Int J Biol Med Res.2017;8(1):5851-5856

Contents lists available at BioMedSciDirect Publications

International Journal of Biological & Medical Research

Journal homepage: www.biomedscidirect.com

Original article

Purification and characterization of *Setaria equina antigen* and evaluate its Efficacy for diagnosis of human filariasis

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ARTICLE INFO

Keywords:
W. bancrofti,
S. equina,
antigens,
polyclonal antibody (pAb)

ABSTRACT

Lymphatic filariasis (LF) is still a public health problem in tropical and subtropical countries. The most common causative agents of human filariasis are Wuchereria (W.) bancrofti, Brugia (B.) malayi and B .timori. This work was designed to evaluate the diagnostic efficacy of 3 types of antigens including crude Setaria (S.) equina, purified S. equina and Dirofilaria (D.) immitis antigens in detecting anti-W. bancrofti IgG polyclonal antibody (pAb) in W. bancrofti infected patients .Crude S. equina antigen from S. equina adult worms was purified by DEAE sephadex G-50 ion exchange chromatography and gel filtration chromatography on Sephadex-G-200 HR column. A total of 92 Egyptian individuals were enrolled in this study and divided into 3 groups. Group I included 42 patients infected with W. bancrofti, group II included 30 patients infected with other parasites as a parasitic control group (10 patients with Schistosomiasis mansoni, 10 patients with Fasciolasis and 10 patients with Hydatidosis) and group III as healthy controls (n= 20). Detection of anti-W. bancrofti IgG pAb in patient's sera by indirect ELISA using crude S. equina, purified S. equina and D. immitis antigens showed 78.6 %, 85.71% and 80.95% sensitivity rates and 68%, 76% and 80% specificity rates, respectively. In conclusion, purified S. equina antigen is promising for diagnosis of W. bancrofti in human. Further study is highly recommended in order to obtain monoclonal antibody against purified fractions of S. equina adult worm antigen to reach higher specificity and sensitivity.

1. Introduction

Lymphatic filariasis (LF) is a globally distributed disease recognized by the World Health Organization (WHO) as one of the most disabling diseases [1]. LF caused by Wuchereria (W.) bancrofti and Brugia (B.) malayi is a major public health and socioeconomic problem in tropical and subtropical countries include central Africa, the Nile delta [2, 3]. An estimated 120 million people in 73 countries are infected with LF, and an estimated 1.4 billion live in areas where the disease is endemic [4, 5].A series of studies revealed that incidence of microfilaria (mf), increased in different governorates in Egypt [6]. Approximately 50 million people in Egypt and Sub-Saharan Africa have bancroftian filariasis and together they represent about a third of all cases of LF worldwide [7].

Serological diagnosis of filariasis is more sensitive than detection of mf in blood samples due to nocturnal periodicity phenomena of mf. Immunodiagnosis is the only possible line of investigation of cases with chronic filariasis in which mf are not detected [8, 9]. Trials were done to explore the potential of heterogeneous filarial antigen isolated from D. immitis [10, 11], S. cervi [12], S. digitata and S. equina [13].

Adult S. equina is a filarial parasite commonly found floating free within the peritoneal cavity of equines in all parts of the world [14, 15]. It infects both human and equines [16, 17]. Cross-reaction has previously been reported between the antigens of S. equina, D. immitis and S. digitata adult worms and sera from human infected with W. bancrofti [18, 19, and 20]. D. immitis adult worm was used as a source of antigens and excretory-secretory (ES) products to detect human filariasis [21, 22]. S. equina adult worms are large and so huge amounts of antigens can be prepared [13]. In addition, S. equina adult worm is more economic than the imported antigens prepared from D. immitis and others. So, diagnosis of human filariasis by indirect ELISA using S. digitata antigen appeared to be sensitive and specific test in surveillance for filariasis instead of traditional night blood surveys and may be helpful for diagnosis of occult filariasis [23, 24].

