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Original Article

Using of paramagnetic nanoparticales with immunomagnetic bead ELISA in diagnosis of hydatidosis (*Echinococcus Granulosus*)

Eglal A. Koura[1], Ibrahim Rabee [2], Reem O. Ahmed[1] and Walaa A. Mohamed[1]

[1] Zoology department, Faculty of women for Arts, Science and Education- Ain Shams University, Cairo, Egypt.
[2]Department of Parasitology, Theodore Bilharz Research Institute, Giza, Egypt.

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ABSTRACT

Cystic echinococcosus (CE) is a serious public health problem in sheep-raising regions. Detection of *E. granulosus* antigens is a better immunodiagnostic tool than determination of the antibody level. The circulating protoscolex antigen (CPA) used was obtained from lung and liver cysts of sheep and camel and injected in rabbits to raise specific polyclonal antibodies (pAb) against *E. granulosus*. A novel immunomagnetic bead ELISA based on immunoglobulin G (IgG) for detection of CPA in sera of rabbit infected with *E. granulosus* was developed. Detection of CPA in serum of human patients by sandwich ELISA gave a sensitivity of 90.48% and a specificity of 91.3% compared to other parasitic infections group and 100% compared to healthy control group. On the other hand, detection of CPA in serum of human patients by sandwich ELISA with paramagnetic nanoparticles gave a sensitivity of 95.2% and a specificity of 95.5% compared to other parasitic infections group and 100% compared to healthy control group. In conclusion, sandwich ELISA with paramagnetic nanoparticles techniques appears to be sufficiently sensitive assays for the detection of human echinococcosis antigen and valuable to judge the efficacy of chemotherapy than sandwich ELISA.

Key words: Echinococcosis – Immunomagnetic bead ELISA technique – Paramagnetic nanoparticles - Circulating protoscolex antigen (CPA)

INTRODUCTION

Human cystic echinococcosis (CE) is a zoonosis caused by the larval stage of *Echinococcus granulosus* and the most common sites affected are the liver and lung in approximately 80–90% of cases. The hydatid bone represents the 0.5–2.5% of all cases **[1]**. Hydatid disease is a rare pathology, but relatively common in the Mediterranean, the Middle East, Central Asia, and East Africa **[2]**.

Clinical diagnosis of CE is frequently difficult, hence always supported by imaging and immunological methods. The immunodiagnostic methods detecting the antibodies have the disadvantages of low specificity and sensitivity and the inability to differentiate between recent and past infections **[3]**. The diagnosis of hydatidosis is based on immunodiagnostic methods along with radiological and ultrasound examinations **[4, 5]**. A great number of immunological assays have been developed for detection of anti-hydatid cyst antibodies and recently, hydatid antigens in the serum **[6]**.

Therefore, immunodiagnosis remains an important tool in the diagnosis of the disease **[6]**. **Chordi and Kagan [7]** used immunoelectrophoresis to identify the antigenic components of sheep hydatid cyst fluid (HCF) and subsequently determined which antigenic components were active in detecting antibodies in the sera of patients with hydatid cysts. A successful immunodiagnostic test depends on the use of highly specific and sensitive antigens, as well as the detection of the appropriate antibody class or subclass **[8, 9]**.

Nanodiagnostics involved the use of nanotechnology in clinical diagnosis to meet the demands for increasing sensitivity and early detection in less time **[10]**. The large surface area of nonmaterial enables attachment of large number of target-specific molecules of interest for ultra-sensitive detection **[10]**. Because of the high sensitivity of nanotechnology, detection of a few

Corresponding Author Address: Reem O. Ahmed,** *Zoology department, Faculty of women for Arts, Science and Education- Ain Shams University, Cairo, Egypt;* ***E-mail: *reemosama12@yahoo.com*

microorganisms or target molecular becomes possible **[11]**.

The use of nanoparticles as tags or labels allows the detection of infections agents with lower costs than current in-use technologies. This advance in early detection enables accurate treatment **[11].** In most magnetic cell separation protocols, target cells are labeled with magnetic nanobeads that are conjugated to specific antibodies [12]. The efficiency of magnetic separation is influenced by factors related to the hardware of the magnetic separation system used and factors determined by the quality of the magnetic labeling of cells which depend on the properties or quality of the magnetic nanobeads employed [13].

