Original Article

Nano-gold based ELISA versus traditional ELISA for early detection of *Trichinella spiralis* crude excretory antigen in experimentally infected mice

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ABSTRACT

Background: The main disadvantage of ELISA in diagnosis of trichinosis is the false-negative result especially during early infections. Nanoparticle-based immunoassays were developed for serodiagnosis of several parasitic diseases.

Objective: Comparison of nano-gold based ELISA (NGB-ELISA) to the traditional sandwich ELISA for the early detection of *T. spiralis* crude excretory antigen (CEA) in serum and fecal samples of *T. spiralis*-experimentally infected mice.

Material and Methods: In the present study, 180 mice were distributed in three groups. In GI (*T. spiralis*-infected group) 108 mice were infected. Infection was confirmed by the presence of encysted *T. spiralis* larvae in excised muscle specimens on the 28th day post-infection (dpi). For microscopic examination by direct smear and MIFC methods and detection of *T. spiralis* CEA, stool samples were collected on the 2nd, 4th, 6th, 8th, and 10th dpi; then mice were sacrificed, and blood samples were collected on the 2nd, 4th, 6th, 8th, 10th, 12th, 14th, 21st, and 28th dpi. The study included two control groups; GII (specificity group) included 18 mice infected with *Cryptosporidium* spp., *T. gondii*, and *S. mansoni* (6 mice each) for testing cross-reactivity. Stool and serum samples were collected on the day of sacrifice (30th dpi for *Cryptosporidium*, 60th dpi for *T. gondii* and *S. mansoni*). The GIII (sensitivity control group) included 54 non-infected mice. Mice were subjected to stool and blood samples collection, and sacrification as described in *T. spiralis*-infected group.

Results: While microscopic examination of direct smear proved negative for detection of *T. spiralis* diagnostic stages, MIFC technique showed diagnostic larvae and adult worms in only 5% of mice sacrificed on the 6th dpi. For *T. spiralis* CEA detection, the difference between NGB-ELISA and traditional ELISA was not significant in fecal samples. In contrast, NGB-ELISA showed significantly higher number of positive serum samples than traditional ELISA, detecting the infection earlier (2nd versus 10th dpi) with a diagnostic accuracy of 92.78% compared to 67.78%.

Conclusion: The NGB-ELISA for detection of *T. spiralis* CEA in serum samples was superior to the traditional ELISA as it revealed a significantly higher number of positive cases. It verified the infection earlier and was more sensitive until the 14th dpi with a specificity of 100%. Regarding coproantigens, no significant differences were noticed between either ELISAs.

Keywords: coprodiagnosis; crude excretory antigen; ELISA; experimental; nano-gold; serodiagnosis; trichinosis.

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INTRODUCTION

Trichinosis caused by *T. spiralis* is a worldwide foodborne parasitic zoonosis. Infection occurs after eating undercooked or raw meat of pigs and horses infected with infective larvae, producing enteral and muscular/parenteral phases. The enteral phase is asymptomatic in mild infections but can cause gastroenteritis with diarrhea and abdominal pain 2 dpi[1]. Larvae migrate and encapsulate in skeletal muscles during muscular phase of infection and produce severe allergic and inflammatory responses with fever, facial and eyelid edema, and eosinophilia. This phase is usually complicated by thromboembolic manifestations, encephalitis, and myocarditis[2].

Unfortunately, clinical diagnosis of trichinosis is difficult as it has no precise pathognomonic clinical presentation[3]. Muscle biopsy and detection of specific anti-*Trichinella* IgG are the most common methods of diagnosis. In addition, being invasive, it is difficult to detect the larvae during the mild and early stages of infection[4]. Utilizing *T. spiralis* excretory-secretory (ES) antigens to detect anti-*Trichinella* IgG by ELISA is the most common serological method for diagnosis. However, a false-negative result is the main disadvantage of this method, especially in the early stage of infection[4], as there is a window period between the antibody positivity and patent infection. Anti-*Trichinella* IgG was only detectable 8-10 dpi by ELISA using *Trichinella* ES antigens

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extracted from intestinal worms, while ELISA using *Trichinella* ES antigens extracted from muscle larvae (ML) was unable to detect the IgG before the 12th dpi[4]. In addition, IgG antibody levels measured by ELISA may persist for 30 years post infection (PI). Hence, it is difficult to differentiate active from past infections because of consistent IgG levels[3]. Moreover, the ELISA sensitivity for detection of anti-*Trichinella* IgG reaches its maximum level (100%) 1–3 months PI[5]. The low sensitivity of traditional ELISA assay was due to the small loading capacity and enzyme inactivation[6].

