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# DETERMINAÇÃO DA PREVALÊNCIA E VARIABILIDADE GENÉTICA DE *Entamoeba histolytica* e *Entamoeba dispar* EM HABITANTES DE PERNAMBUCO

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# DETERMINAÇÃO DA PREVALÊNCIA E VARIABILIDADE GENÉTICA DE *Entamoeba histolytica* e *Entamoeba dispar* EM HABITANTES DE PERNAMBUCO

Tese apresentada ao Curso de Doutorado em Ciências Biológicas da Universidade Federal de Pernambuco, para obtenção do título de Doutor em Ciências Biológicas, área de concentração em Biotecnologia.

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# RESUMO

Vários relatos da literatura revelam ser a prevalência de *Entamoeba dispar* maior do que *Entamoeba histolytica* nos indivíduos que vivem no Nordeste brasileiro, a partir de estudos utilizando Enzyme Linked Immnosorbent Assay (ELISA), imunodifusão em gel e zimodemos. Este trabalho consistiu em determinar a prevalência dessas formas de amebas mediante o uso de detecção imunocoprológica de antígeno específico para *E. histolytica* e Reaction Chain Polimerase (PCR) do DNA genômico extraído de trofozoitos cultivados de amostras de fezes. A presença de amebas tetranucleadas foi investigada em 1437 amostras de fezes de indivíduos vivendo em Macaparana, cidade da zona da mata norte de Pernambuco; em 346 amostras de escolares com idades de 3 a 14 anos morando em uma favela do Recife e em 109 amostras de imunodeprimidos (104 HIV positivos e 05 transplantados) atendidos no Hospital das Clínicas da UFPE (2002 e 2003). Dessas amostras, 59 (4.1%) e 45 (13%) foram positivas para aquelas coletadas das populações de Macaparana e das crianças do Recife, respectivamente, enquanto que nenhuma foi positiva para as obtidas dos imunodeprimidos. Todas as amostras foram negativas para a presença de adesina galactose específica de *E. histolytica*, inclusive as amostras dos pacientes imunosuprimidos. As amostras com amebas tetranucleadas cultivadas em meio de Robinson foram positivas para trofozoítos em 31 daquelas coletadas das populações de Macaparana e 21 das de Recife. A partir da amplificação por PCR de seqüências espécie-específicas de DNA genômico, extraído desses trofozoítos, foi possível identificar *E. dispar* em 23 e 19 amostras dos habitantes de Macaparana e das crianças do Recife, respectivamente, enquanto que nenhuma amplificação foi observada para *E. histolytica*. As demais amostras (08 e 02 para

Macaparana e Recife, respectivamente) foram negativas para ambas as espécies. Estes resultados corroboram aqueles previamente descritos que mostravam a prevalência de *E. dispar* (ameba não patogênica) nestas populações. Ademais, validam o emprego do kit imunocoprológico como alternativa a PCR na identificação de *E. dispar* e *E. histolytica*. Finalmente, o polimorfismo genético das cepas de *E. dispar* das amostras coletadas da população de Macaparana e de crianças do Recife foi investigado com o uso de marcadores moleculares específicos para *E. dispar*, Dsp1/Dsp2 e Dsp5/Dsp6. Das 42 amostras analisadas, 39 amplificaram os loci 1-2 e 5-6. O dendrograma resultante desta análise revelou uma alta variabilidade entre os isolados para esta região. Entretanto, uma comparação entre as freqüências dos produtos de amplificação para as duas localidades, através de teste de Qui-quadrado, mostrou que a incidência de uma banda obtida do *locus* 5-6, foi significativamente diferente entre Recife e Macaparana, evidenciando a potencialidade desta técnica para abordar questões relativas à distribuição geográfica.

**Palavras chave**: *Entamoeba histolytica*, *Entamoeba dispar*, PCR, caracterização genética, ELISA.
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**ABSTRACT**

Previous studies using methods varying from traditional serological test to molecular biology have shown that in Northeast Brazil *Entamoeba dispar* was more prevalent than *Entamoeba histolytica*. In this work the prevalence was established by using *E. histolytica* stool antigen detection kits and Reaction Chain Polimerase (PCR) of genomic DNA extracted from cultured trophozoite in all four nuclei amoeba positive samples from individuals living in Northeast Brazil: Macaparana (1,437 samples); 3-14 years old school children from a Recife slum community (346 samples) and immunocompromised individuals attending the Hospital das Clínicas of the Universidade Federal de Pernambuco (109 samples). Among theses samples 104 were positive for the presence of tetranuclei ameba in those from Macaparana and Recife, respectively, whereas no one was found among from the immunocompromised individuals. However, all of these samples were negative towards the immunoenzymatic assay for the presence of *E. histolytica*-specific galactose adhesin. Out of the 103 tetranuclei ameba positive cultivated samples, only 52 showed trophozoites. DNA extraction of these samples, followed by PCR, showed that 42 samples were positive to *E. dispar* and no amplification was observed to the pathogenic *E. histolytica*. The remaining 10 samples were negative for both species. These findings are in accordance to previous studies performed in our laboratory based on gel diffusion precipitin, ELISA using *E. histolytica* trophozoite HM-1 IMSS antigen and Zymodemes. Furthermore, the genetic variability of *Entamoeba dispar* strains obtained from this survey (1783 samples) was investigated using two polymorphic species-specific loci (locus 1-2 and locus 5-6) with primers Dsp1/Dsp2 and Dsp5/Dsp6. A

combinatory clustering analysis revealed no geographical correlation and a remarkable genetic polymorphism among 39 isolates examined. Nevertheless, a comparison of the frequency of 8 alleles, shared by both populations for the loci, showed that only one allele of locus 5-6 was highly significantly different between the two cities. These results suggested that Macaparana population is infected by similar strains and that locus 5-6 showed potential in assaying questions related to the molecular epidemiology of this region.

**Key words**: *Entamoeba histolytica*, *Entamoeba dispar*, PCR, genetic characterization, ELISA.

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# INTRODUÇÃO

*Entamoeba histolytica* é um protozoário responsável pela amebíase no homem, uma doença infecciosa acompanhada ou não de sintomatologia clínica (WHO, 1997). Foi inicialmente descrita por Fedor Lösch (Lösch, 1875). Possui morfologia idêntica à outra ameba tetranucleada, *Entamoeba dispar*, considerada não patogênica. Ambas parasitam humanos e alguns primatas, sendo o homem o principal reservatório (Smith e Meerovitch, 1985). Vivem no trato intestinal humano e apresentam duas principais formas morfológicas no ciclo evolutivo: cisto, ou forma de resistência, e trofozoíto, ou forma vegetativa. Os cistos, ao serem ingeridos juntamente com alimentos ou água contaminados, passam pelo estômago, resistindo à ação do suco gástrico, chegando no intestino, onde ocorre o desencistamento e posterior multiplicação. Cada cisto origina oito trofozoítos que, em geral, aderem à mucosa do intestino, vivendo como comensal. Estes trofozoítos, sob condições adversas, transformam-se em cistos mononucleados que depois de divisões nucleares se tornam tetranucleados (cistos maduros) e são eliminados juntamente com as fezes normais ou formadas. Os indivíduos infectados podem excretar até 45 milhões de cistos por dia. Em caso de infecção por cepa patogênica, os trofozoítos podem invadir a mucosa intestinal, multiplicar-se no interior das úlceras e, através da circulação sanguínea, alcançar outros órgãos, causando amebíase extra-intestinal, onde não formam cistos e são hematófagos.

A infecção amebiana possui distribuição universal com diferenças na prevalência da infecção e incidência da doença. É difícil estimar a taxa de morbidade e mortalidade, devido a variações na distribuição geográfica da parasitose, na diversidade

das amostras populacionais, nas metodologias e na completa ausência de padronização das técnicas empregadas nos estudos epidemiológicos (Walsh, 1986; Acuna-Soto et al., 1993). A amebíase constitui um problema de saúde pública em muitos países em desenvolvimento, onde é fácil a transmissão fecal oral de cistos, devido principalmente à deficiência higiênico-sanitária. Estima-se que aproximadamente 500 milhões de pessoas são parasitadas pela espécie *E. histolytica*, causando em torno de 100 mil mortes por ano, ocupando o segundo lugar em mortalidade devido a protozoários parasitos, sendo apenas superada pela malária (WHO, 1997). Apenas 10% das pessoas parasitadas desenvolvem sintomas clínicos e destes 1% eventualmente apresentam complicações graves, tais como, abscesso hepático amebiano e colites fulminantes, responsáveis pelo alto índice de mortalidade (WHO Meeting, 1985; Wash, 1986, 1988; Martínez-Palomo, 1987; Acuna-Soto et al., 1993). Tal fato deu origem a possíveis explicações que resultaram em três importantes hipóteses para explicar estes diferentes comportamentos clínicos na amebíase: 1) *E. histolytica* seria normalmente um protozoário comensal no intestino humano e em uma determinada ocasião, por razões desconhecidas, converter-se-ia em uma forma patogênica invasiva (Kuenen & Swellengrebel, 1913); 2) *E. histolytica* seria uma espécie única patogênica, identificada microscopicamente e que todos indivíduos parasitados por ela teriam lesões intestinais, que poderiam apresentar sintomas clínicos reconhecíveis ou não (Dobell, 1919) e 3) *E. histolytica* compreenderia duas espécies morfologicamente idênticas: uma patogênica invasiva, exibindo diferentes graus de virulência, e a outra patogênica não invasiva, que no máximo teria a capacidade de produzir uma lesão superficial na mucosa intestinal (Brumpt, 1928). Estudos posteriores confirmaram esta última hipótese (Diamond e Clark, 1993) a qual foi proposta inicialmente por Brumpt (1925), baseado na

patogenicidade em humanos e animais infectados experimentalmente. A espécie patogênica invasiva foi identificada anteriormente como *Entamoeba dysinteriae,* mas de acordo com Dobell (1919) tratava-se apenas de uma sinonímia de *Entamoeba histolytica* Schaudinn, 1903. A ameba não invasiva foi denominada de *Entamoeba dispar* por Brumpt (1925), porém, a impossibilidade de se distinguir morfologicamente as duas espécies propostas fez com que a sua explicação, na época, tivesse pouca credibilidade e fosse ignorada, até que Sargeunt (1978) sugeriu a existência de duas espécies distintas dentro da que foi originalmente conhecida como *Entamoeba histolytica.*

