ORIGINAL ARTICLE

WESTERN BLOT ANALYSIS OF POLYCLONAL ANTIBODIES FROM EXPERIMENTAL ANIMALS AGAINST *BLASTOCYSTIS* ANTIGENS

Init I, [1]Prummongkol S, [2]Gan CC, Nissapatorn V and Khairul Anuar A
Department of Parasitology, University of Malaya Medical Centre, 50603 Kuala Lumpur, Malaysia.
[1]Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Rd, Bangkok 10400, Thailand.
[2]Division of Parasitology, Institute for Medical Research, Jln Pahang 50588 Kuala Lumpur.

Abstract: **One (1) anti-*Blastocystis* serum from a monkey naturally infected with isolate M12 and four (4) hyperimmune sera raised in inbred Balb/c mice against crude antigens of two *Blastocystis* isolates (C and KP1), one each of *Entamoeba histolytica* (HK9) and *Giardia lamblia* (7404) were used to react with several homologous and heterologous *Blastocystis* isolates, *E. histolytica*, *G. lamblia*, *Endolimax nana* and Bac-4 (*Escherichia coli* isolated from culture medium of a *B. hominis* isolate KP1). All anti-*Blastocystis* sera did not show cross-reactivity with *E. histolytica* and *G. lamblia* by western blotting. Similarly, anti-*E. histolytica* and anti-*G. lamblia* sera also did not react against all *Blastocystis* isolates tested, even though these three protozoa are known to produce diarrhoea in humans. Polyclonal sera raised against antigen prepared from xenic culture of *Blastocystis* produced a smear reaction on the immunoblot, while antibodies raised against antigens prepared from axenic culture (isolate C) gave prominent reaction bands. This may be due to the purity of the immunogen used in inducing the immune response. The cross-reactions of sera from mice immunised with the xenic *B. hominis* isolates may also due to antibodies against *E. coli*. Anti-*Blastocystis* serum from monkey's with natural infection showed several prominent reaction bands together with a smear at above 40 kD were most probably induced by the excretory-secretory antigens of the parasite. A variety of reaction patterns were obtained with these anti-sera and the antigens from different *Blastocystis* isolates. These may be reflects from differences in antigenic components from various strains of this parasite. *(JUMMEC 2002; 2:142-146)***

KEYWORDS: *Blastocystis*, **polyclonal antibodies, immunoblot, experimental animals**

Introduction

B. hominis is often found together with other parasitic protozoa (mainly *Entamoeba histolytica* and *Giardia lamblia*) and produce similar symptoms (1) but single infections are also seen. In healthy individuals, *B. hominis* infection is often seen without symptoms or associated with diarrhoea, which is usually self-limiting. However, many cases of recurrent diarrhoea associated with *B. hominis* resolve only on elimination of the parasite after anti-protozoan treatment with metronidazole (2), ketoconazole (3), iodoquinol, quinacrine and paromomycin (4). *B. hominis* is emerging, as a potential pathogen, and further investigations of this ubiquitous protozoan is warranted (5).

Immulogical studies using ELISA and immunoblot analyses against sera from immunized mice were reported by (6) who showed that there is no cross-reactivity between *B. hominis* and other protozoa. In this study, hyperimune mouse sera raised against *Blastocystis* isolates (immunized mice and infected monkey) were used in Western blotting experiments for identification, characterization and study of the antigenic relationship between several homologous and heterologous *Blastocystis* isolates and other protozoa.

Materials and Methods

Organism isolates

In all, 14 isolates of *Blastocystis*; one isolate of *Escherichia coli* (Bac-4), a common bacteria obtained from the culture medium of *Blastocystis* isolate KP1; and each one isolate of *Entamoeba histolytica* (HK9), *Giardia lamblia* (7404) and *Endolimax nana* (EN1). The *Blastocystis* in-

Correspondence:
Init I
Department of Parasitology, Faculty of Medicine
University of Malaya, 50603 Kuala Lumpur,
Malaysia

cluded 11 isolates (H2, H4, H6, H7, 6105, 10203(1), 27B05(1), KP1, Y51, DJ1, RN) were in xenic culture after isolation from Malaysians; 2 axenic isolates (C, H) supplied by the National University of Singapore; and 1 xenic isolate *Blastocystis* (M12) obtained from soft stool of a monkey kept in the Institute for Medical Research animal house. Isolates KP1, Y51, DJ1, RN were obtained from Malaysian patients with diarrheoa; while isolates H2, H4, H6, H7, 6105, 27B05(1), 10203(1) were isolated from healthy Malaysian Aborigines (Orang Asli).

