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EVALUATION OF NANO-GRAPHENE BASED SANDWICH AND DOT-ELISA AS PROMISING TECHNIQUES FOR DIAGNOSIS OF HUMAN INTESTINAL GIARDIASIS

By

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Abstract

Giardia intestinalis nowadays is recognized as the most common parasitological cause of diarrhea, with 280 million infections per year. Microscopic examination of faecal samples has the advantage of low cost and the ability to simultaneously identify other parasitic infections. However, analysis of single stool sample and the skill of the microscopist can affect the accuracy of detection.

As an attempt to improve the sensitivity of laboratory diagnosis of giardiasis, the present study aimed to evaluate the diagnostic performance of different parasitological techniques (Mini Parasep, MIFC and direct smear) and to evaluate a novel antigen capture immunoassays based on IgG polyclonal antibody conjugated with nanoparticles (Nano graphene based Sandwich ELISA and Dot-ELISA) for detection of *Giardia* antigen in stool samples. A total of 96 human stool samples were collected and classified into three groups according to stool examination results, (GI), 61 *Giardia* infected patients, (GII), 20 samples collected from patients infected with other parasites and (G III), 15 healthy individuals (negative control).

In the current study, *Giardia* antigen detection was carried out by several steps including preparation of *Giardia* antigen, production, purification and labeling of rabbit anti-*Giardia* IgG polyclonal antibodies.

The Mini Parasep was the best followed by MIFC and direct smear. The study demonstrated that Nano Sandwich ELISA was higher than Traditional Sandwich ELISA regarding sensitivity, PPV, NPV and diagnostic accuracy with statistically significant difference between them, while specificity in Traditional Sandwich ELISA was higher than Nano Sandwich ELISA. Dot ELISA and Nano Dot ELISA had the same sensitivity, while Dot ELISA was higher than Nano Dot ELISA regarding specificity, PPV, NPV and diagnostic accuracy without significant difference. This means that the use of graphene nanoparticles improved the diagnostic testing of human giardiasis.

Key words: Giardiasis, *Giardia*, Sandwich ELISA, Dot-ELISA, Graphene Nanoparticles, Diagnosis, Mini Parasep.

Introduction

Giardia intestinalis (*G. intestinalis* or *G. duodenalis, G. lamblia*) is a flagellate intestinal protozoan that infects man and many animal species (Adam, 2001; Bey-han and Cengiz, 2017). It is considered one of the major causes of enteritis in humans worldwide (Klotz and Aebischer, 2015). It is particularly common in developing countries, where poor sanitation and bad hygiene are major problems. The prevalence of infection was 2%-5% in developed countries and up to 30% in developing countries. Additionally, the highest percent of infection occurs in children younger than 10 years old (Caccio and Sprong, 2011; Diagbouga *et al*, 2017). Giardiasis is still a significant public health problem. It is included in the 'Neglected Diseases Initiative' of the World Health Organization (WHO) in 2004 (Diagbouga *et al*, 2017).

Giardiasis is endemic in Egypt (Eissa and Amer, 2010). It was considered one of the most common causes of chronic diarrhea in infants and children (13.6%). It plays an important role in stunting and cognitive im-

pairment in poorly nourished children (El-Deeb and Abdel-Hamid, 2012).

The clinical manifestations of giardiasis vary from asymptomatic infection to acute or chronic diarrhea with abdominal pain, flatulence, weight loss and malabsorption that can last for several months (Romero *et al*, 2015).

Laboratory diagnosis of giardiasis depends generally on microscopic detection of cysts and/or trophozoites of the parasite in stool samples (Hanson and Cartwright, 2001; Beyhan and Cengiz, 2017). It is effective, cheap and has the advantage of detecting large variety of parasitic infections (Mendonça *et al*, 2017).

However, sensitivity is poor when only a single sample is analyzed, particularly in low parasite density or intermittent excretion of cysts. Microscopic examination must be performed on three stool samples within 3-5 days to increase the sensitivity (El-Nahas *et al*, 2013).

The antigen detection immunoassays such as Enzyme Linked Immunsorbant Assay (ELISA) and immunochromatography (IC) were developed to detect *Giardia* antigen in feces (Arora and Arora, 2015). ELISA is a rapid, sensitive and economic method for detection of specific antigens in stool and confirmation of certain infection (Barazesh *et al*, 2010).

Nanotechnology is important for medical diagnosis. The development of nanotechnology architecture and materials could potentially extend sub-cellular and molecular detection beyond the limits of conventional diagnostic modalities (Hu *et al*, 2011). Nanotechnology may improve the sensitivity, specificity, speed, cost and convenience of diagnostic tests. Furthermore, nanotechnology has also opened up the possibility of other screening strategies (Hegazy *et al*, 2015).

In the past few years, graphene and its derivatives have attracted considerable attention because of their excellent thermal and electrical conductivity, biocompatibility, high specific surface area and various surface oxygen-containing groups (Wang *et al*, 2017). Mechanically, graphene also appears to be one of the strongest materials ever tested, with high elasticity, flexibility and adaptability to flat or irregular surfaces (Liu *et al*, 2012).

