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Molecular diagnosis of amoebiasis

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Abstract

Amoebiasis is an intestinal parasitosis caused by the protozoan Entamoeba histolytica that represents the third leading cause of mortality due to parasitosis. It is a prevalent disease in tropical climate regions with poor or absent sanitary services. Microscopy and antigen detection techniques are routinely used to diagnose amoebiasis because of their low cost and ease of application. However, these techniques do not differentiate E. histolytica infections and other potentially pathogenic species such as Entamoeba moshkovskii or Entamoeba bangladeshi. Therefore, in the last decades, molecular tests that allow correct identification of the causal agent of amoebiasis and the establishment of the prevalence of the infecting species have been developed. Techniques based on nucleic acids, such as conventional, multiplex, or real-time polymerase chain reaction (PCR), are being seriously considered in clinical laboratories, because they detect the etiologic agent directly from the sample without the need for previous prolonged culture, thus reducing diagnostic time. Also, the nested PCR test and the sequencing of ribosomal markers have allowed the identification of new parasitic species in humans, such as E. moshkovskii and E. bangladeshi, and an improved characterization of the known infecting species. The application of multiplex platforms allows the simultaneous identification of infecting species, increasing the sensitivity and specificity of these techniques. Therefore, the molecular diagnosis of amoebiasis is projected as an innovative tool in the fight against this parasitosis.

Keywords: *Amoebiasis. Entamoeba spp. Diagnostic. Molecular biology. PCR.*

Diagnóstico molecular de la amebiasis

Resumen

La amebiasis es una parasitosis intestinal causada por el protozoario Entamoeba histolytica y representa la tercera causa de mortalidad por parasitosis. Es una enfermedad prevalente en regiones de clima tropical con deficientes o nulos servicios sanitarios. Las técnicas de microscopía y detección de antígenos se emplean sistemáticamente para el diagnóstico de la amebiasis por su bajo costo y fácil aplicación. Sin embargo, no permiten diferenciar entre infecciones por E. histolytica y otras especies de potencial patogenicidad como Entamoeba moshkovskii o Entamoeba bangladeshi. Ante ello, en las últimas décadas se han desarrollado pruebas moleculares que permiten una correcta identificación del agente causal de la amebiasis y el establecimiento de la prevalencia de la especie infectante. Las técnicas basadas en ácidos nucleicos, como la reacción en cadena de la polimerasa (PCR) convencional, múltiple o en tiempo real, están siendo seriamente consideradas en los laboratorios clínicos, porque detectan al agente etiológico de manera directa en la muestra sin necesidad de cultivo

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prolongado previo, disminuyendo de esta forma el tiempo del diagnóstico. Asimismo, la PCR anidada sumada a la secuenciación de marcadores ribosomales ha permitido la identificación de nuevas especies parasitarias, como E. moshkovskii y E. bangladeshi en humanos, y una mejor caracterización de las especies infectantes ya conocidas. La aplicación de las plataformas multiplex permite la identificación simultánea de especies infectantes, aumentando la sensibilidad y la especificidad de estas técnicas. Por esto, el diagnóstico molecular de la amebiasis se proyecta como una verdadera herramienta innovadora en la lucha contra las parasitosis.

Palabras clave: *Amebiasis. Entamoeba spp. Diagnóstico. Biología molecular. PCR.*

Introduction

Amoebiasis is an infection in humans caused by the protozoan *Entamoeba histolytica*, an extracellular parasitic species classified as a category B biodefense priority pathogen by the National Institute of Allergy and Infectious Diseases (NIAID)[1,2]. This parasitosis, considered the third leading cause of death by protozoa (resulting in 40,000 to 100,000 deaths per year)[3-5], is prevalent in countries that have not yet achieved optimal health services. In other regions, such as Europe, *Entamoeba* infections are caused by travel activities to endemic regions or immigrants[6]. Worldwide, amoebiasis is considered one of the 15 leading causes of childhood diarrhea[1] (in children < 2 years of age) because its primary involvement is at the colon (amoebic colitis).

The World Health Organization (WHO) estimates that 500 million people worldwide may be infected with *Entamoeba*, but only 10% are infected with *E. histolytica*[2]. Also, about 80-90% of individuals with amoebiasis are asymptomatic[7]. For symptomatic patients, amoebiasis severity is influenced by the patient's genetics[8,9], the genotype of the parasite[10], and the microbiota or pathogenic microorganisms present in the gut[11,12]. These factors contribute to the spread of *E. histolytica*, leading to intestinal mucosa inflammation and tissue damage. Intestinal (dysentery) and extraintestinal (hepatic abscesses) complications are associated with mortality[2].

Routine diagnosis of amoebiasis is based on microscopic techniques, such as observing tetranucleated cysts or hematophagous trophozoites (Figure 1), or immunological techniques, such as antigen or antibody detection. Unfortunately, the existence of other non-pathogenic infecting *Entamoeba* species (with cysts morphologically identical to those of *E. histolytica*) or the inability to differentiate a recent infection from previous ones makes both techniques ineffective for specific diagnosis[13,14].

For this reason, molecular tests have been developed based on the detection of parasite nucleic acids by the polymerase chain reaction and its variants (nested, multiplex, and real-time PCR). These techniques resolve aspects of identification, taxonomy, epidemiology, and clinical importance; they also provide knowledge on the genetic diversity of *Entamoeba* species[15,16], which are associated with pathogenic ambiguity[17,18]. The application of this knowledge provides guidelines for the appropriate clinical management of amoebiasis.