Preparation of crude antigens from protozoan parasite

The antigens were prepared as previously reported (7). The antigen concentration was estimated by BioRed assay. The concentrations of antigen used in immunization and Western blotting assay were adjusted to 50-100 mg/ml and 150-250 mg/ml respectively.

Immunization and collection of sera from mice

Four to eight weeks old mice with negative protozoa in stool were used in this study. The pre-immunization sera were obtained from the tail. Antigens of *B. hominis* isolates (C and KP1), *E. histolytica* (isolate HK9) and *G. lamblia* (isolate 7404) were used to immunize inbred Balb/C (10 mice were immunized with each isolate). Each mouse was immunized with three doses of antigens subcutaneously at 2-weekly intervals, each dose consisting of 50-100 µg/ml antigens emulsified with Freund's Complete Adjuvant (FCA). Two weeks after the third immunization dose, the mice were anesthetized with diethylether and blood samples were collected by intracardiac puncture. The blood specimens were then transferred to eppendorf tubes and left at room temperature for 30 minutes to one hour, followed by centrifugation at 4000 rpm for 15 minutes at 4°C. Sera from 10 mice were pooled in one tube, aliquoted in new eppendorf tubes and stored at -20°C until use.

Collection of serum from a monkey with blastocystosis

Anti-M12 serum was collected from a monkey naturally infected with *Blastocystis* (isolate M12), while kept in the animal house at the Institute for Medical Research. Blood sample from this monkey was obtained by vane-section and the blood was allowed to clot at room temperature. The serum was separated by centrifugation, aliquoted and kept as above.

Analysis of Polyclonal Antibodies by Western Blotting

The western blotting technique used was as previously described (8) using the above collected antigens and animals sera.

Results

Reactivity patterns of sera from immunized inbred Balb/c mice

Immunized mice sera against *B. hominis*, isolates C (anti-C), showed four reacted patterns against the antigens of all *Blastocystis* isolates tested (Fig. 1). The first pattern is that against isolate C (lane 1); the second pattern against isolate H (lane 2); the third pattern against isolates H2, H4, H6, H7, 6105, INDO (lane 3-8); and the fourth against 27B05(1), KP1, RN, Y51, 10203(1), M12 (lane 9-14). Recognition bands against Bac-4 were at 26.6, 38, 40 kD with a weak smear at high molecular weight (lane 17). Recognition bands appeared in the first pattern were of 18, 36.5, 42.7, 55.6, 66.4 and 68 kD with a striking smear from 36.5 and 66.4 kD. Reaction bands in the second pattern were at 15.5, 23, 26.6, 32, 36, 40, 48, 55.6, 60 and 64 kD with a smear from 36 and 68 kD. Those in third pattern were at 23.5, 26.6 and 38 kD; and for the fourth were at 23.5, 26.6, 38, 48, 64.4 and 68 kD. There was an extra major band of 55.6 kD in isolate M12 distinguishing this isolate from the other *Blastocystis* isolates. No reaction against *E. histolytica*, HK9 (lane 15) and *G. lamblia*, 7404 (lane 16).

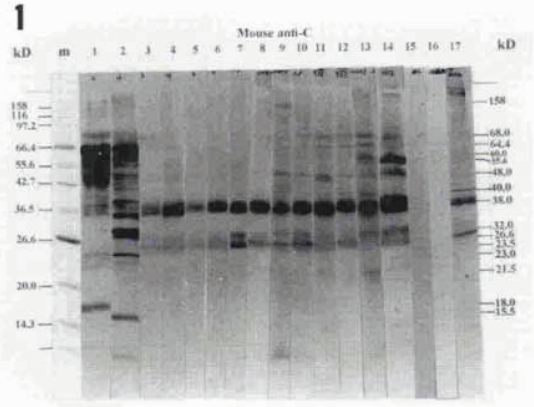

Figure 1. Immunoblot patterns of SDS-PAGE separated polypeptides of *Blastocystis* and other organisms on analysis with mouse sera immunized against isolate C antigens. Standard protein marker (lane m), *Blastocystis* isolates C (lane 1), H (lane 2), H2 (lane 3), H4 (lane 4), H6 (lane 5), H7 (lane 6), 6105 (lane 7), INDO (lane 8), 27B05(1) (lane 9), KP1 (lane 10), RN (lane 11), Y51 (lane 12), 10203(1) (lane 13), M12 (lane 14), *E. histolytica* isolate HK9 (lane 15), *G. lamblia* isolate 7404 (lane 16), *E. coli* isolate Bac-4 (lane 17).