No Brasil, estudos da prevalência de *E. histolytica/E. dispar* em população de baixa renda têm demonstrado diferenças entre a região Norte e Nordeste. No Norte, existem ambas as espécies, com alta prevalência de *E. histolytica*, enquanto que no Nordeste a prevalência de *Entamoeba* com cistos tetranucleados tem sido alta, mas a incidência de *E. histolytica* baixa. Em Pernambuco, estudos para estabelecer a etiologia de diarréia em crianças, inclusive analisando as amebas tetranucleadas através de zimodemos, revelaram ser todas elas do tipo I, não patogênicas (Magalhães et al., 1990; Oliveira et al.,1992). Nesses estudos surgiram dificuldades em estabelecer a etiologia da diarréia em face do grande número de infecções mistas observadas, bem como a elevada ocorrência de enteropatógenos potenciais em indivíduos assintomáticos. Vários outros relatos mostram a prevalência da espécie não patogênica *E. dispar* (Okazaki et al., 1988; Nozaki et al., 1990; Aca et al., 1993; 1994; Tachibana et al., 1992). Vale ressaltar ainda que *E. histolytica* não tem sido detectada em abscesso hepático (Lima et al., 1998) e em fezes de pacientes aidéticos homossexuais masculinos (Alencar et al., 1996). Entretanto, Braga et al. (1996) através de testes sorológicos,

constataram uma alta incidência de *E. histolytica* na população no Estado do Ceará, semelhante às descritas para outros países em desenvolvimento. Recentemente, eles confirmaram esses resultados através de testes sorológicos e pesquisas de coproantígeno a presença de *E. histolytica* em mais de 10,6% dos indivíduos em estudo (Braga et al., 1998, 2001). No entanto, nesses estudos nenhuma correlação foi encontrada entre soropositividade, colonização do parasito no intestino e sintomatologia clínica, pois todos eram assintomáticos.

Atualmente, com base em evidências bioquímicas, genéticas e imunológicas, a *E. histolytica* (Schaudinn, 1903) é reconhecida como a espécie patogênica, diferente da *E. dispar*, considerada não patogênica (Diamond & Clark, 1993). A espécie invasiva usualmente penetra na mucosa destruindo o tecido do hospedeiro, causando doenças como colites hemorrágicas e abscessos extra-intestinais, evidenciando diferentes graus de virulência (Clark & Diamond, 1994), enquanto a espécie não invasiva vive como comensal na cavidade intestinal (Leippe et al, 1993).

O mecanismo de virulência de *E. histolytica* é pouco entendido, observa-se um reduzido número de indivíduos parasitados (5 a 10%) que desenvolvem doenças e sintomas (Ackers, 1996; Britten et al., 1997; Haghighi et al., 2002). Entretanto, várias moléculas têm sido sugeridas como responsáveis pelos danos causados às células e tecidos por estas amebas, incluindo adesinas (lectinas), amebaporos, fosfolipase A, colagenases e cisteina proteinases (Muñoz et al., 1984; Yi et al*.,* 1998; Nickel et al; 1999, Vargas-Villarreal et al., 1995; Xuchu & Sharon, 2000). Os leucócitos polimorfonucleares em contato com trofozoítos morrem e desintegram-se liberando enzimas lisossomais, contribuindo também para intensificar o dano ao tecido, embora trabalhos posteriores sugerem que os trofozoítos sejam capazes de produzir lesões na

ausência de células inflamatórias (Pérez-Tamayo,1986; López-Vancell, et al., 2000; Moncada etal., 2003). O tecido danificado *in vivo* envolve diferentes estruturas celulares e intercelulares que, provavelmente, requerem ação simultânea ou seqüencial de várias ou mesmo diferentes moléculas amébicas. Estudos demonstram a adesina específica para galactose existente em *E. histolytica* como principal responsável pelo início do efeito citotóxico, promovendo inicialmente a aderência, considerando-se esta capacidade um pré-requisito para a patogenicidade da amebíase invasiva (Petri et al., 1987, 2002).

A amebíase é considerada também como uma doença sexualmente transmissível (Sargeunt et al., 1983; Wash et al., 1986) e acomete, principalmente, homossexuais do sexo masculino, constituindo um grupo de estudo importante em várias partes do mundo (Lowther et al., 2000; Ravdin et al., 2002). Na Europa e Estados Unidos, quase todos os isolados de homens homossexuais foram identificados como *E. dispar*, enquanto que no Japão a *E. histolytica* é predominante entre homossexuais e populações reclusas em instituições diversas, principalmente de pacientes com retardo mental (Kobayashi et al., 1992; Tachibana et al. 2000).

A diferenciação das duas espécies é de grande importância clínica, desde que são morfologicamente indistinguíveis, e ambas podem infectar a cavidade intestinal do homem (Ravdin, 1995, Rivera et al.,1996). A infecção por *E. histolytica* produz freqüentemente doenças extra-intestinais, sendo o abscesso hepático a complicação mais comum, que pode ser letal se o tratamento adequado não for instituído a tempo (Tachibana et al. ,1992). Embora existam drogas eficazes para o tratamento da amebíase invasiva, como metronidazol (com baixa atividade frente às formas intestinais do parasita), eles possuem efeitos colaterais que devem ser considerados, principalmente,

em pacientes especiais, como mulheres grávidas, indivíduos HIV positivo ou com infecção persistente após tratamento (Troll et al., 1997; Irusen et al., 1992). Além disto, não só o custo é considerado significante em muitos países e não se pode perder de vista a possibilidade de desenvolvimento de resistência à droga com o uso indiscriminado e desnecessário (Clark, 1998; López-Camarillo et al., 2003).

As evidências bioquímicas que diferenciam as duas espécies de *Entamoeba* foram inicialmente baseadas na mobilidade eletroforética das isoenzimas glicolíticas (glucose-6-fosfato isomerase, EC 5.3.1.9, malato desidrogenase - oxaloacetate-descarboxilase - NADP, EC 1.1.1.40, fosfoglicomutase, EC 5.4.2.6 e hexoquinase, EC 2.7.1.1) de trofozoítos obtidos em meio de cultura, provenientes de portadores assintomáticos e de outros com amebíase invasiva (Sargeaunt, 1978). Estas isoenzimas foram agrupadas em zimodemos (população de enzimas), que diferenciam a espécie, patogênica e não patogênica, sendo útil em estudos epidemiológicos iniciais (Sargeaunt, 1988; Aca et al., 1993, 1994). O comportamento eletroforético das isoenzimas fosfoglicomutase e hexoquinase são determinantes na identificação da patogenicidade (Otner et al., 1997a, 1997b). Apesar desta técnica ser considerada padrão para validação dos demais testes de diagnóstico, sua eficiência é limitada pelo cultivo de trofozoítos, que não ocorre em aproximadamente 30% das amostras de fezes cisto-positivas (Pillai, 1999). Além de que, no caso de infecção mista, poderá ser obtido resultado falso negativo para uma das espécies. Esta técnica é cara, demorada e de difícil aplicabilidade em diagnóstico rotineiro (Sehgal et al., 1995).

O diagnóstico coproparasitológico na amebíase intestinal é impreciso e depende, principalmente, da identificação de cistos ou trofozoítos de *E. histolytica/E. dispar* nas amostras fecais, através do exame microscópico, que é incapaz de diferenciar as duas

espécies morfologicamente idênticas. Além disso, os cistos podem ser facilmente confundidos com leucócitos polimorfonucleares e os trofozoítos com macrófagos em fezes liquefeitas, devido às semelhanças morfológicas dessas células (Bruckner, 1992; Gonzalez-Ruiz et al., 1994). Outras técnicas têm sido utilizadas para a identificação e diferenciação de *E. histolytica* e *E. dispar*, tais como, anticorpos monoclonais (Tachibana et al., 1997) e sondas de DNA (Bracha et al., 1990).

