



## Research Article

# Hydatid Cyst Germinal Layer Purified Glycoproteins for Diagnosis of Camel Cystic Echinococcosis

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### ABSTRACT

Using Con-A affinity Colum chromatography, N- acetylglucosamine, glucose and galactose fractions were isolated from *Echinococcus granulosus* germinal layer. Glc NAc isolated fraction proved diagnostic potentials of cystic echinococcosis in camels by ELISA. The electrophoretic profile of Glc NAc fraction showed two bands of 30 and 77 kDa. Galactose isolated fraction showed also, two bands but of molecular weight 17and 77 kDa. While glucose fraction contained three bands of 30, 43, and 77 kDa. Using western blotting technique the 30, 77 kDa of Glc NAc reacted with camel naturally infected serum samples and hyper immune sera of rabbit. Subsequently, serodiagnostic performance of Glc NAc was evaluated using enzyme-linked immunosorbent assays (ELISAs) by using sera from 98 camel serum 22 were healthy and 76 were with hydatidosis. The sensitivity of Glc NAc was found to be 97.3 % with relatively low specificity 54.5% based on the ability of isolated fraction to differentiate between true positive and true negative. In conclusion, the isolated Glc NAc fraction showed diagnostic potency in serodiagnosis of camel cystic echinococcosis.

**Key words:** Cystic echinococcosis, Glycoprotein, Germinal layer, Diagnosis, Affinity column chromatography, ELISA

### INTRODUCTION

Cystic Echinococcosis (CE) is socioeconomic zoonotic infection caused by *Echinococcus granulosus* taenid cestodes parasite affecting camels in Egypt and other Middle East countries (WHO/OIE 2002). CE causes weight losses, decrease in milk production and fertility. CE is listed of the eighteen Neglected Tropical Diseases according to WHO liste (WHO 2017 a). The global financial load of CE in evaluation of purchasing power parity is 4.1 billion dollars annually, about 46% is related to human treatment and morbidity and 54% is due to animal-health costs (WHO, 2016 and 2017 b). WHO is working on control strategies of cystic echinococcosis by 2020. One of the most productive animals in Egypt is camel where these are producing milk and meat as they up an important part of the dietary proteins especially for the lower income groups. In Egypt there is increase in number of import camels (95,080) than other livestock (FAO statistic database, 2016 <http://www.faostat.fao.org>). Also, camel CE is highly prevalent in Egypt (Kandil et al., 2008). There has, however, to date been little or no assessment of immunodiagnosis of CE in dromedary camels.

WHO Informal Working Group on Echinococcosis (WHO-IWGE) approved the use of ultrasound in diagnosis of CE and classification of the cyst (WHO Informal Working Group. 2003), but this diagnosis should be completed by serology. CE serological diagnosis is usually based on detection of antibodies against hydatid fluid antigen but it shows several drawbacks, including false negative and positive results and detectable antibody level was long persistence in cured patients. Search about the alternative diagnostic candidate to improve test performance and standardization is essential issue. Glycoproteins isolated fractions were used to develop a sensitive diagnosis and quantitation of *E. granulosus* (Kamel et al., 2006) and of *E. multilocularis* (Kouguchi et al., 2011) infections in human and animal hosts. The role of glycans in diagnosis has also been shown in other helminths such as *toxocara vitilorum* (El Shanawany et al., 2019) and *Fasciola gigantica* (Abdel-Rahman et al., 2016).

The hydatid cyst wall consists of an outer laminated layer and an inner germinal layer from which protoscoleces germinate into the cyst lumen (Korc et al., 1967). Furthermore, March et al. (1991) illustrated that one of the antigens common to the germinal membrane,

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protoscoleces and fluid possesses peptide N-glycosidase F (PNGase F) sensitive complex N-glycans. Glycosylated moieties are considered to be immunodominant in natural and experimental hydatid infections (Carmena *et al.*, 2006 and Di'az *et al.*, 2015). The carbohydrate moiety of the *E. multilocularis* was found to be composed of galactose, N-acetylgalactosamine, and N-acetylglucosamine with a ratio of 2.4:1.0:0.5 as determined by gas-chromatography/mass spectrometry (Husmeier *et al.*, 2002). Moreover, in larval, adult worm antigens and adult excretory-secretory extract the Tn antigen (GalNAc-O-Ser/Thr) was expressed also, it has been detected with high level in serum of hydatidosis patients (Alvarez-Errico *et al.*, 2001).

