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Paleoparasitology of Chagas disease revaled by infected tissues from Chilean mummies

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Abstract

Mummified tissues were sampled from bodies stored at the Museo Arqueologico de San Pedro de Atacama, northern Chile, dated from 2000 years BP–1400 AD, and *Trypanosoma cruzi* DNA was recovered using polymerase chain reaction (PCR) methodology. Amplification of the conserved region of the minicircle molecule of *T. cruzi* was achieved in four of the six samples tested. Amplified products corresponding to genetic fragments of the parasite were tested by hybridization experiments with positive results for *T. cruzi* specific molecular probe. The origin and dispersion of *T. cruzi* human infection is discussed as well as the molecular paleoparasitological approach, and what it may represent in an evolutionary perspective.

Keywords: Paleoparasitology; Paleopathology; Mummies; *Trypanosoma cruzi*; Ancient DNA; PCR

1. Introduction

Paleoparasitology and paleoepidemiology allow inferences about disease patterns in the past by studying coprolites, skeleton remains and lesions in mummies, thus contributing to the knowledge of diseases in prehistoric populations. In the last 20 years, molecular approaches allowed the recovery of ancient DNA (aDNA) from archaeological material, opening the possibility for the study of human-parasite relationship through the finding of genetic traces of ethiological agents of diseases in mummified tissues (Brown and Brown, 1994; Handt et al., 1994; Hauswirth, 1994).

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The use of aDNA, revealed by polymerase chain reaction (PCR) technique, promises to clarify the evolution of parasite genomes and their respective association with human beings. Nowadays, molecular paleoparasitology represents a new tool to the discernment of infectious diseases in the past, as well as it points to future studies on parasite virulence and pathogenicity on an evolutionary perspective (Spigelman and Lemma, 1993; Rafi et al., 1994; Salo et al., 1994).

The present paper deals with the recovery of *Trypanosoma cruzi* DNA using the PCR methodology in Chilean mummies dated from 2000 BP–1400 AD. Six mummified tissues were tested and amplification of the conserved region of the minicircle molecule of *T. cruzi*, one constituent of the kinetoplast genome of this protozoan, was achieved in four. Further hybridization experiment with *T. cruzi* specific molecular probe proved that the amplified products correspond to genetic fragments of the parasite.

2. Material and methods

The Atacama Desert is one of the driest region of the Earth, with extremely low air humidity and with rainfall near zero. The soil concentration of mineral salts is high, and thawing forms rivers and lakes near the Andes Cordillera. Temperature ranges are characteristic of deserts, high some hours after sunrise falling to near 10°C or less at night (Berenguer et al., 1985). Environment conditions are favourable for the conservation of organic remains, and mummified bodies are a common finding in archaeological excavations.

The first hunter-gatherers human groups occupied Atacama Desert since 11 000 years ago. Sedentarism began at 5000 years ago with the first dwellings in the oasis. Herding and agriculture were the subsistence way of life until the Spanish conquest. The region presently known as San Pedro de Atacama (22° 55′ S, 68° 12′ W) was an important trade route, from the coast to the mountains (Junqueira and Llagostera, 1994; Nuñez and Dillehay, 1995; Neves and Costa, 1998).

In 1994, samples from 37 human mummified bodies stored at the Museo Arqueologico de San Pedro de Atacama were collected. The mummies, from different cultural periods, were dated between 2000 years ago to 1400 A.D. From the 37 bodies, six (M10, M11, M17, M21, M24, M34, three adults and three children) had their abdominal/chest opened and tissues from the cavity were sampled.

The PCR technique presented herein was used in archaeological material only after proper standardization in laboratory infected and experimentally desiccated mice (Bastos et al., 1996).

Fragments of the mummified tissues were rehydrated during 24 h in a 0.05% trissodium phosphate solution. After that procedure, the tissues were centrifuged at $1500 \times g$ for 15 min. The pellet was resuspended in 2.5 ml of TENS Buffer (50mM Tris–HCl pH 8.0, 50 mM EDTA pH 8.0, 100 mM NaCl, 0.5% SDS), and incubated at 37°C for 5 days. A volume of 5 μl of Proteinase K (100 μg/ml) were added twice every day during the incubation period. The lysate was then submitted to phenol-chloroform extraction and the DNA was precipitated in the presence of sodium acetate and ethanol. The final pellet was resuspended in 30 μl of double distilled water and 10 μl was used for DNA amplification.

Oligonucleotides (Fig. 1A, TGGTTTTGGGAGGGG(G/C)(G/C)(G/T)TCAA(A/C)TTT and TATATTACACCAACCCCAATCGAACC) that flank the conserved region of the minicircle molecule were used to amplify the 121 bp DNA fragment by hot-start PCR methodology (Chou et al., 1992). The PCR thermal profile consisted of an initial step of 94°C for 4 min in order to denature the DNA strands, followed by 30 cycles of 50, 72, and 94°C, lasting 30 s each step, and the final extension at 72°C for 10 min. The reaction was performed in the presence of 3.5 mM $MgCl_2$, 0.25 mM of each dNTP, 100 pmol of each oligonucleotide and 2.5 units of Taq DNA polymerase in the buffer recommended by the manufacturer (Perkin Elmer–Cetus, Norwalk, CT). In every assay, two distinct controls were performed: a negative control consisting of all the PCR reagents without the addition of DNA and a

positive control, where DNA extracted from blood of a chagasic patient that showed previously to be positive by PCR, was added.

