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Laboratory Diagnostic Techniques for *Entamoeba* Species

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INTRODUCTION

The genus *Entamoeba* contains many species, six of which (*Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii, Entamoeba polecki, Entamoeba coli* and *Entamoeba hartmanni*) reside in the human intestinal lumen. *Entamoeba histolytica* is the only species definitely associated with pathological sequelae in humans; the others are considered nonpathogenic (31, 57). Although recent studies highlight the recovery of *E. dispar* and *E. moshkovskii* from patients with gastrointestinal symptoms (52, 73, 130, 189, 201), there is still no definitive evidence of a causal link between the presence of these two species and the symptoms of the host.

Entamoeba histolytica is the causative agent of amebiasis and is globally considered a leading parasitic cause of human mortality (77, 81, 210). Clinical features of amebiasis due to *E. histolytica* range from asymptomatic colonization to amebic dysentery and invasive extraintestinal amebiasis, which is manifested most commonly in the form of liver abscesses. Approximately 50 million people have invasive disease, resulting in 100,000 deaths per year (81, 210). Although the parasite has a worldwide distribution, high prevalence rates of more than 10% of the population have been reported from various developing countries (173). *Entamoeba histolytica*-related diarrheal illnesses have recently been reported to have a negative impact on the growth of children (114). Despite the availability of effective therapy, morbidity and mortality associated with amebic infection have persisted, suggesting that interventions designed to limit or to eliminate disease are ineffective. As humans appear to be the only host, an appropriate control program could potentially eradicate amebiasis.

New approaches to the identification of *E. histolytica* are based on detection of *E. histolytica*-specific antigen and DNA in stool and other clinical samples. Several molecular diagnostic tests, including conventional and real-time PCR, have been developed for the detection and differentiation of *E. histolytica*, *E. dispar*, and *E. moshkovskii* in clinical samples. These molecular methods have led to a reevaluation of the epidemiology of amebiasis in terms of prevalence and morbidity, particularly in those geographical areas with high endemic rates.

The purpose of this review is to discuss the methods that exist for the identification of *E. histolytica*, *E. dispar*, and *E. moshkovskii* which are available to the clinical diagnostic lab-

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oratory. To address the need for a specific diagnostic test for amebiasis, a substantial amount of work has been carried out over the last decade in different parts of the world, and molecular diagnostic tests are increasingly being used for both clinical and research purposes.

Entamoeba histolytica

Entamoeba histolytica was first described by Fedor Lösch in 1875 in St. Petersburg, Russia. He described intestinal amebiasis in detail, and the species name *E. histolytica* was first coined by Fritz Schaudinn in 1903 (155). *Entamoeba histolytica* is the pathogenic species of *Entamoeba* that causes amebic dysentery and a wide range of other invasive diseases, including amebic liver abscess, respiratory tract infections, and cerebral and genitourinary amebiasis.

Entamoeba dispar

In 1925, Brumpt formulated the theory that the difference between many asymptomatic amebic infections and those of individuals with amebic disease could be correlated with the existence of two distinct but morphologically identical species, namely, *E. histolytica* (which is capable of causing invasive disease) and *E. dispar* (which never causes disease). This hypothesis was dismissed at that time, but subsequently evidence which gave support to Brumpt's findings began accumulating. In 1993, 68 years after the original discovery of *E. dispar, E. histolytica* and *E. dispar* were formally accepted as different yet closely related species on the basis of extensive genetic, immunological, and biochemical analyses (43, 177, 188).

Although *E. dispar* was previously considered to be nonpathogenic *E. histolytica* and was regarded as a commensal species, intestinal symptoms in patients infected with this species have been reported (95). In a recent study from India by Parija and Khairnar (130), 68 fecal specimens in which *Entamoeba* species were demonstrated on microscopy were tested using PCR. Eleven patients positive for *E. dispar* and *E. moshkovskii* (in association) had mild gastrointestinal discomfort; however, the study failed to clarify whether other parasites or bacterial or viral pathogens were detected in these 11 samples.

Entamoeba dispar can produce variable focal intestinal lesions in animals (28, 48, 202) and can destroy epithelial cell monolayers in vitro (49). There is also some evidence that following infection with *E. dispar*, pathological changes may occur in some humans (111). However Koch's postulates have not been fulfilled, and no large case-controlled studies have been undertaken to assess the true pathogenic potential of this organism.

Entamoeba moshkovskii

Entamoeba moshkovskii is another species of *Entamoeba* and is morphologically indistinguishable from *E. histolytica* and *E. dispar*. This species was first described from Moscow sewage by Tshalaia in 1941 (193) and was thereafter reported to occur in many different countries (30, 160). *Entamoeba moshkovskii* was initially thought to be a free-living environmental strain. However in 1961 an *E. histolytica*-like strain was isolated from a resident of Laredo, TX, who presented with diarrhea, weight loss, and epigastric pain (46). This strain was named the *E. histolytica* Laredo strain and shared many biological features with *E. moshkovskii*. Both the Laredo strain and *E. moshkovskii* grow at room temperature, are osmotolerant, and are resistant to emetine. These characteristics distinguished them from *E. histolytica* and *E. dispar* (30, 34). Subsequent molecular studies have confirmed that the *E. histolytica* Laredo strain is a strain of *E. moshkovskii* (30). The exact taxonomic classification of the species has yet to emerge, as *E. moshkovskii* seems to be a complex of at least two species (30). Although the early isolations of this species have been from sewage, recent studies have reported the recovery of *E. moshkovskii* from human feces (8, 30, 52, 73, 130, 189).

Reports on detection of *E. moshkovskii* from human specimens to date have come from North America, Italy, South Africa, Bangladesh, India, Iran, Australia, and Turkey (8, 30, 52, 73, 130, 171, 176, 189). Although previous reports on the identification of *E. moshkovskii* in fecal samples have not shown any association with clinical illness (30), recent studies from Bangladesh and India have reported *E. moshkovskii* as a sole potential enteropathogen in patients presenting with gastrointestinal symptoms and/or dysentery, highlighting the need for further study to investigate the pathogenic potential of this organism (73, 130).

IMPORTANCE OF DIAGNOSIS

The epidemiology of *E. histolytica*, *E. dispar*, and *E. moshkovskii* parasitoses remains uncertain, because most of the existing data were obtained using methods incapable of distinguishing among the three morphologically identical species. *Entamoeba dispar* appears to be about 10 times more common than *E. histolytica*, with most of the 500 million people infected with *E. histolytica*/*E. dispar* carrying *E. dispar* (91). Little is known about the epidemiology and incidence of *E. moshkovskii* infections, as only a few studies have used molecular methods to identify this parasite.

Most morbidity and mortality due to amebiasis occur in developing regions such as Central and South America, Africa, and the Indian subcontinent (203). In Bangladesh, where diarrheal diseases are the leading cause of childhood death, approximately 50% of children have serological evidence of exposure to *E. histolytica* by 5 years of age (74).

In developed countries, high-risk groups include travelers, immigrants from areas of endemicity, and men who have sex with men (MSM) (122, 125–127, 185, 186). It is estimated that 20% to 30% of MSM are colonized with *E. dispar* in Western countries, which is attributed to oral-anal sex practices (10). In addition, a few reports describe cases of invasive amebiasis in homosexual men from Taiwan and Korea (88, 124) and Australia (52, 175). Early detection of infection in these high-risk individuals by using molecular diagnostic methods will improve understanding of the public health issues and expedite the initiation of control measures (125–127, 175, 176).

The existence of these morphologically indistinguishable species of *Entamoeba* led the World Health Organization (WHO) to recommend the development and application of improved methods for the specific diagnosis of *E. histolytica* infection (210). Epidemiological surveys of amebiasis should include tools to diagnose *E. histolytica* and *E. dispar* individu-

ally, simultaneously, and accurately. Identification of *E. histolytica* remains an important goal of the clinical parasitology laboratory, and molecular diagnostics represent an important confirmatory diagnostic step in the management of patients who may be infected with *E. histolytica* and require specific therapy (210).

Techniques developed for the identification of *E. histolytica* include the detection of *E. histolytica*-specific antibodies and specific antigen in stool and other clinical samples. In addition, several molecular diagnostic tests, including conventional, nested, and real-time PCR, have been developed for diagnosis of *E. histolytica, E. dispar*, and *E. moshkovskii* by clinical laboratories.

CLINICAL MANIFESTATIONS

Asymptomatic Colonization

Asymptomatic cyst passage, with 90% of human infections either asymptomatic or mildly symptomatic, is considered to be the most common manifestation of *E. histolytica*. However, these studies have been based on the microscopic examination of fecal samples (203, 210). Patients can clear their infection without any signs of disease. In stool samples, cysts are usually detected, and trophozoites, which are rarely seen, lack ingested red blood cells (RBCs). Individuals harboring *E. histolytica* (asymptomatic carriers) can develop antibody titers in the absence of invasive disease (60, 93, 145). Asymptomatic colonization with *E. histolytica*, if left untreated, can lead to amebic dysentery and a wide range of other invasive diseases, but more often the infection resolves spontaneously without the development of diseases (19, 20, 60, 75).

Dysentery/Amebic Colitis

When followed for 1 year, 4 to 10% of asymptomatic individuals colonized with *E. histolytica* developed colitis or extraintestinal disease (60, 75); therefore, it is recommended that asymptomatic cyst carriers should be treated. Symptoms commonly attributed to *E. histolytica* colitis include abdominal pain or tenderness with watery, bloody, or mucous diarrhea. Eighty percent of patients complain of localized abdominal pain; some patients may have only intermittent diarrhea alternating with constipation. Microscopically, trophozoites are readily detected in submucosal tissue or fecal samples by permanent stains. Since *E. histolytica* invades the colonic mucosa, feces are almost universally positive for occult blood. The presence of Charcot-Leyden crystals and blood is the most common finding in the acute stage. In addition to the RBCs, macrophages and polymorphonuclear cells (PMNs) can also be seen on microscopy in cases of amebic dysentery. Fever is unusual, occurring in <40% of patients (4). Occasionally individuals develop fulminant amebic colitis, with profuse bloody diarrhea, fever, pronounced leukocytosis, and widespread abdominal pain, often with peritoneal signs and extensive involvement of the colon (184). Toxic megacolon, ameboma (5), cutaneous amebiasis (112), and rectovaginal fistulae (108) can occur as complications of intestinal amebiasis.

