A serological study of tuberculosis patients for *Dirofilaria immitis* infection in Malaysia

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Abstract

In this study we investigated evidence of *Dirofilaria immitis* infection among TB patients admitted to the Medical Respiratory Institute of the Ministry of Health Malaysia. Three hundred blood sera samples were analysed using enzyme-linked immunosorbent assay and Western Blot methodology. Six positive cases were detected based on antibodies reactive to polypeptides of *D. immitis* at MW 212, 116 and 77kDa using Western Blot analysis.

Key words: Dirofilariasis, human, diagnosis

Introduction

In serological surveys by Ambu *et al.* (1977; 2002) positive reactors to *Dirofilaria immitis* antigens were detected in the general population, cardiovascular disease patients admitted to the National Heart Institute and respiratory infection patients admitted to the Institute of Repiratory Medicine, Ministry of Health Malaysia. Enzyme-linked immunosorbent assay (ELISA) and Western Blot analysis were used for detection of infection.

Various studies in the United States (Kochar, 1984), Puerto Rico (Villanueva & Rodriquez-Perez, 1993) and Japan (Kondo & Fujita, 1991) have shown that human dirofilariasis is present, both in urban and peri-urban areas. A study by Lee *et al.* (2000) in Korea, identified a patient with a coin lesion in the lung, which on exploratory resection showed a degenerate worm identified to be *D. immitis*.

An increase in the population of possible vectors could lead to an increased exposure of the general population to dirofilariasis. In Malaysia, Cheong *et al.* (1981) identified *Armigeres subalbatus*, which is found in abundance in urban areas, to be a potential vector for *D. immitis*. In another study Lai *et al.* (2000), implicated low microfilarial density in infected dogs to be the source of infection for vector mosquitoes. Similar findings were seen in another study in Brazil (Ahid & Lourenco, 2000), where *Aedes taeniorhynchus* and *Culex quinquefasciatus* were identified as potential vectors of *D. immitis*. An increased urban transmission of dirofilariasis can occur in the urban, affluent society of Kuala Lumpur and other Malaysian cities where dogs and the mosquito vector, *Ar. subalbatus* are abundant.

Material and Methods

A total of 300 blood samples (from 100 females and 200 males) were collected from the Institute of Respiratory Medicine, Ministry of Health Malaysia and analysed using ELISA (Voller *et al.*, 1976) and Western Blot (Towbin *et al.*, 1979) techniques. The blood samples were collected from April 1997 to May 1997.

The age range of these patients was between 7 – 88 years (mean age 48.8 years). Of the 300 there were 112 Malays, 87 Chinese, 57 Indians, 20 Indonesians, 7 Bangladeshi, and 17 others.

Adult *D. immitis* worms were homogenised and sonicated at 4°C, using 6 kilocycle MHz thrice (3 minutes on-off cycles) and stored overnight at 4°C. The homogenate was centrifuged at 2,000 rpm for 10 minutes and the supernatant again centrifuged at 14,000 rpm at 4°C for 20 minutes. The protein concentration (4 µg/ml) was determined using the BIORAD Protein Assay kit and the sample was aliquoted and stored at -20°C for use as antigen. The serum was diluted at 1:40,000 (KLP, Maryland, USA) and the substrate used was orthophenelene-diamine (OPD).

In order to establish the minimum ELISA-positive absorbance optical density (OD) reading of the 286 sera, the mean OD of 0.771 + 3SD (standard deviation = 0.556) was used to establish a positive cut-off value of 2.439. Based on these readings 10 extreme OD value cases were excluded from the study. Those excluded

were patient sera number 8, 58, 83, 94, 241, 255, 260, 262, 268 and 303.

The somatic antigens of other helminths used for cross-reaction studies were prepared and their protein concentration estimated as described above. Thirty µl of each of *Toxocara canis* (180 µg), *Toxocara cati* (150 µg), *Ascaris suum* (240 µg), *Dirofilaria immitis* (120 µg), *Angiostrongylus malaysiensis* (180 µg) and *Brugia malayi* (60 µg) antigen concentrations were separated on SDS-polyacrylamide gel (SDS-PAGE) using the discontinuous system of Laemmli (1970). These proteins were electrophoretically transferred to a nitrocellulose membrane using the Western blotting technique. The *D. immitis* ELISA-positive human sera were then allowed to react with these proteins. The positive serum dilution used in the reaction was 1:100 and the anti-human peroxidase conjugate dilution was 1:3,000.