The products were submitted to agarose gel (2%) electrophoresis, stained with ethidium bromide and visualized under UV light. The gel was photographed and the products were capillary blotted to nylon membrane using 0.4 N NaOH (Sambrook et al., 1989). The membrane was further hybridized with kinetoplast DNA isolated from the Y strain (Gonçalves et al., 1984). The probe was radiolabeled with α-32P-dATP through a random priming reaction and P10 column purification. The hybridization experiment was carried out in BLOTTO (1.5X SSC, 1% SDS and 0.05% nonfat dry milk), at 60°C for 16 h (Johnson et al., 1984). The membrane was washed at 65°C, 0.5X SSC and exposed to X-ray film with intensifying screen at −70° for 12 h. To discard the putative presence of contamination in the negative control that could not be previously evidenced, the membrane was also overexposed to X-ray film for a period of four days.

In order to investigate the presence of eventual inhibitors in the DNA preparation that could prevent the amplification of *T. cruzi* DNA giving rise to false negative results, amplification of human mitochondrial sequences and a fragment of the β globin gene were also carried out, maintaining the same PCR reagent conditions. For this purpose, the following sets of oligonucleotides were used, respectively: ACAGTTTCATGCCCATCGTC and ATGCTAAGTTAGCTTTACAG (Anderson et al., 1981; Wrischnik et al., 1987) and ACACAAACTGTGTTCACTAGC and CAACTTCATCCACGTTCACC (Saiki et al., 1985). The PCR thermal profile for the amplification of both human sequences were: two cycles at 98°C for 1 min and 64°C for 1 min, followed by 33 cycles at 94 and 64°C, lasting 1 min each step, and a final extension at 72°C for 10 min.

3. Results

Tissues from six Chilean mummies were rehydrated and submitted to DNA extraction and PCR amplification of the conserved region of the minicircle molecule. This component of the mitochondrial genome is a pre-amplified intracelular target that has been used successfully for diagnostic purposes (Avila et al., 1991; Britto et al., 1993;

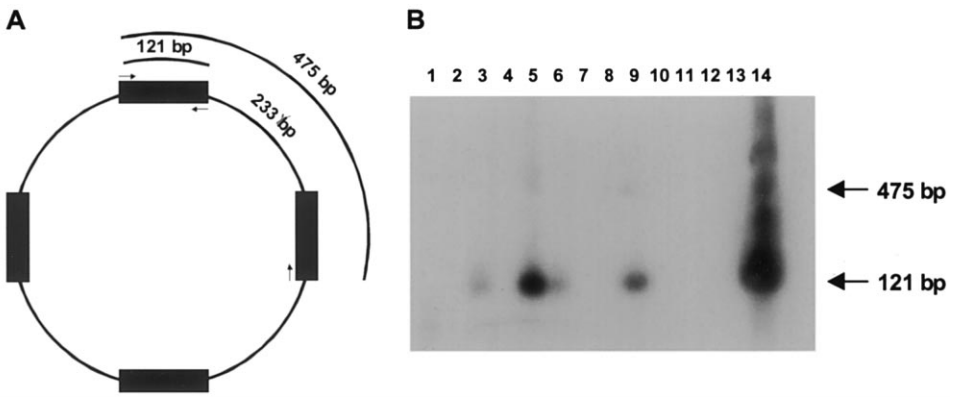

Fig. 1. Detection of *T. cruzi* DNA in mummified tissues. Schematic representation of a minicircle from *T. cruzi* kDNA showing the strategy for PCR amplification of 121 bp fragments containing the conserved regions of the molecule (black rectangles). Hybridization experiment. Amplified products were submitted to 2% agarose gel electrophoresis and transferred to a nylon membrane. The filter was hybridized with *T. cruzi* kDNA. Lane 1, negative control (no DNA added); lanes 2, 4, 10, 11, 12, 13 (empty lanes); lane 3, sample # M11; lane 5, sample # 21; lane 6, sample # M17; lane 7, sample # M10; lane 8, sample # M34; lane 9, sample # 24; lane 14, positive control (DNA extracted from blood of a confirmed chagasic patient). The arrows indicate the expected sizes of the amplified fragments, bp = base pairs.

Wincker et al., 1994a,b; Britto et al., 1995; Wincker et al., 1997). The DNA extracted from the six mummies proved to be in satisfactory conditions to be submitted to PCR amplification. No inhibitory factor could be evidenced in the DNA sample preparations. This fact could be proven by the possibility of amplifying both sequences from the human mitochondrial genome and β globin gene from all the six samples.

