Diagnostic Microbiology and Infectious Disease 80 (2014) 197–199

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Parasitology

Freezing and storage at −20 °C provides adequate preservation of *Toxoplasma gondii* DNA for retrospective molecular analysis

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ARTICLE INFO

Article history:
Received 4 April 2014
Received in revised form 6 July 2014
Accepted 21 August 2014
Available online 29 August 2014

Keywords:
Real-Time PCR
DNA storage
Toxoplasma gondii
Amniotic fluid
Congenital toxoplasmosis

ABSTRACT

Nucleic acid-based testing has become crucial for toxoplasmosis diagnosis. For retrospective (forensic or scientific) studies, optimal methods must be employed for DNA long-term storage. We compared *Toxoplasma gondii* detection before and after DNA storage using real-time PCR. No significant differences were found depending on duration or storage conditions at −20 °C or −80 °C.

Molecular diagnosis (PCR) is now recognized as an essential tool for the diagnosis of congenital toxoplasmosis (Bastien, 2002). In this condition, the search for *Toxoplasma gondii* DNA is done prenatally using amniotic fluid (AF) samples. The sensitivity of this molecular diagnosis is not 100%, as the best current PCR assays do not detect 10–20% of the congenital toxoplasmosis cases (Delhaes et al. 2013; Sterkers et al. 2012), and parasitic loads in AF may indeed be extremely low, with a median below 10 *T. gondii* cells per mL according to previous publications (Bastien et al., 2007; Costa et al. 2001; Kaiser et al., 2007; Romand et al. 2004). DNA extraction from such samples having low parasitic loads may have a low yield (Yera et al., 2009), leading to difficulties in diagnosis that are enhanced if some DNA degradation occurs after extraction. Consequently, it is critical that optimal methods are employed either for short-term storage (if diagnostic analysis is delayed on the short-term) or long-term storage (compulsory conservation in France, retrospective analysis). For example, retrospective examination of a previously extracted DNA sample may be necessary for forensic reason or for further scientific studies. Here, we report a multicenter study that aimed at evaluating the effect of long-term conservation of *Toxoplasma* DNA extracted from AF by comparing the parasite detection before and after DNA storage using real-time PCR, in order to propose suitable and documented recommendations.

A total of 53 PCR-positive AF sample DNAs were retrospectively recruited from documented congenital toxoplasmosis cases in 8 proficient centers that form part of the molecular biology 'pole' of the French National Reference Center for Toxoplasmosis (http://www.chu-reims.fr/professionnels/cnr-toxoplasmose-1). These centers assess molecular methods used for the diagnosis of congenital toxoplasmosis and propose technical recommendations to the medical and scientific community (Sterkers et al., 2010).

The AF samples were isolated by amniocentesis performed at a mean of 18 ± 10 weeks of amenorrhea (WA). DNA extractions from AF were done according to manufacturers' recommendations, using either QIAmp DNA Mini Kit (Qiagen®, Courtaboeuf, France) in 6 centers, High Pure Template Preparation kit (Roche Diagnostics®, Meylan, France) in 1 center, or the Tween-Nonidet-NaOH (0.5% Tween 20, 0.5%

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http://dx.doi.org/10.1016/j.diagmicrobio.2014.08.007
0732-8893/

Table 1
Protocols used in each participating center for DNA extraction and real-time PCR for the detection of *Toxoplasma gondii*.

Centers	DNA extraction		Storage condition	DNA detection method to quantify *T. gondii* DNA	
	Kit	Elution buffer		Reference of the primers and probes used	Method and device used to quantify *T. gondii* DNA
A	QIAmp DNA Mini Kit (Qiagen®)	Kit buffer	−80 °C	(Reischl et al., 2003)	FRET probes on a LightCycler 1.0, or 2.0 or 480 automate (Roche®)
B			−80 °C		
C			−80 °C		
D		Sterile water	−80 °C		
E	High Pure Template Kit (Roche®)	Kit buffer	−80 °C	(Cassaing et al., 2006)	
F	Tween-Nonidet-NaOH lysis buffer (Hohlfeld et al. 1994)	TNN lysis buffer	−20 °C	(Reischl et al., 2003)	
G	QIAmp DNA Mini Kit (Qiagen®)	Sterile water	−20 °C	(Talabani et al., 2009)	TaqMan probe detection on an ABI Prism 7000 or 7500 automate (Applied 119 Biosystems®)
H	QIAamp DNA mini Kit (Qiagen®)	Kit buffer	−80 °C	(Fekkar et al., 2008)	

