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ADAPTING A CONVENTIONAL PCR ASSAY FOR *TOXOPLASMA GONDII* DETECTION TO REAL-TIME QUANTITATIVE PCR INCLUDING A COMPETITIVE INTERNAL CONTROL

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Summary:
We have developed a quantitative PCR assay (LightCycler®) using the pair of primers JW58 and JW59 for the detection of the 35-fold repeated B1 gene of *Toxoplasma gondii*. This real-time PCR, using fluorescence resonance energy transfert (FRET) hybridization probes, allows the quantification of *T. gondii* with several technical requirements not previously described: *i*) an internal amplification control (co-amplified in a single tube with the same primers), *ii*) Uracil-N-Glycosylase and *iii*) a standard curve corresponding to a serial dilution from a calibrated suspension of *T. gondii* ranging from 40 to 4.10^6 parasites in one ml of amniotic fluid (1 to 10^5 *T. gondii*/PCR). In artificial samples, one parasite could be detected if at least three reactions were performed.

KEY WORDS : *Toxoplasma gondii*, LightCycler®, real-time PCR, B1 gene, internal amplification control.

Résumé : ADAPTATION D'UNE PCR QUANTITATIVE EN TEMPS RÉEL POUR LA DÉTECTION ET LA QUANTIFICATION DE *TOXOPLASMA GONDII*

Nous avons développé une PCR quantitative en temps réel (LightCycler®) utilisant les amorces JW58-JW59 pour la détection du gène B1, répété 35 fois chez Toxoplasma gondii. *Plusieurs exigences techniques ont été associées :* i*) un contrôle interne d'amplification compétitif pour la détection des inhibiteurs d'amplification,* ii*) l'Uracil-N-Glycosylase pour prévenir les faux positifs, et* iii*) une droite d'étalonnage réalisée à partir de suspensions calibrées de* Toxoplasma gondii *(de 40 à 4.10^6 parasites dans un ml de liquide amniotique correspondant 1 à 10^5* T. gondii*/PCR) pour permettre une véritable quantification de la charge parasitaire. Les amplicons (cible et contrôle interne) sont détectés par deux couples de sondes marquées selon le principe FRET (Transfert d'énergie par résonance de fluorescence). Dans les prélèvements artificiels, un parasite peut être détecté si au moins trois réactions de PCR sont réalisées.*

MOTS CLÉS : Toxoplasma gondii, *LightCycler®, PCR en temps réel, gène B1, contrôle interne d'amplification.*

INTRODUCTION

Toxoplasmosis is a protozoan infection caused by *Toxoplasma gondii*. This infectious disease can cause congenital toxoplasmosis when maternal infection occurs during pregnancy and severe infections in immunocompromised patients (mainly transplant and AIDS patients). Current diagnosis of toxoplasmosis is based on the results of several techniques: serological detection, cell culture and molecular methods (Switaj *et al.,* 2005). PCR is the major breakthrough for the diagnosis of this infection due to *T. gondii*. Moreover, real-time PCR has recently emerged as an improvement in the reliability of PCR assays (Lin *et al.*, 2000; Costa *et al.*, 2001; Kupferschmidt *et al.*, 2001; Reischl *et al.*, 2003; Romand *et al.*, 2004). Since real-time PCR does not require the tubes to be opened after amplification, the risk of contaminating environment and the risk of false positive results are decreased. Furthermore, real-time PCR gives quantitative data on the parasitic load (Switaj *et al.,* 2005). A conventional PCR using a pair of primers JW58-JW59 has previously been developed for the detection of the B1 gene of *T. gondii* (Pelloux *et al.,* 1996). This conventional PCR, associated with a digoxigenin labeling in ELISA microplates for analysis of amplicon, has been used for routine diagnosis since 1995 (Pelloux *et al.,* 1998; Pelloux *et al.*, 1996). The objective of this work was to adapt this conventional PCR to real-time PCR (LightCycler®, Roche Molecular Biochemicals, France) with the association of several technical requirements: *i*) competitive Internal Amplification Control (cIAC, plasmid pSYC44, containing an insert amplified with the same primers as the target but 59 bp smaller) (Pelloux *et al*, 1996), *ii*) Uracil-DNA-Glycosylase (UNG), to avoid carryover contamination, and *iii*) real quantification of parasitic load. The technological approach presented in this work has not been reported in literature. Indeed, no real-time PCR described for *T. gondii* quantification used this type of internal control together with a true quantification of parasitic load from a standard curve corresponding to parasite dilution (not diluted DNA).

