

Performance of quantum dot nanobeads-based immunoassay in diagnosis of toxoplasmosis

Original
Article

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ABSTRACT

Background: Diagnosis of toxoplasmosis relies mainly on determination of IgG and IgM using ELISA. However, inter-laboratory evaluation of performance of many commercial kits gave unsatisfactory agreements. Merging the innovative use of quantum dots nanobeads (QDNBs) immunoassay may yield higher sensitivity and specificity than the conventional used methods.

Objective: The present study aims to evaluate the performance of QDNBs based immunoassay in serodiagnosis of human toxoplasmosis.

Subjects and Methods: The study included 175 subjects divided into three categories; 122 patients with clinical manifestations suggestive of toxoplasmosis; 32 patients proved positive for other parasitic infections, and 21 healthy control subjects. In comparison to Sabin Feldman dye test (SFDT), as a gold standard test, serum samples were examined in an immunoassay utilizing QDNBs for detection of *T. gondii* specific IgG and IgM antibodies.

Results: Compared to SFDT, combined QDNBs-IgG/IgM immunoassay exhibited sensitivity of 95.4%, specificity of 98.9%, positive predictive value (PPV) of 98.8%, and negative predictive value (NPV) of 95.6%, with relative agreement, validity, and accuracy of almost 97%.

Conclusion: It was concluded that QDNBs-based immunoassay improves the performance of ELISA particularly sensitivity as well as shorten the assay time.

Keywords: diagnosis; human toxoplasmosis; immunoassay; quantum dots; *T. gondii*.

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INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan that can infect any nucleated cell in nearly all warm-blooded animals, including human^[1]. In Egypt, sero-prevalence rates of 68% and 26% have been recorded among pregnant females and meningoencephalitic patients, respectively^[2]. Toxoplasmosis in neonates may result in severe neurological birth defects, including mental retardation and blindness^[3]. Also, infection in immunocompromised patients can lead to serious complications as encephalitis, chorioretinitis, pneumonitis and disseminated infection with multiple-organ damage^[4].

Serodiagnosis by detection of specific antibodies remains the cornerstone for diagnosis of toxoplasmosis. However, serodiagnosis is confronted with many problems due to difficulty of interpretation of the tests' results and in determination of infection status; acute, chronic, congenital, reactivated. Sometimes, conventional assays may require complementary testing in reference laboratories

where sophisticated techniques and experienced personals are available^[5,6]. Moreover, false positive, negative, and discrepant results between different commercial kits were recorded^[7-9].

In an attempt to identify the interassay variability for quantification of anti-*Toxoplasma* IgG, no perfect correlation was found between any two of five immunoassays used^[10]. Large differences in anti-*Toxoplasma* IgG titers were observed among the 5 immunoassays using serum samples from individual patients, from WHO international reference standards, and from standards and calibrators provided by the different five manufacturers. This highlights the importance of an accurate and reliable test for accurate diagnosis of toxoplasmosis, and hence the development of new serological assays.

Nanotechnology is of great use for medical diagnosis, and various nanoparticles (NPs) have exhibited tremendous potential for detecting disease markers^[11]. The convergence of nanotechnology and

immunoassay has led to remarkable improvements in assay sensitivity and ease of operation^[12]. Quantum dots (QDs) are NPs with a size of 2-10 nm and characterized by a large surface area to volume ratio, high loading capacity, facile synthesis, chemical stability, and ease of bioconjugation^[13].

Aly *et al.*^[14] used QDNBs ELISA for diagnosis of cystic echinococcosis and recorded a sensitivity of 91.8%, and a specificity of 88.8% in comparison to 85.7% and 84%, respectively for traditional ELISA. Also, QDNBs based immunoassay was evaluated for detection of histidine rich protein-2 antigen for diagnosis of *falciparum* malaria and the results showed that the test allowed sensitive and quantitative measurement of the target antigen^[15]. In the same context, NPs were generally utilized in order to improve the serological diagnosis of toxoplasmosis; e.g., the use of gold nanoparticles (AuNPs) was reported several times in the development of new diagnostic tools for toxoplasmosis^[16,17]. The aim of the present study is to evaluate the performance of QDNBs based immunoassay, as novel test, in serodiagnosis of human toxoplasmosis.

MATERIAL AND METHODS

This descriptive analytical study was conducted in Medical Parasitology Departments of Ain Shams, and Alexandria Universities, and Theodor Bilharz Research Institute, during the period from June 2018 to April 2021.

Study design: Blood samples were collected from patients with suggestive clinical manifestations of toxoplasmosis, and patients proved positive for other parasitic infections, as well as apparently healthy controls. Sera were examined using SFDT, and accordingly the study subjects were divided into five groups. Sera were also examined utilizing a prepared immunoassay composed of a probe formed of cadmium selenide/zinc sulfide (CdSe/ZnS)-QDNBs conjugated to goat polyclonal antihuman IgG and IgM-horseradish peroxidase (HRP) labeled conjugates for detection of *T. gondii* specific IgG and IgM antibodies, respectively. Sensitivity, specificity, PPV, NPV, relative agreements, validity, and accuracy of QDNBs were calculated in comparison to SFDT.

