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Alena Mayo Iniguez
Instituto Oswaldo Cruz

Karl J. Reinhard
University of Nebraska at Lincoln, kreinhard1@mac.com

Luiz Fernando Ferreira
Escola Nacional de Saude Publica Sergio Arouca

Adauto Araujo
Fundação Oswaldo Cruz, adauto@ensp.fiocruz.br

Ana Carolina
Fundação Oswaldo Cruz

See next page for additional authors

Enterobius vermicularis ancient DNA in Pre-Columbian Human Populations

Alena Mayo Iñiguez[1], Karl Reinhard[2], Luiz Fernando Ferreira[3], Adauto Araújo[3] and Ana Carolina Paulo Vicente[1]

1 Instituto Oswaldo Cruz, Laboratório de Genética Molecular de Microorganismos (IOC-FIOCRUZ), Rio de Janeiro. Brasil.
2 School of Natural Resource Sciences, University of Nebraska-Lincoln, Lincoln. USA.
3 Escola Nacional de Saúde Pública Sérgio Arouca, Laboratório de Paleoparasitologia (ENSP-FIOCRUZ), Rio de Janeiro. Brasil.

ABSTRACT: In prehistoric populations the paleoparasitological findings show an Enterobius vermicularis homogeneous distribution among North American hunter-gatherers intensified with the advent of agriculture. The same occurred in the transition from nomad hunter-gatherers to sedentary farmers in South America, although *E. vermicularis* infection encompasses only the ancient Andean peoples. Since molecular techniques are sensitive in detecting ancient DNA (aDNA), in this work we have performed a molecular paleoparasitological study of *E. vermicularis*. aDNA was recovered from North and South American coprolites (4110 BC-AD 900). Human (cox 2 and HVR) and pinworm (5S rRNA spacer) sequences were determined. The sequence analysis confirmed *E. vermicularis* identity and revealed polymorphisms in independent coprolite samples from Tulán, San Pedro de Atacama, Chile (1080-950 BC). We were successful in detecting *E. vermicularis* aDNA even in coprolites without direct microscopic evidence of the eggs, improving the diagnosis of helminth infections in the past and further pinworm paleoepidemiological studies.

KEYWORDS: Ancient DNA; Enterobius vermicularis; Coprolites; 5S rRNA spacer; Paleoparasitology.
PALABRAS CLAVE: ADN antiguo; Enterobius vermicularis; coprolitos; región intergénica 5S rRNA; Paleoparasitología.

Introduction

E. vermicularis, pinworm, is one of the most common helminths worldwide, infecting nearly a billion persons of all socio-economic levels and is knows to have a major impact on the well-being of infants (Lukes *et al.*, 2005). Parasite transmission has no environmental restrictions and the parasite can be transmitted from host to host without an obligatory stage in soil or intermediary hosts. It is considered that the human-*E. vermicularis* relationship was originated in pre-hominid times, having evolved in Africa and dispersed to other continents through pre-historic human migrations (Ferreira *et al.*, 1997; Hugot *et al.*, 1999). Paleoparasitological findings and the parasite biological cycle suggest that pinworms crossed the Bering Land Bridge with human hosts during the first migratory movements into the Americas. However, transpacific routes have also been postulated (Ferreira *et al.*, 1997).

The presence of pinworm eggs was shown in 10.000-year-old human coprolites from Utah, U.S.A., one of the oldest human coprolites found (Fry & Hall, 1969). Mummies and coprolites from several North American archaeological sites were positive for pinworm infection (Reinhard, 1990; Gonçalves *et al.*, 2003), delineating a homogeneous distribution among hunter-gatherers, in North America, intensifying with the advent of agriculture. This same increase also occurred in the transition from nomad hunter-gatherers to sedentary farmers in South America. *E. vermicularis* infection encompasses the ancient Andean peoples, with no record among the

pre-Colombian populations in the South American lowlands. Pinworm eggs were observed in coprolites from localities in Chile dating from 4100 BC (Before Christ) to 8000 AD (Anno Domini) (Araújo *et al.*, 1985; Ferreira *et al.*, 1989); in Perú dating from 2277 ±180 BP (Before Present) (Patruco *et al.*, 1983) and in pre-Columbian human remains from Argentina (Zimmerman & Morilla, 1983). Old World data on this subject is curiously scarce (Bouchet *et al.*, 2003). Herrmann (1985) found *E. vermicularis* eggs in Roman latrines, and Horne (2002) recorded eggs in an Egyptian mummy.

