

Characterization of *Blastocystis hominis* antigens by the enzyme-linked immunosorbent assay (ELISA) and immunoblotting

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Abstract

Blastocystis hominis antigens were characterized by ELISA and immunoblotting and compared with those of *Giardia lamblia* and *Entamoeba histolytica* to identify species-specific antigens which may be useful for the development of diagnostic assays. SDS-PAGE analysis revealed differences in the polypeptide patterns of the three protozoan antigens. The ELISA performed with homologous and heterologous antigens on mice hyperimmune sera raised against *B. hominis* was specific as no cross reactions were observed with either *G. lamblia* or *E. histolytica* antigens. *B. hominis* antigens did not cross react with antisera produced against the two heterologous antigens indicating the high specificity of the ELISA. Immunoblotting identified specific antigenic polypeptides for each parasite species and these may be useful for diagnosis. For *B. hominis*, antigenic polypeptides of molecular weights 66.2 and 57.5 kD were most reactive with homologous mice hyperimmune sera while for *G. lamblia*, the most reactive polypeptides were of molecular weights 74, 66.2 and 50 kD. For *E. histolytica*, polypeptides of molecular weights 66.2 and 57.5 kD were highly reactive. Both the ELISA and immunoblotting can detect specific antibodies and are therefore potentially useful for the diagnosis of *B. hominis* infection.

Key Words: *Blastocystis hominis*; immunodiagnosis; immunoblotting

Introduction

Blastocystis hominis (Zierdt *et al.*, 1967) is an intestinal protozoan parasite of both humans and animals (Boreham & Stenzel, 1993). In human infections symptoms reported include diarrhoea, abdominal discomfort, anorexia, flatulence and non-specific gastrointestinal effects. Several reports have suggested that this parasite is associated with a variety of diseases such as irritable bowel syndrome (Drossman, 1979), tropical pulmonary eosinophilia (Enzenauer *et al.*, 1990), ulcerative colitis (Jeddy & Farrington, 1991) and reactive arthritis (Lakhanpal *et al.*, 1991). It is also becoming an important cause of diarrhoea in HIV-infected patients (Albrecht, 1995; Storgaard, 1996).

The most common method for diagnosis of this parasite is through microscopic examination of stool samples. However, due to its wide spectrum of clinical presentations, it may be useful to include serological assays to detect infections by this parasite. Studies by Zierdt & Tan (1976) using infected patients' sera showed negative results with the immunofluorescence test while Chen *et al.* (1987) reported no serum antibody response in immunoblot analysis with sera from four blastocystis infected patients. A more recent study by Zierdt *et al.* (1995) demonstrated that ELISA could potentially be used to detect antibodies in sera of blastocystis patients. More studies should therefore be carried out to determine the usefulness of ELISA for the detection of blastocystis infection particularly when the parasite is

absent in the patients' stool samples. As this parasite is often found together with other common parasitic protozoans such as *Giardia lamblia* and *Entamoeba histolytica* in the same patients and produce similar symptoms (Zeidt *et al.*, 1995), it would be relevant to determine the antigenic differences and the extent of their cross reactivity in the ELISA. Another objective of the study is to determine whether immunoblotting can serve as an alternative confirmatory test for blastocystis infection.

Materials and Methods

Protozoan Parasites

The three protozoan parasites used in the study included *Blastocystis hominis* (Strain C) which was obtained from the Department of Microbiology, National University of Singapore, *Giardia lamblia* (Strain 7404) isolated from a Malaysian patient and *Entamoeba histolytica* (HK9 strain).

Preparation of parasite antigens for immunization and ELISA

The three parasites from *in vitro* cultures were harvested by centrifugation at 1000 rpm for 20 minutes, the supernatant discarded and the parasite peller washed once with 0.85% normal saline. The washed peller was then resuspended in an equal volume of 0.85% normal saline, the suspension placed in an ice-bath and sonicated at 7 kilocycles per second at one minute in-

tervals for five minutes. The supernatant was left overnight at 4°C, then centrifuged at 8000 rpm for 20 minutes at 4°C and the supernatant harvested, aliquoted into smaller volumes and kept at -20°C until used for immunization or in the ELISA. The protein content of the parasite antigens was determined using the Biorad® protein assay kit.

