

# DIAGNOSIS OF INTESTINAL AMEBIASIS USING SALIVARY IgA ANTIBODY DETECTION

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**Abstract.** Attempts were made to use soluble antigen extract of strain HK-9 of *Entamoeba histolytica* to detect salivary IgA antibodies in intestinal amebiasis patients by using ELISA. Total salivary samples of 109 individuals were divided into four groups. Group I comprised 32 patients whose stools were positive only for *E. histolytica* cysts and/or trophozoites. Group II comprised 12 individuals whose stools were positive for *E. histolytica* and other intestinal parasites. Group III comprised 36 individuals whose stools were negative for *E. histolytica* but contained other intestinal parasites such as *E. coli*, *E. nana*, *Blastocystis hominis*, *Trichomonas hominis*, *Giardia lamblia*, *Opisthorchis viverrini*, and hookworm. Group IV comprised 29 healthy individuals whose stools were free from any intestinal parasitic infections. Based on the mean optical density, OD + 2SD of the results from 29 parasitologically negative healthy individuals, the cut-off OD value for salivary IgA antibodies was 1.265. Therefore, the assays were positive in 14 out of 32 (43.75%) of group I and 2 out of 12 (16.6%) of group II. The assays were positive in 16 out of 36 (44.44%) for group III whereas 2 out of 29 (6.90%) for group IV were positive. The overall sensitivity and specificity of the assays were 36% and 72%, respectively. The false positive rate was 28% and the false negative rate was 64%. The predictive values of positive and negative results were 47% and 63%, respectively. The diagnostic accuracy of ELISA for the presence of salivary IgA antibodies was 58%.

## INTRODUCTION

*Entamoeba histolytica* is a protozoan parasite that infects the large intestine of man. The disease caused by this protozoan is called amebiasis and has a worldwide distribution. The prevalence rate is high in some tropical regions and is a public health problem in many developing countries such as Mexico, India, countries of northern and southern Africa, and Southeast Asia (Elsdon-Dew, 1968). It has been estimated that 10% of the world's population is infected with *E. histolytica* and that about 36 million suffering from amebic colitis and extraintestinal amebiasis. At least 40,000 to 100,000 deaths are attributable to amebiasis every year (Walsh, 1986). Amebic abscesses are found in the liver, lung and brain (Merritt *et al*, 1982). Many individuals harbor the parasites and pass cysts without any signs of illness, while others manifest symptoms ranging from mild diarrhea to fulminant, bloody mucous dysentery and amebic liver abscess.

Thailand, one of the tropical countries, is regarded as an endemic area in which the survey study of Department of Parasitology, Faculty of Public Health, Mahidol University during 1968-1973 found the parasite all over the country. The prevalence rate was between 1.4-4.2% depending on areas and socioeconomic status (Harinasuta and Charoenlarp, 1971; Chullaruk, 1977; Bunnag *et al*, 1982). Due to lacking a sanitary standard system and health education during that time period, the people have opportunity to acquire this parasite from contaminated food and drinking water. Although the rate of infection is quite low but it is the cause of potential lost of life. Metronidazole, a good effective drug can treat amebiasis efficiently (Maltz and Knauer, 1991). However, the treatment is always done after the obvious symptom have been shown which indicated that the patient is in an invasive stage.

One of the traditional methods in circulating antibody determination is serological assay techniques. Serological assays use many methods such as immunoelectrophoresis (IEP) (Savanat and Chaicumpa, 1969) where circulating antibody in patients with liver abscess and acute diarrhea could be detected for 85-97% and 66-80%, respectively, complement fixation (CF) (Jatinandana and Tharavanij, 1975), latex agglutination (LA) (Savanat *et al*, 1974), indirect hemagglutination (IHA)

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(Soavana *et al*, 1981) and enzyme-linked immunosorbent assay (ELISA) (Samrejongroj and Tharavanij, 1985), cellulose acetate precipitating (CAP) test (Thammapalerd *et al*, 1981). However, there are differences with each method (Tharavanij, 1975; Voller and De Savigny, 1981).

