

# Laboratory diagnosis of amebiasis in a sample of students from southeastern Brazil and a comparison of microscopy with enzyme-linked immunosorbent assay for screening of infections with *Entamoeba* sp.

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## ABSTRACT

**Introduction:** Epidemiological studies on amebiasis have been reassessed since *Entamoeba histolytica* and *E. dispar* were first recognized as distinct species. Because the morphological similarity of these species renders microscopic diagnosis unreliable, additional tools are required to discriminate between *Entamoeba* species. The objectives of our study were to compare microscopy with ELISA kit (IVD®) results, to diagnose *E. histolytica* infection, and to determine the prevalence of amebiasis in a sample of students from southeastern Brazil. **Methods:** In this study, diagnosis was based on microscopy due to its capacity for revealing potential cysts/trophozoites and on two commercial kits for antigen detection in stool samples. **Results:** For 1,403 samples collected from students aged 6 to 14 years who were living in Divinópolis, Minas Gerais, Brazil, microscopy underestimated the number of individuals infected with *E. histolytica/E. dispar* (5.7% prevalence) compared with the ELISA kit (IVD®)-based diagnoses (15.7% for *E. histolytica/E. dispar*). A comparison of the ELISA (IVD®) and light microscopy results returned a 20% sensitivity, 97% specificity, low positive predictive value, and high negative predictive value for microscopy. An ELISA kit (TechLab®) that was specific for *E. histolytica* detected a 3.1% (43/1403) prevalence for *E. histolytica* infection. **Conclusions:** The ELISA kit (IVD®) can be used as an alternative screening tool. The high prevalence of *E. histolytica* infection detected in this study warrants the implementation of actions directed toward health promotion and preventive measures.

**Keywords:** *Entamoeba histolytica*. *Entamoeba dispar*. Microscopy. ELISA.

## INTRODUCTION

Amebiasis is a human infection caused by *Entamoeba histolytica*, a protozoan of cosmopolitan distribution, with or without clinical manifestations<sup>1</sup>. Infection by the species *Entamoeba dispar* is approximately 10 times more common than infection by *E. histolytica*<sup>2</sup>. Given the morphological similarity of these species, diagnosis based on light microscopy can yield either under- or overestimation of infection rates, leading to unnecessary treatment<sup>3</sup>. The sensitivity of microscopy ranges from 5% to 60%, and its specificity ranges from 10% to 50%<sup>4</sup>.

Due to the invasive behavior of *E. histolytica* and the noninvasive nature of *E. dispar*, coupled with the inability of microscopy to distinguish between the species, the World Health Organization (WHO) recommends that diagnoses attained by microscopy be recorded as "*E. histolytica/E. dispar*"<sup>1</sup>.

In 1997, the WHO also advocated procedures capable of ensuring differentiation between these species so that treatment is restricted to confirmed cases of *E. histolytica* infection. Biochemical, immunological, and molecular biology methods are now capable of differentiating between *Entamoeba* species<sup>5</sup>. Among these methods, tests for antigen detection in stool samples are advantageous in terms of speed, accuracy, and reliability<sup>3,5</sup>.

The objectives of our study were to compare the parasitological examination of stools with ELISA kit (IVD®) results as a screening test for the diagnosis of infections by *Entamoeba* sp., to diagnose *E. histolytica* using an enzyme immunoassay for the detection of a specific antigen, and to determine the prevalence of amebiasis in a sample of students from southeastern Brazil.

## METHODS

This cross-sectional epidemiological study with a stratified-sampling design included a total of 1,403 male and female students aged 6 to 14 years who attended 15 public schools in Divinópolis county, State of Minas Gerais, Brazil. The subjects lived in urban neighborhoods and rural communities, thus representing all of the county's 11 geographical areas. In the

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study period, Divinópolis had 10,656 students aged 6 to 14 years who were enrolled in 36 municipal schools. The city is approximately 100km from Belo Horizonte, the state capital. Of its 213,016 residents, 207,516 live in urban neighborhoods, and 5,500 live in rural areas<sup>6</sup>.

