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Diagnostic Potential of Skin Test Using *Brugia malayi* L_3 Antigen in Human Filariasis

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Abstract

The reliability of filarial skin test (FST) using *Brugia malayi* larval (L_3) antigen was validated in 722 patients from endemic areas using improved parasitological techniques and ELISA.

Out of 722 subjects studied from endemic area, FST was positive in 446 (61.8%) cases, while mf was demonstrated in 205 (28.39%) cases. A comparison of results of mf demonstration by various techniques was made in 443 subjects; presence of mf was demonstrated in 25 cases by conventional 20 cmm blood smear, while using 5 ml blood by filtration technique mf could be demonstrated in 129 cases. Of 50 FST positive cases who were mf negative even in 5 ml blood, mf could be demonstrated in 18 after DEC provocation (10 of 22 cases showing filarial manifestations, 6 out of 25 with filaria unrelated symptoms and 2 of 3 healthy subjects from endemic area) and 1 out of 6 subjects with filaria unrelated symptoms showed mf in pleural exudate. Thus, of the 443 subjects 148 cases demonstrated mf.

Out of the 199 FST positive cases, 154 were positive to ELISA using adult *B. malayi* antigen and 78 were positive for mf. Further ELISA was positive in 71 out of 78 mf psoitive and 83 out of 121 mf negative cases. Thus, evidence of filariasis could be established in 161 out of 199 FST positive subjects and the value of FST could be validated in 81% subjects.

It is inferred that the conventional blood examination (20 cmm) yields extremely poor results and should not be relied upon as an epidemiological index of prevalence of filariasis while FST which is easier to perform and quicker to read, is a reliable diagnostic test, irrespective of microfilaraemic status.

Key words: *Brugia malayi*, Filarial skin test, Enzyme linked immunosorbent assay, DEC provocation, filarial diagnosis

Introduction

The filarial skin test (FST), using *B. malayi* larval (L_3) antigen in the diagnosis of human filariasis, has been found to be sensitive and specific (Grove *et al.*, 1977; Chandra *et al.*, 1978 & 1986; Katiyar *et al.*, 1985). In a previous study (Chandra *et al.*, 1978), we have found the test superior to microfilaria demonstration. However, a high percentage of amicrofilaraemic subjects by the conventional night blood smear from filaria endemic area elicited positive reaction to this region. To examine the validity of FST, improved parasitological techniques using larger volume of blood, pleural aspirate and DEC provocation were employed. Selected cases from this population who were positive to FST were also subjected to Enzyme Linked Immunosorbent Assay (ELISA). Results have been compiled in this communication.

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Materials and Methods

Test subjects

The study was carried out on the outdoor and hospitalized patients from various medical colleges and hospitals from Northern Indian region, known to be endemic for bancroftian filariasis. A total of 722 subjects between age group 13 and 70 years were screened, of which

457 were males and 265 females. Majority of the subjects were from Lucknow (443). There were 219 subjects who presented with filaria related symptoms (pyrexia of unknown origin, chyluria, hydrocele, painless haematuria without any obvious cause, lymphadenitis (biopsy non-specific), lymphangitis, eosinophilia (>1000/cmm) and edema on legs), 274 had filaria unrelated diseases (diabetes, spinal injury, pain in abdomen, rheumatic and coronary heart disease, viral hepatitis, backache etc.) and 229 subjects were totally asymtomatic (healthy relatives of patients, healthy medical and Lucknow University students). Control subjects (615) were chosen from a known filaria non-endemic area, situated in the Kashmir valley at an altitude of 2400 meter, surrounded by high mountains. Care was taken to ensure that they had not travelled to the endemic areas of plains of India and they were all negative for microfilaria in their peripheral blood.

Filaria Skin Test (FST)

The FST with *B. malayi* L_3 antigen has been developed and standardized by us (Chandra *et al.*, 1978; Singh *et al.*, 1990). Briefly, the L_3 collected from mosquitoes (*Aedes aegypti*) fed on microfilaraemic *Mastomys natalensis* were homogenized, sonicated and centrifuged under chilled conditions. The soluble fraction was finally sterilized by filtration (0.22 μm filter). The protein content of this fraction was determined (Lowry *et al.*, 1951) and used as antigen. Merthiolate was added in concentration of 1:10,000 as preservative, which when used as control did not elicit a positive skin reaction. The antigen solution was distributed in several sterilized ampoules and lyophilized. The ampoules were sealed under vacuum at 10^{-2} Torr (Lyophilab-80C; The Scientific Instrument Co. Ltd.). The lyophilized antigen could be stored at 37°C for more than 18 months without loss of biological activity, but the reconstituted antigen lost activity at this temperature within 48 hours. However, the reconstituted antigen solution stored at 4° to 8°C could be preserved for about 15 days (Murthy *et al.*, 1988). A dose of 2 μg protein, injected intradermally, was found optimum to elicit positive skin reaction. Injection of antigen in volumes of 0.05 ml was made intradermally. Care was exercised not to deposit antigen in deeper tissues and avoid any spilling of the antigen solution.

