Accurate laboratory diagnosis of human intestinal and extra-intestinal amoebiasis

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ABSTRACT

Accurate diagnosis of *Entamoeba histolytica* is important, as it is known as a causative agent for both invasive intestinal and extra-intestinal amoebiasis. Amoebiasis has a worldwide distribution, especially in developing countries, and it is responsible for up to 100,000 fatal cases annually. A number of diagnostic methods, including microscopy, culture, antigen detection, molecular based methods, and serological assays have been proposed to assist in diagnosing amoebiasis. The present study aimed to gather new data and review the available diagnostic tests of both intestinal and extra-intestinal amoebiasis and to highlight pitfalls and challenges of each of them. A broad literature of electronic databases was conducted and covered articles published up to March 2022. For laboratory diagnosis of intestinal amoebiasis, direct microscopy stool examinations and cultures should be held as the high-performance diagnostic strategies. Molecular and immunological-based assays are also recommended as complementary tests. To diagnose extra-intestinal infection, the use of serological tests is still considered the method of choice. However, serodiagnosis requires further improvement for the accurate differential diagnosis of active infection from past infections.

Keywords: Amoebiasis, *Entamoeba histolytica*, Diagnostic tests, Conventional methods, Serology, Molecular tests.

Introduction

Amoebiasis caused by the protozoan parasite *Entamoeba histolytica* is an important enteric and extra-enteric infection worldwide. It was estimated that approximately 50 million people around the globe are infected with *E. histolytica* (1). Approximately 10% of these infected individuals show clinical symptoms of both intestinal and extra-intestinal amoebiasis, and the other 90% are asymptomatic carriers, acting as cyst passers. Amoebiasis is responsible for the deaths of up to 100,000 humans annually worldwide (1, 2). Amoebiasis is ranked as the third leading parasitic cause of human deaths worldwide (2, 3).

Amoebiasis is prevalent in South and Central America, Asia, Africa, and other tropical areas. In developed countries, this infection is seen mostly among returning travelers or immigrants from endemic regions (4). *E. histolytica* has a simple life cycle, including two active trophozoite and cystic forms. Parasite transmission occurs via the fecal-oral route by the direct or indirect ingestion of mature infectious cysts (1, 5).

Most infections caused by *E. histolytica* are cyst passer and asymptomatic (carrier), but in some cases, the trophozoites invade the intestinal mucosa and cause cramping, abdominal pain, dysentery, and ulcers that may threaten the patients' lives. In the extra-intestinal invasion of trophozoites, amoebic liver abscess (ALA), pneumonia, purulent pericarditis, and even cerebral amoebiasis can be observed. The amoeba may be carried into any organ in the body (1, 6).

Accurate diagnosis of both intestinal and extra-intestinal amoebiasis plays a very important role in the treatment of patients and in controlling the disease. A number of diagnostic methods have been proposed for

the correct diagnosis of amoebiasis, including microscopy, antigen detection, molecular tests, serology, sigmoidoscopy/colonoscopy, and abdominal imaging (such as ultrasound, CT or MRI). However, in poor countries where amoebiasis is endemic, the identification of cysts or trophozoites in stool examinations is the method of choice for diagnosing intestinal amoebiasis. The use of serological methods is recommended for the diagnosis of extra-intestinal amoebiasis cases in these regions.

The correct laboratory diagnosis of amoebiasis was found to be dependent on some factors such as the skill of the operators, the selected method of testing, and the stage at which the test is performed. In the majority of cases, a skilled microscopist and a combination of tests are often required to establish the diagnosis. Unfortunately, most medical diagnostic laboratory workers have difficulty in diagnosing amoebiasis, and false positive and negative laboratory reports are common. For example, a study performed in central Iran showed that among 53 dysentery cases reported as positive for *E. histolytica* by laboratory personnel, only 12 (22.6%) cases were truly positive, and 41 (77.4%) were misdiagnosed (7). Therefore, there is a need for the accurate identification of this amoeba in clinical specimens for diagnostic purposes, the management of patient care, and to prevent the unnecessary treatment with antiamoebic drugs of nonpathogenic parasite-infected individuals (8).

The present study aimed to gather new data and review the available tests used in diagnostic and research laboratories for the accurate diagnosis of both intestinal and extra-intestinal amoebiasis.

Methods

Electronic searches were performed in several available national and international electronic databases and journals to identify articles reporting on amoebiasis diagnostic methods. The search covered articles and some textbooks published up to March 2022. The articles reported the use of at least one method, such as stool examination, immunodiagnostic methods, molecular methods, antigen detection, staining, or culture for diagnosis of intestinal and extra-intestinal amoebiasis. The international databases of ISI Web of Science, PubMed, EMBASE, Scopus, Science Direct, Research Gate, and Google Scholar were electronically searched as well as the national databases Iran Medex, Iran Doc, Magiran, and Scientific Information Database (SID). The words and phrases "amoebiasis OR *Entamoeba*," "amoebic liver abscess," "diagnosis," "immunodiagnosis," "molecular," "serology OR serodiagnosis," "culture," and "stool examination" comprised the panel of keywords. Searches were restricted to articles written in the English and Persian languages, and the references of selected papers were checked for more accuracy. Published abstracts of parasitology, microbiology, and infection diseases congresses were also included in the search.

Results
A: Intestinal amoebiasis
Identification of asymptomatic cyst passers

Approximately 90% of infected individuals are asymptomatic carriers acting as cyst passers. Therefore, the correct diagnosis of asymptomatic carriers is critical for controlling the spread of this amoeba (7). Asymptomatic healthy carriers are usually diagnosed by identifying cysts in stools. In such people, the one to four nucleated cyst stages are more likely to be observed in stool samples. Routine fecal suspension in saline (0.85 NaCl) or Lugol's solution or in a fixative solution may also be used for cyst identification (7). Cysts can be detected by unstained direct wet saline, because they are refractile bodies, but their differentiation from other nonpathogenic cysts needs to be stained by iodine (2-5% Lugol's solution).

Direct microscopy is an economical, rapid method for sample diagnosis in a medical diagnostic laboratory. However, the direct examination of a fecal suspension in either saline or Lugol's solution under a microscope is not known as a sensitive method. Under Lugol's solution, the chromatoidal bodies of a cyst may not be clearly visible, as they are in the saline wet mount.