Immunization of BALB/c mice

Eight BALB/c mice, four to six weeks old, were immunized with each antigen. Pre-immunization sera were obtained from the tail vein and screened by ELISA for antibodies to the three protozoan antigens used in the study. Each mouse was inoculated with three doses of antigen subcutaneously at fortnightly intervals, each dose consisting of 1 mg antigen in 1 ml of normal saline emulsified with an equal volume of Freund's complete adjuvant. Serum was collected from each mouse two weeks after each immunizing dose and the antibody levels determined with the ELISA.

ELISA Procedure

The ELISA procedure used was based on the original method of Engvall & Perlmann (1972) with the microplate modification of Voller *et al.* (1976). The method used was the indirect assay for antibodies using the horseradish peroxidase enzyme system and orthophenylenediamine as the enzyme substrate. A checkerboard titration was performed to determine optimum concentrations of antigens, sera and conjugate using reference positive and negative sera. The absorbance (OD) of the samples were read using an ELISA reader (Dynatech Laboratories, USA) at 492 nm. For each ELISA, reference positive and negative controls were included. In the study, three ELISA experiments were performed, one for each mouse antiserum produced against each of the three protozoans, reacted with both homologous and heterologous antigens. For each serum sample, duplicate tests were made and the average OD value calculated.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Antigenic proteins of the three protozoans were separated on SDS-PAGE using the discontinuous system of Laemmli (1970). The gel used in this study consisted of a stacking gel (4% acrylamide/N,N'-methylene bisacrylamide, 0.125M Tris-HCl, pH 6.8 and 10% (w/v) SDS) and a separation gel (12% acrylamide/N,N'-methylene bisacrylamide, 0.375 M Tris-HCl, pH 8.8 and 10% (w/v) SDS). Samples were denatured by boiling for 5 minutes in Laemmli sample buffer and electrophoresed at room temperature at a constant voltage of 200V in buffer (pH 8.3) consisting of 0.025M Tris base, 0.125M glycine and 0.1% SDS. The molecular weights of the parasite proteins were estimated by comparing their electrophoretic mobilities with

standard molecular weight markers (Sigma) after electrophoresis in the same gel.

Immunoblotting

Individual polypeptides separated on gels were transferred electrophoretically to nitrocellulose paper (NCP) (Toyo Co. Ltd, Japan) by the method of Towbin *et al.* (1979) using the trans-blot cell (Biorad Laboratories, USA) at 100V for 3 hours with cooling of transfer buffer at 4°C. The NCP filter was incubated in different sera samples, then reacted with diluted peroxidase conjugated goat anti-mouse immunoglobulins (Cappel Laboratories, USA) for three hours at room temperature followed by washing. Bound antigen-antibody complexes were visualised by the addition of 4-chloro-1-naphthol substrate and the blots were rinsed in distilled water, air-dried and photographed.

Results

The results of the three ELISA experiments performed are as shown in Figs. 1-3. In Fig. 1, mice antisera produced against *B. hominis* reacted only with the homologous antigen, no cross reactivity was observed with *G. lamblia* and *E. histolytica*. Antibody titres to *B. hominis* antigen was observed to increase after each immunizing dose. A similar antibody response was observed with mice antisera produced against *G. lamblia* (Fig. 2) and *E. histolytica* (Fig. 3) when reacted with the respective homologous antigens. Also, *B. hominis* did not cross-react with either heterologous antisera in the ELISA.

