

Surface Coat Differences between Invasive *Entamoeba histolytica* and Non-Invasive *Entamoeba dispar*

Arturo GONZÁLEZ-ROBLES*, Bibiana CHÁVEZ-MUNGUÍA, Lizbeth SALAZAR-VILLATORO, Anel LAGUNES-GUILLÉN, Verónica Ivonne HERNÁNDEZ-RAMÍREZ, Patricia TALAMÁS-ROHANA, Adolfo MARTÍNEZ-PALOMO

Departamento de Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados, México DF, México

Abstract. Using ultrastructural cytochemical techniques we have found differences in the distribution of surface coat components between the invasive protozoan parasite *Entamoeba histolytica* and the non-invasive *Entamoeba dispar*. Carbohydrate-containing components and anionic sites in the cell surface of both species were detected by staining with ruthenium red and cationized ferritin, respectively. Ruthenium red staining revealed a thicker surface coat in *E. histolytica* trophozoites, whereas trophozoites of *E. dispar* showed a higher concentration of cationized ferritin particles on its surface. Mannose or glucose residues were found at the plasma membrane of both parasites treated with Concanavalin A (Con A)-peroxidase; the surface reaction product was more evident in *E. dispar*, compared with *E. histolytica*. Con A rapidly produced surface caps in *E. histolytica* trophozoites, whereas *E. dispar* showed a much less efficient mobilization of surface Con A receptors. Agglutination with Con A produced much larger clumps in *E. histolytica* in comparison with *E. dispar*. In turn, biotinylation assays revealed striking differences in the composition of surface membrane proteins in both amebic species. Overall, these results further emphasize the phenotypic differences between these two common parasites of the human intestinal tract, once considered to be the same protozoan.

Key words: Amebiasis, *Entamoeba histolytica*, *Entamoeba dispar*, Cell surface, Concanavalin A.

INTRODUCTION

Various *Entamoeba* species may inhabit the human intestinal tract. *Entamoeba histolytica* (Shaudinn 1903) produces amebic dysentery and invasive amebiasis, while *Entamoeba dispar* (Brumpt 1925) is found in asymptomatic infections. Once considered to be

the same ameba species, we found the first evidence of consistent biological differences between *Entamoebae* isolated from invasive cases, in comparison to parasites obtained from human carriers (Martínez-Palomo *et al.* 1973). Besides, the studies carried out by Sargeant (1992) on electrophoretic enzyme mobilities between isolates of *E. histolytica* and *E. dispar* and the redescription of both species achieved by Diamond and Clark (1993) supported the existence of two morphologically identical species, one an invasive pathogen, and the other noninvasive. Subsequently, the existence of two distinct species of ameba was again clearly demonstrated by biochemical, immunological and genetic

Address for correspondence: Arturo González-Robles, Departamento de Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados, Av. Instituto Politécnico Nacional 2508 San Pedro Zacatenco 07360 Mexico, D. F. Mexico; E-mail: goraa@cinvestav.mx

data (Ackers *et al.* 1997, Makioka *et al.* 2007). We have also reported differences in microscopic appearance and cytopathic capacity between both species (Espinosa-Cantellano *et al.* 1998).

It has been proposed that the virulence capacity of parasitic protozoa such as *E. histolytica* may be related to its surface characteristics (Martínez-Palomo 1973), and differences in the chemical structure and electric charge of the cell surface have been identified in pathogenic and non pathogenic strains of various protozoa (De Souza *et al.* 1978, González-Robles *et al.* 2002, 2007). While cytochemical properties of the cell surface have been analyzed in *E. histolytica* trophozoites (Pinto da Silva *et al.* 1975), similar data on *E. dispar* is lacking. In this study, we have used ultrastructural cytochemical to analyze the surface characteristics of these two *Entamoebae* species.

MATERIAL AND METHODS

Amoebae

Trophozoites were cultured in borosilicate glass tubes under axenic conditions. *E. histolytica* trophozoites of the HM-1:IMSS strain were grown to logarithmic growth phase (72 h) in TYI-S-33 medium at 36°C (Diamond *et al.* 1978), and *E. dispar* SAW 760 strain trophozoites in LYI-S culture medium (Diamond *et al.* 1995), for 72 h at 36°C. Both culture media contained 10% bovine serum and a vitamin mixture. Parasites were harvested by chilling the culture tubes to 4°C in an ice-water bath for 10 min and centrifuged at 900 g for 5 min.

Cell surface markers

Ruthenium red

Trophozoites were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, washed with PBS, and post-fixed for 1 h with 1% osmium tetroxide in the same buffer containing 5 µg/ml ruthenium red (Luft 1964).

Concanavalin A

Amoebae were washed and fixed with 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at room temperature. Trophozoites were washed twice with PBS and incubated with 100 µg/ml Con A (Calbiochem, La Jolla, CA, USA) for 15 min. Following incubation, cells were washed twice and treated with 50 µg/ml peroxidase for 15 min. After washing with the same buffer, parasites were incubated with 0.5 mg/ml diaminobenzidine + 50 µl H₂O₂ for 15 min at room temperature (Bernhard and Avrameas 1971).

