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Diagnosis of Multiple Enteric Protozoan Infections by Enzyme-Linked Immunosorbent Assay in the Guatemalan Highlands

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Abstract. We tested a prototype stool enzyme-linked immunosorbent assay (ELISA) (*TRI-COMBO*) that is simultaneously diagnostic for *Giardia lamblia*, *Cryptosporidium parvum*, and *Entamoeba histolytica* in a rural pediatric clinic in Guatemala. We compared its results to those of three individual ELISAs for these parasites, assessed the prevalence of these parasites, and compared our findings to those found by stool microscopy. We tested 620 non-diarrheal stools. The *TRI-COMBO* diagnosed 57 positive samples and 52 (91%) had a correlating positive result in an individual assay, giving a kappa coefficient of 0.90. *Giardia* spp., *E. histolytica*, and *Cryptosporidium* spp. were detected in 52 (8.4%), 2 (0.3%), and 3 (0.5%) samples, respectively. Twenty-three (40%) samples positive by ELISA for *Giardia* spp. were identified by microscopy. This study is the first to test the *TRI-COMBO* in this setting and, to our knowledge, represents the first assessment of these parasites in Guatemala by stool ELISA.

INTRODUCTION

Diarrheal illness is a significant source of morbidity and mortality in the developing world. It is one of the primary causes of mortality in children less than five years of age in developing countries, where it accounts for 1.8 million deaths annually.[1] Diarrheal illness in early childhood also contributes to future physical stunting and cognitive impairment.[2] There are an estimated 58 million cases of childhood protozoal diarrhea annually with *Giardia lamblia*, *Cryptosporidium parvum*, and *Entamoeba histolytica* responsible for most of these cases.[3,4] These parasites are transmitted by the fecal-oral route and are consistently found with higher prevalence in developing countries in association with poor water and sanitation.

Our understanding of these parasites and their epidemiology has advanced greatly over the past 20 years. *Cryptosporidium parvum* was first reported as a human pathogen in 1976, and what was formerly named *Entamoeba histolytica* was determined to be two separate but morphologically indistinguishable species in 1993: the pathologic *E. histolytica* and non-pathologic *E. dispar*.[5–7] *E. moshkovskii* is a third morphologically identical species that is being increasingly recognized as a human colonizer with unclear pathogenicity.

Along with these developments have come improved diagnostic capabilities. Whereas 10 years ago most studies involving these parasites were conducted using stool microscopy, current stool antigen enzyme-linked immunosorbent assay (ELISA), immunochromatography, immunofluorescence, and polymerase chain reaction have made diagnosis of these pathogens far more sensitive and specific. The ELISA has potential as a useful tool in the developing world because of its ease of use, transportability, and relatively low cost. Sensitivities of stool antigen ELISAs for *G. lamblia, C. parvum*, and *E. histolytica* are reported to be 96–100%, 91–97%, and 90%, and specificities are reported to be 100%, 99–100%, and > 90%, respectively.[8,9] These values compared favorably with stool microscopy sensitivities of 50–70%, 84%, and 5–60% for *G. lamblia, C. parvum*, and *E. histolytica*, and specificities of 99% for *C. parvum* and 10–50% for *E. histolytica* (the test characteristics of stool microscopy for *C. parvum* assume use of acid-fast staining).[9–11] A new prototype screening stool ELISA, *TRI-COMBO PARASITE SCREEN* (TechLab, Inc., Blacksburg, VA) (*TRI-COMBO*) has been developed that is simultaneously diagnostic for *G. lamblia, E. histolytica*, and *C. parvum*.[12] It is specific for *E. histolytica* and does not cross-react with *E. dispar* or *E. moshkovskii*. This ELISA cannot distinguish between the three protozoa, but would provide an easier less expensive screening tool for identifying the presence of enteric infections.

There is little current data using these modern diagnostic techniques to assess the burden of *G. lamblia, E. histolytica*, and *C. parvum* in Guatemala. The current study determines the point prevalence of these pathogens in a primarily indigenous and rural population in the Palajunoj Valley in the western highlands of Guatemala, assesses the feasibility of using stool antigen ELISA as a diagnostic technique in a small rural clinic, and compares the results of the *TRI-COMBO* Test to Food and Drug Administration–approved individual ELISAs for these three pathogens.