SDS-PAGE analysis (Fig. 4) revealed differences in polypeptide patterns among the three protozoans. Immunoblots performed with the three antisera against both homologous and heterologous antigens are as shown in Fig. 5-7. Mice antisera against *B. hominis* (Fig. 5) reacted intensely with *B. hominis* antigens but had very low or no reactivity with *G. lamblia* and *E. histolytica* antigens. All the 8 antisera recognized a 66.2 kD polypeptide while 6 recognized a 57.5 kD polypeptide. Other reactive polypeptides ranged from 56 to 21 kD molecular size. Antisera against *G. lamblia* (Fig. 6) and *E. histolytica* (Fig. 7) had very low or no reactivity against *B. hominis* antigen in the immunoblots. *G. lamblia* antigens reacted strongly with the homologous antisera and the most reactive polypeptides were 74 kD, 66.2 and 50 kD. For antisera against *E. histolytica*, the most reactive polypeptides were 66.2, 57.5 and 52.5 kD present in *E. histolytica*.

Discussion

There are very few reports on the immune response against *B. hominis* (Kukosche & Muller, 1991; Zierdt, 1991). Zierdt (1991) successfully showed immunofluorescence with the central body, amoeboid and granular forms of *B. hominis* using rabbit antisera prepared against the whole cell of the protozoan while

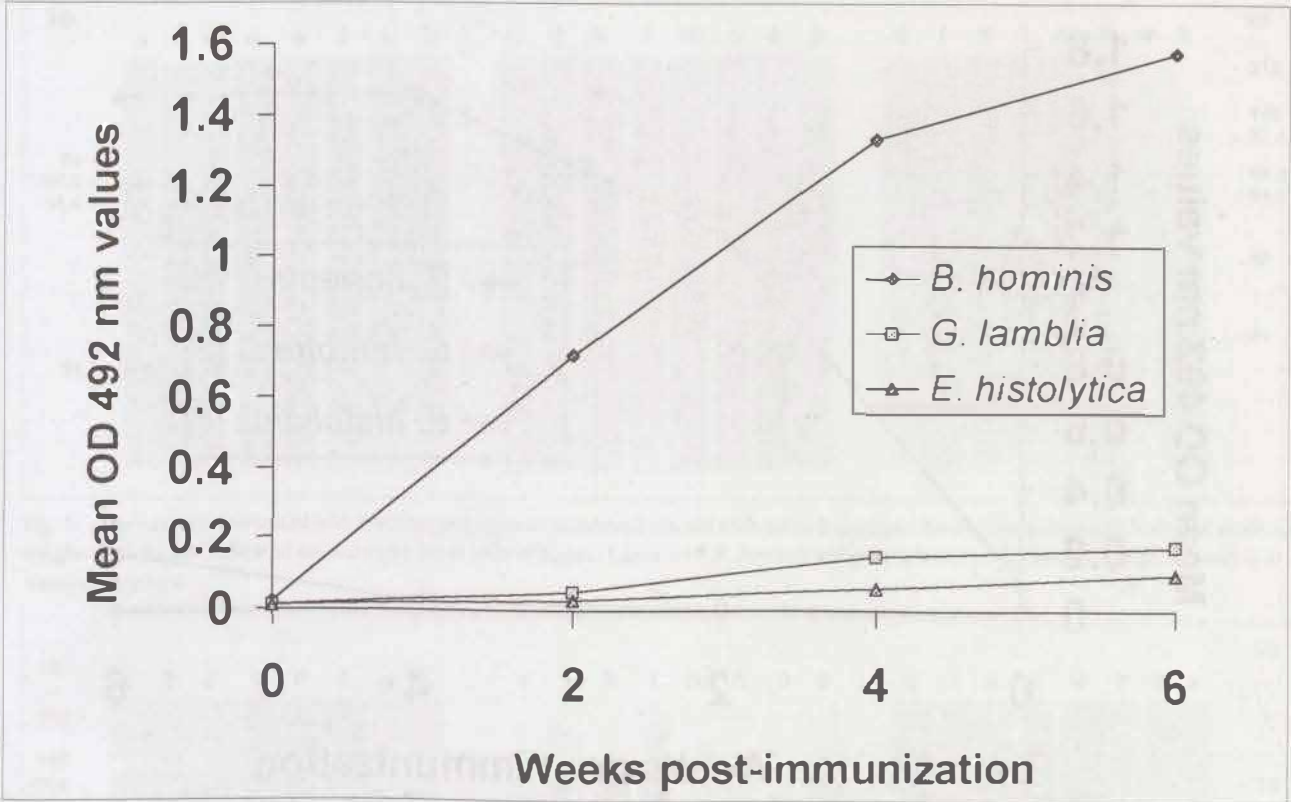


Fig. 1. Mean OD at 492 nm values obtained in the ELISA with mice antisera against *Blastocystis hominis* reacted with homologous and heterologous antigens.

