ORIGINAL ARTICLE

Diversity and evolution of methods and practices for the molecular diagnosis of congenital toxoplasmosis in France: a 4-year survey

Y. Sterkers[1], E. Varlet-Marie[1], P. Marty[2] and P. Bastien[1], on behalf of the ANOFEL *Toxoplasma*-PCR Quality Control Group*

1) *Centre Hospitalier Universitaire (CHU) of Montpellier/University of Montpellier 1, Laboratoire de Parasitologie-Mycologie, Laboratoire Associé (Pôle 'Biologie Moléculaire') of the National Reference Centre for Toxoplasmosis,* 2) *CHU of Nice, Laboratoire de Parasitologie-Mycologie, France*

Abstract

The prenatal diagnosis of congenital toxoplasmosis is currently based upon molecular biology using a sample of amniotic fluid. The vast majority of centres globally (and all centres in France) performing this diagnosis use 'in house' or laboratory-developed PCR assays. This may be the source of considerable inter-laboratory variation in the performances of the assays, hampering any valuable comparison of data among different centres. The present study was based upon questionnaires that were sent to 21–25 centres between 2002 and 2005 enquiring about methods and practices of the PCR-based prenatal diagnosis of congenital toxoplasmosis. An extreme diversity of PCR methods and practices was observed. Thus, in 2005, 35 PCR methods, differing in one of the main steps of the whole process, were reported as being in use for routine diagnosis, with nine centres using two or three methods. We provide comprehensive information on the extraction methods, DNA targets, primer pairs and detection methods used for this diagnosis, as well as their evolution, during the period of study. Interestingly, in this period (2002–2005), a rapid progression of the number of laboratories using real-time PCR technology, which increased from four to 19, was observed. We also studied general PCR practices concerning, for example, the number of reaction tubes used for each biological sample and the inclusion of controls. The return of information in a yearly report provided the opportunity for writing proposals aiming to improve laboratory practices for this diagnosis at the national level. The high diversity of methods and practices currently used emphasizes the need for external quality assessment of the performances of the molecular diagnostic methods.

Keywords: Diagnosis, external quality assessment, molecular methods, PCR, toxoplasmosis
Original Submission: 4 February 2009; **Revised Submission:** 9 October 2009; **Accepted:** 15 October 2009
Editor: D. Raoult
Article published online: 2 November 2009
Clin Microbiol Infect 2010; **16:** 1594–1602
10.1111/j.1469-0691.2010.03101.x

Corresponding author: P. Bastien, Laboratoire de Parasitologie-Mycologie, UMR2724 CNRS/
Université Montpellier 1/IRD, C. H. U. de Montpellier, 39 Av. Charles Flahault (site Antonin Balmès) 34295 Montpellier cedex 5, France
E-mail: p-bastien@chu-montpellier.fr

*The other members of the ANOFEL *Toxoplasma*-PCR Quality Control Group who participated in the present study were: B. Cimon (Angers), P. Millet (Bordeaux), D. Quinio (Brest), M. Vergnaud (Caen), S. Bretagne and F. Botterel (Créteil), A. Bonnin and F. Dalle (Dijon), H. Pelloux and M. P. Brenier-Pinchart (Grenoble), L. Delhaes (Lille), M. L. Dardé (Limoges), F. Peyron (Lyon), J. Franck (Marseille), B. Fortier (Nancy)† (in memoriam), M. Miegeville (Nantes), H. Yera (Paris-Cochin), P. Thulliez (Paris-Institut de Puériculture), S. Brun and L. Paris (Paris-Pitié Salpétrière), P. Roux (Paris-Saint Antoine), I. Villena (Reims), J. P. Gangneux and F. Robert-Gangneux (Rennes), P. Flori (Saint-Etienne), D. Filisetti (Strasbourg), M. H. Bessières and S. Cassaing (Toulouse), and T. H. Duong (Tours).

Introduction

Toxoplasmosis is an endemic protozoan disease whose prime public health importance is the result of possible vertical transmission from an infected mother to her foetus during pregnancy. Prenatal diagnosis (PND) of congenital toxoplasmosis (CT), wherever it has been implemented, has considerably improved the prognosis and outcome of infected children. Prevention of CT, including PND, has become a national policy in France ever since 1978 [1]. This national policy requires: (i) the detection and follow-up of non-immunized women as soon as possible during pregnancy with a series of serological tests; (ii) appropriate counselling aiming at limiting the risks of contamination; (iii) the detection and treatment of toxoplasmosis as early as possible aiming to prevent or limit transmission to the foetus and its consequences; (iv) PND of CT associated with monthly ultrasound examinations in case of a seroconversion; (v) combined

sulfadiazine-pyrimethamine treatment during pregnancy if CT is detected; and (vi) clinical, radiological and serological surveillance of neonates and infants at risk. This prevention programme is justified by the high prevalence of acquired toxoplasmosis in adults in France (approximately 44%) [2] and by the estimated yearly incidence of contamination in women during pregnancy (six or seven per 1000) and of congenital toxoplasmosis (approximately 0.1% of births) [3]. The programme was recently reinforced by the creation of a National Reference Centre for Toxoplasmosis (http://www.chu-reims.fr/professionnels/cnr-toxoplasmose-1/) which includes a 'pole' of molecular biology whose objectives include the improvement and standardization of the molecular diagnosis of CT, and whose coordinator is one of us (PB).