Cationized ferritin

Amoebae were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 h at room temperature. To avoid non-specific labeling with cationized ferritin particles, free alde-

hyde groups of glutaraldehyde-fixed cells were blocked with 0.5 M NH₄Cl for 1 h at room temperature. Afterwards, trophozoites were washed twice with PBS and incubated with 1.5 mg/ml cationized ferritin for 15 min (Danon *et al.* 1972).

Fluorescence assays

Interaction of Con A with trophozoites of *E. histolytica* and *E. dispar* was carried out as described earlier (Espinosa-Cantellano and Martínez-Palomo 1994). After 15 min of interaction with fluorescein-tagged Con A (Vector Laboratories, Burlingame, Ca. USA), parasites were fixed with a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde for 30 min. Observations were carried out in a Leica AF confocal microscope (Leica Microsystems Heidelberg GmbH).

Agglutination reaction

Trophozoites in the exponential phase of growth were washed three times with D-PBS and centrifuged at 3,500 g for 5 min. Cell viability was determined by exclusion of trypan blue. Trophozoites from both *Entamoebae* species ($0.5 \times 10^6/500 \mu\text{l}$) were incubated with 100 µg/ml Con A (Calbiochem, La Jolla, CA, USA) at 37°C for 30 min. The specificity of the agglutination reaction was tested by blocking the process by previous incubation of the amoebae with Con A containing 0.2 M α -methyl-D-mannoside (Sigma-Aldrich, St. Louis, Missouri, USA).

Biotinylation

Trophozoites harvested in logarithmic phase of growth were washed twice with sodium bicarbonate buffer (0.1 M NaHCO₃/0.8%NaCl, pH 8.3) and incubated with Biotin 3-sulfonylhydroxysuccinimide ester (1×10^6 cells/10 µg biotin) (Sigma, Milwaukee, WI, USA) for 1 h at room temperature with occasional rocking. The reaction was stopped with washing buffer. To verify membrane staining, a sample of labeled cells was stained with streptavidin-FITC (1 : 100) for 30 min and observed by confocal immunofluorescence microscopy. Cell viability was determined by exclusion of trypan blue.

Biotinylated cells were centrifuged and lysed with lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl) and protease inhibitors (1 mM Iodoacetamide, N-ethylmaleimide, phenylmethylsulfonyl fluoride, tosyl lysyl chloromethyl ketone). The cell lysate was centrifuged at 24,500 g for 20 min at 4°C, and the pellet containing the total membrane fraction was obtained. The total membrane fraction (10 µg) was run in a 10% SDS-PAGE electrophoresis gel under reducing conditions and blotted onto nitrocellulose paper. The blot was incubated with streptavidin-HRP (1 : 1000) for 1 h, washed thoroughly with TBS-Tween and revealed by chemiluminescence (Hernández-Ramírez *et al.* 2007).

Electron microscopy

Trophozoites were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 60 min and post fixed for 1 hr with 1% (w/v) osmium tetroxide. After dehydration in increasing concentrations of ethanol and propylene oxide, samples were embedded in Polybed epoxy resins and polymerized at 60°C for 24 h. Thin sections (60 nm) were contrasted with uranyl acetate and lead citrate and examined in a JEOL JEM-1011 electron microscope (Tokyo, Japan).

RESULTS

Ruthenium red staining revealed fine structural surface coat differences between *E. histolytica* and *E. dispar* trophozoites. While in the invasive ameba the electron dense reaction product was observed as a continuous electron dense layer approximately 70–80 nm thick (Fig. 1A), in the non invasive ameba the surface coat was less electron dense, 30–40 nm in thickness (Fig. 1B). When both species were treated with cationized ferritin, few of particles were observed in the surface of *E. histolytica* (Fig. 1C), while abundant anionic components were identified on the plasma membrane of *E. dispar* (Fig. 1D). In thin vertical sections of amebas treated with the Con A peroxidase method, a positive reaction was observed on the surface of both strains. The electron dense precipitate was observed in *E. histolytica* (Fig. 2A) as a relatively uniform layer, in contrast with the thicker, denser and irregular coat deposit seen in *E. dispar* (Fig. 2B).

Cap formation. A rapid displacement of surface lectin receptors forming well-defined caps at the posterior pole of trophozoites was observed only in *E. histolytica* (Fig. 2C, D). In contrast, *E. dispar* showed a less efficient mobilization, with the Con A receptors distributed all around the amebic surface with small irregular patches seen in some areas. Caps were not formed (Fig. 2E, F).

E. histolytica and *E. dispar* trophozoites agglutinated after treatment with 100 µg/ml Con A for 30 min at 37°C. Clusters of amebas were clearly visible in the periphery of a central clump formed in both species. *E. histolytica* clearly showed larger cell clusters (Fig. 3A) compared with the less intense agglutination reaction of *E. dispar* (Fig. 3C). The Con A-induced agglutination of both species was inhibited by the addition of excess α -methyl-D-mannoside (Fig. 3B, D).

