DETERMINATION OF THE PREVALENCE OF ENTAMOEBA HISTOLYTICA AND E. DISPAR IN THE PERNAMBUCO STATE OF NORTHEASTERN BRAZIL BY A POLYMERASE CHAIN REACTION

SANDRA M. B. PINHEIRO, ROSA M. CARNEIRO, IVANISE S. ACA, JOÃO I. IRMAO, MARCOS A. MORAIS JR., MARIA R. M. COIMBRA, AND LUIZ B. CARVALHO JR.
Laboratório de Imunopatologia Keizo Asami; Departamento de Medicina Tropical; Departamento de Medicina Social; Departamento de Genética, e Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil; Departamento de Pesca, Universidade Federal Rural de Pernambuco, Recife, Brazil

Abstract. Previous studies using methods varying from traditional serologic tests to molecular biology techniques have shown that in northeastern Brazil, Entamoeba dispar was more prevalent than E. histolytica. In this study, the prevalence was established by using E. histolytica stool antigen detection kits and a polymerase chain reaction (PCR) with genomic DNA extracted from cultured trophozoites in all four-nuclei, amoeba-positive samples from a population living in Macaparana in northeastern Brazil. Among 1,437 stool samples analyzed, only 59 (4.1%) were positive for four nuclei amoeba. However, all of these samples were negative in an immunoenzymatic assay for the presence of E. histolytica-specific galactose adhesin. Of 59 cultivated samples, only 31 showed trophozoites. Extraction of DNA from these 31 samples, followed by the PCR, showed that 23 samples (74.1%) were positive for E. dispar and no amplification was observed for pathogenic E. histolytica. The remaining eight samples were negative for both species. These findings are consistent with those previously reported.

INTRODUCTION

The protozoa Entamoeba histolytica is an intestinal parasite infecting 500 million people worldwide. Up to 100,000 deaths per year have been attributed to complications of amebiasis, notably amoebic liver abscesses. The prevalence of E. histolytica in developing countries is often assumed to be high, frequently without supporting data. Studies on E. histolytica carried out at the Laboratório de Imunopatologia Keizo Asami in Recife between 1988 and 1994 among low-income populations have shown differences in the prevalence of this organism in the northern, northeastern, southeastern regions of Brazil. The methodology used in these studies has ranged from traditional serologic tests, such as gel diffusion precipitin (GDP) and zymodemes, to molecular biology techniques such as restriction endonuclease digestion of amplified genomic DNA. These investigations showed the presence of both E. histolytica and E. dispar in the Amazon region (northern area) with a higher prevalence of E. histolytica, while E. dispar predominated in the northeastern region.

In contrast to these findings, E. histolytica has been reported in a community in Fortaleza in northeastern Brazil. The investigators detected the presence of serum antibodies specific for the Gal/GalNAc lectin of E. histolytica and suggested that this community was highly endemic for E. histolytica with infections rate similar to other developing nations. Despite this result, which differs from those obtained at the Laboratório de Imunopatologia Keizo Asami, the northeastern region seems to have a diverging parasitologic profile with regard to the presence of E. histolytica and E. dispar.

In recent years, a number of methods have been developed for the clear distinction of these two species. Immunoassays have been widely used in routine laboratory analysis. The gel diffusion precipitation test is considered by some researchers to be one of the most reliable serologic tests for the diagnosis of amebiasis. The enzyme-linked immunosorbent assay (ELISA) is also used in serodiagnosis method. However, this method cannot differentiate between a current and previous parasite infection, and is of limited value when examining individuals from endemic areas with high levels of circulating antibodies. Many antigens have been reported as being specific for the diagnosis of amebiasis. These include E. histolytica trophozoite antigens (HM-1 IMSS), pathogen-specific epitopes of the galactose adhesin of E. histolytica, single recombinant E. histolytica antigen (P1-EIA), and the 170-kD antigenic subunit of the amebal Gal/GalNAc-lectin. Although the use of a stool ELISA has been shown to be useful in routine diagnostic procedures, a comparative study on the use of this ELISA and a polymerase chain reaction (PCR) for the detection of E. histolytica and E. dispar indicated that the PCR was more useful than the ELISA.