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MATERIALS AND METHODS

T. gondii DNA was extracted using the QIAmp DNA mini kit (Qiagen, Courtaboeuf, France) according to manufacturer's instructions. The elution volume was 200 µl and the DNA concentration was estimated by spectrophotometric absorbance. This DNA was used to perform a standard curve and the latter was expressed in *T. gondii* genome equivalents. Indeed, Cornelissen *et al.* (1984) calculated that one tachyzoite corresponded to 0.1 pg of DNA. In second step, a calibrated suspension of *T. gondii* was also used to perform a standard curve. Briefly, tachyzoites of RH strain were harvested from the peritoneal ascitic fluid of mice infected three days earlier. For a multiplication cycle, parasites were passed *in vitro* on confluent MRC5 cells and then counted in Malassez cell counter (Brenier-Pinchart *et al.*, 2002). Tachyzoites were resuspended in amniotic fluid (AF). This AF was obtained from a *Toxoplasma* seronegative women after amnioreduction for treatment of hydramnios.

Quantification PCR tests were carried out with LightCycler® instrument (Roche Molecular Biochemicals, Meylan, France). Real-time PCR was performed using forward primer JW58 5'-AAGGGCTGACTCGAACCAGATGT-3' and reverse primer JW59 5'-GGGCGGACCTCTCTTGTCTCG-3' amplifying a 301bp fragment of the B1 gene (GeneBank accession nos. AF179871) (Pelloux *et al.,* 1996). Two hybridization probes, Tg1 5'-GTCTAGGGCACCCTTACTGCAAGAGAAG-3' (labelled at the 3' end with fluorescein) and Tg2 5'-TTTGAGGTCATATCGTCCCATGAAGTCGACC-3' (labelled at the 5' end with LC-Red 640) derived from the *T. gondii* B1 gene, were designed. Furthermore, a second set of hybridizations probes CI1 5'-ACTGAGTCCAATACATAAGCACAGTCTTCCC-3' (labelled at the 3' end with fluorescein) and CI2 5'-AAGTTGGGTATTTCGTATGCGTAGCGATCC-3' (labelled at the 5' end with LC-Red 705) derived from the specific sequence of internal control plasmid (cIAC), pSCY44, was associated to specifically detect this control. Real-time PCR was set up in a final volume of 20 µl with 2 µl of LightCycler FastStart DNA Master^{PLUS} Hybridation probes or LightCycler FastStart DNA Master^{PLUS} SYBR Green (Roche Molecular Biochemicals, Meylan, France), each primer at a concentration of 0.5 µM, each probes at 0.2 µM, 1 µl of IAC and 5 µl of extracted DNA sample. Carryover contamination was prevented using 1 U of heatlabile UNG (Roche Molecular Biochemicals, Meylan, France). The quantitative interpretation of Lightcycler® results was assisted by the "second derivative maximum" algorithm. The reaction mixture was initially

A. Intra-assay reproducibility

PCR No of capillary	Cp according to the number of tachyzoites/PCR reaction					
	100,000	10,000	1,000	100	10	1
1	20.20	20.57	23.34	27.26	29.54	30.86
2	19.50	20.37	23.72	27.02	30.03	30.67
3	19.00	20.18	23.52	26.88	30.17	31.00
4	20.04	20.12	23.27	26.74	30.22	31.22
5	18.88	ND	23.85	27.02	30.50	30.64
Mean	19.538	20.310	23.540	26.984	30.092	30.899
Standard deviation	0.615	0.205	0.246	0.193	0.353	0.211
Variation coefficient	0.031	0.01	0.01	0.007	0.012	0.007