Serum levels of *T. spiralis* circulatory antigen (CAg) are usually low, which renders the detection of *Trichinella* antigens in serum less sensitive than the detection of antibodies. The typical recorded success rates of detecting serum CAg were 19-47% in human patients and 56% in naturally infected pigs[7]. A previous study showed that *T. spiralis* adult worm crude antigens were detectable in the serum of mice at the 7th dpi[4]. Coproantigen detection has an advantage over other diagnostic methods as it indicates only the current infection, and its excretion is closely related to the presence of mature and immature parasite stages in the intestine and their numbers. Hence, it could be detected before the appearance of antibodies in serum and it does not depend on the host's ability to produce antibodies[8].

To overcome the drawbacks of traditional ELISA, nanoparticle-based immunoassays received great attention due to their sensitivity, cost-effectiveness, tunable catalytic activity, and longer shelf life. Besides, nanomaterials improve the detection limit of traditional ELISA by increasing the binding sites for the detection of antibodies. Because of these advantages, several metal nanoparticles have been studied to replace traditional enzymes, such as horseradish peroxidase (HRP) in ELISA[9]. Replacement of HRP by a fabricated nanozyme-based immunosensor increases the biochemical detection signals such as color changes of chromogenic substrates (colorimetric), or optical radiation (chemiluminescence), or electrochemical changes. Additionally, these nanozymes exhibit better stability, easier production, and lower cost besides the original advantages of the natural enzyme-based such as good selectivity, and high sensitivity[10]. With the increased applications of nanotechnology in biomedicines, gold nanoparticles (AuNPs) were the most studied metal nanoparticles for their applications in immunoassays and PCR techniues[9]. Thus, nano-gold beads-based ELISA (NGB-ELISA) was widely investigated for diagnosis of *falciparum* malaria, toxoplasmosis, cryptosporidiosis, and visceral leishmaniasis as previously reviewed[11]. Recent studies were also conducted investigating their efficacy in diagnosis of hydatid cyst[12], toxoplasmosis[13], and leishmaniasis[14]. Moreover, NGB-ELISA showed higher sensitivity and accuracy compared to the standard ELISA assay for the detection of *T. spiralis* CAg in mice[15]. Accordingly, there is a need to develop an ELISA technique that overcomes the problem of early diagnosis of trichinosis. The present study was designed to compare NGB-ELISA with the traditional ELISA for early detection of the crude excretory antigen (CEA) of *T. spiralis* in serum and stool samples of experimentally infected mice.

MATERIAL AND METHODS

This descriptive analytical study was conducted at Parasitology Departments, Faculty of Medicine, Menoufia University, Shebin El-Koum, and Theodor Bilharz Research Institute, Giza, Egypt, during the period from March 2022 to October 2022.

Study design: One hundred and eighty mice were classified as experimental *T. spiralis*-infected mice (GI); and two control groups: mice infected with other parasites (GII) and non-infected mice (GIII). Stool and serum samples were taken on different days for GI and GIII, and on the sacrifice day for GII. All stool and serum samples were examined by the traditional ELISA and NGB-ELISA for detection of *T. spiralis* CEA.

Study animals: Two hundred and ten pathogen-free Swiss albino mice aged 6–8 w and weighing 20±2 g were used in this work. Among them, 30 mice were used for preparation of *T. spiralis* CEA. Mice were housed under controlled temperature and humidity (25±2°C and 70%), and fed on standard pellet food and water under strict hygienic conditions.

Parasite and infection: Infective *T. spiralis* ML were recovered from experimentally infected mice obtained from Theodor Bilharz Research Institute, Giza, Egypt. Infected mice were sacrificed 5 w PI by cervical dislocation after ether inhalation, skinned, and eviscerated. According to Korenaga *et al*.[16], all muscular parts were cut into small pieces with scissors, and added to a glass beaker, filled with artificial gastric juice (1% pepsin and 1% concentrated HCL in warm tap water). The mixture was incubated at 37°C for 2 h under continuous agitation using an electric stirrer. The digested product was filtered by a 50 mesh/inch sieve. The collected larvae were washed with tap water and left for about half an hour to sediment. The supernatant fluid was discarded, and larvae were counted microscopically, and adjusted to the desired number of ML/ml.

Study groups: Mice were divided into the following three groups:

1. **Group I** (*T. spiralis*-infected group): Mice (No.=108) were infected with *T. spiralis* at a dose of 300 *T. spiralis* ML. Mice were subdivided according to the sacrifice time into 9 subgroups (12 mice each). Stool and blood samples were collected on the 2nd, 4th, 6th, 8th, and 10th dpi from five subgroups (60 mice).

Only blood samples were collected from the other 4 sacrificed subgroups (48 mice), on the 12th, 14th, 21st, and 28th dpi. Besides, muscle specimens were excised from mice that were sacrificed at the end of the experiment (28th dpi) i.e., the 9th subgroup.