MATERIALS AND METHODS

Study population. The study took place in a non-profit health clinic (Primeros Pasos) in the Palajunoj Valley in the western highlands of Guatemala during August 4–October 28, 2010. This clinic serves rural, indigenous families living in the valley, located near Quetzaltenango, the second largest city in Guatemala. In this region 95% of the persons are indigenous Quiche Maya and 92.7% of the population is rural.[13] Students from the valley's 10 communities around the clinic are invited annually to have health screenings and receive nutrition and health education. There are 5,228 children 5–15 years of age in the valley (2004 census data) and 56.8% of these children were enrolled in public schools. Of the children enrolled in public schools, 91% were seen in the Primeros Pasos clinic.[13]

Study design. Students from kindergarten through sixth grade attend the clinic for health screening. They are routinely given an empty plastic container 1–2 days before their visit and are asked to bring a stool sample with them to clinic

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for detection of parasites by microscopy. In 2007, approximately two-thirds of the students provided stool samples.[13] The clinic also provides services to daycare centers in the valley where children are 18 months through 6 years of age. Patients also attend the clinic outside of these screenings because of acute medical issues and occasionally to provide stool samples.

In this cross-sectional study, all stool samples brought to the clinic or obtained at daycare centers during the study period were examined by ELISA stool antigen detection by the study investigators. Samples were studied according to the clinic's usual procedure using normal saline wet mount microscopy by the clinic's laboratory technician. When cysts were noted on microscopy iodine staining was used to further identify pathogens. The samples were coded and the study investigator did not have access to information about the patients giving the samples. After completion of the ELISAs, the investigator learned the results of microscopy, and results of the individual ELISAs were given to the clinic.

Stool antigen ELISA. Stools were collected 1–2 days before clinic visit by patients. Samples were not fixed in formalin or other preservatives or refrigerated. Samples were tested immediately upon arrival at the clinic except in a few cases where samples were brought in late in the day. These samples were tested the next business day and were stored at room temperature. Stool samples were tested with individual stool antigen ELISA (*CRYPTOSPORIDIUM II*, *E. HISTOLYTICA II*, and *GIARDIA II*), as well as the *TRI-COMBO* ELISA, all produced by TechLab per manufacturer's instructions. During the study period, because certain reagents ran out and could not be immediately replaced, some samples were only tested on a subset of the four types of ELISAs. Water used for buffer solution was de-mineralized, purified water as opposed to de-ionized water. Because there was no refrigeration available, ELISA kits were stored in coolers with ice replaced Monday through Friday. Because there was no optical density reader available, results were read visually in accordance with manufacturer's instructions.

Statistical analyses. Results of ELISA and microscopy were recorded by using Microsoft (Redmond, WA) Excel. The point prevalence of *G. lamblia, E. histolytica*, and *C. parvum* was calculated for patients seen. Using the *GIARDIA II* ELISA as the reference, we determined the specificity and sensitivity of microscopy for *G. lamblia* detection. Using the three individual ELISAs, *GIARDIA II, E. HISTOLYTICA II,* and *CRYPTOSPORIDIUM II* together as the reference, we determined the specificity and sensitivity of the *TRI-COMBO* Test. For this calculation, a stool sample that was positive by any of the ELISAs was considered positive and a stool sample that was negative by all three ELISAs was considered negative.

Sensitivity, specificity, positive predictive value, and negative predictive value were calculated as follows: sensitivity = 100 [a/(a + c)], specificity = 100[d/(d + b)], negative predictive value = 100[d/(c + d)], and positive predictive value = 100[a/(a + b)], where sample a is a true positive, b is a false positive, c is a false negative, and d is a true negative. Cohen's Kappa coefficient and McNemar's Test were used to determine the level of agreement between the *TRI-COMBO* Test and individual ELISAs and between microscopy and individual ELISAs. Using Cohen's Kappa magnitude reflects the strength of agreement where 1 implies perfect agreement. Using McNemar's test $P < 0.05$ indicates that a difference between the two testing methods is statistically significant.

Ethics. The study protocol was submitted to the Institutional Review Board of the of University of Virginia Health System and was determined to not require further Institutional Review Board approval because it involved coded biological specimens that were not acquired for this study.

TABLE 1

Case detection of *Giardia* spp., *Entamoeba histolytica*, and *Cryptosporidium* spp. by ELISA and microscopy, Guatemala*

Organism	ELISA	Microscopy	ELISA and microscopy
Giardia lamblia	52 (8.4)	35 (5.7)	23 (3.7)
Entamoeba histolytica	2 (0.3)	3 (0.5)	0
Cryptosporidium parvum	3 (0.5)	0	0

*Values are no. (%). ELISA = enzyme-linked immunosorbent assay.

TABLE 2

Test parameters of microscopy for *Giardia* spp. detection using ELISA as a reference, Guatemala*

Parameter	ELISA positive	ELISA negative
No. microscopy positive	23	12
No. microscopy negative	29	556
Specificity	98%	NA
Sensitivity	44%	NA
Positive predictive value	66%	NA
Negative predictive value	95%	NA

*ELISA = enzyme-linked immunosorbent assay; NA = not applicable.

RESULTS

The laboratory received 723 samples during the study period, and 620 samples were tested by all ELISAs and microscopy. Statistical results were calculated for these 620 samples. Samples were received primarily from students 18 months to 12 years of age and a small number were collected from children outside this age range.