B. Inter-assay reproducibility

PCR No of capillary	r	Cp according to the number of tachyzoites/PCR reaction					
		100,000	10,000	1,000	100	10	1
1	0.98	20.15	20.55	24.01	27.80	30.97	31.44
2	0.98	19.80	20.58	24.27	27.57	30.59	31.22
3	0.99	19.54	20.26	23.98	27.10	30.23	31.93
4	0.98	19.65	20.47	23.69	27.55	30.68	31.67
5	0.98	19.49	20.81	24.55	27.59	30.52	32.02
Mean	0.982	19.726	20.534	24.100	27.522	30.598	31.656
Standard deviation	0.004	0.265	0.199	0.325	0.256	0.268	0.333
Variation coefficient	0.003	0.013	0.010	0.013	0.009	0.009	0.011

Each point of standard curve was tested five times in the same run of PCR (A). Five different PCRs were performed to evaluate the variation coefficient of crossing point (Cp) values for each point of the standard curve (B). 10^3 copies of the Internal Amplification Control were added/capillary and were co-amplified. ND: note done.

Table I. – Intra- and inter-assay reproducibility crossing point values for each point of the standard curve.

incubated for five min at room temperature to allow the UNG to act, and followed by a 10-min step at 95° C. PCR was performed for 40 cycles of denaturation (95° C, 10s, ramp rate 20° C/s), annealing (62° C, 10s, ramp rate 20° C/s) and extension (72° C, 10s, ramp rate 20° C/s).

RESULTS

First, the adaptation of primers JW58 and JW59 was performed on *T. gondii* DNA with SYBR green assay. The hybridization temperature was optimized to 62° C, since the former was 70° C in conventional PCR and is not compatible with FRET detection. Ten-fold serial dilutions of *T. gondii* DNA, ranging from 1 to 10^5 *T. gondii* genome equivalents per reaction, were used to give the standard curve (r = 0.998). Melting curve analysis verified correct product amplifications. Two sets of hybridization probes (Red 640 for *T. gondii* target and Red 705 for cIAC) were designed and tested for FRET assay to differentiate the target amplicons from cIAC amplicons. Three different concentrations of cIAC (10^5, 10^3 and 10^2 copies/reaction) were tested with each point of standard curve. The optimal concentration of cIAC was chosen at 10^3 copies/reaction. Indeed, at this concentration, the IC was detected in negative control and did not inhibit the detection of *T. gondii* DNA corresponding to 1, 10, 100 or 1,000 *Toxoplasma* genomes.

Second, a serial 10-fold dilution from a calibrated initial suspension of tachyzoites in amniotic fluid (RH strain) was performed. Each *T. gondii* dilution (ranging from 40 to 4.10^6 parasites in one ml of AF), corresponding to a point of this standard curve (ranging from 1 to 10^5 *T. gondii*/reaction), was independently extracted. The cIAC was added in the mix before being amplified with each point of the standard curve (Fig. 1). Inter- and intra- assay reproducibility of the six points of the standard curve showed that the variation coefficient of crossing point values varied from 0.9 % to 1.3 % and from 0.7 % to 3.1 %, respectively (Table I). Furthermore, the sensitivity of this real-time PCR assay was evaluated in artificial samples: 1 ml of AF was spiked with 1 to 20 *T. gondii*. Table II shows that one parasite in one ml of AF could be detected if at least three PCR were performed in each sample. The specificity was tested, the DNA of several micro-organisms involved in diseases during pregnancy (*Listeria monocytogenes, Escherichia coli K1*, cytomegalovirus, herpes simplex virus type 2) or other protozoa (*Plasmodium falciparum, Leishmania infantum*) were not detected. We have also confirmed that this real-time PCR could detect the Prugniaud strain (cystogenic strain, type 2).

DISCUSSION

Several real-time PCR assays have been developed to quantify the *Toxoplasma* load in different types of clinical samples. However, the development of quantitative PCR was not accompanied with an increased standardisation. On the contrary, several requirements usually developed in conventional PCR were not found in published quantitative PCR. In this study, we adapted a conventional PCR using a pair of primers JW58-JW59 for the detection of the B1 gene of *T. gondii,* because this gene is present in 35-fold repeats, to a quantitative PCR with several technical features. A cIAC was co-amplified simultaneously with the target sequence using the same set of primers and was detected with specific hybridization probes. Moreover, this control was included in the six points of the standard curve as in each sample. This type of IAC is used to detect false negative results due to PCR inhibitors and was reported in real-time PCR assay-SYBR Green to *T. gondii* quantification only by Edvisson *et al.* (2006).

