



Assessment of *Entamoeba histolytica* Coproantigen and Specific Salivary IgA in Relation to Real-Time PCR for Detection of Intestinal Amoebiasis

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ABSTRACT

Despite the *Entamoeba histolytica* was first discovered more than 160 years ago, it remains a major health problem in developing countries, including Egypt. Discriminating the morphologically similar pathogenic species from the non-pathogenic one is a challenging task, specifically when relying on the traditional diagnostic tools as microscopy. The objective of the current study was to assess the usefulness of detecting *E. histolytica* coproantigen and specific salivary IgA for proper identification of intestinal infection with *E. histolytica*, using ELISA, in relation to the gold standard real-time PCR technique. 38 stool samples were proved positive for *E. histolytica*-like stages by microscopy and subsequently exposed to molecular analysis, using specific primers and probes related to *E. histolytica* which excluded 8 out of these 38 samples, indicating their relation to non-pathogenic species. All diagnostic tests achieved 100% specificity and relatively good sensitivity of 93.3 and 86.6% for specific coproantigen and salivary IgA respectively. Conclusively, ELISA-specific coproantigen or secretory salivary IgA are rapid reliable cost-effective and relatively sensitive diagnostic tools that can discriminate between pathogenic *E. histolytica* from those of non-pathogenic *E. dispar*, thus helpful in epidemiological surveys. The short duration of the secretory IgA may pose additional advantages as it can diagnose active infection, besides its ability to diagnose amoebic liver abscess.

INTRODUCTION

Entamoeba histolytica is a common worldwide intestinal protozoan that affects more than 500 million subjects annually. Yet, only 10% of these affected persons suffer from colonic manifestations or/and extra-intestinal extension with subsequent complications resulting in about 100,000 deaths per year (Gunther *et al.*, 2011). This enteric infection was first discovered more than 160 years ago, but it remains a major health problem in developing countries, including Egypt (Carrero *et al.*, 2020). A relatively high rate of asymptomatic carriers (21%) was reported in Egypt by Stauffer *et al.* (2006). These carriers can transmit the infection to other subjects who may develop intense serious manifestations, yet the true burden of such enteric infection is not well known.

Much of the documented epidemiological information relied on microscopic findings which are challenged by many obstacles, including the difficulty concerning the identification of the pathogenic strain from the non-pathogenic morphologically similar species, resulting in biased microscopic results. Many cases diagnosed microscopically as *Entamoeba histolytica* proved to be related to other non-pathogenic species when tested immunologically. False-negative findings may ascend due to inadequate sensitivity of microscopy and as a result of irregular shedding of parasitic stages in the stool (Jelinek *et al.*, 1996 and Othman *et al.*, 2019). Thus, direct microscopic examination is not currently the method of choice to diagnose intestinal amoebiasis (Quispe-Rodríguez *et al.*, 2020).

Stool culture with isoenzymes analysis is an accurate tool, but it takes several weeks to be completed. Molecular methods are also achieved excellent results and are currently considered a gold standard for the detection of amoebiasis infection, yet unfeasible to be performed routinely in the developing world (Shirley *et al.*, 2019). Thus, more attention is needed to establish cost-effective, reliable and rapid diagnostic tools to facilitate effective control strategies (Shirley *et al.*, 2019). *E. histolytica* infection intensifies the mucosal production of specific secretory IgA, which has been immunologically detected in variable body fluids including saliva. Detection of *Entamoeba histolytica* coproantigen is another immunological assay that can be used for the diagnosis of such intestinal protozoan (Navarro-Garcia *et al.*, 1997).

The objective of the current study was to assess the usefulness of detecting *E. histolytica* coproantigen or specific salivary IgA for proper identification of intestinal infection with *E. histolytica*, using ELISA, in relation to the gold standard real-time PCR technique.

