Original Research Article

The Egyptian Journal of Immunology

The Egyptian Journal of Immunology
Volume 29 (4), 2022: 84–93.
www.Ejimmunology.org

Conjugated silver nanoparticles as a diagnostic tool for circulating hydatid antigens

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Abstract

Cystic hydatid disease is one of the most significant worldwide zoonotic diseases. The causative agent is the larval stage of *Echinococcus granulosus*. The diagnosis of cystic echinococcosis by clinical symptoms and scanning alone is often difficult and confusing. The definite diagnosis needs sensitive and reliable serological tests. This study aimed to evaluate a nano silver-based enzyme linked immunosorbent assay (ELISA) in the detection of circulating hydatid antigen in human serum samples. The study included 66 human serum samples (36 hydatidosis confirmed cases, 15 cases infected with other parasites, and 15 normal subjects as negative control). The circulating protoscolices antigen was assayed by the nano-silver dot ELISA, nano-silver sandwich ELISA and the traditional methods (dot ELISA and sandwich ELISA). Our study revealed that the sensitivity and specificity of the nano-silver dot ELISA were 97.2% and 93.3%, respectively. The sensitivity and specificity of nano-silver sandwich ELISA were 94.4% and 96.7% respectively. The nano-silver-based ELISA showed higher sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy than the traditional ELISA. In conclusion, the nano-silver-based ELISA can be proposed as a confirmatory test in the diagnosis of cystic echinococcosis.

Keywords: Hydatid disease, Nanosilver, Protoscolices antigen & ELISA

Date received: 20 June 2022; **accepted:** 17 August 2022

Introduction

Cystic Hydatid disease is a worldwide zoonotic disease.[1] It has significant economic and public health problems especially in rural areas in several countries of the world,[2] it has a wide variety of domestic and wild intermediate and definitive hosts. [3] In Egypt, cystic hydatid disease is an endemic disease in animals and humans.[4]

Human infection occurs by direct contact with infected dogs that harbor adult *Echinococcus granulosus* in their intestine. Human and other intermediate hosts get infected by ingestion of *E. granulosus* eggs that contaminate food, water, hands, and the environment in general.[5] In the intestine, the eggs hatch, and the released embryos penetrate the intestinal mucosa, spread through the portal venous system, and lodge in different tissues, mainly

the liver and lungs as the main filtering organs, and the embryos transform into hydatid cysts.[6]

The fluid-filled hydatid cyst consists of an inner germinal layer surrounded by a very thick carbohydrate-rich acellular laminated layer. Various daughter cysts (brood capsules) bud off from the inner germinal layer and may remain attached or float free in the interior of the fluid-filled cyst. The individual protoscolices bud off from the inner wall of the daughter cysts; these protoscolices and free daughter cysts are called hydatid sand. The cyst induces an immune response, which triggers the formation of a host-derived adventitious capsule surrounding the laminated layer.[7]

The diagnosis and follow-up of cystic echinococcosis (CE) are first approached with imaging techniques. The World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) published standardized ultrasonography (US)-based classification of stage-specific cystic images with detailed cyst status as follows: active (CE1, CE2), transitional (CE3a and CE3b), and inactive (CE4 and CE5).[8]

The assessment of hydatid cyst activity level is through viability testing with light microscopy to observe the presence of integrity (eosin staining), motility of protoscolices (flame cell activity), the presence of intact brood capsules, and the intact architecture of the cyst, in particular of the germinal layer.[9] The parasite viability and cyst morphology are not always matching with the US-based classification, as shown through many studies of metabolite profiles by high-field magnetic resonance spectroscopy (MRS) in different cystic stages.[10]

Several studies that used the detection of IgG antibodies for CE diagnosis reported variable sensitivity and false-negative results. The false results may be due to several factors described by the different authors, including: early (CE1) and inactive (CE4 and CE5) cyst stages, cyst location other than the liver, single and small cysts, the antigenic source variability, and the parasite genotype.[11] The antigen detection in hydatidosis is useful for serodiagnosis and post-treatment follow-up.[12]
The application of nanotechnology in immunodiagnostic studies improves the sensitivity for the detection of pathogens and microorganisms.[13] Nanoparticles have proven to be useful for detecting infectious agents with low costs.[14] Nanoparticles are tiny materials having sizes ranging from 1 to 100 nm and possess unique chemical and physical properties due to their structure, large surface area, shape, and nanoscale size.[15] Nanomaterials provide interesting properties for sensing applications like the large surface area to volume ratio, high reactivity, enhanced electrical conductivity, and analyte-transducer contact. All these properties enable the quantification of low analyte concentrations in assays with improved performance.[16] Therefore, the present study aimed to evaluate the nano-silver-based enzyme-linked immunosorbent assay (ELISA) for the detection of hydatidosis antigen in human serum samples.