Antigen detection has been developed as an alternative for echinococcosis diagnosis [14]. Detection of parasite antigen also helps to demonstrate the effect of treatment, and has a high specificity [15]. The present study was conducted to evaluate the role of prepared protoscolex antigen in the detection of the infection through raising anti-*E. granulosus* immunoglobulin G polyclonal antibody (IgG pAb). Comparative evaluation of protoscolex antigen with sandwich enzyme linked immunosorbent Assay (ELISA) in relation to sandwich ELISA with paramagnetic nanoparticles for the detection of circulating antigen in sera of tested samples was done using rabbit anti-*E. granulosus* IgG pAb.

MATERIALS AND METHODS

Animals: Two New Zealand white male rabbits, weighting approximately 1.5 Kg and about 2 months age, purchased from rabbit research unit (RRU), Agriculture Faculty, Cairo University. They were examined before the start of the experiments and were used in the production of the antibodies **[16]**. They were housed in the animal house in Theodore Bilharz Research Institute (TBRI), Giza, Egypt. They were kept for 4 weeks (experiment duration) under standard laboratory care at 21°C, 16% moisture, filtered drinking water with additional salt (1cm/5 liter) and vitamin (1cm/10 liter). Diet contains 15% protein, 3% fat and 22% fiber purchased from RRU. Animal experiments were carried out according to the Internationally Valid Guidelines.

Parasite: Hydatid cysts were removed from sheep and camel liver and lungs from an abattoir in Cairo Governorate and were transferred to our laboratory in TBRI in Hanks' buffer (Hanks' Balanced Salt Solution) (HBSS) to stimulate normal ion concentration under physiological tissue conditions **[17].**

Sera samples: Forty two *E. granulosus* infected human patients from highly endemic areas in Dakahlia Governorate who were admitted to tropical and surgical departments of Zagazig and El-Azhar University Hospitals were diagnosed by sonography, Computed tomography scan (CT) and Magnetic resonance imaging (MRI) to have CE in their livers. Besides, 24 patients infected with other parasites (*Schistosoma mansoni*, *Fasciola gigantica* and Hookworms) were included. In addition, 20 individuals of the medical staff at (TBRI) served as parasite free-healthy negative controls. Blood samples were collected from all cases and sera were separated, aliquoted and kept at -20 ˚C until used.

Preparation of parasite antigen: *Echinococcus granulosus* hydatid fluid was collected from ovine fertile cysts for subsequent use as a specific parasite antigen and clarified by centrifugation at 10,000 g at 4°C for 60 min., dialyzed against phosphate buffer saline (PBS) pH 7.2. Protoscoleces were prepared following the method of Rafiei and Craig **[18]**. In brief, protoscoleces were collected and the viability was determined by the vital coloration approach with 0.2% eosin staining. The protoscoleces were subjected to three cycles of freezing and thawing and suspension in 10 times their volume of 0.15 M PBS, pH 7.2. Subsequently, the protoscoleces were suspended in 4 times their volume of PBS containing 0.1 mg aprotinin/ mL, then sonicated on ice in a 150 W ultrasonic disintegrator, until no intact protoscoleces were visible microscopically and the supernatant solution was split into aliquots and stored at −20°C until further processing.

Purification and Characterization of parasite antigen: Diethyl[2-hydroxypropyl] aminoethyl (DEAE) -Sephadex A-50 and gel filtration chromatography on Sephacryl-S-200 high resolution (HR) column, these techniques for purification of antigen by separating proteins on the basis of charge and molecular size according to Smith et al., [19]. Then protein content was estimated by a Bio-Rad protein assay as shown by Bradford **[20]**. Finally characterization of protoscolex antigen by Sodium dodecyl sulfate-polyacrylamid (SDS-PAGE) was detected according to Laemmli **[21]**.

Assessment of Reactivity of Protoscolex Antigen by Indirect ELISA: This method was performed, with some modifications from the original method of Engvall E and Perlman **[22]**.

Production and Purification of Polyclonal Antibodies: Before immunization, rabbits were assayed by ELISA for hydatid antibodies (Abs) and

cross reactivity with other parasites. Rabbits were injected intramuscularly (i.m.) at four sites with 100µg of purified protoscolex mixed with equal volume of complete Freund's adjuvant (CFA) (Pierce, Rockford, IL, USA) according to Fagbemi et al., **[23].** Then, 3 booster doses (0.5 mg of purified protoscolex with equal vol. of incomplete Freund's adjuvant (IFA) (Pierce) were given at one week intervals. One week after the last booster dose, the rabbit's sera were obtained and pAb fraction was purified by 50% ammonium sulfate precipitation method **[24]**. More purification of pAb was performed by 4% caprilic acid method **[25]**. The reactivity of anti-protoscolex antigen IgGpAb against *Echinococcus* antigens was assessed using indirect ELISA.

