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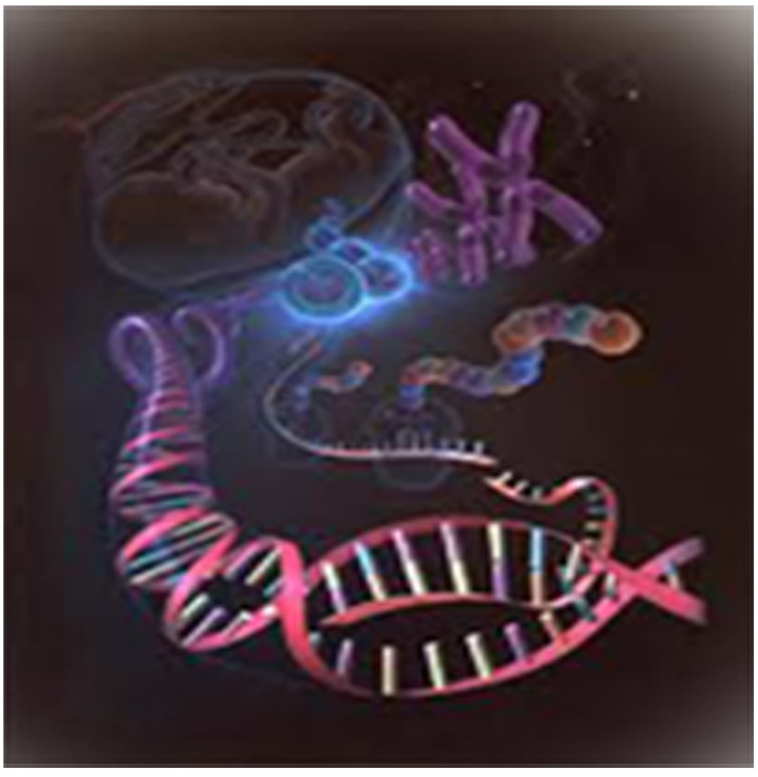

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Research Paper

SETARIA CERVI ANTIGEN FRACTION FOR SERODIAGNOSIS OF HUMAN LYMPHATIC FILARIASIS BY DOT-ELISA

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A partially purified antigen fraction of *Setaria cervi* was used in Dot-enzyme linked immunosorbent assay (Dot-ELISA) for detection of filaria-specific antibodies in *Wuchereria bancrofti* infected patient sera. The *S. cervi* adult antigen extract was fractionated by gel filtration on Sephacryl S-500 column and antigen fractions (obtained just after the void volume) showed high reactivity with polyclonal antibodies against *S. cervi* excretory-secretory antigen fraction-I (having potential for immunodiagnosis of human filariasis) as well as with filarial patient serum pools. The partially purified *S. cervi* antigen fraction (ScAS-1) was used in Dot-ELISA for detection of antibodies in filarial patient sera. The Dot-ELISA was optimized in terms of optimum antigen concentration, antibody dilutions and secondary antibody conjugate and 50 ng of antigen per dot, serum dilution of 1:250 and 1:1000 dilution of secondary antibody conjugate were found optimal for Dot-ELISA. The optimized Dot-ELISA was evaluated using 30 filarial patients sera, 20 sera with other parasitic infections and 20 normal healthy individuals. The partially purified *S. cervi* antigen fraction could detect anti-filarial antibodies in filarial patients sera by Dot-ELISA and thus can be used for serodiagnosis of human filariasis.

Keywords: Filariasis, Bovine filarial parasite, Diagnosis, Dot-ELISA, *Setaria cervi*

INTRODUCTION

Lymphatic filariasis (LF) or elephantiasis is the most debilitating scourge among all the mosquito-borne diseases caused by nematode parasites. Although filariasis does not kill the individual, it causes disfiguration and imposes severe social and economic burden to the affected individuals, their families and the communities. Human lymphatic filariasis is caused by the nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. A billion people are estimated to be at risk of infection, with 120 million already infected and 40 million seriously incapacitated or disfigured by the disease (WHO, 2010). In India 553 million people are at risk, 31 million people are estimated to be microfilariae carriers and over 23 million suffer from filarial disease manifestations (Sabesan *et al.*, 2010). According to WHO,

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lymphatic filariasis is the second most common cause of long term disability after mental illness. The aim of filarial control program is the elimination of circulating microfilariae (mf) so that transmission can be reduced in an endemic area and, therefore, detection of microfilariae in night blood smears is very important for the success of such control programs, however, night blood collection is a problematic and labour intensive (Ottesen, 2000). These problems make it necessary for the development of more efficient, sensitive serodiagnostic methods which can be performed during day time.

