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EXPRESSION OF GLUCOSAMINE-6-PHOSPHATE ISOMERASE AND JACOB GLYCOPROTEIN DURING CULTURE OF ISOLATES OF *ENTAMOEBA HISTOLYTICA*

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ABSTRACT: The glucosamine-6-phosphate isomerase and Jacob glycoprotein gene expression levels were assessed during *E. histolytica* excystation from the feces of five patients. Excystation was performed at 0, 24, 48 and 96 h in Robinson medium, and RNA was extracted from the cysts and trophozoites. cDNA was synthesized to amplify the genes using real-time RT-PCR. The results demonstrated differences according to the incubation time and/or conversion of the cystic phase to the trophozoite, where the most important expression in the genes was observed at 96 h excystation. The expression levels of the genes studied positively correlated with the population of trophozoites during excystament.

INTRODUCTION*: E. histolytica* is an intestinal protozoan parasite and the causative agent of amebic dysentery and liver abscess in humans. This parasite remains a significant cause of morbidity and mortality in developing countries and is responsible for up to 100,000 deaths worldwide each year. *E. histolytica* has a two-stage life cycle that consists of the infective cyst and colon invasive trophozoite forms [1, 2]. The metabolic pathways that determine the conversion from one state to the other remain unknown. *E. histolytica* cysts have an extracellular rigid wall that primarily consists of chitin, a homopolymer of N-acetyl-D-glucosamine ß-(1.4) linkages [3].

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Chitin polymerization requires the presence of specific enzymes called chitin synthases that belong to the family of ß-glucosyl-transferases. Recent reports have identified a cysteine-rich protein found in abundance in the cyst wall of *E. invadens*, a reptilian parasite similar to *E. histolytica* that has been used as a model of in vitro encystment [4-6]. This protein is referred to as Jacob glyprotein and is a chitin-binding protein [7]. Chitin has been proposed to directly deposit on Jacob proteins during the formation of the cyst wall [8].

The cyst wall also includes 100- to 150-kDa glycoproteins, which can specifically bind to wheat germ agglutinin (WGA) and certain uncharacterized surface antigens that may react with anti-cyst monoclonal antibodies [9, 10]. The first reaction of the biosynthetic pathway from trophozoite to cyst conversion is catalyzed by the inducible enzyme glucosamine-6-phosphate isomerase. This enzyme reversibly isomerizes fructose 6-phosphate and the ammonium ion to glucosamine 6-phosphate, which is acetylated to

form N-acetylglucosamine-6-phosphate. The process of encystment of *E. histolytica* has not been determined. However, two proteins that are specific to this process in *E. invadens* have been described: Jacob glycoprotein and glucosamine-6-phosphate isomerase.

Moreover, the transcripts that encode ubiquitin in *E. invadens* are detectable in trophozoites stage cells as well as during encystation. Therefore, the proteasome/ubiquitin system is essential for maintaining the differentiation state [11]. The purpose of this study was to determine the expression of the glucosamine-6-phosphate isomerase and Jacob glycoprotein genes during the excystation at 24, 48 and 96 h.

MATERIAL AND METHOD*:* *E. histolytica* cysts from each of the five patients with gastrointestinal disorders were obtained in 5 g of feces, which were homogenized with 50 mL of water and filtered three times with two meshes of different diameters (1 and 0.2 mm). The samples were then washed five times with water and centrifuged at 1000 x g for 5 min to concentrate the cysts, which were finally quantified per cubic millimeter (mm3) and analyzed with an optical microscope to observe their integrity. Three hundred cysts per milliliter of *E. histolytica* were excysted in Robinson culture medium [12] and subcultured every 24 h.

The sediment was examined under an optical microscope at a magnification of 40X at 24, 48 and 96 h. Subsequently, the number of excysted trophozoites was determined. At time 0, the same number of cysts (300cysts/mL) was incubated with 0.8 U of chitinase (Sigma, St. Louis, MO) in 50 mM PBS at pH 6.1 for 2 h at room temperature. The RNA trophozoites were then obtained with the FastRNA®ProRed Kit, (Qbiogene, Inc., CA) in the FastPrep™ Instrument (Qbiogene, Inc, CA).