Microscopic examination is useful in paleoparasitological diagnosis only when the recovered specimens are in good quality. Consequently, the prevalence of pinworm infection in ancient populations may have been underestimated (Reinhard, 1990; Araújo & Ferreira, 2000). Therefore, molecular biology approach provides specific and sensitive diagnostic tool and the opportunity to access parasite ancient genetic information. The 5S rRNA intergenic spacer was successfully used as a PCR target to *E. vermicularis* diagnosis in Amerindian coprolites (Iñiguez *et al.*, 2003a).

In order to verify the retrieval of *E. vermicularis* aDNA sequences directly from human coprolites and as well to investigate pinworms genetic diversity, and the relationship among geographically and temporally distinct populations of human pinworm, we have determined 5S rRNA spacer DNA sequences from 27 pre-Columbian coprolites that range a age from about 1100 to at least 6110 years and originate from four different North and South American archaeological sites. We have also characterized the presence of the SL1 RNA gene in the ancient pinworm populations.

Materials and Methods

Twenty-seven coprolites from Chilean and United States archaeological sites, previously examined microscopically for helminth eggs and larvae by microscopic techniques (Gonçalves *et al.*, 2003) were used for aDNA extraction. Information about coprolite samples is found on Table 1. The human origin of coprolites was suggested by archaeological context and confirmed by the finding of parasites specific to human. One sample from each 27 coprolites was rehydrated by immersion in a 0.5% aqueous solution of trisodium phosphate for 72 hours, following the technique of Callen & Cameron (1960). The material was submitted to spontaneous sedimentation following the technique proposed by Lutz (1919). A portion of sediment was used for microscopic examination. The material was placed on a slide and examined for the presence of parasites. Twenty slides for each sample were examined at magnification of X100 and X400.

Throughout the research, we implemented standard procedures for aDNA work in order to avoid contemporary DNA contamination and thus to obtain authentic ancient sequences (Hofreiter *et al.*, 2001a; Marota & Rollo, 2002; Drancourt & Raoult, 2005). Ancient DNA was manipulated in the Paleoparasitology Laboratory (ENSP/FIOCRUZ) and later reproduced at the Molecular Genetics Laboratory (IOC/FIOCRUZ), geographically distant from each other. Non-disposable tools and equipment was frequently pretreated with 2.5% NaOCl and work areas were irradiated by ultraviolet light for some hours. Handling of coprolite samples, aDNA extraction and purification was carried out in a physically separate environment than PCR and electrophoresis. DNA extractions were performed in the horizontal laminar flow equipped with ultraviolet germicidal lamps. Pipette filter tips and exclusive pipettes were used at each stage. All extraction reagents and primer solutions were separated into small aliquots. Negative controls, without coprolite samples or aDNA, were included in each step. Positive PCR controls were not included. All PCR positive results were reproduced from DNA extraction step in a second laboratory, and the aDNA sequences were determined both by direct sequencing of amplicons and clones.

Coprolite samples were exposed to 254 nm of ultraviolet light at a distance of 50 cm for 10 minutes. Fine sheets of the surface of coprolites were eliminated and the core was ground. Samples of 0.5 to 2.0 g were hydrated at room temperature for 3 days in TE buffer (Tris-HCl 10mM, EDTA 1mM, pH 8.0) or 0.5% trisodium phosphate aqueous solution. Sediments of 150µl were treated with 400µl digestion buffer (NaCl 100mM, Tris-HCl 50mM, SDS 1%, EDTA 50mM, pH 8.0), 20µl DTT 1M, 60µl proteinase K 10mg/ml (Invitrogen) and 100µl SDS 10% (Iñiguez *et al.*, 2003a). The reactions were incubated at 55-60 °C for 72h with occasional homogenization. DNA extraction was performed by phenol/chloroform/isoamyl alcohol

(25:24:1) and purified using a silica resin column Glass Max DNA Isolation Spring Cartridge System (Invitrogen).