Indeed, molecular diagnostic tests, based upon PCR using amniotic fluid, have become essential in the diagnosis of CT; they have in great part superseded more classical methods, and have also led to the elimination of the need for cordocentesis [4]. In France, the PND of CT is made essentially in university hospitals, as well as in two large private biological diagnosis centres. Not all university hospital centres perform such testing because the centres and practitioners concerned need official authorization from the national health authorities to estabish this diagnosis, which is granted for 5 years.

However, despite their wide use, all PCR assays used for this application are still 'in-house'- or laboratory-developed methods (i.e. they have been set up independently in each laboratory using different targets and customized primers and reaction conditions). In addition, 'in-house' methods can largely differ at any step during the diagnostic process, such as the extraction method, the number of PCR tubes used for diagnosis, the inclusion of an internal control for the detection of inhibitors of the reaction, etc. These differences may be a source of considerable inter-laboratory variation in the performances of the assays, influencing the quality of the diagnosis and hampering any valuable comparison of data among centres. Previous studies have highlighted the lack of homogeneity and performance in European countries and underlined the need for guidelines [5,6]. In view of this heterogeneity, standardization of PCR methods and practices has become a strong desire for both health authorities and the community of clinical microbiologists. Such a standardization should in turn lead to improvement of the diagnosis of CT at a more global level, particularly regarding sensitivity, because parasite loads in this affliction are often very low [7].

To implement the harmonization of PND of CT in France, an early initiative for quality assurance in the molecular PND of toxoplasmosis was launched by the French association of hospital practitioners and teachers in Parasitology-Mycology (ANOFEL) in 2002. Briefly, a panel of *Toxoplasma gondii*-positive and -negative amniotic fluid samples prepared in Montpellier was sent blinded to participating centres for PCR testing on a yearly basis, allowing each centre to assess and follow its own performances in the molecular detection of CT [8].

A national survey was conducted in parallel from 2002 to 2005 aiming to assess the diversity and evolution of methods and practices used in this molecular diagnosis in France. The survey focused exclusively on the molecular PND of CT. The analysis of the data reported here provides an almost comprehensive description of these activities in France during the study period. It revealed a surprisingly high degree of diversity and the absence of any spontaneous trend toward standardization. Also, a massive introduction of quantitative 'real-time' PCR (qrtPCR) technology was observed during the study period, as opposed to 'conventional' PCR (cnPCR), a term used here for any form of end-point detection.

Materials and Methods

All laboratories of Parasitology–Mycology belonging to university hospitals, as well as one of the two officially authorized private diagnosis centres, were informed of the yearly external quality assessment (EQA) described previously [8]. Participating laboratories were free to enroll, anonymity of results was guaranteed, and no fees were imposed for participation. A questionnaire was sent every year to each participant, together with the EQA panel.

Participation in the EQA was anonymized through the use of letter codes and double-blinded cross-reading between the laboratories in Montpellier and Nice. An analysis of the questionnaires was performed after transcription of the data into spreadsheet software. The questionnaires included 11 queries concerning what we considered to be the most critical points of the PCR process. The query items are described below in the Results section. All answers to queries had to be given considering the routine practice of PND of CT, and not the procedures that could have been performed for the EQA only.

Results and Discussion

General observations

Between 2002 and 2005, the number of centres participating in the PND of CT increased from 21 to 25. All participants were from French University hospitals; none of the two private centres accredited for this diagnosis was involved. Most participants (23/25, i.e. 92% in 2005) were officially authorized

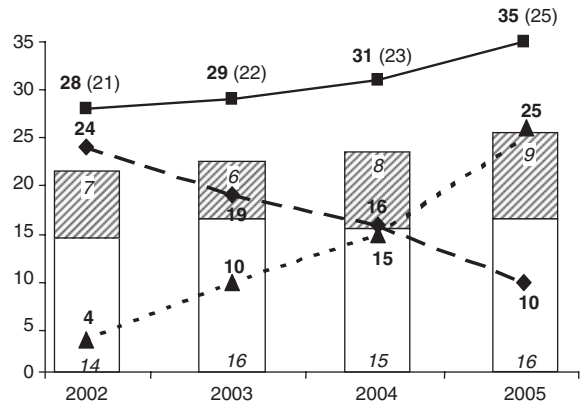

FIG. 1. Evolution of the number of methods used for molecular prenatal diagnosis of congenital toxoplasmosis between 2002 and 2005 in France. Closed squares indicate the total number of methods and centres; the number of centres is indicated in brackets. Closed diamonds and closed triangles indicate the number of cnPCR and qrtPCR methods, respectively. Histograms show the number of centres that used one method (bottom, white area) and two or three methods (top, hatched area); the corresponding figures are indicated within the histogram; only one laboratory used three methods in 2003 and 2005. Different methods were defined as differing by one of the main steps of the process (DNA extraction method, DNA targets/primers or type of PCR technology).

