Diagnosis of Invasive Amebiasis by Enzyme-Linked Immunosorbent Assay of Saliva To Detect Amebic Lectin Antigen and Anti-Lectin Immunoglobulin G Antibodies

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Saliva from subjects with amebic liver abscess (ALA), acute amebic colitis, asymptomatic infection with *Entamoeba histolytica* or *Entamoeba dispar*, and uninfected controls was tested by enzyme-linked immunosorbent assay (ELISA) for the presence of *E. histolytica* galactose-inhibitable lectin antigen and salivary immunoglobulin (IgG) antibodies to a recombinant cysteine-rich lectin-derived protein (LC3). Salivary lectin antigen was found in 65.8% of subjects with acute colitis, compared to 22.2% of those convalescent from ALA, 10.0% with asymptomatic *E. histolytica* infection, 9.8% with *E. dispar* infection, and 2.6% of controls (subjects from the United States and study patients with nonamebic diarrhea) (*P* < 0.001 for each compared to values for subjects with colitis). Salivary anti-LC3 IgG antibodies were found in 92% of ALA patients regardless of duration of illness and in 83.3% of colitis patients who were symptomatic for at least 7 days (*P* < 0.001 compared to other study groups). Serum anti-LC3 IgG antibodies were detected in 56.3% of subjects with acute colitis, 100% of subjects with ALA or prolonged colitis, 45% of subjects with asymptomatic *E. histolytica* infection, 32.3% of subjects with *E. dispar* infection, and 23.4% of diarrhea controls. In comparison to ELISA for serum anti-LC3 IgG antibodies, the salivary lectin antigen assay is a more sensitive and specific test for acute amebic colitis. Detection of salivary anti-LC3 IgG antibodies by ELISA is an effective means for the diagnosis of ALA and prolonged cases of amebic colitis.

Entamoeba histolytica infection most commonly results in asymptomatic colonization. While invasive amebiasis presents as amebic colitis or amebic liver abscess (ALA) (18). Currently, diagnosis requires skilled microscopy and a variety of serologic methods. Infection by *E. histolytica*, but not *Entamoeba dispar*, elicits a serum antiamebic immunoglobulin (Ig) antibody response (19). LC3 is a recombinant cysteine-rich portion of the *E. histolytica* galactose-inhibitable lectin's 170-kDa subunit (23). Anti-LC3 IgG and IgA antibodies are found in serum and anti-LC3 IgA antibodies in saliva and stool of subjects with invasive amebic disease or asymptomatic *E. histolytica* infection (4, 23). The recombinant LC3 protein is immunogenic and effective as a subunit vaccine against experimental ALA in a gerbil model (23). Early during the course of invasive amebiasis, detection of serum lectin antigen (2, 4) and anti-LC3 IgM antibodies (3, 22) is helpful in diagnosis. Seroconversion with antiamebic IgG antibodies occurs after 1 week of invasive disease symptoms and is diagnostic (1, 15, 19, 22).

Differentiation of *E. histolytica* from *E. dispar* in stool is accomplished by culture and zymodeme analysis (21), recognition by species-specific monoclonal antibodies (16), and DNA hybridization (6). However, detection of *E. histolytica* intestinal infection does not differentiate invasive disease from noninvasive colonization. Detection of amebic antigen (2, 4, 8) and antiamebic antibodies in either serum (1, 3, 19) or other body fluids, like saliva (4, 5, 9), plays a critical role in estab-

lishing the diagnosis and differentiating asymptomatic *E. histolytica* infection from invasive amebiasis.

Analysis of saliva can provide useful information for a wide range of diseases and is a good alternative to collection of venous blood, as it has some of the features of plasma and urine (12). Salivary antibody responses have been studied in a variety of infectious diseases; salivary IgG antibodies were found to have varied sensitivity for detection of infection by *Toxoplasma gondii* (10), *Schistosoma mansoni* (7), *Helicobacter pylori* (11), and hepatitis A virus (13).