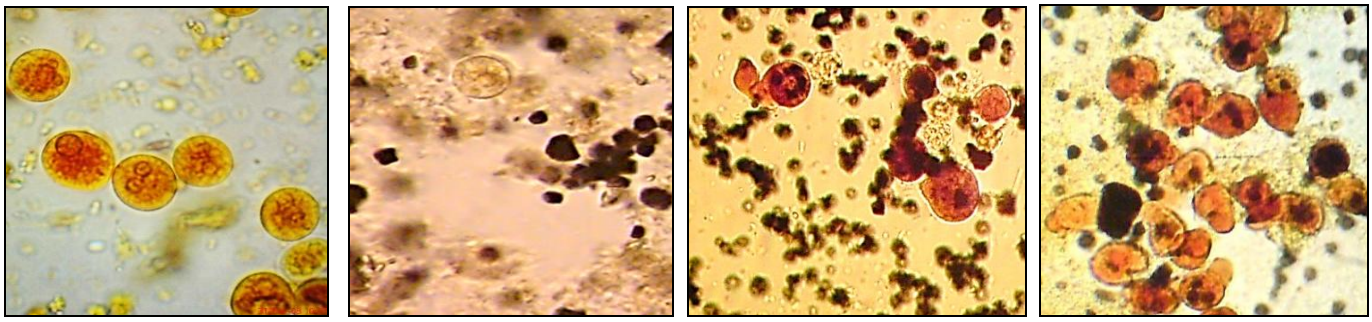
The trophozoites were subcultured in Robinson medium for the indicated times, and 1.0 mL of Trizol (Invitrogen) was then added to each sample. The RNA isolation procedure was continued according to the manuacturer's instructions. To validate the real-time RT-PCR, RNA was extracted from the *E. histolytica* trophozoite strain HM-1: IMSS with the RNAqueous Kit (Ambion, Inc, Austin, TX). The RNA was quantified by spectrophotometry at 260 and 280 nm, and its integrity was observed in a 1.5% agarose gel stained with ethidium bromide. Moreover, genomic DNA was obtained from isolates Robinson culture medium, and the 530 bp *enhhic* gene that encodes for the 30 kDa protease and restriction patterns that identify *E.histolytica* were amplified[13]. A standardization curve was generated with the reference strain using four serial dilutions of cDNA (1:625, 1:125, 1:25 and 1:5) with three replicates each of the glucosamine 6 phosphate isomerase and Jacob glycoprotein genes. After obtaining 5µg of RNA from each isolate during the three incubation times, cDNA was synthesized using M-LVRT and the Taqman® Gold RT-PCR Taq Man Rev Transcriptase Kit (Applied Biosystems, Foster City, CA). The cDNA synthesized from the cysts and trophozoites of the isolates from *E. histolytica* obtained from the patients was diluted to amplify the genes.

The primers were Glucosamine 6 phosphate isomerase gi 67475221 F5'-CT CGAGCTAT TGAAGGCGAATCA-3', R5'-GAA GCAGGT TCATCAATAAAAACAGTAGTATTT-3', Fam 5'-ATGCTGCACATAATTC-3; Jacob glycol protein gi 67484051 F5'-G C A A C A A C A T T CAAAG GATTTA GACCAT-3' R 5′- GCCAG ACACATGGGTTATATTGGTA-3', Fam 5'-CTTGGCACCAGGTTCC-3'. And constitutive gene we use ubiquitin gi 67479594 F5'-ATGCTATTAAGGCTAAGATCCAAGAAAAAG A-3', R5'-TTTTTCCTTCTTCTAA TTGTTTT CCTGCAA-3', Fam 5'-ATCTGGTGGAATTCC-3'; the amplification was carried out using the 7500 Fast Real Time PCR system v.2.0.6.

RESULTS: Fig. 1a shows the integrity of the *E. histolytica* cysts by light microscopy at the start (0 h) of culture. **Fig. 1b** shows the culture at 24 h; one or two trophozoites were observed in some of the fields. **Fig 1c** shows the culture at 48 h, indicating four or more trophozoites per field, and Figure 1d shows the culture at 96 h, indicating that all trophozoites were excysted. The trophozoites per field were observed and characterized based on their amorphous shape, starch cytoplasmic granules and some emitting pseudopods. The isolates