TABLE 1. Diversity and evolution of PCR technology and amplicon detection methods used for molecular prenatal diagnosis of congenital toxoplasmosis between 2002 and 2005[a]

	2002	2003	2004	2005
cnPCR	24 (19)	19 (15)	16 (12)	10 (9)
Ethidium bromide staining	21 (16)	18 (13)	16 (12)	10 (8)
PCR-ELISA[b]	6 (6)	4 (4)	3 (3)	2 (2)
Southern blot or sequencing[c]	3 (2)	4 (2)	4 (2)	0
qrtPCR[d]	4 (4)	10 (8)	15 (13)	25 (19)
Roche	1 (1)	5 (4)	7 (6)	15 (11)
SybrGreen	0	1(1)	0	3 (2)
Fluorescent probes[e]	1 (1)	4 (3)	7 (6)	12 (9)
Applied	2 (2)	3 (2)	5 (4)	5 (4)
Fluorescent probes[e]				
Bio-Rad	1 (1)	1 (1)	1 (1)	3 (2)
Fluorescent probes[e]				
Rotorgene 2000	0	1 (1)	1 (1)	1 (1)
Fluorescent probes[e]				
Stratagene MX400	0	0	1 (1)	1 (1)
Fluorescent probes[e]				
Total	28 (21[f])	29 (22[f])	31 (23[f])	35 (25[f])

[a]The number of methods is given preceding the number of centres (in parenthesis) that use the particular method.
[b]PCR-ELISA was used as the sole detection method in 2002 and 2003 by, respectively, three centres and one centre; otherwise, it was used in association with ethidium bromide staining of agarose gels.
[c]Both used in complement to ethidium bromide-stained gels. Sequencing was used by one centre only in 2003 and 2004.
[d]Roche® (Meylan, France): Light-Cycler 1.0, 2.0 and 480; Applied® (Courtaboeuf, France): ABI Prism 7000 and 7700, Bio-Rad® (Marnes-la-Coquette, France): one iQ and one iCycler.
[e]The type of fluorescent probes used (FRET, Taqman, etc.) could not be specified in this study.
[f]The sum of numbers of centres using 'conventional' PCR (cnPCR) and quantitative 'real-time' PCR (qrtPCR) does not account for the total because one or two centres used both technologies

to establish this diagnosis; this represented 100% and 92% of the public centres and of all centres, respectively, that had received official authorization to establish this PND. Unauthorized laboratories about to request authorization to establish PND of CT were also accepted to perform the EQA and participated in the enquiry.

All questionnaires were returned; only a few queries were not answered by some laboratories. In all, 988 answers vs. an expected total number of 1001 answers could be analysed. Hence, the enquiry was highly representative of the national practices of PND of CT during the study period. During this period, the number of molecular diagnostic methods used in routine practice increased from 28 to 35. Indeed, some laboratories used more than one method for routine diagnosis (Fig. 1). Different methods were defined here as differing by a major step of the diagnostic process, such as the DNA extraction method, or with respect to DNA primers or DNA target and type of PCR technology.

Use of a second molecular diagnostic method

Out of ten (40%) of the participating centres, nine used routinely two and one used three variant methods in parallel at one stage during the study period (Fig. 1). These variations concerned any of the major steps of the molecular diagnostic method defined above. A fundamental distinction should be made here between the centres using two variant methods during a transitory switch period (usually <1 year) and those deliberately choosing to use two methods. The latter represented about 30% of the total number of laboratories.

The interest and rationale behind such a practice are discussed in the Supporting Information (Data S1).

Type of PCR technology and amplicon detection method

Our data show the considerable progression of real-time PCR technologies over the study period (Fig. 1 and Table 1). During these 4 years, the number of laboratories using qrtPCR increased from four out of 21 to 19 out of 25 (19–76%) and the number of methods based on qrtPCR increased from four out of 28 to 25 out of 35 (14–71%). Among the 19 centres that used cnPCR in 2002, 13 had changed for qrtPCR in 2005; nine laboratories still used cnPCR in 2005, among which three also used a qrtPCR method. The four centres that used qrtPCR in 2002 still used it in 2005, and the four centres that joined the network during the study period used qrtPCR technology. These data

TABLE 2. Diversity and evolution of DNA extraction methods used for the molecular prenatal diagnosis of congenital toxoplasmosis[a]

	2002	2003	2004	2005
Laboratory- developed[b]	7 (7)	6 (6)	5 (5)	4 (4)
Tween-Nonidet-NaOH	6 (6)	5 (5)	5 (5)	4 (4)
cnPCR	6 (6)	4 (4)	4 (4)	3 (3)
qrtPCR	0	1 (1)	1 (1)	1 (1)
Chelex	1 (1)	1(1)	0	0
cnPCR				
Commercial kits[c]	21 (15)	23 (17)	26 (19)	31 (22)
Qiagen	17 (12)	17 (13)	20 (15)	21 (16)
cnPCR	14 (11)	11 (9)	10 (8)	5 (4)
qrtPCR	3 (3)	6 (5)	10 (9)	16 (14)
GeneReleaser	1 (1)	1 (1)	0	0
qrtPCR				
Epicentre	2 (1)	2 (1)	2 (1)	2 (1)
qrtPCR				
Roche	1 (1)	3 (2)	4 (3)	4 (3)
qrtPCR				
Magnapure	0	0	0	2 (2)
qrtPCR				
QbioRobot	0	0	0	2 (1)
qrtPCR				
Total	28 (21[d])	29 (22[d])	31 (23[d])	35 (25[d])
cnPCR	24 (19)	19 (15)	16 (12)	10 (8)
qrtPCR	4 (4)	10 (8)	15 (13)	25 (19)

