Detection of *Toxoplasma gondii* DNA by Polymerase Chain Reaction in Experimentally Desiccated Tissues

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Despite toxoplasmosis being a common infection among human and other warm-blooded animals worldwide, there are no findings about Toxoplasma gondii *evolutionary forms in ancient populations. The molecular techniques used for amplification of genetic material have allowed recovery of ancient DNA (aDNA) from parasites contained in mummified tissues. The application of polymerase chain reaction (PCR) to paleoparasitological toxoplasmosis research becomes a promising option, since it might allow diagnosis, acquisition of paleoepidemiological data, access to toxoplasmosis information related origin, evolution, and distribution among the ancient populations. Furthermore, it makes possible the analysis of parasite aDNA aiming at phylogenetic studies. To standardize and evaluate PCR applicability to toxoplasmosis paleodiagnostic, an experimental mummification protocol was tested using desiccated tissues from mice infected with the ME49 strain cysts, the chronic infection group (CIG), or infected with tachyzoites (RH strain), the acute infection group (AIG). Tissues were subjected to DNA extraction followed by PCR amplification of* T. gondii *B1 gene. PCR recovered* T. gondii *DNA in thigh muscle, encephalon, heart, and lung samples. AIG presented PCR positivity in encephalon, lungs, hearts, and livers. Based on this results, we propose this molecular approach for toxoplasmosis research in past populations.*

Key words: paleoparasitology - toxoplasmosis - ancient DNA - mummies - parasitism - *Toxoplasma gondii*

The DNA of intestinal parasites as well as from systemic ones recovered from archaeological remains has been increasingly described in the literature. DNA from *Ascaris lumbricoides* eggs (Loreille et al. 2001, Loreille & Bouchet 2003) and *Enterobius vermicularis* (Iniguez et al. 2003a, b), found in sediments from latrines or coprolites, were used in studies comparing the present parasite genome to sequences of millions of years ago. *Mycobacterium tuberculosis* DNA, extracted from Egyptian mummies was compared across different periods of time (Zink et al. 2003) and *Trypanosoma cruzi* DNA, retrieved from Chilean and Peruvian mummies, dated of up to 6000 years old has allowed some hypotheses about the origin and dispersion of Chagas disease among American pre-Colombian populations (Ferreira et al. 2000, Guhl et al. 2000, Madden et al. 2001, Araujo et al. 2003). However, *Toxoplasma gondii*, one of the most prevalent parasites worldwide, has never been detected in archaeological material. *T. gondii* infection is found on several vertebrate hosts (Frenkel et al. 1970, Tenter et al. 2000), and might be associated to the ancestors of the human species. Nothing is known in respect to the origin, distribution, and epidemiology of toxoplasmosis in ancient populations, since some of its pathologic alterations may be confused with some other diseases and are hard to be detected in mummified tissues. In the present study we have devised an experimental approach to evaluate the application of the polymerase chain reaction (PCR) technique in the recovery of *T. gondii* DNA from ancient remains, using as models chronically and acutely infected mice which tissues were experimentally mummified aiming to standardize protocols for DNA extraction and amplification, as the scarcity of archaeological material poses a serious problem for availability and extensive testing of these sort of biological samples.

MATERIALS AND METHODS

Animals and parasites - Twenty five female C57Black/6 mice, 12-15 g, were obtained from the Laboratory Animal Facility/Fiocruz and infected intraperitoneally with *T. gondii* tachyzoites RH strain or tissue cysts ME-49 strain. *T. gondii* RH tachyzoites were grown in Swiss mice by intraperitoneal injection. After 3 days, mice were sacrificed, and parasites were washed in phosphate-buffered saline (PBS), pH 7.2, and then counted in a Neubauer chamber. The ME-49 strain of *T. gondii* was cryopreserved after sucessive passages from infected brains of mice C57/Bl/6 that had been previously infected with *T. gondii* tissue cysts (Freyre 1995, Popiel et al. 1996)

Experimental mummification protocol - Four mice were infected with 10 *T. gondii* ME-49 tissue cysts, com-

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posing the chronic infection group (CIG). The acute infection group, (AIG) infected with 1 x 10^4 *T. gondii* RH tachyzoites, was composed by another four mice and two mice maintained without infection were called negative control group (NCG). On the 45th day of infection to CIG and on 3rd to AIG, all animals were sacrificed and encephalon, heart, liver, lung, and thigh muscle were removed and layered on sheet of filter paper submitted to the experimental desiccation by dry heat at 39ºC as previously described (Bastos et al. 1996) for 45 days.

