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PATHOGENICITY OF *Entamoeba dispar* UNDER XENIC AND MONOXENIC CULTIVATION COMPARED TO A VIRULENT *E. histolytica*

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SUMMARY

Two xenic isolates and cloned cultures of *Entamoeba dispar* were submitted to monoxenization using *Crithidia fasciculata* as the associated organism. Growth in monoxenic cultivation and ability of xenic and monoxenic trophozoites to destroy VERO cells and produce lesions in hamster livers were compared to those of a virulent *E. histolytica*. Parental and cloned *E. dispar* under monoxenic cultivation showed a remarkable lower growth than the monoxenic *E. histolytica* and were avirulent in both *in vivo* and *in vitro* tests. When xenically cultured, trophozoites of *E. dispar* showed a moderate lytic activity against VERO cells (1.5 to 41.8% of destruction) but caused severe hepatic lesions in hamsters as those caused by the virulent *E. histolytica* (29 to 100% in prevalence and 0.86 to 4.00 in lesion degree). Although *E. dispar* has not been associated with invasive disease in men, the ability of xenic trophozoites to produce prominent tissue damage in experimental conditions has indicated that some strains have a considerable pathogenic potential when in presence of bacteria.

KEYWORDS: *Entamoeba dispar; Entamoeba histolytica;* Cultivation; Pathogenicity.

INTRODUCTION

The existence of *Entamoeba dispar*, a species morphologically similar to *E. histolytica*, was first proposed by BRUMPT in 1925[4] to explain asymptomatic cases of amoebiasis. Most researchers, however, rejected this idea at that time, mainly because the proposed organism could not be differentiated from *E. histolytica*. Some decades later, studies on the isoenzymatic profile (zymodemes) of several isolates[22,23] allowed *E. histolytica* to be classified into pathogenic and non-pathogenic forms. Correlation of zymodemes with other biological and molecular data[8] led researchers to reconsider Brumpt's theory. The non-pathogenic form of *E. histolytica* was then renamed *E. dispar*, a non-invasive parasite of the human gut[28].

It is known that under certain conditions *E. dispar* may be pathogenic[8], based on studies that demonstrated trophozoites ability to produce focal lesions in experimental animals[6,27]. A moderate lytic activity of an axenic *E. dispar* strain was also detected[11]. Nevertheless, studies on *E. dispar* pathogenicity remain scarce. Isolation of new *E. dispar* strains is necessary for a better characterization of this species.

In the present study, two xenically cultured *E. dispar* isolates and cloned cultures obtained from them were subjected to monoxenization using *Crithidia fasciculata* as the associated organism. Growth of monoxenic trophozoites, cytopathic effect and ability to produce lesions in hamster livers of either xenic and monoxenic cultures were compared to a virulent isolate of *E. histolytica*.

MATERIAL AND METHODS

Parasites: The *Entamoeba* isolates ICB-MGL2 (MGL2) and ICB-ADO (ADO) were obtained from cysts from an asymptomatic carrier and a symptomatic patient, respectively, both residents in Brazil. A physician examined the symptomatic patient in order to determine the clinic form. Cultures were established xenically in Pavlova's medium modified by SILVA[13] with bacterial flora. As soon as sufficient growth had occurred, trophozoites were grown in culture flasks to produce lysates for isoenzyme typing[22]. Genetic identification was also confirmed by polymerase chain reaction through analyses of conformational polymorphism in the 482 bp fragment, as previously described[14]. Trophozoites of each isolate were cloned using a dilution method[26] and the clones ADO-A3, ADO-B3 and MGL2-A3 were used in the experiments. The same bacterial flora from the parental strains was maintained in the clones. The *E. histolytica* strain ICB-EGG (zymodeme XIX), used as the virulent reference strain, was obtained from a symptomatic patient[24].