Hyperimmune sera raised against *B. hominis* isolate-KP1 (Fig. 2), showed reactivity against all *Blastocystis* isolates (lane 1-10) tested, *E. nana* (lane 13) and Bac-4 (lane 14) but did not react with *E. histolytica*, HK9 (lane 11) and *G. lamblia*, 7404 (lane 12). The reactivity was seen as a striking/weak smear or with several prominent bands. The antigens from isolates H2, H4, 6105, 7106, 10203(1) were excluded in this study. The reactivity consisted of

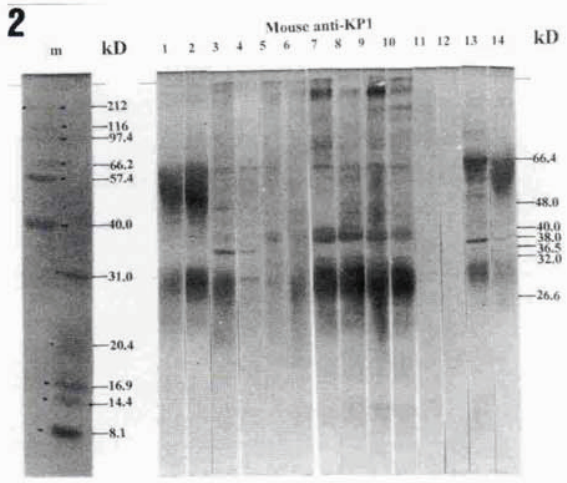

Figure 2. Immunoblot patterns of SDS-PAGE separated polypeptides of *Blastocystis* and other organisms on reaction with polyclonal mouse antisera against isolate KP1 antigens.
Standard protein marker (lane m), *Blastocystis* isolates C (lane 1), H (lane 2), H6 (lane 3), H7 (lane 4), KP1 (lane 5), Y51 (lane 6), DJ1 (lane 7), RN (lane 8), 27B05(1) (lane 9), M12 (lane 10), *E. histolytica* isolate HK9 (lane 11), *G. lamblia* isolate 7404 (lane 12), *E. nana* isolate EN1 (lane 13), *E. coli* isolate Bac-4 (lane 14).

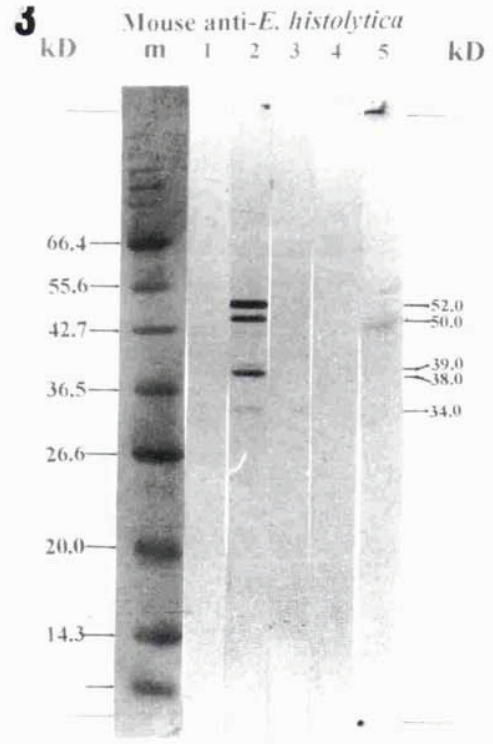

Figure 3. Immunoblot patterns of SDS-PAGE separated polypeptides of *Blastocystis* and other organisms on reaction with polyclonal mouse antisera against *E. histolytica* isolate HK9 antigens. Standard protein marker (lane m), no reaction pattern against *Blastocystis* isolates C (lane 1) (similar result when reacted with H, H2, H4, H6, H7, 6105, INDO, 27B05(1), KP1, RN, Y51, 10203(1) and M12), *E. histolytica* isolate HK9 (lane 2), *G. lamblia* isolate 7404 (lane 3), *E. nana* isolate EN1 (lane 4), *E. coli* isolate Bac-4 (lane 5).