Extraintestinal Amebiasis

The most common extraintestinal manifestation is amebic liver abscess (ALA), which is associated with significant morbidity and mortality. This was a progressive and almost invariably fatal disease little more than a century ago, but since the introduction of effective medical treatment and rapid diagnosis, mortality rates have fallen to 1 to 3% (22, 166). ALA is caused by hematogenous spread of the invasive trophozoites from the colon, which reach the liver via the portal vein. This explains the frequent occurrence of abscesses in the right hepatic lobe, which receives most of the blood draining the cecum and ascending colon (154). Some individuals presenting with ALA have concurrent amebic colitis, but more often they have no bowel symptoms, and stool microscopy is usually negative for *E. histolytica* trophozoites and cysts (5, 152, 190). Individuals can present with ALA months to years after travel or residency in an area of endemicity, so a careful travel history is mandatory (14, 102, 166). The disease should be suspected in anyone with an appropriate exposure history (residency or travel in an area of endemicity) presenting with fever, right upper quadrant pain, and substantial hepatic tenderness. Cough may be present, and dullness and rales in the right lung base are not infrequent (5, 14, 166, 190). Jaundice is unusual. Symptoms are usually acute (<10 days in duration) but can be chronic, with anorexia and weight loss as prominent features. Leukocytosis without eosinophilia, mild anemia, a raised concentration of alkaline phosphatase, and a high rate of erythrocyte sedimentation are the most common laboratory findings (5, 14, 166, 190). The most serious complication of ALA is rupture, particularly into the pericardium, and superinfection with bacteria. Rupture into the pleura is relatively common and usually has a good prognosis. With early diagnosis and therapy, the mortality from uncomplicated ALA is less than 1% (5). Complications of extraintestinal amebiasis include pleuroplumonary amebiasis secondary to ALA rupture through the diaphragm, brain abscess, and genitourinary amebiasis. Diagnosis of brain abscess is usually made by the microscopic detection of parasites on brain biopsy or at autopsy; however, a recent study has highlighted the first diagnosis of *E. histolytica* encephalitis using PCR (172).

Diagnosis of liver abscess is confirmed by a positive serological test, as amebic serology is highly sensitive (>94%) and highly specific (>95%) for diagnosis. A false-negative serological test can be obtained early during infection (within the first 7 to 10 days), but a repeat test is usually positive. Abdominal ultrasound or computed tomography scan does not provide specificity for ALA. However, a positive serological test in combination with abdominal imaging is helpful for diagnosis where PCR is not routinely available. A recent study confirms that in the majority of successfully treated ALA patients the abscess completely resolves; however, in 7.1% of patients residual lesions are detected, with the unique sonographic appearance of round or oval hypo- or isoechoic areas surrounded by the hyperchoic wall (21). The successful use of PCR methods in detection of *E. histolytica* DNA in patients with ALA has shown high sensitivity (100%) (52, 182, 214, 215).

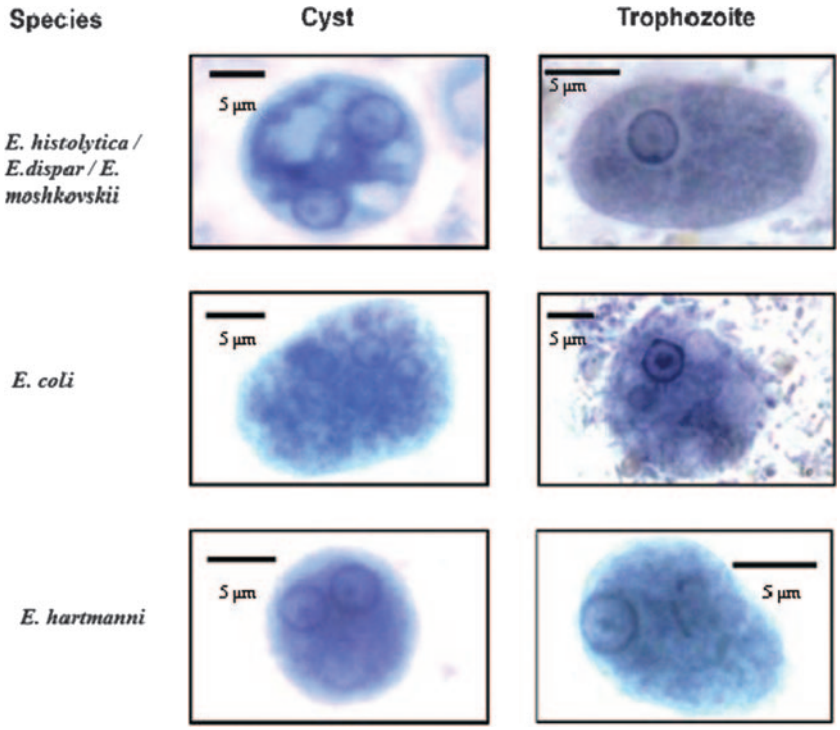

FIG. 1. Cysts and trophozoites of *Entamoeba* species.

LABORATORY DIAGNOSIS

Microscopy

Microscopic techniques employed in a diagnostic clinical laboratory include wet preparation, concentration, and permanently stained smears for the identification of *E. histolytica*/*E. dispar*/*E. moshkovskii* in feces. Microscopic examination of a direct saline (wet) mount is a very insensitive method (<10%) which is performed on a fresh specimen (90). The sample should be examined within 1 h of collection to search for motile trophozoites which may contain RBCs. However, in patients who do not present with acute dysentery, trophozoites will not contain RBCs. Patients with asymptomatic carriage generally have only cysts in the fecal sample. Although the concentration technique is helpful in demonstrating cysts, the use of permanently stained smears (trichrome or iron hematoxylin) is an important method for recovery and identification of *Entamoeba* species.

Microscopy is a less reliable method of identifying *Entamoeba* species than either culture or antigen detection tests (80, 104). The sensitivity of microscopy can be poor (60%) and confounded with false-positive results due to misidentification of macrophages as trophozoites, PMNs as cysts (especially when lobed nuclei of PMNs break apart), and other *Entamoeba* species (67, 72, 76, 80, 188) (Fig. 1; Table 1).

As *Entamoeba* trophozoites generally degenerate rapidly in unfixed fecal specimens (137) and refrigeration is not recommended, specimens should be preserved with a fixative which prevents the degradation of the morphology of the parasite and allows concentration and permanent smears to be performed. Fixatives used for the concentration procedure include Schaudinn's fluid, merthiolate iodine-formalin, sodium acetate-acetic acid-formalin (SAF), or 5% or 10% formalin. The fixatives for the permanently stained smears include trichrome, iron hematoxylin, Ziehl-Neelsen stains, modified polyvinyl alcohol (PVA) (containing mercury compounds), and SAF.

Examination for ova and parasites in a minimum of three stool samples over no more than 10 days is recommended, as these organisms may be excreted intermittently or may be unevenly distributed in the stool. This improves the detection rate to 85 to 95% (107). The presence of RBCs in the cytoplasm is still considered diagnostic for *E. histolytica* in patients with dysentery and may be used to distinguish between *E. histolytica* and *E. dispar*. However, trophozoites containing ingested RBCs are not present in the majority of patients (67, 178). The specificity of this finding was further reduced when it was demonstrated that in some patients *E. dispar* also contains RBCs (80). In vitro studies have also confirmed the ability of *E. dispar* to ingest RBCs (191). In one study, the specificity of *E. histolytica*/*E. dispar* as determined by microscopy (formalin-ether concentrates and permanent stains) was only 9.5% in community laboratories compared with the *Entamoeba* test and ProSpecT enzyme immunoassay (EIA) antigen detection tests (134).

Culture Methods

Culture techniques for the isolation of *Entamoeba* species have been available for over 80 years. Culture media include xenic (diphasic and monophasic) and axenic systems. Xenic cultivation is defined as the growth of the parasite in the presence of an undefined flora (35). The xenic culture of *E.*

TABLE 1. Characteristics of trophozoites and cysts of common intestinal *Entamoeba* species[a]

Characteristics	*E. histolytica*/*E. dispar*/*E. moshkovskii*	*E. hartmanni*	*E. coli*	*E. polecki*
Size, nuclei, and motility				
Trophozoites	15–20 μm, 1 nucleus (difficult to see in unstained preparations), actively motile with finger shaped pseudopodia	8–10 μm, 1 nucleus (usually not seen in unstained prepn), usually unprogressive	20–25 μm, 1 nucleus (often visible in unstained prepn); sluggish, short, and blunt pseudopodia	15–20 μm, 1 nucleus (occasionally seen on wet prepn), sluggish
Cysts	10–15 μm, mature cyst with 4 nuclei, immature cyst has 1 or 2 nuclei (nuclear characters difficult to see on wet prepn)	6–8 μm, mature cyst with 4 nuclei, immature cyst has 1 or 2 nuclei, two nucleated cysts very common	15–25 μm, mature cyst has 8 nuclei, occasionally 16 or more nuclei	10–15 μm, mature cyst with 1 nucleus, rarely 2 or 4 nuclei
Other features				
Trophozoites				
Chromatin (stained)	Chromatin peripheral, may have beaded appearance	Nucleus may stain more darkly than *E. histolytica*, chromatin may appear as solid ring rather than beaded (trichrome)	Chromatin clumped and unevenly arranged, appears as solid ring with no beads	Chromatin finely granular, chromatin may also be clumped at one or both edges of membrane
Karyosome (stained)	Karyosome small, compact, centrally located but may be eccentric	Karyosome usually small and compact; centrally located or eccentric	Karyosome large, not compact, may or may not be eccentric, may be diffuse or darkly stained	Karyosome small and usually centrally located
Cytoplasm (stained)	Cytoplasm is fine, granular, may contain bacteria; presence of RBCs diagnostic for *E. histolytica*, although some *E. dispar* strains may very occasionally contain RBCs	Cytoplasm finely granular, may contain bacteria, no RBCs	Cytoplasm granular with differentiation into cytoplasm and endoplasm, vacuolated; bacteria, yeast, and other debris may be present	Cytoplasm is finely granular, may contain ingested bacteria
Cysts				
Chromatin (stained)	Chromatin peripheral with fine uniform granules, evenly distributed	Chromatin granules evenly distributed (nuclear characteristics may be difficult to see)	Chromatin coarsely granular, may be clumped and unevenly arranged	Chromatin finely granular
Karyosome (stained)	Karyosome is small, compact, usually centrally located but occasionally eccentric	Karyosome is small, compact, usually centrally located	Karyosome large, eccentric, occasionally centrally located	Karyosome small and usually centrally located
Cytoplasm (stained)	May be present; chromatoidal bodies usually elongate with blunt, rounded, smooth edges; may be round or oval; chromatin may be diffuse or absent in mature cyst; clumped chromatin mass may be present in early cysts	Usually present; chromatoidal bodies usually elongate with blunt, rounded, smooth edges; may be round or oval; chromatin may or may not be present	May be present (less frequent than in *E. histolytica*), splinter shaped with rough pointed ends, may be diffuse or absent in mature cysts, clumped mass occasionally seen in mature cysts	Abundant chromatoidal bodies with angular pointed ends, thread-like chromatoidal bodies may also be present, half of the cysts contain spherical or ovoidal inclusion mass