The present review provides an overview of methodological strategies for diagnosing amoebiasis, a disease still considered undertreated in tropical and subtropical regions, by identifying *E. histolytica*, a species with morphology indistinguishable from other non-pathogenic species. We highlight the molecular techniques that have led to a better understanding of this parasitosis and the causative species.

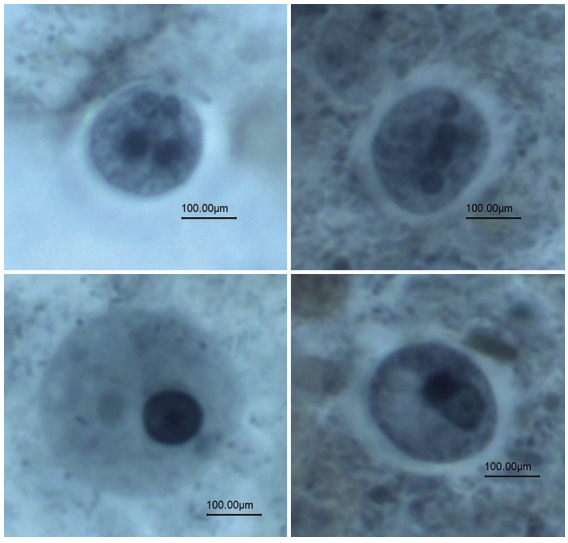

Figure 1. Forms of *E. histolytica*. Light microscopy images of tetranucleated cysts (top) and trophozoites (bottom) are shown.

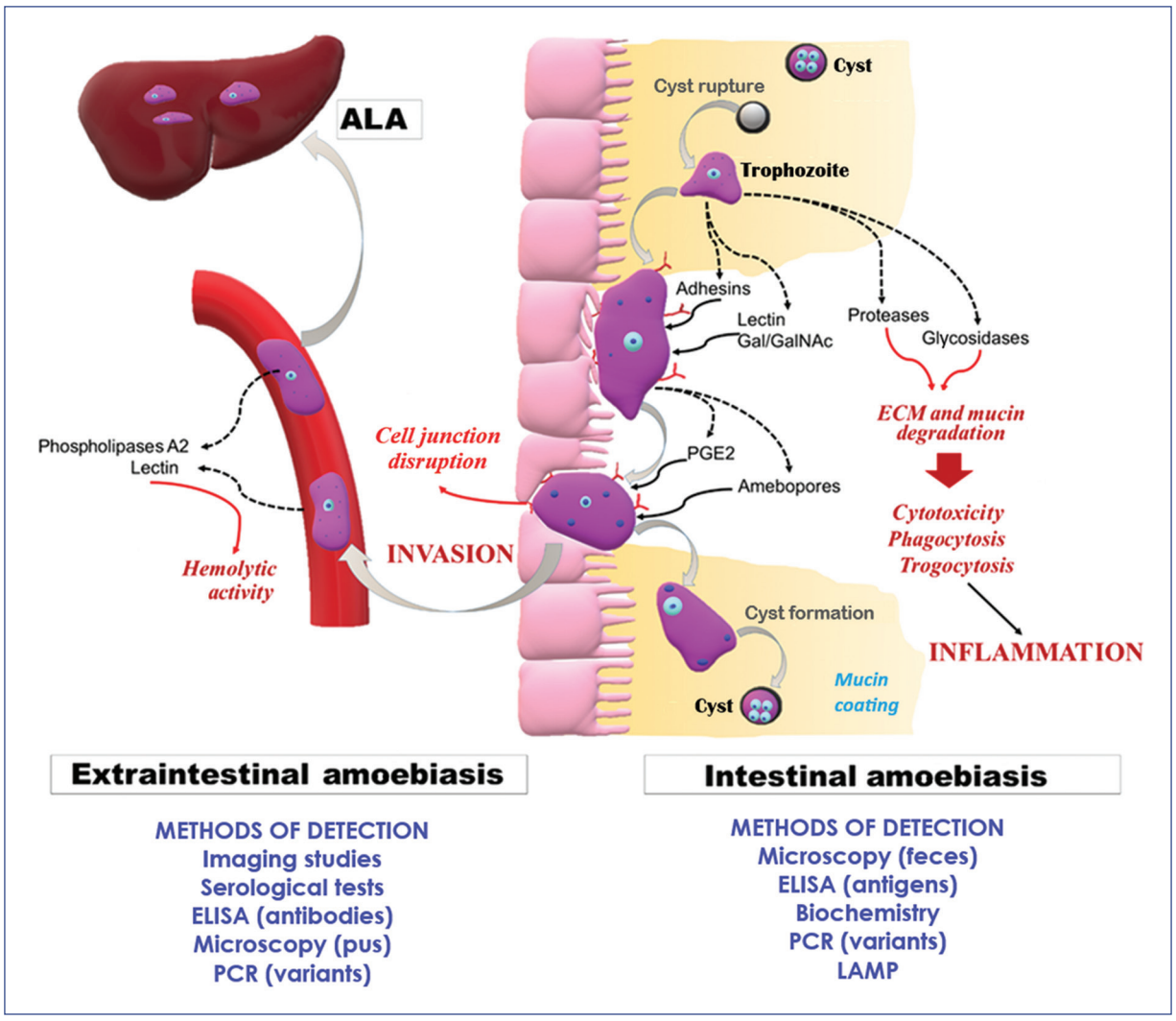

Figure 2. Steps in the infectious process of *E. histolytica*. Amoeba cysts reach the intestinal lumen, develop into trophozoites and colonize the intestinal mucosa and epithelium. The release of amoebic components (dashed black arrows) leads to the pathogenesis of *E. histolytica*: cell death, inflammation (red line), and invasive colonization (solid black arrows). Occasionally, access to the bloodstream allows dissemination into specific organs, including the liver, producing amoebic liver abscesses (ALA). The techniques commonly used for diagnosis both types of amoebiasis are also listed. ECM, extracellular matrix; PG2, prostaglandins E2.

