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Detection of *Giardia duodenalis* antigen in coprolites using a commercially available enzyme-linked immunosorbent assay

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Abstract

The objective of this experiment was to assess the utility of a commercially available enzyme-linked immunosorbent assay (ELISA) kit for diagnosis of giardiasis in archaeological human remains. The kit, a monoclonal antibody assay, is used to detect the presence of *Giardia*-specific antigen 65 (GSA65) in human faeces. We utilized the assay in ancient faecal material. The material included desiccated faeces found in mummies or in archaeological sites, and sediments from latrines. A total of 83 specimens, previously examined microscopically for parasites, were examined. The ELISA detected 3 positive samples, dated to about 1200 AD, 1600 AD and 1700 AD. The ELISA was superior to direct observation. It was possible to identify *G. duodenalis* cysts by direct microscopy in only one of these samples. The results did not show cross-reactivity between this protozoan and helminths. The use of ELISA to detect *G. duodenalis* coproantigen could help the diagnosis of giardiasis in ancient human remains.

Keywords: giardiasis, diagnosis, ELISA, paleoparasitology, coprolites, ancient faeces

Introduction

Paleoparasitology is the study of parasites in archaeological material. Normally, paleoparasitologists in the Americas analyse coprolites which are desiccated faecal material found in mummies or in archaeological sites. Detection of protozoa in coprolites poses a challenge to paleoparasitology. Unlike helminth eggs and larvae, protozoan cysts are not so resistent to the environmental conditions of archaeological sites. The vulnerability of cysts to decay may result in artificially low estimations of protozoa in paleoparasitology as indicated by the infrequent finding of protozoan cysts in coprolites compared to helminth eggs (REINHARD *et al.*, 1986). Also there is considerable day to day variation in cyst excretion that decreases the sensitivity of conventional coprolite examination. A single microscopical examination of fresh faeces from patients infected with *Giardia duodenalis* (syn. *G. lamblia, G. intestinalis*) yields false negative results in up to 50% of patients (BURKE, 1977; WOLFE, 1978). Therefore, it is particularly difficult to identify *G. duodenalis* in archaeological sites.

Some *G. duodenalis* antigens appear to remain detectable for long periods of time. FAULKNER *et al.* (1989) succeeded in obtaining positive indirect immunofluorescent antibody test to *G. duodenalis* in a coprolite 2177 years old. ALLISON *et al.* (1999), using fluorescent monoclonal antibody and enzyme-linked immunosorbent assay (ELISA), obtained positive results in coprolites 500 to 3000 years old.

ELISA for the detection of *G. duodenalis* antigens in fresh or preserved faeces has been useful in the diagnosis of giardiasis. Several studies have found ELISA to be a tool with very high sensitivity and specificity for *G. duodenalis* coproantigen detection (JANOFF *et al.*, 1989; ROSOFF *et al.*, 1989; ADDISS *et al.*, 1991; SCHEFFLER & VAN ETTA, 1994; ALDEEN *et al.*, 1998; GARCIA *et al.*, 2000).

We conducted a study to assess the utility of a commercially available ELISA kit for the detection of a *G. duodenalis*-specific stool antigen, the *Giardia*-specific antigen 65 (GSA65), in archaeological samples. ELISA results were compared with those from conventional coprolite examination. The detection of *G. duodenalis* coproantigen in coprolites could be a method for giardiasis diagnosis in ancient human remains.

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Materials and Methods

Specimens

A total of 83 coprolites and sediment samples, previously examined microscopically for helminth eggs and larvae at the paleoparasitology laboratory, National School of Public Health, FIOCRUZ, Brazil, were tested for *G. duodenalis* antigen. Their archaeological origin and dates are listed in Table 1. The samples were dated either by ^{14}C method or by cultural context. A sample of fresh faeces, positive for *G. duodenalis* cysts by direct wet mount method, was used as a positive control. It was desiccated at 37° C for 2 weeks (an experimental coprolite) and was tested following the same procedures used for coprolite samples. This method of experimental production of coprolites has proven useful in identifying diagnostic criteria for other parasites (CONFALONIERI *et al.*, 1985). All specimens were also re-examined for helminth eggs and larvae, as well as for protozoan cysts.