Immunohistochemistry - Fifteen mice were used in this study: five were infected with 10 *T. gondii* ME-49 tissue cysts (CIG), another five were infected with 1 x 10^6 *T. gondii* RH tachyzoites (AIG) and five non-infected animals (NCG). After 45 days for CIG infection and 3 days for AIG all animals were sacrificed. Anatomical pieces were fixed in 10% formalin buffered solution and embedded in paraffin blocks. Three µm sections from blocks, after mounting on silan (Sigma) coated glass slides, were submitted to immunoperoxidase technique. Tissue sections were incubated with biotinylated anti-mouse/anti-rabbit antibody (Dako, LSAB Kit, US) and then with estreptavidin-peroxidase complex (Dako). Afterwards, they were analyzed by avidin-peroxidase, using primary antibody anti-*Toxoplasma* (Neomarker, Fermont, CA, US) with posterior incubation with diaminobenzidin (DAB) developer (Dako). Sections were stained with Meyer hematoxylin and analyzed to find *T. gondii* components. A brain section from a known infected animal was used as positive control.

DNA extraction - DNA isolation and purification was carried out according to the protocol of Zhu et al. (1998) with modifications. Each desiccated tissue sample, with a maximum weight of 50 mg, was macerated on Petri dishes with the aid of glass slides, placed into microtube with the addition of 0.8 ml of DNAzol (Gibco BRL, US) and 2 mm glass beads. Then, it was homogenized for 2 min using Vortex (Fisher Scientific, US) and heated at 90ºC for 30 min. The homogenate was centrifuged for 5 min at 3000 x g to collect the lysate, followed by another spin at 14,000 x g. Ten µg of glycogen were added to the supernatant, and the DNA was precipitated by the addition of 0.5 ml of absolute ethanol. The pellet was washed with 95% ethanol, air dried, dissolved in 0.5 ml DNAzol, and centrifugated for 10 min. Afterwards, it was washed twice with 95% ethanol, dissolved in 90 µl of 8 mM NaOH, neutralized by the addition of 10 µl of 0.1 M HEPES buffer (pH 8.3) and stored at –20ºC. Tissues of non-infected mice were included as negative controls of DNA extraction.

PCR and hybridization assay - PCR was performed using the 35-fold repetitive *T. gondii* B1 gene as target for amplification previously described (Burg et al. 1989). Oligonucleotide primers used to initiate DNA amplification were GGAACTGCATCCGTTCATGAG (oligo-1) and TCTTTAAAGCGTTCGTGGTC (oligo-4). The PCR mix contained at final concentrations: 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Gibco), 8 ng/µl of each primer and 2,5 units of recombinant Taq DNA polymerase in the buffer recommended by manufacturer (Gibco). Distilled water was added up to the final volume of 50 µl. The mix was layered with 50 µl of mineral oil, 10 µl of each DNA sample was added. Amplifications were carried out on a Perkin Elmer Cetus 480 DNA Thermal Cycler as follows: an initial hot-start step of 94ºC for 5 min in order to denature the DNA strands, followed by 30 cycles of 94ºC, 55ºC, and 72ºC each for 1 min, and final extension at 72ºC for 10 min. The amplicons were submitted to electrophoresis in a 2% agarose gel, afterwards stained with ethidium bromide (0.5 µg/ml) and visualized on a UV light transiluminator. Each assay contained a negative control, in which no DNA was added to the reaction mixture, and a positive control, in which parasite DNA was included as a template in the PCR. All products were applied to a GeneScreen nylon membrane (NEN) by capillary transfer (20X SSPE), hybridized with probes from *T. gondii* B1 gene PCR amplicons radiolabeled with a α-32P-dCTP trough a random priming reaction. The membrane was sequentially washed at 65ºC in 2X SSPE, 0.1% SDS, and at 1X SSPE, 0.1% SDS and autoradiographed with intensifying screen at –70ºC for 7 days.