Tachyzoites/1 ml of AF[a]	B1 gene No of positive amplification (channel F2/back F1)[c]	cIAC[b] No of positive amplification (channel F3/back F1)[d]	Results Overall interpretation No of positive PCR
1	3/10	9/10[e]	3/9
5	6/10	9/10	6/10
10	6/10	10/10	6/10
20	10/10	10/10	10/10

Tachyzoites were spiked in 1 ml of amniotic fluid. IAC (10^3 copies/reaction) was added to the mix and co-amplified with the specific target.

[a] Amniotic fluid.

[b] Competitive internal amplification control.

Fluorescence detection : [c] B1 gene DNA is detected in channel 2 and [d] cIAC in channel 3.

[e] When neither cIAC signal nor target signal is produced, the PCR has failed and its result is not interpretable.

These results are from a representative experiment out of three independent experiments. In the two other experiments, three PCRs were performed on each sample.

Table II. – Sensitivity and biological detection limit of real-time PCR.

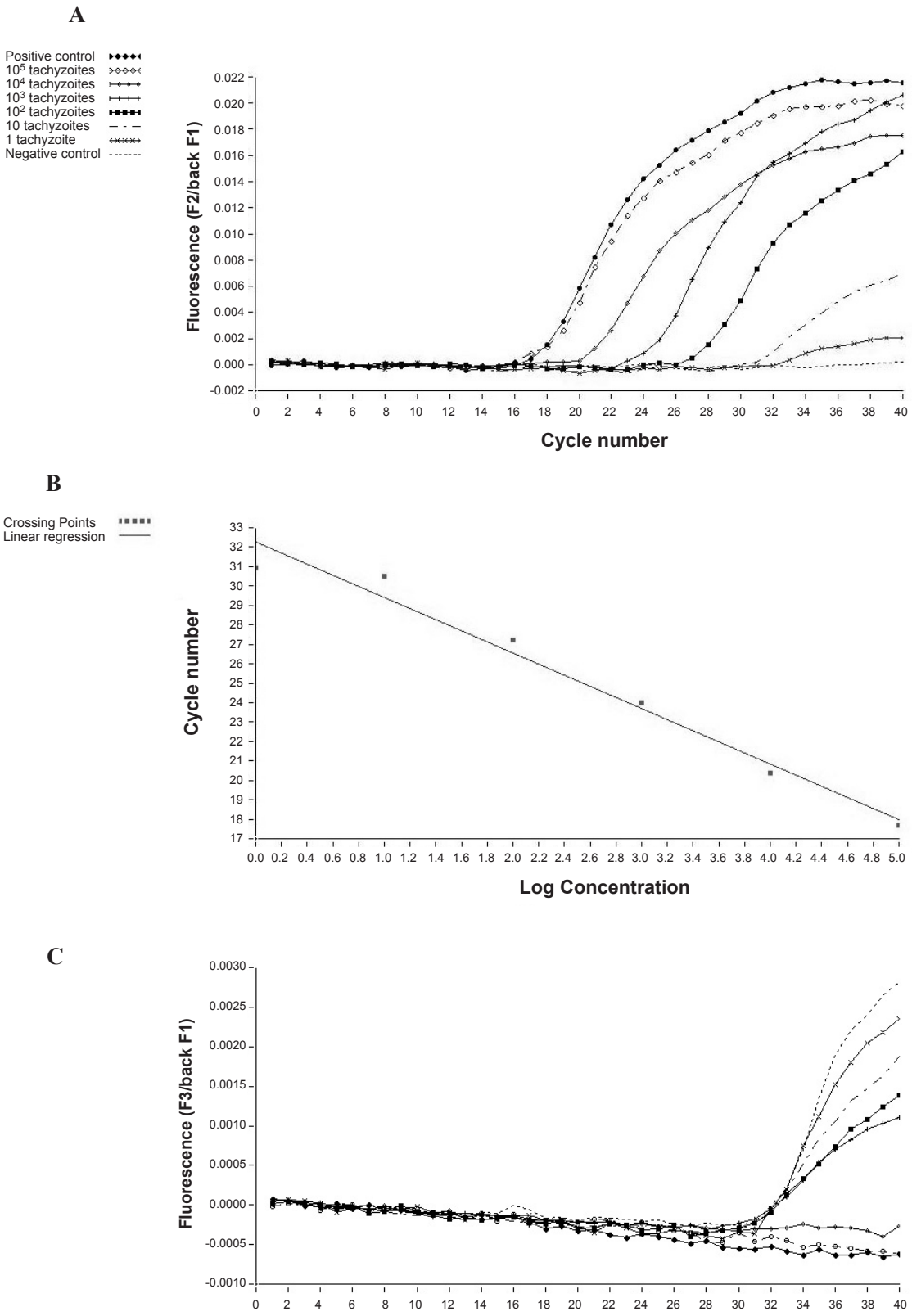

Fig. 1. – Establishment of the standard curve for quantification of *T. gondii* and co-amplification of Internal Amplification Control. Amplification plot obtained from serial dilutions of *T. gondii* (A) in order to construct a standard curve (B) Amplification plot of cIAC in the same run (C).
Serial dilutions of *T. gondii* ranging from 40 to 4.10^6 in one ml of AF were independently extracted and 5 µl of each were amplified corresponding to 1 to 10^5 tachyzoites. cIAC was added at a concentration of 10^3 copies/reaction and was detected from 1 to 10^3 tachyzoites. A positive control corresponding to *T. gondii* DNA was performed in each run.

(Hoorfar *et al*., 2003; Edvinsson *et al.,* 2006). To date, only one real-time PCR assays for quantification of *T. gondii* contains a competitive internal amplification control (Edvinsson *et al.,* 2006). Only amplification of the human beta-globin gene or the mouse GALT gene was used as internal positive control or no amplification control was performed in most publications (Costa *et al.,* 2000; Romand *et al*., 2004; Maubon *et al*., 2007). We chose to perform an external reference curve (standard curve) based on an individual DNA extraction of each dilution of *T. gondii* corresponding to each