PCR primers targeting a fragment of *E. vermicularis* 5S ribosomal RNA spacer region, which contained the Splicing Leader 1 RNA (SL1 RNA) gene were used. The protocol specificity was first tested in DNA extracted from modern human feces and *E. vermicularis* experimental coprolites (Iñiguez *et al.*, 2002; Iñiguez *et al.*, 2003a). *E. vermicularis* amplification was performed by nested PCR targeting a 420 bp and 198bp. Alternatively, the DNA was submitted to reconstructive polymerization pretreatment before PCR reactions (Golenberg *et al.*, 1996; Iñiguez *et al.*, 2003b). The final volume of the PCR reaction was 25 µl and included 20 mM Tris-HCl, 50 mM KCl (Invitrogen 10X Buffer), 2 mM $MgCl_2$, 0.2 mM each dNTPs, 1mg/ml bovine serum albumin (BSA) and 500ng of each primer. The reaction mixture was exposed for 30min to UV radiation before the addition of 2.5 U of *Taq* polymerase (Invitrogen) and 50-100 ng DNA extract or 3-5 µl of PCR product. The reactions were subjected to an initial cycle of 5 min at 94 °C, followed by 40 cycles of 94 °C for 1min, 50-55 °C for 30s and 72 °C for 30s in a programmable thermal controller PTC100 60v (MJ Research, Inc). Extraction and PCR negative controls were included for each sample or reaction. PCR products were submitted to electrophoresis in 1.5% and 2% agarose for 420pb and 198pb products, respectively. Gels were stained with ethidium-bromide and observed over a UV transluminator. In order to verify the presence of PCR inhibitors in the aDNA extracted from coprolites, human mitochondrial DNA (mtDNA) fragments were also amplified by PCR using two approaches: by conditions described by Pääbo (1990) for 92 bp, 121 bp and 471 bp DNA fragments, and the conditions described by Handt *et al.*, (1996) targeting a 184 bp amplicon.

PCR products were cloned into *pGEM-T Easy Vector System* (Promega) and clones were purified with *QIA prep Spin Miniprep Kit* (QUIAGEN). PCR products were purified using *Qia quick PCR Purification Kit* (QUIAGEN) and utilized in direct sequencing reactions with ABI PRISM *Big Dye Terminator Cycle Sequencing Ready Reaction Kit* (Applied Biosystems-Perkin Elmer). Samples were sequenced on both strands in an ABI PRISM 377 *Automated DNA Sequencer* (Applied Biosystems-Perkin Elmer) as described by suppliers. Chromas program version 1.45 (School of Health Science, Griffith University, Queensland, Australia), Bio Edit version 5.0.9 (Departament of Microbiology, North Carolina State University), and the package of

Archaeological site Nº/PCR	Date	References	
Chile			
Tiliviche, Iquique	4110-1950 BC	Araújo et al., 1983 Gonçalves et al., 2002	2/50%
Tulan, San Pedro de Atacama 20/20%	1080-950 BC	Ferreira et al., 1989	
Caserones, Tarapaca Valley	400 BC-800 AD	Araújo et al., 1985	2/50%
USA			
Antelope House, Arizona 3/100%	900 AD	Reinhard 1996	
All 27/33.3%	4110 BC-900 AD		

BC: Before Christ; AD: Anno Domini; Nº: The total number of coprolites analyzed; PCR: The percent of tested samples that were PCR positive from *E. vermicularis*.

TABLE 1. Coprolites locality, date and molecular diagnosis results from *E. vermicularis*

programs GCG (Genetic Computer Group, version 9,1, Madison Incorporation, WI, the USA) were used for the sequence analysis. The *E. vermicularis* ancient sequences were aligned with the *E. vermicularis* sequences available in Gen Bank U65495/U65496 from USA and from Brazil (AF493944-AF493944).

The SL1 nematode motif was identified by BLAST command whereas the spliced donor and the *Sm* antigen binding sites were localized manually (Blaxter & Liu, 1996; Dassanayake *et al.*, 2001). The secondary structure of SL RNA was inferred by the mfold program version 3.1 (Michael Zuker, Rensselaer Polytechnic Institute; Zuker 2003) and previous conditions used to analyze *E. vermicularis* SL RNA secondary structure (Iñiguez *et al.*, 2002).

Results

Nested PCRs were performed using DNA from coprolites as a substrate. We were successful in obtaining the 198 pb DNA fragments in 9/27 coprolites. However the 420 pb target was not amplified, even when reconstructive polymerization pretreatment was used. No DNA signal was observed in negative PCR and extraction controls. Three samples (706, 716 and 719) that were negative for microscopic analysis yielded an *E. vermicularis* diagnostic amplicon (Tab. 1). Dilutions of aDNA sample and/or new aDNA purifications were carried out to prevent *E. vermicularis* false negative PCR. Human mtDNA was also tested and mtDNA fragments were retrieved from all coprolites except samples 384 and 721.

