JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1990, p. 680–684
0095-1137/90/040680-05$02.00/0

Vol. 28, No. 4

Differentiation of Clinical Isolates of *Entamoeba histolytica* by Using Specific DNA Probes

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Received 10 October 1989/Accepted 26 December 1989

Most individuals infected with *Entamoeba histolytica* are reported to be clinically asymptomatic. On the basis of the electrophoretic migration of hexokinase and phosphoglucomutase isoenzymes, two groups of *E. histolytica* isolates have been classified. Those derived from symptomatic cases were found to have fast-migrating hexokinase bands and were labeled pathogenic. The others, isolated from cyst passers, had (in most cases) slow-migrating bands and were called nonpathogenic. Differences between these two groups of *E. histolytica* were found recently at the DNA level. Two sets of different DNA probes derived from tandemly repeated sequences present in extrachromosomal circular DNA elements in each group of *E. histolytica* were characterized. Using these probes with procedures for direct hybridization of trophozoites on nylon membranes, we could correctly correlate hexokinase electromobility with the DNA hybridization signal of 81 different isolates of *E. histolytica*. The advantages of using DNA probes lie in their sensitivity (fewer than 200 trophozoites can be detected) and specificity. The probes hybridized only with amebae from the *E. histolytica* species and not with other enteric protozoa and can thus be useful as a diagnostic tool.

Most individuals infected with *Entamoeba histolytica* are reported to be asymptomatic carriers. Invasive amebiasis occurs in a small proportion of cases (16). Biochemical and immunological differences between isolates of *E. histolytica* obtained from symptomatic and asymptomatic cases have been found. Martinez-Palomo and Gonzales-Robles (7) first showed differences in agglutinability by the lectin concanavalin A. Reeves and Bischoff (10) and later Sargeaunt and Williams (14) showed that the band migration in gel electrophoresis of hexokinase and phosphoglucomutase isoenzymes correlated very closely with the clinical situation of the patient. On the basis of their analyses of hundreds of *E. histolytica* isolates, they named those having fast-migrating hexokinase band patterns and missing the α band of phosphoglucomutase (all originating from symptomatic cases) as pathogenic strains and those with slow-migrating hexokinase patterns as nonpathogenic.

Additional differences between these two forms of *E. histolytica* were recently detected. Strachan et al. (15) have described two monoclonal antibodies which react only with invasive (pathogenic) isolates; more recently, Garfinkel et al. (4) have reported on two types of DNA probes which can selectively hybridize with each form of *E. histolytica*. These DNA probes originate from tandemly repeated sequences found in extrachromosomal circular DNA molecules (5) which are present in each of the two forms of *E. histolytica*. Only a very limited number of laboratory strains was initially tested with these DNA probes (4). We now report the successful identification and differentiation of 81 different isolates of *E. histolytica* from several parts of the world.

MATERIALS AND METHODS

Isolates of *E. histolytica*. Isolates were collected and obtained from various locations: Thailand, Germany, England, and the United States. Isolates obtained from the first two locations were taken from fecal samples at local hospitals, inoculated either in Robinson medium (11), Dobell-Laidlaw medium (3), or TYSGM-9 monophasic medium (1), and cultured at 37°C. Isolates used in this study obtained from the United States were originally collected from different locations during various periods. They were all subcultured at the National Institutes of Health (NIH) and later cryopreserved. For this study, most of the strains were recovered from liquid nitrogen and grown either in TYSGM-9 xenic culture medium or under axenic conditions in TYI-S-33 (2). Most of the isolates were subcultured until sufficient growth was obtained for isoenzyme electrophoretic analysis (8) and for preparation of DNA dot blots (4). Details on the number of isolates, their sources, and their zymodemes are presented in Tables 1, 2, and 3. Non-*E. histolytica* strains were obtained from the well-characterized collection cryopreserved at NIH.

