Entamoeba histolytica: Efficacy of Microscopic, Cultural, and Serological Techniques for Laboratory Diagnosis

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Of 110 subjects with clinical evidence of amebiasis, 15 (14%) were shown to be infected with Entamoeba histolytica. Microscopic examination of stool specimens rendered a diagnosis in all eight cases of localized intestinal infection, but in only one of seven patients with invasive amebiasis. Culture was concomitantly diagnostic in six patients with intestinal amebiasis and in one patient with extraintestinal infection. Assay for antibody to E. histolytica by counterimmunoelectrophoresis and indirect hemagglutination were each 100% effective in all cases of invasive amebiasis and in diagnosing two of eight patients with intestinal infection. Stool specimens of 15 patients revealing intestinal parasites other than E. histolytica failed to demonstrate cultural or serological evidence of amebiasis. Low levels of antibody were observed in the indirect hemagglutination assay in four patients with disease other than amebiasis and in three control sera positive for rheumatoid factor. By counterimmunoelectrophoresis, reactive sera were only encountered among those derived from patients with amebiasis. Six of seven patients with hepatic amebiasis may have gone undiagnosed if not for serology.

Amebiasis, a disease caused by the protozoan parasite Entamoeba histolytica, ensues after ingestion of mature four-nucleated cysts. After excystment, the emerging trophozoites can exist as harmless commensals in the lumen of the bowel, cause a chronic or acute dysentery, or invade through the intestinal mucosa to cause extraintestinal infections, especially in the liver (7).

Intestinal amebiasis is diagnosed by identifying E. histolytica cysts or trophozoites in fecal specimens or, histologically, by visualizing amebae in biopsy specimens or secretions of intestinal mucosa. The detection of this parasite in feces, however, is often difficult in a heterogeneous background containing numerous morphologically similar structures, i.e., other protozoa, artifacts, and phagocytic cells (1). Additionally, in subacute infections, the sparsity of E. histolytica cysts in stool specimens precludes diagnosis in many instances despite the availability of various staining techniques. In the absence of visualizing the parasitic agent in stools or other clinical materials, culture of the specimen may serve as a diagnostic aid. Culturing provides an environment conducive for excystment and multiplication of trophozoites which are then readily observed microscopically.

In symptomatic patients, the failure to identify E. histolytica in specimens does not necessarily rule out amebiasis. Passage of cysts in stools can vary from none to millions per gram of feces (1). Additionally, interfering substances such as antiparasitic drugs, laxatives, antacids, barium, and antidiarrheal preparations make it almost impossible to identify trophozoites (5), whereas in cases of extraintestinal infection, the parasite is often absent from the intestinal tract (7). In the latter instance, the application of serology can be an invaluable aid in achieving diagnosis.

Invasive amebiasis provokes humoral antibodies that can be detected serologically. The standard serological test for amebiasis has been the indirect hemagglutination assay (IHA) (6). Counterimmunoelectrophoresis (CIE) has only recently been adapted for the diagnosis of amebiasis, and its utility has yet to be fully established.

The present study was designed to assess the relative merit of three diagnostic endeavors, namely, microscopic examination, culture, and serology for achieving the laboratory diagnosis of amebiasis. The efficacy of the recently introduced CIE technique was studied concomitantly with that of the IHA.

MATERIALS AND METHODS

Source of clinical specimens. Serum and stool specimens from 110 subjects seen at The Mount Sinai Hospital, New York, New York, were used in this study.

METHODS

Microscopy. Fecal specimens were examined for the presence of Entamoeba histolytica using standard procedures for stool examination. The wet mount method was used for detection of trophozoites, and the formalin-ether concentration method for detection of cysts. The presence of amebae in stool was considered diagnostic for amebiasis.

Culturing. Fecal specimens were cultured on standard media for E. histolytica. Cultures were incubated for 14 days at 37°C and observed microscopically for the presence of trophozoites and cysts. The presence of amebae in stool was considered diagnostic for amebiasis.

Serology. Serum specimens were tested for the presence of amebiasis by the IHA and the CIE. The IHA was performed using the method described by Reddy et al. (10). The CIE was performed using the method described by Young et al. (12).