Conversely, a number of DNA sequences have been used as targets for specific detection of E. histolytica using PCR technology. Ribosomal RNA molecules are the most commonly used targets, followed by restriction pattern analysis. In addition, genomic DNA has also been used in a diagnostic PCR. The primers specific for E. histolytica and E. dispar (P11 plus P12 and P13 plus P14, respectively) were found to show a sensitivity of 100%. The PCR technique is fast, safe, and constitutes an approach to obtain information about the occurrence of E. histolytica or E. dispar in the northeastern region of Brazil.

The purpose of this study was to determine the prevalence of E. histolytica and E. dispar by using E. histolytica stool antigen detection kits and a PCR with genomic DNA extracted from cultured trophozoites in a population located in Pernambuco State in northeastern Brazil.

MATERIALS AND METHODS

Samples. Aliquots of fresh, unpreserved stool obtained from 1,437 randomly selected individuals living in Macapa, Brazil were stored at 4°C. A one-gram portion was stored at −20°C for subsequent immunoenzymatic analysis. Macaparana is located in Pernambuco State, Brazil, on the limits of a sugarcane plantation area, 118 km from Recife (the capital of Pernambuco). It has a population of 22,494 inhabitants (13,518 and 8,976 in the urban and rural areas, respectively) occupying an area of 103 km². Illiteracy is very high (65.1%) among the population more than 10 years old. Young
people represent most of the population (46.5% of the population is less than or equal to 20 years old and 75% is less than 42 years old). The estimated family income is approximately U.S. $480 per year. The study protocol was reviewed and approved by the Hospital das Clínicas Ethics Committee of the Universidade Federal de Pernambuco, Brazil, and informed consent was obtained from each individual or responsible guardian.

**Microscopy analysis.** The presence of parasites in the samples was determined by various concentration methods.

**Immunoenzymatic assay.** The presence of *E. histolytica*-specific galactose adhesin was determined among the samples that were stored at −20°C and positive for the presence of four-nuclei amoeba with a commercially available kit (ELISA kit E. histolytica-II; Techlab, Inc., Blacksburg, VA). This kit is based on the monoclonal antibody–peroxidase conjugate specific for *E. histolytica* adhesin. According to the manufacturer’s instructions, a positive result was defined as an optical density reading > 0.05 after subtraction of the negative control optical density.

**Extraction of genomic DNA.** All four-nuclei, amoeba-positive samples were incubated with the Robinson’s medium at 37°C for 48 hours. Cultured trophozoites were centrifuged and resuspended in ethanol. Subsequently, trophozoites were centrifuged and resuspended in 200 μL of the solubilizing agent (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.5 mg of proteinase K) for two hours at 60°C. Genomic DNA was extracted with phenol-chloroform, precipitated with ethanol and 3 M sodium acetate, resuspended in TE buffer (0.01M Tris-HCl, pH 7.4, 2.5 mM EDTA) and stored at −20°C until PCR amplification.

**Polymerase chain reaction.** The PCR was conducted in a volume of 25 μL containing (final concentrations) 20 mM Tris-HCl, pH 8.4, 3.0 mM MgCl2, 50 mM KCl, 2.0 mM each of the four dNTPs, 10 pmol of each specific primer (p11 plus p12 and p13 plus p14), 2.0 units of Taq polymerase (Invitrogen, Carlsbad, CA), and approximately 50 ng of genomic DNA. The thermal cycles consisted of an initial denaturation at 94°C for one minute, followed by 30 cycles at 94°C for one minute, 59°C for 90 seconds, 72°C for 90 seconds, and a final extension at 72°C for five minutes. The PCR products were isolated by electrophoresis on 2% agarose gels containing ethidium bromide and the gels were photographed under ultraviolet light. Two DNA samples testing positive for each species were used as positive controls.

