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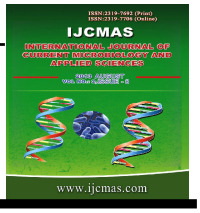

Original Research Article

Encystment and encystations of *Entamoeba invadens* In-Vitro as a model to test amebicides against *Entamoeba histolytica* to the public health problem *Entamoeba dispar*

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Keywords

Entamoeba invadens; cysts; trophozoites; *E. dispar*; exposue to drugs; axenic conditions; *E. histolytica*

A B S T R A C T

The life cycle of *Entamoeba invadens* implies the cyst formation who infest to new guests and that excystation and reproduction cause amebiasis; the excystation process is poorly understood and it hasn`t been systematically studied in vitro.The objective of this study was to quantify the excystation of cysts of *E. invadens* IP-1 strain, previously obtained in axenic conditions for incubation in medium AEM with inoculums of 2 x 10^5 trophozoites / ml for 72 hours. The excystation was caused to transfer the cysts to the growth medium Bl-S-33, the first trophozoites were observed after 12 hours of incubation and after 72 hours, the 13% of the inoculated cyst were excystation: the most range of excystation was observed at 48 hours. It was obtained as cultives of trophozoites, who after six weeks it had similar growth curves to the original strain. These results demonstrated the quantitatively excystation of *Entamoeba invadens* and confirmed the possibility of provoke the life cycle in vitro of this amoeba in axenic conditions for facilitating the study, and it was necessary to determine the optimum conditions to induce the massive and synchronize excystation. The model obtained in *Entamoeba invadens* IP-1 could even reduce unnecessary exposure to drugs, in the case of *E. dispar* and *E. histolytica*, increases the costs to the patient and family, health institutions, and the risks of exposure to drugs, as *E. dispar* is not parasitic amoeba.

Introduction

The species pathogenic of the amoeba *Entamoeba invadens* have two stages or phases in the life cycle: the trophozoite (Figure. 1), which is the form that causes causes disease and cyst resistant to physical and chemical changes that the trophozoite does not support , the first is mainly involved in the spread of the parasite to new hosts (Figure. 2), this unicellular parasite of direct cycle has

Figure.1 Trophozoites (t) and cysts (arrows) of *Entamoeba invadens*. The trophozoites present the amoeboid shape characteristic; the cysts are rounded and measured - 20 um of diameter. Differential interference contrast.