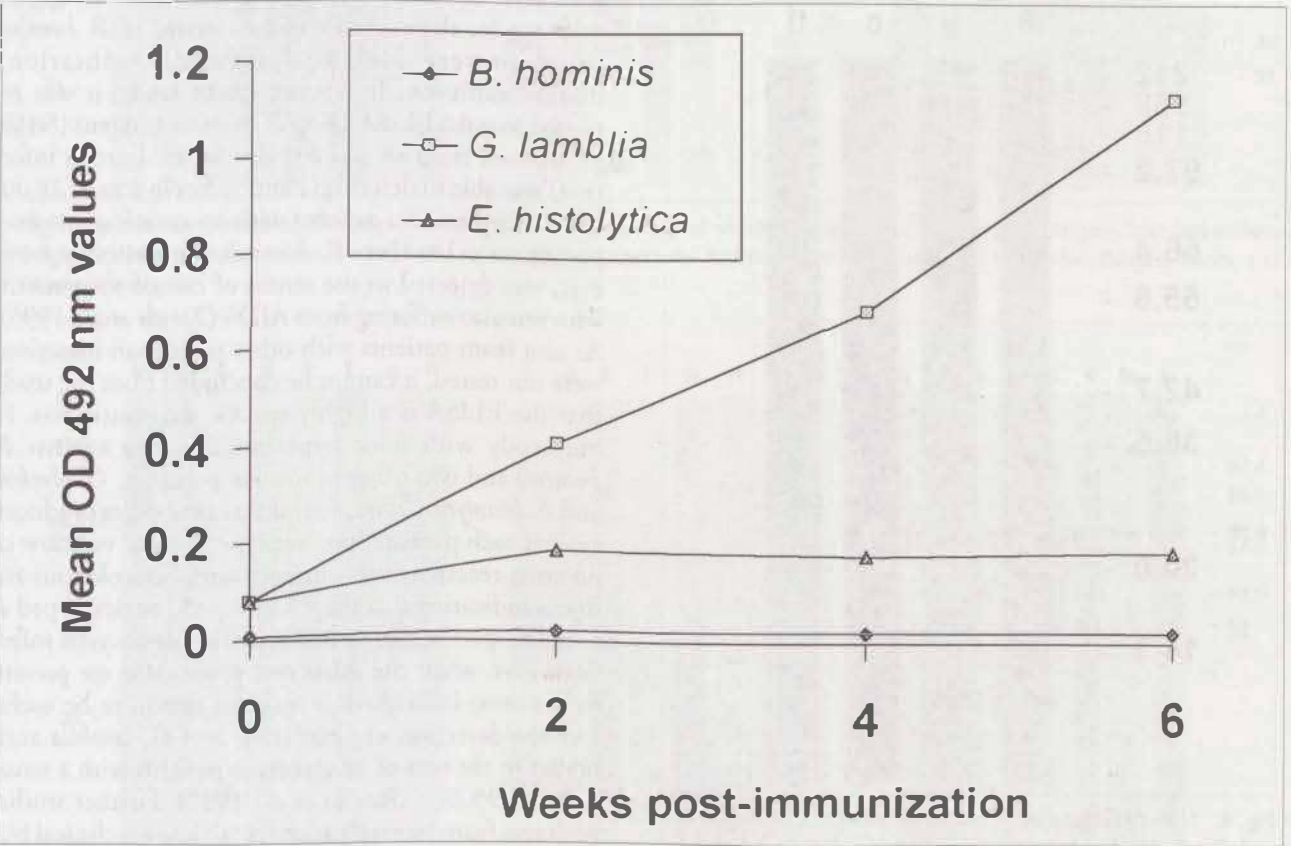


Fig. 2. Mean OD at 492 nm values obtained in the ELISA with mice antisera against *Giardia lamblia* reacted with homologous and heterologous antigens.

