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Modified dot-ELISA for diagnosis of human trichinellosis☆

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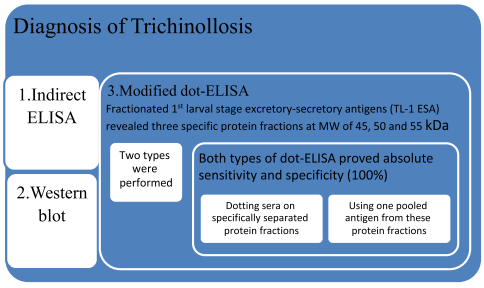
HIGHLIGHTS

- ELISA/TL-1 ESA was used for primary diagnosis compared to EITB gold standard test.
- Sensitivity and specificity of ELISA were 100% & 79.5%.
- Two types of dot-ELISA were applied, both gave 100% Sensitivity and specificity.
- 1st was performed with individual separated specific fractions 45, 50, 55 kDa.
- The 2nd was a typical dot-ELISA using the eluted pooled specific fractions.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aimed to modify Dot-Enzyme-linked immunosorbent assay (dot-ELISA) for the diagnosis of human trichinellosis and to compare its performance with indirect ELISA and Western-blot assay (EITB).

A total of 175 human serum samples were enrolled in the study. Indirect ELISA was used for the primary diagnosis. EITB versus fractionated 1st larval stage excretory-secretory antigens (TL-1 ESA) revealed three specific protein fractions at MW of 45, 50, and 55 kDa (kDa). Dot-ELISA was performed in two ways. In the first one, sera were dotted on the separated three specific protein fractions, while in the second one the three fractions were eluted, concentrated at one pooled antigen that used in classic dot-ELISA.

Both types of dot-ELISA proved absolute (100%) sensitivity and specificity in comparison with the gold standard EITB reaction. While sensitivity of ELISA was 100% and its specificity was 79.5%. The fraction at 45 kDa was the most sensitive one. The use of the pooled antigen improved the test results. The described dot-ELISA is an easy applicable diagnostic tool gathering the benefits of both ELISA and EITB.

1. Introduction

Trichinellosis is a serious zoonotic disease caused by the nematode genus *Trichinella*. Human disease has been documented in 55 countries, with health, social, and economic impacts forming a

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public health hazard (Sun et al., 2015).

Early diagnosis is critical for effective treatment because anti-helminthic drugs are much more effective during the intestinal phase than they are during the muscular phase. This is because the drugs are more accessible to the parasites in the intestine than when they are inside the muscle cells. A definitive diagnosis depends on history of exposure, clinical evaluation, laboratory tests, including serology and/or muscle biopsy for larvae detection (Gottstein et al., 2009). However, the clinical manifestations of the disease are nonspecific and muscle biopsy is painful, invasive and it has low sensitivity (Sun et al., 2015).

ELISA is an easily applied test suitable for evaluating a large number of samples simultaneously. ELISA using ESA is recommended by the International Commission on Trichinellosis for the diagnosis of trichinellosis. It allows detection of *Trichinella* infection as low as 1 larva per 100 g of muscle tissue (Sun et al., 2015). However, the main disadvantage of detection of anti-*Trichinella* antibodies (ATAb) is the high rate of false negative results during the early stages of infection and the cross-reactions with other parasitic diseases (Sun et al., 2015). In addition, most of the commercial ELISA kits for human serology are unreliable (Gómez-Morales et al., 2008). Now there are some ELISAs which have been standardized (Taylor et al., 2009).

Fractionation of proteins using Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis SDS-PAGE and Western blot (EITB) techniques are important for the detection of specific diagnostic parasitic fractions. EITB assay is a confirmatory test for ELISA-positive sera (Gómez-Morales et al., 2008). However, EITB technique is non-applicable in field settings.

Another modification of ELISA technique is dot-ELISA. Both techniques can be considered paired in terms of simplicity, low cost, direct visualization, time, speed and number of samples to be tested and both need minute volume of reagents to perform. Dot-ELISA can be used as an accurate test, gathering the benefits of both ELISA and EITB, and it can be applied for many samples (Shalaby and El-Bahy, 2002).

The aim of this study is to develop two forms of modification dot-ELISA using specific identified TL-1 ESA protein fractions for the diagnoses of trichinellosis. Sensitivity and specificity of both forms of dot-ELISA were evaluated in comparison with indirect ELISA and EITB techniques with the aid of reference positive and negative pigs' sera.

