCR	+	+	+ (14/14)		
Fetuses (n= 14)	26	TBO	+ (3/3)	ND	ND	ND	ND
		Mab	+ (3/3)	ND	+ (3/3)	ND	ND
		PCR	+ (14/14)	ND	+ (14/14)	+ (3/3)	ND

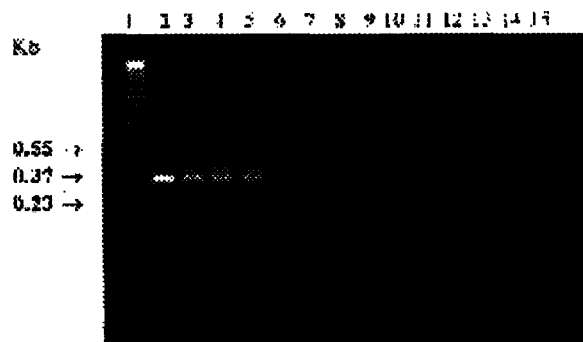
* Détection methods were the following : toluidine blue O (TBO), fluorescent specific monoclonal antibody staining (Mab) or PCR assay (see "Materials and Methods").

** Whole body = PCR was carried out from a total DNA extract of each fetus.

*** In bracket: number of positive embryos or foetuses / number of tested ones,

(+) = *Pneumocystis* positive sample; (-) = *Pneumocystis* negative sample; ND = Not done.

Figure 1 : PCR detection of *Pneumocystis* DNA in maternal, foetal or young rabbit tissues



Pneumocystis mitochondrial DNA (0.346 Kb) amplification from ten samples of young rabbits, maternal blood and foetuses. Lane 1 , Raoul genetic marker (Appligene); lanes 2, 3, 4 and 5, lung samples of young rabbits aged 28, 10, 5 days and a few hours respectively; lane 6, buffy coat extract of a pregnant rabbit; lanes 7, 8, 9, 10

and 11, lung, liver, spleen, placenta and amniotic fluid of foetuses respectively; lane 13, negative control DNA of rabbit sperm and lane 15, negative control water.

Figure 2 : DNA sequence of a 237-bp fragment of the *Pneumocystis* large subunit mitochondrial ribosomal RNA from rabbit- and rat-derived *Pneumocystis*

<i>P. carinii</i>		10		20		30
host :	A A T C G G A C T A G G A T A T A G C T G G T T T T C T G C					
rabbit						
	I I					
host : rat	A A T C G G A C T A G G A T A T A N C T G G T T T T C T G C					
		40		50		60
host :	G A A A T T T G T A T T A G C A A A G T G T T T A T G T A A					
rabbit						
	I I					
host : rat	G N A A T C T A T A T T G G T A G A T G A C T ° ° T G T T A					
		70		80		90
host :	T A A A T ° ° T A G T A G G T A T A G C A C T G A A T A T °					
rabbit						
	I I					
host : rat	T T A T T T G T A G T G G G T A T A G C A C T G T A T A T C					
		100		110		120
host :	° ° ° ° ° ° ° T C A T T C A G A G G A G T A C G T A A G T A					
rabbit						
	I I					
host : rat	T A A C T T A T G T T A G A C G G G A G T A T G A A G G T A					
		130		140		150
host :	T T T A T C T C G G A T A T T T A A T C T C A G A A T G A C					
rabbit						
	I I					
host : rat	C T T A C T T T G G A T A T T T A T A C T C A G A A T A G C					
		160		170		180
host :	T A A T T T T A T A A G A T A A A T G G A T G G A C T T T T					
rabbit						
	I I					
host : rat	T A T T A A T A T A T G A T G A G T T A T C A G A C T T C T					
		190		200		210
host :	T G C G A G A ° A G G T G G A A A G T C A A G A G G G A A A					
rabbit						
	I I					
host : rat	T G C G A T A T A G G T G A G G A G T C G A G A G T G A A A					
		220		230		
host :	C A G C C C A G A A C A G A A T ° G A A A G C T C C C					
rabbit						
	I I					
host : rat	C A G C C C A G A A T A A T A T A T A A A G T T C C C					

The dots indicate gaps to allow alignment of the sequences. N: not determined.

