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ENTAMOEBA HISTOLYTICA: FECAL ANTIGEN CAPTURE IMMUNOASSAY FOR THE DIAGNOSIS OF ENTERIC AMEBIASIS BY A MONOCLONAL ANTIBODY

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SUMMARY

Amebiasis continues to be of epidemiological importance in underdeveloped countries. Clinical diagnosis and epidemiological setting in a region are based on the fecal microscopic identification of cysts or trophozoites. This procedure requires well trained personnel, is laborious, of low sensitivity and frequently yields false-positives results. The present study was designed to develop an immunoenzymatic fecal 96 kDa antigen capture test (COPROELISA-Eh) more sensitive and specific than microscopic diagnosis of amebiasis. Triplicates of 177 stool samples processed by the formol-ether concentration method, were defined as positive or negative by three experienced microscopic observers. Another aliquot was submitted to the antigen capture test by a monoclonal antibody against a specific membrane antigen of pathogenic strains of *Entamoeba histolytica*. Optical densities were interpreted as positive when they exceeded the mean value of negative samples plus two standard deviations. COPROELISA-Eh showed a 94.4% sensitivity, 98.3% specificity, 96.2% positive predictive value and 97.6% negative predictive value for the detection of *E. histolytica* in feces. COPROELISA-Eh is more sensitive and specific than microscopic examination, does not require specially trained personnel and allows the simultaneous processing of a large number of samples.

KEYWORDS: *Entamoeba histolytica*; Amebiasis, Coproantigens; Immunodiagnostic; Monoclonal antibodies; Capture ELISA.

INTRODUCTION

In the underdeveloped world, amebiasis is still very important among the parasitic diseases because it holds third place in morbidity and mortality after malaria and schistosomiasis [16]. Epidemiological estimates made since 1986 state that approximately 500 million people worldwide are infected by *Entamoeba histolytica* each year [38]. About 10% of this infected population shows clinical symptoms, some 80% to 90% with intestinal and 2% to 20% with extraintestinal symptoms [7]. Also, the mortality reaches figures between 40,000 to 110,000 cases each year [39].

Amebiasis is diagnosed by microscopic identification of cysts or trophozoites in the stool [6]. The main drawback of this procedure is the low sensitivity and specificity of the procedure because of the possibility of confusion with plasma cells and non-pathogenic amebas [4]. The possible confusion with *Entamoeba hartmanni* is especially important [15, 26]. It has also been considered that they are between 30% to 40% false negative results due both to the intermitent excretion and to a non-homogeneous distribution in the stool sample [5, 33]. To overcome the aforementioned problems,

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up to 6 exams of serial stool samples have been recommended [3, 22].

Restrictions are found for defining sensitivity and specificity of other procedures for the laboratory diagnosis of intestinal amebiasis [12, 14]. Serologic tests have shown low sensitivity and specificity [15] due to the presence of antibodies in 6 to 20% of healthy subjects in endemic regions, probably as a consequence of continuous reinfection with *E. histolytica* [8, 20], and also, due to the impossibility to establish the difference between the antibodies produced during an active infection and those that persist after treatment.

Most immunoenzymatic tests for diagnosis that emply *E. histolytica* purified antigenic fractions have being designed for the detection of serum antibodies in extraintestinal amebiasis [17, 21, 25, 27-29, 31, 32, 41] and are not useful for the diagnosis of intestinal amebiasis. On the other hand, molecular biology techniques detecting *E. histolytica* DNA [9, 13] are not yet commercially available. Fecal antigen capture immunoassays using monoclonal and polyclonal antibodies have demonstrated high sensitivity and specificity in the diagnosis of several infectious diseases [1, 2, 10, 11, 19, 23, 24, 35, 37, 40]. This is a more objective diagnostic approach because it is designed to detect the parasites or their metabolic products.

This paper describes COPROELISA-Eh, a fecal antigen immunoassay specific for *E. histolytica*, using two antibodies: HU5-11 [34] specific for a 96 kDa antigen, obtained from Balb/c mice immunized with pathogenic *E. histolytica*, strain ICB-CSP [30], outer membrane, and a polyclonal antibody purified from patients with extraintestinal amebiasis. The described test is proposed as a highly sensitive, specific and nonlaborious technique that could help solve the limitations of the conventional microscopic examination.

MATERIALS AND METHODS

Samples: 117 stool samples obtained from the same number of patients were processed up to two hours after collection. Each sample was divided into two aliquots: one diluted in water for the immunoenzymatic technique and the other in 10% formaldehyde for microscopic examination. Samples diluted in water were centrifuged at 250g for 10 minutes and the supernatants frozen at -20°C. For microscopic analysis of formaldehyde-fixed samples, these were aliquoted and each processed by three independent technicians with expertise in the method. After being concentrated by the formol-ether technique, the material was stained with methylene-blue and iodine, and examined by three independent observers with expertise in this method. Samples were considered positive for *E. histolytica* when there was coincidence among the three observers. Samples showing discrepant results were sent for new observation with a new label and a note telling the observers about the discrepancies. After the second examination the result for which at least two observers were coincident was taken as definitive.