Preparation of DNA samples. Culture tubes containing approximately 10^5 trophozoites were chilled in an ice-water bath for 10 min and sedimented by centrifugation (600 × *g*, 5 min). The sediment containing the trophozoites was suspended in cold 10-ml phosphate buffered saline (pH 7.2) and sedimented again by centrifugation as described above. The sediment was suspended in a solution (200 μl) containing NaOH (0.5 M) and NaCl (1.5 M) for 30 min, which denatures the trophozoite DNA (6). Portions (20, 10, and 5 μl) were spotted onto a nylon membrane filter (Zetapore) and allowed to dry. The nylon membrane containing the denatured DNA was then placed on a Whatman 3MM filter paper that was presoaked with a neutralizing solution consisting of Tris buffer (0.5 M, pH 7.2) containing NaCl (1.5 M). The nylon membrane was blotted and dried over a Whatman 3MM paper a number of times to ensure neutralization. In some experiments, the denaturation and neutralization steps were done directly on the nylon filter. A suspension of trophozoites in culture medium was passed through a Buchner funnel containing a nylon membrane filter (0.45 μm). The nylon

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TABLE 1. Differentiation of *E. histolytica* and other *Entamoeba* strains by DNA probes[a]

Strain or isolate	Source	Hexokinase migration pattern	Interaction with MAbs[b]	DNA hybridization with probe:		Comments
				P145	B133	
NIH:200	NIH	Fast	+	+	−	Axenic culture
HM-1:IMSS	IMSS, NIH	Fast	+	+	−	Axenic culture
Rahman	LSHTM	Fast	ND	+	−	Axenic (Avirulent) culture
HK-9	NIH	Fast	ND	+	−	Axenic culture
CDC:0784:4	CDC	Fast	ND	+	−	Axenic, zymodeme switch (8)
SAW 1734R clAR	LSHTM	Fast	+	+	−	Axenic, zymodeme switch (9)
CDC:0784:4	CDC	Fast	ND	+	−	Reassociated with bacteria
CDC:0784:4	CDC	Slow	−	−	+	Original xenic culture
SAW 1734R clAR	LSHTM	Slow	−	−	+	Original cloned culture
SAW 142RR clA	LSHTM	Slow	ND	−	+	Original cloned culture
SAW 755 CR clB	LSHTM	Fast	ND	+	−	Original cloned culture
SAW 891 R clB	LSHTM	Fast	ND	+	−	Original cloned culture
HI 1295:AIIMS	AIIMS	Fast	ND	+	−	
NIH:0283:1	NIH	Fast	ND	+	−	
HI-1372:AIIMS	AIIMS	Fast	ND	+	−	
Rustom	LSHTM	Slow	ND	−	+	
SAW 937R clA	LSHTM	Slow	ND	−	+	Original cloned culture
NIH:0581:1	NIH	Slow	ND	−	+	
Dientamoeba fragilis Bi/Pa	NIH	ND	ND	−	−	Xenic culture, non-*E. histolytica*
Entamoeba moshkovsky FIC	NIH	ND	ND	−	−	Axenic culture, non-*E. histolytica*
Entamoeba invadens 165	NIH	ND	ND	−	−	Axenic culture, non-*E. histolytica*
Blastocystis hominis	NIH	ND	ND	−	−	

[a] Abbreviations: LSHTM, London School of Hygiene and Tropical Medicine; AIIMS, All India Institute of Medical Sciences, New Delhi, India; CDC, Centers for Disease Control, Atlanta, Ga; IMSS, Instituto Mexicano del Seguro Social; ND, not done.
[b] Reaction with monoclonal antibodies 22.3 and 22.5, which are specific for pathogenic zymodemes (15).

TABLE 2. Differentiation between pathogenic and nonpathogenic *E. histolytica* isolates[a]