Giardia spp., *E. histolytica*, and *Cryptosporidium* spp. were detected in 52 (8.4%), 2 (0.3%), and 3 (0.5%) samples, respectively, and no sample was positive for more than one pathogen when assessed by individual ELISA. *Giardia* spp., *E. histolytica*/*dispar*, and *Cryptosporidium* spp. were detected in 35 (5.7%), 3 (0.5%), and 0 samples, respectively, when assessed by stool microscopy (Table 1). Of the 57 positive samples detected by individual ELISAs, only 23 (40%) were also positive by microscopy. For the 52 samples positive for *Giardia* spp. by ELISA, 23 were identified by microscopy, giving a Kappa coefficient of 0.49 and indicating only fair agreement. McNemar's test had a *P* value of 0.012, indicating that there was a significant difference between the two tests. There were few samples positive by microscopy or ELISA for *E. histolytica*, and there was no correlation between ELISA and microscopy for this pathogen. Because acid-fast staining

TABLE 3

Test parameters of *TRI-COMBO* for all parasites using individual ELISA as a reference, Guatemala*

Parameter	Individual ELISA positive	Individual ELISA negative
No. *TRI-COMBO* positive	52	5
No. *TRI-COMBO* negative	5	558
Specificity	99%	NA
Sensitivity	91%	NA
Positive predictive value	91%	NA
Negative predictive value	99%	NA

*ELISA = enzyme-linked immunosorbent assay; NA = not applicable.

TABLE 4
Parasite case detection and prevalence by light microscopy, Guatemala

Parasite	No cases (% of total)
Endolimax nana	22 (3)
Trichomonas hominis	2 (0.3)
Hymenolepsis nana	57 (7.9)
Entamoeba coli	27 (3.7)
Blastocystis hominis	17 (2.4)
Ascaris lumbricoides	111 (15.4)
Strongyloides stercoralis	1 (0.1)
Enterobius vermicularis	1 (0.1)
Trichuris trichiura	2 (0.3)
Iodamoeba butschlii	10 (1.4)
Taenia saginata	1 (0.1)

was not performed in this laboratory, *Cryptosporidium* spp. was not detected by microscopy. Using ELISA as the reference test for *Giardia* spp., we determined that microscopy had a specificity of 98%, a sensitivity of 44%, a positive predictive value of 66%, and a negative predictive value of 95% (Table 2).

Using the *TRI-COMBO*, we found 57 positive samples. Of those samples positive by *TRI-COMBO*, 52 (91%) had a correlating positive individual assay. This finding gave a Kappa Coefficient of 0.90, which indicated good agreement between the *TRI-COMBO* and the individual assays (Table 3). McNemar's test had a *P* value of 1.000 , indicating that there was no significant difference between the two testing methods. Using individual ELISA as the reference test, we found that the *TRI-COMBO* had a specificity of 99%, a sensitivity of 91%, a positive predictive value of 91%, and a negative predictive value of 99% (Table 3).

Other parasites were detected by microscopy beyond those being tested for using ELISA. Other parasites and their prevalence noted in the 723 samples examined by light microscopy during the study period are shown in Table 4.

Few studies have examined the prevalence of enteric protozoa in Guatemala. A PubMed search of "Guatemala" and each of the three parasites yielded a total of 15 articles with epidemiologic data over the past 40 years (Table 5). Most of these studies determined prevalence by using microscopy with or without acid-fast staining or immunofluorescence. One study used serologic analysis to assess seroconversion. This is the first study that has attempted to assess prevalence of these pathogens by using stool antigen ELISA.

DISCUSSION

We found a surprisingly low prevalence of all parasites, including the protozoa of interest. A retrospective analysis published in 2009 reported rates of parasitic infection based on stool microscopy at this same clinic of 13.3% and 18.1% of children for *G. lamblia* and *E. histolytica* infections, respectively.[13] That study compiled data from an entire year and all of the 10 communities that visit the clinic, whereas our study was more limited. Seasonal variability or the individual communities tested may have played a role in the difference in rates of infection between our study and theirs.

As expected, there was only fair agreement between microscopy and ELISA, represented by the low Kappa coefficient of 0.49. We had anticipated that microscopy would miss cases of *Giardia* spp. detected by ELISA, but we also found samples were reported positive by microscopy and not by ELISA. Because microscopy has traditionally been the gold standard for diagnosis of *Giardia* spp., its specificity is unclear from the literature, but is in part related to the skill and experience of the microscopist. However the specificity of stool ELISA is reported to be 100%, and this testing modality is less user dependent.[8] Unfortunately, we had no facilities to perform the polymerase chain reaction, but given the test parameters of stool ELISA for detecting *Giardia* spp., results by this method are likely more reliable.