[a]The number of methods is given preceding the number of centres (in parenthesis) that use the particular method.
[b]TNN, Tween-Nonidet-NaOH [10]; Chelex resin (Bio-Rad, Marnes-la-Coquette, France).
[c]QiaAmp DNA minikit, Blood, tissue or nonspecified (Qiagen, Courtaboeuf, France); Genereleaser (Bioventures Inc., ATGC Biotechnologie, Croissy-Beaubourg, France); MasterPure DNA purification (Epicentre, Tebu-Bio, Le Perray-en-Yvelines, France); HighPure PCR template preparation kit (Roche, Meylan, France) Two methods are automated: Magnapure (Roche) and QbioRobot (Qiagen).
[d]The sums do not account for the total because one centre used both a laboratory-developed method (Tween-Nonidet-NaOH) and a commercial kit (Qiagen). Similarly, the sum of the numbers of centres using 'conventional' PCR (cnPCR) and quantitative 'real-time' PCR (qrtPCR) does not account for the total, because one or two centres used both technologies.

indicate that the change to qrtPCR reflects an inevitable trend, which, we believe, is desirable. It is noteworthy that the advent of this new technology did not reduce the heterogeneity of the methods used at the national level as a result (among other factors) of the diversity of the real-time PCR equipment used, as well as of the methods of detection of amplified DNA (Table 1).

Regarding for the amplicon detection method, most cnPCRs were used with subsequent ethidium bromide-stained agarose gels and only few used PCR-ELISA (Table 1; see also Supporting Information, Data S2). For qrtPCR, specific fluorescent oligo-probes were used instead of Sybr-Green in all centres but one in 2003 and two in 2005 (Table 1). The interest of these different methods is discussed in the Supporting Information (Data S2).

Finally, it is noteworthy that, as opposed to the situation in other countries [6], nested PCR was not in use in routine practice in France during the study period, probably because of the high contamination risks that are typically associated with this type of PCR.

DNA extraction methods

Regarding DNA extraction, a minority of laboratories (7/21 in 2002 to 4/25 in 2005) preferred 'laboratory-developed' methods, including Tween-Nonidet-NaOH (TNN) and Chelex resin, to commercialized kits (Table 2). TNN comprises a simple, inexpensive and highly efficient DNA isolation method first described in 1994 [9]; its main drawback is that it is not standardized; also of note is that it is not applicable to blood-containing samples. The Chelex resin method was rapidly abandoned, probably because of its well-known low capacity for removing PCR inhibitors. Commercialized kits from as many as six different manufacturers were used. Qiagen products were by far the most frequently used, although it is not known whether this was based on scientific grounds or a result of the better commercial strategy of this supplier. What is needed to allow the microbiologist to make evidence-based choices in this respect are comparative studies of the extraction methods; however, few such studies are available because they imply particularly complex and highly standardized protocols [10].

Only one centre used two extraction methods (TNN and a commercial kit) for a single PCR assay during the whole period; the logic behind this practice was to ensure a better quality of diagnosis in case of a fault in the DNA extraction.

DNA target and PCR primers

The diversity observed among the participating centres was extremely high, with 17 primer pairs targeting four DNA sequences (Table 3). The B1 gene [11] remained the most popular target over the 4 years. However, we observed a rapid increase in the use of the noncoding repetitive sequence described by Homan *et al.* [12] that we termed rep529. This may be related to the improved sensitivity compared to that obtained with the B1 gene, as reported by several authors [12–14]. The use of rRNA gene sequences [15] decreased over the 4 years; and $TGR1_E$, another repetitive element described by Cristina *et al.* [16,17], was rapidly abandoned.

Primers are generally chosen using software according to specific criteria. The profusion of primer sets designed here for the two major DNA targets cannot be justified on scientific grounds but rather on personal preferences. In 2002, 12 different primer pairs were being used for four DNA targets, and as many as 15 were used for three targets in 2005 [11–15,17–24] (Table 3). For the B1 gene alone, nine different primer pairs were used in 2002 and eight in 2005. At the same time, the number of primer sets designed for rep529 (particularly for qrtPCR) increased from one to six, two of them yet unpublished (Table 3; see also Supporting Information, Fig. S1 and Table S1). Several of these primer pairs

TABLE 3. Diversity and evolution of DNA targets and primer sets used for the molecular PND of congenital toxoplasmosis in France (2002–2005)[a]