Account for the fact that most of the circulating antibody type is immunoglobulin-G (IgG), the result of the examination may be false positive although the disease has been eradicated at that time, IgG still exist for a long time. The examination result, also, could be false negative if the antibody system of patient is deficient. (Healy, 1986). The weak points of the serological assay technique mentioned above can mislead in treatment. Detection of amebic antigen is the method of choice to assess recent infection and test of cure after successful treatment as has been shown clearly in feces of all 13 patients harboring *E. histolytica* trophozoites by MAb-PAb-based ELISA (Thammapalerd and Thararanij, 1991; Wonsit *et al*, 1992), circulating amebic antigens in infected hamsters (*Mesocricetus auratus*) (Thammapalerd *et al*, 1996) and intrahepatic localization of trophozoite antigens by MAb-based IFA and MAb-based immunoperoxidase (Ipx) tests (Sherchand *et al*, 1994).

The precision examination for treatment should be the methods that can show the real causes of sickness. Another one method, antiamebic antibody examination of secretory IgA (sIgA) type to detect intestinal amebiasis caused by *E. histolytica*, has been reported by del Muro *et al* (1990). The sIgA antibody is local secretory immune response as an indicator of infectious diseases affecting mucosal sites. It can prevent the attachment of *E. histolytica* in trophozoites stage on intestinal wall which is the first step before invasive process and it is the important cause of intestinal amebiasis. (Leyva *et al*, 1992). It is, therefore, the objective of this study to attempt to use soluble antigen of *E. histolytica* for detecting secretory IgA antibody in saliva of patients with intestinal amebiasis.

## MATERIALS AND METHODS

### Subjects

There are four groups of subjects whose stools were examined for parasites, when positive for *E. histolytica* either trophozoites and/or cysts, or negative as control group, their saliva were then collected.

**Group I. Clinical specimens from amebiasis patients:** Stool samples from patients with symptomatic and/or asymptomatic amebiasis were obtained from wards of Siriraj Hospital; laboratory unit of the Bangkok Hospital for Tropical Diseases, Faculty

of Tropical Medicine, Mahidol University and from stool surveying at Kog Sabang village, Thai-Cambodian border, Sakaew Province. These stool samples were examined by a single fresh smear technique. The examination revealed cysts and/or hemophagous trophozoites of *E. histolytica*, Thirty-two stool samples positive for only *E. histolytica* were classified in this group and their corresponding saliva were collected.

**Group II. The specimens from patients infected with *E. histolytica* and other parasites:** This group comprised 12 patients whose stools were positive with *E. histolytica* and other parasites, and their saliva were collected accordingly.

**Group III. The specimens from people infected by parasites other than *E. histolytica*:** Thirty-six saliva were collected from people who lived at Kog Sabang village, Thai-Cambodian border, whose stool examinations were negative for *E. histolytica* but contained other intestinal parasites.

**Group IV. Healthy controls:** Twenty-nine saliva were obtained from volunteers whose stool examinations were free from any parasites.

### Collection and preparation of saliva specimens

Five ml of nonstimulated saliva was obtained from each group of the subjects. The saliva was collected into the cylindrical plastic box, 3x5 cm in size, and centrifuged at 13,000 rpm 10 minutes and the supernatant was frozen at -20°C until used.

### Preparation of *E. histolytica* antigen

Antigen was prepared from axenically grown of *E. histolytica* strain HK-9. The amebae were maintained axenically in TPS-1 medium according to the technique described by Diamond (1968). The medium consists of a nutrient broth supplemented with 2.5% vitamin mixture and 10% heat inactivated horse serum. Subculture was done at 48-72 hours of incubation at 37°C. The amebae were washed 3 times in physiological saline by centrifugation at 1,000 rpm for 5 minutes at room temperature. The ameba suspension was chilled and disrupted by an ultrasonic disintegrator (M and SE Ltd, London.) for 3-5 minutes. Total disruption of the ameba was ascertained by examination under a light microscope. The disrupted ameba suspension was centrifuged at 3,000 rpm for 10 minutes. The opalescent supernatant was distributed in small aliquots, lyophilized and kept at -20°C for being used as the antigen. The protein content of antigen was determined by the method of Lowry *et al* (1951) using bovine serum albumin as a standard.

