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Profiles of *Entamoeba histolytica*-specific immunoglobulins in human sera

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ABSTRACT

Objective: To determine the profiles of anti-*Entamoeba histolytica* (*E. histolytica*) IgA, IgG, and IgM in sera of diarrheic and non-diarrheic individuals and partially characterize target antigens. **Methods:** Serum samples from thirty diarrheic and thirty non-diarrheic individuals were subjected to IgA, IgG, and IgM profiling through enzyme-linked immunosorbent assay (ELISA), flow cytometry, and immunoblot. **Results:** ELISA titer results showed that both diarrheic and non-diarrheic individuals possess high levels of *E. histolytica*-specific IgG compared to IgA and IgM. Flow cytometry data showed that diarrheic serum samples had higher mean reaction percentages against *E. histolytica* cells compared to non-diarrheic samples. Immunoreactive *E. histolytica* proteins with molecular weights ranging between 7 kDa and 292 kDa were recognized by diarrheic serum IgG, and 170 kDa and 250 kDa by non-diarrheic serum IgG. **Conclusions:** Our findings suggest that serum anti-*E. histolytica* IgG, compared with serum anti-*E. histolytica* IgA and IgM responses, was generally high in both diarrheic and non-diarrheic sera, indicating a past exposure to the organism both in symptomatic patients as well as in asymptomatic carriers, respectively. In addition, serum IgG from diarrheic and non-diarrheic patients were able to detect immunogenic *E. histolytica* proteins.

1. Introduction

Amebiasis is an infection with the protozoan *Entamoeba histolytica* (*E. histolytica*) which causes 100 000 deaths per annum, placing it second only to malaria based on mortality caused by protozoan parasites[1]. *E. histolytica* is an enteric tissue-invasive protozoan parasite that causes amebic colitis and occasionally, liver abscess in humans[2]. Disease occurs when trophozoites invade the mucosa to cause colitis or subsequently spread to the liver or other organs where they can cause abscesses[3]. A number of immunological methods such as enzyme-linked immunosorbent assay (ELISA) and antigen kits have been utilized to diagnose invasive amebiasis[4] which predominantly affects individuals particularly those who live in developing countries. In the Philippines, *E. histolytica* infection has not been well-characterized. Serological studies of antibody responses to *E. histolytica* antigens are lacking. This study aimed to conduct profiling of IgA, IgG, and IgM antibodies in humans infected with *E. histolytica*.

2. Materials and methods

2.1. Collection of human serum samples

Serum samples were collected from diarrheic and non-diarrheic individuals from patients in Makati Medical Center, Makati City, Metro Manila, Philippines. Criterion for selecting diarrheic and non-diarrheic individuals was based on the clinical definition by WHO wherein diarrheic study subjects were those experiencing a passage of three (3) or more loose or liquid stools per day, or more frequently than what is normal for an individual[5]. On the other hand, non-diarrheic individuals were those not suffering from this medical condition at the time of the study. Informed consent was obtained from study participants. We tested 30 serum samples from diarrheic and another 30 serum samples from non-diarrheic individuals. All serum samples were heat-inactivated at 56 ℃ for 30 minutes before use.

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2.2. Preparation of E. histolytica antigen

Soluble antigen was prepared for ELISA. Seventy-eight (78)-hour axenic cultures of *E. histolytica* strain HK9, cultured in BI-S-33 medium supplemented with 10% bovine serum and vitamins[6], were chilled on ice and centrifuged at 2 500 rpm for 5 minutes. The parasites were then collected and washed twice with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4; pH 7.2) by centrifugation at 2 500 rpm for 5 minutes. *E. histolytica* cells were then suspended in a solution containing 10 mM Tris-HCl (pH 7.5), 2 mM PMSF and 1 mM $MgCl_2$ after which the cells were homogenized in ice bath. The cell lysate was then centrifuged at 13 000 rpm at 4 ℃ for 1.5 hours, then the pellet was resuspended in 2x PBS prior to storage at −20 ℃. Total protein concentration was determined by the method of Bradford[7].

