Polyclonal IgG Response of Balb/c Mice to the 23 kDa Antigen of *Entamoeba histolytica*

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ABSTRACT

Entamoeba histolytica is one of the most significant protozoan pathogens found in developing countries like the Philippines. This intestinal parasite causes the disease amebiasis, which has a yearly average mortality of about 100,000 people worldwide. Thus, it is essential to develop new diagnostic markers and possible treatment against this disease. The crude cell extract of *E. histolytica*, was used to induce polyclonal antibody response in mice. Balb/c mice were given immunizations of the prepared crude *E. histolytica* antigens for a period of twelve weeks. Indirect fluorescent antibody test showed the specificity of polyclonal IgG in recognizing the cytosolic components of *E. histolytica* trophozoites. Enzyme-linked immunosorbent assay was performed to determine the antibody titers in sera collected at various time intervals. Antibody titers for the mouse serum taken 10 and 20 days after the third booster immunization were known to be 16,384 and 4,096 respectively. SDS-PAGE profile of the crude *E. histolytica* antigens revealed three bands with molecular weights of 23, 41, and 47 kDa. Western immunoblot results indicated that the polyclonal IgG produced by mice targets the potentially novel 23 kDa antigen from an axenic *E. histolytica* culture.

Key words: amebiasis, *Entamoeba histolytica*, IgG, immunoblot, indirect fluorescent antibody test, polyclonal antibodies

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INTRODUCTION

Amebiasis, an infection with the protozoan *Entamoeba histolytica,* causes 100,000 deaths per annum, placing it second only to malaria based on mortality caused by protozoan parasites (WHO, 1997). *E. histolytica* is normally found in the large intestine, occasionally penetrating the intestinal mucosa and may disseminate into other organs such as the liver. Initially, it was believed that the virulence of this parasite is variable due to the common observation that many people are apparently infected with it but never develop symptoms and spontaneously clear the infection. To answer this issue, Emile Brumpt in 1925 suggested the existence of two species of *Entamoeba – E. histolytica* that causes the invasive disease and *E. dispar* which never causes disease. After which, years passed without any support to this view until the 1970s when studies began to arise giving way to the formal redescription of *E. histolytica* separating it from *E. dispar* in 1993 (Diamond & Clark, 1993).

Factors that trigger invasion are still not very clear. It was concluded in a study of Mondal (Mondal, et al., 2006) that ill-nutrition does not considerably contribute to *E. histolytica*-associated diarrhea. However, proteins associated with the virulence have already been identified - prominent are the lectin that is responsible for the epithelial adherence of the parasite, pore-forming peptides lysing host cells, and secreted proteases degrading host tissues. Clearly, the pathogenic mechanism of *E. histolytica* is a multifactorial phenomenon that occurs in 3 steps: adhesion, cytolytic and cytotoxic effect, and phagocytosis (Horstmann et al., 1992).

Trophozoite surface antigens recognized by immune sera that have been identified may represent important components of the host-parasite interaction. The 29-kDa surface antigen (thiol-dependent peroxidase; Eh29) of *E. histolytica* exhibits peroxidative and protective antioxidant activities (Torian et al., 1990a, Sen et al., 2007). Several studies identified different surface antigens of different sizes and proposed these antigens as bases for differentiating isolates, whether pathogenic or nonpathogenic: 125-kDa (Edman et al., 1990), 30-kDa (Blakely et al,. 1990), 96-kDa (Torian et al., 1990b), 170- and 35-kDa subunits of a surface glycoprotein galactose-inhibitable lectin (Petri Jr. et al., 1990a; Ravdin et al., 1990). A 260-kDa galactose-inhibitable adherence protein (GIAP) of *E. histolytica* is a mucosal antigen in naturally occurring invasive infection inhibited by monoclonal antibodies (Kelsall et al., 1994), which could also be used to distinguish pathogenic from nonpathogenic amebae in culture (Petri Jr. et al., 1990b). An intermediate subunit (Igl) of Gal/GalNac (galactose *N*-acetyl-D-galactosamine) lectin, a 150-kDa surface antigen has also been identified (Cheng et al., 1998) and was well recognized in not only symptomatic but also asymptomatic patients with *E. histolytica* infection and that the carboxyl terminus of Igl is a especially useful antigen for the serodiagnosis of amebiasis.

