Antigenic Characterization of Embryo Stage of Setaria Cervi

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Abstract*—Filariasis is a serious health hazard affecting millions of people in tropical and subtropical regions of developing countries including India. The specific diagnosis and control measures are essential for effective management of the disease. It has been shown earlier that the excretory-secretory products of filarial parasitescontain diagnostically important embryo antigens; therefore, it would be important to identify and characterize the embryo stage antigens having potential for serodiagnosis of human filariasis. In the present study efforts were made to analyse the protein and antigenic pattern of embryo stage of *Setaria cervi*, the bovine filarial parasite. The *S. cervi* embryos (ScEmb) were isolated by dissecting adult female worms and purified using Percoll gradient. The SDS-Polyacrylamide gel electrophoresis revealed15-18 protein bands in *S. cervi* embryo somatic extract in molecular weight range of 6.5 – 200 kDa. The polyclonal antibodies produced against *S. cervi* embryo showed significantly high reactivity with ScEmbantigens as well aswith antigens fromadult and microfilarialstages. The 10-14 protein bandsof *S. cervi* embryo were recognized by anti-ScEmb polyclonal antibodies in immunoblotting.These studies revealed some qualitative and quantitative differences among the antigenic pattern of three stage of *S.cervi*.The *S.cervi* embryos exhibited high reactivity with antibodies present in filarial patients' serathereby suggesting the presence of some antigens common/cross-reactive with human filarial parasite. The protein bands of 60 kDa, 45 kDa, 29 kDa and 27 kDa of *S. cervi* embryo stage were recognized by filarial patients serum pool and may have diagnostic utility in human lymphatic filariasis

***Index Terms*—Filariasis, setaria cervi, Embryo, SDS-PAGE, immunoblotting**

I. Introduction

Lymphatic filariasis is a parasitic infection caused by the filarial nematodes Wuchereriabancrofti, Brugiamalayi, and Brugiatimori. The disease is rarely fatal, but it is the second leading cause of permanent and long-term disability in the world [1,2].Over 120 million people are infected, with about 40 million disfigured and incapacitated by the disease [3].Thus lymphatic filariasis has remained an important public health problem in India contributing about 44.3% of the global burden of the disease [4].The lymphatic filariasis infection rate in India has been reduced from 1.24% in 2004 to 0.29% in 2013[5].

The effective control of filariasis depends on early and specific diagnosis of the disease. There is still a need of better diagnostic techniques with better sensitivity that can be applied under field conditions, as lymphatic filariasis elimination program has emphasized the need for tools that can be used to monitor progress of control programmes. The disease is diagnosed by screening night blood smears for circulating microfilariae; however, this is not an optimal monitoring or surveillance tool because of its relative insensitivity and requirement for nocturnal blood collection [6]. Immunological tests offer the convenience of daytime testing and greater sensitivity than night blood examination [7].

Excretory- secretory product from different parasite have been characterised and found to be of great importance in immunomodulation, diagnosis and other biological activities [8-10]. Earlier studies have shown

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that the excretory- secretory antigen (diagnostically important antigens) from filarial parasite contains egg/embryo antigens [11]. The main source of these antigens was found to be the fluid present between egg and embryo (amniotic fluid) [12]. Embryo antigens have also been found of a great importance (high specificity) in the diagnosis of Schistosoma mansoni infection as well as for the chronological evolution of S. mansoni infection in murine schistosomiasis [13, 14]. Therefore, it will be important to characterize the embryo stage antigens of filarial parasites for identifying the antigens having diagnostic potential for lymphatic filariasis. Because of less availability of human filarial parasite, Setaria cervi(bovine filarial parasite) has been used for the identification and isolation of diagnostically important antigens common/cross-reactive with human filarial parasites [15-17]. Setariacervi, a parasitic nematode that infects and resides in the peritoneal cavity of cattle, buffalo and deer etc [18]. The parasites are transmitted between mammals (definite host) and insects (intermediate host) by the bite of Stable fly Haematobiastimulans. Little is known about the pathogenicity of Setaria Sp., but some are known to affect nervous system and eye. Setaria cervi are capable of migrating to central nervous system causing serious neurological disease [19]. Analysis of protein and antigenic composition of adult and microfilarial stages of S. cervi has already been done earlier [20] but, little information is available on the antigenic proteins of embryo stage of the S.cervi.

In the present study, efforts have been made to analyse the protein and antigenic make up of embryo stage of S. cervi andto identify the antigens that show reactivity with filarial patient sera and are important for the diagnosis of the disease.