2.3. ELISA for the detection of E. histolytica-specific immunoglobulins G, M and A in serum specimens

For IgG ELISA, 96-well flat-bottom polystyrene microtiter plates were incubated with 10 μg/mL *E. histolytica* membrane antigen in 0.1 M carbonate buffer (pH 9.6). Antigen sensitization was carried out by adding 100 μL of the antigen solution per well followed by incubation at 4 ℃ overnight. Unbound antigens were removed by washing the wells thrice in PBS containing 0.05% Tween 20 (TPBS). Nonreactive sites were blocked with 100 μL per well of PBS containing 3% skim milk (skim milk-PBS) at 37 ℃ for 1 hour. The blocking reagent was removed and the wells were incubated with 100 μL of serum diluted four-fold in PBS for 2 hours at 37 ℃. The plates were washed thrice in TPBS, and then a 1:2 000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (Santa Cruz Biotechnology, USA) was added to each well. The plates were incubated for 1 hour at 37 ℃, washed three times in TPBS, and then developed with o-phenylenediamine and hydrogen peroxide (Sigma-Aldrich, USA). The reactions were allowed to develop for 30 minutes in the dark at 37 ℃ and were then terminated by the addition of 50 μL of 4.5 M sulfuric acid per well. The degree of color change (optical density at 492 nm) was measured in an ELISA plate reader (Thermo Multiskan, USA). Antibody titer of the serum samples was determined following the statistical procedure designed by Frey and colleagues[8]. PBS was included as the negative control for every 96-well ELISA plate.

For IgM ELISA, all methods were performed as described. Horseradish peroxidase-conjugated mouse anti-human IgM (Beckman Coulter, Inc., USA) diluted at 1:2 000 in blocking buffer was used as the secondary antibody.

For IgA ELISA, all methods were performed as described above with some modifications. The wells were incubated with 100 μL patient serum (four-fold dilutions in PBS). Horseradish peroxidase-conjugated mouse anti-human IgA (Beckman Coulter, Inc., USA) diluted at 1:1 500 in blocking buffer was used as the secondary antibody.

2.4. Flow cytometry

Non-aggregated, viable, 78-hour-culture of *E. histolytica* cells were suspended and washed with 1 mL cold buffer composed of PBS, 0.2% bovine serum albumin (BSA), and 0.1% sodium azide. A suspension of 10^7 cells/mL was prepared. Fifty (50) μL of the cold buffer was mixed with 50 μL serum sample in a sample tube. Afterwards, 100 μL of the cell suspension was added at the bottom of the tube. The solution was vortexed and incubated for 30 minutes at 4 ℃ in the dark. Then, it was washed twice with cold buffer and centrifuged at 2 500 rpm for 5 min. The pellet was then suspended in 100 μL anti-mouse IgA, IgG, and IgM conjugated to fluorescein isothiocyanate (FITC) having a 1:50 dilution. It was again vortexed and incubated in the refrigerator for 20 minutes. The sample was washed twice with 1 mL cold buffer and centrifuged at 2 500 rpm for 5 minutes. The cells were resuspended in 1 mL cold buffer and transferred to a 5 mL polystyrene round-bottom centrifuge tube. Samples were read using a flow cytometer (Becton-Dickinson, USA).

2.5.Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and immunoblot

SDS PAGE was performed using 1.5 mm slab gels consisting of 10% stacking gel and 10% separating gel. Electrophoresis was carried out at 30 mA both in the stacking and separating gels. *E. histolytica* antigens were solubilized in equal amount of Laemmli sample buffer and β-mercaptoethanol. Samples were loaded at approximately 10 μg of protein per well as determined by the method of Bradford[6]. The resolved amebic polypeptides were transferred onto nitrocellulose membrane for 1.5 hours at 100 V with transfer buffer. Immunological detection was performed following the standard procedure. Briefly, nonspecific binding was blocked with 3% skim milk-PBS overnight at 4 ℃. Human serum sample (1:250 final dilution in 3% skim milk-PBS) was overlaid onto the membrane and incubated for 1.5 hours at room temperature. The blots were then washed thrice with TPBS and incubated for 1.5 hours with horseradish peroxidase-conjugated mouse anti-human IgG antibody (Sigma-Aldrich, USA), diluted 1:1 000 in 3% skim milk-PBS. After three washings with TPBS, a solution of 0.5 mg 3,3′-diaminobenzidine-4-hydrochloride (Sigma-Aldrich, Germany) and 0.02% H_2O_2 per 1 mL PBS was added and allowed to react for 5 minutes. Molecular weights of the visible bands were interpolated from known molecular weight standards (Invitrogen, USA).

