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Comparison of Various Methods in the Diagnosis of Entamoeba histolytica in Stool and Serum Specimens

Dışkı ve Serum Örneklerinde Entamoeba histolytica Tanısında Çeşitli Tanı Yöntemlerinin Karşılaştırılması

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Abstract

Objective: *Entamoeba histolytica* is indistinguishable from *Entamoeba dispar* in direct microscopic examination. A definitive diagnosis of *E. histolytica* is important in terms of the treatment of the patient and to avoid unnecessary costs. This study's aim is to determine the prevalence of *E. histolytica* and to make a comparison of the different diagnostic tests in the patients specimens defined as *E. histolytica/E. dispar* infection.

Materials and Methods: Faecal and serum specimens of 90 patients defined as *E. histolytica/E. dispar* with microscopy (wet mount examination with 0.85% saline and Lugol's iodine) were examined. Stool samples were examined by trichrome staining for trophozoites and cysts and by immunoassay methods for specific adhesin antigens (Wampole ® *E. histolytica* II antigen testing) and for specific serine-rich 30 kD membrane protein (Serazym® *E. histolytica* antigen testing). Anti-*E. histolytica* antibodies were investigated using a latex slide test and indirect hemagglutination methods in serum specimens.

Results: Presence of *E. histolytica* was not confirmed in 31.1% cases with trichrome staining, 62.2% of the Wampole antigen test, 64.4%, of the Serazym antigen test, 73.3% of the indirect hemagglutination test and 75.6%. of the latex agglutination. Considering the common results from Wampole and Serazym antigen testing as a reference standard, the specificity/sensitivity is 100/53.85% for trichrome staining, 75.00/98.11% for the latex agglutination test and 78.57/96.77% for the indirect hemagglutination test.

Conclusion: It has been shown that investigation of *E. histolytica* in stools by direct wet-smear microscopy alone can cause significant false positive results. To obtain a reliable diagnosis for *E. histolytica* and to avoid unnecessary treatment for this parasite, at least one more specific assay, particularly an antigen testing and microscopy, is required.

Keywords: *Entamoeba histolytica/E. dispar*, amoebiasis, *Entamoeba* antigens, ELISA, IHA

Öz

Amaç: *Entamoeba histolytica* direkt mikroskobik incelemede patojen olmayan *Entamoeba dispar* ile ayırt edilemez. Hastanın tedavisi yönünden olduğu kadar gereksiz ekonomik kayıpları önlemek açısından da *E. histolytica*'nın kesin tanısının yapılması gerekir. Bu çalışmanın amacı *E. histolytica/E. dispar* enfeksiyonu olarak tanımlanmış hastalarda *E. histolytica* sıklığını belirlemek ve tanıda kullanılan farklı testlerin karşılaştırmasını yapmaktır.

Gereç ve Yöntem: Nativ incelemeyle (dışkı örnekleri serum fizyolojik ve lügolde süspansiye edilerek) *E. histolytica/E. dispar* tanısı konulmuş 90 olgunun dışkı ve serum örnekleri incelenmiştir. Dışkı örnekleri; trikrom boyama ile direkt mikroskobik olarak; özgün adezin antijeni Wampole® *E. histolytica* II kitleriyle ve spesifik antijen Serin-rich 30kD membran proteinleri Serazym® *E. histolytica* (Seramun Diagnostic Gmbh, Almanya) kitleriyle immunoassay yöntemiyle incelenmiştir. Serum örneklerinde anti-*E. histolytica* antikorları lateks lam testi ve indirekt hemaglütinasyon yöntemiyle araştırılmıştır.

Bulgular: *E. histolytica* tanısı trikrom boyama yöntemiyle olguların %31,1'inde, Wampole antijen testiyle %62,2'sinde, Serazym antijen testiyle %64,4'ünde, indirekt hemaglütinasyon testiyle %73,3'ünde ve lateks lam aglütinasyon testiyle %75,6'sında örnekte doğrulanmamıştır. Wampole antijen testi ve Serazym antijen testi ortak sonuçları referans alındığında testlerin duyarlılık/özgüllükleri sırasıyla trikrom boyama için %100/53,85, lateks aglütinasyonu için %75,00/98,11 ve indirekt hemaglütinasyon testi için %78,57/96,77 olarak bulunmuştur.