Scientists start to think about tuning the properties of graphene by controlling the size of graphene. Thus, nano-sized graphene (nanographene, NG) was developed and attracted attention (Dai *et al*, 2018). The sensitivity of laboratory diagnosis of *G. intestinalis* infection was improved by including alternative diagnostic procedures which are more rapid and reliable (El-Nahas *et al*, 2013).

The present study aimed to compare the diagnostic performance of different parasitological methods and to evaluate Nano graphene based Sandwich ELISA and Dot-ELISA for detection of *Giardia* antigen in stool samples of infected patients as promising non-invasive techniques to diagnose giardiasis.

Materials and Methods

Study population: This study was performed from December 2016 to April 2018. It was carried out on patients complaining of gastrointestinal symptoms suggestive of intestinal giardiasis. Fresh fecal samples were collected from patients attending the outpatient clinics of Al-Zahraa University hospital, Abo El Reesh Pediatric hospital and Theodor Bilharz Research Institute (TBRI). Verbal consent was taken from each one.

This study was conducted on 96 stool samples. Grouping of the participants was done as follows based on stool examination (direct and concentration) results: GI: 61 patients positive to *G. intestinalis*. This group was subdivided into: GIa: 49 patients positive to *Giardia* cyst and GIb: 12 patients positive to *Giardia* trophozoite. GII: 20 patients harboring other parasites than *G. intestinalis*: 5 patients infected with *Blastocystis* hominis, 2 patients infected with *Hymenolepis nana* and 13 patients infected with *Entamoeba histolytica/dispar*. GIII: 15 appar-

ently healthy individuals free from giardiasis and other parasitic infections served as negative control.

The fecal samples were collected in clean wide mouth containers. All samples were divided into 3 portions as soon as they were received: a- A small part of each specimen for direct smear examination, b- Another part preserved in 10% formalin for merthiolate iodine formaldehyde concentration method (MIFC) and Mini Parasep method, and c- The majority of fecal samples were freezed at -20°C for immunological techniques (Traditional and Nano based Sandwich ELISA & Dot ELISA).

Parasitologic examinations: **A-** Direct smear method (Garcia, 2007): A sample of about 2mg of stool was taken and simply emulsified in a drop of normal saline, placed on a dry clean glass slide using wooden applicator. Cover slip was applied to make a thin film free of air bubbles, examined by 10X & 40X by light microscope. Presence or absence of *Giardia* cysts/trophozoite was recorded. B- Merthiolate iodine formaldehyde concentration method (MIFC) (Blagg *et al*, 1955): Approximately 1gm of fecal specimen was emulsified in a tube containing 5ml of merthiolate iodine formaldehyde (MIF) mixed well and filtered in other cup, and followed by addition of 7ml ether. The prepared specimen was centrifuged for 5min at 3500xg. A drop of sediment was put on a slide, covered and examined under light microscope, (MIF= a mixture of 2 solutions with ratio 4:1. A composed of 0.1% merthiolate, 36-40% formaldehyde, glycerin and distilled water; B composed of potassium iodide, iodine and distilled water). **C-** Mini Parasep concentration test (APACOR Ltd, England) after Sanprasert *et al.* (2016): The Mini Parasep® solvent free (Mini Parasep® SF) tubes and the sedimentation cones (fig.1) were labelled with the specimen identification numbers. The procedure was used according to manufacturer's directions. The stool sample was introduced to the mixing tube using a spoon on the end of the filter. It was then mixed with 3.3 ml of 10% formalin in mixing tube. Mini Parasep tube was sealed by screwing in filter/ sedimentation cone unit, vortexed to emulsify its content with sedimentation cone pointing upwards, and then inverted and centrifuged at 200xg for 2min. The mixing chamber and filter were then unscrewed and the suspension in the sedimentation cone was discarded. The sediment was microscopically examined using physiological saline.

NB: Comparison of diagnostic performance of direct smear, Mini Parasep & MIFC used for detecting *Giardia* cyst in stool samples of GIa was done for only 43 stool samples due to inadequate quantity of other samples to perform all techniques.

Preparation and purification of *Giardia* antigen by two-phase sucrose gradient technique (Moss *et al*, 1991): The samples chosen for cyst purification were heavily infected with high number of the cysts (more than 8 cysts in each microscopic field with 40X), also free from any other gastrointestinal parasites or yeast contamination.

Samples were diluted with distilled H_2O and filtered through four layers of gauze to remove the course materials. The filtrate was centrifuged at 800×*g* for 5min. The supernatant was eliminated and the deposit was washed again. In the first phase the pellet was re-suspended in 20ml of distilled H2O and divided into several aliquots of 5ml Each of these aliquots was placed over 3ml of cold 1.5 M sucrose and centrifuged at 600×*g* for 10min. The interfaces were removed carefully. In the second phase, the obtained portions were dissolved in distilled H2O and the suspensions were added to 0.75 M sucrose solution followed by centrifugation at 1500×*g* for 10min. So, cysts settled at tubes' bottom. The entire procedure was conducted at room temperature. The purified cysts were then stored at-40°C.

Protein content determination: Protein content determination was based on Bradford dye-binding procedure which was dependent on the color change of Coomassie brilliant

blue G-250 dye in response to various concentrations of proteins (Bradford, 1976).