RESULTS

Table 1 summarizes the results of the microscopic, cultural, and serological examinations of the fecal specimens from 110 subjects. The microscopic examination of stool specimens was diagnostic for amebiasis in all eight cases of localized intestinal infection and in one case of invasive amebiasis. The cultural examination of stool specimens was diagnostic for amebiasis in six cases of localized intestinal infection and in one case of invasive amebiasis. The serological examination of serum specimens was diagnostic for amebiasis in all cases of localized intestinal infection and in one case of invasive amebiasis.

DISCUSSION

Microscopic examination of stool specimens is a sensitive and specific method for the diagnosis of amebiasis. However, it is often difficult to identify the parasitic agent in stools or other clinical materials. Culture of the specimen provides an environment conducive for excystment and multiplication of trophozoites which are then readily observed microscopically.

In symptomatic patients, the failure to identify E. histolytica in specimens does not necessarily rule out amebiasis. Passage of cysts in stools can vary from none to millions per gram of feces (1). Additionally, interfering substances such as antiparasitic drugs, laxatives, antacids, barium, and antidiarrheal preparations make it almost impossible to identify trophozoites (5), whereas in cases of extraintestinal infection, the parasite is often absent from the intestinal tract (7). In the latter instance, the application of serology can be an invaluable aid in achieving diagnosis.

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Hospital with clinical evidence of amebiasis were evaluated based on their respective modalities. A total of 40 sera obtained from the arthritis and serology laboratories served as controls. Of these, five had heterophile antibody (1:448 to 1:1,792), five showed anti-salmonella "febrile agglutinins" (1:400 to 1:1,600), five were reactive to both the Venereal Disease Research Laboratory assay and the Treponema pallidum hemagglutination assay, and ten were positive for rheumatoid factor. Sera from five healthy individuals and ten patients suspected of having autoimmune disease were also studied.

Microscopic methods. Freshly evacuated fecal specimens (<30 min old) were examined by direct smears initially prepared in saline and then in buffered methylene blue. Subsequently, all stools were processed by the Formalin–ether sedimentation technique (13), utilizing D’Antoni’s iodine. For additional confirmation of suggestive trophozoites or cysts of *E. histolytica*, permanent trichrome staining was carried out.

Cultural method. Culturing was performed by the method of Edelman and Spingarn (2), using Entamoeba slants (Difco Laboratories, Detroit, Mich.), 50 mg of rice powder (Difco), and a 1:6 horse serum/saline overlay. The original technique was modified by incorporating 3 mg of streptomycin and 3,000 U of penicillin into the overlay in a single tube. The culture was incubated at 37°C, and material from the bottom of the liquid phase of the culture was aspirated and examined microscopically each day for a period of 7 days.

Serological methods. IHA was performed by the method of Kessel et al. (6). The protein antigen (*E. histolytica* HK-9) was purchased from ICN Pharmaceuticals, Portland, Ore. Positive reactions were indicated by hemagglutination, resulting in a "blanket" of cells covering the entire curved surface of the well. Negative reactions were indicated by the formation of a circumscribed "button" of erythrocytes on the bottom of the well. Based on the criteria of the manufacturer, a titer of 1:64 was considered borderline, whereas those of 1:128 or greater were considered diagnostically high (1:2,048) and low (1:64)-titer reactive sera, a nonreactive serum, and diluent controls were incorporated in each assay.

CIE. Amebiasis test kits and electrophoresis unit (REC-300) were purchased from Cordis Labs, Miami, Fla. CIE was performed by the specifications of the manufacturer. The antigen, prepared from axenic cultures of *E. histolytica* HK-9, was reacted at room temperature against inactivated serum in 1% agarose plates at 20 mA for 1 h. A barbital (0.04 M)–acetate (0.02 M) buffer, pH 8.2, was used. After the prescribed hour, the current was terminated, and the plate was gently rinsed with cold running tap water. The agar plate was then observed against a dark, nonreflecting background with indirect illumination and twofold magnification. The visualization of a white precipitin band(s) between the antigen and antibody wells was interpreted as a positive reaction (Fig. 1). In the absence of a visible precipitin line, the plate was placed in a humid chamber at room temperature and reexamined after 18 h. This procedure was also followed to permit better visualization of initially weak precipitin band(s). Positive sera were serially diluted in saline and titrated. The absence of a precipitin band was interpreted as a negative reaction. Positive and negative controls were included in all tests.