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The focus of previous studies was toidentify antigens from available filarial parasites including those in animals in order to diagnose W. bancrofti infection [20, 25], using ELISA and Dot ELISA tests, in addition to immunoblotting technique which revealed presence of antigenic sharing between W. bancrofti and S. equina antigens. This antigenic similarity of whole S. equina worm makes it an ideal molecule for discovery of a new easily available diagnostic marker and monoclonal antibody production for human LF especially after its purification and fractionation[18].However the possibility of human infection with those parasites andsubsequent misdiagnosis has receiv ed little attention.

This work was designed to evaluate the diagnostic efficacy of crude and purified S. equina and D. immitis antigens in detecting anti- W. bancrofti IgG pAb in W. bancrofti infected patients using indirect ELISA.

MATERIALS AND METHODS

Study population and collection of samples: A total of 92 individuals were chosen for the present study. Forty-two patients with chronic filariasis (group I) were diagnosed clinically and by detection of microfilaria in blood film by Giemsa stain. Thirty patients with parasitic diseases other than filariasis (group II) were chosen and served as a parasitic control group (schistosomiasis =10, fasciolasis =10, hydatidosis =10). Twenty individuals free from any parasitic infection served as normal control (group III). All cases were subjected to complete history and clinical examination. All patients gave informed consents before inclusion according to Institution's human Research Ethics Committee. Microfilaria diagnosis: Thick and thin blood film examination by fresh finger-prick blood samples were done by using day time blood sample collected after one hour (1 hr)of taking a single dose of 100 mg diethylcarbamazine citrate (DEC, filaran) as a provocative test. Blood smears were stained using Giemsa and examined microscopically for detection of W. bancrofti mf. All the examined patients of group I had mf in their blood samples.

Preparation and characterization of different filarial antigens:

Crude S. equina antigen: The adult worms of S. equina were collected from the abdominal and peritoneal cavity of infected donkey. Briefly, worms were washed several times with 0.9% saline solution to get rid of mucus and debris then washed in phosphate buffer saline (PBS, pH 7.2), and grounded in cold PBS using homogenizer. The homogenate was kept overnight at 4oC and centrifuged at 12000 rpm for 30 minutes (min.). The supernatant was used as crude adult worm antigen after addition of few drops of 0.1% merthiolate as preservative. The protein content of the antigen was estimated by Bio-Rad kit (Richmond, CA, USA) according to [26], aliquoted and stored at -20oC until use.

Purified S. equina antigen: Crude S. equine antigen were concentrated using an Amicon 8400 ultra filtration unit with membrane (3000 Da cut-off) and applied to a 120 ml DEAE SephadexG-50-ion exchange chromatography equilibrated in 0.1 M Tris-HCl, pH 7. Fractions of 5 ml were collected and applied to gel filtration chromatography on sephadex-G-200 HR column[27].Absorbance of each fraction was measured at 280 nm.

D. immitis adult worm antigen: Lyophilized adult D. immitis worm antigens were purchased from Seroimmunodiagnostic Inc. (Georgia, USA). They were homogenized in physiological saline, and centrifuged for 10 min. The supernatant was collected for protein content assessment using Bio-Rad kit (Richmond) then aliquoted and kept at -20˚C till use.

Sodium dodecyle sulphate- polyacrylamide gel electrophoresis (SDS-PAGE):-

The two antigens were resolved by SDS-PAGE under reducing conditions using 12.5% gel by a casting apparatus (Hoefer Scientific Instruments San Francisco, California, USA). The gel was stained with commasie blue stain to visualize the antigenic pattern of the prepared antigens [28].

