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FIELD STUDY ON THE DISTRIBUTION OF *ENTAMOEBA HISTOLYTICA* AND *ENTAMOEBA DISPAR* IN THE NORTHERN PHILIPPINES AS DETECTED BY THE POLYMERASE CHAIN REACTION

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Abstract. We used the polymerase chain reaction (PCR) to study the distribution of *Entamoeba histolytica* and *E. dispar* in 1,872 individuals in 14 communities in the northern Philippines. Here we report a field study using a DNA extraction protocol from formalin-fixed stool specimens as previously reported. This assay detected 137 stools (7.318%) containing *E. dispar* and 18 stools (0.961%) containing *E. histolytica.* The most affected age group for *E. histolytica*/*E. dispar* infections were those 5–14 years of age. There was no significant difference in the sex distribution of *E. histolytica,* while in the case of *E. dispar,* a higher prevalence was observed in females (9.186%) than in males (5.731%) ($P < 0.01$). An apparent clustering of stool-positive cases of *E. histolytica* and *E. dispar* was also observed in the northern part of the study area. The results of this survey demonstrate that *E. dispar* is highly prevalent in the communities studied. Moreover, it offers promise for the PCR using DNA extracted from formalin-fixed stools as a sensitive epidemiologic tool for detecting *E. histolytica* and *E. dispar* infections.

The intestinal protozoan parasite *Entamoeba histolytica* is the causative agent of human amebiasis. *Entamoeba histolytica* is responsible for up to 100,000 deaths per year, placing it second only to malaria in mortality due to protozoan parasites.[1] The infection is common in developing countries and predominantly affects individuals with poor socioeconomic conditions, nonhygienic practices, and malnutrition.

A number of epidemiologic studies of *E. histolytica* infection were performed before the clear distinction of two separate species, *E. dispar* and *E. histolytica,* was established.[2] Because they are morphologically indistinguishable, studies based on stool surveys have raised the question on the validity of most of these studies. Moreover, seroepidemiologic studies that were carried out in a number of endemic countries[3–8] usually reflected the seroprevalence of the disease even in the near past. Also, the major problem with current serologic test results is that they remain positive for years after an episode of amebiasis. To date, the standardization of antigen preparation and the relative measurement of positive cut-off antibody titers for use in serodiagnosis still pose a problem.

In recent years, a number of methods such as isoenzyme typing,[9] DNA probes for the polymerase chain reaction (PCR) and hybridization,[10–13] restriction pattern analysis,[14–19] and monoclonal antibodies[20–24] have been developed for the clear distinction of the two species. Clearly, accurate diagnostic tools are required for the clinical and public health management of the disease. The use of a stool ELISA has recently been shown to be useful in routine diagnostic procedure and epidemiologic studies.[25,26] However, a comparative study on the use of the ELISA and PCR for the detection of *E. histolytica* and *E. dispar* indicated that the PCR was more advantageous than the ELISA in epidemiologic studies.[27] It is therefore important to assess the usefulness of the PCR in accumulating data on the prevalence of *E. histolytica* and *E. dispar* in the field that are more accurate.

We previously reported that a successful extraction of DNA from formalin-fixed stool samples for the PCR is a useful tool for rapid diagnosis of both *E. histolytica* and *E. dispar* infections.[28] Here we report the application of this method to document the prevalence of the two species in a number of communities in the northern Philippines. Our preliminary hospital-based studies showed that amebic diarrheal diseases in Baguio City and nearby communities in the northern Philippines are frequent; however, their prevalence and distribution are not well-characterized. We therefore conducted a large-scale field study to determine the distribution of *E. histolytica* and *E. dispar* in these communities.

MATERIALS AND METHODS

Study sites. The study was conducted during the months of December 1995, January and December 1996, and January 1997 in the communities (locally called barangays) within and nearby Baguio City, The Philippines (Figure 1). This study area belongs to the Cordillera Autonomous Region and is the highest city in The Philippines, with an average altitude of 1,500 meters above sea level. Three major land forms characterize the relief features of the city: valley, plateau, and steep slopes. Throughout the year, the weather is cool but is coolest during the months of December to February with an average temperature of 18°C.

