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Methodological innovations for the study of irreplaceable samples reveal giardiasis in extinct animals (*Nothrotherium maquinense* and *Palaeolama maior*)

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ABSTRACT

The use of diagnostic methods that prevent irreplaceable samples (from museum collections, archaeological and paleontological samples) of being consumed or that increase their yield is relevant. For museum collections, archaeological and paleontological samples it is essential to conserve samples, subsamples or portions for future research. We are addressing methods for conservation of irreplaceable samples that could be fully consumed. Innovations in methodologies that are used in studies of Paleoparasitology and Paleomicrobiology will contribute to the preservation of collections. Therefore, to the development of archaeology and paleontology in the future, we evaluated whether the discarded material of the immunochromatography test could be used for molecular diagnosis and vice versa. We used a genotyped experimental coprolite positive for *Giardia duodenalis*. The diagnosis was positive for giardiasis in both cases. This methodology can be corroborated with the coprolite of a *Paleolama maior* (extinct llama) previously diagnosed for *G. duodenalis* with an immunoenzymatic test. The residue of the pre-digestion step of the DNA extraction before adding Proteinase K was confirmed positive with the immunochromatographic test. Also, the DNA extraction residue from a coprolite of *Nothrotherium maquinense* (ground sloth) was tested positive with immunochromatographic test for *G. duodenalis*. These are the oldest findings for *G. duodenalis* confirming that this intestinal parasite occurred among Northeastern Brazilian Megafauna animals from the late Pleistocene period, correlated to human occupation. The relevance of these results will allow the study by different methodological approaches from a small amount of material, reusing discarded materials.

1. Introduction

Apart from challenges to legitimate parasite and microorganism findings in archaeological and paleontological samples, there is the issue with the rarity and small quantities available for research. By rarity we mean samples that are irreplaceable that come from unique context. Thus, methodological innovations that aim for material preservation and optimization using the same sample aliquot are needed for the future archaeology and paleontology. Paleoparasitology and Paleomicrobiology have demonstrated the occurrence of several pathogens of the past in humans and also extinct host species [1]. Optical microscopy was the methodology used in most of these findings due to the age of the material, and in some cases the only one capable of detecting any parasite vestige, especially those that lived in the remote past, such as the dinosaurs. Research analyses by Poinar Jr. [2–4] have detected in hematophagous insects preserved in amber parasitic structures identified as primitive forms of *Trypanosoma* sp., *Leishmania* sp., and *Plasmodium* sp. by microscopy. These hematophagous insects probably fed on extinct animals, some of them since the early Cretaceous period. Researchers have identified *Eimeria macusainiense* oocysts in *Paleolama* coprolites by microscopic analysis and because of the size difference in the oocysts, inferences were made about the evolution of

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parasitism in camelids during the period transition from Pleistocence to Holocene [5, 6]. In the 50s, Ringuelet [7] described nematode eggs from coprolites of *Myladon histai*, the ground sloth of Chilean Patagonia; Schimit et al. [8] described helminth eggs and protozoa cysts of *Nothrotheriops shastensis* from Rampart Cave, USA.

Other methodologies have also contributed to this research area, such as the isolation of *Mycobacterium tuberculosis* ancient DNA (a DNA) from a 17 thousand yr. old extinct bison in North America revealing the history of this bacteria [9]. Nunes et al. [10] have detected *Cryptosporidium* sp. in coprolites of an extinct caprine (*Myotragus balearicus* Bate, 1909) of Mallorca region by immunoenzymatic methods. However, as of today science is in an era that it is not possible to exhaust rare archaeological or paleontological samples for one research topic or technique. In many countries samples are deposited in scientific collections and in some cases, they are rare samples that are to be preserved. Even with new generation methodologies for the study of parasites and microorganisms in which little material is used, these technologies are still destructive and many times the aliquot for that experiment is exhausted. The objective of this research was to evaluate whether the discarded material of the immunodiagnostic could be used for molecular diagnosis and vice versa. We experimented with fresh samples and experimental coprolite samples, and lastly in extinct animal coprolites using as model the zoonotic protozoa *Giardia duodenalis*.

