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Enterobius vermicularis: Ancient DNA from North and South American Human Coprolites

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A molecular paleoparasitological diagnostic approach was developed for Enterobius vermicularis. *Ancient DNA was extracted from 27 coprolites from archaeological sites in Chile and USA. Enzymatic amplification of human mtDNA sequences confirmed the human origin. We designed primers specific to the* E. vermicularis *5S ribosomal RNA spacer region and they allowed reproducible polymerase chain reaction identification of ancient material. We suggested that the paleoparasitological microscopic identification could accompany molecular diagnosis, which also opens the possibility of sequence analysis to understand parasite-host evolution.*

Key words: ancient DNA - *Enterobius vermicularis* - coprolites

Based on several lines of evidence including archaeoparasitology and cladistic analysis, the pinworm *Enterobius vermicularis* is one of the most ancient parasites of humans and has a pre-hominid evolutionary origin (Ferreira et al. 1997, Hugot et al. 1999). Paleoparasitological studies showed the presence of pinworm eggs in 10,000-year-old human coprolites from United States and in coprolites from Chile and Peru dating from 2200 to 400 BC (Ferreira et al. 1997). In some prehistoric cultures, *E. vermicularis* reached very high prevalence as indicated by the numbers of coprolites that contain eggs (Reinhard 1998). Until now, diagnosis of pinworms in archaeological remains was dependent on microscopic examination. This is a particularly poor method of identifying prehistoric pinworm infections (Reinhard 1990, Araújo et al. 1998). Recent work revealing the ancient DNA (aDNA) of parasites such as *Trypanosoma cruzi* in mummies and *Ascaris* in coprolites (Ferreira et al. 2000, Loreille et al. 2001) opened this area of investigation to other organisms. This paper presents a molecular diagnosis of *E. vermicularis* using the conserved region from 5S ribosomal RNA (rRNA) intergenic spacer as target.

The coprolites (n = 27) from archaeological sites in Chile and North America were analyzed. Samples from Chile were collected in archaeological sites of Caserones (n = 2), Tarapacá Valley, dating from 400 BC to 800 AD (Ferreira et al. 1984); of Tulan (n = 20), San Pedro de Atacama dating to 1000 BC (Ferreira et al. 1989); and of Tiliviche (n = 2) dated from 4110 to 1950 BC (Araújo et al. 1983). USA samples (n = 3) were from Antelope House, an Anasazi village site in Canyon de Chelly, Arizona dating from 900 AD (Reinhard 1996) (Table). DNA extraction and amplification were performed under the procedures established for working with aDNA to avoid contamination with modern molecules (Hofreiter et al. 2001, Marota & Rollo 2002). The surface of the samples was exposed to UV light and the coprolite core was ground. Coprolite powder (5-2 g) was hydrated in ddH_2O or TE buffer (Tris-HCl 10mM, EDTA 1mM, pH 8.0). Coprolites, formerly used in microscopic diagnostic in 0.5% trisodium phosphate aqueous solution (Ferreira et al. 1989), also were analyzed. Sediments of 150 µl were treated by 72 h with 400 µl digestion buffer (NaCl 100 mM, Tris-HCl 50 mM, SDS 1%, EDTA 50 mM, pH 8.0), and added 20 µl DTT 1M, 60 µl proteinase K 10 mg/ml (Gibco BRL) and 100 µl SDS10%. The reactions were incubated at 55-60°C for 3-24 h with occasional homogenization followed by the phenol/chloroform extraction and purified using silica resin column (Glass Max DNA Isolation Spring Cartridge System Gibco-BRL).

In order to determine the nature of the coprolites, human mitochondrial DNA (mtDNA) amplification was done using the procedure described by Pääbo (1990) and Handt et al. (1996). *E. vermicularis* amplification was performed by nested PCR targeting *E. vermicularis* 5S rRNA spacer region: Entf (5'-CACTTGCTATACCAACAACAC-3') and Entr (5'-GCGCTACTAAACCATAGAG-3'); and internal Eva (5'-ACAACACTTGCACGTCTC-3') Evb (5'-GAATTGCTCGTTTGC-3'). PCR final volume reaction was: 25 µl using 20 mM Tris-HCl, 0.5 µM KCl (Gibco BRL 10X Buffer), 2 mM $MgCl_2$, 0.2 mM each dNTPs, 1mg/ml bovine serum albumin (BSA) and 500 ng of each oligonucleotide. The mixture was exposed to 30 min UV radiation before the 2.5 U of *Taq* polymerase (Gibco BRL) and 50-100 ng DNA extract addition. The reactions were subjected to an initial cycle of 5 min at 94°C, followed by 35 cycles of 94°C for 1min, 50-55°C for 30 sec and 72°C for 30 sec in a programmable thermal controller (PTC100 60 v, MJ Research, Inc). Extraction and negative PCR controls were included. Amplicons were hybridized with a radiolabeled probe from the same region amplified from a modern sample (Sambrook et al. 1989).