No.	Strain or isolate	Source	Hexokinase migration pattern	Interaction with MAbs[b]	DNA hybridization with probe:		Zymodeme pattern	Comments
					P145	B133		
1	NIH:200	NIH	Fast	+	+	−	II	Dysentery
2	SI	LSHTM	Fast	+	+	−	II	Hematophagous amebae in stool
3	8672	LSHTM	Slow	−	−	+	I	NIS
4	0478	LSHTM	Fast	+	+	−	II	Dysentery
5	C29	LSHTM	Slow	−	−	+	I	NIS
6	88/230	LSHTM	Slow	−	−	+	I	NIS
7	88/216	LSHTM	Slow	ND	−	+	ND	NIS
8	X2	LSHTM	Fast	+	+	−	II	Diarrhea
9	S1	LSHTM	Fast	ND	+	−	XIV	India; gastroenterology clinic; abdominal pain
10	S2	LSHTM	Fast	ND	+	−	XIV	India; dysentery
11	379	LSHTM	Slow	−	−	+	I	NIS
12	S10	LSHTM	Slow	ND	−	+	I	India; dysentery. Also contains *Blastocystis hominis*
13	S3	LSHTM	Fast	+	+	−	XIV	India; dysentery
14	S11	LSHTM	Slow	ND	−	+	I	India; gastroenterology clinic; dysentery
15	C1	LSTHM	Fast	ND	+	−	XIV	India; dysentery
16	C2	LSHTM	Fast	+	+	−	XIV	India; dysentery
17	S8	LSHTM	Fast	+	+	−	XIV	India; gastroenterology clinic; irritable bowel syndrome
18	S5	LSHTM	Fast	+	+	−	XIV	India; dysentery
19	S7	LSHTM	Fast	+	+	−	XIV	India; gastroenterology clinic; abdominal pain
20	419	LSHTM	Slow	ND	−	+	I	NIS
21	395	LSHTM	Slow	ND	−	+	I	NIS
22	S6	LSHTM	Fast	+	+	−	XIV	India; dysentery
23	414	LSHTM	Slow	ND	−	+	I	NIS
24	390	LSHTM	Slow	ND	−	+	I	NIS
25	418	LSHTM	Slow	−	−	+	I	NIS

[a] Abbreviations: LSHTM, London School of Hygiene and Tropical Medicine; NIS, no intestinal symptoms; ND, not done.
[b] Reaction with monoclonal antibodies 22.3 and 22.5, which are specific for pathogenic zymodeme (15).

TABLE 3. Differentiation between pathogenic and nonpathogenic *E. histolytica* isolates obtained from patients[a]

Strain or isolate	Source	Hexokinase migration pattern	DNA hybridization with probe: P145	DNA hybridization with probe: B133	Comments
070	Thai	Fast	+	−	Bloody stool
071	Thai	Fast	+	−	Jaundice patient, NIS
078	Thai	Fast	+	−	Malaria patient, NIS
080	Thai	Fast	+	−	Minor symptoms
090	Thai	Fast	+	−	Bloody stool
1-HAN	BNTM	Slow	−	+	NIS
2-KOR	BNTM	Fast	+	−	Liver abscess
3-DAL	BNTM	Slow	−	+	NIS
4-MOR	BNTM	Fast	+	−	Diarrhea
5-KAM	BNTM	Slow	−	+	NIS
6-SCHU	BNTM	Slow	−	+	NIS
7-MON	BNTM	Slow	−	+	NIS
8-WEN	BNTM	Slow	−	+	NIS
10-PESCH	BNTM	Slow	−	+	NIS
11-HER	BNTM	Slow	−	+	NIS
12-KUE	BNTM	Slow	−	+	NIS
13-COD	BNTM	Slow	−	+	NIS
15-Scho	BNTM	Fast	+	−	Bloody diarrhea
16-MUT	BNTM	Fast	+	−	Diarrhea
17-BER	BNTM	Slow	−	+	NIS
18-RUE	BNTM	Slow	−	+	NIS
19-WOL	BNTM	Slow	−	+	NIS
21-SCHMI	BNTM	Slow	−	+	NIS
22-THE	BNTM	Slow	−	+	NIS
23-REI	BNTM	Fast	+	−	Asymptomatic, seronegative against *E. histolytica*
24-KAN	BNTM	Slow	−	+	NIS
25-GUE	BNTM	Fast	+	−	Chronic diarrhea
26-BECH	BNTM	Slow	−	+	NIS
27-GROE	BNTM	Fast	+	−	Bloody diarrhea
28-ECK	BNTM	Slow	−	+	NIS
29-WIE	BNTM	Slow	−	+	NIS
31-RHO	BNTM	Slow	−	+	NIS
33-BRUE	BNTM	Fast	+	−	Chronic diarrhea
34-MEY	BNTM	Slow	−	+	NIS

[a] Abbreviations: NIS, no intestinal symptoms; BNTM, Bernhard-Nocht Institute for Tropical Medicine, Hamburg, Federal Republic of Germany; Thai, Mahidol University Medical School, Bangkok, Thailand.

