

ENTAMOEBA HISTOLYTICA: DETECTION OF COPROANTIGENS BY PURIFIED ANTIBODY IN THE CAPTURE SANDWICH ELISA

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SUMMARY

A sensitive and specific Capture Sandwich ELISA (CSE) was developed using polyclonal purified rabbit antibodies against three different axenic strains of *Entamoeba histolytica*: CSP from Brazil and HM1 - IMSS from Mexico, for the detection of coproantigens in fecal samples. Immunoglobulin G (IgG) against *E. histolytica* was isolated from rabbits immunized with trophozoites whole extract in two stages: affinity chromatography in a column containing *E. histolytica* antigens bound to Sepharose 4B was followed by another chromatography in Sepharose antibodies 4B-Protein A. A Capture Sandwich ELISA using purified antibodies was able to detect 70ng of amebae protein, showing a sensitivity of 93% and specificity of 94%. The combination of microscopic examination and CSE gave a concordance and discordance of 93.25% and 6.75%, respectively. It was concluded that CSE is highly specific for the detection of coproantigens of *E. histolytica* in feces of infected patients, is quicker to perform, easier and more sensitive than microscopic examination.

KEYWORDS: *Entamoeba histolytica*; Amebiasis; Coproantigens; Immunodiagnostic; Purified antibody; Capture Sandwich ELISA (CSE).

INTRODUCTION

The incidence of infection with *E. histolytica* is high as 29% in some endemic areas of the world and leads to about 40,000 - 75,000 deaths per year^{20, 21}.

Traditionally, the diagnosis of amoebiasis is made by the identification of the parasite (trophozoite and/or cyst forms) in the stool, but this is not easy, requiring in many cases examination of multiple specimens, a well trained technician, vital staining, and microscopy and may have limited use in large scale screening.

Early attempts to diagnostic *E. histolytica* by serological technics gave poor results because the majority is incapable of distinguishing between circulating antibodies produced during active amebic infection and those persisting after treatment.

In view of the difficulties of microscopic and serological diagnosis, some efforts have been made to design a practical method for detecting *E. histolytica* antigens in stools by ELISA assay^{6, 10, 11}. In many of these assays the coproantigens were detected by using

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total immunoglobulins which could increase the cross reactions with antigens of other intestinal protozoans. In human amebiasis IgG appear in significant quantities and react with *E. histolytica* or its products.

In the present study aiming at increasing specificity and sensitivity of ELISA method for coproantigens detection, we decided to isolate immunoglobulins G(IgG) from hyperimmune sera of rabbits immunized with and test it in a capture sandwich ELISA to detect coproantigens of *E. histolytica* in fecal samples from patients with amebic liver abscess, patients with amebic colitis, individuals without symptoms but passing amebic cysts, patients infected with parasites other than *E. histolytica* and healthy individuals.

MATERIAL AND METHODS

Antigen preparation

Trophozoites used were from two strains of *E. histolytica*: CSP, isolated by SILVA et al.¹⁸ and HM1-IMSS isolated by de la TORRE et al.¹. Other *Entamoeba* isolates were also used: *E. moshkovskii*, *E. invadens* and *E. histolytica* like Laredo. All were grown axenically in TPS-I medium, according to DIAMOND³. Mass culture of amebae was performed at 37°C in 250ml culture plastic flasks. Briefly, 72 to 96 hours cultures of amebae were washed five times in Phosphate Buffered saline (PBS) pH 7.2. The trophozoites masses were treated with protease inhibitors (2mM phenylmethyl chloromethyl Ketone; 2mM iodoacetamide and 2mM p-hydroxy mercuribenzoate), and then individually disrupted by ultrasonication. The sonicated material was centrifuged at 20,000 x g at 4°C during 2 hours. The supernatant protein content of this trophozoites total extract was determined by the method of LOWRY et al.¹².

Immune sera

Immune sera against *E. histolytica* was produced by immunizing New Zealand rabbits with four doses of antigen from each ameba strain (two animals/strain), with fifteen days intervals. In the first dose, animals were given 5×10^6 whole trophozoites by intraperitoneal infection. In the second dose, whole trophozoite extract (5mg protein), emulsified in Freund's complete adjuvant (v/v), was injected in the rabbit's back. For the third dose, 5mg of trophozoite protein in Freund's incomplete adjuvant (v/v) was given on the haunch. The booster dose of 1.5mg of trophozoite protein with isotonic saline was given subcutaneously in the last

third of the pads. The animals were bled to death 10 days after the booster dose, and the presence of antibodies to *E. histolytica* determined by ELISA according to GUIMARÃES et al.⁸.

