

Hydrocele fluid: Can it be used for immunodiagnosis of lymphatic filariasis?

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ABSTRACT

Background & objectives: Diagnosis of lymphatic filariasis using serum has been established but the utility of hydrocele fluid for the purpose is not exactly known. Since, hydrocele is a chronic form of the disease manifestation in a variety of situations and often poses difficulty in diagnosing its origin, we have evaluated the usefulness usage of hydrocele fluid for diagnosis of filarial origin of hydrocele in this study.

Methods: Paired samples of serum and hydrocele fluid from 51 individuals with hydrocele, living in an endemic area of *Wuchereria bancrofti* were assessed. Circulating filarial antigen, filarial specific antibody and cytokine assay were performed in both serum and hydrocele fluid of patients.

Results: Og4C3 assay detected circulating filarial antigen (CFA) in serum and corresponding hydrocele fluids. The level of IgG, IFN- γ and IL-10 were found to be high in CFA-negative, while IgM and IgE were high in CFA-positive hydrocele fluid and serum samples associated with hydrocele. On the other hand neither CFA-positive nor CFA-negative hydrocele fluid and serum samples associated with hydrocele showed any difference in IgG4 level.

Interpretation & conclusion: This study showed that the filaria related antigens and antibodies found in serum can be detected with equal sensitivity in hydrocele fluid. Therefore, it can be used as an alternative to serum for immunodiagnosis of filariasis, and help monitoring the filariasis elimination programme.

Key words Hydrocele fluid; immunodiagnosis; lymphatic filariasis

INTRODUCTION

Hydrocele, swelling of the testicle, is caused due to the accumulation of the lymphatic fluid in the scrotum. This might be due to abnormal absorption and secretion secondary to various pathologic processes such as trauma, ischemia, testicular tumor, increased intra-abdominal pressure and infections like STD (sexually transmitted diseases) and *Wuchereria bancrofti*. According to an estimate about 40–50% of males living in highly filarial endemic areas (~ 20% of the world population) develop testicular hydrocele¹. Though, other clinical manifestations due to filarial infection such as acute attacks of filarial fever and chronic manifestations of limb lymphoedema/elephantiasis are known to be influenced by the anatomic location of adult filarial worms, presence or absence of microfilaria (mf), immune responses and secondary bacterial infections², but contribution of *W. bancrofti* in pathogenesis of hydrocele is not exactly known³. However, it is a fact that the mf causes nodal and lymphatic wall damage by impairing lymphatic valve patency and ultimately propulsion of lymph to the para-aortic lymph nodes in testes and epididymis⁴ and normally disappears after the onset of local lymphatic im-

pairment. A number of studies have shown filarial attributed immunological parameters in the serum of hydrocele patients^{5–7}. But, very few studies are available on immunological composition of hydrocele fluid with respect to filariasis^{8–9}. Most importantly anti-microfilarial immunity in human lymphatic filariasis though believed to operate through antibodies to the microfilarial sheath, the presence of anti-sheath antibodies in hydrocele fluid has not yet been studied. In the present study, we have made an attempt to assess the usefulness of hydrocele fluid (which is usually discarded after surgical operation) for immunodiagnosis of lymphatic filariasis by detecting different filarial antibody isotypes and cytokine profiles.

MATERIAL & METHODS

Study population

About 10% of the individuals from among the patients attending for treatment during the study period (2010–12) to filarial clinic, Govt. Hospital, Bajapur of Khurda district, Odisha state, India, a filaria endemic area were selected for the study. The selection criteria were: (i) the patients should have only hydrocele and no other signs/symptoms of chronic filariasis, and (ii) the patients

should not have symptoms of any other chronic disease/illness like leprosy, tuberculosis and malnutrition. Accordingly, 51 male individuals aged 16–65 yr (mean age 32 yr) were enrolled in the study. At least 5 ml of venous blood samples was collected aseptically between 2000 and 2200 hrs from each participant and the hydrocele fluid from corresponding patient was collected by needle aspiration by the treating physician of the hospital after informed consent. Blood and hydrocele fluid samples were immediately transferred to the laboratory of Regional Medical Research Centre (RMRC), Bhubaneswar for further processing. Both buffy coat concentration and Giemsa stained thick smear techniques were performed for detection of mf. Serum sample and fluids were stored at -20°C until further use. The study has been approved by the ethical committees of RMRC, Bhubaneswar.

