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Production and Characterization of Polyclonal Antibodies Specific for *Entamoeba histolytica*

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Murine polyclonal antibodies (pAbs) were produced by immunizing BALB/c mice with trophozoites of *Entamoeba histolytica* HK9 in Freund's adjuvant. Using indirect fluorescent antibody test (IFAT), we have developed polyclonal IgG antibodies that specifically reacted with formalin-fixed *E. histolytica* trophozoites, but failed to bind with live trophozoites, suggesting the epitope to be cytoplasmic and not cell surface located. *Entamoeba dispar*, *Entamoeba coli*, and *Blastocystis hominis* showed no reactivity with the pAbs. Western immunoblot analysis revealed the reactivity of the pAbs with 95 kDa and 103 kDa major proteins, antigens that we consider as novel as there are no reports to date on these molecules. The pAbs developed in the present study are valuable, particularly when used in IFAT in the accurate detection and differentiation of the pathogenic *E. histolytica* from the non-invasive *E. dispar*.

Key Words: amebiasis, indirect fluorescent antibody test, immunoblot, murine antibodies

INTRODUCTION

Amebiasis, a disease caused by the enteric protozoan *Entamoeba histolytica*, is a major public health problem, especially, in developing countries. *E. histolytica* is responsible for up to 100,000 deaths per year, placing it second only to malaria as a cause of mortality due to protozoan parasites (World Health Organization 1997). Because of this, the detection of *E. histolytica* is an important goal of the clinical microbiology laboratories throughout the world (Tanyuksel & Petri 2003).

Laboratory diagnosis of amebiasis based on direct microscopic observation of stool samples is still by far the most widespread method. This approach, however, is labor-intensive and unreliable because it is unable to differentiate *E. histolytica* from morphologically identical species that are nonpathogenic, such as *Entamoeba dispar* and *Entamoeba moshkovskii* (Diamond & Clark 1993; Ackers 2002; Tanyuksel & Petri 2003). This may lead to false positives for amebic infection and subsequent wrong treatment of the actual disease. More recent methods for the diagnosis of amebic infection are growth culture, stool antigen and antibody detection through enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) detection, indirect hemagglutination assays (IHA) and indirect fluorescent antibody test (IFAT) (Tanyuksel & Petri 2003). Studies on the application of monoclonal antibodies in IFAT to detect *E. histolytica* have been reported (Tachibana et al. 1990; Gonzales-Ruiz et al. 1992; Walderich et al. 1998; Yau et al. 2001). However, monoclonal antibodies are very expensive to produce and are not readily available in developing countries such as the

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Philippines. In addition, there have been no reports on the development of polyclonal antibodies (pAbs) being applied in IFAT to detect *E. histolytica*. If pAbs that have comparable specificity and sensitivity to monoclonal antibodies can be produced, it is possible to generate low-priced detection kits for amebiasis.

MATERIALS AND METHODS

Parasites and culture conditions

Trophozoites of *E. histolytica* HK9 were axenically grown at 35.5º C in BI-S-33 medium (consists of Biosate [a peptone mixture with 2 parts trypticase and 1 part yeast extract] and a vitamin-Tween 80 mixture) supplemented with 10% heat-inactivated bovine serum (Diamond et al. 1978). Cultures of enteric protozoans such as *E. dispar*, *E. coli* and *B. hominis* were also used as controls in the IFAT experiment. Three day-old parasite culture suspensions were washed 3 times with ice-cold phosphate-buffered saline (PBS; pH 7.4) by centrifugation at 400 × g for 2 min at 4º C before being used.

Production of polyclonal antibodies (pAbs)

Three 7-week old female BALB/c mice were injected intraperitoneally with 4 × 106 live *E. histolytica* HK-9 trophozoites in complete Freund's adjuvant (CFA) according to the method described by Coligan (1991).

Prior to immunization, blood was collected to serve as a control. The serum was prepared as follows: the blood samples, collected from the mice through venous puncture, were allowed to stand for an hour at room temperature and another hour at 4º C; it was centrifuged at 10,000 rpm for 10 minutes. The prepared serum was stored in 0.5 ml polypropylene microcentrifuge tubes at -20º C until use. The antibody titer of the serum was assayed using IFAT as described below.