Técnicas sorológicas também, têm sido utilizadas para a diferenciação das espécies, principalmente a imunodifusão em gel (Maddison, 1965; Takeuchi et al., 1985) e o ELISA, "Enzyme Linked ImmunoSorbent Assay" (Takeuchi et al.,1998). *E. histolytica* é a única, entre as amebas que parasitam o homem, que é invasiva e induz a produção de anticorpos detectáveis (Sargeunt, 1992). A desvantagem das técnicas sorológicas é que o paciente continua soropositivo mesmo anos depois de curado (Rivera et al.,1998). Muitos antígenos têm sido descritos na literatura como específicos para o diagnóstico da amebíase, tais como: o antígeno HM-1 IMSS do trofozoíto de *E. histolytica* (Okazaki et al., 1988) e a subunidade antigênica 170 kDa da lectina GAL/GALNAC da ameba que constitui a cadeia pesada da lectina de aderência de 260 kDa localizada na superfície de *E. histolytica*, responsável pela sua interação com a mucosa intestinal (Petri & Schnaar, 1995., Shenai et al., 1996).

A evidência de que existem diferentes lectinas nas espécies *E. histolytica* e *E. dispar* (Aswell & Morrel, 1974) resultou no seu uso na diferenciação das mesmas, bem como contribuiu ao entendimento do processo invasivo da espécie patogênica (Chadee et al*., 1987;* Rosales-Encina et al.,1987; Saffer & Pettri, 1991; Yi et al*.,* 1998). As lectinas representam uma classe de proteínas que reconhecem seletivamente a estrutura de carboidratos (Kristensen et al., 2000; Nakamura et al., 2001) e são expressas em uma

variedade de diferentes organismos, animais e vegetais. Possuem grande importância, entre outras, pela capacidade de proporcionar a ligação especifica nas superfícies biológicas com resíduos de açúcar que se encontram ligados a proteínas e lipídios (Wang et al., 2001). Esta interação específica entre carboidratos e lectinas proporcionou o estabelecimento de vários testes de diferenciação utilizando técnicas imunoenzimáticas (Abd-Alla et al*.,* 1993; Haque et al., 1993, 1994, 2000). Existem diversos Kits disponíveis comercialmente para diferenciação das espécies *E. histolytica* e *E. dispar,* entre eles, aquele utilizado neste trabalhado, denominado "ELISA kit E. HISTOLYTICA-II". Seu princípio reside na capacidade de detectar antígeno nas fezes, lectina específica para galactose/N-acetilgalactosamina (Gal/GalNac), mediante o uso de um antígeno anti-lectina. A microplaca constante do Kit contém anticorpos policlonais imobilizados que reconhecem os epítopos 1 e 2 das lectinas comuns às duas espécies. Outro anticorpo monoclonal, ligado à peroxidase, reconhece os epitopos 3 e 6 existentes apenas em *E. histolytica*. O complexo ternário anticorpo policlonal - *E.histolytica*/*E.dispar* – anticorpo monoclonal conjugado à peroxidase será revelado pela adição dos substratos desta enzima. Este teste detecta aproximadamente 0,2 a 0,4 ng de lectina específica para *Entamoeba histolytica* presente na amostra fecal, possibilitando a distinção dos antígenos de *E. histolytica e E. dispar,* diretamente nas fezes (Haque et al*.,* 1995, 2000). Graças a sua utilização, *E. dispar* tem sido detectada em aproximadamente 95% das infecções anteriormente referidas como *E. histolytica* em áreas não endêmicas (Pillai et al., 1999). Sua sensibilidade, especificidade, simplicidade e rápida execução tem sido útil na realização de estudos epidemiológicos (Haque et al., 1995, 1998, 2000, 2003; Gonin & Trudel, 2003), particularmente, para notificar a prevalência de portadores assintomáticos, dos quais têm-se poucas informações (Clark,

1998). A melhor exposição dos antígenos, com ruptura dos cistos, mediante congelamento e descongelamento das amostras fecais, constituiu-se em um importante acréscimo ao procedimento.

Diferenças biológicas são observadas no cultivo de *E. histolytica* e *E. dispar*. *E. dispar* é difícil de ser cultivada axenicamente, sem nenhum outro organismo. Entretanto, o seu crescimento é favorecido na presença de um outro parasita, como *Crithidia fasciculata* ou *Pseudomonas aeruginosa*, indicando que fatores essenciais para o crescimento são fornecidos pelo microrganismo simbiótico.Tais fatores ainda não foram identificados (Clark,1998). Por outro lado, *E. histolytica* cresce normalmente em meio de cultivo axênico, sem a presença de outro microrganismo (Clark,1995). Investigações ultraestruturais da relação ameba/bactéria revelaram que a bactéria é encontrada somente dentro do vacúolo fagocítico dos trofozoítos de *E. histolytica*, enquanto que em *E. dispar* bactérias vivas são encontradas no citoplasma (Pimenta et al., 2002).

Quanto ao cariótipo, o genoma haplóide de *E. histolytica* é composto de 14 cromossomos, com aproximadamente 20 Mb, com a maioria (se não todas) das seqüências codificadoras de proteínas localizadas em grandes fragmentos de DNA de várias centenas de kilobases, sugerindo que estes genes estão localizados em cromossomos e não em plasmídios. As análises de hibridização de DNA indicaram a existência de ploidia funcional em alguns cromossomos neste parasita (Willhoeft et al., 1999).

Análises de DNA revelaram que *E.histolytica* e *E.dispar* são dois organismos sintênicos, sendo os grupos de ligação (cromossomos) altamente conservados entre as duas espécies. Em média, o grau de similaridade entre seqüências ortólogas é de

aproximadamente 95% para região codificante e 80% para região intergênica (Willhoeft et al., 2000).

No entanto, diversas abordagens genéticas revelaram uma diferença de 5% na seqüência de nucleotídeos do DNA genômico entre *E. histolytica* e *E. dispar* (Tannich et al., 1989; Tachibana et al., 1991). Esta diferença vem sendo aplicada na construção de iniciadores ("primers") que amplificam regiões espécie-específicas, por PCR, e tem sido utilizada em estudos de diferenciação das duas espécies de *Entamoeba* (Tachibana et al., 1991; Sanuki, et al., 1997; Zaman et al., 2000; Zindrou et al., 2001).

A técnica de PCR possibilita a amplificação *in vitro* de uma determinada seqüência de DNA a partir da utilização da enzima DNA polimerase termoresistente, desoxirribonucleotídeos trifosfatados (dNTP) e iniciadores que flanqueiam a região alvo, pareando-se às fitas opostas. A mistura destes componentes mais o DNA molde é colocada em um termociclador e os produtos amplificados são observados, após eletroforese, geralmente em géis de agarose corados com Brometo de Etídio.

Além do diagnóstico molecular, vários grupos de pesquisa vêm se dedicando ao estudo da variabilidade genética de espécies de *E. histolytica*, através de técnicas como LSSP-PCR (Gomes et al., 1997), AFLP (Maji et al., 1999), RAPD (Gomes et al., 2000) e *loci* de seqüências repetidas *in tandem* (Zaki & Clark et al., 2001), na tentativa de buscar uma relação entre o genótipo e a virulência. Recentemente, "primers" espécie-especifícos, que amplificam regiões de repetições polimórficas *in tandem* (*locus* 1-2 e 5-6) do genoma de *E. dispar,* permitiram o estudo da diversidade genética em populações da África do Sul (Zaki et al. 2002; 2003).

O *locus* 1-2 caracteriza-se por possui 495 pb, contendo dois blocos principais maiores que têm entre eles sete blocos menores compostos de sete motivos de repetição

similares alinhadas *in tandem*. Apenas um dos sete motivos está repetido nos dois blocos principais. Similarmente, a seqüência completa do *locus* 5-6 é de 510 pb e consiste de seis blocos principais alinhados *in tandem*, contendo seis motivos de repetição. O primeiro e o segundo blocos são formados pelo mesmo motivo e estão separados por 41 pb. O *locus* 5-6 contrasta com o *locus* 1-2 por possuir quatro dos cinco blocos principais compostos de um único motivo (Figura 1). As unidades repetidas *in tandem* variam não somente na seqüência, mas também no número e arranjo em ambos os *loci* e os produtos de amplificação destes *loci* podem ser facilmente identificados em géis de agarose que exibem uma única banda polimórfica para o *locus* 1-2 e mais de duas para o *locus* 5-6 (Zaki et al., 2002).