Nonidet P40, 10 mmol/L NaOH) lysis buffer method (Hohlfeld et al., 1994) in another center. DNA samples were then stored in the center, which had realized the extraction at −80 °C in 6 centers or at −20 °C in 2 centers. The duration of storage varied from 6 to 1884 days, with a mean of 518 ± 353 days, a median of 419 days, and interquartile values (IQ1–IQ3) of 272 and 769 days, respectively. The methods used for *Toxoplasma* DNA amplification all targeted the 529-bp genomic repeat element described by Homan et al. (2000) and were described previously (Sterkers et al., 2010, Yera et al., 2009). Briefly, they used either TaqMan probe detection on an ABI Prism 7000 or 7500 automate (Applied 119 Biosystems®, Villebon-sur-Yvette, France) in 2 centers or FRET probes on a LightCycler 1.0 or 2.0 or 480 automate (Roche®) in 6 centers (Table 1). For each sample and in each center, *Toxoplasma* DNA was amplified and quantified using the same protocol of real-time PCR after storage than the one used for initial detection (Table 1). As quantification of *Toxoplasma* DNA is not standardized and may be subject to large variations among different centers (our unpublished data), we chose to rather compare the quantification cycle (Cq) values obtained before and after storage. Statistical analysis was performed using the Wilcoxon signed rank test with continuity correction, which is a nonparametric test able to compare 2 repeated measurements on a single sample and to assess differences between the population mean rank, yet remaining suitable when the population (here *Toxoplasma* DNA loads) cannot be assumed to be normally distributed (Bastien et al., 2007; Costa et al. 2001; Kaiser et al., 2007; Romand et al. 2004).

Whatever the duration of the sample storage period and for each of the 53 samples, there was no significant difference between Cq values observed at the initial DNA amplification and after thawing at the end of the storage period (Fig. 1A, Fig. 2). We wished to determine if a difference could be more perceptible for samples containing low amounts of *Toxoplasma* DNA, therefore, at higher Cq values. For 21 samples with a Cq ≤30 at the initial testing, corresponding to high amounts of parasite DNA, the Cq means were 27.6 ± 1.7 and 27.8 ± 1.7 before and after storage, respectively. For 32 samples with Cq >30, corresponding to low amounts of *Toxoplasma* DNA, these values were 34.2 ± 2.4 and 33.9 ± 13.2 before and after storage, respectively (Fig. 1B). Despite larger variations between Cq values of samples with low DNA amounts after storage (Fig. 1A and B), the difference was not found statistically significant. In fact, this greater dispersion corresponds to the usual Cq results that we observe in routine practice when analyzing the same type of samples with low DNA amounts in multiplicate. With respect to temperature, we did not observe any significant difference between Cq values obtained after storage at −20 °C (Fig. 1A, samples 1–10 and 30–33) or −80 °C (Fig. 1A, samples 11–29 and 34–53).

Only a few studies have analyzed the effect of storage conditions of biological samples on the sensitivity of molecular detection of *T. gondii* (James et al., 1996, Joss and Ho-Yen, 1997), *Schistosoma mansoni* (Van den Broeck et al., 2011), and malaria parasites (Hwang et al., 2012). With respect to *T. gondii*, the study of storage conditions of biological samples containing *Toxoplasma* such as AF led to contradictory results: before DNA extraction freezing samples at −20 °C resulted in a loss of