Monoxenization: Trophozoites of xenic cultures were washed three times in sterile PBS (pH 7.2) and transferred to YI-S medium[9] containing *Crithidia fasciculata* as the associated organism (*C. fasciculata*

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Anopheles American Type Culture Collection [ATCC] Accession Number 11745, stock maintained in YI-S at room temperature). Bacteria were eliminated by addition of ceftazidime (Fortaz®, Glaxo Wellcome), ofloxacin (Floxtat®, Janssen-Cilag) or amicacine sulphate (Amicacin®, Neoquimica) to the medium at final concentrations of 660 µg/mL, 40 µg/mL and 333 µg/mL, respectively. Antibiotic use was halted when absence of bacteria was confirmed by microbiological assays. Viable *C. fasciculata* was added only on the day of sub-culturing. Monoxenic samples were also submitted to isoenzyme typing and analysis of conformational polymorphism in the 482 bp fragment[14].

Growth curves: After more than a year of monoxenic cultivation, the growth of *Entamoeba* was compared. Sixteen to 18 tubes containing 6.5 mL of YI-S medium were inoculated with 700 trophozoites/mL and 1.4 x 10^6 forms of *Crithidia fasciculata.* Every 24 hours, trophozoites from two tubes were quantified and the average was recorded. Growth curves were delineated with the average of three independent experiments. Differences in growth among the samples were evaluated by the Kruskal-Wallis test.

Cytopathic effect: The cytopathic effect of trophozoites on VERO cells monolayers was measured by a quantitative assay[3]. Briefly, confluent VERO cells monolayers (2 x 10^5 cells) were incubated for one h at 37 °C in a 5% CO_2 atmosphere with 1 x 10^5 trophozoites washed twice in PBS (pH 7.2) and transferred to DMEM (Dulbecco's Modified Eagle Medium). The interaction was stopped by ice bath for 10 min, the wells were carefully washed with cold saline, the remaining cells were fixed with 4% formaldehyde for 10 min and washed again with saline (two times). Methylene blue 0.1% in borate buffer (0.1 M pH 8.9) was added to the wells to stain the cells for 10 min. The wells were washed three times with borate buffer and the stain of each well was extracted with 1 mL of HCl 0.1 M. The solution was spectrophotometrically measured at 660 nm; the dye extracted from cells that did not interact with trophozoites (0% of destruction) was used as control. The bacterial flora of each xenic strain (seeded in 10 mL of Pavlova medium, grown for 24 hour at 37 °C) and *C. fasciculata* (1 x 10^6 grown in YI-S medium) were also tested. The Kruskal-Wallis test was used to determine the significance of differences in the destruction.

Hepatic inoculation in hamsters: Thirty-day-old hamsters (*Mesocricetus auratus*) obtained from a biotery (Biotério do Departamento de Parasitologia ICB/UFMG, Belo Horizonte, Brazil) were anesthetized with sodium pentobarbital and subjected to laparotomy. Trophozoites (2.5 x 10^5 or 5.0 x 10^5) washed twice in PBS (pH 7.2) were inoculated directly into the left lobe of the liver. Bacterial flora (seeded in 10 mL of Pavlova medium, grown for 24 hour at 37 °C) and *C. fasciculata* (1 x 10^6 grown in YI-S medium) were inoculated separately as controls. Animals were sacrificed six days later and results were determined by prevalence (number of animals with lesions divided by number of animals inoculated, expressed as a percentage) as well as the individual and average severity of lesions, based on criteria described previously[10], with some modifications. Fragments of the lesion were inoculated into culture medium or macerated in PBS for microscopic examination in order to confirm the presence of amoebae. Fixation and processing for haematoxylin and eosin staining (H&E) were performed to allow histopathological examination of the material. Statistical significance of prevalence and severity of lesions were determined by Pearson's X^2 and Kruskal-Wallis tests, respectively.

RESULTS

Zymodeme, specific characterization by PCR and clinical form of the subjects from whom the strains were isolated are shown in Table 1. Complete monoxenization of *E. dispar* and *E. histolytica* cultures was accomplished after treatment with antibiotics for three or four days. At this stage, the majority of trophozoites was rounded and did not attach to the glass of the culture tubes. After approximately two months, the monoxenic cultures became stable and trophozoites multiplied adhered to the glass. Cultures did not grow in YI-S without *C. fasciculata*, even after a year had passed. No alterations in the zymodemes of the parental and cloned *E. dispar* were detected after monoxenization, and neither did the amplification profile 482 pb fragment vary (Fig. 1).

