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Comparison of Stool Antigen Detection Kits to PCR for Diagnosis of Amebiasis▽

D. Stark,[1]* S. van Hal,[1] R. Fotedar,[1] A. Butcher,[2] D. Marriott,[1] J. Ellis,[3] and J. Harkness[1]

St. Vincent's Hospital, Department of Microbiology, Sydney, Australia[1]; Institute of Medical and Veterinary Science, The Queen Elizabeth Hospital, Woodville, South Australia, Australia[2]; and University of Technology Sydney, Department of Medical and Molecular Biosciences, Broadway, Australia[3]

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The present study was conducted to compare two stool antigen detection kits with PCR for the diagnosis of *Entamoeba histolytica* infections by using fecal specimens submitted to the Department of Microbiology at St. Vincent's Hospital, Sydney, and the Institute of Medical and Veterinary Science, Adelaide, Australia. A total of 279 stool samples containing the E complex (*E. histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii*) were included in this study. The stool specimens were tested by using two commercially produced enzyme immunoassays (the Entamoeba CELISA PATH and TechLab *E. histolytica* II kits) to detect antigens of *E. histolytica*. DNA was extracted from all of the samples with a Qiagen DNA stool mini kit (Qiagen, Hilden, Germany), and a PCR targeting the small-subunit ribosomal DNA was performed on all of the samples. When PCR was used as a reference standard, the CELISA PATH kit showed 28% sensitivity and 100% specificity. The TechLab ELISA (enzyme-linked immunosorbent assay) kit did not prove to be useful in detecting *E. histolytica*, as it failed to identify any of the *E. histolytica* samples which were positive by PCR. With the TechLab kit, cross-reactivity was observed for three specimens, one of which was positive for both *E. dispar* and *E. moshkovskii* while the other two samples contained *E. moshkovskii*. Quantitative assessment of the PCR and ELISA results obtained showed that the ELISA kits were 1,000 to 10,000 times less sensitive, and our results show that the CELISA PATH kit and the TechLab ELISA are not useful for the detection of *E. histolytica* in stool samples from patients in geographical regions where this parasite is not endemic.

Amebiasis is a parasitic infection caused by *Entamoeba histolytica* and is one of the most common parasitic infections world-wide, infecting about 50 million people and resulting in 10,000 to 40,000 deaths per annum (20). Manifestations of amebiasis include dysentery and extraintestinal invasive disease (18). The diagnosis of *E. histolytica* infection has traditionally relied upon microscopic examination of fresh or fixed stool specimens (5). However, microscopy has several limitations (4, 8, 16), most importantly, the inability to distinguish the pathogenic species *E. histolytica* from the morphologically identical nonpathogenic species *E. dispar* and *E. moshkovskii* (1, 3, 4, 9–11, 16). The sensitivity of microscopy is approximately 60% and is confounded with false positives due to misidentification of the other morphologically similar *Entamoeba* species (5, 9, 10, 16). It is important to correctly diagnose patients not only to reduce the morbidity and mortality of amebiasis but also to minimize the undue treatment of patients infected with *E. dispar* and *E. moshkovskii* with antiamebic therapy. The reference standard used to differentiate *E. histolytica* from *E. dispar* is amebic culture with isoenzyme analysis; however, this method is not widely available and is not practical for routine diagnostic laboratories (5, 11). In addition, the common occurrence of *E. dispar* and *E. moshkovskii* in human populations has led to the need for newer detection methods able to identify and detect several species of *Entamoeba*.

Several newer diagnostic tests are now available which surpass the microscopic detection of these parasites and facilitate a more accurate diagnosis. These approaches include PCR and antigen-based enzyme-linked immunosorbent assays (ELISAs) (7, 10, 12, 13, 16). Stool antigen assays have been reported to outperform microscopy and to be as sensitive (80 to 85%) and specific (99%) as culture with isoenzyme analysis for the detection of *E. histolytica* in areas of endemicity (10, 11). Recently, molecule-based PCR assays have been reported to demonstrate excellent sensitivity and specificity compared with microscopy (4, 5, 9, 15). In several evaluation studies, similar sensitivities and specificities were reported for PCR and ELISA (11, 14). As PCR techniques are not widely available and remain impractical tools in many developing countries, stool antigen assays are considered valid alternative diagnostic methods for the diagnosis of *E. histolytica* infections.