The goal of our study was to determine whether detection by enzyme-linked immunosorbent assay (ELISA) of salivary lectin antigen and salivary anti-LC3 IgG antibodies will be effective in diagnosis of invasive amebiasis, differentiating it from noninvasive *E. histolytica* intestinal infection.

MATERIALS AND METHODS

Study populations. In the outpatient clinic of the Tropical Medicine Department at El-Hussein University Hospital, Cairo, Egypt, salivary samples were obtained from 38 patients with acute diarrhea who had stool ELISA positive for *E. histolytica* lectin and occult fecal blood (32 patients symptomatic for <7 days and 6 symptomatic for more than 1 week), 16 patients with acute diarrhea who were positive for *E. histolytica* fecal lectin but without occult blood, 7 subjects positive for *E. dispar* fecal lectin, and 29 patients with acute diarrhea who had negative stool antigen studies for *E. histolytica* or *E. dispar*. In South Africa, saliva was obtained from 36 ALA patients 1 week after treatment with metronidazole, 4 subjects with asymptomatic *E. histolytica* infection, and 24 individuals with *E. dispar* colonization. ALA cases were diagnosed by sonography and abscess aspiration, while asymptomatic *E. histolytica* and *E. dispar* colonization were identified by stool culture and zymodeme analysis. Control saliva was collected from 48 employees of the University of Minnesota without any history of amebic infection or a recent visit to an area of endemic amebiasis. Five milliliters of saliva was collected from each subject and kept on ice for up to 2 h before being stored at −70°C. All samples were shipped frozen in dry ice to the United States. The present studies were approved by the Institutional Review Boards at the Uni-

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versity of Minnesota, the University of Natal (Durban, South Africa) and Al-Azhar University (Cairo, Egypt).

ELISA for determination of salivary 170-kDa lectin antigen. Flat-bottomed microtiter polystyrene ELISA plates (96 wells; Corning Glass Works, Corning, N.Y.) were coated with monoclonal antibody 3F4 (1.6 μg/well), which is specific for epitopes present in both *E. histolytica* and *E. dispar*, in coating buffer (80 ml of 0.2 M Na$_2$CO$_3$ and 170 ml of 0.2 M NaHCO$_3$). Nonreactive sites were blocked with 1% bovine serum albumin (BSA) in coating buffer. Saliva samples were mixed in an equal volume of phosphate-buffered saline (PBS) containing 2 mM phenylmethylsulfonyl fluoride (United States Biochemical Corp., Cleveland, Ohio) and added at 100 μl/well for incubation for 2 h at room temperature or overnight at 4°C. Alkaline phosphatase-conjugated 8A3 (which recognizes lectin epitopes of *E. histolytica* and *E. dispar*) was added in a concentration of 0.5 μg/ml at 100 μl/well of a 1:1,000 dilution in PBS–Tween–1% BSA for incubation at room temperature for 2 h. Developing solution (1 M diethanolamine and 1 mM MgC$_{12}$ [pH 9.8] with *p*-nitrophenyl phosphate disodium [1 mg/ml]) was added, and the reaction developed for 2 h at room temperature. The degree of color change (optical density at 405 nm) was measured in an ELISA plate reader (Bio-Rad microplate reader; Benchmark, Hercules, Calif.). Results were corrected for nonspecific background by subtracting the optical density of paired wells not coated with a primary antibody but otherwise subjected to the identical procedure. Each sample was studied in duplicate. We have previously demonstrated the lack of cross-reactivity of these antibodies with other parasitic antigens (2, 4).