Detection of Circulating Protoscolex Antigen in Human Sera by Sandwich ELISA: The microtitration plates were coated with 100 µl/well of 1/25 anti-purified protoscolex IgG pAb, incubated overnight at room temperature and washed 3 times with 0.1 M PBS/T, pH 7.4. Wells were blocked with 100 µl/well of 2.5% Fetal Calf Serum per Phosphate Buffer Saline per Tween (FCS/PBS/T), incubated for 2 hr. at 37°C and washed 3 times with PBS/T. 100 µl of human serum samples was pipetted into the wells in duplicate, incubated for 2 hr. at 37°C and washed 3 times. 100 µl/well of peroxidase-conjugated pAb of 1/100 for IgG was then added, plates were incubated for 1 hr. at room temperature. The plates were washed 5 times with washing buffer. 100 µl of substrate solution were added to each well and the plates were incubated in the dark at room temperature for 30 min., 50 µl/well of 8 N H_2SO_4 were added to stop the enzyme substrate solution. The absorbance was measured at 492 nm using ELISA reader **[22]**.

Detection of Circulating Protoscolex Antigen in Human Sera by Sandwich ELISA with nanoparticals: The above sandwich ELISA procedure was repeated by using peroxidase-conjugated nano-pAb.

Statistical analysis: The data are presented as mean ± standard deviation of mean (X ± SD). The mean values of each group were calculated from the mean values of individual patients. The mean groups were compared by analysis of variance **[26]**. This may be accomplished by changing the selection of the reference value (i.e. cut-off) for the particular test **[27]**.

a. Sensitivity= (no. of true +ve cases/ no. of true +ve cases + no. of false -ve cases).
b. Specificity= (no. of true -ve cases/ no. of true –ve cases + no. of false +ve cases).
c. Positive predictive value (PPV) = (No. of true +ve cases/ no. of true +ve cases + no. of false + ve cases).
d. Negative predictive value (NPV) = (No. of true –ve cases/ no. of true -ve cases + no. of false -ve cases).

RESULTS

Calculation of Total Protein Content of Protoscolex Antigen: The protoscolex antigen obtained from hydatid cyst fluid contains 8 mg/ml of total protein as measured by Bio-Rad protein assay while, it was 4.6 mg/ml after precipitation.

Purification by DEAE-Sephadex A-50 and gel filtration chromatography on Sephacryl-S-200 HR column: The fractions collected from sephadex A-50 ion exchange chromatography were further purified by DEAE sephacryl S-200 gel filtration column chromatography and one peak (a) was obtained represents the column elution volume fractions which contain protoscolex antigen with optical density (OD) value 1.30 at fraction number 11 (fig.1).

Characterization of protoscolex antigen by SDS-Gel electrophoresis: The eluted protein fractions resulted from the different purifications methods were analyzed by 12.5% SDS-PAGE under reducing condition and stained with Coomassie Blue. Protein bands were appeared at 3 different bands at 27.5, 50 and 65 kilodalton (kDa) which representing purified protoscolex antigen (fig. 2).

Reactivity of protoscolex antigen by Indirect ELISA: The *E. granulosus* antigen level was measured as OD reading at 492 nm. As mean (X) OD of each group ± standard deviation (SD). Serum samples from human infected with *E. granulosus* gave a strong reaction against protoscolex antigen with mean OD reading equal to 2.093 ± 0.402 and no cross reactions were recorded with sera of animals or patients infected with other parasites e.g. *S. mansoni*, *F. gigantica* and hookworms (table 1).

Production and Purification of Polyclonal Antibodies: Test blood samples were withdrawn from rabbit before the injection of each immunizing dose. They were tested for the presence of specific anti-*E. granulosus* antibodies by indirect ELISA. Three days after the 2nd booster dose immune sera gave a high titer against protoscolex antigen with OD of 2.97. The total protein content of crude rabbit serum containing anti- *E. granulosus* antibody was 16.3 mg/ml. The yield of purified anti-*E. granulosus* IgG pAb following each purification step was determined by

the assessment of protein content. Purification using the 50% ammonium sulfate precipitation the protein content was 10 mg/ml, while following 7% caprylic acid the content dropped to 6.4 mg/ml.