2. Materials and methods

This study was assessed and approved by ethical committee of the Research Institute of Ophthalmology in Cairo, in compliance with the Declaration of Helsinki. This study was conducted for a year in the period from January to December 2015. The diagnostic procedures were performed in the laboratory of Parasitology, Faculty of Veterinary Medicine, Cairo University.

2.1. Larval recovery and collection

Trichinella larvae were detected by trichinoscopy examination of slaughtered pigs' muscles, Cairo abattoir, Egypt. For larvae extraction, 25 gm of heavily infected muscles were subjected to HCl-pepsin artificial digestion with magnetic stirrer method according to IQACA (2012).

2.2. Preparation of larvae ESA (Gómez-Morales et al., 2008)

About 2000 1st larvae were washed 10 times by sedimentation in Phosphate Buffer Saline (PBS) pH 7.4 with added 1 gm of streptomycin sulphate and 1,000,000 I.U. of penicillin G sodium/liter. The settled larvae were suspended in RPMI 1640 Medium with added L-Glutamine, 20 mM HEPES buffer, and antibiotics (500 I.U./ml penicillin and 500 I.U./ml streptomycin). The larvae were cultured for 48 h at 37 °C, 90% humidity and 10% CO_2. The medium containing the ES products was collected by centrifugation at 1,000 g, then dialyzed against PBS and filtered through a 0.2-μm YM-5 filter, and the supernatant was concentrated 100 times in an Amicon pressure concentrating chamber (Amicon, Inc., Billerica, MA). Protein contents were estimated by the Bradford method (Bradford, 1976). The ESA was liquated and stored at −20 °C until use.

2.3. Human and pigs' sera

A total of 175 human serum samples were included in this study:

(G-1): People under high risk of *Trichinella* infection: 75 samples collected from subjects from both sexes. They were members of 15 families inhabiting an endemic area, Al-Mokatum locality in Cairo, Egypt, having regular consumption of pork.
(G-2): Suspected Trichinellosis (ST) cases: These were 40 samples collected from attendance of tropical medicine outpatient clinic, Cairo University hospitals, complaining of myalgia, fever accompanied with leukocytosis, eosinophilia and increased levels of muscle enzymes. Twenty cases gave a positive history of pork consumption (ST-PC) and another twenty cases with a negative history of pork consumption (ST-NPC).

For the above mentioned groups, confirmation of being free from other parasitic infections was required for inclusion in the study.

(G-3): Sera used to assess cross-reactivity including 60 persons, of different age and sex without history of pork consumption. They were 20 healthy control subjects, and forty cases with other parasitic infections, previously confirmed by blood smears, stool examinations, and/or other specific tests. These forty cases were divided into; *Schistosoma mansoni* (*S. mansoni*) infected cases (n = 20), and mixed enteric protozoal infected cases with blastocystosis, entamoebiasis, and cryptosporidiosis (n = 20).
(G-4): Reference control sera used in the present study were aspirated from young pigs' free from any other parasitic infection. Animals were inspected by trichinoscopy after slaughtering, and then artificial digestion with magnetic stirrer method was used to check for *Trichinella* spp. larvae (IQACA, 2012). Positive 20 serum samples from infected pigs and another 20 samples from non-infected pigs were stored at −20 °C until used.

2.4. Indirect ELISA

ELISA was used to detect the level of specific IgG ATAb in examined sera. After checkerboard titration, an in-house ELISA was used in accordance with a previously published validated protocol (Liu et al., 2015; Gómez-Morales et al., 2009) with modifications. Briefly, microtiter plates were coated with 100μl/well of TL-1ESA (4 μg/ml) in carbonate-buffer (pH 9.6) for 1 h at 37 °C. After washing three times with washing buffer (0.5% Tween 20 in PBS pH

7.3 ± 0.2), wells were blocked with 200 μl of PBS-0.05% Tween 20 (PBST) containing 0.5% bovine serum albumin (BSA), and incubated at 37 °C for 1 h. After another washing, the following reagents were sequentially added and incubated at 37 °C for 1 h: (1) 100 μl/well of sera diluted 1:100 in PBST, and (2)100 μl/well horseradish peroxidase HRP-conjugated anti-Human or anti-Pig IgG (Sigma, USA) diluted 1:5000. The reactions were detected by the addition of 100 μl/well of the substrate O-phenylene diamine dihydrochloride (OPD), Sigma, USA) plus H_2O_2 and stopped with 50 μl/well of 1 N H_2SO_4. Optical density (OD) values at 450 nm were measured with a micro plate reader (Titerteck multiskan ELISA reader). Serum samples were tested in duplicate and each plate contained 3 positive and 3 negative reference serum samples.

