MODELS OF INFECTION

Experimental model of congenital toxoplasmosis in guinea-pigs: use of quantitative and qualitative PCR for the study of maternofetal transmission

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Maternofetal transmission of *Toxoplasma gondii* was assessed in pregnant guinea-pigs, with a gestational period of 65 ± 5 days. A total of 56 female guinea pigs was infected by the intraperitoneal route (RH strain), by the oral or the intraperitoneal route (Prugniald strain; PRU) or by the oral route (76K strain). Inoculation was performed 90 ± 18 days or 30 ± 9 days before the onset of gestation or 20 ± 6 days or 40 ± 6 days after. Gestational age was determined by a progesterone assay. Parasite loads (fetal brain and liver) were assessed by nested PCR and real-time PCR quantification on Light Cycler® was performed with a SYBR Green I® technique. The 76K strain appeared to be the most virulent in the model: the neonatal survival rate was 31%, in contrast to 53% and 68% for the PRU and RH strains, respectively. The percentage of survival of neonates for all strains taken together was lower after inoculation at 40 days' gestation (25%) than at 20 days' gestation (77%). Whatever the strain, maternofetal transmission determination was greater with nested PCR (54% for RH, 84% for PRU and 86% for 76K strains) than with real-time quantitative PCR (31% for RH, 66% for PRU and 76% for 76K strains). However, real-time quantitative PCR showed that neonatal parasite load was greater with the cystogenic strains (76K, PRU) and that high hepatic load ($>10\,000$ parasites/g) was often associated with disease severity (11 of 12 cases). Therefore, this technique may provide an important element in understanding this congenital disease.

Introduction

Toxoplasmosis, caused by *Toxoplasma gondii*, is widespread in man and warm-blooded animals. Although usually asymptomatic in immunocompetent individuals, toxoplasmosis may cause severe disorders in immunocompromised patients and in pregnant women because of the high risk of transplacental transmission and the occurrence of multiple congenital lesions in the fetus [1, 2]. Physiological, immunological and therapeutic knowledge of maternofetal transmission is limited because experimental models are few [3]. Several models of congenital toxoplasmosis in rodents such as mouse, rat, guinea-pig and rabbit (few studies) have been described [4]. The mouse is the oldest and best

studied experimental model of congenital toxoplasmosis [5–7]. In most cases, mice are very sensitive to *T. gondii* infection and fetal contamination could occur during chronic maternal toxoplasmosis and even through successive generations of mice [6, 8]. In contrast, the rat model has been only described more recently. Its main advantage lies in the fact that rats, like man and other primates, are resistant to *T. gondii* infection. Reported maternofetal transmission varies according to study, route, load, time of inoculation and strain of *T. gondii* [9–11]. Fetuses are hardly ever seriously ill and little or no fetal transmission occurs during chronic maternal toxoplasmosis [11–13]. Half-way between the rat and mouse model, the guinea-pig model of *T. gondii* infection shows several features relevant to the study of congenital toxoplasmosis: (i) as in man, the placenta is haemomonochorial [4]; (ii) the duration of gestation is 65 days (45 days longer than in rat and mouse models) [8–10, 14]; (iii) the sensitivity of guinea-pigs to *T. gondii* is medium. However, studies

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in pregnant guinea-pigs are mostly outdated [14–16]. Only one recent vaccine study has used this model, but does not completely describe it [17]. Basically, the older experiments consisted of challenging the mother during pregnancy and assessing the fetal transmission rate either by direct observation or, more often, by subinoculation of mice with fetus homogenates. The present study describes the development of a model of guinea-pig congenital toxoplasmosis with molecular techniques. These technologies (nested PCR and real-time quantitative PCR) are very sensitive [18–20] and can be adapted for extensive study.

Materials and methods

Animals and parasites

Female and male Dunkin Hartley guinea-pigs were purchased from Harlan (Gannat, France). Fifty-six female guinea-pigs weighing 600–800 g were transferred to our animal facilities. Their sera were obtained by intracardiac puncture and the absence of *Toxoplasma* antibodies was verified by indirect immunofluorescence antibody test (IFAT).