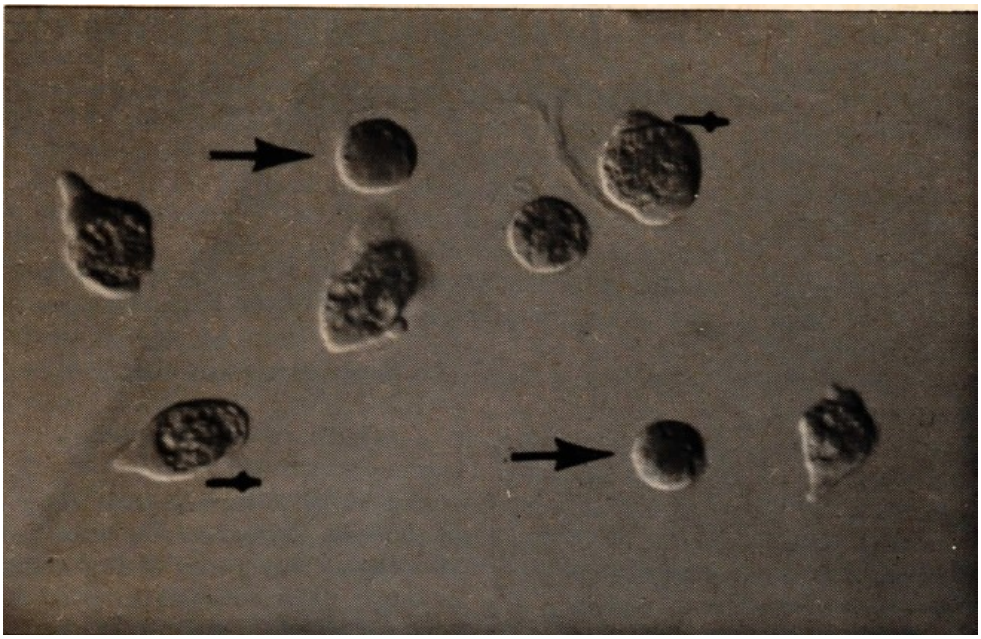
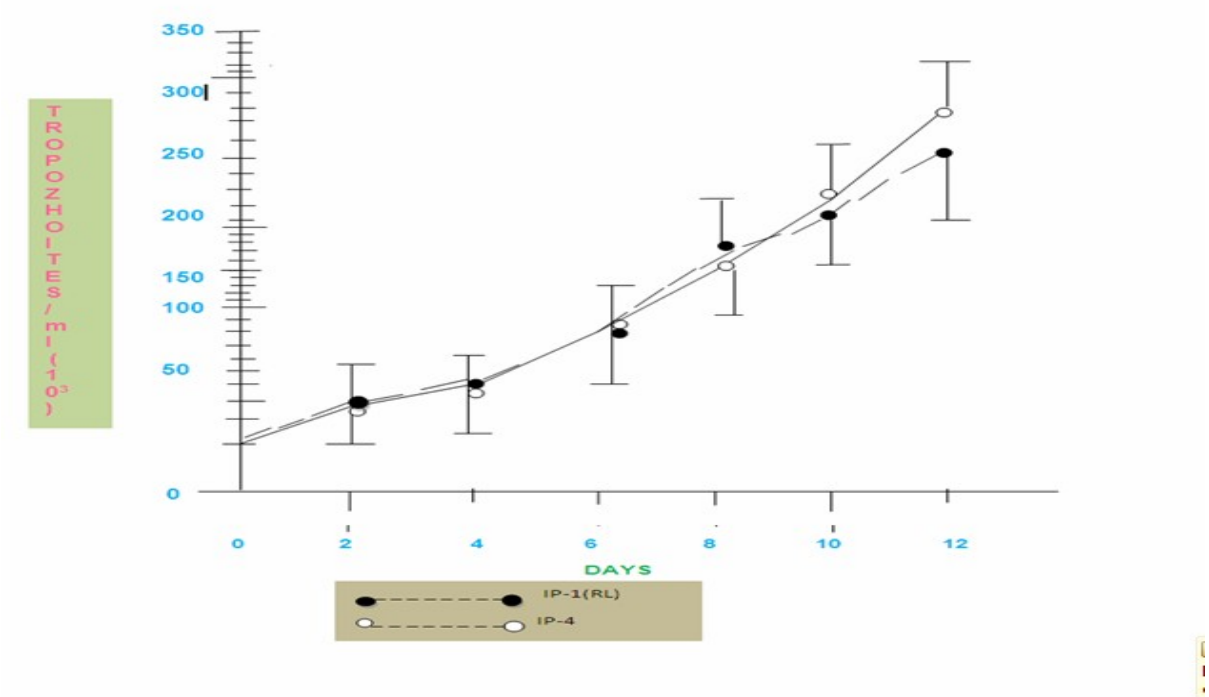


Figure.2 Growth curve of *E. invadens.*



an infectious stage comprising a period of 1 to 14 days, and their eggs can be transported from terrarium to terrarium by vectors such as cockroaches and flies.

However *E. Invadens* hasn't public health importance, as the mainly natural guests are snakes and not present safe for humans, but this species of amoebae has gained importance because of its similarity with *Entamoeba histolytica* (causative agent of human amebiasis) in relation with his morphology, life cycle, the cultive requirements and pathogenesis, (Geiman and Ratcliffe, 1936; McConnachie, 1955; Diamond *et al.,* 1978) but mainly because it is possible to obtain mature cysts of E. invadens in axenic conditions, (Rengpien and Bailey, 1975) which to date has not been achieved in the case of *E. histolytica*. Thanks to this it is known the ultrastructure of cysts (Chavez *et al.,* 1978), the ribosome's characteristics, the variations in the membrane components (Chayen *et al.,* 1985) and the wall quistic composition (Arroyo-Begovich *et al.,* 1980). Similarly, we have continued to study the life cycle of *E. invadens* by inducing the encystment and encystment in vitro.