When PCR amplification of conserved regions of the *T. cruzi* minicircle molecule was first performed using the same paleological material, three tissue samples turned out to be positive (M11, M21, and M24, data not shown).

The complete procedure-from the very first step of rehydration till the PCR amplification-was repeated once with all the samples, and another one appeared as a positive result (M17). The hybridization of the amplified products with a specific *T. cruzi* probe could demonstrate that the products really correspond to *T. cruzi* genomic fragments and that the negative control of the assay is reliable (Fig. 1B).

4. Discussion

Nowadays, DNA can be obtained from fossils and the recovery of ancient DNA from a variety of samples and organisms is technically feasible (Pääbo et al., 1988; Höss and Pääbo, 1993; Stone and Stoneking, 1993; Salo et al., 1994; Araújo et al., 1998). This has opened a new window for research at that level of molecular history. Success has been attributed to the initial studies of Higuchi et al. (1984), Pääbo (1985, 1989), Thomas et al. (1989) who dealt with mammalian genomes. Persing et al. (1990) used the PCR technique to search *Borrelia burgdorferi*, etiological agent of Lyme disease, in ticks, their natural reservoir.

Despite the fact that several reports describe successfull attempts, meticulous care should be taken in order to avoid false results. Especially when dealing with the PCR technique, due to the extreme sensivity of the methodology and the rarity of ancient target molecules. Unfortunately, the first description of a Cretaceus DNA turned out to be in fact a human contamination (Allard et al., 1995; Hedges, 1995; Zischler et al., 1995).

In the present study the PCR methodology was used to detect *T. cruzi* DNA in tissues from Chilean mummies. All precautions were taken in order to avoid PCR contaminations. Furthermore, the experiments were repeated twice, giving rise to coherent results, diverging in only one sample, which might be attributed to sampling prior to DNA extraction.

Trypanosoma cruzi minicircle molecule is composed of four sets of conserved (121 bp) and variable regions (233 bp). Usually, for routine molecular diagnosis of Chagas disease the chosen target is the variable region of the molecule, amplifying a 330 bp product containing the entire variable region and part of the conserved one. Therefore, eventual contamination phenomena that could occur during the sample preparation would be caused by those amplicons. In this study, the conserved region of this mitochondrial molecule was amplified generating a 121 bp product. Multimer of ≈ 475 bp (Fig. 1B), corresponding to the amplification of one conserved region (121 bp) followed by one variable region (233 bp) and another conserved one (121 bp), due to non-hybridization of the primers in every annealing site (Fig. 1A), discard the possibility of contamination of PCR by pre-amplified material, either of 330 bp or 121 bp.

Chagas disease was found in the Andean region during pre-Columbian times. Chilean and Peruvian mummies were found with lesions of the disease, and the parasite was identified in histological analysis (Rothhammer et al., 1985; Fornaciari et al., 1992). Furthermore, Guhl et al. (1997, 1999) described the presence of amplified products corresponding to *T. cruzi* genomic sequences in Chilean mummies dated of 4000 years BP.

Nowadays, San Pedro de Atacama is considered an endemic area for Chagas disease, with a seroprevalence, estimated in 1988, of 16.8% of the human population (Buchard et al., 1996). What happened, in terms of how the dispersion of the parasite occurred during this period is still unknown.

Zeledón and Rabinovich (1981) argue that *T. cruzi* infection was prevalent in the sylvatic cycle, as a pure enzooty, long before the human peopling of the Americas. Coimbra (1988) discuss

triatomine adaptation to Lowland South American Indians, especially in the Amazonian region, concluding that ecological aspects of Indian dwellings and their nomad habits, prevent the insects to colonize and to maintain the infection in the human host.

However, some triatomine species adapted to live in rocks maintained the parasite life cycle by feeding mostly in rodents. One possibility regarding the contact between *T. cruzi*-human being is that prehistoric groups inhabited these kind of shelters in some regions of the American continent, long before dwellings were introduced (Araújo et al., 1998). Recent archaeological findings point to old dates for peopling of the Americas and the use of rocky shelters for funeral ceremonies and for rock paintings and engravings (Guidon & Arnaud, 1991).

Another hypothesis that could have promoted this contact between humans and the protozoa is described by Rothhammer et al. (1984), who believe that Chagas disease began earliest than 5000 years ago with the domestication of pre-infected mammals, such as camelids and rodents, in the Andean region. The contact of these infected reservoirs with triatomines adapted to primitive dwellings allowed the appearence of the first cases of Chagas disease.

Our findings show that *T. cruzi* was indeed living together with human beings in America for a vast period of time and that the pure enzootic cycle, without the human environment, was precociously abolished in some primitive areas. Considering all the aforementioned and the advances of paleoparasitology, it is not unexpected the finding of Chagas disease in prehistoric population out of the Andean environment.

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