Assessment of reactivity of *Giardia* antigen by triple rapid diagnostic test (Cer-Test Biotec S.L. Spain): CerTest *Crypto+ Giardia+Entamoeba* one step combo card test (Fig. 2), a colored chromatographic immunoassay for simultaneous qualitative detection of *Cryptosporidium*, *Giardia, Entamoeba histolytica*/*dispar* in stools.

Card test, test sample (*Giardia* antigen) & stool collection tubes with diluent were allowed to reach room temperature (15-30ºC) prior to testing. The rapid test was used according to manufacturer's directions. 1- Sufficient test sample quantity (approx. 125mg) was picked up by stick and added to the stool collection tube with diluent. Tube was closed and shaked well in order to assure good sample dispersion. 2- Cer-Test *Crypto+Giardia+Entamoeba* combo card test was removed from sealed bag just before use. 3- Stool collection tube was opened to dispense 4 drops in each of circular windows marked with the letter A, B & C. 4- Results read within 10 min.

Production and purification of polyclonal antibodies (PAb): 1. Immunization of the rabbit for production of polyclonal antibodies: Production of pAb was obtained by immunizing New Zealand white rabbit with purified *Giardia* antigen through primary dose in the form of intramuscular injection at two sites 1ml *Giardia* antigen mixed 1:1 with complete Freund's adjuvant (CFA), (Sigma) this was followed by three booster doses, each was 0.5 ml antigen emulsified in equal volume of incomplete Freund's adjuvant (IFA), (Sigma). The first booster dose was two weeks after the primary dose. The following booster doses were given at weekly intervals (Fagbemi, 1995; Guabadia and Fagbemi, 1997). Rabbit was sacrificed three days after the last dose and it's serum was obtained and pAb fraction was purified by 50% ammonium sulfate precipitation method (Nowo-tny, 1979). More purification of pAb was done by 7% caprilic acid method (Mckinney and Parkinson, 1987). Anti-*Giardia* IgG pAb was kept at -20°C till needed.

2- Conjugation of anti-*Giardia* IgG polyclonal antibodies with Horseradish Peroxidase (HRP): Five mg HRP, (Sigma) was resuspended in 1.2ml distilled water followed by addition of 0.3 ml freshly prepared sodium periodate and incubation at room temperature for 20min. HRP solution was dialyzed against 1mM sodium acetate buffer (pH 4) at 4°C overnight (Tijssen and Kurstak, 1984). Anti-*Giardia* IgG pAb solution (5mg/ml in 0.02M carbonate buffer, pH 9.6) was prepared. The HRP was removed from dialysis tube and was added to 0.5ml of antibody solution. The mixture was incubated at room temperature for 2h, 100µl sodium borohydride was added and the solution was incubated at 4°C for 2h. HRP conjugate pAb was dialyzed with several changes against 0.01 M PBS (pH 7.2).

3- Conjugation of anti-*Giardia* IgG polyclonal antibodies with graphene nanoparticles (GrNPs): Conjugation of antibodies to the GrNPs based on following protocol: Five ml GrNPs aqueous solution was diluted by 2 in PBS, and then bath sonicated for 1h to make a clear solution. 1.2g NaOH and 1.0g chloricacetic acid (Cl-CH2-COOH) was added into the 10ml GrNps suspension (2mg/ml) and bath sonicated for 1-3 h to convert the -OH groups to -COOH via conjugation of acetic acid moieties (named GrNps-COOH). The GrNps-COOH solution was neutralized and purified by rinsing and filtration. GrNps-COOH suspension was diluted by water to optical density OD=0.4 at 808nm (1mm optical path). 2mg/ml pAb was added to GrNps -COOH suspension & sonicated for 5min. N-(3-Dimethylam-inopropyl-N’-ethylcarbodiimide hydrochloride (EDC, Sigma Inc.) was added twice to reach 4mM, reaction overnight, and quenched by Mercapto-ethanol (Fluka Inc.). Final product (GrNps-Ab) was obtained by ultra-centrifugation at 45k rpm in 2× phosphate buffered saline (PBS) for 1h to save supernatant (yield ~50%).

Application of the prepared antigen and

polyclonal antibodies in immunological techniques:

1- Preparation of Fecal Samples (Mezo *et al*, 2004): Individual fecal samples were processed by mixing the fecal material in a 1:9 proportion with PBS. Samples were mixed using a vortex to form slurry then centrifuged at 3000rpm for 30min at 25°C. Supernatant was recovered and stored at -20°C until used. This step was done to make a solution (containing parasite antigens) from each faecal sample to be tested by the immunological methods.