point of this standard curve. This original approach allows us to quantify the number of parasites really present in the sample tested and not a load of *T. gondii* DNA (expressed in equivalent genomes). Indeed, in literature, the standard curve is usually constructed from a serial dilution of *T. gondii* DNA or plasmid containing a known number of the target sequence (Reischl *et al.,* 2003; Romand *et al.,* 2004). Only Kupferschmidt *et al.* (2001) have reported a standard curve from a serial-dilution of tachyzoites in human body fluids. The real quantification proposed in this report seems particularly interesting to us if it is confirmed that the quantification of *T. gondii* is useful to establish a prognosis of congenital toxoplasmosis (Costa *et al*., 2001; Romand *et al.,* 2004). The analytical sensitivity of the real-time PCR described here is one tachyzoite in a-20 µl reaction volume. We also evaluated the biological sensitivity in artificial sample and showed that one parasite in one ml of AF could be detected if at least three reactions were performed. Only four different PCR protocols using the B1 gene as a target have been described in the literature and can detect the DNA equivalent from 0.05 to 2.5 tachyzoite per PCR reaction (analytical sensitivity) (Lin *et al.,* 2000; Costa *et al*., 2000, Romand *et al*., 2004; Buchbinder *et al*., 2003; Reishl *et al*., 2003; Edvinsson *et al.,* 2006). These PCR protocols are now used by several teams to quantify *T. gondii* DNA in different types of samples (Costa *et al*, 2001; Cassaing *et al*., 2006; Martino *et al.,* 2005). However, the biological sensitivity in artificial samples was only reported by Kupferschmidt *et al.* (2001) and was 10 tachyzoites/ml of human body fluids by TaqMan® PCR (18S DNA as target gene). The biological sensitivy and the accuracy of quantification of these different quantitative PCR protocols could be specifically evaluated in multicenter studies (Kaiser *et al.,* 2006). In conclusion, we have developed a real-time PCR to quantify *T. gondii* load with several technical features, not previously described in literature, which allow its use in clinical laboratories.

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