2.6. Indirect fluorescent antibody test (IFAT)

E. histolytica antigen slides were blocked with 1% bovine

serum albumin (BSA) in PBS for 1 hr in a moist chamber at room temperature. Then, heat-inactivated diarrheic and non-diarrheic sera diluted four-fold in PBS were added. The wells were washed thrice in 15 min using PBS. Afterwards, the anti-mouse IgG conjugated with FITC in 1% BSA (1:50) was applied to each well for 30 min at room temperature. The slides were then rinsed with PBS prior to viewing under a fluorescence microscope (Olympus®).

2.7. Statistical analysis

The paired Student's *t* test was used to determine the significance between the reaction percentage values of the different antibody isotypes in both diarrheic and non-diarrheic samples. The same statistical analysis was also used to determine the differences in reaction percentage values in all isotypes between diarrheic and non-diarrheic samples. Results with *P* values less than or equal to 0.05 are considered statistically significant.

3. Results

3.1. ELISA

In the present study, both diarrheic and non-diarrheic individuals possess high levels of *E. histolytica*-specific IgG compared to IgA and IgM as determined through ELISA (Figure 1). Both diarrheic and non-diarrheic study participants demonstrate high titers of serum anti-amebic IgG antibodies, with a minimum titer value of 1:1 024. On the other hand, it was observed that serum samples from both diarrheic and non-diarrheic individuals possess low levels of IgA and IgM antibodies against *E. histolytica*. Results for *E. histolytica*-specific IgA assay showed that 10 diarrheic samples and all 30 non-diarrheic samples were non-reactive to the parasitic cells. Meanwhile, anti-IgM assay showed intermediate titer values ranging from 1:16 to 1:262144 for both diarrheic and non-diarrheic samples. No correlations were observed between anti-amebic IgG and IgA titers in serum samples from both diarrheic and non-diarrheic subjects.

3.2. Flow cytometry

Viable *E. histolytica* cells were gated based on the forward scattering and side scattering parameters. The forward and side scatter profiles refer to the cell size and cell granularity, respectively. These parameters were used for preliminary identification of viable *E. histolytica* cells, to exclude debris and dead cells. Fluorescence emission was also used as a parameter wherein FITC-positive fluorescent viable cells found within R1 were counted and the event percentage was reported (Figure 2).

Flow cytometry analysis of the serum immunoglobulins in diarrheic and non-diarrheic samples was perfomed. Data showed that anti-*E. histolytica* immunoglobulin response was higher in diarrheic (79.16%) compared to non-diarrheic samples (20.53%). The mean percent reaction values for IgA, IgG, and IgM in diarrheic samples are 79.36%, 74.79% and 83.33%, respectively. On the other hand, the mean percent reaction values for IgA, IgG and IgM in non-diarrheic samples are 10.45%, 25.24%, and 23.97%, respectively.

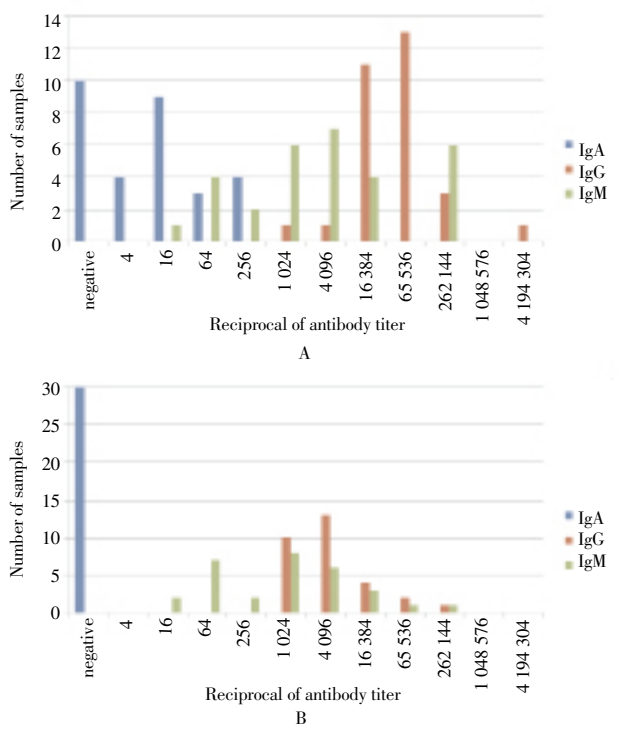

Figure 1. Profiles of anti-*E. histolytica* IgA, IgM and IgG antibody titers of (A) diarrheic and (B) non-diarrheic patients determined through ELISA.

