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Full Length Research Paper

Quantitative estimation of *toxoplasma* B1gene using real time polymerase chain reaction (PCR) in infected symptomatic and asymptomatic clinical cases

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Serological diagnosis of active *Toxoplasma* infection is unreliable because reactivation of latent, hidden infection is not always accompanied by changes in antibody levels, and the presence of IgM does not necessarily indicate recent infection. Data concerning the relation between the severity of infection and the parasitic load especially at human level is limited. A trial was done in this work to study the relationship between the virulence of the parasite in different clinical forms and parasitic genomic load, via SYBR® Green quantitative PCR amplification assay and primers targeting *Toxoplasma* B1 gene. Fluorescence signals were generated from 91 samples, which were as follow; 7 cases with congenital manifestations, 19 cases with neurological disorders, and 65 asymptomatic cases. The seven congenitally infected cases showed significantly higher parasite load ($5.15x\ 10^8$ to $9x\ 10^{10}$). Other clinical forms showed genomic concentration ranged from $1.7x\ 10^4$ to $4.1x\ 10^8$. Equal copy numbers of template did not produce equal parameters on derivative melting curve plots. In conclusion, quantitative polymerase chain reaction (PCR) provides a sensitive and practical method not only for gene quantification, but also for gene scanning by melting curve analysis which is highly recommended for different *Toxoplasma* genes especially those with known pathological functions.

Keywords: Toxoplasma- Real time polymerase chain reaction (PCR)-B1 gene-clinical forms.

INTRODUCTION

Toxoplasma gondii (T. *gondii*) is an intracellular parasite, has very low host specificity, and it will probably infect almost all mammal and birds (Adriana et al., 2006). It is endemic worldwide and 15 to 85% of the human populations are asymptomatically infected. Most cases of human infection are mild, but devastating disease can occur in immune compromised individuals and congenitally infected fetuses in which serious neurological or ocular problems that appear either early after labour or later on during life and may not become manifested until the second or third decade of life. The progression and severity of the disease differ in patients due to several variables, including host and parasite genetics (Flegr, 2007). Prospective and retrospective studies of infants with subclinical toxoplasmosis at birth have revealed late onset squeals, including visual and hearing deficits, neurological deficits (such as seizures and micro-encephaly) and low IQ scores (Wilson et al., 1999; Jeffery, 2003). Schizophrenia is also reported to be correlated with *Toxoplasma* infection by Flegr et al. (2007).

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Serological diagnosis of active infection is unreliable because reactivation of hidden infection is not always accompanied by changes in antibody levels, and evolution of a latent or dormant toxoplasmosis is highly unpredictable (Flegr, 2007). Moreover, reactivation is not always accompanied by changes in antibody levels, and the presence of IgM does not necessarily indicate recent infection (Reischl et al., 2003). Application of polymerase chain reaction (PCR) has evolved as a sensitive, specific, and rapid method for the detection of *T. gondii* Deoxyribonucleic acid (DNA) in different samples, accordingly serving confirmation of active infection **(**Yang et al., 2009). Accelerating the molecular diagnosis of toxoplasmosis by performing quantitative real time PCR (qPCR) protocols has been reported (Lin et al., 2000; Costa et al., 2001; Bell and Cartwright, 2002; Reischl et al., 2003; Contini et al., 2005; Adriana, 2006; Mesquita et al., 2010; Pignanelli, 2011). However, data concerning the relation between the severity of infection and the parasitic load especially at human level is limited.

The aim of the present work was to study the relation between the severity of *Toxoplasma* infection and parasite genomic concentration of recently infected symptomatic and asymptomatic clinical cases, via SYBR® Green quantitative PCR amplification assay

METHODOLOGY

Sample collection and ethical issue

DNA templates extracted from blood of 92 positive *Toxoplasma* samples were kindly provided by Dr. Fawzia habib, Vice Dean of Taibah University, Saudi Arabia. These cases were serologically IgG negative and were diagnosed by detection of *Toxoplasma* B1 gene using a nested–PCR.

The extracted DNA samples were stored at –70°C until used. These samples were collected from patients attending the outpatient clinic of Taibah University as well as governmental hospitals in Almadinah, Saudi Arabia the period from January 2007 to February 2010 and supported by 2 projects.