A recent study conducted by Nlinwe and Kumla (2020) showed the sensitivity rate of direct wet mount examination for the diagnosis of *E. histolytica* / *E. dispar* as 61.54% (42.53% to 77.57%) (9). Another study reported only 34.7% sensitivity for wet mount smear for one or more intestinal parasites (10).

Examination of a single stool sample by wet mount smear may not detect the parasite; thus, it has been

recommended that two or more wet mount smears be examined to increase the chance of finding parasitic cysts. However, due to the intermittent or low levels of cyst shedding of some intestinal parasites, such as *Giardia lamblia* and *E. histolytica*, examining more than three stool samples may be necessary to detect parasitic cysts (11).

The concentration methods, especially sedimentation procedures, as well as permanently stained smears are sometimes needed and also recommended for the accurate identification of cysts in feces. Moreover, using concentration methods to detect *Entamoeba* cysts may be necessary when the cysts are few in number or when it is necessary to check whether treatment has been successful. Concentration methods should also be used to investigate the accurate prevalence or incidence rate of intestinal parasitic infection as a part of an epidemiological survey.

Zing sulphate flotation and formalin-ether sedimentation techniques are two types of concentration procedures used in parasitological laboratories to detect *Entamoeba* cysts. The formalin-detergent sedimentation technique can be considered as a replacement when a centrifuge is not available. Overnight sedimentation is required for obtaining results using this method. Of note, flotation techniques are not recommended as routine concentration techniques for the detection of *Entamoeba* and *Giardia* cysts, as they may result in collapse of the cyst wall (11). Sedimentation procedures, especially the formalin ether/formalin-ethyl acetate technique, are the recommended methods, because they are rapid, easy to perform, and less prone to technical errors (11-12). Different studies have previously shown the superiority of the sedimentation technique over the direct smear preparation method. Moreover, the sensitivity of the formal ether method has been found to be twice that of routine saline or iodine preparation direct smear methods (11, 13-14).

Although the *E. histolytica* cyst can be morphologically differentiated from other common human intestinal amoebas (including *E. coli*, *E. hartmanni*, *E. nana* and *I. butschlii*), three species, namely *E. dispar*, *E. moshkovskii* and *E. bangladeshi*, are morphologically indistinguishable from *E. histolytica*. A WHO expert consultation on amoebiasis stressed the need for the development of simple methods for the specific diagnosis of potentially pathogenic *E. histolytica* from similar nonpathogenic species (8). In addition, this meeting recommended that the cysts should be reported as *E. histolytica* / *E. dispar* in light microscopy diagnosis (8, 15).

Ultimately, laboratory personnel must pay special attention to any unusual findings in cyst figures and to a wide variety of objects resembling *Entamoeba* cysts or trophozoites that may be found in stool specimens.

Amoebic dysentery diagnosis
Microscopic observation

The laboratory diagnosis of amoebic dysentery, especially using traditionally methods, is challenging, because the identification of *Entamoeba* trophozoite depends on morphological criteria. Inexperienced laboratory personnel may not differentiate between macrophages and amoebae trophozoites or degenerated polymorphonuclear cells from cysts. Some variables can affect the outcome of microscopic examination, such as time spent on sample processing, storage conditions, whether the samples are fixed or fresh, parasite density, method selection, and personnel training.

Inexperienced laboratory personnel find it difficult to differentiate *E. histolytica* from other non-pathogen amoebae of the human colon and may even be confused in distinguishing macrophages from trophozoites of amoebae and neutrophils from cysts.

Laboratory personnel should be trained in the selection and performing of appropriate methods for finding and identifying this parasite. In dysentery cases of amoebiasis, concentration methods are not suitable for confirming the existing infection. Using these methods, centrifugation destroys the motile trophozoites of *Entamoeba* and other intestinal parasites in fecal specimens.

Microscopic examination of direct wet saline preparations from fresh stool specimens is appropriate for observing motile trophozoites, but in Lugol's solution-stained smears, trophozoites will be non-motile. The examination of three or more smears is recommended for the detection of organisms when suspecting amoebiasis. Fecal specimens must be collected in a clean container and must not be contaminated with either water or urine to prevent the destruction of motile trophozoite.

If amoebic dysentery is suspected, the feces sample should be examined as soon as possible within a few hours after sampling. The motility of trophozoites can be observed if the sample is kept in a warm environment (35-37 °C) until examination. If a dysenteric stool sample containing trophozoites is left too long before examination, the organisms tend to degenerate. Samples must be kept at 4 °C to avoid autolysis of trophozoites and to reduce the growth and action of bacteria, which may consequently destroy amoeba trophozoites (16). Furthermore, fecal specimens should never be frozen prior to their examination.

If a dysentery specimen needs to be referred to a specialist, a suitable fixative such as polyvinyl alcohol (PVA), merthiolate-iodine-formalin, or sodium acetate–acetic acid–formalin (SAF) solution, should be used to preserve the parasite's trophozoites during transportation. PVA and SAF are suitable for preparing smears for permanent staining (11). The other fixative solutions allow the examination of a stool specimen as a wet mount smear only. The most reliable diagnostic index in the direct microscopy examination of a dysentery specimen is the presence of motile hematophagous trophozoites of *E. histolytica*. However, in the majority of patients, trophozoites containing ingested RBCs may not be seen (17).

Although the direct microscopic examination of a saline wet mount of a dysentery specimen is not a sensitive method (approximately 60%), it is still frequently used in many diagnostic laboratories (18). This method is incapable of distinguishing between pathogenic *E. histolytica* and the morphologically identical but nonpathogenic *E. dispar*, *E. moshkovskii* and *E. Bangladeshi* (15, 19).

Staining

The hematophage trophozoites of *E. histolytica* are identified through morphological criteria. This trophozoite may be problematic or impossible to identify for at least one week following the use of barium for radiologic studies, mineral oil, bismuth non-absorbable antidiarrheal compounds, and certain antibiotics (7). In these cases, or for educational goals, the use of permanent-staining methods such as iron hematoxylin, Wheatley's trichrome, and iodine-trichrome is recommended to observe nuclear detail and confirm specific identification.