2. **Group II** (Specificity control group): This group included 18 mice infected with *Cryptosporidium* spp., *T. gondii*, and *S. mansoni* (6 mice each) to examine the specificity of both ELISA techniques. Mice were infected then sacrificed on the 30th dpi for *Cryptosporidium* spp.[17], and 60th dpi for *T. gondii*[18] and *S. mansoni*[19]. Stool and serum samples were collected from each mouse on the sacrifice day. *Cryptosporidium* and *S. mansoni* infections were confirmed by stool examination, and *T. gondii* infection was confirmed by Giemsa-stained impression smears and histopathology of brain tissue.
3. **Group III** (Negative control group): It included 54 non-infected mice, and each mouse received 0.2 ml of physiological saline solution. Mice were subdivided according to the sacrifice time into 9 subgroups (6 mice each). Sacrifice time and sampling of stool and blood were scheduled as in GI.

Stool samples: From each mouse, the collected fecal sample was divided into two parts. The first part was prepared for microscopic examination by direct smear and merthiolate iodine formaldehyde concentration (MIFC) method and examined by 10× and 40× magnifications[20]. The second part was prepared for ELISA where samples were weighed and mixed with 3 volumes of cold phosphate-buffered saline (PBS) and 0.05% Tween-20, then centrifuged at 1200 ×g at 4°C for 15 min. The supernatants were collected and kept at -20 °C[21].

Blood samples: Blood samples (0.5 ml) were centrifuged at 1000 ×g for 20 minutes for serum separation. The sera were stored at –20°C until use.

Muscle specimens: Collected muscle samples were subjected to acid-pepsin digestion and microscopically examined for *T. spiralis* ML to confirm infection[15].

Materials: Table (1) describes all compounds used in the study.

Preparation of *T. spiralis* CEA: Thirty mice were used; each was orally infected with 5000 *T. spiralis* ML[15]. Mice were then euthanized on the 5th dpi for collection of small intestines that were opened and washed in pre-warmed PBS (pH 7.2), then divided into small pieces and incubated in PBS at 37°C for 1.5 h. The intestinal contents were filtered through 200 µ sieves and precipitated for 30 min to separate the adult worms. The worms were washed in PBS supplemented with penicillin (100 U/ml) and streptomycin (100 µg / ml), and centrifuged at 600 ×g for 10 min. Adult worms were suspended in deionized water, subjected to 5 freeze-thaw cycles, and ground on ice using a tissue grinder. The ground worms were subjected to further homogenization with ultra-sonication (99 times of 3 seconds cycle on ice), and centrifugation at 15000×g

Table 1. Compounds used in the study according to their prescribed method.

Methods	Chemicals	Company/Country of manufacture
Preparation of *T. spiralis* CEA	**Penicillin** **Streptomycin**	Sigma-Aldrich®, USA
Production of anti-*T. spiralis* polyclonal antibodies (pAbs)	**Complete Freund's adjuvant** **Ammonium sulfate**	Santa Cruz Biotechnology, Germany Thermo-Fisher Scientific, USA
	FCS **Caprylic acid**	Sigma-Aldrich®, USA
Conjugation of goat anti-rabbit pAbs with HRP	**HRP** **Sodium acetate buffer** **Carbonate buffer**	Thermo-Fisher Scientific, USA
	Goat anti-rabbit pAbs **Sodium periodate** **Sodium borohydride**	Abcam, USA Santa Cruz Biotechnology, Germany MyBiosource.com, Canada
Conjugation of goat anti-rabbit pAbs with HRP	**Polystyrene microplate wells** **OPD**	TECAN, Austria Sigma-Aldrich®, USA
	Tween-20 **Phosphate citrate buffer** **Sulfuric acid**	Thermo-Fisher Scientific, USA
Synthesis of AuNPs	**Hydrogen tetrachlorocuprate** **Trisodium citrate**	Sigma-Aldrich®, USA Santa Cruz Biotechnology, Germany
Conjugation of anti-*T. spiralis* pAbs with AuNPs	**PEG, 20000** **Sucrose**	Sigma-Aldrich®, USA

for an hour at 4°C to separate the supernatant that was stored at -20°C[22].