2- Detection of *Giardia* Antigen in stool samples by Home-Made Sandwich ELISA: After several optimization trials of sandwich ELISA to detect the optimum dilution of coating Ab and detecting Ab (Venkatesan and Wakelin, 1993): Micro titer plates were coated with 100 µl/well of purified IgG pAb as a capture antibody at dilution of 1/50 in 0.1M carbonate buffer, pH 9.6 and the plates were incubated at 4°C overnight. Plates were washed 3 times with washing buffer 0.1 M PBS/T, pH 7.4. Free sites were blocked with 200µl/well of blocking buffer (0.1% BSA in 0.1M PBS/T) and incubated for 2h at 37°C. Plates were washed with washing buffer 3 times. 100µl of fecal samples were added to each well and incubated for 2h at 37°C and plates were washed 3 times with washing buffer. 100 µl/well of anti-*Giardia* IgG pAb conjugated with HRP was added at dilution of 1/200 in 0.1M PBS to all wells and plates were incubated for 1h at 37°C. Plates were washed 5 times with washing buffer,100µl of substrate solution [a tablet of O-phenylenediamine dihydrochloride (OPD, Sigma)] dissolved in 25ml of 0.05M phosphate citrate buffer, pH 5 with peroxidase H_2O_2,were added to each well and plates were incubated in dark place at room temperature for 30min, 50µl/well of 8 N H2SO4 were added to stop the enzyme substrate solution. Absorbance was measured at 492 nm using ELISA reader (Bio-Rad microplate, Richomond, Co.).

3- Detection of *Giardia* Antigen in stool samples by Home-Made Nano graphene based Sandwich ELISA: Micro titer plates were coated with 100µl/ well of purified IgG pAb conjugated with Nano graphene as a capture antibody at dilution of 1/200 in 0.1M carbonate buffer, pH 9.6 and plates were incubated at 4°C overnight. Steps were completed as mentioned in sandwich ELISA.

4- Detection of *Giardia* antigen in stool samples by Home-Made Dot-ELISA (Yamamoto *et al*, 1998): a. Five µl of the purified anti-*Giardia* IgG-pAb (coating antibody) diluted in carbonate buffer (1/50) was dotted on nitrocellulose (NC) membrane discs and allowed to air dry thoroughly. They were incubated at 4°C overnight. b. In next morning, membrane was washed 3 times with PBS/T. Then blocking solution was applied, incubated at room temperature for 45min. After that the membrane was washed 3 times with PBS/T. Stool samples (5µl) were dotted on NC discs then incubated for 30min and washed 3 times with PBS/T. c. Five µl of HRP conjugated anti-*Giardia* IgG-pAb (detecting antibody) diluted in PBS buffer (1/200) was dotted on NC discs and incubated for 30min, then washed 5 times with PBS /T. d. DAB (Diamino-Benzidine tetrahydrocholoride) substrate (0.5mg/ml DAB 0.03% H_2O_2 in PBS) was applied by dotting 5µl/ disc. NC discs that gave visible brown spots were considered positive. Reaction was stopped with cold distilled H_2O just after color development.

5- Detection of *Giardia* antigen in stool samples by Home-Made Nano grapheme based Dot-ELISA: As with Dot ELISA but using Nano graphene conjugated PAb (at dilution of 1/200 in carbonate buffer) as coating antibodies and HRP conjugated PAb as detecting antibodies.

Statistical analysis: Data were presented as mean±standard deviation (SD) of (X±SD). Cut off value =mean OD readings of negateve controls+2SDs of mean. Sensitivity (%) = A/(A+C)x100, specificity(%)= D/(B+D) x 100, PPV(%) =A/(A+B)x 100 & NPV(%)=

D/(C+D)x 100, as A=true positive, B=false positive, C=false negative & D=true negative.

Results

Stool examination of 43 samples positive to *Giardia* cyst by different parasitological methods (direct smear, Mini Parasep & MIFC) showed that all samples (43) were positive to *Giardia* cyst by Mini Parasep and MIFC methods (Figs. 4&5). But, by using direct smear method, 41 samples were positive to *Giardia* cyst (Fig.3).

Comparison of the diagnostic performance of these methods (direct smear, Mini Parasep & MIFC) used for detecting *Giardia* cyst in stool samples of GIa (Tab. 1, Chart 1) showed that Mini Parasep concentration technique was the best followed by MIFC technique. The least result was obtained from direct smear. This table shows statistically significant difference between them with the highest mean number of *Giardia* cyst in 3 different microscopic fields that was recorded from Mini Parasep technique (7.78) followed by MIFC technique (5.86) and direct smear method (3.52).

Assessment of *Giardia* antigen reactivity by rapid diagnostic test (Fig. 1) showed that *Giardia* antigen was reactive only against *Giardia* Ab (detected by presence of 2 bands; control and test bands) while it was not reactive against *Cryptosporidium* and *E. histolytica/dispar* Abs (detected by presence of one band; control band).

Total protein content of *Giardia* antigen was 5.1mg/ml as measured by Bradford method. Protein content of anti-*Giardia* IgG Polyclonal antibodies: Total protein content was 1.8 mg/ml as measured by Bradford method after purification by 50% ammonium sulphate and dropped to 1.4 mg/ml after 7% caprylic acid purification method of rabbit's serum.

Traditional Sandwich ELISA for detection of *Giardia* antigen in stool samples (Tab. 2), showed 35 positive cases (57.4%) in GI out of 61. In GII, 8 cases were positive out of 20 while 12 cases were negative. In GIII, all cases were negative, and calculated cut off value was 0.44.