		2002	2003	2004	2005
Bl gene[b]		**22 (19**[c]**)**	**21 (19)**	**17 (14**[c]**)**	**20 (15**[c]**)**
	cnPCR	***19 (17)***	***14 (12)***	***11 (9)***	***7 (6)***
	qrtPCR	***3 (3)***	***7 (7)***	***6 (6)***	***13 (10)***
Burg *et al.*, 1989[11]	*cnPCR*	4 (3)	3 (2)	3 (2)	3 (2)
Bretagne *et al.*, 1993[18]		8 (8)	8 (8)	6 (6)	8 (7)
	cnPCR	*8 (8)*	*8 (8)*	*6 (6)*	*4 (4)*
	qrtPCR	*0*	*0*	*0*	*4 (3)*
Foudrinier *et al.*, 1996[20]		1 (1)	0	0	1 (1)
	cnPCR	*1 (1)*	*0*	*0*	*0*
	qrtPCR	*0*	*0*	*0*	*1 (1)*
Pelloux *et al.*, 1996[22]	*cnPCR*	2 (2)	1 (1)	1 (1)	1 (1)
Robert *et al.*, 1996[23]	*cnPCR*	3 (3)	2 (2)	1 (1)	1 (1)
Costa *et al.*, 2000[19]	*qrtPCR*	1 (1)	4 (4)	3 (3)	3 (3)
Lin *et al.*, 2000[20]	*qrtPCR*	1 (1)	2 (2)	2 (2)	2 (2)
Morin and Miegeville, unpublished	*cnPCR*	1 (1)	0	0	0
Unpub 1	*qrtPCR*	1 (1)	1 (1)	1 (1)	1 (1)
rep529		**1 (1)**	**5 (5)**	**12 (12)**	**13 (12**[c]**)**
	cnPCR	***1 (1)***	***3 (3)***	***3 (3)***	***2 (2)***
	qrtPCR	***0***	***2 (2)***	***9 (9)***	***11 (11)***
Homan *et al.*, 2000[12]		1 (1)	3 (3)	3 (3)	2 (2)
	cnPCR	1 (1)	3 (3)	2 (2)	1 (1)
	qrtPCR	0	0	1 (1)	1 (1)
Reischl *et al.*, 2003[14]		0	1 (1)	6 (6)	6 (6)
	cnPCR	0	1 (1)	1 (1)	10)
	qrtPCR	0	0	5 (5)	5 (5)
Cassaing *et al.*,2006[13]	*qrtPCR*	0	1 (1)	2 (2)	2 (2)
Fekkar *et al.*, 2008[24]	*qrtPCR*	0	0	0	1 (1)
Unpub 2	*qrtPCR*	0	0	1 (1)	1 (1)
Unpub 3	*qrtPCR*	0	0	0	1 (1)
rDNA					
Cazenave *et al.*, 1993[15]		**4 (3**[c]**)**	3 (3)	**2 (2)**	**2 (2)**
	cnPCR	***3 (3)***	***2 (2)***	***2 (2)***	***1 (1)***
	qrtPCR	***1 (1)***	***1 (1)***	***0***	***1 (1)***
$TGR1_E$					
Cristina *et al.*, 1992[17]	*cnPCR*	**1 (1)**	**0**	**0**	**0**
Total		**28 (21**[c]**)**	**29 (22**[c]**)**	**31 (23**[c]**)**	**35 (25**[c]**)**
	cnPCR	***24 (19)***	***19 (15)***	***16 (12)***	***10 (8)***
	qrtPCR	***4 (4)***	***10 (8)***	***15 (13)***	***25 (19)***

[a]In the whole table, the number of methods is in clear and the number of centres that use that particular method is between brackets. For example, in 2002, 22 diagnostic methods in 19 centres were based upon the Bl gene, overall using nine different primer pairs (listed with their bibliographic references in the far-left column, under the DNA target).
[b]Bl gene: 35-copy number repetitive gene (GenBank Accession N° AF179871) from Burg *et al.* [11]; rep529: 200-300 fold repeated 529-bp non-coding element identified by Homan *et al.* [12] (GenBank AccessionN° AF146527) and Reishl *et al.*[14] (GenBank Accession N° AF487550); rDNA: ribosomal DNA; $TGR1_E$: a member of a family of repeated DNA elements in *T. gondii* described by Cristina *et al.* [16].
[c]The sums do not make the total as several centres used two or three different primer pairs. Similarly, the sum of numbers of centres using cnPCR and qrtPCR does not make the total, as one to two centres used both technologies.

overlap, in both targets, which renders even more questionable any interest in this diversity. The percentage of primer pairs that were used by only one laboratory among the participants was high, in the range 33–58% during the study. This proportion rises to 50–73% when considering primers used by up to two laboratories only. Two factors should have reduced this diversity in the transition period that this study reflected, although they did not. (i) During the change from cnPCR to qrtPCR, only a few new primer sets were reported and could have been chosen because they appeared highly efficient in amplifying the smaller products required for qrtPCR [7,14,21]. Still, six laboratories out of 13 kept using the same primer set when realizing this change. (ii) This was particularly true for the DNA target rep529 [12] that was introduced during this period, which could have reduced the number of primers used. By contrast, several laboratories developed their own primer pair for this target, perhaps as a result of the publication of nucleotide variations

TABLE 4. Diversity and evolution of certain good PCR practices used for the molecular prenatal diagnosis of congenital toxoplasmosis in France[a]

