Echinococcus granulosus protoscolex antigen used in serodiagnosis of hydatidosis by nano-gold dot-ELISA

Original Article

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ABSTRACT

Background: *Echinococcus granulosus* protoscolex antigen (PSAg) is a protein with significant immunological properties having higher sensitivity and specificity in ELISA. It lowers cross-reaction with antibodies of other parasites and thus its application is recommended in serological diagnosis. Labelling of ELISA with nano-gold particles improved the diagnostic abilities of the laboratory technique in hydatidosis detection.

Objective: To evaluate the use of nano-gold dot-ELISA for isolation of *E. granulosus* PSAg and its application in serodiagnosis of hydatidosis in humans and animals in comparison with dot-ELISA.

Material and Methods: Hydatid cyst PSAg was isolated and used for immunization of rabbits to raise IgG polyclonal antibodies (pAb) in antisera. These sera were labeled with horseradish peroxidase (HRP) and used for detection of circulating PSAg in sera of human cases and camels and sheep by dot-ELISA and nano-gold dot-ELISA.

Results: Conjugation of the anti-protoscolex pAb with gold nano-particles increased the sensitivity of antigen detection by nano-gold dot-ELISA to 94.4% and specificity to 90%, with positive and negative predictive values of 94.4% and 90%, and an accuracy of detection of 92.9% in both human and animal sera.

Conclusion: Nano-gold dot-ELISA technique is more sensitive than dot-ELISA for detection of hydatidosis antigen both in human and animal samples.

Keywords: dot ELISA, hydatidosis, nano-gold, protoscolex antigen.

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INTRODUCTION

Considering the importance of cystic echinococcosis in human health because of vital organs involvement, rapid and precise diagnostic methods are greatly needed[1]. Since hydatidosis may remain silent for years before the enlarging cysts cause symptoms, clinical diagnosis is difficult requiring a combination of physical examination, imaging techniques, and serology. Serodiagnostic tests play a supportive role in diagnosis with the use of different antigen sources[2]. Antibody detection assays do not distinguish between active and past infections[3]. Assays for antigens are preferred, as they are more specific, and their levels reflect improvement in surgically treated patients[4]. PSAg is a protein with significant immunological properties, believed to play an important role in the interaction between parasites and hosts. This occurs through inhibiting the chemotaxis of polymorphonuclear leukocytes and shifting the immune response to a non-protective Th2 response[5]. The antigen has higher sensitivity and specificity in ELISA and lower cross reaction against nematode, cestode and trematode antibodies than other antigens like Ag5. Thus, the WHO recommended its application in serological methods[6].

The use of semi-purified antigens of hydatid cyst fluid (Ag5 and B) in ELISA tests gave better diagnostic value than crude hydatid cyst fluid antigens for cystic echinococcosis diagnosis, confirming that the most immunogenic component of hydatid cyst fluid is Ag 5[7]. Dot-ELISA was used as a solid phase diagnostic method for detection of both antigens and antibodies. Its application validates the diagnosis of human and animals' protozoan and metazoan diseases particularly by accomplishing higher specificity and sensitivity rates in early detection of *E. granulosus* PSAg in patients' sera[8].

Comparison between native and recombinant antigens of *E. granulosus* by dot-immunogold filtration assay revealed 77.9% sensitivity and 98.3% specificity of recombinant AgB, while the crude fluid antigen B showed 92.9% sensitivity and 81% specificity. While these results apparently signified the advantage of absent cross-reaction of recombinant AgB with other parasitic diseases thus improving the specificity, yet the sensitivity was decreased[9]. Another advantage for using dot-ELISA is the employment of very small amounts of reagent and test sample required for dotting

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on nitrocellulose membranes to bind the proteins; while standard ELISA utilizes a relatively higher amount of reagent and sample. Enzyme detection is completed by addition of a substance that triggers a signal from the enzyme in dot-ELISA, but standard ELISA utilizes spectrophotometer examination for enzyme detection[10]. Nano-technology provides dramatic alterations in biotechnological tools thus helping in improvement of diagnosis and treatment of human diseases, allowing cheaper, safer, easier and portable interventional tools[11]. Also, as an immunological diagnostic laboratory technique, nano-ELISA proved to have higher sensitivity than ELISA and is therefore more useful in early detection of protein markers of tumors or other diseases predicting early diagnosis[12]. Nano-gold was later used seeking a more rapid method for diagnosis of hydatidosis by using antigen B labelled with gold nano-particles in a dot-immunogold staining technique for detection of antibodies[13]. Conjugation of IgG fractions with gold nano-particles for specific antigen detection was developed using a combination of anti-native antigen B and gold-conjugated anti-hydatid cyst fluid IgG; this assay once evaluated, again gave a specificity of 82% for antigen B[14]. Therefore, this study investigated using nano-gold dot-ELISA for isolation and evaluation of *E. granulosus* PSAg and its application in serodiagnosis of hydatidosis in humans and animals' samples.

