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Analysis of the *Echinococcus granulosus* Laminated Layer Carbohydrates by Lectin Blotting

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ABSTRACT

Cystic hydatid disease is caused by cystic larval (metacestode) stages of *Echinococcus granulosus*. This parasite can potentially occur all over the world especially in Mediterranean and Middle East countries and some parts of Africa, Latin America and China which have major foci of human infections. The cyst wall of metacestodes consists of inner, middle and external layers. To date, little attention has been paid to the immunological studies of the laminated (middle) layer. Because of the presence of diagnostic carbohydrate antigens, this layer is important. In this report, the characterisation of the laminated layer carbohydrate of the *E. granulosus* was investigated by peroxidase-labelled lectin conjugate blots. The comparison of SDS-PAGE and Western blot analysis confirms that many laminated layer bands are glycoproteins, especially 50-66 kDa and 25-29 kDa bands. These glycoproteins contain ~-Methyl-D-mannoside, N-Acetyl- -D-glucosamine, ~-L-Fucose and N-Acetyl- -D-galactosamine. *Iran. Biomed. J. 5 (1): 47-51, 2001*

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INTRODUCTION

Echinococcus granulosis is causative agent of cystic hydatid disease (CHD) in humans. Natural transmission of the parasite occurs between carnivorous as definitive hosts and herbivorous as intermediate hosts. Humans become accidentally infected through hand and mouth contact with fecally contaminated dog hair. CHD also is an endemic disease in some countries.

The cyst wall of metacestodes consists of inner, middle and external layers. The middle (laminated) layer is unique to the genus Echinococcus in comparison with other larval cestodes. This layer is not present in very young cysts until it is about 14-18 days old when it first appears as a thin, clear layer on its outer margin. This layer is an acellular, polysaccharide protein complex that stains strongly by periodic acid, Schiff's reagent (PAS) and that provides a useful diagnostic marker in histological studies [1, 2]. This layer is originated by the germinal (inner) layer [3-5]. Host material may also contribute to its structure [6, 7]. It has been shown that the laminated layer (LL) of *E. granulosus* contained more galactosamine than glucosamine. However, in protoscoleces (Px) and hydatid cyst fluid (HCF) there are more glucosamine than galactosamine. Acid muco-polysaccharide also demonstrated in this layer [8].

In terms of antigenicity, Gottstein *et al.* [9] isolated a specific fraction containing a lectin-binding carbohydrate antigen (Em2) from crude metacestode of the *E. multilocularis* by immunoaffinity chromatography against anti *E. granulosus*hydatid fluid IgG coupled to CNBr-Sepharose 4B. This antigen has a molecular mass of 54 kDa [10-13]. Migues *et al.* [14], by studying carbohydrates on the surface of *E. Granulosus* protoscoleces (Pxs) in mice showed that carbohydrates were predominantly bound to parasite surface and were highly immunogenic.

Although, the laminated layer of *E. granulosus* has large carbohydrate components, the role of these components in immunogenicity has received little attention. Recently, Taherkhani and Rogan [15], by using SDS-PAGE and Western immunoblotting reported that the most important antigenic molecules of the laminated layer of *E. granulosus* were confined to two regions (50-66 kDa and 25-29 kDa). Since the lectins are carbohydrate binding protein that interacts with specific sugar, we decided to characterise the carbohydrate components in these regions using a series of lectins and to detect whether these molecules are glycoprotein.

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MATERIALS AND METHODS

Parasitic Material. The primary sources of Pxs and LL were from natural infections in the liver and lungs of sheep slaughtered at a local abattoir (Manchester, UK).

The laminated layer extracts. Cyst fluid was removed from the cysts and stored untreated at -20°C. The cyst walls (sheep) were cut and placed in phosphate buffer saline (PBS, pH 7.4). The germinal layers were carefully scraped from the LLs with forceps. The separated LL extracts were washed several times in PBS and microscopically examined to confirm the absence of traces of the germinal layers or the Pxs. The tissues were incubated in 1M NaCl (1 litre) at 4°C for 30 min and washed with PBS. The LLs were first homogenised with an equal volume of PBS. The preparations were then disrupted by sonication in a 150-W ultrasonic disintegrator, (10 s on and 5 s off), on ice for 15 min. Some of the sonicated materials (milky suspensions) were kept at -20°C. The remainder of the suspensions were centrifuged at 10,000 rpm at 4°C for 10 minutes. The suspensions were used for SDS-PAGE and Western blotting techniques. The samples were stored in 200 µl aliquot at -20°C.

Protoscolex extract antigen. 1 ml of Pxs (approximately 10^5Pxs) of *E. granulosus* derived from cysts in the liver and lungs of the sheep were freeze-thawed 3 times and mixed with four volumes of PBS, pH 7.4, containing aprotinin at 0.1 mg/ml. The mixture was sonicated in a 150-W ultrasonic disintegrator, 10 second on and 5 second off on ice until no intact Pxs were visible microscopically (approximately 15 min). The preparation was then left on ice for 1 hour and centrifuged at 10,000 g for 30 minutes. The sonicated Pxs were employed without centrifugation. The samples were stored in 200 µl aliquot at - 20 °C.

