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Efficiency of Direct Microscopy of Stool Samples Using an Antigen-Specific Adhesin Test for Entamoeba Histolytica

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Background: *E. histolytica* is among the common causes of acute gastroenteritis. The pathogenic species *E. histolytica* and the nonpathogenic species *E. dispar* cannot be morphologically differentiated, although correct identification of these protozoans is important for treatment and public health. In many laboratories, the screening of leukocytes, erythrocytes, amoebic cysts, trophozoites and parasite eggs is performed using Native-Lugol's iodine for pre-diagnosis.
Aims: In this study, we aimed to investigate the frequency of *E. histolytica* in stool samples collected from 788 patients residing in the Anatolian region of İstanbul who presented with gastrointestinal complaints. We used the information obtained to evaluate the effectiveness of microscopic examinations when used in combination with the *E. histolytica* adhesin antigen test.
Study Design: Retrospective cross-sectional study
Methods: Preparations of stool samples stained with Native-Lugol's iodine were evaluated using the *E. histolytica* adhesin test and examined using standard light microscopy at ×40 magnification. Pearson's Chi-square and Fisher's exact tests were used for statistical analysis. Logistic regression analysis was used for multivariate analysis.
Results: Of 788 samples, 38 (4.8%) were positive for *E. histolytica* adhesin antigens. When evaluated together with the presences of erythrocytes, leukocytes, cysts, and trophozoites, respectively, using logistic regression analysis, leukocyte positivity was significantly higher. The odds ratio of leukocyte positivity increased adhesin test-positivity by 2,530-fold (95% CI=1.01–6.330). Adhesin test-positivity was significant (p=0.047).
Conclusion: In line with these findings, the consistency between the presence of cysts and erythrocytes and adhesin test-positivity was found to be highly significant, but that of higher levels of leukocytes was found to be discordant. It was concluded that leukocytes and trophozoites were easily misjudged using direct microscopy. Although microscopic examination of samples stained with Native-Lugol's iodine is a cheap and simple method, the confusion of trophozoites with leukocytes may direct the clinician toward an incorrect pre-diagnosis. Because trichrome staining is difficult and time consuming, and results may vary depending on the technician, this method is not preferred in most laboratories. Therefore, an enzyme-linked immunosorbent assay method, which is a more advanced method than polymerase chain reaction, should be used to distinguish between *E. histolytica* and *E. dispar* in order to achieve an accurate diagnosis.
Keywords: Adhesin test, Microscopic examination, *Entamoeba histolytica*

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Entamoeba histolytica, which is the causative agent of amoebic dysentery and an important health problem for developing countries, is an important parasite responsible for deaths worldwide. The genus Entamoeba contains following species: *Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkowskii, Entamoeba polecki, Entamoeba coli* and *Entamoeba hartmannii*. While pathogenic species *E. histolytica* causes abscess in various internal organs most notably liver with tissue invasion, *Entamoeba dispar* which is considered to be nonpathogenic species remains confined to the intestinal lumen without tissue invasion. It is estimated that 500 million people are infected with *E. histolytica* around the world annually; approximately 50 million result in colitis and liver abscess and approximately 100,000 of them in death due to complications resulting from invasive amoebiasis (1). It is observed that infection rate increases in societies with lower socioeconomic status and in crowded living areas due to personal hygiene. Recently, World Health Organization (WHO) recommends to make a specific diagnosis of *E. histolytica* in the developing countries by using improved diagnostic methods specific for *E. histolytica* and technologies developed for these countries (2). Currently, specific methods to detecting *E. histolytica* specific adhesin antigen in the stool sample show higher specificity and sensitivity in differential diagnosis (3-5). Rapid and definitive diagnosis of amoebiasis is important for administering treatment in the early stage. In our study, it was aimed to perform microscopic examinations of stool samples sent from various departments to Microbiology Laboratory of our hospital regarding 788 patients presenting due to gastrointestinal complaints and to evaluate the results of ELISA adhesin antigen test specific for *E. histolytica* together.

