A pioneer usage of nanogold sandwich ELISA in the detection of hydatid antigen in humans as a tool for improvement of serodiagnostic testing.

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Abstract

Background: Human cystic echinococcosis is a zoonosis occurring due to dogs handling and exposure to *Echinococcus granulosus* ova in their stools being an accidental intermediate host. Serology remains the only sure and important tool to diagnose this disease. Our objectives in this research are to improve the serodiagnostic abilities of sandwich ELISA through its binding to gold nanoparticle and proving the role of minute nanoparticles in serodiagnosis.

Methods: gold nanoparticles (AuNPs) were employed for the capture of the antigens of protoscolices (pAg) in the patient's serum by sandwich ELISA. Cross-reactivity for antigens from *Hymenolepis nana*, *Enterobius vermicularis*, and *Fasciola gigantica* was ruled out by using anti-protoscolices polyclonal antibodies (ppAb). (pAg) of sonicated protoscolices which were removed from camel lung cysts, was purified by diethyl aminoethyl Sephadex (DEAE-Sephadex) and injected to a New Zealand white rabbit giving ppAb which then conjugated to horseradish peroxidase (HRP) and loaded on AuNPs being used as a diagnostic indicator for circulating pAg by both sandwich ELISA and nanogold sandwich ELISA techniques.

Results: Nanogold sandwich ELISA was able to give positive results with 96.3% of hydatid patients and 5% of non-hydatid patients while sandwich ELISA showed 81.4% and 20% positive cases of the same groups respectively. The sensitivity and specificity of nanogold sandwich ELISA were 96.3% & 95% against 81.5% & 80% for those of sandwich ELISA respectively.

Conclusion: The conjugation of AuNPs to anti- *Echinococcus* IgG antibodies seems to be beneficial to maximize the sensitivity of sandwich ELISA, reduce the overall cost of the assay as less antibody was needed, solve harsh buffering conditions and achieve greater stability which provides a reliable quantitation of analytes.

Background

Hydatidosis (cystic echinococcosis) has worldwide distribution representing a major public health problem especially those concerning sheep and cows grazing, so its diagnosis represented an important issue and continuous improvement is needed. Hydatidosis diagnosis is dependent on clinical presentation and serodiagnosis so the development of the serodiagnostic technique is a necessary request [1]. Monitoring of hydatid fluid to react against IgG antibodies was a guide for hydatidosis diagnosis but on following up, it was shown to be useless as the anti-hydatid fluid antibodies persist for long time in patients' sera in addition to the appearance of false positive and negative results [2].

Clinical diagnosis of hydatidosis remains vague and is not accurately leading evidence in diagnosis, hence always supported by imaging and serodiagnosis but depending on immunodiagnostic methods that detect the anti-hydatid antibodies thus having insufficient specificity and sensitivity and the inability to pick up recently infected cases among old infections [3].

Due to the lack of target antigen standardization in hydatid infections which is generally formed of crude antigen preparation of hydatid fluid inside cysts, there was an urgent need for the development of new tools among many immunodiagnostic tests which are commercially available to overcome this lack [4]. The use of purified hydatid cyst fluid antigen is reliable in the hydatidosis serodiagnosis compared to the use of whole hydatid cyst fluid [5].

The use of nanotechnology in the field of immunodiagnostic studies provides high sensitivity in the detection of target analytes specific to pathogens and micro-organisms in addition to the advantages of nanomaterial properties thus allowing rapid detection of pathogens in small biological sample volumes [6]. The use of nanoparticles as labels facilitate the detection of infectious agents at lower costs than already used technologies [7].

Antigen detection in hydatidosis became useful for serodiagnosis and also can be used for post-treatment follow up hence it improves the diagnostic abilities when used together with antibody detection tests [8]. Latex agglutination test was used as a diagnostic test for cystic echinococcosis and the results revealed that it could be functionalized as a simple and rapid diagnostic tool to confirm that a suspected cyst is of hydatid origin [8]. Sandwich ELISA with paramagnetic nanoparticles provided an extraordinary tool for improving the sensitivity of hydatidosis antigen detection thus making benefits of the high binding capacity of magnetic beads in enhancing the antigen detection sensitivity [3]. The aim of this present work was to improve the capacity of sandwich ELISA in the detection of hydatid protoscolices antigen in human serum samples by labeling anti-Echinococcus IgG polyclonal antibody on gold nanoparticles.