Detection of circulating filarial antigen (CFA) assay

Detection of CFA was carried out in serum samples using Og4C3 enzyme linked immunosorbent assay test kit (JCU Tropical Biotechnology, Queensland, Australia) according to the manufacturer's instructions. Serum sample from each individual was tested and the optical density values were used to determine the antigen concentration in units from the standard curve prepared using seven standard antigens supplied in the kit. Serum samples with an antigen unit of 128 (>titre of standard No. 3) were considered as antigen positive.

Determination of antibody isotypes

Antibody isotypes (IgG, IgM and IgE) to filarial antigen (*Setaria digitata* antigenic extract) were determined by enzyme linked immunosorbent assay (ELISA) following a published procedure¹⁰. Briefly, polystyrene microtitre plates were coated overnight with *S. digitata* antigens (2 mg/ml) in alkaline buffer pH 9.2. Plates were saturated with 0.4% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. Sera for the detection of IgG and IgM were diluted 200-fold and for IgE sera were diluted to 100-fold, then added to the plate and kept at 37°C for 3 h. Following incubation with test sera, 1000-fold diluted anti-human IgG peroxidase (Sigma) or 2000-fold diluted anti-human IgM peroxidase (Sigma) or 1000-fold diluted anti-human IgE peroxidase (Sigma) were used for detection of filarial-specific antibodies. For the determination of IgG4 subclass, monoclonal antibodies to IgG4 (1/2000) (Sigma) were incubated for 3 h at room temperature after washing. Then plates were incubated with goat anti-mouse IgG Fc specific conjugated to horseradish peroxidase (HP) (1:5000) for 1 h at room temperature. The incubation continued for 3 h and, after washing, the pres-

ence of antibodies was detected with OPD substrate (Sigma, O-phenylene diamine containing H_2O_2). The enzymatic reaction was stopped by adding a drop of 4N sulphuric acid. The absorbance was read at 492 nm using an EIA reader (Bio-Rad, Richmond, USA). The positivity of each isotype was calculated by taking the high-titred chronic serum pool as positive control (100 antibody unit). A serum was considered seropositive if its absorbance exceeded a threshold value of the mean O.D.+3 S.D. of 14 non-endemic normal sera.

Assessment of filarial specific IgG4 subclasses by immunoblotting

About 30 μg of *S. digitata* antigen was loaded into a single well and separated on a gradient (4–20%) SDS PAGE under reducing conditions and transferred onto a poly vinylidene difluoride (PVDF) membrane. After blocking with 3% bovine serum albumin in tris buffer saline tween-20 buffer (TBST: 0.02 M Tris-HCl with 0.15 M NaCl, 0.05% Tween-20, pH 8) the strips were probed with hydrocele serum and fluid (1:50) for overnight at 4°C followed by monoclonal antibodies to IgG4 (1/200) (Sigma) for 3 h at room temperature after washing with TBST. Then strips were incubated with goat anti-mouse IgG Fc specific conjugated to horseradish peroxidase (HP) (1:5000) for 1 h at room temperature followed by vigorous washing with TBST and addition of chemi-luminescent substrate (Vector Lab) for HP. Bands were depicted on a Hyper-Film (Kodak) in a dark room using developer and fixer. Sera used in the qualitative assessment were prepared by pooling equal volumes of 20 samples from each group.

Immunoperoxidase assay

Anti-sheath antibodies to mf sheath of *W. bancrofti* were detected by indirect immunoperoxidase assay (IPA) as described by Ravindran *et al*¹¹. Briefly, acetone fixed mf purified from peripheral blood using 3 μm polycarbonate membrane (Nucleopore Corporation, USA) were used for the assay. The antigen slides were pretreated for 20 min with 0.5% H_2O_2 in methanol to inactivate endogenous peroxidase activity. Approximately, 15–20 μl serum (1:5 diluted in PBS) was applied in the slides which were incubated in a humid chamber for 2 h at 37°C . The slides were then washed three times with PBS and 15 μl of goat anti-human polyvalent IgG-peroxidase conjugate (Sigma) was added to the spots for detecting bound antibody activity. After washing the slides, the reaction was visualized using a light microscope after staining the slides with the substrate, diaminobenzidine (50 mg/100 ml) in Tris-HCl buffer pH 8.6 with 1 $\mu\text{l}/\text{ml}$ H_2O_2 . All the samples

were tested using mf of the same donor for uniformity and standard positive and negative controls were taken for batch of testing.