Sonuç: Dışkıda tek başına direkt mikroskopi ile *E. histolytica* aranması önemli ölçüde hatalı pozitif sonuçların alınmasına neden olabileceği görülmüştür. *E. histolytica*'nın güvenilir tanısı ve hastalara gereksiz tedavi uygulanmaması için mikroskopinin yanı sıra en az bir özgün testin daha yapılması gerekmektedir.

Anahtar Kelimeler: *Entamoeba histolytica/E. dispar*, amibiyazis, *Entamoeba* antijenleri, ELISA, IHA

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Introduction

In a 1997 report involving the World Health Organization (WHO), it is explained that *Entamoeba histolytica* infections result in more than 100,000 deaths worldwide per year, putting it in second place after malaria within the protozoal diseases [1]. It has also been reported that 10% of the world's population is infected with this protozoa [2]. However, some authors exaggerated the ratio because of possible confusion with *E. histolytica* and other *Entamoeba* species [3]. *E. histolytica* caused abscesses in liver, lungs and brain, and infection in skin or perianal regions other than the intestine. It lives in the intestinal lumen without any symptoms, and its cysts pass in faeces. It is stated that 4-10% of the asymptomatic carriers develop an invasive disease [4]. Trophozoites, even if taken orally, die in gastric acid. Amoeba infections are formed by cysts taken with raw food and water contaminated with human faeces. The parasite can be transmitted directly sexually, including anal intercourse [5]. *E. histolytica* having various virulence factors, such as adhesins, toxins, amoebapores and proteases is a powerful pathogen that leads to the lysis, death and destruction of the host tissues [6].

Entamoeba dispar and *E. moshkovskii* are only detected by molecular-based techniques; they are non-pathogenic and non-invasive protozoa, and they cannot be distinguished morphologically from *E. histolytica*. Therefore, WHO recommends the treatment of *E. histolytica* [1]. The native-lugol method, condensation method with merthiolate-iodine formaldehyde (MF), Ritchie's formaldehyde ethyl acetate method and Otto's zinc sulphate flotation method are used in the direct diagnosis of *E. histolytica* in fresh faeces [7]. In cases where the appropriate direct diagnosis of *E. histolytica* is made, other methods, such as immunoelectrophoresis, complement fixation, indirect hemagglutination (IHA), indirect fluorescent antibody test, ELISA, latex agglutination (LA) and cellulose acetate precipitation are applied [8]. Searching for the antibodies against the amoeba antigens in serum is important in the diagnosis of intestinal amoebiasis. Many commercial kits have been developed for the investigation of the amoeba antigen in stools.

It was expressed that amoebic culture with isoenzyme analysis was considered to be a reference standard to differentiate *E. histolytica* from *E. dispar*. However, this method is not practical for routine diagnostic laboratories [9].

Routine diagnosis of amoebic infection in most clinical laboratories is made by an examination of the stool stained or unstained in the slide-coverslip preparation, because results are available quickly. However, this examination is not sufficient for the differentiation of cysts and trophozoites of *E. histolytica* from non-pathogenic *Entamoeba* species. Some researches show that almost half of the cases of suspected *E. histolytica* do not exist [10]. A correct diagnosis of *E. histolytica* is also important to avoid unnecessary costs and to implement appropriate treatment. This study was planned to determine, using different methods, the existence of *E. histolytica* in patients diagnosed with an *E. histolytica/E. dispar* infection. It then compared these methods.

Materials and Methods

Patient Samples and Pre-processing

Ethics committee approval was received for this study from the ethics committee of Atatürk University Health Sciences Institute. Informed consent was not necessary for this study, because all clinical specimens used in the study had been anonymized. Our study was carried out on the stool and serum samples obtained from 90 patient diagnosed as *E. histolytica/E. dispar*. One teaspoon of stool sample from each was taken in stool containers and formaldehyde-free containers with filtration system (Para-Pak Plus ecofix TM, Meridian, Bioscience Europe, Villa Cortese, Italy). Normal stool sample collection containers were stored at -20°C until needed for antigen tests; samples in the filtration system containers were examined for diagnosis of the *E. histolytica / E. dispar* cysts with permanent staining. For *E. histolytica* antibody assays, 3-4 mL of the patients' serum samples, which were to be sent to biochemistry laboratory, were collected and stored at -20°C until testing day.