Reading of results

The reaction being immediate hypersensitivity type, full wheal develops within 15 minutes of antigen injection. The initial wheal (due to volume injected) immediately after the antigen injection was marked with ball pen. Similarly, the wheal area after 15 minutes of injection was also encircled. The impressions were picked up on tracing paper and then translated onto mm^2 graph paper. The area covered by the original wheal and that by the final wheal were determined.

To assess the specificity and sensitivity of the test, the results of FST were compared between 153 microfilaria positive subjects from endemic area (positive) and 415 microfilaria negative subjects from area non-endemic for filaria, i.e., control subjects (negative). McNeMars test and Youden's index were determined for the various wheal sizes. Wheal area of more than twice the area of original injection gave the least false positive and false negative results. On this basis the antigen gave positive response in 92.3% of the mf positives and only 1.9% false positive in non-endemic controls (Singh *et al.*, 1990). Therefore, a reaction ratio of 2 or more in the wheal was taken as positive reaction for filarial antigen.

In filarial patients treated with Diethylcarbamazine citrate (DEC), there was marked suppression of skin reaction to the antigen and this effect lasted for over a year (Murthy *et al.*, 1978). Hence, care was taken out to conduct the skin test in those having received DEC in the recent past or those on corticosteroids or antihistaminic therapy.

Enzyme Linked Immunosorbent Assay (ELISA)

Out of the 722 subjects, 199 sera samples were examined by ELISA test. Twenty sera samples from European subjects with no parasitological background (received through the courtesy of

Richard Lucius, Institute for Tropical Hygiene, Heidelberg, West Germany) and 34 subjects from filaria non-endemic area (Kashmir, India) harbouring *Ascaris* (10), hookworm (8), *Giardia* (8) and *E. histolytica* (8) served as control.

The technique based on the original method of Engvall and Perlmann (1972) was used except that the horse-radish peroxidase conjugate of anti-human IgG (Sigma) was employed in place of alkaline phosphatase conjugate, and O-phenylene diamine as substrate. The optimal concentration of antigen and dilution of test sera and conjugate were determined by chequer board titration. *B. malayi* adult worm (obtained from infected mastomys) soluble fraction (5 ng protein) in aliquote of 200 μl of 0.05M sodium carbonate buffer (pH 9.6) was used as the antigen. Intensity of colour developed, by the enzyme reaction was measured spectrophotometrically at 492 nm (E/492). The titre of antibody in test sera has been expressed as ELISA values. ELISA values higher than mean $\pm$ 2SD of endemic controls has been taken as positive for filariasis.

Microfilaria demonstration

In night blood: Different amounts (20 cmm and 5 ml) of venous blood were drawn between 10 p.m. and 12 midnight. The 20 cmm blood was made into thick smear for microfilaria (mf) demonstration. The 5 ml blood sample was taken in heparinized tubes and were filtered through millipore filters (5 μm porosity) to separate the mf which are seen live.

After DEC provocation: 50 patients found positive for FST but negative for mf (in 5 ml night blood) were given 100 mg of diethylcarbamazine citrate (Hetrazan^R, Cyanamide India Ltd.) orally at 10 p.m.; 30 to 35 minutes later 5 ml blood was drawn and the number of mf was counted by the concentration technique as above.

In pleural aspirate: Six cases presenting with exudative pleural effusion without obvious evidences of tuberculosis, were examined for mf in 5 ml night blood and in pleural aspirates (10–40 ml). Pleural fluid was also filtered through millipore filter to count the mf.

Results

Out of total 722 cases screened for mf and FST, 205 revealed bancroftian microfilariae in the peripheral blood and 446 subjects reacted positively to *B. malayi* larval antigen (Table 1). The mf positivity rate in asymptomatic individuals and those presenting with filaria related symptoms was nearly the same. Similar results were obtained for FST with marginal enhance-

Table 1. Microfilaria demonstration and FST in different clinical groups

Group	No. of cases	No. of mf* positive	No. of FST positive
Controls from non-endemic area (Kashmir)	615	Nil	9 (1.46%)
Subjects with filaria related symptoms	219	78 (35.62%)	162 (73.97%)
Subjects with non-filaria related symptoms	274	50 (18.25%)	137 (50%)
Symptoms and signless subjects from endemic	229	77 (33.6%)	147 (64.19%)
Total	722†	205 (28.39%)	446 (61.77%)

*Microfilaria
†Excluding subjects from non-endemic area

ment of FST positivity in the later group. In contrast, out of 615 control subjects from non-endemic area, only 9 subjects (1.46%) showed false positive FST, though all were negative for mf in blood.