Cytokine assays

Cytokine levels (IL-10 and IFN- γ) in plasma were measured by using ELISA kits and expressed in pg/ml by interpolation from standard curve as described by manufacturer's instruction. Briefly, the capture antibody was diluted in PBS to a concentration of 1 μ g/ml and added 100 μ l to each well of the ELISA plate. The plate was sealed and incubated overnight at room temperature. The plate was washed with PBST thrice and blocked with 1% BSA (PBS) and incubated for 1 h at room temperature. The plate was again washed and the standard and serum samples were added to a dilution of 1:5 and the plate was incubated for 2 h at room temperature. The detection antibody was added to each well after washing and incubated for 2 h at room temperature. The plate was washed and 100 μ l of avidin-HP conjugate was added to each well at a dilution of 1:2000 and incubated for 30 min at room temperature. The plate was again washed and 100 μ l of ABTS substrate solution was added and the colour development was monitored with an ELISA plate reader.

Statistical analysis

Statistical comparison between the two groups was made by two tailed unpaired *t*-test with Welch's correction (95% CI) and $p < 0.05$ was considered as statistically significant.

RESULTS

Out of total 51 individuals enrolled in the study, 4 (7.8%) were found to be positive for mf by both night blood examination and concentration technique, while none of the hydrocele fluid revealed presence of mf. But Og4C3 assay could detect circulating filarial antigen in 11(21%) of the serum samples and 5 (9.8%) of the hydrocele fluids, which includes both serum and hydrocele fluid of all four microfilaremic patients (Table 1).

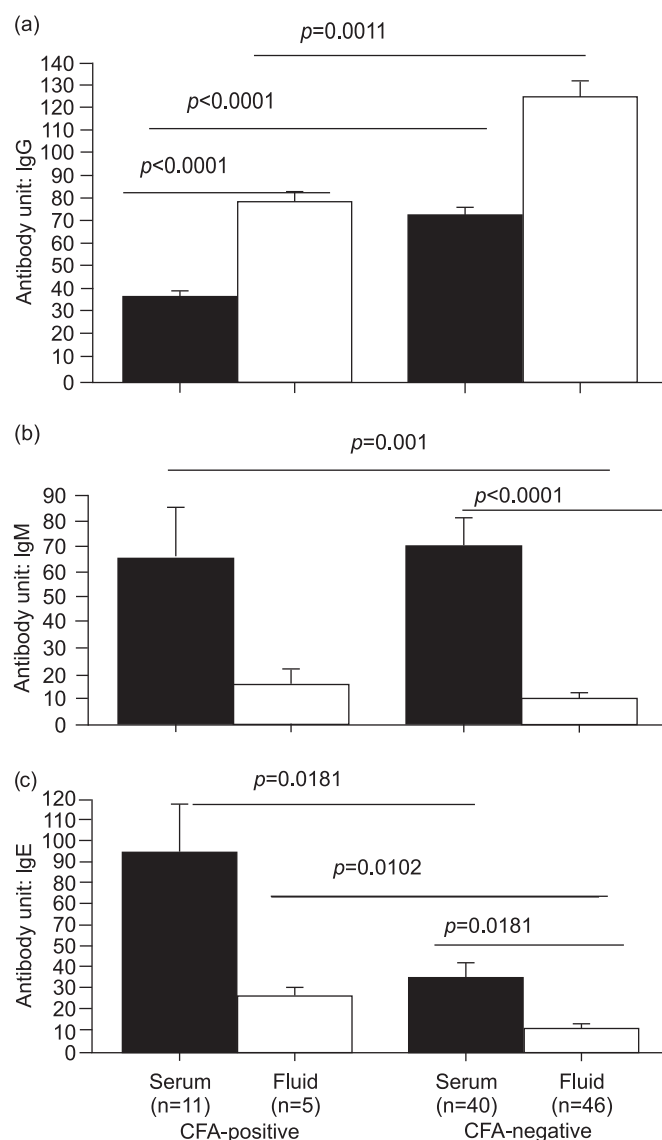
As would appear from Fig. 1 (a), the level of IgG was