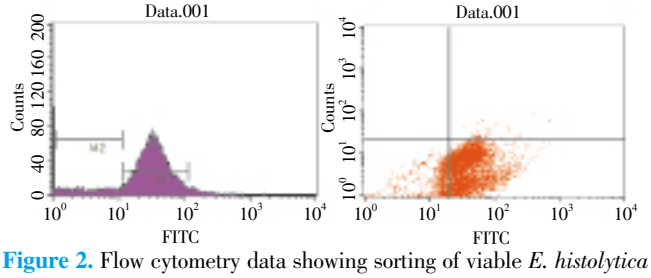

Figure 2. Flow cytometry data showing sorting of viable *E. histolytica* cells based on fluorescence.

In diarrheic samples, IgM was the most reactive among the antibody isotypes, having a significant difference compared to IgG (*P<0.05) but not with IgA (*P>0.05). A different trend was observed in non-diarrheic samples, having IgG reaction to *E. histolytica* cells as the highest, followed by IgM (*P > 0.05) and then by IgA (*P < 0.05).

3.3. Immunoblot

It was determined that diarrheic serum IgG targets several

antigens with molecular weights ranging between 7 kDa and 292 kDa through immunoblot (Figure 3). Non-diarrheic serum IgG was also able to recognize several *E. histolytica* antigenic proteins with molecular weights ranging between 170 kDa and 250 kDa. These antigens may be membrane-associated as determined from micrographs of indirect flourescent antibody test (Figure 4).

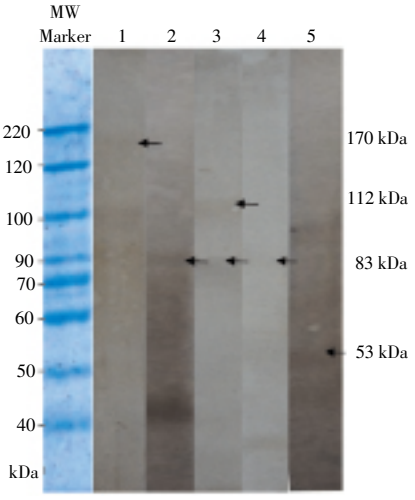

Figure 3. Immunoblot analysis of diarrheic serum IgG against *E. histolytica* soluble antigens as indicated by arrows.

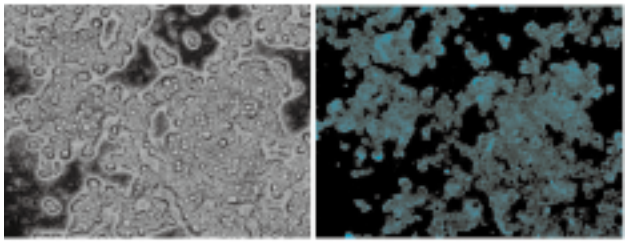

Figure 4. Photomicrographs of fixed-*E. histolytica* trophozoites on slides reacted with diarrheic serum under (A) bright field and (B) fluorescence microscope.

4. Discussion

It has been reported that symptoms are absent or very mild in up to 90% of *E. histolytica* infections[9]. Therefore, examination of anti-amebic antibodies in serum samples from non-diarrheic patients is imperative. In flow cytometry, all three isotypes reacted with *E. histolytica* and were detected in both diarrheic and non-diarrheic samples. Diarrheic samples were more reactive against *E. histolytica*. However, all non-diarrheic samples also reacted against the parasitic cells, indicating that the subjects may be asymptomatic carriers of the protozoon. Asymptomatic carriers in endemic areas develop serum antibody responses to the parasite even in the absence of the invasive disease. It is also important to note that most previous studies have shown that majority of the individuals infected with *E. histolytica* but not the morphologically similar species, *E. dispar*, develop serum antibody responses to the parasite even in the absence of invasive infection[10].