It has been well known that there are a number of antigenic markers in *E. histolytica* that play crucial roles in its pathogenic effects. This study aimed to develop and partially characterize polyclonal antibodies in mice against a crude preparation of cellular antigens of *E. histolytica*. This could be a precedent for the development of new diagnostic markers and possible treatment against amebiasis.

MATERIALS AND METHODS

Animal care and handling

Four 7-week old Balb/c mice were kept in separate cages in a well-ventilated Animal House of the Natural Sciences Research Institute, University of the Philippines, Diliman, Quezon City. The mice were provided with sterile water, food, and beddings. They were exposed to the normal daylight conditions. The cages were regularly cleaned during the immunization period.

Axenic cultivation of E. histolytica

E. histolytica was cultured axenically using BI-S-33 medium (Diamond et al.*,* 1978). Antibiotic solution containing 100 U/ml penicillin and 100 µg/ml streptomycin (1% of the volume of the medium) was also added. Culture tubes were incubated at $35.5°C$ in slanted ($5°$ from the horizontal) position. Subculturing was done every 3-4 days.

Cell counting

Trophozoites of *E. histolytica* were harvested during logarithmic phase of growth (3-4 days) and the cells were counted using hemocytometer. Several culture tubes were pooled to have a total of 10^5 to 10^7 cells/ml.

Preparation of cell lysates

The collected trophozoites were centrifuged at 2,500 rpm for 3 min at 4°C. Then, the medium was decanted. After which, the collected trophozoites were washed two times with 1X phosphate buffered saline (PBS). After washing, the trophozoites were resuspended in a solution containing 10mM Tris-HCl (pH 7.5), 2mM phenylmethylsulfonyl fluoride (PMSF), and 1mM $MgCl_2$. Using a clean glass homogenizer, lysis of the cells was done (40 strokes) in ice-cold bath. The cell lysates were transferred into 1.5 ml microfuge tubes, centrifuged at 13, 200 rpm for 1.5 hr. Finally, it was resuspended in 1X PBS prior to storage at -20°C.

Determination of protein concentration

The concentrations of the cell lysates were measured using Bradford reagent at an optical density (OD) of 595 nm through a spectrophotometer (Bradford, 1976). Bovine serum albumin (BSA) was used as protein standard. Several increasing concentrations of the BSA protein standard were measured through the spectrophotometer to generate a linear curve in relation to the absorbance readings. The crude antigen prepared was then quantified using the linear equation.

Immunizations

Blood samples from Balb/c mice were collected from the tail vein. Serum was separated from the whole blood by centrifugation (2,500 rpm for 10 min). Then, it was stored in the freezer until use for antibody assay.

Prior to immunization, the mice were weighed individually. To prepare the antigen emulsion, complete Freund's adjuvant (CFA) was initially shaken to disperse the insoluble components. After which, the same proportion of antigen (212 µg/µl) in PBS to the CFA was added and both were mixed thoroughly until homogeneous by attaching two syringes to each other's

end and repeatedly forcing the mixture back and forth from one syringe to another. The CFA/antigen emulsion was injected into the mice intraperitoneally. After 10-14 days, blood samples were again collected from the mice. Serum was prepared as mentioned above and likewise stored in the freezer.

Indirect fluorescent antibody test (IFAT)

E. histolytica antigen slides were blocked with 1% BSA in PBS for 1 hr in a moist chamber at room temperature. Afterwards, heat-inactivated serum samples in PBS at four-fold dilutions were added. The wells were washed 4x in 15 min using PBS. Then, the anti-mouse IgG conjugated with flourescein isothiocyanate (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 UV microscope (Zeiss® Axiovert).

Enzyme-linked Immunosorbent Assay (ELISA)

Microtiter plates were coated with 10µg/ml of the *E. histolytica* antigen in 0.1 M carbonate buffer at 4°C overnight. The wells were washed with 0.05% Tween-PBS (TPBS) thrice. Then, blocking was done using 1% BSA in PBS at 37°C for 1 hr. Subsequently, the plates were incubated with the serum in 4-fold dilutions at 37°C for 2 hr. Washing using TPBS was again done thrice. Alkaline phosphatase conjugated-anti-mouse IgG in 1% BSA (1:1000) was then added at 37°C for 1 hr followed by washing with TPBS thrice. Then, 1 mg/ml of the substrate, p-nitrophenylphosphate in 1M diethanolamine buffer (pH9.8) was coated at 37°C for 30 min. Finally, the samples were read at 405 nm using an ELISA plate reader (Multiskan EX, Thermo Electron Corp.).

