

Novelties on Amoebiasis: A Neglected Tropical Disease

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ABSTRACT

In accordance with the 1997 documents of the World Health Organization (WHO), amoebiasis is defined as the infection by the protozoan parasite *Entamoeba histolytica* with or without clinical manifestations. The only known natural host of *E. histolytica* is the human with the large intestine as major target organ. This parasite has a very simple life cycle in which the infective form is the cyst, considered a resistant form of parasite: The asymptomatic cyst passers and the intestinal amoebiasis patients are the transmitters; they excrete cysts in their feces, which can contaminate food and water sources. *E. histolytica sensu stricto* is the potentially pathogenic species and *E. dispar* is a commensal non-pathogenic *Entamoeba*. Both species are biochemical, immunological and genetically distinct. The knowledge of both species with different pathogenic phenotypes comes from a large scientific debate during the second half of the 20th century, which gave place to the rapid development of diagnostics technology based on molecular and immunological strategies. During the last ten years, knowledge of the new epidemiology of amoebiasis in different geographic endemic and non-endemic areas has been obtained by applying mostly molecular techniques. In the present work we highlight novelties on human infection and the disease that can help the general physician from both endemic and non-endemic countries in their medical practice, particularly, now that emigration is undoubtedly a global phenomenon that is modifying the previous geography of infectious diseases worldwide.

Key words: Amoebiasis, Diagnosis, Treatment

INTRODUCTION

In accordance with the 1997 documents of the World Health Organization (WHO), amoebiasis is the infection by the protozoan parasite *Entamoeba histolytica* with or without clinical manifestations.^[1]

The only known natural host of *E. histolytica* is the human body with the large intestine as the major target organ. This parasite has a very simple life cycle in which the infective form is the cyst that is considered a resistant form of the parasite. The asymptomatic cyst passers and the intestinal amoebiasis patients are the natural transmitters; they excrete cysts in their feces, which can contaminate food and water sources.

The cysts are round structures around 10–16 µm in diameter. However, estimation of cyst diameter in *Entamoeba* spp. in the old and recent literature is highly variable. The cyst has four visible nuclei when mature and only one when immature, and the nuclei are spherical with a membrane displaying small chromatin granules and a central karyosome.

When in excystation, each cyst produces eight vegetative forms or trophozoites, which are the motile form of the parasite; they are 20–40 µm in diameter. Life cycle and the relevant structures of both forms of parasite are shown in Figure 1. Cysts are resistant to desiccation in soil and can survive in humid environments and in water for several weeks. Susceptible hosts exposed to the aforementioned infection sources ingest the cysts, which then undergo excystation during their pass through the gastrointestinal tract. Amoebiasis is also considered a sexually transmitted disease, particularly in sexual relationships between men and in individuals with sexual anilingus practices.^[2] Clinical and etiological diagnosis of intestinal and extra-intestinal

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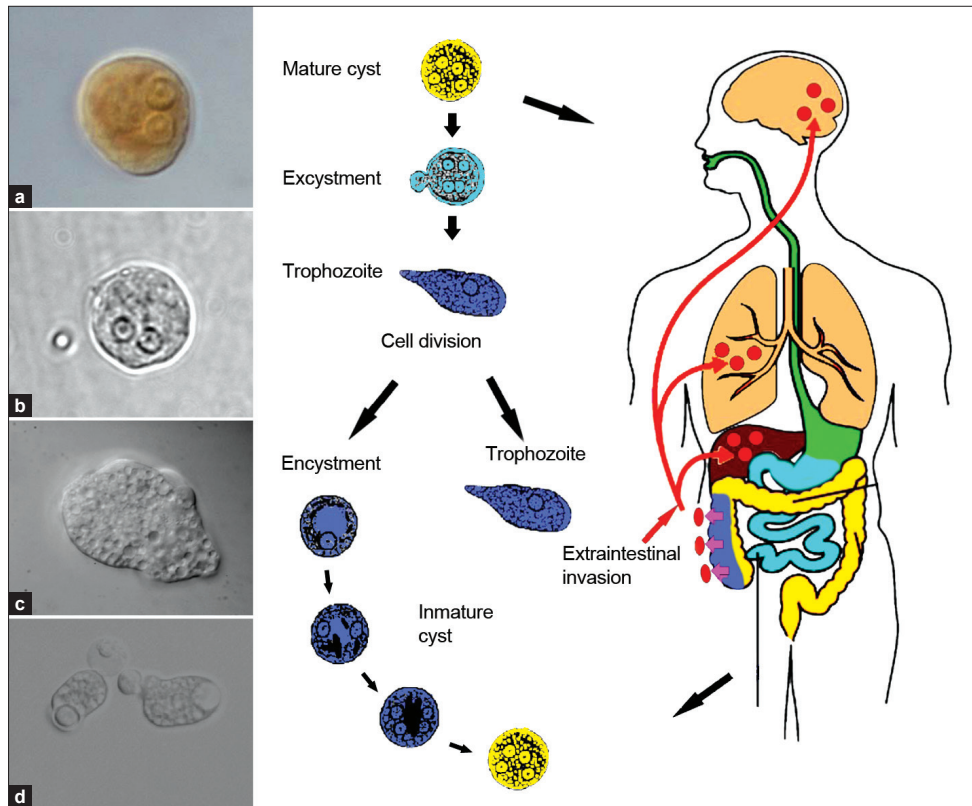