Three reactive bands identified in an earlier study by Ambu *et al.* (1997) were used as the identifying bands for positive identification of patients' sera for *Dirofilaria* infection. The bands used were 77, 116 and 212 kDa and the positive cases were numbers 13, 46, 100, and 238 while two borderline cases were 84 and 261.

Results

SDS-PAGE analysis of *D. immitis* adult worm antigens showed a protein profile of bands ranging from 212kDa to 6.5 kDa. The analysis of the 300 cases using ELISA showed 16 positive cases (5.33%) (Table1), and 10 cases with extreme values were excluded from the study. The other six cases were confirmed using Western Blot analysis (Fig. 1). The 3 reactive bands 212, 116 and 77 kDa used for identifying the positive cases did not show any cross-reactivity to other heterologous antigens. The case history of each positive patient is described in Table 2. The positive cases were between the ages of 23- 65, and all were males (2 Malays, 2 Indians, 1 Indonesian and 1 Chinese).

The results of the Western Blot analysis carried out with *D. immitis, T. canis, T. cati, A. suum, A. malaysiensis* and *B. malayi* antigens on the ELISA (*Dirofilaria*) positive human sera (Nos. 100 and 238) are shown on Fig. 2 and Table 3. Cross-reactions were seen with 185, 158, 93, 64, 52 and 47 kDa molecular weight polypeptides. The sera from these 16 patients were also tested for *Brugia malayi* infection using the Indirect Fluorescent Antibody Test (IFAT) and there were 6 positive cases for filarial infection (Table 1). In this case bands 93, 64, 52 and 47 kDa with strong reactivity for dirofilariasis were also found to react strongly with *B. malayi* positive sera. Reactive bands, 185 and 158 kDa were found to cross-react with *Toxocara canis* antigen.

Discussion

The possible emergence of dirofilariasis as a public health problem is a concern for all health authorities. As mentioned earlier, dogs as pets in the affluent urban population and the presence of potential mosquito vectors can result in increased human exposure and infection with dirofilariasis in Malaysia. Some 60 species of mosquitoes in six genera are capable of supporting the development of *D. immitis* larvae to the 3rd infective stage (Otto, 1969 and Ludlam *et al.*, 1970). Infection in dogs is common in Japan, China, Southeast Asia, Australia and generally throughout the Pacific Islands (Beaver *et. al.*, 1984).

In an earlier study by Ambu *et al.* (1997), 11 putative cases of dirofilariasis were identified from 300 sera samples collected randomly from the Blood Bank, General Hospital, Kuala Lumpur. In another study, Ambu *et al.* (2002) analysed 229 sera samples from patients admitted to the National Heart Institute, Kuala Lumpur and found 8 positive cases. Ethnic composition of positive cases in the previous 2 studies were 8 Malays, 1 Chinese, 1 Indian and 1 Javanese (Ambu *et al.*, 1997) and 4 Malays, 3 Indian and 1 Chinese (Ambu *et al.*, 2002) respectively. In this study, most of the positives were among the Malays (6), followed by Indians (5), Indonesians (4) and Chinese (1). The Malays and Indians for some reason have higher infection rates than the Chinese or others. The reasons could be attributed to environmental conditions such as vector breeding grounds and socio-economic background rather than rearing of dogs. A more detailed study on the disparity in infection rates among the various ethic races should be undertaken to determine the underlying causes.

In our studies we have used 3 reactive polypeptide bands at 212, 116 and 77 kDa as markers for *Dirofilaria* infection. Similarly Perera (1994) found that the 44 kDa protein antigen of *D. immitis* to be a good marker for dirofilariasis in his study.

Further studies have to be carried out on patients with pulmonary and cardiovascular disease to assess the value of non-invasive

serological techniques for detection of *D. immitis* infection in human.