Methods

1. *Protoscolices antigen production:*

Deposit of hydatid fluid from camel lung cysts containing protoscolices was exposed to freezing and thawing 3 times and use 10 times of its volume of 0.15 M phosphate buffer saline (PBS) to wash, pH 7.2 then sonicated by 150 W ultrasonic disintegrator and the sonicate was sedimented at 10000 rpm for 30 minutes, the supernatant was split into aliquots and stored at - 20°C [9].

2. *Purification of pAg by DEAE-Sephadex A 50 chromatography:*

Removal of cross-reactive proteins and host components from *Echinococcus* antigens is a mandatory to be done by purification [10]. DEAE Sephadex A 50 chromatography has been used to purify the antigens of camel hydatid cysts based on their charges thus the DEAE group maintains positive charges that are neutralized by negative chloride charges [11]. Anion exchange chromatography was done by adding 0.5 gm DEAE Sephadex A 50 powder (Pharmacia, Uppsala, Sweden) to 200 ml of 0.5 M Tris-HCL buffer adjusted at pH 7 slowly for 1-2 days at 4°C that this Sephadex was swollen to 22 ml beads. In a 30x2.5 column, the swollen beads suspension was poured using a glass rod to avoid air bubbles trapping, then the swollen beads were covered by binding buffer (20 mM Tris-HCl) and the hydatid antigen sample was

dialyzed versus the binding buffer and allowed to penetrate the beads which were then washed by eluting buffer (20 mM Tris-HCL/150 mM NaCl) and the serum sample protein was calculated by collecting 10 fractions each is 2 ml and by using spectrophotometer (*Perkin Elmer Lambda 1 A*), absorbance at 280 nm for each fraction was measured and peak fractions of high absorbance were pooled together and the protein content was estimated by *BioRad protein assay, USA* [12], [13].

Indirect ELISA was used to assess the reactivity of pAg according to [14] and the absorbance was measured at 492 nm using ELISA reader (*BioRad microplate reader, Richmond co., Wilmington, USA*), measurement of the cutoff point for positivity was an indicator for mean optical density reading.

3. Immunization of a rabbit and construction of ppAb

A New Zealand white male rabbit free from parasitic infections, purchased from Rabbit Research Unit, College of Agriculture, Cairo University. The experiment duration was 4 weeks under standard laboratory care at 21°C, 16% moisture, filtered drinking water with additional salt (1cm/5 liter) and vitamins (1cm/10 liter). Diet contains 15% protein, 3% fat and 22% fiber. A rabbit was immunized by i.m injection of a priming dose of 1 mg purified pAg mixed with a similar volume of complete *Freund's* adjuvant (*Sigma*), then a first booster dose of 0.5 mg pAg was given two weeks after priming dose and two booster doses of 0.5 mg pAg at weekly intervals [15]. Three days after the last immunization, the blood sample was taken and centrifuged at 4000 rpm for 15 min. and serum IgG ppAb was fractionated, then kept at -20°C. Protein content was estimated by Bradford dye-binding procedure thus using the color change of Coomassie Brilliant Blue G-250 dye by reference to a standard curve consisting of known concentrations of purified protein by using a protein assay kit (*Bio-Rad, Richmond, CA, USA*) [12]. The rabbit was exposed to euthanasia by I.V injection of phenobarbital sodium after the experiment to end the animal's suffering due to severe pain during experiment and it was not allowed to be re-used it again.

