Detection of hydatid fluid and protoscolices antigens in sheep with hydatidosis

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Summary

Hydatidosis is a disease caused by infection with the metacestode stage of the dog tapeworm, *Echinococcus granulosus*. This is recognised as one of the world's most important zoonoses, affecting both humans and their domestic animals. In the current study, 20 sheep cystic livers or lungs were collected. Hydatid fluid and protoscolices were isolated and the soluble protein was prepared. Polypeptide profile of hydatid fluid and protoscolices was analysed by SDS-PAGE with 12.5% acrylamide concentration. Humoral immunity and antigenic pattern were evaluated by Western blotting. In electrophoresis of hydatid fluid, five bands with molecular weight of 84, 68–70, 55, 27–28 and 16–17 kDa were observed; the 68–70 and 55 kDa bands were noticeably dominant. In Western blot of hydatid fluid protein, only 68–70 and 55 kDa bands had conciderable positive reaction. A total of 12 bands were also observed in protoscolices polypeptide profile. The molecular weight of the bands were 120, 88–89, 84, 66, 55–56, 49–50, 43–44, 36, 30–31, 24, 20 and 15–16 kDa. Seven bands with molecular weight of 66, 55–56, 49–50, 43–44, 36, 30–31 and 24 kDa had positive reaction in Western blot. We concluded that the determination of specific antigenic bands for sheep hydatid fluid and protoscolices was successfully achieved in this study.

Key words: Hydatidosis, Hydatid fluid, Protoscolex, SDS-PAGE, Western blotting

Introduction

Hydatidosis, caused by infection with larval *Echinococcus granulosus*, has public health importance not only in endemic areas but also in non-endemic regions where migration of infected people and livestock exchanges are happen. Movement of infected livestocks also increases the potential for transmission, and may create new endemic areas (Mamuti *et al*., 2002). Natural intermediate hosts, particularly cattle and sheep, become infected after ingestion of eggs released in the faeces of infected dogs. Humans are accidental hosts of this parasite, usually becoming infected through contact with infected dogs (Thompson, 1995). The definitive hosts of *E. granulosus* are carnivores such as dogs and wolves, which are infected by ingestion of offal containing hydatid cysts with viable protoscolices. After ingestion, the protoscolices evaginate, attach to the canine intestinal mucosa and develop into adult stages (McManus *et al*., 2003). Its larva is a hydatid cyst in sheep, swine, cattle, man, mice, caribou, kangaroo, etc, and is found in a wide range of anatomical sites such as the lungs, liver, heart and brain. The disease is widespread and reported from many countries round the globe (Morris and Richards, 1992). Although several serologic tests have been used for the diagnosis of hydatidosis in animals, it is very difficult to diagnose the newly-formed cyst by radiography or ultrasound.

Early diagnosis of this disease is very important for successful treatment. Currently, indirect haemagglutination (IHA), indirect fluorescence antibody test (IFAT), immunoelectrophoresis (IEP), counter-immunoelectrophoresis (CIEP), double diffusion (DD) and enzyme-linked immunosorbent assay (ELISA) are used for early diagnosis of this disease. Nevertheless, these tests have some disadvantages such as cross-

reactions with other *Taenia spp*. and *Hymenolepis nana*, leading to false positive results, hence, low specificity (March *et al*., 1991; Heath and Lawrence, 1996). Recently, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting have created a new area in immunodiagnosis, which greatly reduces cross-reactions (Burgu *et al*., 2000). These techniques have been used in the field of parasitology. In this study, hydatid fluid and protoscolex antigens were identified by SDS-PAGE and Western blotting in sheep with hydatid cyst.

Materials and Methods

Preparation of hydatid fluid and protoscolex antigens

After preparation of cystic livers and lungs taken from infected sheep, hydatid fluid (HF) aspirated from cysts was centrifuged at 5000 g for 30 min (4°C) to remove protoscolices and stored at -20°C until used. Protoscolices were washed three times with Hank's solution. Sample was freeze-thawed three times and mixed with four volumes of PBS (pH = 7.4), containing 0.1 mg/ml sodium azide. The sample was then sonicated in a 170 W ultrasonic disintegrator (Hielscher, Germany), 2×15 sec on ice until no intact protoscolices were visible. The preparation was then left on ice for one hr, centrifuged for 30 min at 10,000 g and then filtered (0.22 µm) (Ahmad *et al*., 2001). Protein concentration of HF and protoscolex samples was determined by Bradford method (Bradford, 1976). Blood were taken from infected sheep. Sera were separated by centrifugation, pooled and kept at -20°C.