Production of anti-*T. spiralis* pAbs: Two rabbits were kept under standard laboratory conditions (21°C, and 45–55% humidity), provided with tap water, and a standard diet. Stool samples were examined to ensure that they were free from gastrointestinal helminths. Rabbits were then intramuscularly injected at four sites with the priming dose of CEA (1 mg CEA mixed with complete Freund's adjuvant; 1:1). Two booster doses of 0.5 mg emulsified in an equal volume of complete Freund's adjuvant were then given. The first booster dose was injected 2 w after the priming dose. The second booster dose was given one week after the first booster dose. The rabbits were bled for collection of serum one week after the last booster dose. Rabbit sera were purified by 50% ammonium sulfate precipitation method[23], and 7% caprylic acid purification method[24]. To eliminate any non-specific antigen binding from the purified anti-*Trichinella* antibody, 100 µl/well of 2.5% fetal calf serum (FCS) was used.

Conjugation of goat anti-rabbit pAbs with HRP enzyme as a secondary antibody[25,26]: Five mg of HRP was suspended in 1.2 ml distilled water, and 0.3 ml freshly prepared sodium periodate was added. The mixture was incubated at room temperature for 20 min and dialyzed against 1 mM sodium acetate buffer (pH 4) at 4°C overnight. Then it was added to 0.5 ml goat anti-rabbit pAbs that was prepared by adding 5 mg/ml to 0.2 M carbonate buffer. The HRP mixture was incubated at room temperature for 2 h, then 100 µl of sodium borohydride was added followed by incubation at 4°C for 2 h, and dialysis against 0.01 M PBS (pH 7.2). A stock solution was prepared for conjugation of anti-*T. spiralis* pAbs with AuNPs.

Traditional sandwich ELISA[27,28]: Before performance, a checkerboard titration was carried out in which different concentrations of polyclonal *T. spiralis* IgG (as capture and detector antibody) and goat anti-rabbit polyclonal IgG-HRP were titrated against serial dilutions of positive and negative control stool and sera. The selected concentrations and dilutions, at which there were no false positive or false-negative results were 10 µg/ml polyclonal *T. spiralis* IgG (as capture and detector antibody), 1/5000 dilution of goat anti-rabbit IgG/HRP conjugate (as a secondary antibody), and 1/10 dilution of the studied stool and serum samples. Polystyrene microplate wells were coated with polyclonal *T. spiralis* IgG (as capture antibody). The wells were blocked with PBS (pH 7.2) containing 1% FCS and 0.05% Tween-20. Stool and serum samples from all mice groups were separately added in duplicates. Polyclonal IgG against *T. spiralis* CEA (as detector antibody) was added, then goat anti-rabbit polyclonal IgG/HRP conjugate (as secondary antibody) was added to each well. A tablet (30 mg) of orthophenylene diamine (OPD) substrate was dissolved in 25 ml of 0.05 M phosphate citrate buffer (pH 5) then 100 µl/well of OPD solution was added for 30 min. The reaction was stopped by adding 50 µl/well of 2 M sulfuric acid and the optical densities (OD) were read at a wavelength of 492 nm using an ELISA reader[29].

Synthesis of AuNPs: Briefly, this was performed by a chemical reduction method[30], in which one ml 1% hydrogen tetrachlorocuprate solution was added to 100 ml distilled water in a clean 500 ml Erlenmeyer flask and heated to boiling, with continuous stirring. One ml 1% trisodium citrate was added quickly to the boiling solution with constant stirring. The color of the gold solution was transformed from blue to dark red after approximately 3 min, indicating the formation of AuNPs. The solution was left to boil for 2 min more, then allowed to cool and stored in a brown bottle.

Characterization of AuNPs: Samples of AuNPs were examined by transmission electron microscopy (HR-TEM: JOEL JEM-2100 operating at 200 Kilovolt equipped with Gatan digital camera Erlangshen ES500) to determine the morphological properties including shape, surface, and mean size of both AuNPs and gold nanoprobes. A characteristic surface plasmon resonance band of gold NPs was observed in the ultraviolet-visible spectrum, confirming the presence of spherical AuNPs. The analysis of the particle size showed that most of the gold NPs (~90%) were in the 4-5 nm acceptable range for use[31].

Conjugation of anti-*T. spiralis* pAbs with AuNPs[32]: The previously prepared stock solution of HRP-goat anti-rabbit pAbs (1 mg/ml) was added to AuNPs pellet previously prepared by centrifugation of one ml AuNPs solution at 9000 rpm for 50 min. Supernatant was removed and the final volume was adjusted to 100 µl by adding distilled water. The final solution was stirred for 10 min and set at room temperature. Then 0.5% polyethylene glycol 20000 (PEG, 20000) was added to stabilize AuNPs. The solution was centrifuged at 9000 rpm for 50 min and washed three times with a solution composed of PBS (10 mM), FCS (0.5%), sucrose (2.5%), and PEG, 20000 (0.1%). The prepared Au nanoprobes were then dispersed in the washing solution (100 µl) and stored at 4°C.