2. Material and methods

2.1. Samples

Fresh modern feces, already identified for the presence of *Giardia duodenalis* by optic microscopy, were previously genotyped by the specific target B-giardin. DNA extraction and PCR (Polymerase Chain Reaction) conditions were performed following Sudre et al. [11] and Alves [12]. We included in the experiment modern fecal samples of all genotypes available in the laboratory: human samples (n = 2, A and B genotypes); feline (n = 1, F genotype); sheep (n = 1, E genotype) (Fig. 1). Ancient samples include: Two coprolites from *Palaeolama maior* (sample code A364 and A520) and a coprolite from extinct Ground Sloth *Nothrotherium maquinense* (A121) provided by the Collection of Coprolites and Paleoparasitological Materials Luiz Fernando Ferreira, Fiocruz, Brazil. Samples A364 and A520, from *Paleolama maior*, were previously tested with immunoenzymatic method following the methodology described by Gonçalves et al. [13], in which 100 µl of the sediment were used with a commercial kit (MEDIVAX). Sample A364 was tested positive for *G. duodenalis* with a value of 0.247 (cut-off 0.08) [14].

2.2. Methodology applied to fresh samples and experimental coprolites

We applied a rapid immunochromatographic test strip for the qualitative antigen detection of *G. duodenalis* as indicated by the manufacturer to be applied in human feces (Giardia Eco Teste®, Eco Diagnóstica, nova Lima, Minas Gerais, Brazil). As our final goal was to study coprolites of either human or animal origin it was necessary to verify test sensitivity for detection of *G. duodenalis* despite the genotype or sample origin; due to the possibility of other zoonotic types other than A and B, such as specie-specific animal genotypes. Thus, we evaluated all fecal samples (human, genotypes A and B; feline, genotype F; and sheep, genotype E) following the manufacturer protocol that recommends 100 µl sample without a pre-heating process. We replicated the sheep fresh feces in order to evaluate a protocol modification with a pre-heating process of 40 °C for 10 min. Subsequently, a total of four aliquots of the sheep sample were prepared for dehydration to be turned into experimental coprolites. The experimental coprolites were prepared by mimicking the desiccation process, a taphonomic process responsible for coprolite conservation of humans and extinct animals. Experimental coprolites remained at the oven with temperature variations of 38–44 °C during few hours per day. Samples were weighed weekly until completely dehydration. Also, in order to analyze the increase in sensitivity for antigen detection 200 µl of the sheep experimental coprolite sample were added instead of the 100 µl

