Diagnosis of Lymphatic Filariasis Using Nano-Chip CA-RD Assay Compared To Nano-Based Enzyme Linked Immunosorbent Assay

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Abstract

Filariasis is a parasitic infection caused by small roundworms that live in humans' blood and tissues. This study developed a modified nano enzyme-linked immunosorbent test (ELISA) for detection of filarial antigen in human sera. The assay's limit of detection (LOD) and cost were dramatically decreased as a result of this improvement. Serum samples from affected persons were gathered across Egypt's governorates. Membrane filtration was used to determine the individuals' microfilarial status, which was then examined under a light microscope. Sera were collected from 27 microfilariae patients (MF), 21 with chronic lymphatic illness (CL), and 20 microfilariae negative healthy people living in non-endemic areas, as well as sera from 20 other parasites affected patients, after informed consent was obtained. Following their consent, all of these individuals underwent the following procedures: history taking, clinical examination, and laboratory investigations, which included examination of blood samples for microfilaria using thick blood film and serological tests for detection of the circulating filarial antigen (CFA) using sandwich ELISA, Home-made rapid card test, and nano-based ELISA. All non-endemic normal sera were negative for filarial antigen using different applied techniques. On the other hand, 23/27 microfilaraemia sera and 7/21 chronic lymphatic patients' sera were found using Home-made rapid card test. Filarial antigen was found in the serum of 18/27 microfilaraemia patients and 9/21 chronic lymphatic patients using sandwich ELISA. Filarial antigen was found in 25/27 microfilaraemia sera and 6/21 chronic lymphatic patients' sera using nano-based ELISA. Finally, utilizing a modified enzyme-linked immunosorbent assay (ELISA) approach, compinednanoparticle are effective nano-analytical tools for detecting human filariasis.In addition home-made rapid card test was effective diagnostic method for filariasis.

Keywords Human Filariasis - Rapid card test – Filarial Antigen - ELISA

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INTRODUCTION

Lymphatic filariasis, commonly known as elephantiasis, is a neglected tropical disease. Infection occurs when filarial parasites are transmitted to humans through mosquitoes. Infection is usually acquired in childhood causing hidden damage to the lymphatic system (Kabatereine, et al., 2010; Gilabert and Wasmuth, 2013; Mwakitalu, et al., 2013). Accurate and brief determinations are essential for management of filariasis at the individual level and controlling diseases populations in endemic areas. Normally, filariasis analysis relies on the identification of microfilaria in streaked blood and the microscopic evaluation of red thick blood. According to World Health Organization (WHO) regulations, it may take 12 hours to dry thick blood smears during the dry season and 24 to 48 hours during the windy season with high humidity(Shenoy, 2008). In 2018, 893 million individuals in 49 countries need preventative chemotherapy to prevent the spread of illness (Fimbo, et al., 2020). The global baseline affected by lymphatic filariasis is estimated to be 25 million men with hydrocele and more than 15 million patients with lymphedema (Malecela, et al., 2021). At least 36 million people still have manifestations of these chronic diseases (King, et al., 2018). Eliminating lymphatic filariasis can save lives and assist to alleviate poverty. Lymphatic filariasis infections include asymptomatic, acute, and chronic diseases. Most infections are asymptomatic, with no visible evidence of illness, and contribute to parasite propagation**.** These silent infections can nevertheless harm the lymphatic system and the kidneys and damage the body's immune system (Shenoy, 2008).The World Health Organization hopes to achieve the goal of global eradication of lymphatic filariasis through mass drug administration using one of three anthelmintic regimens: diethylcarbamazine plus albendazole in endemic countries. Cancer and filariasis are not co-endemic, ivermectin plus albendazole in African countries where the cancer is endemic and albendazole alone in areas of Africa where filariasis and lymphatic filariasis are circulating together. Compared with the standard two-drug regimen of diethylcarbamazine and albendazole used outside of sub-Saharan Africa, the triple regimen consisting of ivermectin, diethylcarbamazine and albendazole was more effective in the treatment of lymphatic filariasis .(King, *et al.,* 2018). Observing the presence of microfilariae (adult worms) in thick film blood collected between 10:00 p.m. and 2:00 a.m., with or without DEC excitation, using Giemsa or hematoxylin and eosin staining to determine the presence of microfilariae (Eberhard and Lammie, 1991). When adult microfilariae develop, antigens appear, resulting in a rise in IgG4 levels (Adjobimey and Hoerauf, 2010). For decades, antibody detection has been the basis of filariasis diagnostic analysis, especially natural antigens. The best of these tests are sensitive to infection, but cannot distinguish the current infection from previous infections or exposure to other parasites (Weil, et al., 1999). For the diagnosis of *W. bancrofti*, circulating filarial antigen (CFA) tests have been established**.** Because these tests identify antigens secreted by mature filarial worms, they can be positive even in microfilaremic people**.** Furthermore, because antigen levels stay consistent during the day and night, these tests may be conducted at any time (Chesnais, et al., 2016)**.** Commercially available CFA tests for the detection of *W. bancrofti* include a monoclonal antibody-based enzyme-linked immunosorbent assay

