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VIRULENCE PARAMETERS IN THE CHARACTERIZATION OF STRAINS OF *Entamoeba histolytica*

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SUMMARY

Differences in virulence of strains of *Entamoeba histolytica* have long been detected by various experimental assays, both *in vivo* and *in vitro*. Discrepancies in the strains characterization have been arisen when different biological assays are compared. In order to evaluate different parameters of virulence in the strains characterization, five strains of *E. histolytica*, kept under axenic culture, were characterized in respect to their, capability to induce hamster liver abscess, erythrophagocytosis rate and cytopathic effect upon VERO cells. It was found significant correlation between *in vitro* biological assays, but not between *in vivo* and *in vitro* assays. Good correlation was found between cytopathic effect and the mean number of uptaken erythrocytes, but not with percentage of phagocytic amoebae, showing that great variability can be observed in the same assay, according to the variable chosen. It was not possible to correlate isoenzyme and restriction fragment pattern with virulence indexes since all studied strains presented pathogenic patterns. The discordant results observed in different virulence assays suggests that virulence itself may not the directly assessed. What is in fact assessed are different biological characteristics or functions of the parasite more than virulence itself. These characteristics or functions may be related or not with pathogenic mechanisms occurring in the development of invasive amoebic disease.

KEYWORDS: *Entamoeba hystolytica*; Virulence assays; Izoenzyme patterns; Restriction fragments analysis.

INTRODUCTION

Strains of *E. histolytica* obtained from different patients have been long known to present great variability in their pathogenic behavior. Virulence, conceived as degree of pathogenicity, must take into account the circumstances under which it is determined[8]. To the present, several parameters have been employed in the assessment of virulence of *E. histolytica* strains. Among the most employed ones are capability to produce lesions in experimental animals[9], capability to destroy cultured mammal cell monolayers[2], erythrophagocytosis[22], isoenzyme patterns[25], restriction fragment analysis of genes[7, 28, 30] and proteinase and collagenase activities[15, 33].

The comparison of these parameters for each strain has arisen puzzling results. TSUTSUMI et al.[33] did not found correlation between erythrophagocytosis rate with collagenolytic activity or production of lesions in hamster liver. MONFORT et al.[19] was also not able to demonstrate correlation between erythrophagocytosis (assessed as percent of phagocytic amoebae) and virulence to hamster liver. The correlation between zymodemes and virulence *in vivo* was evaluated by GIBODA et al.[12] who found that a strain with pathogenic patterns was not able to produce lesions in hamster liver. Taking cytopathic effect as parameter, some strains with non pathogenic zymodeme may behave more virulent than strains with pathogenic zymodeme; and the reverse also seems to occur[3].

In order to evaluate the behavior of axenic strains of *E. histolytica* in different virulence assays, five strains were characterized in respect to their ability to produce lesions in hamster liver, cytopathic effect upon VERO cells, erythrophagocytosis rate, isoenzyme pattern and restriction fragments analysis of genes. Although good correlation was observed between *in vitro* assays, these did not correlate with *in vivo* assay.

MATERIAL AND METHODS

Strains of *Entamoeba histolytica*

Strains of *E. histolytica* employed in this study and some of their relevant characteristics are summarized in Table 1. All strains were kept under axenic cultivation in TYI-S-33 medium [9] and they are used in exponential growth phase.

***In vivo* virulence**

The ability of trophozoites to induce liver abscess formation in the golden hamster was taken as a parameter for *in vivo* virulence. Groups of ten golden hamsters, four weeks old, mean weight 70g were used for each strain. Under Pentobarbital anesthesia, laparatomy was performed, and 1 x 10^6 amoebae sus-

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TABLE 1
Strains of *Entamoeba histolytica* and its relevant characteristics

Strain	Clinical evaluation	Serological results*	Virulence†	Pattern of Isoenzyme‡ and RFLP §
HM-1: IMSS	amoebic dysentery	Pos.	attenuated	Pathogenic
ICB-CSP	amoebic dysentery	Pos.	attenuated	Pathogenic
ICB-462	asymptomatic	Neg.	low virulence	Pathogenic
ICB-32	asymptomatic	Neg	avirulent	Pathogenic
ICB-RPS	asymptomatic	Neg	avirulent	Pathogenic

* Sera were tested with ELISA except for HM-1 tested with IFAT

† Evaluated by inoculation into hamster liver

‡ Pathogenic isoenzyme pattern was characterized by abscence of an alpha band and presence of a beta band in PGM and two fast bands for HK.