RESULTS

Serum and stool specimens derived from 110 subjects were evaluated by the three modalities: microscopic, cultural, and serological. Of these specimens, 76 lacked evidence of amebiasis based on the test parameters. Fifteen patients whose stool microscopy revealed intestinal parasites other than *E. histolytica*, i.e., *Entamoeba coli*, *Endolimax nana*, *Giardia lamblia*, *Ascaris lumbricoides*, *Trichuris trichiura*, and *Schistosoma mansoni*, failed to show cultural or serological evidence of amebiasis.

Of the 110 patients, 15 proved to have some form of amebiasis on the basis of the test modalities. Eight had intestinal infection, and seven had extraintestinal involvement. Patients with intestinal carriage of *E. histolytica* included an asymptomatic carrier, five with acute dysentery, and two patients with chronic intestinal amebiasis (Table 1). Of the seven patients with extraintestinal infection, five had a solitary hepatic abscess, one had multiple liver abscesses, and one had an amoebic liver abscess which eroded through the diaphragm and into the lung parenchyma (Table 2).

Microscopic examination of smears of either direct or concentrated stool specimens detected trophozoites or cysts in all eight patients with intestinal infections. In six of these eight instances, the parasite was demonstrated by culture of the stool as well. Serologically, two of these eight patients (no. 1 and 4) showed a moderate antibody response to *E. histolytica*. By CIE, a single precipitin band was detected in a 1:2 dilution of the serum from the asymptomatic carrier and with undiluted serum (1:1).
derived from the dysenteric patient. By IHA, the first serum rendered a titer of 1:256, whereas the second was nonreactive. Serum obtained 1 week later from the patient with amoebic dysentery reflected a rise in antibody response as evidenced by an increase in the IHA titer from negative to 1:64 and in the CIE titer from undiluted (1:1) to 1.2. Serologically, antibodies could not be detected in the sera of the two patients with chronic intestinal amebiasis and in four patients with acute amoebic dysentery.

For the seven patients (no. 9 to 15) with extraintestinal infection, the three test parameters were concomitantly diagnostic in only one instance (no. 9). Serologically, however, antibodies were present in the serum of each of the seven patients. By CIE, titers were obtained which ranged from 1:1 to 1:128. Five of these seven sera showed multiple precipitin bands with initial or follow-up samples, or both. One of these sera (patient no. 14, with multiple hepatic abscesses) rendered the highest IHA titer (1:32,768) recorded in this series, whereas the remainder had IHA titers ranging from 1:64 to 1:2,048 (Table 2).

In three instances in which follow-up serum samples were obtained after anti-*E. histolytica* chemotherapy, decreasing antibody titers were noted. Sera from the patient with multiple liver abscesses (no. 14) showed decreasing CIE titers.

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<th>Table 1. Results of stool and serum specimens derived from patients with intestinal amebiasis</th>
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* SNO, Sample not obtained; Neg, negative; Pos, positive.

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<th>Table 2. Results of stool and serum specimens derived from patients with extraintestinal amebiasis</th>
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* SNO, Sample not obtained; Neg, negative; Pos, positive.

* Multiple bands.
over a 3-month period from the initial 1:128 (multiple bands) to 1:4 (multiple bands). A parallel decrease in the IHA titer from 1:32,768 to 1:1,024 also occurred. No evidence of a prozone phenomenon occurred with any of the sera tested.

Purulent abscess exudate was obtained from three patients (no. 11, 12, and 14) with hepatic involvement and was processed microscopically, culturally, and serologically. Trophozoites of *E. histolytica* were observed microscopically and in culture of samples derived from two patients (no. 11 and 12) with a single hepatic abscess. These results were in contrast to the negative findings obtained with the exudate from the patient with multiple abscesses. Antibody could not be detected in any of these samples by either CIE or IHA.