Study subjects: Blood samples (5 ml) were collected from inpatients and outpatients attending Ain Shams University Hospitals who had signs and symptoms suggestive of toxoplasmosis such as generalized and cervical lymphadenopathy, complicated pregnancies, delivery of infants with congenital malformations and immunocompromised patients. Blood samples were also collected from patients with collagen diseases and schizophrenic patients. This category of patients included 122 subjects. Study subjects also included patients with known other parasitic infections (32

control positive), as well as apparently healthy individuals (21 control negative). Sera were separated from all samples, aliquoted and labeled and kept frozen at -20°C until tested.

Study groups: According to results of SFDT the patients and control subjects were classified into the following groups:

- **Group 1a:** Symptomatic patients with sera positive for *Toxoplasma* antibodies with titers $>1:16$ - $<1:1024$ suggestive of chronic infections.
- **Group 1b:** Symptomatic patients with serum positive for *Toxoplasma* antibodies with titers $\geq 1:1024$ suggestive of acute infections.
- **Group 2:** Symptomatic patients with serum samples negative for *Toxoplasma* antibodies.
- **Group 3:** Negative for *Toxoplasma* antibodies and proved positive for other parasitic diseases by serology and/or direct parasitological methods (cystic echinococcosis, cryptosporidiosis, malaria, microsporidiosis, amoebiasis, giardiasis, urinary and intestinal schistosomiasis, and fascioliasis).
- **Group 4:** Apparently healthy non-symptomatic control subjects.

SFDT: All collected sera were first screened by SFDT^[18] with modifications according to Hoshina *et al.*^[19].

Sera examination for rheumatoid factor (RF): As RF is known to give false positive results in indirect IgM-ELISA assays, all sera were examined for RF by latex quick agglutination test (Avitex, Omega Diagnostics, UK). Positive samples were subjected to adsorption of RF using Serion RF adsorption kit according to the manufacturer specification (Serion Immunodiagnostica GmbH, Germany).

Synthesis and characterization of CdSe/ZnS-QDNBs: CdSe QDs were prepared according to Xing *et al.*^[20], followed by the shell formation step according to Dabbousi *et al.* and Xie *et al.*^[21,22], annealing at 260°C for 20 min, then cooling to room temperature. Finally, core/shell QDs were isolated from solution with chloroform (Pubchem, USA) and methanol by centrifugation, washed three times in deionized water, and stored overnight in chloroform. Then the resulting NBs with polystyrene-co-maleic anhydride (PSMA) as matrix polymer were pelleted at 12,000 rpm for 10 min and the supernatant was discarded. The pellet of NBs was washed three times in deionized water.

QDNBs were characterized by high resolution transmission electron microscope HR-TEM (Joel-Jem- 2100, Japan), Fourier-transform infrared (FTIR) spectroscope (Thermo fisher scientific, USA), powder X-Ray crystallography (Thermo fisher scientific, USA), Zeta potential analyzer (Jinan Winner Particle Instrument Stock Co., China) and Ultraviolet/ visible (UV/Vis) spectrophotometry (Schimadzu UV-1280, Japan).

QDNBs-anti-human-IgG/IgM-HRP conjugates: Goat polyclonal antihuman IgG and antihuman IgM were conjugated to HRP using periodate method according to Tijssen and Kurstak^[23]. This was followed by conjugation of anti-human(IgG/IgM) HRP conjugates to QDNBs according to Zhang *et al.*^[24]. The antihuman IgG and IgM-HRP conjugates (120 µg) were added to activated QDNBs and the mixture was incubated at room temperature for 2 h. To confirm the binding of the QDNBs to HRP labeled antibodies, the fluorescence spectrum of QDNBs was measured before and after coupling with the antibodies.

Optimization of the immunoassay: Preliminary check board titrations were done to determine optimal antigen concentration, serum dilution and QDNBs anti-human-IgG and IgM-HRP conjugates dilution using antigen concentrations of (1, 5, 10, 15, 20, 25 and 30) µg /ml, serum dilutions of (1/25, 1/50, 1/100, 1/200, 1/400, 1/600 and 1/1200) and QDNBs conjugates dilutions of (1/250, 1/500, 1/1000, 1/1500, 1/2000, 1/2500 and 1/3000)^[25]. Optical density (OD) signal developing time was detected after adding the QDNBs conjugates for 1 h and 30 min^[26]. Different incubation time-periods with the o-phenylenediamine dihydrochloride (OPD) were tested for satisfactory development of the color reaction.