Conventional examination of coprolites

The specimens were rehydrated by immersion in a 0·5% aqueous solution of trisodium phosphate for 72 h, following the technique of CALLEN & CAMERON (1960). The rehydrated sample solutions were mixed approximately 10:1 in acetic formalin solution (Raillet Henry solution) to retard fungal and bacterial growth (REINHARD *et al.*, 1986). The material was allowed to sediment following the technique proposed by LUTZ (1919). A portion of each sediment was used for microscopical examination. The material was placed on a slide and covered with a coverslip (22 × 22 mm) and examined for the presence of parasites. Twenty slides for each sample were examined at magnification of × 100 and × 400.

A portion of each rehydrated sample was also examined by the zinc–sulphate flotation method (FAUST *et al.*, 1938). Five slides for each sample were examined at magnification of × 100 and × 400. All wet preparations were examined by at least 2 of the authors.

Enzyme-linked immunosorbent assay

The ProSpecT *Giardia* microplate assay (Alexon, Inc, Sunnyvalle, CA, USA), a monoclonal antibody assay, was performed on each sample (100 µL of rehydrated sample solution, as described above), according

Table 1. Country of the archaeological site, type, and age of archaeological samples examined for *Giardia duodenalis*

Country	Archaeological samples
Brazil	39 coprolites from archaeologic layers and 3 coprolites from colon contents of mummies, dated from 5230 BC to 1730 AD
Chile	21 coprolites from archaeologic layers and 7 coprolites from colon contents of mummies, dated from 4100 BC to 800 AD
USA	5 coprolites from archaeologic layers, dated from 1200 AD to 1300 AD
Germany	3 medieval latrine soil samples, dated from 1500 AD to 1600 AD
Argentina	2 coprolites from colon contents of mummies, pre-Colombian time
France	1 coprolite from archaeologic layer, dated to about 400 000 BC and 1 medieval latrine soil sample, dated to about 1400 AD
Belgium	1 medieval latrine soil sample, dated to the 18th century

to the manufacturer's directions. It detects the GSA65 *G. duodenalis*-specific antigen in stools. Results were interpreted by visual inspection, following the colour panels included in the kit. Any detectable colour generation was regarded as a positive result. Following visual inspection, the absorbance of each specimen was measured at 450 nm wavelength with a spectrophotometer. The absorbance values were adjusted by subtracting the optical density (OD) of the negative control from the OD of the samples. Specimens which produced an adjusted OD $\geq 0{\cdot}050$ were considered positive, according to the manufacturer's instructions.

Results

The immunoassay and the conventional examination results are given in Table 2. The microscopical examination was positive for *G. duodenalis* in only 1 of the 3 archaeological samples positive for *G. duodenalis* antigen detection by immunoassay. Two of the 3 positive samples (latrine soils) were retested using the same steps and procedures as above and the ELISA was concordant in both samples. Visual and spectrophotometer readings were concordant in all samples.

The faeces used for the experimental coprolite had abundant cysts of *G. duodenalis* before desiccation. After desiccation and rehydration, identifiable cysts were very scarce.

Discussion

The observed decline of identifiable cysts in the experimental coprolite is very important. This indicates that the dehydration process, which is the reason coprolites preserve, and the rehydration process used for the analysis of most coprolites, destroys the majority of cysts. Therefore, the near absence of protozoa in coprolite studies is not surprising.

The results show no cross-reactivity between *G. duodenalis* and helminths, as can be seen in Table 2.

Table 2. Results of microscopic examination and enzyme-linked immunosorbent assay for GSA65 *Giardia duodenalis* antigen in human coprolites from archaeological sites