**RESULTS**

Among 1,437 stool samples analyzed by optical microscopy, only 59 (4.1%) were positive for the presence of four-nuclei amoeba, namely, *E. histolytica* or *E. dispar*. However, all 59 samples were negative in the immunoenzymatic assay for the presence of *E. histolytica*-specific galactose adhesin. Microscopic analysis also showed the presence of the following other microorganisms: *Entamoeba coli* (27), *Ascaris lumbricoides* and *Entamoeba coli* (4), *Ascaris lumbricoides* (3), *Entamoeba coli* and *Endolimax nana* (3), *Iodameba bütschlii* (2), *Trichuris trichiura* (1), *Endolimax nana* (1), *Ancilostomídeos* (1), *A. lumbricoides* and *T. trichiura* (1), *A. lumbricoides* and *Enterobius vermicularis* (1), *A. lumbricoides*, *I. bütschlii*, and *Entamoeba coli* (1), *Entamoeba coli*, Ancilostómeidos, and *I. bütschlii* (1), *Entamoeba coli* and *Schistosoma mansoni* (1), *I. bütschlii* and *Giardia lamblia* (1), *I. bütschlii* and *Entamoeba coli* (1), and no other parasites (10).

Of 59 cultivated samples positive for the presence of four nuclei, only 31 showed trophozoites. This was expected because there are reports describing the impractical and time-consuming nature of obtaining cultures from a large number of microscopy-positive samples.

Extraction of DNA from 31 samples, followed by the PCR, showed that 23 samples (74.19%) were positive for *E. dispar* as demonstrated by amplification of the species-specific fragment (100 basepairs). Conversely, no amplification was observed for pathogenic *E. histolytica* (Figure 1). The remaining eight samples were negative for both species. The absence of amplification of these samples indicates either the presence of PCR inhibitors in the stool samples or DNA from trophozoites of *Entamoeba* species other than *E. dispar* or *E. histolytica*.

**DISCUSSION**

These findings are consistent with those previously reported for the Pernambuco State of Brazil. They...
showed a high incidence of four-nuclei Entamoeba and the presence of E. dispar (non-pathogenic amoeba) in this population. Furthermore, the E. histolytica-specific ELISA was shown to be a sensitive and specific method for the rapid differentiation of the two species because its results were comparable to those obtained with the PCR.

These results indicate that in communities in northeastern Brazil, the presence of either four-nuclei amoeba or trophozoites in the stool of a patient with diarrhea is not equal to amebiasis (the presence of the pathogenic E. histolytica). This is important when one considers the currently available treatments, some of which have undesirable side effects. A diagnosis of amebiasis should also be considered when red blood cells are observed within trophozoites in stool specimens.1

We recommend that ELISA procedures based on reliable antigens or antibodies be used in this region. Unfortunately, PCR methods are still too sophisticated and expensive for the public health systems of these communities.

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Authors’ addresses: Sandra M. B. Pinheiro, Ivanise S. Aca, and João I. Irmão, Departamento de Medicina Tropical, Universidade Federal de Pernambuco, Campus Universitário, 50670-901, Recife, Pernambuco, Brazil. Rosa M. Carneiro, Departamento de Medicina Social, Universidade Federal de Pernambuco, Campus Universitário, 50670-901, Recife, Pernambuco, Brazil. Marcos A. Morais Jr., Departamento de Genética, Universidade Federal de Pernambuco, Campus Universitário, 50670-901, Recife, Pernambuco, Brazil. Maria R. M. Coimbra, Departamento de Pesca, Dom Manoel de Medeiros, Dois Irmãos, 52171-900, Universidade Federal Rural de Pernambuco, Recife, Brazil. Luiz B. Carvalho Jr., Departamento de Bioquímica, Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Campus Universitário, 50670-901, Recife, Pernambuco, Brazil.

Reprint requests: Luiz B. Carvalho Jr., Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Campus Universitário, 50670-901, Recife, Pernambuco, Brazil, Telephone: 55-81-3271-8484, Fax: 55-81-3271-8485, E-mail: lbcjr@liku.ufpe.br.

REFERENCES


25. Zaman S, Khoo J, Ng SW, Ahmed R, Khan MA, Hussain R,