Subjects and Methods

The present study was carried out on 66 human patients attending El-Hussein University Hospital, Sayed Galal University Hospital, and Theodor Bilharz Research Institute during the period from April 2019 to June 2021. The samples were classified into 3 groups: Group I: 36 samples of hydatidosis confirmed cases by ultrasound, computed tomography (CT) scan, positive indirect heamaglutination test (IHAT), and surgical removal of hydatid cyst. Group II: 15 samples from cases infected with other parasitic infection (seven cases positive for *Hymenolepis nana*, three cases positive for Fasciola, five cases positive for *Ascaris lumbricoides*). The infection was confirmed by stool analysis. This group aimed to avoid cross-reactivity in the results. Group III: 15 samples of normal individuals as negative control. Cases of hydatidosis were inquired about the following data: Name, age, sex, residence, occupation, washing hands before eating, proper washing of row vegetables, fruits, and history of contact with animals such as dogs. The data were gathered in a questionnaire.

Serum samples collection

Five ml of blood was collected from each study participant. Blood samples from group I (hydatidosis confirmed cases) were collected

before treatment. They were allowed to clot at room temperature for 2 hours then, centrifuged at 2000 xg for 10 minutes. The serum was divided into small aliquots and stored at -20 °C until used.

Preparation of Protoscolices antigen and Purification

The hydatid fluid was collected from hydatid cysts dissected from the liver and lung of recently slaughtered sheep at El-Basatin abattoir. The Hydatid fluid was centrifuged at 1500 g for 30 min at 4 °C. The deposit was exposed to three freezing and thawing cycles, then washed with 0.15 M phosphate buffer saline (PBS), pH 7.2 and sonicated by 150 W ultrasonic disintegrator (Hielscher, Germany), for 10 sec with 5 sec intervals on ice until no intact protoscolices were visible. The preparation was then left on ice for one hour, and centrifuged for 30 min at 10,000 xg. The supernatant was split into aliquots and stored at - 20 °C.[17] Purification of the protoscolices antigen was done by using Diethyl-amino-ethyl Sephadex chromatography (DEAE-Sephadex G-50; Pharmacia, Uppsala, Sweden), binding buffers, NaCl salt, and Tris-HCL.[18] Then the protoscolices proteins were dialyzed versus the binding buffer. The protein content of the sample was estimated by Bio-Rad protein assay[19] and then passed through gel filtration chromatography on a Sephacryl-S-200 HR column.

Production and purification of polyclonal antibodies

The purified protoscolices antigens (1 mg) were emulsified with an equal volume (v/v) of Freund's complete adjuvant, and injected intramuscularly into a non-infected white male rabbit (2.5 kg).[20] A second injection of 0.5 mg of protoscolices antigens was given two weeks later. Two booster doses of 0.5 mg protoscolices antigens at weekly intervals were given. One week after the last booster dose, a blood sample was taken and centrifuged at 4000 xg for 20 min. The protein content was estimated by Bradford dye-binding procedure (Bio-Rad, Richmond, CA, USA). The serum containing polyclonal antibodies (IgG) was divided in aliquots and kept at -20°C. The reactivity of the antibodies against *Echinococcus* protoscolices antigens was assessed by indirect ELISA.[21] The absorbance was measured at 492 nm using an ELISA reader (BioRad microplate reader, Richmond co., Wilmington, USA). The cutoff point for positivity was defined by the mean optical density reading.