Detection of circulating filarial antibody by different filarial Antigens:

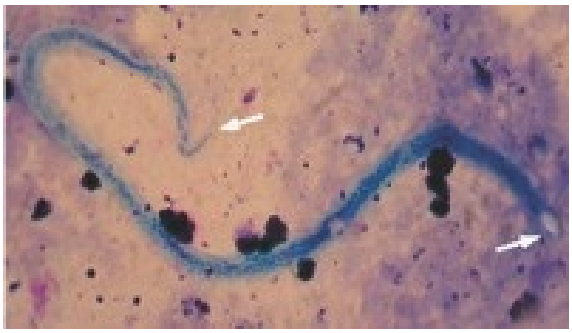
Wells of polystyrene microtitre plates (96-flat bottomed wells, M 129A Dynatech) (Costar, Corporate Headquarters, Cambridge, MA, USA) were coated with 100 µl/well of crude and purified S. equina and D. immitis antigens (10 µg protein in 100 µl coating buffer/well) pH 7.4, overnight at 4°C. The wells were washed 3 times with washing buffer, and then blocked with 200 l/well of blocking buffer for 1 hr. at 37°C. The plate wells were washed with washing buffer 5 times. Hundred µl of human sera infected with filariasis, schistosomiasis, fasciolasis and hydatidosis diluted 1/100 in washing buffer were added to each well and incubated for 1 hr. at 37°C. The plates were washed 3 times with washing buffer. Hundred µl of polyvalent anti-human peroxidase conjugate (Sigma) diluted in washing buffer (1/1000) was dispensed into each well and the plates were incubated for 1 hr at 37°C. The plates were washed 5 times with washing buffer. Hundred µl per well of substrate solution one tablet of OPD (Sigma) dissolved in 25 ml of 0.05 M. Phosphate citrate buffer, pH 5 with peroxidase H2 O2 (Sigma) was added and the plates were incubated in the dark at room temperature for 30 min. Fifty µl/well of 8 N H2SO4 was added to stop the enzyme substrate solution. The absorbance was measured at 492 nm using ELISA reader (Bio-Rad microplate reader).

Statistical Analysis: Data are expressed as mean ± standard deviation (SD). Receiver operating curve (ROC) is used to determine the cutoff values and diagnostic indices (sensitivity, specificity, positive and negative predictive values and efficacy) of different assays. Statistical analysis was performed with the aid of the SPSS computer program (version windows 16.0).

RESULTS

Parasitological parameters in studied patients: According to parasitological and clinical examination, a total of 92 individuals were chosen for the present study. Forty two patients with chronic filariasis (group I) were diagnosed clinically and by detection of mf in blood film by Giemsa stain. Thirty patients with parasitic diseases other than filariasis were chosen as other parasitic infected patients (schistosomiasis =10, fasciolosis =10 and hydatidosis = 10). Twenty individuals are free from any parasitic infections served as normal control (Fig.1).

Figure 1: Thick blood film showing W. bancrofti mf of with unstained sheath, bluish green nuclei and free anterior and posterior ends (Giemsa stain X400).



Purification of S. equina antigens: The eluted S. equine antigen after purification by DEAE-Sephadex G-50-ion exchange chromatography was represented by a single peak with maximum OD value equal to 1.3 at fraction number (6)(Fig.2).The partially purified antigen eluted by DEAE sephadex G-50 ion exchange chromatography (peak a) was further purified by DEAE sephadex G-200 gel filtration column chromatography and two peaks (b and e) were obtained represents the column elution volume fractions which contain S. equina antigen with OD values 0.7 and 1.0 respectively (Fig. 3).

Figure 2: OD280 profile of the antigen fractions following purification by DEAE Sephadex. G-50 ion exchange chromatography.