Serological methods such as agglutination, immunoprecipitation and immunofluorescence assay were used earlier for diagnostic purpose; however, these techniques were found to be of low sensitivity (Dissanayake *et al.,* 1984; Fletcher and Wu, 1992). The technique of radioimmunoassay (RIA) and Enzyme Linked Immunosorbent Assay (ELISA) have been considered as highly sensitive technique for measuring low levels of antibodies. The ELISA and its modifications have been used for measuring antibody response to a number of parasite antigens including filarial antigens (Voller *et al*., 1976; Dissanayake and Ismail, 1980, Ndao, 2009). Antibody assays based on purified and recombinant antigens from human filarial parasites have been found to be more specific for diagnosing filarial infections (Harinath *et al.,* 1984; Kaushal *et al.,* 1982; 1984). The Dot-ELISA test has been described as being an appropriate method for the detection of antigens or antibodies in several human and animal parasitic and infectious diseases (Coelho *et al*., 2007; Blanco *et al.,* 2009; Ndao, 2009; Bojanich *et al.,* 2012). Due to the non-availability of human filarial parasitic antigenic material in sufficient amount and also in view of the antigenic sharing of homologous and heterologous filarial parasites, antigens from different animal filarial parasites were used for immunodiagnostic purposes by number of investigators (Ottesen, 1984, Dissanayake and Ismail, 1980; 1981; Kaushal *et al.*, 1987; Keiser and Nutman, 2002; Riyong *et al.*, 2005). In earlier studies from our lab, *S. cervi* adult and microfilariae specific antigens as well as common or cross-reactive antigens between bovine and human filarial were identified and characterized using immune rabbit and filarial patient sera (Malhotra *et al.,* 1986; 1987; Kaushal *et al.,* 1987; 1994). In the present study, we have partially purified *S. cervi* adult antigen fraction on Sephacryl S-500 and used for the serodiagnosis of human filariasis by Dot ELISA that can be done rapidly and the data can be recorded visually. The Dot-ELISA was based on the same principle as that for standard plate ELISA and has been shown to be useful for rapid detection of anti-filarial antibodies in serum of patients with lymphatic filariasis.

MATERIALS AND METHODS

Parasites

Adult worms of *Setaria cervi* (bovine filarial parasite) were collected from the peritoneal folds of freshly slaughtered buffaloes at a local abattoir and transported to the laboratory in normal saline. The parasites were washed extensively with normal saline before use.

Setaria cervi Adult Antigen Extract

Antigen extract was prepared by grinding adult *S. cervi* worms in pestle-mortar at 4^{o}C by adding extraction buffer (20 mM Tris HCl buffer, pH 8.0, containing 10 mM EDTA, 0.1 mM PMSF and 100 mM NaCl) followed by sonication at 20 KHz for 5

min under ice cold conditions. After sonication, the extraction was carried out at 4°C for 2-3 h with intermittent vortexing. The extract was centrifuged at 12000 g for 30 min at 4°C. The protein content of supernatant was estimated by the Lowry's method (1951) with BSA as the standard protein.

Antibodies and Sera Samples

The polyclonal antibodies against purified fraction-1 of *S. cervi* excretory-secretory products (ScES-I) were produced earlier in our lab (Mustafa *et al.,* 1996) and were used in the present study.

Sera samples from bancroftian filarial patients (both microfilariae positive individuals and patients with clinical manifestations like elephantiasis, hydrocele, etc.) were collected from areas endemic for *Wuchereria bancrofti.* A total of 30 samples were collected from patients having apparent symptoms of lymphoedema and elephantiasis (did not have microfilariae). 20 samples were also taken from subjects who are defined as individuals having other parasite infections such as ascaris and hookworms but do not have history of filariasis. 20 samples were collected from Non-Endemic Normal (NEN) individuals who had no history of the disease and had never been to filaria-endemic areas. These sera samples were used as negative controls. The human sera samples were collected under the guidance of a medical doctor and with the consent of the individuals as per the guidelines approved by the Institutional Ethics Committee.