The first to study the life cycle of *E. invadens* were Geiman and Radcliffe, one its discoverers, (Ratcliffe and Geiman, 1934; Ratcliffe and Geiman, 1933), who unable to induce the massive encystment in cultivation medium, based his studies in observations of samples in inoculated snakes experimentally with amoebic cysts, they found that are required five to seven hours for undertaking the excystation.

They also observed that the emergence of trophozoites was affected in periods as short as 15 minutes or as long as 80 minutes; they concluded that the encystment of *E. Invadens* is similar in detail to describe for *E. Histolytica*. Subsequently McConnachie obtained Trophozoites by subculturing of cysts in the growth medium of amoebas, 2 procedure used by others to induce the encystement (Cervantes-Mamoa and Martínez-Palomo, 1980; Barrer and Svihla, 1964; Thepsuparangskul *et al.,* 1971).

However, knowledge about the encystment is scarce. Knowing the factors involved in the induction about encystation as the encystment, besides being an inherent basic knowledge which will introduce new strategies parasite control by blocking their life cycle avoiding the infestation of new hosts, particularly of *E. Histolytica* contributing in directly relation to decreasethe morbidity and mortality triggers of this infestation.(Avron *et al.,* 1982; Mora-Galindo *et al.,* 1986).

The limited knowledge about encystment process is due to the lack of a system who permit the massive encystment in reproducibly form, because in the most cases only has been executed qualitative analysis (Geiman and Ratcliffe, 1936; McConnachie, 1955; Cervantes-Mamoa and Martínez-Palomo, 1980; Thepsuparangskul *et al.,* 1971) In this work is evaluated quantitatively the excystation of *E. invadens* cysts in between Bl-S-33.

Materials and Methods

Strain and cultivation conditions

This work was used trophozoites of *Entamoeba invadens* of the strain IP-1, grown in axenic growth medium BI-S-33 to 25 ° C, in glass tubes with screw cap 16

X100 mm. This strain was originally isolated from a snake (Natrix ciclopiori) in 1952 and since then has been in several laboratories in different culture media. The strain was maintained in our laboratory by replanting of 1x104 trophozoites / ml every seven days.

Encystment

To Inducing the encystment were used trophozoites harvested in logarithmic growth phase, which were reseeded in tubes with seven ml of encystment medium AEM (Rengpien, S. y G.B. Bailey, 1975), in the appropriate number for obtain 2x105 trophozoites / ml. Were incubated at 25°C and to the 24, 48 and 72 hours were quantified cysts and trophozoites in Neubauer chambers and we proceeded to calculated the percentage of encysted amoebae in relation to the total cells obtained (trophozoites and cysts).
The viability of cysts obtained at 72 hours was determined by the exclusion of tripartite blue.

Encystation

The excystation of cysts was induced on the same day who they were obtained, for this it was incubated in growth medium BI-S-33 at 25 ° C after washing the amoebae with Sarkosyl (N-lauryl sarkosyl) 0.2% to removing trophozoites.

Were performed observations of the tubes in the inverted microscope every 12 hours up to 72 hours for detect the presence of trophozoites; every 24 hours were quantified the trophozoites and cysts in Neubauer chambers. For the quantitative analysis, we calculated the percentage of trophozoites and cysts in the different periods indicated, in relation to the total cells in each period studied. For the statistical evaluation we used the Student T test.

In another series of encystment experiments, the cultivation were washed with 0.2% Sarkosyl for 24 hours and the subcultivation in growth medium, to determine if the process was continued in a period of 72 hours and was determined semiquantitatively in what period occurred an increased encystation; in this case was considered as positive the encystment observing through a microscope the presence of motile trophozoites in culture tubes.

Result and Discussion

The Cultives of *E. Invadens* had exponential growth and yields were 3X105 trophozoites / ml after 12 days of culture (Figure. 3), after which it started the declining cell period. For induction of encystment, the amoebae were harvested after seven to eight days of to be replanted, as during this period were found in the logarithmic growth phase.

As to encystment, we observed an increase in the concentration of cysts according to the incubation time, the first cells in encystement were of the lumps are normally formed in the cultives. After 48 hours, the average of the encystment was to the 50% and not significantly increased at 72 hours (Figure. 4).