Fourteen E. vermicularis sequences were obtained and the comparison with the GenBank database revealed their identity to *E. vermicularis* 5S rRNA spacer. The presence of the 22-nucleotide motif, corresponding to the SL1 RNA sequence in nematodes, which is in opposite direction (Liu *et al.*, 1996) of transcription from the 5S rRNA gene, was verified. The alignment of contemporary and ancient *E. vermicularis* sequences showed a high degree of

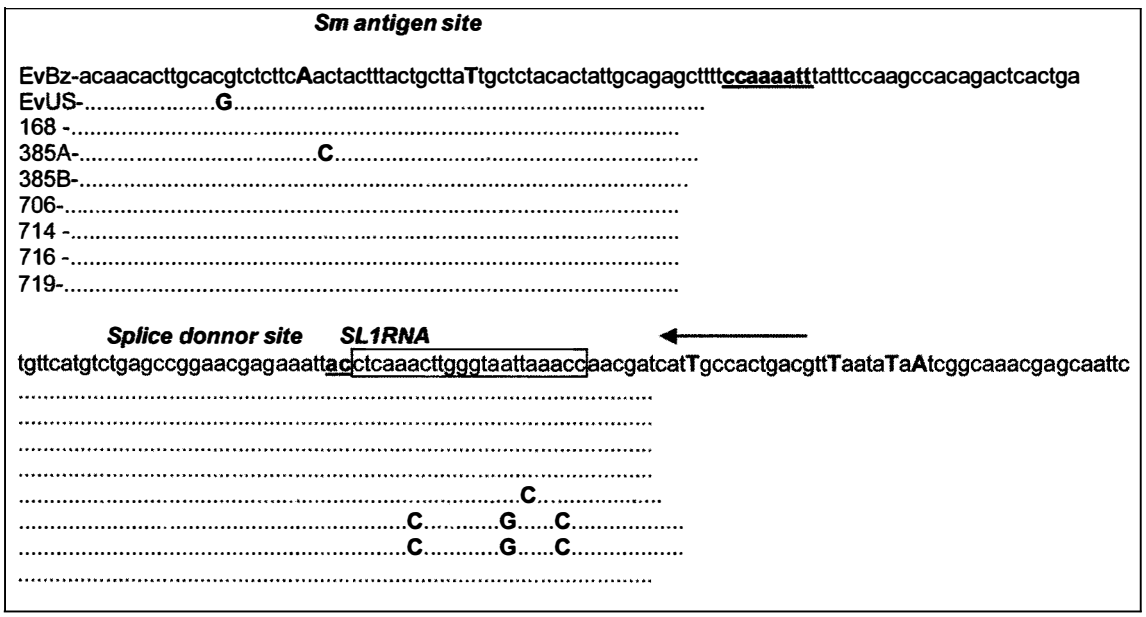

FIGURE 1. *E. vermicularis* 5S rRNA spacer region alignment. One sequence of each archaeological site is represented and identical sequences were omitted. The nucleotide substitutions are shown in capitals. The position of SL1 RNA sequence is in box. The spliced donor and Sm antigen-binding sites are bolded and underline. Ev-Bz corresponding to modern pinworm Brazilian sequences (GenBank AF493945 to AF493949) and Ev-US is American sequence (GenBank U65495). Ancient sequences from Chilean coprolites are 168 from Televiche, Iquique (4110-1950 BC); 385 from Caserones, Tarapaca Valley (400 BC-800 AD), and 714-719 from Tulan, San Pedro de Atacama (1080-950 BC). Ancient North American sequence is 706 from Antelope House, Arizona, USA (900 AD)