II. Material and Methods

A. Parasites

Adult motile S.cervi were collected from the peritoneal folds of freshly slaughtered buffaloes at a local slaughterhouse and brought to the laboratory in normal saline. The parasites were washed extensively with normal saline before use.

The S. cervi embryo and microfilariae were obtained by dissecting longitudinally the motile gravid female worms and incubating the uteri in Ringer's solution at 37˚C for 3-4 hrs. The embryo and microfilariae released into the medium, were purified on two step Percoll gradient(30% and 60% Percoll) followed by washing with phosphate buffered saline and stored at -70˚C until used.

B. Preparation of Antigens

The somatic extracts from S.cervi embryo (ScEmb) and microfilariae (ScMf) were prepared as described by Singh et al [21]. Briefly, 10 batches each of purified ScEmb and ScMf were pooled separately, suspended in 50mM phosphate buffer pH8.0 and subjected to sonication on ice using Misonix ultrasonic sonicator (10 cycles of 30 s each at 20% amplitude) and extracted on ice for 1.5 h with occasional vortexing. The somatic extract from S.cervi adult (ScA) was prepared as described by Kaushal et al [16]. Briefly, the adult female worms were cut into small pieces, ground to a fine paste in a pestle and mortar, sonicated and extracted as described for embryo and microfilariae. The extracts were centrifuged at 18,000xg for 45 min at 4˚C and the supernatant obtained were stored at -70˚C until used.

C. Immunization of Rabbit

The polyclonal hyper immune rabbit serum against embryo stage antigen of S.cerviwas produced by immunizing two rabbits intra muscularly with appropriate amount of antigens (0.2 mg ScEmb/rabbit) emulsified with Freund's complete adjuvant. The second injection was done 3 weeks after the first injection and the immunization was done over a period of 4 months. The rabbits were bled from marginal ear vein, 1 week after each injection starting from the second injection. The sera were collected and stored at -20˚C until used.

D. Patient Sera

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 W. Bancrofti infection. A total of 125sera samples were collected froman endemic area. Twenty 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. Twenty 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 patient/ normal serum pools were made by mixing equal volume of respective sera. 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.

E. SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide gel electrophoresis (SDS- PAGE) was carried out according to Laemmli [22]. A 10% gel with an acrylamide/ bis ratio of 37.5:1, 10% sodium dodecyl sulphate (SDS) in the presence of N,N,N',N', tetra methylene ethylene diamine (TEMED) and ammonium per sulphate in Tris-HCl buffer pH 8.8 was prepared.. The antigens (ScA, ScMf and ScEmb, 45 µg protein/ lane) were diluted in 2x SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 5% w/v sodium dodecyl sulphate, 5% V/V beta mercaptoethanol, 10% glycerol, pH 6.8, 5% w/v sodium dodecyl sulphate, 5% V/V beta mercaptoethanol, 10% glycerol and .001% bromophenol blue), kept in boiling water bath for 5 min, centrifuged and loaded onto the gel containing 4% stacking gel . After electrophoresis, the gels were fixed and subjected tosilver staining following the method of Morrissey etal [23]

F. Enzyme Linked Immunosorbent Assay

The enzyme linked immune sorbent assay (ELISA) was performed in 96 well flat bottom microtitre plates according to the method of Volleret al [24]with slight modifications [25].Briefly, thewells of micro titre plate were coated with appropriate concentrations of antigenin100µl of phosphate buffered saline (PBS, pH 7.4) by incubation overnight at 37˚C. The unbound antigen was removed by washing the plate three times with PBS-Tween (PBS containing 0.05% Tween-20) and the wells of the plate were blocked by adding 300 µl of 3% non-fat dry milk powder (W/V) prepared in PBS (PBS-milk) and the plate was kept at 37˚C for 2 h.After 3 washes with PBS- Tween, 100 µlof appropriately diluted immune rabbit serum/ filarial patient serum(diluted in 1% PBS-milk) were added to the wells and incubated for 2 h at 37˚Cfollowed by washing thrice with PBS-Tween. To this100µl of peroxidase conjugated secondary antibody (1: 4000 for anti-rabbit IgG conjugate and 1:2000 for anti-human IgG conjugate, diluted in 1 % milk- PBS) was added and incubation was done for 90 min at 37˚C. The plate was finally washed with PBS-Tween and the colour was developed by adding 100µl of substrate solution (1 mg/ml O-phenylenediamine in citrate phosphate buffer pH 5.0, containing 1µl/ml H_2O_2). The reaction was stopped, after 10-15 min by adding 50µl of 5 N H_2SO_4 and the absorbance was read at 490 nm using Molecular Devices Spectramax 190 plus microplate ELISA Reader.