Nano graphene based Sandwich ELISA to detect *Giardia* antigen in stool samples (Tab, 3), showed 51 positive cases (83.6%) in GI out of 61. In GII, 10 cases were positive out of 20 and 10 cases were negative. In GIII all cases were negative. Calculated cut off value was 0.43.

Nano graphene based Sandwich ELISA was higher than Traditional Sandwich ELISA on detecting *Giardia* antigen in stools (Tab. 4) as to sensitivity (83.6% vs. 57.4%), PPV (83.6% vs. 81.4%), NPV (71.4% vs. 50.9%) and diagnostic accuracy (79.2% vs. 64.6%). Traditional Sandwich ELISA was higher than Nano graphene based Sandwich ELISA; specificity (77.1% vs. 71.4%), with significant difference (p- <0.05).

Dot ELISA for *Giardia* antigen in stool samples (Tab. 5) showed 56 positive cases (91.8%) in GI out of 61. In GII, 7 cases were positive out of 20 and 13 cases were negative. In GIII all cases were negative.

Nano graphene based Dot ELISA for detection of *Giardia* antigen in stool samples (Tab. 6), showed 56 positive cases (91.8%) in GI out of 61. In GII, 11 cases were positive out of 20 and 9 cases were negative. In GIII all cases were negative.

Dot ELISA and Nano graphene based Dot ELISA had same sensitivity (91.8%) for *Giardia* antigen in stool samples (Tab. 7). Dot ELISA was higher than Nano graphene based Dot ELISA as to specificity (80% vs. 68.6%), PPV (88.9% vs. 83.6%), NPV (84.8% vs. 82.8%) and diagnostic accuracy (87.5% vs. 83.3%), but without significant difference (p-value >0.05).

Results

The results were in tables and figures

Table 1: Comparison of the diagnostic performance of different methods (direct smear, Mini Parasep & MIFC) used for detecting *Giardia* cyst in stool samples of GIa using mean±SD and ANOVA test

	Giardia cyst under microscope					ANOVA	
	Positive	Mean	±SD	Min.	Max.	F	p-value
Direct	41	3.52	3.14	0.67	12	6.167	0.003*
MIFC	43	5.86 (a)	6.23	0.3	30		
Mini Parasep.	43	7.78(a,b)	6.58	1	25		

a: significant difference direct <0.05, b: significant difference MIF <0.05

Table 2: Detection of *Giardia* antigen in stool samples using Traditional Sandwich ELISA in groups

Groups	Cut-off=0.44: Traditional Sandwich ELISA				Total	
	Positive		Negative			
	No.	%	No.	%	No.	%
Group I: *Giradia*	35	57.40%	26	42.60%	61	100.00%
a: Cyst	27	55.1%	22	44.9%	49	100.0%
b: Trophozoite	8	66.7%	4	33.3%	12	100.0%
Group II: Other parasites	8	40.0%	12	60.0%	20	100.0%
Group III: Healthy Control	0	0.0%	15	100.0%	15	100.0%

Table 3: Detection of *Giardia* antigen in stool samples by Nano graphene based sandwich ELISA in groups

Groups	Cut-off=0.43: Nano Sandwich ELISA				Total	
	Positive		Negative			
	No.	%	No.	%	No.	%
Group I: *Giradia*	51	83.60%	10	16.40%	61	100.00%
a: Cyst	40	81.6%	9	18.4%	49	100.0%
b: Trophozoite	11	91.7%	1	8.3%	12	100.0%
Group II: Other parasites	10	50.0%	10	50.0%	20	100.0%
Group III: Healthy Control	0	0.0%	15	100.0%	15	100.0%

Table 4**:** Sensitivity, specificity, PPV, NPP and diagnostic accuracy percentage of Traditional Sandwich ELISA and Nano graphene based Sandwich ELISA for detection of *Giardia* antigen in stool samples

Techniques	Sens.	Spec.	PPV	NPV	Accuracy
Traditional Sandwich ELISA	57.4%	77.1%	81.4%	50.9%	64.6%
Nano Sandwich ELISA	83.6%	71.4%	83.6%	71.4%	79.2%

Table 5: Detection of *Giardia* antigen in stool samples using Dot ELISA in groups

Dot ELISA	Positive Cases		Negative Cases		Total	
	No.	%	No.	%	No.	%
Group I: *Giradia*	56	91.8%	5	8.2%	61	100%
a: Cyst	47	95.9%	2	4.1%	49	100%
b: Trophozoite	9	75.0%	3	25.0%	12	100%
Group II: Other parasites	7	35.0%	13	65.0%	20	100%
Group III: Healthy Control	0	0.0%	15	100.0%	15	100%

Table 6: Detection of *Giardia* antigen in stool samples using Nano geaphene based Dot ELISA in groups

Nano Dot ELISA	Positive Cases		Negative Cases		Total	
	No.	%	No.	%	No.	%
Group I: *Giradia*	56	91.8%	5	8.2%	61	100%
a: Cyst	45	91.8%	4	8.2%	49	100%
b: Trophozoite	11	91.7%	1	8.3%	12	100%
GII: Other parasites	11	55.0%	9	45.0%	20	100%
GIII: Healthy Control	0	0.0%	15	100.0%	15	100%