Characterization and reactivity of anti- *E. granulosus* IgGpAb: The purity of IgG after each step of purification was assayed by 12.5% SDS-PAGE under reducing condition. The purified pAb IgG was represented by H- and L-chain bands at 50 and 31 kDa respectively. The pAb appears free from other proteins. Reactivity of anti- *E. granulosus* antibodies against protoscolex antigen and other parasitic antigens (*Schistosoma mansoni*, *Fasciola gigantica* and hookworms) was determined by indirect ELISA. The produced anti-*E. granulosus* antibodies diluted in PBS buffer gave strong reactivity to protoscolex antigen. The OD reading at 492 nm for E. granulosus was 2.11 ± 0.152 compared to 0.352 ± 0.201, 0.263 ± 0.224 and 0.217 ± 0.112 for S. mansoni, F. gigantica and hookworms respectively (table 2).

Detection of circulating protoscolex (E. granulosus) antigen in human serum using sandwich ELISA and sandwich ELISA with paramagnetic nanoparticals: In order to determine the cut off value for positivity or the line of demarcation between positive and negative results, the mean OD reading of negative controls ±SD of the mean were estimated. Tested samples showing OD values > cut off value were considered positive for *E. granulosus* patients.

The cut off value was 0.26 when detecting in *Echinococcus granulosus* antigen in serum by using sandwich ELISA. The results were positive in 38 cases, while 4 cases were negative in sera of patients with *Echinococcus granulosus*. In patients with other parasitic infections 8 cases were detected as positive (1 with *S. mansoni*, 1 with *F. gigantica*, 0 with Hookworms infection), while the other 22 cases were negative. All healthy control patients were negative. The sensitivity, the specificity, PPV and NPV were 90.48%, 91.3%, 95% and 91.3% respectively (tables 3, 5).

The cut off value for positivity was 0.27 when detecting in *Echinococcus granulosus* antigen in serum, by using sandwich ELISA with paramagnetic nanoparticles. 40 cases gave positive results, while 2 cases were negative in sera of patients with *Echinococcus granulosus*. In patients with other parasitic infections 8 cases were detected as positive (1 with *S. mansoni*, 0 with both *F. gigantica* and Hookworms infection), while the other 23 cases were negative. All healthy control patients were negative. The sensitivity, the specificity, PPV and NPV were 95.2%, 95.5 %, 97.6% and 95.5% respectively (tables 4, 5).

DISCUSSION

Human cystic echinococcosis (CE) is regarded as a significant public health problem with high morbidity and mortality rates in endemic areas worldwide **[28]**. Cystic hydatid disease (CHD) is detectable clinically through various imaging techniques such as ultrasonography or radiology. The primary diagnosis must be confirmed by more specific testing, such as serological tests based on the discovery of antibodies against the organismal antigens in the patient's serum **[29]**. Ordinary serological tests such as immunoelectrophoresis, double diffusion in agar, or indirect hemagglutination are being replaced by more sensitive assay methods such as enzyme linked immunosorbent assay (ELISA), immunoblot (IB), and indirect immunofluorescent antibody test (IFA) **[30]**. ELISA is a high-sensitivity test that is strongly recommended for the detection of specific antibodies in cystic human disease (CHD) cases **[31]**. Moustafa et al. [32] reported that antigen detection assays may facilitate earlier diagnosis than antibody tests, as production of detectable levels of specific immunoglobulin needs time. Antigen detection assay in serum is generally performed by sandwich ELISA **[11]**.

Nanodiagnostics can be defined as the use of nano-sized materials, devices or systems for diagnostics purposes. It is a burgeoning field as more and improved techniques are becoming available for clinical diagnostics with increased sensitivity at lower cost **[33]**.

In this study, two immunological methods were used in diagnosis of human cystic hydatidosis (sandwich ELISA and sandwich ELISA with paramagnetic nanobeads). We developed an antigen-based ELISA using crude antigen to measure the levels of circulating protoscolex antigens (CPAs) in infected serum samples collecting from highly endemic areas in Egypt. The antigen used was a crude hydatid fluid isolated from living crude protoscolex cysts and used in production of pAb it. The protein content of the produced protoscolex antigen was 4.6 mg/ml after precipitation.