### Enzyme-linked immunosorbent assay (ELISA) for anti-amebic salivary IgA detection

The indirect ELISA was done on 96-wells flat bottomed polyvinyl microtiter plate (Nunc-Immuno Plate) as described previously (Tharavanij, 1975). The conjugates were horseradish peroxidase labeled goat-antihuman IgA conjugated (A-07032, Sigma). The wells of the Nunc polyvinyl microtiter plate were precoated with 100  $\mu$ l of amoebic antigen in a dilution of 5  $\mu$ g/ml in carbonate buffer 0.1M, pH 9.6 and incubated at 37°C for 1 hour and overnight at 4°C in refrigerator, usually 18 to 20 hours. The unbound antigens were washed away excessively three times with phosphate buffer saline (PBS), pH 7.2, containing 0.05% Tween 20 (PBS-T). After washing the unbound surfaces were saturated with PBS-T containing 5% non-fat dry milk. The plate was incubated at 37°C for 1 hour and washed as above. After three additional washing, 100  $\mu$ l of undiluted clarified saliva was added into the wells. The plate was further incubated for 2 hours at 37°C, and then washed. One hundred  $\mu$ l of diluted horseradish peroxidase labeled goat anti-human IgA conjugate (1:1,000 dilution in PBST-0.1% BSA), was added to each well and the plate incubated at 37°C for 1 hour. The wells were washed and 100  $\mu$ l of the freshly prepared substrate (orthophenylenediamine dihydrochloride : OPD in phosphate-citrate buffer, pH 5.0) were added, the reaction was allowed to occur at 37°C. After 30 minutes, the enzyme reaction was stopped by adding 50  $\mu$ l of 12.5% H<sub>2</sub>SO<sub>4</sub> into each well. The optical density (OD), represented the substrate conversion by the enzyme was measured by ELISA reader (Titertek Multiskan, MCC/340, Flow Laboratories) at 492 nm.

## RESULTS

### ELISA for salivary IgA anti-amebic antibody detection

The cut-off OD value was obtained by taking 2SD (2 x 0.588) above mean value (0.677) of healthy subjects. Thus, the test was considered positive with OD of 1.265 or above. Therefore, with the cut off value of OD 1.265 (Mean + 2SD), the assay was positive only 16 out of 44 cases (36%) in groups I and II. In 65 of stools negative examination for *E. histolytica*, 20 out of 36 cases (56%) in group III and 27 out of 29 (93%) in group IV were negative by ELISA, respectively (Fig 1).

There were other parasites present in three *E. histolytica* positive stool samples of group II, one each of *E. coli*, *B. hominis* and *H. nana*, and 6 samples of *O. viverrini*. There were mixed parasitic infections in 2 samples with *A. lumbricoides*, *T. trichiura* ova and

*O. viverrini*, *S. stercoralis*, and hookworm respectively.

In group III, 36 stool samples were negative for *E. histolytica* but contained other parasites as follow: *E. coli*, *E. nana*, *B. hominis*, *T. hominis*, *G. lamblia*, *O. viverrini*, hookworm and intestinal fluke.

### Comparison of salivary IgA ELISA and microscopic stool examination for diagnosis of intestinal amebiasis

The salivary IgA detection was compared to microscopic stool examination. It was found that IgA antibodies to *E. histolytica* were present in saliva samples from 36% (16 of 44) of cases infected with this parasite. The overall sensitivity of IgA ELISA was 36% (16 of 44) and specificity 72% (47 of 65). The false positive rate was 28% (18 of 65) and the false negative 64% (28 of 44). The predictive value of a positive ELISA was 47% (16 of 34) and the predictive value of a negative ELISA was 63% (47 of 75). The diagnostic accuracy for the presence of salivary IgA antibodies to *E. histolytica* was 58% (63 of 109) (Table 1).