Standardization of Sensitivity and Specificity of Monoclonal HU5-11: HU5-11 monoclonal antibody was produced in a hybrid cell line obtained from a fusion of SP_2 0 mouse plasmocytoma cells with total spleen cells from Balb/c mice immunized with *E. histolytica* plasma membranes and a monoclonal antibody panel because it recognized a 96kDa membrane antigen [34]. To evaluate HU5-11 sensitivity and specificity to water suspensions of 30 stools previously demonstrated to be negative for *E. histolytica* by microscopic examination, different concentrations (100 µg to 10 ng/ml) were added of crude extracts obtained from several strains of *E. histolytica*: HMI-IMSS, ICB-CSP, HK9, IULA: 1092-1 and IULA: 0593-2 (the last two strains were isolated and axenizated in our laboratory) [36] as well as of *Giardia lamblia*, *Entamoeba invadens*, *Entamoeba moshkouskii* and *E. histolytica* like *Laredo*. All these stool suspensions were then submitted to COPROELISA-Eh.

Human polyclonal anti-ameba antibody: Polyclonal human antibody anti *E. histolytica* was obtained from patients with *E. histolytica* hepatic abscess as follows: patient sera were pooled and affinity purified using a Sepharose 4-B column according to manufacturer's protocol (Pharmacia) with a crude extract of axenic ICB-CSP *E. histolytica*. Antibodies bound to Sepharose were eluted using 0.2M Glycine, pH 2.8, neutralized immediately with 1M NaOH and dialyzed against PBS. The same procedure was performed to obtain polyclonal antiserum from previously immunized rabbits.

Capture sandwich ELISA (COPROELISA-Eh): HU5-11 monoclonal antibody against 96kDa *E. histolytica* external membrane antibody was obtained from Balb/c mouse ascitic liquid [34]. This antibody was purified by affinity chromatography using Protein A Sepharose columns.

The COPROELISA-Eh was performed as follows: 96-well microplates (Dynatech) were coated with 100µl of the HU5-11 monoclonal antibody (1µg protein/ml carbonate buffer, pH 9.6) incubated 1h at 37°C, incubated overnight at 4°C and blocked with skim milk at 2% in PBS for 1h at room temperature. After two new washes with PBS containing 0.05% Tween 20 (PBS-T), 100µl of the stool suspension were added to each well, the plates were incubated for 3 hours at room temperature, washed 5X with PBS-T and incubated for 1h with 100µl of the second antibody (human anti-*E. histolytica*) at 1:1000 dilution with PBS-T. Microplates were washed 5X and all washes were performed with PBS-T to eliminate excess of the second antibody, and 100µl of anti human IgG peroxidase conjugates (ATAB) diluted 1:1000 were added to each well and incubated for 1h at room temperature. After this last incubation, microtiter plates were washed 5X with PBS-T, and 100µl of 0.002% O-phenylendiamine (SIGMA) in a citrate-phosphate solution (40mM Na_2HPO_4 plus 100 mM citric acid and H_2O_2) were added to each well and incubated at room temperature for 45 minutes; then 30µl of a 2N solution of H_2SO_4 were added to each well in order stop the reaction. Optical density (OD) was recorded in an automatic ELISA spectrophotometer (organon Tecnika). Two positive and negative controls were added to each plate. For COPROELISA-Eh the cut off was established as the OD corresponding to the mean value observed for negative stool samples plus two standard deviations.

Statistics: For statistical analysis of variance, the chi square and Student's t test were used. Specificity, sensitivity, agreement and reproducibility were calculated when needed.

RESULTS

In terms of COPROELISA-Eh specificity, there was a statistically significant discrimination between samples containing virulent *E. histolytica*, antigens and samples containing low virulent *E. histolytica* antigens, or pathogenic or non-pathogenic protozoa different from *Entamoeba* (Fig. 1). The OD of *E. histolytica* positive and negative samples showed a highly significant difference ($p<0.001$). The test was able to detect up to 31 ng/ml of antigen. Analysis of variance for OD at 31 ng protein content per well showed a significant difference ($0<0.05$) between HMI, ICB-CSP, IULA-1092-1 (high virulence strains) and IULA-0593-2, HK9, ICB-462 (low virulence strains).

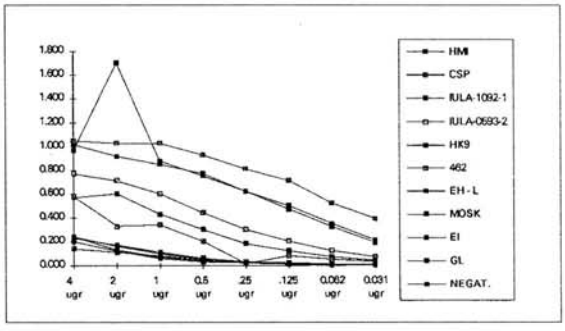

Fig. 1 - Optical densities of COPROELISA-Eh after adding decreasing concentrations of soluble extracts of pathogenic and non-pathogenic protozoan strains.