Performance of NGB-ELISA[29]: The selected concentrations and dilutions were 10 µg/ml pAbs *T. spiralis* IgG, 1/5000 Au nanoprobe, and 1/10 dilution of the investigated stool and serum samples. Briefly, each polystyrene microplate well was coated by pAbs *T. spiralis* IgG (capture antibody), and blocked with PBS (pH 7.2) containing 1% FCS and 0.05% Tween-20. Samples from all groups were separately performed in duplicates. The pAbs IgG against *T. spiralis* CEA (detector antibody), Au nanoprobes, and the OPD substrate dissolved in phosphate-citrate buffer (100 µl) were added to each well. The reaction was stopped

by sulfuric acid (50 µl) and the OD was read at a wavelength of 492 nm using an ELISA reader.

Calculation and interpretation of ELISA readings: For discrimination between positive and negative samples, the cut-off point was calculated using ROC curve according to the following equation: Cut-off value = mean OD reading of negative controls + 2 standard deviation (SD) of negative results. Analyzed samples showing OD values more than cut-off values were considered positive, and vice versa. The diagnostic indices (sensitivity, specificity, positive (PPV) and negative (NPV) predictive values, and diagnostic accuracy) were calculated[31]. Sensitivity=(TP×100)/(TP+FN), Specificity=(TN×100)/(TN+FP), PPV=(TP×100/(TP+FP), NPV=(TN×100)/(TN+FN), and diagnostic accuracy=[(TN+TP)×100]/N. Where TP: true positive; TN: true negative; FP: false positive; FN: false negative; and N is the total sample number.

Statistical analysis: Data entry, coding, and analysis were conducted using JMP pro-16.2 (SAS Institute, Cary, NC). Quantitative data were expressed in mean ($\bar{x}$), and standard error of mean (SEM), while qualitative data were expressed in frequency (number), and percent (%). Tests of significance were the student's *t*-test to estimate the difference between two means of two groups, the one-way ANOVA test to estimate the difference between more than two means, the *Chi*-square (X^2) test to assess the relationship between two or more qualitative parameters, and receiver operator characteristic (ROC) curve to estimate cut-off points. A *P* value of ≤ 0.05 was considered statistically significant.

Ethical consideration: The study was approved by the Scientific Research Ethical Committee, Faculty of Medicine, Menoufia University (IRB:4/2018PARA15). All procedures involving mice complied with the international guidelines for laboratory animals.

RESULTS

Microscopic examination of muscle specimens from *T. spiralis*-infected mice sacrificed on the 28th dpi showed moving, transparent *T. spiralis* larvae (~ 1 mm) that were either lightly or tightly coiled with rounded ends and smooth cuticles (Figure 1). The diagnostic stages of *T. spiralis* were not detected by direct smear examination in all stool samples of all the studied groups while MIFC technique showed larvae and adults in 5% of *T. spiralis*-infected group sacrificed on the 6th dpi (Figure 2).

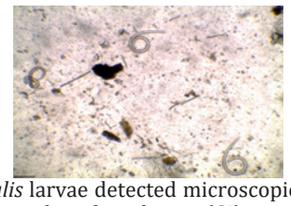

Fig. 1. *T. spiralis* larvae detected microscopically in digested muscles of *T. spiralis*-infected mice (GI) sacrificed at 28th dpi (x100).

Fig. 2. (a) *T. spiralis* larvae detected microscopically in a stool sample of a mouse of GI (*T. spiralis*-infected group) at 6th dpi (MIFC, x400). **(b)** *T. spiralis* dead adult male was detected microscopically in a stool sample of a mouse of GI (*T. spiralis*-infected group) at 6th dpi (MIFC, x100).

Detection of *T. spiralis* CEA in stool samples using traditional ELISA and NGB- ELISA was at cut-off values 0.242 and 0.11, respectively (Figure 3). Results showed that both ELISA techniques could detect *T. spiralis* CEA in 100% of the examined stool samples of infected mice (GI) as early as the 2nd dpi and on all subsequent days till the 10th day. Comparison of the performance characteristics of traditional ELISA and NGB-ELISA for the detection of *T. spiralis* in fecal samples showed no false-positive results in stool samples of the specificity control group (GII) and negative control group (GIII). Accordingly, the sensitivity, specificity, PPV, NPV, and accuracy were 100% for both ELISA techniques throughout the experiment. Figure (4) showed that both ELISA techniques gave negative results in all stool samples collected from GII.

Detection of *T. spiralis* CEA in serum samples using traditional sandwich ELISA and NGB-ELISA was at cut-off values 0.848 and 0.719, respectively (Figure 5).