Affinity purification of serum

E. histolytica antigen (10 mg) were coupled to a Sepharose 4B column according to the manufacturer's protocol (Pharmacia). The rabbit antisera (5mg) was applied to the column, and recirculated overnight through the column. The bound immunoglobulins were eluted with 10ml of 0.2M glycine, pH 2.8, and neutralized immediately. The eluted immunoglobulins were applied to a protein A Sepharose affinity chromatography column. The bound anti-amebic antibodies (IgG) were eluted with 10ml of 0.2M glycine (pH 2.8) neutralized immediately and stored at -70°C. The purification of IgG was routinely monitored by polyacrylamide gel electrophoresis. Purified IgG and total immunoglobulins were measured by ELISA with the antigen of CSP strain according to GUIMARÃES et al.⁸.

Fecal specimens

One hundred and sixty three stool samples from *E. histolytica* infected individuals were collected at the Clinics Hospital in Belo Horizonte, Brazil, and examined microscopically by trained technicians. The techniques used were: a) direct stools examination; b) centrifugation in formaldehyde-ether; c) MIFC and iron-haematoxin staining. Control samples of *E. histolytica* non-infected individuals were obtained at the Amebiasis Laboratory, Parasitology Department, of the Minas Gerais Federal University, Brazil.

Stool suspensions were prepared by vortexing the samples in PBS containing 0.05% Tween 20 (0.5g feces/ml), and centrifuging them at 1000 x g for 10 minutes. The supernatants were stored at -20°C for a maximum of 6 months before ELISA testing.

Antigen-capture sandwich ELISA (CSE)

Purified IgG (20µg per ml) was used to coat microtiter plates by overnight incubation at 4°C. The plates were washed three times with PBS-Tween 20, and incubated for 45 minutes with PBS containing 0.3% casein (PBS-C). After washing, 30µl of the stool suspension and 70µl of PBS-C were added and incubated at 37°C, for two hours. Plates were washed and 100µl of a 1:1000 dilution of human anti-amebae sera (second antibody) was added to each well and allowed to incubate at 37°C for one hour. Plates were washed

and a peroxidase conjugate goat anti-human IgG in PBS-C (1:1000) was added to each well and incubated as above for one hour. After washing, 100µl of 0.002% O-phenylenediamine in citrate solution pH 5.0 (13mM Na₂HPO₄, 24mM citric acid and 0.012% H₂O₂) was added to each well. The reaction was interrupted after 30 min at room temperature by the addition of 20µl of 4N H₂SO₄. Intensity of colour developed in each well was recorded as optical density (OD) at 492nm. The same human negative and positive controls feces were used in all assays.

The sensitivity, specificity, positive and negative predictive values of this capture sandwich ELISA were calculated as previously described ⁴.

RESULTS

It was observed that the purified IgG shows a higher reactivity than total immunoglobulin from hiperimmune rabbit sera (Fig. 1). From the dilution 1/40 until 1/5120 the purified IgG always presented higher readings.

The specificity of the purified IgG was clearly seen in a capture sandwich ELISA, using antigens from several *Entamoeba* isolates (Fig. 2).

The sensitivity of purified IgGs was tested in

ELISA with serial dilutions of trophozoites total extract of CSP strain. For comparison we used purified IgG for CSP strain and also IgG produced in rabbits against HM1 strain.

A cross reactivity between anti-HM1 antibodies and CSP antigen was observed in higher concentrations of the antigen. In higher antigen dilutions, the anti-CSP antibodies showed more binding to the CSP antigen. Purified IgG anti-CSP was able to detect up to 0.075µg/ml of amebae proteins of CSP strain. This is the estimated amount of protein of only one throphozoite (Fig. 3).

As the purified IgG anti-CSP showed good sensitivity it was decided to use it in a diagnostic test. A capture sandwich ELISA was done to detect cysts and or throphozoites of *E. histolytica* in fecal samples. Purified IgG bound to the plates were incubated with fecal samples containing cysts and/or throphozoites by microscopic examination of stool concentrates and iron-hemotoxylin stain. For comparison, negative stool and samples presenting other intestinal parasites were used. In figure 4 it is that the test used is able to detect a difference between stool samples from negative and positive individuals. Antigens in the stool samples from patients with other intestinal parasites present some cross reactivity with the purified IgG used.