For the priming immunization, 1 ml of PBS with 20 x 106 live *E. histolytica* trophozoites was added in portions of 0.2 ml to 1 ml of CFA at 4º C. Each mouse was injected intraperitoneally with 0.2 ml of the CFA antigen emulsion. Throughout the process, the mixture was chilled on a bed of ice from time to time to keep the mixture as close to 4º C as possible to prevent denaturation of proteins. Blood was collected and the serum was prepared and assayed, 10 days after the priming immunization.

Four weeks after the priming immunization, the mice were boosted with same number and concentration of trophozoites in emulsion but instead of using CFA, incomplete Freund's adjuvant (IFA) was used. The mice were boosted again after two additional weeks. The mice were bled 10 days after each booster immunization and the serum was prepared and assayed using IFAT.

Isotyping of polyclonal antibodies

The pAbs produced were assayed for specific isotype using Rapid Mouse IsoGoldTM (BioAssay Works, L.L.C). The procedure was performed according to the manufacturer's instructions.

IFAT using formalin-fixed trophozoites

IFAT was done according to the methods previously described by Tachibana et al. (1990) with some modifications. Trophozoites in cultures were fixed with 3% formalin in PBS for 30 min at 4º C and washed three times with PBS. The parasites were suspended in PBS to a concentration of 106 cells/ml. Two microliters of the suspension was added to the wells of multispot glass slides, which were air-dried at room temperature. The prepared slides were wrapped in aluminum foil and kept at -20º C until use.

The IFAT was carried out at 23º C in a moist chamber. The antigen slides were allowed to thaw before use. The wells of the multispot slides were treated with 50 µl of 3% skim milk in PBS (PBS-SKM) for 30 minutes. Thereafter, the PBS-SKM solution was aspirated. For the purpose of titration, the purified antiserum was serially diluted starting at a ratio of 1:16, followed by fourfold stepwise dilutions (1:64, 1:256, 1:1024, 1:4096, 1:16384, and 1:65,536). Then, 50 µl of different dilutions of the antiserum were added to each well and were incubated for 1 h. Three wells were used for each antiserum dilution and three trials were done. The pre-immune serum served as a negative control. The slides were washed five times with PBS by immersing the slide in chambers filled with PBS (each washing was done in separate chambers; 1st wash: immediately; 2nd wash: 4 min; 3rd wash: 4 min; 4th wash; 3 min; 5th wash; 4 min). Afterwards, the wells were allowed to dry and were treated with 40 µl of commercially available fluorescein isothiocyante (FITC)-labeled goat anti-mouse IgG (MBL, Japan), diluted 1:40, in PBS-SKM for 30 min. The slides were again washed with PBS and incubated for 30 min. The slides were mounted with 50% glycerol in PBS and were observed under a Zeiss Axiovert fluorescence microscope.