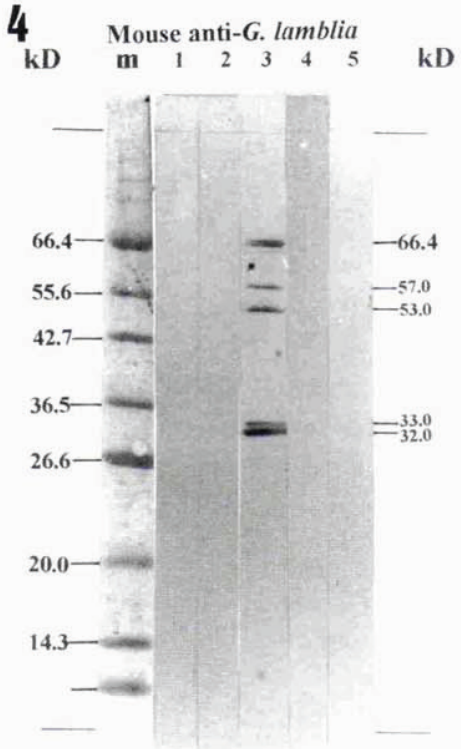

Figure 4. Immunoblot patterns of SDS-PAGE separated polypeptides of *Blastocystis* and other organisms on reaction with polyclonal mouse antisera against *G. lamblia* isolate 7404 antigens. Standard protein marker (lane m), no reaction pattern against *Blastocystis* isolates C (lane 1) (similar result when reacted with H, H2, H4, H6, H7, 6105, INDO, 27B05(1), KP1, RN, Y51, 10203(1) and M12), *E. histolytica* isolate HK9 (lane 2), *G. lamblia* isolate 7404 (lane 3), *E. nana* isolate EN1 (lane 4), *E. coli* isolate Bac-4 (lane 5).

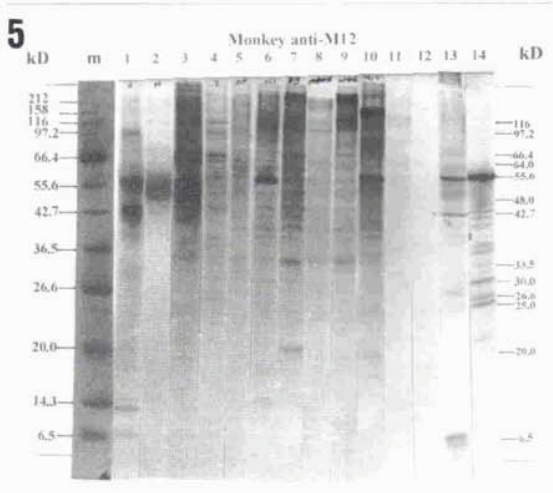

Figure 5. Immunoblot patterns of SDS-PAGE separated polypeptides of *Blastocystis* and other organisms on reaction with serum from a monkey with *Blastocystis* (isolate M12) infection. Standard protein marker (lane m), *Blastocystis* isolates C (lane 1), H (lane 2), H6 (lane 3), H7 (lane 4), KP1 (lane 5), Y51 (lane 6), DJ1 (lane 7), RN (lane 8), 27B05(1) (lane 9), M12 (lane 10), *E. histolytica* isolate HK9 (lane 11), *G. lamblia* isolate 7404 (lane 12), *E. nana* isolate EN1 (lane 13), *E. coli* isolate Bac-4 (lane 14).

a striking smear at 26.6 to 32 kD against *Blastocystis* isolates C, H, H6, KP1, Y51, DJ1, RN, 27B05(1), M12 as well as *E. nana* and Bac-4. Another striking smear was present between 48 to 66.4 kD against isolates C and H (lane 1 and 2) as well as against *E. nana* and Bac-4. Several prominent bands at 36.5 and 48 kD were present against H6 (lane 3); 31 and 36.5 kD against H7 (lane 4); 38 kD against KP1, DJ1, RN, 27B05(1), M12 (lane 5, 7, 8, 9, 10), *E. nana* and Bac-4. Isolates RN, 27B05(1) and M12 (lane 8, 9 and 10) also showed a weak smear between 40 and 212 kD.

Mouse anti-*E. histolytica* sera showed major bands at molecular weight 38, 50, 52 kD and minor bands at 34 and 39 kD against

E. histolytica (HK9) antigens (lane 2). The sera did not show reactivity against all *Blastocystis* isolates (lane 1), *G. lamblia* (lane 3), *E. nana* (lane 4) and Bac-4 (lane 5) antigens tested (Fig. 3).

Mouse anti-*G. lamblia* sera showed reactivity against *G. lamblia* antigen at molecular weight 32, 33, 53, 57 and 66.4 kD (lane 3). No reaction was seen against all *Blastocystis* isolates (lane 1), *E. histolytica* (lane 2), *E. nana* (lane 4), and Bac-4 (lane 5) antigens tested (Fig. 4).