These projects (number 27 to 33 and 421 to 430) were funded by King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia and Deanship of scientific researches, Taibah University, Kingdom of Saudi Arabia respectively. In this project, there was a collaboration with many Egyptian researchers, including our team, therefore, they supplied us with these positive samples. According to the clinical data of these *Toxoplasma* positive cases were as follow; seven cases of severe clinical forms of toxoplasmosis in the form of congenital anomalies, abortion or still birth, 19 cases with neurological disorders of unknown etiology (8 adults and 11 children) and 65 asymptomatic cases (diagnosed during pregnancy).

Quantitative real time polymerase chain reaction (PCR) protocol

qPCR was performed with the LightCycler® fastStart DNA Master SYBR Green dye, using the LightCycler® 1.x/ 2.0/ 480 instrument (Roche Diagnostics, Hoffmann-La Roche Ltd, USA). Primers from bases (5´-CCG TTG GTT CCG CCT CCT TC-3´) and (5´-GCA AAA CAG CGG CAG CGT CT-3´) were used to amplify *Toxoplasma* B1 gene of 35-fold repeats. The resulting PCR fragment of *T. gondii* was analyzed using the LightCycler® Red 640 (detected in channel 640). The supplied standard row was used to determine the linear range of the reaction and to estimate the quantity of the target sequence in unknown samples. Software data analysis version 3.5.3 was applied as described in the LightCycler® instrument operator's manual. The reaction mixture (20 µl; Master SYBR Green kit; Roche Diagnostic) contained 0.5 µM of each primer, 5 mM $MgCl_2$ and 5 µl templete DNA. All capillaries were sealed, centrifuged at 500 *g* for 5s, and then amplified in a LightCycler instrument. Amplification was performed for 50 cycles: 5 s denaturation at 95°C, 10 s annealing at 61°C and 15 s extensions at 72°C, with an overall ramp rate of 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 crossing points (Cps) were plotted against the known parasite concentration to obtain a standard curve. The parasite count for a given sample was calculated by extrapolation from this standard curve (positive control). Positive sample specificity was confirmed by determining the melting curve with different values of melting temperatures (T_m) (95°C, 4.40°C/s ramp rate; 40°C, 2.20°C/s ramp rate; 65°C, 4.40°C/s ramp rate, 95°C, 0.02°C/s ramp rate continuous measurement). All data concerning Cps, melting curve parameters of the amplified products were calculated. Statistical analysis was done using the SPSS program version 0.13. ANOVA followed by post Hock test were applied and interpreted at the 5% level of significance.

Positive and negative control

A standard row was generated using the provided cloned and purified Toxoplasma DNA (Roche Diagnostics), allows for the absolute quantification of the unknown samples. The standard was prepared with concentration in the range from 10^6 copies/rxn to 10 copies/rxn of *T. gondii* and the CPs calculated with second derivative

Table 1. Range of *Toxoplasma* genomic concentrations and crossing point in different clinical forms of *Toxoplasma* diseased cases.

Clinical forms	NO. of cases	Parasite genomic concentrations	CPS
Group (1): Manifested congenital infection	7	$5.15x\ 10^{8}$- $9x\ 10^{10}$	19.33 – 12.88
Group (2): Cases with Neurological disorders	19	$3.37x\ 10^{5}$- $4.1x\ 10^{8}$	27.71- 19.33
Group (3): Asymptomatic cases	65	$1.7x\ 10^{1}$ -$1.5x\ 10^{8}$	31.42- 19.33
Total	91	$1.7x\ 10^{1}$- $9x\ 10^{10}$	31.42-12.88

maximum method. To prepare the standard row: 6 different quantities provided with the kits to yield 10 to 10^{6} target molecules in 5µl once resuspended. A hole through the sealing foil was punched. 45 µl PCR- grade water was added to each vial of the row. The target DNA was mixed by pipetting the solution up and down 10 times. Negative control: By replacing the template DNA with water. PCR product was obtained within one hour.