4. Isolation of rabbit ppAb

As a result of polar and ionic groups of proteins, their solutions form hydrogen bonds in water and on the addition of highly charged ions such as ammonium of sulfate, they compete with proteins to bind water; this removes water molecules from proteins being precipitated [16]. Saturated ammonium sulfate solution $(\text{NH}_4)_2\text{SO}_4$ was mixed with rabbit serum, centrifuged for 20 min. at 3500 rpm at 4°C, the supernatant was discarded and after repeated precipitations, the final precipitate was dissolved in 0.01 M PBS, pH 7.2 and $(\text{NH}_4)_2\text{SO}_4$ was dialyzed against 0.01 M PBS, pH 7.2 for 72 hours to obtain pure protein [16]. Protein content in isolated IgG ppAb after ammonium sulfate treatment was determined by the *Bradford* dye-binding procedure [12].

5. Labeling of ppAb with HRP

IgG ppAb molecules are conjugated to HRP enzyme exploiting its glycoprotein nature thus the saccharide residues of the enzyme are oxidized by sodium periodate giving rise to aldehyde groups which react with the IgG molecule amino groups which in turn lead to the production of Schiff bases being reduced to

produce high molecular weight conjugate [17]. According to [18] labeling was done on two successive days; on the 1st day, 5 mg of HRP was dissolved in 1.2 ml distilled water (dist. H₂O) then 0.2 ml sodium periodate was added for 20 min. at room temperature and dialysis against 1 mM sodium acetate buffer, pH 4 at 4°C overnight to give solution (A), 5 mg/ml of IgG ppAb was dialyzed against 0.02 M carbonate buffer, pH 9.6 at 4°C overnight to give solution (B). In the 2nd day, solution (A) was mixed with the solution (B) at room temperature for two hours and 0.1 ml of sodium borohydride was added to the new mixture which finally dialyzed against 0.01 M of PBS, pH 7.2 at 4°C overnight and the resulting conjugate was stored at – 20°C till used.

6. Synthesis of antibody-AuNPs conjugates

- a. *Source of AuNPs:* AuNPs (40 nm/particle) were in the form of gold HCL solution with a concentration of 3.08×10^8 particles/ml, they were purchased from (*Tech. Egypt Co., 6 October, Egypt*).
- b. *Method of loading of IgG ppAb on AuNPs:* according to [19], all glasswares were soaked with Aqua Regia (*PanReac, Germany*) for overnight and in the next day rinsed with tap water, the hydrogen tetrachloroaurate III (HAuCl₄) (*Sigma, Aldrich, Germany*) solution was vortexed and an amount of 250 ml of it was added in a round bottom flask, condensed for 1 hour and refluxed using a sand bath then 25 ml of 38.8 mM sodium citrate were added and the flask was left to reflux again for 15 min. and stored in a dark place. Functionalization of 30 ml of AuNPs solution with 45 µl of 1mM mercaptoundecanoic acid (MUA) in ethanol (*Sigma, Aldrich, Germany*) for overnight at 4 °C and the AuNPs concentration was determined in the solution was determined before and after functionalization by UV/vis spectrophotometry using Beer's law [20].
Conjugation of ppAb with AuNPs-MUA; 5 ml of AuNPs-MUA was added to 2 ml of ppAb in presence of N-hydroxy Succinimide/1-Ethyl 3-Dimethylaminopropyl Carbodiimide (NHS/EDC) as cross-linkers (*Fluka, Germany*) in order to obtain powerfully built AuNPs-MUA conjugates, this was achieved by addition of 5 ml of ppAb-AuNPs conjugates to 5 ml of a mixture of (5 mM sodium phosphate buffer = pH 7, 1.2 mM NHS and 2.8 mM EDC) then left overnight at 4°C so as to elicit electrostatic binding between ppAb and AuNPs-MUA [21].

7. Quantitation of protein after conjugation of ppAb with AuNPs

Based on the *Bradford* dye-binding procedure, quantitation of protein content in ppAb-AuNPs conjugates was done by preparing standards of protein samples in the form of serial dilutions of Bovine Serum Albumin (BSA) with dist. H₂O as (BSA: 1, 5, 10, 15, 25, 50 and 80 µl against dist. H₂O: 99, 95, 90, 74, 50 and 20 µl respectively), then preparing standards of ppAb-AuNPs conjugates samples in the form of conjugates with dist. H₂O as (conjugates 3, 10, 15 and 20 µl against dist. H₂O: 97, 90, 85 and 80 µl respectively) and then 5 ml of diluted dye (phosphoric acid and methanol) was mixed with both preparations and the absorbance of color was measured at 595 nm for each standard tube being planned on standard curve for protein content measurement calculation.