This study was undertaken to use Con-A affinity chromatography for purification of different glycoprotein fractions from *E. granulosus* cyst germinal layer and immune-characterization of them. Also, comparative assess of the diagnostic potency of crude germinal layer and different isolated Con A glycoprotein fraction in diagnosis of camel hydatidosis in Egypt.

## MATERIALS AND METHODS

### Ethical approval

All animal experiments were done according to National Research Centre institutional guide lines after permission of the Animal Research Committee under number (18/199). All experiments were performed in specially designed safety facility in National Research Centre, Veterinary division, parasitology and animal diseases department.

### Parasite

Large lung and viscera *E. granulosus* cysts were obtained from naturally infected camels collected from Cairo, Egypt abattoir. Cyst fluid was removed by aspiration with a syringe. Germinal layers were detached from hydatid cyst used as the whole entity washed extensively with PBs pH (7.2) and kept freeze until use.

### Antigen preparation

The crude hydatid germinal layer antigen was prepared by homogenizing germinal layer in 0.1 M PBs buffer pH 7.2 with glass homogenizer at 4°C. Homogenate was then centrifuged at 14000 rpm for 30 m. at 4°C. The protein content of the supernatant was collected and assayed by the method of Lowry *et al.* (1951) and was stored at -20°C until use.

### Serum samples

A total of 98 camel serum samples; 22 healthy and 76 diseased with CE were collected from slaughtered camels at El Basatine abattoir, Cairo, Egypt. Its negativity and positivity were confirmed by the presence or absence of visible cysts in their visceral organs. Serum samples were labeled in serial numbers and stored at -20°C until use. A rabbit hyperimmune serum to crude germinal layer antigen of *E. granulosus* was prepared by repeated subcutaneous immunization with 40 µg/ kg of antigen. The initial dose was emulsification in Freund's complete adjuvant and the second dose was emulsified in incomplete Freund's adjuvant. At 21 and 28 days respectively third and fourth booster doses were given without adjuvant according to Fagbemi *et al.* (1995).

After 4 days post last injection blood samples were collected from the animal's ear vein and sera were stored at -20°C until use.

### Purification of *E. granulosus* germinal layer glycoproteins

Purification using lectin affinity chromatography was performed according to (Abdel-Rahman *et al.*, 2016). Briefly, the dissolved crude germinal cell wall antigen extract was mixed with 2 ml of poured *Concanavalin ensiformis* agarose (Con A) agarose purchased from Sigma Chem. Co. St. Louis, USA, incubated overnight at 4 C ° on a rotator. The bound glycoproteins were eluted using 50mM Tris-HCL pH (7.5), 300 mM Nacl and 300mM galactose to isolate Gal fraction. The column was also eluted with 50 mM Tris-HCL pH (7.5), 300 mM Nacl and 300 mM N-acetyl glucose eluting buffer the yield glycoprotein fraction was designed as N- acetyl glucosamine fraction (Gluc NAc). Gluc glycoprotein fraction was eluted using 50mM Tris-HCL pH (7.5), 300 mM Nacl containing 300mM α-D glucose. The fractions were checked for protein content by the method of Lowery *et al.* (1951).

### SDS-PAGE

Equal amounts (50 µg) of crude hydatid cyst germinal layers antigen and isolated glycoprotein fractions were loaded separately on 10% SDS polyacrylamide gel electrophoresis under reducing conditions (Laemmli 1970). The gel was then silver stained (Wray *et al.*, 1981). Gels photos were analyzed by Lab Image software (BioRad).

### Western blotting

SDS-PAGE-separated proteins were electro blotted onto a nitrocellulose membrane (Sigma, USA) then incubated in blocking buffer (125 mM Tris, containing 5% skim milk and 0.1% Tween 20) for 1 h at room temperature. The membrane was incubated with infected camel serum with hydatidosis (1:200 dilutions in 0.01 M Tris buffer saline, pH 7.5, containing 0.5% bovine serum albumin) overnight at 4°C. The membrane was washed several times to remove excess antibodies, and then it was incubated with Protein A IgG conjugate 1:2000 dilution (Sigma, USA). Immunoreactive bands were visualized with 4-chloro-1-naphthol as a substrate (Towbin *et al.*, 1979).