Housing is inadequate in the city, given a rapid population increase and inadequate area for settlement expansion aside from poor economic conditions. Ninety-nine percent of the total number of households in the city have access to potable water. Only 0.95% of the total population have doubtful water sources, most of which are places where water pipes are inaccessible in view of the high elevation of the city.

Collection of samples. The day before the scheduled sample collection, the research team visited the randomly selected households, explained the purpose of the study and the requirements for sample collection, and distributed specimen cups. Particular care was taken in explaining the need to label the specimen cups with name, age, and sex and to avoid sample contact with soil and with samples from other individuals. Informed consent was obtained from all individuals. This protocol was approved by the Ethical Committees for Human Studies of the Institute of Tropical Medicine at Nagasaki University and Baguio General Hospital and Med-

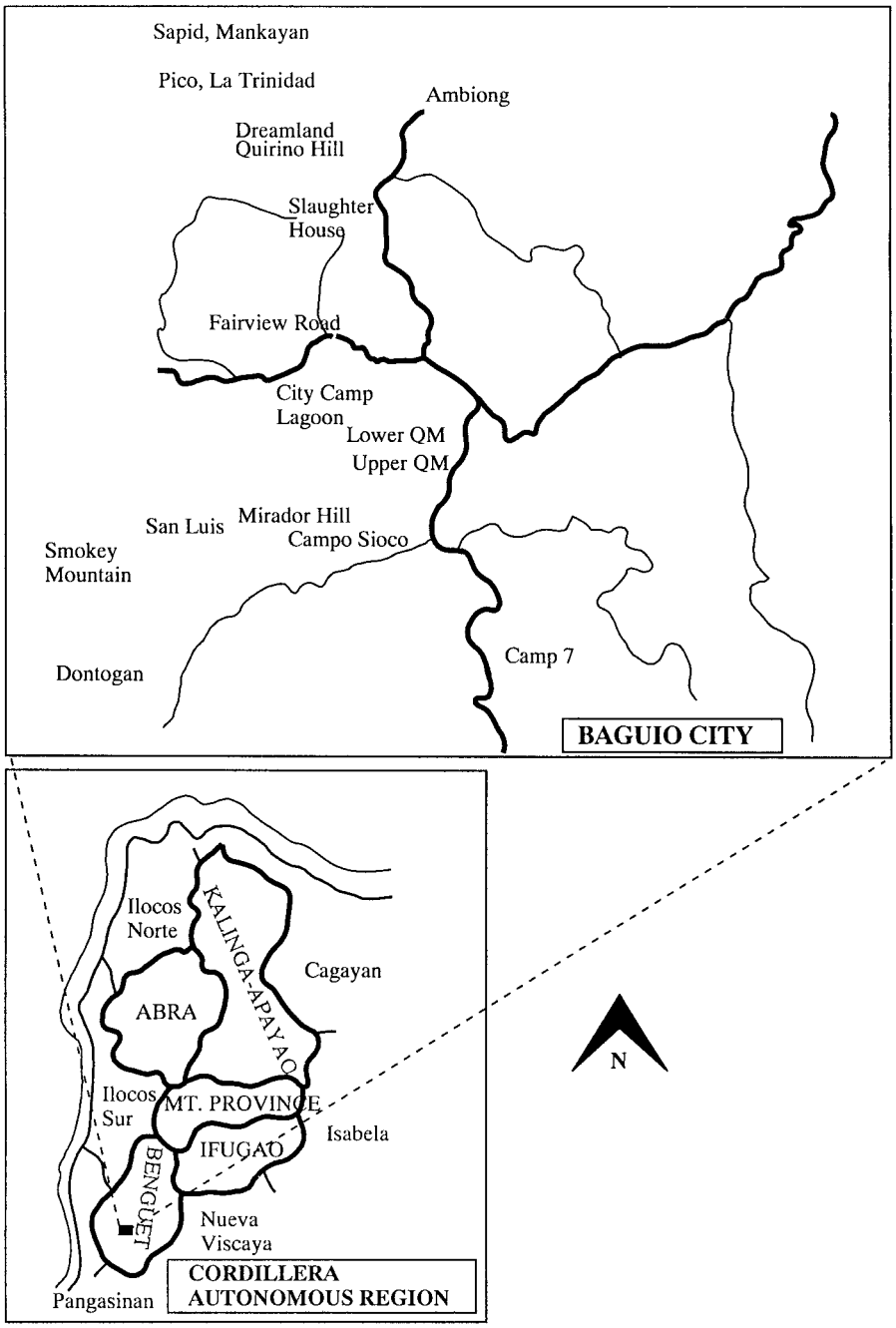

FIGURE 1. Map of Baguio City, The Philippines, showing the study areas.

ical Center (Baguio City, The Philippines). The samples were then collected the next morning and immediately transported to the laboratory. Each sample was macroscopically inspected for its consistency and for the presence of soil contamination, blood, or mucus. Routine microscopic diagnosis of parasites was then performed.

Microscopic examination of parasites. The presence of parasites was determined by microscopic examination of fresh stools and formalin-ether concentrated specimens.