[a] Data are from references 57, 73, and 188.

histolytica was first introduced by Boeck and Drbohlav in 1925 in a diphasic egg slant medium, and a modification of this medium (Locke-egg) is still used today (35). Different monophasic media that were developed for *E. histolytica* are the egg yolk infusion medium of Balamuth (12), Jones's medium (96), and TYSGM-9 (41). Of the different media developed for the xenic cultivation of *E. histolytica*, only three media, diphasic Locke-egg, Robinson's medium (150), and the monophasic TYSGM-9 (41), are in common use (for details, see reference 35).

Axenic cultivation involves the cultivation of parasites in the absence of any other metabolizing cells (35). The axenic cultivation of *E. histolytica* was first achieved by Diamond in 1961 (39). The monophasic medium TP-S-1 was developed and used widely for culture of *E. histolytica* in different research laboratories (35, 40). Currently TYI-S-33 (45) and YI-S (44) are the most widely used media for axenic cultivation of *E. histolytica* (35).

Culture of *E. histolytica* can be performed from fecal specimens, rectal biopsy specimens, or liver abscess aspirates. As the liver abscess aspirates of ALA patients are usually sterile (98% cases) (19), addition of a bacterium or a trypanosomatid is necessary before inoculation of amebae into xenic culture (35, 53, 204).

The success rate for culture of *E. histolytica* is between 50 and 70% in reference laboratories (35). As culture of *E. histolytica* from clinical samples such as feces or liver abscesses has a significant false-negative rate and is technically difficult, it is not undertaken in a routine clinical laboratory.

Entamoeba dispar can be grown in xenic culture; however, most isolates grow poorly in monoxenic culture, and the growth of only a few strains has been reported to be viable in

axenic culture, suggesting that *E. dispar* may be less able than *E. histolytica* to obtain nutrients in a particle-free medium (29, 103). The use of different media for the culture of *E. dispar* has been investigated, and these studies indicate that YI-S may not be a suitable medium for the culture of *E. dispar* (35, 103).

For *E. moshkovskii* strains, culture media employed include TTY-SB-monophasic with the trypanosomatid, TP-S-1-GM monophasic for the axenic culture of amebae (40), and the TP-S-1-GM monophasic medium (42). Other media containing bovine serum used for culture of *E. moshkovskii* include axenic medium TYI-S-33 with 10% bovine serum at 24°C (45) or xenic medium TYSGM-9 with 5% bovine serum at either 24°C or 37°C (41).

Culture of *E. histolytica* in a clinical diagnostic laboratory is not feasible as a routine procedure and is less sensitive than microscopy as a detection method (35). Parasite cultures are difficult, expensive, and labor-intensive to maintain in the diagnostic laboratory (35). Overgrowth of bacteria, fungi, or other protozoans during culture is the main problem encountered, and therefore culture is not recommended as a routine diagnostic procedure for the detection of *Entamoeba* species (35).

Isoenzyme Analysis

The pioneering work of Sargeaunt et al. (158) demonstrated that isoenzyme analysis of cultured amebae would enable the differentiation of *Entamoeba* species. A zymodeme is defined as a group of ameba strains that share the same electrophoretic pattern and mobilities for several enzymes. Zymodemes consist of electrophoretic patterns of malic enzyme, hexokinase, glucose phosphate isomerase, and phosphoglucomutase isoenzyme (159). A total of 24 different zymodemes have been described, of which 21 are from human isolates (9 of *E. histolytica* and 12 of *E. dispar*). The presence of starch in the medium influences the most variable zymodeme patterns (16), and many zymodemes "disappear" upon removal of bacterial floras, suggesting that at least some of the bands are of bacterial rather than amebal origin (94). If the zymodemes defined by stable bands alone are counted, only three remain for *E. histolytica* (II, XIV, and XIX) and one for *E. dispar* (I). Isoenzyme (zymodeme) analysis of cultured amebae enables differentiation of *E. histolytica* from *E. dispar* and was considered the gold standard for diagnosing amebic infection prior to development of newer DNA-based techniques.

Zymodeme analysis has a number of disadvantages, including the difficulty of performing the test. It is a time-consuming procedure and relies on establishing the amebae in culture, with a large number of cells needed for the enzyme analysis. This process is not always successful. The cultivation of amebae may lead to selection bias, and one species or strain may outgrow the other, which is not desirable when studying zymodemes. Furthermore, the amebic cultures and therefore isoenzyme analyses are negative for many microscopy-positive stool samples (67, 76, 80, 178). Zymodeme analysis is not easily incorporated into routine clinical laboratory work because of the expertise required to culture the parasites, the complexity of the diagnostic process, and the cost. Isoenzyme analysis has been superseded by DNA-based methods as the method of choice for studying *Entamoeba* species.

Antibody Detection Tests

Serological tests for the identification of *E. histolytica* infection may be helpful from a diagnostic perspective in industrialized nations, where infections due to *E. histolytica* are not common (127, 207). However, in areas where infection is endemic and people have been exposed to *E. histolytica*, the inability of serological tests to distinguish past from current infection makes a definitive diagnosis difficult (26, 60).

Detection of antibodies can be helpful in the case of ALA where patients do not have detectable parasites in feces. The sensitivity for detection of antibodies to *E. histolytica* in serum in patients with ALA is reported to be about 100% (215). In contrast, a study from a area of high endemicity, Hue in Vietnam, revealed that 82.6% (38/46) of individuals who were infected with *E. histolytica* even when asymptomatic had significant antiameba antibody titers. These results were confirmed by real-time PCR studies (18, 19).

Many different assays have been developed for the detection of antibodies, including indirect hemagglutination (IHA), latex agglutination, immunoelectrophoresis, counterimmunoelectrophoresis (CIE), the amebic gel diffusion test, immunodiffusion, complement fixation, indirect immunofluorescence assay (IFA), and enzyme-linked immunosorbent assay (ELISA). A variety of antibody assays for detection of *E. histolytica* antibodies in human serum are also commercially available (Table 2).

Complement fixation tests appear to be less sensitive than others, cost more to perform, and are not used by most laboratories. IHA is simple to perform and has been shown to be a highly specific (99.1%) diagnostic tool in human immunodeficiency virus-infected patients presenting with gastrointestinal symptoms (88). However, the lower sensitivity may lead to false-negative results compared to ELISA (156). The latex agglutination test appears to detect the same antibody as IHA. Commercial kits are available, and the test can be performed in 10 min. However, due to nonspecific reactions, the specificity of this test appears to be disappointing (156).

Immunoelectrophoresis, CIE, and immunodiffusion use the property of antibody and antigen precipitation in agar gel membrane. Sheehan et al. (168) reported that detection of antibody to extraintestinal *E. histolytica* by CIE is time-consuming but has a high sensitivity (100%) in patients with invasive amebiasis.

Detection of antibodies using the IFA test was shown to be rapid, reliable, and reproducible and helps to differentiate ALA from other nonamebic etiologies (56). In addition to this, IFA tests have been shown to differentiate between past (treated) and present disease (56). A study conducted by Jackson et al. (92) indicated that monitoring of immunoglobulin M (IgM) levels using the IFA can be of clinical value in cases of invasive amebiasis. The IgM levels become negative in a short period of time after infection, with more than half of the subjects having negative results at 6 months or 100% becoming negative by 46 weeks after treatment. In ALA the sensitivity of the IFA is reported to be 93.6%, with a specificity of 96.7%, making it more sensitive than the ELISA (165). A negative test therefore indicates that a patient never had invasive amebiasis. However, this test requires skills in culture and subsequent antigen preparation, making it difficult to undertake in a routine clinical laboratory (131).

