IN VITRO DIVISION MODALITIES DEVELOPED BY BLASTOCYSTIS HOMINIS EXAMINED WITH THE ACRIDINE ORANGE STAIN

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Abstract. Blastocystis hominis is a common enteric protozoan in humans and animals, with a high rate of zoonotic transmission. The morphology and the division modalities of Blastocystis hominis parasite have been studied both in vitro and in vivo, between the two media being differences on the division modalities developed by this parasite. This study reveals that this common parasite exploits in a different manner the environmental resources, adapting it to the existing conditions. From our study resulted that on the medium used in \textit{in vitro}, Gibco\textsuperscript{®}RPMI 1640, the budding division is predominant, with formation of one or more daughter cells (asymmetrical division), while \textit{in vivo}, in the feces multiplication, the dominant form is the binary division, resulting two identical daughter cells (symmetrical division). In order to emphasize the division forms present in the culture and feces, we used Acridine-Orange (AO) staining. AO is a cell-permeant nucleic acid binding dye that emits a green fluorescence bounding dsDNA and a red fluorescence bounding ssDNA or RNA. This unique characteristic makes the AO stain useful for cell-cycle studies and particularly for our study, showing the structural differences determined by the cell division.

Keywords: Blastocystis hominis, Acridine Orange (AO), binary division, daughter cells.

Rezumat. Modalități de diviziune \textit{in vitro} dezvoltate de Blastocystis hominis examine cu colorația Acridin Orange. \textit{Blastocystis hominis} este un protozoar enteric comun la om și animale, cu rată înaltă de transmitere zoonotică. Morfologia și modalitățile de diviziunea ale parazitului \textit{Blastocystis hominis} au fost studiate atât \textit{in vitro} cât și \textit{in vivo}, între cele două medi de viață existând diferențe din punct de vedere al modalităților de înmulțire pe care le dezvoltă parazitul. Studiul evidențiază că acest parazit comun exploatează în mod specific resursele de mediu, adaptându-se la condițiile de viață existente. \textit{In vitro}, pe mediul utilizat în acest studiu, (Gibco\textsuperscript{®}RPMI 1640), predomină înmulțirea prin înmulțire, cu formare de celule fiice (diviziune asimetrică), în timp ce \textit{in vivo}, în fecale, forma dominantă de înmulțire este diviziunea binară, rezultând două celule identice (diviziune simetrică). Pentru evidențierea modalităților de diviziune prezente în materiile fecale și în cultură, s-a utilizat colorația Acridin Orange (AO), colorație a acizilor nucleici, împreună cu care emite fluoroscență verde atunci când se leagă de dsADN și fluoroscență roșie atunci când se leagă de ssADN sau ARN. Această caracteristică unică face colorația Acridin Orange utilă pentru studiul ciclului celular și evidențiază, particular în acest studiu, diferențele structurale determinate de diviziune.

Cuvinte cheie: Blastocystis hominis, Acridin Orange (AO), diviziune binară, celule fiice.

Introduction

\textit{Blastocystis hominis} represents even nowadays a challenge for the parasitologists. Morphologically, it is the protozoan with the highest polymorphism, and the most diverse division modalities observed both \textit{in vivo} and \textit{in vitro}. From its discovery, different
cultivation, staining and identification techniques were elaborated and even PCR genotyping (Noël et al., 2005). Its reproduction was an enigma for a long time, at least four division modalities being described in the last decades: binary fission, plasmotomy, endogeny, and schizogony (Zierdt, 1991).

The purpose of this study was to describe in vitro behavior of Blastocystis hominis cultivated on a liquid media, morphological and division characteristics and to compare with his division ways developed in feces. Consequently, we used xenic cultures on the Gibco® RPMI 1640 medium (Zhang et al., 2012) and feces, with more than 5 parasites/microscopical field (400x). We used Acridine-Orange (AO) staining (Suresh et al., 1993; Suresh et al., 1994). This fluorochrome highlight the ADN concentration from cellular structures especially the nuclei and it is used for cell-cycle and detection of various stages of development of cells. In our study we use the AO to highlight the Blastocystis cells division.

Material and Methods

The origin of isolates

The isolates for the culture and also from feces were provided by patients with irritable colon syndrome (IBS) and colitis, and also by asymptomatic patients, where the parasite meets the quantitative criterion (over 5 parasites/microscopical field (400X)).

Work protocol

The Gibco® RPMI 1640 is a basic culture medium containing vitamins, aminoacids, salts, glucose, glutathione and a pH indicator. It does not contain proteins or growth agents. Therefore, it needs to be enriched, in order to become a “complete” medium.

The cultivation was realized in sterile test tubes with cotton stoppers, in which we distributed, with a sterile pipette, 5 ml Gibco® RPMI 1640 medium with an addition of fetal bovine serum (5 ml/100 ml RPMI medium) and antibiotics (1 ml mix of penicillin and streptomycin/100 ml RPMI medium). The cultivation was made in the aerobiosis.