The present study was designed to compare two commercially available stool antigen detection kits, namely, the *Entamoeba* CELISA PATH kit (Cellabs, Brookvale, Australia) and the TechLab *E. histolytica* II kit (TechLab, Inc., Blacksburg, VA), with conventional PCR amplification of small-subunit ribosomal DNA (rDNA) from fecal samples submitted by patients to St. Vincent's Hospital, Sydney (St. Vincent's), and the Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia. Although the TechLab *E. histolytica* II kit has previously been evaluated, this is the first study to compare it with the *Entamoeba* CELISA PATH kit and PCR.

MATERIALS AND METHODS

Patient samples. All patient fecal samples submitted to St. Vincent's and IMVS for parasitology (ovum, Cyst, and parasite program) investigation (note

* Corresponding author. Mailing address: Division of Microbiology, SydPath, St. Vincent's Hospital, Darlinghurst 2010, NSW, Australia. Phone: 61 2 8382 9266. Fax: 61 2 8382 2989. E-mail: dstark@stvincents.com.au.

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TABLE 1. *E. histolytica* negative control stool samples which contain one or more protozoa other than *E. histolytica*[a]

Protozoa	No. of samples
Blastocystis hominis	5
Entamoeba coli	5
Entamoeba hartmanni	5
Giardia intestinalis	5
Endolimax nana	5
Iodamoeba bütschlii	2
Cryptosporidium sp.	2
Cyclospora sp.	1
Chilomastix mesnili	2
E. hartmanni, *E. nana*, *Enteromonas hominis*, *B. hominis*	2
E. nana, *I. bütschlii*, *C. mesnili*, *B. hominis*	2
Cryptosporidium sp., *B. hominis*	2
E. nana, *I. bütschlii*, *C. mesnili*, *B. hominis*	2
G. intestinalis, *E. nana*, *B. hominis*	1
E. coli, *E. nana*, *I. bütschlii*, *B. hominis*	1
E. coli, *E. hartmanni*, *E. nana*, *I. bütschlii*, *B. hominis*	1
E. hartmanni, *E. nana*	1
B. hominis, *E. nana*	4
C. mesnili, *E. hominis*, *E. nana*	1
G. intestinalis, *E. nana*, *B. hominis*	1

[a] The 50 fecal specimens listed in this table were all negative by PCR for *E. histolytica* but were positive by microscopy for a range of common human protozoa.

that some of the IMVS samples were from asymptomatic refugee patients) between March 2004 and August 2007 were screened for inclusion in the evaluation. A portion of the fecal samples submitted were fixed in sodium-acetate-formalin and permanently stained with a modified iron-hematoxylin stain (Fronine, Australia) according to the manufacturer's instructions. All microscopy-positive samples containing the E complex ($n = 279$) were then used for the evaluation of ELISA and PCR. A negative control group was also included in this study (see below).

ELISA. Antigen-based testing with the ELISA kits was performed with fresh stool samples within 48 h of collection. The two ELISAs (TechLab *E. histolytica* II kit [TechLab, Inc., Blacksburg, VA] and *Entamoeba* CELISA PATH kit [Cellabs, Brookvale, Australia]) were performed according to the manufacturers' instructions. Both tests were designed to detect *E. histolytica* alone. Each test included positive and negative controls. The remaining fresh stool sample was stored at −20°C for PCR amplification at a later date.

PCR and DNA sequencing. DNA extraction; PCR amplification of the small-subunit rDNA of *E. histolytica*, *E. dispar*, and *E. moshkovskii*; and sequencing were carried out as previously described (4, 17).To exclude inhibition from fecal inhibitors, all specimens were spiked with an equal volume of genomic DNA from *E. histolytica* strain HTH-56:MUTM controls and run in parallel with an unspiked specimen.