Bichro-Latex Amibe Fumouze® Test (Fumouze Diagnostics, Levallois-Perret, France)

To search for *E. histolytica* antibodies in the serum, 20 µL of serum from each test were transferred into sterile Eppendorf tubes. The serum specimens were diluted with two drops of diluent in the kit. Then a drop of reagent and a drop of diluted patient serum were added on the test slide, and the mixture was rotated in a rotator for 5 min. Finally, agglutination observed specimens were evaluated as positive.

IHA-Amebiasis Fumouze® (IHA-AF) Test (Fumouze Diagnostics, Levallois-Perret, France)

The quantitative determination of *E. histolytica* antibody in serum was performed by an indirect hemagglutination test. In tests carried out according to manufacturer recommendations, U-based microplates were used, and serum specimens were serially diluted in ratios of 1:80, 1:160, 1:320, 1:1280 and 1:2560 with a phosphate buffered solution. The last dilution showed a large peripheral ring in the hole and was reported as a positive result. Suspected positives in the 1/80 to 1/160 dilutions were evaluated as negative in positive results in other tests.

Table 1. A comparison of the studied methods in the diagnosis of *E. histolytica*

	Positive		Negative	
Tests	n	%	n	%
Direct microscopy (in faeces)*	90	100.0	-	-
Trichrome staining (in faeces)	62	68.9	28	31.1
EH-Wampole antigen test (in faeces)	34	37.8	56	62.2
Serazym antigen test (in faeces)	32	35.6	58	64.4
IHA-AF antibody test (in serum)	24	26.7	66	73.3
Bichro-Latex antibody test (in serum)	22	24.4	68	75.6
All tests**	20	22.4	28	31.1

*Shows the results defined before as E. histolytica /E. dispar
**Shows the common results obtained from all other tests except for direct microscopy

Serazym® *E. histolytica* (Seramun Diagnostic Gmbh) Test (Seramun Diagnostic Gmbh, Heidesee, Wolzik, Germany)

This test is a rapid enzimometric immunoassay based on polyclonal peptide antibodies, which recognizes two different epitopes of serine-rich *E. histolytica*-specific antigen membrane proteins of 30kDa (SREHP) in faecal specimens. This test is intended to directly recognize the specific faecal antigen of *E. histolytica*. Frozen stool samples were dissolved at room temperature before starting the test, and test procedures were performed according to the guidelines in the test kit.

Wampole® E. Histolytica II Test (Techlab., Blacksburg, VA, The Netherlands)

This test is a monoclonal ELISA test that rapidly detects the adhesins of *E. histolytica* (specific antigen) in stools. The monoclonal antibody-peroxidase conjugate used in the test was the specific adhesin for *E. histolytica*. Frozen stool samples were dissolved at room temperature before starting the test, and test procedures were performed according to the instructions in the test kit.

Trichrome Staining

Stool samples were stained with Para-pak™ ECOSTAIN (Meridian Bioscience, Europe, Villa Cortese, Italy). Before the process, the main stain was prepared by mixing the main tube with two units of Trichrome Stain, one unit Trichrome Enhancer A, and one unit Trichrome Enhancer B. Then, stool samples in containers with filtration systems were investigated after staining in accordance with the manufacturer's instructions. Cell components of red-purple and cytoplasm blue green cysts were suspected of *E. histolytica*.

Reference Standard

A reference standard for a positive result was defined as a "consensus positive" result when *E. histolytica* was detected by Wampole and Serazym antigen testing. The reference standard for a negative result was defined as a "consensus negative" result by Wampole and Serazym antigen testing.

Statistical analysis

The sensitivities and specificities of the tests were calculated according to the following Bayes' formula by creating 2x2 tables in Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA) [11].