Labeling of anti-Echinococcal polyclonal antibody (IgG) with horseradish peroxidase

Horseradish peroxidase (HRP) is an enzyme used to amplify the signal in photometric assays. Five mg of HRP (Sigma-Aldrich, Inc.St. Louis, USA) was resuspended in 1.2 ml of distilled water. Sodium periodate (0.3 ml) was added and incubated at room temperature for 20 min. The HRP solution was dialyzed against 1 mM sodium acetate buffer. The HRP was added to 0.5 ml of the antibody solution. The mixture was incubated for two hours at room temperature. Sodium borohydride (100 µl) was added and the solution was incubated at 4 °C for two hours.[22]

Preparation of nano-silver conjugated polyclonal antibodies

Silver nanoparticles (AgNPs) (50 nm/particle) provided in a commercially covalent conjugation kits (Cytodiagnostics Inc. Burlington, Canada) were used to conjugate to IgG polyclonal antibodies. The covalent conjugation can increase the stability of the conjugates. A fresh solution of 1-Ethyl 3-dimethylaminopropyl carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) was prepared. Carboxyl silver nanoparticles solution was mixed with EDC/NHS solution and incubated for 30 minutes at room temperature to activate the particle surface, then centrifugation for 30 min. at 1800 g. The polyclonal IgG antibodies were added to the pellet and incubated for 2 hours on a rotary shaker to conjugate the antibodies to the activated silver nanoparticle surface. The protein content in the polyclonal IgG-AgNPs conjugates was determined by Bradford dye-binding procedure (Bio-Rad, Richmond, CA, USA).

Detection of circulating hydatid antigen in human sera samples by dot ELISA

A nitrocellulose (NC) membrane was transferred to the Bio-Dot apparatus (Bio-Rad. "Bio-Dot® Microfiltration Apparatus, California, USA) and washed, then coated with purified rabbit anti-echinococcal polyclonal IgG (10 µl) diluted (1/500) in carbonate buffer (pH 9.2). The membrane was blocked with 10 ul PBS (pH7.2) containing 2% BSA (HiMedia, Laboratories, GmbH, Einhausen, Germany). Positive and negative human sera were added and incubated for 1.5 h at 37°C with constant shaking. The membrane was incubated with polyclonal IgG conjugated to HRP for 45 min. at 37°C in dark with constant shaking**.** The NC membrane was removed from the Bio-Dot apparatus and washed. The NC membrane was immersed in a Diaminobenzidine substrate. The reaction was stopped just after the development of color**.** [23]

Detection of circulating hydatid antigen in patients' serum samples by nanosilver dot ELISA

The steps were the same as in the dot-ELISA except that the NC membrane was coated with 10 µl of purified polyclonal IgG conjugated with AgNPs.[24]

Detection of circulating hydatid antigen in human sera samples by sandwich ELISA

The wells of a microtiter plate were coated with 100 µl of a 20 µg/ml purified rabbit anti-echinococcal polyclonal IgG, then washed 3 times with 0.1 M PBS/Tween-20 (PBS/T). Free sites in wells were blocked with 200 µl/well of bovine serum albumin (BSA), pH at 7.4 for 2 hours at 37°C, then washed with PBS three times. A serum sample of 100 µl was added to test wells and incubated for 2 hours at 37°C, then wells were washed 3 times. Polyclonal IgG conjugated with HRP in PBS/T (1/10 µg/ml) was added (100 µl/well) and incubated for 1 hour at room temperature, then wells were washed 3 times with washing buffer. A total volume of 100 µl/well of the substrate solution (O-phenylene diamine dihydrochloride in 10 ml of PBS pH 7.2, containing 0.05% Tween-20 and 10 µl hydrogen peroxide) and incubated for 30 min. Sulfuric acid (50 µl/well) was added to stop the enzyme-substrate reaction (change the pH of the medium). The absorbance was measured at 492 nm by an ELISA reader (Bio-Rad microplate reader, Richmond, CA, USA).[25]

Detection of circulating hydatid antigen in patients' serum samples by nanosilver sandwich ELISA

This method followed that described in a previous study by Rashed et al., 2020.[26] Briefly, the wells of the microtiter plate were colored white with 100 µl of a 20 µg/ml of purified polyclonal IgG conjugated with AgNPs and diluted in 0.1 M carbonate buffer, pH 9.6 (Sigma, UK) overnight at room temperature, then washed 3 times. The steps were completed as in sandwich ELISA.[26]

Statistical analysis

Data were collected from all studied groups and statistically analyzed by using a statistical package for the Social Sciences Program, version 18 (SPSS Inc. Chicago, Illinois, USA). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy were calculated. A *P*-value of < 0.05 was considered significant.