### ELISA

The assay was used to compare the diagnostic potentials of the crude and fractions with specific hyperimmune sera prepared in rabbits. The test was also, used to assess the potency of the fractions in the diagnosis of natural camel hydatidosis. All samples were analyzed in triplicate. The test was carried out as described by Oldham (1983).

Briefly, the plates were coated separately with crude *E. granulosus* cyst germinal layer antigen and isolated glycoprotein fractions at a concentration of 10 µg/ml in carbonate buffer (100 µl /well) and incubated overnight at 4°C. After washing, the unbinding sites were blocked with 0.1M bovine serum albumin (BSA) in carbonate buffer and incubated for one hour at room temperature. The plate was washed repeatedly and serum samples in dilution (1: 100) were added (100 µl/well) and incubated for 90

minutes at 37°C. After washing, the conjugate was added (100 µl/well of anti-rabbit IgG horse radish peroxidase or protein A horse radish peroxidase which were purchased from Sigma Chem. Co. St. Louis, USA to each well. The plate was incubated for one hour at 37 °C. Then the substrate Ortho Phenylene Diamine (OPD) was added and the plate was read spectrophotometrically at 450 nm. The cut off values was calculated according to Almazan *et al.* (2001).

**Data analysis**

The following definitions were used to calculate the corresponding diagnostic parameters: true-positive values (tp); sera from camel infected with *E. granulosus* showing positive readings; false-negative values (fn), sera from camel infected with hydatidosis showing negative readings; false-positive values (fp), sera from non-infected camels showing positive result; true-negative values (tn), sera from non-infected camels showing negative readings. Specificity=  $tn / (tn + fp)$  and sensitivity=  $tp / (tp + fn)$  (Parikh *et al.*, 2008).

**RESULTS**

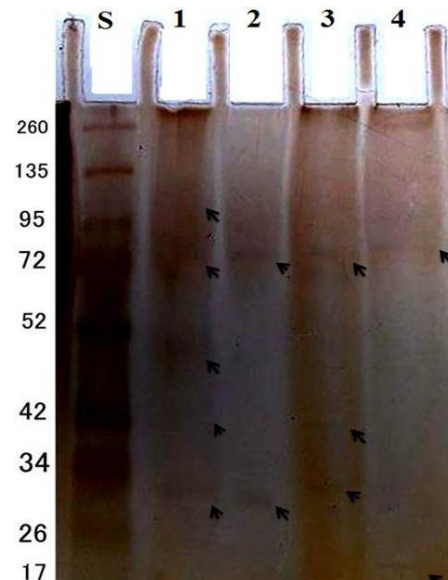
**Electrophoretic profile of isolated glycoproteins**

Gal revealed two bands at 17, 77, kDa (Fig. 1, lane 4). Also, Glc NAc fraction showed two bands but with molecular weights 30, and 77 kDa (Fig. 1 lane 2). Gluc fraction showed three bands at 30, 43, and 77kDa (Fig. 1, lane 3). The crude germinal layer antigen revealed 6 bands at 17, 30, 43, 49, 77, and 99 kDa (Fig. 1, lane 1).

**Screening of diagnostic potency of isolated glycoprotein fractions by ELISA**

In order to determine the promising antigen for use in the serodiagnosis of cystic camel echinococcosis, ELISA was used using each isolated glycoprotein fraction and compared with crude germinal layer antigen. These antigens were tested against positive and negative from camels and rabbits hyperimmune sera was prepared against the germinal layer. ELISA results proved that N-acetyl glucosamine fraction has the highest potency in reaction with rabbit hyperimmune serum. Thus, isolated

N-acetyl glycoprotein fraction was used in serodiagnosis of cystic echinococcosis in camel (Fig. 2 a and b).