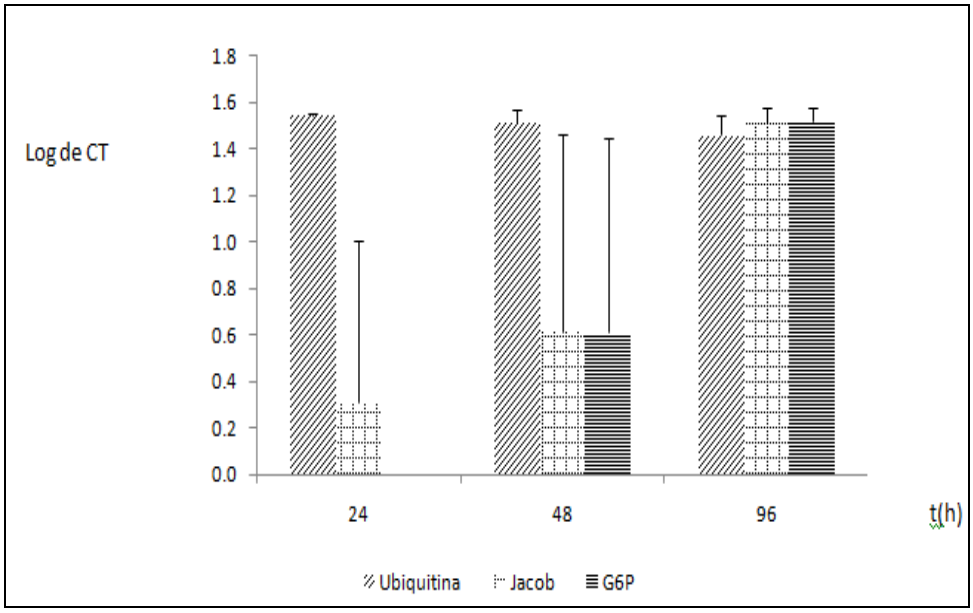
obtained from patients belong to *E. histolytica*, which was demonstrated by the *enhhic* gene amplification and restriction patterns (data not shown). The dynamic range was determined using the standardization curve, which in turn yielded the efficiency of the amplification. The standard deviation between the different values was 0.2 CT units. The CT results were graphed as a function of the log of the concentration, and the slope of the line was close to -3.5.

Fig. 2 shows the average expressions of the genes encoding glucosamine 6-phosphate isomerase and Jacob glycoprotein in the five isolates during each of the intervals of time in culture during excystation. Neither of the genes was expressed in the cystic stage in all isolates. In the throphozoite HM-1:IMSS control axenic strain, the expressions of glucosamine 6 phosphate isomerase and Jacob glycoprotein were log CT 1.40 and 1.45, respectively. The average ubiquitin (reference gene) expression in the five samples was 1.26 at 0 h (data not shown), 1.26 at 24 h, 1.51 at 48 h and1.45 at 96 h. The ubiquitin expression in the thropozoite stage of the HM-1: IMSS control strain was log CT 1.26 (data not shown). The data generated demonstrate the trend of the two studied genes.



a **b** **c** **d**

FIG. 1: KINETIC EXCYSTATION OF *E. HISTOLYTICA* IN ROBINSON CULTURE. CYSTS AND TROPHOZOITES WERE STAINED WITH LUGOL'S IODINE. a) CYSTS AT 0 h; b) CULTURE AT 24 h; c) CULTURE AT 48 h AND d) CULTURE AT 96 h. MAGNIFICATION 40X



t = time
CT= threshold cycle

FIG.2: THE AVERAGE EXPRESSION LEVELS OF THE GENES ENCODING GLUCOSAMINE 6 PHOSPHATE ISOMERASE AND JACOB GLYCOPROTEIN IN THE FIVE ISOLATES DURING EXCYSTATION. GLUCOSAMINE 6 PHOSPHATE ISOMERASE WAS EXPRESSED UP TO 48 H, AND JACOB GLYCOPROTEIN WAS EXPRESSED AFTER 24 H. UBIQUITIN (REFERENCE GENE) WAS EXPRESSED IN THE FIVE SAMPLES AT 24, 48 AND 96 H OF CULTURE IN THE CYST STAGE AS WELL AS IN THE HM-1:IMSS CONTROL STRAIN

DISCUSSION: The data generated were obtained from fresh patient cysts, and the excystament was obtained from starting material. The trend shown is the expression of the two genes studied in *E. histolytica*. At 24 h, the expression levels of glycoprotein Jacob and G6P were detected until 48 h. To date, Jacob glycoprotein has been reported to be present in the cyst stage; however, we did not detect its expression in the cyst stage, but began to see the morphological shift in the trophozoite.