Figure 1: Life cycle of *E. histolytica/E. dispar*. a) Mature cyst stained with 4% Lugol solution (100× magnification). b) Mature cyst without staining (100×). c) Trophozoite observed with differential interference contrast (DIC) (100×). d) Trophozoites of *E. histolytica* species with phagocytosed erythrocytes (DIC 40×)

amoebiasis is neither easy or simple in part because of the discovery of two species made from the previously known *E. histolytica* species, both indistinguishable under microscopy. These two species are biochemical, immunological and genetically distinct.^[1] *E. histolytica sensu stricto* is the potentially pathogenic species and *E. dispar* the commensal non-pathogenic *Entamoeba*.

Knowledge of both species with different pathogenic phenotypes comes from a large scientific debate during the second half of the twentieth century,^[3,4] which gave rise to the rapid development of diagnosis technology based on molecular and immunological strategies.^[5-9] During the last 10 years, knowledge of the new epidemiology of amoebiasis in different geographic endemic and non-endemic areas has been obtained through the application of mostly molecular techniques.^[10-14]

Moreover, these molecular epidemiology studies have unveiled the extraordinary genetic variability^[13-15] of *E. histolytica* and *E. dispar*, allowing the discovery of other *Entamoeba* species, such as *E. moshkovskii*, which can also infect the human intestine with a significant frequency. However, much of the epidemiology and its contribution to morbidity of *Entamoeba* infections remain unknown.

There are excellent recent reviews on the molecular epidemiology and intestinal and extra-intestinal characteristics of amoebiasis in the human host that can be consulted.^[10,11,16] Nevertheless, the major purpose of the present work is to highlight the novelties in regard to human infection and the disease that can help the general physician from both endemic and non-endemic countries in their medical practice. This is especially critical given that emigration is undoubtedly a global phenomenon that is modifying the previous geography of infectious diseases worldwide.

THE THREE SPECIES OF INTESTINAL ENTAMOEBA

The speciation of *Entamoeba* protozoa has been discussed since 1925 when Emile Brumpt proposed the existence of two distinct species that could infiltrate the human intestine: One associated with symptoms of diarrhea with or without dysentery and the other excreted with feces from asymptomatic individuals. The former was called *E. histolytica* and the latter *E. dispar*. While years of scientific discussions have left Brumpt's theory behind, no molecular technology prior to the 1990s allowed clear differentiation of the currently known *E. histolytica* and *E. dispar* species in terms of pathogenic or non-pathogenic phenotypes.^[5,6]

Both species are genetically diverse and this variability allowed for the beginning of studies on molecular epidemiology in different endemic areas. The new data on the epidemiology of amoebiasis based on frontier technology suggests that genetic variability could be an important tool in the study of geographic distribution of both species and particular strains of *Entamoeba*, which may determine the morbidity rates of different forms of amoebic infection in different geographic areas. Some *E. histolytica* genetic variants (strains) have been isolated from asymptomatic cyst passers, patients with invasive intestinal amoebiasis or from samples of amoebic liver abscess material.^[15,17,18] However, *E. dispar* has been mainly isolated in feces samples resulting from asymptomatic cyst passers and displays high genetic polymorphisms,^[15,19] even more than the *E. histolytica* species.^[11] We recently have obtained evidence that in at least two endemic countries (Brazil and Mexico), *E. dispar* genetic variants have been detected in patients with invasive amoebiasis. In Brazil, the ICB-ADO *E. dispar* strain was isolated from a non-dysenteric colitis patient maintained in culture with his own intestinal flora displaying a pathogenic behavior in experimental models of amoebic liver abscess.^[20,21] DNA extracted from hepatic abscess material obtained from six patients in Mexico also clearly showed the presence of *E. dispar* DNA sequences.^[22]