MATERIALS AND METHODS

Stool samples were sent from various departments to the Microbiology Laboratory of our hospital for 788 patients with clinical suspicion of amoebiasis during the period from January 2012 to October 2013. The samples were investigated retrospectively (Ethics Committee Decision No: 18/12 Project No: KOU KAEK 2015/207). At microscopic examinations of stool, approximately 2 g of stool sample was stained with saline solution and Lugol's Iodine Stain. Preparations that were prepared have been investigated with x10 and x40 lenses. Then, *E. histolytica* adhesin ELISA test (a second generation monoclonal antibody-based ELISA, TECHLAB Blacksburg; VA, USA) was studied from these samples in the direction of the recommendations of the manufacturer. Pearson Chi-Square and Fisher's Exact tests were used for statistical analysis and enter logistic regression analysis was used for multivariate analysis. The results were evaluated in 95% confidence interval and at a significance level of $p<0.05$ (Number Cruncher Statistical System; Utah, USA).

RESULTS

Thirty-eight (4.8%) of 788 stool samples studied were determined to be adhesin antigen test positive. Sixteen of the samples determined to be adhesin antigen test positive belonged to the female patients; 22 of them belonged to male patients. The difference between female and male patients was not found to be statistically significant ($p>0.05$). Mean age was 30.1±3.2 years (range:1-75 years). No leukocytes, erythrocytes, cysts or trophozoites were observed microscopically in 23 (60.5%) of 38 stool samples determined to be adhesin antigen test positive. Erythrocyte, leukocyte, cyst and trophozoite test results were determined to be positive in 11 (28.9%), 10 (26.3%), 3 and 2 stool samples, respectively (Table 1). Adhesin test-positivity was found to be significant with Pearson Chi-Square test in the samples determined to be erythrocyte positive ($p=0.003$). Adhesin test positivity was determined to be significant with Fisher's Exact test in the samples determined to be amoeba cyst positive ($p=0.015$). While erythrocyte, leukocyte and amoeba cyst positivity were statistically significant in logistic regression analysis of microscopic evaluation of erythrocyte, amoeba cyst and trophozoite; trophozoite positivity was found to be non-significant (Table 2). It was

TABLE 1. Microscopic evaluation and Adhesin test positivity

Microscopic Evaluation	Total	Adhesin test negative	Adhesin test positivity
Nothing Visible (Leukocyte, Erythrocyte, Cyst, Trophozoite)	685	662	23
Leukocyte	22	19	3
Erythrocyte	27	24	3
Cyst	1	1	0
Trophozoite	3	3	0
Leukocyte + Erythrocyte	34	31	3
Erythrocyte + Cyst	4	3	1
Erythrocyte + Trophozoite	2	1	1
Leukocyte + Cyst	2	2	0
Leukocyte + Trophozoite	3	2	1
Leukocyte + Erythrocyte + Cyst	4	1	3
Leukocyte + Trophozoite + Erythrocyte	1	1	0
Total	788	750	38

TABLE 2. Logistic regression analysis (enter method) of microscopic evaluation of erythrocyte, leukocyte and amebic cyst and trophozoite

	Univariate analysis				Multivariate analysis			
			95% CI				95% CI	
	p	ODDS	Lower	Upper	p	ODDS	Lower	Upper
Erythrocyte	0.001	4.602	2.177	9.726	0.003**	3.465	1.518	7.912
Cyst	0.001	12.487	3.487	44.718	0.015*	5.704	1.402	23.201
Trophozoite	0.066	5.897	1.183	29.403	0.067	4.786	0.897	25.522
Leukocyte	0.001	4.426	2.046	9.575	0.150	2.050	0.772	5.447

ODDS ratio: relative risk; CI: confidence interval; P: p value

observed that leukocyte positivity was significantly higher and it masked erythrocyte positivity. With determination of significantly higher rate of leukocyte in adhesin positive samples, it was concluded that trophozoite and leukocytes could not be differentiated very well.