MATERIAL AND METHODS

This descriptive analytical study was conducted in the Medical Parasitology Department, Faculty of Medicine, Benha University, Benha, during the period from January 2017 to February 2018.

Study design: Hydatid cysts fluids were collected aseptically in tubes by a syringe for subsequent use as a specific parasite scolex antigen. Protoscolices preserved in phosphate buffer saline (PBS)[15] were used to produce rabbit serum containing anti-*Echinococcus* pAb. Reactivity of pAb against PSAg was assessed by indirect ELISA. The antiserum was then conjugated with HRP and used for the detection of specific antigen in sera of infected human cases and animals by dot-ELISA and nano-gold dot-ELISA.

Hydatidosis human serum samples: Twenty-seven blood samples from hydatid infected patients, confirmed by C.T, U/S, and IHAT, were obtained from Benha University Hospital in Kaliobeya Governorate, and Hospital of Theodore Bilharz Institute in Giza Governorate. For positive human control, 20 blood samples were taken from patients infected with *F. gigantica*, *H. nana*, and *E. vermicularis*, confirmed by stool analysis. For negative human control, 20 blood samples were obtained from parasite free individuals. Separated sera were stored at -20°C until use.

Hydatidosis animal serum samples: Thirty-six blood samples were obtained from camels and sheep naturally infected with *E. granulosus* confirmed after slaughtering at the abattoir. Positive control included 20 blood samples collected from cattle with *F. gigantica* infected livers. Negative control included 20 blood samples collected from parasite-free camels and sheep, confirmed by examination of feces samples collected from the intestine of slaughtered animals.

Preparation of PSAg: Hydatid cysts were obtained from lungs of infected slaughtered camels at the Toukh abattoir, in Kaliobeya Governorate. Samples were analyzed in the laboratory of Immunoparasitology Department of Theodore Bilharz Institute. To prepare PSAg[16] collected hydatid fluid was clarified by centrifugation at 10000 rpm at 4^0C for 60 min. The deposit containing protoscolices was subjected to three cycles of freezing and thawing then washed three times in 10 times its volume of 0.15 M PBS, PH 7.2 and sonicated by ultrasonic disintegrator till no intact protoscolices were visible microscopically. The sonicate was left standing for 1 h, sedimented and centrifuged for 30 min at 10000 rpm. The supernatant was stored at -20^0C until further processing.

Purification of PSAg: Using Diethyl-amino-ethyl Sephadex chromatography[17] (DEAE-Sephadex G-50; Pharmacia, Uppsala, Sweden), Tris-HCL, NaCl salt, binding buffers, eluting buffers and a column, stored protoscolices proteins were dialyzed versus the binding buffer. Protein content of the sample was estimated by Bio-Rad protein assay[18] and then passed through gel filtration chromatography on Sephacryl-S-200 HR column.

Production of pAb: A New Zealand white male rabbit, about 1.5 Kg and about 2 months age, was purchased from the Rabbit Research Unit (RRU), Agriculture Faculty of Cairo University. Assessment of reactivity of PSAg using indirect ELISA was performed according to Engvall and Perlmann[19]. Blood samples were collected from the rabbit before injection and checked by indirect ELISA for *Echinococcus* antibodies and cross-reactivity with other parasites; *F. gigantica*, *H. nana* and *E. vermicularis* according to Goubodia and Fagbemi[20]. Purified PSAg mixed with equal volume of complete Freund's adjuvant (Sigma) was injected intramuscularly by a priming dose of 1 mg antigen, followed by the 1st booster dose of 0.5 mg antigen two weeks after priming dose; then two booster doses of 0.5 mg antigen each were injected at weekly intervals[21]. Sacrifice and collection of blood sample was three d after the last dose. Centrifugation of the blood was done at 4000 rpm for 15 min and separated serum containing anti-*Echinococcus* pAb was portioned and kept at -20°C.

