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Hybridization Screening of Very Short PCR Products for Paleoepidemiological Studies of Chagas' Disease

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ABSTRACT

Single strands of very short PCR products can be covalently immobilized to a slide and then easily detected by probe hybridization. In this work, the PCR product was a 70-nucleotide segment of ancient DNA, representing a portion of repeat minicircle DNA from the kinetoplast of *Trypanosoma cruzi*, the infectious agent of Chagas' disease (American Trypanosomiasis). The target segment was initially established to be present in soft tissue samples taken from four "naturally" mummified Andean bodies using PCR followed by cloning and sequencing. Hybridization screening of the covalently immobilized PCR products positively identified products from 25 of 27 specimens of different tissues from these four mummies. The method appears to be ideal for the purpose of screening a large number of specimens when the target PCR product is very short.

INTRODUCTION

The method we developed and report here is part of our major paleoepidemiological study of Chagas' disease (American Trypanosomiasis). It represents the response to our need for a specific screening technique that is capable of identifying the presence of a DNA segment unique to *Trypanosoma cruzi* (the infectious agent of the disease) in hundreds of tissue samples from ancient South American mummies. Chagas' disease is limited to the Americas and is not easily diagnosed by anatomical or microscopic approaches. Fortunately, *T. cruzi* contains several thousand copies of a 1433-nucleotide minicircle DNA in its kinetoplast that can be used to detect its presence. The minicircle contains four highly conserved repeat regions shown schematically in Figure 1, which can be targeted and amplified by the

PCR with specific primers. Others (2,3) have targeted the kinetoplast DNA to identify *T. cruzi* in ancient human remains. Although neither group sequenced their PCR products, Ferreira et al. (2) did hybridization experiments using a total kinetoplast DNA probe. However, the use of such a probe may yield false positives if the interprimer portion of the PCR product is not *T. cruzi* because of hybridization to the exogenously added primer portions. In this paper, we report the extraction of DNA from several human mummy tissue samples up to 2000 years old, which was then amplified with *T. cruzi*-specific primers, cloned, and sequenced.

Having established the presence of Chagas' disease in these ancient populations, we turned our attention to developing a method that was less time consuming while retaining a level of sensitivity and specificity necessary for a paleoepidemiological survey of hundreds of samples from multiple locations. Our initial approach was to dot blot PCR products from many samples and probe them with the labeled *T. cruzi*-specific probes. This approach failed, using a wide variety of membranes and hybridization conditions, probably because the abbreviated length (70 nucleotides) of the DNA products caused them to be so totally adsorbed to the membrane that they were deprived of the freedom to hybridize. Methods have been published for hybridization detection of PCR products, and a few are applicable to short PCR products Jiang et al. (4) immobilized biotinylated PCR products on streptavidin-coated microplates and then probed with europium-labeled probes. The PCR product targeted a 69-nucleotide segment of DNA. Rasmussen et al. (6) immobilized DNA of various sizes by covalent reaction to Covalink NH microwells (Nalge Nunc International, Rochester, NY, USA) with good success and applicability to hybridizing very short PCR products. Finally, Saiki et al. (7) immobilized short oligopolynucleotides to nylon membranes by UV radiation after adding a long oligo dT tail. This left the oligopolynucleotides free to hybridize. Recently, a new product, 3D Link™, from SurModics (Eden Prairie, MN, USA) allowed us to immobilize cova-

lently the 5' end of one strand of the PCR products to a slide, which was then easily detected with the appropriate *T. cruzi*-specific probes. We demonstrate in this report the potential application of this method to screen short PCR products from hundreds of samples with a single hybridization.

MATERIALS AND METHODS

The Mummies

Northern Chile's Pacific coast forms part of one of the world's driest deserts—the Atacama. The arid atmosphere there commonly produces complete desiccation of an interred body's tissues before it decays, resulting in spontaneous mummification. The literally total absence of rainfall in this area thus preserves such tissues for a long time; mummies up to 9000 years old have been excavated there (1). The mummified bodies were excavated by the professional staff of the Department of Archaeology at the University of Tarapaca in Arica, Chile, and the studied tissue samples were obtained by dissection of such mummies by two of the authors of this report (1). All were excavated from burial sites within 15 km of the coast in the Azapa valley, at the mouth of which the city of Arica is located at about 18° 30' south latitude. Features of these mummies are listed in Table 1. Gel electrophoresis had yielded a 330-nucleotide *T. cruzi* kDNA segment extracted and amplified (but not sequenced) from tissues of the first three mummies listed in Table 1 during an earlier study (3), but tissues from mummy AZ141, T-26 had not been studied previously. Because ancient DNA segments longer than 200 nucleotides are often not regularly amplifiable by PCR, we targeted an 85-nucleotide segment for this study. This segment was long enough to be unique to *T. cruzi*; no such sequence could be identified by a Basic Local Alignment Search Tool (BLAST) search in any other animal or human genomic databank.