RESULTS AND DISCUSSION

The immunoperoxidase analysis of infected mice tissue sections was made to observe parasite distribution. Table I shows the immunohistochemistry results from CIG and AIG. Tissue samples from five CIG animals revealed a higher positivity in encephala, corroborating the data showed in the literature that point towards a preferential location of the parasite in rat and mouse brains in an independent way of the administered strain (Dubey 1997, 1998). A frequent presence of the parasite in lungs, hearts and livers was observed for the AIG mice, results that agree with previous studies, suggesting that tachyzoites inoculated by intraperitoneal route in this animal model were first detected in this cavity and later found in adjacent organs, being disseminated by the blood to the whole organism (Zenner et al. 1998). Positive and negative controls were in good agreement with the expected results. We have also observed, by chance, positivity for *T. gondii* at bone marrow cells from a thigh bone fragment of an AIG animal. It raises the possibility for acute toxoplasmosis research in bone remains (Fig. 1).

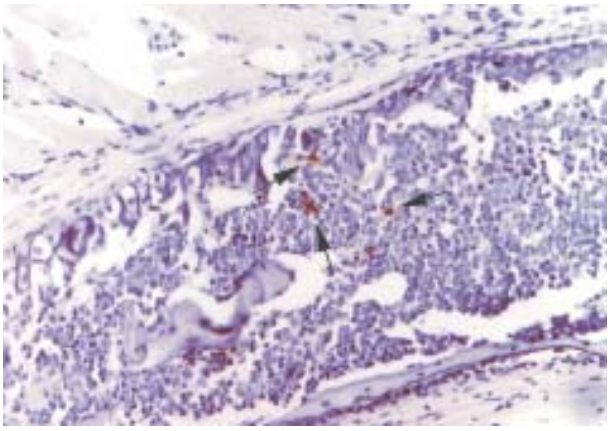

Fig. 1: immunoperoxidase assay for *Toxoplasma gondii* at bone marrow of acute infection group mouse. Arrowheads points the parasites.

TABLE I

Immunoperoxidase results from organ/tissue samples of C57Black/6 mice from five animals of the chronic infection group (CIG) and the acute infection group (AIG) to detect *Toxoplasma gondii* parasitic components

	Animals									
	CIG					AIG				
Organ/Tissue	CI	CII	CIII	CIV	CV	AI	AII	AIII	AIV	AV
Encephalon	+	+	+	+	+	-	-	-	-	+
Lungs	-	-	-	-	-	+	+	+	+	+
Heart	-	-	-	-	+	+	+	+	+	+
Liver	-	-	-	-	-	+	+	+	+	+
Thigh muscle	-	-	-	+	-	-	-	-	-	+

TABLE II

Distribution of *Toxoplasma gondii* B1 gene products in desiccated tissue samples from four animals of the chronic infection group (CIG) and the acute infection group (AIG) mice measured by polymerase chain reaction and hybridization assays

	Animals							
	CIG				AIG			
Organ/Tissue	C1	C2	C3	C4	A1	A2	A3	A4
Encephalon	+	+	+	-	+	-	-	-
Lungs	+	-	+	-	+	-	+	-
Heart	-	+	+	-	-	+	+	-
Liver	-	-	-	-	-	+	-	+
Thigh muscle	+	+	+	+	-	-	-	-

The results of PCR assays displayed in Table II yielded 194 bp *T. gondii* B1 gene amplicons at different samples of CIG and AIG animals. A PCR hybridization assay, representing the set of data using the B1 gene as a probe, is shown on Fig. 2. Encephala samples, as well as lungs and hearts from GIC, displayed a considerable positivity, and the highest rate was observed for the thigh muscles. These results agree with the data shown in studies done with fresh tissues of mice chronically infected by *T. gondii*, which show a consistent positiveness in brain samples (Savva et al. 1990, Owen & Trees 1998, Jauregui et al. 2001), as well as in heart and skeletal musculature (Homan et al. 2000). DNA of the parasite was detected by PCR and