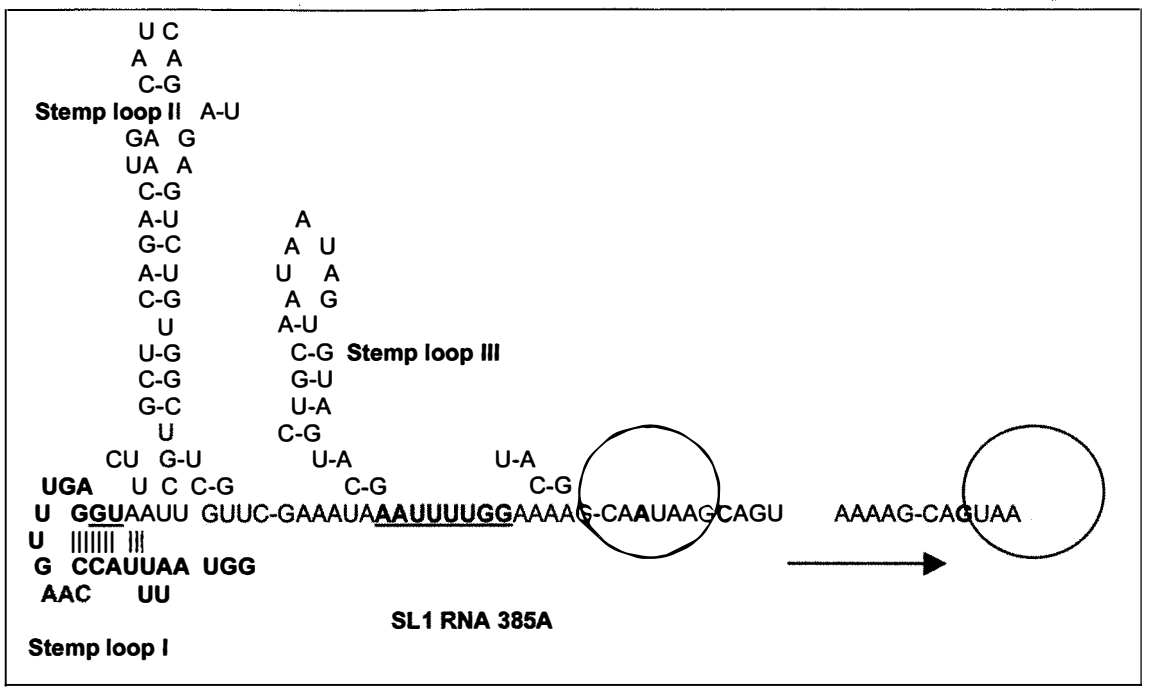

FIGURE 2. The putative secondary structure of *E. vermicularis* SL1 RNA corresponding to ancient sequences. The three stem-loops are indicated. The SL1 RNA sequence in stem-loop I is bolded. The spliced donor dinucleotide GU and the Sm antigen-binding sequence are bolded and underline. In a circle the detail the putative secondary structure of *E. vermicularis* SL1 RNA corresponding to ancient sequence of clone 385A from Caserones, Tarapaca Valley (400 BC-800 AD) and the nucleotide substitution is bolded

conservation within this segment (Fig. 1). Ten sequences from all archaeological sites were found to be identical to *E. vermicularis* contemporary sequences. Two *E. vermicularis* sequences from Tulán, San Pedro de Atacama, Chile, had three nucleotide substitutions at the same positions. Sequences from coprolites collected in Caserones, Tarapaca Valley, Chile, were the same as the modern consensus, except for two clones that displayed one difference at a distinct position (Fig. 1).

In addition to the SL1 RNA motif, the canonical dinucleotide spliced donor and the *Sm* antigen binding sites, were identified in all aDNA sequences, which gives evidence to the presence of the SL1 RNA gene (Stratford & Shields, 1994; Blaxter & Liu, 1996; Favia *et al.*, 2000; Dassanayake *et al.*, 2001). The nucleotide substitutions observed in aDNA *E. vermicularis* sequences were 3 T→C transitions and two T/C→G/C transversions (Fig. 1). All these substitutions were found outside of the conserved regions of SL1 RNA gene.

The sequence 385B display a substitution 28 nucleotides downstrean the SL1 RNA sequence and the first substitution in sequences 714 and 716 occur 10 nucleotide downstrean. The SL1 RNA secondary structure was determined for sequence 385, which has one substitution 26 nucleotides upstream the consensus sequence of *Sm* antigen binding site (Fig. 2). The remaining SL1 RNA gene sequences were identical to the modern *E. vermicularis* sequence and therefore their SL1 RNA gene secondary structures are maintained (Iñiguez *et al.*, 2002; Fig. 2). The potential SL1 RNA secondary structure from 385A sequence was shown to be similar to the SL1 RNA gene three stem-loops previously described in modern *E. vermicularis*, other nematodes and trypanosomatids (Evans *et al.*, 1997; Sturm *et al.*, 1999; Evans & Blumenthal, 2000; Iñiguez *et al.*, 2002). The unique nucleotide substitution in sequence 385A does not interfere in the structure of the stem-loops since it is found in the linear strand downstream of the third stem-loop.

The remaining of the structure of both sequences was shown to be the same: the 22 nucleotides SL1 RNA exon molding the first stem-loop, the *Sm* antigen binding-site situated in the intron moiety, and the stem-loops II and III localized upstream and downstream of *Sm* binding-site, respectively. (Evans *et al.*, 1997; Vandenberghe *et al.*, 2001; Fig. 2).