Genomic DNA extraction and the PCR. Genomic DNA was extracted from cysts present in formalin-fixed stool specimens as previously described[28] with slight modifications. Briefly, the pellet resulting from the sedimentation procedure was washed four times with phosphate-buffered saline. The pellet from the last wash was resuspended in TE buffer (100 mM Tris, pH 8.0, and 25mM EDTA) and subjected to freezing (using dry ice and ethanol for 5 min) and thawing (at 37°C in a water bath for 2 min) six times. After the last treatment, the solution was mixed with 200 ml of 0.2% Triton-X 100 and then heated in 98°C water bath for 10 min. The mixture was then incubated with 200 μg/ml of proteinase K in lysis buffer (100 mM Tris, pH 8.0, 1% sodium dodecyl sulfate, 25 mM EDTA) at 80°C for 2 hr, and the DNA was extracted using the routine phenol-chloroform extraction procedure.[29] The PCR was then carried out using primers specific for *E. histolytica* and *E. dispar* (p11 plus

TABLE 1
Prevalence of single and mixed infections of intestinal protozoans and helminths in the study population based on microscopy*

Species	Total number of positive cases	Prevalence rate (%)	*E. histolytica*/ *E. dispar*	Mixed infections with		
				E. coli	*E. nana*	*Blastocystis* sp.
Protozoa						
Entamoeba histolytica/*E. dispar*	152	8.119	–	21 (3)	3 (3)	8 (1)
Entamoeba coli	126	6.730	21 (3)	–	3 (3)	13 (1)
Endolimax nana	8	0.427	3 (3)	3 (3)	–	2 (1)
Blastocystis sp.	83	4.433	8 (1)	13 (1)	2 (1)	–
Giardia intestinalis	5	0.267	0	1 (0)	0	1 (0)
Helminths						
Ascaris lumbricoides	57	3.044	0	8 (2)	0	15 (2)
Trichuris trichiura	24	1.282	0	6 (4)	0	11 (4)

* Numbers in parentheses represent the total number of mixed infections between species.

p12 and p13 plus p14, respectively) as previously described.[12]

Serology. Eleven serum samples of individuals infected with *E. histolytica* and *E. dispar* and three samples from healthy controls were collected. The commercially available Amoebiasis HA-Test (Japan Lyophilization Laboratory, Tokyo, Japan) and an indirect hemagglutination assay (IHA) were used to detect antibodies in the serum samples.