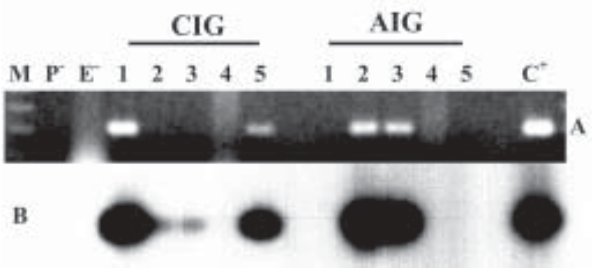

Fig. 2: detection of *Toxoplasma gondii* DNA by polymerase chain reaction (PCR) in desiccated tissue samples from one mouse of the chronic infection group (CIG) and acute infection group (CIG) - A: 2% agarose gel stained with ethidium bromide. Lanes - M: 1 Kb plus DNA ladder molecular weight standard (Life Technologies, Gibco BRL); P^-: negative PCR control (no DNA added); E^-: negative DNA extraction control; 1: encephalon; 2: lung; 3: heart; 4: liver; 5: tight muscle; C^+, positive control (*T. gondii* DNA sample); B: Southern blots prepared with samples from panel A which were hybridized with *T. gondii* B1 gene probes.

hybridization assays in the same kind of tissues that were positive for the immunohistochemistry analysis, including lungs, revealing the ability of the technique in detecting the infection by *T. gondii* in CIG. Concerning the AIG mice, we could verify the diverse distribution of the parasite in several tissues, with positivity in liver, heart and lungs, which is in accordance to the results presented in a previous experimental study (Joseph et al. 2002). Our study showed that the PCR assay was able to recover parasitic DNA from tissue samples subjected to experimental desiccation in the CIG and in the AIG mice. However, false negative PCR results in some samples could eventually occur by the excess of host DNA (Burg et al. 1989), or due the presence of Taq DNA polymerase inhibitors, like heparin, heme radicals or another porphyrin elements that could be present in tissues (Paabo & Wilson 1988, Hitt & Filice 1992). It may eventually be also caused by endonucleases and other autolytic effects from the post-mortem process (Lindahl 1993, Hofreiter et al. 2001). In our study the higher positivity in CIG compared to the AIG animals may have occurred by the fact that parasites on acute phase of *T. gondii* infection are consistently present on lung, heart, and liver of AIG mice which are highly vascularized organs, and could justify the lower number of positive results for the reasons mentioned above. Besides, the number of bradyzoites found inside a cyst has a magnitude order higher than the tachyzoites, favoring the DNA extraction and amplification of the CIG mice organs. No amplification was observed in the NCG animals.

The use of PCR in this study and its usefulness for the research of infections caused by *T. gondii* in past

populations is justifiable by the high sensitivity and specificity of the technique itself, that allows the recovery of parasite DNA at low concentration, from preserved organisms that have undergone special conditions of fast desiccation, low temperatures, high concentration of salt, and neutral pH, conditions which avoid its destruction by the action of the post-mortem autolytic phenomena (Lindahl 1993, Hofreiter et al. 2001). The choice of using the fragment of 194 bp from the B1 gene as target to PCR amplification was based on the observations made by other authors (Paabo & Wilson 1988, Paabo 1989) in studies with DNA, showing a decrease in the efficiency of the amplification process when the DNA sequence target exceeds 200 bp. High specificity, repetitive nature, high level of sequence conservation between the different forms of the parasite and between several strains isolated from clinical samples (Burg et al. 1989), were factors which influenced the selection of B1 in this study. Thus, to minimize the changes that may have occurred at the genetic level during the parasite evolution history, the utilization of highly conserved sequences increase the chances to recover preserved DNA.

Based on the efficiency shown by PCR in the recovery of *T. gondii* DNA from desiccated tissues, we recommend the utilization of this methodology as a tool for the research of this infection in past populations, in order to reach a better understanding about the paleoepidemiology, the origin of the *T. gondii* infection, its distribution in space and along the time, as well as the evolution of this etiologic agent in association with its hosts.

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