8. Patients sampling

Blood samples from human cases were collected from Benha University Hospital, Benha Teaching Hospital, Kasr Eleni Hospital, Pediatrics Abo-Elresh Hospital, Ain Shams University (Al-Damerdash) Hospital, and Theodore Bilharz Institute Hospital during the period from March 2016 to October 2017 and manipulated in the immune-parasitology department of TBRI, Giza Province, Egypt. Samples were divided into 3 groups; group 1 (hydatidosis group), group 2 (cross-reactivity group) and group 3 (control group).

- Group 1: blood samples from patients having hydatidosis proved by U/S, CT scan, IHAT and surgical excision of the cyst being confirmed by microscopic examination of hydatid cyst wall and sand.
- Group 2: blood samples from patients infected by *nana*, *E. vermicularis*, and *F. gigantica*, they are the patients who were negative to investigations in group 1 but stool analysis showed their previous parasitic infections.
- Group 3: blood samples from apparently normal individuals; workers and official personnel of the hospital who showed negative investigations and stool analysis.

Samples each of 5 ml were allowed to clot for 2 hours at room temperature and serum was separated and kept at – 20°C until used.

Data collection sheet was done by collecting data about age, sex, residence, and occupation. Clinical presentation (symptoms & signs) and personal hygiene (washing hands, vegetables & contact with dogs) data were recorded in addition to data collected from patients' sheets as cyst size, treatment, IHAT laboratory result thus titer above 1/160 was considered positive.

9. The Detection of pAg in patients' serum samples by sandwich ELISA

Coating of polystyrene microtiter plates wells (96-flat bottom wells, M 129A Dynatehc, Telangana, India) with 100 µl of a 20 µg/ml concentration of purified IgG ppAb which were incubated at room temperature for overnight, then washing 3 times with 0.1 M PBS/T, and pH was maintained at 7.4. Free sites in wells were blocked with 200 µl/well of BSA, pH at 7.4 for 2 hours at 37°C, then washed with buffer 3 times. Tested serum samples, each of 100 µl/well were added to wells and incubated for 2 hours at 37°C, then wells were washed 3 times. IgG ppAb conjugated with HRP in PBS/T (1/10 µg/ml) was added by 100 µl/well for 1 hour at room temperature, then wells were washed 3 times with washing buffer. Substrate solution was added by 100 µl/well for 30 min. in the dark at room temperature, then 50 µl/well of 8N H₂SO₄ was added to stop the enzyme-substrate reaction and then the absorbance was measured at 492 nm by ELISA reader (Bio-Rad microplate reader, Richmond, CA, USA).

10. Detection of circulating pAg in patients' serum samples by nanogold sandwich ELISA

Coating of polystyrene microtiter plates wells colored white for optical absorbance, was done with 100 µl of a 20 µg/ml concentration of purified IgG ppAb conjugated with AuNPs representing a capture antibody and diluted in 0.1 M carbonate buffer, pH 9.6 (Sigma, UK) for overnight at room temperature, then washed

3 times with 0.1 M PBS/T, pH 7.4. Blocking of wells was done by 100 µl/well 0.1 BSA for 2 hours at 37°C, then washed 3 times with PBS/T. Tested serum samples were pipetted into wells as 100 µl/well of each and incubated for 2 hours at 37°C, then washed 3 times. The remaining steps as above.

11. *Analysis of data*

Chi-square test (χ^2 -value) and Fisher exact test (FET) were used for inter-group comparison of categorical data. ROC curve was used to assess the validity of techniques thus including diagnostic sensitivity, specificity and predictive values [22]. Probability (P-value) was detect to know significance of the results as: if $P > 0.05$ (this was represented as non-significant difference), if $P \leq 0.05$ (significant difference) and if $P < 0.01$ (highly significant difference). Accuracy was detected as the percentage of agreement between both sensitivity and specificity. **Sensitivity (%)** = (No. of true +ve results/No. of +ve results + No. of false – ve results) x100, **specificity (%)** = (No. of true –ve results/No. of –ve results + No. of false +ve results) x 100, **positive predictive value (%)** = No. of true +ve results/No. of true +ve results + No. of false +ve results) x100 and **negative predictive value (%)** = No. of true -ve results/No. of true –ve results + No. of false –ve results) x100.