Pathology of *E. histolytica*

Infection with *E. histolytica* occurs when food or water contaminated with the cyst form of the amoeba is consumed. Typically, the parasite is confined to the intestinal lumen of the host and feeds on bacteria, cellular debris, and food residues. In its trophozoite form, the amoeba can disperse along the intestinal mucosa as polyploid cells that adhere to the mucosa through the action of the lectin galactose/N-acetylgalactosamine (Gal/GalNAc), causing diarrhea and colitis[19] (Figure 2).

The production and secretion of glucosidases and cysteine proteases by *E. histolytica* (EhCPs) confer resistance to physicochemical barriers such as mucins, secreted immunoglobulin A (IgA), and other antimicrobial molecules[20]. Additionally, trophozoites produce several molecules, such as amoebapores, prostaglandin E2, mucopolysaccharidases, and phospholipase A_2[19]. These molecules are implicated in pathogenicity by producing effects such as contact-dependent and contact-independent cytotoxicity, hemolytic activity, phagocytosis, and trogocytosis[19,21]; the latter includes the participation of the AGC 1 family of kinases[22]. *E. histolytica* feeds on phagocytized erythrocytes and apoptotic and necrotic cells outside the intestinal lumen. This process apparently

Table 1. Sensitivity of conventional methods for the diagnosis of amoebiasis

Diagnosis method	Identification of *E. histolytica*	Sample	Sensitivity	Detection	Time for analysis	Reference
Microscopy	No	Feces (fixed)	25-60%	Trophozoites/cysts[a]	1-2 h	35
Culture and isoenzyme analysis[b]	Yes	Feces/ALA aspirate	Gold-standard	Zymodeme	7 days	46
						5
Antigen detection	Yes	Feces	85-100%	Fecal-antigens	15-30 min	46,58
	Yes	Serum	95.7%[c]	Lectin Gal/GalNAc		37
Antibody detection	Yes	Serum	78%[d]	IgM/IgG anti-lectin	10 min	37
	Yes	Serum	> 90[e]			5

[a]Hematophagous trophozoites suggest the presence of *E. histolytica*, usually in patients with acute dysentery.
[b]Axenic culture media TYI-S-33 and YI-S, specific for *E. histolytica*.
[c]Serological analysis was performed before treatment with metronidazole. Sensitivity decreases to 34.8% after treatment.
[d]Serum from patients with acute amebic liver abscess.
[e]Sensitivity of 100% for patients with ALA and > 90% in serum from patients convalescing from infection.
Ig, immunoglobulin.

constitutes a virulence factor in avoiding detection by the immune system during tissue invasion[2,23]. The activity of EhCPs leads to disruption of extracellular matrix components and activation of metalloproteinases that destroy cell junctions to initiate extraintestinal invasion[23]. In some cases, the parasites can enter the portal vein and reach the liver, causing an amoebic liver abscess (ALA). In other cases, they infest the lungs or the brain, mainly in immunocompromised patients[2,19]. Untreated intestinal amoebiasis or ALA can lead to death[2,19].

If not lethal, amoebiasis negatively influences growth and cognitive development in children[24-26]. Therefore, proper diagnosis of this parasitosis is necessary for effective treatment and improvement in the quality of life.

Methods for routine diagnosis of amoebiasis

Table 1 summarizes the methods that have been reported for the routine diagnosis of amoebiasis, indicating the sensitivity of each method. Most of these methods are particular in their methodology and are based on direct visualization of cysts or trophozoites or the presence of antigens or antibodies. Mainly, immunological strategies are not considered the reference technique, although they are widely used and allow the identification of *E. histolytica*[13].

Microscopy

The classic diagnostic technique for parasitic infections is microscopy, used to identify hematophagous trophozoites and tetranucleated cysts in fecal samples[27], and also provides material for teaching and research[28]. Due to its simplicity is the method of choice in rural health centers in developing countries where amoebiasis is prevalent[14]. However, its efficacy depends on the skill of laboratory personnel in the correct identification of trophozoites since, in an immobile state, they can be confused with leukocytes, macrophages, and tissue cells. Additionally, rapid sample handling is required (20-30 min), as the trophozoites are destroyed, resulting in false negatives[29,30].

Microscopy has a sensitivity of 60% because identifications are only assigned as "Entamoeba complex." There is a limitation to differentiate species morphologically identical to *E. histolytica* at the level of the nucleus and tetranucleated cysts, such as *E. dispar* (non-pathogenic) and *E. moshkovskii* (of potential pathogenicity)[18,27].

Innovations in this technique, such as using sample concentration by sedimentation[31] or staining with ferric hematoxylin[13], increase its sensitivity. For example, the use of hematoxylin allowed the differential identification of hematophagous trophozoites of *E. histolytica* in fecal samples and thus measured the prevalence (11%) of this species in rural areas in Lima, Peru[32].