Discussion

The 5S ribosomal RNA spacer region has been particularly used for systematic, diagnosis and phylogenetic inferences in nematodes due to its variability in size and sequence (Stucki *et al.*, 1993; Xie *et al.*, 1994; Liu *et al.*, 1995; Favia *et al.*, 2000; Iñiguez *et al.*, 2002; Veronico *et al.*, 2004; Van der Giessen *et al.*, 2005). Contrasting with the variability of ribosomal spacers in other species, *E. vermicularis* display at most 1% of difference in the nucleotide composition between contemporary North and South American isolates (Liu *et al.*, 1995; Iñiguez *et al.*, 2002). SL1 RNA gene was identified in the 5S rRNA spacer region from *E. vermicularis* ancient DNA. The sequences from all archaeological sites were identical to the consensus 5S rRNA spacer modern sequence. Interestingly all nucleotide substitutions in the 4 *E. vermicularis* sequences from the Caserones and Tulán archaeological sites were outside of the SL1 RNA gene. The high conservation of 5S RNA spacer region in geographically and temporally distant samples revealed in this work is probably consequence of the selective pressure due to the presence of an essential gene in this region. aDNA studies have previously shown that 18S rRNA gene sequence recovered from Middle-Age Ascaris eggs is identical to moderns Ascaris sequences (Loreille *et al.*, 2001). The DNA analysis of bacteria from pre-Columbian mummy coprolite (1170-980 AD) using the 16S rDNA gene, have also revealed a total or high similarity between modern and ancient sequences (Luciani *et al.*, 2006).

The SL RNA secondary structure has a functional role in the trans-splicing process (Sturm *et al.*, 1999; Xu *et al.*, 2000; Nilsen, 2001). Analyses of structure-function of SL1 RNA in nematodes showed that stem-loop II and the Sm binding-site are crucial for trans-splicing (Denker *et al.*, 1996; Evans & Blumenthal, 2000). Of the 22 nucleotides of the SL1 sequence that are totally conserved in all nematodes studied, the first 16 nucleotides are apparently fundamental for transcription and trans-splicing (Xie & Hirsh, 1998; Ferguson & Rothman, 1999; Evans & Blumenthal 2000). The recovery of *E. vermicularis* 5S RNA spacer from coprolites allowed the inference of the secondary structure for pinworm SL1 RNA ancient sequences. The parasites sequences retrieved from coprolites had the same SL1 RNA gene sequence and conformation as the modern pinworms.

Damage in ancient DNA may cause addition of incorrect base during the PCR, but these missincorporations are improbable to arise at the same point in independent samples (Poinar *et al.*, 1998; Hofreiter *et al.*, 2001b). Therefore, the polymorphism in samples 714 and 716 from Tulán, San Pedro de Atacama, Chile are authentic of Tulán *E. vermicularis*. The territory currently known as San Pedro de Atacama region was an important pre-Columbian trade route from the Pacific coast to the Andes Mountains (Ferreira *et al.*, 2000). If the substitutions identified in ~3000-year-old *E. vermicularis* are present in current pinworm populations from San Pedro de Atacama, Chile these polymorphisms would be an evidence of a different parasite lineage. On the other hand, the conservation and length of 5S rRNA spacer target sequences, did not allow any evolutionary inferences.

The recovery of *E. vermicularis* aDNA sequences, directly from coprolites, provides one more approach to perform paleoepidemiological studies considering host-parasite relationship, since human aDNA has been successfully retrieved from coprolites.

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Literature Cited

ARAÚJO, A., L.F. FERREIRA, U.E. CONFALONIERI, L. NUNEZ and F.B.M. RIBEIRO, 1985. The finding of *Enterobius vermicularis* eggs in pre-Columbian human coprolites. *Mem. Inst. Oswaldo Cruz*, 80, 141-143.

ARAÚJO, A. and L.F. FERREIRA, 2000. Paleoparasitology and antiquity of human host-parasite realtionships. *Mem. Inst. Oswaldo Cruz*, 95, 89-93.

BLAXTER, M. and L. LIU, 1996. Nematode Spliced Leaders: Ubiquity, Evolution and Utility. *Int. J. Parasitol*, 26, 1025-1036.

BOUCHET, F., N. GUIDON, D. KATHARINA, S. HARTER, L.F. FERREIRA, S.M. CHAVES, K. REINHARD and A. ARAÚJO, 2003. Parasite remains in archaeological sites. *Mem. Inst. Oswaldo Cruz*, 98, 47-52.

CALLEN, E.O. and T.W.M. CAMERON, 1960. A prehistoric diet as revealed in coprolites. *New Scientist*, 8, 35-40.

DASSANAYAKE, R.S., N.V. CHANDRASEKHARAN and E.H. KARUNANAYAKE, 2001. Trans-spliced leader RNA, 5S-rRNA genes and novel variant orphan spliced-leader of the lymphatic filarial nematode *Wuchereria bancrofti*, and a sensitive polymerase chain reaction based detection assay. *Gene.*, 269, 185-193.

DENKER, J.A., P.A. MARONEY, Y.T. YU, R.A. KANOST and T.W. NILSEN, 1996. Multiple requirements for nematode spliced leader RNP function in trans-splicing. *RNA*, 2, 746-755.