QDNBs-based immunoassay for detection of IgG and IgM antibodies in tested sera: The immunoassay was performed according to Gheshlaghchaei *et al.*^[26] and Ciaurriz *et al.*^[27] with QDNBs used instead of gold NBs. Antigen was prepared according to Hegazy *et al.*^[28]. Antigen concentration, serum, and QDNBs conjugates dilutions and incubation times for each were performed according to results of preliminary check board titrations. Samples were tested in duplicates. The plates were read at 492 nm using ELISA reader and the results were expressed in the form of OD. Cutoff value for best discrimination between positive and negative results was determined by calculating mean and standard deviations for 18 *Toxoplasma* negative IgG and IgM serum samples. Samples that gave OD > mean ± 2 SD were considered positive.

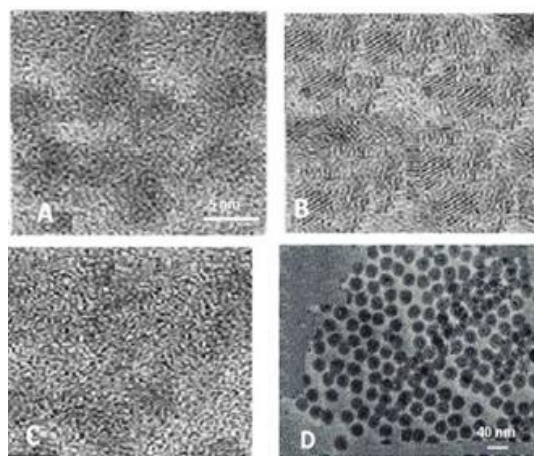


Fig. 1 (A-D). HR-TEM images of CdSe/ZnS-QDNBs. A-C show regularly shaped, sufficiently mono distributed and well separated QDs with a darker core surrounded by the shell. D shows successful encapsulation of QDs into polystyrene-co-maleic anhydride PSMA where QDs were dispersed in each NB.

Statistical analysis: The collected data was revised, coded, tabulated and analyzed by the appropriate statistical tests using Statistical Package for Social Science (SPSS 20). Tests used included Chi-square test and Fisher's exact test. Sensitivity, specificity, PPV, NPV, validity and diagnostic accuracy were calculated for QDNBs immunoassay.

Ethical considerations: All subjects under the study were informed by the objective of the present work and consent was taken from each participant according to the regulations of the Egyptian Ministry of Higher Education and after having the required approval from the ethical committee of the Faculty of Medicine, Ain Shams University.

RESULTS

Characterization of CdSe/ZnS-QDNBs: Studying of CdSe/ZnS by high resolution transmission electron microscopy (HR-TEM) showed that the prepared QDs were regular in shape, sufficiently mono distributed and well separated (Fig. 1 A-C). There was successful encapsulation of QDs into PSMA with estimated average size of about 60 nm and narrow size distribution (Fig. 1D).

The Fourier-transform infrared (FTIR) spectroscopy analysis showed a prominent absorption band at $1702 \pm 10 \text{ cm}^{-1}$ in CdSe/ZnS QDs that was due to the C=O stretch of carboxyl group and vibrations at 4257 and 4300 cm^{-1} due to presence of hydroxyl (-OH) group in CdSe/ZnS. This confirmed that QDs were water soluble and biocompatible (Fig. 2). The core/shell structure was identified by investigating the crystallographic properties by powder X-ray diffraction (XRD) (Fig 3). The zeta potential value was approximately -30.2 mV at peak and it was shifted after successful conjugation to polyclonal antihuman IgG/ IgM antibodies-HRP conjugates (Fig.4). Ultraviolet/visible (UV/Vis) spectrophotometer was used to qualitatively characterize the successful conjugation of QDNBs to antihuman IgG/IgM antibodies HRP conjugates and measure the concentration of QDNBs which was 2.33×10^8 particles/ml as calculated by Beer's law (Fig.5).

Number of QDs in each NB was estimated as 501.9 QDs/bead and the number of HRP conjugated antibodies, per QDNBs (N) was approximately 37 HRP-IgG antibodies/QDNB, and 33 HRP-IgM antibodies/QDNB.

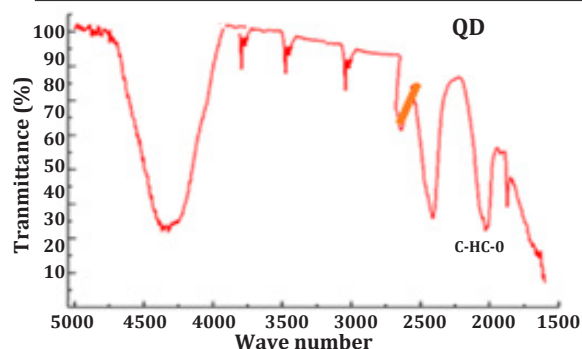


Fig. 2. Result of FTIR spectra analysis of QDNBs.