§ The restriction fragment lenght polymorphism (RFLP) analysis, considered as pathogenic profile the pattern obtained by digestion of the 482 bp gene by the enzymes *Taq* I and *Xmn* I.

pended in 0.1 mL medium was injected directly into the left lobe of the liver. Six days later the animals were sacrificed and examined for abscessses and for the presence of trophozoites.

Cytopathogenicity

The destruction of cell culture monolayers was taken as a semiquantitative assay for the virulence of the trophozoites *in vitro* [2]. VERO cells growing in Dulbecco modified Eagle's medium were assayed. Briefly, the cells attached in microtiter plates were exposed to the amoebae. The cells remaining in the wells were stained with methylene blue. The color intensity developed in that wells which were not added trophozoites was used as control (zero per cent of detachment of cells by amoebae). The color intensity was measured at 660 nm in a Shimadzu UV 160A spectrophotometer.

Erythrophagocytosis

The phagocytic capacity was determined as another marker of virulence. The test for erythrophagocytosis was performed according to the method described ty TRISSL et al. [31]. Briefly, log-phase trophozoites were adjusted to a concentration of 1 x 10^6/mL in phosphate buffered saline (PBS). 0.4 mL of this suspension was left to interact for 10, 20 and 30 min with the same volume of a suspension of 1 x 10^8/mL of heparinazed human erythrocytes, type A, corresponding to a final 1:100 amoeba: erythrocyte ratio. Results were assessed as percent of phagocytic amoebae, as well as the mean number of phagocytized erythrocytes per amoebae.

Isoenzyme Analysis

The zymodeme pattern was analyzed by starch gel electrophoresis for the enzymes: EC1.1.1.40, malic (ME), EC2.7.5.1, phosphoglucomutase (PGM), EC5.3.1.9, glucose phosphate isomerase (GPI) and EC2.7.1.1, hexokinase (HK) which have been