After biotinylation, both amebic species showed 98% cell viability. The protein concentration was normalized, observing a distinctive profile of both amebas. As loading control, densitometry was performed on a 60 kDa band (Fig. 4A). Figure 4B shows the profile of the biotinylated membrane fraction of both species. While the profile of *E. dispar* shows an intense biotinylation band of approximately 97 kDa, this band is barely present in the profile of *E. histolytica*. However, in *E. histolytica* an intense biotin labeling of a 70–50 kDa band, as well as a labeling of a 35–20 kDa band was observed. To validate these assays, confocal

microscopy analysis was performed using FITC-conjugated streptavidin. Biotinylated proteins were clearly found associated to membranous structures in the cytoplasm and on the plasma membrane of *E. dispar* (Fig. 5A, C), while in *E. histolytica* the biotin labeling was found as discrete dots on the plasma membrane, while internal structures were scarcely labeled (Fig. 5B, D). Only one-third of biotin labeling was detected in *E. histolytica*, in comparison with *E. dispar* (Fig. 5E).

DISCUSSION

The contact of protozoan parasites with target cells occurs through recognition of surface molecules present in the surface coat, a dense layer of mainly carbohydrate-containing components located on the external surface of the plasma membrane. This layer, formed by membrane-associated glycoconjugates, mediates the recognition and adhesion of the parasites to target cells (Martínez-Palomo 1970). Recent results by Boetner *et al.* (2005) have shown that externalized phosphatidylserine receptors participate in this recognition process both in *E. histolytica* and *E. dispar*.

E. histolytica damages target cells mainly via direct contact (McCoy *et al.* 1994); therefore the composition and properties of the cell coat of this parasite may play a crucial role in its pathogenicity (Martínez-Palomo 1982). According to Leippe *et al.* (1992) *E. histolytica* produce a pore-forming peptide implicated in potent cytolytic activity, besides Nickel *et al.* (1999) reported in *E. dispar* the presence of homologs amoebapores in lower amount and activity and suggest that differences in the lytic polypeptides may have an impact on the pathogenicity of amoebae. Immunological assays indicate that *E. histolytica* and *E. dispar* differ in cell coat phosphorylated glycolipids and suggest that these components are related to the pathogenicity of *E. histolytica*, being implicated in its ability to evade the innate immune response (Campos-Rodríguez and Jarillo-Luna 2005).

Adhesion and ability to invade tissues are accepted features to distinguish pathogenic from non-pathogenic amebic species (Jamerson *et al.* 2012). In the invasive parasite *E. histolytica*, adhesion is mediated by the 112 kDa amoebic surface adhesin (Arroyo and Orozco 1987), which can be inhibited by N-acetyl-D-galactosamine. The contact of the parasite with target cells occurs through the recognition of surface molecules that

