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SHORT COMMUNICATION

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Characterization of protein profiles and cross-reactivity of *Blastocystis* antigens by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis

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Abstract The protein profiles of *Blastocystis hominis*, *B. lapemi*, and *B. ratti* were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and species could be differentiated by this means as well as by Western-blot analysis with polyclonal antibodies. No intraspecies difference could be distinguished between the two *B. hominis* isolates or the three *B. ratti* isolates. Western-blot analysis showed extensive cross-reactivity of *B. lapemi* and *B. hominis* antigens with anti-*B. ratti* serum. Some of the cross-reactive antigens were glycoproteins as determined on the basis of their sensitivity to periodate treatment.

Blastocystis may be found in a wide range of animals, including birds, reptiles, monkeys, and laboratory rats (Boreham and Stenzel 1993; Singh et al. 1996; Chen et al. 1997). Symptomatic patients infected with *B. hominis* may suffer from abdominal pain, discomfort, diarrhea, cramping, nausea, vomiting, and flatus (Zierdt 1991). There have been few studies on the immunological aspects of this organism (Zierdt and Tan 1976; Chen et al. 1987; Kukoschke and Müller 1991; Boreham et al. 1992; Zierdt et al. 1995; Hussain et al. 1997). A lack of antibody response to *B. hominis* in infected patients has been reported (Zierdt and Tan 1976; Chen et al. 1987). Recently, however, Zierdt et al. (1995) were capable of demonstrating a strong antibody response by enzyme-linked immunosorbent assay (ELISA) in patients infected with *B. hominis*. Four axenic strains of *B. hominis* from different sources were separated into two groups on the basis of consistent major differences by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and immunodiffusion (Kukoschke and Müller 1991). Four serologically different groups were identified when 61 strains of *B. hominis* were studied by immunodiffusion (Müller 1994). A possible link between irritable bowel syndrome (IBS) and blastocystosis was investigated by determination of IgG levels with respect to *B. hominis* in patients with IBS and it was shown that only IgG2 levels were significantly increased in these patients, indicating that the predominant response in IBS may be directed to carbohydrate antigens (Hussain et al. 1997). The present work was undertaken to investigate the antigenic profiles of *Blastocystis* by SDS-PAGE and immunoblotting.

Axenic isolates of *Blastocystis* from humans, a sea snake, and laboratory rats were used in this study. *B. hominis* isolates B and G were isolated from patients in Singapore. *B. lapemi* was isolated from the cecum of a sea snake, *Lapemis hardwickii*. Both *B. hominis* and *B. lapemi* were axenized following procedures previously described elsewhere (Teow et al. 1991). Three isolates of *B. ratti* (WR1 and WR2 from Wistar rats and S1 from Sprague-Dawley rats) were axenized following the methods described by Chen et al. (1997). All the isolates were maintained in Iscove's modified Dulbecco's medium (IMDM) containing 10% horse serum in an anaerobic jar (Ho et al. 1993). The isolates were cultured at 37 °C, except for *B. lapemi*, which grew best at room temperature. Subcultures were carried out twice a week.

Parasites in the log phase of growth were washed five times in sterile saline. Ethylenediaminetetraacetic acid (EDTA) and aprotinin (Sigma) were added as protein inhibitors. After ultrasonication (30 s at 30-s intervals, 6 cycles, MSE) the homogenates were centrifuged at 11 000 *g* for 10 min at 4 °C and the supernatants were used as crude extracts. The protein concentrations were

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determined by the Bio-Rad protein assay. The antigens were aliquoted and stored at −20 °C.

The procedure for immunization generally followed that previously described by Harlow and Lane (1988). To raise polyclonal antibodies against WR1 we injected two 4-month-old male rabbits intramuscularly with 0.5 ml of 400 μg of crude antigen emulsified in an equal volume of complete Freund's adjuvant (Gibco). The rabbits were boosted twice every 4 weeks with emulsified antigen (300 μg) in incomplete Freund's adjuvant. Blood was collected from the marginal ear vein before immunization and at 12 days after the last boost. Antisera were stored at −20 °C.