membrane was then placed on a Whatman 3MM filter paper soaked with the NaOH denaturing solution described above and then placed in the neutralizing solution. After neutralization, the membrane filters were numbered and cut into halves, and each half was hybridized under stringent conditions with either the B133 or P145 radiolabeled probes. After being hybridized and washed, the two halves of each of the membrane filters were rejoined and placed opposite each other for X-ray exposure (4 h) so that the filters in the upper part were those hybridized with P145 and the filters in the lower part were those hybridized with B133. Most of the experiments were done blindly. The different trophozoite samples were placed on the nylon filters by one investigator, and the person who made the hybridization did not know the isoenzyme pattern of the isolate being tested. For quantitative determinations, portions of DNA from different samples containing the equivalent of 10^4, 10^3, and 10^2 trophozoites were spotted on the nylon membranes.

Preparation of DNA probes. The two DNA probes used in this study were obtained as follows. P145 was isolated from the pathogenic strain *E. histolytica* HM-1:IMSS by partial digestion with restriction enzyme *Pvu*I (New England BioLabs, Inc., Boston, Mass.) of the tandemly repeated elements found in the extrachromosomal circular DNA molecules. A restriction fragment (H16) containing these repetitive elements (4) was cloned into *Escherichia coli*, and the released inserts were digested with *Pvu*I. The digest was separated by agarose electrophoresis, and the bands containing the dimer and trimers of 145 base pairs were extracted from the gel and used for labeling. A similar procedure was employed for obtaining the DNA probe B133 from the nonpathogenic strain SAW 1734R clAR (4). A plasmid (B3.0) (4) containing the insert with the repetitive 133-base-pair elements was partially digested with restriction enzyme *Bam*HI (New England BioLabs) and the bands containing the dimers and trimers of the 133 base pairs were extracted.

Labeling of DNA probes. Labeling was done with a random priming DNA labeling kit according to the instructions of the manufacturer (Boehringer GmbH, Mannheim, Federal Republic of Germany). The DNA to be labeled was heated at 100°C for 5 min and cooled in an ice bath for 10 min, and a mixture of three nucleoside triphosphates (0.5 mM each) plus the random primer hexanucleotide mixture was added together with a labeled nucleotide [α-^{32}P]dCTP (3,000 Ci/mM; Dupont, NEN Research Products) and the Klenow enzyme. Incubation was done at 37°C for 60 min. The product was separated by elution from free-labeled nucleotide on a 5-ml Sephadex G-50 column (5.0 by 0.9 cm). The labeled DNA probe was heated to 100°C for 5 min and cooled in an ice bath before being added to the hybridization mixture.

Hybridization of *E. histolytica* trophozoites with DNA probes. DNA samples on nylon membranes were baked for 2 h at 80°C under vacuum. The membranes were then washed in a 1/10 diluted solution of sodium citrate (15 mM) and sodium chloride (300 mM) (SSC) (6) containing 0.5% sodium dodecyl sulfate (SDS) at 65°C for 20 min. (6). Prehybridization was done in a solution consisting of 50% formamide and 6× SSC as well as Denhart solution (6) (consisting of bovine serum albumin, Ficoll, and polyvinylpyrrolidone, 0.025% each; Sigma Chemical Co., St. Louis, Mo.), 0.3% SDS, and 100 μg of herring sperm DNA (Boehringer GmbH) per ml for 4 h at 42°C (4). The solution was then changed to a fresh one, and the radioactively labeled probe was added at a concentration of between 1×10^5 and 2×10^5 cpm/ml of solution. Hybridization was carried out at 42°C for 14 h. The filters were rinsed under stringent conditions three times with 2× SSC containing 0.1% SDS at room temperature and then with 0.1× SSC–0.1% SDS and incubated at 65°C for 20 min in the same solution. After being rinsed further in the solution described above, the filters were dried at ambient conditions and exposed to X-ray film for 4 to 16 h at −70°C.

Hexokinase isoenzyme pattern migration on agarose gel electrophoresis was done as described earlier (4). Some of the trophozoites were also tested for interaction with monoclonal antibodies 22.3 and 22.5 specific for pathogenic zymodemes by immunofluorescence as described previously (15).