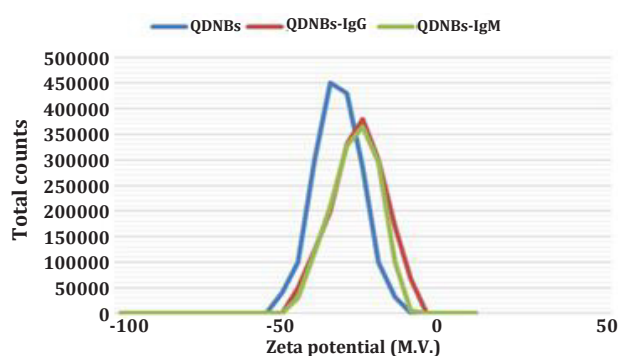


Fig. 4. Graph represents the zeta potential of CdSe/ZnS-QDNBs (blue line), CdSe/ZnS-QDNBs/IgG (red line), and CdSe/ZnS-QDNBs/IgM (green line).

Optimization of the immunoassay: The optimal antigen concentration, serum and conjugates dilutions were determined by preliminary check board titrations. It was observed that antigen concentration of 15 µg/ml coating buffer, serum dilution of 1/200 in PBS-T and QDNBs-antihuman-IgG/IgM-HRP of 1/2000 PBS-T were optimal for running the immunoassay. The optimal incubation time of QDNBs antihuman-IgG/IgM-HRP conjugates with serum samples was 30 min. Five min were found to be optimal for the development of color reaction after the addition of the substrate. Cutoff value for positive samples were estimated for each run by calculating the mean ODs of 18 negative samples + 2 SD.

Inter-assay precision of QDNBs based immunoassay: This was assessed by running the same four serum *Toxoplasma*-IgG antibodies positive samples on four different days. They gave acceptable average coefficient of variation (CV%) of 3.56%.

Screening of patients and control subjects for anti-Toxoplasma antibodies by SFDT and RF by latex agglutination (Table 1): Out of 175 study and control subjects screened by SFDT, 87 (49.7 %) gave positive results. Of these, 27 patients (15.4%) exhibited titers more than 1/1024, and 60 (34.3%): 56 patients and 4 healthy control subjects, exhibited titers ranging from 1/16-1/256. The highest seropositivity was among patients complaining of lymphadenopathy

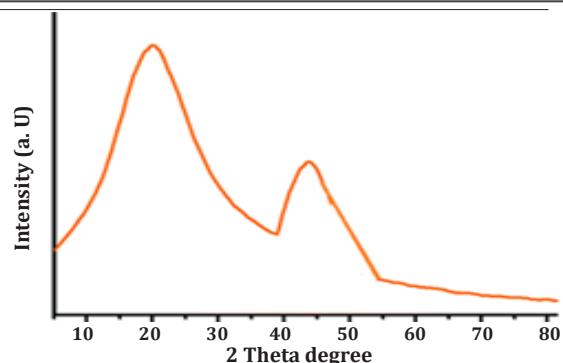


Fig. 3. X-ray powder diffraction patterns of prepared CdSe/ZnS-QDNBs.

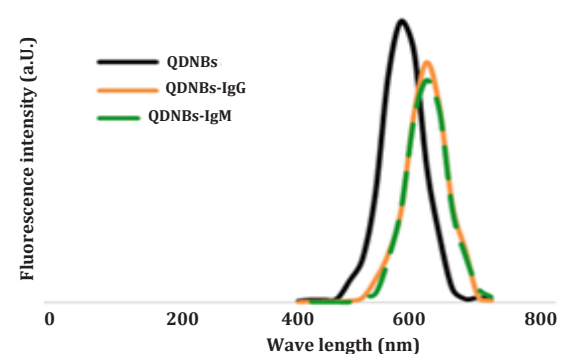


Fig. 5. Graph showing emission spectra of bare QDs (black line), QD-HRP-anti-IgG conjugates (orange line), and QD-HRP-anti-IgM conjugates (dashed green line).

(81.8%), followed by complicated pregnancies (80%), schizophrenia (75%), collagen diseases (69.6%), congenital diseases (60.7%), and immunosuppression (44.4%). The only one case of chorioretinitis examined and 4 cases of the healthy control subjects reacted positively in SFDT with titers 1/16-1/128. Screening of the 175 sera for RF, revealed that 41 (23.4%) were seropositive mainly among patients with collagen diseases (69.6%), followed by patients with schizophrenia (62.5%), lymphadenopathy (45.5%), complicated pregnancies (32%), and immunosuppression (11.1%).

Clinical presentation and demographic data of the groups under study (Table 2): The clinical presentation, age range and median, and gender of the patients and subjects belonging to the five groups under study were recorded.

Comparison of SFDT and QDNBs-IgG/IgM immunoassays (Table 3): For group 1a, out of 56 patients reacted positively in SFDT with titers ranging from 1/16-1/256, 52 (92.8%) reacted positively in QDNBs-IgG immunoassay and none (0%) gave positive reaction in QDNBs-IgM immunoassay. Out of the 27 patients of group 1b who gave titers \geq 1/1024 in SFDT, 27 (100%) and 19 (70.4 %) reacted positively in QDNBs-IgG and QDNBs-IgM immunoassays, respectively. For group 2, out of the 39 symptomatic patients with negative SFDT, only 1 (2.6%) gave positive reaction

Table 1. Results of SFDT and rheumatoid factor screening for patients and control subjects.