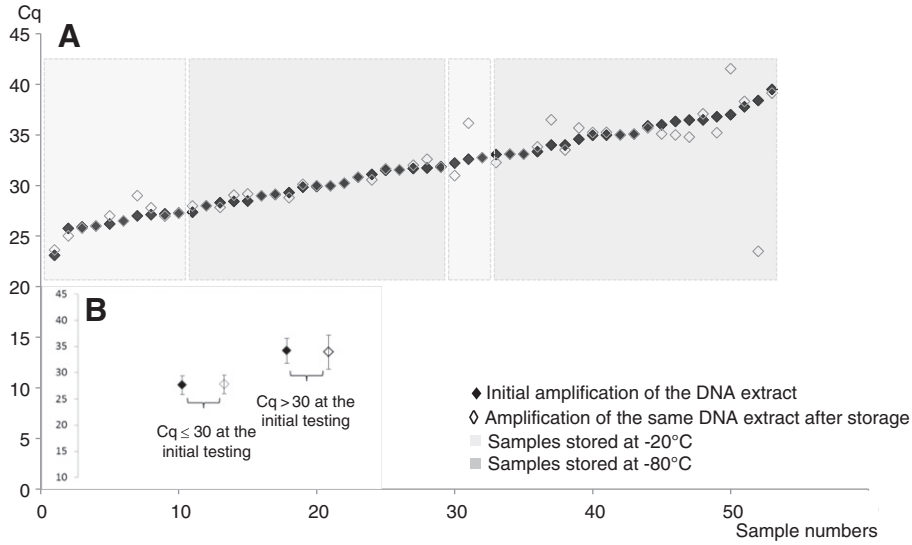

Fig. 1. Cq values recorded by real-time PCR before and after storage of amniotic fluid DNA samples. Abscissa: individual sample code number; ordinate: Cq values. (A) Individual Cq values are shown for each sample; ♦: initial amplification of the DNA extract; ◊: amplification of the same DNA extract after storage; ▫: samples stored at −20 °C; ▪: samples stored at −80 °C. Sample repartition was as follows: samples numbered 9, 18, 20, 24, 25, 29, and 45 were from center A; samples numbered 6, 30, and 42 from center B; samples numbered 39 and 47 from center C; samples numbered 27, 31, 33, 37–40, 50, and 51 from center D; samples numbered 21–23, 26, 32, 35, 36, and 43 from center E; samples numbered 3, 8, 11, and 41 from center F; samples numbered 2, 10, 13–17, 19, 34, 44, 48, and 49 from center G; samples numbered 1, 4, 5, 7, 12, 28, 46, 52, and 53 from center H. (B) Cq values are shown as means ± SD, differentiating between samples with Cqs ≤30 and >30 at the initial testing.

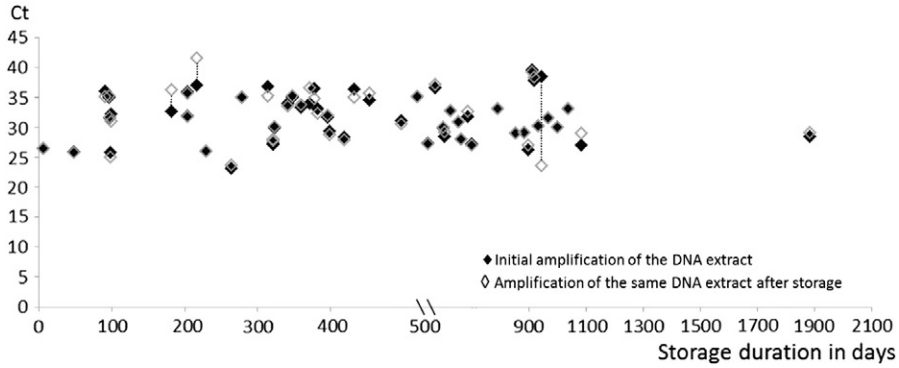

Fig. 2. Cq values observed before and after storage at −80 °C or −20 °C according to the duration of storage. Cq values are shown for each sample, before and after storage (♦ and ◊, respectively). Abscissa: duration of the storage period (days); ordinate: Cq values.

PCR sensitivity (James et al., 1996), while storage AF samples at room temperature did not affect the PCR sensitivity (Joss and Ho-Yen, 1997). Here, for the first time to our knowledge, we assessed whether duration and temperature of storage of DNA extracted from AF samples may impact the ability to detect parasite DNA. Using a diverse panel of PCR assays, we show that the long-term storage of these DNA extracts at −20 °C or −80 °C does not affect PCR sensitivity. We therefore propose that DNA extraction of AF be performed promptly after sampling, and followed by DNA storage at least at −20 °C when the *T. gondii*-PCR is delayed. This information should be of value for the molecular diagnosis of congenital toxoplasmosis and for retrospective scientific studies using DNA extracts as well as forensic studies.

Acknowledgments

The authors are grateful to Florence Robert-Gangneux (Reims) and Jean Menotti (Paris), E. Dei-Cas (in memoriam), E. Fréalle, S. Loridan, A.S. Deleplanque, and E. Dutoit, for their constant interest and fruitful discussions, and to S. Douzou, G. Serres, F. Michel, and B. Sanichanh (Montpellier); C. Barois (Grenoble), V. Bans, E. Duthu, S. Gisquet, and C. Paris (Toulouse); F. Naji and M. Wauquier (Lille); R. Huber and S. Matern (Strasbourg); and D. Mehidi and Y. Garoun (Paris, Hôpital Cochin) for their technical assistance in the routine diagnosis of congenital toxoplasmosis. This study was initiated by the Molecular Biology 'pole' of the French National Reference Centre for Toxoplasmosis (Centre National de Référence de la Toxoplasmose) and funded by the Institut de Veille Sanitaire.

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