Moreover, both the modified iron hematoxylin and Wheatley's trichrome stains are recommended for routine use in medical diagnostic laboratories (20). Among the staining techniques available for the identification of *E. histolytica* and other human intestinal protozoa, the trichrome method generally tends to give the best and most reliable results with both fresh and PVA-preserved specimens (11).

Biochemical methods

Culture

Most routine clinical diagnostic laboratories do not use the culturing technique for diagnosing intestinal amoebiasis. Although the cultivation of dysentery stool specimens is known as a useful method for detecting trophozoites, it is expensive, laborious, and time-consuming (18).

Isolation of the hematophagous trophozoites of *E. histolytica* will be more successful if the specimens are received and cultivated within a few hours of being passed. However, after trophozoite growth and establishment, it must be accurately identified using wet mount examination or examination of permanent stained smear of culture sediment.

Another problem regarding cultivation is the elimination of other unwanted intestinal organisms, like *Blastocystis*, that may contaminate the media. Additionally, maintaining *Entamoeba* in culture is not an easy procedure.

E. histolytica is grown in the mixed and axenic types of the culture system. In the mixed system, the trophozoite is grown in diphasic or monophasic media in the presence of additional flora organism species; in the axenic one, *E. histolytica* is grown without any other accompanied live cell. To date, many different media have been developed and used to culture *E. histolytica* and other intestinal amoeba, some of which are currently commercially available.

Diphasic Locke-egg (HSre + s), Robinson medium (21), and TYSGM-9 medium (22) are usually used for the xenic cultivation of *E. histolytica*. In a previous study, Haghighi and Rezaian showed an 85% sensitivity using the modified HSre + s medium for *E. histolytica* cultivation (23). The reported success rate for culturing *E. histolytica* is between 50% and 70% in medical reference laboratories (17).

The most widely used media for the axenic cultivation of *E. histolytica* are TYI-S-33 and YI-S. The axenic cultivation o f *E. histolytica* is applied in the research laboratory for isoenzyme analysis as well as other research purposes. Accordingly, it is a useful method for distinguishing *E. histolytica* from non-pathogenic *E. dispar*, but axenic culture techniques are not established as usable for routine diagnostic purposes in clinical laboratories (7, 20).

Material aspirated or scraped from the base intestinal ulcer during colonoscopy/sigmoidoscopy can be examined using the direct wet mount preparation, cultivations, or staining methods. Enemas or cathartics should not be used to prepare patients before colonoscopy, because these materials will interfere with the identification of amoeba trophozoites (24).

Immunodiagnostic methods

Some substances interfere with stool examination for intestinal parasites, especially *E. histolytica*, particularly antidiarrheal preparations, antibiotics, and antacids preparations, oily laxatives, and watery, soapy, or hypertonic enema solutions. Therefore, using other diagnostic methods is indispensable. Today, the use of serologic and other testing methods is widely accepted as an adjunctive diagnostic tool.

Immunodiagnostic tests used to identify acute intestinal amoebiasis include immunoassay techniques for antibody detection or titration as well as some methods that are dependent on the detection of specific antigens in human fecal specimens. Immunodiagnostic tests are also useful for seroepidemiological studies.

Antibody detection

Antibody detection tests performed for intestinal amoebiasis are normally not recommended except in patients with truly observed symptoms. Even in these patients, the antibody titer may be low and difficult to interpret. Therefore, the definitive diagnosis of intestinal amoebiasis without the presence of any symptom or demonstrating the organism would not be valuable. A high antibody titer using the ELISA method would also be helpful in diagnosing amoebiasis in cases with detectable *E. histolytica* in fecal specimens, because there is no cross-reaction with other human intestinal parasites (17).

Antibody detection tests might be negative in identifying patients who are asymptomatic carriers. Haghighi and Rezaeian reported that only 15.6% of *E. histolytica* / *E. dispar* asymptomatic cyst passers, diagnosed by both microscopy and culture, showed anti-amoebic antibodies after the ELISA test (25). However, these tests, especially ELISA, are frequently used to detect anti-amoeba antibodies in serums of symptomatic patients. Moreover, this method is the most commonly used assay for clinical purposes, particularly for the diagnosis of patients with extra-intestinal amoebiasis. In addition, it is widely used to study the epidemiology of asymptomatic diseases worldwide. The ELISA test has a high specificity and no cross-reactions with other nonpathogenic human intestinal amoebas (26).

The detection and measurement of serum IgM antibodies to the amoebic Gal or GalNAc-inhibitable adherence lectin using the ELISA method in patients suffering from amoebic colitis have been reported to be sensitive and specific without showing any cross-reactions with other human intestinal amoebas (27).

The measurement of IgA antibodies in saliva using ELISA is also useful in diagnosing intestinal infection amoebiasis, such that it showed an 85% sensitivity and 98% specificity (28). In contrast, another study reported the sensitivity and specificity of the ELISA test for secretory IgA in saliva to be 36% and 72%, respectively (29).

Indirect hemagglutination assay (IHA) has been found to have a good specificity (99.1%) and a high negative predictive value (95.5%) in the diagnosis of invasive amoebiasis in human immunodeficiency virus-infected patients manifesting gastrointestinal symptoms (30). Moreover, IHA is simple to perform. However, its low sensitivity compared to ELISA may lead to false-negative results.

Some commercial and homemade dipstick assays have been introduced for the identification of intestinal and extra-intestinal amoebiasis (31-32). Dipstick assays can be performed rapidly. Additionally, they are easy to use, do not require trained personnel, and can be read without a microscope or other equipment. The sensitivity and specificity of dipstick assays for the diagnosis of intestinal amoebiasis were reported to be 89-100% and 89-95%, respectively (33). It is suggested that antibody detection tests be used as additional useful tools for supporting or rejecting the microscopic diagnosis of *E. histolytica* infection, which is the cause

Table 1. The main types of serological test used in the diagnosis of amoebiasis.