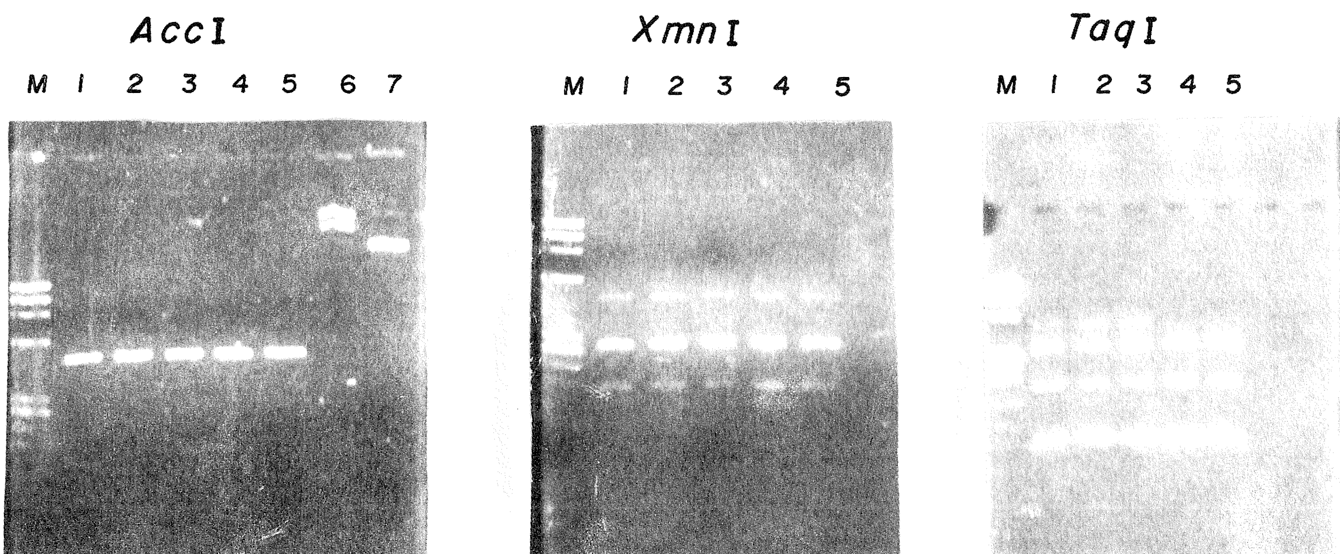

Fig. 1. Electrophoretic separation 2% agarose gel containing ethidium bromide of a 482 bp gene amplified by PCR and digested with restriction enzymes. M = DNA size marker (φx174). Lanes 1-5 is showed the strains of *E. histolytica*: HM1, CSP, 462, RPS and 32 respectively after incubation with restriction enzymes. lanes 6 and 7 are the activity control for *Acc* I enzyme. Blue script digested (7) and not diggested (6) with this endonucleases.

TABLE 2
Virulence characterization of axenic strains of *Entamoeba histolytica*, by means of erythrophagocytosis, cytopathic effect and abscess formation in hamster liver.

	Erythrophagocytosis *						Cytopathic effect†	Abscess formation‡
	Percentage of phagocytic amoebae			mean number of uptaken erythrocytes				
Strain	10	20	30	10	20	30		
HM1:IMSS	89.8	100	99.1	8.1	14.4	13.4	39. 2	0
ICB-CSP	88.4	95.3	98.9	4.9	12.7	15.8	41.0	0
ICB-462	79.1	85.6	85.7	4.6	11.7	12.0	29.0	25
ICB-RPS	46.4	83.0	94.5	1.2	5.7	8.5	7.0	0
ICB-32	50.4	48.2	46.4	0.9	2.3	2.2	4.0	0

* Amoebae and erythrocytes, at a 1:100 ratio, were left to interact for 10, 20 or 30 minutes.
† Percentage of destruction of VERO cells by trophozoites at a ameba: cell ratio of 1:1. Incubation carried out for 1 hour.
‡ Percentage of hamster with abscess observed six days after intrahepatic inoculation. Ten hamsters were inoculated for each strain.

TABLE 3
Correlation coefficient taken as an index concordance between different parameters.

Parameter 1	Parameter 2	r*	P value
cytopathic effect	10 min. % phagocytic amebae	0.98	<0.01
	20 min. % phagocytic amebae	0.83	NS
	30 min. % phagocytic amebae	0.68	NS
	10 min. mean number uptaken erythrocytes	0.91	<0.02
	20 min. mean number uptaken erythrocytes	0.96	<0.01
	30 min. mean number uptaken erythrocytes	0.92	<0.02
	Abscesses in hamster liver	0.16	NS
Abscesses in hamster liver	10 min. % phagocytic amebae	0.25	NS
	20 min. % phagocytic amebae	0.09	NS
	30 min. % phagocytic amebae	0.01	NS
	10 min. mean number uptaken erythrocytes	0.12	NS
	20 min. mean number uptaken erythrocytes	0.26	NS
	30 min. mean number uptaken erythrocytes	0.17	NS

* Correlation coefficient
NS, not significant.

employed as markers for invasive and non-invasive strains [26]. We analyzed the enzymatic pattern using 20 μL of the extracts which had been stored in liquid nitrogen (-196° C) in bead shape. The gel staining was carried out as previously described [25].

Restriction Fragment Lenght Polymorphisms (RFLP)

The DNA polymorphism of the strains were determined by specific amplification of a 482 bp gene described as useful for differentiate pathogenic and nonpathogenic strains of *E. histolytica* allowed by their digestion with restriction endonucleases[30]. Each 20 μL of the reaction mixture contained 0.7 units of *Taq* polymerase (Cenbiot, Rio Grande do Sul, Brazil), 200μM dNTPs, 10 mM Tris/HCl (pH 9.0), 75mM KCl, 3.3 mM $MgCl_2$, 7 pmol of each primer P1S-17 (5'-GCAACTAGTGTTAGTTA) and P1AS-20 (3'GTTAAAACATATCTTGGAGG) and 10 ng of purified *E. histolytica* DNA. This mixture was subjected to 30 cycles of specific PCR as described by TANNICH & BURCHARD[30]. After specific PCR 10 uL of reaction product was digested with restriction endonucleases *Acc* I, *Taq* I and *Xmn* I, under conditions recommended by manufactures (Gibco/BRL). The RFLP was analyzed on 1% agarose gels stained by ethidium bromide.

