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Immunogenicity and protective efficacy of heparan sulphate binding proteins of *Entamoeba histolytica* in a guinea pig model of intestinal amoebiasis

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HIGHLIGHTS

- Heparan Sulphate Binding Proteins (HSBPs) of *E. histolytica* were immunogenic.
- HSBPs elicited both humoral and cellular immune response in guinea pig model.
- Vaccination with HSBPs limits pathology after challenge infection.
- Histopathological studies also supported the protective role.

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ABSTRACT

Entamoeba histolytica infection is associated with considerable morbidity and mortality in the form of intestinal and extraintestinal amoebiasis. No vaccine is yet available for amoebiasis. Heparan Sulphate Binding Proteins (HSBPs) from *E. histolytica* were evaluated for immunogenicity and protective efficacy in a Guinea pig model. Animals were immunized subcutaneously with 30 μg of HSBP by three weekly inoculations. The immunogenicity of HSBP was determined by antibody response (IgG, IgM and IgA), splenocyte proliferation assay and *in vitro* direct amoebicidal assay with splenic lymphocytes and monocytes from vaccinated and control animals. The efficacy of the vaccine was evaluated by challenge infection to vaccinated and control animals by intra-caecal inoculation of *E. histolytica* trophozoites and comparing gross and histopathological findings in caeca of these animals. HSBP was found to induce specific anti-amoebic response as seen by specific antibody production and direct amoebicidal activity of splenocytes. The vaccine also showed partial protection against challenge infection in vaccinated animals as shown by mild/absent lesions and histopathological findings.

1. Introduction

Amoebiasis is one of the most common causes of death from protozoan parasitic diseases, second to malaria (Calderaro et al., 2006). Worldwide 50 million people suffer from amoebiasis, developing disabling colitis or extraintestinal complications leading to 50,000–100,000 deaths every year (Haque and Petri, 2006; WHO, 2007). Ingestion of *Entamoeba histolytica* cysts in faecally contaminated food or water initiates infection and parasite usually resides in the large intestine. Clinical symptoms range from asymptomatic colonization to amoebic dysentery and invasive extra intestinal amoebiasis.

Adherence of the parasite to intestinal epithelial cells is a prerequisite for the pathogenesis of a disease (Ravdin, 1986). A large array of glycoproteins, glycolipids and proteoglycans are present on the surface of eukaryotic cells and several pathogenic organisms use these surface proteoglycans as receptors for attachment, a process that ultimately facilitates tissue colonization and invasion. These proteoglycans include heparan sulphate, dermatan sulphate, chondroitin sulphate, keratin sulphate, heparin etc. A large number of microbial pathogens bind to heparan sulphate on eukaryotic cell surfaces, facilitating the microbial adherence and cellular invasion of the pathogen (Rostand and Esko, 1997). Heparan sulphate occurs as a proteoglycan in which two or three heparan sulphate chains are attached in close proximity to cell surface or extracellular matrix proteins (Gallagher and Lyon, 2000; Lozzo, 1998). Several protozoan parasites such as *Leishmania amazonensis* (Love et al., 1993); *Plasmodium falciparum* (Pancake et al., 1992) and *Trypanosoma cruzi* (Ortega-Barria and Pereira, 1991) have also been reported to bind to

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heparan sulphate on eukaryotic cell surfaces which facilitate the microbial adherence and cellular invasion by the pathogen (Hirmo et al., 1997). However, this has not been demonstrated in *E. histolytica* previously.

We recently identified heparan sulphate binding proteins in *E. histolytica* and non-pathogenic form *Entamoeba dispar*. In *E. histolytica*, two proteins (51.2 and 61.0 KDa) were identified which showed reactivity to heparan sulphate on immunoblotting. The aim of the present study was to evaluate immunogenicity and protective efficacy of heparan sulphate binding proteins of *E. histolytica* in a guinea pig model of intestinal amoebiasis. HSBPs were found to be immunogenic and capable of limiting the pathology of experimental infection in guinea pigs. Therefore HSBPs of *E. histolytica* has the potential as a vaccine candidate against amoebiasis.

2. Materials and methods

2.1. Isolation and purification of E. histolytica Heparan Sulphate Binding Proteins (HSBPs)

The axenic strain of *E. histolytica* (HM1: IMSS) was maintained in TYI-S-33 medium (Diamond et al., 1978) supplemented with 10% heat inactivated horse serum and 100 U/ml of penicillin and 100 mg/ml of streptomycin. Trophozoites were harvested at 48–72 h (mid log phase), by chilling, and pelleted at 150 g for 5 min. Before lysis, the trophozoites were incubated in serum free medium for 48 h and then lysed in 10 ml of buffer containing 150 mM NaCl, 50 mM Tris, 0.5% (v/v) Nonidet P-40 (Sigma, USA) and 20 µl protease inhibitor cocktail (Serine & Cysteine proteases), pH 8.3 (Sigma, USA). The solubilized amoebic trophozoites were microcentrifuged at 10,000 g for 10 min. The supernatant was stored at −20 °C and was used for further protein isolation by ammonium sulphate. Proteins were precipitated by using different concentrations of ammonium sulphate (40%, 60%, 80% & 100%) from the culture lysates of axenically grown *E. histolytica*. The HSBPs were purified by affinity chromatography with Heparin Hi Trap column (Amersham Biosciences, UK). Further, the heparan sulphate binding proteins were identified by SDS–PAGE followed by coomassie blue staining (Fig. 1A). The HSBPs were confirmed by immunoblotting with heparan sulphate (Sigma Aldrich, USA) conjugated with horse radish peroxidase which showed the presence of two proteins with heparan sulphate binding activity at 51.2 kDa and 61.0 kDa in *E. histolytica* in precipitation with 40% and 80% ammonium sulphate (Fig. 1B). As the yield of the protein was maximum with 80% ammonium sulphate, for further studies this concentration was used for protein precipitation.