DNA Extraction

Physically isolated laboratories were used for each step of isolation and am-

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Table 1. Demographic Features of Sampled Individuals

Specimen No.	Age (Years)	Sex ^a	Approximate Time Period	Culture	Tissue
AZ 142, T5	50–60	M	AD 1875	Colonial	Heart
AZ 75, T133	45–55	M	AD 25	San Lorenzo	Lung
AZ 75, T43	35–40	F	AD 76 ^b	San Lorenzo	Esophagus
AZ 141, T26	20–25	M	AD 1000	Cabaza	Lung
EG-2 ^c	14	M	c. AD 300	Egyptian	Lung

^aM = male, F = female.
^bThis mummy was not individually dated, but the AZ-75 burial site contained mummies whose dates ranged from 351 BC to AD 504 with a median value of AD 76.
^cThis body of a 14-year-old boy was excavated from a Roman Period site (AD 200–300) in the Dakhleh Oasis of Egypt's Western Desert. The body had become mummified spontaneously by desiccation without embalming. Because Chagas' disease is limited to South and Central America, a sample of lung tissue from this body was included in our study as a negative control.

plification as well as DNA handling in Clone Zone™ (USA Scientific, Ocala, FL, USA), a DNA-disruptive UV workstation. Control tissue (tissue from a spontaneously mummified Egyptian body) and “water only” were included as negative controls. All pipetting was done

using aerosol-resistant filtered pipet tips.

Tissue samples were pulverized under liquid nitrogen in a Spex Freezer/Mill® (Spex, Metuchen, NJ, USA). DNA was extracted using the GeneClean for Ancient DNA™ kit, (BIO 101, Vista, CA, USA). A 400-mg sam-

ple of pulverized tissue was incubated 16 h in a solution of 5 mL 0.5 M EDTA, 200 µL 10% SDS, 200 µL 20 mg/mL Proteinase K at 37°C, then processed as per the GeneClean for Ancient DNA protocol.

DNA Amplification

PCR was performed using a GeneAmp® PCR System 2400 (Applied Biosystems, Foster City, CA, USA). The final concentrations for the PCR solutions were 1× PCR reaction buffer (Applied Biosystems), 1.5 µM MgCl₂, 1.0 µM upstream/downstream primers, 200 µM each dNTP, 2.5 U AmpliTaq Gold® (Applied Biosystems), and 2 µL isolated ancient DNA extract. The PCR cycling parameters were: denaturation at 95°C for 10 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 70°C for 1 min. One final round was allowed to extend for 10 min at 72°C. To each sample, or a second set of samples, was added 100 fg mimic DNA as previously published (8). The amplification of the mimic target indicates that the mummy extract was not inhibitory to the PCR.

The PCR products were analyzed for size by agarose gel electrophoresis, 3% SeaPlaque™ GTG agarose (BMA, Rockland, ME, USA) in 1× TBE buffer. A 2-µL aliquot of the PCR product was subjected to a second round of heminested PCR with the primer set (primers B and C) using the same cycling conditions. The mimic is not amplified in the second round of PCR. The second-round PCR product was analyzed by agarose gel electrophoresis, and those samples that exhibited PCR products of the correct size were isolated and cloned into a plasmid vector (TA Cloning Kit™; Invitrogen, Carlsbad, CA, USA). Clones were picked and placed into 2 mL LB medium containing ampicillin, incubated at 37°C with shaking at 250 rpm for 18 h. Plasmid DNA was isolated using Wizard® Plus Miniprep DNA purification system (Promega, Madison, WI, USA). Clones were screened by restriction digestion for the presence of the insert.

DNA Sequencing

Clones containing inserts were amplified by PCR as described previously.