Approximately 15 μg of the various protein preparations were electrophoresed in 12% polyacrylamide gels under reducing conditions (Laemmli 1970). After SDS-PAGE the proteins were electrophoretically transferred at 750 mV for 45 min onto a 0.2-μm nitrocellulose membrane (Schleicher and Schuell, Germany) following the method of Towbin et al. (1979). After being blotted, the membrane was blocked in 1% nonfat skim milk in phosphate-buffered saline (PBS), and then incubated overnight with anti-*B. ratti* serum (1:200 in 0.5% bovine serum albumin in PBS-Tween 20) at 4 °C under gentle shaking. Preimmune serum was used as the negative control. The membrane was then washed three times with PBS-Tween 20 and incubated with a 1:200 dilution of horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit immunoglobulin, Dakopatts, Denmark) for 1 h at room temperature under gentle shaking. IMDM supplemented with 10% horse serum was included to ensure that the antibodies were not raised against the culture medium. The membrane was washed three times with PBS-Tween 20 and developed in a solution of 4-chloro-1-naphthol (Sigma) at 0.6 mg/ml in a mixture of 40 ml of PBS and 10 ml of cold methanol after the addition of 30 μl of H_2O_2. The strips were washed extensively with distilled water to stop the reaction.

The method of Voller et al. (1976) was used to detect the reactivity of the antigens in ELISA. Wells of polyvinyl chloride plates were coated with 100 μl of *B. hominis* isolate B, *B. ratti* isolate WR1, or *B. lapemi* crude antigens (5 μg/well) diluted in carbonate-bicarbonate buffer. After being blocked in 1% skim-milk solution in PBS for 1 h the wells were incubated with a 1:200 dilution of antiserum against *B. hominis* or *B. ratti* for 2 h at 37 °C, washed with PBS-Tween 20, and incubated with peroxidase-conjugated secondary antibodies. *ortho*-phenylenediamine (OPD) was added to the wells as a substrate for the enzyme along with 2.5 *M* H_2SO_4 as the stopping solution. Optical density (OD) values were measured with an ELISA reader (Bio-Tek) at 490 nm.

The method of Schallig and van Leeuwen (1996) was employed for the treatment of antigens with sodium periodate. After being blotted, the membrane was incubated in 10 or 20 m*M* sodium periodate in 50 m*M* sodium acetate (pH 4.5) for 30 min at room temperature in darkness. The membrane was washed extensively in 50 m*M* sodium acetate and incubated in $NaBH_4$ in PBS (pH 7.4) for 30 min. After being washed, the membrane was blocked and processed for antibody binding.

Antigens on ELISA plates were treated with periodic acid (Merck) as described by Saito et al. (1994). Periodate concentrations ranging from 0 to 100 m*M* were added to antigen-coated wells before the addition of serum. After incubation at room temperature for 1 h in darkness, the reaction was stopped with 20 m*M* sodium sulfate. The rest of the procedure was the same as that followed for the ELISA described above.

A large number of bands with molecular weights ranging from 25 to 220 kDa were revealed for the different *Blastocystis* species in Coomassie blue-stained SDS-PAGE gels. The protein profiles of *B. hominis*, *B. ratti*, and *B. lapemi* were different in the three species (Fig. 1). *B. hominis* isolate B and G were identical in their banding patterns as shown in lanes 2 and 3 of Fig. 1. The protein banding patterns of *B. lapemi* were rather different from those of the other species (Fig. 1, lane 4). The three isolates of *B. ratti* shared identical protein banding patterns (Fig. 1, lanes 5–7).

Western-blot analysis revealed that the antiserum raised against *B. ratti* isolate WR1 resulted in multiple bands for all three *B. ratti* isolates as well as the two isolates of *B. hominis* and *B. lapemi* (Fig. 2). Rabbit anti-*B. ratti* serum reacted with a large number of antigens from all three isolates of *B. ratti* (WR1, WR2, and S1). The major antigens were of molecular weights 85, 68, 56, 55, 47, 37, and 28 kDa (Fig. 2, lanes 6–8). There was a strong reaction with >100-kDa antigens of *B. ratti*. Anti-*B. ratti* serum reacted with three major (64, 47, and 43 kDa) antigens in *B. hominis* isolates B and G (Fig. 2, lanes 3, 4). It reacted with three (64, 48, and 30 kDa)

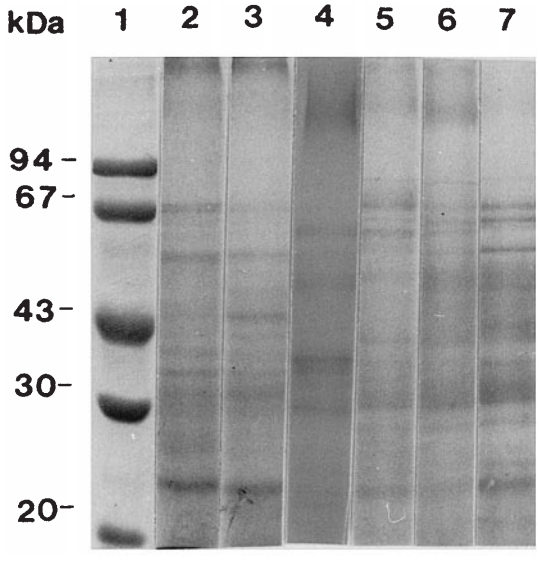

Fig. 1 Coomassie blue-stained SDS-PAGE profile of molecular-weight markers (*lane 1*), *Blastocystis hominis* isolates G (*lane 2*) and B (*lane 3*), and *B. lapemi* (*lane 4*) and *B. ratti* isolates S1 (*lane 5*), WR2 (*lane 6*), and WR1 (*lane 7*)

