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Review Article

Evaluation of Different Techniques in Laboratory Diagnosis of Intestinal Amoebiasis

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Abstract

Entamoeba histolytica is an intestinal protozoan disease that causes amoebiasis, which is a health problem in several developing countries that can lead to 100 000 deaths annually. This parasite is the third cause of death in tropical regions with poor sanitary conditions. An essential part of the treatment of patients is the detection of the pathogenic *E. histolytica* and its differentiation from non-pathogenic *Entamoeba spp*. Because microscopy and antigen detection techniques are inexpensive and readily available, these techniques are commonly used to diagnose amoebiasis. Rapid tests and different ELISAs for antigen detection are some of the more modern and sensitive methods, and some diagnostic methods are not able to distinguish different species of Entamoeba. Techniques for molecular detection are highly specific and sensitive. However, utilizing molecular methods as the usual diagnostic method becomes difficult due to their high cost in most endemic areas. For the diagnosis of intestinal amoebiasis, there is still a need for highly sensitive and specific tests that are quick and affordable to use, especially in developing countries where this disease is common.

Keywords: Evaluation, Techniques, Laboratory diagnosis, Intestinal amoebiasis

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Introduction

Amoebiasis infection is caused by the protozoan *Entamoeba histolytica*, which is an extracellular parasite, and the National Institute of Allergy and Infectious Diseases (NIAID) has classified this parasite as a category B primary biodefense pathogen (1,2). This parasite, which is considered the third cause of death by protozoa, causes 40 000 to 100 000 deaths annually and is common in countries that do not have good health services (3,4). In developed European regions, *Entamoeba* infection occurs as a result of immigrants entering or traveling to endemic areas (5). Patients with amoebiasis are asymptomatic in about 90% of cases. The remaining 10% show symptoms that contain extraintestinal amoebiasis, colitis, and dysentery (3). Amoebiasis severity in patients with symptoms depends on the genotype of the parasite (6), the microbiota (pathogenic microorganisms) in the intestines (7,8), and the patient's genetic history (9,10). There is still no vaccine to prevent this disease (11). Most *Entamoeba* infections are seen in places with low economic and social status, as well as poor health status. People can become infected through water, food, or hands contaminated with feces containing cysts. Both oral and anal contact have been reported as routes of human-to-human transmission (12). Intestinal amoebiasis is diagnosed according to clinical symptoms and laboratory test results. Improving sanitary conditions is necessary to prevent the prevalence of amoebiasis. The current review study with the identification of *E. histolytica*, which is an indistinguishable species from other non-pathogenic species. The molecular techniques and other detection methods that lead to the differentiation of this parasite from its other species were described.

Diagnostic Methods of Amoebiasis

Laboratory diagnosis of amoebiasis is based on parasitological, immunological, and molecular tests. The "gold standard" method of diagnosis is thought to involve microscopically observing the parasite in a sample of tissue, fluids from the body, or stool. Intestinal diseases can be diagnosed by microscopic methods, cultures, isozyme analysis, antigen detection tests, molecular tests, and rapid diagnostic tests. The laboratory diagnosis of extraintestinal amoebiasis differs from that of intestinal amoebiasis in several ways. First, patients with intestinal amoebiasis who have been exposed to *E. histolytica* produce IgG antibodies against this parasite, which may remain in the body for a period of time and challenge the definitive diagnosis of the current and previous infections. Second, most patients with extraintestinal

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amoebiasis, especially amoebic liver abscess (ALA), do not have concurrent amebic colitis. Therefore, stool sample testing for this suspected ALA is not performed unless the patient has intestinal symptoms (13,14).

Intestinal Amoebiasis

Microscopic Examination

The microscopic diagnostic method for parasitic infections is the most prominent method used to identify hematophages trophozoites, and four-nucleate cysts in stool samples (15). Microscopic tests are common in developing countries because they are easy and inexpensive (16). Stool samples should be examined within 1 hour after collection to ensure that the trophozoite does not lose its motility. Also, stool samples should be preserved in polyvinyl alcohol (PVA), Schaudinn's fixative, or sodium acetate-acetic acid-formalin (SAF) if the examination is not possible at this time (17). However, the skill of laboratory personnel to accurately recognize trophozoites is crucial since, if they stay motionless, they can be mistaken for tissue cells, leukocytes, and macrophages (18). Trophozoites are more often seen in feces that contain some blood, mucus, and pus. What is more, cysts are also seen in loose and formed stools (19). To see the size, shape, and number of the nuclei, the permanent stain of the stool smear should be examined. Stains such as Giemsa, Wright, methylene blue, and trichrome iodine can be used for staining. However, iron-modified hematoxylin and Whitley's trichrome stains are recommended for routine use (3). Although microscopic tests allow us to see the parasite, they are not able to identify and differentiate the species. The morphology of *E. histolytica*, *E. dispar*, and *E. moshkovskii* is indistinguishable under the microscope. Therefore, it is thought that microscopic testing has a low sensitivity and diagnostic specificity when it comes to identifying *E. histolytica* in stools (see Table 1) (15, 20).