Figure 1. *T. cruzi* kinetoplast DNA, 1440-nucleotide minicircle. PCR target regions in bold (GenBank accession no. X04680). Primer positions within conserved repeat regions. Primer set A and B generates an 85-nucleotide product. Heminested primer set C and B produces a 70-nucleotide product. Primer A, 85 nucleotides upstream 5'-ACACCAACCCCAATCGAACCC-3'; primer B, downstream 5'-GCGGAAATTCATGCATCTCCC-3'; and primer C, 70 nucleotides upstream 5'-GAACCCACCTCCCCGTAAAA-3'.

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Table 2. Mummy Tissues Tested with Probe and Results

Position No.	Mummy File No.	Tissue Tested	Results
1	AZ75, T 43	Lung	Positive
2	AZ75, T 43	Liver	Positive
3	AZ75, T 43	Muscle	Positive
4	AZ75, T 43	Kidney	Positive
5	AZ75, T 43	Sternum	Negative
6	AZ75, T 43	Rib	Positive
7	AZ75, T 43	Brain	Positive
8	AZ75, T 43	Cartilage	Positive
9	AZ75, T133	Skin	Positive
10	AZ75, T133	Muscle	Positive
11	AZ75, T133	Sternum	Positive
12	AZ75, T133	Rib	Positive
13	AZ75, T133	Stomach	Positive
14	AZ75, T133	Aorta	Positive
15	AZ142, T5	Lung	Positive
16	AZ142, T5	Spleen	Positive
17	AZ142, T5	Brain	Positive
18	AZ142, T5	Carotid artery	Positive
19	AZ141, T26	Lung	Positive
20	AZ141, T26	Liver	Positive
21	AZ141, T26	Coprolite	Positive
22	AZ141, T26	Spleen	Negative
23	AZ141, T26	Skin	Positive
24	AZ141, T26	Muscle	Positive
25	AZ141, T26	Esophagus	Positive
26	AZ141, T26	Kidney	Positive
27	AZ141, T26	Rib	Positive
28	EG-2 (Egyptian)	Lung	Negative
29		Mimic plasmid DNA	Negative
30		No DNA	Negative

Positive test, targeted DNA segment present.
 Negative test, targeted DNA segment absent.
 If individual was parasitemic at time of death, then any blood-containing tissue can be expected to contain some parasites.

They were purified using QIAquick™ PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced with Thermo Sequenase™ radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Fragments were separated through an 8% polyacrylamide sequencing gel; the gel was then dried and exposed to X-ray film. The acquired sequence was com-

pared to sequences in the databanks by a BLAST search, and alignments were made to the known sequences.

DNA Amplification and Immobilization of One Strand of PCR Products

Samples of the first-round PCRs (2 µL) were subjected to a second round

of PCR using primer C and a 5'-amino-derivative of primer B. The PCR conditions were the same as described above. The products were purified using a QIAquick PCR purification kit and precipitated with ethanol/sodium acetate. The precipitated amine-PCR products were dried, dissolved in 1 µL printing buffer and coupled to the 3D Link slides according to the supplier's directions. In this work, the spots were about 2 mm in diameter, but much smaller spots are certainly possible. After coupling, the residual amino-reactive groups on the slide were blocked, the bound DNA was denatured, and the unbound strand was washed away, making the slide ready for hybridization to the probes.

Preparation of *T. cruzi*-Specific RNA Probes

Three oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, IA, USA) that could be amplified by the PCR, and which contained T7 promoter sequences such that they could serve as templates to enzymatically generate ³³P-labeled *T. cruzi*-specific RNA probes for nucleotides 37–65 of target regions 1, 2, 3, and 4 (note that regions 1 and 2 in Figure 3 are identical). The RNA probes were complementary to the interprimer portion of the immobilized PCR strand on the 3D Link slide. The ³³P-labeled RNA probes were synthesized using the Maxiscript™ system (Ambion, Austin, TX, USA) employing T7 RNA polymerase. The reactions were carried out according to the supplier's directions and contained 1 µg template DNA and 50 µCi of α-[³³P]-UTP. The reaction was stopped, treated with RNase-free DNase I, and co-precipitated with glycogen. The precipitate was washed with 70% ethanol and dissolved in 11 µL hybridization solution.

Hybridization

The slides containing the spots of immobilized PCR products from several different mummy samples were hybridized for 16 h to an equimolar mixture of the three ³³P-labeled RNA probes, as described by the SurModics protocol, washed, and exposed to X-ray film.