Limit of detection. Xenic cultures of *E. histolytica* strain HTH-56:MUTM were grown in LE medium by standard procedures (2). Trophozoites were harvested and used as the antigen/DNA to determine the lower limits of detection of the ELISAs and PCR. Trophozoites were counted in a hemacytometer. Duplicate serial dilutions were prepared from suspensions of known concentrations of trophozoites. An aliquot of each sample underwent DNA extraction and was then suspended in 200 μl of a negative control stool sample and tested by PCR as described above. The duplicate sample underwent lysis by freeze-thawing in phosphate-buffered saline containing a mixture of various protease inhibitors as previously described (12). For ELISA, simulated stool samples were prepared by adding the serial dilutions of the trophozoite lysates to 400 μl of a negative control stool sample. The trophozoite lysate-seeded stool samples were then tested with the reagents and protocols of the two ELISA kits as described above. All testing was performed in duplicate at each dilution. The negative control stool sample used in both PCR and ELISA experiments was from a healthy volunteer and was negative for protozoan parasites by microscopy and E complex PCR (as described above).

Control group. In addition, to confirm the absence of cross-reactivity, a control group comprising 100 stool samples that were negative by microscopy for *E. histolytica* were randomly selected. The samples were considered negative after examining approximately 200 to 300 oil immersion fields of view of the stained slides. These samples were further classified into specimens that were negative for all parasites ($n = 50$) and samples that were positive for one or more parasites, excluding the E complex ($n = 50$) (Table 1). All of these samples were included in the test protocol and underwent both ELISA and PCR testing as described above.

TABLE 2. Comparison of results obtained by testing 279 fecal samples positive for E complex by microscopy with the Entamoeba CELISA PATH and TechLab *E. histolytica* II ELISA kits compared with *E. histolytica* PCR

ELISA result	No. of samples PCR:	
	Positive	Negative
CELISA		
Positive	5	0
Negative	13	261
TechLab		
Positive	0	3
Negative	18	258

RESULTS

E. histolytica was detected in 6% (18/279) of the stool samples by PCR (Table 2). *E. dispar* was detected in 136 samples, while *E. moshkovskii* was detected in 73 samples (most of this information has been previously reported elsewhere [4, 15]). All of the DNA sequences revealed 99 to 100% homology with sequences stored in GenBank. PCR inhibition occurred in 1% (3/279) of the samples. All of the control fecal samples *E. histolytica* negative by microscopy were *E. histolytica* PCR negative, and there was no PCR product from any of the control samples which contained protozoa other than *E. histolytica* (Table 1). Similarly, *E. histolytica* antigen was not detected in microscopy-negative (including the control group) fecal samples with either ELISA kit. However, for samples that were microscopy positive for E complex, the TechLab *E. histolytica* II kit failed to detect any (0/18) of the PCR-positive samples. In addition, a false-positive result was obtained in 1% (3/261) of the samples *E. histolytica* negative by PCR. These three samples were found to be positive by PCR for non-*E. histolytica* species (*E. moshkovskii* [$n = 2$] and both *E. dispar* and *E. moshkovskii* [$n = 1$]). This was confirmed by DNA sequencing, which revealed 99 to 100% similarity to the *E. dispar* and *E. moshkovskii* 18S rDNA sequences deposited in GenBank (GenBank accession no. Z49256 and AF149906). Thus, these three samples were considered false positives. All 100 negative control group samples were negative by both ELISA kits. The *Entamoeba* CELISA PATH kit detected 28% (5/18) of the *E. histolytica* PCR-positive samples with no false-positive results (Table 2). Compared to PCR, the sensitivities were 0 and 28% for the TechLab *E. histolytica* II kit and the *Entamoeba* CELISA PATH kit, respectively. In contrast, the specificities of both stool antigen tests were similar at 99 and 100%, respectively.

Quantitative estimates with lysates produced from *E. histolytica* cultures revealed that the *Entamoeba* CELISA PATH kit was able to detect a 10-fold lower concentration of *E. histolytica* trophozoites per well (1, 000) compared with the TechLab *E. histolytica* II kit, which required lysate from 10,000 trophozoites for a positive reaction. In contrast, the *E. histo-*

lytica PCR was able to detect a PCR product from a sample containing one trophozoite per reaction.