IFAT using live trophozoites

The method was done according to the procedure previously described by Tachibana et al. (1991) with modifications. Harvested trophozoites from cultures were washed three times with cold PBS. The parasites were suspended in PBS to a concentration of 106 cells/ml. One milliliter of the suspension was placed in 1.5 ml centrifuge tubes. The suspension was centrifuged for 2 min at 400 x g. It was then blocked with 500 µl 3 % PBS-SKM for 15 min. It was centrifuged for 2 min at 400 x g. The cells were incubated with 300 µl antiserum for 15 min. The cells were washed twice with cold PBS and centrifuged for 2 min at 400 x g. The cells were incubated with the 300 µl FITC-labeled goat anti-mouse IgG (MBL, Japan), diluted 1:50, in PBS-SKM for 30 min. The cells were again washed twice with cold PBS and viewed under Zeiss Axiovert fluorescence microscope. The incubation steps were carried out in 1.5 ml centrifuge tubes on ice.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Trophozoites of *E. histolytica* were solubilized with an equal volume of sample buffer (0.25 mM Tris-HCl [pH 6.8], 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue, and 2 mM EDTA) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM pepstatin, and 4 µM leupeptin for 5 min at 95º C. The supernatant that was obtained after centrifugation at 10,000 × g for 3 min was subjected to denaturing SDS-PAGE, using 10% polyacrylamide separating gel, according to the method of Laemmli (1970). Santa Cruz Biotechnology stained broad range molecular mass markers (sc-2361) were used. Electrophoresed proteins were transferred onto a sheet of 0.45-µm nitrocellulose membrane using the procedure of Towbin et al. (1979). Electrophoretic transfer was carried out at 200 mA for 1 h. Some lanes of the sheet were stained with Coomassie brilliant blue, while the others were incubated first with PBS-SKM for 2 h and then with antiserum (1:200 dilution in PBS-SKM) for an additional 2 h at 23º C. The membrane was washed with PBS containing 0.05% Tween 20 and afterwards, it was incubated with 1:250 commercially available horseradish peroxidase-labeled goat anti-mouse IgG (Sigma) for 1 h. The sheet was washed and developed in a solution of 0.05% diaminobenzidine (DAB) plus 0.03% hydrogen peroxide in PBS for 1h.

RESULTS AND DISCUSSION

We have produced pAbs that are capable of detecting and distinguishing pathogenic *E. histolytica* from non-invasive *E. dispar* and other enteric protozoans like *E. coli* and *B. hominis*. The pAbs isotype was identified as IgG.

From the results of the IFAT, the sera and ascitic fluids rich in pAbs showed a strong positive fluorescence on formalin-fixed trophozoites of *E. histolytica* (Figure 1). The titer of the produced

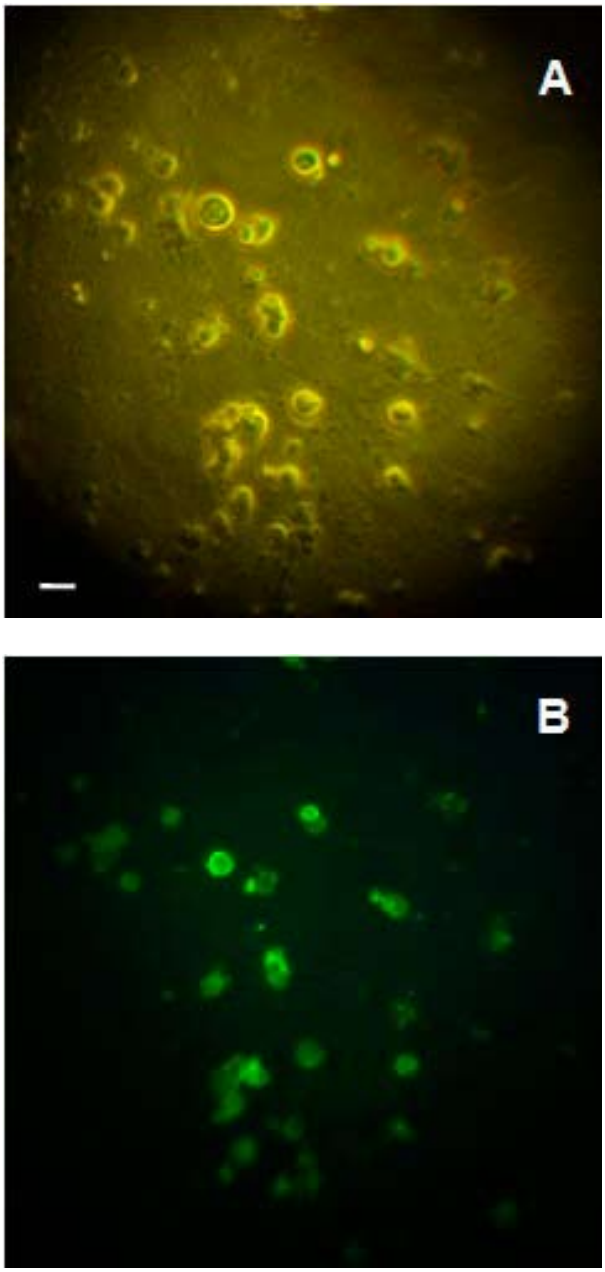

Figure 1. (a) Phase-contrast and (b) Immunofluorescence photomicrographs of formalin-fixed trophozoites of Entamoeba histolytica probed with mouse antiserum (diluted 1:64) containing the pAbs followed by FITC-labeled goat anti-mouse IgG. Fluorescence was observed (localized at cytoplasm). Bar, 20 µm.