Dialysis and concentration of antigens

If the concentration of antigens was low, the samples were dialysed. The dialysis membrane (millipore) was boiled for 5 min to remove a variety of chemicals introduced during manufacturing. The samples were concentrated by dialysis tube (cut-off 12000) with polyethylenglycol (20000 Da).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

For separation of proteins, discontinuous SDS-PAGE with 12.5% concentration under denaturated conditions was carried out as described by Laemmli (1970) using a mini gel. The solution was mixed and then degassed by attaching the flask to a vacuum for 15 min and finally poured into the mould as a separating gel (Biogene). The gel was allowed to set and then the stacking gel was poured on top of the running gel with the comb in place. The gel was run in a Biogene electrophoresis apparatus for four hrs in a gel running buffer. The gel was stained overnight in Coomassie blue stain followed by soaking in destaining solution with multiple changes for four hrs on gentle rocking platform. The gel was stored at 4°C in a solution containing 30% v/v methanol and 10% v/v glycerol. Wide molecular weight (Sigma) was used for determination of molecular weights.

Western blotting

To identify antigenic subunits within parasite, prepared samples run in SDS-PAGE were probed with sera obtained from infected sheep by immunoblotting as described by Towbin *et al*., (1992). Transfer took place at 220–250 mA using a mini blotter (Hoeffer, USA). The nitrocellulose membrane was stained in Ponceau S (0.2% in 3% trichloroacetic acid), (Sigma, UK) for 20–30 sec and the stain was removed in double distilled water (DDW). Non-specific binding sites on nitrocellulose membrane or strips were blocked with PBS containing 2% BSA for one hr at room temperature on a rocker platform. After washing, the nitrocellulose membrane was incubated in suitable dilution (1/50) of sera taken from infected sheep for 90 min at room temperature on a rocker platform. Washing was repeated. The nitrocellulose was then incubated with the alkaline phosphatase conjugated rabbit anti-sheep IgG (Biodesign) with concentration of 1/5000 in 1% BSA for 90 min at room temperature on a rocker platform. After washing, the nitrocellulose membrane was developed at room temperature with agitation until the bands were suitably appeared, using 4-chloro-1-naphtol substrate as prefering the manufacturer instructions (Biogene). The nitrocellulose membranes were washed

(DW) and dried.

Results

In electrophoresis of HF, five bands were observed, molecular weight of these bands were 84, 68–70, 55, 27–28 and 16–17 kDa; the 68–70 and 55 kDa bands were noticeably dominant (Fig. 1). In Western blotting of HF, only 68–70 and 55 kDa bands had positive reaction. A total of 12 bands were observed in protoscolices polypeptide profile. The molecular weight of these bands were 120, 88–89, 84, 66, 55–56, 49–50, 43–44, 36, 30–31, 24, 20 and 15–16 kDa (Fig. 2). Seven bands with molecular weight of 66, 55–56, 49–50, 43–44, 36, 30–31 and 24 kDa were detected in Western blotting of protoscolices (Fig. 3).

Discussion

Accurate serological diagnosis of hydatidosis infection in livestocks is difficult due to serological cross-reactions with several other species of taeniid cestodes including *Taenia hydatigena* and *Taenia ovis*. Furthermore, natural intermediate host animals produce very poor antibody responses to the infection as compared with the relatively high levels of specific antibody observed in human infection (Lightowlers *et al*., 1999). In recent years, SDS-PAGE and Western blotting have been widely used for the diagnosis of parasitic diseases. In our study, a total of 12 bands