Biochemical Methods

Culture and Isoenzyme Analysis

The isozyme cultivation and analysis method used to be considered the gold standard, but nowadays it is mostly used in the research field (18). Stool samples, rectal biopsy or liver abscess aspirate can be used for *E. histolytica* culturing (21). Zymogene enzymes are used as markers for isoenzyme analysis in cultured amoeba (22). These enzymes include hexokinase, decarboxylating malate dehydrogenase, glucose phosphate isomerase and phosphoglucomutase isozyme (23). With this method *E. histolytica* and *E. dispar* are separated because their hexokinase enzymes are different (24). However, in the method of isozyme analysis, culture is required for the growth of the parasite trophozoite, which is a costly, time-consuming and boring method and may not be successful in all cases (19,25). The success rate of this method in the studies reported was 50%-70%. Often, isozyme analysis gives a false negative result (26). In general, this method is used for intestinal amoebiasis (27).

Immunological Detection

In the immunological technique, the ELISA method can be used which detects *E. histolytica* antigen in our target sample. The sensitivity of this method is 80-94% and its specificity 94%-100%, which is higher than the microscopic and culture methods (20). Several ELISA kits are commercially available: the TechLab *E. histolytica* II ELISA kit, the Entamoeba CELISA PATH kit, the Optimum S kit, and the ProSpecT ELISA kit (see Table 2).

Table 1. Sensitivity of Microscopy and Culture Methods for the Diagnosis of Amoebiasis

Diagnosis Method	Sample	Identification of *E. histolytica*	Detection	Sensitivity %	Time for Analysis	Reference
Microscopy	Stool	No	Cyct and trophozoites[a]	25-60	1-2 h	(20)
Culture and isoenzyme analysis[b]	Stool and ALA aspirate	Yes	Zymodeme	Gold standard	7 days	(3)

[a] Hematophagous trophozoites in patients with acute bloody diarrhea indicative of *E. histolytica*.
[b] Axenic culture mediums YI-S and TYI-S-33 for *E. histolytica*.

Table 2. Antigen Detection Assays for the Detection of Intestinal Amoebiasis

Kit	Specimen	Recognition	Species	Sensitivity (%)	Specificity (%)	Reference
TechLab Kit II	Stool	Intestinal amoebiasis	*E. histolytica*	95[a] 79[b] 87/5[c]	93[a] 96[b] 100[c]	(20,31,32)
Entamoeba CELISA-PATH	Stool	Intestinal amoebiasis	*E. histolytica*	27/8[d]	98[d]	(33)
Optimum S kit	Stool	Intestinal amoebiasis	*E. histolytica*	100	Unknown	(34)
ProSpecT ELISA	Stool	Intestinal amoebiasis	*E. histolytica*	78[d]	99[d]	(35)

[a] Compared to culture and microscopy.
[b] Compared to real-time PCR.
[c] Compared to isoenzyme analysis.
[d] Compared to microscopy.

A monoclonal antibody against Gal/GalNac lectin is used in the first and second kits. In the third kit, it detects the serine-rich antigen of *E. histolytica*, and in the fourth kit, it detects the EHSA antigen of *E. dispar* and *E. histolytica* (28). The most commonly used kit is TechLab, which detects both symptomatic and asymptomatic patients (20). During the last two decades, ELISA kits have been used due to the ease and speed of results, the ability to differentiate between *E. histolytica* and *E. dispar*, high sensitivity and specificity compared to microscopy and culture, cost-effectiveness, and the ability to detect on a large scale (29). The ELISA test has a sensitivity of 80%-94% and a specificity of 94%-100% compared to the microscopic and culture methods (20,30). Table 2 shows the sensitivity and diagnostic specificity of ELISA kits obtained from different studies.