2.5. Fractionation of antigens & transferring of protein

SDS-PAGE analysis of TL-1 ESA was carried out under reduced conditions using 12% non-gradient slab gel with 5% stacking gel (Laemmli, 1970). The antigen was electrophoresed at 20 mA current. The gel was calibrated with the molecular weight markers (Mr) (Sigma SDS-100B). TL-1 ESA antigen fractionated proteins were transferred from the gel at 10 V, 100 mA overnight at 4 °C, onto nitrocellulose paper (NC) as described by Towbin et al. (1979). The sheet was dried and stored in freezing until use.

2.6. Determination of specific protein fractions using EITB

Longitudinal NC strips (15 × 0.5 cm) containing the fractionated TL 1ESA were cut out. The nonspecific binding of antibodies was blocked by incubating the strips with 3% BSA TNT at 37 °C for 1 h. After blocking, the strips were incubated versus known positive and negative control serum samples at 1:100 dilution, 0.5 ml of sera/strip for 2 h using Western-blot assay (EITB) according to Towbin et al. (1979). After washing, the strips were exposed to 1:1.000 HRP-conjugated anti-human or anti-pig IgG (Sigma, USA), diluted in blocking buffer, at 37 °C for 1 h. The peroxidase substrate (4-chloro-1-naphthol, Sigma) was then added to reveal the IgG/antigen interaction. Three positive and three negative control pigs' sera were included in each working session. Protein fractions reacting positively with positive control serum, and negatively with negative control one, were considered as specific fractions (Fig. 1). These strips were retained back to its original position on NC sheet for determination of the site of the specific protein bands in association with the MW standard.

2.7. Elution and concentration of the specific protein bands from gel

After SDS-PAGE analysis of TL-1 ESA, and the gel ran its full length, longitudinal strips containing the MW standards and the peripheral part of the fractionated antigen, were cut out. The strips were stained with Coomassie blue according to Tsai and Frasch (1982). Transverse gel strips containing the proteins corresponding to MW of 45–55, were determined then cut out horizontally across the whole gel. The cut gel strips were transferred to elution tube membrane 6–8 MW cut-off (Spectrum Medical Inc., Los Angeles, CA 900060). The tube was filled with PBS (pH 7.4) and kept in Bio-Rad elution unit at 10 V, 100 mA overnight at 4 °C. The gel material was removed and the volume was reduced using poly-ethylene-glycol - as highly as possible-in molecular porous membrane tubing 6–8 MW cut-off according to Katrak et al. (1992) & Bien et al. (2013). Three elution runs were performed to obtain enough amount of pooled antigen to perform this study. The protein content of the eluted concentrated material was determined per each elution process by Bradford method. The protein content (mean ± standard deviations) were 100.96 ± 1.37, 93.7 ± 3.8 & 96.4 ± 2.3 μg/mL for the three runs respectively. The prepared antigen was kept in 1 ml vials at −70 °C till use.

2.8. Dot-ELISA technique

The technique was done as described by Shaheen et al. (1989) after modification. The test was applied in two forms. In the 1st form, the test was directly applied on the whole longitudinal NC strips containing only the transferred fractionated ESA, without treatment with sera (tested strips, Fig. 3, lanes 2–9). The precise positions of specific kDa protein fractions on the tested strips (where the reaction would take place) were visually determined by matching the tested strips with the corresponding high positive EITB-NC strips (treated with sera, with determined specific protein fractions, Fig. 3, lane 1). Using a pencil, two transverse lines were drawn onto the tested strips determining the upper and lower borders of each specific kDa protein fraction (45, 50, and 55), representing the wells where the reaction would take place (Fig. 3).