Results

In the present study, there was a significant difference in residence between hydatid cases and controls (Table 1). A larger number of studied hydatidosis cases were from rural areas than urban areas (77.8 % versus 22.2%, respectively). Consumption of raw unwashed vegetables was 55.6% of hydatidosis patients and 58.3% had positive contact with dogs which are the main sources of infections (Table 2). Of the 36 hydatidosis serum samples, 35 (97.2 %) samples had circulating hydatid antigen as detected by nano-silver dot-ELISA which was significantly higher than these detected by dot ELISA, 32 (88.9%) ($P < 0.001$).

Table 1. Age, sex, and residence distributions of studied groups.

		Group I (Hydatid infected group) (N=36)		Group II (Other parasites infected group) (N=15)		Group III (Control group) (N=15)		*P* value
		No.	%	No.	%	No.	%	
Age mean ± SD		37.4 ± 19.9		31.5 ± 22.7		26.1 ± 14.6		NS
Gender	Male	25	69.4	9	60	8	53.3	NS
	Female	11	30.6	6	40	7	46.7	
Residence	Urban	8	22.2	10	66.7	12	80	0.00014
	Rural	28	77.8	5	33.3	3	20	

P > 0.05 is not significant (NS).

Table 2. Risk factors and personal demographic data of the 36 hydatid infected patients.

Studied parameter		Hydatid infected group	
		NO	%
Age / year	10 - 20	4	11.1
	12 - 30	9	25.0
	31 - 40	15	41.7
	41 - 50	5	13.9
	> 50	3	8.3
Sex	Male	25	69.4
	Female	11	30.6
Residence	Urban	8	22.2
	Rural	28	77.8
Occupation	Farmer	19	52.8
	Employee	5	13.9
	Student	3	8.3
	Housewife	9	25.0
Eating raw unwashed vegetables	Yes	20	55.6
	No	16	44.4
Contact with animals (Dogs)	Yes	21	58.3
	No	15	41.7

Of the 15 serum samples in group II (other parasitic infections) the dot-ELISA was positive in 4 samples (3 cases were infected with *H. nana* and one case infected with *Ascaris lumbricoides*), whereas the nano-silver dot-ELISA was positive in 2 serum samples (one case infected with *H. nana* and the other infected with *Ascaris lumbricoides*). The cross-reactivity was reduced by using the nano-silver dot-ELISA. All 15 samples in group III (negative control), were negative for circulating hydatid antigen by either dot ELISA or nano-silver dot-ELISA.

The nano-silver dot-ELISA had statistically significant higher values than dot-ELISA regarding the sensitivity (97.2% versus 88.9%), the specificity (93.3% versus 86.7%), PPV (94.6% versus 88.9%), NPV (96.6% versus 86.7%) and the diagnostic accuracy (95.5% versus 87.9%) (Figure 1).

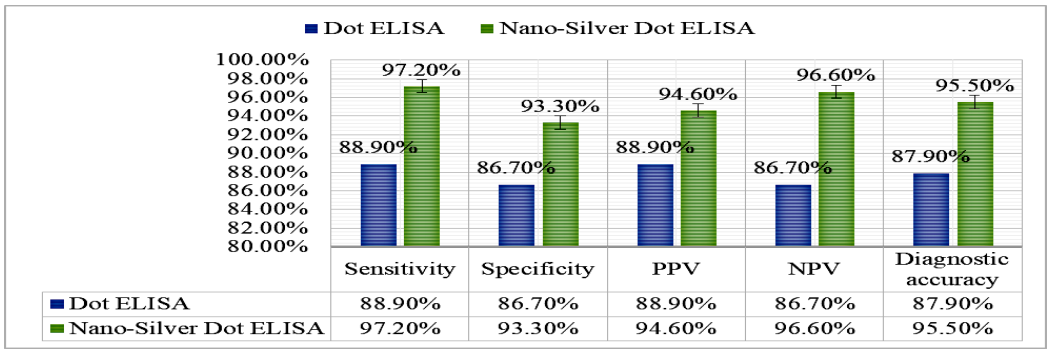

Figure 1. Comparison of the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy of nano-silver Dot ELISA versus Dot ELISA.