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Human mtDNA was retrieved from almost all coprolite samples by using Handt et al. (1996) procedure (Fig. 1). Due to the nature of aDNA, the target choice is a crucial step for the successful sequence retrieval. The complete pinworm highly conserved ribossomal 5S intergenic region is about 800 bp (Liu et al. 1995), with several copies in an organism. We designed primers for a nested PCR targeting this region. The first and second primer pair produced 420 bp and 198 bp specific and unique amplicon, respectively. The control of primer specificity was done previously using DNA extracted from modern feces and *E. vermicularis* experimental coprolites (Iñiguez 1998). We were successful in the molecular paleoparasitological diagnosis of *E. vermicularis* using the specific pinworm 5S rRNA spacer region (Fig. 2). The hybridization result confirmed the specific nature of the diagnostic bands with 198 bp length (data not shown). All positive samples in the microscopic analysis but nine were PCR positive. Two samples, 706 and 716, negative in the microscopic analysis, yielded *E. vermicularis* diagnostic amplicon (Table). The sample 721 was PCR negative either to mtDNA or pinworm target. Considering that during this work the experimental procedure of DNA extraction, purification and the set up of PCR reactions, were done in two different laboratories and repeated at least twice, we concluded that the aDNA 721 was highly degraded (Lindahl 1993, Marota et al. 2002). In this regard, microscopic identification of pinworms eggs is still relevant for paleoparasitological diagnosis. However, molecular approach not only offers a precise identification, but also the opportunity of ancient parasite sequence comparisons with those of contemporary populations. Further analysis of human mtDNA and pinworm aDNAs sequences can provide more comprehension about *E. vermicularis* evolution and their human host.

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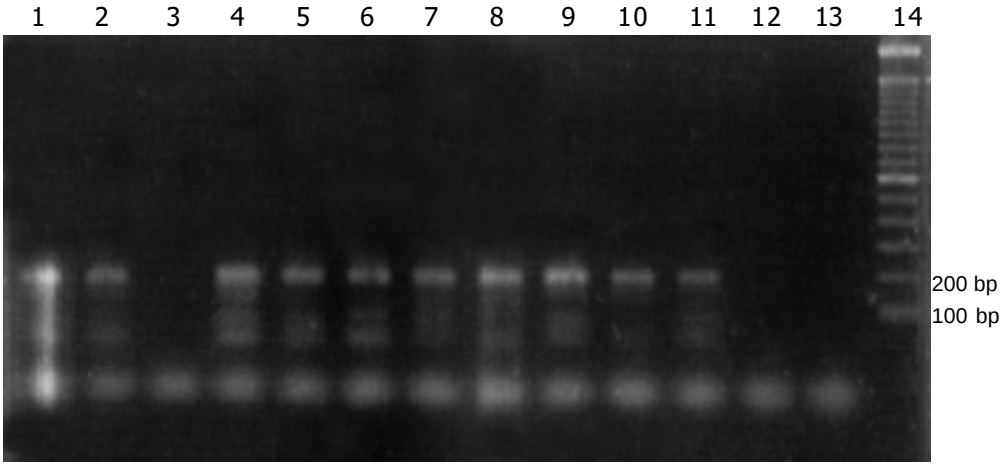

Fig. 1: mitochondrial human DNA amplicom (185 bp): Lanes - 1 to 12: coprolite samples: 168, 170, 384, 385, 704, 706, 708, 714, 715, 716, 719, and 721, respectively. Lane 13: PCR negative control; 14: 100 bp DNA ladder (Gibco BRL). (2 % Agarose gel electrophoresis).

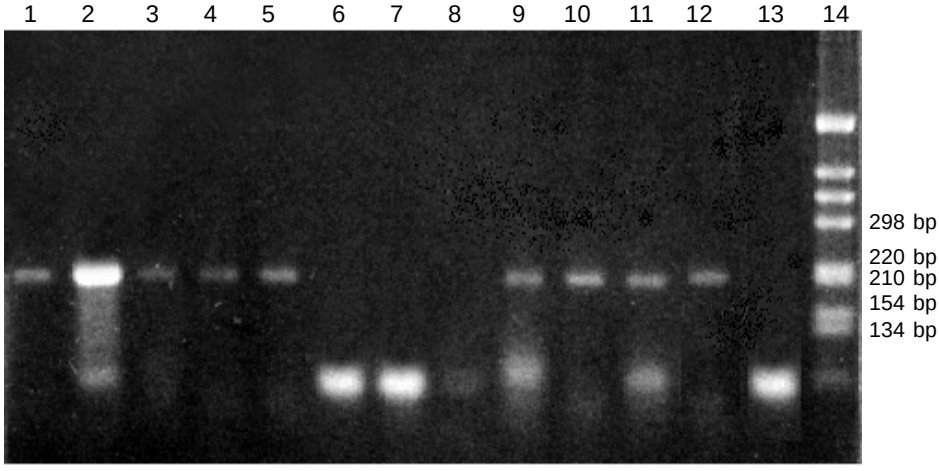

Fig. 2: *Enterobius vermicularis* diagnostic amplicon (198 bp). Lanes - 1 to 12: coprolite samples 170, 385, 704, 708, 714, 721, 168, 384, 706, 715, 716 and 719, respectively; 13: PCR negative control; 14: 1 kb DNA ladder (Gibco BRL). (2 % Agarose gel electrophoresis).

TABLE

Enterobius vermicularis microscopic and molecular paleoparasitological analysis

Coprolite	Archaeological site	Microscopic analysis	Molecular analysis
168	Tiliviche, Chile	-	-
170		+	+
384	Caserones, Chile	-	-
385		+	+
704	Arizona, USA	+	+
706		-	+
708		+	+
710	Tulan, Chile	-	-
710A		-	-
710B		+	-
710C		-	-
711		+ [a]	-
712		+	-
713		+	-
714		+ [a]	+
715		+	+
716		-	+
717		-	-
718		+	-
719		-	+
720		+	-
721		+	-
722		+	-
723		-	-
724		+	-
725		-	-
726		-	-

a: also positive for *Trichuris trichiura*

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