Molecular Methods

The use of molecular methods to diagnose amoebiasis has solved the problem of species differentiation (26). There are different molecular methods that can detect Entamoeba species in different samples, such as stool, aspiration of liver abscesses, or tissues. Including conventional PCR, nested PCR, nested multiplex PCR, real-time PCR, multiplex real-time PCR and loop-mediated isothermal amplification assay (LAMP) can be mentioned among them. In addition to species identification, this molecular technique can identify mixed infections with *E. histolytica*, *E. moshkovskii*, or *E. dispar*, which are associated with gastrointestinal complications (36).

Conventional PCR

The conventional PCR method can determine the actual prevalence of *E. histolytica* and *E. dispar* and provide an effective method for adequate treatment of the infection (29,37). The most commonly used genes for PCR is the small ribosomal unit gene (18S rRNA), which differentiates between *E. histolytica* and *E. dispar* (29). Other genes used are hemolysin (HLY6) (38), 30 kDa antigen (39), serin-rich protein of *E. histolytica* (SREHP) (40), cysteine protease 8 (CP8) (41), actin, and adhesin (adh112) (42). In a study, it was found that there was 100% sensitivity and diagnostic specificity for the identification of *E. histolytica* DNA in stool samples, and the HLY6 gene (38). However, the PCR method targeting small subunit rRNA is more commonly used due to the presence of multiple copies of extra chromosomal plasmids (see Table 3) (43). Conventional PCR has a specificity of 97% and a sensitivity of 99% compared to the ELISA method (37).

Nested PCR

The nested PCR method is used to increase the sensitivity of the PCR method. The first PCR products are used as templates to perform the second PCR, and in this reaction, two sets of external and internal primers are used against a target sequence in two consecutive PCR reactions (46). This method has been carried out in different regions of the world to determine the true prevalence of *E. histolytica* from other species, and it was done for the first time in Bangladesh on stool samples by targeting the 16S rRNA gene, which showed 100% specificity (46). The nested PCR method measures the size difference of the 18S RNA gene from *E. histolytica*, *E. dispar* and *E. moshkovskii* using sequencing and correlates the obtained results using polymorphic ArgTCT tRNA gene sequences from three species (45). Nested PCR actually detected 75% of *E. histolytica* in an outbreak survey conducted in Malaysia, and also detected *E. dispar* (30.8%) and *E. moshkovskii* (5.8%) (51). Although this method is used to distinguish species from each other, it is a time-consuming and tedious process.

Nested Multiplex PCR

Simultaneous detection of species *E. histolytica*, *E. dispar*, and *E. moshkovskii* using nested multiplex PCR makes it easier and shows the sensitivity of the test in complex samples with the lowest concentration of 1000 parasites in 0.05 grams of stool (52). Khairnar K and et al. also found that using the 18S rRNA gene in multiplex nested PCR, three species of *E. histolytica*, *E. dispar*, and *E. moshkovskii* can be distinguished. Moreover this method showed 94% sensitivity and 100% specificity (52). Fallah et al. observed that accurate determination of pathogenic and non-pathogenic species of Entamoeba in stool samples is possible using nested multiplex PCR. Out of 724 stool samples, 31 (4.28%) showed positive for *E. histolytica/E. dispar*, with 8 (25.8%) showing positive for *E. histolytica* and 54.8% positive for *E. dispar* (53). Nested PCR was used in an epidemiological population in Malaysia and showed that *E. histolytica* had a higher prevalence of about 75% than non-pathogenic species (51). On the other hand, in another study in Iran, this method showed *E. dispar* with a higher prevalence of about 0.58% and also reported the *E. moshkovskii* species for the first time in the northwestern region of Iran (54). It allows simultaneous detection and differentiation of *E. dispar* and *E. histolytica* in stool samples that were positive microscopically (44,52).

Real-Time PCR Method

This method is important in the diagnosis of amoebiasis for several reasons: saving time, relative quantification of the number of parasites in the sample, high detection sensitivity and reducing contamination in the results (28,55). In addition, this technique reduces the false positive results that occur in conventional PCR and nested PCR due to electrophoresis. This is so because the risk of contamination is lower. This method provides

