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.

Résumé : TRANSMISSION *IN UTERO* DE *PNEUMOCYSTIS CARINII* SP. F. *ORYCTOLAGI*

Bien que la transmission verticale de Pneumocystis *ait été souvent suspectée, cette modalité d'infection n'a jamais été démontrée. Ce parasite ubiquiste est constamment retrouvé dans les poumons des lapins, qui développent spontanément une pneumocystose bénigne au moment du sevrage. Mais les sources, la voie et les mécanismes de l'infection des lapereaux par* Pneumocystis *ainsi que le moment où la contamination a lieu n'ont pas été déterminés. Comme le parasite a été détecté microscopiquement et par PCR chez des lapereaux à la naissance, l'hypothèse d'une transmission* in utero *de* Pneumocystis *a été émise. Le parasite fut alors recherché chez 16 lapines gestantes, leurs embryons et leurs fœtus en associant plusieurs méthodes de détection.* Pneumocystis *a été détecté par PCR dans le sang maternel et le liquide amniotique, ainsi que dans le placenta et les tissus pulmonaires fœtaux et maternels, en associant des méthodes histologiques, d'immunofluorescence et la PCR. Ces résultats suggèrent que la transmission verticale de* P. carinii *sp. f.* oryctolagi *aurait lieu dès le 10e jour de gestation.*

MOTS CLÉS : Pneumocystis carinii *sp. f.* oryctolagi, *transmission transplacentaire, infection congénitale, lapin, pneumonie à* Pneumocystis carinii.

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.*, 1990*a*) 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.*, 1990*b*). 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.

* INRA, Centre de Tours, Station de Pathologie Aviaire et de Parasitologie, F 37380 Nouzilly.
** Laboratoire de Parasitologie-Mycologie, Faculté de Médecine et CHRU, 1 Place Verdun, F 59045 Lille.
*** Département de Microbiologie des Écosystèmes, Institut Pasteur de Lille, F 59019 Lille Cedex.
Correspondence: Pierre Coudert.
Phone: (33) 2 47 42 77 52. Fax: (33) 2 47 42 77 74.

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 all the 26-day-old fetuses, and PCR testing for *Pneumocystis* was performed. Whole bodies, amniotic fluid and placentas of 10- or 15-day-old fetuses (2 to 7 days after nidation) were tested for *Pneumocystis* as well as blastocystic embryos from the six females (5th day of pregnancy) and the uterine washing fluid using only PCR. In addition, samples of lungs and placentas of six 26-days old fetuses as well as lung samples from 5 pregnant does were frozen (– 80 °C). Sections (5 µm) were made in order to detect *Pneumocystis* by immunofluorescence and toluidine blue O staining (TBO) (Chalvardjian & Grawe, 1963).

Light microscopy assessment of *Pneumocystis* organisms in lungs

Rapid assessment of the level of *Pneumocystis* infection was carried out on lung impression smears stained with TBO. Parasite extraction was performed as described by Aliouat *et al.* (Aliouat *et al.*, 1993) with some modifications. Lungs were washed, finely minced with scissors in phosphate buffered saline (PBS) and homogenized in sterile Dulbecco minimum essential medium (DMEM) (F0455-Sigma, France) with a hand Potter homogenizer (A14.197.31, OSI, France). After centrifugation the pellet was resuspended in a buffered hemolytic solution (150 mM NH_4Cl, 1 mM $NaHCO_3$), incubated for 10 min (4 °C) and centrifuged. Parasite extracts were filtered through sterile stainless steel (250 and 30 µm) and through Nucleopore filters (10 and 8 µm) (Cofralab, Gradignan, France). The number of *P. carinii* was assessed in lung extract smears stained with TBO as previously described (Aliouat *et al.*, 1993).

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 $MgCl_2$, 0.02 mM deoxynucleoside triphosphate, 3.5 µl reaction buffer (750 mM Tris HCl pH 9 at 25 °C, 200 mM $(NH_4)_2SO_4$ 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 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. 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. 1*e*). A *Pneumocystis* specific band of 346 bp (Wakefield *et al.*, 1990) was amplified by PCR (Fig. 2).