Test	Intestinal amoebiasis Specificity / Sensitivity		Extra-intestinal amoebiasis Specificity / Sensitivity		References
Bentonite flocculation (BF)	100%	86%	100%	93%	93
Cellulose acetate membrane precipitation (CAP)	100%	90.9%	100%	87.7-97.5%	95, 104
Counter immune electrophoresis (CIEP)	98.2%	18-96%	98.2%	84-100%	94,103
Enzyme linked immunosorbent assay (ELISA)	97-100%	70-92%	94.8-100%	80-100%	71, 72, 73, 74, 99
Fluorescent immunoassay (FIA)	100%	92.1%	100%	92.1%	102
Immuno electrophoresis (IE)	Unknown	66,7	100	96.8-100%	95
Indirect immunofluorescence assay (IFA)	100%	60- 95%	96.7-98%	93.6-100%	34-80, 98, 100
Indirect haemagglutination (IHA)	96.77-99.1%	75-96.77%	88.7-99.8%	62-99%	76, 77, 78, 30, 35, 92, 97
Latex agglutination (LA)	81.8-98.11%	75-96%	92.5-99.5%	93 -98%	35, 96, 31, 97
Gel diffusion precipitin test (GDP)	99.1%	60-85%	100%	95-97.1%	32, 101

of colitis. Some types of serological tests used in the diagnosis of intestinal amoebiasis are shown in Table 1.

The sensitivity of serologic testing in invasive intestinal amoebiasis has been reported as 60% to 96% (30-31, 34-35). However, the sensitivity of the tests used for distinguishing antibodies in sera from asymptomatic cyst passers has been reported as varying in different countries depending on the disease prevalence and different study group populations. The specificity of most serological tests used for intestinal amoebiasis, even in individuals with inflammatory bowel disease, are high (Table 1).

Antigen detection
(Antigen-based ELISA kits and rapid immunochromatographic assay)

The dependent methods for the detection of specific antigens in human fecal specimens are based on the detection of some specific antigens or epitopes with monoclonal or polyclonal antibodies. These methods are often considered to be quick and convenient, which has resulted in the development of commercial kits. Correspondingly, some of these diagnostic kits allow differentiating *E. histolytica* from *E. dispar*, but they are expensive because they use monoclonal antibodies to identify antigens. These kits are not routinely used in diagnostic clinical laboratories. Among the antigen detection tests, ELISA methods performed based on capturing amoebic antigens in fecal specimens are more successful (18). For example, *E. histolytica* Gal/GalNAc is a highly immunogenic and conserved protein lectin that can be used to detect *E. histolytica* in stool samples specifically. The best known and first-generation kit in ELISA format, namely the *E.*

histolytica TechLab kit, was first produced in 1993 for the specific detection of *E. histolytica* Gal/GalNAc lectin in stool samples (18, 20, 36). The second versions of this kit are called TechLab *E. histolytica* II (Blacksburg, VA, USA) and CELISA PATH kit (Cellabs, Brookvale, Australia), which were developed for the specific detection of *E. histolytica*. Moreover, these kits are used to differentiate between *E. histolytica* and *E. dispar* in stool samples. The specificity rates of TechLab kits I and II vary from 93% to 100%, and their sensitivity rates were reported to be 80–99% and 86–98%, respectively (18, 20, 37-38). In contrast, some studies previously performed on carriers in non-endemic areas, have reported that TechLab II *E. histolytica* has a low diagnostic sensitivity (39, 40, 41).

Importantly, these antigen detection kits are rapid and technically simple to perform, so they can be used in clinical and epidemiological studies, where amoebiasis is most prevalent, but molecular assays cannot be used in this regard. The disadvantages of TechLab and CELISA PATH kits are the need for fresh stool samples and having a limitation for fixed or frozen samples (17).

The ProSpecT (Alexon-Trend, Ramsey, MN.) can be used for both fresh and frozen specimens, but not on formalin-fixed stool samples. Furthermore, this kit cannot distinguish *E. histolytica* from *E. dispar*.

In addition to the above-mentioned kits, some other antigen-based ELISA kits also use monoclonal antibodies against serine-rich antigen of *E. histolytica* (Optimum S kit; Merlin Diagnostika, Bornheim-Hersel, Germany), a lectin-rich surface antigen (24), a lipophosphoglycan (42), and a 170-kDa adherence

lectin amoebic antigen detected in saliva (43), and some other uncharacterized antigens (17).

Rapid diagnostic tests (RDTs) are immunochromatographic assays which help detect antigens of one or more protozoan parasites in a single test. They are easy to perform and provide adequate sensitivity and specificity to be used in endemic areas for large numbers of samples. Additionally, they can be applied as complementary tests in diagnostic laboratories.

The triage parasite panel (TPP) (Biosite Diagnostic Inc., San Diego, CA) is an immunochromatographic assay (EIA) panel applied for the simultaneous detection of *G. lamblia*, *E. histolytica* / *E. dispar*, and *Cryptosporidium parvum* specific antigens in both fresh and frozen but unfixed stool specimens.

The monoclonal antibodies specific to the 29-kDa surface antigen of *E. histolytica* / *E. dispar* are coated on an immunochromatographic strip used in this assay. Using this specific monoclonal antibody, the specific antigen for *E. histolytica* is captured and then immobilized on a membrane (17, 44). High specificity and sensitivity rates were reported for the TTP kit in diagnosing *E. histolytica* / *E. dispar* compared to microscopy stool examination (Table 2). The well-known advantage of the TTP method is that it can be performed within 15 min. However, this test is unable to differentiate among *E. histolytica*, *E. dispar*, and *E. moshkovskii.*

ImmunoCard STAT® CGE is an EIA that uses specific monoclonal antibodies against *C. parvum*, *G. lamblia*, and *E. histolytica* / *E. dispar* simultaneously. This kit can be used for trophozoite or cyst forms of the Entamoeba complex in dysentery or formed stool specimens, but it is not able to distinguish *E. histolytica* from *E. dispar* (45).

Another rapid assay used is a single-step immunochromatographic test, namely "RIDA®QUICK *Cryptosporidium/Giardia/Entamoeba* Combi." In this commercial kit, some specific antibodies which are directed against each parasite attach themselves to green (*Entamoeba* specific), red (*Giardia* specific), or blue (*Cryptosporidium* specific) latex particles.