Then the purification of the protoscolex antigen by DEAE-sephadex A50-ion exchange chromatography and Sephacryl- S-200 HR column gel filtration methods was performed. Protoscolex antigen has OD values 1.145 at fraction number 7 and 1.6 at fraction number 9 respectively. These

yields were reported by Raina et al **[34]** and Rabia et al, **[35].**

The purification was followed by SDS-PAGE (12.5%) analysis. The use of this method proved previously to yield a highly antigen fraction as demonstrated by Sheehan and Gerald **[36].** Many bands were appeared in the SDS-PAGE analysis of protoscolex antigen, the most prominent of which were 27.5, 50 and 65 kDa. Then the antigenicity of the crude antigen was tested by indirect ELISA.

The purification procedures followed in this study were satisfactory, for IgG pAb two purification methods undertaken; ammonium sulfate precipitation which showed that most of albumin was removed from rabbit anti-*E. granulosus* IgG pA band 7% caprylic acid according to Goding **[37].** The purity of IgG pAb was assayed by 12.5% SDS-PAGE. The purified IgG pAb was represented by H- and L- chain bands at 50 and 31 kDa respectively, indicating that, the purified pAb appears free from other proteins. The yield of pAb as protein content by these methods was 6.4 mg/ml IgG from starting protein content of 16.3 mg/ml**.**

In the present study, reactivity of purified pAb demonstrated the reactivity of pAb as determined by indirect ELISA, gave a strong reactivity to protoscolex antigen. The purified pAb was further used as a primary capture to coat ELISA plates. The secondary capture of pAb was by conjugation with Horse-Raddish Peroxidase enzyme (HRP), sandwich ELISA was adopted using a pair of pAbs against protoscolex antigen, anti- *E. granulosus* IgG pAb and peroxidase-conjugated IgG polyclonal antibodies. Sandwich ELISA has been described as a valid test for detection of rabbit antibodies to fluke antigens, and has been the technique receiving most attention as an immunodiagnostic method for various parasitic infections [38].

In the current study, it was found that sandwich ELISA with paramagnetic nanoparticles offer a potential advantage of improving the sensitivity of the assay. The magnetic bead immunoassay combines the use of magnetic beads with a high binding capacity as a solid phase and the rapid reaction kinetics of solutions with the simple separation of bound and unbound materials on the solid phase, which provides the chance of enhancing the sensitivity of antigen detection [39].
In serum samples of the tested groups by using sandwich ELISA with paramagnetic nanoparticles out of 42 *Echinococcus* cases 40 gave positive results, while 2 gave negative results, so the sensitivity, the specificity, PPV and NPV were 95.2%, 95.5%,97.6% and 95.5% respectively .On the other hand, the sensitivity, the specificity, PPV and NPV of sandwich ELISA were 90.48%, 91.3%, 95% and 91.3% respectively Therefore, sandwich ELISA with paramagnetic nanoparticles was found to provide higher specificity and sensitivity compared to a microplate-based ELISA technique

In conclusion, sandwich ELISA with paramagnetic nanoparticles techniques appears to be sufficiently sensitive assays for the detection of human echinococcosis and valuable to judge the efficacy of chemotherapy than sandwich ELISA.

Table 1: Reactivity of purified protoscolex antigen by indirect ELISA.

Serum samples	**OD readings (human) at 492 nm ± SD**
E. granulosus	2.093 ± 0.402
S. mansoni	0.211 ± 0.311
F. gigantica	0.206 ± 0.143
Hook worms	0.225 ± 0.129

OD= optical density, SD= standard deviation.

Table 2: Reactivity of rabbit anti- *E. granulosus* IgG pAb against many parasitic antigens by indirect ELISA .

Parasitic antigen	**OD readings (human) at 492 nm ± SD**
E. granulosus	2.11 ± 0.152
S. mansoni	0.352 ± 0.201
F. gigantica	0.263 ± 0.224
Hookworms	0.217 ± 0.112

OD= optical density, SD= standard deviation.

Table 3: Detection of circulating protoscolex antigen in sera of human subjects infected with *Echinococcus granulosus* or other parasites in comparison to healthy control using sandwich ELISA.

Group (no. of human patients)	Positive cases		Negative cases	
	No.	X± SD	No.	X± SD
Healthy control (n= 20)			20	0.208 ± 0.029
E. granulosus (n= 42)	38	0.811± 0.113	4	0.217± 0.030
S. mansoni (n= 8)	1	1.029 ± 0.143	7	0.343 ± 0.048
F. gigantica (n= 8)	1	0.923 ± 0.032	7	0.226 ± 0.031
Hookworms (n= 8)	-	-	8	0.275 ± 0.038

X= mean; SD= standard deviation.