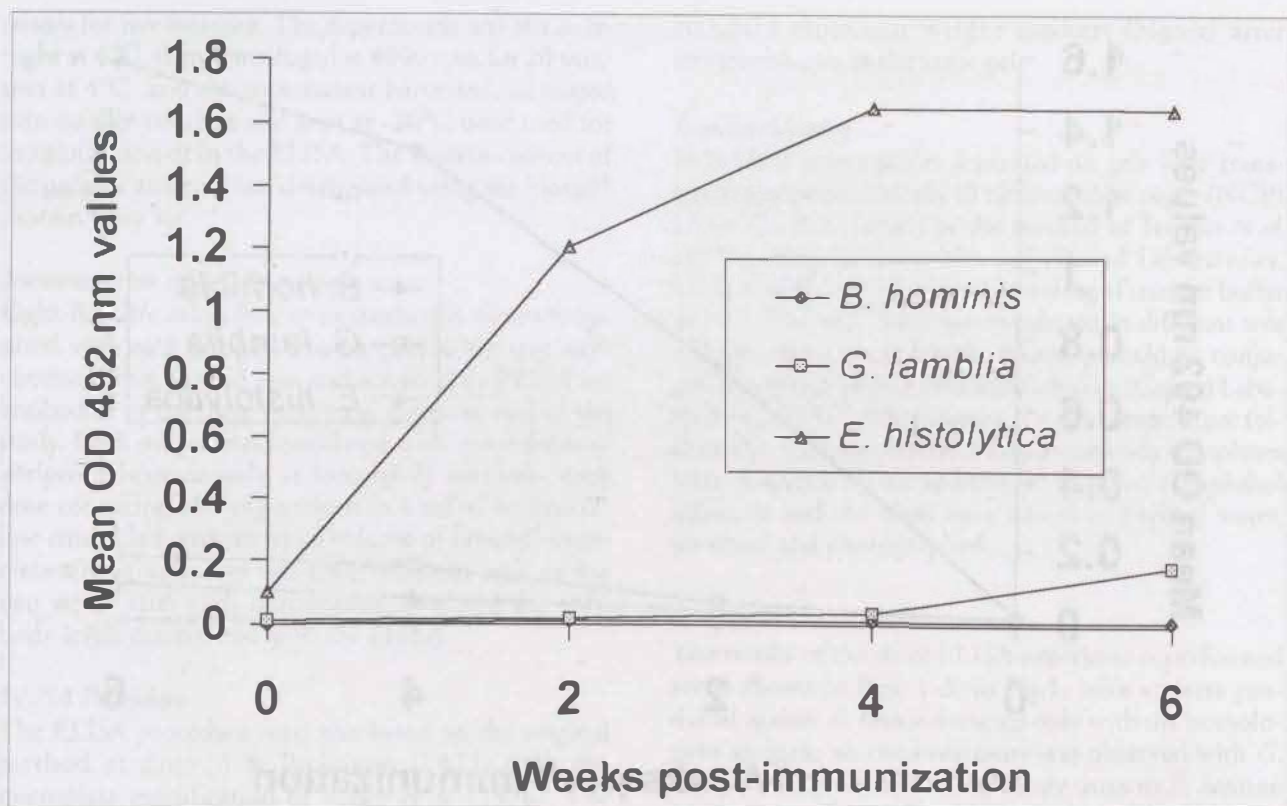


Fig. 3. Mean OD at 492 nm values obtained in the ELISA with mice antisera against *Entamoeba histolytica* reacted with homologous and heterologous antigens.