Students whose parents or guardians agreed to fill in a questionnaire and to sign a consent form were given a collection cup with no preservatives. The samples (one per student) were transported on ice to the Universidade Federal de São João del-Rei (UFSJ) Laboratory of Immunology and Parasitology, prepared on the day of collection, and processed using the Hoffmann-Pons-Janer (HPJ, or Lutz) method<sup>7</sup>. To increase the likelihood of parasite detection, four qualified professional examined each sample (100% of fields read). An aliquot of each sample was stored at -20°C for later coproantigen testing using an *E. histolytica*/*E. dispar* ELISA (Enzyme-linked immunosorbent assay) kit (IVD® Research, Carlsbad, CA, USA) for the *in vitro* detection (but not discrimination) of *E. histolytica* and *E. dispar*. According to the manufacturer's instructions, the immunoassay is based on the interaction of monoclonal antibodies conjugate with peroxidase that bind to antigens of *E. dispar* and *E. histolytica*, and the reaction is revealed by the addition of a substrate containing tetramethylbenzidine and peroxide. The kit has a sensitivity of 88% and a specificity of 100%<sup>8</sup>. In comparison, the *E. histolytica* II kit (TechLab®, Blacksburg, VA, USA) is an immunoassay based on the interaction of monoclonal antibodies with the single antigenic determinant adhesin present at the galactose affinity *E. histolytica*. The kit has a sensitivity of 96.9% and a specificity of 100%<sup>9</sup>. All tests were run and interpreted according to the manufacturer's instructions.

The data were encoded and processed using Statistical Package for the Social Sciences (SPSS) software, version 19.0, American University in Cairo - Department of University Academic Computing Technologies (UACT). The Chi-squared test was used to compare proportions, and the adopted significance level was 5% (p-value < 0.05). To compare the microscopy test and the ELISA kit (IVD®), the sensitivity and specificity, positive predictive value (PPV), and negative predictive value (NPV) were computed, assuming that the ELISA kit (IVD®) can adequately serve as the gold standard, using a dichotomous approach<sup>10</sup>.

### Ethical considerations

The investigation was approved by the research ethics committee (opinion 56/2009) and was performed from February 2010 to October 2011.

## RESULTS

The microscopy results revealed a 5.7% (80/1,403) prevalence of infection for the *E. histolytica*/*E. dispar* complex. ELISA (IVD®) testing returned a 15.7% (220/1,403) infection rate for *E. histolytica*/*E. dispar*. A total of 45 (3.2%) samples were positive by both tests, whereas 35 (2.5%) were positive only by direct microscopy, and 175 (12.5%) were positive only by ELISA (IVD®). Both tests were negative for 1148 (81.8%) samples (**Table 1**).

In comparison with ELISA (IVD®), light microscopy showed 20% sensitivity and 97% specificity, with 56% PPV, 87% NPV, 44% false positives (1 - PPV), and 13% false negatives.

The *E. histolytica* II kit (TechLab®, Blacksburg, VA, USA), specific for *E. histolytica*, returned a 3.1% (43/1403) infection rate for *E. histolytica*. The results of the ELISA (TechLab®) and microscopy were positive in 18 (1.3%) samples (**Table 2**).

Of the 1,403 samples, 52% (728/1403) were from females, and 48% (675/1403) were from males. The ages and genders of the subjects were evenly distributed. A significant association (p-value = 0.01) was observed for *E. histolytica* with females but with not males.

*E. histolytica* infection was detected in all age groups, with the highest number of cases in individuals aged > 9 and ≤ 12 years (**Figure 1**). When the study population was segmented by age range, no significant association was observed for the age groups.

Divinópolis county is composed of 11 so-called planning regions, 2 of which are rural and 9 of which are urban. *E. histolytica* cases were detected in 7 regions (the Southeast, West, Northwest, Far Northwest, Rural Northwest, Far Southwest, and Rural Southeast).