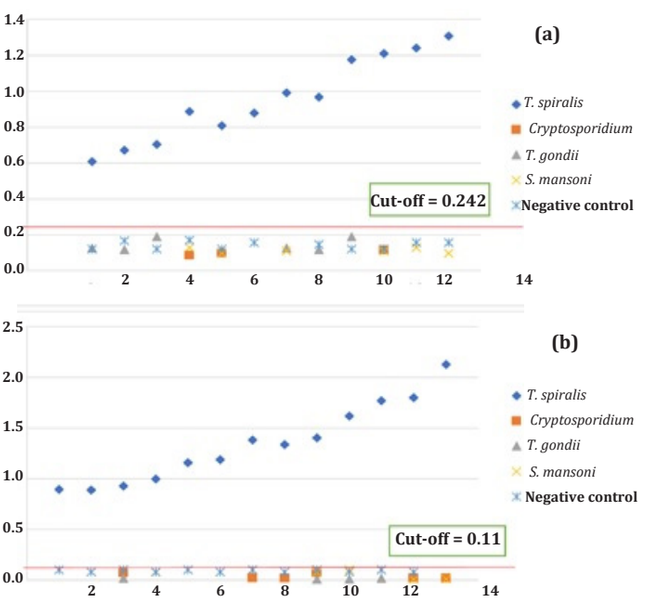

Fig. 3. The cut-off points for (a) traditional ELISA and (b) NGB-ELISA in stool samples.

There was a statistically significant difference between the number of positive serum samples from *T. spiralis*-infected group (GI) sacrificed on 2nd, 4th, 6th, and 8th dpi when NGB-ELISA was compared to traditional ELISA (P=0.000031, 0.00026, 0.0005, and 0.00053, respectively) (Figure 6). Figure (4) showed that both ELISA techniques gave negative results in all serum samples collected from GII.

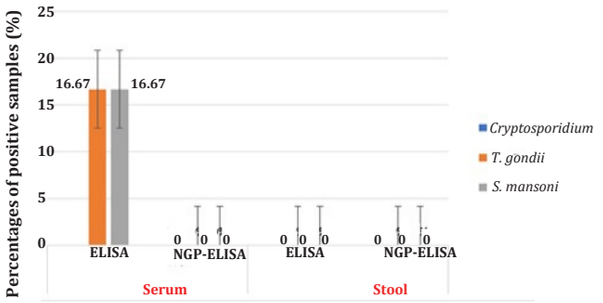

Fig. 4. Comparison between the percentage of false positive samples of traditional ELISA and NGB-ELISA for detection of *T. spiralis* CEA in serum and stool samples of the specificity control group (GII).

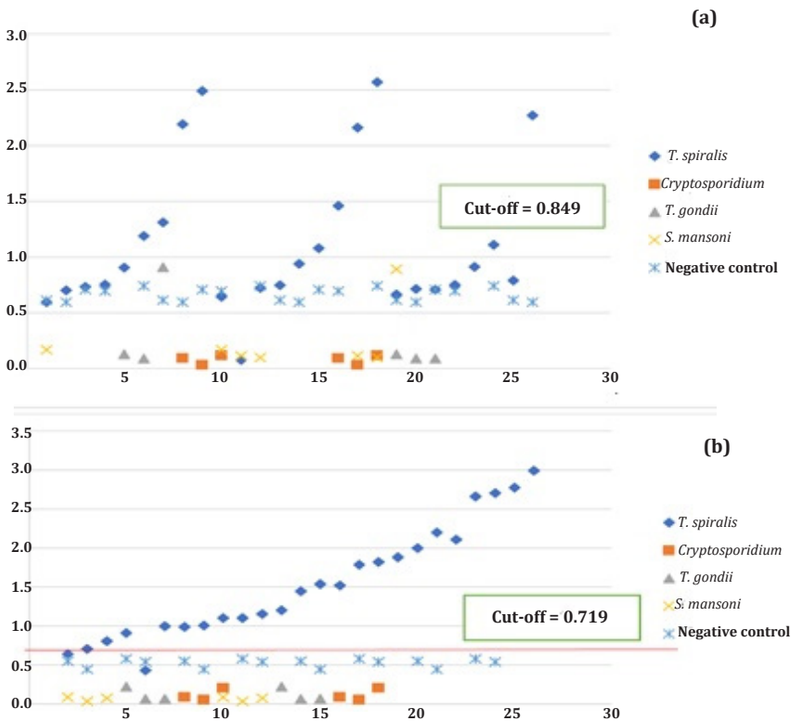

Fig. 5. The cut-off points for (a) traditional ELISA and (b) NGB-ELISA in serum.

