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ARBITRARILY PRIMED PCR FINGERPRINTING OF RNA AND DNA IN *Entamoeba histolytica*

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SUMMARY

Differences were detected in the gene expression of strains of *E. histolytica* using RNA (RAP-PCR) and DNA fingerprinting (RAPD). Analysis of the electrophoretic profiles of the gels revealed some polymorphic markers that could be used in the individual characterization of the strains. The 260 bands generated by using five different primers for RAP-PCR, as well as RAPD, were employed in the construction of dendograms. The dendogram obtained based on the RAPD products permitted the distinction of symptomatic and asymptomatic isolates, as well the correlation between the polymorphism exhibited and the virulence of the strains. The dendogram obtained for the RAP-PCR products did not show a correlation with the virulence of the strains but revealed a high degree of intraspecific transcriptional variability that could be related to other biological features, whether or not these are involved in the pathogenesis of amebiasis.

KEYWORDS: *Entamoeba histolytica*; Virulence; RAP-PCR; RAPD.

INTRODUCTION

The protozoan parasite *Entamoeba histolytica*, causative agent of amebiasis, produces very varied clinical manifestations, ranging from severe symptomatic presentations involving hepatic abscess to asymptomatic cases. This diversity in manifestations of the disease can be explained by the great variety of strains with different degrees of virulence. This variability of virulence is related to factors dependent on the host, the parasite and the interaction between the two of them. In relation to the parasite, many molecules related to this function have already been described[1,2,3,5,7,8,9,12,13] and the mechanisms involved in destruction of the tissues elucidated to some extent. The process begins with adherence of the trophozoite to the target cell and is followed by cellular lysis, which may be preceeded by phagocytosis. The proteins that act in the diverse stages of this process, which ends in the destruction of the host cell, are present in a differentiated form in the diverse isolates of *E. histolytica*. Even so, the relationship between virulence of strains and variability of proteins is still not clear. In this study we analysed the gene expression of some strains of *E. histolytica* showing different degrees of virulence through the RAP-PCR (RNA Arbitrarily Primed – PCR) technique[21]. This technique is carried out in two steps (cDNA synthesis and PCR) and provides a "fingerprinting" of RNA, enabling not only the identification of the products of gene expression, but also the intensity with which they are being transcribed. We can thus evaluate the real influence of a determined gene on the virulence of the strain at the moment of RNA extraction. We also analysed the DNA polymorphism of the strains in this study, using the RAPD (Randomly Amplified Polymorphic DNA) technique[20], for comparison.

MATERIALS AND METHODS

Strains of *Entamoeba histolytica*

The strains of *E. histolytica*, culture conditions and some biological characteristics of the strains are summarized in Table 1.

Nucleic acid extraction

Axenic samples of *E. histolytica* were obtained from a culture in exponential growth phase. The trophozoites were washed with PBS pH 7.2, concentrated by centrifugation at 300 g for 10 min., counted and adjusted to 1 x 10^6 cells. Then, the DNA of these cells was digested with proteinase K, extracted with phenol/chloroform and precipitated with ethanol[14]. The RNA was extracted by the TRIZOL® method according to the manufacturer's protocol (Life-Technologies).

cDNA preparation and RAP-PCR

For cDNA synthesis we used the protocol described by WELSH *et al*. (1992)[21] with some modifications. Each 1 µg of RNA was heated at 65 °C for 10 min. and the following reagents were added: 0.1 mM dNTPs, 100 units of reverse transcriptase (Life-Technologies), 0.5 µg of (oligo dT), in a buffer that consisted of 50 mM Tris/HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT in a final reaction volume of 20 µl. This mixture was incubated at 37 °C for 1 hour and 1 µg of the resulting cDNA was employed in the PCR reaction. In this reaction 0.2 mM of dNTPs and 1.0 unit of Taq DNA polymerase (Cenbiot, RS, Brazil) were

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Table 1
E. histolytica strains used in this study

strains	clinical form*	culture conditions	Virulence**	zymodeme‡
HM1	Sympt.	Axenic	Amoebic abscess Degree IV	Pathogenic
CSP	Sympt.	Axenic	Amoebic abscess Degree IV	Pathogenic
HK-9	Sympt.	Axenic	Amoebic abscess Degree II	Pathogenic
200-NIH	Sympt.	Axenic	Amoebic abscess Degree II	Pathogenic
462	Asympt.	Axenic	Negative	Pathogenic
452	Asympt.	Axenic	Negative	Pathogenic
32	Asympt.	Axenic	Negative	Pathogenic
RPS	Asympt.	Axenic	Negative	Pathogenic