Table 3. Types of Molecular Tests for the Diagnosis of Amoebiasis

Diagnosis Method	Species	Amplification Product (bp)	Target Gene	Primers (5′-3′) used Amplification	Sample	Reference
Conventional PCR	*E. histolytica*	166	18S rRNA	EnF 5′-ATGCACGAGAGCGAAAGCAT-3′ EhR 5′-GATCTAGAAACAATGCTTCTCT-3′	Stool	(44)
	E.dispar	752		EnF 5′-ATGCACGAGAGCGAAAGCAT-3′ Ehd 5′-CACCACTTACTATCCCTA CC-3′		
	E.moshkovskii	580		EnF 5′-ATGCACGAGAGCGAAAGCAT-3′ Enm 5′-TGACCGGAGCCAGAGACAT-3′		
Nested PCR	*E.histolytica/ E.dispar*	268	adh112 gene	5′-CGCCCGCCGCGCGGCCGCGGC-3′ 5′-CGGCCGGGGGCACGCGGCGGC-3′ 5′-AGAAAAAAAATAA TAATAA-3′ 5′-TTCATTTGTTTT ACTTTCA-3′	Stool	(42)
Nested multiplex PCR	*E. histolytica*	900	SSU-rRNA	E-1F, 5′-TTTGTATTAGTACAAA-3′ E-2R, 5′-GTA[A/G] TATTGATATACT-3′	Stool	(45, 46)
	E. histolytica	550		Eh-1F, 5′-AATGGCCAATTCATTCAATG-3′ Eh-2R, 5′-TTTAGAAACAATGCTTCTCT-3′		
	E.moshkovskii	200		Ed-1F, 5′-AGTGGCCAATTTATGTAAGT-3′ Ed-2R, 5′-TTTAGAAACAATGTTTCTTC-3′		
	E.dispar	260		Em-1F, 5′-CTCTTCACGGGGAGTGCG-3′ Em-2R, 5′-TCGTTAGTTTCATTACCT-3′		
Real-time PCR (Light Cycler)	*E.histolytica*	172	18SrRNA	5′-ATTGTCGTGGCATCCTAACTCA-3′ 5′-GCGGACGGCTCATT ATAACA-3′	Stool, Pus of ALA	(47, 48)
Multiplx Real time PCR	*E.histolytica*	110	SSU-rRNA	5′-GGACACATTTCAAT TGTCCTA-3′ 5′-CATCACAGACCTGTTATTGCTG-3′	Stool	(49)
		111		5′-GGACACATTTCAAT TGTCCTA-3′ 5′-CATCACAGACCTGT TATTGCTG-3′		
LAMP	*E.histolytica*	External primers	HLY-6	Eh-2F3, 5′-GCACTATACTTGAACGGATTG-3′ Eh-2B3, 5′-GTTTGACAAGATGTTGAGTGA-3′	Stool	(50)
		Internal primers		Eh-2FIP, 5′-TCGCCCTATACTCAAATATGACA AGACTTTGGTGGAAGATTCACG-3′ Eh-2BIP, 5′-ATCTAGTAGCTGGTTCCACCTGA ACACCTAATCATTATCTTTACCAATC-3′		
		Additional primers		Eh-2F2, 5′-ACTTTGGTGGAAGATTCACG-3′ Eh-2B2, 5-CACCTAATCATTATCTTTACCAATC-3′		

the number of parasites numerically in different samples such as feces, urine, and aspiration of liver abscesses (56). Also, this method measures and detects the fluorescence released after each amplification step using labeled primers and probes that hybridize to specific sequences (57). Many studies have used DNA from stool samples to diagnose *E. histolytica* and *E. dispar* (31,56,58). Probes (like TaqMan) hybridize with the amplified products to diagnose amoebiasis with 100% accuracy in recognizing *E. histolytica* (56). The TaqMan method, which targets the 18S rRNA gene, is more specific than the SYBR Green method for the detection of amoebiasis, according to a comparison of real-time PCR using different probes. Furthermore, they discovered that *E. histolytica* can be found in clinical samples containing very few parasites using probe-based real-time PCR techniques that are undetectable with conventional PCR (59).

Multiplex Real-Time PCR

Multiplex qPCR protocols (duplex, triplex or tetraplex) allow rapid identification, DNA quantification and genotyping of several Entamoeba species simultaneously (60). This approach uses TaqMan probes and primers that are common to all four species to hybridize the products and distinguish them according to fluorescent molecules (FAM, VIC, fluorescein, etc) (57). In a study conducted in Thailand, a multiplex real-time PCR was developed to differentially identify *E. histolytica*, *E. disbar*, and *E. moshkovskii*. *E. histolytica* was detected by this method in four samples out of 32 stool samples that were positive by the microscopic method. Most of the samples were reported as *E. dispar*, and one sample was mixed with *E. moshkovskii* (60). In another study conducted in Egypt, 396 stool samples that were involved in diarrhea were compared with 202 stool samples of healthy controls microscopically, and 43 patient samples tested positive for *E. histolytica/dispar*. However, only eight samples with *E. dispar* were identified with a real-time PCR technique, while no *E. histolytica* was identified at all for the accurate and rapid diagnosis of amoebiasis, Real-time PCR with multiple DNA targets would therefore be advantageous (48). Although real-time PCR is a very specific and sensitive method, its relatively high cost is its main drawback, and it may not be used in most laboratories in developing countries.