The RIDA®QUICK kit can be used for both fresh and frozen specimens with no added preservatives, and the test results can be read after 10 minutes. It is noteworthy that this kit cannot differentiate between *E.*

histolytica and *E. dispar* (46, 47, 48). There is a version of this kit (RIDA®QUICK *Entamoeba*) that can only detect antigens of *E. histolytica* in stool samples. Both the specificity and sensitivity of this kit are reported in Table 2. The *E. HISTOLYTICA* QUIK CHEK™ test is based on EIA used for qualitatively detecting *E. histolytica* in fecal samples with no cross-reactivity with *E. dispar* (49, 50, 51).

The sensitivity and specificity of different commercial kits for *E. histolytica* antigen detection are compared in Table 2.

Molecular methods

Molecular diagnosis of amoebiasis is not performed in routine medical laboratories because most lack the relevant facilities. Molecular assays are mostly limited to research laboratories and core clinical laboratories in many countries. In the last decade, molecular biology-based techniques have been accepted as the gold standard method for diagnosing both amoebiasis and giardiasis because of its advantages of increased sensitivity, specificity, and simplicity (11, 18, 20).

The main limitations of molecular-based methods are their high cost, being time-consuming, and requiring specialized equipment, which restricts their usage in the developing world (18). DNA extraction directly from fecal samples was problematic because of the existence of PCR inhibitor in fecal material. Therefore, today, simple and effective methods have been developed for the recovery of DNA from fecal material. One such method is the QIAamp DNA stool kit (QIAGEN, Hilden, Germany), which attempts to eliminate fecal inhibitors during DNA extraction; thereby increasing the sensitivity of the PCR assay (17, 20).

PCR-based methods are often restricted to core and research laboratories and used primarily for the differential diagnosis of *E. histolytica* from non-pathogenic, morphologically identical *E. dispar* and *E. moshkovskii*. However, these methods are accepted and used in many medical diagnostic laboratories, especially in developed countries.

The most commonly targeted gene sequence, used in different molecular methods for the differential diagnosis of *E. histolyica*, is gene encoding small subunit (SSU) ribosomal RNA (rRNA), because there is a high genetic variation between the 18S rRNA genes of *E. histolytica* and *E. dispar*. Moreover, this gene is

Table 2. Comparison of sensitivity and specificity of stool antigen detection kits for amoebiasis.

kit	Intestinal amoebiasis Sensitivity Specificity		Manufacturer	Compared to	Ref
TechLab Kit II	86-95%	93-100%	TechLab, Blacksburg, VA, USA	Microscopy and culture	20, 38, 105
	71-79%	96-100%	TechLab, Blacksburg, VA, USA	Real-time PCR	20, 37, 106, 107
	87.5%	100%	TechLab, Blacksburg, VA, USA	isoenzyme analysis	38
	14.2%	98.3%	TechLab, Blacksburg, VA, USA	Culture and Isoenzyme	39
Entamoeba CELISA-PATH	95-100%	93-100%	Cellabs Pty Ltd., Brookvale, Australia	isoenzyme analysis	20
	28	100	Cellabs Pty Ltd., Brookvale, Australia	PCR	108
	27.8	98	Cellabs Pty Ltd., Brookvale, Australia	microscopy	3
Optimum S kit	100	Unknown	Merlin Diagnostika, Berheim-Hersel, Germany	ProSpecT *Entamoeba histolytica* microplate assay	109
ProSpecT *Entamoeba histolytica* microplate assay; Alexon-Trend, Ramsey, MN	54.5	94	REMEL Inc., Lenexa, KS	Culture and Isoenzyme	39,110
	78%	99%	REMEL Inc., Lenexa, KS	microscopy	110
Triage parasite panel	100%	100%	BIOSITE Diagnostics, San Diego, CA	microscopy	111
	96%	99.1%	BIOSITE Diagnostics, San Diego, CA	Permanent staining	44
	68.3%	100%	BIOSITE Diagnostics, San Diego, CA	ProSpecT *Entamoeba histolytica* microplate assay	109
RIDA®QUICK *Cryptosporidium/Giardia/Entamoeba* Combi	100%	88%	R-BioPharm, Darmstadt, Germany	microscopy	46
	62.5%	96.1%	R-BioPharm, Darmstadt, Germany	PCR	47
RIDA®QUICK *Entamoeba*	28.6%	86.1%	R-BioPharm, Darmstadt, Germany	Techlab *E. histolytica* II	48
ImmunoCard STAT!® CGE	88%	92%	Meridian Bioscence, Milan, Italy	real-time PCR	45
E. HISTOLYTICA QUIK CHEK™	78.4%	97%	TechLab, Blacksburg, VA, USA	ProSpecT *Entamoeba histolytica* microplate assay	49
	98%-100%	100%	BIOSITE Diagnostics, San Diego, CA	Techlab *E. histolytica* II	49, 50
	44.7%	99.8%	BIOSITE Diagnostics, San Diego, CA	PCR	51

present in multiple copies of the extrachromosomal plasmids (20, 52).

The other PCR-targeting gene sequences currently used are DNA highly repetitive sequences, the hemolysin gene (HLY6), cysteine proteinase, serine-rich *E. histolytica* (SREHP) gene, actin gene, and tandem repeats in extrachromosomal circular DNA and 16S-like rRNA (18, 20, 52, 53, 54).

The technique applied the most for the differential diagnosis as well as detection of the *Entamoeba* species in stools, tissues, and liver lesion samples consist of conventional PCR, PCR-RFLP, nested PCR, multiplex PCR, nested multiplex PCR, real-time PCR, and loop-mediated isothermal amplification assay (LAMP).

There is extensive literature on comparing the sensitivity and specificity of molecular methods and

other diagnostic techniques in the detection of amoebiasis (18, 20, 55). Many researchers have reported the high sensitivity and specificity of PCR-based methods in detecting *E. histolytica* DNA (20, 56-57). A recent comparative analysis of microscopy and nested multiplex PCR infection revealed a sensitivity of 100% and a specificity of 98.36% for nested multiplex PCR in the laboratory diagnosis of *E. histolytica* (16).

A detailed description of both the sensitivity and specificity of molecular available methods for the diagnosis of amoebiasis has been previously reported by Saidin et al., 2019 (20). Currently, because of high costs, being time-consuming, non-quantitative results, and false positives in conventional, nested, and multiplex PCR, using real-time PCR assay is receiving more attention in simultaneous laboratory diagnoses of *Entamoeba* species. It is noteworthy that real time PCR allows the quantification of the relative number of trophozoites or cysts present in clinical samples and also enhances the diagnostic sensitivity and eliminates post-PCR manipulation and false positives from carrying over contamination (6, 20).