The third species of *Entamoeba*, *E. moshkovskii*, has been considered a free-living organism since 1940s^[16] in contrast to *E. histolytica* and *E. dispar*, with a geographic distribution mainly in developing countries. *E. moshkovskii* has been frequently detected in individuals from developed and highly industrialized countries.^[16] Particularly in regard to this species we are at the beginning of the study of its pathogenic potential and the context in which it is expressed and the epidemiologic significance of infection and its contribution to morbidity rates of diarrheic diseases.

INTESTINAL AND EXTRA-INTESTINAL AMOEBIASIS

What doses of cysts are necessary for colonization of large bowel mucosa? For the three *Entamoeba* species this is not known with any certainty. Moreover, we do not know which environmental characteristics are permissive for intestinal colonization, and this could be an interesting field for future research. Finally, until today, the only species recognized as an etiologic agent of amoebic invasive disease is *E. histolytica*, which, once in the colon, undergoes excystation and the generation of trophozoites.

Trophozoites multiply by binary fusion and some of them

may encyst and be excreted with stools. Cyst viability under appropriate conditions of humidity may last as long as several weeks and thus they be available for a new susceptible host. We have to stress that more than 90% of infections have an asymptomatic course and are frequently auto-limited at different periods of time.^[23,24] After intestinal infection there is no evidence of induction of a long-lasting protective immune response, and in endemic areas, individuals may have several periods during the year of re-infection and clearance of infection.^[24] In relation to the susceptibility of HIV⁺ and AIDS patients to the invasive forms of infection, contradictory evidence exists; however, morbidity seems to be more related to the particular prevalence of the *E. histolytica* strain with invasive phenotypes than to the specific immunological status of the patient.^[24-27] As for the invasive behavior of *E. histolytica*, some authors consider this trait not to be typical. On the contrary, it seems to be an aberrant conduct^[16] and we agree with this opinion.

The natural history of invasive intestinal amoebiasis is an acute event, characterized by the presence of diarrhea that occurs days or weeks after exposure—in our personal experience lasting no more than four to five weeks.^[11] Although there are reports of occurrence years after exposure,^[28] in this case we presume the cause and effect relationship is extremely difficult to corroborate.

Intestinal amoebiasis is basically an acute disease in which the most frequent symptoms are abdominal pain (colic) and the presence of diarrhea with mucus and/or blood, or a clear dysenteric syndrome. However, fever and other systemic symptoms are infrequent.

Severe forms of invasive amoebiasis can be observed in young children (<5 years), pregnant woman, the elderly, and particular those with chronic diseases, such as *diabetes mellitus*, and in individuals being treated with immunosuppressants or those with immunodeficiency disorders. Those severe forms of amoebiasis are colon ameboma, fulminant necrotizing colitis, and toxic mega colon.^[11] The appearance of symptoms, such as severe dysentery and pain with signs of peritoneal irritation (rebound), intense tenesmus, fever (>38°C), tachycardia, hypertension, nausea, and anorexia are suggestive of the previously mentioned severe forms of intestinal amoebiasis. The mortality rates of dysenteric syndrome due to *E. histolytica* are less than 1%, but mortality due to complications increases up to 75%.^[29-31] Fortunately in the last few decades such complications are uncommon.

The intestinal amoebiasis form known as chronic non-dysenteric colitis is the most frequent form of amoebiasis