ELISA is the most popular assay in diagnostic laboratories

TABLE 2. Commercially available antibody assays for diagnosis of amebiasis

Antibody assay	Sensitivity, % (reference)	Specificity, % (reference)	Manufacturer
Cellognost-Amoebiasis (IHA)	100[a] (134), 99 (84)	90.9–100[a] (134), 99.8 (84)	Dade Behring Marburg GmbH, Marburg, Germany
Novagnost *Entamoeba* IgG	>95[b]	>95[b]	NovaTec Immundiagnostica GmbH, Dietzenbach, Germany
Bichro-Latex Amibe	93.3 (194), 98.3 (149)	95.5 (194), 96.1 (149)	Fumouze Diagnostics, Levallois-Perret Cedex, France
I.H.A. Amoebiasis	93.4 (149)	97.5 (149)	Fumouze Diagnostics, Levallois-Perret Cedex, France
Amoeba-Spot IF	NA[c] (61)	NA (61)	bioMérieux, Marcy-l'Etoile, France
Amebiasis Serology microplate ELISA	95[b]	97[b]	Light Diagnostics
Amebiasis Serology microwell EIA (HK-9 antigen, axenic)	97.9 (84), 92.5 (169)	94.8 (84), 91.3 (169)	LMD Laboratories, Inc., Carlsbad, CA
RIDASCREEN *Entamoeba* (IgG detection)	100[b], 97.7–100 (100)	95.6[b], 97.4 (100)	R-Biopharma AG, Darmstadt, Germany

[a] For the titer of ≥1:64, 100% sensitive and 90. 9% specific; for the titer of ≥1:512, 100% sensitive and 100% specific.
[b] As recommended by the manufacturer.
[c] NA, not available.

throughout the world and has been used to study the epidemiology of asymptomatic disease (66) and the diagnosis of symptomatic amebiasis after fecal examination. This method is widely thought to be sufficient for clinical purposes, particularly for diagnosis of patients with ALA, and can be easily performed in a clinical laboratory. It may also be useful in the evaluation of intestinal and extraintestinal infections where amebiasis is suspected but organisms cannot be detected in feces (152). A microtiter ELISA to detect antibodies to *E. histolytica* (LMD Laboratories, Inc., Carlsbad, CA) has been shown to be 97.9% sensitive and 94.8% specific for detection of *E. histolytica* antibodies in ALA patients (84).

Serum IgG antibodies persist for years after *E. histolytica* infection, whereas the presence of IgM antibodies is short-lived and can be detected during the present or current infection. An ELISA for detection of serum IgM antibodies to amebic adherence lectin was successfully used with patients suffering from acute colitis for less than 1 week, as 45% had detectable antilectin IgM antibodies (1). In another study, it was shown that an assay based on the detection of anti-LC3 (recombinant cysteine-rich portion of the *E. histolytica* galactose-inhibitable lectin's 170-kDa subunit) antibodies in saliva is a more sensitive and specific test for diagnosis of ALA and acute amebic colitis than detection of serum anti-LC3 IgG antibodies (2). Of the recommended serological tests such as ELISA, those that demonstrate the presence of serum antilectin antibodies are the most frequently used for diagnosis of patients with ALA (145).

A high ELISA antibody titer is helpful in the diagnosis of amebiasis in patients with detectable parasites in stool, as it has a sensitivity of 95%. Since there is no cross-reaction with other, non-*E. histolytica* parasites, it is a useful test for the diagnostic clinical laboratory (23, 64, 68, 144, 165, 188).

Antigen Detection Tests

Several investigators have developed ELISAs for the detection of antigens in fecal samples. These antigen detection tests have a sensitivity approaching that of stool culture and are rapid to perform. Antigen-based ELISA kits that are specific for *E. histolytica* use monoclonal antibodies against the Gal/GalNAc-specific lectin of *E. histolytica* (*E. histolytica* II; TechLab, Blacksburg, VA) or monoclonal antibodies against serine-rich antigen of *E. histolytica* (Optimum S kit; Merlin Diagnostika, Bornheim-Hersel, Germany). Other ELISA kits for antigen detection include the *Entamoeba* CELISA PATH kit (Cellabs, Brookvale, Australia), which uses a monoclonal antibody specific for lectin of *E. histolytica*, and the ProSpecT EIA (Remel Inc.; previously manufactured by Alexon-Trend, Inc., Sunnyvale, CA), which detects *E. histolytica*-specific antigen in fecal specimens (Table 3). In addition to the above-mentioned clinical assays, research-based detection tests have included the use of monoclonal antibodies against a lectin-rich surface antigen (132), a lipophospholglycan (113), a 170-kDa-adherence lectin amebic antigen detected in saliva (2), and an uncharacterized antigen (209).

The *E. histolytica* TechLab kit was designed in 1993 to detect specifically *E. histolytica* in feces (72, 76). This antigen detection test captures and detects the parasite's Gal/GalNAc lectin in stool samples. The lectin is conserved and highly immunogenic, and because of the antigenic differences in the lectins of *E. histolytica* and *E. dispar*, the test enables specific identification of the disease-causing *E. histolytica*. The level of detection of amebic antigens is quite high, requiring approximately 1,000 trophozoites per well (78, 113). However, this test suffers from the disadvantage that the antigens detected are denatured by fixation of the stool sample, therefore limiting testing to fresh or frozen samples. Nevertheless, this test has demonstrated good sensitivity and specificity for detection of *E. histolytica* antigen in stool specimens of people suffering from amebic colitis and asymptomatic intestinal infection (72, 76, 80).

The TechLab ELISA for detection of *E. histolytica* antigen in stool specimens from people suffering from diarrhea was shown to have an excellent correlation with nested PCR (72), and in other studies this test was found to be more sensitive (80 to 94%) and specific (94 to 100%) than microscopy and culture (76, 80). In contrast, Gonin and Trudel (65) found that TechLab ELISA was less sensitive than microscopy (concen-

TABLE 3. Commercially available antigen assays for the diagnosis of amebiasis

Test	Sensitivity, % (reference)	Specificity, % (reference)	Manufacturer	Detection limit
TechLab *E. histolytica* II[a]	96.9–100,[b] 14.2 (61),[c] 87.5 (76),[d] 86 (76),[e] 71 (201), 95 (80),[f] 79 (153)[g]	94.7–100,[b] 98.3 (61),[c] 100 (76),[d] 98 (76),[e] 100 (201), 93.0 (80),[f] 96 (153)[g]	TechLab, Blacksburg, VA	0.2–0.4 ng of adhesion per well
Entamoeba CELISA-PATH[a]	95–100[b]	93–100[b]	Cellabs Pty Ltd., Brookvale, Australia	0.2–0.4 ng of adhesion per well
Optimum S *Entamoeba histolytica* antigen ELISA[a]	100 (134)	NP[h]	Merlin Diagnostika, Berheim-Hersel, Germany	Not given
Triage parasite panel[i]	96.0 (58),[j] 68.3 (133),[k] 100 (167)[l]	99.1 (58),[j] 100 (133),[k] 100 (167)[l]	BIOSITE Diagnostics, San Diego, CA	Not given
ProSpecT *Entamoeba histolytica* microplate assay[i]	87,[m] 54.5 (61),[c] 78 (128)[n]	99,[m] 94 (61),[c] 99 (128)[n]	REMEL Inc., Lenexa, KS[o]	40 ng/ml of *E. histolytica*-specific antigen

[a] Specific for *E. histolytica*.
[b] Sensitivity and specificity compared to culture/zymodeme, as cited by the manufacturer.
[c] Sensitivity and specificity compared to culture and microscopy.
[d] Compared to isoenzyme analysis.
[e] Compared to culture.
[f] Compared to culture and microscopy.
[g] Compared to real-time PCR.
[h] NP, not published.
[i] Cannot distinguish between *E. histolytica* and *E. dispar*.
[j] Compared to permanent staining with trichrome and modified acid-fast stains.
[k] Compared to ProSpecT *Entamoeba histolytica* microplate assay.
[l] Compared to ovum and parasite examination.
[m] As mentioned by the manufacturer, related to ovum and parasite identifications.
[n] Compared to microscopy (wet mounts and concentration).
[o] Previously manufactured by Alexon-Trend, Inc., Sunnyvale, CA.

tration and permanent staining) and PCR in differentiating *E. histolytica* from *E. dispar* from fecal samples. In another comparative study on the use of ELISA and PCR for the detection of *E. histolytica* and *E. dispar*, PCR was found to be 100 times more sensitive than ELISA for the differentiation of the two species (113). This kit has been discontinued by the manufacturer and has been replaced by a second-generation TechLab *E. histolytica* II kit, which has been found to be sensitive (86% to 95%) and specific (93% to 100%) compared with microscopy (wet mount with 0.9% saline and Lugol's iodine) and culture for identification of *E. histolytica* as a screening method in areas of Bangladesh with high endemicity (76, 80). The TechLab II ELISA compared to real-time PCR as a reference test also demonstrated good levels of sensitivity and specificity for the diagnosis of *E. histolytica* (71 to 79% and 96 to 100%, respectively) (153, 201). However, another study demonstrated much lower sensitivity (14.3%) and specificity (98.4%) for *E. histolytica* compared to culture and zymodeme identification (61). In addition to this, cross-reactivity of samples is an issue with this assay, since samples positive by PCR for *E. dispar* may give false-positive results (55, 201). No specific antigen tests are available for the detection of *E. dispar* and *E. moshkovskii* from clinical samples.

The TechLab *E. histolytica* II kit can also be used for the detection of *E. histolytica* lectin antigen in the serum and liver abscess pus of patients with liver disease (79). In Bangladesh, 96% (22/23) and 100% (3/3) of patients with ALA had detectable levels of lectin antigen in their serum and liver abscess pus samples, respectively, before treatment with metronidazole. However, the sensitivities of this method were only 33% (32/98) and 41% (11/27) for serum and liver abscess pus, respectively, after a few days of treatment with metronidazole (79), which is probably associated with a decrease in the amount of antigen in the serum or pus following therapy.

Results of antigen detection using both the TechLab kits suggest that more specific and sensitive diagnostic tests, such as PCR, are needed to establish the actual worldwide distribution of *E. histolytica* and *E. dispar* (61, 65). Detection of specific antigens of *E. histolytica* and *E. dispar* in feces by ELISA could be useful for clinical and epidemiological studies where molecular assays cannot be used (76, 78). Importantly, of the four diagnostic methods, i.e., antigen detection, antibody detection, microscopy, and isoenzyme analysis, only antigen detection using ELISA is both rapid and technically simple to perform and can be used in laboratories that do not have molecular facilities, thus making it appropriate for use in the developing world, where amebiasis is most prevalent. In all cases, the combination of serological tests with detection of the parasite (by antigen detection or PCR) offers the best approach to diagnosis. However, as reported by Mirelman et al. (113), improvements in automation and simplification of PCR procedures for clinical sampling directly from feces suggest that a comparison with ELISA needs to be performed.