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(ELISA) that provides a quantitative result that corresponds with adult worm load (Weil, et al., 1997). Immuno-chromatographic technique (ICT) card-based assay which gives only qualitative results (Makhsin, et al., 2012). Nano-diagnostics involve the use of nanotechnology in clinical diagnosis to meet the demands for increased sensitivity, specificity and early detection in less time. The large surface area of A nanomaterial allows for the attachment of a large number of target-specific molecules of interest for ultrasensitive detection (Buzea, et al., 2007).

Many unique and effective nano-diagnostics for infectious diseases have been created in this field**.** Most significantly, these nanotechnology-based systems have the potential to produce portable, robust, and economical POCT platforms for detecting infectious diseases in underdeveloped nations**.** For example, researchers have created a smartphone dongle that may be used as a POCT device to screen infectious diseases (Gounoue-Kamkumo, et al., 2015). In this work, a modified enzyme-linked immunosorbent test (ELISA) technique based on antibody-functionalized nanoparticles was created for the diagnosis of human filariasis in comparison to the standard methods used.

DETAILS EXPERIMENTAL

2.1. Materials and Procedures

Infected serum was collected from a variety of different regions of Egypt. After heparinized venous blood was filtered by membrane filtration using polycarbonate filters (Nucleopore, Pleasanton, Calif., USA), a light microscope was used to examine it**.** Sera were taken with informed permission from 27 microfilaremic patients (MF), 21 with chronic lymphatic illness (CL), and 20 microfilariae negative healthy persons living in endemic areas (endemic normals [EN]), while sera from 20 additional parasites infected patients were collected in parallel**.** Following their consent, all of these individuals were subjected to the following procedures: history taking, clinical examination, and laboratory investigations, which included an examination of blood samples for microfilaria using thick blood film and serological tests for detection of circulating filarial antigen ELISA, Home-made rapid card test (RCT), and Nano-ELISA.

Thick Blood Smears Test

A thick smear test was performed, following the standard procedure developed by Garcia and Procop (2016), to determine the severity of LF infection. With brief, 50 µL of blood was taken through finger prick, distributed on a slide, de-haemoglobinized in water, and stained with Giemsa. An optical microscope was used to examine these preparations (x100 or x400 magnification). Microfilariae of *W. bancrofti* were detected and counted, and the findings were represented as microfilariae (mf) per milliliter of blood (mf/ml).

Patients' serum: venous blood samples approximately 4 ml were taken from all individuals in EDTA tubes. Sera were separated by centrifugation at 2000 g for 10 minutes and then fractionated into small eppendorf and stored at - 20°C until used.

Home-made rapid card test (RCT).