The18S rRNA gene or species-specific episomal DNA repeat genes are targeted for the recognition and discrimination of the *Entamoeba* species in most of the studies that have used real time PCR (20). It was also reported that real-time PCR assays can be used to detect a very low number of *E. histolytica* in clinical samples, whereas they are not detectable using conventional PCR (58).

A study previously conducted in Bangladesh by Haque et al. using real-time PCR on 28 amoebic colitis patients and 43 control subjects showed that the real-time PCR method could detect *E. histolytica* DNA in 36%, 61%, and 64% of blood, urine, and saliva samples, respectively. Accordingly, all blood, urine, and saliva samples were negative in control subjects (59). The researchers concluded that the overall sensitivity for the diagnosis of amoebic colitis by real-time PCR on urine and saliva was far less than that of other methods such as antigen detection or real-time PCR on stool samples.

Multiplex real-time PCR has been developed for the accurate and rapid differential detection of *E. histolytica*, *E. dispar*, and *E. moshkovskii* simultaneously in a single reaction. In another study,

Roy (2020) successfully developed a highly sensitive and specific hydrolysis probe-based tetraplex real-time PCR assay with the ability to detect and differentiate four morphologically indistinguishable *Entamoeba* species (*E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi*) simultaneously in human clinical samples (60). The tetraplex real-time PCR could also detect *Entamoeba* DNA extracted from the equivalent of 0.1 trophozoites per reaction due to the hundreds of copies of target 18S small-subunit ribosomal rRNA gene molecules in the trophozoite genome. Moreover, a sensitivity rate of 100% and specificity rate of 82.4% have been reported in the detection of *E. histolytica* and *E. dispar* using tetraplex real-time PCR compared to the duplex real-time PCR (60).

The FilmArray™ technology (BioFire Diagnostics, Salt Lake City, Utah) is a fully-automated PCR system that has recently improved the rapid PCR multiplexing. In addition, it can simultaneously detect bacterial, viral, and parasitic pathogens in diarrheal stool samples.

The FilmArray™ gastrointestinal panel was designed to identify 22 of the most common gastrointestinal pathogens simultaneously, namely *Cryptosporidium spp.*, *Cyclospora cayetanensis, E. histolytica, G. lamblia, Campylobacter* (*jejuni*, *coli*, and *upsaliensis*), *Colestridium difficile* (Toxin A/B), *Plesiomonas shigelloides, salmonella, Yersinia enterocolitica, Vibrio* (*parahaemolyticus, vulnificus*, and *cholerae*), *Vibrio cholera*, enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), *Shiga*-like toxin-producing *E. coli* (STEC), *E. coli* O157, *Shigella*/enteroinvasive *E. coli* (EIEC), adenovirus (AdV) F40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus (I, II, IV, and V) (61).

Other fully automated PCR systems are the Verigene® Enteric Pathogens Test (Nanosphere, Northbrook, IL), and Luminex xTAG® Gastrointestinal Pathogen Panel (Luminex Corporation, Canada).

A high specificity (100%) and sensitivity (100%) were reported for the detection of *E. histolytica* using fully automated PCR systems (61-66). However, due to the relatively high cost of using these automated methods, they cannot be established as routine and economical practices in medical diagnostic laboratories in developing countries.

B: Extra- intestinal amoebiasis

The liver is the most frequently involved organ in extra-intestinal amoebiasis, but the amoeba may also be carried to any other organ in the body. Amoebic liver abscess is the most common clinical occurrence of extra-intestinal amoebiasis. The noninvasive identification of ALA is challenging as well. Extra-intestinal infections to *E. histolytica* are mostly secondary to intestinal amoebiasis; however, most patients with ALA are shown to have no *E. histolytica* trophozoites or cysts in their stool examinations. Katzenstein et al. showed that the amoeba was diagnosed in stool samples obtained from less than 10% of ALA patients using microscopy and antigen detection tests (67).

It is currently well-known that fecal microscopy examination or stool antigen detection tests are not useful tools for the diagnosis of ALA (20, 67). Using a combination of imaging, serology, molecular methods, and paying sufficient attention to clinical findings have been recommended as the diagnostic approach for ALA (68). Abnormalities in the complete blood cell count are not specific to the diagnosis of extra-intestinal amoebiasis; however, more than 75% of patients show a leukocytosis with neutrophilia. Eosinophilia is not known as a feature, but erythrocyte sedimentation rate is always increased, usually over 50 mm/h, and hemaglobin is frequently low. Serum alkaline phosphatase is the only consistent abnormality in blood chemistries that was found to be elevated in about 80% of ALA patients. Serum albumin was reduced in some patients and transaminases were elevated only in acute patients or patients with multiple abscesses (7, 68).

Aspiration of pus from hepatic amoebic abscesses is invasive and not recommended for diagnostic purposes. Aspiration can be a part of treatment, especially in patients not responding to medical therapy or those who have large and single abscesses. Microscopic examination of pus from abscesses is accompanied by staining. Moreover, culturing may be helpful in diagnosing ALA, but a very low sensitivity (11-25%) was reported for the examination of pus (69). Most trophozoites are attached to the walls of abscesses. Many polymorphs and deformed hepatocytes are misdiagnosed as amoeba trophozoites in pus staining.

Serology

Serological tests are still considered as methods of choice, and they are the most popular and useful assays, combined with imaging and the existence of clinical manifestations, used to detect extra-intestinal amoebiasis like ALA. Antiamoebic antibodies can also be observed in the serum of 95% of ALA patients. Conversely, a study performed in the endemic area of Vietnam revealed that approximately 83% of asymptomatic individuals had detectable anti-*E. histolytica* antibodies (70). Therefore, serological tests may possibly be useful diagnostic tools in detecting extra-intestinal infections with *E. histolytica* in developed countries, whereas using these tests alone is not recommended to distinguish past from current infections accurately in developing countries where individuals are constantly exposed to *E. histolytica*.