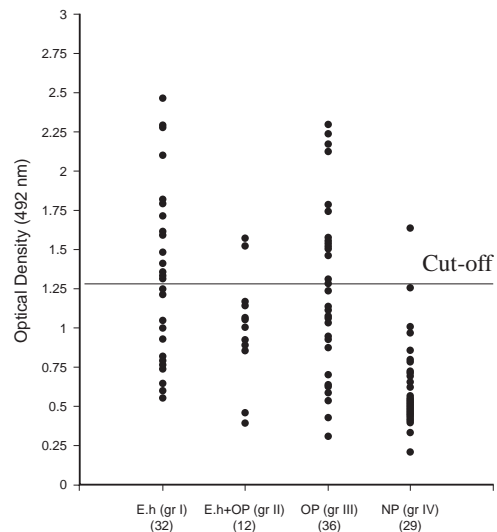


Fig 1- Scatter diagram of the optical density (OD) and cut-off OD from indirect ELISA for detecting sIgA antibodies in saliva samples.

gr I : Stool positive only for *E. histolytica* trophozoites.

gr II : Stool positive for *E. histolytica* and other intestinal parasites.

gr III : Stool negative for *E. histolytica* but containing other intestinal parasites.

gr IV : Stool negative for all parasites.

(—) : Total subjects in each group.

Table 1  
Comparison of salivary IgA ELISA and microscopic stool examination for diagnosis of *E. histolytica*

Tested by IgA ELISA	Positive for <i>E. histolytica</i> by microscopic stool examination	Negative for <i>E. histolytica</i> by microscopic stool examination	Total
Positive for <i>E. histolytica</i> by sIgA ELISA	16	18	34
Negative for <i>E. histolytica</i> by sIgA ELISA	28	47	75
Total	44	65	109

## DISCUSSION

Traditionally, the diagnosis of amebic infection is based on stool examination or specific antibodies detection. Both methods have several inherent problems. Microscopic examination of stool samples is tedious, time-consuming and dependent on a high degree of skill. The cystic stages could not be used to distinguish between *E. histolytica* and *E. dispar*. The most accurate method for diagnosis of invasive amebiasis is microscopic examination, showing hematophagous *E. histolytica* trophozoites. Another group of individuals, so-called asymptomatic cyst passers may be *E. dispar* which are an important group in transmission of the parasite, are not detected by such methods. There have been cases of misidentification of *E. histolytica* cysts, confusing them not only with cysts from other, non-pathogenic ameba, but also with lymphocytes and other cells found in stool samples (Neal, 1988).

There are many serological tests for amebic diagnosis, including the indirect hemagglutination (IHA), latex agglutination (LA), counterimmuno-electrophoresis (CIEP), gel diffusion (GD) and ELISA. These serological tests are particularly useful in the detection of extraintestinal amebiasis such as amebic liver abscess, since in such cases stool examination is often negative for *E. histolytica*. Since positive antibody titers may persist for months or years after successful treatment, it was therefore, difficult to differentiate between present and past infection (Krupp and Powell, 1971).

The detection of salivary IgA antibodies to *E. histolytica* for diagnosis of intestinal amebiasis has been described (del Muro *et al*, 1990). The membrane antigen of *E. histolytica* strain HM1:IMSS was used for plate coating. The sensitivity and specificity of the assays were 85% and 98% respectively. The diagnostic accuracy of salivary IgA antibodies was 91%. Therefore, salivary sampling may be a useful diagnosis of intestinal amebiasis.