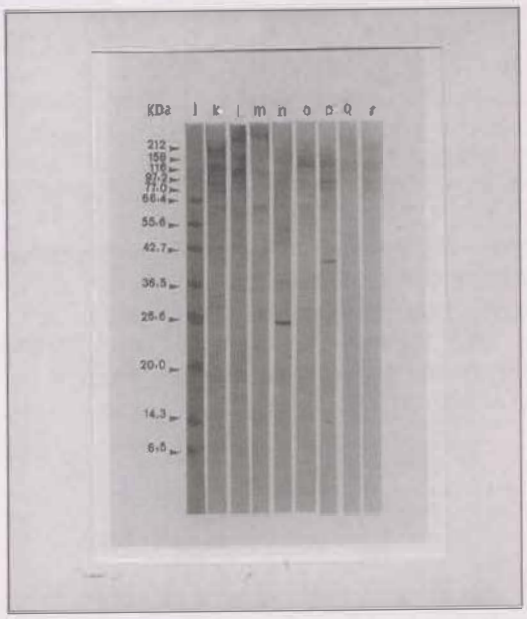

Fig. 1. Western Blot analysis of sera from tuberculosis patients using SDS-PAGE separated *Dirofilaria immitis* antigens. Lane j: molecular weight marker, New England Biolab; Lane k: serum No. 268; Lane l: serum No. 303; Lane m: serum No. 13; Lane n: serum No. 46; Lane o: serum No. 100; Lane p: serum No. 238; Lane q: serum No. 84; Lane r: serum No. 261.

In comparative studies on invasive and non-invasive procedures, Sato *et al.* (2000), found echocardiography studies to be useful in the diagnosis of heartworm disease in ferrets. These appeared as hyperechoic densities within the right arterial and ventricular cavities and on necropsy, four *Dirofilaria immitis* parasites (three females and one male) were found in the right heart, the cranial vena cava and the caudal vena cava. The resemblance of dirofilariasis to primary bronchogenic carcinoma or metastatic tumour (Leonardi *et. al.*, 1977) has placed this disease in the category of an emerging public health problem. Studies have shown that pre-operative diagnosis using non-invasive methods (Kondo & Fujita, 1991) such as serology is a preferred choice. However in many lung involvement cases, lung biopsies have been preformed to locate the parasite in tissues.

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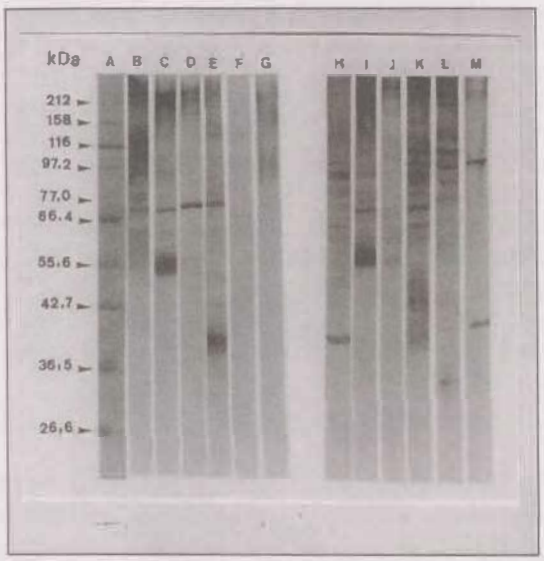

Fig. 2. Western Blot analysis of sera from tuberculosis patients using various nematode SDS-PAGE separated antigens. Lane A: protein marker A (New England Biolabs) Lane B & H: *Dirofilaria immitis* antigen; Lane C & I: *Toxocara canis* antigen; Lane D & J: *Toxocara cati* antigen; Lane E & K: *Ascaris suum* antigen; Lane F & L: *Angiostrongylus malaysiensis* antigen; Lane G & M: *Brugia malayi* antigen. Lanes B-G: ELISA positive serum sample No. 100; Lanes H-M: ELISA positive serum sample No. 238.

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Table 1. ELISA* and Western blot analysis of *Dirofilaria immitis* positive sera using crude *D. immitis* soluble antigen. Positive human sera also tested for *Brugia malayi* using IFAT.

Serum No.	Race	Sex	Age (years)	+ve case	MW kDa			IFAT	
					212	116	77	Sonicated Ag	Papain digested Ag
8	Indonesian	Male	51	2.601		+		-ve	-ve
58	Malay	Male	35	over			+	1:10	1:40
83	Indian	Male	43	over			+	-ve	1:10
94	Indian	Male	71	3.400	+		+	1:80	1:80
241	Indian	Female	60	3.305	+	+	+	1:80	1:40
255	Malay	Female	60	2.576		+	+	1:40	1:80
260	Malay	Male	51	2.850	+	+	+	-ve	-ve
262	Indonesian	Male	25	3.200	+	+	+	1:10	1:20
268	Chinese	Male	62	3.006	+	+	+	-ve	-ve
303	Indonesian	Male	25	2.579		+	+	-ve	-ve
13	Malay	Male	23	2.206		+		-ve	-ve
46	Chinese	Male	62	2.186		+		-ve	-ve
100	Malay	Male	63	2.182			+	-ve	-ve
238	Indian	Male	65	2.241	+		+	-ve	-ve
84	Indian	Male	36	1.962			+	-ve	-ve
261	Indonesian	Male	36	1.882			+	-ve	-ve

*OD readings of positive control = 1.684; negative control = 0.189.