TABLE 1 - Comparison of samples by light microscopy and ELISA (*E. histolytica*/*E. dispar*).

	ELISA					
	<i>E. histolytica</i> / <i>E. dispar</i>				Total	
	Presence		Absence			
Microscopy	n	%	n	%	n	%
Positive	45	3.2	35	2.5	80	5.7
Negative	175	12.5	1,148	81.8	1,323	94.3
Total	220	15.7	1,183	84.3	1,403	100.0

ELISA: Enzyme-linked immunosorbent assay; E: *entamoeba*.

TABLE 2 - Light microscopy and ELISA (*E. histolytica* II) results.

	ELISA					
	<i>E. histolytica</i>				Total	
	Presence		Absence			
Microscopy	n	%	n	%	n	%
Positive	18	1.3	62	4.4	80	5.7
Negative	25	1.8	1,298	92.5	1,323	94.3
Total	43	3.1	1,360	96.9	1,403	100.0

ELISA: Enzyme-linked immunosorbent assay; E: *entamoeba*.

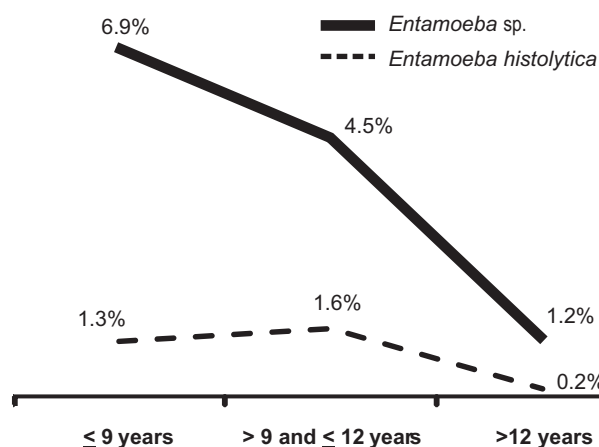


FIGURE 1 - Prevalence of *E. histolytica* and *Entamoeba* sp. in 6- to 14-year-old students, by age. Divinópolis, Minas Gerais, Brazil, from February 2010 to October 2011 (n = 1,403).

## DISCUSSION

Data on the prevalence of *E. histolytica* have emerged from studies on helminths and protozoans that were based on microscopic investigation of cysts and/or trophozoites in clinical specimens. Until relatively recently, *E. histolytica* and *E. dispar* were not differentiated, and infection with either of the two species was referred to as amebiasis, resulting in an overestimation of the true prevalence<sup>11</sup>.

However, the procedure for distinction has been reevaluated since Clark and Diamond<sup>12</sup> demonstrated that *E. histolytica* and *E. dispar* are distinct species that, despite morphological similarities, differ in pathogenicity. The genus *Entamoeba* contains many morphologically similar species, several of which, including *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. polecki*, and *E. hartmanni*, can be found in human stools<sup>13,14</sup>.

Therefore, the epidemiology of amebiasis is confusing, mainly because of the recently appreciated distinctions between *E. histolytica*, *E. dispar*, and *E. moshkovskii*<sup>11</sup>. Because light microscopy does not efficiently identify *E. histolytica*, other tools have been developed to differentiate between *Entamoeba* species, including polymerase chain reaction (PCR), isoenzyme analysis, and ELISA, which are well suited for estimations of true prevalence and the treatment of patients<sup>5</sup>. Malatyali et al.<sup>15</sup> advocated the use of sensitive and effective tests, such as ELISA, for antigen detection in stools.

Several epidemiological studies have been conducted to estimate the incidence and prevalence of amebiasis by testing commercially available antigens from various manufacturers<sup>11</sup>.

Our study distinguished *E. histolytica* from other amebae and strengthened existing epidemiological data. Moreover, the study evaluated the ELISA kit (IVD®) as a screening test.