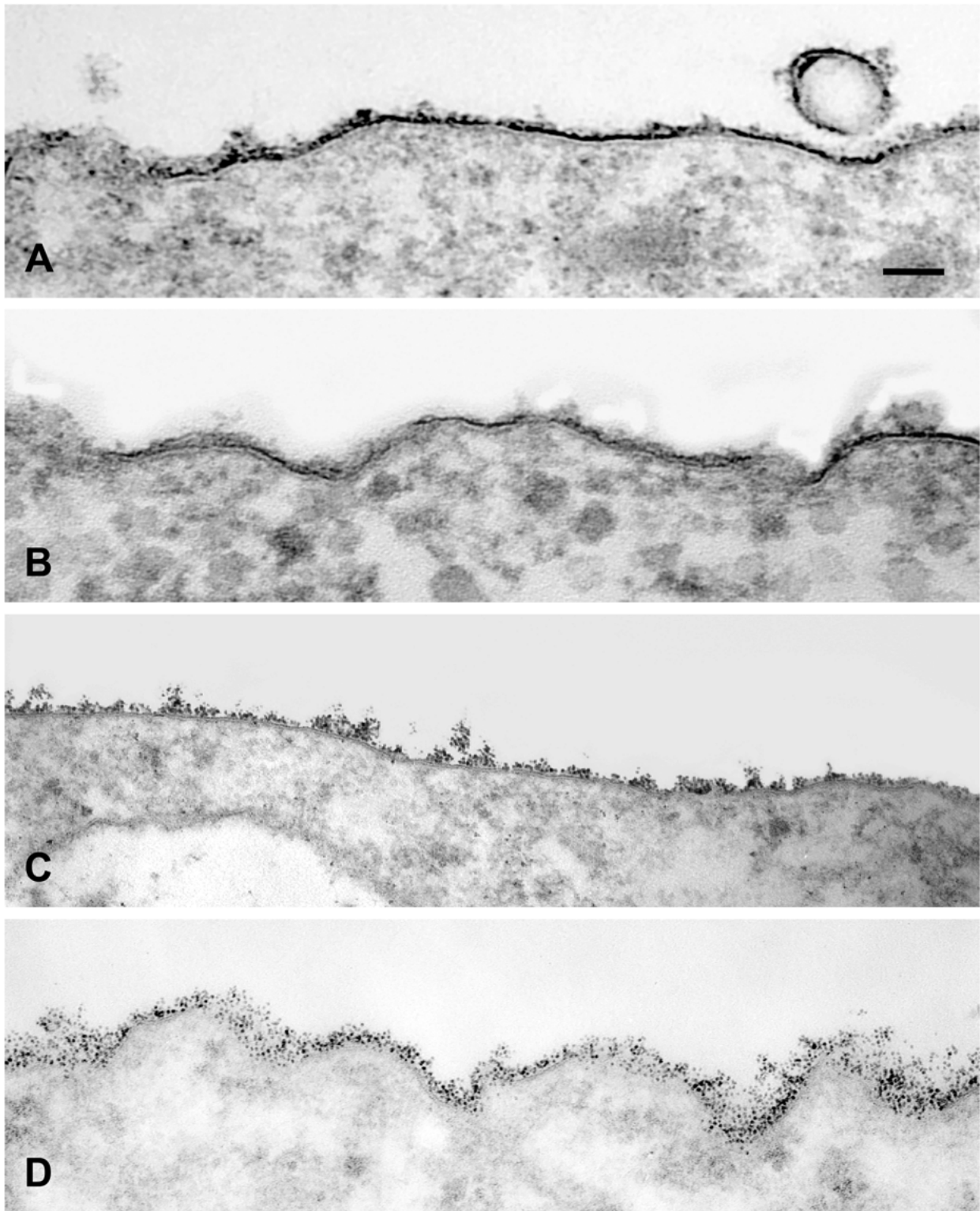


Fig. 1. Transmission electron photomicrographs of amoebas treated with ruthenium red and cationized ferritin. *E. histolytica* (A) and *E. dispar* (B) stained with ruthenium red. The stain on the cell surface of *E. histolytica* is seen as a dense solid layer. In contrast, in *E. dispar* the stain is observed as a slight deposit. Small groups of ferritin particles were observed in *E. histolytica* (C) while substantially large particles clumps were found in *E. dispar* (D). Bar: 0.1 μm .