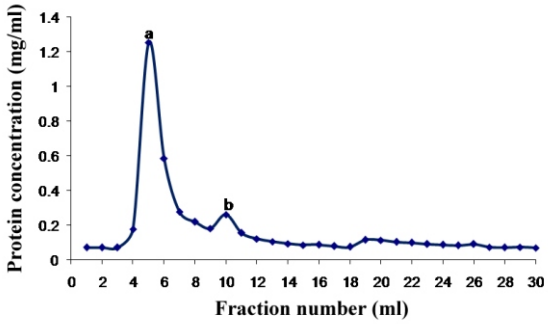
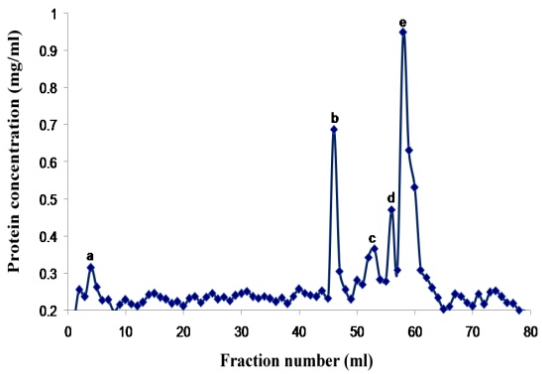


Figure 3: OD280 profile of the antigen fractions following purification by Sephadex G-200 column chromatography.



Estimation of total protein content of S. equina antigens: The protein content of the crude S. equina antigens was 16.6 mg/ml using Bio-Rad Protein assay while it was 4.6 and 1.9mg/ml after purification by DEAE-sephadex G-50 and G-200 ion exchange chromatography, respectively.

Characterization and identification of purified filarial antigens by SDS-PAGE:

The eluted protein fractions resulted from the different purification methods were analyzed by 12% SDS-PAGE under reducing condition and showed only 1 band at about 112 kDa which representing purified S. equina antigens (Fig. 4).

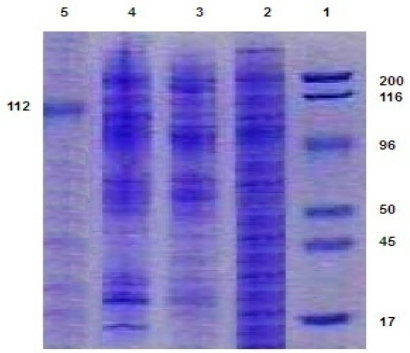


Figure 4: SDS-PAGE (12%, reducing) of target antigens eluted from affinity chromatography columns.

Lane 1: Low molecular weight standard

Lane 2:Dirofilarial antigen

Lane 3: Crude S. equina antigen

Lane 4: Target antigen eluted from sephadex G 50

Lane 5: Target antigen eluted from sephadex G 200

Detection of anti-filarial antibodies in different studied groups: The anti-filarial antibodies level was measured as OD readings at wave length 492 nm. The results were expressed as m ± SD of the mean. The OD values of anti-filarial antibody were 0.92± 0.80, 1.86±0.71 and 1.30± 0.63 using crude S. equina, purified S. equina, and D. immitis antigens, respectively (Table 1).

Table 1: Detection of anti-filarial antibodies in different studied groups using crude S. equina, purified S. equina and D. immitis antigens.

Groups (no. of individuals)	Positive cases of crude *equina* antigens (M SD)		Positive cases of purified *S. equina* (M SD)		Positive cases of *D immitis* antigen (M SD)	
Healthy control (n=20)	No.	0.27 – 0.21	No.	0.21 – 0.21	No.	0.22 – 0.21
Filaria (n= 42)	33	0.92–0.80	36	1.86 – 0.71	34	1.30 – 0.63
S. mansoni (n= 10)	6	0.27 – 0.13	2	0.24 – 0.15	5	0.25 – 0.12
Fasciola (n= 10)	5	0.11 – 0.14	6	0.15 – 0.16	2	0.16 – 0.14
Hydatid (n= 10)	5	0.23 – 0.21	4	0.28 – 0.21	3	0.29 – 0.21