The inoculation was realized by adding directly feces in the culture medium, obtaining a xenic culture due to the dependence of Blastocystis on the commensal flora. The culture was kept in a thermostat at 37°C, and it has been monitored for 14 days, with a daily agitation.

The readings were realized daily, after 48 hours from the inoculation. We notice if the parasites from the inoculation preserved their characteristics (shape, dimensions) and, if they divided, how their division modalities were.

For the microscopic evaluation of the parasite in direct fecal examination and also from culture we used AO stain.

Wet preparations from feces and culture suspension were stained with Acridine-Orange, which we examined under the fluorescence microscope (Leica microscope - 5500Q TCS SPE with a DFC 290 camera). This fluorochrome has affinity for DNA, staining bright yellow the DNA concentrations from the nuclei (Suresh et al., 1994).

Acridine-Orange staining

Acridine-Orange (AO) is a specific staining for the nucleic acids, selectively used to determine the cell cycle. AO interacts with the DNA and RNA by intercalation, or electrostatic attraction, respectively: DNA intercalated with AO – green fluorescence (525 nm); RNA with electrostatic attraction to AO – red fluorescence (>630 nm). There is a
distinction between the passive and the activated, proliferating cells, and also differentiated cells in the G1 phase can be detected. AO can also be useful as a method to identify the apoptosis, and to detect the intracellular pH gradients, and to measure the activity of protons pump related to the age and live activity of the Blastocystis organism.

In our study we used the AO stain for highlight the division modalities developed by the Blastocystis hominis in vitro and in vivo.

The solution prepared was stored at room temperature into dark bottles. We place on each microscopic slide 25 μl of the feces suspension containing Blastocystis, and 25 μl of AO solution or 50 μl AO and a small quantity of feces. We cover it with the 22x22 mm coverslips, waiting for 3-5 min, and then we examined the preparation under the fluorescence microscope (400x) and with immersion (Tan & Suresh, 2006).

At fluorescence, the central body forms have yellow cytoplasm, green vacuoles and bright yellow nuclei.

**Results and Discussion**

At a direct examination with AO stain, after 48 hours in the culture medium, the parasites was perfectly round, with a big and light green central vacuola, with a tight peripheral cytoplasm, and bright yellow nuclei being situated mainly at the periphery of the parasite cell (Fig. 1).

Only the vacuolar form was observed. We interpreted this fact by the adaptation of this population to the environmental stress. Concerning the dimensions, the parasite maintained its pleomorphic characteristics with sizes varying from 5 to 20 μm. With the AO stain, we showed a lot of morphological and division details.

![Figure 1. Xenic culture of Blastocystis hominis -vacuolar forms with large central vacuola and tight peripheral cytoplasm and bright light nuclei.](image-url)
The most frequent \textit{in vitro} division modality is the burgeoning, with the appearance of one or more daughter cells, oriented towards the exterior of the parasite (Figs. 2a-d), while \textit{in vivo} studies, using the same staining, we noticed that the binary division was the main modality.

The formation of the daughter cells is realized by accumulation and differentiation of the cytoplasm at the periphery of nuclei, followed by the appearance of the membrane.

Another asexual reproduction modality was the endogony, with the formation of a division bag inside the parasite, where the daughter cells could be seen (Fig. 2f). Simultaneously, the binary division of the protozoan which already presented daughter cells was also emphasized (Fig. 2e).

\textbf{Figure 2.} \textit{In vitro} xenic culture-division modalities emphasized with AO staining: a. \textit{Blastocystis hominis} with peripheral undifferentiated daughter cells; b. burgeoning forms and young vacuolar forms; c. burgeoning forms with differentiated daughter cells; d. \textit{Blastocystis} with daughter cells and vacuolar degradation; e. binary division and burgeoning simultaneously developed; f. endogony with division bag.
In the feces we notice the binary division predominance with elongation stages and the segregation in two daughter cells (Fig. 3a, b).

**Figure 3.** *Blastocystis hominis* in feces: elongation stage (a) and binary division (b).

The AO staining has the advantage that it emphasizes several development stages of the parasite, especially *in vivo*, allowing to differentiate the life forms, especially the cyst, whose reporting is difficult in common staining. In our study, this fluorochrome emphases the presence of daughter cells by burgeoning or endogony like dominant division modality *in vitro* and evidence of the predominance of binary division *in vivo*.

**Conclusions**

*In vitro*, in RPMI medium, only vacuolar forms was detected.

The main *in vitro* reproduction modality is the appearance of one or several daughter cells.

*In vivo* binary division was observed predominantly.

The life environment influences the division modality, *in vivo* - binary division, while *in vitro* – the budding with daughter cell is predominant.

**References**


