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Longitudinal Study of Intestinal *Entamoeba histolytica* Infections in Asymptomatic Adult Carriers

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To gain insight into the dynamics of intestinal *Entamoeba histolytica* infection, a longitudinal study was performed over an observation period of 15 months with a group of 383 randomly selected adult individuals (mean age, 38.5 years) living in an area of amebiasis endemicity in central Vietnam. Ameba infection was diagnosed by using species-specific PCR and DNA extracted directly from fecal samples. The results indicated an *E. histolytica* prevalence of 11.2% and an annual new infection rate of 4.1% in the study population. Follow-up of the 43 individuals who were *E. histolytica* positive at enrollment suggested a regular exponential decline in infection of about 3% per month and a mean half-life of infection of more than 15 months. However, the reinfection rate for this group of participants was 2.7 times higher than that predicted for the study population as a whole. Both the reappearance of the parasite after successful treatment of *E. histolytica* infection and changes in "genetic fingerprints" of parasites during the course of infection revealed an annual new infection rate of about 11.5%. Thus, the mean half-life of *E. histolytica* infection was calculated to be 12.9 months (95% confidence interval, 10.2 to 15.6 months). Notably, none of the participants developed symptoms compatible with invasive intestinal amebiasis, and only one of the subjects developed an amebic liver abscess during the observation period.

Entamoeba histolytica, the causative agent of human amebiasis, is endemic in most tropical and subtropical countries and is considered responsible for millions of cases of dysentery and liver abscess each year (19). Despite its medical importance, there is a considerable lack of knowledge about the epidemiology of this parasite. The recent identification of *Entamoeba dispar* as a separate but nonpathogenic species which is morphologically indistinguishable from *E. histolytica* has called into question most of the earlier data on the worldwide prevalence of the parasite and its importance as a human pathogen (7, 8, 20). The studies conducted to date that have used methods capable of differentiating between the two species suggest that, in general, *E. dispar* is more prevalent than *E. histolytica* (6, 9, 10, 11, 12, 14, 17) and that only a small proportion of individuals specifically infected with *E. histolytica* will progress to amebic disease (10, 15). However, to reach a definite conclusion on these points, many more studies are required, and these should be performed at various sites where the parasite is endemic and should include different age groups. Nearly all previous studies were performed with children, despite the fact that amebic liver abscess is substantially more prevalent in adults (4).

In addition, reliable data are lacking on the time course of *E. histolytica* infections. On the one hand, evidence exists that ameba infection can persist for a considerable period (1). This is well documented in travelers, who usually develop amebic liver abscess months or even years after returning from an area of endemicity (18, 21). On the other hand, studies from South Africa and Bangladesh suggest that intestinal infections with *E. histolytica* are short-lived, as nearly all of the study subjects spontaneously cleared their infections within a few months (10, 15).

Blessmann et al. have recently reported on an area of amebiasis endemicity in Vietnam with an *E. histolytica* prevalence of about 10% in adults (3, 4). Here we report on a longitudinal study of the infection dynamics in this population over a period of 15 months. This study used a recently developed, highly sensitive and specific direct stool PCR assay for parasite detection as well as for genetic typing of individual *E. histolytica* infections during follow-up.

MATERIALS AND METHODS

Study site and study subjects. The study was performed between April 2000 and September 2002. It was carried out in a specific area of the Phu Cat commune of Hué City, Vietnam, which is known for its high *E. histolytica* prevalence (3, 4). This area comprises the very densely populated triangle between Chi Lang Street, Bach Dang Channel, and the Perfume River. All 961 households within this triangle were randomized, and 20% of the households were visited. A total of 491 adult household members agreed to participate in a longitudinal study of *E. histolytica* intestinal infection. The study was approved by the Scientific Council of Education, Training, and Ethics of Hué Medical School. Written informed consent was obtained from all study participants. During the observation period there was some loss to follow-up. A total of 383 of the 491 participants (78%) provided at least two consecutive stool specimens, collected in April 2000 and April 2001, respectively. From a number of participants, additional specimens were collected in July 2000 and August 2001. In addition to the 383 randomly selected participants, another 57 individuals from Phu Cat, from a group enrolled for a treatment study described previously (5), were included. These 57 individuals were identified as *E. histolytica* carriers in July 2001. Forty-four of them were treated with a luminal antiamebic agent and were parasite negative in August 2001, whereas the remaining 13 refused medication. In September 2002, all 57 carriers were reinvestigated for intestinal *E. histolytica*

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TABLE 1. Results of follow-up of 383 individuals analyzed for intestinal *E. histolytica* infection by fecal PCR in April 2000 and April 2001

Result in April 2001	No. of persons (%) with the following result in April 2000:		Total no. of persons (%)
	E. histolytica positive	*E. histolytica* negative	
E. histolytica positive	28 (7.3)	14 (3.7)	42 (11.0)
E. histolytica negative	15 (3.9)	326 (85.1)	341 (89.0)
Total	43 (11.2)	340 (88.8)	

infection. For all participants with a positive *E. histolytica* fecal PCR at the end of the study, antiamebic treatment was provided.