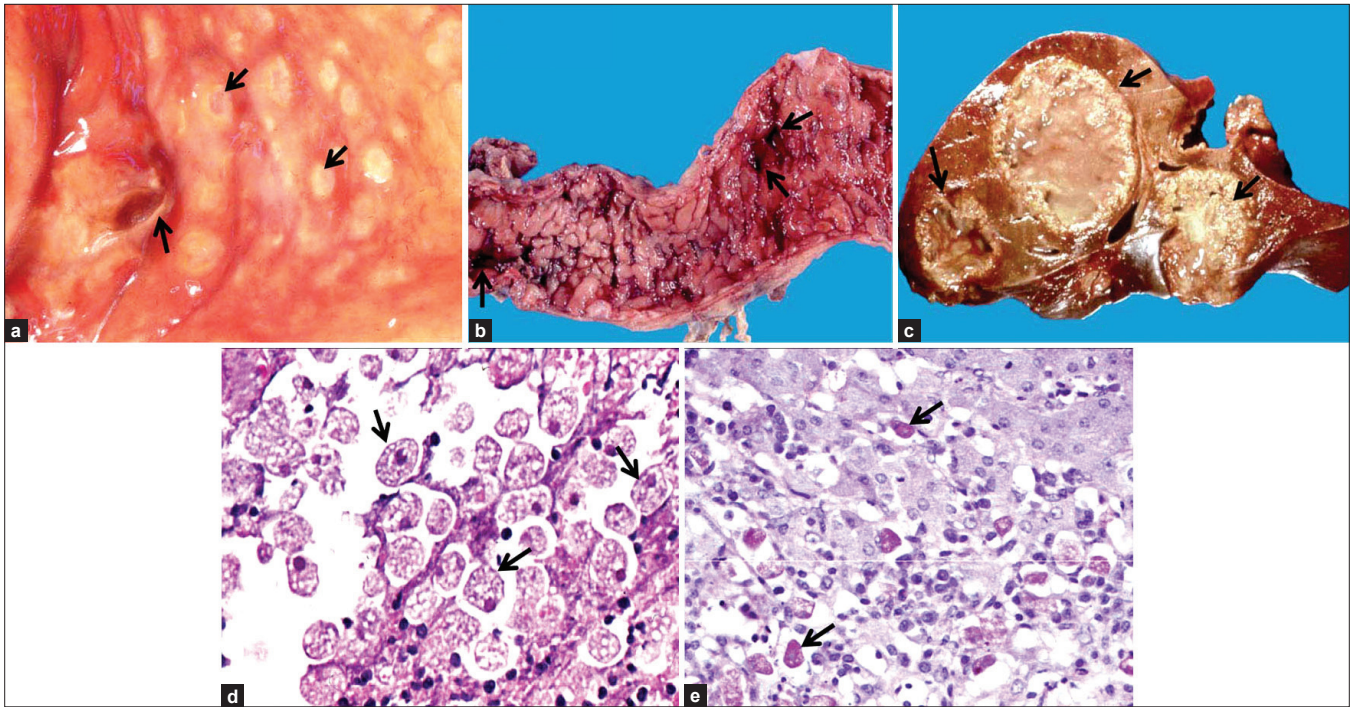


Figure 2: a) Intestinal flask-shaped ulcers observed through rectosigmoidoscopy examination. Arrows indicate the colonic ulcers. b) Large bowel necropsy specimen from a case of fulminant amoebic colitis. Arrows indicate hemorrhagic ulcers and important intestinal mucosa necrosis. c) Necropsy specimen of liver abscesses. Arrows indicate the three large abscesses. d) Intestinal biopsy obtained from the edge of flask-shaped ulcer where large numbers of trophozoites (HE and PAS stained, 60x) are clearly visible. e) Biopsy obtained from the edge of amoebic liver abscess (HE and PAS stained, 20x). Notice the presence of trophozoites, hepatocytes, and the large number of inflammatory cells. Courtesy: Doctor Ruy Pérez-Tamayo

in people of all ages, characterized by non-specific symptoms. The natural history of this clinical form can also mimic irritable bowel syndrome.^[32] Symptoms more relevant in this instance are periods of abdominal pain (colic) and auto-limited episodes of diarrhea alternating with constipation. However, both non-dysenteric amoebic colitis and irritable bowel syndrome are controversial themes in the clinical practice.

Where endoscopy examination is available, colonoscopy can be of great help in clinical diagnosis of invasive intestinal amoebiasis. This procedure allows for microscopic examination of samples taken directly from the characteristic flask-shaped ulcer produced by *E. histolytica* and from other sites of mucosal lesions. Microscopic observations of this type of material are described in the diagnostics section and in Figure 2. On the other hand, colonoscopy detects the presence of lesions related to the mentioned severe forms of intestinal amoebiasis and allows for differential diagnosis of other pathologies, such as inflammatory bowel disease or colon carcinoma.