	2002	2003	2004	2005
Number of reaction tubes				
One tube	3	2	0	0
cnPCR	3	1	0	0
qrtPCR	0	1	0	0
Two tubes	13[b]	11	12	10
cnPCR	11	8	4	1
qrtPCR	4	3	8	9
Three to six tubes	5	9[b]	11[b]	15[b]
cnPCR	5	6	7	7
qrtPCR	0	4	6	10
Extraction control[c]				
Plasmid	0	1	1[b]	0
cnPCR	0	1	1	0
qrtPCR	0	0	1	0
Toxoplasma	5[d]	4[bd]	6[bd]	8[bd]
cnPCR	5	4	4	3
qrtPCR	0	1	3	7
Duplicate	2[d]	3[d]	2[d]	2[d]
cnPCR	1	2	0	0
qrtPCR	1	1	2	2
OD	2[d]	3	3	4
cnPCR	2	2	1	1
qrtPCR	0	1	2	3
β-globin	3	4	4	5
cnPCR	3	3	2	2
qrtPCR	0	1	2	3
Albumin	2[bd]	2	2	1
cnPCR	1	0	0	0
qrtPCR	2	2	2	1
No control	9[b]	6	6	6
cnPCR	9	4	1	1
qrtPCR	1	2	5	5
Negative control				
Yes	17[b]	21[b]	22[b]	25[b]
cnPCR	16	16	13	9
qrtPCR	3	6	11	18
No	1	1	1	0
cnPCR	0	0	0	0
qrtPCR	1	1	1	0
NS[e]	3	0	0	0
cnPCR	3	0	0	0
qrtPCR	0	0	0	0
Inhibition control[f]				
Plasmid	6[b]	8[b]	6[b]	5[bg]
cnPCR	6	8	5	3
qrtPCR	1	2	3	3
Toxoplasma	3	5	8	9[g]
cnPCR	3	5	5	3
qrtPCR	0	0	3	6
Human gene	4[b]	4	2	2
cnPCR	4	1	0	0
qrtPCR	1	3	2	2
Exogenous DNA	3	3	4	5
cnPCR	2	1	2	2
qrtPCR	1	2	2	3
No control	2	1	1	2
cnPCR	2	0	0	0
qrtPCR	0	1	1	2
NS[e]	3[b]	1	2	3
cnPCR	3	1	0	1
qrtPCR	1	0	2	2
Total	21[b]	22[b]	23[b]	25[b]
cnPCR	19	15	11	8
qrtPCR	4	8	14	19

[a]All numbers here represent centres; the same information applied specifically to methods may either be not meaningful or sometimes be lacking as a result of insufficiently detailed questionnaires.
[b]The total does not account for the sum of the numbers below it because one or two centres used both 'conventional' PCR (cnPCR) and quantitative 'real-time' PCR (qrtPCR).
[c]DNA extraction control. Plasmid: PCR amplification after extraction of a plasmid added to the raw amniotic fluid sample. *Toxoplasma*: concomitant extraction of a *Toxoplasma gondii* suspension in parallel to that of the sample. Optical density (OD): extracted DNA quantitation by spectrophotometric measure of the absorbance at 260 nm. β-globin and albumin: amplification of the corresponding human genes.
[d]Some laboratories used two different types of extraction controls.
[e]NS, not specified
[f]Types of inhibition control: for details, see text.
[g]One laboratory used two different types of inhibition control.

between both reported sequences of this repetitive element [14]. The primer diversity probably is the greatest obstacle to standardization. Indeed, once a diagnostic assay has been set up for routine diagnosis using a certain primer pair, difficulties are usually experienced with respective to changing primers in view of the workload that such a change may imply. Our data show that most centres hesitate in making this change (not shown). Hence, comparative studies of primer pairs become a priority in this field. We believe that these should be 'intra-laboratory' comparisons of finely 'optimized' assays: indeed, 'inter-laboratory' comparative studies are useful for assessing PCR practices and method performances [5,6,25,26], but they do not allow conclusions to be drawn regarding the superiority of a given method/primer set whose value in a given application greatly depends upon 'optimization' and technical proficiency [27,28].

Similarly, an 'intra-laboratory' comparison may not inform about the real respective value of the assays if both have not been finely 'optimized'.

Good practices for molecular diagnosis

The adaptation to routine diagnosis that follows the setting up and 'optimization' of a PCR assay should include a number of good general practices [27]. We enquired about some of these, more specifically the number of PCR reaction tubes used for each one patient in routine diagnosis and the use of controls (verification of DNA extraction, negative and positive controls), all of which also appeared highly diverse (Table 4; see also Supporting Information, Data S3).

Conclusion

This 4-year survey allows a detailed and almost comprehensive description of the practices in the molecular diagnosis of congenital toxoplasmosis in a country which attributes a considerable medical importance to this infection. The observed diversity is considerable, essentially because of the use of independent laboratory-developed methods. No consensus exists for any step of the whole process, be it DNA extraction, DNA target/primers, or detection methods; furthermore, there is no sign that this diversity should decrease. There were as many extraction methods in 2005 as there were in 2002, and the introduction of automated extraction may further increase this diversity. Similarly, there were almost as many primers sets used for rep529 in 2005 than for the B1 gene in 2002.

This diversity in itself constitutes an obstacle to the standardization of diagnostic methods as well as to the comparability of results among different laboratories. The question of a correlation between any method and its results and performance (i.e. whether some methods proved to be more efficient than others during the various external quality assessments) [8] cannot be answered here, perhaps precisely because of this diversity. Our experience, as well as that of different groups [5,6,26,29], demonstrate that no link can be made between the methods and the results of comparative assessment studies. This points out the crucial importance of proficiency and optimization of PCR conditions, rather than the method itself, for establishing a solid molecular diagnosis. Thus, the great range of sensitivities of PND observed in a recent multicentric European study [30] may reflect the different performances of the methods, varying proficiency among centres, or true differences in the pathology. In turn, this issue prevents CT control policies from being efficiently evaluated. Moreover, the molecular PND of CT suffers from a relatively high rate of false negative results, approximately 30% (mean) in Europe [30] and in the range 10–35% in France [30–32]; it appears that this rate cannot be reduced to zero, probably essentially for physiopathological reasons, such as delayed transplacental transmission or the high frequency of low parasite loads in amniotic fluid [7]. This implies that the molecular diagnostic method must be highly sensitive. Given that the diversity of methods and practices observed in the present study is far from decreasing, it appears that, rather than to standardize the existing methods, it better assess their performances would be more practical to, using a common and calibrated basic material. This may be achieved through an external quality assessment that would not only aim to verify that the laboratories are able to detect positive samples and to return negative samples as negative, such as those existing in France and in Europe [6,8], but also would allow an estimation of the sensitivity thresholds of their methods.