Use of ELISA enabled detection of an additional 29 cases of *Giardia* infection, more than doubling the true positive case detection by microscopy among 620 children. Increasing the

TABLE 5
Summary of articles with epidemiologic data for Guatemala and *Giardia lamblia*, *Entamoeba histolytica*, or *Cryptosporidium parvum**

Parasite	Year	Location	Technique	Reference
Giardia lamblia, *Entamoeba histolytica*	1963–1972	Santa Maria Cauqué	Weekly stool study†	Melvin and Mata[18]
G. lamblia	1964–1969	Santa Maria Cauqué	Weekly stool microscopy	Farthing and others[19]
G. lamblia	1978–1979	Santa Maria Cauqué	Stool microscopy	Gupta and Urrutia[20]
Cryptosporidium parvum	1985–1986	Guatemala City	Stool microscopy and staining with methylene blue	Cruz and others[21]
E. histolytica, *C. parvum*, *G. lamblia*	1991	Guatemala, Peace Corps	Stool microscopy with AF and IF	Herwaldt and others[22]
G. lamblia, *E. histolytica*	1993	Guatemala City	Stool microscopy	Grazioso and others[23]
C. parvum	1997–1998	Guatemala City	Stool microscopy with AF and UV epifluorescence, confirmed by IF	Bern and others[24]
Cryptosporidium (4 subtypes)	1998–2000	China, Guatemala, India, Kenya, Portugal, Slovenia	Stool PCR for subgenotype analysis	Peng and others[25]
C. parvum	1999	San Juan	Serum IgG by ELISA	Steinberg and others[26]
G. duodenalis, *Entamoeba* sp.	2001	La Mano de Leon, Santa Maria de Jesus	Stool microscopy	Jensen and others[27]
C. parvum, *G. lamblia*	2001–2002	Sacatepéquez	Serum ELISA	Crump and others[28]
C. parvum	2001–2002	Lake Atitlan	Stool microscopy with AF	Laubach and others[29]
C. parvum	2003	Rural areas around Guatemala City	IF of drinking water	Dowd and others[30]
E. histolytica, *G. lamblia*	2004–2007	Palajunoj Valley	Stool microscopy	Cook and others[13]
E. histolytica, *G. lamblia*	2009	Antigua, Rio Dulce	Stool microscopy	Jensen and others[31]

*AF = acid-fast staining; IF = immunofluorescence; UV = ultraviolet; PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
†Details of stool study method not reported.

sensitivity of diagnostics for enteric infections may be important for preventing morbidity. Studies in Brazil have shown reduced physical fitness associated with early childhood diarrhea years afterwards, growth deficits of 3.6 cm at seven years of age, and cognitive deficits.[14–16] Although mortality from diarrheal illness has greatly decreased since 1955, rates of illness have not changed, suggesting that morbidity remains severe.[17]

In performing this study we hoped to determine the feasibility of using ELISA stool detection routinely in a small rural clinic. The laboratory available consisted of a small sink, two desks, small waste basket, and a window. Equipment included a light microscope and a centrifuge. There was no available refrigeration for laboratory use. In this setting, it was possible to perform ELISAs. Because the lack of refrigeration meant that samples had to be tested the day they arrived, batching samples over multiple days was not possible. The samples were collected at home 1–2 days before being brought to the laboratory and rarely were tested the next business day. This limitation may have adversely affected the ELISA results. However, it more likely affected the detection of protozoa by microscopy, which might explain the low sensitivity of microscopy in our study. Conducting four ELISAs required significant space and produced more solid waste than was typical for the laboratory. Limitations affecting the ELISA performance included the type of water used for mixing the buffer solution, lack of an optical density reader, and lack of refrigeration. However, positive and negative controls were consistently within manufacturer's specifications, reassuring us that this did not affect our results.

The *TRI-COMBO* showed a good correlation with individual assays. In locations with high numbers of all three parasites, this correlation would provide a simplified method of screening stools for a variety of infections after which positive samples could be tested further for individual pathogens. The time required for a single laboratory technician to perform three separate ELISAs is substantial and using the *TRI-COMBO* could decrease this time considerably.

The prevalence of *Giardia* spp., *E. histolytica*, and *Cryptosporidium* spp. among school children in the Palajunoj Valley measured by stool antigen ELISA was lower than previously reported at 8.4%, 0.3% and 0.5%, respectively. Use of a stool antigen ELISA can greatly augment case detection of these pathogens and is feasible in a rural laboratory, but does require additional equipment and laboratory space, and produces more solid waste than standard microscopy. Use of a triple-screening ELISA such as the *TRI-COMBO* is accurate and can be used to simplify the ELISA screening process where giardiasis, cryptosporidiosis, and/or amebiasis are suspected.

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