MATERIALS AND METHODS

Stool And Saliva Samples Collection, Processing and Examination:

Stool and saliva samples were collected from each enrolled subject at the tropical diseases outpatient clinic, Faculty of Medicine, Fayoum University, and were classified according to the results of stool and molecular assays into 2 groups; group (1); 30 positive samples in which *Entamoeba histolytica* cysts or trophozoites were detected on stool examination and the species were confirmed by real-time PCR. The other group (2); included 30 positive samples for other intestinal protozoal infections; *Giardia lamblia* (7 cases), *Cryptosporidium* (7), *Dientamoeba fragilis* (4), *Iodamoeba butschlii* (4) and *Entamoeba dispar* (8) to test for any cross-reactivity. Samples related to *Entamoeba dispar* were obtained after exclusion of morphologically similar stages, next to the molecular analysis.

Wet fresh stool smears were examined microscopically in a 0.9% saline and following formol ether concentration for the presence of parasitic stages. Modified Ziehl-Neelsen staining was applied specifically to detect *Cryptosporidium*. As a preparatory step for coproantigen detection, 5 grams from each stool sample were mixed with an equivalent amount of distilled water to be sonicated for 5 minutes, then the mixture was centrifuged at 1900x for 15 minutes, the supernatant was then collected and stored at -20° C until use. For saliva samples, about 5 ml of the whole saliva was collected from each subject, using a test tube which was dipped in ice through a disposable funnel, followed by centrifugation at 2500g. The supernatant was then frozen at -20° C. The sample was thawed at 4° C prior to use and refined by centrifugation at 14,000g. Samples related to any subject received anti-parasitic treatment, and samples containing mixed infection (*E. histolytica*-like stages and any other parasitic stages) were excluded. All enrolled subjects were provided with the results of the

diagnostic tools and were kindly managed according to the institutional protocol.

Immunological Assays:

ELISA coproantigen detection specific for *E. histolytica* was applied according to the manufacturer's instructions [Ready to use Techlab antigen detection kit, Inc., Blacksburg, Va]. Briefly, 0.1ml diluted stool samples were incubated with one drop of monoclonal antibody-enzyme conjugate for 2 hours at room temperature and then washed 4 times, using the washing buffer. Substrate solution was then added and incubated for 10 minutes before adding the intensifier for additional 10 min incubation. Detection of salivary *E. histolytica* secretory IgA was applied using ELISA according to the manufacturers' instructions [Alexon Co., Sunnyvale, Calif.]. Concisely, wells were coated with 100µl of a 1µg/ml solution of specific *E. histolytica* antigen overnight at 4°C. Then the wells were washed 3 times with PBS and blocked for 1hour at 37°C with 1% bovine serum albumin (BSA). The wells were then incubated for 2hours with undiluted refined saliva, followed by washing with PBS-Tween and then incubated with goat anti-human IgA antibody labeled with horseradish peroxidase diluted 1:2000 in PBS-Tween-BSA. Next, the wells were incubated for another 30 min at 37°C with the freshly prepared substrate. ELISA plates were read according to the manufacturers' instructions using ELISA plate reader [Titertek Multiskan, Flow Laboratories, McLean, Va.] at 450nm.

Species Confirmation by Molecular Technique:

For DNA extraction, 200 mg of the fecal samples were exposed to DNA extraction after washing twice with sterile PBS and centrifuged for 5min at 14,000 rpm using the QIAamp DNA stool mini kit [QIAGEN, Hilden, Germany] following the manufacturer's instructions and the extracted DNA was eluted in 0.2 ml AE buffer. Real-time qPCR was done according to the method of Roy, *et al.* (2005), to specifically