Biochemical method

This method was considered the gold standard, although it is currently used more in the research field than in the clinical settings[30]. It employs fecal culture, followed by electrophoretic analysis of some enzymes

(hexokinase, malate dehydrogenase, glucose phosphate isomerase, phosphoglucomutase, among others) to establish zymodemes as markers. The technique can accurately differentiate the presence of *E. histolytica* or *E. dispar* since they have different hexokinase enzymes[33]. However, it has disadvantages for its application in epidemiology due to the long processing time (1 week), the requirement for special laboratory facilities, immediate processing of samples, interference from antiparasitic drugs in treated patients, and inability to identify other infecting *Entamoeba* species[34]. Additionally, the technique can give false-negative results opposite to those obtained by microscopy and has generally been used only for intestinal amoebiasis[29].

Immunological techniques

The enzyme-linked immunosorbent assay (ELISA) technique is based on detecting *E. histolytica* antigens in fresh fecal samples. This technique has higher sensitivity (80 to 94%) and specificity (94 to 100%) than microscopy and culture[35]. The most commonly used antigen is the Gal/GalNac adhesion lectin, detected by monoclonal antibodies in symptomatic and asymptomatic patients[35]. This lectin is highly conserved in *E. histolytica* and has antigenic characteristics different from the *E. dispar* lectin[36]. ELISA also allows the detection of serum antigen levels. However, its sensitivity is reduced (by 16%) when there is prior treatment with antibiotics such as metronidazole, used to treat ALA[37]. Over the past 20 years, the use of ELISA kits has replaced both microscopy and the gold-standard method for clinical purposes because of the rapid results, the ability to differentiate *E. histolytica* and *E. dispar*, sensitivity and specificity, affordability, and large-scale diagnostic capability[38,39].

Furthermore, along with other serological methods such as immunodiffusion, counterimmunoelectrophoresis, indirect hemagglutination, and immunoelectrophoresis, ELISA has been used to detect antibodies in the case of extraintestinal amoebiasis[29]. These methods detect anti-lectin Gal/GalNac IgG antibodies produced at high levels by patients infected with *E. histolytica* and absent in those infected with *E. dispar*. In acute *E. histolytica* infection, about 75-85% of patients develop high levels of antibodies, and more than 90% develop them once the infection is resolved (convalescent titers)[40,41].

The detection of IgG usually favors epidemiological studies in regions with amoebiasis seroprevalences above 50%; however, it limits the diagnosis of acute infections, so the combined application of this method with antigen detection is necessary[37,42]. Alternatively, the detection of IgM antibodies can be used; these antibodies do not persist over time and can be detected in periods of less than one week[43].

Molecular methods

The application of methods based on amplifying DNA fragments to diagnose amoebiasis solved the problem of differentiating *E. histolytica* from other species[44] and determining its prevalence and genetic differences[45]. PCR has greater sensitivity and specificity than microscopy and antigen detection[44,46] and allows early detection of amoebiasis for timely treatment[47]. Species differentiation is achieved by amplifying DNA regions corresponding to single- or multi-copy genes.

The small ribosomal unit gene (*18S rRNA*) is the most widely used PCR marker for taxonomic differentiation between *E. histolytica* and *E. dispar*[6,38]. Other genes used are 30kDa antigen[48], hemolysin (*HLY6*)[49], serine-rich *E. histolytica* protein (*SREHP*)[50], actin[51], cysteine protease 8 (*CP8*)[3], and adhesin (*adh112*)[52].

With the use of PCR, it has been possible to identify new parasitic species in humans, such as *E. moshkovskii* (present among infants in Bangladesh)[53], *E. bangladeshi* (in symptomatic and asymptomatic patients)[54], and the species *E. coli*, *E. hartmanni*, and *E. polecki* (commensal species), with morphology identical to *E. histolytica* and even with shared virulence factors[55,56]. Additionally, this technique detects mixed infections of *E. histolytica* and *E. moshkovskii* or *E. dispar*, the confluence of which is associated with gastrointestinal complications[57]. Table 2 shows the main PCR protocols and variants used as a reference for current studies.

DNA extraction

Like other molecular tests, the diagnosis of amoebiasis requires DNA of high purity and in sufficient quantity. Stool, the primary sample used, is a complex source of contaminants due to the presence of bacteria and human cells and a variety of metabolically derived substances, such as bile salts, which can interfere with or inhibit the amplification process[57]. Pre-incubation with bovine serum albumin is effective in removing