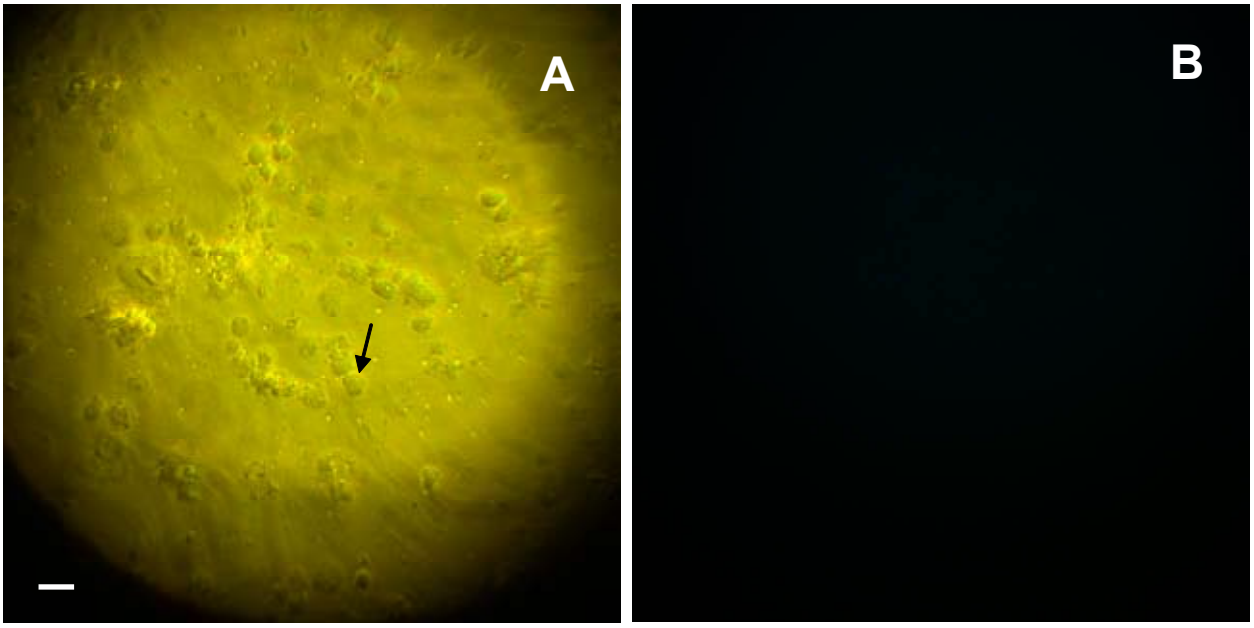

Figure 2. (a) Phase-contrast and (b) Immunofluorescence photomicrographs of formalin-fixed trophozoites of *Entamoeba dispar* probed with mouse antiserum (diluted 1:64) containing the polyclonal antibodies followed by FITC-labeled goat anti-mouse IgG. No fluorescence was observed. Bar, 20 μm.