RESULTS AND DISCUSSION

Samples of DNA from each ameba isolate were hybridized with both of the DNA probes (B133 and P145). Whenever *E. histolytica* trophozoites were present, only one of the probes gave a positive signal (Fig. 1). The signals obtained correlated in all cases with the pathogenic or nonpathogenic hexokinase migration pattern of the different isolates (Tables

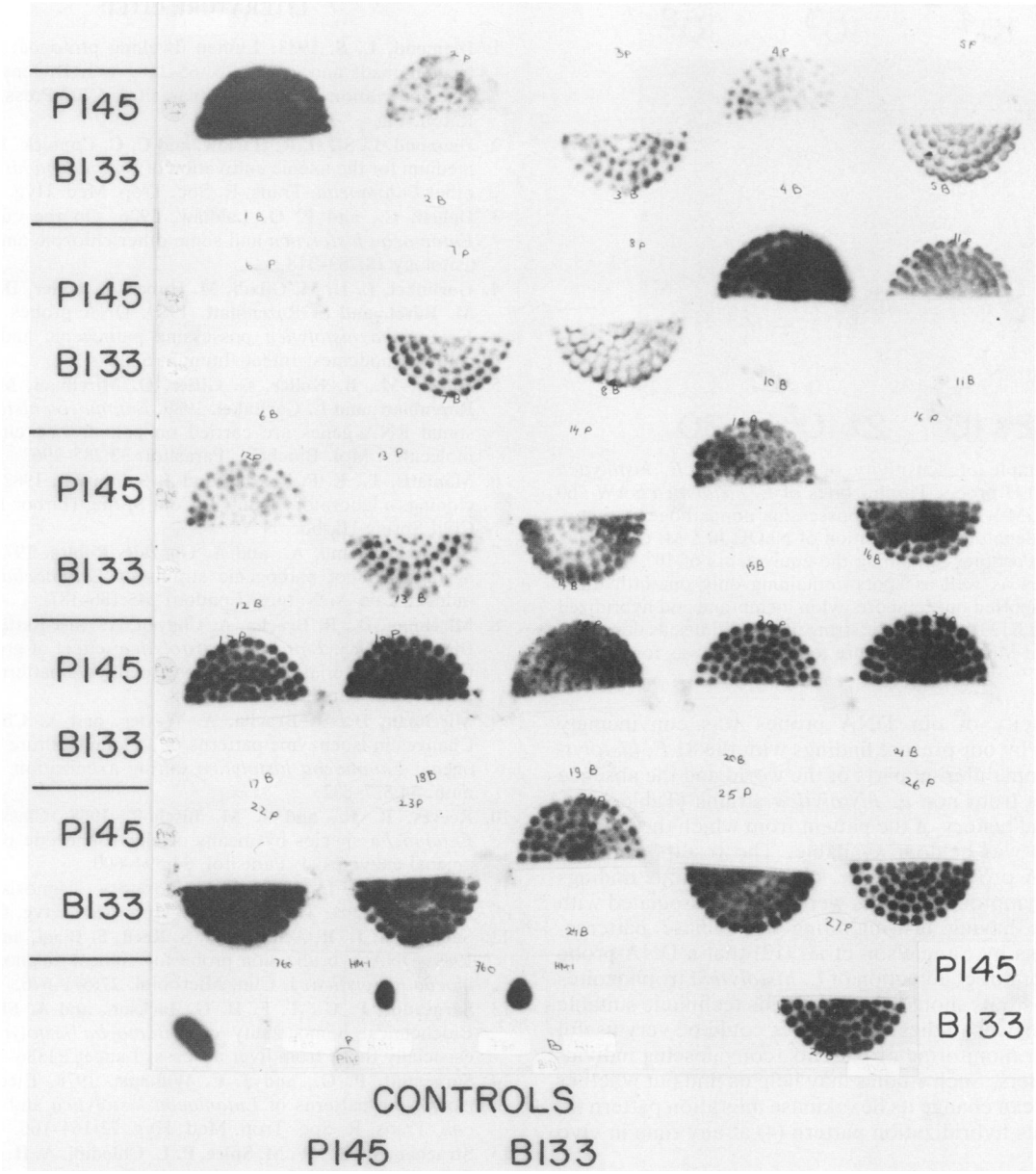

FIG. 1. Differentiation of *E. histolytica* isolates by hybridization with DNA probes. Trophozoites (approximately 5×10^5 in 5 ml of medium) were filtered through a Zetapore nylon membrane with a Buchner funnel and denatured in situ by placing the membrane on top of a Whatman 3MM filter paper soaked with NaOH (0.5 M) and NaCl (1.5 ml) as described in Materials and Methods. After neutralization, the membrane filters were numbered and cut into halves, and each half was hybridized under stringent conditions with either the B133 (B) or P145 (P) radiolabeled probes. After being hybridized and washed, the two halves of each of the membrane filters were rejoined and placed opposite each other for X-ray exposure (4 h) so that the filters in the upper part were those hybridized with P145 and the filters in the lower part were those hybridized with B133. Positive signals were obtained for each isolate with only one of the probes. Controls containing DNA dot spots from the well-characterized pathogenic strain HM-1:IMSS (for probe P145) and SAW 760 (for probe B133) were used as controls for each probe (bottom left).

1, 2, and 3). This was also true for the trophozoites of the two *E. histolytica* strains SAW 1734R clAR and CDC: 0784:4, which had changed their hexokinase pattern (from slow to fast migration) during the process of axenization in culture (8, 9) (Table 1). In all pathogenic isolates that were examined, the three tests coincided: the fast hexokinase migration pattern, the positive interaction with the monoclonal antibodies 22.3 and 22.5, which are specific for pathogenic zymodemes (15), and the hybridization signal with probe P145 (4).