DISCUSSION

It has previously reported that lungs of nonimmunosuppressed young weaning rabbits from conventional breeders are spontaneously and heavily infected with *Pneumocystis* ($68 \pm 50 \times 10^6$ cystic forms per animal) [6, 7]. Then, the microscopic detection of *Pneumocystis* organisms was easy when the infection level was relatively high (weaning rabbits), but the microscopic detection was still more difficult in one-day-old newborns but the PCR technique confirmed that they were already infected. Thus, PCR assay allowed a more accurate detection of *Pneumocystis* than conventional parasitological or IFA methods. On the whole, the sequences of the products

amplified from rat and rabbit-derived *Pneumocystis* mitochondrial rRNA genes were similar to those published by Peters et al [24], who have found a 75,8% sequence identity over 219-bp of the same gene. As rabbits were already infected with *Pneumocystis* at birth, in utero *Pneumocystis* infection was hypothesised. Thus, four pregnant does and their 31 fetuses and 5 embryos were examined to investigate *Pneumocystis* vertical transmission. As *Pneumocystis* rates were highest in non-SPF rabbits, the occurrence of *Pneumocystis* in utero transmission was investigated in pregnant rabbits from a conventional breeder. Four days before birth, *Pneumocystis* was detected by PCR in all tested organs from mothers and fetuses. PCR testing of blood of the pregnant mothers revealed the presence of *Pneumocystis* in the buffy coat, suggesting that parasites could reach the fetuses by hematogenous way. Moreover, the no detection of *Pneumocystis* in blastocysts and its presence in fetuses suggests that placenta was necessary to the foetal infection. Steven [25] has shown that in rabbits the endothelium of maternal capillaries disappears on the 10th day of pregnancy and that the placenta is hemochorial until the 17th day. Then, from the 17th day until birth the placenta is hemoendothelial. Thus, the placenta permeability increases with the stage of pregnancy. In the present work, it was found that fetuses were already infected at 15 days' gestation, which corresponds to the hemochorial stage of placentation. The placental barrier is then relatively permeable and parasites circulating in the maternal blood could reach the foetus. Another way of passage could be the amniotic fluid where *Pneumocystis* was detected by PCR. As humans have also a hemochorial type of placenta, in utero transmission of *Pneumocystis* could also occur [1]. However, no definitive proofs of vertical transmission of *Pneumocystis* neither in humans nor in rats were presented. Ito et al [2] did not find evidences of transplacental infection with *Pneumocystis* in SCID mice. Anyway, in this work, clear evidences of transplacental transmission of *Pneumocystis* in rabbits are presented. At least in rabbits, transplacental passage could be a supplementary route of transmission of *Pneumocystis*, besides the airborne route already shown in rats [1] and in mice [26]. The mechanism of passage of *Pneumocystis* from the mother to the foetus remains to be elucidated. Likewise, it has to be determined whether the transplacental passage of *Pneumocystis* organism infecting the foetus in utero are involved in the spontaneous pneumocystosis observed in rabbits at weaning [6, 7].

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Etude de la transmission transplacentaire de *Pneumocystis carinii* chez le lapin - Quatre-vingts à 100% des lapereaux sont spontanément et intensément parasités par *Pneumocystis* (*P. carinii* sp.f. *oryctolagi*) au sevrage. *Pneumocystis* a été détecté dans le poumon et autres organes par microscopie, immunofluorescence et PCR. Cependant, le moment, la source et le mécanisme de l'infection n'ont pas été établis. L'hypothèse d'une transmission verticale du micro-organisme se posait. Quatre femelles gestantes, leurs embryons et foetus ont été examinés. *Pneumocystis* a été détecté dans le sang maternel et le liquide amniotique par PCR, dans le placenta et les tissus pulmonaires foetaux et maternels par histologie, immunofluorescence et PCR. Ces résultats suggèrent que la transmission transplacentaire de *Pneumocystis* a lieu dès le 15ème jour de gestation.