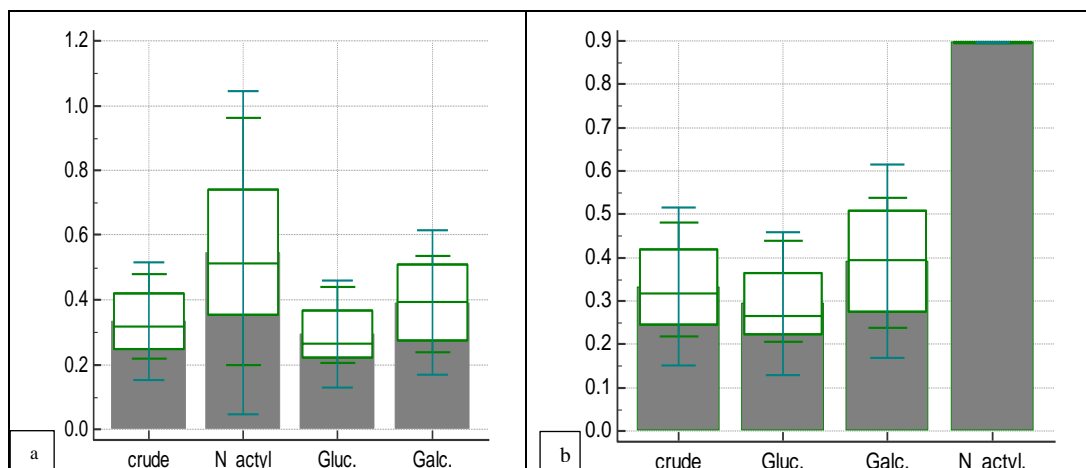
**Fig. 1:** 10% SDS-PAGE of the purified glycoproteins fractions, Glc NAc lane 2, Gluc lane 3 and Gal lane 4, compared with crude *E. granulosus* germinal layer antigen lane 1. Fermentas wide range standards was used to estimate molecularweight lane S.

**Sensitivity and specivcity of Glc NAc fraction**

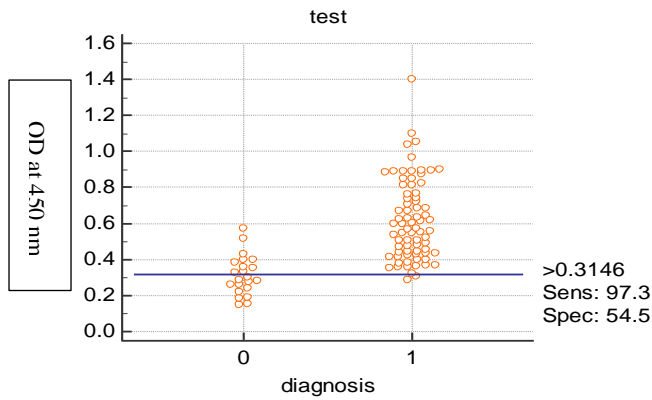
Glc NAc isolated fraction was tested with sera from naturally infected camel and healthy camel to assess the diagnostic potency of the isolated fraction. As shown in Fig. (3) the Glc NAc fraction exhibited 97.3 % sensitivity and 54.5% specificity at cutoff value 0.3145.

**Immunoreactivity of Glc NAc fraction**

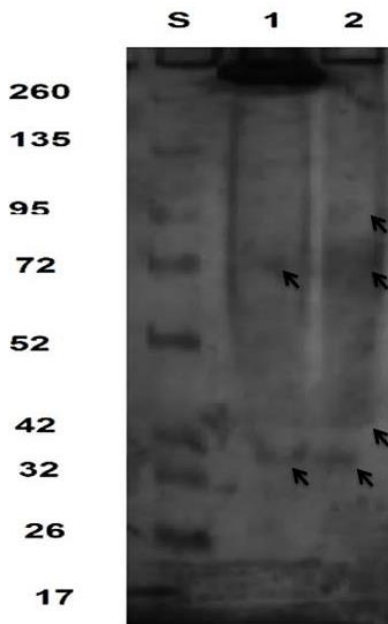
Immunoreactivity of IgG from infected camel sera to crude germinal layer and isolated Glc NAc glycoprotein fraction - was tested by western blot. Glc NAc fraction showed a clear two bands at 30 and 77 kDa on the western blot similar to SDS-PAGE profile (Fig 4 lane 1). The germinal layer showed reactive banding profiles at 30, 49, 77 and 99 kDa, (Fig 4 lane 2).



**Fig. 2:** Serological evaluation of different isolated glycoprotein fractions compared with crude germinal layer antigen tested with rabbit hyper immune sera (a) and camel naturally infected sera (b) against crude germinal layer antigen of *E. granulosus*. The cutoff value estimated as the mean value plus three standard deviations.