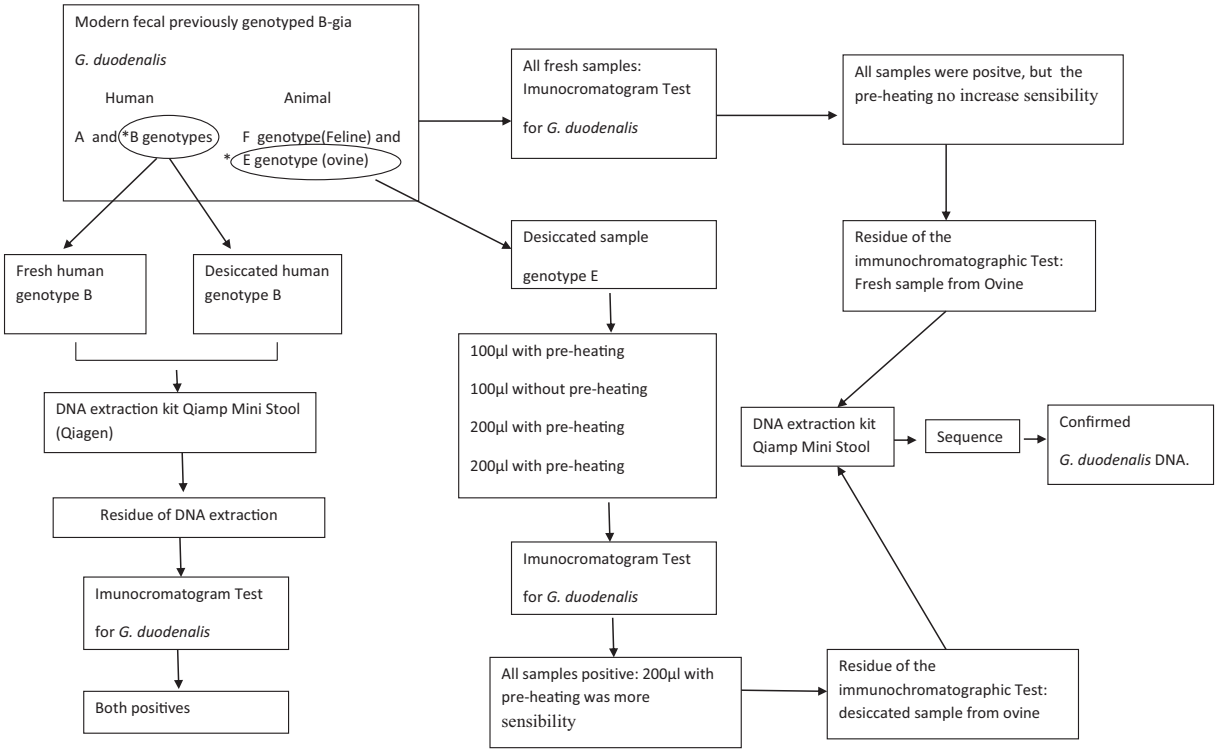

Fig. 1. Description of the experiments with fresh samples and experimental model for evaluation of sample re-usage with both immunochromatographic and molecular methods.

recommended by the protocol. Our goal was to check if the immunochromatographic test would work in desiccated samples and if protocol modifications could increase test sensitivity; then we would attest if the residue of the leftover material of this test could be used in molecular analysis.

We selected 100 μl of leftover sample residue (buffer and sample) previously tested with immunodiagnostic kit of both fresh and experimental desiccated sheep leftover sample. Next, a DNA extraction (QIAmp DNA Stool Mini Kit - Qiagen) of these leftover sheep samples was performed with protocol modifications by Leles et al. [15] and PCR for *G. duodenalis* with target B-giardin [11, 12]. Amplicons were purified and sequenced in both forward and reverse sequences with ABI-Prism 3130 Sequencer (Applied Biosystems) by Genomic Platform-DNA Sequencing-RPT01A/Fiocruz.

Following, we tested the reverse: if the residue of DNA extraction could be used with the immunochromatographic kit. The residue from the DNA extraction consisted in a mixture of feces and buffer. The buffer excess was removed and its supernatant (approximately 200 μl) that would normally be discarded was used with the immunochromatographic kit. DNA was extracted of two aliquots of human sample, B genotype, one freshly processed and the other previously desiccated in the oven, as described above. After the first step of DNA extraction (QIAmp DNA Stool Mini Kit - Qiagen) before adding Proteinase K, the discarded residue was used for immunochromatographic assay. Manufacture's protocol was followed with modifications: a total of 200 μl of sample was used with previous heating process. We added 1 ml of buffer from the immunochromatographic kit to 200 μl of the DNA extraction residue. The mixture was homogenized and after the decantation of particles we pipetted 150 μl to a new tube in order to test the immunochromatographic strip (see Fig. 1 for experimental design synthesis).

2.3. Methodology applied to extinct animal coprolites

The total DNA of coprolite samples: A364, A520b and A121 was extracted for analyses that would be performed in other laboratory facility and were not part of this study; therefore, samples and DNA are not available in our laboratory. However, due to the rarity of these samples we kept frozen the first step residue of DNA extraction before applying Proteinase K, usually discarded. For the immunochromatographic assay we used 200 μl of each sample residue, heating for 10 min in 40 °C. All methodologies applied with ancient samples were performed in a laboratory used only for paleoparasitological analyses.