Table 7: Sensitivity, specificity, PPV, NPP and diagnostic accuracy percentage of Dot ELISA and Nano geaphene based Dot ELISA for detection of *Giardia* antigen in stool samples

Technique	Sensitivity	Specificity	PPV	NPV	Accuracy
Dot ELISA	91.8%	80.0%	88.9%	84.8%	87.5%
Nano Dot ELISA	91.8%	68.6%	83.6%	82.8%	83.3%

Discussion

Microscopic techniques (direct or concentrated) for fecal samples are still commonly used to detect *Giardia* cysts or trophozoites. But, analysis of single stool sample and the skill of the microscopist can affect the detected accuracy (Beyhan and Cengiz, 2017). Given these difficulties the development of sensitive, cost effective and rapid diagnostic methods is of the most importance (Moharam *et al*, 2014).

Single antigen detection technique detected 50% more infections than the routine stool examination (Garcia, 2007).

Nanotechnology may improve sensitivity, selectivity, speed, cost, and convenience of diagnostic tests (Hegazy *et al*, 2015).

The present study revealed that Mini Parasep concentration technique was the best followed by MIFC technique and the least result was obtained from direct smear. There is a statistically significant difference between them.

Sanprasert *et al.* (2016) showed that Mini Parasep® SF is the most sensitive (56.38%) in the intestinal parasites detection among school-age children, followed by direct smear (40.43%) and modified formol ether concentration technique (M-FECT) (37.32%). Zeeshan *et al.* (2011) found that Parasep filters enhanced the ability to detect intestinal parasites in stool; 100 samples were negative for parasites using direct microscopy whereas 13 of them were positive with Parasep.

On the other hand, Kitvatanachai and Rhongbutsri (2017) recorded the highest efficacy of direct smear technique in detecting intestinal parasites (74.62%), followed by modified formol ether concentration technique (65.67%) and Mini Parasep® SF kit (55.22%).

The present study revealed that microscopic examination was simple, non-invasive and allowed the detection of other parasitic infections. This agreed with Lebwohl *et al.* (2003). However, routine microscopic examination for diagnosis of giardiasis is time consuming and relies on the microscopist's skills and experience (Rosoff *et al*, 1989; Scheffler and Van Etta, 1994; Schuurman *et al*, 2007). The sensitivity of laboratory diagnosis of *Giardia* infection can be improved by repeating stool examination on 3 consequative days. But this was not possible in the present study, as the patients were attending the outpatient clinics.

In order to increase the sensitivity, different immunological methods have been developed as an alternative for the diagnosis of giardiasis (Chakarova, 2010).

In this study, a novel antigen-capture immunoassay based on IgG pAb conjugated with graphene nanoparticles was used for detection of *Giardia* antigen in stool samples which was used as a first trial for diagnosis of human giardiasis.

The current study (Tab. 4) demonstrated that Nano Sandwich ELISA was superior to Traditional Sandwich ELISA on detection of *Giardia* antigen in stool samples regarding sensitivity (83.6% vs. 57.4%), PPV (83.6% vs. 81.4%), NPV (71.4% vs. 50.9%) and diagnostic accuracy (79.2% vs. 64.6%), while specificity in Traditional Sandwich ELISA was higher than Nano Sandwich ELISA (77.1% vs. 71.4%). This was a statistically significant difference between them ($P < 0.05$). This means that the use of graphene nanoparticles improved the diagnostic testing of human giardiasis.

Moharam *et al*. (2014) compared Nano gold based ELISA and Traditional Sandwich ELISA in detecting *Giardia* antigen in stool samples; they reported that the sensitivity and specificity of Nano Sandwich ELISA was higher than that of Traditional Sandwich ELISA (95.8% vs. 93% & 95% vs. 92.5%) respectively.

Another study aimed was to detect potential specificity and sensitivity of paramagnetic nanoparticles based ELISA for diagnosis of human giardiasis by detection of *Giardia* copro-antigen, Koura *et al*. (2016) reported that Sandwich ELISA achieved sensitivity of 88% and specificity of 92%, while

immuno-magnetic bead ELISA with paramagnetic nanoparticles achieved higher values of sensitivity and specificity; 92% & 94%, respectively.

The difference between the results of the present work and other studies may be explained by the difference in the method of antigen preparation, the strain of *Giardia* and the number of participating individuals in each study; positive cases, other parasites and control groups.

Several studies compared between Nano based ELISA versus (vs.) Traditional ELISA for detection of parasitic infection. Among them, Naser *et al*. (2017) who compared the sensitivity and specificity of the Traditional Sandwich ELISA vs. Nano gold based Sandwich ELISA for detection of *Cryptosporidium* antigen in stool samples. They reported that the sensitivity of Nano gold based Sandwich ELISA was higher than that of Traditional Sandwich ELISA (85% vs. 68%), while the specificity of Nano gold based Sandwich ELISA was lower than that of Traditional Sandwich ELISA (73.4% vs. 77.3%). Rashed *et al.* (2018) tested the Validity of Sandwich ELISA and Nano-gold Sandwich-ELISA for diagnosis of human hydatidosis. They reported that Nano-gold Sandwich-ELISA had higher values than Traditional Sandwich-ELISA regarding sensitivity (96.3% vs. 81.5%); specificity (95% vs. 80%); PPV (96.3% vs. 84.6%); NPV (95% vs. 88.89 %) and diagnostic accuracy (95.7% vs. 80.9%).