Statistical Analysis

The concordance between parameters of virulence were statistically evaluated by means of correlation coefficient. A p value of 0.05 was regarded as significant [1].

RESULTS

The results of erythrophagocytosis, cytopathic effect upon VERO cells, abscess formation in hamster liver are stated in Table 2. The profile obtained after restriction endonuclease digestion of the 482 bp gene with *AccI*, *Taq* I and *Xmn* I are shown in the figure 1. All strains showed pathogenic profile. The values for the correlation coefficient (r) for the different parameters are shown in Table 3. Good correltion was found between *in vitro* assays. The mean number of uptaken erythrocytes by amoebae significantly correlated with cytopathic effect upon VERO cells.

No significant correlation was found between percentage of phagocytic amoebae and cytopathic effect, particularly if amoebae and erythrocytes are left to interact for more than 10 minutes. There was very poor correlation between *in vitro* and *in vivo* assays. Abscess production in hamster liver did not correlate with mean number of uptaken erythrocytes, percentage of phagocytic amoebae and cytopathic effect upon VERO cells. Isoenzyme and restriction fragments analysis showed all strains in the study to belong to pathogenic pattern (Table 1).

DISCUSSION

The searching for reliable parameters in distinguishing virulent strains of *E. histolytica*, from non-virulent ones [18, 24, 31] is time-consuming and depends on of several intrinsic amebic properties and/or of host factors. Recently, differences at molecular level have been regarded as good parameters, leading to the acceptance of the dualist theory proposed in 1925 by BRUMPT [10]. The problem for phenotypic characterization of virulence of *E. histolytica* "sensu stricto" still deserves investigation since it has been proposed that extreme virulence variability may occur in this parasite [8]. The use of biological parameters, both *in vivo* and *in vitro*, however, has frequently presented conflicting results [19, 33].

Our results clearly show that poor correlation was found between *in vivo* and *in vitro* assays, possibly reflecting that dif-

ferent pathogenetic mechanisms or functions of the parasite are in fact measured. It is understandable the cytopathic effect as a mechanism involved in pathogenicity for animal models with both parenchymal and/or inflammatory cells[32] as target cells. The absence of correlation between cytopathic effect and hamster liver abscess formation may be due to the fact these parameters are related to other parasite functions, which cannot be assessed by the cytopathic effect assay, such as its ability to survive to the immune response including complement lysis[11, 24] or to interact or degrade the extracellular matrix[17, 27, 29]. This suggests that these parasite functions may be independently expressed.