During the process there was a corresponding reduction in the number of trophozoites, those remaining as such were seen primarily attached to the tubes and presented the characteristic amoeboid form. The viability of cysts obtained was always greater to the 70%.

Figure.3 Kinetic encystment of *E. invadens*, strain EP-1.

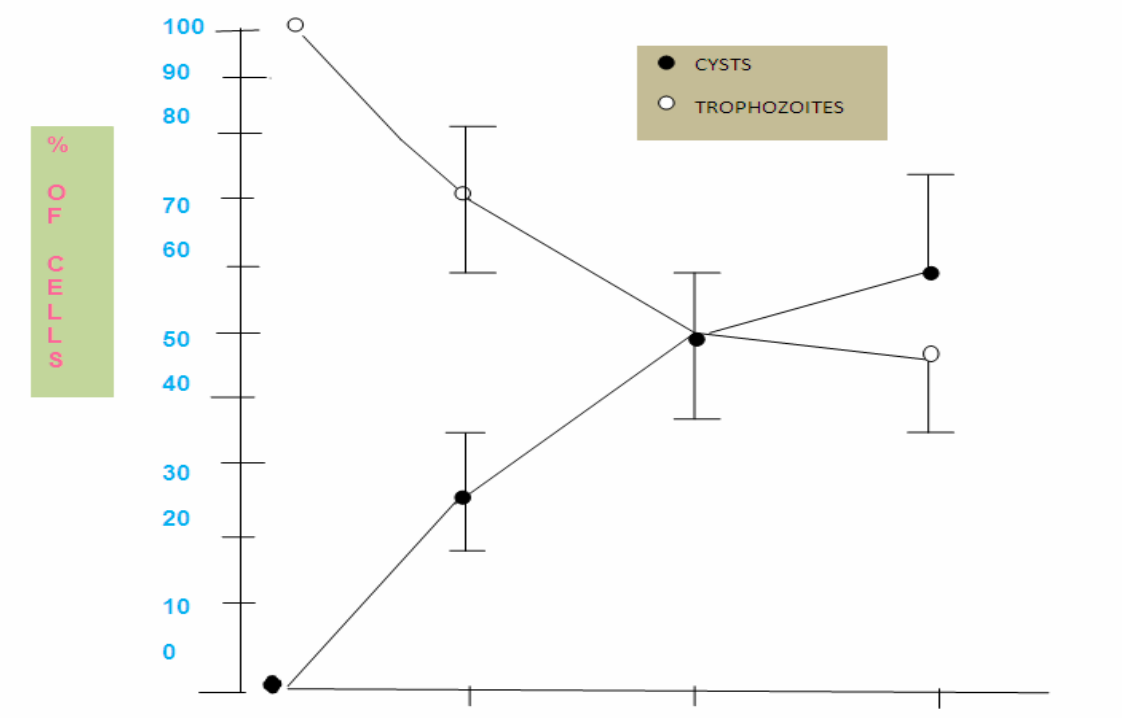
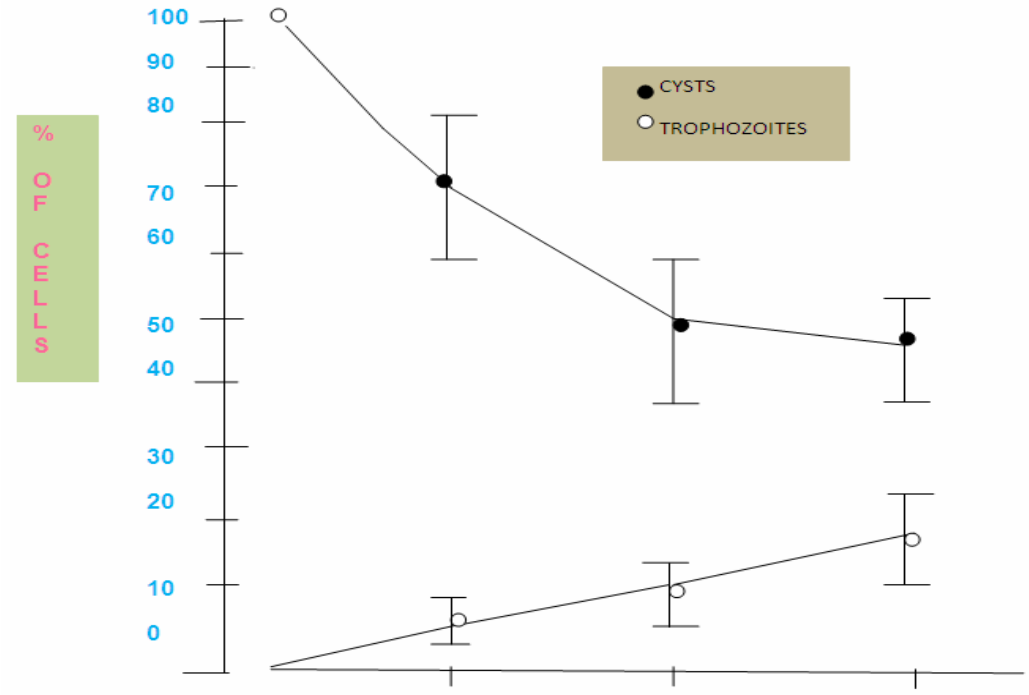