ELISA has been proven useful in the differentiation of *Entamoeba* species particularly in clinical laboratories less equipped with biological and biochemical methods or antigen capture kits[11]. In the present study, conflicting trends were observed between ELISA and flow cytometry data. In the ELISA data, both diarrheic and non-diarrheic individuals possess high levels of *E. histolytica*-specific IgG. It has been found that serum IgGs are present within 1 week after onset of symptoms in over 95% of patients with amebic colitis or liver abscess[12, 13] and may persist for years[14]. Therefore, it should be considered that serum IgGs detected in the current study may reflect immunological memory rather than disease and that boosting may occur during intestinal contact with the agent[15]. However, in flow cytometry, high IgM reactivity was detected in diarrheic sera. This may be attributed to a recent infection by the pathogen.

Meanwhile, both diarrheic and non-diarrheic individuals examined in the present study were found to have low levels of serum anti-amebic IgAs as determined through ELISA. This is in accordance with the results obtained by Shetty and co-workers in a seroprevalence study in 1992 wherein they detected low levels of anti-amebic IgA in children and infants in Southern India[16]. IgA antibodies prevent contact of enteric pathogens with the intestinal epithelial surface due to their agglutination, entrapment within immune complexes, and clearance within the mucous blanket[17]. IgA is mainly seen in mucosal secretions such as tears, saliva, colostrum and secretions from the genitourinary tract, gastrointestinal tract, prostate and respiratory epithelium. Only small amount of IgA can be found in blood which is evident in non-diarrheic samples. However, in the flow cytometry analysis, diarrheic serum IgA reactivity level was observed to be higher than IgG. Shetty *et al* in 1990, detected significantly high IgG and IgA levels in the sera of patients with invasive amebiasis[18]. Grundy *et al*. in 1983 reported low to moderate IgA levels in serum of lactating mothers[19]. Detection of high serum IgA levels might suggest possible invasive infection. Low titer values of serum IgA detected in ELISA compared to flow cytometry may be attributed to the limitations in the sensitivity of the method.

Several published reports identified different immunogenic proteins of *E. histolytica*. A 150 kDa surface antigen, which serves as an intermediate subunit (Igl) of Gal/GalNac (galactose N-acetyl-D-galactosamine) lectin, was a useful target antigen for the serodiagnosis of amebiasis[20]. Rodriguez and co-workers identified seven proteins involved in the adhesion of *E. histolytica* trophozoites to target cells. Target cell-parasite relationship in *E. histolytica* was seen in 210 kDa, 160 kDa, 112 kDa, 90 kDa, 70 kDa, 50 kDa and 24 kDa. These molecules involved in adhesion are important in the virulence of the parasite[21].

In this study, the 170 kDa target antigen detected through immunoblot may correspond to the 170 kDa subunit of the *E. histolytica* galactose-inhibitable lectin. A cysteine-rich portion of this subunit is LC3[17]. It has been reported that anti-LC3 IgG antibodies are found in serum and anti-LC3 IgA antibodies in saliva and stool of subjects with invasive amebic disease or asymptomatic *E. histolytica* infection.

Similarly, in this study, an 83 kDa antigen was detected through immunoblot in both diarrheic and non-diarrheic samples. Previously, proteins ranging between 81 kDa and 84 kDa were strongly recognized by monoclonal antibody in invasive *E. histolytica* isolates[22].

Overall, the immunoglobulin profiles of diarrheic and non-diarrheic sera, generated through ELISA, flow cytometry, and immunoblot suggest a high IgG response against *E. histolytica*. This could be corroborated with the findings of previous studies wherein invasive amebic infections provoke a strong humoral response, especially of the IgG isotype and that these antibody levels may persist for many years without any sign of reinfection[23]. Further understanding of the roles of the three classes of antibodies in the immune response against amebic infection among Filipinos is warranted to provide a better insight on the mechanisms that permit or inhibit invasive amebiasis. This could also be a precedent for the development of new diagnostic markers and possible treatment against amebiasis.

Conflict of interest statement

We declare that we have no conflict of interest.

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