G. Immunoblotting

Immunoblotting was done by the method of Towbinet al [26]with slight modifications [27].The SDS-PAGE separated proteins were electrophoreticallytransferred to nitrocellulose paper (NCP), using the Semi dry transfer apparatus (GE healthcare, UK) for 90 min at a constant voltage of 30Vusing the Transfer buffer (Tris-glycine buffer containing 20% methanol). After transfer, one NCP strip was stained with amido black and rest of the NCP was blocked with 5% non- fat dry milk in Trisbuffered saline (TBS, pH 8.0) for 1 h at room temperature. The membrane was washed with TBS containing 0.05% tween 20 (TBS-Tween) three times and incubated with 1:500 dilution of immune rabbit serum/1:250 of filarial patient serumat room temperature for 1 h and thenat 4˚Cover night. Subsequently, the membrane was washed again and incubated with peroxidase conjugated secondary antibody(1:2000 for anti-rabbit IgG conjugate and 1:1000 for anti-human IgG conjugate)for 1 h at room temperature. The protein bands recognized by antibodies were detected by adding substrate solution (15 mg of 4-chloro 1- nephthol dissolved in 5 ml of methanol and made up to 30 ml with 0.05M Tris-HCl pH7.5, 1µl/ml H_2O_2). The reaction was stopped by washing the strip with distilled water. The density of antigenic bands of interest was analysed using myImage Analysis™ (Version 1.1 Build 753) software of Thermo Fisher Scientific Inc. (MA, USA).

H. Protein Estimation

Protein estimation was done by the method of Lowry et al [28] as modified by Deans et al [29].

III. Results

A. Protein Pattern of S.cerviembryo Antigen

The protein pattern of embryo stage of *S.cervi*was analysed by SDS-PAGE and the protein patterns of adult and micro filarial stages were also analyzed for comparison. The SDS-PAGE analysis followed by silver stainingrevealed15-18 bands in the molecular weight range of6.5-200kDa in ScEmb (Fig. 1).On comparing the protein patterns of different somatic extracts (ScEmb, ScA, and ScMf), 13-15 protein bands in ScA and ScMf, corresponding to the protein bands present in ScEmb, were observed (Fig. 1). The protein bands of molecular weight 100, 65, 60, 50, 45, 29, 24, 15, 10 and 6.5 kDa were present in all the three stages.

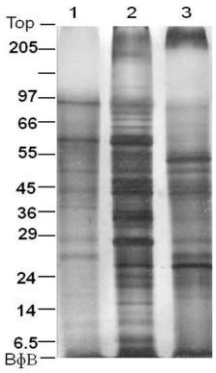

Figure 1. SDS-PAGE analysis of embryo stage of S. cervi. 10% polyacrylamide gel was used followed by silver staining as described in material and method: lane 1= ScEmb, 2= ScA, 3= ScMf and 4= molecular weight marker.

B. Polyclonal Antibodies Against S.cervi Embryo Antigen

The polyclonal antibodies against embryo antigen were produced by immunizing the two rabbits (immune rabbit serum 1, IRS1 and immune rabbit serum 2, IRS2)with *S. cervi* embryo somatic extract and the immune rabbit sera were tested in ELISA. The ELISA was optimized using different concentrations (1.0 µg to 0.015 µg) of ScEmb antigen at two fixed dilutions (1:500 and 1:2000) of anti-ScEmb antibody. The antigen concentration of 0.25µg/well of ScEmb antigen was found to be optimum (Fig. 2).

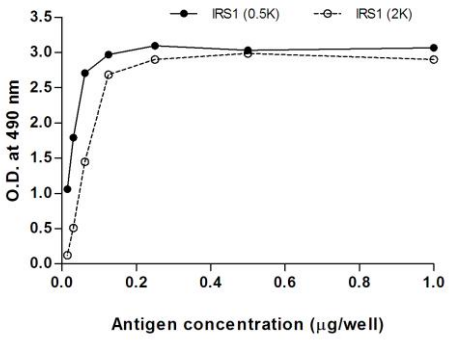

Figure 2. Determination of optimum antigen concentration of S. cervi embryo antigen using immune rabbit serum in ELISA. Different concentrations (0.015 to 1.0 µg) of ScEmb were used at two dilutions (1:500 and 1: 2000) of immune rabbit serum1 (IRS1).