To test for cross-reactivity, the collected serum from mice immunized with *E. histolytica* antigen was reacted with *Blastocystis hominis* antigen using ELISA.

Western immunoblot

Prepared crude antigen was mixed with Laemmli sample buffer and β-mercaptoethanol to make a 13 µg/µl loading sample into sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE). Proteins separated through the gel was transferred to a nitrocellulose membrane (NitroBind, Osmonics Inc.) then immunostained using serum (1:250) collected 10-14 days after booster immunizations as primary antibody and horseradish-peroxidase-conjugated anti-mouse IgG (1:1000) as secondary antibody. The reaction was visualized using diaminobenzine (DAB) with 30% hydrogen peroxide.

RESULTS AND DISCUSSION

Approximately 0.8 µg/µl of the *E. histolytica* crude cellular antigen was injected into mice. Accordingly, the antigen concentration was readjusted to the desired measurements for ELISA (10 µg/ml), and Western blot (13 µg/µl).

IFAT results showed fluorescent reactions of the mouse serum IgG to the trophozoite antigen fixed on slides. The reaction was localized on the cytosolic component of the trophozoite (Figure 1). Immune response to intestinal and extraintestinal infection caused by *E. histolytica* was predominantly composed of IgG class of immunoglobulin in sera. High titers had been reported even long after successful treatment (Shetty et al. 1990). It was determined from the result of the ELISA analysis that the titers for the sera collected 10 and 20 days after third boost were 1:16,384 and 1:4,096, respectively (Figure 2).

To determine cross-reactivity of the serum from the mice immunized with *E. histolytica* antigen, ELISA was also done using *Blastocystis hominis* antigen. *B. hominis* is a protozoan parasite normally found in the gastrointestinal tract and is commonly found in association with *E. histolytica* infections. As shown in Figure 3, the serum antibody taken from the mice reacted with *E. histolytica* antigen coated on ELISA plates at higher dilutions compared to *B. hominis* antigen. Here we can conclude that the antibody produced is specific to *E. histolytica* and does not react with the protozoan, *B. hominis*.

SDS-PAGE separated the crude antigen suspension into three distinct protein bands, with sizes 23, 41, and 47 kDa. Serum which was taken 20 days after the 3rd booster injection had a positive immunoblot on the 23
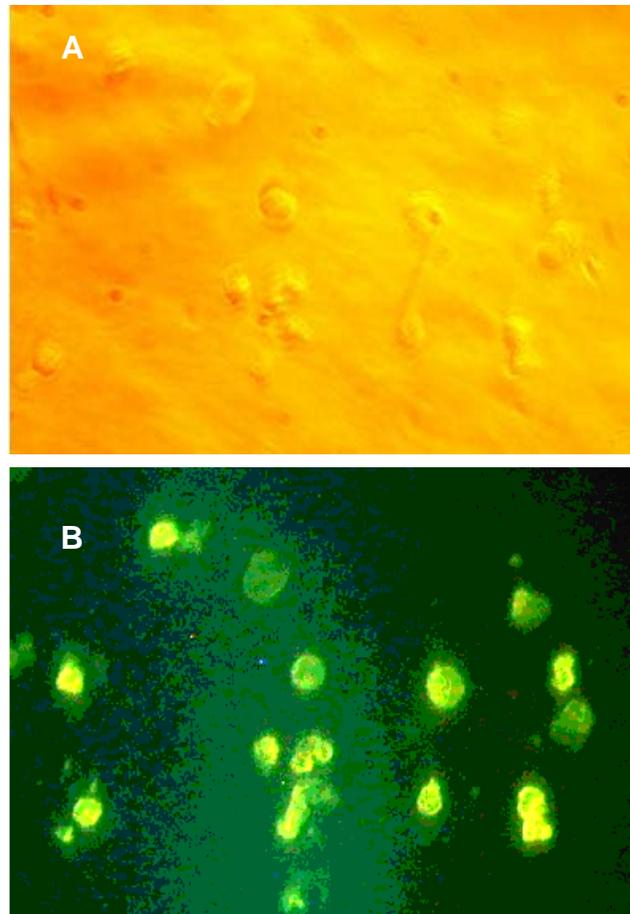
Figure 1. A: Phase contrast photomicrograph of *E. histolytica* trophozoites fixed on slides. B: Immunofluorescence photomicrograph of the *E. histolytica* trophozoites reacted with the serum antibodies produced from mice. (Colored images appear online.)