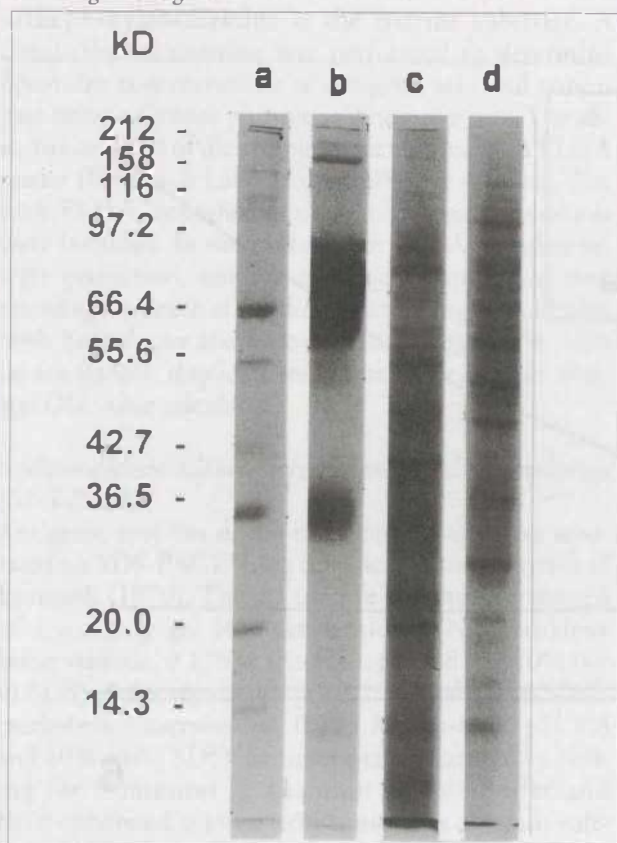


Fig. 4. SDS-PAGE analysis of *Blastocystis hominis*, *Giardia lamblia* and *Entamoeba histolytica* antigens. Lane a: Standard molecular weight markers; Lane b: *B. hominis*; Lane c: *G. lamblia*; Lane d: *E. histolytica*.

Kukosche & Muller (1991) provided immunological evidence for the existence of two strains of *B. hominis* using western blot analysis and Ouchterlony immunodiffusion. In a more recent study, it was reported that the ELISA using *B. hominis* antigens (Strain 91 isolated from an acute symptomatic human infection) was able to detect IgG antibodies in sera of 28 out of 30 symptomatic patients with no enteric protozoan pathogens other than *B. hominis*. No antibody however, was detected in the serum of one of the patients who was also suffering from AIDS (Zierdt *et al.*, 1995). As sera from patients with other protozoan infections were not tested, it cannot be concluded from the study that the ELISA is a highly specific diagnostic test. In our study with mice hyperimmune sera against *B. hominis* and two other protozoan parasites, *G. lamblia* and *E. histolytica*, it was found that antibodies produced against each parasite spp. were specific and very low or no cross-reactivity was obtained with heterologous antigens indicating that the ELISA could be developed as a useful specific diagnostic assay for blastocystis infections even when the other two protozoans are present in the same individual. It was also shown to be useful for the detection of circulating anti-*G. lamblia* antibodies in the sera of 48 giardiasis patients with a sensitivity of 95.8% (Romia *et al.*, 1990). Further studies with sera from human patients with known clinical history are being carried out to confirm the specificity and to determine the sensitivity of the ELISA using crude