The levels of antibodies produced in immune rabbit sera were determined by testing different bleeds of immune rabbit sera (1st bleed to 6th bleed) at different dilutions (1:1000 to 1:256000) using the optimum antigen concentration (0.25µg/well) of ScEmb antigen in ELISA. The ELISA reactivity was found to be increasing up to the 4thbleed and after that it became almost constant (data not shown). The 4thto6th bleeds of immune serum showed antibody titre of 1: 256000. The bleeds of rabbit-anti-ScEmb sera (IRS1 and IRS2) showing high ELISA values were pooled and used for determining the antibody titre. In order to determine the antibody titre of immune rabbit serum pools (IRS1 and IRS2), ELISA was done at different dilutions (1:1000 to 1:512000) of immune rabbit serum pools using the ScEmb antigen. The titration curves obtained with IRS1 and IRS2 serum pools were found to be comparable and are shown in Fig. 3. The immune rabbit serum pools showed an antibody titre of 1:256000 with ScEmb antigen.

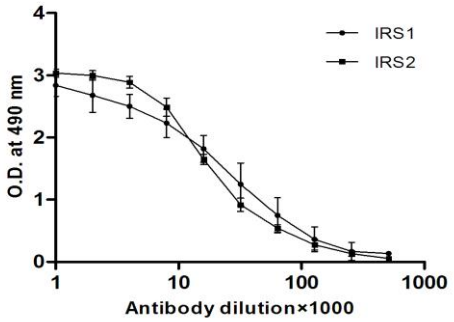

Figure 3. Antibody titration of immune rabbit serum pool with S. cervi embryo antigen in ELISA. Different dilutions (1:1000to 1:512000) of immune rabbit serum pools (IRS1 &IRS2) were tested in ELISA using 0.25 µg per well concentration of ScEmb antigen.

C. Immunoreactivity of immune rabbit serum pool with S. cervi microfilariae and adult antigen:

The Immunore activity of immune rabbit serum pool (rabbit anti-ScEmb serum pool) with *S. cervi* microfilariae (ScMf) and adult (ScA) was tested in ELISA.

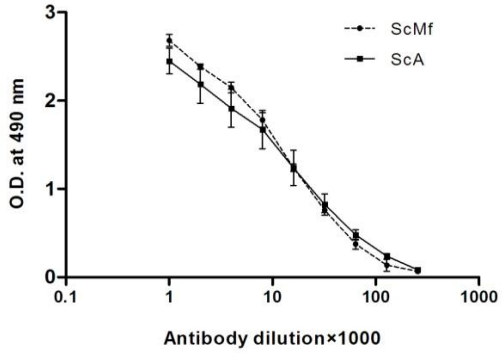

Figure 4. Reactivity of immune rabbit serum pool with S. cervi adult and microfilariae antigen in ELISA. Different dilutions (1:1000 to 1:256000) of immune rabbit serum pool1 (rabbit anti-ScEmb serum pool) were tested with 0.2 µg/well of ScA and ScMf.

Different dilutions (1:1000 to 1:256000) of immune rabbit serum pool were tested using optimum antigen concentrations of *S. cervi* microfilariae and adult somatic antigens (0.25µg/well) in ELISA. The antibody titration curves are shown in Fig. 4and both the titration curves were found comparable. Antibody titres of 1:64000 and 1:128000 were observed with ScMf and ScA respectively.

D. Immunoblotting of S.Cervi Embryo Antigen with Immune Rabbit Serum Pool

Immunoblotting of *S.cervi* embryo antigen was done with immune rabbit serum pool. The *S.cervi* microfilariae and adult somatic antigens were also used for comparison. The immune blotting, using rabbit anti-ScEmb serum pool revealed 10-14 antigenic bands in ScEmbwhile12-14 and 8-11antigenic bands were observed in ScA and ScMf somatic extracts respectively (Fig. 5). A number of protein bands (60, 50, 45, 40, 32, 29, 27, 8 kDa) in were recognized by anti-ScEmb antibody in all the three stages of *S. cervi.*

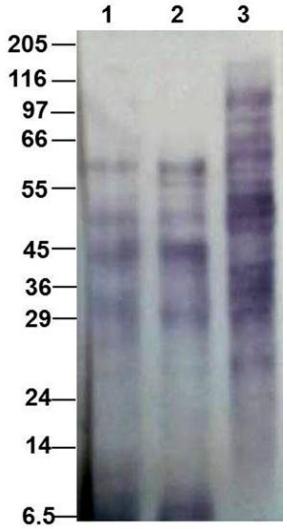

Figure 5. Immunoblotting of S. cervi embryo antigen with immune rabbit serum. The immunoblotting of ScEmb was done using IRS1 pool. Lane 1= ScEmb; Lane 2= ScMf; Lane3= ScA.

