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Amoebiasis distribution in the past: first steps using an immunoassay technique

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Summary The identification of parasites in ancient human faeces is compromised by differential preservation of identifiable parasite structures. However, protein molecules can survive the damage of the environment and can be detected even after centuries. In this paper it is shown that is possible to detect copro-antigen of *Entamoeba histolytica* in historic and prehistoric human faecal remains, using a commercially available enzyme immunoassay (ELISA) kit. The kit uses monoclonal antibody-peroxidase conjugate specific for *E. histolytica* adhesin. A total of 90 specimens of desiccated faeces found in mummies and ancient organic sediment from South America, North America, Africa, and Europe were examined. The ELISA detected 20 positive samples, dated to about 5300 years before present to the 19th Century ad. The positive samples are from archaeological sites in Argentina, USA, France, Belgium, and Switzerland. The detection of protozoan antigen using immunoassays is a reliable tool for the studies of intestinal parasites in the past.

1. Introduction

Different species of parasites have been found in preserved human faecal material from historic and prehistoric times throughout the world. Many findings of nematodes, trematodes, cestodes and acanthocephalan eggs in archaeological material are available in the literature. A review of the paleoparasitological findings has been made by Reinhard et al. (1986), Reinhard (1990) and Goncalves et al. (2003). Such findings have been made possible through the study of organic sediment from ancient cesspits and desiccated faecal material collected from mummies or found during archaeological excavations. Desiccated faeces are called coprolites. Paleoparasitology is the study of parasites in such archaeological material.

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Protozoan detection in coprolites, on the contrary, is not a frequent finding in paleoparasitology (Reinhard et al., 1986; Goncalves et al., 2003). Unlike helminth eggs, cysts quickly decay in the environmental conditions of archaeological sites, and are very difficult to find by direct microscopic examination.

However, protozoan antigens remain detectable for longer periods of time. Faulkner et al. (1989) and Allison et al. (1999) succeeded in finding *Giardia duodenalis* antigen using immunological techniques in material as old as 2177 and 3000 years respectively. The use of enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies has been useful in the detection of protozoan copro-antigen in ancient faecal samples. Goncalves et al. (2002) detected *Giardia* Specific Antigen 65 (GSA65) in coprolites and latrine sediments dated from 1200 ad to 1700 ad.

Several studies have found ELISA to be a tool with very high sensitivity and specificity for *Entamoeba histolytica* copro-antigen detection in fresh faeces (Haque et al., 1995, 1998; Pillai et al., 1999; Garcia et al., 2000; Haque et al., 2000; Sharp et al., 2001). We conducted a study to assess the feasibility of the detection of *E. histolytica* in ancient human remains using a commercially available enzyme immunoassay. The kit uses monoclonal antibody to detect specific *E. histolytica* adhesin in faecal samples.

2. Materials and methods

2.1. Specimens

A total of 90 coprolites and sediment samples from South America, North America, Africa, and Europe were tested for *E. histolytica* adhesin antigen. Their origins are listed in Table 1. The samples have been dated either by ^{14}C method or by cultural context. All samples used were rehydrated by immersion in a 0.5% trisodium phosphate aqueous solution for 72 h (Callen and Cameron, 1960). Raillet—Henry solution

Table 1 Country, archaeological site, type and number of samples, age, and positive results[a] for *Entamoeba histolytica* antigen

Country	Archaeological site	Type of sample	Number of samples	Age	Number of positive samples
Argentina	Fortin Minana	cesspit A	7	19th Century ad	7
		cesspit B	4	19th Century ad	2
	Neuquen	coprolite	2	1000—500 BP[b]	0
Belgium	Namur	cesspit	4	9th—11th Century ad	0
		cesspit	3	14th Century ad	1
		cesspit	2	15th—17th Century ad	0
		cesspit	3	18th Century ad	1
Brazil	Serra da Capivara	coprolite	7	8760 BP[b]	0
	São Domingos	coprolite	1	6000—5000 BP[b]	0
	Santa Elina	coprolite	1	4000—2000 BP[b]	0
Chile	San Pedro de Atacama	coprolite	7	3080—2950 BP[b]	0
	Iquique	coprolite	1	6110—3950 BP[b]	0
France	Castillon-du-Gard	cesspit	14	3rd Century ad	2
	Gresine	sediment	5	2500 BP[b]	1
	Montbeliard	cesspit	1	15th—16th Century ad	0
Germany	Lubeck	cesspit	1	15th Century ad	0
Sudan	Sai	coprolite	5	2700—2330 BP[b] and 1700—500 BP[b]	0
Switzerland	Arbon	sediment	5	5300 BP[b]	3
USA	Canyon De Chelly	cesspit	4	1100—800 BP[b]	0
		cesspit	13	800—700 BP[b]	3

[a] Adjusted optical density $\geq$0.050.
[b] BP, before present.

(920 ml NaCl 0.8%—30 ml formalin 40%—50 ml glacial acetic acid) was added to each rehydrated sample in a proportion of 1:10 to avoid fungal and bacterial growth. All samples were microscopically examined for parasite remains following standard techniques.