In the second form of dot-ELISA, 2μL/dot (about 0.2 μg protein) of the eluted concentrated pooled protein fractions were dotted on circular NC disks laid on the bottom of micro-ELISA plate and left to dry at room temperature. In both forms of dot-ELISA, sera were tested in duplicate. The non-specific binding sites were blocked by 3% BSA TNT, and then washed three times with PBS-T. After drying, 2 μl of the tested sera were spotted on each NC disk and left to dry for 10–15 min then washed. Four μl of 1:1000 HRP-conjugated anti-Human or anti-Pig IgG (Sigma, USA), were doted to each disk and left to dry. After washing, the sheet was immersed in 5 ml of freshly prepared substrate solution (4-chloro-1- naphthol 340 μg/ml substrate buffer with 0.03% hydrogen peroxide solution). Color developed within 15 min, the wet sheets were observed in natural light and a well-defined blue-purple spot was regarded as positive with the naked eye and evaluated in comparison with the reference control & tested sera. Sensitivity and specificity were calculated as before.

2.9. Statistical analysis

Data entry and statistical analysis were calculated using SPSS 16.0 statistical software package. Qualitative results were presented as frequencies and percent, while quantitative data presented as mean ± standard deviation (SD). Comparison between groups was performed using Chi-square and Fischer Exact for qualitative data, *t*-test and ANOVA test for quantitative data.

The receiver-operator characteristic (ROC) curve was built to detect best cut-off point of ELISA that differentiates between positive and negative cases, based on the gold standard. The ROC curve is a graphical plot of the sensitivity versus (1−specificity) for a binary classifier system, using various cut-off values. This allows the cut-off value that gives the best balance of sensitivity and specificity to be selected for the test being considered. The area under curve (AUC), determines the accuracy of the test.

Validity parameters of ELISA and dot ELISA test against gold standard were calculated (sensitivity, specificity, positive predictive value = PPV. Negative predictive value = NPP and accuracy). P-values less than or equal to 0.05 were considered statistically significant.

3. Results

Indirect ELISA tested versus known positive and negative sera (Table 1) gave absolute sensitivity & specificity (100%). ELISA was applied afterwards for the detection of ATAb in human sera (Table 1). The positivity rate of G-1 was 52/75 (69.3%), without any significant difference in relation with the sex of examined cases. Results showed a significant association between mean ELISA OD values and the age of examined cases (P value = 0.013), with an increased ratio of infected cases in the age group 31–50 years old, which was confirmed later by other diagnostic techniques. The detection of ATAb in G-2 was associated with pork consumption, since100% of ST-PC cases were positive with the highest OD values, while, only 20% from ST-NPC were positive, with lower OD values. Concerning G-3, ATAb were positively detected in (30% and 20%) of examined sera of patients suffering from *S. mansoni* and enteric protozoa infections, respectively. Healthy control sera were all negative, indicating absence of ATAb.

Western blot reaction revealed the characteristic profile for ELISA positive and negative groups of sera, with specific molecular weight, in kDa of reactive bands (Fig. 1).

Reaction of positive control pigs 'sera through EITB (Fig. 1, strip no. 1); revealed 11 reactive bands, having MW range at 22, 28, 30, 45, 50, 55, 63, 66, 68, 98 and 135 kDa. EITB reaction of human sera positive by ELISA (e.g. Fig. 1, strip no. 2) revealed 9 protein fractions to MW at 22, 28, 30, 45, 50, 55, 70, 98 and 135 kDa. The triple protein fractions with the MWS of 45, 50 and 55 kDa were considered the TSL-1 specific fractions, since they were recognized by positive control pigs' sera and not recognized by pigs' negative control (Fig. 1 strip no. 6). True positive cases by Western blot (63 cases) were diagnosed by detection of sharp reaction with the triple specific bands irrespective to their ELISA OD values.

Three out of six ATAb ELISA positive cases from *S. mansoni* group reacted positively in EITB reaction with a maximum appearance of two of the triplet protein fractions. Their OD values ranged from 0.469 to 0.476. The other three ELISA positive sera reacted like ELISA negative sera from the same group yielding 6 cross-reactive bands at 30, 63, 66, 70, 98 and 135 kDa (e.g. Fig. 1, strip no. 3).

Serum samples from enteric protozoa cases (e.g. Fig. 1, strip no. 4) revealed 7 cross-reactive bands at 22, 30, 63, 66, 70, 98 and 135 kDa. In addition, five cross-reactive bands were recorded by ELISA negative samples belonged to ST-NPC and to healthy control subjects (e.g. Fig. 1, strip no.5) at the range of 22, 30, 66, 70 and 135 kDa. Only one fraction corresponding to MWS of 65 kDa was recorded with negative control non infected pigs' sera.