MONFORT et al.[19] and MONFORT & PEREZ-TAMAIO[20] recently discussed the role of erythrophagocytosis assay in evaluating virulence of *E. histolytica* strains and were not able to find correlation between erythrophagocytosis, assessed as percent of phagocytic amoebae and virulence to hamster liver, leading the authors to argue its validity in evaluating *E. histolytica* virulence. We demonstrated that when percent of phagocytic amoebae is taken as the variable response, when amoebae and erythrocytes are left to interact for more than 10 minutes, poor correlation is found between erythrophagocytosis and cytopathic effect. Much better correlation is obtained when the mean number of uptaken erythrocytes is taken as variable response for erythrophagocytosis assay. It is not clearly understood how phagocytic function can be related to pathogenic mechanisms involved *in vivo* virulence[20]. It is apparent from our study that phagocytic function itself (assessed by percentage of phagocytic amoebae) is poorly correlated to virulence and differences between strains diminish as time of interaction increases. On the other hand, when mean number of uptaken erythrocytes is taken as the variable response, a good correlation is found with cytopathic effect. This response may estimate the velocity of phagocytosis, reflecting this way, probably indirectly, membrane functions of *E. histolytica*, such as plasticity and remodelling velocity which may be directly related to pathogenic mechanisms, such as, for example, capping and endocytosis of adhered molecules[4]. Also, a more rapid uptake of erythrocytes by amoebae explains the strong correlation between percent of phagocytic amoebae as ten minutes and cytophatic effect. Our results therefore recommend that the mean number of phagocytized erythrocytes should be taken as the variable response in erythrophagocytosis assay. TSUTSUMI et al.[33] considered that phagocytic ability may be taken only as a qualitative marker of pathogenicity of axenic strains of *E. histolytica* whereas a quantitative estimate of degree of virulence is taken by the production of liver abscesses in hamsters. If one takes this assertive as truth, strains RPS and 32 should be regarded as "non-pathogenic". In fact, these strains were obtained from asymptomatic carriers with negative serology and were never able to induce abscesses in hamster liver. A major disagreement would then arise when one compares isoenzyme and restriction fragments analysis with phagocytic ability. Our study demonstrates that, at least under axenic conditions, extreme differences in virulence indexes can be observed among strains presenting pathogenic patterns in isoenzyme and restriction fragments analysis. To the present, however, there is limited experience of isoenzyme analysis in axenic *E. histolytica* strains and, to our knowledge, no strain under this condition has ever presented a non pathogenic zymodeme pattern. The analysis of pathogenicity by RFLP shows a pathogenic pattern for all strains, suggesting that this parameter is not good to detect differences in the virulence of *E. histolytica* even though it may clearly distinguish pathogenic strains (*E. histolytica*) from nonpathogenic ones (*E. dispar*) [7, 28, 30].

The discordance in the results observed in the different virulence assays showed that the analysis of virulence estimates of *E. histolytica*, in biological assays, should take into account probable pathogenic mechanisms or parasite functions assessed. Pathogenic mechanism in amebiasis has been described as a chain of events occurring in the development of invasive disease, which involves different functions such as interaction with intestinal mucus[6], secretion of enzymes and/or toxins[16], adherence to intestinal epithelial cells[21, 23], cytopathic effect upon epithelial[2] and/or host inflammatory cells[13], interaction with or degradation of extracellular matrix components[14, 27, 29], resistance to immune defense mechanisms[4, 5, 11, 24]. The virulence assays, including animal inoculation, erythrophagocytosis, cytotoxic and cytopathic effect, proteinase and collagenase activities, resistance to complement lysis, and others should be taken as direct or indirect estimates of these functions, which can be independently expressed, leading to the puzzling results observed when comparisons are done between different parameters.

RESUMO

Parâmetros de virulência na caracterização de Cepas de *Entamoeba histolytica.*

A caracterização quanto a virulência das diferentes cepas de *E. histolytica* foi avaliada através de ensaios *in vivo* e *in vitro*. Discrepâncias nesta caracterização têm surgido quando se compara os diferentes ensaios. Avaliamos alguns parâmetros de virulência na caracterização de 5 cepas axênicas de *E. histolytica*. Estas cepas foram caracterizadas com relação à sua capacidade para induzir abscesso em fígado de hamster, eritrofagocitose e efeito citopático sobre células VERO. Encontramos correlação significante entre os ensaios biológicos *in vitro*, mas não entre estes com os ensaios *in vivo*. Boa correlação foi achada entre o efeito citopático e o número médio de eritrócitos ingeridos, mas não com a percentagem de amebas que fagocitaram eritrócitos, mostrando que grande variabilidade pode ser observada no mesmo ensaio, dependendo da variável escolhida. Não foi possível correlacionar isoenzima e padrão de fragmento de restrição com o índice de virulência desde que todas as cepas estudadas apresentaram padrão patogênico. Os resultados discordantes observados nos diferentes ensaios de virulência sugerem que a mesma não deve ser avaliada diretamente. O que de fato está sendo avaliado são as diferentes características biológicas ou funções do parasita, mais do que a própria virulência. Estas características ou funções podem estar relacionadas ou não com mecanismos patogênicos que ocorrem no desenvolvimeto de amebíase invasiva.

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