* Sympt. (symptomatic); Asympt. (asymptomatic).
** The virulence was determined by inoculation into hamster liver[4]. The inocula size variation was 5 x 10^3 to 1 x 10^6 amoebae.
‡ The zymodeme was determined as pathogenic or nonpathogenic according to the analysis of hexoquinase and phosphoglucomutase enzymes, as the parameters established by SARGEAUNT *et al.* (1978)[15].

used in a buffer that consisted of 10 mM Tris-HCl, pH 8.5, 50 mM KCl and 1.5 mM $MgCl_2$ in a final volume of 10 µl. One micromol of five different arbitrary primers was used in each amplification reaction. The samples were submitted to 1 cycle of 95 °C for 1 min., 40 °C for 3 min. and 72 °C for 3 min., followed by 29 amplification cycles as follows: 95 °C for 1 min., 55 °C for 1 min. and 72 °C for 2 min.

RAPD

One nanogram of genomic DNA of each strain studied was used as a template for RAPD and as a control in the RAP-PCR reaction. The amplification reaction followed the protocol described by STEINDEL *et al.* (1993)[16]. Each 10 µl of the reaction mixture contained 0.8 U of Taq DNA polymerase (Cenbiot-RS-Brazil), 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 50 mM of KCl, 10 mM of Tris-HCl at pH 8.5 and 1.0 µM of primer. The following five primers were used in the two techniques:

L1-5'a: 5'CGCTGGGAGCTGTAGACCGGAG,
L2401: 5'AATATCCACTTGCAGATTCTAG,
L15: 5'CTCCACCATTAGCACCCAAAGC,
U2355: 5'GTGATGTGTGCATTCATCTCAT and
λgt11: 5'TTGACACCAGACCAACTGGTAATG.

Five microliters of the PCR products were run in 5% polyacrylamide gel and stained with silver salts.

Analysis of the RAP-PCR and RAPD data

The data derived from the five primers, the DNA fragments (bands), were considered for analysis. The genetic distances between the strains were calculated by LINK *et al.* method (1995)[6] based on RAPD and RAP-PCR product. These distances were listed in two matrixes and used to construct dendograms. The LINK *et al.* method (1995)[6] provides the genetic distance through the following mathematical model: $GDxy = (Nx + Ny) / (Nx + Ny + Nxy)$ where Nx is the number of bands in line x and not in line y, Ny is the number of bands in line y and not in line x, and Nxy is the number of bands shared in lines x and y. Tree topologies were established according to the clustering complete linkage method with the data presented on the matrixes. Once the complete linkage method works with cluster analysis, it considers the evolution rate as being the same for each strain, what makes the dendogram ultrameric. This means that the distance from the main root to the end of each branch is the same for each strain. The reason for using the complete linkage type of cluster analysis is that it allows the simultaneous comparison of intraspecific patterns. All the methods applied in the construction of dendograms, from the calculation of genetic distance to the designing of the trees, are part of the "TREECON *for Windows*" software package .

RESULTS

The cDNA and DNA extracted from all the strains were used as a template with five primers chosen at random. The products of the RAP-PCR and the RAPD generated polymorphic patterns (Fig. 1). The polymorphism of bands and its philogenetical meaning are represented as numbers in the matrixes (Fig. 2) that lists the genetic distance between the strains calculated from RAPD and RAP-PCR data. The dendograms (Fig. 3) show a graphical idea of the information presented by the matrixes. The analysis of the tree based on the products of RAP-PCR did not present a correlation with the virulence of the strains (Fig. 3B). The analysis of the dendogram based on RAPD (Fig. 3A) revealed two groups, one formed by the strains isolated from symptomatic individuals and the other consisting of asymptomatic ones. The following ramifications corresponded to a greater or lesser virulence of the strains based on biological studies (Table 1).