Figure.4 Kinetic encystment of *E. invadens* of the strain IP-1



The encystement of the cysts resistant to sarkosyl began 12 hours after transfer the cysts to the medium BIS-33, however, the quantification of trophozoites in Neubauer chambers only was possible after 24 hours (Figure. 4).

However, in three experiments, although there were encystement determined by analysis inverted microscope, it was not possible to quantify the trophozoites even after 72 hours, the same whathappened between these two last.

In other seven experiments the number of trophozoites increased in function on the incubation period to an average of 13%, and concomitantly decreased the number of cysts.

Statistical analysis showed that there significant difference between the percentage of trophozoites at 24 hours compared with the 48 and 72 hours, the same happened between the latter two.

The trophozoites destruction, product of the encystement with Sarkosyl and the later incubation of the cysts resistant in the medium Bl-S-33, indicated who said process of encystement is continuous (Table 1), and that after 48 hours there is a higher concentration of trophozoites in relation to the 24 and 72 hours.

Subsequently was subcultivated of the same way that the original strain and was obtained a cultive with a similar growth curve (Figure 3) to the strain that gave rise through cysts;to differentiate it was called IP-1 strain (RL) and maintained for several months in the laboratory, but was lost by bacterial contamination (Ruvalcaba-Ledezma *et al*., 1989).

According to what was reported by Rengpien and Bailey (1975) were obtained cysts from *E. invadens* in an AEM axenic medium, but the yield obtained was not as high as reported by these authors.

No clutch is possible that our accounts were underestimated, because the cysts obtained was agglutinated making more difficult a precise quantification.

An alternative explanation would be that the nutrient medium or the metabolic state of the cells were not optimal to allow proper cell division to significantly increase the number of trophozoitesand in direct relation was obtained a high population after of the cell lysis who typically occurs in the AEM, due to their low osmolality as compared to Bl-S-33. In previous works from our laboratory (Mora-Galindo *et al.,* 1986)[15] and other authors, (Avron *et al.,* 1982; Avron *et al.,* 1983) the cysts yields were also lower without the authors have found the reasons for this.

Moreover, we unknown if the trophozoites who wasn't encystment after 72 hours in medium AEM,moreover,we unknown if the trophozoites who wasn't encystment after 72 hours in medium AEM can do incubating for prolonged periods in the same medium.

Regarding to the encystement so far only been performed descriptive analysis or semiquantitative, in the latter are reported as negative or positive, indicating with different number of crossings the magnitude of the encystation. The present work report for first time quantitative evaluation of the encystation. The first trophozoites appeared 12 hours after the induction of the encystation, although there is the possibility that it may occur earlier. There is no difference when

compared to the time reported by Geiman and Radcliffe(1936) although in this case the encystation occurred in vivo and with a different strain of amoebae.

Not all the cysts was encystment and the ratio of trophozoites obtained was low, possibly not all cysts were ripe, and might exist anormal encystment.Although it is generally considered that the cysts are resistant to sarkosyl, some might be affected by the washing with detergent. On the other hand, are unknown the causes that provoke of the destruction of cysts in the middle BI-S-33, where in theory are resistant.The destruction of cysts has already been reported by other authors, even at times not achieved trophozoites to from cysts (Chayen *et al.*, 1985).