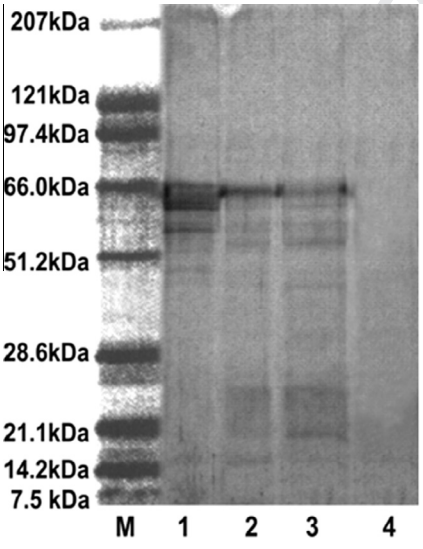

Fig. 1A. SDS PAGE analysis of *E. histolytica* protein preparations purified by affinity chromatography. Lane M: Protein molecular weight marker (Rainbow); Lane 1: 40% $(NH_4)_2SO_4$ precipitate eluted with 0.25 M & 0.5 M NaCl; Lane 2: 60% $(NH_4)_2SO_4$ precipitate eluted with 0.25 M & 0.5 M NaCl; Lane 3: 80% $(NH_4)_2SO_4$ precipitate eluted with 0.25 M & 0.5 M NaCl; Lane 4: 100% $(NH_4)_2SO_4$ precipitate eluted with 0.25 M & 0.5 M NaCl.

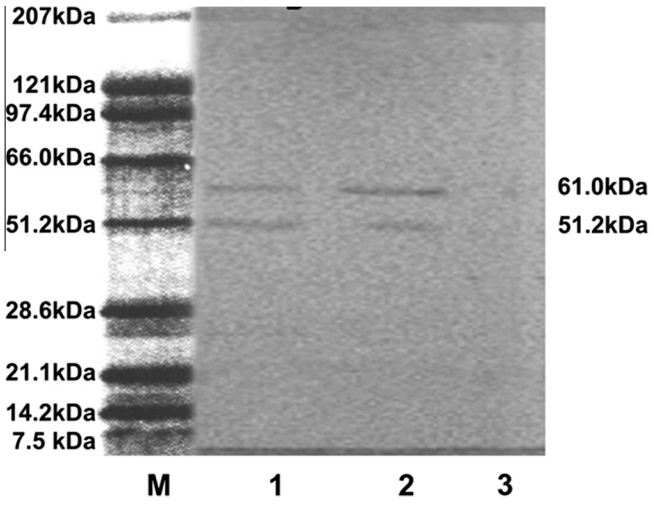

Fig. 1B. Determination of Heparan sulphate binding proteins of *E. histolytica* by immunoblotting with Heparan sulphate- HRP conjugate (Westeren Blot analysis). Lane M: Protein molecular weight marker, Rainbow Lane 1: 40% $(NH_4)_2SO_4$ precipitate; Lane 2: 80% $(NH_4)_2SO_4$ precipitate; Lane 3: 60% $(NH_4)_2SO_4$ precipitate.

2.2. Preparation of antigen (HSBPs) for immunogenicity studies

For immunogenicity and protection studies, large amount of HSBPs were needed. The bulk production of HSBPs was done by mass culturing of parasites. The parasite lysates were precipitated with 80% ammonium sulphate for the isolation of proteins. From these proteins, the HSBPs were purified by affinity chromatography with Heparin Hi Trap column (Amersham Biosciences, UK) and purified proteins were separated by SDS–PAGE on multiple gels. Proteins were eluted electrically from the gels using model 422 Elecro-eluter (Bio Rad apparatus, USA), reconfirmed by immunoblotting and stored at −20 °C for further use.

Following electroelution, salts, SDS and dye were removed by dialysis (Lei et al., 2007).

2.3. Vaccination of animals

A total of 36 healthy, 2–4 weeks old, male guinea pigs (weighing 100–150 g) were used in the study. Animals were fed on standard pellet diet, 30 g/day which gave 100–110 calories/day with supplements of fresh green vegetables and spinach leaves. Twenty-one out of 36 animals were immunized with HSBP (Vaccinated group) and 15 were inoculated with PBS (Non-vaccinated control group). Immunization was done subcutaneously with 30 µg of HSBP of *E. histolytica* by three weekly inoculations, first with Freund's complete adjuvant and subsequent inoculations with Freund's incomplete adjuvant (in equal amounts).

Due to non- availability of adequate number of animals, the control group inoculated with Freund's complete/incomplete adjuvant was not included. However, a prior study using three guinea pigs inoculated with Freund's complete adjuvant/incomplete