E. Immunoreactivity of S.cervi Embryo Antigen with Filarial Patient Sera

The immunore activity of *S. cervi* embryo antigen was tested with filarial patient sera. The ELISA was optimized in terms of optimum antigen concentration and 0.5 µg/ well showed optimum result. Initially the individual filarial patient sera were tested at two different dilutions (1:250 and 1:1000) with optimum concentration of ScEmb in ELISA. The filarial patient sera with significant ELISA reactivity at 1:1000 were used to make IHS pool1 (ELISA OD of 2.0-3.0) and IHS pool 2 (OD490 of 1.0-2.0). The filarial patient serum pools (IHS pool1 and IHS pool2) were tested at different dilutions (1:500 to 1:64000) in ELISA and the titration curves are shown in Fig. 6. Antibody titres of 1: 32000 and 1:16000 were observed for IHS pool1 and IHS pool2 respectively.

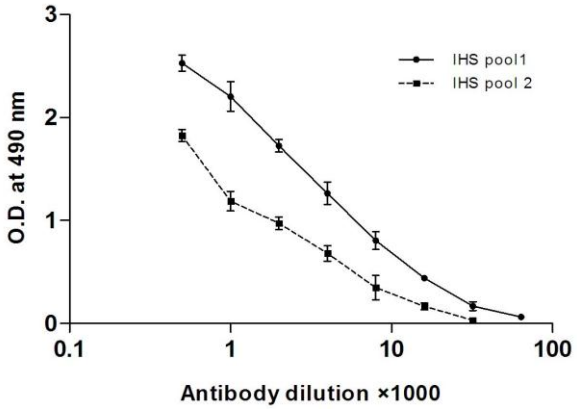

Figure 6. Immunoreactivity of S. cervi embryo antigen with filarial patient serum pools in ELISA.The IHS pool 1 and IHS pool 2 were prepared as described in materials and methods.

F. Immunoblotting of S.cervi Embryo Antigen with Filarial Patient Sera

In order to identify the *S.cervi* embryo antigens recognized by filarial patient's serum, immunoblotting of ScEmb was done using filarial patient sera pool1. The ScA and ScMf antigens were used for comparison.

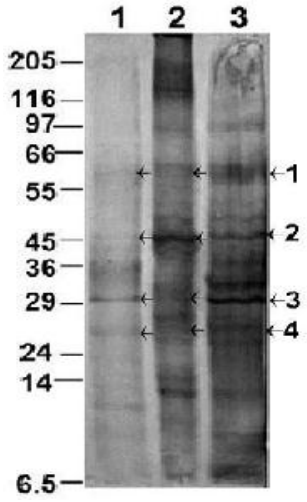

Figure 7. Immunoblotting of S. cervi embryo antigen with filarial patient serum pool. Lane 1= ScEmb, lane 2= ScA and Lane3= ScMf. All the three antigenic preparation was used at 50 µg concentration. The filarial patient serum was used at 1:250 dilutions.

The immunoblotting revealed 7-8 protein bands in ScEmb while 11-12protein bands in both ScA and ScMf(corresponding to protein bands present in ScEmb) were recognized by filarial patient serum pool and the results are shown in Fig. 7.Theprotein bands of molecular weight 60, 45, 29, and 27kDa were found to be strongly recognized by filarial patient serum pool in all the three stages of *S. cervi*(marked in Fig. 7). The densitometric analysis of these antigenic bands was done by myImage Analysis software and the comparative band densities of four antigenic bands (60, 45, 29, and 27 kDa) are given in Table 1.