DISCUSSION

The *E. histolytica* PCR was found to be both sensitive and specific for the detection and differentiation of the E complex. In addition, the PCR was found to have a lower limit of detection of approximately one trophozoite per well. In contrast, both of the stool antigen kits (the *Entamoeba* CELISA PATH kit and the TechLab *E. histolytica* II kit) showed poor sensitivities of 28 and 0%, respectively, compared to PCR, with these results representing the first published standardized evaluation of the *Entamoeba* CELISA PATH kit compared with PCR.

Several ELISA kits have been developed and reported to possess high sensitivity and specificity (7, 10, 12, 16). However, this evaluation has found that both ELISA kits performed poorly compared with PCR when testing routine microscopy-positive stool samples submitted to two diagnostic parasitology laboratories in Australia. The quantification from cultured lysates revealed that the *Entamoeba* CELISA PATH kit was the more sensitive, with the ability to detect approximately 1,000 trophozoites per well compared to the TechLab *E. histolytica* II kit, which required a 10-fold greater load, at approximately 10,000 trophozoites per well for a positive test. Both kits use the same target, a monoclonal antibodies against the Gal/GalNAc-specific lectin (adhesin molecule) of *E. histolytica*. The differences in performance between the two ELISAs may be attributed to the amounts of antibody used to coat the wells of the ELISA plates. The level of detection observed with the antibody-based systems was >1,000-fold less sensitive than that which can be attained by PCR amplification targeting the rDNA. As none of the PCR-positive samples were quantitated, it is unclear whether this is the only reason for the lower detection level. However, it may explain the difference in performance between the two ELISA kits.

The TechLab antigen kit has been used over several years in different laboratories for the detection of *E. histolytica* in regions of the world where it is endemic or nonendemic. The results obtained with the TechLab antigen kit are in conflict with those of studies conducted in countries where *E. histolytica* is highly endemic that reported high sensitivities between 95 and 100% (9, 11, 14). However, a recent study conducted in a region of northern Ecuador where *E. histolytica* is highly endemic found that the TechLab *E. histolytica* II test performed poorly, with a reported sensitivity of 14.3% and a specificity of 98.4% compared to isoenzyme analysis (6). In low-endemicity settings, the TechLab ELISA has been documented to have a sensitivity lower than that of microscopy (7). Similar results were obtained when the TechLab ELISA was compared to real-time PCR as a reference test in a low-endemicity setting (19). Mirelman et al. (1997) were able to quantitate the difference in sensitivity, with the TechLab kit >100 times less sensitive than PCR (12). These previous findings are all supported by our results, which showed that the TechLab ELISA kit was not as sensitive or specific as PCR. In addition, the TechLab ELISA kit was 1,000 times less sensitive than PCR. Gatti et al. proposed that the poor performance of ELISA kits could be due to the fact that the assays recognize the antigens on the vegetative forms only, which are generally found in diarrheal stool samples during an acute amebic infection and not in the cystic stage of the parasite (6). In our study, at least half of the patients were symptomatic and in the majority of the cases both trophozoites and cysts were present, as proven by microscopy, yet the ELISA kits still performed poorly. It should be noted, however, that this study used cell lysates of *E. histolytica* to calculate the analytical sensitivity of the ELISAs, and it is not clear if the ELISAs have comparable limits of detection of trophozoites, cysts, and cell lysates.

In conclusion, antigen detection by ELISA is technically simple to perform, rapid, and cheaper than molecular methods; however, in view of the poor performance of both commercial ELISA kits, it can be argued that they should not be used as the mainstay in the diagnosis of *E. histolytica*. Furthermore, if these tests are used they should first undergo extensive local evaluation compared with PCR as the "gold standard" to determine the level of false-negative results expected in that population when using ELISA for the diagnosis of *E. histolytica*. Both ELISA kits were specific and therefore may still have a place in the differentiation of species of the E complex when large numbers of cysts and/or trophozoites are detected by microscopy. This study clearly demonstrates the advantages of PCR over ELISA-based kits in both sensitivity and specificity. In addition, PCR has the advantage of specifically targeting and detecting *E. histolytica*, *E. dispar*, and *E. moshkovskii* in clinical samples. Given the improvements in the cost of PCR and the advent of automation and simplification of PCR protocols, we believe that all detection and differentiation of *Entamoeba* spp. should be performed by PCR.

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