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Short communication

Insights about echinostomiasis by paleomolecular diagnosis

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ABSTRACT

Echinostomiasis is a zoonosis caused by intestinal trematodes and transmitted by the ingestion of mollusks, crustaceans, fish, amphibians, and reptiles, either raw or poorly cooked. Today human infection is endemic in Southeast Asia and the Far East, but has been reported more recently in other regions of the world. Interestingly eggs identified as *Echinostoma* sp. were found in coprolites from a mummified body human in Brazil, dated 560 ± 40 BP (before present). However, the specific diagnosis based on morphology of the eggs has not been resolved at the species level. As a follow-up to the previous finding, the current study now aims to standardize the methodology for molecular diagnosis and apply it to the coprolite, using current *Echinostoma paraensei*-positive feces as the reference, and also the same fecal material dried in a stove as an experimental coprolite model. Isolated eggs of *E. paraensei* and adult worm were included to verify the sensibility and as positive control, respectively. An adult worm of *E. luisreyi* was used for comparison. PCR using *primers in-house* for ITS1 region (126 bp) and *cox*1 (123 bp) of *Echinostoma* spp. and subsequent nucleotide sequencing were performed. This is the first molecular paleoparasitological diagnosis for echinostomiasis. The methodology was able to amplify specific DNA fragments for the genus *Echinostoma* sp. in all samples: adult worm, feces, and a single egg of the parasite, in both the experimental coprolite and archaeological sample. Additionally we observed that ancient DNA can also be retrieved without rehydrating the material. The nucleotide sequences from *E. paraensei* and *E. luisreyi* are very similar in the fragment analyzed that difficult the differentiation these species, but DNA sequence analysis recovered in the parasite found in the mummy showed more similarity with the species *E. paraensei*.

1. Introduction

Parasite infections have accompanied human and other animal species throughout their evolutionary history. Paleoparasitology is the field of science that studies the origins and evolution of parasites and their relation established with their hosts over time [1,2].

Archaeologists excavating a cave in Lapa do Boquete in the Peruaçu Valley in Minas Gerais State, Brazil, found bodies flexed in the fetal position. Estimates dated these burials at 1200 to 600 before present (BP). One of these bodies (burial IV) was found partially mummified, and the date for a rib from this individual was 560 ± 40 BP (BETA 215193) [3]. The ^{14}C measurements are expressed in "years before present (BP)", where "present" is defined by convention as the year 1950. Thus, with "the present" fixed, it is possible to compare radiocarbon dates without needing to know the year in which the dates were calculated.

This partially mummified body was classified as a male. Interestingly, the body presented an accumulation of hardened feces in the intestine, suggestive of megacolon resulting from Chagas disease. Fernandes et al. [4] confirmed that hypothesis by molecular diagnosis.

Sianto et al. [5] found parasites and food remains in the coprolite removed from this same mummy's pelvic cavity. Paleoparasitological examination of the coprolite showed ancylostomid eggs and eggs identified morphometrically as genus *Echinostoma* sp.

Echinostomiasis is a zoonosis attributed to at least 20 species of trematodes. The human hosts, as well as rodents, aquatic birds, and carnivores, are definitive hosts of the parasite [6]. The first intermediate host of the infection is a mollusk, and various animals can serve as the second intermediate host, including gastropods, bivalves, fish, and amphibians. For humans, this is a food-borne infection, transmitted by ingestion of the second intermediate host. Human infection is endemic in Southeast Asia and the Far East, encompassing China, Taiwan, India, Korea, Malaysia, Philippines, and Indonesia [7,8].

In Brazil, the gastropod *Biomphalaria glabrata* has been found naturally infected with *E. paraensei* in the State of Minas Gerais, while

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its definitive host includes a rodent with semi-aquatic habits, *Nectomys squamipes*, or scaly footed water rat. This parasite infection has been reported consistently in these animals in Southeast Brazil, especially in the states of Rio de Janeiro and Minas Gerais [9].

Microscopic examination, based on morphological and morphometric analysis, had already allowed the diagnosis of parasitism by trematodes of genus *Echinostoma* in the coprolite from the mummified body. Molecular technology was now used to refine the diagnosis and reach the species-specific diagnosis. Therefore, the purpose of this study was to establish and standardize a molecular diagnostic methodology applied to modern fecal samples. Next, *Echinostoma*-positive experimental coprolites were used to standardize the specific diagnosis to be applied to archaeological material, since there was no previously existing methodology for the paleoparasitological molecular diagnosis of this helminth infection.

2. Materials and methods

2.1. Samples

The archaeological sample used here was a fragment of the coprolite from the partially mummified body (burial IV) dated 560 ± 40 BP, found in the Peruaçu Valley, Minas Gerais State, Brazil, and deposited in the Paleoparasitology Collection at ENSP-FIOCRUZ [4,5]. Standardization of the methodology employed in the ancient material used modern samples: one adult worm, uterine eggs (1–5 eggs), and feces from experimental infection of *Mesocricetus auratus* (hamsters) with the species *E. paraensei* from the Laboratory of Wild Mammal Reservoir Biology and Parasitology/IOC-FIOCRUZ. DNA from *E. luisreyi* one adult worm was also used for sequencing and comparisons.