Diagnosis of *E. histolytica* infection. Stool samples no older than 24 h were collected and either processed immediately or stored at −20°C. Infection was diagnosed by use of a species-specific real-time PCR, as previously described (3). DNA for PCR was extracted directly from fecal samples by using the QIAamp DNA stool minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

DNA typing of *E. histolytica*. Genetic typing was performed by PCR using the DNA directly extracted from fecal samples. Two pairs of primers were used, both of which flank polymorphic short-tandem-repeat-containing regions of the *E. histolytica* genome: locus 5-6 (primers 5A and 6A [23, 24]) and locus NK (primers NK5 and NK3 [I. K. M. Ali, M. Zaki, and C. G. Clark, unpublished data]). Amplification used BioTaq polymerase (Bioline, London, England) and consisted of 30 cycles of 1 min at 94°C, 1.5 min at 56 or 55°C for 5-6 or NK, respectively, and 2 min at 72°C, with a final extension of 5 min at 72°C. Products were separated in 1.8% agarose gels and Tris-borate-EDTA buffer, stained with ethidium bromide, and photographed. A 100-bp ladder (Promega UK, Ltd., Southampton, England) was included in all gels as a size marker.

Statistical analysis. All data collected were computer coded and analyzed by use of Sigma-Stat (SAS software; Jandel Scientific, Erkrath, Germany). Binomial or χ^2 tests were used for comparisons between two groups. A P value of <0.05 was considered significant.

RESULTS

To evaluate the dynamics of intestinal *E. histolytica* infection, a longitudinal study was performed with a group of 383 randomly selected adult individuals (177 males and 206 females) living in the Phu Cat commune of Hué City, Vietnam. Stool samples were collected from all of the subjects in April 2000 and April 2001 and were analyzed for the presence of *E. histolytica* by direct fecal PCR. The results indicated a relatively high prevalence of the parasite and a rather stable epidemiological situation: 43 (11.2%) of the subjects were parasite positive in April 2000, and 42 (11.0%) were positive in April 2001 (Table 1). There were minor differences in prevalence between males and females (10.2 versus 12.1%), but there were no age differences between infected and noninfected individuals (mean age, 38.5 years; range, 21 to 60 years). Interestingly, only 4.1% (14 of 340) of the subjects negative for *E. histolytica* in April 2000 were found to be infected 1 year later, whereas 65.1% (28 of 43) of the subjects positive in April 2000 were also positive in April 2001 ($P < 0.001$), suggesting a relatively low annual rate of new infection in the study population but a considerable period of parasite persistence in the infected individuals. The latter conclusion was further supported by analysis of additional samples. Thirty-four of the 43 individuals positive in April 2000 were also investigated in August 2000, at which time 29 (85.3%) were found to be infected. Of the 28 individuals positive in both April 2000 and April 2001, 25 were still positive in July 2001. Moreover, of the 14 individuals who were parasite negative in April 2000 but positive in April 2001, 12 (85.7%) were also positive in July 2001. Taken together, these results indicate a regular exponential decline of intestinal *E. histolytica* infections over time, at a rate of about 3% per month (Fig. 1, curve a). Notably, despite the high *E. histolytica* prevalence of about 11%, none of the 383 study participants developed clinical symptoms compatible with invasive intestinal amebiasis. However, one individual developed an amebic liver abscess during the observation period. This 45-year-old man was parasite negative in April 2000 but positive in April 2001. One month later (May 2001), he developed fever and abdominal pain. Hepatic ultrasound revealed an abscess within the right liver lobe, which was cured by oral administration of metronidazole.