Table 2. Types of PCR tests and parameters used for the diagnosis of amoebiasis

Method	Species in which it is used	Target gene	Product (bp)	Primers (5′-3′) used	Amplification protocol	Ref	Disadvantages
Conventional PCR	*E. histolytica*	*30 kDa antigen*	100	P-11 5′-GGAGGAGTAGGAAAGTTGAC-3′	Denaturation: 94°C x 2 min 45 cycles (denaturation: 94°C x 60 s; hybridization: 55°C x 90 s; extension: 72°C x 90 s)	48	Electrophoresis-dependent technique, with risk of contamination and unquantifiable results
				P-12 5′-TTCTTGCAATTCCTGCTTCGA-3′			
	E. histolytica	*HLY 6*	256	Eh6F 5′-GACCTCTCCTAATATCCTCGT-3′	Denaturation: 94°C x 2 min 35 cycles (denaturation: 94°C x 60 s; hybridization: 55°C x 60 s; extension: 72°C x 60 s)	49	
				Eh6R 5′-GCAGAGAAGTACTGTGAAGG-3′			
	E. histolytica	*18S rRNA*	166	EnF 5′-ATGCACGAGAGCGAAAGCAT-3′	Denaturation: 94°C x 3 min 35 cycles (denaturation: 94°C x 60 s; hybridization: 58°C x 60 s; extension: 72°C x 60 s)	67	
				EhR 5′-GATCTAGAAACAATGCTTCTCT-3′			
	E. dispar		752	EnF 5′-ATGCACGAGAGCGAAAGCAT-3′			
				Ehd 5′-CACCACTTACTATCCCTA CC-3′			
	E. moshkovskii		580	EnF 5′-ATGCACGAGAGCGAAAGCAT-3′			
				Enm 5′-TGACCGGAGCCAGAGACAT-3′			
Conventional multiplex PCR	*E. histolytica*	*18S rRNA*		EntaF, 5′-ATGCACGAGAGCGAAAGCAT-3′	Denaturation: 94°C x 3 min 30 cycles (denaturation: 94°C x 60 s; hybridization: 58°C x 60 s; extension: 72°C x 60 s)	62, 64, 67	
			166	EhR, 5′-GATCTAGAACTCACACTTATGT-3′			
	E. dispar		752	EdR, 5′-CACCACTCCCTACTATTATC-3′			
	E. moshkovskii		580	EmR, 5′-TGAGCCCCAGAGGAGACAT-3′			

(Continues)

Table 2. Types of PCR tests and parameters used for the diagnosis of amoebiasis *(continued)*

Method	Species in which it is used	Target gene	Product (bp)	Primers (5′-3′) used	Amplification protocol	Ref	Disadvantages
Multiplex nested PCR		*SSU-rRNA*	900	E-1F, 5′-TTTGTATTAGTACAAA-3′	Denaturation: 92°C x 60 s 30 cycles (denaturation: 92°C x 60 s; hybridization: 55°C x 60 s; extension: 72°C x 60 s) [a]	53, 54	Under the single format, it is tedious for each amplification process. Under the multiplex format, there is a possibility of false positives. In both cases, the results are not quantifiable
				E-2R, 5′-GTA[A/G] TATTGATATACT-3′			
	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′			
		18S rRNA	900	E-1F, 5′-TAAGATGCACGAGAGCGAAA-3′	Denaturation: 96°C x 2 min 30 cycles (denaturation: 96°C x 60 s; hybridization: 56°C x 60 s; extension: 72°C x 90 s) [b]	68-71	
				E-2R, 5′-GTACAAAGGGCAGGGACGTA-3′			
	E. histolytica		439	EH-1F, 5′-AAGCATTGTTTCTAGATCTGAG-3′			
				EH-2R, 5′-AAGAGGTCTAACCGAAATTAG-3′			
	E. moshkovskii		553	Mos-1F, 5′-GAAACCAAGAGTTTCACAAC-3′			
				Mos-2R, 5′-CAATATAAGGCTTGGATGAT-3′			
	E. dispar		174	ED-1F, 5′-TCTAATTTCGATTAGAACTCT-3′			
				ED-2R, 5′-TCCCTACCTATTAGACATAGC-3′			
Real-time PCR with multiplex platform		*18S rRNA*	231	Ehd-239F, -ATTGTCGTGGCATCCTAACTCA [c]	Denaturation: 95°C x 3 min 40 cycles (denaturation: 95°C x 15 s; hybridization: 60°C x 30 s; extension: 72°C x 30 s)	75	High implementation and operational costs. Highly trained personnel
				Ehd-88R, -GCGGACGGCTCATTATAACA [c]			
	E. histolytica			FAM-UCAUUGAAUGAAUUGGCCAUUU-BHQ1			
	E. dispar			HEX-UUACUUACAUAAAUUGGCCACUUUG-BHQ1			

(Continues)

Table 2. Types of PCR tests and parameters used for the diagnosis of amoebiasis *(continued)*