2.2. Enzyme-linked immunosorbent assay

The *E. histolytica* II assay (TechLab, Blackburg, VA, USA) was used according to the manufacturer's directions. Each test well was inoculated with 400 μl of sediment from a rehydrated sample solution. Results were interpreted by visual inspection and by a spectrophotometer. A test sample was considered positive if it had an obvious yellow color when compared to the negative control well. A test sample was considered negative if it was colorless or less yellow than the negative control well. The absorbance of each specimen was measured at 450 nm wavelength. The values were adjusted by subtracting the optical density of the negative control from the optical density of the samples. According to the instructions, samples with adjusted optical density $\geq$0.050 were considered positive.

3. Results

A total of 20 samples (22.2%) were positive for *E. histolytica* antigen. The positive immunoassay results are shown in the Table 1. Visual and spectrophotometer readings were concordant in all samples. All samples but 1 were negative for protozoan cysts by microscopy. As previously reported, *G. duodenalis* were found in 1 sample from the archaeological site of Namur, Belgium (Goncalves et al., 2002).

4. Discussion

The former species *E. histolytica* was separated in 1993 into two morphologically identical species, *E. dispar*, a non-pathogenic intestinal parasite and *E. histolytica*, that can cause dysentery and extra-intestinal disease in humans (Diamond and Clark, 1993).

Although microscopy can not differentiate between these two species, isoenzyme analysis and specific molecular techniques are able to distinguish *E. histolytica* from *E. dispar* (Haque et al., 1998; Huston et al., 1999). Molecular techniques include polymerase chain reaction (PCR) and detection of *E. histolytica*-specific antigen using enzyme-linked immunosorbent assay. Antigenic differences between the lectins of each species make possible the differentiation. Stool amoebic lectin detection by ELISA has been shown to be a cost-effective and rapid method for detection of *E. histolytica* in stool specimens (Huston et al., 1999; Haque et al., 2000).

We used a commercially available immunoassay to detect an amoebic adherence lectin that is inhibitable by D-galactose (Gal) or N-acetyl-D-galac - tosamine (GalNAc). This high immunogenic molecule is a major amoebic surface protein (Petri et al., 1988; Gaucher and Chadee, 2002). Antigenic differences in Gal/GalNAc lectins of *E. dispar* and *E. histolytica* make possible the specific diagnostic by using monoclonal antibodies (Huston et al., 1999). The kit uses a monoclonal antibody against the *E. histolytica* adhesin. The sensitivity and the specificity of the method varies from 96.9% to 100% and 94.7% to 100%, respectively, when compared with culture of stool samples and isoenzyme analysis, according to the manufacturer. Haque et al. (2000), in a cross-sectional study, report that the kit identified all 16 of the culture-positive samples in a 1164 preschool children, as well as 34 negative culture samples. Using a PCR technique, they detected *E. histolytica* in 27 of these 34 samples.

According to the kit manufacturer, the immunoassay is able to detect approximately 0.2 to 0.4 ng of adhesin per well. The protein epitopes from the *E. histolytica* lectin can remain stable for at least 5300 years, as assessed by our findings from Switzerland. However, caution should be exerted in the interpretation of negative results in ancient samples as antigen structure may be compromised due to uncontrolled environment conditions.

Beck et al. (2002) have demonstrated genetic conservation of the Gal/GalNAc lectin between *E. histolytica* isolates from Bangladesh and Georgia. They argued that *E. histolytica* could be a clonal population. Similar studies using samples five thousand years apart could clarify such evolutionary questions.

We could detect *E. histolytica* antigen in human faecal residues from a wide array of times throughout the world, reflecting the ubiquity of this parasite. *E. histolytica* can infect apes and other primates. However, it is unlikely that we confused non-human primate faeces with human faeces since humans are the only primates that inhabited the study areas in Europe and North America.

In the New World, we detected positive samples in cesspits from Fortin Minana (Argentina), a small military settlement at the countryside in Buenos Aires Province, used during the second half of 19th century. Samples from cesspits of Anasazi culture, Canyon De Chelly (USA), dated from 1200 to 1300 ad, were also positive.

In the Old World, we detected positive samples from cesspits of a roman villa called La Gramiere, at Castillon-du-Gard, France, occupied from the Roman Period until the High Medieval Period, and also from medieval latrines from Place D'Armes, in Namur, Belgium. *E. histolytica* antigen was also found in organic sediment of a Neolithic settlement close to Lake Constance, in Arbon, Switzerland, and from a Bronze Age settlement in Bourget lake, Gresine, France. References about the archaeological sites can be found in the paleoparasitological review of Goncalves et al. (2003).

Although a very common parasite worldwide, the finding of amoebic cysts in ancient human faeces is very rare, probably due to a faster decay than helminth eggs and larvae. The utility of this technique, combined with a new generation of molecular methods, will reveal the temporal and spatial distribution of protozoan parasites in ancient times.

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