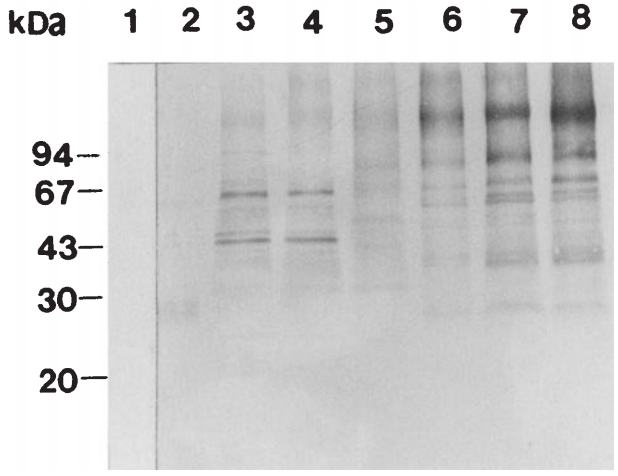

Fig. 2 Western-blot patterns of the reactivity of *B. hominis* isolates G (*lane 3*) and B (*lane 4*) and of *B. lapemi* (*lane 5*) and *B. ratti* isolate S1 (*lane 6*), WR2 (*lane 7*), and WR1 (*lane 8*) antigens with anti-*B. ratti* serum. *Lanes 1 and 2* are the results of the reaction of culture medium with preimmune (*lane 1*) or anti-*B. ratti* serum (*lane 2*)

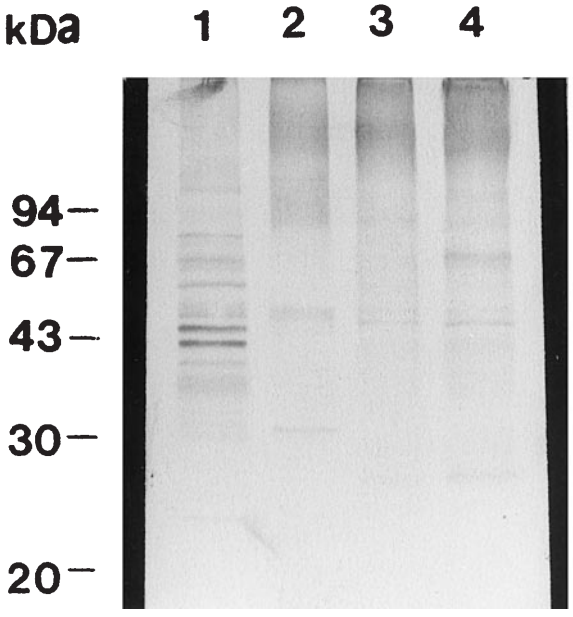

Fig. 3 Western-blot analysis of the reactivity of *B. hominis* isolate B (*lane 1*) and of *B. lapemi* (*lane 2*) and *B. ratti* isolates S1 (*lane 3*) and WR1 (*lane 4*) with anti-*B. ratti* serum after treatment with 20 m*M* periodate

antigens of *B. lapemi* (Fig. 2, lane 5). An intraspecies difference within *B. hominis* or *B. ratti* in the reaction patterns with anti-*B. ratti* serum was not seen.

To characterize the chemical nature of antigenic determinants recognized by anti-*B. ratti* serum we examined the effect of periodate treatment on the reactivity. In immunoblotting, some of the reactive bands remained after treatment with 10 or 20 m*M* sodium periodate (Fig. 3). In *B. ratti*, three bands at 68, 47, and 28 kDa remained visible. Reactions with a smear of >100 kDa antigens were apparent in *B. ratti* isolates WR1 and S1 as well as in *B. lapemi*. Two bands at 48 and 30 kDa remained detectable in *B. lapemi*. For *B. hominis*, most of the bands persisted, but the reaction with antigens of >100 kDa was weaker.

ELISA was performed to study the reactivity before and after periodate treatment. The reactivity of antigens of all the species with anti-*B. ratti* serum decreased after periodate treatment (Fig. 4). The OD readings dropped gradually following the increase in periodate concentration from 0 to 100 m*M*. However, some reactivity remained even after treatment with 100 m*M* periodate.