The current work showed that Dot ELISA and Nano graphene based Dot ELISA had the same sensitivity (91.8%) on detecting *Giardia* antigen in stool samples while Dot ELISA was higher than Nano Dot ELISA on detecting *Giardia* antigen in stool samples regarding specificity (80% vs. 68.6%), PPV (88.9% vs. 83.6%), NPV (84.8% vs. 82.8%) and diagnostic accuracy (87.5% vs. 83.3%), but there is no statistically significant difference between them (P >0.05).

Referring to hydatidosis diagnosis; Rashed *et al.* (2018) tested the validity of Dot-ELISA and Nano-gold Dot-ELISA for detection of human hydatidosis. They reported that Nano-gold Dot-ELISA had higher values than Traditional Dot-ELISA regarding sensitivity (95.7% vs. 88.9%); specificity (95% vs. 80.7%); PPV (96.3% vs. 85.7%); NPV (95% vs. 84.2 %) and diagnostic accuracy (96.3 vs. 85.1%).

The low sensitivity and specificity by using ELISA methods in the current work than some other studies may be due to dilution of stool samples during the preparation, which may be not adequate for carrying enough number of the cysts/trophozoites to be detected especially in cases with light infection; when few numbers are present in the specimen. This agreed with Naser *et al*. (2017).

Conclusion

Microscopic examination is reliable in diagnosis of human giardiasis as a first choice especially concentration techniques such as Mini Parasep and MIFC. In addition, this study using the prepared anti-*Giardia* IgG pAb that was carried out for detection of *Giardia* antigen in stool samples of patients infected with *Giardia* revealed that the use of graphene nanoparticles improved the diagnostic testing of human giardiasis.

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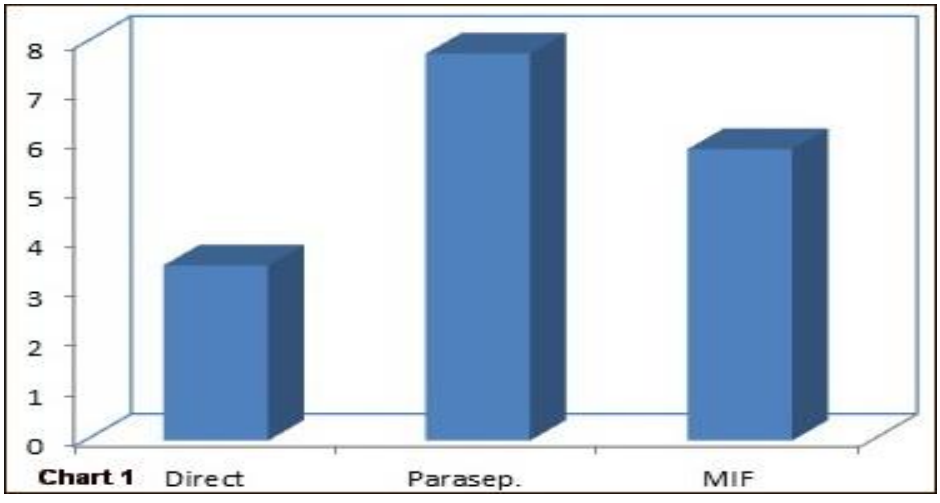
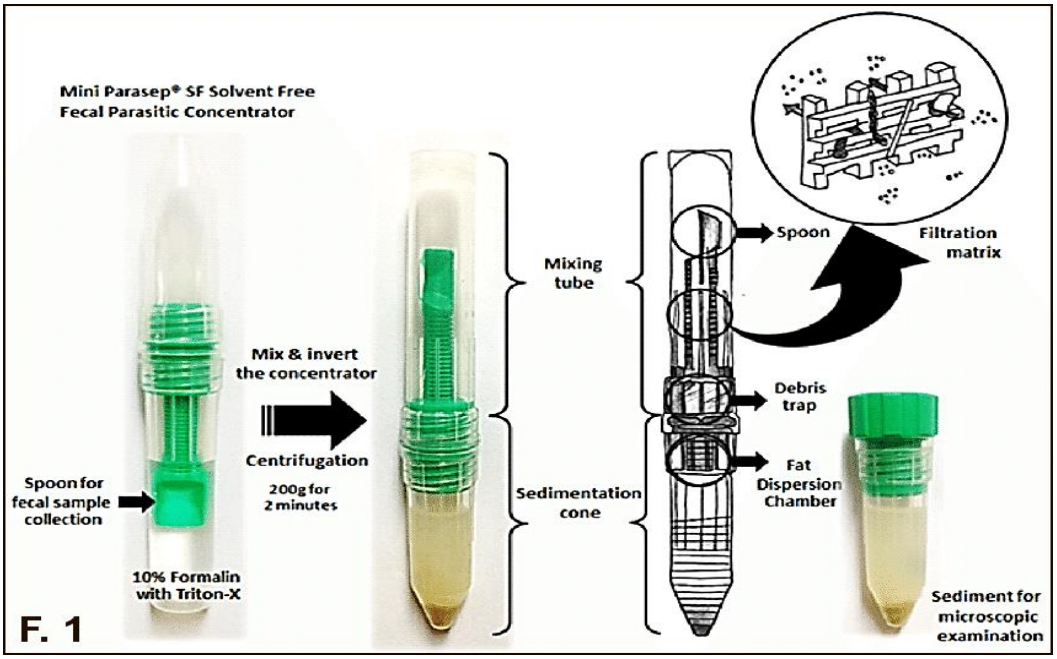
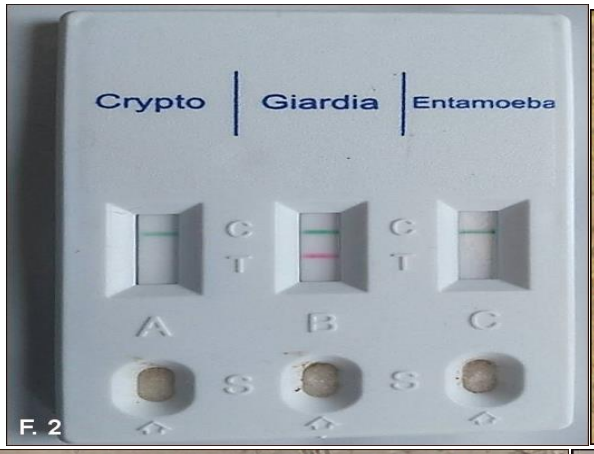
Explanation of figures