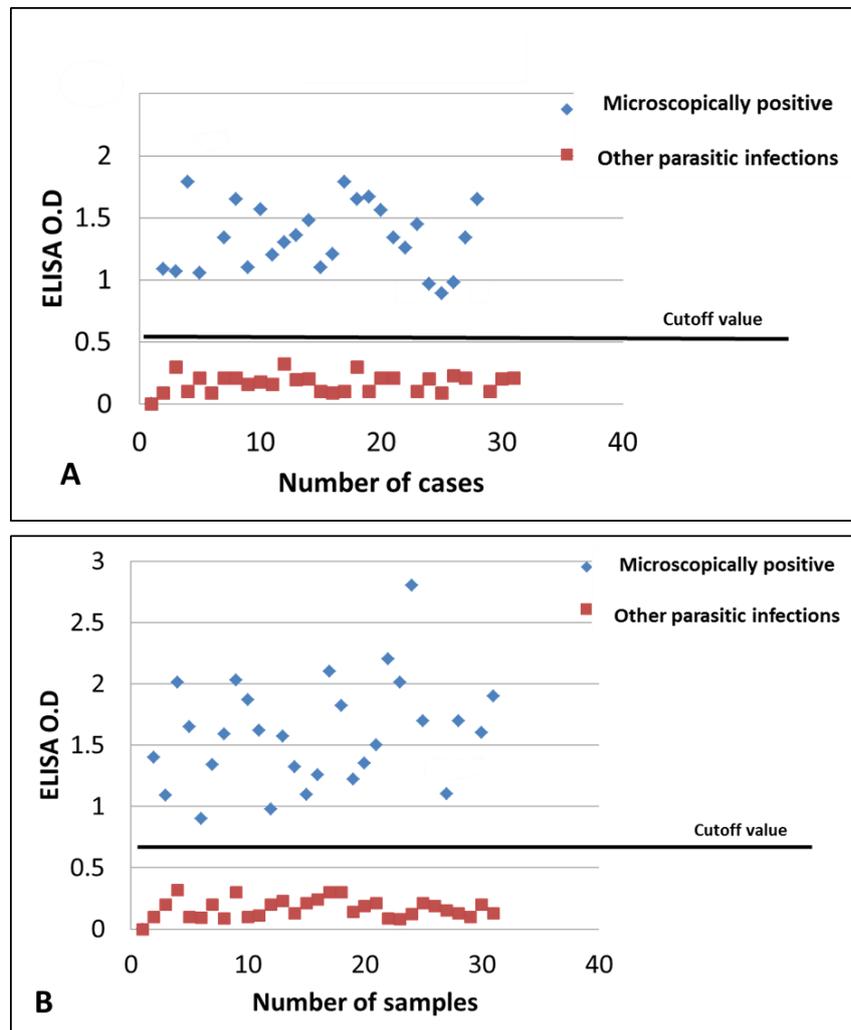
amplify a genetic fragment within the 16S-like small-subunit rRNA gene of *E. histolytica*. *E. histolytica*-specific primers and double-labeled molecular-beacon probe set involved the forward primer (Ehf) 5-AAC AGT AATAGT TTC TTT GGT TAG TAA AA-3 and the reverse primer (Ehr) 5-CTT AGA ATG TCA TTT CTC AAT TCA T-3 [Eurogentec, UK.]. Reactions were performed in a total volume of 25 µl with Bio-Rad's IQ super mix; 2.0 µl of the genomic template, 25 pmol of each primer, 6.25 pmol of the specific probe, 100 mM KCl; 40 mM Tris-HCl, 1.6 mM dNTPs; *iTaq* DNA polymerase and 7.75 µl of double-distilled water. the amplification cycle involved 45 cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 15 seconds at 72°C using a real-time detection system [Bio-Rad laboratories].

RESULTS

Direct microscopic examination identified 38 samples positive for *Entamoeba histolytica* parasitic stages (cysts or/and trophozoites). Subsequent molecular analysis was done for these 38 samples using real-time PCR. Fluorescence signals were initiated from 30 samples out of these 38 samples, confirming their specific relation to *E. histolytica* species. Stool and salivary samples related to the 30 confirmed positive samples for *E. histolytica* were exposed to subsequent immunological assays. No false-positive results were reported with any sample related to other parasitic infections. Thus, both immunological techniques reported 100% specificity. The mean optical density value related to ELISA coproantigen was 1.57 ± 0.42 and 1.368 ± 0.29 for ELISA salivary-specific IgA without a statistically significant difference ($P \geq 0.05$) (Fig. 1). ELISA coproantigen succeeded to diagnose 28 samples out of the 30 samples, thus achieving a sensitivity of 93.3%. While 4 false-negative results were obtained with ELISA salivary specific IgA which attained 86.6% sensitivity (Table 1).