Table 1. Characteristics of study population according to circulating filarial antigen and parasitological status

Samples	No.	Mean age (yr)	CFA (+)ve MF (+)ve	CFA(+)ve MF(-)ve	CFA(-)ve MF(+ve)	CFA(-)ve MF(-)ve
Serum	51	32	4	7	0	40
Fluid	51		0	5	0	46

high in fluid than serum in both CFA-positive and CFA-negative patients. But the level of IgG was significantly more in both hydrocele fluid and serum in CFA-negative compared to the CFA-positive hydrocele fluid and serum. In contrast, filarial specific IgM and IgE were significantly (IgM : $p < 0.001$; and IgE : $p = 0.01$) higher in serum than the fluid in CFA-positive and CFA-negative hydrocele patients. However, the level of IgM and IgE were significantly higher in serum and fluid of CFA-positive patients compared to corresponding fluid and serum of CFA-negative hydrocele patients (Figs. 1b and 1c).

The level of filarial specific IgG4 in hydrocele patients has shown a significant increase in serum of CFA-



Figs. 1 (a-c): Filarial specific antibody units: (a) IgG; (b) IgM; and (c) IgE to hydrocele fluid and serum of human lymphatic filariasis. Bars represent geometric mean \pm SEM values; and p -values are the statistical analysis between the two groups.

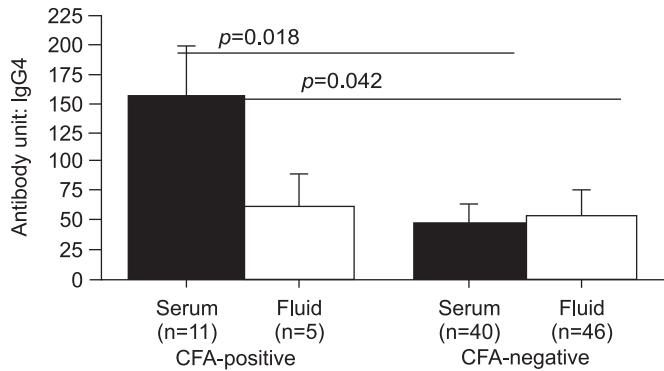


Fig. 2: Filarial specific IgG4 antibody unit in hydrocele fluid and serum of human lymphatic filariasis. Bars represent geometric mean \pm SEM values; and p -values are the statistical analysis between the two groups.

positive patients than the serum as well as fluid of CFA-negative subjects (Fig. 2). Though elevated level of IgG4 was observed in the fluid of CFA-positive subjects than seven CFA-negative fluid, but it was nonsignificant. However, the level of IgG4 does not differ within the groups among fluid and serum in both CFA-positive and CFA-negative hydrocele patients.

The qualitative assessment of IgG4 done by western blotting in the hydrocele fluid and serum pools of CFA-positive and CFA-negative individuals is shown in Fig. 3. The recognition of stronger bands was found in CFA-positive serum and fluid than that of CFA-negative serum and fluid of hydrocele patients. The serum of CFA-

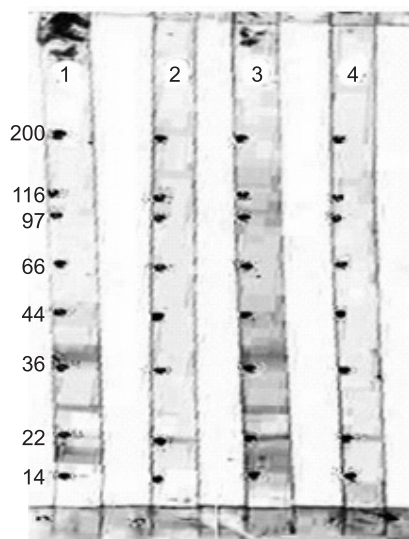


Fig. 3: Recognition profile of antifilarial IgG4 antibody responses against *S. digitata* antigen by immunoblotting in fluid and serum of hydrocele patients. Lane 1: CFA-positive hydrocele fluid; Lane 2: CFA-negative hydrocele fluid; Lane 3: CFA-positive hydrocele serum; and Lane 4: CFA-negative hydrocele serum.

