Molecular paleoparasitological diagnosis of *Ascaris* sp. from coprolites: new scenery of ascariasis in pre-Columbian South America times

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Paleoparasitological studies using microscopy showed that Ascaris *and* Trichuris trichiura *are the human intestinal parasites most found in archaeological sites. However, in pre-Columbian South American archaeological sites,* Ascaris *is rare. In this work we standardized a molecular methodology for* Ascaris *diagnosis directly from ancient DNA retrieved from coprolites. Using cythochrome b gene (142 bp) target, ancient DNA sequences were retrieved from South American samples, negative by microscopy. Moreover, the methodology applied was sensitive enough to detect ancient DNA extracted from 30* Ascaris *eggs from an European coprolite. These results revealed a new scenery for the paleodistribution of* Ascaris *in South America.*

Key words: *Ascaris* - molecular paleoparasitological diagnosis - paleodistribution

The human intestinal parasite *Ascaris lumbricoides* has a cosmopolitan distribution affecting about 1.4 billion people (Lukes et al. 2005). The infection by *A. lumbricoides* and *Trichuris trichiura* is one of the most common parasitic associations, probably due to similar transmission cycles and ecological niches occupied. Paleoparasitological studies using microscopy for detecting parasite egg morphology showed that these intestinal parasites are the most commonly found in archaeological material (Bouchet et al. 2003, Gonçalves et al. 2003). Based on data from Gonçalves et al. (2003), the association of *A. lumbricoides* and *T. trichuris* eggs was found in over 80% of European archaeological sites, and in approximately 100% of ancient remains from the Medieval Age. However, *A. lumbricoides* eggs in pre-Columbian South American samples are uncommon. The only finding dated of 4,227 Before Present (BP) was found in the Huarmey Valey site, Peru (Patrucco et al. 1983). In contrast, *T. trichiura* eggs are frequently found, having been identified in seven archaeological sites in Brazil, Chile, and Peru, dated of 8000 - 950 BP (Table). The absence of any morphological characteristics to distinguish *A. lumbricoides* from *Ascaris suum* eggs (parasite of swine) makes difficult the diagnosis in archaeological material, especially those from the Medieval Age when swine had already been domesticated and lived in close relation to humans (Loreille & Bouchet 2003, Rocha et al. 2006). Nuclear and mitochondrial DNA sequences have been used as molecular targets in order to discriminate these two related species (Zhu et al. 1999, Anderson 2001, Peng et al. 2005). However, there is no clear genetic definition yet between the two *Ascaris* species parasitizing humans and those of pigs (Anderson 2001).

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The first molecular paleoparasitological study of *Ascaris* sp. recovered ancient DNA (aDNA) from parasite eggs from samples of the Middle Age site "Place d´Armes" in Namur (XIV century) (Loreille et al. 2001). This successful study was possibly due to a parasite egg high concentration in the latrine coprolites (Loreille & Bouchet 2003). However, this is not the scenery for South American archaeological sites where the findings of *Ascaris* sp. are less frequent and the amount of recovered helminth eggs is low. Recently, Iñiguez et al. (2006) succeeded in recovering *Enterobius vermicularis* aDNA sequences from pre-Columbian samples, opening the possibility of molecular diagnosis using aDNA extracted directly from coprolites. In order to study the temporal and spatial distribution of *Ascaris* in the past, the objective of this work was to develop a methodology of DNA extraction and *Ascaris* molecular diagnosis, sensitive enough to be applied to aDNA extracted directly from coprolites.

Six coprolites samples, which four were positive through microscopy for *T. trichiura*, and all negative for *A. lumbricoides*, were selected for this study (Ferreira et al. 1980, 1982, 1983, Gonçalves et al. 2003). One sample was from Brazilian Northeast (Toca do Meio, National Park of Serra da Capivara, state of Piauí) and four from the state of Minas Gerais, Southeast region (one from Lapa Pequena, one from Boqueirão Soberbo and two from Gruta do Gentio II archaeological sites), and one sample was from Tulán, San Pedro de Atacama, Chile (Table). The human origin of coprolites was suggested by archaeological context and/or confirmed by the finding of *E. vermicularis* and *T. trichiura* eggs, parasites specific to humans (Confalonieri et al 1985, Confalonieri 1988, Iñiguez et al. 2003b). The absence of parasite was considered after examination of forty slides for each sample at magnification of 100 X and 400 X. Precautions to prevent contamination by modern DNA and thus to obtain authentic ancient sequences were followed as described elsewhere (Drancourt & Raoult 2005). Preparation of coprolites samples, aDNA extraction, and polymerase chain reaction (PCR), were carried out at the Paleogenet-

TABLE

Ascaris lumbricoides and *Trichuris trichiura* finds in pre-Columbian South American archaeological sites and morphological and molecular paleoparasitological analysis