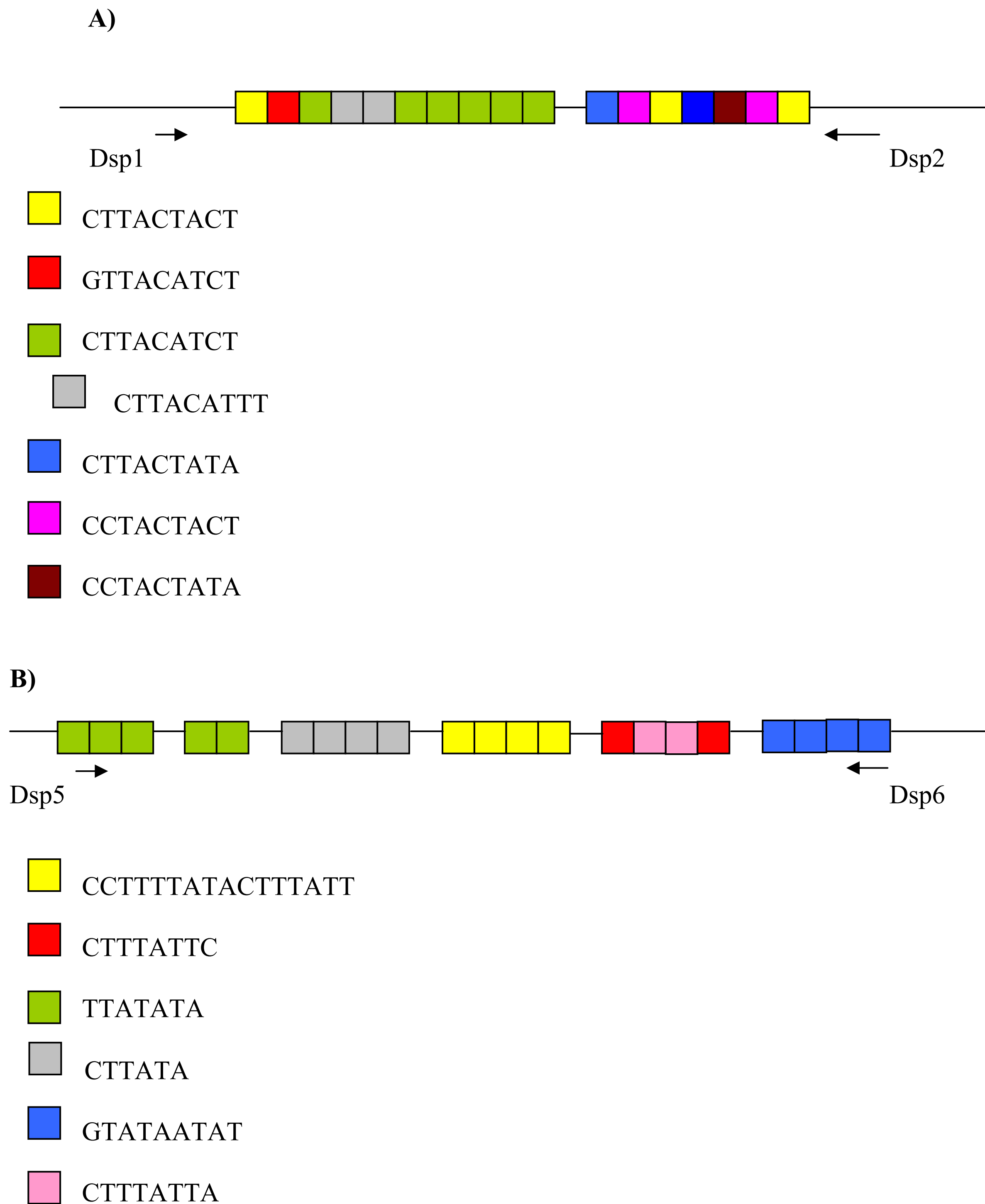


**Figura 1.** Representação esquemática dos *loci* 1-2 (**A**) e 5-6 (**B**) do DNA de *Entamoeba dispar*. Os iniciadores Dsp1, Dsp2, Dsp5 e Dsp6 amplificam as regiões indicadas (setas) contendo várias seqüências repetidas *in tandem* (quadrados coloridos). As linhas finas representam seqüências de DNA não repetido (adaptado de Zaki et al., 2002).

# JUSTIFICATIVA

O estudo da amebíase consolidou o conceito de que além de seu agente etiológico, *E. histolytica*, há que se levar em consideração à existência nas fezes dos pacientes de outra espécie de *Entamoeba*, morfologicamente idêntica, não patogênica, *E. dispar*. A maioria dos estudos sobre a prevalência de *E. histolytica* desconsidera este detalhe. Estudos conduzidos no Laboratório de Imunopatologia Keizo Asami entre 1988 e 1994 à luz dessa informação, em populações de baixa renda, revelaram diferenças epidemiológicas nas regiões Norte, Nordeste e Sudeste. A metodologias empregadas variaram da tradicional sorologia, como difusão de precipitinas em gel (GDP), aos zimodemos e biologia molecular, mediante a digestão do DNA genômico amplificado por endonucleases de restrição. Esses estudos mostraram que na Amazônia (Norte) existe maior prevalência de *E. histolytica*, enquanto que no Nordeste *E. dispar* predomina. Dados contraditórios têm sido relatados para uma comunidade pobre de Fortaleza, empregando técnicas que detectam a presença de anticorpos anti-lectina Gal/GalNAc no sangue. Recentemente, “primers” específicos para *E. histolytica* (P11 mais P12) e *E. dispar* (P13 mais P14) permitiram a distinção dessas espécies por PCR com muito maior sensibilidade e especificidade, representando importante ferramenta para esclarecer as dúvidas sobre a ocorrência dessas duas espécies em nossa região. Paralelamente, o método imunocoprológico tem sido proposto para a identificação de antígenos específicos de *E. histolytica* capaz de distinguir as duas espécies. Justifica-se, deste modo, retomar os estudos sobre a prevalência de *E.histolytica* e *E. dispar* em populações nordestinas utilizando-se este novo instrumento de investigação. Os relatos

contraditórios destas duas regiões despertam a necessidade de se aprofundar no estudo da caracterização de *E. dispar* no estado de Pernambuco. Soma-se a isto, a disponibilidade de “primers” polimórficos espécie-específicos que eliminam a necessidade de se utilizar culturas axênicas e tornam as análises mais rápidas.

# OBJETIVOS

## Objetivo Geral:

Determinar a prevalência de *E. histolytica* e *E. dispar* em habitantes de Pernambuco, mediante o emprego de técnicas de biologia molecular e imunológica.

## Objetivos Específicos:

1- Determinar a prevalência de *E. histolytica* e *E. dispar* em indivíduos residindo em Pernambuco (habitantes de Macaparana, escolares de uma região periférica da cidade do Recife e pacientes imunodeprimidos atendidos no Hospital das Clínicas-UFPE) mediante a técnica PCR;

2- Determinar a prevalência de *E. histolytica* e *E. dispar*, em indivíduos residindo em Pernambuco, mediante técnica de detecção de antígeno nas fezes;

3- Comparar a eficiência da técnica de PCR em relação ao teste imunocrológico e

4- Investigar a variabilidade genética de *E. histolytica* e *E. dispar,* através do polimorfismo de dois *loci,* espécie-específicos, contendo repetições *in tandem.*

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# CAPÍTULO I

# PREVALENCE OF *ENTAMOEBA HISTOLYTICA* AND *ENTAMOEBA DISPAR* BY USING PCR IN PERNAMBUCO STATE, NORTHEAST BRAZIL.

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# PREVALENCE OF *ENTAMOEBA HISTOLYTICA* AND *ENTAMOEBA DISPAR* BY USING PCR IN PERNAMBUCO STATE, NORTHEAST BRAZIL.

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**Abstract.**

Previous studies using methods varying from traditional serological test to molecular biology have shown that in Northeast Brazil *Entamoeba dispar* was more prevalent than *Entamoeba histolytica*. In this work the prevalence was established by using *E. histolytica* stool antigen detection kits and PCR of genomic DNA extracted from cultured trophozoite in all four nuclei amoeba positive samples form a population living in Macaparana, Northeast Brazil. Among 1,437 stool samples analyzed only 59 (4.1%) were positive for four nuclei amoeba. However, all of these samples were negative towards the immunoenzymatic assay for the presence of *E. histolytica*-specific galactose adhesin. Out of 59 cultivated samples, only 31 showed trophozoites. DNA extraction of the 31 samples, followed by PCR, showed that 23 samples (74.19%) were positive to *E. dispar* and no amplification was observed to the pathogenic *E. histolytica*. The remaining eight samples were negative for both species. These findings are in accordance with those previously reported.

**Introduction.**

The protozoon *Entamoeba histolytica* is an intestinal parasite infecting 500 million people worldwide.[1] Up to 100,000 deaths per year around the world have been attributed to complications of amebiasis, notably amoebic liver abscess.[2] The prevalence of *E. histolytica* in developing countries is often assumed to be high, frequently without supporting data.[3] In Brazil, studies on *E. histolytica* carried out at the Laboratório de Imunopatologia Keizo Asami- LIKA, between 1988 and 1994, among low-income population have shown differences in regions of the Northern, Northeastern and Southeastern Brazil. The used methodology in these studies varied from traditional serological test, such as Gel Diffusion Precipitin (GDP) and zymodemes to molecular biology by restriction-endonuclease digestion of amplified genomic DNA.[3-7] These investigations showed that the Amazon region (North) presented both *E. histolytica* and *E. dispar* with higher prevalence for *E. histolytica*, while in the Northeast the *E. dispar* predominated.

Contradictory to these findings, the occurrence of *E. histolytica* has been described among a community in Fortaleza, Northeast Brazil.[8] Authors detected the presence of serum antibodies specific for the Gal/GalNAc lectin and suggested that this community was highly endemic for *E. histolytica* with infections rate similar to other developing nations.