kDa subunit of the crude *E. histolytica* cellular antigen (Figure 4). The same serum contains highly specific antibodies against *E. histolytica* antigens since it did not react with any of the protein bands of *Candida albicans* nor of *Pseudomonas aeruginosa* from whole cells (data not shown). This protein is presumed to be a novel antigen since this is the first report of a 23 kDa *E. histolytica* antigen. Thus, it is warranted that further characterization of this antigenic protein reactive to the murine polyclonal IgG be conducted. This is significant because this protein from an axenic culture may be another immunogenic marker which may be used for improving diagnosis and treatment of amebic infection.

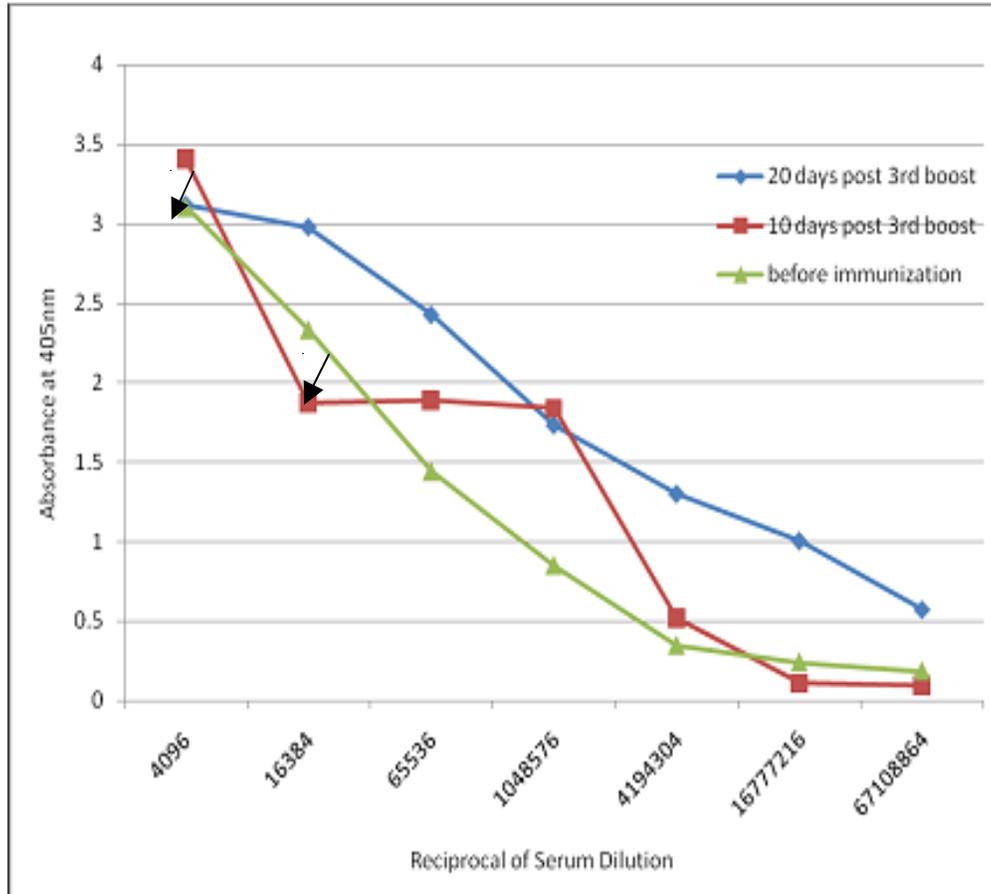Axenized strains of *E. histolytica* may lose their virulence to various degrees during adaptation to culture