Sensitivity=True Positive / [(True Positive + False Negative)] x 100

Specificity=True Negative / [(True Negative + False Positive)] x 100

Positive Predictive Value (PPV)=True Positive / [(True Positive + False Positive)] x 100

Negative Predictive Value (NPV)=True Negative / [(True Negative + False Negative)] x 100

Results

In our study, 90 stool samples and their serum samples belonging to patients diagnosed as *E. histolytica/E. dispar* by direct microscopy in stool specimens were investigated using different methods. Stool samples with trichrome staining microscopic cysts and trophozoites of *E. histolytica* were serologically investigated by using two different *E. histolytica* antigen-specific ELISA tests. Serum samples latex agglutination and IHA were investigated for serological tests with *E. histolytica* antibodies. These results showed that 20 (22.2%) patients were found positive, while 28 (31.1%) were negative in the sample. However, 62 were found positive by microscopy trichrome, and *E. histolytica*-specific antigen presence was found in 34 using the EH-Wampole and 32 using Serazym tests. *E. histolytica* antibody positivity was found in 24 samples by IHA and in 22 samples by LA (Table 1). EH-Wampol,

Table 2. Sensitivity and specificity of the tests used in the diagnosis of *E. histolytica* in stool and serum

Tests	Nu. of true positive samples	Nu. of false positive samples	Nu. of false negative samples	Nu. of true negative samples	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Trichrome staining	28	24	0	28	100.00	53.85	53.85	100.00
Bichro-Latex antibody test	21	1	7	52	75.00	98.11	95.45	88.14
IHA-AF antibody test	22	2	6	60	78.57	96.77	91.67	90.91

PPV: positive predictive value; NPV: negative predictive value

Serazym, IHA-AF and latex agglutination tests were also positive in the trichrome staining for all samples. EH-wampol antigen positive samples were also positive in IHA-AF and latex agglutination tests. Consequently, presence of *E. histolytica* was not confirmed in 31.1% cases by trichrome staining, in 62.2% with the Wampole antigen test, in 64.4% with the Serazym antigen test, 73.3% with the indirect hemagglutination test and 75.6% with the latex agglutination test.

Considering the common results from Wampole and Serazym antigen testing as a reference standard, the sensitivity/specificity is 100/53.85% for trichrome staining, 75.00/98.11% for the latex agglutination test and 78.57/96.77% for the indirect hemagglutination test (Table 2).

Discussion

Most of the laboratory, routine kopro-parasitological examinations for helminths and protozoa are made by direct microscopy using physiological serum or saline-iodine preparations. Microscopy is preferred because it is cheap and easy to apply. However, it is not possible to say that this method is as reliable for the diagnosis of protozoa as it is in the diagnosis of helminths. Most studies support this argument. Dogan et al. [12] has shown that direct microscopic examination of intestinal parasites results differ according to the education level of the person who made this examination. Therefore, they suggest a direct microscopic examination of intestinal parasites must be done by experienced persons having adequate training, and at least two different methods must be used if possible.

For algorithms used in the diagnosis of intestinal amoebiasis, the following information must be obtained by the clinician: (i) if there is a subacute onset of the disease, (ii) whether the stools contain blood and mucus and (iii) whether the patient travelled to endemic areas. Then, clinical signs and symptoms, such as the patient's diarrhoea/dysentery, abdominal pain, weight loss and fever over 38°C, are evaluated. Then, lactoferrin, leukocytes and occult blood are tested in the stool to eliminate secretory and invasive diarrhoea. Finally, a combination of *E. histolytica* stool antigen tests or serological tests with stool PCR is recommended in the algorithm [13].