Assessment of rabbit pAb reactivity against PSAg using indirect ELISA: Wells of polystyrene microtiter

plates were coated with purified PSAg, washed with washing buffer, blocked with bovine serum albumin, then washed for 5 times. One hundred µl of pAb were dispensed into wells and incubated for 1h. After washing for 3 times, 100 µl of polyvalent anti-rabbit peroxidase conjugate (Sigma) were dispensed in wells, incubated for 1h, and then washed. One tablet of O-phenylene-diamine-dihydrochloride substrate was dissolved in 25 ml of 0.05 M phosphate citrate buffer and 100 µl were added to each well and the plate was incubated in the dark for 30 mins. Fifty µl/well of 8 NH2SO4 were then used to stop the enzyme substrate reaction. Absorbance was measured at 492 nm using ELISA reader (Bio-Rad, Richmond, VA 'Virginia', USA)[19].

Conjugation of rabbit pAb with HRP: Prepared buffers were in the form of 1mM Na acetate (pH 4.4), 0.2 M carbonate buffer, 0.1 M Borate, 0.1 M PBS, 0.1 M Na metaperiodate, Na borohydride. Conjugation on the 1st d was by: (A) 5mg HRP suspended in 1.2 ml distilled H_2O plus 0.2 ml freshly prepared sodium periodate followed by dialysis against 1 mM sodium acetate buffer; (B) 5mg/ml of pAb dialyzed against 0.02 M carbonate buffer. On the 2nd d; (A) and (B) were mixed and incubated at room temperature for 2 h followed by addition of 100 µl freshly prepared Na borohydride for 2 h at 4°C, and dialysis of the mixture against 0.01 M PBS (pH 7.2) overnight at 4°C. The conjugate was stored at -20°C until used.

Conjugation of rabbit pAb with gold nano-particles: Gold nano-particles were obtained as a solution. Before use, all glass wares used were soaked with Aqua Regia then rinsed with deionized water. Gold trichloride $HAuCl_4$ in a dilute HCl solution was added to sodium citrate solution. The concentration of gold nano-particles was determined by UV/vis spectrophotometry using Beer's law[22]. The solution was mixed with mercaptoundecanoic acid (MUA) and then conjugated with pAb and left to incubate overnight at 4°C. This allows for the electrostatic binding between pAb and gold nano-particles-MUA to occur. Protein content determination after conjugation was based on Bradford gel filtration chromatography[18].

Detection of circulating PSAg in serum samples by dot-ELISA: Dot-ELISA was performed according to Boctor *et al.*,[23] by means of a Bio-dot apparatus (BIO-RAD, USA) for detection of circulating PSAg by double antibody sandwich procedures. The pre-wetted nitrocellulose membrane was transferred to the Bio-dot apparatus and washed once with coating buffer for 5 min, coated with 10-50 µl/well of the purified pAb, diluted in carbonate buffer (1/250, 500 and 1000), then incubated. Excess solution was removed, the membrane was washed 3 times with 100 µl PBS-T/well, followed by application of blocking solution (10-50 µl/well), and incubation at room temperature for 15-45 min. Positive and negative control reference samples were added, diluted 1/1-1/32 in the diluent-blocking buffer then incubated for 15-45 min and washed. HRP-conjugated pAb was diluted in the diluent-blocking solution and incubated for 15-45 min, then the nitrocellulose membrane was removed and washed 5 times with 100 µl PBS-T/well. Diaminobenzidine substrate was then applied by immersing the membrane in substrate solution. The reaction was stopped with cold distilled H_2O, just after development of the color.

Detection of circulating PSAg in serum samples by nano-gold dot-ELISA: The same method as mentioned with dot-ELISA was applied but with the use of gold nano-particles-pAb as coating antibody and HRP-pAb as the conjugate antibody.

Statistical analysis: The statistical analysis was performed using statistical package for social science (SPSS) version 20. Descriptive statistics were calculated for the data in the form of frequency and distribution for qualitative data. For analytical statistical comparison between different groups, the significance of difference was tested using chi square test (X^2 value) and fisher exact test (FET) and ROC curves. Diagnostic sensitivity, specificity and accuracy as well as positive and negative predictive values were calculated. *P* value of < 0.05 was considered significant.

Ethical considerations: Human serum samples were taken after explaining the investigation to the patients and obtaining a written informed consent. The study protocol was approved from the Scientific Research Ethical Committee in Faculty of Medicine in Benha University. The experimental animal studies followed the ethical guidelines of the Medical Ethical Committee of Theodor Bilharz Research Institute (TBRI) in Egypt.