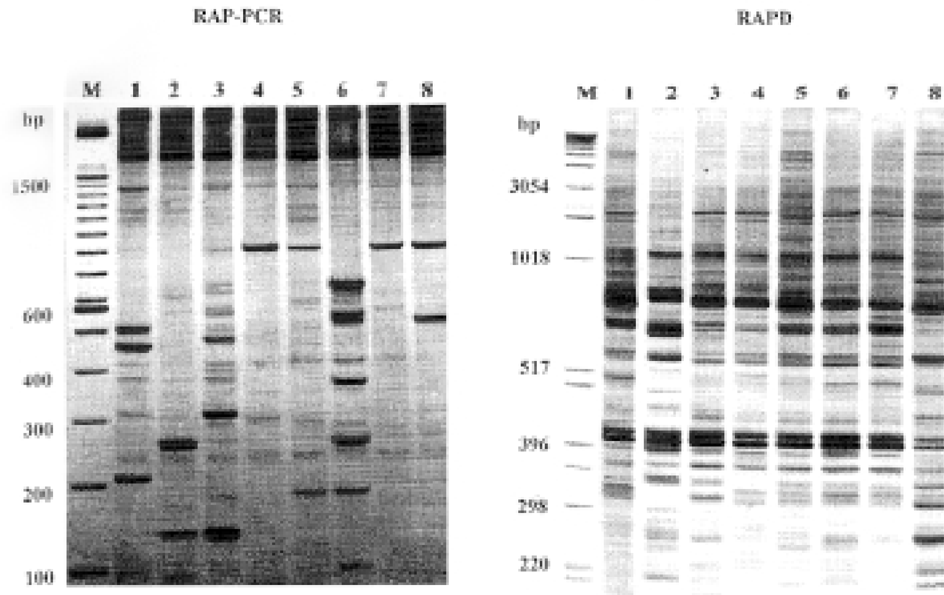

Fig. 1 - 5% polyacrylamide silver stained gels showing fingerprints generated by analysis of RNA (RAP-PCR) and DNA (RAPD) of strains of *E. histolytica* using the primer L15. Lanes 1-8 as follows: HM1, CSP, HK-9, 200-NIH, 462, 452, RPS and 32.

RAPD matrix

	HM1	CSP	HK-9	200-NIH	462	452	RPS	32
HM1	0.000	50.714	53.472	57.500	52.632	56.494	57.407	61.392
CSP	50.714	0.000	59.627	57.059	51.553	53.416	56.140	57.317
HK-9	53.472	59.627	0.000	43.949	46.497	43.421	52.381	60.355
NIH-200	57.500	57.059	43.949	0.000	37.736	33.333	45.930	50.296
462	52.632	51.553	46.497	37.736	0.000	26.897	35.849	47.239
452	56.494	53.416	43.421	33.333	26.897	0.000	24.490	44.304
RPS	57.407	56.140	52.381	45.930	35.849	24.490	0.000	37.975
32	61.392	57.317	60.355	50.296	47.239	44.304	37.975	0.000

RAP-PCR matrix

	HM1	CSP	HK-9	200-NIH	462	452	RPS	32
HM1	0.000	47.771	40.000	52.703	46.104	49.359	57.246	64.000
CSP	47.771	0.000	51.786	48.507	48.980	53.333	53.226	58.955
HK-9	40.000	51.786	0.000	52.903	48.466	48.765	63.816	66.250
NIH-200	52.703	48.507	52.903	0.000	56.429	54.745	53.636	60.000
462	46.104	48.980	48.466	56.429	0.000	47.552	56.000	63.504
452	49.359	53.333	48.765	54.745	47.552	0.000	50.420	50.420
RPS	57.246	53.226	63.816	53.636	56.000	50.420	0.000	51.515
32	64.000	58.955	66.250	60.000	63.504	50.420	51.515	0.000

Fig. 2 - Quadratic and symmetric matrixes of genetic distances calculated between strains of *Entamoeba histolytica*.