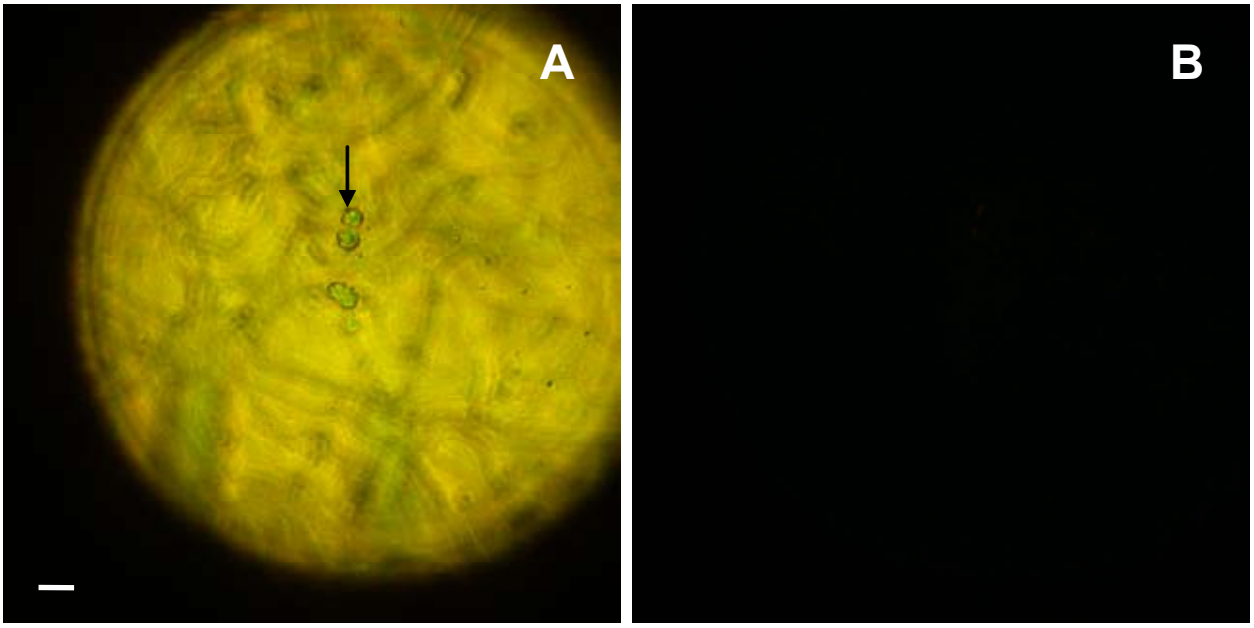

Figure 3. (a) Phase-contrast and (b) Immunofluorescence photomicrographs of formalin-fixed trophozoites of *Blastocystis hominis* probed with mouse antiserum (diluted 1:64) containing the polyclonal antibodies followed by FITC-labeled goat anti-mouse IgG. No fluorescence was observed. Bar, 20 μm.