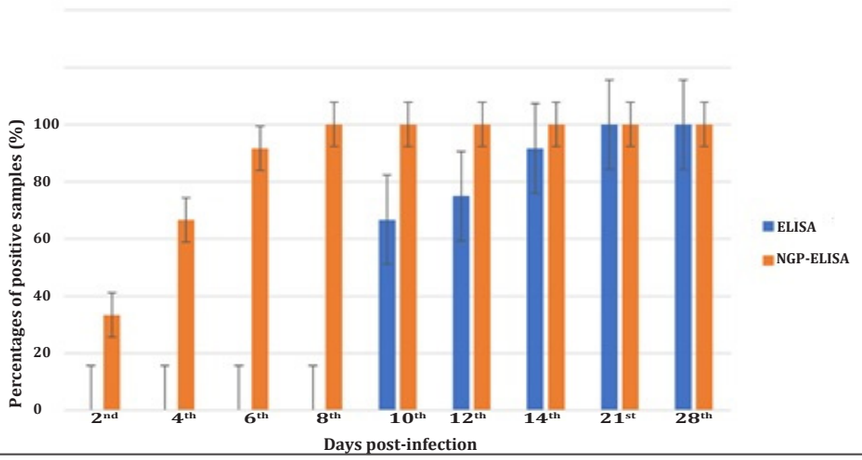

Fig. 6. Comparison between the percentage of positive samples of traditional ELISA and NGB-ELISA for detection of *T. spiralis* CEA in serum samples of *T. spiralis*-infected group (GI) on different days of examination.

In addition, it was observed that NGB-ELISA detected the *T. spiralis* CEA in serum samples earlier than traditional ELISA, as early as the 2nd dpi versus the 10th dpi (Figure 6). The NGB-ELISA showed sensitivities of 33.33%, 66.67%, 91.67%, and 100% on 2, 4, 6, and 8 dpi, compared to sensitivities of 0% on the same days for traditional ELISA. On the following days, NGB-ELISA continued to have a 100% sensitivity while the traditional ELISA started to display increasing sensitivities of 66.67%, 75%, and 91.67% on the 10th, 12th, and 14th dpi, respectively and reached a 100% sensitivity on the 21st and 28th dpi. Besides, NGB-ELISA showed overall specificity, PPV, NPV, and diagnostic accuracy of 100%, 100%, 84.7%, and 92.78%, respectively compared to 97.2%, 96.29%, 55.56%, and 67.78% respectively for traditional ELISA (Table 2).

Table 2. Performance characteristics of ELISA and NGB-ELISA in serum samples at different dpi.

Dpi	Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
2nd (n=18)	**ELISA**	0	100	NA	33.34	33.34
	NGB-ELISA	33.33	100	100	42.86	55.56
4th (n=18)	**ELISA**	0	100	NA	33.34	33.34
	NGB-ELISA	66.67	100	100	60	77.8
6th (n=18)	**ELISA**	0	100	NA	33.34	33.34
	NGB-ELISA	91.67	100	100	85.72	94.45
8th (n=18)	**ELISA**	0	100	NA	33.34	33.34
	NGB-ELISA	100	100	100	100	100
10th (n=18)	**ELISA**	66.67	100	100	60	77.78
	NGB-ELISA	100	100	100	100	100
12th (n=18)	**ELISA**	75	100	100	66.67	83.34
	NGB-ELISA	100	100	100	100	100
14th (n=18)	**ELISA**	91.67	100	100	85.72	94.45
	NGB-ELISA	100	100	100	100	100
21st (n=18)	**ELISA**	100	100	100	100	100
	NGB-ELISA	100	100	100	100	100
28th (n=36)	**ELISA**	100	91.67	85.7	100	94.4
	NGB-ELISA	100	100	100	100	100
Total (n=180)	**ELISA**	48.15	97.2	96.29	55.56	67.78
	NGB-ELISA	87.96	100	100	84.7	92.78

NA: Not applicable.

DISCUSSION

The results of the current study revealed that direct smear examination did not detect *T. spiralis* diagnostic stages in stool samples from all infected mice. This agreed with Ashour *et al*.[21] who also reported absence of *T. spiralis* larvae or adults in the stool samples of all examined animals. On the other hand, *T. spiralis* larvae and adults were detected by MIFC technique in 5% of the infected mice sacrificed on the 6th dpi. This difficulty in finding *T. spiralis* adult worms and larvae in the stool could be attributed to their tendency to degenerate rapidly[33].