It is considered that the lack of glucose (Vázquez De Lara-Ceneros and Arroyo-Begovich, 1984) and the low osmolarity in the medium (Rengpien and Bailey, 1975) are the main factors that induce the encystment, and it is possible that the presence of glucose and osmolarity of the medium BI-S-33 cause the encystation of the encysted amoebae.

The analysis of the results corresponding in a way to the generally proposed in the sense that the encystement occurs in adverse conditions and the encystation is performed when the conditions are own for developing trophozoites; however, it is not known which are the factors that induce the encystment, as well as at the molecular mechanisms involved in these processes of cell differentiation.

The results in Table 1 demonstrate that the encystement is a continuous process and the trophozoites increasing Figure. 5 results from the sum of newly hatched trophozoites more the appeared in the previous time, may be can join the amoebas that have resulted from the division of the trophozoites that hatched early. Moreover, this series of experiments demonstrated that the highest encystement percentage occurred at 48 hours.

The obtaining of the trophozoites cultives from cysts confirms the fact that the life cycle of *E. invadens* can be caused in vitro, using medium of axenic cultivation. Although in the present work it was performed a quantification of encystation, it was required a procedure who the provoke in the synchronized form as well as of the massive induction of the same, for that later can be evaluated the effect of the different factors, both on the encystement as well as on the encystation, with the goal of blocking the life cycle of these parasites and prevent infection to new hosts.

Asymptomatic individuals with documented *E. histolytica* infection should be treated with a luminal agent to eradicate infection; this recommendation is based both on the known risk for the development of invasive disease in such patients, and the fact that individual shedding *E histolytica* cysts are a risk to public health. (Gathiram and Jackson Tfhg, 1987; Haque *et al.*, 2001) *E. dispar* infection does not require treatment, but should alert the physician that the infected person has been exposed to faecally contaminated food or water.

The results of the table one explain the reason for the importance of further research respect to *E. dispa* (Samuel L Stanley, 2003) as though it is a kind of amoeba comennsal represents a public health problem that could intervener in patients being exposed to medical treatment without needing it.

En The University of Munich between 2005 and 2009, 103 laboratory-confirmed amebiasis cases were detected. The studycompares the results of various diagnostic tests amongthese patients, analyzes data on co-infections and clinicalsymptoms, and determines the risk for acquiring amebiasis.Results Initial screening tests (stool microscopy, coproantigen enzyme-linked immunosorbentassay (ELISA) were positive in 82.5 and 93.9%, respectively. Fecal samplesfrom patients with positive screening test results were subjected to polymerase chain reaction (PCR), which detected *E. histolytica* in 9.7% and E. dispar in 88.3% of the cases.

The majority of *E. histolytica* cases and morethan half of the *E. dispar* cases had intestinal symptomstypical for amebiasis. In 53.4% of the cases, intestinal coinfections were found, mostly *Blastocystis hominis* (39.8%), *Giardia lamblia* (10.7%), *Campylobacter* spp. (4.9%), and *Salmonella typhi* (2.9%). The risk for travelersto be infected with *E. histolytica* or *E. dispar* was highestfor destinations in West Africa, East Africa, and South and South-East Asia. (Herbinger *et al.,* 2011) In general terms it is necessary to continue investigating the matter and that is why we consider it important to review this article again.

Using the methodology was possible to cultivate, encysting *Entamoeba invadens* and desenquistar in axenic in vitro. The cysts and trophozoites obtained both in-vitro use of *Entamoeba invadens* allows amoeba that species as a model to test the effect of drugs amebicides as *E. invadens* represents a model for studies of amebiasis caused by *E. histolytica*. The model obtained in *Entamoeba invadens* IP-1 could even reduce unnecessary exposure to drugs such as metronidazole in our country is still used as a treatment for amebiasis, since in the case of being mistakenly diagnosed with this disease in the case of *E. dispar* and *E. histolytica*, increases the costs to the patient and family, health institutions, and the risks of exposure to drugs, as *E. dispar* is not parasitic amoeba.

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The authors declare that no conflict of interests for the publication of this research paper.

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