Clinical presentation (No.)	SFDT		SFDT Titers					RF
	Positive	1/16	1/32	1/64	1/128	1/256	>1/1024	Positive
	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)
Complicated pregnancies (25)	20 (80)	2 (8)	1 (4)	1 (4)	0 (0)	0 (0)	16 (64)	8 (32)
Congenital diseases (28)	17 (60.7)	4 (14.3)	5 (17.9)	0 (0)	1 (3.6)	0 (0)	7 (25)	0 (0)
Chorioretinitis (1)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Lymphadenopathy (11)	9 (81.8)	1 (9.1)	0 (0)	3 (27.3)	1 (9.1)	2 (18.2)	2 (18.2)	5 (45.5)
Immunosuppression (18)	8 (44.4)	1 (5.6)	0 (0)	2 (11.1)	0 (0)	4 (22.2)	1 (5.6)	2 (11.1)
Schizophrenia (16)	12 (75)	4 (25)	2 (12.5)	3 (18.8)	2 (12.5)	1 (6.25)	0 (0)	10 (62.5)
Collagen diseases (23)	16 (69.6)	6 (26.1)	4 (17.4)	3 (13.1)	2 (8.7)	0 (0)	1 (4.3)	16 (69.6)
Other parasitic infections (32)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Apparently healthy controls (21)	4 (19)	2 (9.5)	0 (0)	1 (4.8)	1 (4.8)	0 (0)	0 (0)	0 (0)
Total (175)	87 (49.7)	21 (12)	12 (6.9)	13 (7.4)	6 (3.4)	8 (4.6)	27 (15.4)	41 (23.4)

SFDT: Sabin Feldman dye test; RF: Rheumatoid factor.

Table 2. Clinical presentation and demographic data of the groups under study.

Groups (No.)	Clinical presentations: No.(%)	Age		Gender	
		Range	Mean ± SD	Male	Female
		D: Day; M: Monthe; Y: Year		No.(%)	No.(%)
1a (56)	Complicated pregnancy: 4 (7.1)	18-30 Y	21.5 ± 4.4 Y	0 (0)	4 (7.1)
	Congenital diseases: 10 (17.8)	3 D-1 Y	16 D +0.28 Y	7 (12.5)	3 (5.4)
	Chorioretinitis: 1 (1.8)	10 Y	-	0 (0)	1 (1.8)
	Lymphadenopathy: 7 (12.5)	20-46 Y	36 ± 8.23 Y	6 (10.7)	1 (1.8)
	Immunosuppression: 7 (12.5)	18-47 Y	28+10.7 Y	1 (1.8)	6 (10.7)
	Schizophrenia: 12 (21.4)	3-48 Y	34.5 ± 8.9 Y	11 (19.6)	1 (1.8)
	Collagen diseases: 15 (26.8)	19-48 Y	32 ± 8.3 Y	5 (7.7)	10 (17.8)
1b (27)	Complicated pregnancy: 16 (59.3)	17-35 Y	24 ± 5.4 Y	0 (0)	16 (59.3)
	Congenital diseases: 7 (25.9)	1 D -5 m	18 ± 49.2 D	5 (18.5)	2 (7.4)
	Lymphadenopathy: 2 (7.4)	47-53 Y	50 ± 3 Y	1 (3.7)	1 (3.7)
	Immunosuppression: 1 (3.7)	20 Y	-	0 (0)	1 (3.7)
	Collagen diseases: 1 (3.7)	36 Y	-	0 (0)	1 (3.7)
2 (39)	Complicated pregnancy: 5 (12.8)	20-39 Y	23 ± 6.9 Y	0 (0)	5 (12.8)
	Congenital diseases: 11 (28.2)	3 D-6 Y	6 M ± 1.1 Y	5 (12.8)	6 (15.3)
	Lymphadenopathy: 2 (5.2)	42-63 Y	52 ± 10 Y	1 (2.6)	1 (2.6)
	Immunosuppression: 10 (37.0)	18-48 Y	41 ± 9.6 Y	5 (12.8)	5 (12.8)
	Schizophrenia: 4 (10.4)	17-34 Y	26 ± 6 Y	2 (5.1)	2 (5.1)
	Collagen diseases: 7 (25.9)	23-36 Y	27 ± 4 Y	0 (0)	7 (17.9)
3 (32)	Cystic echinococcosis: 12 (37.5)	29-65 Y	44 ± 11 Y	9 (28.1)	3 (9.4)
	Intestinal schistosomiasis: 5 (15.6)	19-63 Y	49 ± 14 Y	4 (12.5)	1 (3.1)
	Urinary schistosomiasis: 2 (6.2)	33-47 Y	40 ± 7 Y	2 (6.3)	0 (0)
	Fascioliasis: 3 (9.4)	29-45 Y	33 ± 7 Y	2 (6.3)	1 (3.1)
	Amoebiasis: 3 (9.4)	35-65 Y	51 ± 12 Y	1 (3.1)	2 (6.3)
	Cryptosporidiosis: 2 (6.2)	23-36 Y	30 ± 7 Y	0 (0)	2 (6.3)
	Microsporidiosis: 2 (6.2)	21-30 Y	26.5 ± 5 Y	2 (6.3)	0 (0)
	Malaria: 2 (6.2)	33-35 Y	34 ± 1 Y	2 (6.3)	0 (0)
	Giardiasis: 1 (3.1)	20 Y	-	1 (3.1)	0 (0)
4 (21)	Apparently healthy control subjects	21-67 Y	23 ± 13.5 Y	7 (33.3)	14 (66.7)