Results of the present work showed that there was a significant difference between the numbers of positive serum samples for *T. spiralis* CEA detected by NGB-ELISA in the *T. spiralis*-infected group sacrificed on the 2nd, 4th, 6th, and 8th dpi when compared to the traditional ELISA. Moreover, NGB-ELISA detected *T. spiralis* CEA earlier than traditional ELISA (2nd dpi versus 10th dpi, respectively). Similarly, Gomaa[15] recorded that the standard ELISA couldn't detect *T. spiralis* CEA in sera collected on the 6th, 8th, and 10th dpi. However, the nano-based assay detected the antigen from the 6th dpi. Sun *et al*.[34] and Xu *et al*.[35] demonstrated some antigenic epitopes of *T. spiralis* adult worm soluble proteins from the 8th to the 10th dpi. They suggested that excretory/secretory (E/S) or surface antigens might be secreted into the peripheral blood at the early stage of infection which may induce an early antibody response. On the contrary, Wang *et al*.[36] detected *T. spiralis* circulating antigen of adult worms later on the 13th and 24th dpi. Zumaquero-Ríos *et al*.[29] also reported that the circulating E/S antigens of *T. spiralis* ML were detected after 3 w of experimental rats infection. The difference between the findings of our study and the other studies may be due to the different types of antigens used (CEA in the current work versus the adult worm[36] and E/S antigens of ML[29], respectively).

The NGB-ELISA showed statistically higher sensitivities than the traditional ELISA in early examination days before the 21st dpi with overall sensitivity of 87.96% and 48.15% respectively. Gomaa[15] also recorded a significantly higher sensitivity of NGB-ELISA than the standard ELISA on the 8th and 10th dpi. Regarding specificity, the traditional ELISA

showed a total specificity of 97.2% with false-positive results in the *T. gondii* and *S. mansoni*-infected subgroups compared to a specificity of 100% for the NGB-ELISA. Cross-reactivity is a major obstacle in the serological diagnosis of parasitic infections because of the shared epitopes. The presence of shared antigens with other parasites has been widely documented in several serological studies which showed cross-reactions of *Trichinella* antigens with other helminths such as *Ascaris, Trichuris, Clonorchis, Paragonimus, Schistosoma*, and *Anisakis* in serum samples[4,37].

Coproantigen detection for diagnosis of trichinosis is a simple useful technique during the intestinal phase of the infection. Besides, coproantigens quickly decrease after treatment, presenting a good indicator of current infection and a good monitor for the efficacy of treatment[29]. In the current work, traditional ELISA and NGB-ELISA detected CEA of *T. spiralis* in 100% of the stool samples of *T. spiralis*-infected mice as early as the 2nd dpi. Similarly, Ashour *et al*.[21] recorded a sensitivity for determining *Trichinella* coproantigen by capture ELISA of 60%-80% in the first 3 dpi and reached 100% from the 4th to the 9th dpi. They detected these antigens as early as the 1st dpi and reached the peak on the 5th and 6th dpi. Besides, Zumaquero-Ríos *et al*.[29] detected the coproantigens of *T. spiralis* on the 1st dpi by ELISA. This early presence of the antigen was attributed to the direct passage of *Trichinella* larvae in stool[21]. Notably, the activated first stage larvae undergo their first molt at 10 h PI, then the adults develop by molting three times during 10–31 h PI[38]. This is supported by Liu *et al*.[39] who recorded that *T. spiralis* DNA could be detected in the stool of the infected mice as early as 3 h PI.

In this study, there were no false-positive results in stool samples of the negative control group and the specificity control group either by traditional ELISA or by NGB-ELISA; thus, the specificity was 100% for both tests. This was comparable to the results obtained by Zumaquero-Ríos *et al*.[29] who reported no cross-reactivity between *T. spiralis* ML coproantigen and the antigenic extracts of other parasites including *A. cantonensis, A. suum, E. granulosus, F. hepatica, S. stercoralis, T. solium, T. canis* and *T. trichiura* by using a sandwich ELISA.

The promising high performance of NGB-ELISA was explained by Tabatabaei *et al*.[9] who documented that AuNPs in NGB-ELISA provide a nano-platform to conjugate multiple enzymes for the antibody on their surface. It is worth mentioning that AuNPs promote more than one enzyme per antibody, give a higher colorimetric signal, and can easily detect positive samples with a few analyses. The improved sensitivity of NGB-ELISA could be related to the high stability and strong peroxidase-like catalytic activity of AuNPs which makes these nanozymes ideal alternatives to HRP[10]. Additionally, Zhao *et al*.[40] stated that antibody conjugated AuNPs as detection labels accelerated the catalysis of chromogenic substrates and increased the detection sensitivity.

In conclusion, no significant differences were noticed between the NGB-ELISA and the traditional ELISA regarding the detection of *T. spiralis* CEA in fecal samples. However, both ELISA techniques should be considered to diagnose the intestinal phase of trichinosis instead of microscopic stool examination. In contrast, NGB-ELISA for the detection of *T. spiralis* CEA in serum samples showed higher performance indices regarding sensitivity, specificity, PPV, NPV, and accuracy when compared to the traditional ELISA.

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