Interlaboratory Comparison of Polymerase Chain Reaction for the Detection of *Toxoplasma gondii* DNA Added to Samples of Amniotic Fluid

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To investigate the accuracy of the polymerase chain reaction (PCR) method for the detection of *Toxoplasma gondii* in clinical specimens, aliquots of amniotic fluid to which known amounts of *Toxoplasma gondii* DNA had been added were tested by five European Centres. Four laboratories were able to detect DNA at levels equivalent to ten tachyzoites or less, including two that detected DNA equivalent to a single parasite. Two laboratories erroneously found one of eight negative control samples to be positive. These findings confirm that the high level of sensitivity associated with the PCR method can be readily achieved under routine laboratory conditions, but they also underscore the potential for both false-positive and false-negative findings to occur. Furthermore, the results confirm the urgent need for an external quality assurance scheme to support laboratories employing PCR in a clinical context for the detection of *Toxoplasma gondii.*

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Direct detection of *Toxoplasma gondii* DNA using the polymerase chain reaction (PCR) has been shown to be helpful in the diagnosis of toxoplasmosis (1-7). In a recent comprehensive study incorporating 339 cases in which maternal *Toxoplasma* infection occurred during pregnancy, testing of amniotic fluid indicated PCR to be significantly more sensitive than other currently available laboratory tests for the diagnosis of congenital toxoplasmosis (8). As the value of PCR for the diagnosis of toxoplasmosis is investigated further, the use of this method among European diagnostic laboratories is likely to increase.

Despite the widespread use of PCR, there is, at present, no external quality assurance scheme available by which individual laboratories can assess and directly compare the performance of their respective in-house PCR methods. This is particularly important because PCR protocols can vary significantly between laboratories – for example, in sample preparation, design of PCR primers, PCR reaction conditions, and amplicon detection.

Under the auspices of the European Union-funded Research Network on Congenital Toxoplasmosis, an interlaboratory comparison was carried out in five participating laboratories, all performing *Toxoplasma* testing on a regional or national basis and all experienced in the use of PCR for the detection of *Toxoplasma gondii* in clinical specimens.

Materials and Methods. Each of the participating laboratories employed the PCR method it had developed (9, 10) and was experienced in using the method for the detection of *Toxoplasma gondii* in clinical specimens. The protocols for PCR employed by each laboratory differed in a number of ways. Initial preparation of DNA from amniotic fluid samples incorporated either extraction using phenol/chloroform or affinity purification using silica beads. Because the design of PCR primers differed, the precise reaction conditions varied, too. Some laboratories employed a single PCR system, whereas others used nested PCR. Amplicon detection methods included either direct visualisation of DNA by ethidium bromide staining after agarose gel electrophoresis or Southern blot hybridisation using a labelled DNA probe. A summary of the precise methodology for each laboratory is shown in Table 1.

Two types of amniotic fluid samples were used: some were not infected with *Toxoplasma gondii*

Table 1: Summary of polymerase chain reaction (PCR) protocols in five European laboratories.

Laboratory	*Sample preparation*		Target gene	PCR protocol		Uracyl-N-glycolase	Positive controls	
	Petreatment	DNA extraction		Single/nested PCR	Amplicon detection		Internala	Externalb
A	NH_4OH/100°C	silica beads	B1	nested (50/35 cycles)	gel electrophoresis/ ethidium bromide staining	no	no	yes
B	proteinase K	phenol/ chloroform	B1	single (40 cycles)	Southern blot hybridisation	no	ß-globin	yes
C	proteinase K	phenol/ chloroform	B1	nested (30/25 cycles)	gel electrophoresis/ ethidium bromide staining	no	M13 flanked by B1 primer sequences	yes
D	proteinase K	phenol/ chloroform	Bi	single (40 cycles)	gel electrophoresis/ ethidium bromide staining	yes	pUC18 clone containing amplicon with 59bp fragment excised	no
E	centrifugation, NaOH/100°C		B1	single (40 cycles)	gel electrophoresis/ silver staining	no	no	yes

A positive control amplicon was produced concurrently and in the same reaction tube as the test amplicon by the addition of a control sequence of target DNA. This control DNA may either be an artificial DNA construct containing the primer sequences (amplified by the test primers) or a defined sequence of DNA amplified by a second primer pair, e.g.

[b] *Toxoplasma gondii* DNA was added to a second aliquot of each specimen. Sample preparation and amplification of the test and positive control samples took place in parallel.

and others had known amounts of *Toxoplasma gondii* DNA added to them. Samples of amniotic fluid collected for reasons other than the investigation of suspected congenital toxoplasmosis by the Department of Cytogenetics, Centre Hospitalier, University of Grenoble, were tested by PCR to exclude the presence of *Toxoplasma gondii* DNA and were then pooled. Aliquots were prepared to which purified *Toxoplasma gondii* DNA (RH strain) was added corresponding to either 1 (100 femtograms DNA), 10,100, or 1,000 tachyzoites per 500 µl of amniotic fluid. A total of 16 aliquots (2 each of the above, and 8 to which no DNA was added) were prepared for each laboratory. Samples were randomised and coded by staff members not participating in the study and then stored at -20°C until dispatch. Samples were transported in dry ice to avoid thawing before arrival.