Pneumocystis IN PREGNANT FEMALES, EMBRYOS AND FETUSES (Table I)

Pneumocystis organisms were detected in the maternal lungs of all pregnant females tested using microscopy and IFA (Fig. 1*a*). At the 26th day of pregnancy, *Pneu-*

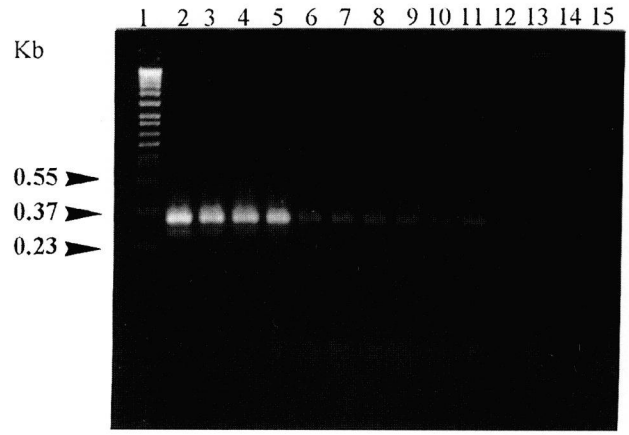

Fig. 2. – PCR detection of *Pneumocystis* DNA in maternal, fetal or young rabbit tissues. *Pneumocystis* mitochondrial DNA (346 bp) amplification from ten samples of young rabbits, maternal blood and fetuses. Lane 1, Raoul genetic marker (Appligene); lanes 2, 3, 4 and 5, lung samples of young rabbits 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 fetuses, respectively; lane 13, negative control DNA of rabbit sperm; lane 15, negative control water.

Day of pregnancy	Host	Detection (1)	*P. carinii* in: Lung	Blood	Placenta	Amniotic fluid	Whole body (2)
5	Blastocysts (3) (n = 29) (from 6 pregnant rabbits)	PCR				0/6 (4)	0/7
10	Fetuses (n = 12) (from 4 pregnant rabbits)	PCR			0/12	0/12 12/12	12/12
15	Fetuses (n = 20) (from 4 pregnant rabbits)	PCR	20/20	6/10	20/20		
26	Fetuses (n = 28) (from 2 pregnant rabbits)	TBO	6/6	ND	ND	ND	ND
		Mab	6/6	ND	6/6	ND	ND
		PCR	28/28	ND	28/28	6/6	ND

(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.