Results

1. *Microscopic examination:*

Surgically removed cysts from different organs (liver, spleen, and lung) of patients attending to the above-mentioned hospitals, were sent to the pathology department of TBRI for histopathological examination of cyst wall and hydatid fluid. Hydatid cyst wall showed protoscolices, germinal layer and laminated layer while the fluid showed scattered hooks, multiple invaginated protoscolices with hooks and evaginated protoscolices appearing with the suckers, rostellum with its hooks and body region (Figure 1).

2. *History and clinical data:*

Hydatid infection in males (51.9%) was higher than in females (48.1%), the highest age group acquired the infection was in group 21-30 years by total percentage 37.03% in both males and females and the lowest infected age groups were group 11-20 years and group 51-60 years by total percentage of 3.7% in both also (Table 1).

According to residence, the rural areas cases record higher percentage of infection (70.37%) than the urban areas cases (29.63%), in relation to animal contact the infected cases represented 59.26% of total hydatid infected cases while patients who were used to eat unwashed raw vegetables represented 55.56% of total hydatid cases. Farmers represented a higher percentage to get an infection (48.15%). On basis of IHA test reports associated with the cases, they revealed positivity (titer is 1/160 or more) in 51.9% of the total 25 hydatid infected cases as there are 2 cases that had no IHA test reports (Table 2).

The main symptom of hydatid infection in these cases was right hypochondriac pain with liver affection in 77.78% of cases being followed by spleen affection in 14.81% of cases, lung affection in 7.41% of cases (Table 3).

3. Results of ELISA techniques:

The results of sandwich ELISA technique in detection of protoscolices antigen in group 1 (hydatidosis group) showed positivity in 22 serum samples (81.48%) that is statistically significant while by using the nanogold sandwich ELISA technique the positivity in hydatidosis group reached to 26 serum samples (96.3%) which were statistically significant also. In group 2 (cross-reactivity group) sandwich ELISA gave 20% positive results while the nanogold sandwich ELISA gave only 5% positive results (Table 4)

The sensitivity of nanogold sandwich ELISA was higher than that of sandwich ELISA in detection of protoscolices antigen being (96.3% versus 81.5%), also the other validity tests were higher in nanogold sandwich ELISA technique than those in sandwich ELISA showing specificity (95% versus 80%), positive predictive value (96.3% versus 84.6%), negative predictive value (95% versus 76.2%) and accuracy (95% versus 80.9%) (Table 4, Figure 2).

Discussion

This study was aimed at detecting circulating hydatid protoscolices antigen using sandwich ELISA and highlighted the importance of nanogold sandwich ELISA in serodiagnosis after collecting clinical and historical data from patients involved in the study.

According to history and clinical data in our study, male infections was recorded by the percentage of 51.9% which was higher than female infection (48.1%) and these results agreed with those of [23] who said that hydatid infection was higher in males (72.03%) than in females (27.96%). This was explained by the many times of male exposure to infection sources as soil, vegetables, and dogs, which was reported by [24] that males were 2.5 times at higher risk than females in areas where males were in more contact with risk factors.

According to the age groups in this study, hydatidosis was the highest in age group 21–30 years (37.03%) followed by age group 41–50 years (33.33%) and these results were in the same line of [25] and [26] who revealed that the third decade of age was a marked period of life in some patients to show hydatid infection by percentages of 27.29% and 25.42% respectively. On the other side, it was found that the fourth decade recorded the highest age group of infection [23] but really it is challenging to detect the true age group of infection with hydatidosis as it is a chronic disease thus taking 20–30 years of incubation period to appear as clinical problem in the patient due to its slow progression [27].