Despite this result, different from those conducted at LIKA, the Northeast region seems to have a diverging parasitologic profile concerning 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 employed in the

laboratorial routine. Gel diffusion precipitation test (GDP) was considered by some researchers to be one of the most reliable serological tests for diagnosis of amebiasis.[9-10] Enzyme-linked immunosorbent assay (ELISA) is also a tool for serodiagnostic method, nevertheless, this method have problems once it is difficult to differentiate between a current and previous parasite infection, and it is of limited value when examining individuals from endemic areas with high circulating antibodies.[11] Many antigens have been reported as specific for diagnosis of amebiasis such as *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 antigenic 170-Kda subunit of the amebal Gal/GalNAc-lectin.[3,13-15] Although the use of a stool ELISA has been shown to be useful in routine diagnostic procedure, 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.[16]

On the other hand, a number of DNA sequences have been used as targets for specific detection of *E. histolytica* using PCR technology. Ribosomal RNA molecules were the most commonly used targets, followed by restriction pattern analysis. [16, 17-19] In addition, genomic DNA has also been used in diagnosis by PCR.[20-23] The primers specific for *E. histolytica* and *E. dispar* (P11 plus P12 and P13 plus P14, respectively) were found to give 100% sensitivity. [24,25]

The PCR technique is fast, safe and constitutes an outstanding approach to overcome doubts and to answer questions about the occurrence of *E. histolytica* or *E. dispar* in the Northeast Brazil.

This contribution aimed to determine the prevalence of *E. histolytica* and *dispar* by using *E. histolytica* stool antigen detection kits and PCR of genomic DNA extracted

from cultured trophozoite in a population located in Pernambuco State, Northeast Brazil.

## Materials and Methods.

*Samples.*

Aliquots of fresh unpreserved stool obtained from randomly selected 1,437 individuals living in Macaparana were kept at -4$^{o}$C and one gram at -20$^{o}$C for subsequent immunoenzymatic analysis. Macaparana is located in Pernambuco State, Brazil, on the limits of a sugarcane plantation area, 118 km far away from Recife (capital of Pernambuco). It has a population of 22,494 inhabitants (13,518 and 8,976 in the urban and rural area, respectively) occupying an area of 103 km$^2$. Illiteracy rate is very high (65,1%) among population older than 10 years. Young people represent most of the population, provided that 46,5% of the population is no older than 20 years old and 75%, than 42 years old. The estimated familiar income is about US$ 480 per year.

***Microscopy analysis.***

The presence of parasites in the samples was determined by different methods of concentration. [26,27]

***Immunoenzymatic assay.***

The presence of *E. histolytica*-specific galactose adhesin (ELISA kit E.HISTOLYTICA-II, Techlab, Inc., Blacksburg, VA) was investigated among the samples that were kept at –20$^{o}$ C and positive for the presence of four nuclei amoeba. 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 of >0.05 after subtraction of the negative control optic density.

***Genomic DNA extraction.***

All four nuclei amoeba positive samples were incubated with the Robinson's medium at 37°C for 38 h.[28] The cultured trophozoites were centrifuged and resuspended in ethanol. Subsequently, trophozoites were centrifuged and resuspended in 200 µl of the solubilising agent containing Tris-HCl (pH 7.5), 10 mM, EDTA 10 mM and SDS 0.5% and 0.5 mg proteinase K for 2h 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, 2.5mM EDTA pH 8.0) and stored at -20° C until PCR amplification.

***PCR.***

PCR was performed in a 25 µl solution containing (final concentration) 20 mM Tris-HCl (pH 8.4), 3.0 mM MgCl2, 50 mM KCl, 2.0 mM each of the four dNTP, 10 pmol of each specific primers (p11 plus p12 and p13 plus p14), 2.0 U of *Taq* Polymerase (Invitrogen, California-USA) and approximately 50 ng of genomic DNA. The thermal cycles consisted of an initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 59°C for 90 sec, 72°C for 90 sec and a final extension of 5 min at 72°C. PCR products were electrophoresed in 2 % agarose containing ethidium bromide and the gel was photographed under UV light. Two DNA samples, testing positive for each species, were used as positive controls.

**Results and Discussion.**

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 of these latter samples were negative towards the immunoenzymatic assay for the presence of *E. histolytica*-specific galactose adhesin. It is worthwhile to register that these samples microscopy analyzes also showed the following additional microorganisms: *Entamoeba coli* (27); *Ascaris lumbricoides plus Entamoeba coli* (4); *Ascaris lumbricoides* (3); *Entamoeba coli plus Endolimax nana (*3); *Iodameba bütschlii* (2); *Trichuris trichiura* (1); *Endolimax nana* (1); *Ancilostomídeos* (1); *Ascaris lumbricoides plus Trichuris trichiura* (1); *Ascaris lumbricoides plus Enterobius vermicularis* (1); *Ascaris lumbricoides plus Iodameba bütschlii plus Entamoeba coli* (1); *Entamoeba coli plus* Ancilostomídeos *plus Iodameba bütschlii* (1); *Entamoeba coli plus Schistosoma mansoni* (1); *Iodameba bütschlii* plus *Giardia lamblia* (1); *Iodameba bütschlii* plus *Entamoeba coli* (1) and no other parasites (10).

Out of 59 cultivated samples, positive for the presence of four nuclei, only 31 showed trophozoites. This result was expected, once there are many records describing the impracticability and time-consuming of obtaining cultures from a large number of microscopy-positive samples. [29,30]

The DNA extraction of the 31 samples, followed by PCR, showed that 23 samples (74.19%) were positive to *E. dispar* as proved by the amplification of the species-specific fragment (100 pb). On the other hand, no amplification was observed for the pathogenic *E. histolytica* (Figure). The remaining eight samples were negative for both species. The absence of amplification among these samples indicates either the

presence of PCR inhibitors in the stool samples or DNA from trophozoites *Entamoeba* species, other than *E. dispar* or *E. histolytica*.

These findings are in accordance with those previously reported for Pernambuco State. [4,6,7,31,32] They showed high incidence of four nuclei Entamoeba, but prevalence of *E. dispar* (non-pathogenic amoeba) in this population. Furthermore, the *E. histolytica*-specific ELISA showed to be a sensitive and specific means for the rapid differentiation of the two species since its results were comparable to the PCR ones

The importance of these results lies on the fact that, for Northeast Brazil communities, should be reviewed the common practice that the presence of either tetra nuclei amoeba or trophozoites in the stool of a patient with diarrhea is equal to amebiasis, namely, the presence of the pathogenic *E. Histolytica*. Furthermore, if you consider that the available treatment is based on chemicals with undesired side effects. The amebiasis diagnosis should be considered in the presence of red blood cells inside the trophozoites under the stool examination. [2] It is worthy of being recommended to use ELISA procedures based on reliable antigens or antibody. Unfortunately, PCR methods are still too sophisticated and expensive for the public health system of these communities.

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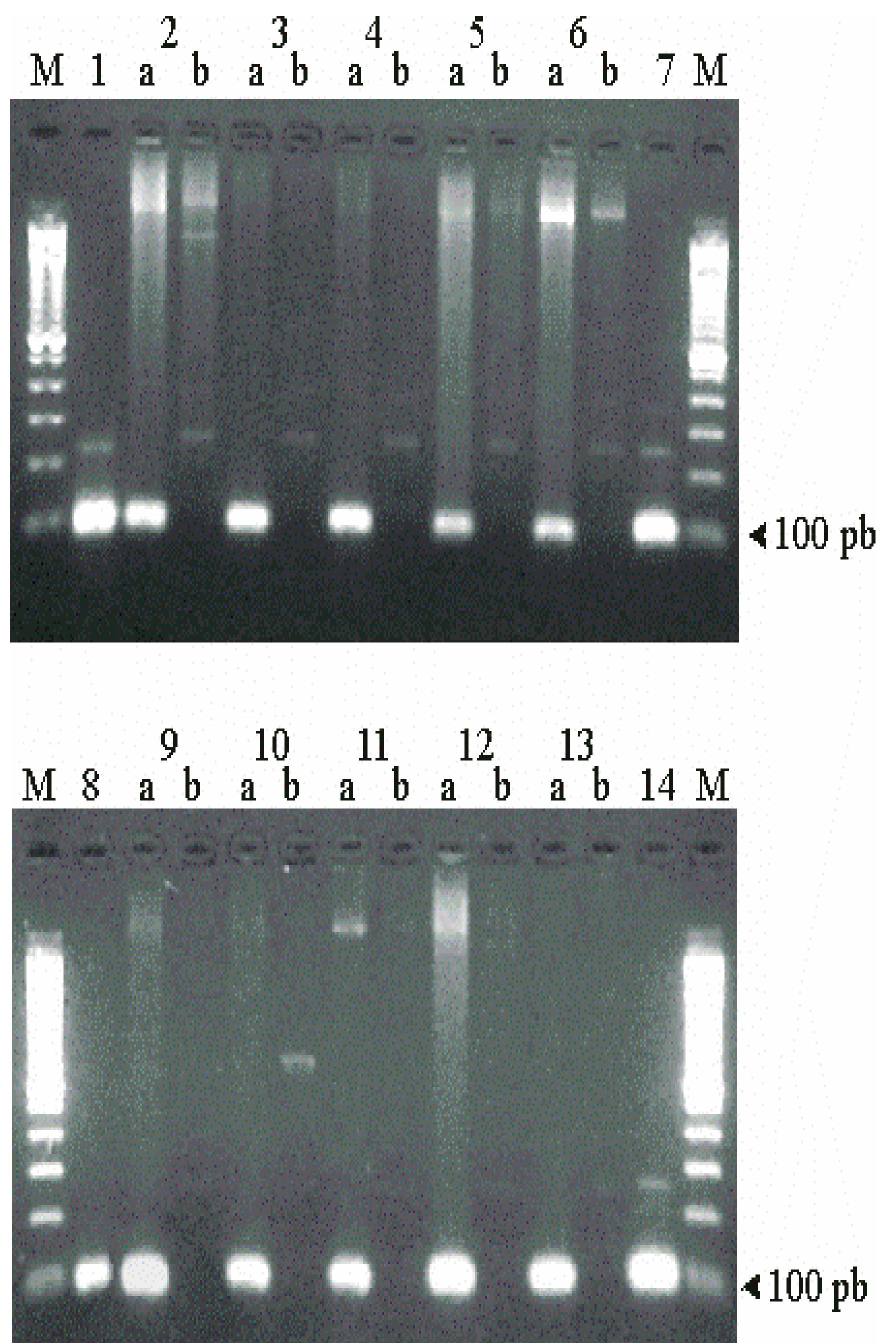


Figure

Legend of Figure.