DRANCOURT, M. and D. RAOULT, 2005. Palaeomicrobiology: current issues and perspectives. *Nat. Rev. Microbiol.*, 3, 23-35.

EVANS, D., D. ZORIO, M. MACMORRIS, C.E. WINTER, K. LEA and T. BLUMENTHAL, 1997. Operons and SL2 trans-splicing exist in nematodes outside the genus *Caenorhabditis*. *PNAS*, 94, 9751-9756.

EVANS, D. and T. BLUMENTHAL, 2000. Trans-splicing of polycistronic Caenorhabditis elegans pre-mRNAs: analysis of the SL2 RNA. *Mol. Cell. Biol.*, 20, 6659-6667.

FAVIA, G., C. BAZZOCCHI, G. CANCRINI, C. GENCHI and C. BANDI, 2000. Unusual organization of the 5S ribosomal spacer in *Dirofilaria repens*: absence of a canonical spliced leader 1 sequence. *Parasitol Res.*, 86, 497-499.

FERGUSON, K.C. and J.H. ROTHMAN, 1999. Alterations in the conserved SL1 trans-spliced leader of *Caenorhabditis elegans* demonstrate flexibility in length and sequence requirements in vivo. *Mol. Cell. Biol.*, 19, 1892-1900.

FERREIRA, L.F., A. ARAÚJO, U. CONFALONIERI and M. CHAME, 1989. *Enterobius vermicualris* infection in pre-Columbian population from San Pedro de Atacama, Chile. *Mem. Inst. Oswaldo Cruz*, 84, 197-199.

FERREIRA, L.F., K.J. REINHARD, A. ARAÚJO and L.C. COURA, 1997. Paleoparasitology of oxyuriasis. *An. Acad. Nac. Med.*, 157, 20-24.

FERREIRA, L.F., C. BRITTO, M.A. CARDOSO, O. FERNANDES, K. REINHARD and A. ARAÚJO, 2000. Paleoparasitology of Chagas disease revaled by infected tissues from Chilean mummies. *Acta Trop.*, 75, 79-84.

FRY, G.F. and H.J. HALL, 1969. Parasitological examination of prehistoric human coprolites from Utah. *Proc. Utah Acad. Sci. Art Letters*, 46, 127-154.

GOLENBERG, E.M., A. BICKEL and P. WEIHS, 1996. Effect of highly fragmented DNA on PCR. *Nucleic Acids Res.*, 24, 5026-5033.

GONÇALVES, M.L.C., A. ARAÚJO and L.F. FERREIRA, 2003. Human Intestinal Parasites in the Past: New Findings and a Review. *Mem. Inst. Oswaldo Cruz*, 98, 103-118.

HANDT, O., M. KRINGS, R.H. WARD and S. PÄÄBO, 1996. The retrieval of ancient human DNA sequences. *Am. J. Hum. Genet.*, 59, 368-376.

HERRMANN, B., 1985. Parasitologisch-epidemiologische auswertungen mittelalterlicher kloaken. Z. *Archaol. Mittelalters*, 13, 131-161.

HIGUCHI, R., B BOWMAN, M. FREIBERGER, O.A. RYDER and A.C. WILSON, 1984. DNA sequences from the quagga, an extinct member of the horse family. *Nature*, 312, 282-284.

HOFREITER, M., D. SERRE, H.N. POINAR, M. KUCH and S. PÄÄBO, 2001a. Ancient DNA. *Nature Reviews Genetics*, 2, 353-359.

HOFREITER, M., V. JAENICKE, D. SERRE, A.V.A. HAESELER and S. PÄÄBO, 2001b. DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Res.*, 29, 4793-4799.

HORNE, P.D.J., 2002. First evidence of enterobiasis in ancient Egypt. *Parasitol.*, 88, 1019-1021.

HUGOT, J.P., K.J. REINHARD, S.L. GARDNER and S. MORAND, 1999. Human enterobiases in evolution: origin, specificity e transmission. *Parasite*, 6, 201-208.

Iñiguez, A.M., A.C.P. Vicente, A. Araújo, L.F. Ferreira and K.J. Reinhard, 2002. *Enterobius vermicularis*: specific detection by amplification of an internal region of 5S ribosomal RNA intergenic spacer and trans-splicing leader RNA analysis. *Exp. Parasitol.*, 102, 218-222.

Iñiguez, A.M., K.J. Reinhard, A. Araújo, L.F. Ferreira and A.C.P. Vicente, 2003a. *Enterobius vermicularis*: ancient DNA from North and South American Human Coprolites. *Mem. Inst. Oswaldo Cruz*, 98, 67-69.