Table 1
Entamoeba dispar and *E. histolytica* isolates

Isolate	Species [a]	Zymodeme	Origin	Clinical form [b]
ADO	*E. dispar*	I	Brazil	NDC
MGL2	*E. dispar*	I	Brazil	Asymptomatic
EGG	*E. histolytica*	XIX	Brazil	DC, HA

[a] Genetic identification by conformational polymorphism in the 482 bp gene; [b] NDC: non dysenteric colitis; DC: dysenteric colitis; HA: hepatic abscess.

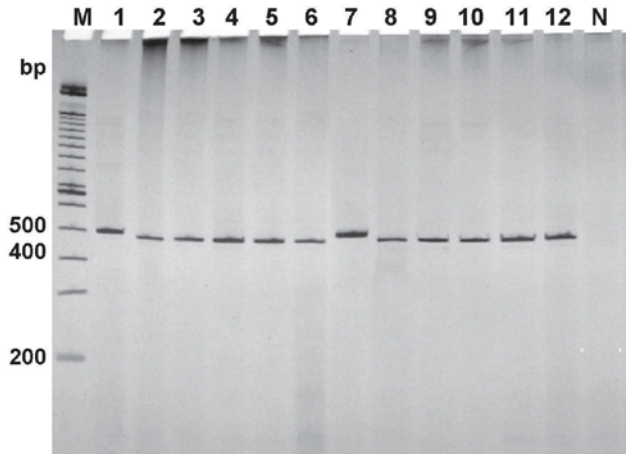

Fig. 1 - Silver-stained 5% polyacrylamide gel electrophoresis showing the conformational polymorphism of a 482 bp fragment amplified in strains of *Entamoeba histolytica* (EGG) and *E. dispar*. M- 100 Kb ladder, 1- EGG xenic, 2- ADO xenic, 3- ADO-A3 xenic, 4- ADO-B3 xenic, 5- MGL_2 xenic, 6- MGL_2-A3 xenic, 7- EGG monoxenic, 8- ADO monoxenic, 9- ADO-A3 monoxenic, 10- ADO-B3 monoxenic, 11- MGL_2 monoxenic, 12- MGL_2-A3 monoxenic, N- Control without DNA.

Exponential growth of monoxenic cultures is represented in Fig. 2. After 48 hours of cultivation, the *E. histolytica* EGG strains surpassed the *E. dispar* strains, the difference reaching a significant point at 72 hours, a significance level of 10% (p = 0.089). While the *E. dispar* strains suffered growth decrease between 48 and 72 hours, the EGG *E. histolytica* kept its exponential growth until 120 hours of cultivation.

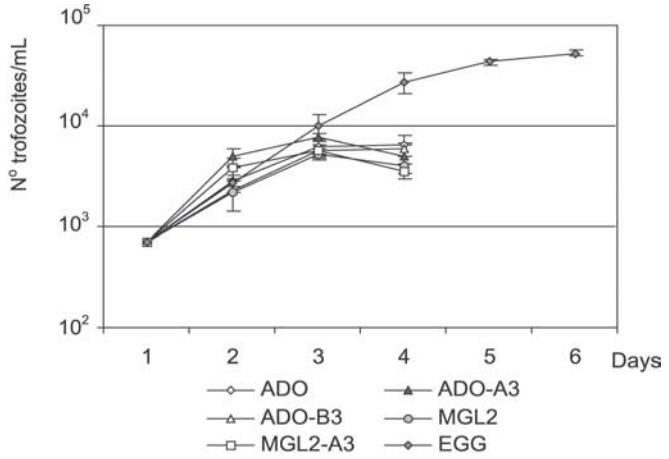

Fig. 2 - Growth curves of the *Entamoeba dispar* and *E. histolytica* (EGG) strains, in monoxenic cultivation with *Crithidia fasciculata* in YI-S medium, with initial inocula of 700 trophozoites/mL.

There was no significant growth difference when the *E. dispar* original and cloned strains were compared.

Most xenic *E. dispar* cultures presented a low to mild lytic activity against VERO cells (1.5 to 41.8% destruction), whereas the monoxenic cultures caused very little or no damage (Fig. 3). Destruction rates of *E. dispar* were significantly lower than those of *E. histolytica*, regardless the culture condition ($p < 0.05$). Bacterial flora or *C. fasciculata* alone did not destroy VERO cells.

