

## Use of Real-Time Polymerase Chain Reaction to Differentiate between Pathogenic *Entamoeba histolytica* and the Nonpathogenic *Entamoeba dispar* in Ecuador

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**Abstract.** Microscopic examination of stool samples has been considered to be the “gold standard” for diagnosis of intestinal parasites. Recently, polymerase chain reaction (PCR) has been approved by the World Health Organization as the method of choice for the diagnosis of *Entamoeba histolytica*. Of the 106 stool samples collected from the Esmeraldas and Pichincha provinces of Ecuador, all (100%) were positive for *E. histolytica/Entamoeba dispar* by light microscopy, whereas using real-time PCR (RT-PCR) DNA amplification, 74 (69.8%) were positive for *E. dispar* and only three (2.8%) were positive for *E. histolytica*. Some 29 (27.4%) samples were negative for the presence of either *E. histolytica* or *E. dispar*, this may be due the presence of *Entamoeba moskovskii*, which is morphologically identical to *E. histolytica/ E. dispar* and not specifically targeted by the RT-PCR used. These results indicate the necessity of reevaluating the epidemiology of amebiasis in Ecuador as the prominent species found are nonpathogenic.

Amebiasis is a common intestinal parasitic infection caused by *Entamoeba histolytica*, approximately 500 million people are estimated to be infected worldwide.<sup>1</sup> The prevalence of *Entamoeba* infection varies being more common in developing countries where poor sanitation leads to ingestion of food or water contaminated with *Entamoeba* spp. cysts. Generally, infections are asymptomatic but in some cases, colitis and extraintestinal infection can occur.<sup>2</sup>

The diagnosis of intestinal amebiasis is based on the microscopic examination of stool samples in search of cyst and/or the trophozoite stages of the parasite. At least six different species of the genus *Entamoeba* can be found in human intestinal lumen; two of them, *Entamoeba dispar* and *Entamoeba moshkovskii*, are morphologically identical to *E. histolytica*, thus making it high impossible to differentiate them by microscopy. In addition, *E. histolytica* is the only recognized pathogenic species, although the potential pathogenicity of *E. dispar* and *E. moshkovskii* has not been completely ruled out.<sup>3,4</sup> Therefore, new diagnostic techniques are needed to differentiate *Entamoeba* species; a rapid diagnostic immunochromatographic antigen test to differentiate *E. histolytica* from the nonpathogenic species exists but has a low specificity for *E. histolytica* sensu stricto.<sup>5</sup> Molecular DNA-based tools have been shown to be useful to differentiate between *E. histolytica*, *E. dispar*, and *E. moshkovskii*.<sup>2,6</sup>

Previous studies in Ecuador showed a higher prevalence of *E. dispar* infections in comparison with *E. histolytica*.<sup>7,8</sup> In the present study, 106 samples from patients from the provinces of Esmeraldas (rural area) and Pichincha (urban area) in Ecuador, previously positive for *E. histolytica/E. dispar* infection by microscopy, were collected to confirm the presence of infection using real-time polymerase chain reaction (RT-PCR).

A portion of the stool sample was prepared for observation by light microscopy in search of intestinal parasites. Another part of the sample, approximately 200 mg, was preserved in