Out of 175 examined human sera, 63 (36%) diagnosed positive by EITB compared to 86 (49%) by ELISA. EITB cases were 40 cases that belonged to pig contacts group, 20 cases from ST-PC group and three cases from *S. mansoni* group (Table 1). There was a significant association between trichinellosis and history of eating pork, since 100% of true positive cases gave history of pork consumption (P-value = 0.000).

In order to evaluate the performance of ELISA in comparison with the gold standard EITB technique, ROC curve was built for serum samples from cases with confirmed trichinellosis according to EITB (63) to negative serum samples (112). The statistical indices for ELISA results are shown in (Table 2).

The AUC, indicating accuracy, was determined for the mean of the OD duplicates and was equal to 0.983 (Fig. 2). The best cut-off point, determined by ROC analysis, was 0.413 for the OD values. Based on this cut-off value, ELISA sensitivity and specificity were 100% and 79.5%, respectively. The test reported 73.3% PPV, 100% NPV and 86.8% level of accuracy.

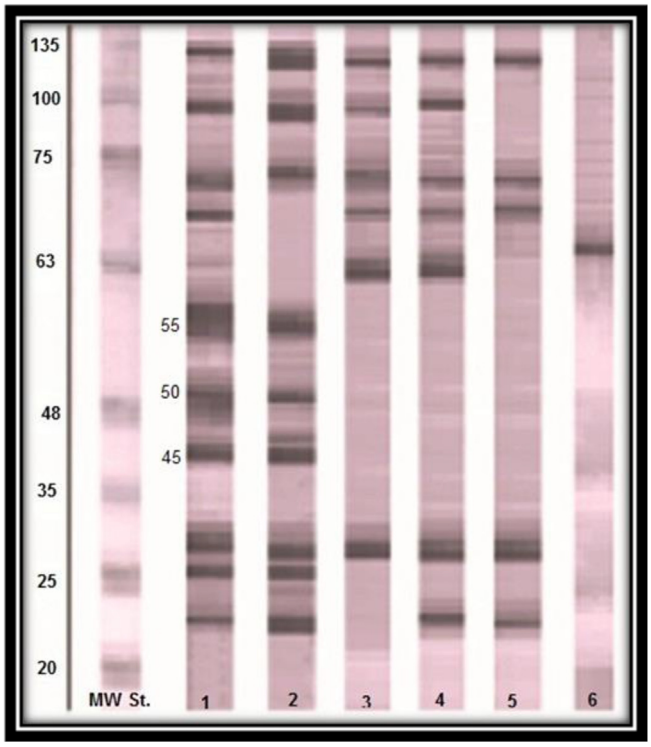

Fig. 1. Western blot profile obtained by reaction of NC strips with TL-1 ESA protein fractions with *Trichinella* positive & negative sera identified by ELISA;

Lane (1): Strip reacted with infected pig sera.
Lane (2): Strip reacted with a high positive ELISA OD.
Lane (3): Strip reacted with sera from a case infected with *S. mansoni*; ELISA negative OD.
Lane (4): Strip reacted with sera from a case infected with enteric protozoa; ELISA negative OD.
Lane (5): Strip reacted with sera from cases of ST-NPC or healthy human control; ELISA negative OD.
Lane (6): Strip treated with non-infected pig sera, ELISA negative OD.

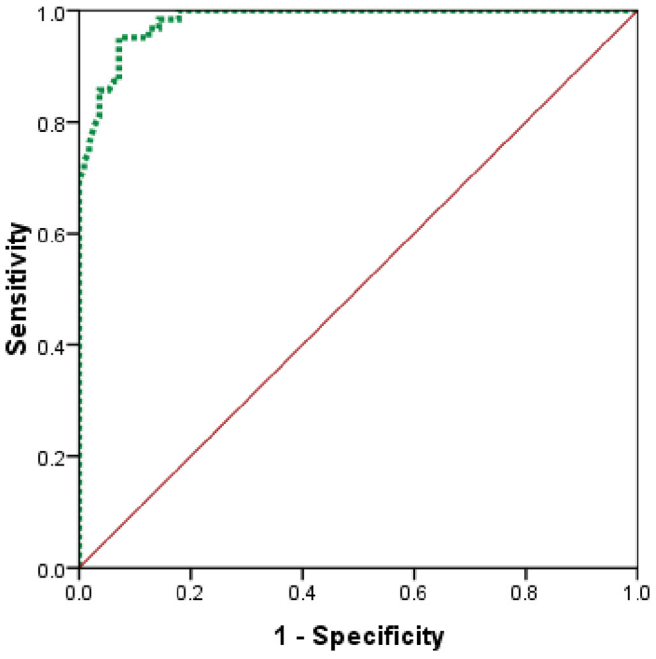

Fig. 2. ROC curve according to Western blot golden standard.