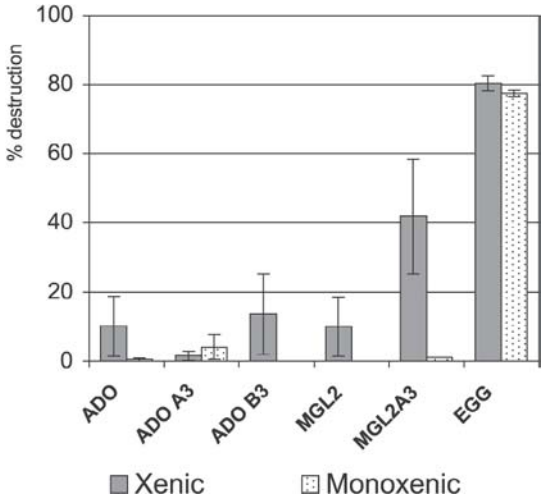

Fig. 3 - Cytopathic effect against VERO cells of *Entamoeba dispar* cultures under xenic and monoxenic cultivation in comparison to an *E. histolytica* strain (EGG).

Xenically cultivated trophozoites of both *E. histolytica* and *E. dispar* were able to produce lesions in hamster livers, with variable prevalence and severity (Table 2). Parental xenic strains of *E. dispar* showed similar prevalence and lesion grades ($p = 0.396$) to those of the xenic strain of *E. histolytica* and higher values than their respective clones ($p < 0.05$). Macroscopically, the lesions caused by xenic *E. histolytica* and *E. dispar* were similar, characterized by a yellowish necrotic area with a friable aspect. Microscopic examination of unfixed fragments revealed the presence of bacteria and motile trophozoites, the lesions being characterized as mixed. H&E stained slides demonstrated areas with complete destruction of parenchyma and predominantly chronic inflammatory infiltrate on the border between necrotic and preserved parenchymal tissue (Figs. 4 A-B). Trophozoites were barely detected in H&E slides of xenic *E. dispar*, whereas they were abundant in slides of xenic *E. histolytica* (Fig. 4 C). Hamsters inoculated with bacterial flora presented normal livers or bacterial lesions smaller than 4 mm in diameter, limited by a fibroid wall enclosing purulent material (Fig. 4 D); perivascular and periportal, predominantly chronic, infiltrate was observed in less damaged areas of tissue.

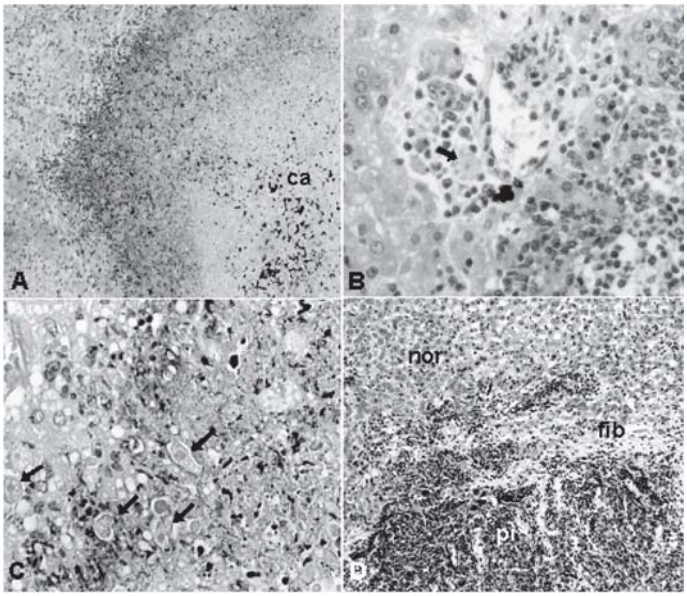

Fig. 4 - Histopathological aspects of hepatic lesions in hamster. A) Necrotic area and calcification foci (ca) of liver inoculated with a xenic *E. dispar* strain ADO. 100X. B) Inoculation with a xenic *E. dispar* (strain MGL_2). Detail of an area between the normal parenchyma and necrotic focus showing a predominantly mononuclear infiltrate and a trophozoite (arrow). 400X. C) Necrotic area with several trophozoites (arrows) of the xenic *E. histolytica* (strain EGG). 400X. D) Bacterial abscess caused by inoculation of ADO bacterial flora. Normal parenchyma (nor) has a lesion limited by a fibroid wall (fib) enclosing pyocites (pi). 100 X.