Fractionation of *S. cervi* Adult Antigen on Sephacryl S-500 Column

Sephacryl S-500 beads were first washed with triple distilled water and then packed in glass column (20 x 1.25 cm) and the bed volume of the column was 100 ml. The Sephacryl S-500 column was equilibrated with 20 mM Tris HCl buffer, pH 8.0 and 2.5 ml *S. cervi* adult antigen extract was applied to the column at the flow rate of 0.5 ml/min. The Sephacryl S-500 column was washed at flow rate of 1 ml/min with the same buffer and 2 ml fractions were collected. The absorbance of the Sephacryl S-500 fractions was measured at 280 nm and all column fractions were tested in ELISA with polyclonal antibody against ScES-I fraction prepared earlier in our lab (Mustafa *et al.*, 1996). The reactivity of different fractions were tested with ScES-I polyclonal antibody and filarial patient serum pool in ELISA/Dot-ELISA.

Enzyme Linked Immunosorbant Assay

The enzyme linked immunosorbant assay was performed according to the procedure of Voller *et al.* (1976) with slight modification (Kaushal *et al.*, 1994). Briefly, wells of the polystyrene microtitre plates were sensitized with appropriate concentrations of *S. cervi* adult antigen/ScAS-1 (100 µl/well) diluted in 0.05 M phosphate buffered saline, pH 7.4 (PBS) at 37°C overnight. The uncoated sites were blocked by incubation with 3% non-fat milk in PBS for 2 h at 37°C. The microtiter plate was washed three times with PBS containing 0.05% Tween-20 (PBS-T) and then appropriately diluted antibodies (100 µl/well) were added to the wells followed by incubation for 2 h at 37°C. The plate was washed three times with PBS-T and then 100 µl of appropriately diluted peroxidaise conjugated secondary antibody were added to each well. After 90 min incubation, the plate was washed three times with PBS-T and 100 µl of substrate solution (1 mg/ml orthophenylenediamine in citrate phosphate buffer, pH 5.0, containing 1 µl/ml H_2O_2). After 5-10 min the reaction was stopped by adding 5 NH_2SO_4

and plate was read at 490 nm in microtiter plate reader.

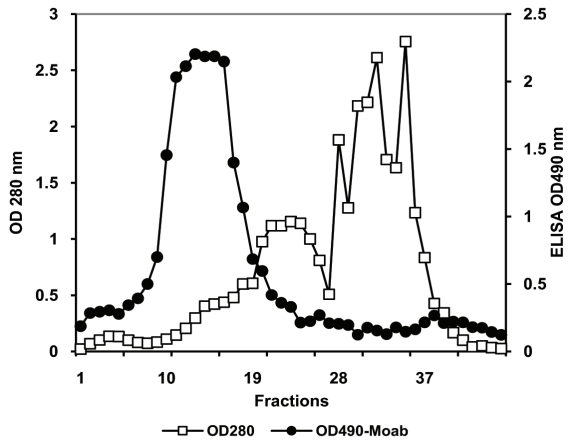
Dot-ELISA

The Dot-ELISA was done according to the procedure described by Bojanich *et al.* (2012) with some modification. Two microliter of different concentrations of *S. cervi* adult antigen/ScAS-1 fraction were spotted on nitrocellulose paper (NCP). After drying, the free sites on NCP strips were blocked by incubation with 3% skimmed milk in phosphate buffered saline (PBS, pH 7.4) at 37°C for 1 h. Then the strips were washed three times with PBS-T and incubated with appropriate dilution of antibodies at 37°C for 1 h. The strips were washed three times with PBS-T followed by incubation with peroxidise conjugated secondary antibody at 37°C for 1 h. The strips were washed and incubated with freshly prepared substrate solution (3 mg of 4-chloro-l-naphthol dissolved in 1 ml of methanol, made up to 10 ml with 50 mM Tris-HCl buffer, pH 7.5, 5µl of H_2O_2) for 15 min. The reaction was stopped by washing the strip in distilled water, air dried and the appearance of purplish blue spot indicate a positive reaction. The intensity of the color was judged visually and scored as +++ (intense); ++ (moderate); + (fair) in reference to the negative control (-).