Among the main types of serological tests used in diagnosing amoebiasis (Table 1), ELISA is a rapid, easy-to-perform, and reliable technique which can be used to diagnose extra-intestinal amoebiasis, especially in routine medical diagnostic laboratories in developing countries. The diagnostic sensitivity and specificity of ELISA for the detection of extra-intestinal amoebiasis were reported to be in the range of 80 to 100% (71-74).

Due to the persistence of anti-amoebic IgG antibodies in sera for years following infection with invasive amoebiasis, only a high ELISA antibody titer can be helpful in diagnosing invasive amoebiasis. The presence of anti-amoebic IgM antibodies is short-lived and can be used for the detection of current infection. The detection of anti–amoebic IgM antibodies combined with the results of imaging procedures or the existence of clinical manifestations can be used as an important strategy for the early diagnosis of extra-intestinal amoebiasis.

Levels of specific IgA antibodies to *E. histolytica* in saliva and serum have been used in diagnosing invasive amoebiasis. Sehgal et al. showed a high level of specific secretory anti-amoebic IgA levels in patients with ALA. Furthermore, they observed a significant difference in terms of the specific secretory IgA levels between ALA patients and patients with other parasitic infections or healthy controls (75).

Indirect hemagglutination is a very simple technique which can be performed without any special equipment in diagnostic medical laboratories. Hira et al. reported

99% sensitivity and 99.8% specificity rates for commercial IHA tests in ALA patients (76). However, the 62% sensitivity and 96% specificity of IHA by the recombinant calcium binding domain containing protein have also been reported for the diagnosis of ALA (77).

There are some commercially available antibody assays kits, like IHA Cellognost-Amoebiasis (Dade Behring Marburg GmbH, Germany), for the detection of *E. histolytica* antibodies in human serum. Mohammed et al. (2009) reported a 70% sensitivity and an 88.7% specificity for the cellognost commercial IHA kit for diagnosing ALA (78). Hira et al., however used a commercial ELISA kit and reported a sensitivity of 97.9% and a specificity of 94.8% in this regard (76).

The commercial ELISA kits had higher sensitivity when compared to IHA, but IHA tests can be employed in medical laboratories that have minimal facilities for measuring antibodies as well as in epidemiological studies. The sensitivity and specificity rates of different commercial antibody assay kits for the diagnosis of extra-intestinal amoebiasis were previously compared by Saidin et al. (20).

Garcia et al. also reported that the detection of antibodies using the IFA test is rapid and reliable, which makes reproducible differentiation of ALA from other nonamoebic etiologies. The IgM antibody level becomes negative in a short period following therapy, so monitoring IgM levels using the IFA can be of great clinical value in cases of amoebiasis (79). The sensitivity of the IFA in diagnosing ALA was reported to be 93.6%, with a specificity of 96.7% (34).

Motazedian and Rezaian's study on sera from 91 patients who were clinically suspected of extra-intestinal amoebiasis in Iran showed the sensitivity and specificity of the IFA test as being 100% and 98%, respectively, compared to those of gel diffusion and counter immune-electrophoresis tests (80). However, the IFA is a time-consuming method that requires skills in culturing and antigen preparation (34). The cut-off point titer is better determined and adjusted for each country, especially in endemic areas. In Iran, IFA titers of > 1:640 were reported to be valuable for the diagnosis of ALA and as significant for the differentiation of the present infection from a past one (80). Rezaian and Hamzavi reported that the IHA test in a cut-off titer of >2560 had good diagnostic value in detecting ALA (88.8% sensitivity and 97.2% specificity) in Iran (35).

The latex agglutination test is considered suitable for field and epidemiological studies, because this test can be performed in 10 minutes and does not need any expensive equipment. A sensitivity of 94.4% and specificity of 92.5% were also reported for this assay (35). However, nonspecific reactions were shown for this test (17).

Bentonite flocculation, cellulose acetate membrane precipitation, counter-immune electrophoresis, fluorescent immunoassay, immunoelectrophoresis, immunodiffusion, and tube precipitin are other diagnostic procedures used for the serodiagnosis of amoebiasis. The sensitivity rates of these methods are compared in Table 1.

Rapid tests (dipstick) using serum specimens have been developed for the detection of *E. histolytica* antibodies in serum. Their sensitivity and specificity rates were reported to be similar to those of the ELISA method (31, 33).

Using well-defined *E. histolytica* antigens such as lipophosphoglycan, lectin-rich surface antigen, and pyruvate, phosphate dikinase (PPDK) have been developed for the standardization of serological tests, and some limitations, such as distinguishing between past and current infections, have been overcome. A rapid dipstick test for the detection of ALA based on the detection of anti-PPDK IgG4 antibodies was developed by Saidin et al. This test showed 87% sensitivity and 100% specificity (81).

Antigen detection

Although TechLab *E. histolytica* II ELISA is a kit recommended for the detection of specific Gal/GalNAc lectin antigen in intestinal amoebiasis, it has been reported as having been used for the detection of specific antigens in serum samples of ALA patients as well. Haque et al. used TechLab Kit II in their study to diagnose amoebic liver abscess in serum samples obtained from patients before treatment with metronidazole and showed a 96% sensitivity for the detection of lectin antigen in samples from these patients (82). The sensitivity increased to 100% when pus from the abscess was used for examination. After one week of treatment with metronidazole, 82% of patients were shown as negative in serum examinations using TechLab Kit II. The sensitivity of this kit

decreased to 33% when it was used in patients receiving therapy for several days (82). The rapid reduction of lectin in serum samples after treatment may possibly be the reason for this result. However, the results obtained by Haque et al. are contrary to those of Zeehaida et al., who found that TechLab *E. histolytica* II ELISA is not sensitive in detecting amoebic antigens in samples obtained from ALA patients; thus, it was determined to be not useful for the diagnosis of ALA. Additionally, Zeehaida et al. found that 72.4% of patients were found to be positive by IHA, but only 8.6% of them were found to be positive by TechLab *E. histolytica* II ELISA. The agreement between the IHA and ELISA kits was poor (83).