RESULTS

Fluorescence signals were generated from all samples when analyzed by SYBR® Green qPCR except one sample. Test for inhibitors was not done; therefore, the negative sample was excluded during further analysis. Quantitative genomic estimation of these positive samples in qPCR was ranging from $1.7x\ 10^{1}$ to $9x\ 10^{10}$. The symptomatic infected *Toxoplasma* cases (7 congenitally infected and 19 with neurological manifestation) showed significant higher parasitic load ($P< 0.05$) (from $5.15x\ 10^{8}$ to $9x\ 10^{10}$) than those with asymptomatic infection in which parasitic load was ranging from $1.7x\ 10^{1}$ to $1.5x\ 10^{8}$ (Table 1). No significant difference was noticed between the symptomatic groups ($P> 0.05$). Crossing points (Cps) showed different values ranging from 39.68 to 12.88 reflecting the different DNA quantities (from $1.7x\ 10^{1}$ to $9x\ 10^{10}$) respectively (Table 1). Applying gene scanning option and high resolution melting curve analysis, the software of the programmed LightCycler® 1.x/ 2.0/ 480 instrument category, positive samples in different groups showed different values of T_m that were varied from 84.2 to 87.07 (Figure 1). The means of T_m were estimated to be 85.6362 ± 0.57950 SD, 85.7467± .44802 SD and 85.8640 ± 0.68857 SD. The maximum and minimum values in the different groups were 84.20 to 86.76, 85.27 to 86.49 and 85.37 to 87.07 respectively. The ranges of different melting curve parameters (area, width and heights) were 2.56 to 67.14, 3.16 to 4.92, and 0.73 to 15.4 respectively. Equal copy numbers of template did not produce equal parameters on derivative melting curve plots for example, samples obtained concentration around 10^{7} showed different heights ranging from 12.5 to 2.8. There was significant difference concerning areas of melting curve ($P < 0.05$). Otherwise, no significant correlation was noticed between parasite load and different T_m parameters (area width and height), ($P> 0.05$).

DISCUSSION

T. gondii is an ubiquitous parasite found in all classes of warm-blooded vertebrates. Nearly one-third of humans have been exposed to this parasite (Yang et al., 2009). Factors that regulate the virulence and pathogenesis of *Toxoplasma* are still poorly understood. When acquired during pregnancy, toxoplasmosis can be disastrous, leading to fetal loss or conversely to subclinical disease. In congenitally infected infants, evolution is highly unpredictable (Peyron et al., 2004). These increase the demand of quantitative assays to monitor the severity of the parasitic infection in different clinical situations. Recently, assays targeted LightCycler instruments have been greatly developed and provided with variable software technologies which expand the performance on different optional analytic parameters.

Unfortunately, no commercially available PCR kits for routine diagnosis of toxoplasmosis, but only limited for research purposes. Even so, we could not start working in this study, except after receiving positive *Toxoplasma* samples from our colleagues in Saudi Arabia who have finished a funded project in which hundreds of human blood samples were screened for the presence of *Toxoplasma* B1 gene using nested PCR. In general there is limited data concerning the relation between the intensity of infection and the parasitic load especially at human level. A trial was done in this work to study the relationship between the severity of infection and parasitic load in blood applying SYBR® Green quantitative PCR amplification assay.

The present study included 91 cases ranging between asymptomatic and sever *Toxoplasma* infection that were previously diagnose by nPCR. Quantitative *Toxoplasma* genomic estimation was performed on LightCycler® 1.x/ 2.0/ Roche 480 instruments with a SYBR Green

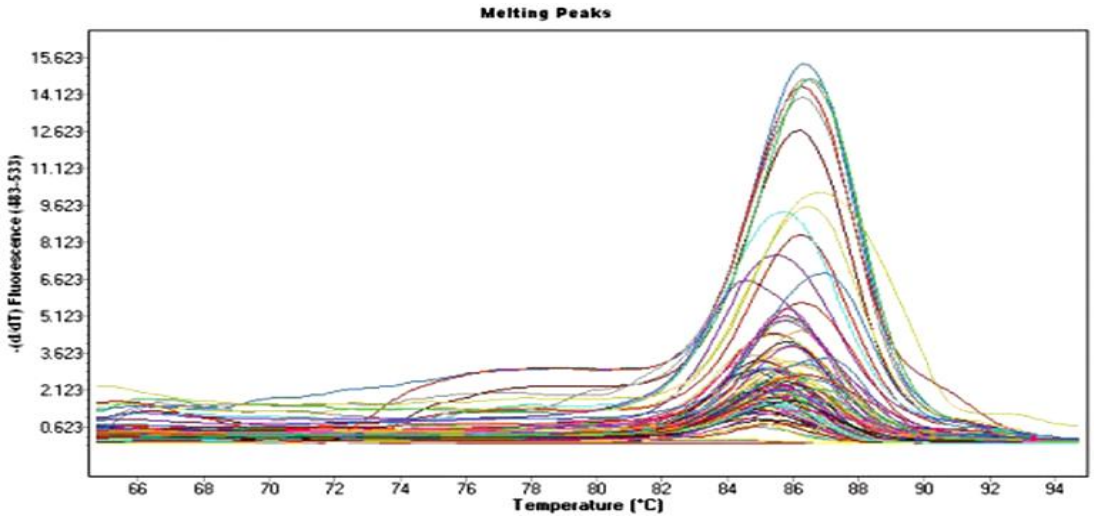

Figure 1. High resolution melting curve analysis of different samples, in which positive samples showed different patterns.