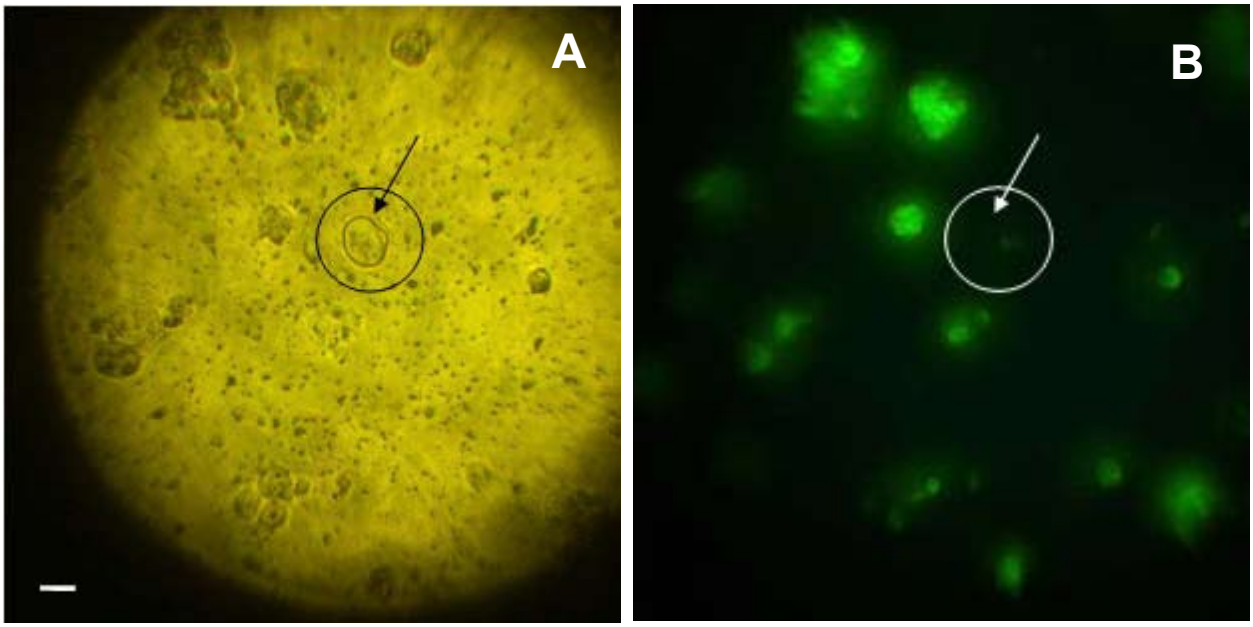

Figure 4. (a) Phase-contrast and (b) Immunofluorescence photomicrographs of a live trophozoite of *Entamoeba histolytica* (inside the circles) probed with mouse antiserum (diluted 1:64) containing the polyclonal antibodies followed by FITC-labeled goat anti-mouse IgG. No fluorescence of live trophozoite was observed (white arrow). Note that cells outside the circle that fluoresced were dead trophozoites of *E. histolytica*. Bar, 20 μm.

antibodies was observed at the 16,384 dilution. In contrast, the pAbs did not react with formalin-fixed trophozoites of *E. dispar*, *E. coli* (data not shown) and *B. hominis* as shown by the absence of fluorescence even at the 1:64 dilution of sera and ascitic fluids (Figures 2 and 3). Absence of fluorescence was also observed at the minimum 1:16 dilution. These suggest that the pAbs are very sensitive and specific to *E. histolytica*.

Live trophozoites of *E. histolytica* were also used to localize the epitopes recognized by the antibodies, i.e. if they are surface immunogens or not. From the results, no fluorescence was observed in IFAT of live trophozoites (Figure 4) suggesting that the epitopes recognized by the pAbs are not exposed on the surface of the parasite. This was supported by the observed fluorescence localized in the cytoplasm in IFAT using formalin-fixed trophozoites (Figure 1).

To characterize the antigenic molecules recognized by the pAbs, SDS-PAGE under reducing conditions and Western immunoblot analysis were used. The pAbs recognized two major protein bands with estimated molecular masses of 95 kDa and 103 kDa, respectively (Figure 5).

The production of a number of monoclonal antibodies that are specific to *E. histolytica* have already been reported (Petri et al. 1990; Tachibana et al. 1990, 1991, 1997; Gonzalez-Ruiz et al. 1992, 1994; Sengupta et al. 1993; Leon et al. 1994; Yau et al. 2001) but this is the first report concerning the production of antibodies specific for the 95-kDa and 103-kDa antigenic molecules of *E. histolytica*.

In the review made by Espinosa-Cantellano and Martinez-Palomo (1991), they have reported that most research groups have observed 11 principal antigenic proteins of *E. histolytica* having molecular weights of 220, 170, 150, 125, 96, 80, 60, 45, 20 and 9 kDa. It is notable that the 95 kDa molecule that was observed in the study has almost the same molecular mass as the 96 kDa molecule reported by Torian et al. (1990). This 96 kDa molecule is reported to have two forms in *E. histolytica*: an integral plasma membrane protein and a soluble form in cytosol. It is possible that the 95 kDa molecule described here is the same as the 96 kDa molecule reported by Torian et al. (1990) although it remains to be established.

The results indicate that there are immunological differences between *E. histolytica* and *E. dispar,* and the produced pAbs applied in IFAT can be useful in the

accurate detection and differentiation of *E. histolytica* from *E. dispar*. Since the method used can detect formalin-fixed trophozoites, this will be an important tool in epidemiological studies especially in far-flung areas where the samples cannot be kept fresh or frozen and are rather fixed with formalin or methanol. This will be useful in the establishment of an accurate prevalence data of *E. histolytica* infections in many parts of the world. Furthermore, these pAbs may be used commercially in immunodiagnostic kits for *E. histolytica* infections. These future diagnostic kits can provide a fast, easy, cheap and accurate detection of *E. histolytica*. The pAbs and the epitopes that they recognize may also contribute to the development of a potential vaccine for amebiasis. Lastly, the produced antibodies can be sold commercially as a positive control for immunological tests in laboratories.

