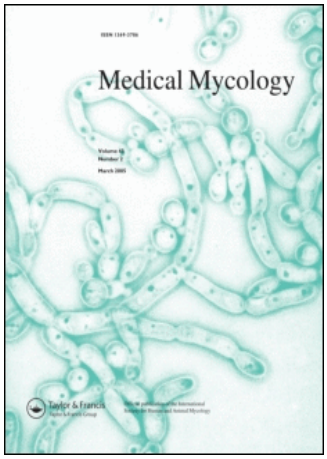

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Exploring transplacental transmission of **Pneumocystis oryctolagi** in first-time pregnant and multiparous rabbit does

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Exploring transplacental transmission of *Pneumocystis oryctolagi* in first-time pregnant and multiparous rabbit does

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Pneumocystis sp. is transmitted through the airborne route and presents a high host-species-specificity. Occasional reports of *Pneumocystis* pneumonia in still births and newborn infants suggest that other routes of transmission, e.g. transplacental might occur. The latter has been reported in rabbits but available data indicate that transplacental transmission of *Pneumocystis* seems not to occur in corticosteroid-treated rats and in SCID mice. The present study was undertaken to evaluate transplacental transmission of *Pneumocystis oryctolagi*. The spontaneously-acquired pneumocystosis rabbit model using hybrid California/New Zealand white female rabbits was selected because of similarities among rabbit and human placentas. Three different experiments were conducted in France and Chile. *Pneumocystis* organisms were detected by microscopy in the lungs of pregnant does and *Pneumocystis* DNA was found in the lungs of fetuses from the multiparous does from the second week to the end of gestation. *Pneumocystis* DNA was not detected in fetuses from primiparous does. Detection of *Pneumocystis oryctolagi* – DNA in fetuses of multiparous does and not in those of primiparous ones, suggests that transplacental transmission may be favored by multiple gestations. Whether *Pneumocystis*-DNA in fetal tissues from multiparous does resulted from transplacental passage of viable transmissible forms requires further investigation.

Keywords *Pneumocystis oryctolagi*, transplacental transmission

Introduction

Documentation of new routes of *Pneumocystis* species transmission may lead to the development of new strategies for disease prevention. Cross infection experiments are unequivocal in documenting that these organisms are highly species-specific indicating that human *Pneumocystis* pneumonia (PcP) is not a zoonosis [1]. It is also well known that *Pneumocystis* intra-species transmission can occur by the airborne route [2–5]. In addition, evidence of vertical hematogenous transmission has been presented [6].

The respiratory route has long been recognized as a common and important mode of transmission of *Pneumocystis* in animal models [2]. The more recent detection of *Pneumocystis* DNA in healthcare workers

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that had been in contact with a patient with PcP [7] and demonstration of similar *Pneumocystis* genotypes in isolates from a mother and her newborn infant [8] suggest this route of transmission is also common in humans. However, other modes of transmission such as transplacental deserve investigation. The early age of acquisition of *Pneumocystis* in different species warrants the study of vertical/transplacental as an additional route of transmission of this microorganism. Documenting vertical transmission could reveal a route that can secure the persistence of *Pneumocystis* independent of environmental hazards as occurs with *Toxocara* nematods, the protists *Neospora caninum* and *Toxoplasma,* and other eukaryotic pathogens. Interestingly, vertical transmission may not necessarily be hematogenous. A controversial report of *Pneumocystis* cysts found solely in the lungs of a fetus from an HIV-positive mother, suggests inhaling contaminated amniotic fluid as a possible non-hematogenous mechanism of vertical transmission [9,10].

Transplacental transmission was first suggested by a few reports of PcP in neonates or still born infants before the AIDS epidemic [11,12] and by descriptions of *Pneumocystis* infection in neonates [8,13,14]. More recent studies have documented that children may frequently harbour *Pneumocystis* in their lungs at an early age [15,16]. Similarly, animal experiments document that neonatal or young rodents, pigs and rabbits can harbor substantial numbers of *Pneumocystis* organisms [17–20]. The infection can be detected in neonatal rats as soon as 1 hour after birth [19].

Available studies on transplacental transmission of *Pneumocystis* suggest that this varies among mammal species. For example, it seems not to occur in corticosteroid-treated rats and in SCID mice [19,21]. However, this route was reported by Cere *et al*. to be present in rabbits that develop spontaneous PcP, usually at weaning, that heals spontaneously about one month later [6]. This initial report motivated us to undertake this study to further characterize transplacental transmission in the rabbit model of spontaneously acquired pneumocystosis [22,23].