Previous epidemiological studies have shown that for people living in Phu Cat, a number of variables such as level of education or living conditions have a considerable impact on the risk of *E. histolytica* infection (4). Thus, some individuals in the study area may have a higher probability than others of becoming infected with the parasite. Accordingly, the reinfection rate in subjects previously infected with *E. histolytica* might be substantially higher than that predicted for the study population. To test this, a group of 57 individuals from Phu Cat who were *E. histolytica* positive in July 2001 were reinvestigated in September 2002. Of these, 44 had been treated with a luminal amebicidal agent and were subsequently parasite negative, as revealed by two consecutive fecal PCRs performed in August 2001, 10 and 20 days after termination of therapy. In contrast, the remaining 13 individuals refused medication and therefore did not receive any antiamebic treatment during the 14-month observation period. The results of fecal PCR indicated that 11.4% (5 out of 44) of the successfully treated patients were again parasite positive in September 2002, suggesting an annual new infection rate in this group that is about 3 times higher than that predicted in the study population (11.4 versus 4.1%). However, a significantly higher rate of infection was found in the group of individuals who refused treatment, as 8 out of 13 (61.5%; $P < 0.001$) were positive in September 2002, which again is consistent with a decrease in the number of intestinal *E. histolytica* infections of about 3% per month.

To further investigate whether the high infection rate during follow-up was indeed the result of parasite persistence and not due to reinfection, we made use of highly polymorphic loci within the *E. histolytica* genome that have been shown to allow typing of individual ameba strains (22, 23). These loci consist of variable numbers of short tandem repetitive sequences that can be amplified by PCR using primers derived from regions flanking the repeats. Two different loci (NK and 5-6) were examined by using extracted fecal DNA from all of the 28 individuals who were *E. histolytica* positive in two consecutive samples obtained in April 2000 and April 2001. Additional samples from August 2000 and July 2001 from eight of the subjects who were parasite positive in all four consecutive samples during the 15-month observation period were also included. Thus, a total of 144 PCRs were performed, of which 138 (95.8%) revealed specific amplification products (Table 2). As expected, a considerable degree of fragment length polymorphism among the amebae of the various subjects was detected. A total of 11 different patterns for locus NK and 14