Ozer et al. [14] detected *E. histolytica/dispar* cysts and/or trophozoites in 2.2% of stool samples by direct examination using the saline-iodine method and detected 0.7% in samples using the ELISA method. They pointed out that direct microscopic method was not effective in the diagnosis of amoebiasis and reported that the use of stool antigen tests would be appropriate in clinical laboratories because they are more sensitive, more specific and more practical than the direct microscopic method. Saeed et al. [15] believed that PCR-based tests should be used to obtain reliable results for the diagnosis of *E. histolytica* in stools. Gözkenç et al. [16] detected it in 1.1% of specimens using direct native-iodine preparations methods and in 1.3% using saline-iodine preparation methods after sedimentation. These researchers also stated that the native-lugol method used in the diagnosis of amoebiosis is the easiest, least time-consuming and cheapest method, but they stated that trichrome staining was necessary for specific diagnosis. Tuncay et al. [17] investigated the stool samples of patients using the saline-iodine, trichrome staining culture and/or the stool antigen searching methods. They drew attention to the fact that the saline-iodine and trichrome staining positivity are very low compared to other test and emphasized the necessity of working with specific ELISA for *E. histolytica*. Zeyrek et al. [18] detected specific *E. histolytica* antigen positivity in 21.7% of cases using ELISA and microscopy positivity in 26.4%. They said it would be correct to use the ELISA method because these assays are cheap and do not require experienced staff, unlike other specific tests. Aykan et al. [19] indicated that trichrome staining is successful in diagnosing 87.9% of intestinal protozoa. These researchers reported that using trichrome staining and direct examination in the diagnosis of protozoa is more reliable. Tüzemen and Dogan [20] detected positivity in 54.7% of the samples by seeing suspected amoeba cysts/trophozoites using direct microscopy, in 39.3% using trichrome stain, in 15.5% using ELISA and in 7.1% using culture. These two researchers reported that the prevalence of *E. histolytica/E. dispar* ranged from 0.2-45.9%. In different regions between the years 2008-2013, they suggested using combined methods and evaluating them together with the clinical findings in the laboratory diagnosis of patients with amoebiasis.

The antigen test of Yuksel et al. [21] found that 7% of the stool samples of the patients with clinical gastroenteritis symptoms were positive for *E. histolytica/E. dispar*. They also reported that, due to the low sensitivity of direct microscopy, the use of antigen detection methods by ELISA would be appropriate to confirm diagnosis in patients with suspected amoebiasis. Aydin et al. [22] stated that the preferred method is permanent trichrome staining because it allows faeces to be examined later for the identification of the internal structure of the protozoa.

Gonzalez-Ruiz et al. [23], in the early 1990s, found 87% sensitivity and 100% specificity in stool antigen testing by ELISA for the diagnosis of amoebiasis, and they suggested the use of this method as a diagnostic test. Kraoul et al. [24] compared the IHA, latex agglutination and the ELISA test. Sensitivity and specificity of the tests were found as 97.6% and 97% for IHA, 90.7% and 95% for latex agglutination and 93% and 100% for ELISA, respectively. Singh et al. [25] divided stool samples into direct microscopy to detect the trophozoites and erythrocytes and trichrome and/or lugol staining to detect cysts and trophozoites. Singh et al. [25] expressed that the presence of trophozoites in red blood cells differentiates *E. histolytica* from *E. dispar*. Tanyüksel and Petri [13] reported that sensitivity and specificity were about 60% positive and between 10-50% for microscopy, 95% for an ELISA test based on the antigen in the stool and between 90%-85% for the ELISA test based on the antibody in serum. Goñi et al. [26] argued that microscopy and PCR are the gold standard reference techniques. In their studies that took microscopy as the gold standard, they found 17.1% and 96.6% for antigen testing sensitivity and specificity and 24.4% and 97.5% for ELISA, respectively. Tüzemen and Dogan [20] took multiplex PCR for a reference, and they found sensitivity and specificity at 66.7% and 77.4% for direct microscopy, 44.4% and 83.5% for trichrome staining and 11.1% and 91.3% for ELISA, respectively.

In our study, which showed similar results to the studies referred to above, stools were investigated for *E. histolytica* using two different antigen tests. We found a positive result in 34 patients using the EH-Wampole antigen test and in 32 patients using the Serazym antigen test. Common positive results were obtained from 28 patients in the tests. Considering as a reference standard the common results from the Wampole antigen testing and Serazym antigen testing, the sensitivity was high (100%) but specificity was too low (53.85%) for trichrome staining. The latex agglutination test (98.11%) and the indirect hemagglutination test (96.77%) had a higher specificity than trichrome staining.

It was seen that there was misdiagnosis of *E. histolytica* in half of the patients with the direct microscopic examination and one-third of the patients with trichrome staining. Specificity of commonly-used laboratory methods based on routine microscopic examinations, such as direct native microscopy and trichrome staining, were low, although they had high sensitivity. These situations show that a high rate of false positive results is obtained from microscopic examination. Consequently, the preferred tests for a reliable diagnosis of *E. histolytica* are specific antigen tests in the stool and a microscopic examination.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Atatürk University Health Sciences Institute (No: 2007.4.2./18).

Informed Consent: Informed consent was not necessary for this study, because all clinical specimens used in the study had been anonymized.

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