TABLE I. DENSITOMETRIC ANALYSIS OF IMMUNOBLOTS OF S. CERVI EMBRYO, ADULT AND MICROFILARIAE SOMATIC ANTIGENS

Mol. wt. (kDa)	Antigen band density*					
	Immunoblot with IRS pool			Immunoblot with filarial patient serum		
	ScEmb	ScA	ScMf	ScEmb	ScA	ScMf
60	5033	10489	4494	7647	8340	12682
45	2945	14699	6123	11331	11560	14755
29	173	14773	2909	17326	11250	22041
27	123	10539	1111	12276	13476	18021

*Background corrected densities of bands of interests were analyzed using myImage Analysis™ (Version 1.1 Build 753) software of Thermo Fisher Scientific Inc. (MA, USA).
Protein bands of ScA and ScMf are given corresponding to the protein bands present in ScEmb.

IV. DISCUSSION

Characterisation of antigens of differentlife stage of filarial parasites is a fundamental and essential step towards the identification of diagnostically important antigensfor developing improved diagnostic methods. The protein and antigenic composition of a number of helminth parasites have been analysed to identify the antigens of diagnostic significance [10-13]. In our earlier studies we have investigated the protein and antigenic patterns of somatic extracts as well as E-S product from adult and microfilarial stages of *S. cervi*[11,17, 20].In the present study, we have done the antigenic analysis of somatic extracts from *S. cervi* embryo using polyclonal antibodies and filarial patient'ssera.

The identification of 15-18 protein bands in ScEmb compared to 30-34 bands in ScA and 24-27 bands in ScMf, identified in our earlier studies [20], revealed a simpler nature of ScEmb as compared to adult and microfilarial stages. The polyclonal antibodies produced against *S. cervi* embryo somatic antigen in rabbits using the optimized protocols and the levels of antibodies in immune rabbit serum were determined by ELISA. The ELISA was optimized in terms of optimum antigen concentration and antibody dilutions. On increasing the antigen concentration above optimum antigen concentration, no further increment of ELISA values was observed; however, lowering the antigen concentration resulted in reduction of ELISA values. The *S. cervi* embryo antigens exhibited optimum ELISA reactivity at 0.25 µg/well antigen concentration.

The immune rabbit serum, obtained after different injections, showed an increase in the titer value with increasing number of injections and significantly high antibody responses were obtainedafter4thinjection.In the present study,immunization of rabbits with ScEmb resulted in the production of hyper-immune polyclonal antibody thereby suggesting the antigenic nature of *S. cervi* embryo stage somatic antigenic preparation.

The polyclonal antibody (rabbit anti-ScEmbserum),produced against the ScEmb somatic extracts in the present study, recognized almost same number of protein bands (10-14 antigens) in all the three

stages (ScEmb, ScA and ScMf somatic extracts) with difference in the intensities of different protein bands. Out of the antigens recognized by the immune rabbit serum pool, four antigens of 60 kDa, 45 kDa, 29 kDa and 27kDa were found to be strongly recognized inthe three stages thereby suggesting the sharing these of antigens among the three stages (embryo, microfilaria and adult) of *S.cervi.* The intensities of all the four antigens were found to be almost similar in the three stages (ScEmb, adult and microfilariae) of *S. cervi* as revealed by densitometric analysis. Studies on the microfilariae of human as well as canine filarial parasites have shown the diagnostic potential of 64 kDa antigen [30]. In the present study, we have identified 60 kDa antigen present on all the developmental stages of *S. cervi* may have a diagnostic significance.

In the present study, the high immunore activity of *S. cervi* embryo antigen with filarial patient sera suggests the presence of antigens/antigenic epitopes having diagnostic significance. The antigens of 60, 45, 29, and 27 kDa, recognized by the filarial patient serum, were found to be common among the three stages of *S. cervi* and the 29 kDa antigen was found to be more prominent. Similarly John et al [31] have isolated a 29 kDa protein from *S. digitata* and shown its diagnostic potential for bancroftian filariasis. Tang et al [32] and Rathauret al [33] have shown that a 29 kDa recombinantcu/zn superoxide dismutase is actively secreted in filarial infection and may have a role in diagnosis of filarial infection. Cooksen and colleague have cloned and sequenced a protein of 29 kDa from lymphatic filarial parasite and found it a Cuticular glycoprotein [34]. They have proposed a role of this protein in resistance of these parasites to immune effector mechanisms and their persistence in the mammalian host [34].

Therefore, in the present study, we have been able to identify antigens (60 kDa, 45 kDa, 29 kDa and 27 kDa) of*S. cervi* embryo stage that may have diagnostic utility in human lymphatic filariasis. Out of these antigens, 29 kDa antigenof *S. cervi* embryo was most prominently recognized by filarial patient serum pool. Further characterisation of this protein as well as its gene may help in understanding its usefulness for diagnostic and therapeutic purpose.

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