Statistical analysis. Prevalence levels were estimated according to each of the variables studied. For measuring the strength of association, we used the Mantel-Haenszel odds ratio (OR) with a 95% confidence interval (CI).[30]

RESULTS

Microscopic examinations. The prevalence of all parasites found in the sample of individuals is shown in Table 1. In the course of this study, 1,872 fecal samples were examined. One hundred fifty-two persons were infected with *E. histolytica*/*E. dispar*, resulting in a prevalence of 8.119%. Few cases of *Endolimax nana* and *Giardia intestinalis* were found, with prevalence rates of 0.427% and 0.267%, respectively. Infection with *E. histolytica*/*E.dispar* was found to be strongly associated with infection by *Entamoeba coli*. Helminthic eggs of *Ascaris lumbricoides* (3.044%) and *Trichuris trichiura* (1.282%) were also observed and found to be associated only with *E. coli* and *Blastocystis sp.* but not with *E. histolytica*/*E. dispar* (Table 1).

Polymerase chain reaction. Eighteen of the 152 *E. histolytica*/*E. dispar* microscopically positive samples were identified as *E. histolytica* using PCR-specific primers. The remaining 134 samples were found to be *E. dispar* based on the PCR. However, the PCR detected three samples from *E. coli*-positive samples as *E. dispar*. Although we found 21 *E. coli*-positive samples in close association with *E. histolytica*/*E. dispar* based on microscopy, we failed to identify these three samples coinfected with *E. dispar*. Moreover, no mixed infections of *E. histolytica* and *E. dispar* was detected.

Age, sex and geographic distribution of *E. histolytica* and *E. dispar* infections in the study area. The age-specific prevalence is presented in Table 2. Both *E. histolytica* and *E. dispar* were most common for those 5–14 years of age, with prevalence rates of 1.509% (OR = 2.396, 95% CI = 0.658–8.730) and 9.056% (OR = 1.467, 95% CI = 0.915–2.352), respectively. These prevalence rates did not show a statistically significant difference when compared with those of other age groups for either of the two species. However, analysis of the combined prevalence rates of *E. histolytica* and *E. dispar* in this age group showed that these were statistically significant ($P < 0.05$). On the other hand, subjects less than 1–4 years of age had prevalence rates of 0.635% for *E. histolytica* and 6.355% for *E. dispar*. In those greater than 45 years of age, the prevalence rates were 0.523% (OR = 0.823, 95% CI = 0.085–7.945) for *E. histolytica* and 6.806% (OR = 1.076, 95% CI = 0.548–2.111) for *E. dispar*.

There was no statistically significant difference between the prevalence rates of *E. histolytica* in males (1.086%) and females (0.813%) while in *E. dispar*, a higher prevalence rate was observed in females (9.186%) than in males (5.731%) (OR = 1.664, 95% CI = 1.174–2.359, $P < 0.01$) (Table 3).

The study was carried out in 12 communities of Baguio City and two nearby communities of La Trinidad and Mankayan. The map of the study area is shown in Figure 1 and the prevalence of *E. histolytica* and *E. dispar* by geographic

TABLE 2
Age distribution of *Entamoeba histolytica*– and *E. dispar*-positive samples as determined by the polymerase chain reaction

Age group (years)	Sample size	No. of *E. histolytica*	Prevalence rate (%)	OR (95% CI)*	No. of *E. dispar*	Prevalence rate (%)	OR (95% CI)*
<1–4	472	3	0.635	1.00†	30	6.355	1.00†
5–14	530	8	1.509	2.396 (0.658–8.730)	48	9.056	1.467 (0.915–2.352)
15–24	251	3	1.195	1.891 (0.389–9.205)	18	7.171	1.138 (0.621–2.086)
25–34	258	1	0.387	0.608 (0.064–5.754)	17	6.589	1.039 (0.561–1.924)
35–44	170	2	1.176	1.861 (0.317–10.941)	11	6.470	1.019 (0.499–2.083)
>45	191	1	0.523	0.823 (0.085–7.945)	13	6.806	1.076 (0.548–2.111)
Total	1,872	18	0.961		137	7.318	

* Odds ratio (OR) with 95% confidence interval (CI).
† Reference point for comparison with other groups.