Another study which compared antigen detection and PCR for the diagnosis of amoebic liver abscess in patients receiving anti-amoebic treatment showed that 75.5% were classified as ALA using PCR examination on the liver aspirate of 200 patients, but with the *E. histolytica* lectin antigen test (TechLab *E. histolytica* II), only 11.0% of patients were classified as ALA (84). Therefore, the researchers concluded that PCR may be used as an alternative test to serology in the diagnosis of ALA, but TechLab *E. histolytica* II kit is not suitable for the diagnosis of ALA patients with a prior history of anti-amoebic therapy.

Molecular methods

Molecular methods were indicated to have high sensitivity and specificity for the diagnosis of extra-intestinal amoebiasis, but they are expensive and require technical expertise, which may limit their usage in medical diagnostic laboratories.

Molecular methods have been evaluated in the diagnosis of ALA by detecting E. histolytica DNA in serum, liver pus, saliva, and urine samples (85). Using molecular methods on liver puncture allows a rapid diagnosis of ALA and helps differentiate it from bacterial liver abscess (86, 87). The sensitivity rate of the conventional PCR in the diagnosis of ALA samples ranges from 80% to 100% (86, 88).

A study conducted on the direct amplification of *E. histolytica* DNA from amoebic liver abscess pus using the PCR method showed that two pairs of primers (P1 + P2 and P11 + P12) of the ten pairs of previously published primers had 100% sensitivity. The targets of these primers were the extrachromosomal circular DNA of *E. histolytica* and the 30-kDa antigen gene, respectively (52).

A recent study conducted on serum samples of 19 ALA patients and 57 non-ALA patients using quantitative PCR (qPCR) assay showed positive results in 17 of the 19 ALA patients and in none of the control patients (89.5% sensitivity and 100% specificity). Additionally, by examining five abscess pus aspirates from ALA patients and seven others from non-ALA patients, all ALA patient samples were found to be qPCR positive, and all samples from non-ALA patients were found to be qPCR negative. Accordingly, these results indicate a strong argument between pus aspirate qPCR and serum qPCR. Thus, qPCR on serum could be known as a substitute for pus aspirate in the early stage of the disease when abscess puncture aspiration is not possible (89).

Parija and Khairnar evaluated a nested multiplex PCR targeting 16S-like rRNA gene for the detection of *Entamoeba* DNA excreted in urine samples from ALA patients. They reported that this method detected *E. histolytica* DNA in 39.6% of urine specimens of ALA patients. The sensitivity of the detection of *Entamoeba* DNA secreted in urine was found to be significantly lower than that of PCR for liver abscess pus (80.4%) in this study (90).

In another study conducted in Bangladesh by Haque et al., a total of 98 blood, urine, and saliva specimens obtained from amoebic liver abscess patients were examined. The results showed that *E. histolytica* DNA was detected in 49%, 77%, and 69% of blood, urine, and saliva specimens obtained from ALA patients and in no samples from healthy controls (59). They concluded that the detection of *Entamoeba* DNA in urine and saliva specimens using real-time PCR assay is a sensitive and noninvasive tool for the diagnosis of ALA.

Loop-mediated isothermal amplification (LAMP) assay for the detection of *E. histolytica* in ALA cases is known as a one-step amplification with higher yield and as an immediate visual detection diagnostic tool that has rapidity, operational simplicity, and high specificity and sensitivity. Singh et al. reported that the LAMP assay successfully detected *E. histolytica* DNA in 82% of 50 liver abscess samples, whereas conventional PCR only detected the presence of *E. histolytica* DNA in 72% (36/50) of samples. Moreover,

the LAMP assay showed 100% specificity when tested with sera from pus samples obtained from known cases of pyogenic liver abscess (91). However, pus aspiration is not recommended for diagnostic proposes, because there is a risk of spillage of abscess content or contamination with bacterial infections. Pus aspirations can be performed if the size of the abscess is greater than 10 cm in diameter or if there is a possibility of its rupture.

Conclusion

Amoebiasis remains one of the most common parasitic infections in human beings worldwide, mainly in developing countries. According to the World Health Organization (WHO), amoebiasis is responsible for 40,000–100,000 human deaths annually (1-2). Therefore, the accurate and rapid diagnosis of amoebic infections, especially in extra-intestinal cases, is of great importance to reduce the number of deaths. Differential diagnosis and the treatment of healthy cyst passers also play important roles in the prevention of disease spread and transmission.

To diagnose both symptomatic and asymptomatic intestinal infections, microscopic stool examination accompanied with culture method is the most frequently used laboratory procedure worldwide. Due to the inability of microscopic examination in differential diagnosis of the three morphologically identical but genetically distinct species of *Entamoeba*, non-microscopy diagnostic methods, and particularly molecular assays, are needed to achieve this purpose. However, the use of these methods cannot be established in routine medical diagnostic laboratories, especially in developing countries.

For laboratory diagnosis of amoebic dysentery, culturing, staining, and direct microscopy stool examination are good-performance diagnostic strategies. Moreover, molecular methods and immunological-based assays are recommended to be used as complementary tests to traditional techniques. The fully automated PCR system for the identification of the most common gastrointestinal pathogens simultaneously can be established as a diagnostic tool in medical diagnostic laboratories in developed countries.

For the diagnosis of extra-intestinal infections such as ALA, serological tests have been highlighted, and

they are still considered to be the methods of choice. Moreover, they are the most popular and useful assays in the diagnosis of extra-intestinal amoebiasis in combination with imaging and existence of clinical manifestations. These methods should still be held as the golden standard in this field. However, serodiagnosis needs to be improved for the accurate differential diagnosis of active infections from past infections. Both molecular methods and rapid diagnostic tests seem to be good potential methods, as complementary of clinical findings, for the diagnosis of ALA. Finally, we conclude that today, laboratory diagnosis of amoebiasis has improved due to the development of more sensitive and specific tests.

Concluding remarks and future perspective

It is concluded that for the laboratory diagnosis of intestinal amoebiasis, culture, staining, and direct microscopy stool examinations are considered to be well-performing diagnostic strategies. Furthermore, molecular methods and immunological-based assays are recommended to be used as complementary tests. To diagnose extra-intestinal infection, the use of the serological tests has been highlighted and are still considered the methods of choice. Serological methods in combination with imaging and the existence of clinical manifestations should also be used; however, serodiagnosis still needs to be improved for the accurate differential diagnosis of active infection from past infections.

Conflict of interests

All authors declare that they have no conflict of interest.

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