Of the 177 fecal samples, 51 were positive by both microscopic examination (ME) and COPROELISA-Eh. An additional two samples were positive only by COPROELISA-Eh; three samples were positive only by ME, and 121 were negative by both tests (Table 1).

The negative samples detected in COPROELISA-Eh were the ones which presented values equal to or below the mean of the negative stool samples plus two standard deviations. Statistical comparison of these two tests showed 97.2% concordance and 91% reproducibility.

COPROELISA-Eh showed 94.4% sensitivity and 98.3% specificity. Predictive positive value was 96.2% and predictive negative value was 97.6%.

DISCUSSION

Application of the COPROELISA-Eh to the 117 stool samples showed that this technique can detect the existence of *E. histolytica* alone or in the presence of other parasites.

TABLE 1
Comparison of the diagnosis of amebiasis by COPROELISA-Eh and microscopic examination for *E. histolytica*.

COPROELISA	Microscopic Examination Positive	Negative	Total
Positive	51	2	53
Negative	3	121	124
Total	54	123	177

There were no statistically significant differences (p=0.1488) between the negative samples and those which had the most common intestinal parasites such as *E. hartmanni, B. hominis, G. lamblia, E. coli, E. nana, A. lumbricoides, T. trichiura, S. stercoralis, E. vermicularis* and *A. duodenalis*. This allows us to confirm the absence of cross-reaction with other parasites.

It is very important to stress the capacity for discrimination between *E. histolytica* and *E. hartmanni*, because of the well known morphological confusion between this Entamoebas [15, 26] which could be responsible for overestimation of the prevalence of amebiasis. By using the HU5-11 monoclonal antibody we were able to find as much as 31 ng of crude protein extract of *E. histolytica*, enough antigen to be detected by this ELISA test. This could represent less that the amount of antigen present in a single trophozoite. This is important because in a given sample, trophozoites and cysts could be present at very low concentrations due to the irregular way the parasites are excreted from the intestine [33], which makes the microscopic examination very difficult.

The sensitivity of this method was increased by defining the cut off as two standard deviations above the mean value of the negative controls. Figure 1 shows that OD values obtained with strains HMI, ICB-CSP and IULA: 1092-1 (high virulence) were different from those obtained with strains IULA:0593-2, HK9 and ICB-462 (low virulence strains). These differences may be attributed to the difference in the pathogenicity of these two groups of Entamoebas [11]; the first three strains are considered to be more pathogenic and we found higher OD values for them.

Taken together, these results allow us to confirm that COPROELISA-Eh can positively detect the presence of cysts or trophozoites in minimal quantities or even antigenic fractions that are out of scope for microscopic observers. Also we have shown the ability of this test to detect strains of high and low virulence.

Another advantage of COPROELISA-Eh is that a single operator can test 40 samples on a single plate and process 4 simultaneous plates, which add up to a total of 160 samples within a period of 3 hours, plus the statiscally demonstrated fact of a sensitivity of 94.4%, specificity of 98.4%, with a concordance of 97.2% when compared to microscopic examination.

Our results allow us to propose this method as a very safe tool for epidemiological studies involving large amounts of samples.

RESUMO

Entamoeba histolytica: imunodiagnóstico, através de captura de antígeno fecal em amebíase entérica por um anticorpo monoclonal.

A amebíase mantém sua importância epidemiológica em países subdesenvolvidos onde sua prevalência a converteu na parasitose de maior morbidade e mortalidade após malaria e esquistosomose. Em regra, tanto o diagnóstico clínico como os levantamentos epidemiológicos assentam na identificação microscópica de cistos e/ou trofozoítos em extractos fecais. Este procedimento requer pessoal muito bem treinado, é laborioso, e frequentemente fornece resultados contraditórios. Para obviar estas dificuldades, no presente trabalho montamos uma técnica de diagnóstico imunoenzimático baseado na captura de um antígeno de 96 kDa presente nas fezes de indivíduos infectados pela *E. hystolytica* (COPROELISA-Eh). Triplicatas de 117 amostras fecais processadas pelo método de concentração do formol-éter foram definidas como positivas ou negativas por três microscopistas especialistas em amebíase. Outra alíquota foi submetida ao teste de captura de antígeno por um anticorpo monoclonal contra um antígeno de 96 kDa específico de membranas de cepas patogênicas de *E. histolytica*. Consideramos como positivos aqueles resultados em que densidades óticas estavam acima do valor dos controles negativos mais 2 desvios padrões. COPROELISA-Eh mostrou 94.4% de sensibilidade, 98.3% de especificidade, 96.2% de valor preditivo positivo e 97.6% de valor preditivo negativo para a deteção de *E. hystolytica* nas fezes. Em conclusão, o método COPROELISA-Eh é mais sensível e específico do que o exame microscópico dos extratos fecais, não requer pessoal especializado na identificação morfológica das amebas, e permite o processamento de um grande número de amostras simultâneamente.

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