Figure. Typical PCR amplifications of trophozoites DNA harvested from 10 stool samples (2-6 and 9-13) and using primers for *E. histolytica* (p11/p12) and *E. dispar* (p13/p14). Columns **a** and **b** represent amplifications for the primers p13/p14 (≅ 100pb) and p11/p12, respectively. Columns 1 and 8 represent controls for positive *E. dispar* whereas 7 and 14 for positive *E. histolytica*, respectively.

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# CAPÍTULO II

# Absence of *Entamoeba histolytica* in immunocompromised patients of Recife, Brazil.

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**Absence of *Entamoeba histolytica* in immunocompromised patients of Recife, Brazil.**

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## Abstract

The presence of *Entamoeba histolytica*-specific galactose adhesin was immunologically investigated and not detected in feces of 109 immunocompromised individuals attending the Hospital das Clínicas of the Universidade Federal de Pernambuco. Although 55.9% of the samples contained multiple parasites such as *Cryptosporidium parvum, Isospora belli, Cyclospora cayetanensis, Blastocistis hominis, Strongyloides stercoralis, Schistosoma mansoni,* hookworm and *Giardia lamblia*, no one was positive to either tetra nuclei ameba or *Entamoeba histolytica*-specific galactose adhesin. This result is in accordance to previous studies performed in our laboratory based on gel diffusion precipitin, ELISA using *E. histolytica* trophozoite HM-1 IMSS antigen and Zymodemes.

The association of infectious diseases and immunocompromised individuals has been recognized as an important issue regarding those suffering from Acquired Immunodeficiency Syndrome (AIDS), with hematologic cancers (leukemias and lymphomas), kidney, bone marrow and heart transplantation, and in individuals using high doses of corticosteroids and other immunosupressors. This combination is one of the most important death causes among them (Dietrich et al. 1999, Zambrano-Villa et al. 2002)

Classical protozoa such as *Entamoeba histolytica* is less frequent as cause of severe illnesses in HIV-infected patients, when compared with Microsporidia, *Isospora belli* and *Cryptosporidium parvum* and it is not considered as opportunistic infection in AIDS (Cimerman et al. 1999). However, one should not neglect its occurrence, particularly, in areas under impaired public health conditions. Studies performed in HIV-posi tive patients have been shown prevalence rates of about 0.2% for amebiasis in USA (Esfandiari et al. 1995, Lowther et al. 2000). In Japan rate of more than 8% has been reported for male homosexuals (Nozaki et al. 1989, Takeuchi et al. 1990, Nozaki 2000). In Brazil, examination of 771 fecal samples from AIDS patients living in São Paulo, performed under the program for the control and prevention of AIDS, has shown rate of 5.18% of amebiasis (Dias et al. 1988) Another study in this city, analyzing patients with more severe immunodeficiency, *E. histolytica* was not observed (Cimerman 1998). In Recife, an investigation to evaluate invasive amebiasis in 74 AIDS patients, 54 with diarrhea, *E. histolytica* was found in only one patient but its zymodemes was characterized as belonging to a non-pathogenic ameba (Alencar et al. 1996). Furthermore, Arcoverde et al. (2003) studying 110 diarrheic feces samples from HIV-positive patients did not find *E. histolytica* suggesting that coccidios are more relevant cause of diarrhea. The reclassification of *E. histolytica* into two species, *E. histolytica* and *E. dispar*, established by Diamond & Clark (1993) gave rise to the necessity of a worldwide prevalence reevaluation (WHO, 1997).

Although the prevalence of *E. histolytica* is low among HIV/AIDS patients the occurrence of amebic liver abscess is increasing suggesting that these individuals are more susceptible to invasive amebiasis (Shamsuzzaman & Hashiguchi, 2002).

Therefore, this work aimed to determine the presence *E. histolytica*-specific galactose adhesin in the feces of 109 immunocompromised patients (104 HIV-positives and 05 kidney transplanted) attending the Serviço de Doenças Infecciosas e Parasitárias of the Hospital das Clínicas of the Universidade Federal de Pernambuco, Brazil, from January 2002 to January 2003.

Aliquots of fresh unpreserved diarrheic stools were kept at 4$^{o}$C and one gram at -20$^{o}$C for subsequent immunoenzymatic analysis. Firstly, the presence of parasites in the samples was investigated for the presence of either trophozoites or cysts (Hoffman et al. 1934, Ritchie 1948) as well as for coccidian (Shimizu 1992). The presence of *E. histolytica*-specific galactose adhesin (ELISA kit E.HISTOLYTICA-II, Techlab, Inc., Blacksburg, VA) was investigated among the samples that were kept at -20$^{o}$ C. 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 of >0.05 after subtraction of the negative control optic density.

All stool samples were negative for nuclei amoeba under microscopy analysis. However, they presented 55.9% multiple other infections and showed the following parasites: *Cryptosporidium parvum* (29,3%), *Isospora belli* (18,3%), *Cyclospora cayetanensis* (3,6%), *Blastocistis hominis* (3,6%), *Strongyloides stercoralis* (3,6%), *Schistossoma mansoni* (0,9%), hookworm (1,8%) and *Giardia lamblia* (5,5%). No parasite was found in 45% of the samples. They were also negative under the immunocoprologic procedure, confirming the negative results of the microscopic analysis. These results are in accordance to those previously reported in AIDS patients from Recife (Alencar et al. 1996, Arcoverde et al. 2003).

It is worthwhile to register that previous studies carried out in our laboratory have reported higher prevalence of *E. dispar* compared to *E. histolytica* in Northeast Brazil (Aca et al 1993; 1994; Nozaki et al. 1990; Okasaki et al. 1988; Tachibana et al 1992). The prevalence of both *Entamoeba* was recently established using stool antigen detection (the same procedure used in this work) and PCR of genomic DNA extracted from cultured trophozoite in four nuclei amoeba positive samples from a population living in Macaparana, Northeast Brazil (Pinheiro et al. 2003). Among 1,437 stool samples analyzed only 59 (4.1%) were positive for four nuclei amoeba. However, all of these samples were negative towards the immunoenzymatic assay for the presence of *E. histolytica*-specific galactose adhesin. Out of 59 cultivated samples, only 31 showed trophozoites. DNA extraction of the 31 samples, followed by PCR, showed that 23 samples (74.19%) were positive to *E. dispar* and no amplification was observed to the pathogenic *E. histolytica*. The remaining eight samples were negative for both species. Therefore, the results here described for immunocompromised patients corroborate the intriguing finding that *E. histolytica* is not readily demonstrated in Northeast Brazil.

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# CAPÍTULO III

# GENETIC CHARACTERIZATION OF *Entamoeba dispar* ISOLATES IN NORTHEAST BRAZIL

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**Abstract**

The genetic variability of *Entamoeba dispar* strains obtained on a survey of 1783 individuals from two different cities of the Northeast Brazil was investigated using two polymorphic species-specific loci (locus 1-2 and locus 5-6). A combinatory clustering analysis revealed no geographical correlation and a remarkable genetic polymorphism among 39 isolates examined. Nevertheless, a comparison of the frequency of 8 PCR products, shared by both populations for the loci, showed that only one product of locus 5-6 was highly significantly different between the two cities. These results suggested that Macaparana population is infected by similar strains and that locus 5-6 showed potential in assaying questions related to the molecular epidemiology of this region.

**Keywords**

*Entamoeba dispar*, molecular characterization, Northeast Brazil.

## 1. Introduction

Amebiasis is an infection caused by the microscopic parasite *Entamoeba histolytica*. Ninety percent of the time, this parasite causes no symptoms, but in 10% of those infected the amoebas invade deeply into the intestinal wall, causing amebic colitis and liver abscess [1].

The haploid genome of *E. histolytica* comprises 20 Mb of DNA with most (if not all) of the protein encoding genes located on 14 large DNA fragments of several hundred kilobases each, suggesting that these genes are located on chromosomes and not on plasmids [2]. *Entamoeba dispar* is morphologically similar to *E. histolytica* but is not pathogenic to man because it is unable to penetrate tissues and produce invasive amebiasis. Direct sequencing of genomic DNA has revealed a 5% difference in the nucleotide sequences of the two organisms [3].

The reclassification of *E. histolytica* into two species, *E. histolytica* and *E. dispar,* established by Diamond and Clark [4], gave rise to the need for a worldwide prevalence reevaluation [5].