Antibody conjugated with graphene oxide (GO) was prepared two steps. Briefly, 1 mg of GO was ultrasonically dispersed in 5 mmolof (4-(2-hydroxethyl)-1-piperazineethanesuulfonic acid (HEPES) pH 5.5, then centrifuged and washed 3 times with 0.05M of phosphate buffersolution (PBS, pH = 7.4).then mixing with 25ul of filarial antibodies, vortex for 5 seconds and rotate the mixture for 2 hours at 15 °C. subsequently 30ul of 10 % Bovine Serum Albumin (BSA) in 0.05 M PBS was added to block the residual surface of the GO. A volume of 50µL of each individual plasma sample was placed to the sample pad of the

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home- prepared rapid card. This pad includes a polyclonal anti-filarial antibody that is GO-labeledand binds to filarial antigen in the plasma. When the card is closed, the pad comes into contact with a nitrocellulose strip.The antibody-antigen combination then proceeds along the strip, where it is captured by an immobilized anti-filarial monoclonal antibody (AD12.1) included in the strip's coating. At 10 minutes, all legitimate positive tests have dark gray control (C) and test (T) lines visible, whereas antigen-negative persons only have the control pink line visible.

NANO-ELISA

Graphene loaded anti-FA ELISA: Microtitration plates were covered with 100 µl/well anti-FA pAbs combined with Graphene nanoparticles (20 ng/ml carbonate buffer, pH 9.6) and incubated at room temperature overnight. Plates were rinsed three times with 0.1 MPBS/T at a pH of 7.4. The remaining sites were blocked with 100 µl/well 2.5 percent FCS/PBS/T and incubated at 37 °C for 2 hours. PBS/T was used to wash the plates three times. Serum samples (100 µl) were pipetted into duplicate wells and incubated at 37°C for 2 hours. After three washes, 100 µl/well of peroxidase-conjugated pAbs were added and incubated for 1 hour at room temperature. (Zhang, et al., 2013).

RESULTS

Study population:

In this study, the microfilarial patients were 27 with mean age (41±2.4), the percent of females was 55.6 % versus 44.4% male. While in CL group the percent of female versus male was 38.1% and 61.9% respectively. Table1. After the parasitological screening using thick blood smears Test, the individuals were classified into four parasitological categories: group I - 27/88 (30.7%) MF positive; group II - 21/88(23.9%) chronic LF; group III - 20/88 (22.7%) MF–and group IV 20/104 (22.7%) MF negative (Table 2&4).

Table (1): Demographic characteristics of study population

Groups	Number	Age	Sex M/F
microfilaremic patients (MF)	27	15-55	12/15
chronic lymphatic patients (CL)	21	17-60	13/8
healthy individuals	20	20-45	8/12
other parasites	20	15-50	9/11

Table (2): Thick blood smears Test

Groups	Number	Positive	Negative
microfilaremic patients (MF)	27	27	-
chronic lymphatic patients (CL)	21	2	19
healthy individuals	20	-	20
other parasites	20	-	20

Table (3): Detection of CFA in Filarial Sera using commercial kit ELISA

Groups	Positive cases			Negative cases		
	Mean (OD) ± SE	No.	% positivity	X (OD) ± SE	No	% negativity
healthy individuals	-	20	0	0.326± 0.03	20	100%
microfilaremic patients (MF)	2.12 ± 0.22	23	85.2 %	0.179± 0.14	4	14.8%
chronic lymphatic patients (CL)	1.17 ± 0.21	7	33.3 %	0.211± 0.12	14	66.7 %
Other parasites (n= 20)	0.521 ±0.03	2	10 %	0.191 ±0.11	18	90%

Detection of CFA Using Home made Rapid card

Using home-made rapid card for detection of CFA 23 cases gave positive results in MF group, while 4 cases were negative in sera of patients with microfilaremic (85.2% incidence). Seven patients were positive within chronic group (33.3% incidence).In patients with various parasite illnesses, two instances were found to be positive, while the remaining 18 were found to be negative. All of the healthy control patients tested negative. The ICT test had a 90% specificity for detecting human filariasis (Table 3&4 and Fig. 1).