We believe that the cyst stage was not contaminated in the samples studied after 24 h because the gene expression positively correlated with the population of trophozoites until 96 h of culture. We do not know if this glycoprotein Jacob is another structural feature in addition to the cyst wall; however, the expression levels of both isolated genes were higher at 96 h in the five samples than in the control strain HM -1. According to proteomic data isolated from partial samples from patients who are at least 78, proteins are reportedly localized in both the cyst and trophozoite; however, these proteins have not been individually described [14].

Our experience in the in vitro excystation of *E. histolytica* allowed us to determine the expression of glucosamine-6-phosphate isomerase in the trophozoite stage at 48 h. Therefore, this gene was not expressed during the cystic phase, possibly because it was inactive. This finding was contrary to what occurred in the trophozoite stage in all isolates expressed at up to 96 h as well as the HM-1:IMSS control strain. Once the cystic wall is formed, the expression of the glucosamine-6-phosphate isomerase gene is not detectable, or the gene gi 67475221 of the isomerase is not functional at this stage, as demonstrated by the gene gpi2 in *G. intestinalis* [15, 16].

Glucosamine-6-phosphate isomerase reportedly is the first enzyme induced in the synthesis of the cyst wall, similar to what has been described in *Giardia intestinalis*, a parasite in which in vitro encystment may take place [17].

In *E. invadens*, the Jacob and Jessie lectins and chitinase are absent in the trophozoite [18]. However, the expression of the Jacob lectin was detected too during the excystation process of the clinical samples in our study from 24 to 96 h, demonstrating differences in the culture time of each isolate of *E. histolytica* and the axenic HM-1: IMSS strain. A possible explanation is that this gene is present in the trophozoite because it is used to form the cyst wall, which shows that the data obtained in *E. invadens* cannot always be extrapolated to *E. histolytica*. Despite belonging to the family Amoebidae, the two species are phylogenetically different based on RNA sequencing studies [19].

Factors that influence the encystation of *E. invadens* appear to be different those of *E. histolytica.* By applying the same methodology as well as other factors, such as the presence of metal ions in the serum [20], osmotic shock [21, 22] and fasting glucose [23], the presence of mucin glycoprotein acts as a ligand for the lectin on the membrane and results in the aggregation of *Entamoeba* trophozoites and short-chain fatty acids [24].

Modulating factors that regulate the cell cycle [25], composition of the culture media and bacterial flora [26, 27] has not yet successfully yielded infective cysts of *E. histolytica*. Despite carrying out the *in vitro* excystation of *E. histolytica*, factors that induce this process remain poorly understood. Only the presence of bacteria on a biphasic media of saline agar with a pH 6.8 and rice starch as a carbon source are needed. Subsequently, peptone has been used as a nitrogen source for enrichment, and bacterial growth is controlled with antibiotics. However, our results showed that the process depends on the isolate and the viability and environmental characteristics that induce protein synthesis for trophozoite formation. Parasite excystation was carried out in the presence of the enterobacteria *E. coli* ATCC 35218, which is classified as nonpathogenic.

This process has been postulated to influence the cyst-to-trophozoite conversion for the production of short-chain fatty acids [28]. Bacteria like Proteus sp. have been isolated from the microbial flora in patients' stool samples containing *E. histolytica* cysts. Other investigators demonstrated that enteropathogenic bacteria modulate their response to epithelial cellular damage [25] and possibly

stimulate other genes involved in encystation. Ineffective structures of the parasite and the axenization of the parasite, which is a complicated process, have been obtained. However, this phenomenon allowed us to adapt an isolate of *E. histolytica* to the culture media TYI-S-33 in the laboratory.

To date, only one transcript profile has been described using microarrays with functions associated with the cyst, such as proteins with a chitin-binding domain, like chitinases and Jessie and Jacob lectins, cysteine proteinases EhCP-B1 and EhCP-B8. However, glucosamine 6 phosphate isomerase did not localize to a transcript of a gene specific to the cyst. The expression profiles may also vary among laboratories, even when cultures are grown in well-defined media and compared in an in vivo system [30]. Determining how to conduct excystment and vice versa would be a major contribution to the control of amebiasis caused by *E. histolytica.*

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