Monoxenic *E. dispar* cultures did not cause lesions in hamster livers, even when higher concentrations (5.0×10^5) of trophozoites were used. Hamsters inoculated with the monoxenic *E. histolytica* presented lesions when 5.0×10^5 trophozoites were inoculated, but not with 2.5×10^5 (Table 2).

DISCUSSION

The two isolates obtained in this study, identified as *E. dispar* by PCR and isoenzyme typing, were monoxenized in YI-S medium, which has been demonstrated as being appropriate for this species[15] . Unlike previous reports showing zymodeme alterations after modification of the cultivation system[1,17,18,26], monoxenization did not affect this parameter in our *E. dispar* isolates.

Table 2
Intra-hepatic inoculation of hamsters with *Entamoeba dispar* and *E. histolytica* under xenic and monoxenic cultivation

Strains	Xenic							Monoxenic	
	Prevalence[a]	Grade[b]						Prevalence[a]	
	$2.5x10^5$ troph.	0	I	II	III	IV	Average	$2.5x10^5$ troph.	$5.0x10^5$ troph.
ADO	8/8 (100)	0	1	1	1	5	3.25	0/5 (0)	0/4 (0)
ADOA3	3/6 (50)	3	1	0	2	0	1.17	0/4 (0)	0/4 (0)
ADOB3	2/7 (29)	5	0	0	2	0	0.86	0/4 (0)	0/4 (0)
MGL_2	5/5 (100)	0	0	0	0	5	4.00	0/5 (0)	0/4 (0)
MGL_2A3	4/7 (57)	3	1	1	2	0	1.29	0/4 (0)	0/4 (0)
EGG	6/6 (100)	0	1	0	0	5	3.50	0/6 (0)	4/4 (100)[c]

a - Prevalence: number of animals with lesions divided by number of animals inoculated, also expressed as percentage; b - Lesion grade evaluated as follows: 0 - Normal liver or bacterial lesion less than 4 mm in diameter (absence of amoebae confirmed by inoculation of a fragment in culture medium); I - Primary lesion at the site of inoculation, less than 15 mm in diameter; II - Primary lesion larger than 15 mm in diameter, no metastasis; II - Lesion in primary lobe, some metastasis to other areas of liver; IV - Large primary lesion, extensive metastasis, minimum of 50% of liver involved; c - Three animals with lesion in primary lobe, some metastasis to other areas of liver (grade III) and one animal with primary lesion at the site of inoculation, less than 15 mm in diameter (grade I).

When it comes to the clinical form of the ADO strain carrier, other causes for the symptoms - such as pathogenic bacteria, virus and *Blastocystis hominis* - should be considered. The latter has been associated to symptoms in some infections[21] and was detected in the parasitological examination of the patient (data not shown). However, considering that the isolation of strains with nonpathogenic zymodeme from symptomatic patients has already been described in Brazil[2], the hypothesis that the symptoms could be due to *E. dispar* ought not to be discarded. More clinical and biological studies are necessary in order to further investigate such possibility.

The *E. dispar* growth in monoxenic cultivation was clearly lower than the *E. histolytica* strain utilized for comparison. Maximal density obtained for *E. dispar* occurred in one of the experimental repetitions of ADO strain, which reached 9,000 trophozoites/mL at 72 hours (data not shown). This density was six times lower than the density of *E. histolytica* EGG at the same time. Some hypothesis suggested explaining the difficulty in axenizing *E. dispar* strains are the inability of trophozoites to obtain enough nutrients through pinocytose or their lower tolerance to toxic metabolites produced in the medium[7]. These characteristics could also explain the lower duration time of the *E. dispar* growth curves in monoxenic cultivation, compared to the *E. histolytica*.

As expected, *E. histolytica* exhibited high lytic activity and caused lesions in hamster livers, regardless the cultivation condition. Hepatic lesions by monoxenic *E. histolytica* were induced only when inocula containing the highest concentrations of the parasite were used, suggesting a decrease in virulence *in vivo* due to axenic cultivation. The phenomenon has usually been observed in studies with axenic *E. histolytica* cultures[16].