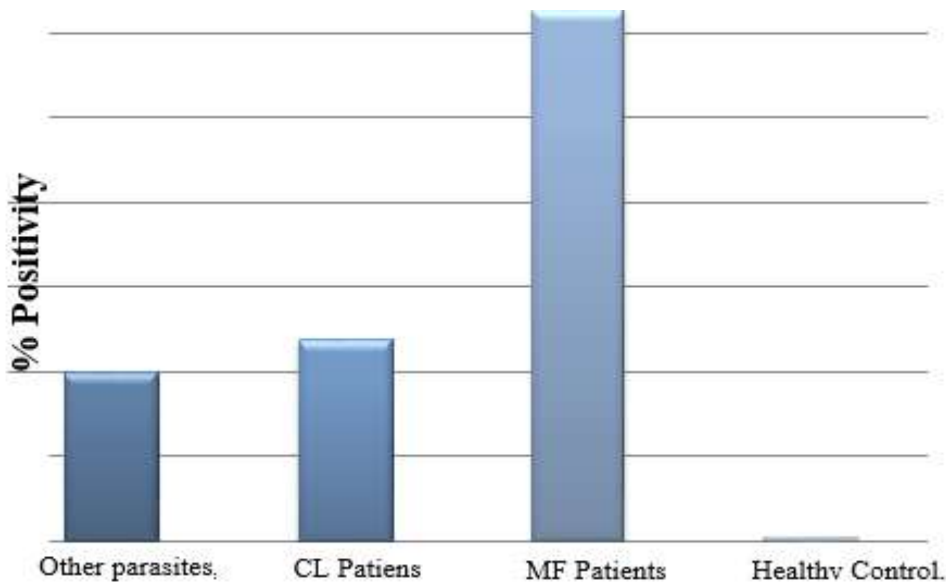

Fig.1 Detection of CFA Using Home made Rapid card

Detection of CFA Using Sandwich ELISA

The mean OD reading of negative controls SD of the mean was computed to define the cut-off value for positivity or the line of demarcation between positive and negative findings. Tested samples with OD values greater than the cut-off value were deemed positive for filariasispatients.The cut-off value was 0.263 when detecting in circulating filarial antigenin serum by using sandwich ELISA. The results were positive in 21/27, while 6 cases were negative in sera of patients with filariasis (77.8% incidence). Nine patients were positive within chronic group (42.9% incidence). In patients with other parasitic infections 8 cases were detected as positive, while the other 12 cases were negative. All healthy control patients were negative. The specificity was 80.0%. (Fig. 2).

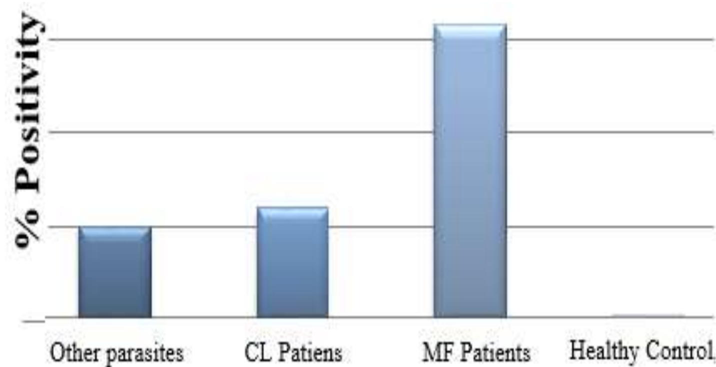

Fig. 2 **Detection of CFA Using Sandwich ELISA**

Detection of CFA Using Nano-Sandwich ELISA

The cut-off value for positivity was 0.448when detecting in circulating filarial antigen in serum, by using nano-ELISA. Twenty-five cases gave positive results, while 2 cases were negative in sera of patients with filariasis (92.6% incidence). Ten patients were positive within chronic group (47.6% incidence).In patients with other parasitic infections 4 cases were detected as positive, while the other 16 cases were negative. All healthy control patients were negative. The specificity was 95.1% (Fig. 3).