This work revealed high incidence of hydatid infection in rural areas by 70.37%, which was reported before by [27] thus recording 54% of cases from rural areas and was said before by [28] that the ideal conditions of maintenance of *E. granulosus* life cycle were found in rural areas as these areas contain

large numbers of sheep and goats associated with stray dogs and home slaughters offering a fertile soil for hydatidosis spread.

Consumption of unwashed raw vegetables was a source of hydatid infection as proved by our study that 55.56% of hydatidosis patients were following this bad habit. Although washing of vegetables with water only didn't intercede to avoid hydatid infection [29], in the time that dogs were left to round freely in vegetable fields and contaminate these vegetables so eating raw vegetables carries a lot of risk of hydatid infection [30].

Studying the most common site in the body being affected by hydatidosis, we found in this work that it is the liver 77.78%, this was in agreement with [27] who ensured that the liver was the most commonly affected organ in addition to [25] who observed higher rate of cystic echinococcosis in the liver 46.3%, also the variation in clinical data of hydatidosis cases was represented in this work revealing that right hypochondriac abdominal pain as the most common symptom 51.85% while 14.8% of cases were asymptomatic especially splenic hydatidosis cases. This was reported by [23] and [31] who said that the right upper quadrant of the abdomen was the source of pain in hydatid infection cases.

Asymptomatic hydatidosis cases were the commonest finding by 22.03% in the study of [26] and all variations in clinical data were due to variable sizes and sites of hydatid cysts exerting different mass effects on surrounding organs and complications were related to cysts rupture [32]. Other studies like [33] showed that the patient's reaction to hydatid infection ranged from tolerance for a long period without any symptoms to sudden, intense acute symptoms.

All above mentioned clinical and history data didn't intercede for accurate diagnosis of hydatidosis so serological tests improvement is still needed especially detecting the circulating Echinococcus antigen in cystic echinococcosis patients [34] and introducing new materials like nanogold particles for functionalization of detecting antibodies used in ELISA techniques enhanced the sensitivity and reduced the analysis time of these techniques [35].

We used in this study nanogold particles to load the detecting ppAb in sandwich ELISA technique for the first time in hydatidosis serodiagnosis to detect diagnostic circulating antigens, lateral flow dipstick assay was used by [34] to prepare three hydatid antigens including hydatid cyst fluid, native antigen B and recombinant antigen B to raise polyclonal rabbit antiserum against each antigen and the IgG fractions were conjugated on gold nanoparticles to detect those antigens. The dot-immunogold staining method was used to detect antibodies against hydatid cyst disease in sheep by preparing antigen B which was labeled with gold nanoparticles and analyzed by SDS-PAGE thus nanoparticles produced a typical purple color by binding to the strip at the site of immunoreaction [36]. The mentioned two studies were the only studies used gold nanoparticles in serodiagnosis of hydatid disease so our work is considered as a pioneer in using nanogold sandwich ELISA for diagnosis of human hydatid disease.

Our study revealed positivity in the detection of protoscolices antigen in human serum samples by sandwich ELISA in 81.48% of cases and the results were improved on using the nanogold sandwich

ELISA technique in the same cases to reach 96.3%. Also on the scale of improving sensitivity and specificity of the serodiagnostic tests, it was found that the nanogold sandwich ELISA achieved 96.3% and 95% versus sandwich ELISA which achieved 81.5% and 80% for both sensitivity and specificity respectively. Our results were greatly higher than those of [37] which showed a sensitivity of ELISA in detecting circulating protoscolices antigen in humans as (52.5%). But we agreed with [3] who used paramagnetic nanoparticles in detection of protoscolices antigen in human serum samples and revealed that paramagnetic sandwich ELISA showed a sensitivity of 95.2% and a specificity of 95.5% while ordinary sandwich ELISA showed a sensitivity of 90.48% and a specificity of 91.3% for detection of human hydatid antigen. This proved that the usage of paramagnetic nanoparticles as protein conjugate achieved improvement in sandwich ELISA technique nearer to our results by using gold nanoparticles based sandwich ELISA although our study achieved higher sensitivity of the test.