The traditional thick night blood smear (20 cmm) examination and filtration technique (5 ml) could be compared only in 443 of total 722 subjects (Table 2). While conventional 20 cmm blood smear was positive for mf in 25 subjects, the filtration technique yielded mf in 129 subjects over five fold rise in mf recovery rate (details of subject wise break up as in Table 2). All those positive for mf in thick smear were also positive in 5 ml blood.

DEC provocative test carried out in 50 FST positive cases, who were initially negative for mf, revealed 18 additional mf carriers. Pleural aspirate examination in 6 cases of exudative pleural effusion with a presumptive diagnosis of tubercular effusion revealed mf in 3 cases, of which 2 were already positive for mf in blood, thus one extra case was identified as mf positive. The mf positivity in 148 cases by different parasitological techniques performed in 443 cases has been detailed in Table 2.

In 199 subjects with positive FST, filaria specific antibodies by ELISA could be detected in 71 out of 78 microfilaraemic subjects and in 83 out of 121 mf negatives. Thus, sensitivity of ELISA was 95.2% and 97.4% respectively in mf negative and mf positive cases of filariasis showing manifestations. However, 36 mf positive cases showing sign and symptoms of other than filaria, ELISA titre could be detected in 31 (86.1%) subjects. Among mf negative subjects without any sign and symptoms, 14% also showed filaria specific circulating antibodies. Thus out of these 199 FST positive cases, the evidence of filariasis could be established in 161 cases, 78 by parasitological examination and in 83 by ELISA (Table 3).

The sera from control European subjects did not yield positive ELISA response. Cases from filaria non-endemic area and having one or the other intestinal parasite also did not cross-react in ELISA except for 1 out of 10 *Ascaris* positive cases.

Table 2. Filaria positivity by different parasitological techniques and FST

	Mf demonstration in blood				Total mf positive cases	No. of FST positive
	20 cmm	5 ml	DEC* provocated	Pleural aspirate		
Subjects with filaria related symptoms (n = 208)	11	72	10 (22)†	–	82	146
Subjects with non-filaria related symptoms (n = 156)	14	38	6 (25)	3 (6)	45	97
Symptoms and signless cases from endemic area (n = 79)	Nil	19	2 (3)	–	21	58
Total (n = 443)	25	129	18	1‡	148	301

*Night blood examination after DEC provocation in cases positive for FST but negative for mf initially.
†No. of cases who have been subjected for provocative test.
‡Out of 3 cases shown above two were positive in 5 ml blood also.
n – No. of cases

Table 3. Results of ELISA and mf demonstration in FST positive cases

Group	Mf	No. of cases	ELISA (values Mean ± SD) positive	Negative
Subjects with filaria related symptoms	Positive	42	40 (0.545 ± 0.293)	2 (0.131 ± 0.030)
	Negative	79	77 (0.650 ± 0.280)	2 (0.136 ± 0.036)
Subjects with non filaria related symptoms	Positive	36	31 (0.340 ± 0.124)	5 (0.102 ± 0.020)
	Negative	–	–	–
Symptoms and signless subjects from endemic area	Positive	–	–	–
	Negative	42	6 (0.200 ± 0.099)*	36 (0.116 ± 0.050)*
	Total	199	154	45

*Combined mean ± SD = 0.122 ± 0.040

Table 4. FST positive subjects in different age groups

Age in yr	No. of cases		No. FST positive (%)	RR (Median with range)
Upto 15	M	22	20 (90.9)	3.5 (2.0 – 12.0)
	F	28	21 (75.0)	3.3 (2.0 – 8.1)
16 – 30	M	114	89 (78.1)	3.3 (2.0 – 10.6)
	F	71	47 (66.2)	3.7 (2.3 – 10.7)
31 – 45	M	64	38 (59.4)	3.3 (2.0 – 14.2)
	F	45	37 (82.2)	3.8 (2.0 – 10.8)
46 –60	M	56	35 (62.5)	3.5 (2.1 – 14.7)
	F	48	31 (64.6)	3.7 (2.0 – 14.7)
60	M	22	9 (40.9)	3.6 (2.2 – 5.3)
	F	17	11 (64.7)	2.4 (2.4 – 6.7)
	Total	487	338 (69.4)	

RR – Reaction Ratio, M – Male, F – Female

Discussion

Conventionally the diagnosis of lymphatic filariasis is established by demonstration of mf in 20 cmm night blood. This method besides being cumbersome, yields extremely poor results (Grove *et al.*, 1977; Chandra *et al.*, 1978 & 1986). Improved parasitological methods also do not yield desired results because of non-availability of mf in blood (prepatent, latent and occult filarial cases).