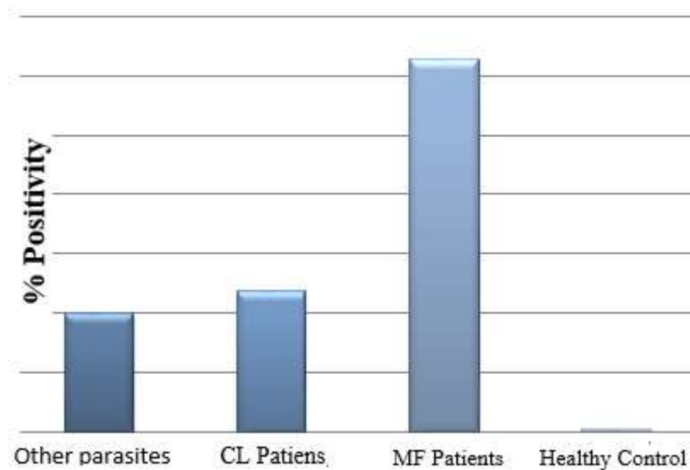

Fig. 3 Detection of CFA Using Nano-Sandwich ELISA

DISCUSSION

Lymphatic filariasis (LF) is one of the ignored tropical diseases that are transmitted by mosquitoes. It is endemic in 81 countries and puts 1.2 billionpeople at risk, of which 120 million are expected to be infected (Chesnais, Vlaminck, Kunyu-Shako, Pion, Awaca-Uvon, Weil, Mumba and Boussinesq, 2016; Manyeh, et al., 2019). Adult long-filament nematodes, specifically *W. bancrofti*, *Brugiamalayi*, and *Brugiatimori*, are largely accused of "domestic"lymphatic murmur (lymphedema, elephantiasis, and hydrocele) in the human lymphatic system.

Table (3): Summarized of sensitivity and specificity for different techniques.

Techniques	Sensitivity%		Specificity%
	MF Patients	CL Patients	
commercial kit ELISA	85.2 %	33.3 %	95%
ICT Kit	62.9 %	23.8 %	77. 5 %
Sandwich ELISA	77.8 %	42.9 %	80 %
Nano-Sandwich ELISA	92.6 %	47.6 %	95.1 %

For detecting filariasis, antigenic detection is a very sensitive and specific technique. Even if a patient has extremely low levels of microfilariae that cannot be identified by microfilariae testing, antigen detection will almost always discover filariasis. It is also specific in that it identifies particular antigens for lymphatic filariasis and differentiates between microfilarial infections. The most successful antigen detection test is the antigen card test. Furthermore, this test is very specific and sensitive, as well as rapid (available in 10 minutes), portable, and low-cost. In addition, unlike other tests that need blood samples at periods when the number of microfilariae in the blood is high, this test may be done at any time of day or night (Mohanty, et al., 2001). The antigen detection assay, however, cannot distinguish between active and inactive microfilariae. Despite a good ICT test, the patient may or may not have living, active microfilaria. If some dead microfilaria remain in the bloodstream, antigens may still be present (Chesnais, et al., 2013). After informed consent, we collected sera fr om 27 microfilaremic patients, 21 patients with chronic lymphatic disease, 20 healthy individuals who reside in the endemic areas of microfilariae positivity and 20 other parasitically infectedindividuals.

Male and female patients in the study were equally represented by microfilarial infections (55.6%) and 44.4%, respectively. The difference in percentages was not statistically significant. However, in chronic lymphatic patients a significant increase amongst examined male patients than that in female (*P <0.05*). On previous study by Alexander and Grenfell (1999) that females have a lower mean prevalence of infection than men. Females are less exposed to

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infective vectors, resulting in lower rates of infection and clinical pathology. Other study by Maciel, et al. (1996) In terms of sex differences, rates of microfilaremia are equal for both sexes in certain places, while males are more afflicted in others.