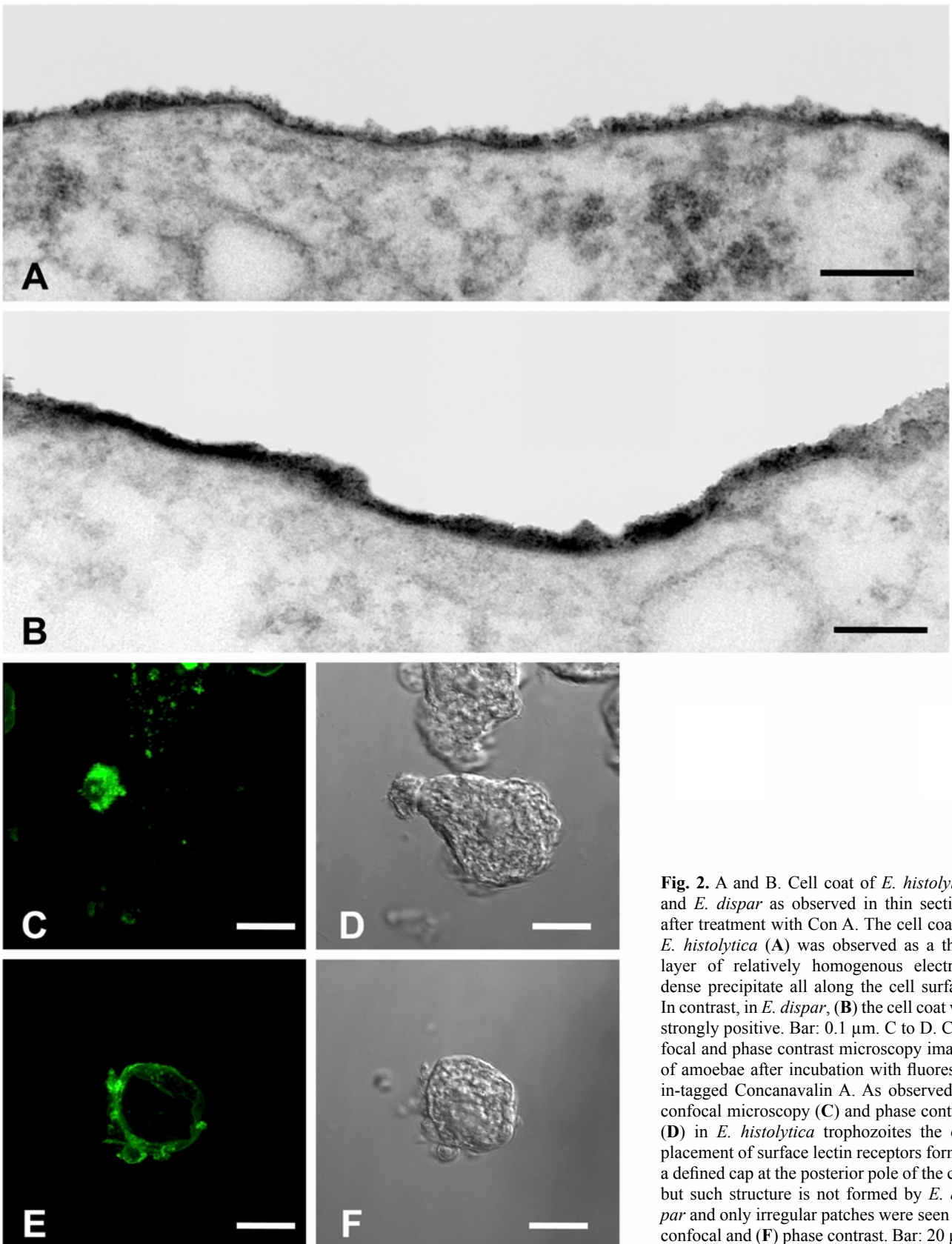
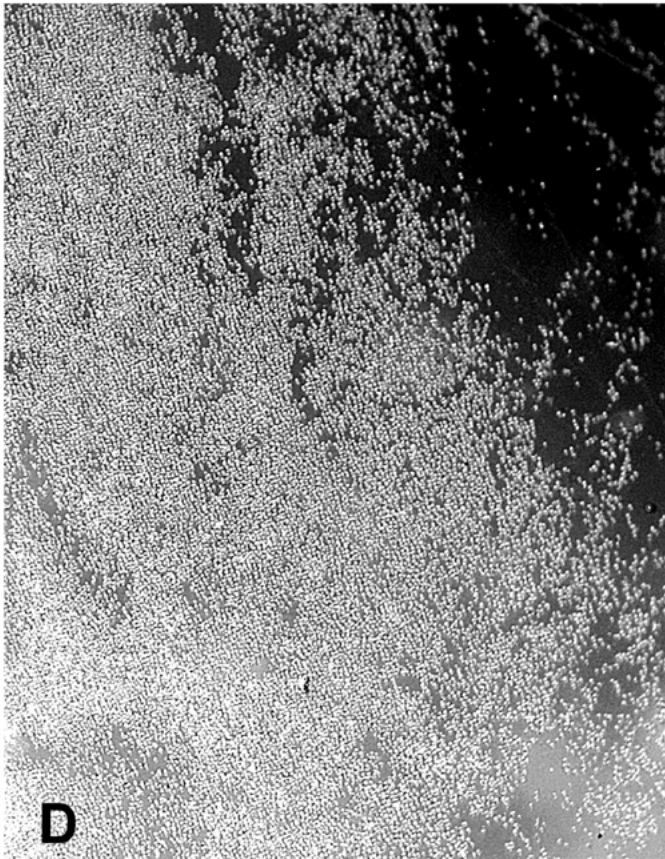