Fig. 1: Polypeptide profile of hydatid fluid analysed by SDS-PAGE. Five bands with molecular weight of 84, 68–70, 55, 27–28 and 16–17 kDa were observed. MW and HF indicate molecular weight marker and hydatid fluid, respectively

Fig. 2: Polypeptide profile of protoscolices analysed by SDS-PAGE. A total of 12 bands were also observed in protoscolices polypeptide profile. The molecular weight of the bands were 120, 88–89, 84, 66, 55–56, 49–50, 43–44, 36, 30–31, 24, 20 and 15–16 kDa. MW and Pr indicate molecular weight marker and protoscolex, respectively

were observed in protoscolices protein profiles. Heath and Lawrence (1996) obtained bands with molecular weight of 23 and 25 kDa from egg onchospheres of *E. granulosus* by SDS-PAGE, and then tested these proteins on sheep. Specific antibodies which cause lysing of oncospheres were developed in the sera of these sheep. Maddison *et al*., (1989) reported that specific 8 kDa is the most specific band in the diagnosis of hydatidosis in humans. In addition, some researchers have pointed out that antibody responses to bands with molecular weight of 12, 16, 20, 37, 38 and 48 kDa in the sera of patients with hydatidosis are also specific (Chamekh *et al*., 1990; Leggatt and McManus, 1994; Profumo *et al*., 1994). Twenty bands in the range of 8–120 kDa have been detected in HF of human, sheep and cattle by SDS-PAGE (Burgu *et al*., 2000). Kanwar and Kanwar (1994) identified that 15 protein fractions with molecular weights of 8–116 kDa were detected in hydatid cyst fluid taken from sheep, goats, pigs and humans, and antibody responses were developed against 12 protein fractions of sheep HF in humans infected with hydatidosis. Researchers (Chamekh *et al*., 1990; Sanchez *et al*., 1991) reported that immune responses to antigenic bands of 14, 16, 20, 37, 38 and 48 kDa were specific to patients with hydatid disease. Woollard *et al*., (1998) identified a total of five fractions with molecular weight of 6, 12, 13, 21–22 and 24 kDa in HF antigen. Gonzalez *et al*., (2000) reported a novel 29 kDa antigen from *E. granulosus* by characterization of P-29. Using a monoclonal antibody prepared against protoscolices components, they revealed the localization of P-29 to the tegument and rostellum of protoscolices and to the germinal layer of the cyst; it was however, absent in HF. The presence of a 27 kDa band in sheep laminated layer and protoscolices extracts in SDS-PAGE and immunoblotting experiments, and its absence in sheep serum supports the suggestion that this protein is probably derived from the parasite (Taherkhani and Rogan, 2000). Thompson (1991) reported differences between protoscolices from different cysts even within the same individual host. On the other hand, the fertility of the cysts may influence the number of protein bands. Although, Lightowlers and Gottstein (1995) suggested that antigenicity is not exclusively associated with cyst fertility, fertile cysts contain higher concentrations of antigens than sterile cysts. Immunoblotting was used by Ortona *et al*., (2000) as the basis of serodiagnostic method due to its simplicity and sensitivity. Furthermore, immuno-blotting directly shows the reactivity of target protein with a positive serum sample. Antigen B, banding at approximately 8 kDa in SDS-PAGE, was used for comparative purposes as it has been shown to be a useful marker for the diagnosis of hydatidosis (Li *et al*., 2004). Fu *et al*., (1996) also described that a 66 kDa band was present in the germinal layer of metacestode and protoscolices. In this study, antigenic profiles of HF of cattle and sheep were studied by Western blot. Antigenic determinants (8, 20, 45, 57 and 68 kDa)

Fig. 3: Protoscolices Western blot. Molecular sizes (in kDa) are indicated on the right

were recognized by antibodies. Even those who studied with the same antigens, found that it is possible to get different results in the fractionating of proteins by SDS-PAGE. These differences may be due to preparing antigenic solutions, chemical reagents of different quality and quantity or application procedures used (Burgu *et al*., 2000). Moreover, the nature and quality of the antigens are variable among the host species. This may be one of the reasons why different laboratories obtain different results for the detection of anti HF antibodies with antigens prepared from different host species (Poretti *et al*., 1999).

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