RESULTS AND DISCUSSION

The use of specific, sensitive, and reliable techniques to detect the presence of antibody in body fluid of infected individuals is important for serodiagnosis of filariasis. Antibody tests have widely been used for serodiagnosis of filarial infections and have a role in epidemiological and disease surveillance program. ELISA is the most commonly utilized serologic test for human lymphatic filariasis and other parasitic diseases (Keiser and Nutman, 2002; Ndao 2009; Piña *et al.*, 2011). The Dot-ELISA is a qualitative ELISA utilizing antigen dotted onto nitrocellulose paper strips and can be performed more quickly with the end detection done visually. Because of its relative speed and simplicity, the dot ELISA is an attractive alternative to standard ELISA for serodiagnosis of parasitic diseases (Ndao, 2009). In the present study, we have evaluated partially purified *S. cervi* antigen fraction (ScAS-1) in Dot-ELISA for serodiagnosis of human filariasis. In view of antigenic sharing among different filarial parasites, antigens from heterologous filarial parasites have been used for immunodiagnostic purposes, however, use of crude somatic extract from filarial parasites gave false positive reactions due to the extensive cross-reactivity with other nematode parasites (Kaushal *et al.*, 1984; Riyong *et al.*, 2005). Therefore, isolation of filarial specific antigens, cross-reactive and common between human and animal filarial parasites, could help in isolating specific immunodiagnostic antigens from heterologous filarial parasites. In the present study, *S. cervi* adult antigen extract was fractionated by gel filtration using Sephacryl S-500 in order to isolate the antigens having potential for diagnosis of human filariasis. Fractionation of *S. cervi* adult antigen extract on Sephacryl S-500 column resulted in the separation of four protein peaks and the elution profile is shown in Figure 1. On testing the immunoreactivity of column fractions with ScES-I polyclonal antibody in ELISA, the column fraction numbers 11-16 (after the void volume and before 1st protein peak) showed high reactivity with ScES-I polyclonal antibody. These column fractions (11-16) were pooled and designated as ScAS-1 antigen

Figure 1: Fractionation of *S. cervi* Adult Extract on Sephacryl S-500 Column. *S. cervi* Adult Antigen Extract was Applied to the Column and 2 ml Fractions were Collected. Other Details are Given in Materials and Methods



fraction. The ScAS-1 fraction (0.015 µg/well to 1 µg/well) was tested in ELISA against ScES-I polyclonal antibody and an antibody titre of 1:64000 was obtained at 50 ng/well optimum antigen concentration of ScAS-1 antigen fraction (data not shown).

The ScAS-1 antigen fraction was then tested for reactivity with filarial patients serum pools in ELISA and Dot ELISA. The Dot-ELISA was standardized in terms of optimum antigen concentration. Out of different concentrations of ScAS-1 antigen fraction (1, 0.5, 0.25 0.125, 0.062, 0.031 and 0.015 µg) tried at three fixed dilutions (1:500, 1:1000 and 1:2000) of ScES-I polyclonal antibody, 50 ng of ScAS-1 antigen was found to be optimum and this antigen concentration (50 ng/dot) was used in further experiment. The ScES-I polyclonal antibody when tested in Dot-ELISA at different dilutions starting from 1:2000 upto 1:128000, significant intensity dots were observed upto 1:32000. The optimum antigen concentration of ScAS-1 50 ng/dot was used to test the filarial patients serum pools (IHS pool1, IHS pool2, IHS pool3) at two dilutions (1:250 and 1:1000) in Plate ELISA and one dilution (1:250) in Dot-ELISA. The ScAS-1 showed significantly high reactivity with filarial patient serum pools 1 and 2 (IHS pool1, IHS pool2) and slightly lower with filarial patient serum pools3. In Dot ELISA, intense dots were observed with IHS pool1 and IHS pool2 while faint dot was observed with IHS pool3 (Figure 2). These findings suggest that the plate ELISA results co-related well with the results obtained with Dot-ELISA. Individual filarial patients sera were tested against ScAS-1 antigen fraction in Dot-ELISA in comparison to Plate ELISA. Fifteen filarial patients sera (having ELISA OD 490 values between 0.8 to 3.0) were tested in Dot-ELISA at 1: 250 serum dilution and the results are given in Table 1. The Dot-ELISA results were visually recoded and scored as + for positive and – for negative. The Dot ELISA data of individual patients sera showed correlation with the Plate ELISA data. The filarial patients sera showing high ELISA OD values (OD 490 of 1.9-3.3) gave intense dots (+++) in Dot-ELISA, sera with ELISA of 1.8.0-2.1 gave moderate intensity dot (++) while ELISA OD of 0.8-1.2 gave faint dot (+). No dots were