1a: SFDT positive with titres >:16-<1:1024; **1b:** SFDT positive with titres >1:1024; **2:** SFDT negative; **3:** Control positive; **4:** Control negative.

Table 3. Comparison between SFDT and QDNBs-immunoassay for detection of anti-*Toxoplasma* antibodies.

Groups (No.)	SFDT		QDNBs-immunoassay positive	
	Titer	No. (%)	IgG No. (%)	IgM No. (%)
1a	1/16	22 (39.3)	20 (35.7)	0 (0)
	1/32	18 (32.1)	16 (28.5)	0 (0)
	1/64	10 (17.9)	10 (17.9)	0 (0)
	1/128	5 (8.9)	5 (8.9)	0 (0)
	1/256	1 (1.8)	1 (1.8)	0 (0)
1b	>1/1024	27 (100)	27 (100)	19 (70.4)
2	<1/16 (Negative)	39 (100)	1 (2.6)	1 (2.6)
3	<1/16 (Negative)	32 (100)	0 (0)	0 (0)
4	1/16	2 (9.5)	2 (9.5)	0 (0)
	1/64	1 (4.8)	1 (4.8)	0 (0)
	1/128	1 (4.8)	1 (4.8)	0 (0)
	<1/16 (Negative)	17 (80.9)	0 (0)	0 (0)

1a: SFDT positive with titres >:16-<1:1024; **1b:** SFDT positive with titres >1:1024; **2:** SFDT negative; **3:** Control positive; **4:** Control negative.

in QDNBs-IgG and QDNBs-IgM immunoassays. Among the 32 patients of group 3 (control positive) with other serologically and parasitological confirmed parasitic infections and who gave negative reaction in SFDT, none (0%) reacted positively in QDNBs IgG or IgM immunoassay, even with the 8 samples that had given positive reactions in IgM-parasite specific immunoassays. Only four subjects of the 21 (19.1 %) apparently healthy control negative subjects in group 4, reacted positively in SFDT with titers of 1/16-1/128

and in QDNBs-IgG immunoassay, but not (0%) in QDNBs-IgM immunoassay.

The agreements between SFDT and QDNBs IgG and IgM immunoassay are shown in tables (4 and 5), respectively. Significant agreements between SFDT and QDNBs immunoassays ($P < 0.05$) were found, although it was higher for QDNBs-IgG than QDNBs-IgM, with Kappa values of 0.94 and 0.76, respectively.

Table 4. Agreement between SFDT and QDNBs based immunoassay in detection of *T. gondii* IgG antibodies.

QDNBs IgG	SFDT			Agreement
	Positive	Negative	Total	
Positive	83 (47.43%)	1 (0.57%)	84 (48%)	Kappa: 0.943 P value <0.001 Significant
Negative	4 (2.29%)	87 (49.71%)	91 (52%)	
Total	87 (49.71%)	88 (50.29%)	175 (100%)	

Table 5. Agreement between SFDT and QDNBs based immunoassay in detection of *T. gondii* IgM antibodies.

QDNBs IgM	SFDT			Agreement
	Positive	Negative	Total	
Positive	19 (15.96%)	1 (0.84%)	20 (16.81%)	Kappa: 0.763 P value <0.001 Significant
Negative	8 (6.72%)	91 (76.47%)	99 (83.19%)	
Total	27 (22.68%)	92 (77.32%)	119 (100%)	

QDNBs-IgG/IgM immunoassay diagnostic performance for detection of anti-*Toxoplasma* antibodies in comparison with SFDT (Table 6): The sensitivity specificity, PPV, NPV, relative agreement, validity, and accuracy of QDNBs combined IgG/IgM immunoassay in comparison with the SFDT gold standard test for serodiagnosis of toxoplasmosis were estimated. Combined QDNBs-IgG/IgM immunoassay exhibited sensitivity of 95.4%, specificity of 98.9%, PPV of 98.8% and NPV of 95.6%.

Table 6. Diagnostic performance of combined QDNBs IgG/IgM immunoassay for diagnosis of toxoplasmosis in comparison with SFDT as gold standard.