Three strains of *T. gondii* were used: the highly virulent RH strain, the mildly virulent 76K strain and the slightly virulent Prugniald (PRU) strain as previously used by Zenner *et al.* [10]. The RH strain (tachyzoite form) was maintained through successive intraperitoneal tachyzoite passages in mice every 3 days as described previously [21]. Briefly, tachyzoites obtained from ascitic fluid of OF1 mice (Charles River, L'Arbresle, France) infected 3 days earlier were used for intraperitoneal infection after counting and dilution as necessary (10^5 – 10^6 factor dilution). The 76K and

PRU strains (cystogenic strains) were maintained by oral inoculation of cysts in mice every 3 months as described previously [22]. Briefly, cysts obtained from the brains of OF1 mice infected 3 months earlier were used for oral or intraperitoneal infection. Under sterile conditions, brains from infected mice were homogenised in a 20-ml potter's tube, and the cysts were counted and diluted as necessary (5–20 factor dilution).

Mating and infection of female guinea-pigs

Groups of three, five or seven female guinea-pigs were kept in separate cages with one or two males for mating. After the 18th day of the menstrual cycle, the female guinea-pigs were housed in individual cages and grouped according to the time, strain and route of inoculation. On day 20 or 27 after the beginning of mating, two serum progesterone (Pg) measurements (Access[®] Beckman-Coulter, Villepinte, France) were performed to confirm and date the gestational age [23].

In all, 32 animals were infected by the intraperitoneal route (IP) with 100 tachyzoites of the RH or 100 cysts of the PRU strains. Another 24 animals were infected by the oral route (OR) with 100 cysts of the 76K or PRU strains. Animals were infected by the IP or OR before or after the beginning of gestation as follows: six guinea-pigs on the 90th day (G-90 $\pm$ 18 days) and six guinea-pigs on the 30th day (G-30 $\pm$ 9 days) before the beginning of gestation; 22 guinea-pigs on the 20th day (G20 $\pm$ 6 days) and 22 guinea-pigs on the 40th day (G40 $\pm$ 6 days) after the beginning of gestation (Table 1). To confirm infection, *T. gondii* IFAT was performed on the 14th and the 30th days after inoculation.

Table 1. Experimental congenital toxoplasmosis in female guinea-pigs inoculated with different strains of *T. gondii* 30 (G-30) or 90 (G-90) days before pregnancy and 20 (G20) and 40 (G40) days after the beginning of pregnancy

Strain (route)	Inoculation day	Number of guinea-pigs assessed/inoculated	Total offspring	Offspring 55–G70 Viability + recovered immature embryos	Number (%) of infected offspring determined by		
					nested PCR	real-time quantitative PCR	
RH (IP)	G-30	2/3	6	6	2]	0]	0 (0)
	G-90	2/3	5	4	0]	0]	
PRU (IP)	G-30	2/3	7	4	0]	0]	0 (0)
	G-90	3/3	11	10	3]	0]	
RH (IP)	G20	4/5	13	8 (of 10) + 3 dead embryos (G30)	5]	3]	8 (31)
				5 (of 9) + 4 dead embryos (G52)	9]	5]	
	G40	4/5	13	12	10]	6]	
				3 (of 11) + 3 dead embryos (G53)	14]	12]	
PRU (IP)	G20	4/5	13	12	10]	6]	29 (66)
	G40	4/5	14	3 (of 11) + 3 dead embryos (G53)	14]	12]	
	G20	3/5	9	6	6]	6]	
	G40	4/5	8	0 (of 7) + 1 dead embryo (G53)	7]	5]	
76K (OR)	G20	3/7	8	5	8]	7]	22 (76)
	G40	5/7	21	4	17]	15]	

OR, oral route; IP, intraperitoneal route; G55–G70 offspring, offspring of gestational age 55–70 days.

Organ harvesting of newborn guinea-pigs

A large number of newborn guinea-pigs were either stillborn (SB) or non-viable (NV), perhaps because of the congenital toxoplasmosis. The other newborns were killed with phenobarbital 15 days after birth. Stillborns were thoroughly washed with distilled water to avoid possible contamination with circulating parasites from maternal blood and amniotic fluid. Liver and brain were removed separately with sterile instruments, washed in phosphate-buffered saline (PBS), wiped and weighed and then parts of the organs were placed in 1.5-ml Eppendorf tubes. They were homogenised and ground with a pestle in the Eppendorf tube.