3. Results

All fresh samples tested with immunochromatographic test presented the positive control line, showing that the test works for different sample origins; also, all samples presented the antigen presence line for *G. duodenalis* despite the genotype, being able to detect not only zoonotic A and B genotypes but also specie-specific such as F and E found regularly in felines and ruminants. There was no increase in sensitivity observed by the pre-heating process in the replicate fresh sheep sample. In experimental coprolites there was efficient antigen detection by the immunochromatographic test of *G. duodenalis*. However, the intensity of the strip was higher with the pre-heating step and high quantity sample (Fig. 1).

The DNA extracted from the residue of the immunochromatographic kit, composed of buffer and sheep sample resulted in amplification for the detection of *G. duodenalis*. The PCR product was sequenced and genotype E of *G. duodenalis* was confirmed by sequence analysis of both fresh and desiccated samples. However, the desiccated sample presented nucleotide differences that resulted in a non-synonymous mutation when translated to amino acids (Figs. 2, 3). When we compared the fresh sample to the previous genotyped and not tested with immunochromatography assay sample, we observed identical sequences. Moreover, *G. duodenalis* was detected in the DNA extraction residue (digestion buffer and human sample genotype B) in both fresh and desiccated aliquots (Fig. 1). The extinct animal coprolite residue from DNA extraction showed positivity for the *Paleolama maior* sample, A364 already positive for ELISA (Enzyme Linked ImmunonoSorbent Assay) in a previous study [13]; and also for the extinct Ground Sloth *Nothrotherium maquinense*, A121.

4. Discussion

Studies that recovered a DNA from ancient hominids and extinct animals of Pleistocene sediments showed the importance to study “discarded” materials [16]. Vast amount of museum and collection materials have been studied for a long time by different methodological approaches [17–21]. Besides the rarity of the material, in some countries it is bureaucratic to access and analyze it, especially when the methodology involves in its destruction, the quantity is minimum to analyze, or as stated by Gibbons [22]: these studies are not a priority, and receive less funds for research. These circumstances not only reflect on research advance in some countries, especially with new generation sequencing methodologies, but also on the number of publications.

Even in other countries that do not consider this research as a priority there are paleontological and archaeological sites of worldwide importance. Therefore, finding low cost methodologies that would add information and prevent rare samples from being exhausted or that increase their yield is relevant for this research area, especially in countries for example in South America, where New Generation Sequencing (NGS) technology such as metagenomics is not available for all researchers.

Nowadays, immunological tests for the detection of coproantigens in clinical samples are more popular, for example for the detection of *G. duodenalis*. This intestinal protozoa of fecal and oral transmission is considered zoonotic and leaves in the host's feces the resistance structure that propagates the infection to other hosts as it occurs in the environment. However, most tests were created for the study of human or animal feces, but not for both. Until now, 8 genotypes are known for *G. duodenalis* (A-H): genotypes A and B are considered zoonotic; genotypes C—H that are specie-specific to some animal groups.

In this study we did not have all 8 genotypes available for the analyses. Except for genotype F, usually detected in felines and available for analysis in this study, we prioritized those that could be present in extinct animal coprolites. These would include: zoonotic genotypes A and B; genotype E, detected in ruminants and camelids and therefore present in coprolites of *Paleolama maior*. However, recently this genotype (E) was identified in children from impoverished communities from the State of Rio de Janeiro, Brazil [23]. Thus, nowadays there are predicaments in establishing specie specific genotypes, allowing the possibilities of cross transmission of, what seemed as, unlikely genotypes.

Although some studies used immunodiagnostic kits designed for human samples in animal samples, and vice versa [24, 25], (acknowledging that some kits´ protocols do not specify whether it is for animal or human samples), there is a lack in most studies about the association of the genotypes of the parasite in the sample. In this study it was important to verify the sensitivity of the immunochromatographic kit for the different *Giardia* genotypes, especially A, B and E, due to the reason that it was not possible to know which ones would be present in ancient samples, since they were already used up.