HEPATIC INVASION

What circumstances define the extra-intestinal invasive

behavior of some *E. histolytica* strains? This remains unknown today. For example, we do not know the frequency of extra-intestinal invasion after intestinal colonization with virulent *E. histolytica*. However, this seems to be an infrequent event as suggested by the low morbidity rates of amoebic liver abscess and other extra-intestinal forms of invasive amoebiasis compared with the prevalence rates of asymptomatic infections and intestinal disease. While amoebic liver abscess is a disease that can affect individuals of all ages, in some endemic areas the incidence rates are higher in both children under 5 years and young adults (20–45 years).^[11,33] Males are also more prone to developing amoebic hepatic abscess than females (1 female for 4–6 males).^[11,16,22]

After exposure, 80% of patients display symptoms over a few days to 4–6 weeks.^[11,16,18,22] The most common symptoms suggestive of amoebic liver abscess are fever (38°C), chills and diaphoresis, anorexia and abdominal pain in the right upper quadrant that increases during inspiration. Pain also frequently radiates to shoulder and back. Nausea has also been referenced but diarrhea is only occasionally mentioned (50% of cases).

Hepatomegaly can be detected during digital percussion of the hepatic area and is always related to the dimensions

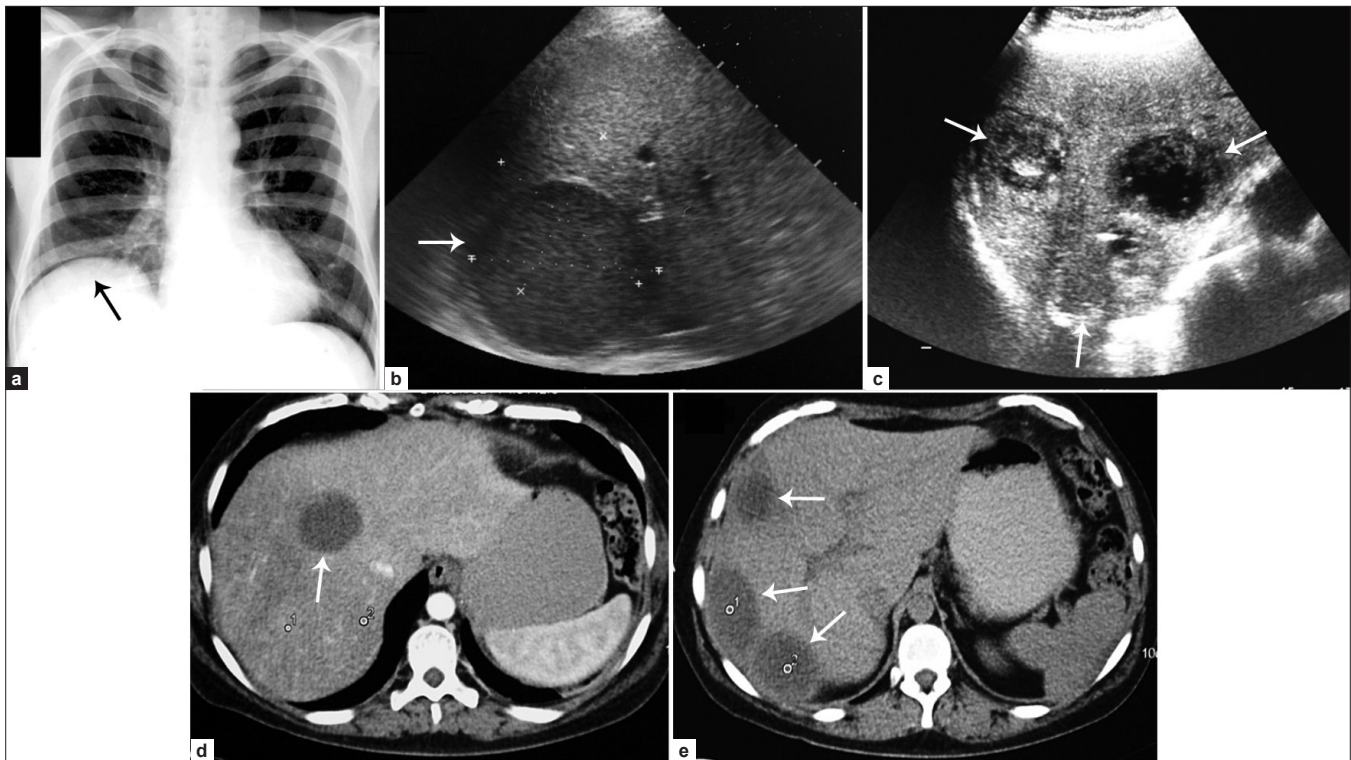


Figure 3: a) Thoracic X-ray of a patient with amoebic liver abscess showing the elevation of the right hemi-diaphragm. Ultrasound images of: b) Single large amoebic abscess and c) Three amoebic hepatic abscesses. d) Contrast computed tomography (CT) scan of a single abscess and e) Three clear amoebic liver abscesses