Materials and methods

Animals and experimental plan

This report is the result of a scientific collaboration between research teams in France (MC, EMA, ID-J and ED-C) and in Chile (CAS, CL and SLV). The conditions for care of laboratory animals stipulated in European guidelines were followed in both countries (See: Council directives on the protection of animals for experimental and other scientific purposes, and J. Off. Communautés Européennes, 86/609/EEC, 18 December 1986, L358).

Experiments with multiparous *Oryctolagus cuniculus* does were conducted in France, and with never-pregnant and primiparous does in Chile. Hybrid California/New Zealand white female rabbits were used throughout the investigation. The animals were obtained from the CEGAV (St Mars d'Egrenne) in France, and from the animal facilities 'Lo Chena' of the Institute of Public Health of Chile in Santiago, Chile. In total, 8 multiparous pregnant female rabbits and their 42 fetuses were studied in France and 30 virgin does (14 allowed to get pregnant and their 24 fetuses and 16 never-pregnant controls) and their fetuses were studied in Chile. Animals were bred and housed in conventional animal facilities with food and water *ad libitum*. The presence of *Pneumocystis* organisms or DNA was explored in pregnant and non-pregnant does' lungs and fetuses at different times of the gestation.

Experiment 1: Four multiparous does were euthanatized respectively at weeks 1, 2, 3 and 4 of their pregnancy. Their lungs were microscopically examined for *Pneumocystis* organisms and single round and nested PCR was used to detect *Pneumocystis* DNA in the amniotic fluid, placentas, lungs and whole bodies of the fetuses.

Experiment 2: Two multiparous pregnant does were euthanatized after three weeks and two more at four weeks of gestation. *Pneumocystis* DNA was identified by PCR in their lungs and in the amniotic fluid, placenta, lungs, heart, kidneys, liver and whole body of the fetuses.

Experiment 3: Thirty virgin female rabbits were distributed in two groups, i.e., 16 non-pregnant and 14 pregnant does. Mating day was designated as day 1 of the experiment, at which time two pregnant and four non-pregnant does were euthanatized. Thereafter, four does from each group were euthanatized respectively on days 8, 16, and 25 of the experiment. Their lungs were microscopically examined for *Pneumocystis* organisms and single round and nested PCR was used to detect *Pneumocystis* DNA. *Pneumocystis* DNA was studied by single round and nested PCR in the lungs and liver of the fetuses.

Sampling

Female rabbits were euthanatized by intra-peritoneal injection of sodium thiopental. They were placed inside a biosafety cabinet immediately after sacrifice and their chest and abdomen sprayed with 70% alcohol prior to dissection. Surgical instruments were autoclaved prior to their use in the studies. To avoid contamination different sets of instruments were used for each rabbit and for each necropsy step, e.g. to open the thoracic and abdominal cavities, to remove the lungs and, then to remove the uterus. Female rabbit lungs were placed in individual sterile Petri dishes before parasite extraction and 100 mg samples of the organ were obtained and kept frozen at −20°C for DNA extraction. In addition, hysterectomies were performed under aseptic conditions and uteri placed in separate sterile plastic plates before dissection using a different sterile set of instruments. Lungs, liver, heart, kidneys, placenta, amniotic fluid and whole bodies from the fetuses were collected when possible relative to their maturity or from newborn rabbits. All samples were placed in individual sterile tubes and frozen at −20°C for DNA extraction.

Detection and counting of Pneumocystis *organisms by light microscopy in the lungs of does*

Pneumocystis organisms were detected in impression smears of lungs from pregnant or non-pregnant does using Gomori-Grocott silver methenamine or toluidine blue O (TBO) stain. Parasite extraction and quantification were performed as described elsewhere [24]. Briefly, *Pneumocystis* cysts and total forms were counted in 5-µl air-dried lung homogenate smears stained with Gomori-Grocott or TBO and a rapid Giemsa-like stain (RAL-555, Réactifs RAL, Paris).

PCR analysis of Pneumocystis *DNA in the lungs of does and fetuses*

DNA was extracted using QIAamp© DNA minikit (QIAGEN, Courtaboeuf, France, or QIAGEN, Valencia, CA, USA) in all cases. A PCR method which amplified a portion of the large sub-unit of the mitochondrial ribosomal RNA gene (mtLSU rRNA) of *Pneumocystis* was performed with all samples using primers pAZ102-H and pAZ102-E [25]. When the amount of *Pneumocystis* organisms in the samples was low and insufficient to produce visible bands on an ethidium bromide-stained gel after a single round of amplification, a nested-PCR was used with the internal primers pAZ102-X and pAZ102-Y [26], or primers pAZ102-X and pAZ102-Z (5′-AAG CCC ACT TCT TRA CTG TC-3′) [27]. Reaction mixtures and PCR conditions were the same in all the experiments and are described elsewhere [5]. PCR products were analyzed by electrophoresis in 2% agarose gels and visualized with ethidium bromide. Positive and negative *Pneumocystis* DNA controls were included in the DNA extraction step and for each amplification.