Loop-Mediated Isothermal Amplification Assay (LAMP)

In 2000, Notomi et al developed the LAMP method for the detection of the hepatitis B virus and this method was able to improve the diagnostic limits of up to six copies of DNA in 45 minutes using four specific primers (61). This technique is based on nucleic acid sequence-based amplification (62), self-stable sequence repeats (3SR) (63) and strand displacement amplification (64). This technique increases sensitivity and specificity by using a series of transcription digestion, reverse transcription or restriction enzyme reactions to reduce detection time (61). In addition to high sensitivity and specificity, this method is a good choice for molecular detection in developing regions due to its high speed and simplicity (61). More than one parasite per reaction can be detected with LAMP in the diagnosis of amoebiasis (65). In a study, an LAMP assay was developed for the detection of intestinal amoebiasis and the results were compared with the nested PCR method. Results were detected in the nested PCR method for E. histolytica DNA in 33% of samples (10/33) but detected in 60% of samples (18/30) using LAMP, Therefore, the LAMP method showed more sensitivity (66). The LAMP technique allows the detection of about 10 trophozoites of *E. histolytica* in each reaction with 100% specificity, but the ability of this method to identify infected species should be improved (40), although it has shown better performance than other methods such as PCR, qPCR, and nested PCR (67).

Rapid Diagnostic Test

Rapid diagnosis kits have been used as amoebiasis point-of-care (POC) tests in recent years (68). This method is a superior diagnostic tool in developing countries with restricted resources and is more rapid than other laboratory methods such as ELISA and PCR. Also, it does not require expensive equipment (69). Some of these tests that are available on the market for the diagnosis of gastrointestinal amoebiasis include the Triage Parasite Panel (TPP) which is the first diagnostic test that simultaneously detects specific antigens for *E. histolytica/E. dispar*, *G. lamblia* and *C. parvum*. This test uses monoclonal antibodies specific for *G. lamblia* alpha-1-giardin, the surface antigen of 29 kDa *E. histolytica/E. dispar*, and *C. parvum* protein disulfide isomerase. The TPP kit has been shown in studies to have high specificity (99.1%-100%) and high diagnostic sensitivity (96%-100%) compared to the microscopic method for the diagnosis of *E. histolytica/E. Dispar* (70, 71). The next kit is RIDA®QUICK Cryptosporidium/Giardia/Entamoeba Combi, used to check the lateral flow of parasites in stool samples, which was conducted in an outpatient clinic in Belgium. The kit showed 100% sensitivity and 80%-88% specificity for the diagnosis of *E. histolytica* (72). Another test is ImmunoCard STAT!® CGE, which qualitatively shows the antigens *C. parvum, G. lamblia*, and *E. histolytica* in feces. Compared to real-time PCR, this test showed 88% sensitivity and 92% specificity in the detection of E. histolytica, but it also displayed cross-reactivity with *E. dispar* (73,74). The next generation that was recently introduced to the market is a rapid test called the RIDA Quick Entamoeba test *E. histolytica* Quick Chek, the antibody used in this kit is specifically against the adhesive lectin of *E. histolytica* (75). This rapid test was evaluated in Bangladeshi children and showed 100% sensitivity and specificity compared to the ELISA antigen detection method (76,77). The next test, prototype of lateral flow dipstick test, detects *E. histolytica* PPDK in stool samples (78,79). In comparison to real-time PCR, the sensitivity of this test was 65.4% ($n=17/26$), but its specificity was 92% (23/25) when tested on stool samples that included different enteric pathogens (80).

Conclusion

According to the above-mentioned points, it can be concluded that microscopic methods do not have the necessary efficiency due to the lack of species differentiation, despite their wide application in developing regions. Antigen identification methods in stool samples are valuable, but they are much less sensitive than molecular methods. What is more, molecular methods are recommended for diagnosing intestinal amoebiasis in areas that have developed, as well as the LAMP method for areas that are developing. With correct and early diagnosis, it is possible to prevent excessive use of drugs and the creation of drug-resistant strains.

Authors' Contribution

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Competing Interests

The authors of this review declare that they have no conflict of interest.

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