During the present study, annual reports were sent to the participants, including recommendations drawn from the analysis of both the external quality assessment results [8] and the accompanying questionnaires, with a view to improving laboratory practices for CT diagnosis at the national level. Without knowing whether this is the fruit of technical and proficiency evolution or the consequences of these recommendations, we observed an improvement of certain practices over the years: in particular, evolution toward abandoning less efficient DNA targets (i.e. $TGR1_E$ and rDNA) and generalization of certain good practices. For example, (i) the proportion of laboratories that did not verify their DNA extraction decreased from 45% to 24%; (ii) all centres included negative controls in 2005; (iii) the median of the number of reaction tubes per biological sample increased from two to three and all laboratories have been using more than one tube subsequent to 2004. By contrast,

the number of centres that do not use any inhibition control remained stable at approximately 12%. The objective here should be that any laboratory involved in PND of CT should include inhibition controls. In summary, we strongly consider that such surveys, accompanying the assessment of the performances of PND methods and practices (which is a major objective of the French National Centre for Toxoplasmosis), should be performed wherever a routine diagnosis of CT is made.

Acknowledgements

We thank all the members of the ANOFEL *Toxoplasma* Quality Control Group for their participation. We also wish to thank I. Villena, H. Pelloux and M. P. Brenier-Pinchart, D. Filisetti, H. Yera, F. Dalle, S. Cassaing, B. Cimon, T. Feriel, M. Machouart and L. Delhaes for fruitful discussions. We are also indebted to D. Chabasse and J. M. Pinon for their support to the EQA initiative. We finally acknowledge the assistance of E. Chabbert as well as the technical help of G. Bresson, S. Douzou, B. Sanichanh and F. Michel.

Transparency Declaration

The study received financial support from ANOFEL and the National Reference Centre for Toxopalsmosis. The authors do not have any dual or conflicting interests to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Position of the PCR primers used for the molecular diagnosis of congenital toxoplasmosis in France (2002–2005) on the two main DNA targets (B1 gene and rep529).

Table S1. Nucleotide sequences and localization of the primer pairs used to amplify different DNA targets of *Toxoplasma gondii*.

Data S1. Use of a second method.

Data S2. Type of PCR technology and amplicon detection method.

Data S3. Good practices for molecular diagnosis.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1. Thulliez P, Daffos F, Forestier F. Diagnosis of *Toxoplasma* infection in the pregnant woman and the unborn child: current problems. *Scand J Infect Dis Suppl* 1992; 84: 18–22.
2. Berger F, Goulet G, Le Strat Y, Desenclos J. Toxoplasmosis in pregnant women in France: trends in seroprevalence and incidence, and associated factors, 1995–2003. *Bull Epidémiol Hebdomadaire* 2008; 14–15: 117–121.
3. King L, Villena I, Ancelle T *et al.* Congenital toxoplasmosis: implementation of a surveillance system in france. *Bull Epidémiol Hebdomadaire* 2008; 14–15: 122–123.
4. Bastien P. Molecular diagnosis of toxoplasmosis. *Trans R Soc Trop Med Hyg* 2002; 96 (suppl 1): S205–S215.
5. Pelloux H, Guy E, Angelici MC *et al.* A second european collaborative study on polymerase chain reaction for *Toxoplasma gondii*, involving 15 teams. *FEMS Microbiol Lett* 1998; 165: 231–237.
6. Kaiser K, Van Loon AM, Pelloux H *et al.* Multicenter proficiency study for detection of *Toxoplasma gondii* in amniotic fluid by nucleic acid amplification methods. *Clin Chim Acta* 2007; 375: 99–103.
7. Costa JM, Ernault P, Gautier E, Bretagne S. Prenatal diagnosis of congenital toxoplasmosis by duplex real-time PCR using fluorescence resonance energy transfer hybridization probes. *Prenat Diagn* 2001; 21: 85–88.
8. Bastien P, Jumas-Bilak E, Varlet-Marie E, Marty P. Three years of multi-laboratory external quality control for the molecular detection of *Toxoplasma gondii* in amniotic fluid in france. *Clin Microbiol Infect* 2007; 13: 430–433.
9. Hohlfeld P, Daffos F, Costa JM, Thulliez P, Forestier F, Vidaud M. Prenatal diagnosis of congenital toxoplasmosis with a polymerase-chain-reaction test on amniotic fluid. *N Engl J Med* 1994; 331: 695–699.
10. Yera H, Filisetti D, Bastien P, Ancelle T, Thulliez P, Delhaes L. Multicenter comparative evaluation of five commercial methods for toxoplasma DNA extraction in amniotic fluid. *J Clin Microbiol* 2009; 47: 3881. 3886.
11. Burg JL, Grover CM, Pouletty P, Boothroyd JC. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J Clin Microbiol* 1989; 27: 1787–1792.
12. Homan WL, Vercammen M, De Braekeleer J, Verschueren H. Identification of a 200- to 300-fold repetitive 529 bp dna fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. *Int J Parasitol* 2000; 30: 69–75.
13. Cassaing S, Bessieres MH, Berry A, Berrebi A, Fabre R, Magnaval JF. Comparison between two amplification sets for molecular diagnosis of toxoplasmosis by real-time PCR. *J Clin Microbiol* 2006; 44: 720–724.
14. Reischl U, Bretagne S, Kruger D, Ernault P, Costa JM. Comparison of two DNA targets for the diagnosis of toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. *BMC Infect Dis* 2003; 3: 7.
15. Cazenave J, Cheyrou A, Begueret J. Improvement of rDNA-based PCR assay to detect toxoplasma. *Prenat Diagn* 1993; 13: 543.
16. Cristina N, Liaud MF, Santoro F, Oury B, Ambroise-Thomas P. A family of repeated DNA sequences in *Toxoplasma gondii*: cloning, sequence analysis, and use in strain characterization. *Exp Parasitol* 1991; 73: 73–81.
17. Cristina N, Derouin F, Pelloux H, Pierce R, Cesbron-Delauwn MF, Ambroise-Thomas P. Detection of toxoplasma gondii by "Polymerase chain reaction" (PCR) technique in aids infected patients using the repetitive sequence TGR1E. *Pathol Biol (Paris)* 1992; 40: 52–55.
18. Bretagne S, Costa JM, Vidaud M, Tran J, Nhieu V, Fleury-Feith J. Detection of *Toxoplasma gondii* by competitive DNA amplification of bronchoalveolar lavage samples. *J Infect Dis* 1993; 168: 1585–1588.