Results

Pneumocystis *in multiparous pregnant females and in their fetuses*

In experiment 1, *Pneumocystis* organisms were detected in the lungs of the four multiparous pregnant females, i.e., 5.5×10^5–1.5×10^6 total parasite counts (Table 1). *Pneumocystis* DNA was amplified in the lungs of fetuses from the second week to the end of gestation. No *Pneumocystis* DNA was detected in amniotic fluids or placentas (Table 1).

In experiment 2, *Pneumocystis*-DNA was detected in three of the four female rabbits euthanatized at 3 or 4 weeks of gestation (Table 2). The whole bodies of fetuses, amniotic fluids and placentas were found free

Table 1 Total number of *Pneumocystis* organisms in the lungs of multiparous rabbit does euthanatized at different stages of gestation and detection of *Pneumocystis*-DNA in fetal tissues (Experiment No. 1, see text).

Does			Fetuses			
			*No. of positive/No. of samples tested for *Pneumocystis* by nested-PCR			
No.	Day of pregnancy at euthanasia	No. of total parasites/lung	Whole body	Lungs	Amniotic fluid	Placenta
1	7	5.5×10^5	0/3	ND	ND	ND
1	14	6.5×10^5	ND	2/3	0/3	0/3
1	21	1.5×10^6	ND	4/4	0/4	0/4
1	28	7.5×10^5	ND	5/5	ND	ND

*All samples tested negative by single round PCR. ND, not done.

Table 2 *Pneumocystis*-DNA as detected in the lungs of multiparous pregnant rabbits does by single round PCR and in fetal tissues by using nested-PCR (Experiment No. 2, see text).

Rabbit does			Fetuses						
			*No. of positive samples/No. of samples tested for *Pneumocystis* by nested-PCR						
No. of does	Day of pregnancy at euthanasia	*Pneumocystis*-PCR of lungs	Lungs	Heart	Liver	Kidneys	†Rest of the body	Amniotic fluid	Placenta
1	21	positive	ND	ND	ND	ND	0/7	0/3	ND
1	21	negative	ND	ND	ND	ND	0/7	0/4	0/7
1	28	positive	2/7	1/7	0/2	0/2	ND	ND	0/7
1	28	positive	2/6	5/6	ND	ND	6/6	ND	0/6

*All samples tested negative by single round PCR. †Rest of the body = the remaining organs after dissection. ND, not done.

of *Pneumocystis* DNA in the two does sacrificed at 3 weeks of gestation. However, *Pneumocystis*-DNA was detected in the lungs, hearts and whole bodies of fetuses from the other two does sacrificed at 4 weeks of gestation (Table 2).

Pneumocystis *in control does, first-time-pregnant does, and in their fetuses*

Pneumocystis organisms were found in the lungs of the 14 first-time pregnant does and in 15 of the 16 non-pregnant controls, with respectively a mean of 1.5×10^5 and 2.1×10^5 total parasites. Therefore pulmonary load of *Pneumocystis* in the does was independent of their pregnancy status. Fetal livers and lungs were all found to be free of *Pneumocystis* DNA (Table 3).

Discussion

A mild *Pneumocystis* pulmonary infection was identified by microscopy and/or nested PCR in all the rabbit does regardless of their number of pregnancies, as well as in the lungs of non-pregnant controls. Housing conditions probably favored airborne acquisition of *P. oryctolagi* and accounted for this mild *Pneumocystis* carriage as the organism is highly prevalent in rabbit colonies [20,22,23]. However, the mean number of parasites in the lungs of multiparous pregnant does was up to 10 times higher and detectable by means of just single round PCR, when compared with the results found with primiparous or non-pregnant does (Tables 1–3). This data suggest that the physiologic immunodepression associated with pregnancy may favor *Pneumocystis* growth as reported in humans [28].

This study documented *Pneumocystis*-DNA in the lungs of fetuses from multiparous does starting from the second week of pregnancy but not in the lungs of fetuses from primiparous does. *Pneumocystis*-DNA has been previously documented in the buffy coats of blood samples from pregnant rabbit does [6]. Therefore our findings suggest that transplacental transmission of *P. oryctolagi* may be favored by multiple gestations and eventually by a higher number of parasites as detected in the lungs of the multiparous pregnant does. We found no data to support that placental permeability can increase with successive pregnancies. It can be hypothesized that a minimum number of *Pneumocystis* organisms in the lungs may be needed to reach a threshold to cross physiologic barriers (and pass to the blood) as occurs for example with *Candida* sp. [29]. Unfortunately, rabbits usually carry *P. oryctolagi,* which impedes an experimental infection with the fungus. Furthermore, *Pneumocystis*-free rabbits are not available to determine the minimum number of *Pneumocystis* organisms required to initiate infection.