Luciani S., G. Fornaciari, O. Rickards, C.M. Labarga and F. Rollo, 2006. Molecular characterization of a pre-Columbian mummy and in situ coprolite. *American Journal of Phisical Anthropology*, 129, 620-629.

Lukes, J., A. Horák and T. Scholz, 2005. Helminth genome projects: all or nothing. *Trends in Parasitology*, 21(6), 265-266.

Liu, L.X., M.L. Blaxter and A. Shi, 1996. The 5S ribosomal RNA intergenic region of parasitic nematodes: variation in size and presence of SL1 RNA. *Mol. Biochem. Parasitol.*, 83, 235-239.

Liu, L.X., J. Chi, M.P. Upton and L.R. Ash, 1995. Eosinophilic colitis associated with larvae of the pinworm *Enterobius vermicularis*. *Lancet*, 346, 410-412.

Lutz, A., 1919. O *Schistosomum mansoni* e a schistosomatose segundo observações feitas no Brasil. *Mem. Inst. Oswaldo Cruz*, 11, 121-155.

Marota, I. and F. Rollo, 2002. Molecular paleontology. *Cell. Mol. Life Sci.*, 59, 97-111.

Nilsen, T.W., 2001. Evolutionary origin of SL-addition trans-splicing: still an enigma. *Trends in Genetics*, 17, 678-680.

Odile L., E. Roumat, O. Verneau, F. Bouchet and C. Hänni, 2001. Ancient DNA from Ascaris: extraction amplification and sequences from eggs collected in coprolites. *Int. J. Parasitol.*, 31(10), 1101-1106.

Pääbo, S., 1990. Amplifying ancient DNA. In: M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White (eds.). *PCR Protocols: A guide to methods and applications*. California: Academic Press, 159-166.

Patruco, R., R. Tello and D. Bonavia, 1983. Parasitological studies of coprolites of pre-hispanic Peruvian populations. *Curr. Antropol.*, 24, 393-394.

Poinar, H.N., M. Hofreiter, W.G. Spaulding, P.S. Martin, B.A. Stankiewicz, H. Bland, R.P. Evershed, G. Possnert and S. Pääbo, 1998. Molecular coproscopy: dung and diet of the extinct ground sloth *Nothrotheriops shastensis*. *Science*, 281, 402-406.

Reinhard, K.J., 1990. Archaeoparasitology in North America. *Am. J. Phys. Anthropol.*, 82, 145-162.

Stratford, R. and R. Shields, 1994. A trans-spliced leader RNA sequence in plant parasitic nematodes. *Mol. Biochem. Parasitol.*, 67, 147-155.

Stucki, U., R. Braun and I. Roditi, 1993. *Eimeria tenella*: Characterization of a 5S ribosomal RNA repeat unit and its use a species-specific probe. *Exp. Parasitol.*, 76, 68-75.

Sturm, N.R., M.C. Yu and D.A. Campbell, 1999. Transcription termination and 3'-End processing of the spliced leader RNA in kinetoplastids. *Mol. Cell. Biol.*, 19, 1595-1604.

Van der Giessen, J.W.B., M. Fonville, I. Briels and E. Pozio, 2005. Phylogenetic analysis of encapsulated and non-encapsulated Trichinella species by studying the 5S rDNA tandemly repeated intergenic region. *Vet. Parasitol.*, 132, 51-55.

Vandenberghe, A.E., T.H. Meedel and K.E.M. Hastings, 2001. mRNA 5'-lader trans-splicing in the chordates. *Genes Dev.*, 15, 294-303.

Veronico, P., F. De Luca and C. De Giorgi, 2004. Molecular dissection of the rDNA array and of the 5S rDNA gene in Meloidogyne artiellia: phylogenetic and diagnostic implications. *Mol. Cell. Probes*, 18, 177-183.

Xie, H., O. Bain and A.S. Williams, 1994. Molecular phylogenetic studies on filarial parasites based on 5S ribosomal spacer sequences. *Parasite*, 1, 114-151.

Xie, H. and D. Hirsh, 1998. In vivo function of mutated spliced leader RNAs in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA*, 95, 4235-4240.

Xu, Y., L. Liu and S. Michaeli, 2000. Functional analyses of positions across the 5' splice site of the trypanosomatid spliced leader RNA: implications for base-pair interaction with U5 and U6 snRNAs. *J. Biol. Chem.*, 275, 27883-27892.

Zimmerman, M.R. and R.E. Morilla, 1983. Enterobiasis in pre-Columbian America. *Paleopathol. News*, 42, 8.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.*, 31, 3406-3415.