In the present investigation, microscopy underestimated the number of subjects infected with *E. histolytica/E. dispar* (5.7%),

in contrast to the *E. histolytica/E. dispar* ELISA kit (IVD®) (15.7%). Whereas the ELISA (IVD®) was positive for 220 of the patients who had *E. histolytica/E. dispar* cysts in their stools, only 45 samples were positive in both tests. Additionally, 175 samples with negative results by direct microscopy were positive in the ELISA (IVD®) antigen detection test. This difference may be attributed to the quantity of the pathogen in the samples. Stools with a low number of cysts may be negative by direct microscopic examination but may yield positive results using ELISA<sup>3</sup>.

A comparison of the samples by light microscopy and ELISA (IVD®) revealed a low sensitivity (20%) and a high specificity (97%) for light microscopy. The high NPV of 87% reduced the likelihood of false-negative results, yet the low PPV of 56% rendered the test unreliable. However, positivity on microscopy does not rule out the possibility that 44% of the samples are negative. Delialioglu et al.<sup>3</sup> reported that microscopy provided 53.8% sensitivity and 94% specificity, with 78% PPV and 17% NPV, relative to an ELISA kit (Ridascreen *Entamoeba*, R-Biopharm AG, Darmstadt, Germany). In another study, compared with an ELISA triage kit (ProSpecT EIA, Alexon Inc., Sunnyvale, CA, USA), microscopy was more specific (92.1%) but less sensitive (68.4%)<sup>16</sup>. The ELISA kit (Alexon-Trend, Inc., Sunnyvale, CA, USA) had a sensitivity of 54.5% and a specificity of 94%. If matched with culture and microscopy, the sensitivity of direct microscopic examination was 66%, and the specificity was 83.7%. However, the results should be confirmed with a larger number of fecal samples<sup>17</sup>.

Considering these data, the low sensitivity of microscopy may have been influenced by the collection of a single stool sample per student. According to Ravdin<sup>18</sup>, the examination of three separate stool specimens is required to attain 90% sensitivity, and a single examination identifies only 40% to 60% of infections. If feces were collected more than once and were fixed in preservatives, a higher prevalence of *E. histolytica/E. dispar* would be expected<sup>11</sup>. These data suggest that the ELISA (IVD®) can be used as a screen for the immediate testing of stools. The performance of antigen detection assays suggests that they may be considered as reference standards for the detection of *E. histolytica* and *E. dispar*<sup>5,19</sup>.

However, microscopy should still be considered as a screening method for the detection of *Entamoeba* found in human stools, despite the fact that this technique cannot differentiate between *E. histolytica*, *E. moshkovskii*, and *E. dispar*, although *E. polecki*, *E. coli*, and *E. hartmanni* can be differentiated morphologically from *E. histolytica*<sup>20</sup>.

One of the problems with screening kits is that they cannot differentiate between the amebae. However, the ELISA kit (TechLab®) is commercially available for the specific, direct detection of an *E. histolytica* antigen in stool specimens<sup>5,19</sup>. In the 1,403 samples subjected to ELISA (TechLab®), the prevalence of *E. histolytica* was 3.1%. Haque et al.<sup>21</sup>, based on isoenzyme analysis of 202 samples from symptomatic individuals seen at the International Center for Diarrheal Disease Research in Dhaka, Bangladesh, obtained 52 culture-positive results using the *E. histolytica* II ELISA kit (TechLab®), a method that

ensured faster diagnosis than when using isoenzyme analysis and achieved a higher sensitivity and specificity than did microscopy. The *E. histolytica* II ELISA (TechLab®) correctly identified 21 of 22 cases of *E. histolytica* infection and 28 of 30 for *E. dispar* cases. Haque et al.<sup>19</sup>, examining 2000 samples using two TechLab® kits (the *E. histolytica* II ELISA kit and an *Entamoeba* ELISA kit), reported prevalence rates of 4.2% for *E. histolytica* and 6.5% for *E. dispar* in children aged 1 to 14 years who were living in the vicinity of Dhaka and presenting with diarrhea. In contrast, in asymptomatic children, the percentages were 1% for *E. histolytica* and 7% for *E. dispar*. Following the same strategy, Nesbitt et al.<sup>22</sup> examined 842 samples from Kilimanjaro, Tanzania, and detected prevalence values of 1% for *E. histolytica* and 7.3% for *E. dispar*. The high prevalence of *E. histolytica* infection detected in the present study warrants the implementation of actions directed toward health promotion and preventive measures.