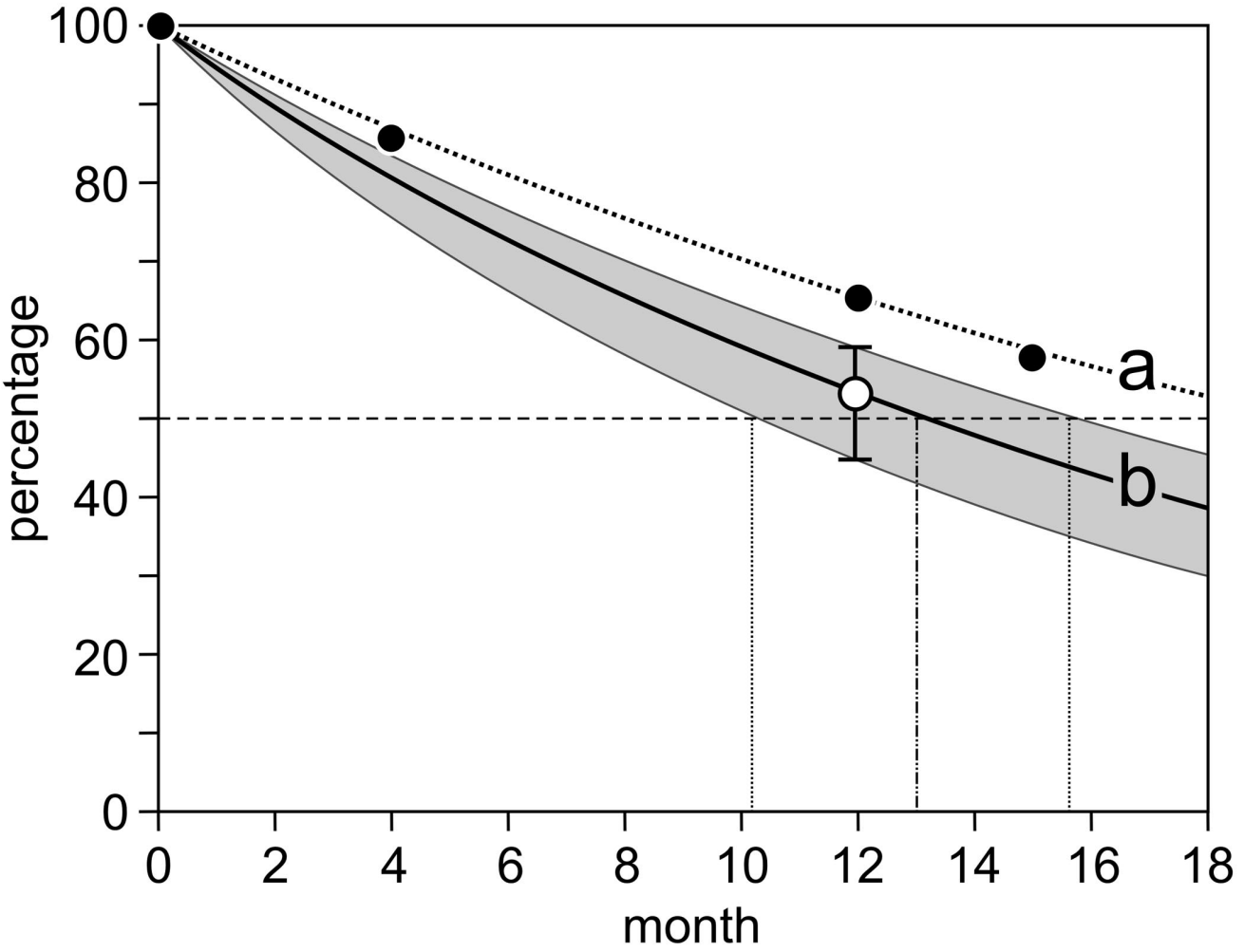

FIG. 1. Time course of intestinal *E. histolytica* infection in asymptomatic carriers and half-life of infection. A total of 46 *E. histolytica* carriers (100%) were identified by fecal PCR in April 2000 (time point, 0 months) and were monitored over a 15-month observation period. Curve a represents the relative number of individuals infected with the parasite at various time points according to the PCR results obtained during follow-up in August 2000 (4 months), April 2001 (12 months), and July 2001 (15 months). Curve b is a modification of curve a, which has been corrected for reinfection; in addition, the 95% confidence intervals have been included. The annual reinfection rate was determined by two independent approaches: (i) analysis of changes in DNA fingerprints of *E. histolytica* infections during follow-up and (ii) analysis of new infections in previous *E. histolytica* carriers 13 months after successful antiamebic therapy. Time points at which 50% of individuals have lost their infections are indicated by dotted lines.

different patterns for locus 5-6 were found. When the results from both loci were combined, a total of 20 different genotypes were identified. However, despite the high degree of variability between samples from different individuals, the DNA patterns obtained by using consecutive samples from the same individual were found to be identical in most cases (Table 2 and Fig. 2). Only in 5 of the 28 subjects did the pattern change between April 2000 and April 2001, and in 1 of the 8 cases in which four samples were investigated (subject 72-1) an additional change was observed between April 2001 and July 2001 (Table 2). These data strongly suggest that the vast majority of individuals had persistent infections with the same *E. histolytica* variant and that only a small proportion were reinfected during the observation period. Taking into account that 15 of the 43 individuals (34.9%) infected with *E. histolytica* in April 2000 lost the infection and 5 (11.6%) were reinfected within 1 year, 23 of the subjects (53.5%) are considered to have been persistently infected, which indicates a mean half-life of infection of 12.9 months (95% confidence interval, 10.2 to 15.6 months) (Fig. 1, curve b).

DISCUSSION

This survey revealed three major findings. First, there was a considerable period of parasite persistence in adults asymptomatically infected with *E. histolytica*. Second, the vast majority of carriers remained asymptomatic during the 15-month observation period. Third, the various *E. histolytica* genotypes were stable during the course of the infection.

The study was performed primarily to determine the dynamics of *E. histolytica* infection in a population from a setting in which the parasite is endemic rather than to determine vari-