DISCUSSION

Eighty-five to ninety percent of *E. histolytica* infections are asymptomatic. No clinical manifestation is observed. The World Health Organization reported that cases determined to have *E. histolytica* should be treated, whether or not there are clinical symptoms (2). Prevalence is 1-21% in asymptomatic cases in developing countries. While it is prevalent in all of temperate and tropical regions, it is essentially prevalent in Africa, middle South America and India. Prevalence is reported to be 4% in high risk groups in developed countries (6). In a study performed in the population of North Eastern Indian population, the prevalence rates of *E. histolytica*, *E. dispar* and *E. moshkowskii* in 1260 fecal specimens between 2011 and 2014 with molecular methods were determined to be 13.7%, 11.8% and 7.8; respectively (7). In a study performed with 500 subjects in Malaysia, the rates of *E. dispar*, *E. histolytica* and *E. moshkowskii* in 93 stool samples considered to be amoebiasis microscopically were determined to be as followings with molecular studies: 13.4%, 3.2% and 1%; respectively (8). In a study performed in Bangladesh, the prevalence of *E. histolytica* in the children living in rural areas was determined to be 8% with ELISA method (9). In a study performed in Egypt, the prevalence of *E. histolytica* in 600 children with gastroenteritis was reported to be 20% with antigen ELISA method (10) and again in a study performed in Pakistan, the prevalence of *E. histolytica* was reported to be 21.69% (11). Since the discrimination of *E. histolytica* from the other nonpathogenic species of the genus *Entamoeba* has not been made in the backdated publications in Turkey, it is difficult to make interpretation regarding its prevalence. However, the prevalence in Turkey is estimated to be 0.4-18.4% (5,12). *E. histolytica* is endemic in south and southwest regions of our country. In our study, the prevalence of *E. histolytica* in the cases with clinical suspicion of amoebiasis was determined to be 4.8%. Consistent with the other studies, no statistically significant difference was found regarding gender in positive cases (6,12,13). Since it is cheap and easy to administer, direct wet mount examination method is the most commonly used method in the diagnosis of amoebiasis. Since it necessitates experienced healthcare professional, the sensitivity of Native-Lugol method changes between 10% and 60%, amebic cyst may be confused with macrophages in stool sample or amebic species such as *E. dispar* and *E. moshkovskii* that cannot be differentiated but considered to be nonpathogenic may be present in stool sample; false-positive results can be obtained. Additionally, because the nucleus structure of *Entamoeba* species cannot be discriminated with precision in some preparations using the direct wet mount examination method, advanced methods such as Trichrome staining or *E. histolytica* antigen detection can be used. The most reliable discriminative methods for *E. histolytica* and *E. dispar* are reported to be demonstration of specific antigens with different methods and determination of specific DNA regions. In evaluation performed with advanced methods, it has been reported that causative agent was *E. dispar* in 90% of the people determined to be amoebiasis and *E. histolytica* in 10% of them (14,5). While *E. histolytica* positivity has been determined to be at a rate of 43.2%, 54.5% and 72.4% with antigen-specific ELISA in the samples determined to have amoeba in different studies performed, the rate of ELISA positivity is not known in the samples that are microscopically negative (5,15,16). Since both microscopic method and adhesin ELISA methods were studied in 788 samples in our study, it was determined that 23 (3.3%) of 685 samples that were microscopically negative were ELISA adhesin test-positive (Table 1). This is an important rate and therefore trichrome staining should be performed together with adhesin antigen specific ELISA method or PCR method in all of samples with clinical suspicion of amoebiasis.

Although direct wet mount microscopic examination of the stool is inexpensive and easy, while the identification of leu-

kocytes in the stool may distract the clinician from the correct diagnosis due to an inability to discriminate trophozoites and leukocytes, the confusion of *Entamoeba histolytica* with *E. dispar* and *E. moshkowskii*, which do not require treatment, directs the clinician toward an incorrect pre-diagnosis. Therefore, methods should be used in combination for the diagnosis of *E. histolytica*, and in case of need ELISA method which can make discrimination between *E. histolytica* and *E. dispar* or more advanced method of PCR should be applicable routinely. In this way, it will be possible to report higher reliability results.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Kocaeli University School of Medicine (Date of approval:18 December 2015 Project No: KOU KAEK 2015/207.

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