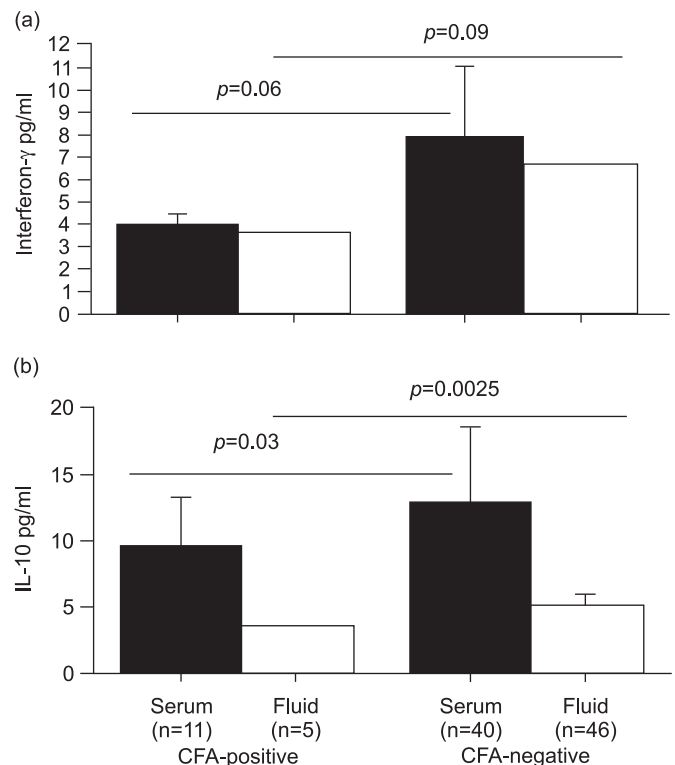
Table 2. Prevalence of anti-sheath antibodies and CFA in serum and fluid of hydrocele patients

Infection status	Serum			Fluid		
	No.	Anti-sheath antibodies		No.	Anti-sheath antibodies	
		(+)ve	(-)ve		(+)ve	(-)ve
CFA (+)ve	11	1 (9)	10 (91)	5	0 (0)	5 (100)
CFA (-)ve	40	34 (85)	6 (15)	46	39 (84.7)	7 (15.3)

Figures in parentheses indicate percentages.

positive hydrocele patients recognize bands of both high and low molecular weight range from 14–116 kDa. In contrast, fluid of CFA-positive hydrocele patients recognize only low molecular weight antigen from 14–44 kDa. However, filarial specific IgG4 does not recognize any clear cut band (except 22 kDa) in CFA-negative hydrocele serum and fluid.

The prevalence of anti-sheath antibodies in hydrocele fluid and serum has been shown in Table 2. Only one CFA-positive hydrocele serum showed anti-sheath antibody, in contrast none of the hydrocele fluids showed anti-sheath antibody in CFA-positive hydrocele patients. However, in CFA-negative hydrocele patients, almost equal percentage



Figs. 4 (a & b): Plasma level of (a) IFN- γ ; and (b) IL-10 in serum and corresponding fluid of CFA-positive and CFA-negative hydrocele patients. Bars represent geometric mean \pm SE values; and p -values represent the statistical significance between the two groups.

(85%) of individuals showed anti-sheath antibody in fluid and serum. Those individuals in CFA-negative group showed anti-sheath antibody in serum also showed anti-sheath antibody positive in the corresponding hydrocele fluid.

Figure 4 shows the plasma level of Th1 and Th2 (IFN- γ and IL-10) cytokines in serum and fluid of hydrocele subjects. An elevated level of IFN- γ was observed in CFA-negative serum and fluid than the CFA-positive serum and fluid. In contrast, IL-10 level was significantly high in fluid and serum of CFA-negative hydrocele patients than the CFA-positive hydrocele patients. The difference among the serum and fluid of both groups (CFA-positive and CFA-negative) of hydrocele patients were nonsignificant.