of the abscess; patients can also display peritoneal signs (abdominal guarding or rebound). Absence of intestinal noises, jaundice, and pleural or pericardial rub are symptoms that should elicit alarm related to the rupture or imminence of rupture of the hepatic abscess.

In general, the right hepatic lobule is the most frequently affected due to the portal circulatory system of the right colon. However, the left lobule can be also affected. Laboratory findings suggestive of amoebic liver abscess are the presence of leukocytosis, neutrophilia, increased globular sedimentation velocity, and high levels of alkaline phosphatase.

Thoracic X-ray data useful in the diagnosis of amoebic abscesses, as well as other types of hepatic abscesses, include elevation of the right hemidiaphragm, atelectasis, or pleural effusion [Figure 3a]. Ultrasound is the gold standard technique for diagnosis of amoebic liver abscess, as its positive predictive value (PPV) is around 95% (85–100% depending on analyzed series). Although contrast computed tomography (CT scan) [Figures 3d and 3e] have a PPV up to 95% due to a higher definition capacity, ultrasound is considerably less expensive compared to CT scan technology, which is of tremendous importance to countries with limited medical and economic

resources.^[10,11,16] Ultrasound reveals hypoechoic areas that can be single or multiple with round edges [Figures 3b and 3c]. Several authors have mentioned the presence of a large single abscess as a frequent characteristic of a amoebic liver abscess. However, this characteristic is not a *sine qua non* of amoebic abscesses, and in our medical practice we have seen multiple abscesses more frequently than we had first assumed. Pyogenic abscesses are also not characteristic of multiple abscesses (personal observation). Early lesions due to amoebic invasion of hepatic parenchyma are multifocal in nature (micro-abscesses) as a consequence of tissue destruction and necrosis by proteases from *E. histolytica* and neutrophil recruitment to the site of infection. The advantage of CT scans and magnetic resonance is detection of small abscesses and the high definition of the images. Moreover, other techniques not always available in endemic countries (e.g. gallium scans) can help differentiate between amoebic (cold images) and pyogenic abscesses (hot images). Thus, the difference is based on the absence (amoebic) or presence (pyogenic) of white blood cells in the abscess. Additionally, we have to mention that in endemic countries the frequency of mixed abscesses (pyogenic and amoebic) is considerable; in our practice this frequency is approximately 17% (non-reported data). Another important fact in medical practice is the coincidence of previous symptoms with the presence of

high levels of serum anti-amoebic antibodies—more than 90% of amoebic liver abscess patients develop this type of antibody response. However, in cases of fast development of amoebic abscess these antibodies may not be present.^[11]

CURRENTLY AVAILABLE DIAGNOSTIC TESTS FOR INVASIVE AMOEBIASIS

Etiological diagnosis of intestinal parasitic diseases has been mainly performed using direct or concentration techniques for microscopic examination of fecal specimens.^[34] While technically simple in approach, these techniques require the expertise of highly qualified technicians in the morphological identification of ova, cysts, and helminths to be feasible, and the sensitivity and specificity is no more than 80%. While this technique cannot differentiate between the three *Entamoeba* species already mentioned, in some endemic communities this is the only diagnostic technology available. On the other hand, during the last 10 years diagnostics in amoebiasis have changed dramatically, considerably improving sensitivity and specificity. Some of the current techniques are based on immunological strategies, such as ELISA^[11,35,36] and different modalities of polymerase chain reaction (PCR), with clear advantages in bedside diagnosis and in clinical laboratories of health institutions.^[10,11,17,36] Even though some of these diagnosis procedures are also suitable for large epidemiologic trials, it is mandatory to make a careful selection of the diagnosis test when the test has to be applied in the field. In particular, the election has to be directed to those procedures that do not need special conditions for sample preservation or pretreatment of specimens in the working field. In our experience, immunologically based diagnostics tests for detection of anti-amoebic secretory antibodies in feces or detection of intestinal amoebic antigens through polyclonal or monoclonal specific antibodies (ELISA) are excellent tests in hospitals where fresh specimens (feces) can be obtained. Results are reproducible and reliable. In contrast, in epidemiological trials, these techniques can be

biased when samples are more than 18 hours old. Tables 1 and 2 have a list of diagnostic tests that have proven to be useful in clinical and laboratory diagnosis of intestinal and extra-intestinal amoebiasis in first and second level health institutions.