Nested PCR and real-time quantitative PCR

Extraction and purification. To detect *T. gondii* DNA in the organ homogenate, 50 µg of brain or liver were used for DNA extraction with the High Pure PCR Template Preparation Kit[®] (Roche Molecular Biochemicals, Meylan, France) according to manufacturer's recommendations. Briefly, after homogenisation and grinding with the pestle for the Eppendorf tube (sterile, DNA-free material for single use), cells were lysed by a short incubation with proteinase K (included in the kit) in the presence of a chaotropic salt (guanidine-HCl). Cellular DNA binds selectively to special glass fibres pre-packed in the High Pure Purification filter tube. Bound DNA was purified in a series of rapid 'wash-and-spin' steps to remove contaminating cellular components. Finally, 200 µl of low salt elution fluid (included in the kit) released the DNA from the glass fibre. The concentration was determined to be 100–300 mg/L (100–200 mg/L for brain and 150–300 mg/L for liver extracts). Purity of DNA was measured by UV spectrophotometry (OD₂₆₀/OD₂₈₀ between 1.6 and 1.8).

Nested PCR. Nested PCR was performed in a final volume of 50 µl containing 10 pmol of each primer, 200 µM deoxynucleoside triphosphates (dATP, dCTP, dGTP, dUTP ×2; Roche), 50 mM MgCl₂ (Life Technologies, Cergy Pontoise, France) 3 µl, PCR buffer 10× (200 mM Tris-HCl, 500 mM KCl, pH 8.3 (Life Technologies) 5 µl, Taq DNA polymerase (Life Technologies) 1.25 U and 4 µl of purified DNA. The denaturing, annealing and extension times were 1 min each at 95°C, 58°C and 72°C, respectively. The final extension step continued for a further 10 min. In the first round, DNA samples were amplified for 40 cycles with 5'-GGAAGTGCATCCGTTTCATGAG-3' and 5'-TCTTTAAAGCGTTCGTGGTC-3' primers derived from the *T. gondii* B1 gene [24] to produce a 194-bp fragment. In the second round, a 1-µl portion of the first amplification product was amplified for only 25 cycles, under the same conditions as in the first round but with 5'-TGCATAGTTGCAGTCACTG-3' and 5'-GGCGACCAATCTGCGAATACACC-3' primers [24] to produce a 97-bp internal fragment. The products were separated on agarose (Metaphor[®] agarose, Tebu)

3% and detected after staining with ethidium bromide under UV illumination.

Development of real-time quantitative PCR. PCR was performed with the LC FastStart DNA Master SYBR Green I[®] (Roche) in a standard PCR reaction containing 0.4 µM of each selected primer 5'-TGAAGAGAG-GAAACAGGTGGTTCG-3' and 5'-CCGCCTCCTTCG TCCGTCGTA-3' [25] derived from the *T. gondii* B1 gene, 3 mM MgCl₂ and precisely 50 ng of genomic DNA in 4 µl of sample dilution. To prevent potential carry-over contamination of the amplified target DNA from previous reactions, the PCR reaction mix was incubated initially with uracyl-DNA-glycosylase (Roche) for 5 min at room temperature. Amplification and detection were performed in a LightCycler[®] instrument[®] (Roche) as follows: 20 µl of reaction mixture were incubated initially for 10 min at 95°C to denature the DNA and activate the Fast Start Taq DNA; amplification was performed for 40 cycles of denaturation (95°C, 15 s, ramp rate 20°C/s), annealing (68°C, 4 s, ramp rate 20°C/s) and extension (72°C, 8 s, ramp rate 20°C/s). A single fluorescence reading for each sample was taken at the extension step. Quantitative results were expressed by determination of the detection threshold or the crossing point (Cp), which marked the cycle when the fluorescence of the given sample significantly exceeded the baseline signal. They were expressed as a fractional cycle number. Then, the Cps were plotted against the known parasite concentration to obtain a standard curve. The parasite count for a given tissue sample was calculated by extrapolation from this standard curve. Positive sample specificity was confirmed by determining the melting curve (95°C, 0 s, ramp rate 20°C/s; 68°C, 15 s, ramp rate 20°C/s; 95°C, 0 s, ramp rate 0.1°C/s, continuous measurement). The melting point of the specific amplification product (131 bp) was 84 ± 0.5°C.