On the scale of studying the predictive value of an immunodiagnostic test, its importance manifested in community screening and because of the low positive predictive value recorded for most of the serodiagnostic tests in human hydatidosis infection, this led to lack of confidence for community screening [38]. In this scope, this work achieved a high positive predictive value of sandwich ELISA in hydatid antigen detection as 84.6% which improved by nanogold sandwich ELISA to be 96.3% thus recommending this test for field study and community screening.

Concerning cross-reactivity in human hydatid cases with other parasitic infection, the cross-reactivity group in our study showed two *Hymenolepis nana* infected cases, one *Entrobis vermicularis* infected case and one *Fasciola gigantica* gave false-positive results by sandwich ELISA (20% of cross-reactivity group) and only one *Hymenlepis nana* infected case by nanogold sandwich ELISA (5% of cross-reactivity group), this was attributed to the common antigens shared between *Hymenlepis* and *Echinococcus* protoscolices antigens. Cross-reaction was noticed in few non-hydatidosis patients when indirect ELISA was used for antibody detection using antigen B [39]. Overestimation of the patients became a must due to cross-reactivity with other helminths to detect the parasite's true prevalence [40]. Sometimes false cross-reactivity may occur as in a case of taeniasis which showed cross-reaction with all hydatid antigens; hydatid cyst fluid, antigen B and recombinant antigen but this case was considered to be co-infected with *Taenia saginata* and *E. granulosus* so this leads us to exclude carefully the cross-reactivity cases from co-infection with hydatidosis [41]. Hydatid fluid immunochromatography showed the highest cross-reactivity (64.3%) in hydatidosis patients while those with alveolar echinococcosis showed the lowest cross-reactivity (9.5 to 16.7%) [2]. On the other side, no cross-reaction was observed by the study of [42] on using patients' sera from infected toxoplasmosis cases compared to *E. granulosus* infected patients' sera.

Conclusion:

The labeling of gold nanoparticles to sandwich ELISA test improved its sensitivity and specificity in the detection of hydatid protoscolices antigen in human serum samples and hence raised the serodiagnostic

confirmation of clinically infected patients. The nanogold sandwich ELISA improved the predictive value of the test and so became recommended for field study and community screening.

List Of Abbreviations

Abb.	Description	Abb.	Description
ELISA	Enzyme Linked ImmunoSorbent Assay	AuNPs	Gold nanoparticles
pAg	Protoscolices antigen	ppAb	Protoscolices polyclonal antibodies
DEAE-Sephadex	Diethyl amonoethyl Sephadex	HRP	Horseradish peroxidase
IgG	Immunoglobulin G	M	mole
PBS	Phosphate buffer saline	W	watt
°C	Degree centigrade	gm	gram
ml	milliter	HCL	Hydrochloric acid
mM	millimole	NaCl	Sodium chloride
nm	nanometer	USA	United states of America
i.m	Intramuscular injection	CA	California
(NH ₄) ₂ SO ₄	Ammonium sulfate solution	rpm	Round per minute
min.	minute	HAuCl ₄	Hydrogen tetrachlorlaurate
H ₂ O	water	UV/vis	Ultraviolet/visual
NHS	N-hydroxy Succinimide	EDC	1-Ethyl, 3-Dimethylaminopropyl Carbodiimide
MUA	Mercapto-undecanoic acid	BSA	Bovine serum albumin
μl	microliter	U/S	Ultrasound
IHAT	Indirect haemagglutination test	CT	Computerized topography
H. nana	Hymenolepis nana	E. vermicularis	Entrobisus vermicularis
F. gigantica	Fasciola gigantica	PBS/T	Phosphate buffer saline/Twin
UK	United kingdom	TBRI	Theodor Bilharz Research Institute
FET	Fisher exact test	ROC curve	receiver operating characteristic curve
SDS-PAGE	Sodium dodecyl sulfate- Polyacrylmide based discontinous gel	E. granulosus	Echinococcus granulosus

Declarations

Ethics approval and consent to participate

The Scientific Research Ethical Committee, College of Medicine, Benha University approved this work in 2016. Before taking samples, a written informed consent was obtained from all patients. The animal involved in this experiment was in line with the approval of the Medical Ethical Committee of Theodor Bilharz Research Institute (TBRI) in Egypt and per under the ethical guidelines approved by the Ethical Committee of the Federal Legislation and National Institutes of Health Guidelines in the USA.