Results and Discussion. Four of the five laboratories were able to detect levels of DNA equivalent to ten or fewer *Toxoplasma gondii* cells in 0.5 ml of amniotic fluid (Table 2). In the other laboratory, all samples tested were found negative. This last laboratory employed a protocol not considered optimal for the detection of free DNA in the artificial samples included in this study (samples were centrifuged and the supernatant was discarded before subsequent DNA extraction); we concluded this to be the most likely explanation for the failure to obtain any positive results.

Two of the four laboratories that were able to detect *Toxoplasma gondii* DNA also obtained one positive result among the eight negative control samples included in the study. In both cases, the false-positive sample corresponded to a tube processed in sequence immediately after a sample containing *Toxoplasma gondii* DNA equivalent to 1,000 tachyzoites. Thus, carryover of target DNA between adjacent tubes is a likely explanation. Anecdotally, one of these two laboratories reported difficulty in opening some of the flip-top lids of the microfuge tubes employed in the study. This problem raises the possibility that gloves may have been contaminated with tube contents.

A number of previous studies have indicated that PCR has great potential for the detection of *Toxoplasma gondii* infection in a variety of clinical scenarios. However, PCR is a difficult technique to implement and carries a significant risk of producing false-positive as well as false-negative results. Thus, in addition to the sensitivity and specificity of individual in-house PCR protocols for the detection of *Toxoplasma gondii* DNA, overall performance and, hence, the diagnostic value of PCR will likely depend on the technical ability and experience within each laboratory.

The present study indicates that a high level of sensitivity in the detection of *Toxoplasma gondii* DNA can be achieved reliably under the conditions applied by laboratories for the use of PCR

Table 2: Summary of results for the detection of *Toxoplasma gondii* DNA in amniotic fluid using the polymerase chain reaction.

Laboratory*	No. of *T. gondii* cells in 0.5 ml amniotic fluid (DNA equivalents)								No. of negative control samples found positive
	1	1	10	10	100	100	1000	1000	
A	-	+	+	+	+	+	+	+	0
B	-	-	+		-	+	+	+	1
C	_	_	+	+	+	+			0
D	+	+		+		+		+	1
Total no. of samples positive	3/8		8/8			7/8		8/8	2 32

* One laboratory (E), not shown, determined all samples to be negative. +, positive PCR result; -, negative PCR result.

in a diagnostic context. However, the incidence of one false-positive result among eight negative control samples in two of four such laboratories underscores the importance of stringent protocols for sample preparation and manipulation to avoid contamination. For example, all of the laboratories in this study routinely confirm PCRpositive results by repeating the procedure using a second aliquot of the original clinical specimen to minimise the possibility that a false-positive result might occur. However, our findings indicate that an additional strategy to achieve this may be to replace flip-top microfuge tubes with screw-cap tubes in sample preparation.

Because it is technically difficult to dispense small numbers of tachyzoites accurately into amniotic fluid samples, the addition of purified DNA was chosen. However, this approach may be incompatible with some PCR sample preparation methods, such as those optimal for the detection of DNA in intact cells, as used by laboratory E in the present study. Thus, we conclude that artificial samples of the type used in this study can be employed as standards to determine the sensitivity of a particular PCR method and to indicate the potential for cross-contamination between samples. However, it is likely that naturally infected specimens will be more helpful and more appropriate for ensuring the quality of the overall diagnostic PCR service offered. A study to address this issue is now being organised by the European Unionfunded network.

Although PCR appears to be a promising technique for [illegible] diagnosis of congenital toxoplasmosis, a number of points should be considered when determining its application in clinical investigations. First, as has been demonstrated in the present study, the PCR test is not yet standardised. A number of different techniques and primers are in use, some of which have yet to be evaluated fully. Multicentre studies are required to compare these methods and to ensure that laboratories are able to provide a PCR test of high quality and technical accuracy. This issue is underscored by preyious multicentre quality control studies of PCR methods used to detect other pathogens in clinical specimens. These studies have revealed significant variation in performance – for example. in the detection of hepatitis C virus (11). Until the outcome of such studies for toxoplasmosis is known. we believe that the use of PCR for diagnosis should be restricted to specialist centres. Further. we suggest that the use of PCR results alone to determine clinical management is unacceptable based on current knowledge; many countries would regard such a practice as contrary to medical and ethical standards.

We conclude that an external reference system is urgently required to provide materials and Support for proficiency testing for laboratories attempting the diagnosis of toxoplasmosis using PCR.

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