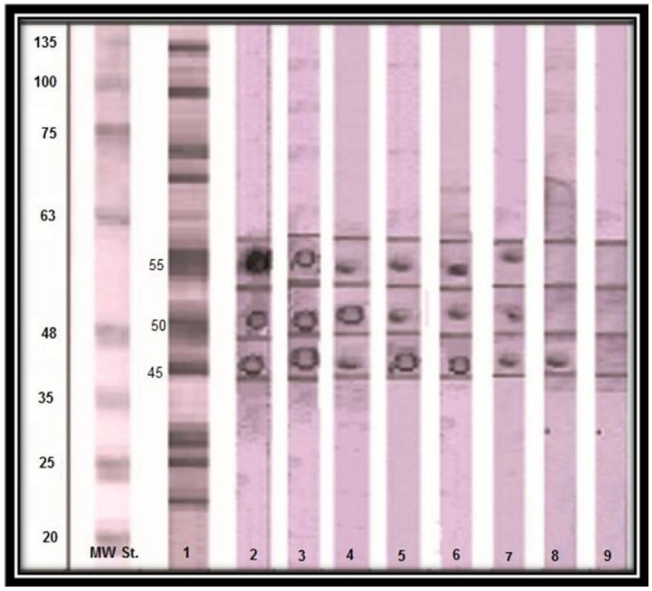

Fig. 3. NC strips containing specific protein fractions of TL-1 ESA versus + Ve & -Ve sera using dot ELISA technique;

Lane (1) EITB Reaction with infected pig sera ELISA + Ve (OD 0.987) serum.
Lane (2): Dot ELISA reaction with high ELISA + Ve (OD 0.799) serum.
Lane (3): Dot ELISA reaction with medium ELISA + Ve (OD 0.641) serum.
Lane (4&5): Dot ELISA reaction with medium ELISA + Ve (OD 0.555) serum.
Lane (6, 7&8): Dot ELISA reaction with low ELISA + Ve (OD 0.455–0.433) serum.
Lane (9): Dot ELISA reaction with sera of healthy people ELISA -Ve (OD 0.085) serum.

Application of both forms of dot-ELISA gave the same results as EITB, without any clear difference concerning number of diagnosed cases, with 100% sensitivity, specificity, and accuracy (P = 0.000). Both dot-ELISA demonstrated no false positive reactions versus negative control human and pig sera. Both tests succeeded to confirm true ELISA positive *Trichinella* infected cases and to exclude false positive cases. Results are presented in comparison with results of ELISA & EITB for the same serum samples (Table 1 and Fig. 3).

For both forms of dot-ELISA, it was important to demonstrate the significant direct relation between the degree of darkness and the mean OD value of ELISA (p = 0.00), which reflects the level of ATAb in the tested sera. Positive serum samples were classified into, strong, moderate or weak positives by judging their color reactions with known strong and weak positive control sera (Table 3 and Fig. 3). The 45 kDa protein fraction and, the pooled antigen were both sensitive as they demonstrated clear reactions even with sera of low ELISA OD values.

Use of the pooled antigen improved the degree of darkness of the reaction. It increased the number of high positive (5.7%) and decreased the number of weak positive (3.4%), while the number of moderate positive remained as that obtained with 45 kDa fraction (26.8%). At the same time, the sensitivity was still as that of each separate fraction (Table 3).

4. Discussion

All diagnosed true positive cases were clinically positive, had positive epidemiologic data and history of pork consumption, and they were ELISA/ESA positive, and EITB positive with the specific kDa (45–55), by this way we followed the ICT serological guidelines for diagnosis of human trichinellosis, including the algorithm for serological diagnosis of human trichinellosis (ICT Recommendations for Serology, Page 15).

In the present study, EITB reaction was adopted as the golden standard for serological diagnosis according to Gómez-Morales et al. (2008). The sensitivity and specificity of ELISA, in comparison with EITB, agreed with (Gottstein et al., 2009).

The absolute sensitivity of ELISA is related to sensitivity of TL-1 ESA. TSL-1ESA is the most abundant stage-specific antigen, and originates from the “stichocytes” occupying the anterior half of the L1 larva, in the larval cuticular surface, directly exposed to the host's immune system (Wang et al., 2014). TSL-1 antigen epitopes are highly conserved, allowing serology to detect infection with any of *Trichinella* species (Gottstein et al., 2009).