In Ceará, a State located in the Northeast Brazil, more than 10% of slum-dwelling individuals are colonized with *E. histolytica* based on results obtained with ELISA antigen detection kits [6, 7]. In contrast, in Pernambuco, another State in the Northeast, a recent survey showed an absence of *E. histolytica* in the local population [8].

Notwithstanding the consensus that *E. dispar* is a non-pathogenic parasite, there is evidence that *E. dispar* is capable of producing intestinal lesions in animals [9], of destroying epithelial cell monolayers in vitro [10] and to cause pathological changes in some humans [11]. More recently, a Brazilian strain of *E. dispar* was found to interact with indigenous bacteria in hamsters, playing an important role in the pathogenesis of amebiasis [12]. These facts suggest that the non-pathogenicity of some strains of this species should not be completely ruled out and further investigations are required.

Different molecular techniques, such as LSSP-PCR [13], AFLP [14] RAPD [15] and tandemly repeated loci [16], have been used to characterize intraspecific variation,

mostly in *E. histolytica*. Recently, species-specific primers were designed for two polymorphic DNAs containing tandemly repeated sequences from *E. dispar* by Zaki et al. [17], allowing population variability to be assessed and patterns of transmission to be followed.

In this paper, these two polymorphic loci were used to better understand the genetic diversity of *E. dispar*, as well as to determine the geographic origins of isolates. Moreover, this study provides the basis for future investigations of the epidemiology of *E. dispar*, as well as the reasons for the high prevalence of this non-pathogenic species in the State of Pernambuco.

## 2. Material and Methods

### *2.1 Samples*

Stools were collected from 346 children, ranging in age from 3 to 14 years old, from an urban slum community of Recife, which is the capital city of Pernambuco State, Brazil, and constitutes one of the biggest metropolises of Northeast Brazil. Samples were also obtained from 1,437 individuals living in Macaparana, a city located in Pernambuco State, on the limits of a sugarcane plantation area, 118 km far away from Recife. These individuals belonged to a low-income population earning approximately US$ 480 per year (family income).

### *2.2 Microscopy analysis*

The presence of parasites in the samples was determined by different methods of concentration [18, 19]. After microscopic examination and ethanol-alcohol

sedimentation, trophozoites samples were grown xenically at 37ºC for 38 h in Robinson's medium [20].

*2.3 Genomic DNA preparation*

Cultured trophozoites were washed in ethanol and suspended in 200 µl of the solubilizing agent containing 10 mM Tris-HCl (pH 7.5), 10 mM EDTA and 0.5% SDS, and incubated with 0.5 mg Proteinase K for 2h at 60$^{o}$C. DNA was extracted with phenol-chloroform, precipitated with ethanol and 0.3 M sodium acetate, suspended in TE buffer (0.01M Tris-HCl pH 7.4, 2.5mM EDTA pH 8.0) and stored at -20$^{o}$ C until used in PCR amplification.

*2.4 Differentiation of E. histolytica and E. dispar*

In order to verify that all trophozoite cultures were *E. histolytica* or *E. dispar* positive, we amplified a 100-bp *E. histolytica*-specific and a 101-bp *E. dispar*-specific fragment by PCR with a set of species-specific primers (P11/P12 and P13/P14 for *E. histolytica* and *E. dispar*, respectively) under the conditions described by Tachibana et al. [21].

*2.5 PCR conditions for genotyping E. dispar*

Genomic DNA was subjected to PCR by using primers that detect intraspecific polymorphism, Dsp1/Dsp2 and Dsp5/Dsp6, designed by Zaki et al. [17]. Amplification was performed in a 25 µl solution containing 20 pmol of each primer, 1 X PCR buffer (100 mM Tris-HCl pH 8.3; 500 mM KCl; 0.01% gelatin), 2.5mM of $MgCl_2$, 200 µM of each dNTP. Thermal cycling consisted of an initial denaturation at 94ºC for 2 min,

followed by 30 cycles of 94ºC for 1 min, primer-dependent annealing temperature for 1.5 min and 2 min at 72ºC, with a final extension of 5 min at 72ºC. PCR products were analyzed by electrophoresis using NuSieve 3:1 agarose (BMA, Rockland-USA) gel 2%. Alleles sizes were determined using two standard ladders (100 bp and 50 bp).

*2.6 Genetic analysis*

A variable binary similarity matrix was prepared with Jaccard coefficient by the NTSYS v2.1 (Numerical Taxonomy System of Multivariate Program) computer program [22] used to produce a dendrogram by UPGMA (Unweighted Pair Group Method With Arithmetical Average).

**3. Results and Discussion**

Out of 45 cultivated samples positive for the presence of cysts with four nuclei among the 346 childrens' stool samples analyzed, only 21 gave rise to trophozoites. Among the 1,437 stool samples from Macaparana 59 were positive for cysts with four nuclei amoeba and 31 gave rise to trophozoites. The DNA extraction of these samples followed by PCR showed all samples were positive for *E. dispar* (19 and 23 among Recife and Macaparana samples, respectively), as proved by the amplification of the species-specific fragment (100 bp). The remaining samples (2 and 8 for Recife and Macaparana samples, respectively) were negative for both species, indicating either the presence of PCR inhibitors in the stool samples or DNA from trophozoites of *Entamoeba* species other than *E. dispar* or *E. histolytica*.

Most of the positive samples obtained in Recife and Macaparana were successfully amplified at loci 1-2 and 5-6. However, some of them did not give amplification products for both loci, perhaps suggesting mutation within the DNA sequence complementary to one of the primers. These mutations may inhibit or completely prevent primer binding, resulting in either reduced or complete loss of product, acting as a "null allele". For this reason, we were only able to amplify a total of 39 samples at both loci.

Amplification of locus 1-2 (primers Dsp1 and Dsp2) generated one single major polymorphic band for all isolates, ranging in size from 410 to 470 bp (Figure 1-A). Four variants were observed in the samples collected in Macaparana and nearly 65% of the individuals shared the same sized band (450 bp). In contrast, in the samples collected in Recife seven variants were detected and 42% of the students displayed the same sized band (450 bp), thus reflecting the homogeneity of the population at this locus. A single major polymorphic band was also reported for most of the *E. dispar* and *E. histolytica* South African samples [17, 23] as well as for *E. histolytica* Japanese isolates investigated [24].

The maximum number of bands observed for locus 5-6 (primers Dsp5 and Dsp6) was three, whereas the size of the amplified fragments varied from 390 to 650 bp (Figure 1-B). It is noteworthy that the variation of the amplified fragments for this locus is much higher than for locus 1-2, which is consistent with the results of Zaki and Clark [16]. Out of the six different bands observed in the isolates from Macaparana, a product of 390 bp was present in 65% of the isolates. An interesting observation is that the majority of these isolates also showed a second band, in addition to that at 390 bp.

Among the scholars of Recife, nine different bands were seen and among these the most common one (430 bp) was found in 52% of the students.

When we compared the frequency of the 8 bands, shared by both populations for the two loci, by using chi-square test, only the incidence of the locus 5-6 band of 390 bp was found to be significantly different between Recife and Macaparana ($\chi^2$= 8.326 $P<0.01$). We therefore speculate that the Macaparana population is infected by related strains carrying the locus 5-6 390 bp length variant.

However, a combinatory clustering analysis using the two loci revealed no geographical correlation, and, at the same time, a remarkable genetic diversity among the isolates from this restricted geographic area (Figure 2). The exceptions to this are isolates MA155/ MA918/ RE17/ RE175/ RE165, RE16/ RE116, MA841/ RE6 and MA28/ MA925/ MA932 that were clustered in separate groups with 100% similarity. These independent groups could represent different clonal lineages infecting unlinked individuals in the State of Pernambuco, which is reasonable considering that Macaparana and Recife are just 100 km from each other.

The fact that amplification of locus 5-6 gave a triple-band pattern may, at first glance, be interpreted as co-infection with multiple strains of *E. dispar*. However, locus 1-2 presented only a single band for all isolates studied, thus rejecting this hypothesis. Southern Blot analyses indicate that *E. histolytica* likely has a functional ploidy of at least four for some chromosomes [2]. In light of this, it can be speculated that, in contrast to locus 1-2, locus 5-6 might be located on triploid or polyploid chromosomes. Alternatively, the presence of multiple bands could be explained by the existence of these repeat loci at multiple locations in the *Entamoeba* genome, each with a

characteristic PCR product size [16]. Further investigation will be needed to answer these questions.

Mixed infections are rare and, in culture, it is likely that one strain would outgrow any others that are present and thus give the appearance of a single genotype (C.G. Clark, personal communication). It is not likely to be significant that we have used DNA from trophozoites in culture rather than DNA extracted directly from stool samples [25]. Indeed, our aim was to detect population diversity among *E. dispar* strains occurring in Pernambuco, regardless of the source of DNA used.

The genetic polymorphism of *E. dispar* reported in this paper is extremely high, meaning that many different strains are present in Pernambuco State. This study concludes that only locus 5-6 shows potential in investigating questions related to the molecular epidemiology of *E. dispar* in this region.