19. Costa JM, Pautas C, Ernault P, Foulet F, Cordonnier C, Bretagne S. Real-time PCR for diagnosis and follow-up of toxoplasma reactivation after allogeneic stem cell transplantation using fluorescence resonance energy transfer hybridization probes. *J Clin Microbiol* 2000; 38: 2929–2932.
20. Foudrinier F, Aubert D, Puygauthier-Toubas D *et al.* Detection of *Toxoplasma gondii* in immunodeficient subjects by gene amplification: influence of therapeutics. *Scand J Infect Dis* 1996; 28: 383–386.
21. Lin MH, Chen TC, Kuo TT, Tseng CC, Tseng CP. Real-time PCR for quantitative detection of *Toxoplasma gondii*. *J Clin Microbiol* 2000; 38: 4121–4125.
22. Pelloux H, Weiss J, Simon J *et al.* A new set of primers for the detection of *Toxoplasma gondii* in amniotic fluid using polymerase chain reaction. *FEMS Microbiol Lett* 1996; 138: 11–15.
23. Robert F, Ouatas T, Blanche P, Tourte-Schaefer C, Sicard D, Dupouy-Camet J. Retrospective evaluation of the detection of *Toxoplasma gondii* by polymerase chain reaction in AIDS patients. *Presse Med* 1996; 25: 541–545.
24. Fekkar A, Bodaghi B, Touafek F, Le Hoang P, Mazier D, Paris L. Comparison of immunoblotting, calculation of the goldmann-witmer coefficient, and real-time PCR using aqueous humor samples for diagnosis of ocular toxoplasmosis. *J Clin Microbiol* 2008; 46: 1965–1967.
25. Guy EC, Pelloux H, Lappalainen M *et al.* Interlaboratory comparison of polymerase chain reaction for the detection of toxoplasma gondii DNA added to samples of amniotic fluid. *Eur J Clin Microbiol Infect Dis* 1996; 15: 836–839.
26. Costa JM, Munoz C, Kruger D *et al.* Quality control for the diagnosis of *Toxoplasma gondii* reactivation in SCT patients using PCR assays. *Bone Marrow Transplant* 2001; 28: 527–528.
27. Apfalter P, Reischl U, Hammerschlag MR. In-house nucleic acid amplification assays in research: how much quality control is needed before one can rely upon the results? *J Clin Microbiol* 2005; 43: 5835–5841.
28. Bastien P, Procop GW, Reischl U. Quantitative real-time PCR is not more sensitive than 'conventional' PCR. *J Clin Microbiol* 2008; 46: 1897–1900.
29. Pelloux H, Dupouy-Camet J, Derouin F, Aboulker JP, Raffi F. A multicentre prospective study for the polymerase chain reaction detection of *Toxoplasma gondii* DNA in blood samples from 186 AIDS patients with suspected toxoplasmic encephalitis. Bio-toxo study group. *AIDS* 1997; 11: 1888–1890.
30. Thalib L, Gras L, Romand S *et al.* Prediction of congenital toxoplasmosis by polymerase chain reaction analysis of amniotic fluid. *BJOG* 2005; 112: 567–574.
31. Romand S, Wallon M, Franck J, Thulliez P, Peyron F, Dumon H. Prenatal diagnosis using polymerase chain reaction on amniotic fluid for congenital toxoplasmosis. *Obstet Gynecol* 2001; 97: 296–300.
32. Bessieres MH, Berrebi A, Cassaing S *et al.* Diagnosis of congenital toxoplasmosis: prenatal and neonatal evaluation of methods used in Toulouse university hospital and incidence of congenital toxoplasmosis. *Mem Inst Oswaldo Cruz* 2009; 104: 389–392.