TABLE 2. DNA fingerprints of *E. histolytica* infections during follow-up

Subject ID[a]	Stool collection date[b] (mo/yr)	PCR band(s) (bp)	
		NK	5-6
10-2	4/2000	550, 780	330, 420
	4/2001	550, 780	330, 420
20-2	4/2000	550, 750	480
	4/2001	550, 750	480
46-5	4/2000	550, 720	480
	8/2000	550, 720	480
	4/2001	550, 720	480
	7/2001	550, 720	480
72-1	4/2000	550, 620	430, 520
	8/2000	550, 620	430, 520
	4/2001	550, 620	430, 520
	7/2001*	570, 620	400
72-2	4/2000	550, 780	350, 450
	8/2000	Negative	Negative
	4/2001	550, 780	350, 450
	7/2001	550, 780	350, 450
84-3	4/2000	550, 750	480
	4/2001	550, 750	480
85-3	4/2000	550, 750	330, 420
	8/2000	550, 750	330, 420
	4/2001	550, 750	330, 420
	7/2001	550, 750	330, 420
92-1	4/2000	550, 780	350, 450
	8/2000	550, 780	350, 450
	4/2001	550, 780	350, 450
	7/2001	550, 780	350, 450
94-6	4/2000	550, 780	330, 480, 680
	4/2001*	550, 700	330, 670, 740
97-2	4/2000	550, 750	480
	8/2000	550, 750	480
	4/2001	Negative	Negative
	7/2001	550, 750	480
136-1	4/2000	550, 780	480, 550
	4/2001*	550, 780	330, 420
148-5	4/2000	540, 690	460, 520
	8/2000	540, 690	460, 520
	4/2001	540, 690	460, 520
	7/2001	540, 690	460, 520
148-7	4/2000	550, 750	330, 420
	8/2000	550, 750	330, 420
	4/2001	550, 750	330, 420
	7/2001	550, 750	330, 420
215-2	4/2000	500	Negative
	4/2001	500	Negative
215-4	4/2000	550, 720	500
	4/2001	550, 720	500
217-10	4/2000	550, 780	350, 450
	4/2001	550, 780	350, 450
226-2	4/2000	550, 750	500, 550
	4/2001	550, 750	500, 550
226-3	4/2000	550, 680, 850	350, 420, 450
	4/2001*	550, 800	350, 450
231-1	4/2000	550, 750	500
	4/2001	550, 750	500
233-7	4/2000	500	350, 450
	4/2001*	550, 780	350, 450
242-1	4/2000	550, 700	780
	4/2001	550, 700	780
484-1	4/2000	550, 780	350, 450
	4/2001	550, 780	350, 450
489-4	4/2000	550, 780	350, 450
	4/2001	550, 780	350, 450
493-4	4/2000	550, 750	340, 430
	4/2001*	550, 620	420, 500
493-5	4/2000	550, 620	420, 500
	4/2001	550, 620	420, 500
498-2	4/2000	550, 720	500
	4/2001	550, 720	500
499-2	4/2000	500, 700	Multiple bands
	4/2001	500, 700	Multiple bands
509-2	4/2000	550, 630	450, 520
	4/2001	550, 630	450, 520

[a] ID, identification number.
[b] Asterisks next to dates indicate changes in DNA fingerprints.

ables that may influence clinical outcomes. Previous studies have already shown that about 10% of the residents from Phu Cat, Hué, are infected with *E. histolytica* but that the annual incidence of amebic liver abscess is only about 0.7% in adult males and considerably lower in children and adult females (3, 4). Thus, the single amebic liver abscess observed during the study was within the range of what was expected for a sample size of 383 individuals of which only 177 were males.

To our knowledge only two studies of the time course of intestinal *E. histolytica* infection in untreated carriers have been reported (10, 15). These studies, conducted in areas of amebiasis endemicity in South Africa and Bangladesh, respectively, suggested a much faster clearance of the parasite and a considerably higher rate of reinfection. However, besides differences in geographic and local conditions, the individuals enrolled in the South African and Bangladeshi studies were considerably younger, comprising mainly children and young adults aged ≤20 years, whereas our study subjects from Vietnam were all >20 years old (mean age, 38.5 years). Whether children are able to clear *E. histolytica* more rapidly remains to be determined. However, children in areas of endemicity usually have considerably more episodes of diarrhea, which may