TABLE 3
Sex distribution of *Entamoeba histolytica*– and *E. dispar*-positive samples as determined by the polymerase chain reaction

Sex	Sample size	No. of *E. histolytica*	Prevalence rate (%)	OR (95% CI)*	No. of *E. dispar*	Prevalence rate (%)	OR (95% CI)*
Male	1,012	11	1.086	1.00†	58	5.731	1.00†
Female	860	7	0.813	0.747 (0.289–1.929)	79	9.186	1.664 (1.174–2.359)
Total	1,872	18	0.961		137	7.318	

* Odds ratio (OR) with 95% confidence interval (CI).
† Reference point for comparison with other groups.

area is listed in Table 4. *Entamoeba histolytica* was found mostly in the northern part of Baguio City (Ambiong, 3.389%; Dreamland, Quirino Hill, 1.526%; Slaughter House, 3.529%) and in the communities of La Trinidad (Pico, 1.652%) and Mankayan (Sapid, 2.439%). *Entamoeba dispar*, on the other hand, was widespread in the whole city but higher prevalence rates were also observed in the northern communities.

Development of antibodies against *E. histolytica*. Of the 155 individuals positive for *E. histolytica* and *E. dispar* by the PCR, only 11 agreed to provide serum samples. Using the commercially-available Amoebiasis HA-Test, we found three samples reactive to *E. histolytica* antigen. Stool samples from the same individuals showed *E. histolytica* positivity in the PCR, while the remaining eight samples were identified as *E. dispar*. The three serum samples collected from healthy controls did not show any reactivity to *E. histolytica* antigen.

DISCUSSION

Infection with *E. histolytica* is a severe health problem in many tropical and subtropical areas of the world, especially in developing countries. In The Philippines, studies on the prevalence and distribution of *E. histolytica* are not well-documented. A previous epidemiologic study using an IHA showed stool positivity prevalence rates of 2–8% and seropositivity rates of 1–13%.[4] However, surveys that determine the prevalence of infection by stool examination of parasites measure predominantly *E. dispar*, since this species is more common, while serologic surveys reflect the incidence of *E. histolytica* infection because *E. dispar* does not show a positive serologic test result.[31]

In this study, we sought to determine the prevalence of *E. histolytica* and *E. dispar* with our previously reported technique that uses genomic DNA extracted directly from formalin-fixed specimens for the PCR. To our knowledge, this is the first report on the distribution of *E. histolytica* and *E. dispar* in The Philippines since the description of the two species was established. This survey showed prevalence rates of 0.961% and 7.318% for *E. histolytica* and *E. dispar*, respectively, in the study population. It is interesting to note that there is an apparent clustering of stool-positive cases of *E. histolytica* in the northern part of the study area. *Entamoeba dispar*, on the other hand, was widespread throughout the study sites except in one community where no cases were found. However, high prevalence rates for *E. dispar* were also found in the northern region. Several factors may account for the high prevalence rates of protozoan and helminthic parasite. These include urbanization, availability of potable water, and population density. In this study, intrafamilial infection with *E. histolytica* was observed in at least

TABLE 4
Geographic distribution of *Entamoeba histolytica*– and *E. dispar*-positive samples as determined by the polymerase chain reaction

Community	Sample size	No. of *E. histolytica*	Prevalence rate (%)	OR (95% CI)*	No. of *E. dispar*	Prevalence rate (%)	OR (95% CI)*
North							
Sapid, Mankayan	123	3	2.439		14	11.382	
Pico, La Trinidad	121	2	1.652		17	14.049	
Ambiong	118	4	3.389		13	11.016	
Dreamland, Quirino Hill	131	2	1.526		11	8.396	
Slaughter House	170	6	3.529		10	5.882	
Fairview Road	137	0	0		15	10.948	
Subtotal	800	17	2.125	1.00†	80	10.000	1.00†
Central							
City Camp Lagoon	123	0	0		10	8.130	
Lower/Upper QM	163	0	0		12	7.361	
Mirador Hill	110	0	0		0	0	
San Luis	161	0	0		6	3.726	
Campo Sioco	140	0	0		4	2.857	
Subtotal	697	0	0	0.032 (0.005–0.194)	32	4.591	0.433 (0.286–0.655)
South							
Smokey Mountain	135	1	0.740		11	8.148	
Camp 7	128	0	0		9	7.031	
Dontogan	112	0	0		5	4.464	
Subtotal	375	1	0.266	0.123 (0.023–0.673)	25	6.666	0.643 (0.404–1.022)
Total	1,872	18	0.961		137	7.318	