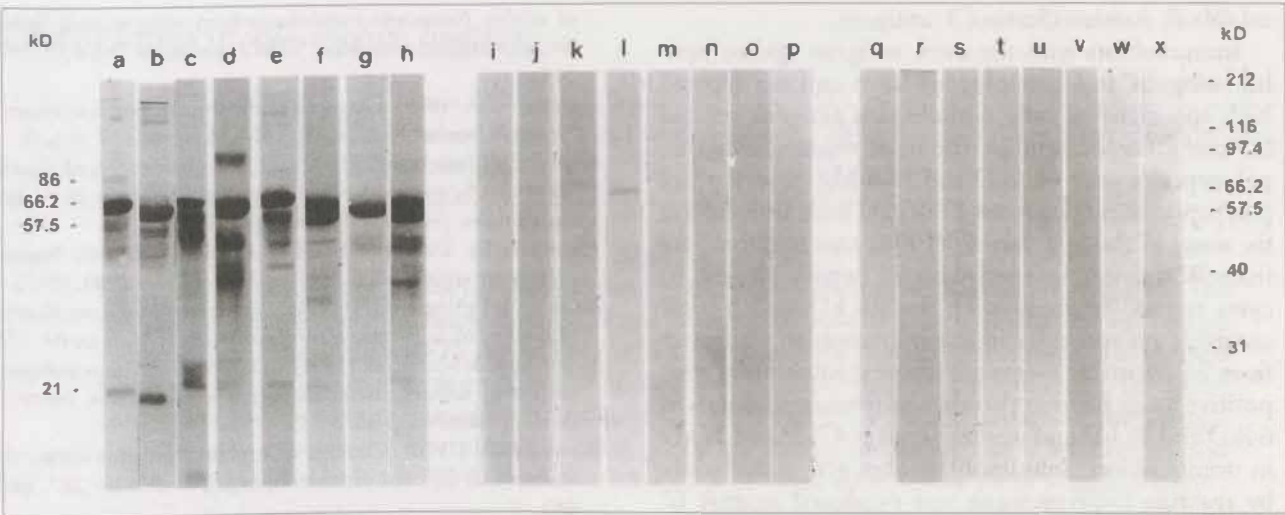


Fig. 5. Immunoblots obtained with mice antisera against *B. hominis* reacted with homologous and heterologous antigens. Standard molecular weight markers are indicated on the right hand side of figure. Lanes a-h: *B. hominis* antigen; Lanes i-p: *G. lamblia* antigen; Lanes q-x: *E. histolytica* antigen

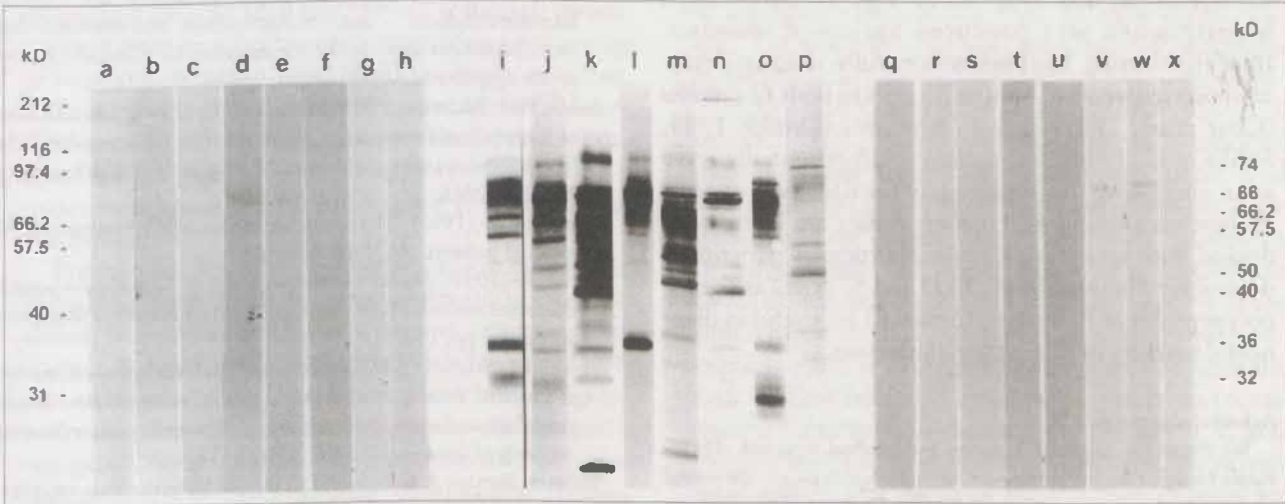


Fig. 6. Immunoblots obtained with mice antisera against *G. lamblia* reacted with homologous and heterologous antigens. Standard molecular weight markers are indicated on the left hand side of figure. Lanes a-h: *B. hominis* antigen; Lanes i-p: *G. lamblia* antigen; Lanes q-x: *E. histolytica* antigen