Basic technique. Lectin binding assays of the crude sheep laminated layer (SLL) and Pxs antigens were done according to Shimizu *et al.* [16] with modifications as follows: The crude SLL and Pxs antigens were electrophoresed on 12% polyacrylamide gel [17] and transferred onto nitrocellulose papers [18]. After washing with PBS, the nitrocellulose strips were treated with 0.15 M NaCl, 0.01 M Tris-HCl, 0.5 mM $CaCl_2$ (pH 7.4) containing 1% bovine serum albumin (BSA) at RT for 1 h. Then the nitrocellulose strips were washed with PBS for a few min. Different peroxidase-labelled lectins were dissolved in the above buffer at a concentration of 50 µg/ml. These were used as follows: Con A, L-6397. DBA, L-4258.WGA, L-3892.SBA, L-2650. PNA, L-7759.APA, L-5759 and ATA, L-3139 (Sigma, UK). The lectins and their sugar specifications are shown in Table 1. Each strips was incubated with 2 ml of these mixtures at RT for 1 h. All of the lectin-reacted nitrocellulose strips were washed three times with 20 ml of 0.01 M Tris-HCl, 0.15 M NaCl (pH 7.4) containing 0.1% BSA and once with the same buffer without BSA. The presence of the lectin conjugates was visualised by the incubation of the NCP strips in 10 ml of 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2 in 0.1 M Tris-HCl (pH 7.4) at RT for 1 - 2 min.

RESULTS AND DISCUSSION

In order to analyse and visualise the carbohydrate component of glycoprotein bands in the 50-66 kDa and 25-29 kDa regions in the LL, the crude SLL and Pxs antigens were probed with various peroxidase-labelled lectin conjugates as shown in Table 1. The results are summarised in Figure 1 and Table 2.

No attempt has been made to study *E. granulosus* carbohydrates on blots using peroxidase-labelled lectin conjugates. In this study, analysis of the laminated layer carbohydrates by lectin blotting probes showed that the carbohydrates include; α-Methyl-D-mannoside, N-Acetyl-β-D-glucosamine ,α-L-Fucose and N-Acetyl-β-D-galactosamine in the 66-60 kDa, 55-50 kDa and 25-31 kDa regions. The bands of particular interest were at 25, 27 and 31 kDa, stained heavily for the presence of α-Methyl-D-mannoside and N-Acetyl-β-D-galactosamine being absent from the 25-kDa band. The presence of the glycoproteins in LL were in agreement with previous reports using fluorescent lectin analysis [1, 6]. All the laminated layer polypeptide bands identified by SDS-PAGE did not have reaction with any particular lectin. This suggests that the profile was made up both glycoproteins and proteins without carbohydrate chains.

In relation to Px antigen, Miguez *et al.* [14] reported the presence of carbohydrate antigens with a molecular weight higher than 45 kDa, by an antisera raised against a carbohydrate enriched soluble fraction. This result is similar to the result of current study where carbohydrates were present on bands in the 50-55 kDa (N-acetyl-β-D-glucosamine and/or α-Methyl-D-mannoside) and in the 60-66 kDa (N-

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Table 1. Different peroxidase-labelled lectin conjugates and sugar specificities which used in this study

Lectin	Carbohydrate specificities*	
	Major	**Minor**
Concanavalin A (Con A) *(Canavalia ensiformis)*	α-Methyl-D-mannoside	α-D-glucose, N-Acetyl-α-D-glucosamine
Horse gram agglutinin (DBA) *(Dolichos biflorus)*	N-Acetyl-α-D-galactosamine	α-D-galactose
Wheat germ agglutinin (WGA) *(Triticum vulgaris)*	N-Acetyl-β-D-glucosamine	None
Soybean agglutinin (SBA) *(Glycine max)*	N-Acetyl-α-D-galactosamine N-Acetyl-β-D-galactosamine	α-D-galactose
Peanut agglutinin (PNA) *(Arachis hypogaea)*	β-D-galactose-(1-3)-N-acetyl-D-galactosamine linkage	None
Asparagus pea agglutinin (APA)	α-L-Fucose	α-Methyl-D-mannoside, N-acetyl-α-D-glucosamine, α-D-glucose
Winged bean agglutinin (PTA) *(Psophocarpustetragonolobus)*	N-Acetyl-α-D-galactosamine	D-galactose

* [16]