The ProSpecT EIA (Remel Inc.) is a microplate EIA which detects both *E. histolytica* and *E. dispar*. However, this assay cannot differentiate between *E. histolytica* and *E. dispar*. The advantage of this test is that it can be performed on fresh, frozen, or Cary-Blair specimens but not on formalin-fixed fecal samples. The sensitivity of the ProSpecT EIA was compared with that of conventional microscopy (using wet mounts and concentration methods) for the diagnosis of *E. histolytica*/*E. dispar*, and a sensitivity of 78% and specificity of 99% were reported (128). In another study, by Gatti et al. (61), the reported sensitivity and specificity of ProSpecT ELISA were

54.5% and 94%, respectively, compared to culture and zymodeme identification for *E. histolytica*/*E. dispar*.

Immunochromatographic Assays

The Triage parasite panel (TPP) (Biosite Diagnostic Inc., San Diego, CA) is the first immunochromatographic assay for the simultaneous detection of antigens specific for *Giardia lamblia*, *E. histolytica*/*E. dispar*, and *Cryptosporidium parvum*. The immunochromatographic strip used in this assay is coated with monoclonal antibodies specific for the 29-kDa surface antigen (*E. histolytica*/*E. dispar*), alpha-1-giardin (*G. lamblia*), and protein disulfide isomerase (*C. parvum*). By using specific antibodies, antigens specific for these organisms from the stool samples are captured and immobilized on a membrane. A high sensitivity (96% to 100%) and specificity (99.1% to 100%) of the TPP kit compared to microscopy (stool ovum and parasite examination) for *E. histolytica*/*E. dispar* were reported (58, 167). In another study, although the specificity of the Triage kit was high (100%), the sensitivity was low (68.3%) compared to that of the ProSpecT test (133). A recent study from Sweden has compared the TPP test with PCR and demonstrated a low sensitivity for TPP assay (106).

The advantage of the TPP method is that it can be performed in approximately 15 min with fresh or frozen, unfixed human fecal specimens. The TPP provides diagnostic laboratories with a simple, convenient alternative method for performing simultaneous, discrete detection of *Giardia*-, *Cryptosporidium*-, and *E. histolytica*/*E. dispar*-specific antigens in patient fecal specimens. However, the inability of this test to differentiate between *E. histolytica, E. dispar*, and *E. moshkovskii* makes it not a method of choice for the diagnostic laboratory. Only fresh or fresh-frozen unpreserved stool samples can be tested by the Triage assay, and to maintain the integrity of the specimens, they need to be frozen or transported to the laboratory for testing as soon as possible after collection.

DNA-BASED DIAGNOSTIC TESTS

DNA-based assays are limited to research laboratories and clinical laboratories in developed countries. In most tropical and subtropical countries where amebiasis is responsible for significant morbidity and mortality, the diagnosis is still made by microscopic examination due to the lack of facilities to conduct DNA-based tests.

Complexity of Fecal Samples

Over the last decade, many DNA-based methods for the detection of viral, bacterial, and parasite DNA have been published. Fecal samples are considered to be among the most complex specimens for direct PCR testing because of the presence of PCR inhibitors, such as heme, bilirubins, bile salts, and complex carbohydrates, which are often coextracted along with pathogen DNA (85). Therefore, optimization of the fecal DNA extraction procedure is critical to the success of PCR studies.

In the past, the isolation of DNA directly from fecal samples was problematic and laborious. Recently, simple and effective methods for the isolation of parasitic DNA from feces have been developed, which enhance detection and increase the sensitivity of the PCR assay when used directly from clinical samples. Recent approaches that attempt to eliminate fecal inhibitors which copurify with the DNA consist of purification methods prior to DNA extraction and/or direct removal of inhibitors during DNA extraction (83, 206). However, many of these methods include multiple steps that are time-consuming and expensive, and so only a limited number of samples can be processed at a time.

The QIAamp DNA stool kit (QIAGEN, Hilden, Germany) has proved to be a successful and reliable method for the recovery of DNA from fecal material (196). Improvement in reproducibility and sensitivity has been obtained by modifying the extraction kit method by optimizing the duration and temperature of proteinase K digestion and by adding an additional wash step prior to DNA elution (153).

Transportation of fecal samples containing parasites at ambient temperatures may result in the rapid degeneration of parasite DNA, especially for highly labile stages such as trophozoites. Consequently, the sensitivity of DNA assays using unpreserved fecal specimens is time dependent (105). Specimens may be preserved by refrigeration or in PVA fixative, SAF, or formalin. PVA and SAF preserve trophozoites and cysts, and formalin preserves cysts for examination in wet mounts. However, methods of fixation of feces with fixatives or preservatives may result in a decreased sensitivity of PCR with time (143, 192). A few groups have, however, shown good results using formalin-fixed samples for PCR (147, 157). Ethanol is a simple transport medium that preserves amebic DNA. The most widely used reagent for the preservation of fecal samples is 10% buffered formalin solution (120); however, this solution is reported to hamper product amplification by PCR because of the interfering nature of the fixative, which perfuses the organisms and reacts with DNA (143). Consequently, freezing a fresh fecal specimen at −20°C before extraction of DNA is a better strategy, as it does not affect the sensitivity of the molecular assays (52, 105, 123).

Methods of DNA Extraction

Manual methods. Earlier methods of DNA extraction relied on the culture of microscopy-positive fecal samples in Robinson's medium followed by subsequent extraction of DNA from cultured trophozoites by the phenol-chloroform method (135, 181, 183). Later methods included direct extraction of DNA from microscopy-positive fecal samples (151), and with modification this proved to be a successful strategy for formalin-ether-concentrated samples (3, 116, 147, 148, 179) and for unpreserved stool samples and stool samples stored at 4°C or −20°C (123, 141).

QIAamp tissue kit spin columns (QIAGEN, Hilden, Germany) have been used for the purification of DNA from microscopy-positive samples stored at −20°C in phosphate-buffered saline (196, 199) and for DNA isolation using other modifications (such as treatment with 2% polyvinylpolypyrrolidone [Sigma]) which improve the sensitivity of the PCR (197). The use of the QIAamp stool kit for the extraction of DNA from fecal samples was a major advance, and this has proven to be the most widely accepted method for DNA ex-

traction. Formalin-fixed fecal samples have also been used for DNA extraction without a reduction in the ability to perform amplification of *E. histolytica* and *E. dispar* (143). Other kits used for the direct extraction of DNA from fecal samples include the XTRAX DNA extraction kit (Gull Laboratories, Salt Lake City, UT) (50), the Extract MasterFaecal DNA extraction kit (Epicenter Biotechnologies, WI), and the Genomic DNA Prep Plus kit (A&A Biotechnology, Gdansk, Poland) (118). Of the methods for DNA extraction from feces, those based on the QIAamp stool kit (QIAGEN) have predominated (50, 51, 54, 65, 82, 129, 196, 199) and are now used widely in clinical research laboratories in developed nations, as they minimize the extraction time and the DNA can be extracted directly from the feces without the need to culture the parasites.

Automated methods. A number of automated methods are available for the extraction of DNA from fecal samples. The MagNA Pure LC workstation is an automated "walkaway" system for nucleic acid extraction. With a MagNA Pure LC DNA isolation kit, genomic DNA from organisms lysed in buffer containing guanidine isothiocyanate is bound to magnetic glass particles under chaotropic conditions. The magnetic particles are washed to remove unbound substances and impurities. The washed DNA is eluted from the magnetic particles under conditions of low salt concentration and elevated temperature. MagNA Pure LC total nucleic acid isolation kit (Roche Applied Sciences) extraction technology has successfully been used for DNA extraction from microsporidia in fecal specimens (208) and for extraction of bacterial and viral DNA from clinical samples (87, 101). However, a reduction in PCR sensitivity was reported using DNA extracted from whole-blood samples for detection of viral pathogens (161). This reduction of the PCR activity was related to problems with retrieval of DNA from the magnetic glass particles, where up to 60% of the DNA could not be retrieved by use of the MagNA Pure extraction system (161). Other available automated methods include the QIAGEN automated BioRobot M48 (QIAGEN) and Nuclisens easyMAG (bioMerieux, Marcy, l'Etoile, France), but so far there have been no published protocols using these automated systems for the successful recovery of *Entamoeba* DNA from feces.

Conventional PCR

PCR-based approaches are the method of choice for clinical and epidemiological studies in the developed countries (27, 71, 81, 212) and have been strongly endorsed by the WHO. *Entamoeba histolytica* can be identified in a variety of clinical specimens, including feces, tissues, and liver abscess aspirate (188). PCR of the small-subunit rRNA gene (18S rDNA) is reported to be approximately 100 times more sensitive than the best ELISA kit currently available (113, 192).

Edman et al. (47) used PCR to amplify the gene which encodes the 125-kDa surface antigen, and this was subsequently adapted to distinguish among *Entamoeba* species by restriction digestion (187). The initial studies by Edman et al. (47) and Tannich and Burchard (187) were performed with DNA extracted from laboratory-maintained control isolates of *Entamoeba* species. PCR was subsequently used in an epidemiological study of *E. histolytica*/*E. dispar* infection, using DNA extracted from cultured trophozoites from feces, and the PCR was performed using primers specific for highly repetitive sequences present in pathogenic and nonpathogenic *E. histolytica* (now identified as *E. dispar*) strains defined previously through their respective isoenzyme patterns (59, 151).

There is now a wide variety of PCR methods, targeting different genes, which have been described for detection and differentiation of the three *Entamoeba* species (Table 4). The consistent genetic diversity detected between the 18S rDNAs of *E. histolytica* and *E. dispar* initiated the use of 18S rDNA as a target for differentiation of the two species (31, 32, 36, 138). DNA extracted from laboratory-cultured trophozoites and DNA recovered directly from microscopy-positive fecal samples using the manual and automated methods were tested, and the PCR methods proved to be highly sensitive and specific for detecting *Entamoeba* DNA (33, 34, 82, 116, 117, 141, 142, 192, 196, 199). PCR assays targeting 18S rDNA are widely used for the detection and differentiation of *Entamoeba* species, as these targets are present on multicopy, extrachromosomal plasmids in the amebae (15), making the 18S rDNA more easily detected than a DNA fragment of a single-copy gene.