2.2. Prior treatment of samples

2.2.1. Eggs

For the *E. paraensei* analysis, 1, 3, and 5 eggs were isolated and obtained by dissection of the uterus of adult worms placed in 0.2 ml micro-tubes containing 5 μl of H_2O Milli-Q, and these were submitted to three thermal shock cycles (liquid nitrogen/boiling for 2 min) to break the egg shells as previously tested by our research group with other parasites [10].

2.2.2. Adult worms

One adult worm was macerated in liquid nitrogen, and distilled water was then added to remove 200 μl of the macerated product.

2.2.3. Fecal samples

The fecal samples were diluted in distilled water and then submitted to spontaneous overnight sedimentation at 4 °C, and 200 μl of the sediment was transferred to a 2 ml micro-tube, which was submitted to the same thermal shock as the eggs.

2.2.4. Experimental coprolite

With the aim of reproducing the conditions of desiccation that the eggs underwent over the years in the coprolite found inside the mummy, and to evaluate the effect of this desiccation on the molecular diagnosis, an experimental coprolite was prepared. A recent fecal sample containing the parasite's eggs was dried in an oven at 37 ºC, weighing it daily until there was no longer any weight loss. Rehydration was performed in Na_3PO_4 at 0.5% for 72 h, followed by spontaneous sedimentation for 48 h at 4 °C as recommended for coprolites. A 200 μl sample was removed from this sediment and placed in a 2 ml micro-tube, which was submitted to the same thermal shock described earlier.

2.2.5. Archaeological sample

Two aliquots of sediment (~200 mg each) were separated from the archaeological material, one was rehydrated and the other non-rehydrated (we decided to make an aliquot not rehydrated to verify if this could influence the yield of ancient DNA). Both were submitted to the same procedures performed with the experimental coprolite.

2.3. DNA extraction

2.3.1. Eggs

DNA extraction from the eggs consisted only of the thermal shock described earlier.

2.3.2. Adult worms, fecal samples, and experimental coprolite

DNA extraction followed the manufacturer's suggested DNA extraction protocol for fecal samples (Kit QIAamp DNA Stool - QIAGEN®), with the following modifications: the samples were submitted previously to thermal shock or were macerated in liquid nitrogen. The digestion phase with proteinase K was performed at 60 ºC and incubated under agitation for 2 h, and the final elution was performed with 50 μl.

2.3.3. Archaeological sample

Both aliquots from the archaeological material were extracted in a separate environment from that in which the assays were performed with the modern material. A 45-day interval was left between the experiments with the modern and ancient materials. To attest the authenticity of the results from extraction of the ancient DNA, a negative DNA extraction control was monitored, which was left open throughout the entire time in which the experiments were performed and underwent the same stages as the archaeological material. The same extraction applied to the experimental coprolite was performed on the archaeological material.

2.4. PCR

2.4.1. Description of primers

The *primers* were designed using the Primer-Blast, available at: www.ncbi.nlm.nih.gov/tools/primer-blast. For *Echinostoma* sp., *primers* were designed to amplify a fragment of the ITS1 (Internal Transcribed Spacer 1) region: forward ECHF: 5′-GCCGAAATCCTAAYCTGGCA-3′ and reverse EHCR: 5′-AYACGGGTAGGCACCCAGTC-3′, which amplify a 126 bp fragment, and to amplify a fragment of the mitochondrial gene cox1 (cytochrome oxidase 1), the following were used: forward Ech-cox1-123F 5′-ATTGTGTGTTTGGGTAGTGT-3′ and reverse Ech-cox1-123R 5′-MACCTTWATACCCGTTGGAA-3′, which amplify a 123 bp fragment.

2.4.2. PCR conditions

In the PCR for modern material the DNA extracted from the adult worm was used as a positive control in all the reactions with the modern material. Initially, two PCR conditions were tested, and all the PCRs were accompanied by a negative control. Each reaction was prepared in a final volume of 50 μl (buffer 1×; Mg (3 mM or 1.5 mM); 0.2 mM dNTP; 100 ng of each primer; 2 U taq *Platinum* — Invitrogen), and 3 μl of DNA. The reactions were performed in a programmable thermocycler using an initial 5-min cycle at 94 ºC followed by 35 cycles 94 ºC for 20 s, 55 ºC for 20 s, and 72 ºC for 30 s, and extension for 7 min.

In the PCR for the archaeological sample, initially to verify whether there was any inhibition, a PCR was performed for human target using the hypervariable segment 1 (HVS1). Having verified the absence of inhibiters, the experiment proceeded with the target for the parasite *Echinostoma* sp. The PCR was performed with a final volume of 50 μl under the same conditions described earlier, except for the amount of enzyme (2.5 U) and DNA (5 μl). The cycling conditions were the same, except that the number of cycles for the archaeological material was 45. All the PCR reactions were accompanied by a negative control (PCR and extraction). The PCR products were submitted to agarose gel electrophoresis at 3%, stained with ethidium bromide, and viewed under an ultraviolet (UV) transilluminator and subsequently photographed.