Country	Microscopic examination	ELISA result (OD)[a]
Brazil	13 samples with *Trichuris trichiura* eggs + Ancylostomidae larvae and/or eggs	neg. (<0·050)
	12 samples with *T. trichiura* eggs	neg. (<0·050)
	8 samples with Ancylostomidae larvae and/or eggs	neg. (<0·050)
	3 samples with free-living larvae	neg. (<0·050)
	6 negative samples	neg. (<0·050)
	(FERREIRA *et al.*, 1980, 1983, 1987)	
Chile	6 samples with *Diphyllobothrium pacificum* eggs	neg. (<0·050)
	1 sample with *Enterobius vermicularis* eggs	neg. (<0·050)
	1 sample with *T. trichiura* eggs	neg. (<0·050)
	20 negative samples	neg. (<0·050)
	(FERREIRA *et al.*, 1984; ARAÚJO *et al.*, 1985)	
USA	2 samples with *E. vermicularis* eggs	pos. (1 sample) (0·092)
		neg. (1 sample) (<0·050)
	3 negative samples	neg. (<0·050)
	(REINHARD, 1985)	
Germany	3 samples with *T. trichiura* + *Ascaris lumbricoides* + *D. latum* + *Fasciola hepatica* eggs	pos. (1 sample) (0·237)
	(HERRMANN, 1985)	neg. (2 samples) (<0·050)
Argentina	1 sample with Ancylostomidae eggs	neg. (<0·050)
	1 sample with free-living larvae	neg. (<0·050)
	(unpublished data)	
France	1 sample with *T. trichiura* + *A. lumbricoides* + *D. latum* + *Schistosoma haematobium* eggs	neg. (<0·050)
	1 negative sample	neg. (<0·050)
	(BOUCHET & PAICHELER, 1995)	
Belgium	1 sample with *T. trichiura* + *A. lumbricoides* + *D. latum* + Ancylostomidae larvae and eggs + *G. duodenalis* cysts	pos. (0·496)
	(unpublished data)	
Positive control	1 sample with *G. duodenalis* cysts (experimental coprolite)	pos. (0·741)

[a]Enzyme-linked immunosorbent assay; optical density. Reader adjusted by subtracting the OD of the negative control (OD = 0·019) from the OD of the samples.

The use of 0·5% aqueous solution of trisodium phosphate and acetic formalin solution in coprolites did not prevent positive results in the immunological test.

The ELISA was superior to direct observation. The imunoassay detected *G. duodenalis* in 2 samples in which the direct examination was negative. In one sample the results were concordant. The finding of human-specific parasites in these samples confirms their human origin.

The sensitivity of direct microscopy to detect protozoa in coprolites is poor. There are not many well-documented papers referring to protozoa in coprolites (WITENBERG, 1961; FOUANT *et al.*, 1982; FAULKNER *et al.*, 1989; FERREIRA *et al.*, 1992; ALLISON *et al.*, 1999). Even in fresh faeces, in order to obtain a reliable result of giardiasis by direct examination, one should examine at least a series of 3 or more stool samples, as cysts of *G. duodenalis* are shed from the intestinal tract on a periodic basis. Antigens, however, are shed in a more continous way (ROSOFF & STIBBS, 1986a).

The previous use of ELISA for protozoan detection in coprolites has not been encouraging. FOUANT *et al.* (1982) observed possible *Entamoeba* sp. cysts in 4 coprolites from pre-Colombian Indians from Chile. None were reactive to an ELISA utilized to detect *E. histolytica* antigens. They argued that the cysts found could be from *E. coli* species rather than *E. histolytica* species. Alternatively, they argued that the negative ELISA findings could result from decay of cyst antigenicity with time. ALLISON *et al.* (1999) compared a fluorescent antibody kit (Meridian Diagnostics, Cincinnati, OH, USA) to an ELISA kit (Meridian Diagnostics) for *G. duodenalis* detection in coprolites from pre-Colombian South American mummies 500 to 3000 years old. The fluorescent antibody kit yielded more positive results than the immunosorbent assay. It was not stated if the ELISA kit used monoclonal or polyclonal antibodies. The findings from FOUANT *et al.* (1982) and from ALLISON *et al.* (1999) could be explained by the relative low sensitivity of old generation enzyme immunoassays.

The assay evaluated here utilizes monoclonal antibody against GSA65 antigen. The GSA65 *G. duodenalis*-specific stool antigen is a glycoprotein. It is stable and is secreted in large amounts by encysting trophozoites (ROSOFF & STIBBS, 1986b). According to ALDEEN *et al.* (1998), the sensitivity and specificity of the assay is 100% in fresh faeces. The immunological test performed was rapid and simple, although expensive. According to our results, GSA65 survives the destruction of cysts and trophozoites. It was shown to be present for at least 800 years in human remains.