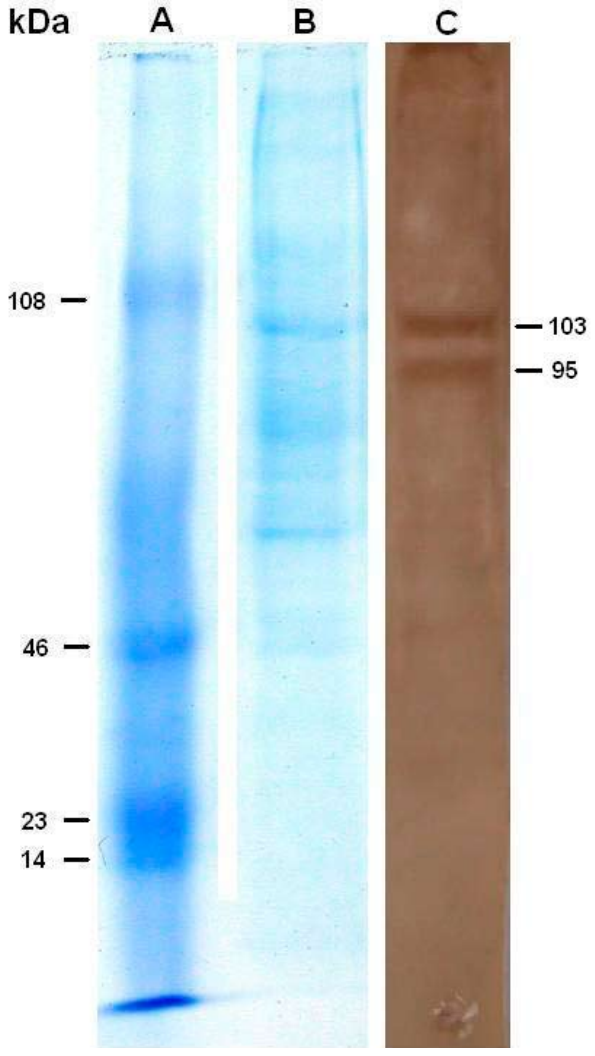

Figure 5. Western immunoblot analysis of the reactivity of pAbs to trophozoites of Entamoeba histolytica HK9. Cell lysates were subjected to SDS-PAGE under reduced conditions and transferred to nitrocellulose membrane. Protein bands in lane b were stained with Coomassie brilliant blue. Lane c was treated with polyclonal antibodies, followed by horseradish peroxidase-labeled goat anti-mouse IgG. Lane a shows molecular mass standards.

In summary, we have produced murine polyclonal antibodies that are specific to *Entamoeba histolytica*. These antibodies can detect and distinguish *E. histolytica* and *E. dispar*. The major epitopes that are detected by the produced pAbs are located in the cytoplasm and have molecular masses of 95 kDa and 103 kDa, respectively. These data demonstrate that IFAT with the produced pAbs can be useful in the accurate detection and differentiation of the pathogenic *E. histolytica* from non-invasive *E. dispar*.

In this study, the produced pAbs were only used in the detection of *E. histolytica* trophozoites from axenic cultures. In order to assess its applicability in field studies, the evaluation of the effectiveness of the produced pAbs in the detection of *E. histolytica* in stool samples is highly recommended. The molecular analysis of the 95 kDa and 103 kDa major antigens and determination of their biological functions deserves further study. Other strains of *E. histolytica* and more enteric protozoans (e.g. *Entamoeba moshkovskii* and *Cryptosporidium parvum*) should be tested for cross-reactivity with the produced pAbs using IFAT. The application of the produced pAbs in the detection of *E. histolytica* using ELISA should also be evaluated.

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