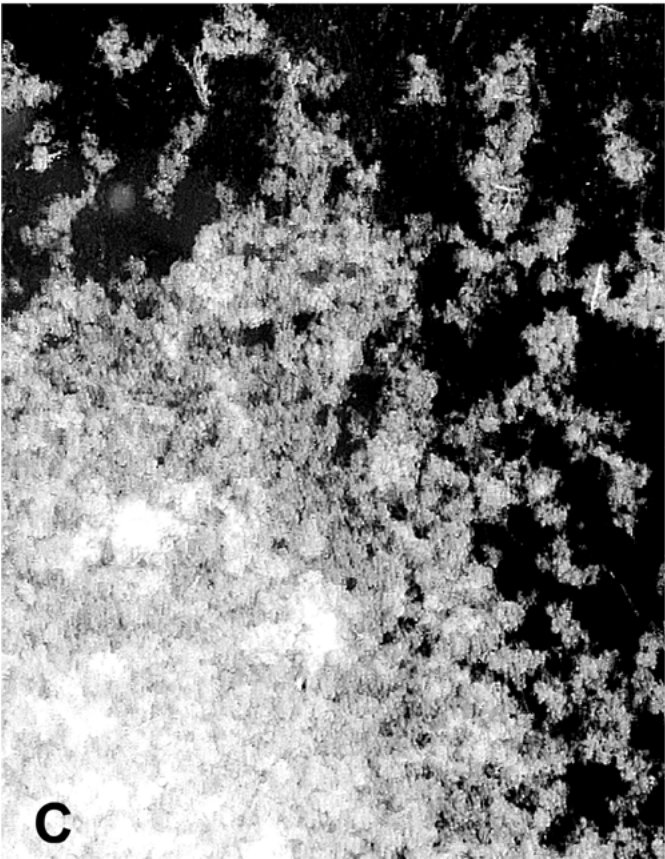
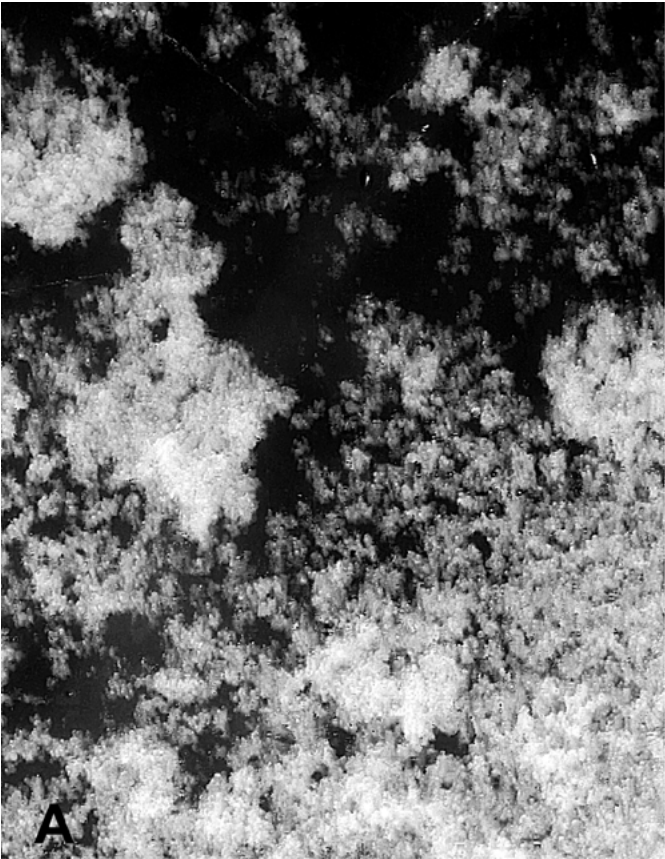