phosphate buffered saline/2% polyvinylpyrrolidone and frozen at  $-20^{\circ}\text{C}$  till DNA extraction using QIAamp<sup>®</sup> DNA stool kit (Qiagen N.V., Hilden, Germany) according to the manufacturer's instructions. In each sample, 2  $\mu\text{L}$  of exogenous Phocine Herpesvirus type-1 DNA (PhHV-1) was added as an internal control. The RT-PCR targets have been described previously by Verweij et al.<sup>9</sup> Amplification reactions for all the samples were performed in a 25- $\mu\text{L}$  reaction mixture containing PCR buffer (SsoFast master mix; Bio-Rad Laboratories<sup>®</sup>, Milan, Italy), 2.5  $\mu\text{g}$  of bovine serum albumin (Sigma-Aldrich<sup>®</sup>, St. Louis, MO), 80 nM of each of the PhHV-1-specific primers, and 200 nM of PhHV-1 CY5-BHQ2 labeled probe, 60 nM of each *E. histolytica/E. dispar* specific primers, and 200 nM of *E. histolytica* fluorescein amidite-minor groove binder-labeled probe and *E. dispar* 2'-chloro-7' phenyl-1,4-dichloro-6-carboxy-fluorescein-minor groove binder-labeled probe; RT-PCR cycles consisted of 3 minutes at  $95^{\circ}\text{C}$  followed by 40 cycles of 15 seconds at  $95^{\circ}\text{C}$  and 30 seconds at  $60^{\circ}\text{C}$ , and 30 seconds at  $72^{\circ}\text{C}$ . Reactions, detection, and data analyses were performed with the DA7600<sup>®</sup> RT PCR (DaAn Gene Co. Ltd., Guangzhou, China). Positive and negative controls were included in all the experiments. One of the two positive controls had a low cycle threshold (Ct) ( $30 < \text{Ct} < 36$ ) and the other a high Ct ( $37 < \text{Ct} < 39.9$ ); for all the RT-PCR analysis, the threshold was set at 200.

Using light microscopy, *E. histolytica/dispar* cysts were observed in all samples along with other intestinal parasites (Table 1). However, using RT-PCR DNA amplification, few stool samples ( $N = 3$  [2.8%]) were positive for *E. histolytica* (Table 2). Given that the method of light microscopy is the standard laboratory method used for diagnosing amoeba, 97.2% of patients are potentially being misdiagnosed and unnecessarily treated. Because of the lack of molecular diagnostics in rural areas, the attending physician must decide if treatment is necessary based more on clinical evidence than laboratory results. The presence of *E. histolytica/dispar* cysts in stool samples does not necessarily warrant treatment. In conclusion, a reevaluation of the epidemiology of amebiasis in Ecuador is necessary to minimize these unnecessary treatments for the presence of nonpathogenic species.

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TABLE 1  
Intestinal parasites in stool samples examined by light microscopy examination

	Eh/d, n (%)	Ec, n (%)	lb, n (%)	En, n (%)	Gl, n (%)	Ei, n (%)	Enb.i, n (%)	Bh, n (%)	Tt, n (%)	Al, n (%)	Sst, n (%)
Rural area, N											
78	78 (100)	53 (67.9)	5 (6.4)	3 (3.8)	11 (14.1)	6 (7.6)	7 (8.9)	4 (5.1)	26 (33.3)	48 (61.5)	4 (5.1)
Urban area, N											
28	28 (100)	11 (39.2)	1 (3.5)	1 (3.5)	–	–	–	3 (10.7)	–	–	–
Total											
106	106 (100)	64 (60.38)	6 (5.6)	4 (3.8)	11 (10.4)	6 (5.6)	7 (6.6)	7 (6.6)	26 (24.5)	48 (45.2)	4 (3.8)

Al = *Ascaris lumbricoides*; Bh = *Blastocystis hominis*; Ec = *Entamoeba coli*; Eh/d = *Entamoeba histolytica/dispar*; Ei = *Entoromonas intestinalis*; En = *Endolimax nana*; Enb.i = *Entadomonas intestinalis*; Gl = *Giardia lamblia*; lb = *Iodamoeba butschlii*; Sst = *Strongyloides stercoralis*; Tt = *Trichuris trichiura*. In all cases, cysts were observed except eggs for the helminths Al and Tt. In Sst, the parasite stage detected was larva.

TABLE 2  
RT-PCR results

RT-PCR	<i>Entamoeba histolytica</i>	<i>Entamoeba dispar</i>
	n (%)	n (%)
Rural area, n		
78	0	62 (79.48)
Urban area, n		
28	3 (10.71)	12 (42.85)
Total, n		
106	3 (2.83)	74 (69.81)

RT-PCR = real-time polymerase chain reaction.

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