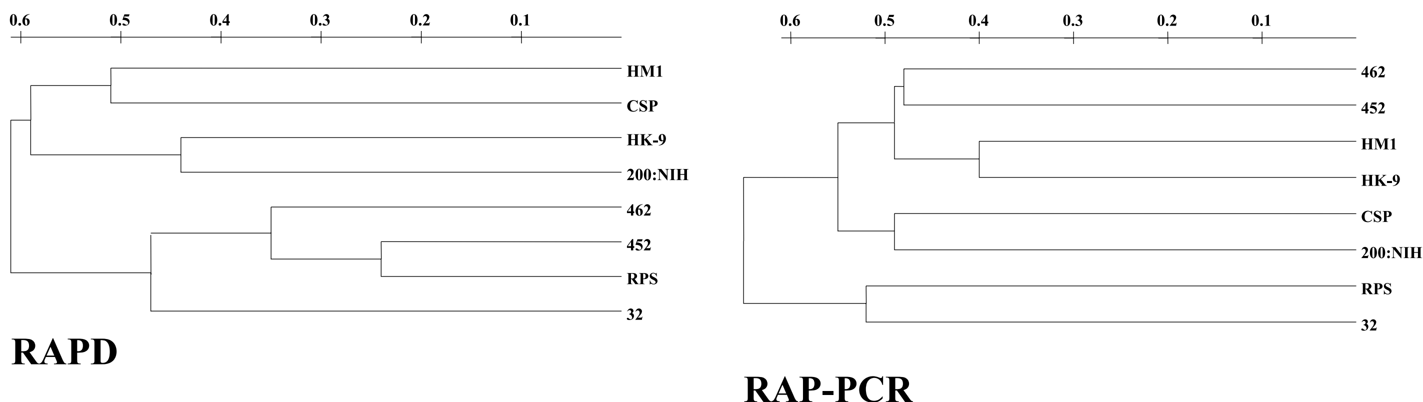

Fig. 3 - Phylogenetic tree of *E. histolytica* strains derived from RAPD (A) and RAP-PCR (B) data. The genetic distance was calculated by LINK *et al* (1995)[6] method and the tree constructed by clustering complete linkage method[19].

DISCUSSION

The protozoan parasite *E. histolytica* possesses an exceptional capacity for tissue destruction that can be considered as its principal pathogenic characteristic[11]. This capacity, however, is not found in all isolates. The identification and characterization of the molecular bases involved in its cytotoxicity have been sought, and several molecules were discovered as responsible for this function[1,7,8,9,13]. Different amebae molecules, have been found in virulent strains, being present at lower levels or even absent in strains presenting low virulence[2,3,5,12,17,19]. Considerable diversity has also been found in the genes that code for some of these proteins[10]. Nevertheless, there is no clear correlation between this protein diversity and the capacity to produce tissue damage.

In view of the above we sought to evaluate the virulence of *E. histolytica* using the RAP-PCR technique as an approach to the differentiation of genes expression. We compared the results obtained with this method with the well-established RAPD technique. It was believed that the results obtained using RAP-PCR with arbitrary primers, capable of evaluating RNA variability, would be more indicative of the virulence of the strains, since this technique analyses the product of gene transcription, determining the phenotype of the strains, and would thus presumably be more sensitive.

The variability of RNA and DNA was analysed using dendograms and a greater variability in the RNA was observed with the primers employed here. However, this greater variability did not correlate with the virulence of the strains. On the contrary, the DNA polymorphism visualized by RAPD revealed a better concordance with strain virulence. These results suggest that, at least with the primers used, RAPD may have amplified neutral regions of the DNA of the samples that in their phylogenetically conserved form did not suffer pressures of axenic culture or prolonged manipulation in the laboratory. By contrast, RAP-PCR should have amplified individual-specific regions, revealing the great protein variability within the species. These differentially amplified regions may constitute important molecules involved in the pathogenesis of amebiasis or could be the result of mutations due to prolonged maintenance in the laboratory, and may thus constitute important potential targets for the understanding of the still-obscure aspects of the biology of this parasite. Nevertheless, the RAP-PCR analysis did not differentiate between symptomatic and asymptomatic strains, as did the RAPD one.

RESUMO

Impressões digitais de DNA e RNA através de AP-PCR em *Entamoeba histolytica*

Diferenças na expressão gênica de cepas de *E. histolytica* foram obtidas pelo “fingerprinting” de RNA (RAP-PCR) e DNA (RAPD). A análise do perfil eletroforético do gel revelou alguns marcadores polimórficos que poderiam ser usados na caracterização individual das cepas. As 260 bandas geradas pela utilização de cinco primers diferentes, tanto no RAP-PCR, quanto no RAPD foram empregadas na construção de dendogramas. O dendograma obtido com os produtos do RAPD permitiu a distinção das cepas isoladas de pacientes sintomáticos e assintomáticos, além de correlacionar o polimorfismo exibido com a virulência das mesmas. O dendograma obtido com os produtos do RAP-PCR não apresentou correlação com a virulência das cepas, mas revelou uma exuberante variabilidade transcricional intra-específica, que pode estar relacionada a outros caracteres biológicos envolvidos, ou não, na patogênese da amebíase.

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