ELISA for detection of salivary anti-LC3 IgG antibody. Flat-bottomed microtiter polystyrene ELISA plates (96 wells; Costar, Cambridge, Mass.) were coated with recombinant LC3 lectin (0.2 μg/well) by incubating overnight in buffer (80 ml of 0.2 M Na$_2$CO$_3$ and 170 ml of 0.2 M NaHCO$_3$). Plates were washed five times with PBS-Tween. Nonreactive sites were blocked with 1% BSA in coating buffer for 2 h at room temperature. Saliva was mixed in an equal volume of PBS containing 2 mM phenylmethylsulfonyl fluoride and added at 100 μl/well for incubation for 2 h at room temperature or overnight at 4°C. Wells were washed (with PBS-Tween), and alkaline phosphatase-conjugated goat anti-human IgG antibodies, diluted in PBS–Tween–1% BSA (1:5,000), were added at 100 μl/well for incubation at room temperature for 2 h. Plates were washed in PBS-Tween and a developing solution (1 M diethanolamine and 1 mM MgC$_{12}$ [pH 9.8] with *p*-nitrophenyl phosphate disodium [1 mg/ml]) was added. The reaction was developed in the dark for 2 h at room temperature, and the degree of color change (optical density at 405 nm) was measured in an ELISA plate reader. Results were corrected for nonspecific background by subtracting the optical density of paired wells not coated with primary antibody but otherwise subjected to the identical procedure. Each saliva sample was studied in duplicate.

Statistics. Results are expressed as means ± standard deviations, percents positive, and percents negative. The *Z* test (converted to *P* value) and unpaired Student *t* test were used to determine the significance of differences (24). Sensitivity was calculated as follows: number of patients with positive test results/total number of patients × 100. Specificity was calculated as follows: number of controls with negative test results/total number of controls × 100. The positive predictive value was calculated as follows: number of true positives/(number of true positives + number of false positives) × 100. The negative predictive value was calculated as follows: number of true negatives/(number of true negatives + number of false negatives) × 100. The positive predictive value defines the probability of patients having a disease if the test is positive. The negative predictive value defines the probability of patients not having invasive amebiasis if the test is negative.

RESULTS

Salivary 170-kDa adherence lectin. Table 1 summarizes the mean of ELISA readings for detection of lectin antigen and anti-LC3 IgG antibodies in saliva and anti-LC3 IgG antibodies in serum for all subjects studied. None of the U.S. controls had positive assays for salivary lectin antigen, compared to 9.8% of those with *E. dispar* infection, 10% of those with noninvasive *E. histolytica* infection, and 6.9% of those with nonamebic diarrhea (no significant differences between these groups; *P* > 0.1). Amebic colitis subjects were more likely to have positive assays for salivary lectin (65.8%), compared to those convalescent for ALA (22.2%) (*P* < 0.001) or any of the control groups (*P* < 0.001 for each compared to colitis). ALA patients were more likely to be positive than U.S. controls (*P* = 0.002), but their results were not significantly different from subjects with nonamebic diarrhea (*P* = 0.176), *E. dispar* infection (*P* = 0.301), or noninvasive *E. histolytica* infection (*P* = 0.436).

Salivary anti-LC3 IgG antibody response. As summarized in Table 1, anti-LC3 IgG antibodies were found in the saliva of 92% of all ALA subjects and in 83.3% of patients with amebic

TABLE 1. Detection of salivary 170-kDa adherence lectin and salivary anti-LC3 IgG antibodies, with comparison to the serum anti-LC3 IgG antibody response

Study group (no. of subjects)	Mean OD reading g (% positive ELISA) for:		
	Salivary 170-kDa lectin	Salivary anti-LC3 IgG	Serum anti-LC3 IgG
Amebic colitis[a] (32)	0.662 (65.8)	0.13 (18.4)	0.357 (56.3)
Amebic colitis[b] (6)	0.915 (66.7)	0.257 (83.3)	0.399 (100)
ALA (36)	0.318 (22)	0.756 (92)	0.611 (100)
Noninvasive *E. histolytica* (20)	0.241 (10)	0.140 (10)	0.365 (45)
E. dispar (31)	0.271 (12.9)	0.115 (6.4)	0.304 (32.3)
Nonamebic diarrhea (29)	0.265 (6.9)	0.11 (6.9)	0.324 (62.1)
U.S. controls (48)	0.174 (0)	0.081 (0)	0.125 (0)