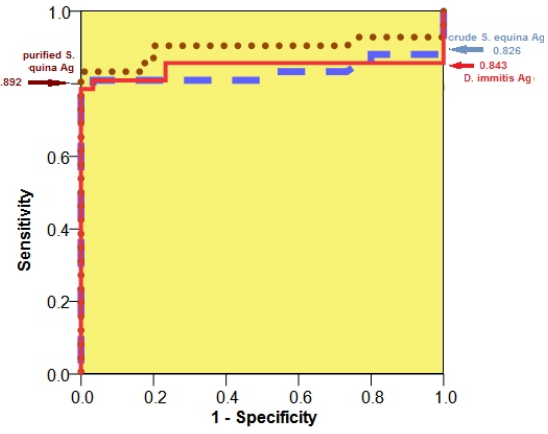
M= Mean; SD= Standard deviation; No.= Number.

The sensitivity and specificity of anti-filarial antibodies against crude S. equina, purified S. equina or D. immitis antigens by indirect ELISA were 78.6 %, 85.71% and 80.95% sensitivity rates and 68%, 76% and 80% specificity rates, respectively(Table 2). A significance in the level of anti-filarial antibodies was observed in the filarial group than the other studied groups represented by the area under the curve reached to 0.826 (crude S. equina antigen), 0.892(purified S. equina antigens) or 0.843 (D. immitis antigen) respectively (Fig. 5).

Table 2: Sensitivity and specificity of different studied antigens.

	Area under Roc curve	Cut□off	Sensitivity	Specificity
Crude *S. equina* Ag	0.826	0.482	78.6%	68.0%
Purified *S. equina* Ag	0.892	0.495	85.71%	76.0%
D. immitis Ag	0.843	0.512	80.95%	80.0%

Figure 5: ROC curve for antibody against crude S. equina, purified S. equina and D. immitis antigens



Discussion

Filariasis is a vector-borne parasitic disease that is endemic in many tropical and subtropical countries [29]. The disease is endemic in 80 countries and 1.1 billion people are at risk from infection [30]. In Egypt, nocturnally periodic LF caused by W. bancrofti infection has been endemic in rural areas for centuries [31, 32, 33& 34].

Various parasitological examination techniques such as thick smear, counting chamber procedure, and concentration test and nucleopore membrane filtration are not useful in low MF, occult or chronic infection. Hence there is a need for simple, sensitive and specific immunodiagnostic test which would be of great value in nocturnally periodic filarial infection as a mean of avoiding laborious night blood surveys and in serology for early detection [35, 11, 36 & 37] and loss of mf from the film during processing especially if anti-coagulated blood is used [38].

Immunological diagnosis using antibodies detection techniques were useful for identifying different filarial Igs such as IgG, IgM, IgE and IgG4 [39]. Moreover, it is used to confirm the absence of infection, and to identify recent infections [24].

Until then, infected mosquitoes and humans are the only sources for filarial infective larvae and mf, respectively, making it difficult to get required parasite material. W. bancrofti mf can be maintained in culture or isolated from blood of infected individuals and used to prepare antigens for diagnostic studies [40, 41].The viability of separated or cultured mf can be ascertained by means of a tetrazoliumformazan assay [42]. The only animals that can be infected with W. bancrofti are the leaf monkeys Presbytis (P.) cristata [43, 44], P. melalophos [45] and Taiwan monkeys Macacacyclopis [46]. These animals are expensive and difficult to maintain in captivity. Hence studies using homologous W. bancrofti antigens are scanty [40].

Due to comparatively easy availability of heterologous antigens, a good amount of work has been done to explore immunodiagnostic potential of heterologous filarial antigens isolated from D. immitis [10, 11]. D. immitis adult worm was used as a source of antigens and ES products to detect human filariasis [22].

Moreover, Kaushal et al. [19] revealed the presence of antigenic sharing between W. bancrofti and S. equina antigens by using ELISA, in addition to/or immunoblotting techniques.