Chart 1: comparison between direct smear, Mini Parasep & MIFC techniques used for detecting *Giardia* cyst in stool samples of GIa
Fig. 1: Mini parasep
Fig. 2: Assessment of reactivity of *Giardia* antigen by Rapid diagnostic test
Fig. 3: *Giardia* cyst, direct smear, stained with iodine (X1000)
Fig. 4: *Giardia* cyst (prepared with MIFC, X1000)
Fig. 5: *Giardia* cysts (prepared with Mini Parasep, unstained, X1000)




Mini Parasep® SF Solvent Free
Fecal Parasitic Concentrator
Spoon for fecal sample collection
10% Formalin with Triton-X
Mix & invert the concentrator
Centrifugation
200g for 2 minutes
Mixing tube
Sedimentation cone
Spoon
Filtration matrix
Debris trap
Fat Dispersion Chamber
Sediment for microscopic examination
F. 1


Crypto
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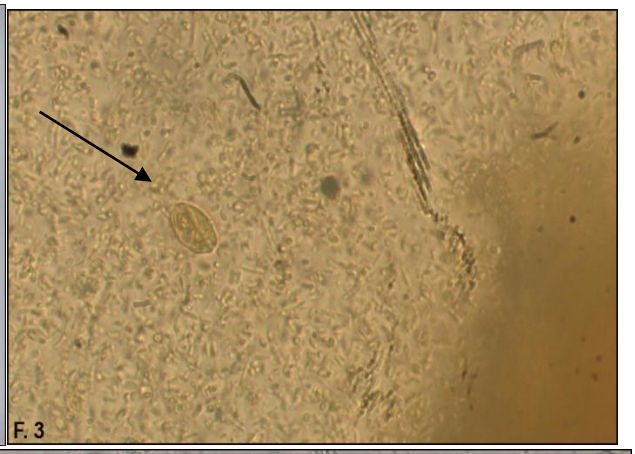
F. 2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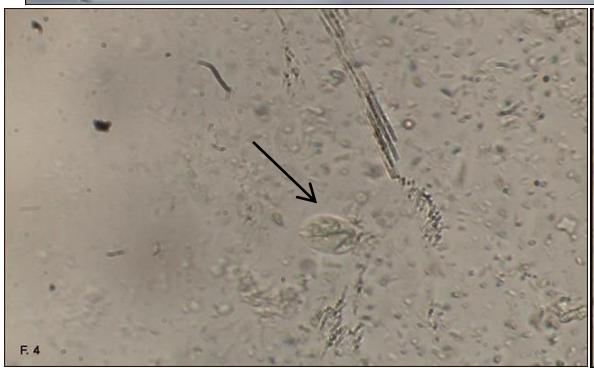
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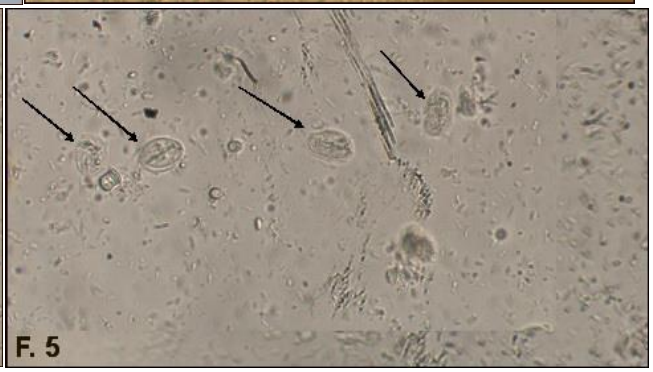
F. 5