The successful use of PCR in studying the epidemiology of *Entamoeba* infection was first reported by Acuña-Soto et al. (3). Those authors used DNA extracted directly from feces, avoiding the need to culture trophozoites, and the primers were targeted to amplify the extrachromosomal circular DNA. This gene target was subsequently used by other researchers (6, 24). This PCR target, with colorimetric detection of the product, was also used with DNA extracted from fecal samples, using a modification of the QIAGEN kit (6, 196, 199).

Primers for the 29-kDa/30-kDa antigen gene have been used for distinguishing among pathogenic and nonpathogenic species of *Entamoeba* using conventional PCR (183). In research laboratories, this target has been used for analyses of microscopy-positive feces which have been cultured in the laboratory (135, 181) as well as formalin-fixed fecal samples (146, 147, 148, 179).

Other gene targets for PCR include two protein-encoding genes which have been shown to exhibit polymorphism in the coding region. These are the serine-rich *E. histolytica* protein (SREPH) gene (174) and the chitinase gene (38). SREPH as a target was reported for the amplification of DNA recovered from laboratory cultures and microscopy-positive feces concentrated by the zinc-sulfate gradient floatation technique (141). A nested SREPH PCR approach was recently used to investigate *E. histolytica* diversity in a single human population, using DNA extracted from microscopy-positive feces (11). PCR using the cysteine proteinase gene and actin genes as targets was also used to study the epidemiology of amebiasis (54). In addition, a novel PCR assay based on the *E. histolytica* hemolysin gene HLY6 (hemo-PCR) was developed for the detection of *E. histolytica* DNA with fecal and ALA samples and was shown to have 100% sensitivity and specificity (216).

In an attempt to increase the sensitivity of the PCR assay, a nested multiplex PCR was developed for the simultaneous detection and differentiation of *E. histolytica* and *E. dispar* from DNA extracted from microscopy-positive fecal samples (50, 72, 89, 97). Utilizing this multiplex technique, the sensitivity and specificity were increased to 94% and 100%, respectively (123). This method has been successfully used for detection of *E.*

TABLE 4. Primers used for conventional PCR for *E. histolytica*, *E. dispar*, and *E. moshkovskii*

PCR assay	Gene target	Amplification product (bp)	Primers	Sequence (5′→3′)	Reference(s)
Single tube	M17	482	P1-S17[a] P1-AS20[a]	GCAACTAGTGTTAGTTA CCTCCAAGATATGTTTTAAC	63, 187, 214
	30-kDa protein	100	P11[a] P12[a]	GGAGGAGTAGGAAAGTTGAC TTCTTGCAATTCCTGCTTCGA	69, 119, 135, 146, 147, 148, 157, 179, 181, 182, 183, 214, 216
		101	P13[b] P14[b]	AGGAGGAGTAGGAAAATTAGG TTCTTGAAACTCCTGTTTCTAC	
	DNA highly repetitive sequences	145	EHP1[a] EHP2[a]	TCAAAATGGTCGTCGTCTAGGC CAGTTAGAAATTATTGTACTTTGTA	151
		133	EHNP1[b] EHNP2[b]	GGATCCTCCAAAAAATAAAGT CCACAGAACGATATTGGATACC	
	Small-subunit rRNA	876	Psp F[a] Psp R[a]	GGCCAATTCATTCAATGAATTGAG CTCAGATCTAGAAACAATGCTTCTC	32, 105, 106, 116, 117 118, 119, 141, 142, 196
			NPspF[b] NPspR[b]	GGCCAATTTATGTAAGTAAATTGAG CTTGGATTTAGAAACAATGTTTCTTC	
	Extrachromosomal circular DNA	145	P1[a] P2[a]	TCAAAATGGTCGTCGTCTAGGC CAGTTAGAAATTATTGTACTTTGTA	3, 6, 24, 214
		133	NP1[b] NP2[b]	GGATCCTCCAAAAAATAAAGTTT ATGATCCATAGGTTATAGCAAGACA	
	Small-subunit rRNA	1,950	RD5[c] RD3[c]	GGAAGCTTATCTGGTTGATCCTGCC AGTA GGGATCCTGATCCTTCCGCAGGTTCAC CTAC	141, 214
	Small-subunit rRNA	880	Eh5[a] Eh3[a]	GTACAAAATGGCCAATTCATTCAATG CTCAGATCTAGAAACAATGCTTCTCT	82, 192
		880	Ed5[b] Ed5[b]	GTACAAAGTGGCCAATTTATGTAAGT ACTTGGATTTAGAAACAATGTTTCTTC	
	Hemolysin gene (HLY6) LSU rRNA	256	EH6F[a] Eh6R[a]	GACCTCTCCTAATATCCTCGT GCAGAGAAGTACTGTGAAGG	216
	30-kDa protein	374	HF[c] HR[c]	AAGAAATTGATATTAATGAATATA ATCTTCCAATTCCATCATCAT	86
Duplex single step	Cysteine proteinase	242	Ehcp6F[a] Ehcp6R[a]	GTTGCTGCTGAAGAAACTTG GTACCATAACCAACTACTGC	54
	Actin gene	300	Act3F[c] Act5R[c]	GGGACGATATGGAAAAGATC CAAGTCTAAGAATAGCA TGTG	
Nested	Small-subunit rRNA	135	EH1[a] ED1[b] EHD 2[c]	GTACAAAATGGCCAATTCATTCAATG TACAAAGTGGCCAATTTATGTAAGTA ACTACCAACTGATTGATAGATCAG	27, 65
	Small-subunit rRNA	900	EH-1[c] EH-2[c]	TTTGTATTAGTACAAA GTA(A/G)TATTGATATACT	11, 72, 97, 214, 216

Continued on following page

TABLE 4—*Continued*

PCR assay	Gene target	Amplification product (bp)	Primers	Sequence (5′→3′)	Reference(s)
		900	EHP-1[a]	AATGGCCAATTCATTCAATG	
			EHP-2[a]	TCTAGAAACAATGCTTCTCT	
		900	EHN-1[b]	AGTGGCCAATTTATGTAAGT	
			EHN-2[b]	TTTAGAAACAATGTTTCTTC	
	Small-subunit rRNA	1,076	E1[c]	TGCTGTGATTAAAACGCT	50, 51, 129
			E2[c]	TTAACTATTTCAATCTCGG	
		427	Eh-L[a]	ACATTTTGAAGACTTTATGTAAGTA	
			Eh-R[a]	CAGATCTAGAAACAATGCTTCTCT	
		195	Ed-L[b]	GTTAGTTATCTAATTTCGATTAGAA	
			Ed-R[b]	ACACCACTTACTATCCCTACC	
	Small-subunit rRNA	823	Outer 1F[c]	GAAATTCAGATGTACAAAGA	89
			Outer 1R[c]	CAGAATCCTAGAATTTCAC	
		447	Eh1[a]	AAGCATTGTTTCTAGATCTG	
			Eh2[a]	CACGTTAAAAGAGGTCTAAC	
		603	Ed1[b]	AAACATTGTTTCTAAATCCA	
			Ed2[b]	ACCACTTACTATCCCTACC	
		553	SRPEh F	CCTGAAAAGCTTGAAGAAGCTG	141
			SRPEh R[c]	AACAATGAATGGACTTGATGCA	
		452	nSRPEh F[a]	TGAAGATAATGAAGATGATGAAGATG	
			nSRPEh R[a]	TATTATTATCGTTATCTGAACTACTT CCTG	
		567	SRPEd F[b]	GTAGTTCATCAAACACAGGTGA	
			SRPEd R[b]	CAATAGCCATAATGAAAGCAA	
	Small-subunit rRNA	260	Em-1[d]	CTCTTCACGGGGAGTGCG	8, 52, 130, 171
			Em-2[d]	TCGTTAGTTTCATTACCT	
			nEm-1[d]	GAATAAGGATGGTATGAC	
			nEm-2[d]	AAGTGGAGTTAACCACCT	
Multiplex	Small-subunit rRNA	166	EntaF[d]	ATGCACGAGAGCGAAAGCAT	71
		752	Ehr[d]	GATCTAGAAACAATGCTTCTCT	
		580	Edr[d]	CACCACTTACTATCCCTACC	
			Emr[d]	TGACCGGAGCCAGAGACAT	
	Tandem repeats in extrachromosomal circular rDNA	132	EhP1[a]	CGATTTTCCCAGTTAGAAATTA	123
			EhP2[a]	CAAAATGGTCGTCGTCTAGGC	
		96	EdP1[b]	ATGGTGAGGTTGTAGCAGAGA	
			EdP2[b]	CGATATTGGATACCTAGTACT	

[a] Specific for *E. histolytica*.
[b] Specific for *E. dispar*.
[c] Specific for *E. histolytica* and *E. dispar*.
[d] Specific for *E. moshkovskii*.

histolytica and *E. dispar* in formalin-fixed stool samples (129). A PCR solution hybridization enzyme-linked immunoassay targeting extrachromosomal circular DNA from *E. histolytica* and *E. dispar* with specific primers and a biotin-conjugated probe was shown to be sensitive for detection and differentiation of the two *Entamoeba* species in clinical samples (7, 11, 199).