**Acknowledgement**

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Figure 1- Polymorphic DNA analysis of *Entamoeba dispar* isolates in the State of Pernambuco. (A) Locus 1-2 amplification products, M1 (Ladder 50 bp), M2 (Ladder 100 bp), Lane 1 (RE 4), Lane 2 (MA 559), Lane 3 (RE 82), Lane 4 (MA155), Lane 5 (MA 260), Lane 6 (RE 224), Lane 7 (RE 221), Lane 8 (RE 77), Lane 9 (MA 841), Lane 10 (negative control of *E. histolytica*). (B) Locus 5-6 amplification products, M1 (Ladder 50 bp), M2 (Ladder 100 bp), Lane 11 (RE 15), Lane 12 (RE 16), Lane 13 (RE 77), Lane 14 (RE 33), Lane 15 (RE 36), Lane 16 (RE 98), Lane 17 (RE 116), Lane 18 (RE 115), Lane 19 (MA 918), Lane 20 (RE 285), Lane 21 (negative control of *E. histolytica*).

Figure 2- Dendrogram of 39 *Entamoeba dispar* isolates, constructed by UPGMA method using a binary similarity matrix and Jaccard coefficient obtained from two species-specific polymorphic loci (Dsp1-2 and Dsp5-6). Macaparana and Recife isolates are represented by MA and RE, respectively. Cophenetic correlation r = 0.99.

**Figure 1**

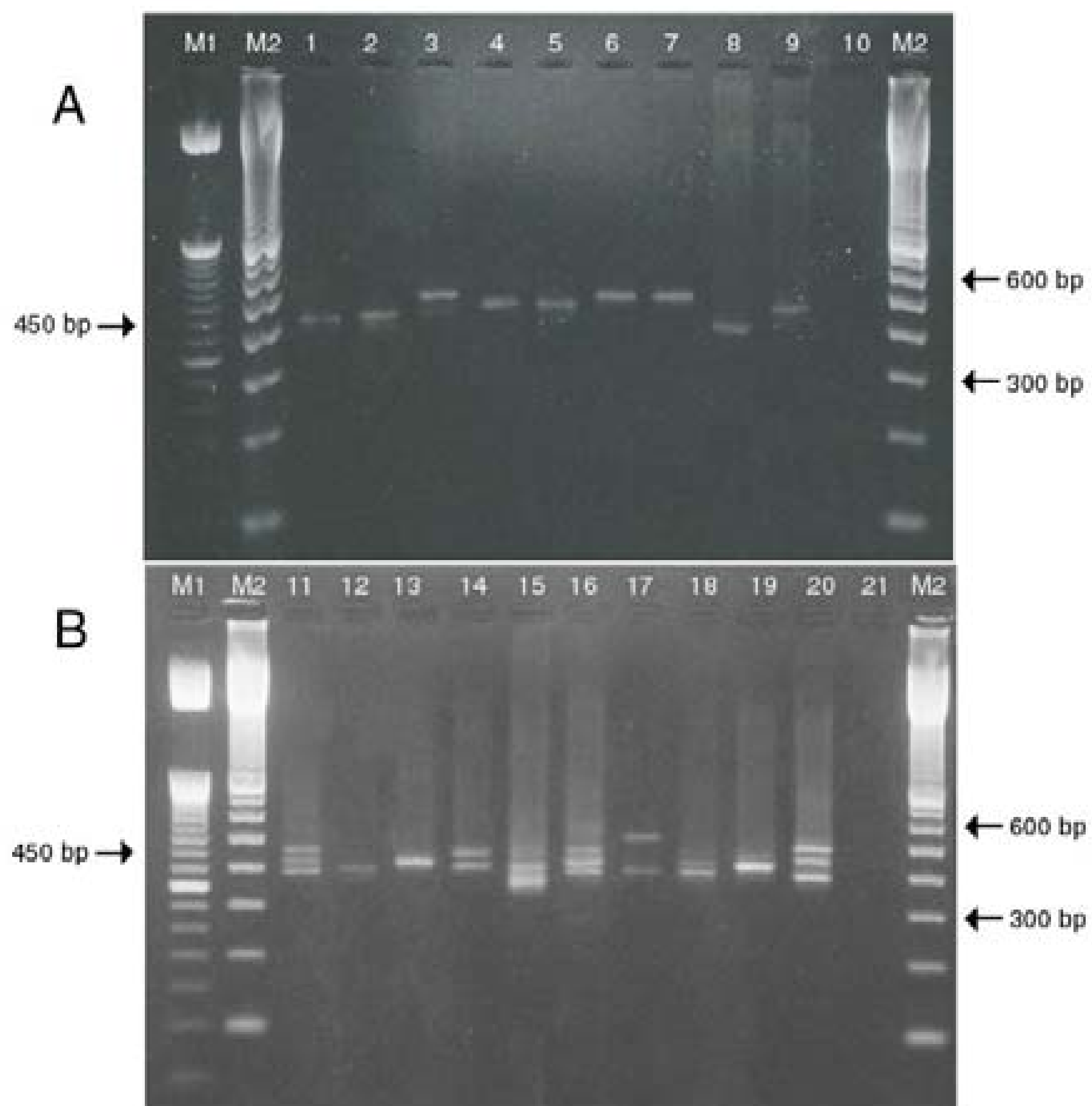
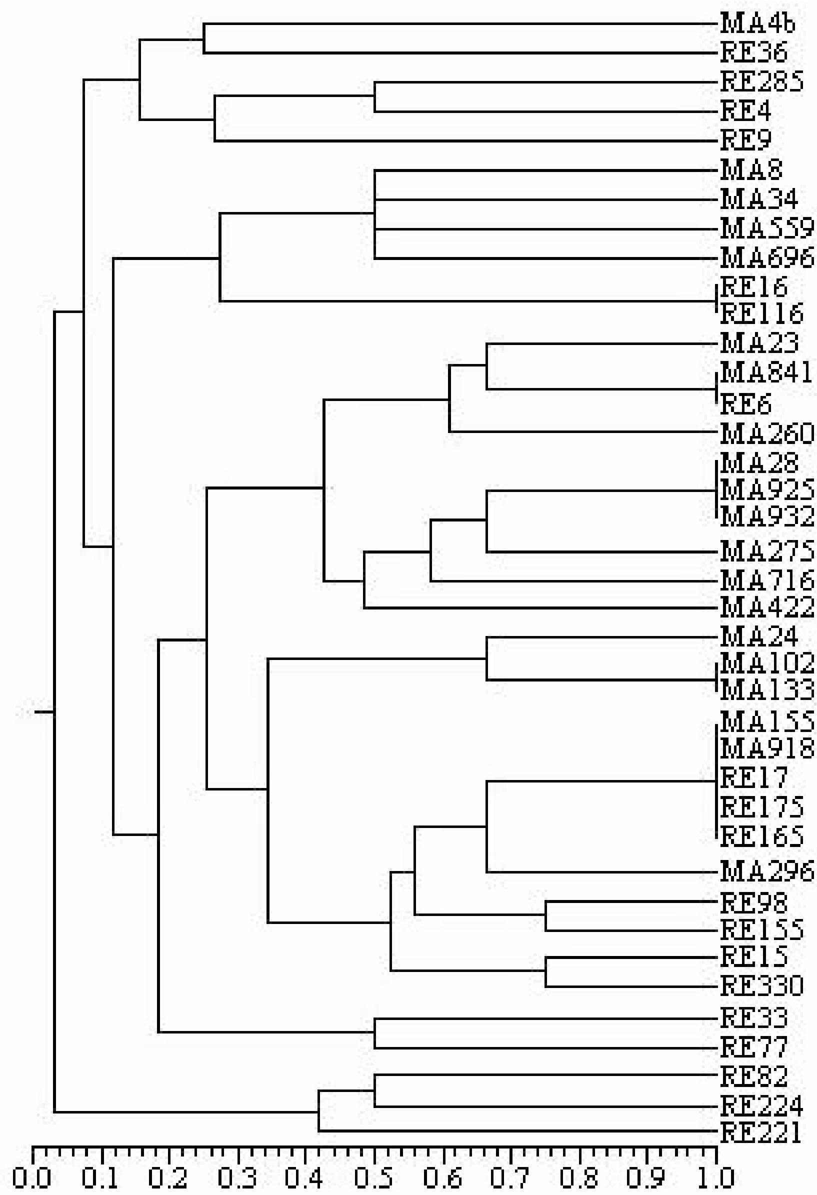


**Figure 2**



# CONCLUSÕES GERAIS

Os resultados dos trabalhos desenvolvidos nesta tese permitem concluir:

1. *Entamoeba histolytica* está ausente nos três grupos populacionais de Pernambuco estudados: habitantes da cidade de Macaparana, escolares do Recife (Várzea) e imunossuprimidos atendidos no Hospital das Clínicas da UFPE;

2. *Entamoeba díspar* foi identificada nos habitantes de Macaparana e nos escolares do Recife;

3. Houve correlação entre os resultados das técnicas imunocoprológicas e de genética usadas;

4. Existe alta variabilidade genética nos dois *loci* analisados para a espécie *Entamoeba dispar* e

5. Potencialidade do *locus* 5-6 em posteriores investigações sobre sua epidemiologia molecular na região;

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Genetic nomenclature for Trypanosoma and Leishmania should follow the guidelines proposed by Clayton et al (1998), Mol Biochem Parasitol 1998; 97:221-224 (http://www.elsevier.nl/cas/tree/store/molbio/free/1998/97/1-2/3178.pdf).

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