	Combined QDNBs IgG/ IgM immunoassay
Sensitivity	95.4%
Specificity	98.9%
PPV	98.8%
NPV	95.6%
Relative agreement	97.1%
Validity	97.2%
Accuracy	97.1%

DISCUSSION

Serological diagnosis of toxoplasmosis and interpretation of the tests' results are confronted with many problems especially in diagnosis of acute infections in pregnant women, congenital infections in fetuses and newborns, infections in immunosuppressed patients and patients with chorioretinitis and collagen diseases^[5,6,10,29]. ELISAs for detection of IgG or IgM are the most widely used tests for serodiagnosis of toxoplasmosis having several advantages including reproducibility, cost effectiveness, and flexibility in adapting desired research interests^[30,31]. However, whether manual or automated, ELISAs are subjected to false negative results due to low levels of antibodies. Also false positive results, especially with IgM, may occur due to interference from RF, antinuclear antibodies, and other viral and bacterial diseases, or due to persistence of IgM from old infections^[5,10,32]. Moreover, inter-laboratory evaluation of performance of many commercial kits for quantification of IgG antibodies gave unsatisfactory agreements^[6,10]. In many instances, the results of ELISA should be confirmed in reference laboratories where other tests as SFDT, indirect fluorescent antibody test (IFAT), immunosorbent agglutination assays (ISAGA), serum IgG avidity test, and western blotting (WB) of serum from mother-baby pairs are available^[5,6,10,29]; which would improve the diagnostic ability and reporting of the infection status. This necessitates the improvement of the sensitivity and specificity of routinely used ELISA.

The use of NPs in immunoassays may enhance the sensitivity, by increasing the number of enzyme molecules in the final antigen-antibody-enzyme complex. This will increase the catalysis of the substrate and lead to signal amplification in a single recognition reaction^[12]. QDs are fluorescent inorganic semiconductor NPs with diameters in the range of 2–10 nm^[33] which can offer high luminescence with great long term colloidal stability and significant signal amplification. Also, the polymer matrix material facilitates the conjugation of biomolecules to the probes. Finally, by the encapsulation of several QDs into one NB as a single probe, the reporting signal can be directly amplified, without the need for additional signal enhancing steps^[24].

In the present study the diagnostic performance of CdSe/ZnS-QDNBs based immunoassay in serodiagnosis of human toxoplasmosis was studied to improve the efficiency of the present serological diagnostic methods. During optimization of the test, it was found that only 5 min incubation time with OPD substrate were needed to develop optimal color reaction. This fast response is apparently directly related to the increased concentration of enzyme activity in HRP-QDNB conjugate when used instead of HRP only. This agrees with the study of Ambrosi *et*

al.^[34] who showed that only a 5 min incubation period of the enzyme with 3,3',5,5'-tetramethylbenzidine was needed when a Au-nano bioconjugate was used, while in the conventional ELISA test about a 20 min incubation was necessary to develop the color. Also, Gheshlaghchaei *et al.*^[26] showed the same effect, where the time needed for the enzyme incubation with OPD substrate was reduced from 20 to 9 min for optimal color attainment, in comparison to traditional ELISA.

SFDT is the gold standard test for serodiagnosis of toxoplasmosis due to its high sensitivity. It is recommended as reference test for the confirmation of acute infection and for the validation of commercial kits^[35]. As SFDT detects whole IgG, IgM, IgA immunoglobulins, it permits very early diagnosis^[36]; but the achieved antibody titers cannot accurately differentiate between acute or chronic infection^[37]. In our study five groups classified on the basis of their SFDT results, were tested by QDNBs IgG/IgM-immunoassay. The results showed significant agreement between SFDT and both IgG and IgM QDNBs immunoassays. The sensitivity of IgM immunoassay in detecting acute cases of group 1b (19/27; 70.4%) corresponds to $\geq 1:1024$ titre in SFDT suggesting acute infection in immunocompetent patients, but doesn't confirm the presence of IgM antibodies^[38]. On the other hand, Remington *et al.*^[29] stated that SFDT detects mainly IgG antibodies, which may explain why, in the present study, the agreement was higher between SFDT and QDNBs-IgG than with QDNBs-IgM, with Kappa values of 0.94 and 0.76, respectively.

As recommended by Petersen *et al.*^[39], SFDT measures all immunoglobulin subclasses and therefore its results cannot be compared to those of isolated IgG or IgM assays, but only to combined IgG/IgM evaluation. In the present study the diagnostic performance of combined QDNBs-IgG/IgM immunoassay compared with SFDT revealed sensitivity of 95.4%, specificity of 98.9%, PPV value of 98.8%, NPV of 95.6%, relative agreement, validity, and accuracy of almost 97%. It is worth noting that none of the included sera from patients with other parasitic infections reacted positively in either QDNBs-IgG or IgM immunoassay denoting high specificity of the tests. Only four healthy control subjects gave positive reaction in QDNB-IgG which is expected as patients infected with *Toxoplasma* may appear healthy and are discovered by chance to have anti-*Toxoplasma* antibodies.