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RESULTS AND DISCUSSION

The PCR amplification target sequence was the repeat region of *T. cruzi* minicircle DNA (Figure 1). The minicircle DNA, a 1433-nucleotide DNA

segment that contains four repeated regions, is present in 20 000–25 000 copies in the kinetoplast of each parasite cell (9). Primers were synthesized (Integrated DNA Technologies) to amplify an 85-nucleotide DNA target of

the repeat region, as well as a heminested target of 70 nucleotide (Figure 1). The heminested primer set allowed for increased specificity of the PCR fragment with decreased background. Figure 2 demonstrates the presence of PCR products of the appropriate size for our target sequence by gel electrophoresis from tissues of all four mummies. Figure 3 reveals the sequence of four clones prepared from these bands. The cloned PCR products have sequences that compare favorably with the known sequences of the four conserved segments in modern *T. cruzi* kinetoplast DNA (GenBank®). Note the variability present in the segment from nucleotides 37–53 of the contemporary sequences for regions 1–4 as well as in the ancient sequences.

The results of the hybridization screening of different tissues from four mummy samples using 3D Link slides are shown in Figure 4. The different intensity levels of the spots may be the result of varying efficiencies of amplification of the four target regions, hybridization, or the number of *T. cruzi* organisms in those tissues.

The DNA target has been shown to be present in all four of these mummies as determined by sequencing. Probe tests on PCR-amplified extracts of multiple tissues from each of these four

Figure 2. Amplification of ancient DNA. Second round of PCR using primers B and C (heminested). Lane 1, AZ142, T5, Heart (clone 2-4); lane 2, AZ141, T26, Lung (clone 3-1); lane 3, AZ141, T26, Lung (clone 3-3); lane 4, AZ75, T133, Lung (clone 4-1); lane 5, AZ75, T133, Lung (clone 4-2); lane 6, AZ75, T43, Esophagus (clone 5-6); and lane 7, PCR negative control (no target DNA).

Figure 3. DNA sequence alignments of the *T. cruzi* repeat regions and ancient DNA. Heminested primer set C and B is located at positions 16–36 for upper primer C and 65–85 for lower primer B. The target region is located at positions 37–65. No. 1, *T. cruzi* repeat region 1; no. 2, *T. cruzi* repeat region 2; no. 3, *T. cruzi* repeat region 3; no. 4, *T. cruzi* repeat region 4; no. 5, AZ142, T5, Heart (clone 2-4); no. 6, AZ141, T26, Lung (clone 3-1); no. 7, AZ141, T26, Lung (clone 3-3); no. 8, AZ75, T133, Lung (clone 4-1); no. 9, AZ75, T133, Lung (clone 4-2); and no. 10, AZ75, T43, Esophagus (clone 5-6).

mummies reacted positively. Individuals with the chronic form of Chagas' disease may or may not be parasitemic (i.e., have trypanosomes in the circulating blood) at the time of death. If so, then organisms can be expected to be present in every blood-containing organ or tissue; if not, autopsy studies on modern victims of Chagas' disease have revealed that the trypanosomes are often concentrated primarily in certain organs, especially the heart, esophagus, or colon (5).

Figure 4. Hybridization screening of different tissues of mummies identified in Table 1. AZ75, T43 nos. 1–8: lung, liver, muscle, kidney, sternum, rib, brain, and cartilage, respectively. AZ75, T133 nos. 9–14: skin, muscle, sternum, rib, stomach, and aorta, respectively. AZ142, T5 nos. 15–18: lung, spleen, brain, and carotid artery, respectively. AZ141, T26 nos. 19–27: lung, liver, coprolite, spleen, skin, muscle, esophagus, kidney, and rib, respectively. EG-2 no. 28: lung from Egyptian mummy, a negative control. Mimic PCT target, no. 29: a negative control; Ø DNA, no. 30: a negative control.

We sampled 14 different organs or tissues for a total of 27 different specimens from these four mummies. Of these 27 samples, 25 reacted positively with the probes (Table 2). One spleen and one bone (sternum) sample from two different mummies were probe-negative (nos. 5 and 22); samples from each of these two tissue types from the other mummies were probe-positive. Given that the number of trypanosomes/U blood varies considerably among Chagas'-afflicted persons and even from day to day within the same person, and given the broad range of vascularity in the different sampled tissues, it is encouraging that almost all samples reacted positively with the probes. Quantitative sensitivity tests of the probes will be determined before they are widely applied on our specimen collection.

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