Method	Species in which it is used	Target gene	Product (bp)	Primers (5′-3′) used	Amplification protocol	Ref	Disadvantages
		18S rRNA		EntaF, 5′-ATG CAC GAG AGC GAA AGC AT-3′	Denaturation: 94°C x 3 min 30 cycles (denaturation: 94°C x 60 s; hybridization: 58°C x 60 s; extension: 72°C x 60 s)	67	
	E. histolytica		166	EhR, 5′-GAT CTA GAA ACA ATG CTT CTC T-3′			
	E. dispar		752	EdR, 5′-CAC CAC TTA CTA TCC CTA CC-3′			
	E. moshkovskii		580	EmR, 5′-TGA CCG GAG CCA GAG ACA T-3′			
	E. histolytica	*18S rRNA*	133	HEX -GTTTGTATTAGTACAAAATGGC -BHQ1	Denaturation: 95°C x 2 min and 50°C x 2 min 40 cycles (denaturation: 95°C x 15 s; extension: 55°C x 60 s)	81	
	E. dispar		134	Cy5 -GTATTAGTACAAAGTGGCCAA -BHQ3			
	E. moshkovskii		145	FAM -CTCGAGGTGGTTAACTCCAC -BHQ1			
	E. bangladeshi		132	TAMRA -GGGTGTTTAAAGCAAAACATTAA-BHQ2			
LAMP	*E. histolytica*	*SSU-rRNA*	166	EhF, 5′-ATGCACGAGAGCGAAAGCAT-3′	Amplification: 63°C x 120 min, and final heating of 90°C x 1 min[d]	94	The critical point of T° must be estimated for the correct performance of the test. It depends mainly on the specificity of the primers
				EhR, 5′-GATCTAGAACTCACACTTATGT-3′			
			External primers	Ehd1-F3-5′-AAAGATAATACTTGAGACGA TCC-3′			
				Ehd1-B3-5′-TCGTTATCCGTTATAATCTTGG-3′			
			Internal primers	FIP-5′-CATCCTAACTCACTTAGAATGTCAAGTACAAAATGGCCAATTCATTC-3′			
				BIP-5′-CACGACAATTGTAGAACACACAGTTCCTC GATACTACCAACTGAT-3′			
	E. histolytica	*HLY6*	External primers	Eh-2F3, 5′-GCACTATACTTGAACGGATTG-3′	Amplification: 63°C x 60 min, and final heating of 90°C x 60 s[e]	93	
				Eh-2B3, 5′-GTTTGACAAGATGTTGAGTGA-3′			
			Internal primers	Eh-2FIP, 5′-TCGCCCTATACTCAAATATGACAAGACTTTGGTGGAAGATTCACG-3′			
				Eh-2BIP, 5′-ATCTAGTAGCTGGTTCCACCTGAACACCTAATCATTATCTTTACCAATC-3′			
			Additional primers	Eh-2F2, 5′-ACTTTGGTGGAAGATTCACG-3′			
				Eh-2B2, 5×-CACCTAATCATTATCTTTACCAATC-3′			

(Continues)

Table 2. Types of PCR tests and parameters used for the diagnosis of amoebiasis *(continued)*

Method	Species in which it is used	Target gene	Product (bp)	Primers (5′-3′) used	Amplification protocol	Ref	Disadvantages
LAMP-NALFIA triplex	*E. histolytica*	*SREHP*	Internal primers	Eh-FIP-SER GCTTCGTTCTTTAAAAATACACCGTCATTCTTGATTTGGATCAAGAAGT	Amplification: 63°C x 60 min, and final heating of 80°C x 5 min[f]	50	
				Eh-BIP-SER-FITC 5′-AGTAGCTCAGCAAAACCAGAATCACTTGCTTTTTCATCTTCATCA-3′			
			External primers	Eh-F3-SER 5′-TGCATTCACTAGTGCAACT-3′			
				Eh-B3-SER 5′-GCTTGATTCTGAGTTATCACTTG-3′			
			Primer loop	Eh-LB-SER-Biotin 5′-AAGTTCAAATGAAGATAATGAA-3′			
	E. dispar-E. moshkovskii	*LSU-rRNA*	Internal primers	Eh-FIP-HLY 5′-TACGCCATTTCGTTTCCTTACTCGATTTCTTAACTGATACTCGACCG-3′			
				Eh-BIP-HLY-FITC 5×-AGATTGAAACTGTCCTTAGTGCAGCAGTTCTAAGATGTTTTTTTCCTC-3′			
			External primers	Eh-F3-HLY 5′-CCTGAAAATGGATGGCATTA-3′			
				Eh-B3-HLY 5′-CCCTAATCCAAGTAATGTTGTT-3′			
			First loop	Enta-LB-HLY-Tex 5′-CTTGGTGGTAGTAGCAAATACTAAG-3′			

[a]The nested PCR used 1.0 µL of product from the first PCR under the same conditions, but changing the hybridization temperature to 62°C.
[b]Multiplex nested PCR used a set of multiple primers under the same conditions as the initial PCR, but changing the hybridization temperature to 48°C.
[c]General primers for *E. histolytica/E. dispar.*
[d]Products were evaluated by agarose gel electrophoresis (2.0%) and fluorescent detection.
[e]Products were detected by turbidity, SYBR green staining change, fluorescence, and agarose gel electrophoresis (1.5%).
[f]The products were captured by proteins fixed on a nitrocellulose membrane.
HLY 6, hemolysin gene; LAMP, loop-mediated isothermal amplification; LSU-rRNA, long subunit of ribosomal RNA; NALFIA, nucleic acid lateral flow immunoassay with dry reagent; PCR, polymerase chain reaction; SREHP, serine-rich protein gene; SSU-rRNA, small subunit of ribosomal RNA.

some of these contaminants[55]. Another factor to consider is the thickness of cyst walls, which makes them resistant to chemical and physical lysis[58]. For example, in the case of *Cryptosporidium* spp., the combination of thermal treatments (freezing and rapid thawing) showed good results in the fragmentation of the cyst walls[59].

As a general protocol, the combined use of cetyltrimethylammonium bromide (CTAB), proteinase K, and heat treatments effectively destroy cysts and trophozoites. The resulting DNA can then be precipitated with phenol/chloroform/isoamyl alcohol[60].

In addition, there are commercial stool DNA extraction systems, such as the QIAamp Stool Mini Kit (QIAGEN), used for the differential diagnosis of *Entamoeba* species, which can correctly identify samples in 88% of cases[57].

Conventional PCR

The use of conventional PCR marked two fundamental milestones in the diagnosis of amebiasis: 1) the ability to determine the actual prevalence of the species *E. histolytica* and *E. dispar*, which routine methods had not been able to resolve[61], and 2) to provide an effective diagnosis for the adequate treatment of the infection[38]. In fact, in patients with a positive microscopy diagnosis of *E. histolytica-E. dispar*, PCR—through the *18S rRNA* gene—allowed the identification of *E. dispar* as the most prevalent species[61].