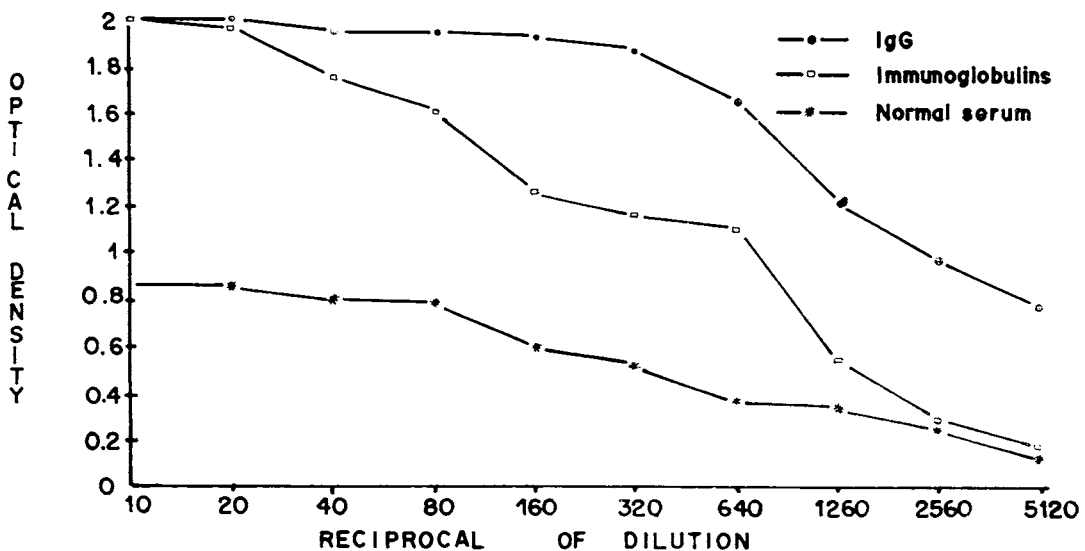


Fig. 1 - Reactivity of purified IgG and total immunoglobulins from hiperimmune rabbit sera in ELISA, using trophozoites whole extract from strain CSP, as antigen.

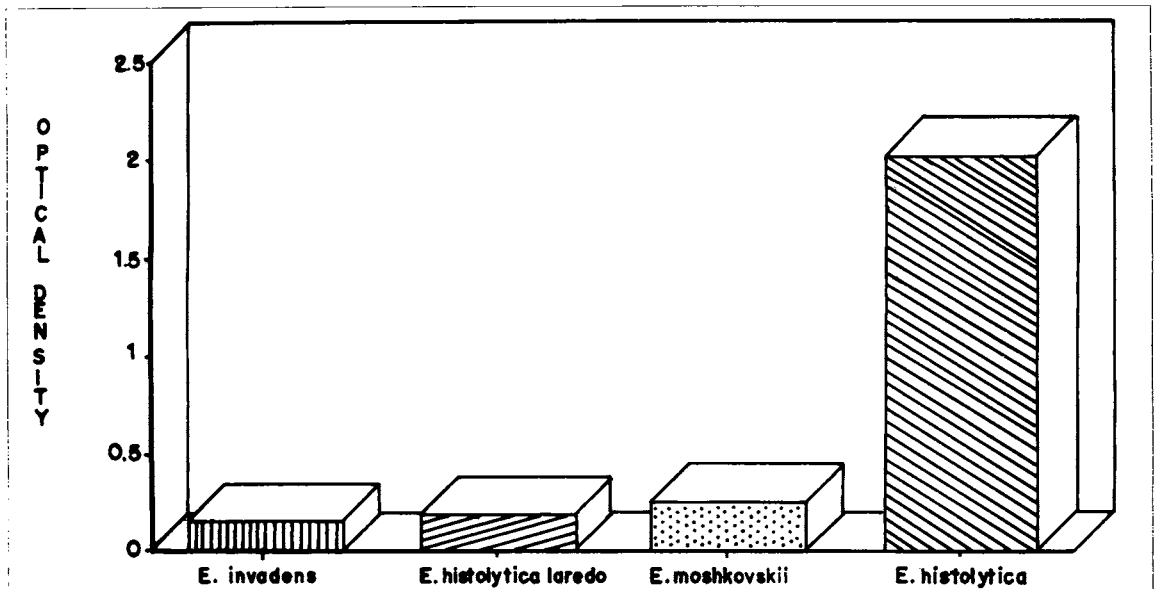


Fig. 2 - Purified IgG from rabbit immune sera reacting in ELISA with antigens from several *Entamoeba* isolates.

To calculate the sensitivity and specificity of the CSE technique with purified IgG, the data of figure 4 are presented in table 1.