**Fig. 3:** Dot diagram representing the plot of sensitivity versus 1-specificity for the N-acetyl (Glc NAc) isolated glycoprotein of *E. granulosus* germinal layer antigen in the diagnosis of cystic camel echinococcosis. Disease statues were represented by (0) healthy and (1) diseased.



**Fig. 4:** Immunoblotting analysis of GlcNac fraction lane 1 and crude hydatid cyst germinal layer antigen lane 2 reacted with camel sera naturally infected with *Echinococcosis*. Lane S: fermentas wide range prestained molecular weight standard.

**DISCUSSION**

The development of diagnostic candidate to demonstrate the presence of antibodies in sera from infected animals and human with confirmed hydatidosis is an important step for improving diagnosis. In the present study, successfully purified three different glycoprotein fractions using Con A affinity chromatography fraction was designed as Gal, Gluc, Glc NAc. The three isolated glycoprotein fractions were characterized by SDS-PAGE and revealed 17 and 77kDa for Gal fraction, 30, 77 kDa for Glc NAc fraction and 30, 43, and 77 kDa for Gluc fraction. However, crude germinal layer resolved into 6 bands at 17, 30, 43, 49, 77, and 99 kDa. The immunogenicity of Glc NAc isolated fraction and the crude germinal layer was tested using immunoblotting technique and the result showed that bands of Glc NAc were immunogenic. However, the crude germinal layer

showed that only 30, 49, 77, and 99kDa were immunogenic when reacted with camel naturally infected sera. The current study isolated glycoproteins differ in the molecular weight from Con-A-binding 80, 110 kDa camel *E. granulosus* fluid glycoprotein isolated by Kamel *et al.*, (2006). This difference may be attributed to the difference in the source antigen used in the isolation of glycoprotein. Also, Kouguchi *et al.*, (2011) purified glycoprotein fraction from *E. multilocularis* protoscolex using Con A-agarose and subsequent gel filtration chromatography with molecular weight >4000 kDa upon gel filtration this difference in molecular weights from our isolated glycoproteins may be related to the difference in *Echinococuse Spp.* Where, suspected differences between *E. granulosus* and *E. multilocularis* glycan size was confirmed (Díaz *et al.*, 2015).

In order to compare the diagnostic performance of crude camel hydatid germinal layer and Con-A purified glycoprotein fractions in the diagnosis of camel echinococcosis ELISA was used. The Glc NAc showed high reactivity with rabbit hyperimmune sera and naturally infected camels than a crude germinal layer, Gal, and Gluc antigens. Kamel *et al.*, (2006) was also, purified *E. granulosus* glycoprotein fraction using Con A purification method and showed high diagnostic efficacy. Moreover, Kouguchi *et al.*, (2011) isolated glycoprotein component of *E. multilocularis* protoscolex, and discuss that it was useful as a serodiagnostic antigen.

To our knowledge, studies concerning the development of serodiagnostic techniques for a camel with *E. granulosus* infection are limited. In the present study we present Glc NAc isolated glycoprotein fraction as the diagnostic candidate of camel echinococcosis which showed 97.3% sensitivity and 54.5% specificity. However, Kamel *et al.*, (2006) proved that isolated glycoprotein from hydatid fluid revealed higher specificity; 98.4% and lower sensitivity; 94.5%. Also, Kouguchi *et al.*, (2011) isolated Con A glycoprotein from *E. multilocularis* protoscolex showed 100% sensitivity and 83% specificity. The difference in sensitivity and specificity between presented result and these results may be related to the difference in the isolated glycoprotein nature and also, may be attributed to different species of *Echinococcus* used as they used *E. multilocularis* protoscolex rather than the presented study used *E. granulosus* germinal layer.

**Conclusion**

GlcNac fraction of hydatid cyst germinal layer proved potency in the diagnosis of cystic echinococcosis than crude antigen, glucose, and galactose fractions.

Development of Glc NAc -based serodiagnosis of camel cystic echinococcosis would be confirming technique to infection beside ultrasonography. Further evaluation of this antigen should be performed using sera from camel infected with closely-related Platyhelminthes which are expected to prove that this serodiagnostic system is sufficiently specific for clinical and field applications.

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