To obtain standard ranges for nested PCR and real-time quantitative PCR assays, the trophozoites of strain RH from mouse peritoneal fluid were quantified with Nageotte cells. The DNA of the control was extracted under the same conditions and then a series of 10-fold dilutions were made. Different negative extracts (from liver or brain) were loaded with known dilutions and this series (equivalent extract tissue) was included in each amplification run (Fig. 1).

Statistical analysis

Statistical assessment of differences in mean viability and maternofetal transmission was done by the χ^2 test ($p < 0.05$ was considered significant).

Results

Follow-up of female guinea-pigs

Only 45 of the 56 guinea-pigs could be included in the

Fig. 1. (a) Real-time quantitative LC-PCR test with SYBR Green I[®]. Amplification plots obtained for *T. gondii* DNA from 5×10^5 , and serial 10-fold dilutions from 10^5 to 10 parasites in extract. Each slope corresponds to a particular input target quantity: **1**, from 5×10^5 parasites/extract; **2**, 10^5 parasites/extract; **3**, 10^4 parasites/extract; **4**, 10^3 parasites/extract; **5**, 10^2 parasites/extract; **6**, 10^1 parasites/extract. (b) Plot of crossing points against the input target quantity (common log scale) showing the linearity of the results. The computer-calculated correlation coefficient (r) is 1, the variations coefficient is 26.7% (error = 0.267); slope = -3.204 ; intercept = 35.28.

protocol; the other 11 were not gestating (6) (Pg concentration < 10 ng/ml on two samples), not infected (3) (inoculation not leading to seroconversion) or dead (2) secondary to intracardiac puncture.

Of the 45 guinea-pigs, 6 aborted early (gestation < 35 days) and offspring could not be counted except for one. Thus, 40 guinea-pigs remained, with 128 fetuses or neonates (number of neonates per litter = 3.2 SD 0.97) from which 249 samples (121 brain and 125 liver samples from neonates or fetuses ≥ 35 days and 3 fetal samples from the fetuses aborted at < 35 days) were taken and analysed.

Offspring viability according to strain and time of inoculation

For inoculation during gestation, percentage viability of offspring at a gestational age of 55–70 days was 68%

(13/19) for strain RH, 53% (21/40) for PRU and 31% (9/29) for 76K. Thus the number of viable offspring was significantly lower with strain 76K than with the other strains (χ^2 test, $p = 0.019$). It was also found that early abortions were more frequent after G20 inoculation with 76K (4/7 gestating females) than with PRU and RH strains (2/12 gestating females).

Inoculation was done at 20 days or 40 days after onset of gestation (G20 and G40, respectively). Percentage viability of offspring at gestational age > 55 days (all strains taken together) was 77% (31/40) for G20, but only 25% (12/48) for G40 (χ^2 test, $p < 0.001$). However, this difference in viability was less marked with strain RH than with the cystogenic PRU and 76K strains (Table 1).

There was also a small number of pre-gestational inoculations (on G-90 days and G-30 days); in these

cases offspring viability remained high at 83% (24/29) and this percentage was similar to those of the G20 post-gestation inoculations (77%).

Two different inoculation routes were tested (IP and OR) only for strain PRU and no significant difference in offspring viability was found (χ^2 test, $p = 0.19$).

Development of real-time quantitative PCR

Fig. 1 shows the amplification of the purified *T. gondii* DNA in 10-fold serial dilutions with fluorescence plotted against cycle number. The higher the initial copy number, the earlier the fluorescent signal appears. The sensitivity of the system is such that as few as two parasites/extract can be distinguished from the background signal. However, because of the dilution required by the assay (1 in 10 or 1 in 20) to avoid an excess of non-specific DNA, the sensitivity of the assay was 20–40 parasites/50 μ g of tissue. The intra-assay variation coefficient was 5% (high concentration DNA = 1800 parasites) and 24% (low concentration DNA = 25 parasites). The inter-assay variation coefficients were 73%, 35%, 24% and 21% for samples with 10, 25, 110 and 1800 parasites, respectively.