Fig. 2. A and B. Cell coat of *E. histolytica* and *E. dispar* as observed in thin sections after treatment with Con A. The cell coat of *E. histolytica* (A) was observed as a thick layer of relatively homogenous electron-dense precipitate all along the cell surface. In contrast, in *E. dispar*, (B) the cell coat was strongly positive. Bar: 0.1 μm . C to D. Confocal and phase contrast microscopy images of amoebae after incubation with fluorescein-tagged Concanavalin A. As observed by confocal microscopy (C) and phase contrast (D) in *E. histolytica* trophozoites the displacement of surface lectin receptors formed a defined cap at the posterior pole of the cell, but such structure is not formed by *E. dispar* and only irregular patches were seen (E) confocal and (F) phase contrast. Bar: 20 μm .



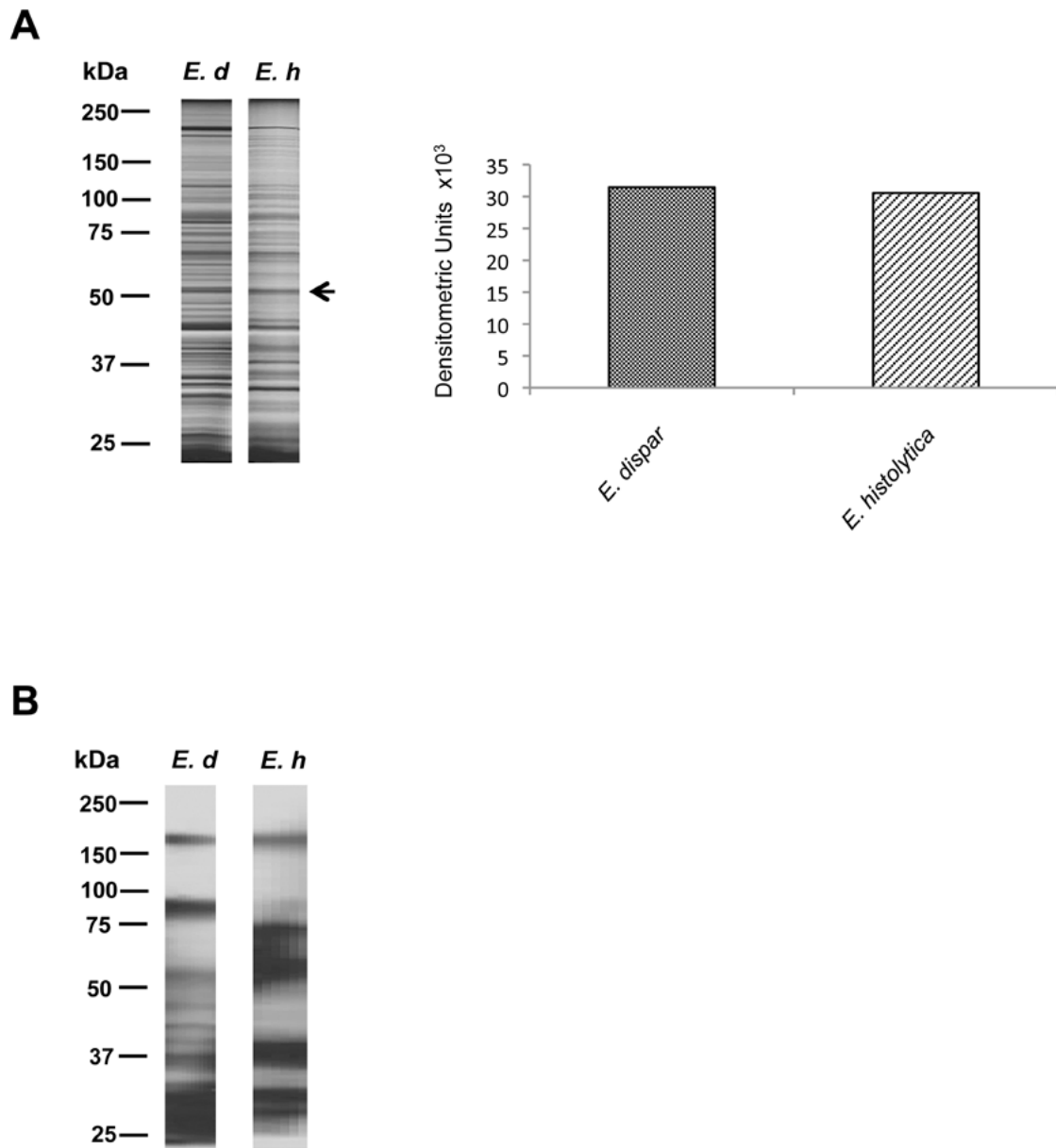


Fig. 4. (A) Silver staining of the protein profile of *E. dispar* and *E. histolytica*, and densitometry analysis of the 60 kDa band as loading control. (B) Biotinylated patterns of membrane proteins of *E. dispar* and *E. histolytica*.

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Fig. 3. Agglutination and inhibition processes in *E. histolytica* and *E. dispar*. A. Trophozoites of *E. histolytica* produced large clumps of cells when induced to agglutinate with 100 μ m /ml Con A at 37° C for 30 min. (C). Similar cell aggregation was produced by *E. dispar* but these were smaller in size. The specificity of the agglutination effect in both strains was inhibited by incubation of the amoebae with Con A containing 0.2 M α -methyl-D-mannoside where individualized cells are clearly visible (Figs. B and D).

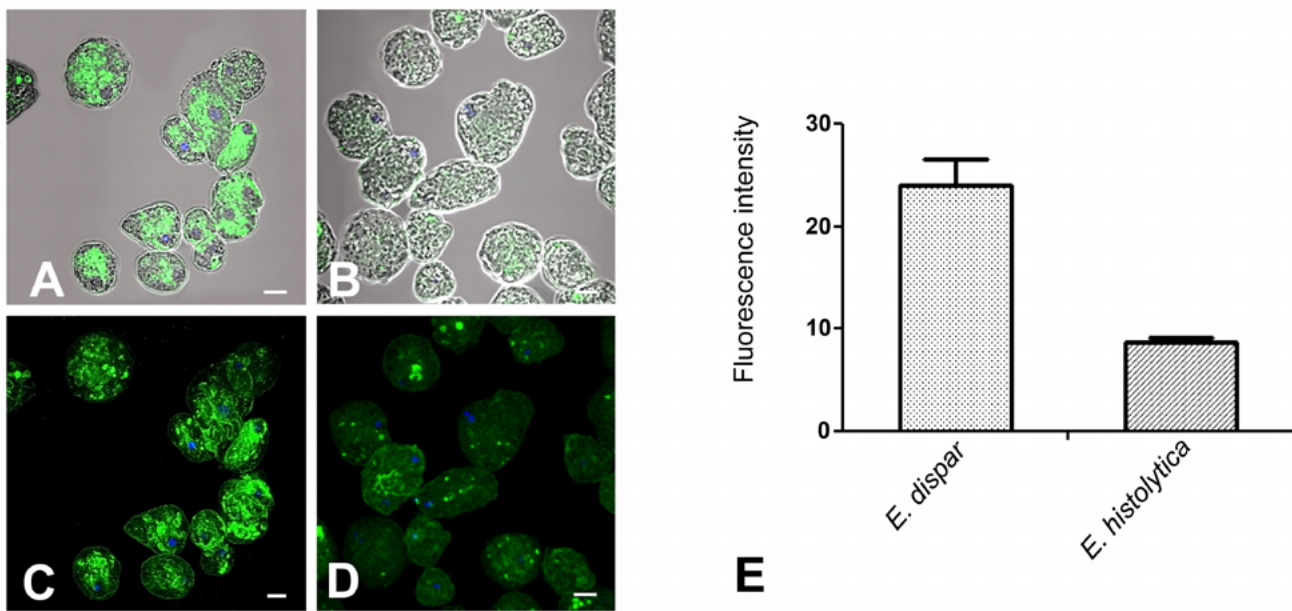


Fig. 5. Confocal, and fluorescence intensity assays were carried-out using FITC-conjugated streptavidin (1 : 100) for the localization of biotinylated proteins in *E. dispar* (A, C) and *E. histolytica* (B, D). Observation conditions were the same for both species, but had to be modified for *E. dispar* to improve image quality, since the high intensity of fluorescence produced distortion of the image. **E** – Graphical representation in arbitrary fluorescence units. Bar: 20 μ m.