Our research has shown that immunochromatographic test is an important tool in diagnosing positive samples for *G. duodenalis* for both human and animal, being sensitive for genotypes A, B, E and F. Previous immunoenzymatic and immunofluorescence analyses in archaeological samples have detected intestinal protozoa, including *G. duodenalis* [26], but not by immunochromatographic tests. Additionally, in these studies most samples are of human origin, some are of animal origin, but rarely from extinct animals. Nunes et al. [10] showed the potential of

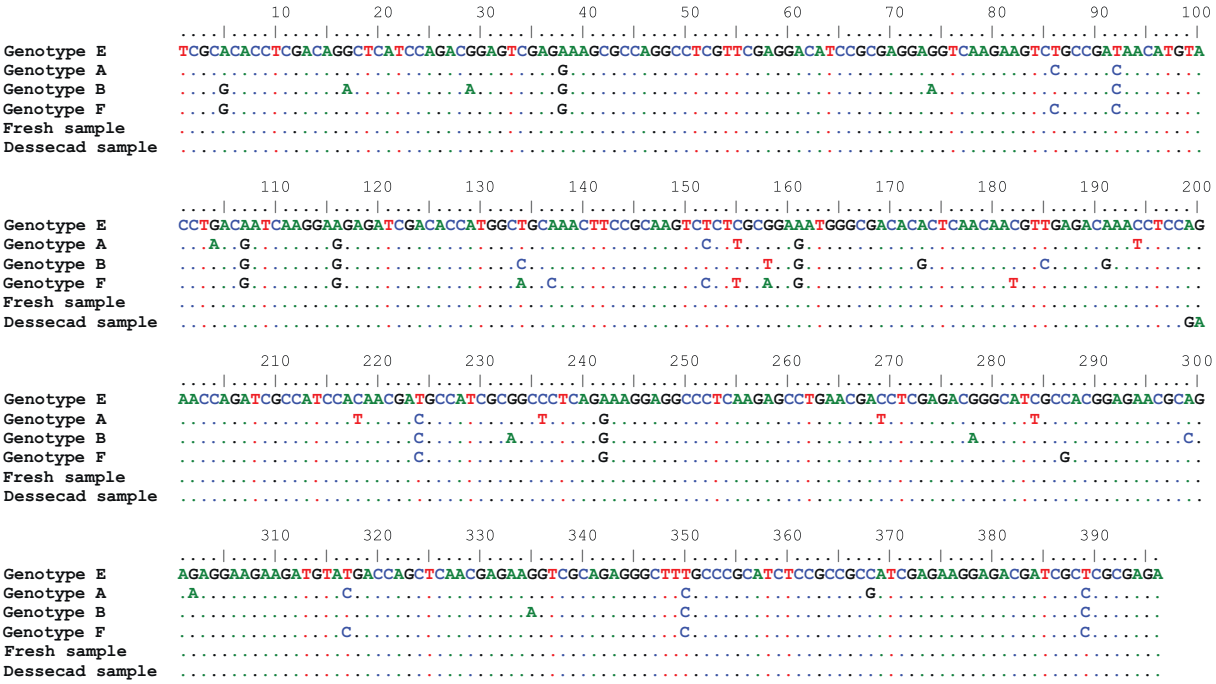

Fig. 2. Nucleotide sequences resulted from B-giardin target for the detection of *Giardia duodenalis*.

immunodiagnostics for the study of extinct animal coprolites in detecting *Cryptosporidium* antigens in *Myotragus balearicus* Bate 1909, and hypothesized the introduction of these parasites by humans due to its zoonotic characteristic.

In this study we used samples from the same archaeological site of the northeast region in Brazil of two different host species, both positive for *G. duodenalis*, which one sample was previously diagnosed by other immunological method - ELISA. Even though it is not possible to verify the genotypes of *Giardia* in the samples, the immunochromatographic assay has shown the presence of *G. duodenalis*, a zoonotic parasite, among extinct animals. The result shows that it was probable that this parasite circulated among megafauna animals, a period in which humans inhabited that area. Also, *G. duodenalis* was not extinct with the megafauna animals but adapted to several other host species, even though there were several climate changes in that area.