technique. Negative control did not generate any fluorescence signal, while positive samples showed signals at different crossing points (CPs) depending on the number of genomic materials which were ranging from 10^1 to 10^{10} reflecting different parasite load. The severely manifested, congenitally infected (7 cases) showed significantly higher DNA concentrations (from 5.15x 10^8 to 9x 10^{10}) and other clinical forms showed different concentrations of the genomic materials (from 1.7x 10^1 to 4.1x 10^8). Therefore, high parasite load does not always progress to active infection that resulting in serious illness. This was explained by some authors being as a result of different genotypes of *Toxoplasma* parasite that lead to different clinical forms of the disease (Howe et al., 1997; Fuentes et al., 2001; Saeij et al., 2005).

Understanding the different population of *T. gondii* could enable prediction of the outcome of infection. For example, not all seropositive acquired immunodeficiency syndrome (AIDS) patients develop *Toxoplasma* encephalitis; the ones that do might be infected with a particular subset of parasite strains. Similarly, seroconversion during pregnancy does not always lead to infection of the fetus; this might be a result of variability in the ability of different strains to cross the placental barrier (Saeij et al., 2005). Another explanation was reported by Araujo and Slifer (2003) in an experimental study, in which a role for the parasite in the development of severe forms of the infection through increased production of proinflammatory cytokines was suggested, that might have a relation also with the genotype of the parasite. Therefore, besides the ability of the qPCR assays to accurately estimate DNA concentrations in a particular sample, genotyping has become increasingly important in laboratory diagnostics. The most commonly used method for genotyping is polymerase chain reaction (PCR) based on restriction fragment length polymorphism (RFLP) analysis of single nucleotide polymorphisms (SNPs). However, it is a labor intensive, and only a proportion of the SNPs are recognized by currently available restriction enzymes (Su et al. 2008).

In the present study, melting curve analysis displayed different profiles in positive samples in which the values of T_m were varied from 84.2 to 87.07. Equal copy numbers of template did not produce equal parameters on derivative melting curve plots e.g. samples obtained concentrations around 10^7 showed different heights ranging from 12.5 to 2.8. Targeting *Toxoplasma* B1 gene might be the reason behind the previous observation. Despite that this 35-fold repeats are commonly used in diagnostic studies, it is reported to be of unknown pathological function and with low rate of polymorphism (2 to 4 alleles) (Saeij et al. 2005). Studies on genotyping and melting curve analysis were previously performed concerned a highly infectious agent as hepatitis C virus genes (HCV) using similar approach and known strains as control, however, controversy was highly observed. The described method of Bullock et al (2002) for HCV genotyping using a LightCycler™ instrument was able to distinguish different types with (T_m) predicted to differ by 1 °C. While, T_m analysis which performed by Matthias Schröter et al (2002) also on HCV gave similar T_m for some different genotypes and different T_m with other different types. Intapan et al (2008) applied the technique for identifications of different snails and discrimination between infected and non-infected one and from genomic

DNA of other parasite DNAs. The results concerning T_m were recorded by the previous workers to have 100% specificity and sensitivity. They concluded that, melting curve analysis is a sensitive alternative of benefit for such field; rapid, allows a high
throughput, can be done on small samples and might be of value in epidemiological surveys.

In conclusion, qPCR application provides a sensitive and practical PCR method for gene quantification and scanning. qrt-PCR represents significant progress for all molecular diagnostics in microbiology, and it has been rapidly replacing conventional PCR in the vast majority of its applications. This is due to a number of undisputable technical advantages, such as speed and low contamination risk, in addition to the information provided by quantification and melting curve analysis of the amplified products. Melting curve analysis for different *Toxoplasma* genes especially those with known pathological functions are highly recommended using such technology which might be developed to analyze sample differences, without the need of external standards or an independent reference gene**.**

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