Figure 2. Plot of the absorption at 405 nm versus reciprocal of dilution for mouse 4 sera taken before and after immunization (10 and 20 days after the third boost).

without bacteria (Blakely et al., 1990). However, this study was able to characterize a potentially novel antigen from axenic cultures of *E. histolytica*. The cellular antigen was found to be 23 kDa by Western immunoblot. Immunoassays ELISA and IFAT were also utilized to determine the polyclonal IgG serum antibody response of the Balb/c mice to the *E. histolytica* crude antigen. It is suggested that further characterization of the IgG produced be done by isotype analysis.

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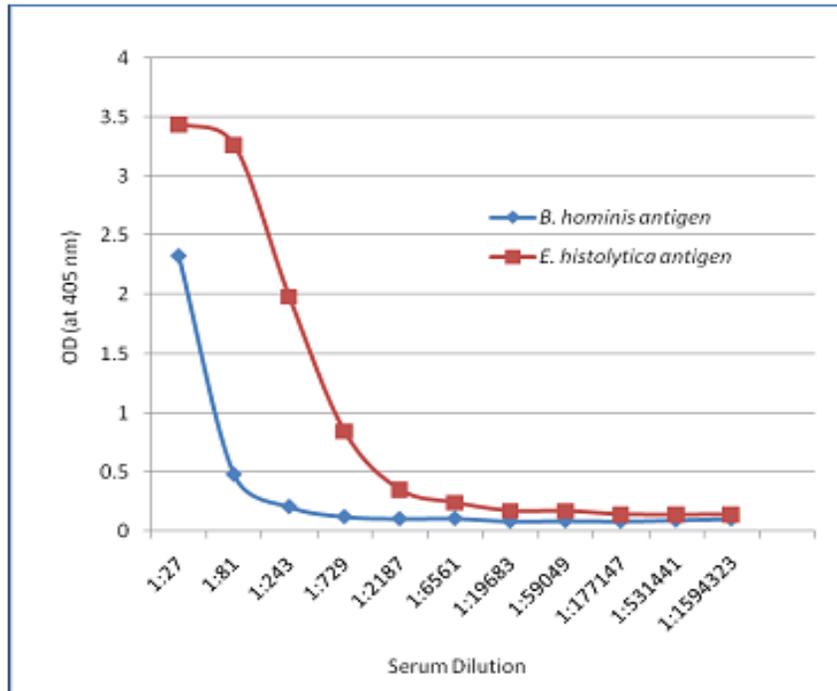
Figure 3. Test of cross-reactivity with *B. homini*s antigen. By ELISA, OD values were taken against increasing dilutions of the mouse serum.
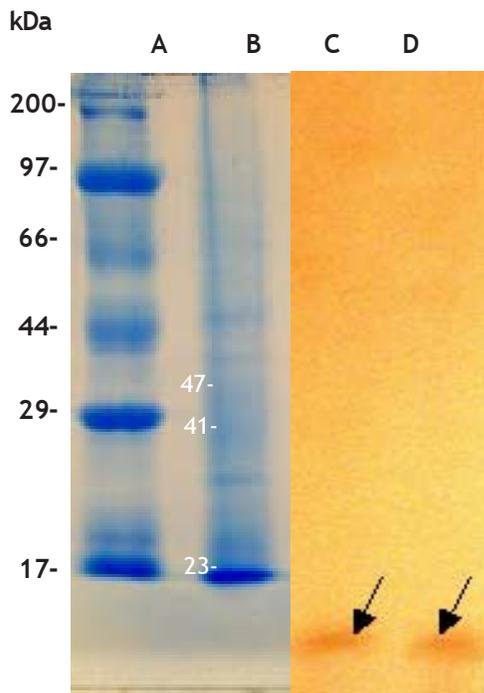

Figure 4. Western immunoblot analysis of the reactivity of polyclonal antibodies to trophozoites of *E. histolytica.* A: molecular weight marker; B: *E. histolytica* protein antigens (47, 41, and 23 kDa); C and D: two lanes of serum IgG antibody targeting the 23 kDa antigen (arrows pointing to the 23kDa *E.histolytica* specific antigen).

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