Besides the evolutionary implications of this study we point out the innovation of the methodology used in this research by using commonly used commercial kits in research. When Slon et al. [16] was able to recover a DNA from hominids and extinct animals in cave sediments, a door was opened: there is a possibility to make a difference for future archaeology and paleontology. With the same goal that is to promote methodological innovations already used in Paleoparasitology and Paleomicrobiology such as immunological and molecular diagnosis, we were able to demonstrate that it is possible to optimize sample usage. The leftover material of immunochromatographic assays for the detection of parasites that would normally be discarded still contains DNA and therefore can be used for molecular diagnosis, as demonstrated by the fresh and experimental coprolite samples. We reiterate that nucleotide alterations observed were not detected in the fresh sample tested with immunochromatographic assay, presenting an identical DNA sequence from the database for this sample before the test. Therefore, nucleotide alterations that resulted in a non-synonymous amino acids were probably caused by desiccation process, calling attention to the fact to the time the sample spent in the oven when compared to “in situ” condition. Still, even with alterations the diagnosis was possible.

Also, the leftover material of the pre-digestion step of DNA extraction contains antigens of parasites and can be used in immunochromatographic assays, as demonstrated for fresh and experimentally desiccated samples, and also for the extinct animal coprolites.

5. Conclusions

In this study we were able to extract DNA, amplify and confirm by sequencing *G. duodenalis* with the leftover material of the immunochromatographic kit; and also, to detect parasite antigens with the immunochromatographic assay using the leftover material of the DNA extraction method in the experimental model. The efficacy of the methodology was corroborated and validated when using the DNA extraction residue of *Paleolama maior* and ground Sloth *Nothrotherium maquinense* coprolites by detecting antigens of *G. duodenalis*. Moreover, this is the oldest record of *G. duodenalis* in samples of extinct animals of the late Pleistocene period that could have zoonotic potential at that time, due to the presence and occupation of humans.

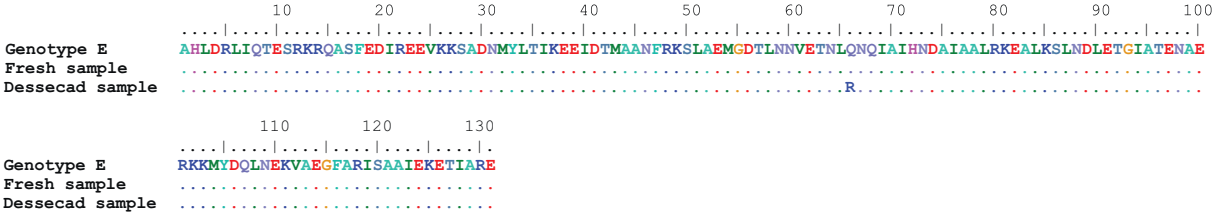

Fig. 3. Aminoacids translation of the nucleotide sequences for the B-giardin target of *Giardia duodenalis*.

We call attention to the validation of this methodology in the experimental model; also, we have shown the presence of the protozoa in both *Paleolama major* and *Nothrotherium maquinense* by immunodiagnosis in a material that would be discarded. Although there are limitations in this study, due to the reason that it was not possible to test the presence of a DNA in the extinct animals' coprolite residue leftover from the immunochromatography by molecular analysis, these results are relevant especially for rare and small quantity samples, as it amplifies sample usage by different methodological approaches. The samples from the extinct animals were exhausted and therefore nucleotide sequences were not recovered from *Giardia duodenalis*, thus it is not possible to identify the genotype of the parasite.

Although the methodology we used is very distinct from the new generation technologies described by Slon and colleagues [16], the common goal is not to “throw out the baby with the bath water”, but to use all the water we can get.

Conflicts of interest

To the best of our knowledge, there is no conflict of interest.

Acknowledgements

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