Additionally, QDs were used in immunoassays several times. Islam *et al.*^[40] improved routine colorimetric detection for the measurement of aflibercept level (vascular endothelial growth factor) in plasma using a unique amplification reagent composed of QD-fluorescent beads conjugated to anti-goat antibodies. The beads offered high

luminescence with relevant long-term colloidal stability and were able to improve sensitivity by 3-fold (0.25 ng/ml), and amplified signal significantly compared to conventional colorimetric methods. Aly *et al.*^[14] evaluated QDNBs immunoassay for diagnosis of human cystic echinococcosis in human serum samples and recorded sensitivity of 91.8%, and specificity of 88.8% in comparison to 75.5% and 60 %, respectively for traditional sandwich ELISA. Lv *et al.*^[25] used CdSe/ZnS-QDNBs in quantitative detection of the C-reactive protein standard antigens by fluorescence linked immunoassay that requires half of the analysis time, and offers a lower limit of detection presenting a wide linear detection range than those of conventional ELISA.

Using anti-schistosomal monoclonal antibodies (MAb) loaded on AuNPs increased the sensitivity and specificity of sandwich ELISA for detection of circulating schistosomal antigen allowing easy detection of active and light infections^[41]. Polystyrene NPs conjugated with polyclonal IgG antibodies specific for *P. falciparum* showed high sensitivity for the detection of *P. falciparum* antigen^[42]. El-Shafey *et al.*^[43] used anti-filarial antibodies IgG conjugated with AuNPs in nano-sandwich ELISA for detection of circulating filarial antigen in patients infected with *W. bancrofti*. They recorded higher sensitivity of 95.1%, specificity of 87.5%, PPV of 92.9%, and NPV 91.3% in comparison with conventional ELISA that exhibited for the same parameters 90.7%, 75%, 84.8% and 84%, respectively.

In the same context, NPs as well as QDs were generally used in order to improve the serological diagnosis of toxoplasmosis. For example, the use of AuNPs was reported in the development of new diagnostic tools for *T. gondii* infections. Al Hamshary *et al.*^[16] evaluated the detection of *Toxoplasma* antigen (SAG1) in serum samples by nano-gold sandwich ELISA. The sensitivity and specificity were 89.2% and 94%, respectively. In patients with other parasites only three cases were positive, two with *G. lamblia* and one with *E. histolytica*. Shaheen *et al.*^[17] evaluated the diagnostic performance of nano-gold beads-based ELISA in serodiagnosis of human toxoplasmosis based on detection of circulating *Toxoplasma* surface antigen (SAG3). The study showed that nano-gold ELISA had higher specificity, PPV, and diagnostic accuracy than conventional sandwich ELISA. While conventional ELISA gave higher sensitivity and NPV than nano-gold. Among 30 samples having other parasitic infections, only two patients with giardiasis gave positive reaction.

Jiang *et al.*^[44] developed a dynamic flow immunochromatographic test (ICT) using AuNPs of 15 nm conjugated to staphylococcal protein A for the detection of *Toxoplasma* IgG antibodies in dogs and cats. The test showed high sensitivity and specificity of approximately 92% and 93.1%, respectively, similar to the ELISA test. However, performance time for ICT

was shorter than that for ELISA and required 10 to 20 times fewer samples versus conventional ICT. Aly *et al.*^[45] used a nano sandwich ELISA based on bioconjugation of polyclonal antibody (pAb) IgG with Silica nanoparticles (SiO₂) for detection of circulating *Toxoplasma* lysate antigen in sera and urine of patients suspected of infection with *T. gondii* and found that the sensitivity of the assay was 90.0%. All the negative controls were below the cutoff value while 4 out of 30 patients with other parasites infections were at the borderline of the cutoff value giving 92.0% specificity. False positive cases belonged to the group of patients infected with *Cryptosporidium* spp. and *G. lamblia*. Medawar-Aguilar *et al.*^[46] developed and applied a laser-induced fluorescence immunosensor, based on the integration of chitosan zinc oxide NPs into the microfluidic channel, to quantify anti-*T. gondii* IgG antibodies. It significantly improved the sensitivity of the method and simplified the operation process decreasing the analysis time. Hegazy *et al.*^[25] studied immunomagnetic bead-ELISA using SAG1 for diagnosis of human toxoplasmosis and showed sensitivity, specificity, PPV, and NPV of 98%, 96.4%, 96%, and 98.1%, respectively. While 2/35 samples from individuals infected with other parasites (one with *E. histolytica* and one with *Cryptosporidium* spp.) were borderline. Li *et al.*^[47] developed a lateral flow immunochromatographic assay (LFIA) for detection of specific IgM to causative agents of TORCH infections during pregnancy (toxoplasmosis, rubella, cytomegalovirus, and herpes simplex virus infections). This LFIA using goldmagnetic NPs modified with polymath acrylic acid, and conjugated with antihuman IgM antibodies, obtained 100% sensitivity and specificity, plus the ability to simultaneously detect multiple pathogens.

In conclusion, QDNBs have large surface area-to-volume ratio that allow the attachment of multiple enzyme-conjugated antibodies which generates an amplified colorimetric signal compared with a single enzyme-conjugated antibody. Therefore, QDNBs apparently improve the performance of ELISA particularly sensitivity as well as shorten the assay time.

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