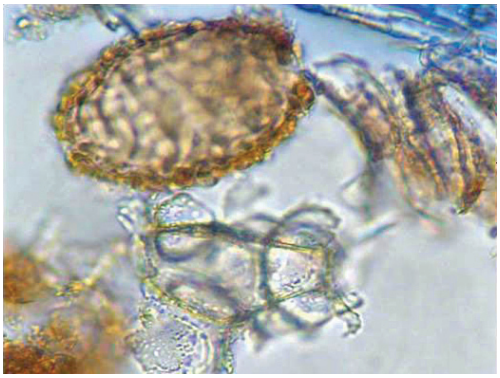
Archaeological sites	Country	Microscopy analysis[a]		Molecular analysis[b]	Date	References
		T. trichiura	*Ascaris*	*Ascaris*		
Toca do Meio, PI	Brazil	–[c]	–	+[e]	8800 ± 60 BP[h]	Gonçalves et al. 2003
Lapa Pequena, MG	Brazil	+	–	+	8000 – 7000 BP	Gonçalves et al. 2003
Boqueirão Soberbo, MG	Brazil	+	–	–	4905 ± 85 – 1325 ± 60 BP	Ferreira et al. 1982
Huarmey Valley	Peru	–	+	NI	4227 ± 181 BP	Patrucco et al. 1983
Santa Elina, MT	Brazil	+	–	NI	4000 – 2000 BP	Gonçalves et al. 2003
Gruta do Gentio II, MG	Brazil	+/–	?[d]	+/–[f]	3490 ± 120 – 430 ± 70 BP	Ferreira et al. 1980, 1983
Tulán, San Pedro de Atacama	Chile	+	–	+[g]	3030 – 2900 BP	Gonçalves et al. 2003
Estrago Cave, PE	Brazil	+	–	NI	2000 BP	Ferreira et al. 1989
Huarmey Valley	Peru	+	–	NI	950 BP	Patrucco et al. 1983

a: examination of 40 slides; *b*: from this study; *c*: parasite eggs absent; *d*: uncertain diagnosis, Ascarididae (Gonçalves et al. 2003); *e*: PCR positive results; *f*: two samples, one PCR positive and one negative; *g*: also positive to *Enterobius vermicularis* (Iñiguez et al. 2006); *h*: BETA 47494. Brazilian states: MG: Minas Gerais, MT: Mato Grosso, PE: Pernambuco, PI: Piaui. BP: before present; NI: Not included in this study.

ic room, physically distant from the Laboratory of Molecular Genetic of Microorganisms (IOC/Fiocruz) where electrophoresis, cloning, and sequencing were performed. The isolation of eggs was conducted at Laboratory of Paleoparasitology (ENSP/Fiocruz), geographically distant to the genetic laboratories. Positive PCR controls with modern *Ascaris* DNA were never included.

The surface of coprolites was removed, and all the samples were ultra violet irradiated (Iñiguez et al. 2003b). The core of coprolites was ground and rehydrated by immersion in a 0.5% aqueous solution of trisodium phosphate for 72 h, following the technique of Callen and Cameron (1960). The aDNA was extracted by physical-chemical treatment. First 150 µl of coprolite sediment were submitted to physical treatment by boiling/freezing, and then proteinase K digestion and phenol/chloroform method extraction were applied as described (Iñiguez et al. 2006). Alternatively, the commercial QIAamp® DNA Stool Mini Kit (Qiagen) was used after physical treatment. In order to verify the presence of PCR inhibitors, human mitochondrial DNA (mtDNA) fragments (92 bp) were also amplified using conditions described by Pääbo (1990). The molecular target for *Ascaris* PCR was a 142 bp fragment of cytochrome b (*cyt* b) mitochondrial gene (Loreille et al. 2001). Re-amplifications and reconstructive PCR reactions were applied when necessary (Golenberg et al. 1996, Iniguez et al. 2003a). After processing all South American coprolites, a sample from Walraversijde site, Belgium (XVI century), which was positive for *A. lumbricoides* by microscopy analyses, was used as positive control (Fernandes et al. 2005). In order to test the sensitivity of this methodology, 30, 50 and 80 eggs were isolated from Walraversijde sample (Figure) and aDNA extraction by physical/chemical treatment was performed. Cloning and nucleotide sequencing of all PCR products were carried out.

The efficiency of the two extractions method employed was quite similar. However, physical treatment seems to be essential to *Ascaris* aDNA extraction. We were successful in retrieving human DNA from all samples, confirming the lack of PCR inhibitors and corroborating their human origin. Four of six samples, which were microscopically negative for of *A. lumbricoides* eggs, were PCR positive for *Ascaris cyt* b segment. The *cyt* b fragment was detected in aDNA extracted directly from coprolite sediment as well as from 30 *Ascaris* isolated eggs. The aDNA sequence analysis of 11 clones samples revealed that all, including from European coprolite sequences, displayed nucleotide T at position 5522, which is characteristic of the *A. lumbricoides* (Loreille et al. 2001), whereas a cytosine (C) is displayed in *A. suum* (Okimoto et al. 1992, access number X54253). New nucleotide substitutions T5492C, C5554G or C5554A, and T5555C were found in aDNA sequences. The sequence translation using the mitochondrial genetic code revealed one synonymous difference (T20) and two nonsynonymous differences at position 41. Clones from *cyt* b protein fragment from Walraversijde-Belgium, Gruta do Gentio II-Brazil, Toca do Meio-Brazil contain the T41S substitution and a clone from Walraversijde-Belgium the T41N substitution (access number EF439709-24).



Ascaris eggs (400X) found in latrine from Walraversijde archaeological site, Belgium, dated of XVI century.

In this study, we demonstrated the ability of a methodology to detect *Ascaris* aDNA without the evidence of parasite eggs and directly from coprolites. The methodology applied was sensitive enough to diagnose the parasite in a sample with only 30 parasite eggs. In spite of few samples analyzed, our results indirectly contributed to design a new panorama of *Ascaris* paleodistribuition, showing that in fact, this parasite has been present in South America since 8,800 years BP in pre-historic populations from Brazil and Chile. Furthermore, our results suggest that the parasitic association *T. trichiura* with *Ascaris*, observed in European historical period and in modern times, also seems to be occurred in South America during pre-Columbian times, possibly with a minor *Ascaris* prevalence. We are currently working to confirm *Ascaris* paleodistribuition, including new South America samples and molecular targets. In addition, we are investigating possible factors influencing *Ascaris* prevalence in pre-Columbian South America, such as pollen of anti-helminths plants and spores of nematophagous fungi.

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