PCR for the detection of *E. histolytica* DNA from liver abscess samples was first employed using the gene encoding the 30-kDa antigen, and 100% sensitivity was reported (182). In another study, PCR performed on liver samples demonstrated only 33% sensitivity for the presence of *E. histolytica* using primers specific for 18S rDNA of *E. histolytica*, whereas the second pair, specific for the 30-kDa antigen gene (182), showed a sensitivity of 100% (215). Direct amplification for detection of *E. histolytica* DNA (without the extraction of DNA) from ALA pus was reported using 10 different previously published primer pairs (used for amplification of *E. his-*

tolytica from liver and stool samples) (214). Of the 10 different primer pairs tested, two pairs, i.e., P1-P2, targeting extrachromosomal circular DNA of *E. histolytica* (3), and P11-P12, targeting the 30-kDa antigen gene (182), gave 100% sensitivity. Another PCR assay (hemo-PCR), based on the novel hemolysin gene HLY6 of *E. histolytica*, was analyzed for the liver abscess samples. The hemo-PCR gave a positive result for 89% of ALA samples, compared to 77% and 28% for the 30-kDa antigen gene and 18S rDNA, respectively (216). The hemo-PCR was found to be a valuable diagnostic tool for identification of *E. histolytica* in liver and fecal samples.

For the identification of *E. moshkovskii* in fecal specimens, a riboprinting method was first reported by Haque et al. (72). Subsequently, a PCR for the identification of *E. moshkovskii* in fecal samples was developed as a nested 18S rDNA PCR followed by restriction endonuclease digestion (8). This method has a high sensitivity and specificity (100%) with DNA extracted directly from stool samples using the QIAGEN stool extraction kit (52).

Although PCR-based methods have been successfully used for detection of all three *Entamoeba* species, their application in routine diagnosis is still very limited. The introduction of PCR-based methods has been hindered by difficulties in DNA extraction from fecal samples (115). Moreover, the amplification and detection of DNA are time-consuming and expensive. The shortcomings of PCR-based assays become apparent during practical applications. The generation of nonspecific DNA fragments from environmental and clinical samples poses a significant problem that often results in false-positive results.

Real-Time PCR

Real-time PCR is a new and a very attractive methodology for laboratory diagnosis of infectious diseases because of its characteristics that eliminate post-PCR analysis, leading to shorter turnaround times, a reduction in the risk of amplicon contamination of laboratory environments, and reduced reagent costs (99). This approach allows specific detection of the amplicon by binding to one or two fluorescence-labeled probes during PCR, thereby enabling continuous monitoring of amplicon (PCR product) formation throughout the reaction. An important aspect of real-time PCR is enhanced sensitivity compared to conventional PCR, with an ability to detect 0.1 cell per gram of feces (18). In addition, real-time PCR is a quantitative method and allows the determination of the number of parasites in various samples.

Distinct real-time PCR protocols have recently been published for identification and differentiation of *E. histolytica* from *E. dispar* (Table 5). These include a Light Cycler assay utilizing hybridization probes to detect amplification of the 18S rDNA from fecal samples (18, 27) and two TaqMan assays, one targeting the 18S rDNA (98, 195, 198) and another targeting the episomal repeats, using DNA extracted from fecal samples collected from primates and humans (198, 200). A molecular beacon-based real-time PCR targeting 18S rDNA of *E. histolytica* for use on fecal and ALA specimens was described (153). A SYBR green real-time assay targeting the 18S rDNA was described by Qvarnstrom et al. (139).

The sequences selected in the majority of these real-time studies have included rDNA as the target for PCR. A recent evaluation of three real-time PCR assays, focusing on the weaknesses and strengths of each assay and their usefulness for clinical laboratory diagnosis, was published by Qvarnstrom et al. (139). This study highlighted major differences in detection limits and assay performance that were observed among the evaluated tests. Two of the assays in this study could not reliably distinguish *E. histolytica* from *E. dispar*, including the Light Cycler assay (17) and the TaqMan assay targeting episomal repeats (198, 200). A multiplex real-time assay was subsequently developed for detection of different intestinal parasites with 100% sensitivity and specificity (195). This assay allows detection of *E. histolytica*, *G. lamblia*, and *C. parvum* and offers the possibility of introducing DNA detection in the routine diagnosis of intestinal parasitic infections. The implementation of such multiplex assays and the development of automated DNA isolation procedures could have a tremendous impact on routine parasitology practice. Accurate diagnosis necessitates that the same reaction conditions are used for a standard and for the sample. Duplex or multiplex approaches with internal standardization provide a solution for this problem.

A real-time PCR for detection of *E. moshkovskii* in clinical samples has not yet been reported. Further research is therefore required to develop these methods for the detection of *E. moshkovskii*.

Although real-time PCR assays are sufficiently sensitive to detect a single cell, the limited number of probes that can be applied in one reaction hinders its utility for confident multitarget detection and genotyping analysis (139). The overabundance of one species to be detected in a real-time PCR can mask the ability to detect a second species when the same amplification primers are shared in a duplex assay. Such duplex (or multiplex) assays that distinguish between targets only by use of different probes are not suitable for simultaneous detection of more than one microorganism in a single reaction. In addition to this, real-time PCR is a costly procedure compared with fecal microscopy and antigen-based detection tests. Thus, poor regions of the world, where *E. histolytica* is most prevalent, will unfortunately be less likely to benefit from real-time PCR. Instead, this technique will be feasible primarily in clinical laboratories in developed countries that need to diagnose amebiasis in high-risk groups such as MSM, travelers, and immigrants from regions of the world where *E. histolytica* is endemic.

Microarray Development

One application that has revolutionized the postgenomic era is the development and use of microarray technology. DNA microarrays are a newly developed technology used for the detection of pathogens and are rapid and sensitive. The method involves four steps: extraction of genomic DNA, amplification of targeted DNA, hybridization of labeled DNA with oligonucleotide probes immobilized on a microarray, and data analysis. Oligonucleotide microarrays have been successfully applied to the diagnosis of many pathogens in recent years. Microarray-based approaches represent an attractive diagnostic tool for detection and identification of parasitic species in clinical and epidemiological investigations.

The first oligonucleotide microarray developed for parallel detection of *E. histolytica*, *E. dispar*, *G. lamblia* assemblages A

TABLE 5. Published real-time PCR assays for *E. histolytica* and *E. dispar*

Assay	Gene target	Amplicon (bp)	Primer or probe	Sequence (5′→3′)	Nucleotide position	Reference(s)
Light cycler	18S rRNA	307	Eh-S26C[a]	GTACAAAATGGCCAATTCATTCAACG	190–216	18, 27, 139
			Ed-27 C[b]	GTACAAAGTGGCCAATTTATGTAAGCA	191–217	
			Eh-Ed-AS25[c]	GAATTGATTTTACTCAACTCTAGAG	497–473	
			Eh/Ed-24LC-Red 640	LC-Red-640-TCGAACCCCAATTCCTCGTTA TCCp	373–350	
			Eh-Ed-25-F[c]	FL-GCCATCTGTAAAGCTCCCTCTCCGAX	400–376	
TaqMan 1	18S rRNA	231	Eh-d-239F[a]	ATTGTCGTGGCATCCTAACTCA	260–239	98, 139, 195, 198
			Ehd-88R[b]	GCGGACGGCTCATTATAACA	88–107	
			Histolytica-96T[a]	VIC-TCATTGAATGAATTGGCCATTT-nonfluorescent quencher	217–197	
			Dispar-96T[b]	FAM-TTACTTACATAAATTGGCCACTTTG-nonfluorescent quencher	218–194	
TaqMan 2	Episomal repeats (SREPH gene)	83	Histolytica-50F[a]	CATTAAAAATGGTGAGGTTCTTAGGAA	50–76	139, 198, 200
			Histolytica-132R[a]	TGGTCGTCGTCTAGGCAAAATATT	132–109	
			Histolytica-78T[a]	FAM-TTGACCAATTTACACCGTTGATTTTCG GA-Eclipse Dark quencher	106–78	
		137	Dispar-1F[b]	GGATCCTCCAAAAAATAAAGTTTTATCA	1–28	
			Dispar-137R[b]	ATCCACAGAACGATATTGGATACCTAGTA	137–109	
			Dispar-33[b]	HEX-UGGUGAGGUUGUAGCAGAGAUAUUA AUU-TAMRA	33–60	
Multiplex real time PCR	18S rRNA	172	Ehd-239F[c]	ATTGTCGTGGCATCCTAACTCA	260–239	195
			Ehd-88R[c]	GCGGACGGCTCATTATAACA	88–107	
			Histolytica-96T[a]	VIC-TCATTGAATGAATTGGCCATTT-nonfluorescent quencher	217–197	
Artus (Hamburg, Germany) real-time LC-PCR kit[d]		230	*E. histolytica*		ND[e]	55
SYBER green	18S rRNA	877	PSP5[a]	GGCCAATTCATTCAATGAATTGAG	200–223	139
			PSP3[a]	CTCAGATCTAGAAACAATGCTTCTC	1076–1052	
		878	NPSP5[b]	GGCCAATTTATGTAAGTAAATTGAG	200–224	
			NPSP3[b]	CTTGGATTTAGAAACAATGTTTCTTC	1077–1052	
Molecular beacon	18S rRNA	134	Ehf[a]	AACAGTAATAGTTTCTTTGGTTAGTAAAA	104–238	153
			Ehr[a]	CTTAGAATGTCATTTCTCAATTCAT		
			Molecular beacon probe	Texas Red-GCGAGC-ATTAGTACAAAATGGCC AATTCATTCA-GCTCGC-dR Elle		

[a] Specific for *E. histolytica*.
[b] Specific for *E. dispar*.
[c] Specific for *E. histolytica* and *E. dispar*.
[d] Discontinued.
[e] ND, not described.

and B, and *C. parvum* types 1 and 2 in a single assay with high specificity and sensitivity was reported by Wang et al. (205). In addition to distinguishing between the principal genotypes, this assay proved to be useful in detecting and differentiating *E. moshkovskii* from *E. histolytica*. However, this study was conducted with purified genomic DNA extracted from standard culture strains of different parasites (205).

A microarray-based genotyping assay (comparative genomic hybridization) technique was later developed using sequenced genomic DNA clones from *E. histolytica* (HM-1:IMSS). This was the first genome-wide analysis of *Entamoeba* strains, and it revealed that this technology can be used to distinguish *E. histolytica* from *E. dispar*, to identify genes restricted to virulent strains, and to find potential genotypic-phenotypic associations (164).