Consent for publication

The Author grants the Publisher the sole and exclusive license of the full copyright in the

Contribution, which license the Publisher hereby accepts. Consequently, the Publisher shall have the exclusive right throughout the world to publish this article.

Availability of data and materials

The data that support the findings of this study are available from [Immunoparasitology department, TBRI] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [Immunoparasitology department, TBRI]. On behave of all authors I requested the data from HOD of immunoparasitology department and am waiting for his response.

Competing interest

The authors declare no conflict of interests.

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Author contributions

All authors contributed to the study design and organized the multicenter study. AE and IS collected specimens and contributed to the practical architecture of the work. SR, MN, NA and MMN shared in data analysis. WE shared in writing the manuscript and data analysis and contributed to the editing of the manuscript for publication. All authors read and approved this manuscript.

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Tables

Table (1): Age and sex distributions of hydatid infected human cases:

Age groups in years	Male		Female		Total No.	Total Percent	FET test	P-value
	No.	Percentage from total	No.	Percentage from total				
11-20	1	3.70%	0	0%	1	3.7%	3.05	0.68
21-30	6	22.2%	4	14.8%	10	37%		
31-40	2	7.4%	4	14.8%	6	22.2%		
41-50	4	14.8%	5	18.51%	9	33.3%		
51-60	1	3.7%	0	0%	1	3.7%		
Total	14	51.85%	13	48.15%	27	100		

Table (2): Personal history and IHA test results of hydatid infected human cases:

Personal data		No.	%	Total hydatid infected cases
Residence	Urban	8	29.63	27
	Rural	19	70.37	
Animal contact (dogs and cats)	Yes	16	59.26	27
	No	11	40.74	
Consumption of unwashed raw vegetables	Yes	15	55.56	27
	No	12	44.44	
Occupation	Farmer	13	48.15	27
	House wife	8	29.63	
	Employee	5	18.52	
	Student	1	3.70	
IHA test	Positive	14	51.9	27
	Negative	11	40.74	
	Not requested	2	7.41	

Table (3): Clinical data of different affected organs in hydatid infected human cases:

Affected organ	No.	Percentage	Symptoms	No
Liver	21	77.78%	Asymptomatic	1
			Right hypochondriac pain	14
			Abdominal colic	3
			Epigastric pain	3
Spleen	4	14.81%	Asymptomatic	3
			Left hypochondriac pain	1
Lung	2	7.41%	Cough, expectoration and wheezes, chest pain	2
Total	27	100%		27

Table (4): Sandwich ELISA and nanogold sandwich ELISA in the detection of protoscolices antigen:

Groups	Sandwich-ELISA				Nano-gold sandwich-ELISA			
	+ve cases		-ve cases		+ve cases		-ve cases	
	No	Percentage from total	No	Percentage from total	No	Percentage from total	No	Percentage from total
Hydatidosis group n=27	22	81.48%	5	18.52%	26	96.3%	1	3.7%
Cross-reactivity group n=20	4	20%	16	80%	1	5%	19	95%
control n=20	0	0%	20	100%	0	0%	20	100%
X2 test	36.37				59.05			
P-value	<0.001**				<0.001**			
Sensitivity	81.5%				96.3%			
Specificity	80%				95%			
PPV	84.6%				96.3%			
NPV	76.2%				95%			
Accuracy	80.9%				95%			

Figures

Figure 1

Microscopic examination of surgically removed cysts. Figure (1) legends: Associated keys/annotations: A) Multiple invaginated protoscolices with their hooks. B) An invaginated protoscolex with hooks (red arrow). C) *Echinococcus granulosus* hooks. D) Hydatid cyst wall: laminated layer (red arrow), germinal layer (blue arrow) and scolices (green arrow). E) Evaginated protoscolex showing the suckers, rostellum with its hooks and body region (the posterior part).

Figure 2

Sensitivity and specificity of sandwich ELISA versus nanogold sandwich ELISA for hydatidosis antigen detection in human cases. Figure (2) legends: ROC curve.

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