S. equina antigens were prepared from the adult worms of S. equina which are available and easily collected from peritoneal cavity of infected donkey and equines; the worms are large and so huge amounts of antigens can be prepared according to Sakla et al. [13], Sonin [16] and Dalai et al. [17]. The antigenicity of the purified S. equina was tested by indirect ELISA, detecting the highly antigenicity as the major factor in the pathogenesis of filariasis.

Accordingly, in the present study, three types of antigens crude and purified S. equina and D. immitis antigens were used for detection of anti-filarial antibodies in different studied groups. The eluted protein fractions resulted from the different purifications methods was analyzed by 12% SDS-PAGE under reducing condition and showed only one band at about 112 kDa which representing purified S. equina antigen.

Zoheiry et al. [18] showed fractionation of both whole S. equina crude and imported D. immitis antigens was done by polyacrylamide gel electrophoresis. The two antigens shared the same three major bands pattern at molecular weights of 112, 65 and 34 kDa, respectively. Also, immunoblotting revealed sharing of two antigens with one common band at 112 kDa when probed with sera of filarial infected patients. Such antigen could be used to elicit specific antibodies which could bind it and it will be useful in filariasis diagnosis.

Indirect ELISA using W. bancrofti mf ES antigen has been found to be quite useful in detection of filarial infection and in better coverage of large population in endemic areas. Sensitivity and specificity of the test may be increased by including additional tests for detection of antigen or immune complexes [47]. Mahanty et al. [48] have shown that anti-filarial IgG4 is a good index of the intensity and

duration of filarial exposure in endemic populations; also Maizels et al. [49] found that the level of IgG4 antibody correlates with MF counts. Several studies reported a sensitive and specific ELISA that detects anti-filarial specific IgG4 in serum samples from W. bancrofti infected patients, the IgG4 responses were significantly higher in early asymptomatic patients whether microfilaremic or amicrofilaremic than in hydrocele or in chronic elephantiasis and higher in the high endemic community than in the low one [6, 24, 50, 51].

So, in the current work, the antigenicity of the three types of filarial antigens was tested by indirect ELISA technique to evaluate their efficacy for diagnosis of human filariasis. Serum samples from human-infected with filarial parasite gave positive reaction against the crude S. equina, purified S. equina and D. immitis antigens and no cross reactions were recorded with sera of patients infected with any other parasites e.g., S. mansoni, Fasciola and Hydatid. The strong reaction was detected against the purified S. equina antigen. The sensitivity of the indirect ELISA using crude S. equina, purified S. equina and D. immitis antigens was 78.6 %, 85.71%and 80.95%, respectively, while specificity was 68%, 76% and 80%, respectively.

Similarly, several studies measured the sensitivity and specificity of antifilarial IgG antibodies against D. immitis adult worm and/or S. equina antigens by using indirect ELISA. Riyong et al. [52] found the sensitivity and specificity of the assay was 80% and 52.8 %, respectively, against D. immitis antigen. While Zoheiry et al. [18] showed 88% and 82.5% sensitivity rates and 92.2% and 87.4% specificity rates, respectively, against D. immitis and S. equina antigens. In addition Rao et al. [53] showed 100% sensitivity and 88% specificity.

The immunoreactivity of crude somatic antigens of male and female D. immitis adult worms was examined against sera from patients with bancroftian filariasis, other parasitic infections and healthy controls at the optimal conditions for the ELISA , sensitivity and specificity values for crude male antigen were 100% and 60.8%, respectively. With crude female antigen, the sensitivity, specificity values were 80 % and 52.8%, respectively [52].

In conclusion, our study demonstrated that, purified S. equina antigen is promising for diagnosis of W. bancrofti in human using indirect ELISA techniques which appear to be sufficiently sensitive and specific for detection of human filariasis. Further study is highly recommended to prepare antibody against purified fractions of S. equina adult worm antigen for detection of circulating filarial antigen in human sera to reach higher specificity and sensitivity.

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