This study demonstrates interspecies but not intraspecies differences in the protein profiles of *Blastocystis* from humans, rats, and a sea snake. The polyclonal antibody raised against *B. ratti* cross-reacted with heterologous proteins. Western blotting showed that some antigens were conserved in different species. Kukoschke and Müller (1991) previously reported that four axenic strains of *B. hominis* showed consistent differences in their patterns as determined by SDS-PAGE, Western blotting, and Ouchterlony immunodiffusion. However, in our study, no difference in the protein profiles determined by SDS-PAGE and Western blotting using a polyclonal antibody against *B. ratti* was detected among isolates of the same species. Our findings are consistent with those of Tan et al. (1996), who showed that the protein profile obtained by SDS-PAGE analysis was similar for all five *B. hominis* isolates examined. Immunoblotting with five monoclonal antibodies directed

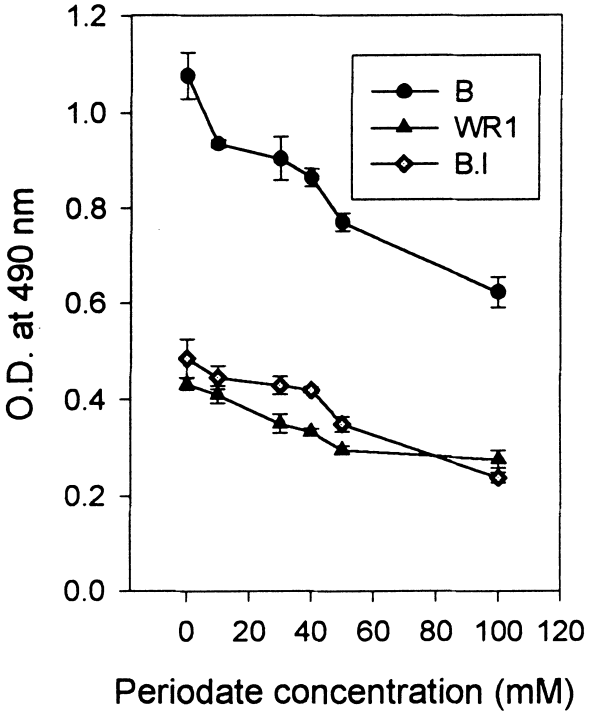

Fig. 4 Graph showing the ELISA results of anti-*B. ratti* serum reactivity with *B. hominis* isolate B and with *B. lapemi* and *B. ratti* isolate WR1 antigens after treatment with varying concentrations of periodate

against one of the isolates resulted in similar bands for all five isolates (Tan et al. 1996). The difference between our results and those of Kukoschke and Müller (1991) may be due to the geographic difference of the isolates. Some minor variations in the position and number of chromosomal bands were revealed by pulsed-field gel electrophoresis (PFGE) for *B. hominis* isolates (Ho et al. 1994). This variation was not reflected by any appreciable antigenic diversity in our study. Similarly, for the rat isolate a slight difference in the chromosomal patterns was seen by PFGE (unpublished data), but this could not be reflected by the banding patterns obtained in SDS-PAGE and Western blotting.

Periodate treatment demonstrated that some of the cross-reactive antigens were glycoproteins. Treatment with periodate decreased the reactivity of antigens with anti-*B. ratti* serum, suggesting that some cross-reactive antigens contain carbohydrate moieties. However, even at a periodate concentration of 100 m*M*, about 50–70% of the reactivity was retained. Increasing the periodate concentration from 50 to 100 m*M* resulted in only slightly lower OD readings. This indicated that the rest of the cross-reactive antigens contained noncarbohydrate moieties. The carbohydrate property of the antigens was further confirmed by pronase treatment, whereby a significant amount of activity remained even after treatment with pronase at 50 μg/ml (data not shown). The presence of large amounts of glycoproteins in *B. hominis* has been shown by fluorescein isothiocyanate-concanavalin A (FITC-Con A) staining (Tan et al. 1996).

The presence of glycoproteins has been described in other protozoans (Mehlhorn 1988). In all, 12 glycoproteins ranging from 12 to 200 kDa were identified by radiolabeling and autoradiography of proteins of *Entamoeba histolytica*, and some of these antigens were recognized by sera from patients with amebic abscess of the liver (Aley et al. 1980). Variant surface glycoproteins (VSG) exist in the metacyclic and bloodstream forms of *Trypanosoma*, which undergo antigenic variation and function as a barrier to protect the plasma membrane from attacks by the complement system and phagocytic cells. The presence of many glycoproteins in *Blastocystis* may function similarly in the protection of the organism in the intestinal environment.

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