In this study, the low specificity of ELISA agreed with Yera et al. (2003) and Gómez-Morales et al. (2008). It was explained with cross-reactivity with other parasitic infections; cysticercosis, hydatidosis, schistosomiasis, fascioliasis, strongyloidosis, toxocariasis, anisakiasis or filariasis (Sun et al., 2015).

The protein fractions obtained by different groups of sera on applying EITB reaction, especially the triple specific bands, were in the range previously mentioned by Gómez-Morales et al. (2008) and Cuttell et al. (2013). Wang et al. (2014) and Sun et al. (2015) identified the 40–70 kDa ES proteins of *T. spiralis*, as the major immunodiagnostic antigenic proteins for trichinellosis.

To overcome the disadvantages of ELISA and the time-

Table 1
Efficacy of three immunoassays in diagnosis of trichinellosis in different groups of serum samples.

Examined Groups		No examined (Male/female)	ELISA		Mean O.D. ± SD	EITB/Dot ELISA	
			No positive (Male/female)	%		No positive (Male/female)	%
(G-1) Pig Contacts (age in years)	31–50	32 (13/19)	26 (13/13)	81.3	0.653 ± 0.094	23 (11/12)	71.9
	13–30	24 (12/12)	14 (5/9)	58.3	0.517 ± 0.052	8 (4/4)	33.3
	≤12	19 (13/6)	12 (8/4)	63.2	0.531 ± 0.114	9 (6/3)	47.4
	Total	75 (38/37)	52 (26/26)	69.3	0.588 ± 0.11	40 (21/19)	53.3
(G-2) Suspected trichinellosis.(ST)	ST-PC[a]	20 (11/9)	20 (11/9)	100	0.790 ± 0.123	20 (11/9)	100
	STNPC[b]	20 (7/13)	4 (4/0)	20	0.452 ± 0.013	0 (0/0)	0
(G-3) Cross- reactivity	*S. mansoni*	20 (15/5)	6 (6/0)	30	0.472 ± 0.035	3 (3/0)	15
	Enteric protozoa	20 (13/7)	4 (0/4)	20	0.440 ± 0.011	0 (0/0)	0
Healthy human control sera		20 (10/10)	0 (0/0)	0	0.082 ± 0.05	0 (0/0)	0
Total		175 (94/81)	86 (47/39)	49	0.613 ± 0.15	63 (35/28)	36
Trichinella spp. infected pigs		20	20	100	0.688 ± 0.08	20	100
Non-infected pig sera		20	0	0	0.115 ± 0.021	0	0

No for number.
[a] ST-PC=Suspected trichinellosis pork consumers.
[b] ST-NPC= Suspected trichinellosis non-pork consumers.

Table 2
Statistical indices of ELISA from ROC curve.

Statistical Indices	Western blot analysis		
	True positive (n = 63)	Negative (n = 112)	P- value
	OD	OD	
Mean	0.666	0.165	0.000
SD	0.141	0.16	
Minimum	0.423	0.074	
Maximum	0.987	0.586	

Table 3
Relation between Dot ELISA and ELISA OD values for 175 samples.

Dot ELISA	Color depth	No. serum samples	%	ELISA OD values	
				Range	Mean ± SD
55 kDa	Negative	112	64	0.074–0.586	0.165 ± 0.16
	Weak positive	38	21.7	0.423–0.897	0.6 ± 0.12
	Moderate positive	22	12.6	0.597–0.987	0.745 ± 0.1
	Strong positive	3	1.7	0.878–0.968	0.911 ± 0.049
50 kDa	Negative	112	64	0.074–0.586	0.165 ± 0.16
	Weak positive	35	20	0.423–0.987	0.576 ± 0.107
	Moderate positive	28	16	0.654–0.974	0.777 ± 0.088
	Strong positive	0	–	–	–
45 kDa	Negative	112	64	0.074–0.586	0.165 ± 0.16
	Weak positive	10	5.7	0.423–0.507	0.470 ± 0.027
	Moderate positive	47	26.8	0.484–0.878	0.673 ± 0.093
	Strong positive	6	3.4	0.889–0.987	0.935 ± 0.046
Pooled fractions (45–55 kDa)	Negative	112	64	0.074–0.586	0.165 ± 0.16
	Weak positive	6	3.4	0.444–0.527	0.482 ± 0.022
	Moderate positive	47	26.8	0.466–0.888	0.665 ± 0.083
	Strong positive	10	5.7	0.788–0.967	0.915 0.036

consuming EITB, this study presented two forms of dot-ELISA for diagnosis of trichinellosis. The 1st technique gave the same results as conventional EITB, with 100% sensitivity, specificity, and accuracy better than ELISA. However, this modified technique is still based on SDS-PAGE and immunoblotting, and needs the same time to complete the whole test procedure. The 2nd dot-ELISA using the pooled antigen overcame the previous problems.