The sensitivity of the hybridization assay using DNA from *E. histolytica* SAW 760 (nonpathogenic) and probe B133 was very good (Fig. 2). Signals could be observed even at dilutions in which DNA from only 200 trophozoites was present after exposures to an X-ray film for 16 h. Long exposures to the X-ray film (more than 72 h) revealed sometimes faint signals with the second probe (P145) also, suggesting that at least one copy of the alternative DNA repetitive sequence is present in all, if not most, of the *E. histolytica* isolates (4).

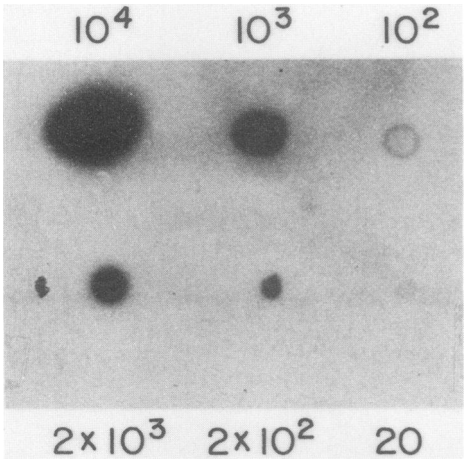

FIG. 2. Example of sensitivity of detection of *E. histolytica* DNA by the B133 probe. Trophozoites of *E. histolytica* SAW 760 grown in TYSGM-9 medium and possessing nonpathogenic zymodeme (4) were denatured in a solution of NaOH (0.5 M) containing NaCl (1.5 M). Portions containing the equivalents of 10^4, 10^3, and 10^2 trophozoites as well as spots containing only one-fifth these amounts were spotted on Zetapore nylon membrane and hybridized with ^{32}P-labeled B133 probe under stringent conditions, as described in Materials and Methods. Exposure to X-ray film was for 16 h.

The specificity of our DNA probes was convincingly demonstrated by our present findings with the 81 *E. histolytica* isolates from different parts of the world and the absence of any signals from non-*E. histolytica* strains (Table 1). A reliable clinical history of the patient from which the amebae were isolated was seldom available. The results obtained with the DNA probes, however, confirm previous findings (13) that the symptomatic cases were always associated with *E. histolytica* having fast-migrating hexokinase patterns. Recent findings by Samuelson et al. (12) that a DNA probe can be used for direct detection of *E. histolytica* trophozoites or cysts from stools should also make this technique suitable for epidemiological studies. The probes could be very useful for the regular monitoring of patients, convalescing individuals, and carriers. Such studies may help us find out whether *E. histolytica* can change its hexokinase migration pattern (8, 9) as well as its hybridization pattern (4) at any time in vivo in a host.

ACKNOWLEDGMENTS

This study was supported by grants from the European Communities Commission, the John D. and Catherine T. MacArthur Foundation, and the Wellcome Trust.

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