Of the 36 hydatidosis serum samples, the nano-silver sandwich-ELISA detected circulating hydatid antigen in 34 (94.4%) serum samples which were significantly higher than those detected by sandwich-ELISA, 31 (86.1%) ($P < 0.001$).

Of the 15 serum samples in group II (other parasitic infections) the sandwich-ELISA was positive for the circulating hydatid antigen in 3 serum samples (2 cases infected with *H. nana* and one case with *A. lumbricoides*), whereas the nano-silver sandwich-ELISA was positive in one serum sample (infected with *H. nana*) that was detected by the sandwich-ELISA.

The nano-silver sandwich-ELISA had statistically significant higher values than sandwich-ELISA regarding the sensitivity (94.4% versus 86.1%), the specificity (96.7% versus 90 %), PPV (97.1% versus 91.2%), NPV (93.5% versus 84.4%) and the diagnostic accuracy (95.5% versus 87.8%) (Figure 2 and Table 3).

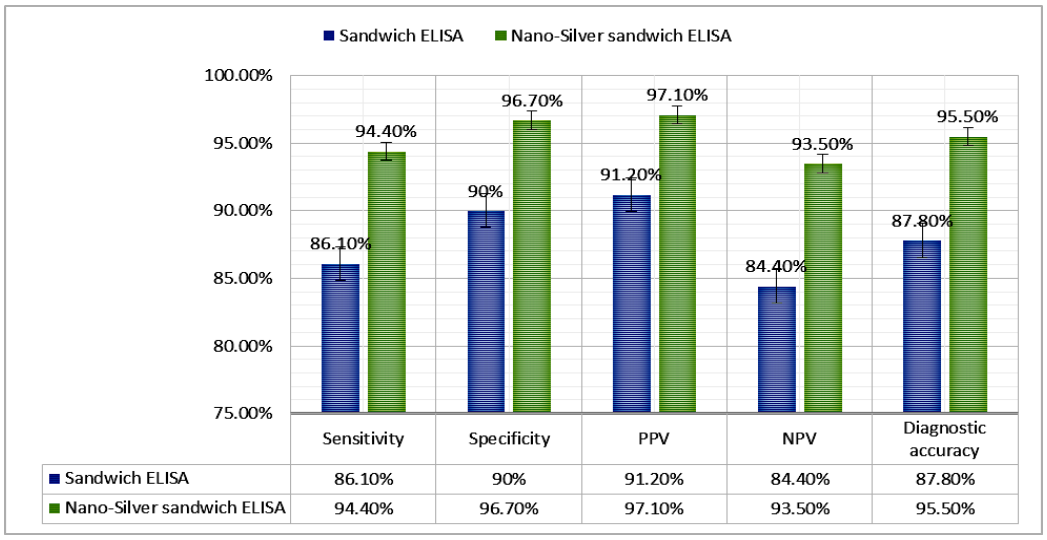

Figure 2. Comparison of the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy of nano-silver sandwich-ELISA versus sandwich ELISA.

Table 3. Comparison between dot-ELISA and nano-silver dot-ELISA versus sandwich ELISA and nano-silver sandwich ELISA for detection of circulating hydatid antigen in studied groups.

		Group I (Hydatid infected) No.=36	Group II (Other parasites infected) No.=15	Group III (Negative control) No.=15	*P value
Dot ELISA	No. (+ve)	32	4	0	< 0.001
	% (+ve)	88.9	26.7	0	
	No.(-ve)	4	11	15	
	%(-ve)	11.1	73.3	100	
Nano-Silver Dot ELISA	No. (+ve)	35	2	0	< 0.0001
	% (+ve)	97.2	13.3	0	
	No.(-ve)	1	13	15	
	%(-ve)	2.8	86.7	100	
Sandwich ELISA	No. (+ve)	31	3	0	< 0.001
	% (+ve)	86.1	20	0	
	No.(-ve)	5	12	15	
	%(-ve)	13.9	80	100	
Nano-Silver sandwich ELISA	No. (+ve)	34	1	0	< 0.0001
	% (+ve)	94.4	6.7	0	
	No.(-ve)	2	14	15	
	%(-ve)	5.6	92.3	100	

*$P \leq 0.05$ is significant.