ELISA established in this study demonstrated the possibility of using soluble antigen preparation from trophozoite extracted of axenic culture of *E. histolytica* strain HK-9, and peroxidase-labeled goat anti-human IgA conjugate (A-7032, Sigma) to detect anti-amebic IgA antibody in saliva from amebiasis patients. The positive results are those greater than 1.265 absorbance units (see Fig 1). Positive ELISA results in which microscopic stool examination were positive for *E. histolytica*, were 36% (16 of 44). The failure of the ELISA to detect the IgA in other specimens (28/44) may be due to either the amount of antibody below the sensitivity of the assay or recently acquired infection in which the antibody response has not yet appeared. In addition, the detecting antigens might not recognize the antibody in these specimens because of their loss in the antigenicity for specific sIgA to *E. histolytica* after long-term storage and repeated freezing and thawing (Root *et al*, 1978). Moreover, it was possible that the false-positive of microscopic examination may be due to misidentification between *E. histolytica*, *E. dispar* and *E. hartmanni* or other nonpathogenic amebae such as *Iodamoeba butschlii* as well as white blood cells (Neal, 1988).

The sIgA ELISA results were positive in 18 out of 65 subjects without demonstrable *E. histolytica* in stools. Therefore, the false positive rate was as high as 28%. The problem might be due to the assays detecting residual IgA antibodies. In addition, the subjects studied were mostly from the Thai-Cambodian border where a high parasitic load and a high risk of infection were expected (Kataitong, 1994). Under these conditions, intestinal parasites in constant contact with the host's tissue may elicit a secretory immune response even in the absence of invasiveness (Mestecky *et al*, 1978). In human, IgA antibodies appeared in external secretion about two weeks after presentation of bacterial antigens to the gut associated lymphoid tissue (Czerkinsky *et al*, 1987) and that will decrease over 45 days after treatment. Furthermore, it was possible that the examination of stool samples might miss the

parasites from collection since the parasites might be variably excreted or unevenly distributed in the stool. Marsden and Smith (1946) reported the probability of diagnosis based on one direct microscopic stool examination as 50% in a person excreting 100,000 cysts daily or 0.45% in a person excreting only 1,000 cysts a day.

The IgA ELISA was found in a salivary sample from one child (OD 1.636) whose stool was negative for all parasites. The false-positive results could be attributed to missing diagnosis of *E. histolytica* in his stool. Unfortunately, we could not follow up this case for further study.

The assay was less satisfactory because of the overall sensitivity and specificity of the IgA ELISA in this study was 36% and 72% respectively. The false positive rate was 28% and the false negative rate was 64%. The predictive value of a positive ELISA was 47% and the predictive value of a negative ELISA was 63%. The diagnostic accuracy for the presence of salivary IgA antibodies to *E. histolytica* was 58%. Therefore, the membrane antigens were more appropriate than those of soluble antigens for the diagnosis of salivary IgA antibodies in intestinal amebiasis.

The presence of secretory immunoglobulin A (sIgA) anti-*E. histolytica* antibodies in the saliva of patients with intestinal amebiasis was demonstrated by immunoblot assay, and the capacity of these antibodies to inhibit amebic adherence to a monolayer of MDCK cells was analyzed. (Carrero *et al*, 1994). Inhibition was due to IgA antiamebic antibodies and in part to anti-Gal-binding-lectin antibodies, as demonstrated by absorption experiments with total amebic extract and with the fraction of Gal-binding lectin (Carrero *et al*, 1994). These results emphasize the relevance of secretory IgA antibodies in the phenomenon of *E. histolytica* adherence to epithelial cells.

The *E. histolytica* surface antigens capable of inducing secretory IgA (sIgA) responses in humans have been cloned and sequenced (Carrero *et al*, 2000). A cDNA library from the strain HM1:IMSS was immuno-screened with saliva from patients with intestinal amebiasis or amebic liver abscess. Clones isolated with sIgA antibodies from patients with intestinal amebiasis corresponded to the known serine-rich protein isoform, a 29 kDa cysteine-rich protein and 1-alpha elongation factor. Clones corresponding to enolase, cyclophilin, ribosomal protein L23a, and an Hsp70 family protein were isolated with sIgA from a patient with amebic liver abscess. A glutamic acid-rich peptide (EhGARP) positive with sIgA from a

patient with amebic liver abscess was also isolated; for EhGARP, no homologs were found in the protein databases (Carrero *et al*, 2000). The antigens isolated are potentially useful in the development of an oral vaccine or new diagnostic tools for amebiasis.

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