activate specific signaling pathways and mediate invasive mechanisms. Among these, the best characterized and apparently the major adhesion surface molecule is Gal/GalNAc lectin, a heterodimeric glycoprotein considered as multifunctional virulence factor (Mann 2002, Petri *et al.* 2002). Surface β 1 integrin-like molecules capable to attach to extracellular matrix fibronectin have also been identified in this invasive parasite (Sengupta *et al.* 2001, Talamás-Rohana *et al.* 1994). Recently, Biller *et al.* (2013) reported the cell surface proteome of *E. histolytica* and found that a larger number of proteins are surface-associated, suggesting that the plasma membrane is a dynamic component partially implicated in the cellular machinery in which intracellular membrane systems are in constant replacement with the plasma membrane.

Ruthenium red binds to cellular components, predominantly to the surface coat rich in glycoconjugates. By cryo-fracture studies, a difference between *E. histolytica* and *E. dispar* plasma membrane has already been reported concerning the structures exposed on the surface and the distribution and arrangement of intramembranous proteins (Pimenta *et al.* 2002). In this study, a notorious difference in cell surface coat thick-

ness between the two amebic species was observed after treatment with ruthenium red. Differences were also evident in the extent of surface anionic sites detected in samples treated with cationic ferritin. Other protozoa, such as the human parasite *Trichomonas vaginalis*, pathogenic *Naegleria fowleri* and non-pathogenic *Naegleria lovaniensis* also have a similar distribution of these cell surface markers (González-Robles *et al.* 2002, 2004, 2007). Sialic acid a common component of the surface coats, along with other carbohydrates that contain carboxyl or sulfonyl groups, as well as phospholipids and dicarboxylic amino acids, all of which contribute to generate the negative surface charge. The negative charge of surface glycoproteins has been associated with cell adhesion capacity (Sumiyoshi *et al.* 2008), and could be related with virulence of parasites such as *T. vaginalis* (González Robles *et al.* 2004). Besides, the presence of profuse anionic components on the surface of *E. dispar* may be related with the low phagocytic capacity of this amoeba to ingest red blood cells compared with *E. histolytica* (Talamás-Lara 2014).

The cell surface of this parasite plays a key function in detection and lysis of target host cells. Diverse cell-surface molecules have been characterized and

their participation in amebic pathogenesis has been recognized (Clark *et al.* 2000). According to Moody *et al.* (1998), cell-surface lipophosphoglycan-like (LPG-like) are apparently limited to virulent strains while lipophosphopeptidoglycans (LPPG_s) are common to both virulent and avirulent strains of *E. histolytica* and *E. dispar*. LPG possible performs a specific function related to virulence while LPPG performs a function that is essential to survival within the host.

Lectins are proteins that bind to carbohydrates and regulate functions such as adhesion. Con A is a lectin with four identical binding sites, capable of bind to α -mannose, α -glucose and their derivatives. Studies in *E. histolytica* demonstrated that Con A treatment induces displacement of membrane structural components (Pinto da Silva *et al.* 1975). In *E. dispar* a deficient capping capacity induced by Con A treatment was previously reported (Chávez-Munguía *et al.* 2012). Recent mass spectrometry studies in *E. histolytica* revealed that Man₅GlcNAc₂, an unusual truncated N-glycan precursor is capped by Con A (Magnelli *et al.* 2008). Likewise, calreticulin, an endoplasmic reticulum chaperone, translocates to the cell surface and may be recruited during capping in *E. histolytica* (Girard-Misguich *et al.* 2008). In the present study, the Con A/peroxidase treatment produced an electron dense surface deposit thicker in *E. dispar* than in *E. histolytica*. In addition, differences were also observed in the agglutination capacity between the pathogenic and the non-pathogenic trophozoites.

Besides, we report here differences in the biotin reaction between the plasma membranes of *E. histolytica* and *E. dispar*. Biotinylation assays displayed clear differences in the distribution of cell surface proteins between the two amebic; similar results were previously obtained between pathogenic and non-pathogenic free-living amoebae (González-Robles *et al.* 2007).

In summary, prominent cell surface differences were found between *E. histolytica* and *E. dispar*. Further studies may point out whether these variations in surface components may be related to the invasive and non-invasive character of these two amebas, commonly present in the human intestinal tract, and once considered to constitute a single species.

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