* Odds ratio (OR) with 95% confidence interval (CI).
† Reference point for comparison with other groups.

three households, indicating the possible involvement of human waste disposal systems, personal hygiene habits, housing conditions, and food preparation habits. Aside from the factors mentioned, the apparent clustering in the northern region may be due to the lack of an adequate water supply due to the elevated topography of the study area. Water pipes are inaccessible to the households; thus, they depend on individuals who collect, deliver, and sell water in containers.

With regard to age, as shown in Table 2, *E. histolytica* and *E. dispar* prevalence rates were particularly high in 5–14-year-old age group, with an observable decrease in positivity with age. A possible reason for this is decreased exposure of individuals to the parasite. Seroepidemiologic studies in endemic areas of Nicaragua,[8] Bangladesh,[7] and Brazil[32] showed peak prevalences of *E. histolytica* in a similar age group. Although the purpose of this study was the detection of parasites, a correlation was observed between the presence of *E. histolytica* in asymptomatic individuals and a positive antibody response in a limited number of serum samples assayed by IHA (one of three seropositive samples from the 5–14-year-old age group). Since only *E. histolytica* elicits the production of serum antibodies,[33] the possibility of prior exposure of the individuals to *E. histolytica* cannot be excluded. However, confirmation of the presence of parasites by the PCR rules out this problem.

Microscopic examinations revealed the presence of other intestinal protozoans as well as helminthic parasites in the study population. A clear association between *E. histolytica*/*E. dispar* and *E. coli* was observed. However, coinfection of three additional samples of *E. coli* with *E. dispar* were detected only after the PCR. This also shows the reliability of the PCR in clearly documenting multiple infections among subjects in epidemiologic studies.

Mixed infections of *E. histolytica* and *E. dispar* were not observed in the samples tested. This observation was also shown in other studies involving the distribution of *E. histolytica* and *E. dispar* in the field.[17,28] Although it is not yet clear if a competitive phenomenon between *E. histolytica* and *E. dispar* is true *in vivo*, an *in vitro* study showed that only a minuscule amount of *E. histolytica* can ultimately outgrow *E. dispar* in culture in a given period of time.[34] Thus, the development of an adequate animal model would be a prerequisite to prove this phenomenon.[35]

It was also observed in this study that all the *E. histolytica*-positive individuals were asymptomatic. This is consistent with reports from endemic areas that showed that most *E. histolytica* infections are asymptomatic.[36,37] Although three bloody stool samples and a few cases of diarrhea were observed among the subjects, based on the PCR results, we could not attribute these to infections with amebae. Moreover, erythrophagocytosis was not observed in bloody stool samples after microscopic examination. Thus, the presence of blood and mucus among individuals with diarrhea maybe due to other factors.[17,26]

This study shows that a clear potential application of PCR-based detection of *E. histolytica* and *E. dispar* directly from stools offers the most promise for epidemiologic studies. An additional benefit is that it can be used to monitor the efficacy of treatment, which is usually the limitation of serologic tests because of the persistence of the antibody response even after successful treatment. Moreover, the use of the PCR in detecting *E. dispar* among cyst carriers can facilitate longitudinal studies to determine the nature of this nonpathogenic species in the hosts.

In conclusion, our results have demonstrated that *E. dispar* infections are predominant in Baguio City and nearby communities in the northern Philippines. However, further studies that include other variables such as socioeconomic conditions, educational backgrounds, and types of settlements are needed to clearly document the epidemiology of amebiasis in the northern Philippines.

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