Although many species of *Giardia* parasitize virtually all classes of vertebrates, only *G. duodenalis*, a species complex, is identified from humans and most other mammals (THOMPSON *et al.*, 1993; THOMPSON, 2000). Molecular characterizations indicate that isolates from humans belong to assemblage A (genetically equivalent to 'Polish' group or Groups 1/2) or assemblage B (genetically equivalent to 'Belgian' group or Group 3). Major assemblages have distinct clusters. These clusters appear to have typical patterns of dispersion and potential for zoonotic transmission (THOMPSON *et al.*, 2000). The use of such molecular markers in coprolites with *G. duodenalis* could raise interesting issues concerning the origin and dispersion of this parasite. MAYRHOFER *et al.* (1995) pointed out the relevance of studying the genetic divergence in *Giardia* assemblages concerning the phylogenetic or ecologic route of the giardiasis in humans.

If further studies confirm our results, the use of immunological methods for antigen detection can become the 'gold standard' for diagnosis of protozoan infection in coprolites. As more sensitive *G. duodenalis* detection tools arrive, a more comprehensive picture of this infection in ancient populations could be done.

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Book Review

Control of Chagas Disease. Second Report of the WHO Expert Committee. WHO Technical Report Series, No. 905. Geneva: World Health Organization, 2002. vi + 109pp. Price Sw.fr. 23/US$20.70 (in developing countries Sw.fr.16.10). ISBN 92-4-120905.4.

On November 20–28 2000, the WHO Expert Committee on the Control of Chagas Disease met in Brasilia. This small book represents a compilation of the topics that were discussed and the resulting consensus. Its relatively short length (109 pages) should not mislead readers to believe that the discussion is cursory. In fact, it contains perhaps the most complete coverage of the epidemiology, prevention, and control of this disease to be found in any single reference. It is an elegant and detailed summary of the current knowledge of Chagas disease diagnostics, pathogenesis, treatment, epidemiology, parasite biology, vector biology, and control. Consequently, the authors should be applauded for the extraordinary time and effort that no doubt went into this undertaking.

Despite the overall excellent quality of this book, it has several shortcomings. Controversies or alternative strategies for specific treatment of chronic Chagas disease, transplantation of organs from seropositive donors to seronegative recipients, and serological diagnosis are not addressed. The addition of more references to the primary literature would support the recommendations made and at the same time assist the reader in drawing his or her own conclusions. Nevertheless, the recommendations will be very useful, knowing that they come from a highly knowledgeable group of experts.

The section on vector population genetics is unclear in parts. There seems to be some confusion in the definitions of taxonomy, systematics, and population genetics and their application to vector biology and control. For example, the authors discuss the domestic triatomine species *Rhodnius prolixus* and the sylvatic species *R. colombiensis* within the context of recently diverged populations. It is well known, however, that while these 2 species are morphologically similar and exist sympatrically, they have very little else in common and are as phylogenetically distant from each other as *R. prolixus* is from *R. pictipes* or *Triatoma infestans* from *T. sordida* (see *American Journal of Tropical Medicine and Hygiene*, 1999: **60**, 377–386 and 2000: **62**, 460–465). Consequently, the issue is not one of population genetics but of molecular taxonomy and systematics. The implications of these distinctions are important and shed light on our understanding of the potential risk of sylvatic species to invade domestic habitats.

A failure to mention the need for alternative control approaches, such as biological, genetic, or environmental control, aimed at peridomiciliary triatomine populations that have been virtually unaffected by the current insecticide-based control methods is overshadowed by the inclusion of very solid recommendations for future work.

On balance, this book makes an extremely valuable contribution toward understanding Chagas disease and promoting solid prevention and control efforts. It will no doubt be an important addition to the library of anyone, student and expert alike, with interests in Chagas disease. The authors are to be congratulated on the completion of a work of excellence—an extremely difficult endeavour at a time when our knowledge of vectors, parasites, and methodologies is expanding so rapidly.

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