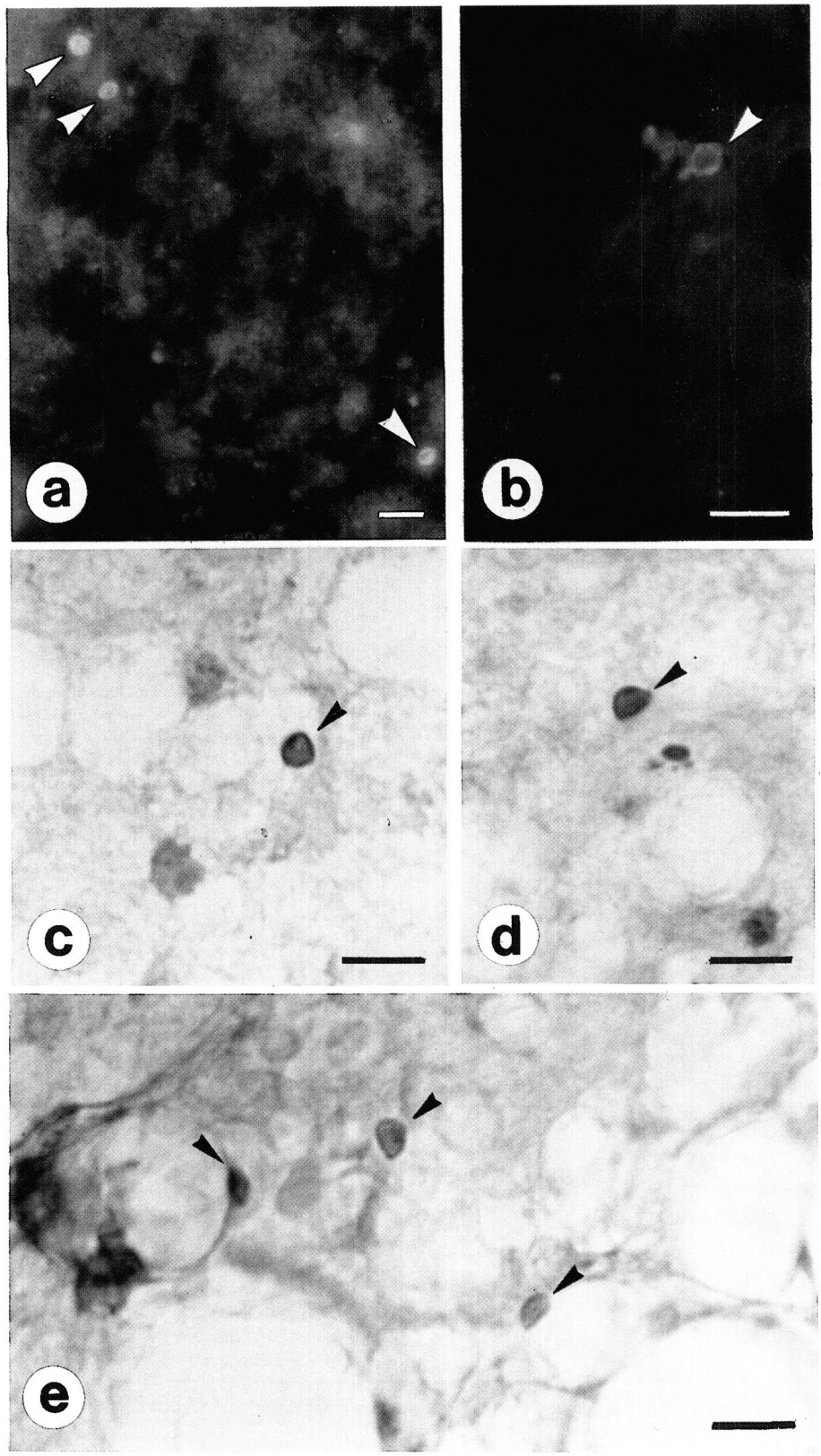

Fig. 1. – *Pneumocystis in utero* transmission in rabbits. *a)* Cystic forms of the parasite in the lung of a pregnant rabbit (26th day of pregnancy); *b), c)* and *d) Pneumocystis* cystic forms (arrowheads) in fetal lungs (26th day of pregnancy); *e) Pneumocystis* cystic forms in the lung of a newborn 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.

mocystis organisms were revealed in fetal (Fig. 1*b, c, d*) lungs and at the fetal side of placentas 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.*, 1990*a*). This natural infection has been used as an experimental model of *P. carinii* pneumonia (PCP) (Goyot *et al.*, 1984; Dei-Cas *et al.*, 1990*a*; Dei-Cas *et al.*, 1994; Akono & Palluault, 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.f. *hominis)* 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 know 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.*, 1990*a*).

ACKNOWLEDGEMENTS

We are grateful to Dr. A.E. Wakefield (Oxford University) and C. Ödberg-Ferragut (INSERM U42, Villeneuve d'Ascq) for their advice. We also thank Y. Breuzin and A.F. Francineau for their valuable technical assistance. This work was carried out in the framework of a European Concerted Action on *Pneumocystis* Research (BMH-1 CT94 PL1118). This work was supported by a grant from the Conseil régional « Région Centre » (France).

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Reçu le 22 mars 1997
Accepté le 22 juillet 1997