To overcome these difficulties, several immunodiagnostic tests have been developed and their usefulness tested in fields (Kagan, 1963 & 1983; Ambroise-Thomas, 1974). Among the two human lymphatic filariids, *W. bancrofti* and *B. malayi,* the former is highly specific to human host (Cross *et al.*, 1979; Dissanaike & Mak, 1980) but the later can be conveniently transmitted to small laboratory rodents (Petranyi *et al.*, 1975; Ash and Riley, 1970). This major advantage and excellent cross reactivity between them (Chandra *et al.*, 1978), justify the usage of *B. malayi* L_3 antigen for the diagnosis of bancroftian filariasis. In skin test, the antigen made from *B. malayi* (L_3) was found to be highly specific and sensitive (Grove *et al.*, 1977; Chandra *et al.*, 1978 & 1986; Katiyar *et al.*, 1985). Data on agewise distribution of FST positive subjects in Table 4 indicate that although the reaction ratio was independent of age, the highest positivity existed in the age group upto 15 years.

Since many amicrofilaraemics with apparent no symptoms reacted positively to the antigen, in the present study membrane filtration technique using 5 ml blood, pleural aspirate examination and DEC provocation were employed to further validate usefulness of FST antigen. A high percentage (73.97%) of population with filaria related manifestations reacted positively to the antigen. However, 50% of subjects with filaria unrelated symptoms and 64.19% healthy subjects also reacted positively to the antigen. Some had mf either in blood or in pleural aspirate. Yet in others, the mf could be demonstrated after DEC provocation. The results obtained further narrowed down the gap between parasitological demonstration and FST. It may be assumed that even higher microfilaria rate would have been recorded by screening still larger volume of blood and examination of all 172 FST positive but mf negatives (301-129) by DEC provocation.

DEC provocation has been successfully used to mobilize mf to peripheral blood (Hawking & Adams, 1964; Katiyar *et al.*, 1973) but the investigators established it's utility during day time only. At night, the mf mobilization from their day hide-outs is due to physiological factors (Hawking 1975) which appear to be very effective. In the present study, DEC administration even at night was successful in driving the mf to peripheral blood exhibiting it's superiority over the forces responsible for nocturnal periodicity.

In occult filariasis, parasitological examinations invariably fail though the patient carry active infection. Pleural cavity appears to be one of the sites for mf in occult filariasis. The finding that out of 6 cases of exudative pleural effusion, 3 were positive for mf in the pleural fluid, raises the possibility of filariasis as a cause of exudative pleural effusion which is assumed to be tubercular in origin in developing countries, though further studies are needed to establish this firmly.

The results of FST were validated by ELISA. Out of 199 FST positive subjects, 78 had bancroftian mf in their blood. In the remaining 121 cases, 83 were positive by ELISA. Thus, among 199 FST positive subjects, the diagnosis of filaria could be established in 161 (81%) cases (78 direct and 83 indirect). The ELISA negativity in 38 among the FST positive subjects could be explained on the basis of different immunological principles involved in these two tests. Out of 78 mf positive, 7 cases were negative by ELISA test, which could be due to immunosuppression (Ottesen, 1984), or due to neutralization of antibodies by the enormous amount of antigen liberated by the parasite.

The cases which had positive FST but were negative for mf, might be having immature infection or very low infection and the mf either have not appeared so far or were below the thresh-hold level to be detected in blood. The

occult filariasis can not be detected by blood examination (WHO, 1984) and even DEC provocative test would prove ineffective in such cases. In animals, it has been observed that at times only a few worms of one sex develop or the worms of different sexes are located at distant places with no production of mf. These cases would go undetected by blood examination but since they carry active infection, will show positivity in FST. This may explain the gap in positivity of mf and FST in the present study.

The nearly same rate of recovery of mf in those presenting with filaria related symptoms and healthy individuals is not surprising as it is well known that there is no relation between microfilaraemia and clinical signs and symptoms (Kazura *et al.*, 1984).

The present diagnostic methods of filariasis grossly exceeds the prevalence rate. Even in the most heavily infected countries, the 'infection rate', which is based on presence of microfilariae in peripheral blood plus microfilaria negative individuals who have clinical signs of disease, does not exceed more than 50% (WHO, 1984). Global infection rate of *W. bancrofit* is mere 9% and that of *B. malayi* and *B. timori* as 0.9% (WHO, 1984). Corresponding figures for South-East Asia are 11.5% and 1.1% respectively. The present study clearly demonstrated that the prevalence rate of filariasis as based on conventional night blood smear is grossly inadequate to establish the epidemiology of the infection in a given population. FST with *B. malayi* (L_3) antigen, as developed by us, is more superior to conventional diagnostic tests. The FST besides being simple and quick, detects filariasis irrespective of the intensity of the infection. The test antigen has long keeping quality (Murthy *et al.*, 1988). It may convenient and suitable for individual as well as community based diagnosis of filariasis.

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