Table (1): Comparison between sensitivity and specificity of different diagnostic tools

			Other parasitic infections	Microscopically & molecularly positive	Sensitivity	Specificity
Gold standard	Real time PCR	Negative	30	0	100.0%	100.0%
		Positive	0	30		
Immunological tools	Coproantigen	Negative	30	2	93.3%	100.0%
		Positive	0	28		
	Secretory IgA	Negative	30	4	86.6%	100.0%
		Positive	0	26		

**Fig. 1:** ELISA quantitative values for specific *E. histolytica* salivary IgA (A) and coproantigen (B).

DISCUSSION

In this study, 8 samples diagnosed by direct microscopy as *Entamoeba histolytica* parasitic stages proved to be related to non-pathogenic *E. dispar* after molecular investigation. Differentiation between the 2 species is necessary to determine the proper management of the pathogenic species, while the non-pathogenic species don't require any treatment (Ackers *et al.*, 1997). Diagnosis of amoebiasis has usually relied on microscopic analysis which cannot differentiate between the morphologically similar species (Pillai *et al.*, 1999 and Othman *et al.*, 2019).

Haque *et al.* (1997) underlined the risk of reliance on microscopy to diagnose intestinal amoebiasis. They studied the infection among symptomatized Bangladeshi children with diarrhoea and only 40% were confirmed to have *E. histolytica* infection when antigen detection and culture-enzyme analysis were performed. The authors also reported a high percentage of false-negative results with microscopy and they concluded that direct microscopy is not useful to diagnose amoebic colitis, being insensitive, unable of discriminating the pathogenic *E. histolytica* from the non-pathogenic *E. dispar*, and thus prone to obtain false-positive outcomes.

In this work, ELISA coproantigen achieved a relatively good sensitivity (90.3%) with 100% specificity which was able to discriminate between the 2 morphologically identical species, excluding the non-pathogenic one. This is going with the report of Haque *et al.* (1998) who recommended its use and found it almost equal to PCR to diagnose *E. histolytica* infection. They added that the reliability of this diagnostic tool is not limited to diagnosis, but it is extremely beneficial in epidemiological studies.

Concerning the specific salivary IgA, the results of this study are more or less similar to that recorded by Adao *et al.* (2022). The authors reported diagnostic accuracy of 85.5 to 90% of this tool,

compared to PCR which was considered the gold standard by the authors who recommended its use, next to criticizing microscopy, as being insensitive. Saliva samples are easier to collect than stool samples and ELISA plates can keep the results for at least one year at - 4°C. The ability to diagnose amoebic liver abscess in addition to amoebic colitis gives the specific salivary IgA test additional significance (Adao *et al.*, 2022).

In general, the technical character of ELISA allows the processing of several specimens without extensive variability in the quality of implementation in a short period of time. Thus, ELISA is potentially more appropriate than microscopy, especially in epidemiological surveys in areas with a high rate of prevalence, and also in follow-up inspections of patients who proved to be *E. histolytica* positive. Furthermore, epidemiological surveys relied on the screening of salivary antibodies can be carried out with relative ease and lower cost than that relied on the stool or on more or less invasive serum analysis (Jelinek *et al.*, 1996 and Adao *et al.*, 2022).

In conclusion, ELISA-specific coproantigen or secretory salivary IgA are rapid reliable cost-effective and relatively sensitive diagnostic tools that can discriminate between pathogenic *E. histolytica* from those of non-pathogenic *E. dispar*, thus helpful in epidemiological surveys. The short duration of the secretory IgA may pose additional advantages as it can diagnose active infection besides its ability to diagnose amoebic liver abscess.

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