Figure 2: Reactivity of *S. cervi* Purified Antigen Fraction with Filarial Patients Sera in Plate ELISA and Dot ELISA. Details are Described in Materials and Methods

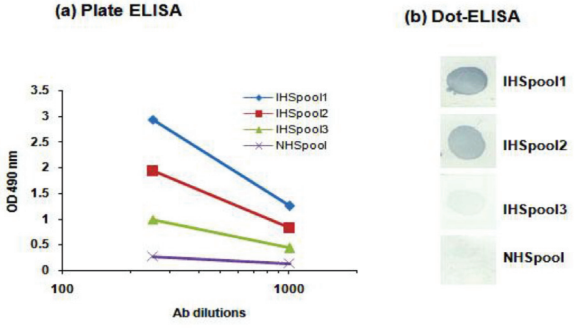


Table 1: Reactivity of *S. cervi* Purified Antigen Fraction with Individual Filarial Patients Sera Using Dot ELISA and Plate ELISA

Sera	Dot ELISA Scale	Plate ELISA (OD490)
IHS1	+++	3.337
IHS3	+++	2.868
IHS4	+++	3.107
IHS5	+++	2.649
IHS6	+++	2.861
IHS2	++	1.976
IHS11	++	2.026
IHS13	++	1.844
IHS14	++	2.134
IHS16	++	2.169
IHS12	+	1.184
IHS15	+	1.027
IHS26	+	0.817
IHS30	+	0.922
IHS31	+	0.846
NHS1	-	0.221
NHS2	-	0.234
NHS3	-	0.186
NHS4	-	0.195
NHS5	-	0.243

Note: +++ = Intense, ++ = Moderate, + = Fair, - = Negative All sera were used at 1:250 dilution in both ELISA and Dot-ELISA against ScAS-1 antigen fraction.

observed with individual normal human sera with OD value of 0.3 or lower. The Dot-ELISA was further evaluated using filarial and non-filarial patients sera against ScAS-1 antigen fraction. Thirty sera from individuals having filarial infection, 20 from other infections (ascaris and hookworm) and 20 sera from non-endemic controls were used in Dot-ELISA. All the 30 filarial patients sera were positive by Dot-ELISA. No positive dots were observed with sera from other infection and non-endemic controls (Table 2). Therefore, in the present study, purified *S. cervi* Sephacryl S-500 fraction has successfully been used in Dot-ELISA for serodiagnosis of human filariasis. Wickremanayake *et al.* (2001) have used Dot-ELISA employing *S. digitata* antigens to detect antibodies in *W. bancrofti* infected patient sera and can be able to differentiate microfilaremics from endemic normals.

Table 2: Detection of Antibodies in Filarial Patients Sera by Dot- ELISA Using *S. cervi* Purified Antigen Fraction

Sera	No. Tested	Positive for Antibodies by Dot ELISA
Filarial	30	30
Other Infections	20	0
Non-endemic controls	20	0

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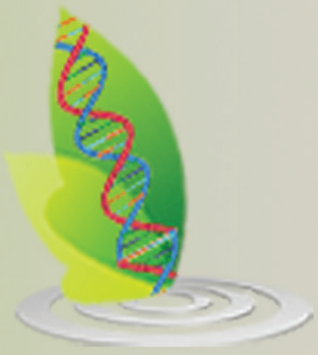

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