Thick blood film was done to all persons after an hour of taking 100 mg DEC to detect microfilaria. 5.1% of filarial diseased persons were positive for microfilaria, while 94.9% were microfilaraemic. The explanation of this result is that most of the patients were chronic obstructive cases (lymphedema and elephantiasis), so lymphatics were obstructed which prevented microfilariae appearance in the peripheral blood. Until the early 1990s, the blood thick smear was the sole parasitological tool available for assessing individuals or communities infected with *W. bancrofti*. However, because of its limited sensitivity, this approach cannot accurately determine the real frequency of infection. (Mohanty, Satapathy, Sahoo and Ravindran, 2001). Mustafa (2013) Antigen detection assays, rather than antibody testing, may allow for quicker diagnosis since the synthesis of measurable amounts of particular immunoglobulin takes time. Sandwich ELISA is commonly used to identify antigens in serum.

In the present investigation using ICT kit for detection of filariasis, the percent of positivity was 62.9% incidence in MF patients. Five patients were positive within chronic group (23.8% incidence).In patients with other parasite illnesses, 9 instances were found to be positive, while the remaining 11 cases were found to be negative. All of the healthy control patients tested negative. 77.5 percent of the time, the specificity was 77.5 percent. When ICT was compared to night blood samples and day samples following DEC provocation, a rate of 38 percent microfilaria positivity was discovered among the ICT test's night blood samples and 57.4 percent microfilaria positivity was detected among the DEC provocation test's samples (Faris, et al., 1993). The results were positive in 21/27, while 6 cases were negative in sera of patients with filariasis (77.8% incidence). Nine patients were positive within chronic group (42.9% incidence). In patients with other parasite illnesses, 8 instances were found to be positive, while the remaining 12 cases were found to be negative. All of the healthy control patients tested negative. The specificity was set at 80.0 percent.

The present study showed that the sensitivity values of sandwich ELISA were 77.8% and 42.9 % within MF and chronic filariasis group respectively, where the specificity was 80.0%. Faris, Ramzy, Gad, Weil and Buck (1993) detected circulating filarial antigen by ELISA with a sensitivity of 97.5%. ELISA gave 94% sensitive and 70% specific for detection of microfilariae antigen. Wattal, et al. (2007) found that ELISA gave 100% sensitivity and 94.12% specificity. Hoti, et al. (2002) by ELISA found specificity (100%) and sensitivity (96.8%) of samples collected at night.

Another study showed that of the 600 test subjects (341 men; 259 women) who were tested for blood circulatory filament antigen (CFA), 141 subjects were antigenemic positive, for an overall CFA prevalence of 23, fifty%; 13.8% men and 9.7% men, was not statistically significant, which suggests that the distribution of antigenemia in the population studied was not a specific gender bias (Ivoke, et al., 2015). In another study, the ELISA was found to be 94% sensitive and 70% specific for the detection of microfilariae antigen. In a similar study by Wattal, Dhariwal, Ralhan, Tripathi, Regu, Kamal and Lal (2007) found that the ELISA test was found to have 100% sensitivity and 94.12% specificity. In another study by Hoti, Elango, Radjame, Yuvaraj and Pani (2002). When comparing the ELISA test between chronic carriers of Mf and acute filariasis, the specificity of the

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assay in serum samples taken overnight was found to be 100% and the sensitivity was 96.8%.

Using the Nano ELISA sandwich, the sensitivity in sera of MF patients was 92.6% incidence and 47.6 % within chronic group, the specificity was 95.1%. Because of their high binding capacity (as a solid phase) and quick response kinetics of solutions (with the simple separation of bound and unbound material in the solid phase), nanobeads may give the chance to increase antigen detection in immunoassay.

CONCLUSIONS

The application of nano-sandwich ELISA for the identification of filarial antigen in blood samples from W. bancrofti-infected individuals leads in increased sensitivity and specificity. The best diagnostic properties of nanoparticles may be their high-binding capacity (as a solid phase) and fast response kinetics of the solutions; hence, they may provide the prospect of increasing antigen detection in immunoassays.

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