RESULTS

Reactivity of rabbit pAb against PSAg was confirmed by indirect ELISA. Detection of *Echinococcus* PSAg in human cases by dot-ELISA and nano-gold dot-ELISA was statistically significant in 24/27 (88.9%) and 26/27 (96.3%) serum samples, respectively ($P < 0.001$) (Table 1). Nano-gold dot-ELISA gave higher sensitivity values than dot-ELISA (95.7% versus 88.9%); specificity (95% versus 80.7%); PPV (96.3% versus 85.7%); NPV (95% versus 76.2%) and accuracy (96.3% versus 85.1%), (Table 1). Of the 20 control positive sera, 4 were positive in dot ELISA (2/7 cases infected with *H. nana*, 1/7 case with *E. vermicularis*, and 1/6 case with *F. gigantica*). Cross reaction was reduced to 1 case with *F. gigantica* in nano-gold dot ELISA.

Detection of PSAg in animals by dot-ELISA and nano-gold dot-ELISA revealed 31/36(86.1%) and 34/36 (94.4%) significantly positive serum samples respectively ($P>0.001$), (Table 2). Nano-gold dot-ELISA showed higher values than dot-ELISA regarding sensitivity (94.4% versus 86.1%); PPV (94.4% versus

93.9%); NPV (78.3% versus 90%); accuracy (92.9% versus 87.5%); and specificity (90%) was the same for both tests (Table 2). In the 20 positive control blood samples collected from cattle with *F. gigantica* infected livers, 2 cross reacted in dot ELISA and 2 in nano-gold dot ELISA.

Table 1: Dot-ELISA and nano-gold dot-ELISA for detection of hydatidosis in human cases.

	Dot-ELISA		Nano-gold dot ELISA	
	Positive	Negative	Positive	Negative
	No. (%)	No. (%)	No. (%)	No. (%)
Positive (No. = 27)	24 (88.9)	3 (11.11)	26 (96.3)	1 (3.7)
Positive control (No. =20)	4 (20.0)	16 (80.0)	1 (5.0)	19 (95.0)
Negative control (No. =20)	0 (0.0)	20 (100.0)	0 (0.0)	20 (100)
Statistical analysis	$X^2 = 42.88$, $P<0.001$*		$X^2 = 59.05$, $P<0.001$*	
Sensitivity	88.9%		95.7%	
Specificity	80.7%		95.0%	
PPV	85.7%		96.3%	
NPV	76.2%		95.0%	
Accuracy	85.1%		96.3%	

* Significant

Table 2: Dot-ELISA and nano-gold dot-ELISA for detection of hydatidosis in animal cases.

	Dot-ELISA		Nano-gold dot ELISA	
	Positive	Negative	Positive	Negative
	No. (%)	No. (%)	No. (%)	No. (%)
Positive (No. = 36)	31 (86.1)	5 (13.9)	34 (94.4)	2 (5.6)
Positive control (No. =20)	2 (10.0)	18 (90.0)	2 (10.0)	18 (90.0)
Negative control (No. =20)	0 (0)	20 (100.0)	0 (0.0)	20 (100.0)
Statistical analysis	$X^2 = 51.15$, $P<0.001$*		$X^2 = 61.2$, $P<0.001$*	
Sensitivity	86.1%		94.4%	
Specificity	90.0%		90.0%	
PPV	93.9%		94.4%	
NPV	78.3%		90.0%	
Accuracy	87.5%		92.9%	

* Significant

DISCUSSION

In this study, the circulating antigens in the sera of humans or animals infected with *E. granulosus* were detected by dot-ELISA and nano-gold dot-ELISA using pAb produced against PSAg. An interesting feature is the recorded high specificity of the nano-gold dot-ELISA in sera of patients, thus promoting early serological antibody diagnosis before surgical intervention and improving the discrimination between recent and past hydatid infections. According to Parija[24], 40% of surgically confirmed patients fail to show antibodies in their serum by various techniques. Moreover, because specific antibodies may persist for a long time, even after the removal of hydatid cyst by surgery or after clinical cure by chemotherapy[25], antibody detection assay cannot discriminate between chronic and acute states in hydatid disease[3]. It was noted that because circulating *Echinococcus* PSAg is present in active or recent infection and becomes absent in patients treated with surgery or chemotherapy, demonstration of circulating antigen in the serum was believed to indicate recent and active infection and helps in monitoring the efficacy of chemotherapy[26].