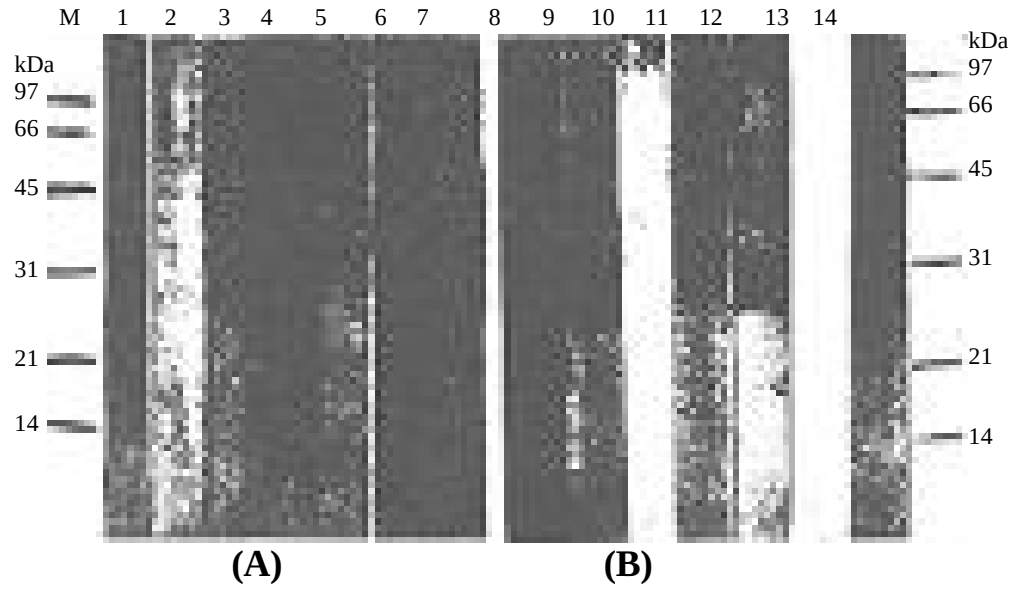

Fig. 1. Analysis of SLL and Pxs carbohydrate components using various peroxidase-labelled lectin conjugates by immunoblotting: Glycoprotein patterns of the Pxs (A) and LL (B) antigens as detected by lectin; 1 and 14, Con A-peroxidase. 2 and 13, DBA-peroxidase.3 and 12, WGA-peroxidase.4 and 11, SBA-peroxidase.5 and 10, PNA-peroxidase.6 and 9, APA-peroxidase.7 and 8, PTA-peroxidase.M, Protein marker.

Acetyl-β-D-galactosamine and/orβ-D-galactose-(1-3)-N-Acetyl-galactosamine linkage). No lectin binding was observed in the 25-29 kDa region in the protoscolex preparation, although, multiple bands from 31 kDaupward were recognised.

Regarding antigenicity, Gottstein *et al.* [9] isolated 54 kDa Em2 as a lectin-binding carbohydrate antigen in the laminated layer of the *E. multilocularis.* On the other hand, Sato *et al.* [19] reported that a monoclonal

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Table 2. Partial carbohydrate bands revealed in the SLL and Pxs using different peroxidase-labelled lectin conjugates and sugar specificity.

kDa	Antigens		Peroxidase conjugated labelled	Sugar specificity
	SLL	Px		
66	+	+	Con A and APA	N-Acetyl-α-D-glucosamine and\or α-D-Glucose (F).
60-66	+	+	SBA	N-Acetyl-β-D-galactosamine (M),
	-	+	PNA	β-D-Galactose-(1-3)-N-Acetyl-Galactosamine linkage (M),
	+	+	APA	N-Acetyl-α-D-glucosamine (F),
	-	+	PTA	D-Galactose (F)
	-	+	WGA	N-Acetyl-β-D-glucosamine (F).
50-55	+	+	Con A	-α-Methyl-D-mannoside (M),
	+	+	WGA	N-Acetyl-β-D-glucosamine (M),
	+	-	APA	α-D-Glucose and\or N-Acetyl-α-D-glucosamine (F).
27-31	+	-	Con A	α-Methyl-D-mannoside (M),
	+	-	WGA	N-Acetyl-β-D-glucosamine (M),
	+	-	SBA	N-Acetyl-β-D-galactosamine (M),
	+	-	APA	α-D-Glucose and\or N-Acetyl-α-D-glucosamine (F).
25-26	+	-	WGA	N-Acetyl-β-D-glucosamine (M),
	+	-	APA	α-L-Fucose.

+, presentation of band; -, no band; M, presentation of major band; F, presentation of minor band

antibody reacts with one of the carbohydrate antigens (C-antigen) located at 30-35 kDa in LL of the *E. multilocularis*. Both reports showed the most effective diagnostic carbohydrate antigens for human alveolar hydatid disease. Previously, we showed that [15], immunoblotting with confirmed human cystic hydatid sera and polyclonal antisera to LL of the *E. Granulosus* identified indicated that the major bands at 50-66 kDa and 25-29 kDa that probably were the most important glycoprotein antigens for serodiagnostic of human cystic hydatid patients.

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