Comparison of sensitivity between different PCR techniques

Nested PCR sensitivity was 1–2 parasites/50 μ g of tissue. Sensitivity of quantitative PCR was 20–40 parasites/50 μ g of tissue because of the dilution necessary in the assay. Comparative sensitivities of these techniques for the 249 tissue extracts are shown in Table 2. Only the nested PCR was positive in 33 samples in contrast to three samples positive only by the real-time quantitative PCR, confirming the better sensitivity of the former technique. The 36 discordant samples were tested in the second series and the first results were confirmed.

Parasite load according to strain, time of inoculation and type of tissue

An offspring was deemed to be affected when there was at least one (liver or brain) positive test. With nested PCR, percentage maternofetal transmission was lower with strain RH (54%) than with 76K (86%) and PRU (84%) (χ^2 test, $p = 0.001$). With real-time

quantitative PCR, regardless of the strain, percentage of maternofetal transmission was lower – 31%, 76% and 66%, respectively (Table 1). Moreover, whatever organ and route was analysed, the mean positive parasite load after inoculation with RH ($\mu = 2240$ SD 1920 parasites/g) was lower than with the cystogenic strains (PRU, $\mu_1 = 5840$ SD 6090 parasites/g; 76K $\mu_2 = 10\,900$ SD 13\,340 parasites/g) (Fig. 2).

Percentage maternofetal transmission rates were significantly lower (19 and 15%) (χ^2 test, $p < 0.001$) when inoculations had been carried out before the onset of gestation (G-90, G-30) than in those inoculated after (67% for G20, 84% for G40). The mean tissue loads showed the same pattern: for all the G-90 and G-30 inoculated offspring tissue loads were unquantifiable (<400 parasites/g), whereas loads after G20 and G40 inoculations were much greater (6880 SD 10\,100 parasites/g).

The qualitative and quantitative analyses of the tissue load were done by comparing the tissue samples from liver with those from the brain. No significant difference was found either in the percentage of samples positive with the nested PCR (50% for liver *versus* 48% for brain samples) or in the mean load (9160 SD 13\,040 parasites/g for liver *versus* 6380 SD 9000 parasites/g for brain samples). However, there was a significant difference in the association of high hepatic load (>10\,000 parasites/g) and offspring viability. Whatever the date of inoculation and the strain, 11 of 12 offspring with high hepatic loads were stillborn, whereas only 2 of 9 offspring with high brain loads were stillborn (Fig. 2).

Discussion

The guinea-pig model has been described in various studies [14–17, 26]. Halfway between the rat and mouse, guinea-pig sensitivity to *T. gondii* is medium. Adult animals may die with infection with an inoculum of ≥ 1000 tachyzoites of strain RH [15, 26], whereas they remain insensitive with the semi-virulent or weakly virulent strains. Nevertheless, whatever the strain of *T. gondii*, fetuses were often seriously ill [14, 26 and present study], an observation not seen in rats. Guinea-pig placenta has a composition close to that of human (i.e., haemomonochorial), suggestive of similar modes of transmission. Hormonal control is cyclic (18 days) and gestation duration is 65 days, long enough to enable comparative studies with different inoculation times (e.g., G20 and G40, in the present study) and comparative chemotherapy studies (satisfactory medicinal impregnation). These reasons formed the rationale for the present study. Therefore the inoculum was adapted to induce a subclinical infection in the female guinea-pigs and the maternofetal transmission in the absence of acute non-controlled maternal infection was studied. The small (100 tachyzoites) inoculum used

Table 2. Comparison of sensitivity of LightCycler® real-time quantitative PCR and nested PCR of 249 extracts of tissue (brain and liver) from guinea-pig offspring infected with different strains of *T. gondii*

Nested PCR result	LightCycler® PCR result		
	Negative	Positive	Total
Negative	128	3	131
Positive	33	85	118
Total	161	88	249

Fig. 2. Parasitic load in brain (a) and liver (b) of guinea-pig offspring from female infected after (G20, G40) and before (G-30, G-90) the beginning of the pregnancy. The inoculation was done by oral route (OR) and intraperitoneal route (IP) with RH, Prugnnaud (PRU) and 76K strains of *T. gondii*. G, date of inoculation; ◆, RH, G20, IP; ■, RH, G40, IP; ▲, PRU, G20, OR; ●, PRU, G40, OR; *PRU, G20, IP; □, PRU, G40, IP; +, 76K, G20, OR; △, 76K, G40, OR; ◇, RH, G-30-90, IP; ○, PRU, G-30-90, IP.

with strain RH may account for the low rate of maternofetal transmission (54% *versus* 86% and 84% with strains 76K and PRU, respectively). Nevertheless, it should be borne in mind that a virulent strain (defined in terms of virulence in adult mice) is not necessarily more liable or able to cross the placenta to induce a higher maternofetal transmission rate [10, 11].