We are aware that these experiments are not standard ones. No efficient culture system is available for *Pneumocystis* and *P. oryctolagi* is not a conventional micro-organism. Furthermore, viability of *P. oryctolagi* organisms can only be assessed using molecular or ultrastructural methods [5,30] and there is no means of evaluating the infectivity of *P. oryctolagi*. Therefore, the detection of *P. oryctolagi*-DNA in the lungs of fetuses in this study does not prove passage of viable and infectious *Pneumocystis* organisms. Previous work by Cere *et al*. documented *Pneumocystis* by microscopy in rabbit fetuses starting at day 10 of pregnancy, as well as in placentas and amniotic fluid samples [6]. However, placentas and amniotic fluids from multiparous does in this study tested negative for *Pneumocystis*-DNA.

Humans have an hemomonochorial type of placenta like guinea pigs and members of the Octodontiae family (South American rodents). Therefore, these animals provide ideal models to study vertical transmission and to relate the results to the human setting [31–33]. Rabbits are better characterized with regard to *Pneumocystis* infection and although they have placentas composed of two layers of trophoblast in the exchange area, these layers are extremely thin in some

areas. Furthermore, placental permeability increases with the stage of pregnancy [31–34]. Therefore, rabbits provide a suitable model to study vertical transmission that may be related to humans. Rats and mice may be inadequate because they have a hemotrichorial placenta. Vertical transmission of *Pneumocystis* does not seem to occur in mice and rats [19,21]. Of interest is the fact that rabbits like rodents have a yolk sac that functions until term of gestation [31–33]. The yolk sac endoderm faces the uterine epithelium and can phagocytize and lyse products present in the uterine lumen. This underscores the need to complement PCR studies with microscopic examinations, as previously done by Cere *et al*. [6]. Furthermore, conducting viability studies would be needed to document vertical transmission.

The mechanisms by which pathogens breach the placental barrier remain to be elucidated. The size of the parasite would not seem to prevent it from passing across the placenta. For instance, the nematode larvae of *Toxocara canis,* that measures 400×20 μm infects fetal dogs by the transplacental route [35]. Alternatively, the intracellular nature of these pathogens may facilitate transfer across placentas. The majority of data on eukaryotic etiologic agents that infect fetuses via the transplacental route are based on works with such pathogens as *Toxoplasma* [36] or *Trypanosoma cruzi* [37]. In contrast, while *Pneumocystis* organisms are relatively small (2–8 μm trophic forms and 3–6 μm cystic forms), they are extracellular pathogens.

In summary, this work documents that *P. oryctolagi* causes a mild infection in adult female rabbits and their susceptibility seems to be favored by the number of their pregnancies. In addition, *P. oryctolagi*-DNA can be detected in fetuses from multiparous does. The data also suggest that transplacental transmission of *P. oryctolagi* may occur in rabbits as reported by Cere *et al*. [6]. However, the latter does not seem to occur in primiparous does and its occurrence in multiparous rabbits is not systematic. Further studies are needed to confirm that the transplacental transmission does involve viable and infective *P. oryctolagi* organisms.

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Table 3 Microscopic and molecular *Pneumocystis oryctolagi* detection in non-pregnant or first-time pregnant rabbit does at different stages of gestation, and *Pneumocystis*-DNA detection (single round and nested-PCR) in fetal tissues (Experiment No. 3, see text).

	Pneumocystis-positive/number of animals examined									
	Lungs of non-pregnant does (controls) $n = 16$				Lungs of first-time-pregnant does $n = 14$				Fetus lungs		Fetus livers	
Age of pregnancy (days)	Gomori-Grocott	Mean of parasite counts (Giemsa)*	Single PCR	Nested PCR	Gomori-Grocott	Mean of parasite counts (Giemsa)*	Single PCR	Nested PCR	Single PCR	Nested PCR	Single PCR	Nested PCR
1			0/4	3/4	ND		0/2	2/2	ND	ND	ND	ND
8	6/16	2.1×10^5	0/4	4/4	2/4	1.5×10^5	0/4	4/4	ND	ND	ND	ND
16		/15 does	0/4	4/4	ND	/14 does	0/4	4/4	0/7	0/7	ND	ND
25			0/4	4/4	3/4		0/4	4/4	0/17	0/17	0/17	0/17

*Mean of total parasite counts in animals with a positive count. ND, not done.

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