Sera from 4 of the 110 subjects with symptomatology suggestive of amebiasis rendered titers of 1:64 to 1:512 when tested by the IHA method. These same sera were negative by CIE. Stool samples from these patients failed to reveal *E. histolytica* by microscopic and cultural techniques. These four patients were eventually diagnosed as having ulcerative colitis, pyelonephritis, rectal cancer, and colitis resulting from psychological disturbance (Table 3). The latter patient, however, had had a past history of amebiasis.

Two patients with bacterial hepatic abscesses lacked microscopic, cultural, or serological evidence of amebiasis.

The 40 sera comprising the control group were all negative when tested by CIE, whereas 37 of these same sera were also negative by IHA. Three sera derived from patients with serum positive for rheumatoid factor gave IHA titers of 1:16, 1:32, and 1:64, respectively (Table 4).

**DISCUSSION**

The diagnosis of amebiasis may be achieved by demonstrating the protozoan agent, *E. histolytica*, in either fecal samples or the purulent exudate derived from sites of invasion, or by culturing the parasite from these sources. In the absence of visualizing the etiological agent, detection of antibody to *E. histolytica* may also render definitive diagnosis.

In the present study, microscopic examination of stool specimens was diagnostic of amebiasis for the eight patients with localized intestinal infection. Culture of these stool specimens for amoebae, however, was effective in only six instances, because excystment failed to occur with two specimens in which cysts were demonstrated microscopically. Reasons for the observed strain variability regarding excystment and proliferation in this biphasic medium are not known. Edelman and Spingarn (3), studying the in vitro cultivation of *E. histolytica*, found that excystment failed to occur with approximately one-third of specimens in which *E. histolytica* cysts were observed. These investigators attributed their results to an insufficient number of cysts present in the original inoculum and to the nature and quantity of bacterial and fungal associates present in the specimen.

In extraintestinal amebiasis, in contrast to results observed with intestinal localization, microscopic analysis of stool specimens proved to be of little diagnostic value. This procedure and the accompanying cultural approach detected the parasite in only one of seven confirmed cases of invasive amebiasis.

CIE and IHA tests, on the other hand, proved diagnostic in 100% of the seven cases of extraintestinal infection, but in only two (25%) of the eight cases of intestinal amebiasis. This latter serological result may reflect the restricted invasive potential of some strains of *E. histolytica*.
which remain confined to the intestinal tract and, consequently, fail to elicit a circulating antibody response. The temporal relationship between the onset of disease and diagnostic endeavors may also be a factor. The possibility exists that antibody either had not been formed by the time sera was collected or the amount present was below the threshold sensitivity of the serological tests.

In one case of acute amoebic dysentery (patient no. 4), antibody was first detected by CIE in the undiluted serum of the patient, collected during the initial stage of disease. Antibody was not demonstrated when this same serum was assayed by IHA. This result not only reflects the difference in the nature of the antibody response (hemagglutinating versus precipitating), but also indicates the possibility that precipitin antibodies may be detected earlier in the disease process than hemagglutinins. Maddison and colleagues (11) demonstrated that hemagglutinins and precipitins are evoked in response to E. histolytica invasion. These authors could differentiate between the two antibodies by removing hemagglutinin antibody from sera by absorption with packed sensitized cells which did not affect precipitin activity. The authors, however, did not comment on which antibody response was primary.

Five of seven patients (no. 9, 10, 13, 14, and 15) with invasive amebiasis yielded IHA titers ranging from 1:1,024 to 1:32,768. These same five sera tested by CIE gave titers of 1:1 to 1:128 with three (no. 13, 14, and 15) showing multiple bands when reacted against the E. histolytica antigen. This latter finding not only facilitated interpretation, but was strongly suggestive of an active infectious process. In this regard, multiple bands may be indicative of antibody response to several E. histolytica antigens released by lysing trophozoites. The fact that the sera of two patients (no. 9 and 10) with liver involvement showed a single precipitin band may be related to the age of the lesion because it may be that antibodies against minor antigens do not persist for an extended period of time. Alternatively, antibody to the minor antigenic stimuli may have decreased with time beyond the threshold sensitivity of the CIE test.