The present results also corroborate previous findings<sup>23</sup> that indicated that females are more prone than males to *E. histolytica* infection. In Brazil, amebiasis rates are highest in the northern region of the country, where both intestinal and extraintestinal forms of the disease exist, with serious public health implications<sup>24</sup>. In Belém, the capital city of the northern state of Pará, a prevalence rate of 29.35% has been reported using the *E. histolytica* II ELISA (TechLab®)<sup>25</sup>. The highest reported *E. histolytica* prevalence rates in Brazil that were detected using the *E. histolytica* II kit (TechLab®) were 36.6% (30/82) for stool samples from the state of Rondônia in Ariquemes and 19.4% (26/134) for stools taken from residents of Monte Negro<sup>26</sup>.

In Pernambuco state, in northeastern Brazil, Dourado et al.<sup>27</sup> detected only *E. dispar*, whereas in Macaparana county, within the same state, all samples investigated by Pinheiro et al.<sup>28</sup> tested negative in an *E. histolytica*-specific ELISA (TechLab®) and positive for *E. dispar* using a molecular biology method.

In southeastern Brazil, using light microscopy, Santos et al.<sup>29</sup> detected a 21% prevalence of the *E. histolytica*/*E. dispar* complex in urban and rural areas of Rio de Janeiro State, yet only two samples tested positive for *E. histolytica* by PCR and *E. histolytica* II ELISA (TechLab®). However, in São Leopoldo, within the southern State of Rio Grande do Sul, Tomé and Tavares<sup>30</sup> found no cases of *E. histolytica* infection using the *E. histolytica* II ELISA (TechLab®).

Light microscopy has several limitations when applied to the diagnosis of amebiasis, given factors such as examiner experience and similarities between *Entamoeba* cysts<sup>3</sup>, which can increase the likelihood of false-positive results<sup>4</sup>. Despite the low cost of light microscopy compared with culture, isoenzyme analysis, antigen detection, and PCR, the method's dependence on subjective diagnosis limits its reliability<sup>3</sup>. Therefore, microscopy is not appropriate for either rapid disease diagnosis or prevalence studies.

According Ngui et al.<sup>31</sup>, molecular techniques are indeed promising tools for epidemiological studies, particularly in discriminating the pathogenic from the non-pathogenic species of *Entamoeba*. *E. moshkovskii*, another morphologically

indistinguishable human parasitic *Entamoeba*, has not been mentioned, nor has it been considered a contributor to prevalence figures in endemic areas<sup>11</sup>. Molecular techniques that can differentiate all studied species of *Entamoeba*, including *E. moshkovskii*, in human specimens have already been reported in Italy, Bangladesh, India, Australia, Turkey, Iran, and Malaysia<sup>31-36</sup>.

It is necessary to use new techniques to differentiate *Entamoeba* diagnoses<sup>37</sup> and to establish a readily available and cost-effective test for the specific diagnosis of amebiasis caused by *E. histolytica* in public laboratories<sup>26</sup>.

Diagnostic methods that are more sensitive and specific than light microscopy are required to establish the true distributions of *E. histolytica* and to reduce the rates of unnecessary treatment, thereby discouraging the development of drug resistance, precluding the risks of side effects, and reducing the costs of hospitalization. The present findings demonstrate that the ELISA kit (IVD®) can be used as an alternative screening tool. In addition, this assay could be utilized by personnel who do not have extensive training in manual parasitological methods. The determination of the true prevalence of *E. histolytica* infection among students from southeastern Brazil is very crucial, as this information will lead to a better understanding of the public health problem and will help outline measures for controlling amebiasis.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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