AMOEBIASIS TREATMENT

The WHO/PAHO recommendations published in 1997^[11] contain in detail the actions that WHO country members have to observe with regard to *E. histolytica* species infection. They highlight the characterization (when possible) of *E. histolytica* and *E. dispar* (we now add *E. moshkowskii*) to be treated properly. In accordance with the WHO recommendation only *E. histolytica* – infected individuals have to be treated regardless of the presence of symptoms. It is also important to remember that in some endemic countries, mixed infections (e.g. *E. histolytica* and *E. dispar*) are frequent, and that only those infected with *E. dispar* should be excluded for anti-amoebic treatment.

Table 3 shows treatment schedules that have proven to

Table 2: PCR assays for *E. histolytica* and/or *E. dispar* detection

PCR Assay	Gene target	Amplification product (bp)	Reference
Single tube	Small-subunit rRNA	1950	Ramos F <i>et al.</i> ^[45] 2005
		880	Heckendorn F <i>et al.</i> ^[46] 2002
	Extrachromosomal circular DNA	145	Aguirre A <i>et al.</i> ^[47] , 1995
Nested	Small-subunit rRNA	133	
		135	Calderaro A <i>et al.</i> ^[48] 2006
Multiplex	Small-subunit rRNA	900	Ayeh- Kumi PF <i>et al.</i> ^[49] 2001
		166	Hamzah Z <i>et al.</i> ^[50] 2006
		752	
	Tandems repeats in extrachromosomal circular rDNA	132	Nunez YO <i>et al.</i> ^[51] 2001
Real-Time	18S rRNA	96	
		172	Verweij JJ <i>et al.</i> ^[52] 2005

Table 1: Microscopy and immunoassays for *E. histolytica* detection

Assay		Sensitivity (%)	Specificity (%)	Manufacturer	Reference
Microscopy	Direct examination methods				Biagi FF and Portilla J, 1957. ^[34]
Antigen detection	Antigen detection				
	TechLab <i>E. histolytica</i> II	14.2-100	94.7-100	TechLab, Blacksburg, VA	Haque R <i>et al.</i> ^[42] 1997;
	<i>Entamoeba</i> CELISA-PATH	95-100	93-100	Cellabs Pty Ltd., Brookvale, Australia	Visser LG <i>et al.</i> ^[43] 2006.
	ProsPecT <i>Entamoeba histolytica</i> microplate assay	54.5-87	94-99	REMEL inc., Lenexa, KS	
Antibody detection	Antibody detection				
	Indirect hemagglutination assay (IHA)	99-100	90.9-100	Dabe Behring Marburg GmbH, Marburg, Germany	Hira PR <i>et al.</i> ^[44] 2001
	Enzyme-linked immunosorbent assay (ELISA)	95	97	Light diagnostics	Morán P <i>et al.</i> ^[45] 2007

be highly effective in both intestinal and extra-intestinal invasive amoebiasis. In cases of large amoebic abscesses in which imminence of rupture or distances of less than 1 cm between the abscess wall and liver surface prevail, ultrasound-guided puncture is indicated. The procedure allows for the establishment of a differential diagnosis with other liver pathologies, especially pyogenic liver abscess, which is common in clinical practice.^[37]

In our experience, patients with amoebic liver abscess may not excrete *E. histolytica*/*E. dispar* cysts in feces. However, some of these patients are asymptomatic cyst passers after treatment with systemic anti-amoebic drugs, such as metronidazole. In such cases, treatment has to include luminal anti-amoebic drugs. At present, evidence of low susceptibility or resistant strains of either *E. histolytica* or *E. dispar* species to metronidazole has not been apparent.