Reactivity patterns of serum from monkey with Blastocystis natural infection

This serum reacted with all *Blastocystis* isolates tested (lane 1-10), *E. nana* (lane 13) and Bac-4 (lane 14), but did not react against *E. histolytica*, HK9 (lane 11) and *G. lamblia*, 7404 (lane 12) antigens. Reactivity against isolates C and H (lane 1 and 2) showed different patterns from the other antigens tested. Anti-M12 produced three major bands at 42.7, 55.6 and 97.2 kD, with a smear between 36.5 and 116 kD and a minor band at around 14.3 kD against isolate C (lane 1); and a smear between 42.7 and 66.4 kD against isolate H (lane 2). Reactivity against local *Blastocystis* isolates (H6, H7, KP1, Y51, DJ1, RN, 27B05(1) and M12) (lane 3-10) was similar. They showed a smear pattern from 33.5 kD and above. The major recognition bands were at 55.6 kD against Y51 (lane 6) and M12 (lane 10); 158 kD against M12; 20 and 35.5 kD against DJ1 (lane 7); 212 kD against DJ1 and 27B05(1) (lane 9); 6.5, 42.7 and 55.6 kD against *E. nana* (lane 13); and 25, 26.6, 30 and 55.6 kD against Bac-4 (lane 14). They also showed many other minor bands (Fig. 5).

Discussion

In our previous SDS-PAGE analysis, several *Blastocystis* isolates, *E. histolytica* (HK9) and *G. lamblia* (7404) showed at least 15 polypeptide bands while *E. nana* (EN1) showed only 4 polypeptide bands ranging from 6.5 to 212 kD. Among these protozoa, several polypeptide bands showed similar molecular weight on SDS-PAGE but gave different polypeptide patterns on immunoblot analysis (7). In this study, immunoblot analyses with polyclonal antibodies (sera) showed that there is no cross-reactivity between the isolates of *Blastocystis*, *E. histolytica* and *G. lamblia* even though these three protozoa are known to produce diarrhoea in humans. The result concurs with the report by (6) and (7).

Cross-reactivity against all *Blastocystis* isolates tested by polyclonal anti-C was showed as clear-cut bands, while polyclonal anti-KP1 gave a smear reaction. This may due to the purity of antigens used in mice immunization; antigen C was prepared from axenic culture while antigen KP1 was from xenic cultures. The differences in polypeptide patterns between the axenic and xenic isolates are probably due to *E. coli* polypeptides (7). Cross-reactivity between the isolates of *Blastocystis* and *E. nana* could be seen in both anti-C and anti-KP1 sera but the reactivity patterns were obviously different. Results also showed that cross-reactivity occurred among isolates of *Blastocystis*; some showed similar cross-reaction patterns, but there were obvious differences in reaction patterns. These patterns are clearly seen with polyclonal anti-C (Fig. 1), which gave four types of patterns; first given with isolate C, second with isolate H, third with isolates H2, H4, H6, H7, 6105; and fourth with isolates 27B05(1), KP1, RN, Y51, 10203(1), M12. These could be due to biochemical differences among different strains of *B. hominis* as previously reported (9, 10).

In this immunoblot study, the reaction pattern type-4 was shown with antigen from *Blastocystis* isolates KP1, RN, Y51 (from patients with diarrhoea), M12 (from a monkey's mushy stool) and isolates 27B05(1), 10203(1) (from aborigines, Orang Asli). In other report, all these *Blastocystis* isolates produced a PCR product at 280 bp, which was suspected to be one of the markers of pathogenic *Blastocystis* (8). Therefore, if this suspected is true then the pathogenic protein produce by the pathogenic *Blastocystis* could be in the range from 48.0 kD and above.

Anti-serum from infected monkey (M12) showed reaction with all the *Blastocystis* isolates tested. This showed that there is immune response against *Blastocystis* in the infected monkey. As with sera from human-*Blastocystis* natural infection (7), this monkey's serum (anti-M12) showed prominent smears reaction together with several clear-cut bands at above 40 kD. We suspect that these reactions at above 40 kD were most probably induced by the excretory-secretory antigens of the parasite. The excretory-secretory antigens are believed to be associated with capsule formation through their continuous accumulation on the surface of the parasite (11).

Acknowledgements

This study was made possible by funds obtained through the Malaysia Government's IRPA grants nos. 06-05-01-0025 and 06-05-01-0108. The authors wish to thank Prof. Mulkit Singh, National University of Singapore, who generously supplied axenic cultures of *Blastocystis hominis*

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