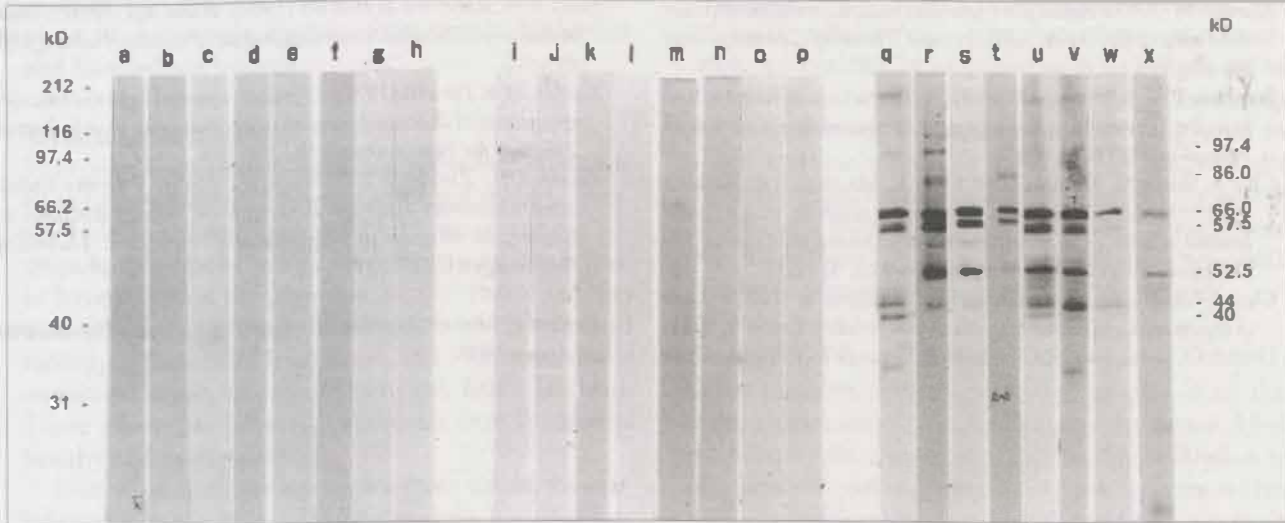


Fig. 7. Immunoblots obtained with mice antisera against *E. histolytica* reacted with homologous and heterologous antigens. Standard molecular weight markers are indicated on the left hand side of figure. Lanes a-h: *B. hominis* antigen; Lanes i-p: *G. lamblia* antigen; Lanes q-x: *E. histolytica* antigen

soluble *B. hominis* (Strain C) antigens.

Immunoblots with the three antigens against both homologous and heterologous mice antisera showed high specificity as only homologous antigens reacted intensely. For *B. hominis*, the most reactive antigenic polypeptides were 66.2 kD and 57.5 kD. Several other polypeptides ranging from 21–56 kD were also reactive for some of the sera. Suresh (1994) reported that sera from Wistar rats infected with *B. hominis* (Strain B) cysts reacted strongly with the 64 kD and 57 kD antigenic polypeptides in the immunoblot while sera from 2 patients in Singapore whose stool samples were positive for *B. hominis* also showed prominent bands of 64 kD and 57 kD and weaker bands at 47 kD and 41 kD in immunoblots. Immunoblots obtained in our study by reacting hyperimmune sera produced against *G. lamblia* and *E. histolytica* antigens with *B. hominis* antigens showed very low or no reactivity indicating the specificity of the assay. Similarly both *G. lamblia* and *E. histolytica* did not react significantly with hyperimmune sera produced against *B. hominis*. Immunoblotting has been successfully used to detect antibody responses of human patients to both *G. lamblia* (Char *et al.*, 1991) and *E. histolytica* (Ditrich 1988; Stibbs *et al.*, 1990) and therefore can potentially serve as an alternative diagnostic assay for detection of anti-*B. hominis* antibodies. Further studies are being conducted with sera from human blastocystis patients to determine whether the 66.2 kD and 57.5 kD antigenic polypeptides of *B. hominis* (Strain C) are useful as diagnostic markers of blastocystis infection.

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