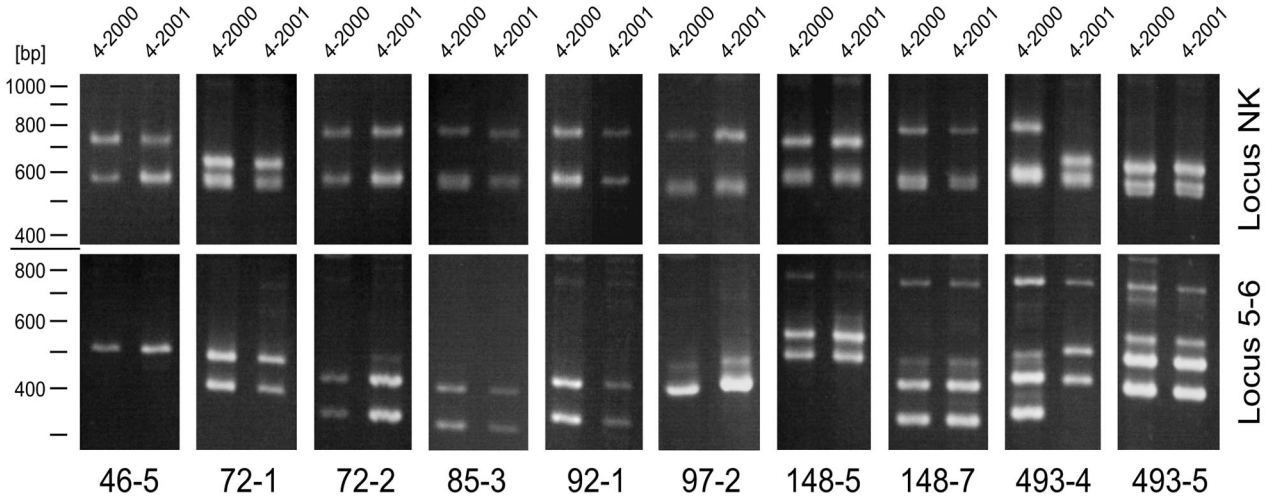

FIG. 2. DNA fingerprints of a representative number of *E. histolytica* organisms during follow-up. Gel photographs of PCR-amplified fecal DNA for locus NK and locus 5-6 of *E. histolytica* from 10 individuals infected with the parasite in April 2000 and April 2001 are shown. Note that the band patterns for different individuals are highly divergent. However, only for one individual (493-4) did the parasite DNA pattern change between April 2000 and April 2001.

help to "flush out" the parasite more rapidly. Ten percent of individuals in the South African study and about 3% in the Bangladeshi study showed symptoms of dysentery associated with amebic infection, but none of the study subjects developed an amebic liver abscess during the 12-month observation period. Thus, in agreement with the results presented here, it can be concluded that the vast majority of *E. histolytica* carriers from areas of endemicity do not develop amebic disease. Whether this is also true for nonimmune travelers remains to be determined. However, even a relatively low risk for the development of amebic disease, in a range of 2 to 10%, argues for treatment of *E. histolytica* carriers, as suggested previously (10, 20).

It has been repeatedly reported that travelers usually develop amebic liver abscess several months after returning from areas of endemicity and in some cases even after several years (18, 21), which suggests a considerable period of parasite persistence. Our average half-life of infection of about 13 months is in agreement with the clinical observations of long latencies between infection and the onset of amebic liver abscess. Extrapolation of the calculated 13-month half-life suggests that even after 5 years, 5% of infected individuals will still harbor the parasite. This long period of persistence may also explain the observed elevated risk for development of recurrent amebic liver abscesses (4), as in most countries where the parasite is endemic, treatment of invasive amebiasis usually does not include a luminal amebicidal agent.

A particular problem for time course studies on parasite infections in areas of endemicity is the correct determination of the reinfection frequency. We have analyzed the reinfection rate by two independent approaches. First, reappearance of the parasite was detected in individuals successfully treated for *E. histolytica* infection, and second, changes in genetic "fingerprints" of *E. histolytica* during the course of infection were analyzed. As both methods revealed rather similar results, the calculated annual reinfection rate of about 11.5% in those individuals previously infected with *E. histolytica* seems to be a reliable value for the population studied. This value is about 3 times higher than the infection rate in individuals not recently infected with the parasite, which is in line with previous findings that the risk of *E. histolytica* infection is not evenly distributed among the study subjects but is dependent on a number of confounding factors, such as level of education or sanitary conditions (4).

Several studies have already demonstrated considerable genetic polymorphism among *E. histolytica* isolates, even from limited geographic areas (2, 13, 16, 22, 23, 24). However, only two of these studies have monitored the parasite isolates longitudinally (16, 24). Thus, it was of particular interest that in addition to a high degree of polymorphism among *E. histolytica* parasites from Phu Cat, Hué, the patterns were genetically stable during asymptomatic infection over an observation period of at least 12 to 15 months. Genetic changes in a very small number of cases were most likely the result of new infections rather than spontaneous mutations, as two different independent genomic loci were investigated, and in five of six cases in which changes occurred, both loci were affected. The high genetic stability of amebae within an infected subject may indicate that *E. histolytica* infections in Phu Cat are clonal or that in the case of mixed infections, one subpopulation outgrows the other and establishes a long-lasting infection. Thus, we cannot completely rule out the possibility that the analysis of genetic fingerprints has missed new infections that were unable to compete with *E. histolytica* parasites already present in the bowel.

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