[a] Symptoms lasting less than 7 days.
[b] Symptoms lasting for at least 7 days.

colitis who were symptomatic for a week or more (compared to 6.4% with *E. dispar* infection, 10% with asymptomatic *E. histolytica* infection, and none of 48 American controls [*P* < 0.005, for each]). Salivary anti-LC3 IgG antibodies were less frequently found in subjects with acute amebic colitis (<7 days in duration) and nonamebic diarrhea (for both, *P* < 0.001 compared to ALA patients and patients colitis for longer than 7 days, respectively) (Table 1).

Serum anti-LC3 IgG antibody response. Serum anti-LC3 IgG antibodies were detected in 100% of ALA patients and amebic colitis patients with more than 7 days of illness (*P* = 0.258 and 0.983 compared to salivary anti-LC3 IgG, respectively) (Table 1). ELISA for detection of serum anti-LC3 IgG antibodies was a more sensitive test for diagnosis of acute amebic colitis (56% positive compared to 18.4%; *P* < 0.05), but had a higher level of false-positive results during *E. dispar* infection (32% positive) and nonamebic diarrhea (62% positive; *P* < 0.05 compared to saliva anti-LC3 IgG antibodies). This is most likely due to the high levels of background positive antiamebic IgG in areas of endemicity due to frequent asymptomatic *E. histolytica* infections (7).

Sensitivity, specificity, and predictive values of ELISA for detection of salivary antigen and anti-LC3 IgG antibodies in comparison with serum anti-LC3 IgG antibody response. As summarized in Table 2, ELISA for detection of salivary 170-kDa lectin antigen demonstrated a 65.8% sensitivity and a 97.4% specificity, with a 92.3% positive predictive value and a 91.5% negative predictive value for diagnosis of acute amebic colitis. In contrast, ELISA for detection of salivary anti-LC3 IgG antibodies in convalescent ALA patients demonstrated 92% sensitivity and a 97.4% specificity, with a 94.3% positive predictive value and a 96.2% negative predictive value. In patients with amebic colitis who are symptomatic for more than one week, ELISA for salivary anti-LC3 IgG antibodies had 83.3% sensitivity and a 97.4% specificity, with a 71.4% positive predictive value and a 98.7% negative predictive value. Salivary 170-kDa lectin detection showed an improved diagnostic capability compared to ELISA for serum anti-LC3 IgG antibodies, which had 56.3% sensitivity and a 76.6% specificity, with a 50% positive predictive value and an 80.8% negative predictive value.

DISCUSSION

There is a broad spectrum of interest regarding the study of secretory IgA antibody responses resulting from amebic infections. Most investigators have studied salivary antiamebic IgA

TABLE 2. Statistical analysis of salivary lectin and anti-LC3 IgG antibody test results and comparison to ELISA for serum anti-LC3 IgG antibodies[a]

Value	Amebic colitis (<1 wk)			Amebic colitis (>1 wk)			ALA		
	Sal. lectin	Sal. IgG	Serum IgG	Sal. lectin	Sal. IgG	Serum IgG	Sal. lectin	Sal. IgG	Serum IgG
Sensitivity (%)	65.8	18.4	56.3	66.7	83.3	100	22.0	92.0	100
Specificity (%)	97.4	97.4	76.6	97.4	97.4	76.6	97.4	97.4	76.6
Positive predictive (%)	92.3	77.8	50.0	66.7	71.4	25.0	80.0	94.3	66.7
Negative predictive (%)	91.5	75.0	80.8	97.4	98.7	100	72.2	96.2	100