After the first report of *E. moshkovskii* in humans, the application of PCR for the diagnosis of amoebiasis became more important[62]. The development of new protocols for the simultaneous detection of *E. histolytica*, *E. dispar*, and *E. moshkovskii* species is based on a differential pattern of the core region size of the *18S rRNA* gene[63]. Studies based on this technique corroborate the high global prevalence of *E. dispar*, followed by *E. moshkovskii* at the regional level[64], the latter being associated with diarrhea in children[65].

Conventional PCR has higher specificity, sensitivity (97-99%), and positivity than routine methods, including ELISA[61,66], even when using small amounts of DNA obtained from fecal or culture samples[44,67].

A variant of conventional PCR, PCR coupled to denaturing gradient gel electrophoresis (PCR-DGGE), employs urea and formaldehyde to create denaturing conditions and reliably discriminates *E. histolytica* from *E. dispar*[52].

Nested PCR

Nested PCR protocols are generally used to increase detection sensitivity. They use previously amplified products as a template to perform a second PCR in which regions are amplified using internal anchor primers.

Nested PCR has been applied in different parts of the world to determine the actual prevalence of *E. histolytica* and the other species. This technique provided the first report of *E. moshkovskii* infection in Bangladesh by diagnosing amoebiasis in fecal samples from children[54]. The technique shows the differential size of *18S rRNA* of *E. histolytica*, *E. dispar*, and *E. moshkovskii* by sequencing and correlating the results with the polymorphic sequences of the Arg^{TCT} tRNA gene of the three species[53].

Based on the sensitivity and specificity of nested PCR in diagnosing amoebiasis, the group of Fotedar et al.[5] developed a protocol including primers for the differential detection of *E. moshkovskii*, with results showing discrimination of the three species. Subsequent sequencing analyses gave 98.5%, 99.7%, and 100% similarity percentages, with the sequences deposited at GenBank of *E. dispar*, *E. histolytica*, and *E. moshkovskii*, respectively[5].

The innovation of multiplex nested PCR facilitates the simultaneous detection of *E. histolytica*, *E. dispar*, and *E. moshkovskii*, increasing the test's sensitivity even in complex samples and minimum concentrations of 1000 parasites/0.05 g of feces[68]. The *18S rRNA* gene (Table 2) in multiplex nested PCR allows differentiation of the three species, with a sensitivity and specificity of 94% and 100%, respectively[68].

In the epidemiological context, nested PCR established a higher prevalence for *E. histolytica* (75%) over non-pathogenic species in Malaysian patients[69]. In contrast, in northwestern Iran, this technique placed *E. dispar* as the species with the highest prevalence (0.58%) and reported the presence of *E. moshkovskii* for the first time in the region[70]. Additionally, in the United Arab Emirates, this technique changed the previously reported prevalence for *E. histolytica* (by microscopy) from 72% to 10%[71].

Real-time PCR

The real-time PCR (qPCR) or quantitative PCR method has gained interest in the field of amoebiasis diagnosis due to the optimization of the time used, the relative quantification of the number of parasites, and

its high sensitivity[72,73]. In addition, this technique reduces the risk of contamination, the leading cause of false-positive results in conventional PCR amplification (dependent on electrophoresis[74]), and allows numerical understanding of the results[75].

The technique employs primers and labeled probes that hybridize to specific sequences and are then detected and quantified through the fluorescence emitted after each amplification step. The probes show high performance in other nucleic acid detection platforms such as LUMINEX, achieving differential detection of Entamoeba species and other human protozoan parasites with a specificity similar to that obtained in simple real-time PCR[76].

In diagnosing amoebiasis, probes (such as TaqMan) hybridize with the amplified products and achieve 100% efficiency in identifying *E. histolytica*[75]. In samples with low DNA concentrations, qPCR can detect up to 0.5 trophozoites/mL of stool, a concentration value that allows calculation of mean C_t values[75]. In the case of fecal samples, the method's efficiency improves after applying freezing steps before extraction(-20°C/-80°C) to maximize DNA detection expressed in decreasing C_t values[77].

The use of Eswab brushes or DNA dilutions in saline phosphate buffer improves the technique's efficiency by reducing contaminants (soluble inhibitors) or normalizing sample volume, respectively[78]. Considering these factors and controlling the quality and quantity of extracted DNA, qPCR achieves remarkably low DNA detection limits of up to 0.2 pg for *E. histolytica* and 2 pg for *E. dispar* and *E. moshkovskii*, varying only the denaturation temperatures[79].

Multiplex qPCR protocols (either duplex, triplex, or tetraplex) allow differential detection of the four *Entamoeba* species (*E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi*)[80]. These protocols use primers common to all four species and Taqman probes that hybridize with the products and differ according to the fluorescent molecules they contain (FAM, VIC, fluorescein, among others)[76]. Currently, primers have been designed in the multiplex qPCR platform that can be applied in conventional versions of PCR, thus maintaining specificity in identification[81]. This strategy would be optimal mainly for sites where qPCR cannot be applied due to its high cost[81].