Of the 163 fecal samples, 62 (38.04%) were posi-

tive by both coproscopic examination and the ELISA test. An additional 06 (3.68%) patients were positive only by the ELISA test; 05 (3.07%) patients were positive only by coproscopic examination; and 90 (55.21%) were negative by both coproscopic examination and the

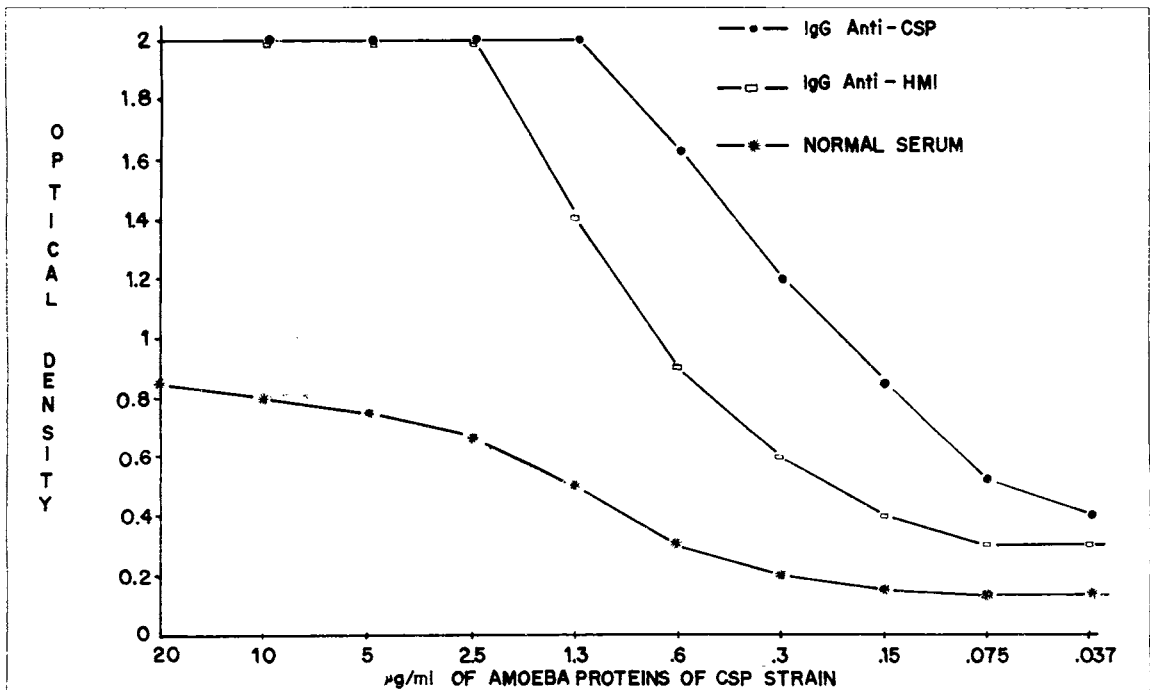


Fig. 3 - Purified IgG anti-CSP and anti-HM1 tested in ELISA against serial dilutions of trophozoites whole extract (µg/ml) of CSP strain.

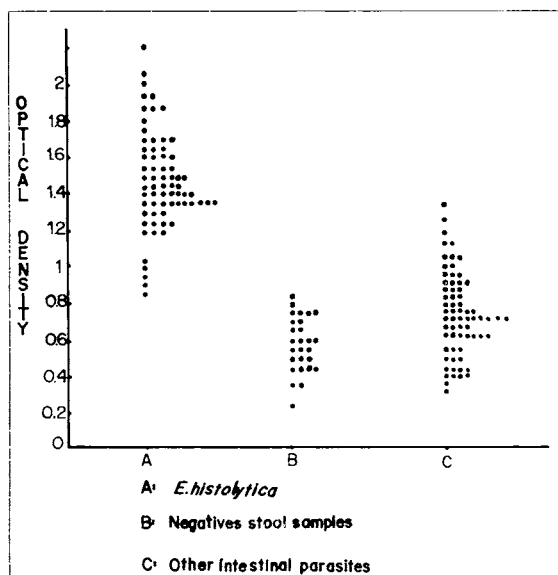


Fig. 4 - Detection of *Entamoeba histolytica* antigens by Capture Sandwich ELISA in Stool samples of patients with and without *E. histolytica*, or other intestinal parasites

ELISA test (Table 1). The negative patients detected in ELISA were the ones which presented values equal or below the mean of the negative stool samples plus standard deviation.

The capture sandwich ELISA showed a sensitivity of 92.50% and specificity of 94.75%. The combination of coproscopic examination and the capture sandwich ELISA gave an improved concordance and a discordance of 93.35% and 6.75%, respectively, and a positive and negative predictive values of 91% and 94%, respectively.