The present study used the same strains as a previous study involving gestating rats [10], which found maternofetal transmission rates of 58% (strain RH), 35% (76K) and 63% (PRU). The results are obviously not directly comparable, yet it is noteworthy that the

guinea-pig model was found to be more sensitive to strain 76K in the present study, with not only a higher rate of congenital toxoplasmosis but also greater incidence of stillbirth and early abortion.

Comparison of the results of the present study with those from other works (with the guinea-pig model) shows four common features: (i) the percentage of stillborn (SB) and non-viable (NV) fetuses remains high even with inoculation in the second part of gestation (G40): 31 (57%) SB/54 and 6 (11%) NV/54 in the present study *versus* 23 (55%) SB/42 and 9 (21%) NV/42 found by Giraud *et al.* [26]; (ii) after

inoculation in the first part of gestation (G20), more offspring reaching term were viable and normal (N): 31 (77%) N/40 in the present study *versus* 3 (100%) N/3 found by Giraud *et al.* [26]; (iii) after pre-gestation inoculation, maternofetal transmission is low: 5 (17%) of 29 in the present study compared with 5 (29%) of 17 for Wright [14]; (iv) abortion, when it occurs, does so between the 8th and the 20th day after inoculation of the mother, as described by these authors [14, 26].

In this study, molecular biology techniques were used because they are more sensitive and rapid than other techniques (cell culture, mouse inoculation, capture ELISA, immunoblotting) [27, 28]. For determining parasite load, it was necessary to use the following two techniques: nested PCR and a quantitative PCR. Nested PCR has a sensitivity of detecting <1 parasite/ml of amniotic fluid [29], making it the most sensitive of the techniques. In this study, the sensitivity was 1–2 parasites/50 µg of tissue. With the single PCR, a decrease in sensitivity of >10 parasites was observed because of the presence of excess leucocyte or tissue DNA [30]. The Light-Cycler[®] quantitative PCR technique is best used only to assess tissue parasite load, because of the reduced sensitivity (20–40 parasites/50 µg of tissue) resulting from the dilution factor involved in the SYBR Green I[®] protocol. However, LightCycler[®] PCR enabled the differential quantification of the tissue load from that in the circulation. Indeed, in another study (data not shown), only 25 parasites/ml were detected (no dilution needed, circulating DNA level low). Very high sensitivity in determining tissue load is not necessarily an advantage as mentioned by Zenner *et al.* [31]; however, it is essential for detecting circulatory or amniotic fluid loads which, in human subjects reach no more than a few parasites/ml [19, 32].

The following points are notable with respect to the quantitative parasite load analysis: (i) there were greater parasite loads with cystogenic strains; (ii) a strong relation was shown between lethal infection and high offspring liver load, probably reflecting generalised systemic infection; and (iii) the absence of any relationship between offspring viability and high brain loads, which were even more frequent in viable offspring, proving that they develop gradually during the latter part of gestation and in the first days after birth. This finding has already been reported [31, 33] in kinetic studies of tissue load, although only in mice and adult rats.

In conclusion, after 30 years of silence regarding the guinea-pig model, we believe that the present results are relevant. Transmission rates were >50% regardless of the strain used. Moreover, this model has some similarities to congenital toxoplasmosis in man (haemomonochorial placenta, partial transmission, variable severity) and some differences too (congenital transmission during chronic maternal toxoplasmosis, high

transmission rates regardless of the time of inoculation during the gestation). Offspring death was frequently associated with high liver load, indicating an uncontrolled systemic infection. Quantitative PCR determination of parasite load may prove to be an important element in the understanding of this congenital disease. Moreover, it could provide an experimental basis for physiopathological, kinetic, therapeutic and vaccine studies.

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