Table 4: Detection of circulating protoscolex antigen in sera of human subjects infected with *Echinococcus granulosus* or other parasites in comparison to healthy control using sandwich ELISA with paramagnetic nanoparticals.

Group (no. of human patients)	Positive cases		Negative cases	
	No.	X± SD	No.	X± SD
Healthy control (n= 20)			20	0.214 ± 0.03
E. granulosus (n= 42)	40	1.413± 0.197	2	0.240 ± 0.033
S. mansoni (n= 8)	1	1.479 ± 0.206	7	0.197 ± 0.027
F. gigantica (n= 8)	-	-	8	0.251 ± 0.035
Hookworm (n= 8)	-	-	8	0.118 ± 0.016

X= mean; SD= standard deviation.

Table 5: Comparison between data of sandwich ELISA and sandwich ELISA with paramagnetic nanoparticles.

***Echinococcus* antigen detected in human serum**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
Sandwich ELISA	90.48%	91.3%	95%	91.3%
Sandwich ELISA with paramagnetic nanoparticles	95.2%	95.5%	97.6%	95.5%

Positive predictive value (PPV)
Negative predictive value (NPV)

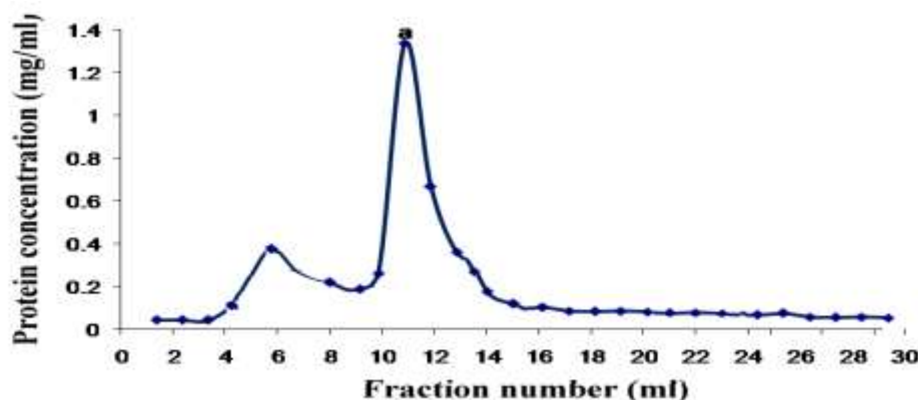

Figure 1: Elute profile for chromatography of protoscoleces on sephacryl S-200 column.

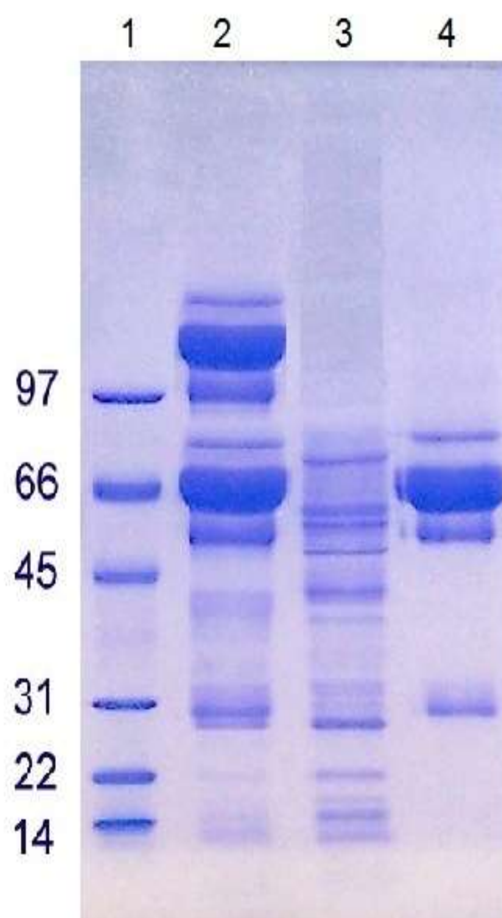

Figure 2: 12.5% SDS-PAGE of protoscolex antigen before and after purification (stained with Coomassie Blue).

Lane 1: Molecular weight of standard protein.
Lane 2: Crude protoscolex antigen.
Lane 3: Purified protoscolex after A-50.
Lane 4: Purified protoscolex after S-200.

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