CONSIDERATIONS OF AMOEBIASIS IN THE CONTEXT OF DIARRHEIC SYNDROME

Gastrointestinal infections are responsible for morbidity and mortality rates of children and young adults worldwide. In Africa, diarrhea is responsible for 25–75% of all childhood illnesses.^[38] Infection and intestinal diseases with ICD10 code A00-A09 are the second cause of disease in children under 10 years old in Mexico, and intestinal amoebiasis ranks with some annual variations 5th and 6th in the list of the 20 major causes of disease in Mexico^[11,39] (<http://www.dgepi.salud.gob.mx/anuario/index.html#>). Causes of diarrhea in endemic areas include a large variety of enteropathogens (viruses, bacteria, and parasites). In Mexico, parasitic intestinal infections are multiple infections

that constitute approximately 40% of analyzed individuals in which it is possible to detect more than one pathogen together with commensal parasites that are an indicator of fecalism. Prevalence of parasitic infection in three different communities in the state of Morelos, Mexico, are shown in Table 4, one of which is a strictly rural population (Amacuzac) and two (Tlaltizapan and Xoxocotla) are suburban communities. The relevance of these results lies in the high frequency of mixed parasitic infections detected in the studied populations. A remarkable low prevalence of soil-transmitted helminthiasis was also observed and could be the consequence of a biannual anti-parasitic treatment of school children with albendazole. This policy was implemented by the Health Ministry since the 1990s. However, there are emerging parasites with an increasing prevalence in the last 10 years, including *Blastocystis hominis* and in some areas *Cryptosporidium* spp. In South Africa intestinal mixed infection is present in 46% of patients with diarrhea and 33% in school children.^[40] Furthermore, mixed intestinal infections due to bacteria, parasites, and viral pathogens are the major forms of intestinal infection in developing countries. Systematic studies in groups of diarrhea and non-diarrhea patients are scarce. However, Cheun et al.^[41] recently published a splendid study on diarrheal patients in hospitals in Korea. The study documented the presence of enteropathogenic bacteria, parasites, and viruses in mixed infections, and highlighted the importance of diarrheal disease associated with protozoan infections. The major association of *E. histolytica* positive samples was with rotavirus type 1 [10.3(6.1–14.6) positivity/100 infected individuals, 95% CI], Astrovirus [9.3(5.2–13.4) positivity/100 infected individuals, 95% CI], pathogenic *Escherichia coli* [7.2 (36–10.0) positivity/100 infected individuals, 95% CI] and *Clostridium perfringens* [10.3(6.0–14.6) positivity/100 infected individuals, 95% CI]. As the authors mentioned, the question is whether mixed infections with protozoa are more likely to induce serious diarrhea.

Efforts in the near future have to be directed on studies focusing the interactions of microorganism in the intestinal environment. This knowledge will have a positive impact

Table 3: Treatment of amoebiasis disease

Antimicrobial therapy	Amoebic liver abscess	Intestinal amoebiasis
Metronidazole	750–800mg three times daily for 10 days	750–800mg three times daily for 5–10 days
Tinidazole	2 g daily for 5 days	2 g daily for 3 days
Paromomycin	–	25–35mg/kg per day, divided into three doses, for 7 days
Diloxanide furoate	–	500mg three times a day for 10 days

Source: Farthing, 2005^[53]

Table 4: Prevalence rates of parasite intestinal infections in Morelos, Mexico*

Community	Non-parasitized	Prevalence (%)	CI 95% [†]	Parasitized	Prevalence (%)	CI 95% [†]	Single infection	Prevalence (%)	CI 95% [†]	Mixed infection [‡]	Prevalence (%)	CI 95% [†]
Tlaltizapan (n=472)	353/472	74.7	70.1-78.2	119/472	25.2	21.0-28.9	81/119	68	59.3-76.9	38/119	31.9	23.1-40.7
Xoxocotla (n=57)	17/57	29.8	17.1-42.6	40/57	70.1	57.4-82.9	19/40	47.5	30.8-64.2	21/40	52.5	35.8-69.2
Amacuzac (n=1138)	470/1138	41.3	38.4-44.2	668/1138	58.6	55.8-61.6	254/668	38	34.3-41.8	414/668	61.9	58.2-65.7

*Prevalence values of intestinal parasite infections in three populations of the State of Morelos, Mexico; [†]Confidence values at 95%; [‡]More than one parasite

in clinical and laboratory diagnosis of diarrheic syndrome, its treatment, and thereafter the implementation of more reliable control schedules.

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