Being based on the same specific triplet protein fractions, the 2nd technique gave same results as EITB and the previous dot-ELISA from the aspect of incidence, sensitivity and specificity. Moreover, both dot-ELISA demonstrated no false positive reactions versus negative control human and pigs' sera.

Dot-ELISA by this way had all the benefits of traditional ELISA, overcame its drawback concerning the degree of purification of antigen and decreased the level of cross-reaction with other parasites. Moreover, it is easy and can be evaluated either directly by the naked eye or by a special reader (densitometer). It facilitated examination of a large number of samples at one time. In addition, it had the same accuracy of EITB technique. Both techniques succeeded in confirming true ELISA positive *Trichinella* infected cases and to exclude false positive cases.

The elution process provided easily handled, concentrated pooled protein fractions, with a protein content reached up to 100 μg/ml. The protein content depended on the thickness of gel and the amount of loaded antigen. The obtained amount was sufficient to perform many samples at one time in typical dot-ELISA. Each dot performed using 2μL/dot (about 0.2 μg protein). In addition, it was easy to prepare any amount of pooled antigen by repeating the elution process.

In comparison with the 1st form of dot-ELISA, where the specific bands are fixed to NC, encroaching on a large space with decreased protein concentration/area leading to relatively faint reaction per band, the pooled protein provided an increased concentration of protein per a small area (dot) leading to more clear reactions. By this way, the pooled antigen had synergistic action, as this high protein concentration increased the number of strong positive and decreased weak positive reactions. The direct relation between intensity of color reaction in dot-ELISA and the OD values of ELISA and sensitivity of 45 kDa agreed with (Manoharan et al., 2004; Gómez-Morales et al., 2008).

Both EITB and dot-ELISA techniques succeeded in excluding 23 false positive cases determined by ELISA. However, three cases from *S. mansoni* group remained positive after performing EITB and dot-ELISA techniques, which may be attributed to either inaccurate history of pork consumption supplied by these cases or to cross-reactivity that may also occur at the triplet bands Gómez-Morales et al. (2008).

The main limitation in defining the cut-off point for this study is the small sample size. Its difficulty in Egypt is to find serum samples from patients with trichinellosis, since the majority of population acts Muslim that forbid pork consumption. In addition to the marked decrease of disease transmission, due to decreased pork consumption, after governmental elimination of most of the pigs in the Egypt in reaction to the 2009 flu pandemic. Another problem is that performing muscle biopsy to define *Trichinella* infection was refused by patients. In this study, due to lack of positive control sera, we used sera from naturally infected animals as control. Many studies used antibodies prepared in experimental animals as control for different serological assays (e.g. using hyperimmune sera prepared in rabbits or in mice). In addition, in many studies, preparing monoclonal antibodies depends upon injection of the prepared antigens in experimental animals, and then these antibodies are used in diagnosis or vaccination. Sun et al. (2015) compared the antibody response developed in humans and experimentally infected mice to three *Trichinella spiralis* antigens. The sensitivity reached 100% for ESA for both human and mice. Yang et al. (2016)

stated that specific antibodies develop in both pigs and humans as a response to infection. However, they differ according to the number of ingested larvae, the *Trichinella* species involved, and the individual immune response. For indirect ELISA, the sensitivity and specificity to ESA change according to the host species, with higher values for humans. In future studies, may the number of the infected cases will be sufficient to meet the recommendation of ICT, and we conduct a larger study to standardize this work.

5. Conclusion

Dot-ELISA proved absolute (100%) sensitivity and specificity in comparison with the gold standard EITB reaction. While sensitivity of ELISA reached up to 100% and its specificity was 79.5%. The fraction at 45 kDa was the most sensitive one. Use of the pooled antigen improved the test results. The described dot-ELISA is an easy applicable diagnostic tool gathering the benefits of both ELISA and EITB.

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