Microarray assays are at this time mostly a research tool and have seldom been used in the clinical diagnostic laboratory for detection and differentiation of parasites. However, with anticipated improvements in the microarray technology along with decreasing cost, it is possible that this technology may become placed at the forefront of parasitic research.

Typing Methods

The observed heterogeneity in virulence among strains, which may determine a strain's ability to cause invasive disease,

TABLE 6. Primers for fingerprinting of *E. histolytica*, *E. dispar*, and *E. moshkovskii*

Gene target	Primer	Sequence (5′→3′)	Reference(s)
Strain-specific gene for *E. histolytica*	SSG5	GGTCTCAAAAAACCCACGAG	33, 213
	SSG3	CAAACGATAAAATCTAGCAAACTAC	
Serine gene for *E. histolytica*	SREHP5 (EHF)	GCTAGTCCTGAAAAGCTTGAAGAAGCTG	11, 33, 69, 146, 170, 213
	SREPH3 (EHR)	GGACTTGATGCAGCATCAAGGT	
	nSREPH5	TATTATTATCGTTATCTGAACTACTTCCTG	
	nSREPH3	TGAAGATAATGAAGATGATGAAGATG	
Serine gene for *E. dispar*	SREHP (F) EDF	AGATACTAAGATTTCAGTC	62
	SREHP (R) EDR	CATAATGAAAGCAAAGAG	
Chitinase gene for *E. histolytica*	EHF	GGAACACCAGGTAAATGTATA	62, 69
	EHR	TCTGTATTGTGCCCAATT	
Chitinase gene for *E. dispar*	EDF	GGAACACCAGGTAAATGCCTT	62
	EDR	TCTGTATTGTGCCCAATT	
Intergenic regions between actin gene and superoxide dismutase gene of *E. histolytica* and *E. dispar*	EH/EDF	TTGGTGGAATGTAGTCAACTG	62
	EH/EDR	AAATCCGGCTTTACACATTCC	
Locus 1-2 (*E. histolytica* and *E. dispar*)	Dsp1	TTGAAGAGTTCACTTTTTATACTATA	136, 211, 212
	Dsp2	TAACAATAAAGGGGAGGG	
Locus 5-6 (*E. histolytica* and *E. dispar*)	Dsp5	CTATACTATATTCTT TTTATGTACTTCCC	
	Dsp6	CTGAGAGCATTGTTTTTAAAGAA	
E. moshkovskii Arg gene	EmR-1	GGCGCCTTTTTTACTTTATGG	8
	EmR-2	GCTAACAAGGCCAATCGATAAA	

has stimulated efforts through molecular epidemiological studies to determine whether some subgroups of *E. histolytica* are more likely than others to cause invasive disease. The parasite and host variables that contribute to the epidemiology of disease are not clear, and there is probably a complex interplay between host genetics, immunity, enteric flora, nutrition, and parasite genetics that occurs and contributes to disease. Whether there are subtypes of *E. histolytica* that have higher or lower virulence potential or a predilection for infection of certain organs is not known. The WHO has prioritized efforts to determine whether functional subgroups of *E. histolytica* exist, which may help address some of the unanswered questions surrounding the virulence of this parasite (210). The strain identification tools available to date are limited. Isoenzyme analysis provided the first markers (159), but it is now known that isoenzyme patterns are not fixed (see "Isoenzyme Analysis" above), and therefore many assigned zymodemes are unreliable (94).

Intraspecific variation in *E. histolytica* was described by Clark and Diamond (33), and their studies on *E. histolytica* cultures (xenic and axenic) from different geographical areas of the world demonstrated the presence of extensive polymorphism in the SREHP gene (174) and the strain-specific gene (SSG) (25) (Table 6). The SREHP gene, which encodes an immunodominant surface antigen, encodes contains 8- and 12-amino-acid tandem repeats. The existence of genetic differences among strains of *E. histolytica* which cause intestinal or liver disease has been demonstrated by the polymorphism exhibited in the SREHP gene using nested PCR performed on DNA extracted from stool and liver samples (11). However, these findings were later contradicted by Haghighi et al. (70). The SSG, which is a noncoding gene and contains tandemly repeated sequences ranging in size from 8 to 16 bp, has been used to differentiate strains by the number of repeats among strains of *E. histolytica* (25, 33). However, the complete absence of this locus in certain strains makes it a poor marker for intraspecies typing (162).

The use of short tandem repeats that are linked to tRNA genes has been developed for genotyping of *E. histolytica* (9). This PCR-based genotyping of *E. histolytica* should allow the investigation of a possible association between the genotype and the outcome of infection (9).

Other DNA markers to distinguish among isolates of *E. histolytica* include the chitinase gene, which encodes tandem repeats of a degenerate 7-amino-acid sequence (38, 69). Studies with the chitinase gene as a marker for studying populations of *E. dispar* have revealed the presence of different strains in

TABLE 7. Sensitivity and specificity of different laboratory tests for diagnosis of amebiasis[a]

Test	Specimen	Sensitivity (%)		Specificity (%)	Time required	Technical expertise required	Cost
		Amebic colitis	ALA				
Microscopy (wet mount/permanent stain)	Stool	25–60	<10	10–50	1–2 h	Yes	Low
	Liver abscess fluid	NA[b]	<25	100			
Antigen detection (ELISA)	Stool	>95	Usually negative	>95	3 h	None	Low
	Serum	65 (early)	75 (late), 100 (early detection, before treatment)	>90			
	ALA fluid	NA	100 (before treatment)	90–100			
Antibody detection	Serum (acute infection)	75–85	70–100	>85	2–3 h	For certain antibody assays	Low
	Serum (convalescent infection)	>90	>90	Yes			
Culture and isoenzyme	Stool	Lower than that of antigen or PCR tests (problems in case of mixed infections)	<25	Gold standard	1–2 wk	Yes	High, labor-intensive
PCR-based assays[c]	Stool	>90	NA	>90	1–2 days	Yes	High
	Liver aspirate	NA	100	90–100			

[a] Data are from references 77, 90, 91, 188, and 215.
[b] NA, not available.
[c] PCR assay for cerebrospinal fluid (172).

different geographical areas by using DNA extracted from fecal samples (62, 140).

Other typing methods targeting repeats include the use of microsatellite typing for detecting intra- and interspecies differences. Microsatellites are segments of DNA that consist of tandem repeats of very simple motifs such as $(CT)_n$. The microsatellite typing is performed by amplifying the microsatellite by PCR using specific primers. Two minisatellite loci containing internal repeats, loci 1-2 and 5-6, have demonstrated variable polymorphism for *E. histolytica* and *E. dispar* (136, 211, 212), indicating that these loci have the potential to be used as molecular markers for investigating the epidemiology of the two *Entamoeba* species.

Riboprinting has revealed considerable genetic divergence among isolates of *E. moshkovskii* (34). Detection of polymorphisms among the *E. moshkovskii* samples was studied using the EmR primers, and this attempt was only partially successful due to the differences in sequence of the primer-binding regions (8). With the increasing reports highlighting the recovery of *E. moshkovskii* from human stool samples, further studies involving typing of *E. moshkovskii* would be helpful for studying the epidemiology of this *Entamoeba* species.

CONCLUSION

Amebiasis caused by *E. histolytica* is one of the most common parasitic infections of mankind. Research on different aspects of the parasite, carried out in various parts of the world, particularly in the last two decades, has provided the basis for breakthroughs such as the discovery of the other closely related *Entamoeba* species, *E. dispar* and *E. moshkovskii*. The redefinition of *E. histolytica*, the redescription of *E. dispar*, and the recent studies of recovery of *E. moshkovskii* from patients have dramatically changed our understanding of the prevalence of different *Entamoeba* species in the community. This has motivated researchers to develop diagnostic tests capable of distinguishing and differentiating the three species of *Entamoeba* encountered in the clinical laboratory (Table 7). The diagnosis of invasive amebiasis is most commonly attempted by a combination of microscopy of a fecal specimen, serological testing, and, where indicated, by colonoscopy and biopsy of intestinal amebic lesions or by drainage of a liver abscess. The inability of microscopic examination to detect and differentiate the three species of *Entamoeba* has been highlighted. While serological testing remains a useful tool for the diagnosis of amebiasis, studies have demonstrated the unreliability of serological tests to correctly diagnose this infection in areas of endemicity, as the level of antiamebic antibodies remains elevated in serum for many years, resulting in the inability to distinguish past from current infection. Antigen detection using fecal ELISA is another diagnostic tool, which could be used in areas of endemicity where diarrheal diseases are common, and it has proven to be useful in the developing world, where most cases of amebiasis occur and where molecular techniques cannot be used because of cost constraints. However, the sensitivity of the fecal antigen test is about 100 times less than that of PCR, and in addition, several studies have highlighted low specificity because of cross-reaction with other *Entamoeba* species. The development of molecular tools, including PCR and real-time PCR, to detect *E. histolytica, E. dispar*, and *E. moshkovskii* DNA in stool or liver abscess samples has led to major advances in making an accurate diagnosis during recent years. This in turn has assisted clinical diagnosis and the appropriate selection of patients for antiamebic therapy. In order to minimize undue treatment of individuals infected with other species of *Entamoeba* such as *E. dispar* and *E. moshkovskii*, efforts have been made for specific diagnosis of *E. histolytica* rather than treatment based on the microscopic examination of *Entamoeba* species in feces. In tropical and subtropical countries where amebiasis is endemic, the standard clinical approach is to treat all asymptomatic individuals with cysts in feces with an antiprotozoal agent. This approach to treatment causes indiscriminate use of antiamebic agents and has led to increased MICs of these therapeutic agents against *E. histolytica*, with a potential for resistant strains to appear (13, 110). These considerations suggest that positive fecal samples should be confirmed with reliable tests prior to initiation of therapy.

Our understanding of different aspects of *Entamoeba* species and the disease they can cause makes this a very exciting and rewarding time for the study of amebiasis. The incorporation of many new technologies into the diagnostic laboratory will represent a challenge to us all. Such studies will then lead to a better understanding of the public health problem and measures to control the disease.

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