Table 2. Chest radiography features of patients showing positive reactivities with *Dirofilaria immitis* antigens on Western blot analysis

No	Age	Sex	Race	*Brugia* +ve	*Dirofilaria* +ve	Chest radiograph findings
1	51	Male	Indonesian	-	2.601	Cavity upper zone, calcification of both hilar areas and reticulo-nodular lesion. Suggestive of old healed pulmonary tuberculosis
2	35	Male	Malay	+	Over	Shadowing both upper zones. Nodular shadow left mid-zone. Suggestive of pulmonary tuberculosis.
3	43	Male	Indian	-	Over	Multiple cavity lesions both upper zones with surrounding nodular shadows. Suggestive of pulmonary tuberculosis.
4	71	Male	Malay	+	3.400	Opacity left lower zone. Cancer of lung
5	69	Female	Indian	+	3.305	Bilateral scattered nodular shadowing both lung fields.
6	60	Female	Malay	+	2.576	Extensive reticulo-nodular shadowing both lung fields.
7	51	Male	Malay	-	2.850	Collapsed consolidation both upper lobes with cavitation. Suggestive of pulmonary tuberculosis.
8	25	Male	Indonesian	+	3.200	Infiltration right upper zone. Suggestive of pulmonary tuberculosis.
9	59	Male	Chinese	-	3.006	Cavitation with scarring both upper zones. Suggestive of pulmonary tuberculosis.
10	25	Male	Indonesian	-	2.579	Infiltration right upper zone with collapse of right upper lobes. Suggestive of pulmonary tuberculosis.
11	33	Male	Malay	-	2.206	Bilateral extensive micro nodular shadows. Suggestive of miliary tuberculosis.
12	62	Male	Chinese	-	2.186	Collapsed right upper lobes with cavitation. Suggestive of pulmonary tuberculosis.
13	63	Male	Malay	-	2.182	Shadowing left upper zone with multiple cavitations. Suggestive of active pulmonary tuberculosis.
14	65	Male	Indian	-	2.241	Diffused nodular shadowing both lung fields. Advanced pulmonary tuberculosis. Patient died.
15	36	Male	Indian	-	1.962	Bilateral diffused miliary shadowing. Suggestive of miliary tuberculosis.
16	36	Male	Indonesian	-	1.882	Partial collapsed right upper lobe with shadowing in both upper zones associated with a right pleural effusion. Suggestive of pulmonary tuberculosis.

Table 3. The ELISA O.D. readings in cross-reactivity studies with *Toxocara canis*, *T. cati*, *Ascaris suum*, *Angiostrongylus malaysiensis*, and *Brugia malayi* antigens using *Dirofilaria immitis* ELISA positive patient sera 8 to 261*

Specimen number	*Dirofilaria immitis*	*Toxocara canis*	*Toxocara cati*	*Ascaris suum*	*Angiostrongylus malaysiensis*	*Brugia malayi*
PBS	0.240	0.129	0.358	0.089	0.459	0.054
8	1.867	2.358	1.419	1.805	1.692	2.703
58	Over	Over	Over	2.640	1.909	Over
83	Over	3.178	2.699	2.067	0.882	Over
94	Over	Over	2.921	2.813	2.056	Over
241	Over	2.891	2.260	2.166	2.336	3.343
255	3.050	3.025	1.071	1.905	1.322	2.441
260	2.656	2.500	1.890	1.482	1.634	2.750
262	Over	2.055	1.364	1.970	0.442	3.328
268	2.976	2.986	2.720	1.928	1.778	Over
303	2.704	2.545	1.761	1.282	2.634	2.578
13	1.411	1.957	1.251	1.616	1.116	1.598
46	2.470	1.098	0.864	1.502	0.308	1.833
100	2.392	1.198	0.661	1.285	0.239	1.588
238	2.471	Over	2.053	2.714	1.643	2.645
84	1.676	0.939	0.409	0.756	1.401	1.498
261	1.954	1.884	0.763	1.346	1.411	2.364

*Test conditions: antigen concentration = 4mg/ml; serum dilution 1: 400; conjugate dilution: 1: 40,000 (KPL Anti-Human Peroxidase)