DISCUSSION

The common clinical manifestations of lymphatic filariasis has severe consequences for affected individuals both physically, socially and economically and makes the infection one of the important causes of disability. Death of the adult filarial worms during the process of development causes an inflammatory reaction that manifests as acute filarial lymphangitis (AFL). Recurrent episodes of AFL impair lymphatic flow, predisposing the host to secondary bacterial infections, which result in fibrosis, lymphatic obstruction, and lymphedema. High-protein lymphedema causes further inflammation and tissue destruction and chronic hydrocele ensures once damage is sufficient to overwhelm the lymphatic system. The elevated level of IFN- γ in CFA-negative serum and fluid of hydrocele patients indicates that there is a polarization of immune responses towards Th1 type of response which help in the clearance of parasite. The high level of IFN- γ along with anti-sheath antibodies in CFA-negative hydrocele patients might be restricting the parasite survival and growth subsequently giving protection to the individuals against the infection.

Significantly high level of IL-10 in CFA-negative hydrocele cases also correlates with the amicrofilaraemic condition in our study as described by Dimock *et al*¹² where they have shown greater T-cell proliferation and interleukin-10 (IL-10) production in amicrofilaraemic patients with hydrocele or elephantiasis than in amicrofilaraemic or microfilaraemic asymptomatic persons. So high levels of IFN- γ and IL-10 in CFA-negative hydrocele patients indicated that there is no clear cut bias of the Th1 or Th2 immune response in these subjects. Some of the subjects cleared their parasites and become CFA-negative and others may in the verge of their parasite clear-

ance. Therefore, the current study has provided an evidence for the existence of Th1 or Th2 type of environment which can play an important role in determining the presence and absence of parasite as well as the symptoms in the hydrocele subjects.

Interestingly, immunoblotting analysis during our study has shown stronger band recognition in serum and fluid of CFA-positive hydrocele patients which indicates the transfer of filarial specific antigen in active filarial infection, which are of only low molecular weight. Recently, the role of T-regulatory cells in modulation of human immune responses, such cells promoting the induction of the noncomplement fixing immunoglobulin G4 (IgG4) in helminthic infections has been demonstrated¹³. The recognition of IgG4 by low molecular weight antigen (22 kDa) by the CFA-negative hydrocele fluid and serum in the present study is at par with the previous findings and could be used as a marker to diagnose the filarial origin of hydrocele. Further, present finding shows only 4 out of 51 individuals are to be microfilaraemic by microscopic method, which is the gold standard for diagnosis of filariasis. However, the advanced method (Og4C3 assay) could detect 11 out of 51 individuals as CFA-positive. In this context, it may be postulated that our study population might have cleared the mf after five rounds of MDA which is being implemented in the state since 2004 for elimination of filariasis¹⁴. Similar to our study Hassan *et al*¹⁵ were able to identify CFA from paired samples of sera and hydrocele fluids in 50 and 66.7% of the mf negative and mf positive men with hydrocele respectively using the mAb AD12-ELISA¹⁶.

Moreover, only 9% from hydrocele serum and none from hydrocele fluid of CFA-positive group showed anti-sheath antibody positive in our study that supports the finding of strong inverse association between sheath antibody positive and the markers of filarial infection¹⁷. However, the higher prevalence of anti-sheath antibodies in both serum and fluid of CFA-negative hydrocele subjects suggests that continuous exposure to infective larvae may induce production of anti-sheath antibodies, which could adversely affect further development of the incoming filarial larvae, keeping the host amicrofilaraemic. Further, this result also emphasizes the filarial origin of hydrocele when the diagnosis was in doubt.

In conclusion circulating filarial antigen (CFA) and other filarial attributed immunological parameters can be measured in both hydrocele fluid as well as serum. This indicates that hydrocele fluid can be used as an alternative to serum for diagnosis of filariasis. Since, filariasis is in elimination phase and hydrocele is a manifestation of a variety of diseases including filariasis the hydrocele fluid

can be used for immunodiagnosis to monitor the filarial elimination programme.

Conflict of interest

The authors have no conflict of interest concerning the work reported in this paper.

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