DISCUSSION

An enzyme-linked-immunosorbent assay (ELISA) test to detect amebae in feces was divided as an alternative approach to coproscopic and serologic diagnosis of intestinal amebiasis. With the purification of immunoglobulins it is possible to increase the sensitivity and specificity of antibodies for the detection of *E. histolytica*.

The method for purification of antibodies enabled the removal of many immunoglobulins which could otherwise compete with IgGs during the antigen-antibody reaction in capture ELISA assay.

The specificity obtained in our results agreed with

JAIN et al. ⁹, KARAPETIAN et al. ¹¹, NEAL et al. ¹⁴ and PARKHOUSE et al. ¹⁵, who found serologic differences when they used antigens from nonpathogenic *E. histolytica* and other amebae such as *Entamoeba moshkovskii*, *Entamoeba* like Laredo and *Entamoeba invadens*. Along with ELISA, these authors used indirect hemagglutination, precipitin tests and fluorescent assay.

Purified polyclonal antibodies obtained by affinity chromatography gave a better sensitivity and specificity for ELISA (93% and 94% respectively), comparable with UNGAR et al. ¹⁹, who using monoclonal antibodies, found sensitivity and specificity of 82% and 98%, respectively. WONSIT et al. ²² reported the development of a double antibody sandwich ELISA involving both monoclonal and polyclonal antibodies to detect amoebic antigens in stool samples that showed sensitivity and specificity values, 77.5% and 97.6%, respectively, similar to those reported by UNGAR et al. ¹⁹ and by us.

The weak cross-reactivity observed when anti-*E. histolytica* antibodies were tested with antigens from other tetranuclear amebae could be explained by the occurrence of common antigens between amebae of the genus *Entamoeba* PARKHOUSE et al. ¹⁵

The sensitivity of our purified IgG tested with ELISA by serial dilutions of the amebic antigen, permitted detection as low as 37-75ng of *E. histolytica* antigen (Fig. 3). GRUNDY ⁷ and JAIN et al. ⁹, using polyclonal rabbit antibody, were able to detect 150-300ng and 20-50 ng protein/well, respectively.

Positive correlation was found between the number of parasites (cysts or trophozoites) and optical density readings, and we observed that the assay was more sensitive in detecting free antigens, than whole parasites, similar data to those of GREEN et al. ⁶, who used the same method for detection of *Giardia lamblia* antigens.

TABLE 1

Comparison of the diagnosis of amebiasis by Capture Sandwich ELISA and stool examination for *Entamoeba histolytica*

ELISA	Stool Examination		Total
	Positive	Negative	
Positive	62	06	68
Negative	05	90	95
TOTAL	67	96	163

Chi-square analyses, $X^2 = 3.84$, $p > .05$

The results presented in this paper demonstrate the utility of the capture sandwich ELISA with purified antibody. It is easy to perform, less time-consuming than coproscopic examination, and should be very useful for the rapid investigation of large numbers of samples in clinics and in field conditions. Considering the possibility that this assay may also serve as a basis for the production of a diagnostic kit, it overcomes many of the problems traditionally associated with laboratory confirmation of the diagnosis of amebiasis by microscopy or by serological methods.

RESUMO

Entamoeba histolytica: Detecção de coproantígenos por ELISA de captura utilizando anticorpo purificado

Foi desenvolvido um teste de ELISA de Captura usando anticorpos policlonais purificados obtidos em coelhos contra três diferentes cepas axênicas de *Entamoeba histolytica* (ICB-CSP and ICB-462 do Brasil e HM1 do México) para detecção de coproantígenos em amostras de fezes de indivíduos: a) sintomáticos, b) assintomáticos, c) com outros parasitos intestinais, e d) sadios. Imunoglobulina G (IgG) contra *E. histolytica* foi isolada de imune soro de coelho, em duas etapas: cromatografia de afinidade em uma coluna contendo antígenos de *E. histolytica* unidos à Sepharose 4B, seguido por outra cromatografia em Sepharose 4B Proteína A. O teste de ELISA usando anticorpos purificados, foi capaz de detectar até um só trofozoíto por lâmina ou 70 ng de proteína de ameba por orifício, apresentando uma sensibilidade de 93% e uma especificidade de 94%. A combinação do exame microscópico com o método de ELISA de Captura teve uma concordância e discordância de 93,25% e 6,75%, respectivamente. Pode-se concluir que o teste de ELISA de Captura utilizando anticorpo purificado é altamente específico para a detecção de coproantígenos de *E. histolytica* em fezes de pacientes infectados, rápido, fácil e mais sensível que o exame microscópico.

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