Currently, there are commercial qPCR panels, such as the singleplex and the arrays-TAG, which use Taqman probes and can detect up to 19 species of enteropathogens ranging from bacteria to helminths[59,82]. The detections are performed under universal conditions and use DNA extracted from bacteriophages to control the correct execution of DNA extraction and amplification, achieving a sensitivity of 85% and a specificity of 77% for detecting *E. histolytica*. Similarly, incorporating probes to detect *E. dispar* and *E. moshkovskii* is possible to provide additional diagnostic support compared to conventional PCR protocols[59,82]. Since *E. histolytica*, *Giardia lamblia*, and *Salmonella* spp. have been detected simultaneously in drinking water samples thanks to the protocols designed, the application of this methodology provides high levels of specificity[83].

Loop-mediated isothermal amplification

Building on molecular methods based on polymerase amplification, researchers have developed other methodologies, such as nucleic acid sequence-based amplification (NASBA)[84], self-sustained sequence replication (3SR)[85], and strand displacement amplification (SDA)[86]. These techniques modify conventional amplification by eliminating heat denaturation and using a set of transcription, reverse transcription, or restriction enzyme digestion reactions to reduce detection times and increase sensitivity and specificity. However, despite the efficiency of these methods (detection of fewer than ten copies of DNA in approximately one hour), they have some shortcomings and require expensive equipment[87].

In 2000, Notomi et al. developed LAMP[87] for the detection of hepatitis B virus, improving detection limits of up to 6 DNA copies in 45 minutes by using a set of four specific primers: two internal direct (FIP) and two internal reverse (BIP) to amplify 6 HBs regions of the virus. Each primer contains two different sequences corresponding to the sense and antisense sequences of the target DNA, which hybridize to different regions of the DNA and are then amplified under isothermal conditions by *Bst* DNA polymerase. The improved specificity, compared to PCR, lies in the use of primers designed explicitly for each reaction, whose t_m are between the optimal temperatures of the *Bst* enzyme (60-65°C) and which also recognize different sequences in the initial steps (without amplification). Subsequently, with two additional primers, the sequences present in the generated stem-loop are recognized[87]. A particular advantage of this technique is that the amplified DNA products can be observed with the naked eye as white precipitates in the reaction tube or by fluorescence if fluorescent intercalating dyes are incorporated.

Since its development, LAMP has been successfully applied to detect different gastrointestinal parasites such as *Fasciola hepatica*, *Opisthorchis* spp.[88], *Schistosoma japonicum*[89], *Taenia* spp.[90], and protozoa such as *Cryptosporidium* spp. in fecal and water samples[91]. The design of specific primers, melting temperatures, and negative controls are critical to ensure that the amplification reaction is effective.

In the diagnosis of amoebiasis, LAMP allows the detection of *E. histolytica* up to one parasite per reaction, amplifying regions of the *18S rRNA* gene (Table 2) with a sensitivity of 15 to 50 parasites compared to nested PCR and a specificity of 92%, which makes it the most uncomplicated technique to apply with high specificity[92]. Another LAMP-compatible marker is the hemolysin gene (*HLY6*), which achieves a sensitivity of five parasites per reaction and whose specificity was tested against *E. dispar*, *Blastocystis hominis*, and *Escherichia coli*, showing no results for these species. Positive reactions for *E. histolytica* were identified as tube turbidity or staining changes using SYBR green. Additionally, LAMP has demonstrated 100% specificity compared to nested PCR from ALA pus samples with detection limits of 1 pg DNA, even detecting new cases beyond those reported by PCR[93].

Currently, LAMP has already been adapted to qPCR protocols[94] and to the thermostabilized triplex strategy, which, together with a dry-reagent nucleic acid lateral flow immunoassay (NALFIA), allows the simultaneous and differential detection of *E. histolytica*, *E. dispar*, and *E. moshkovskii*, facilitating the visualization and interpretation of the amplicons produced by LAMP[50]. In LAMP-NALFIA, the primers for *E. histolytica* correspond to the specific sequences of the *SREHP* gene, while for *E. dispar* and *E. moshkovskii*, they correspond to the large subunit of the ribosomal RNA gene (*LSU-rRNA*) (Table 2), which are double-labeled by haptens and fluorescent molecules. The technique allows detection limits of ten *E. histolytica* trophozoites per reaction to be obtained with a specificity of 100%, although its ability to discriminate infecting species needs to be improved[50]. However, it has shown better performance than PCR, qPCR, and nested PCR[95,96].

Considering that LAMP allows detection with high sensitivity and specificity without the need for expensive equipment compared to the PCR techniques described, its application for the diagnosis of amoebiasis is relevant in the development of protocols that allow differentiation between *E. histolytica* and *E. moshkovskii*. Furthermore, LAMP could be applied in ordinary circumstances, decreasing the risk of disease severity and identifying small outbreaks in countries where amoebiasis is endemic, and resources are scarce.

In conclusion, the impact of *E. histolytica* infections on children's health in rural areas of developing countries requires effective diagnostic methodologies. Molecular methodologies have consistently contributed to the understanding of amoebiasis, for example, the actual prevalence of *E. histolytica* and the clinical significance that *E. moshkovskii* species may have. Furthermore, these techniques significantly reduce the time to obtain an accurate diagnosis with the added benefit of simultaneously detecting a broad panel of gastrointestinal parasites, bacteria, and viruses. However, the need for a practical diagnostic test is linked to the feasibility of its application, so the operational and logistical reality of health centers should be considered. In these circumstances, molecular techniques (PCR, qPCR, and nested PCR) have a restricted mass use. However, innovations such as LAMP offer opportunities for diagnosis with a higher degree of sensitivity and specificity than routine techniques, so it is necessary to evaluate their performance on site.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflict of interest.

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