[a] Specificity, sensitivity, and positive and negative predictive values are expressed in percentages according to the definition described in Materials and Methods. Analyses were carried out using salivary (Sal.) lectin and IgG and serum IgG from patients with amebic colitis (divided into those with symptoms lasting less than 1 week and those with symptoms lasting more than 1 week) and patients with ALA.

antibodies for determination of possible protective immunity or for use as a diagnostic tool (4, 5, 9). Recently, the diagnostic efficacy of salivary antiamebic IgA antibodies was studied in amebic colitis patients, ALA patients, and asymptomatic cyst carriers (4). Investigators reported finding salivary antiamebic IgA antibodies in 90 to 100% of Mexican subjects with amebic colitis and ALA (14, 17). Salivary antiamebic IgA antibody responses were not found during asymptomatic *E. histolytica* infection ($P = 0.25$) (17). An epidemiologic survey in a Mexican village reported finding positive fecal microscopy results for amebic cysts in 83.3% (827 out of 933) of the study population; of those having cysts, 94.9% had salivary antiamebic IgA antibodies. Noncharacterized amebic antigens were used in these ELISA studies, and nonspecific reactions due to other parasites were not excluded (20). Studies of salivary antigen and IgG antibodies have only been reported for the diagnosis of other infectious diseases (7, 10, 11, 13).

We found that ELISA for the detection of *E. histolytica* lectin antigen and anti-LC3 IgG antibodies in saliva provided an effective method for diagnosis of amebic colitis and ALA. Serum lectin antigenemia is detected early during the course of invasive amebiasis (2). Within a week of therapy, lectin antigenemia is cleared (4) and serum antiamebic IgM antibodies began to appear (3, 22). Serum antiamebic IgG antibodies are usually found after 1 week of symptoms due to invasive amebiasis (1, 19, 22). We found that salivary anti-LC3 IgG antibodies were also detected in subjects 1 week after treatment for ALA and amebic colitis patients (≥7 days duration of symptoms) but not in subjects with acute amebic colitis or in subjects having asymptomatic infection with *E. histolytica* or *E. dispar*. Of interest, this is in contrast to studies of serum anti-LC3 or antilectin IgG antibodies (1, 19, 23), which are found during asymptomatic *E. histolytica* infection. The lack of measurable salivary IgG in the setting of a positive ELISA for serum anti-LC3 IgG may be due to a dilutional effect. As expected, *E. dispar* infection did not elicit salivary or serum antiamebic IgG antibodies, confirming the results of serology studies (1, 4, 19).

Salivary lectin was found in subjects with *E. histolytica* colitis, a finding which is in agreement with studies of detection of lectin antigen in serum (3, 4). Subgrouping of patients with amebic colitis by duration of illness (shorter or longer than 1 week) does not change the sensitivity of ELISA for salivary antigen as a diagnostic test. However, the sensitivity of this test is significantly decreased in ALA patients 1 week after starting antiamebic therapy. This might be attributed to the relationship between the availability of amebic lectin antigen in body fluids and elimination or at least reduction of the *E. histolytica* infection load by medical treatment. This hypothesis is supported by the clearance of serum lectin within a few days after administration of antiamebic therapy for amebic colitis (4) and

persistence of salivary lectin in patients with prolonged amebic colitis who have not yet receive amebicidal therapy.

The negative association of asymptomatic *E. histolytica* infection with salivary lectin is supported by the absence of a salivary antiamebic IgA antibody response in the same group (17). This could be attributed to a lack of invasion or a dilutional effect of saliva compared to serum. Absence of salivary lectin in *E. dispar* infection is consistent with its well documented nonpathogenic properties (2, 6, 19).

In summary, ELISA for salivary anti-LC3 IgG antibodies was a highly specific and sensitive diagnostic test for identifying patients with ALA and amebic colitis (duration of symptoms, ≥7 days). Detection of salivary adherence lectin antigen is an effective tool for use in early diagnosis of invasive amebiasis, before the appearance of serum or salivary antiamebic IgG antibodies.

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