Discussion

Protoscolices antigens are native meta cestode-derived antigens that show substantial (more than 90%) sensitivities in the diagnosis of CE. The composition of purified protoscolices is less dependent on the stage of cysts and contains fewer host-derived proteins, therefore yielding a higher reproducibility of results.[27] The antigens prepared from protoscolices of hydatid cyst are suitable for the serological diagnosis of human CE with a sensitivity of 93%.[28]

The positive circulating protoscolices antigen indicates active infection and turns negative in patients treated by surgical removal or chemotherapy.[29] The ELISA yields the best performance as a diagnostic method for the detection of the metacestode antigen.[30]

Some strategies have been explored to enhance the detection limit of ELISA such as redox complexes, electroactive molecules, metal ions, and nanotechnology-based strategies. Nanoparticles can serve as excellent carriers for specific recognition molecules such as probes or reporter molecules as well antibodies. They present more binding sites for capturing elements and for reporting tags leading to amplification of the analytical signal in a single recognition reaction.[31]

In the current study, the circulating protoscolices antigen of *E. granulosus* in the sera of the studied groups was detected by using polyclonal IgG antibodies produced against the protoscolices antigen. The detection of the circulating protoscolices antigen was done by dot-ELISA, nano-silver dot-ELISA,

sandwich-ELISA, and nano-silver sandwich-ELISA.

The present study showed that the detection of circulating hydatid antigen in serum samples by dot-ELISA was 88.9% in hydatidosis confirmed cases. However, the nano-silver dot-ELISA showed significantly higher positivity (97.2 %) ($P < 0.0001$). The dot -ELISA showed cross-reactivity with 4 cases of other parasitic infections, but by using the nano silver-dot ELISA the cross-reactivity was reduced to 2 cases. The validity of our results is supported by the results of another study by Abou-Elhakam et al., 2016.[32] They reported that the hydatid antigen was detected in 96% of patient sera using dot-ELISA, they were positive for *Echinococcus* spp. with sensitivity and specificity of 96% and 94%, respectively.[32]

The current study showed that the detection of the circulating hydatid antigen in sera by sandwich-ELISA, was positive in 86.1% of confirmed cases, whereas nano-silver sandwich-ELISA detected significantly higher positive cases (94.4%) ($P< 0.0001$). The sandwich-ELISA showed cross-reactivity in 3 cases infected with other parasitic infections, but by using the nanosilver-sandwich-ELISA the cross-reactivity was reduced to one case.

Nanomaterials-based detection technology has advanced over the conventional methods in selectivity and sensitivity. Nanomaterials work on the same scale as biological processes with different substrates.[33] Silver nanoparticles are one of the most vital nanomaterials among several metallic nanoparticles that are involved in biomedical applications due to their biological, optical, thermal, and high electrical conductivity properties.[34]

The high sensitivity and specificity of the nano-silver dot-ELISA and nano-silver sandwich-ELISA in comparison with the traditional dot-ELISA and Sandwich-ELISA, promoting the early serological diagnosis of hydatid disease and improves the discrimination between recent and past hydatid infections. The better performance of the nano particles-based ELISA as a diagnostic tool for circulating hydatid antigen is explained by the good utilization of silver nano-particles as antibody carriers. The large surface area of the silver nano-particles enables the capture of a low concentration of circulating Hydatid antigen.

In conclusion, the hydatid disease continues to threaten humans and animals in many countries all over the world, including Egypt. Based on our findings, the nano-silver dot-ELISA and nano-silver sandwich-ELISA techniques, were more sensitive, specific assays than traditional dot-ELISA and sandwich-ELISA for the detection of protoscolices antigen. Therefore, they can be proposed as complementary and confirmatory tests with imaging in the diagnosis of cystic echinococcosis.

Acknowledgements

All authors offer sincere appreciation to staff at El-Hussein University Hospital, Sayed Galal University Hospital, and Theodor Bilharz Research Institute for providing samples.

Author Contributions

GA and MS conceived and designed the research. IS and GA conducted the experiments. MS, IS, and AH analyzed the data and drafted the manuscript. GA, MS, AH and IS made critical reviews and approved the final version.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) denies receipt of any financial support for the research, authorship, and/or publication of this article.

Ethical approval

The protocol of the study was reviewed and approved by the Research Ethical Committee of the Al Azhar Faculty of Medicine, Al Azhar University (approval date; January 2, 2019).

Informed consent

Informed written consent was obtained from all participants included in the study.

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