Nano-technology enables detection of a few microorganisms or targets molecular analyses specific to pathogens. Also, it could allow rapid and real-time detection of the pathogens with relatively small sample volumes[27]. This is because nano-materials have a large surface area that enables attachment of many target-specific molecules (antigens) of interest for ultra-sensitive detection. Other conventional methods are inefficient in attaining this ultra-sensitivity. Reportedly the periodate method is the most common method for labeling anti-*Echinococcus* pAb molecules with HRP utilizing the glycoprotein nature of the enzyme. The saccharide residues of the enzyme are oxidized with sodium periodate to produce aldehyde groups that can react with the amino groups of the IgG molecule and the Schiff bases formed are then reduced to give a stable conjugate of high molecular weight[28].

In our present study, detection of hydatidosis using PSAg in human sera by dot-ELISA showed 88.9% statistically positive serum samples, and nano-gold dot-ELISA gave a 96.3% higher rate of positive cases. Also, the present study using the same antigen showed that nano-gold dot-ELISA gave higher values

than dot-ELISA regarding sensitivity (95.7% versus 88.9%); specificity (95% versus 80%); PPV (96.3% versus 85.7%); NPV (95% versus 76.2%) and accuracy (96.3% versus 85.1%) in human cases. In comparison, the study of Swarna and Parija[29] assessed dot-ELISA serodiagnosis and their results revealed a sensitivity of 96.66%, 86.66% and 93.33% using cyst wall, protoscolex and cyst fluid, antigens respectively, with a specificity of 70% for all antigens. In these researchers' study and analogous to our study using PSAg, the authors reported a similar sensitivity of 86.66% versus 88.9% in our study, and lower specificity of 70% versus 80.7% in our study.

Validity of our results is supported by Abou-Elhakam *et al.*,[8] who reported that *E. granulosus* PSAg was detected in 48/50 patients' sera using dot-ELISA, with sensitivity and specificity of 96% and 94%, respectively; PPV of 94% and NPV of 90%. Using a regular PSAg-based ELISA, the sensitivity for diagnosis of circulating antigens and antibodies in human was only 52.5% while the specificity of the assay was 75%[30]. The authors concluded that using this system, antibody detection assay is superior and more sensitive than antigen detection assay. Another report using hydatid cyst fluid antigen from sheep in a serodiagnostic ELISA system recorded 91.5% sensitivity and 96% specificity for diagnosis of hydatidosis cases[31]. Our study raised the sensitivity of diagnosis by using nano-particles in detection of serum circulating antigens to 95.7%. This improvement in results is explained by the utilization of gold nano-particles as antibody carrier as well as the reporter peroxidase enzyme, which coincides with results of Ciaurriz *et al.,*[32]. It is worth mentioning that with dot-ELISA we recorded 4/20 cross reactions in sera of patients infected with *H. nana* (2/7), *E. vermicularis* 1/7), and *F. gigantica* (1/6). With nano-gold dot ELISA cross reaction was reduced only to the one case infected with *F. gigantica*.

In our study on animal hydatidosis, the validity of dot-ELISA versus nano-gold dot-ELISA for detection of PSAg in animals showed a marked improvement in favor of nano-gold dot-ELISA; NPV and sensitivity improved from 78.3% and 86.1% to 90% and 94.4%, respectively, while accuracy improved from 87.5% to 92.9% but specificity and PPV showed minimal improvement.

Positive control serum from *F. gigantica* infected cattle showed 2/20 positive recordings with PSAg by both Dot-ELISA and nano-gold dot ELISA, indicating cross reactivity between *F. gigantica* and *E. granulosus*. In another study[13], dot-immunogold staining for serologic diagnosis of sheep hydatidosis disease, was reported to provide a rapid, simple, sensitive and specific method. In confirmation, Sangaran *et al.*,[33] in their study on buffaloes and sheep echinococcosis achieved a sensitivity of dot-ELISA that reached 94% and a specificity of 96%.

In conclusion, nano-gold dot-ELISA technique is more sensitive than dot-ELISA for detection of hydatidosis antigen both in human and animals.

Author contributions: All authors contributed to the study design and organized the multicenter study. SM Rashed, ME Nasr, NS Ali, WE Elawamy and SM Kishik shared in writing the manuscript and data analysis. NS Ali, WE Elawamy and AS El Ghanam collected clinical samples and data. WE Elawamy and SM Kishik contributed to editing of the manuscript for publication.

Competing interest: The authors declare no competing interests.

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