The sera of two of the seven patients (no. 11 and 12) with hepatic amebiasis (where one would normally expect to find the highest antibody level) yielded borderline IHA titers of 1:64 and 1:128, respectively, making definitive interpretation of these results difficult. These same sera, however, when tested by CIE gave titers of 1:2 and 1:8 which, based on the criteria of the manufacturer as well as our own experience, are indicative of amebiasis. In addition, each of these sera rendered multiple bands when reacted against the E. histolytica antigen. This finding not only facilitated interpretation, but was strongly suggestive of an active infectious process. Krupp and Powell (8) found that in over 10% of patients with an amebic liver abscess, minimal IHA antibody titers were encountered. These investigators attributed this finding to differences in individual immunological response to amoeic invasion.

Although other investigators have indicated little prognostic value of serological tests for amebiasis (11), our results, although few in number, show that significant decreases in antibody titer occurred with three patients (no. 10, 12, and 14) after administration of anti-E. histolytica chemotherapy. The most marked decline in antibody titer was observed in the patient with multiple amoebic liver abscesses. This patient’s initial IHA titer of 1:32,768 and CIE titer of 1:128 (multiple bands) decreased over a 3-month period to 1:1,024 and 1:4, respectively. Multiple bands were still evident by CIE with the last serum.

In contrast to the results of Mahajan et al. (12), antibody was not detected in three purulent exudates assayed by both CIE and IHA. The apparent discrepancy has not been resolved.

The serological component of the present study assayed the status of 110 subjects residing in a “nonendemic” area. The results disclosed an overall sparsity of E. histolytica antibody in our patient population. Only four patients with diseases other than amebiasis showed low-level IHA antibody ranging from 1:64 to 1:512. The latter result was obtained with the serum of a patient with a previous episode of amebiasis (9). Three of ten additional rheumatoid factor positive sera rendered IHA antibody titers of 1:16 to 1:64. None of these patients, however, had a prior history of amebiasis.

In marked contrast to results obtained by IHA, outside of subjects with known amebiasis, no reactive sera were encountered by CIE. Included among these samples were sera derived from patients with hepatic abscesses of bacterial etiology, as well as samples obtained from patients with other infectious processes and autoimmune disease. Krupp (10) in her survey encompassing 143 sera from patients did not encounter a single false-positive reaction by CIE. F. Aguilar-Torres, E. Ross, R. Niebojeski, L. Jackson, G. Healy, and M. Rytel (Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 17th, New York, N.Y., Abstr. no. 435, 1977), however, encountered two reactive sera with CIE from patients with intraabdominal infection or septicemia.

Subjects residing in an endemic area have
been shown to have higher antibody response than those living in nonendemic areas. Krupp and Powell (8) working with 103 sera obtained from such individuals detected IHA antibody in 47 of these samples. They attributed this finding to either antibody persistence after resolution of disease or a previous asymptomatic infection. In a separate but similar study, Elsdon-Dew (4) ascribed the observed low-level antibody response by IHA to “background noise” from antibody persisting long after cure. This explanation could account for the IHA titer of 1:512 we observed with the sera of a patient who had colitis stemming from a psychological disturbance, and had previously had amebiasis.

To date, the studies of Krupp (10) and Aguil- lar-Torres et al. have addressed themselves to a comparison of IHA and CIE for the diagnosis of amebiasis. These investigators showed comparable test results, except for the ability of IHA to detect low levels of antibody. However, an analysis of our results leads us to view this aspect of the IHA as a disadvantage because of the dubious interpretation of a low-level antibody response. Results obtained by CIE offer a distinct advantage over IHA, because the demonstration of a precipitin band when undiluted serum is reacted with an *E. histolytica* antigen is indicative of infection. High titers and multiple bands are diagnostic as well as suggestive of active extraintestinal infection. Another, perhaps more important, value of serology, particularly of CIE, resides in the ability of this serological test to differentiate patients with actual invasive amebiasis from those with similar symptomatology, but whose etiology is due to other incitants.

The importance of routine amoebic serology cannot be over-emphasized. Six of seven patients with hepatic amebiasis might have gone undiagnosed if not for this modality. At The Mount Sinai Hospital, we are recognizing a substantial increase in disease due to this “tropical” protozoan parasite. Tourism, overseas business ventures, and homosexuality are all contributing to the increased incidence, which will ultimately dictate expanding the diagnostic approach to include serology as a major component.

**LITERATURE CITED**