When monoxenic *E. dispar* were tested, biological tests were practically negative. Concerning to the hepatic inoculation, we also used 10^6 trophozoites of the parental monoxenic strains ADO and MGL2 (data not shown) and obtained no lesion whatsoever. These results are similar to those of ESPINOSA-CANTELLANO *et al.*[12], who showed that an axenic *E. dispar* was unable to cause lesions in hamster livers. Therefore, the monoxenic cultures can exhibit behavior similar to the axenic ones. Since axenization is a time-consuming and difficult process and *Crithidia fasciculata* has no pathogenic effect, monoxenic cultures could be an alternative for the biological evaluation of *E. dispar* in the absence of bacteria.

Nevertheless, the interaction of trophozoites and bacteria must be considered in the determination of the pathogenic potential of *E. dispar*. In our experiments, the cytopathic effect of *E. dispar* was moderate for xenic cultures, in contrast to the absent or minimal destruction when monoxenic strains or bacteria were used alone. Differences in results obtained from parental and cloned cultures had also occurred in the hepatic inoculation test, and can indicate population variability, which has already been detected in *E. histolytica*[20]. No correlation was found between the *in vitro* and *in vivo* assays with xenic *E. dispar,* suggesting that the mechanisms of pathogenicity *in vivo* can be quite different from the *in vitro* ones when bacteria are present in the culture.

Concerning the hepatic inoculation, the prominent lesions induced by xenic *E. dispar* could not be attributed only to associated bacteria, since bacterial flora alone did not cause important lesion. Thus, a synergistic effect between *E. dispar* and bacteria must be considered. Presence of inflammatory cells around the necrotic foci, lysis of these cells by trophozoites and release of lytic enzymes could be involved in the tissue damage. This mechanism has been shown in experimental hepatic amoebiasis induced by axenic *E. histolytica*[25]. Another report also showed the migration of inflammatory cells around trophozoites in the hepatic parenchyma inoculated with an axenic *E. dispar*[12], but no necrotic lesion was developed. In our experiments, it is possible that the presence of bacteria could enhance the inflammatory process, maintaining a propitious environment that allows trophozoites to multiply and spread along the hepatic parenchyma.

Although *E. dispar* cannot cause invasive disease to man, the experimental induction of lesions in animals is a way to access its pathogenic potential. Previous reports showed the ability of xenic strains with nonpathogenic zymodeme in causing lesions in hamster liver[5,19]. Our studies evaluated quantitatively not only the prevalence, but also

the degree of the lesion, showing that some *E. dispar* can experimentally cause severe tissue damage *in vivo* as that caused by a virulent *E. histolytica.* It remains to be investigated whether the pathogenic potential of some strains of *E. dispar* is enough to determine symptoms on the human host.

RESUMO

Patogenicidade de *Entamoeba dispar* em cultivo polixênico e monoxênico comparada a uma cepa virulenta de *E. histolytica*

Dois isolados de *Entamoeba dispar* em cultivo polixênico e culturas clonadas deles obtidas foram submetidos à monoxenização utilizando *Crithidia fasciculata* como organismo associado. O crescimento em cultivo monoxênico dos isolados e clones, bem como sua capacidade de destruir células VERO (efeito citopático) e de produzir lesões hepáticas em hamster foram comparados a uma cepa virulenta de *E. histolytica*. Os trofozoítos de *E. dispar* em cultivo monoxênico apresentaram um crescimento nitidamente menor que o de *E. histolytica* e foram avirulentos tanto no teste *in vivo* quanto *in vitro*. Entretanto, isolados e clones de *E. dispar* em cultivo polixênico exibiram uma atividade lítica moderada sobre as células VERO (1,5 to 41,8% de destruição) e causaram lesões hepáticas em hamster (29 a 100% em prevalência e 0,86 a 4,00 no grau de lesão) tão extensas quanto aquelas causadas pela *E. histolytica*. Embora *E. dispar* não seja associada à doença invasiva no homem, a ocorrência de lesões teciduais significativas, causadas por trofozoítos em condições experimentais, indica que esta espécie pode apresentar potencial patogênico considerável quando em presença de bactérias intestinais.

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