# Immunodiagnosis of human dirofilariasis in Malaysia - a preliminary study

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## Abstract

*Dirofilaria immitis*, the dog hearrworm has been reported in humans in the United States of America, Puerto Rico and Japan. This is the first study that is being carried out in Malaysia to identify human infections. The study is based on 300 random blood sera collected from the Blood Bank, General Hospital, Kuala Lumpur. A total of 11 putative positive cases were identified based on enzyme-linked immunosorbent assay and the presence of antibodies reactive to polypeptides of *D. immitis* at MW 212, 116 and 77 kDa on Western Blot analysis.

Key words: *Dirofilaria immitis* - immunodiagnosis

## Introduction

*Dirofilaria immitis* until recently has been considered to be a parasite of dogs. However, in the last two decades, human dirofilariasis have been reported from the United States (Kochar, 1984), Puerto Rico (Villanueva & Rodriguez-Perez, 1993) and Japan (Kondo & Fujita, 1991). The involvement of the human lung in the form of a focal pulmonary infarct due to a filarial parasite was first reported by Dashiell (1961). Although the infection in human is generally benign, it may be difficult to distinguish from lung cancer or tuberculosis. The primary threat to human health from pulmonary dirofilariasis is not caused by the parasites, but rather by invasive procedures that may be required to obtain a definitive diagnosis (Glickman *et al.*, 1986). Many attempts have been made to develop and improve immunodiagnostic tests using somatic adult worm antigens (Yamashiro *et al.*, 1989; Sun & Sugane, 1992; Villanueva & Rodriguez-Perez, 1993). In this paper we report the first study in Malaysia on immunodiagnosis of human dirofilariasis using enzyme-linked immunosorbent assay and Western Blot analysis.

## Materials and Methods

In the present study a serological survey was carried out for *D. immitis* infection in humans from randomly collected sera from the Blood Bank, General Hospital, Kuala Lumpur. There were 123 Malays, 140 Chinese, 33 Indians, 2 Javanese, and 1 each Iban and Munit, with the age range between 16 - 57 years. The 300 sera samples were analysed using the enzyme-linked immunosorbent assay (ELISA) (Voller *et al.*, 1976). For the ELISA, somatic antigen was prepared from *D. immitis* adults using a glass homogenizer at 4°C, followed by sonication at 6 kilocycle mHz 3 times on a 3 minutes off-3 minutes on cycle. The homogenate was kept overnight at 4°C and centrifuged at 2,000 rpm for 10 minutes. The supernatant was centrifuged at 14,000 rpm at 4°C for 20 minutes. The protein concentration (4µg/ml.), was determined using the BIORAD® Protein Assay and the sample was aliquoted and stored at -20°C. The serum dilution used was 1:400, the anti-human peroxidase conjugate diluted at 1:40,000 (KPL® Maryland, USA) and orthophenelenediamine (OPD) substrate was used. In order to establish the minimum ELISA-positive OD (Dynatech® Reader, USA) value, the mean OD of 293 sera were used (7 sera samples with extreme values, >1.31 were excluded from the calculation of the mean). The mean OD of 0.38 + 3SD (standard deviation =0.20), resulted in a positive cut-off value of 0.98.

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 the following antigen concentrations, *Toxocara canis* (180µg), *Toxocara cati* (150µg), *Ascaris suum* (240µg), *Angiostrongylus malaysiensis* (180µg), *Brugia malayi* (60µg) and *D. immitis* (120µg) were separated on SDS-polyacrylamide gel (SDS-PAGE) using the discontinuous system of Laemmli (1970). These proteins were electrophoretically transfered to a nitrocellulose membrane using the Western blotting technique (Towbin *et al.*, 1979) and ELISA-positive (*Dirofilaria*) human sera were then allowed to react with these proteins. The *Dirofilaria* positive serum dilution used in the reaction was 1:100 and the anti-human peroxidase conjugate dilution was 1:3,000.

## Results

There were 11 ELISA-positive cases (3.66%) and cross-reactivity studies showed the sera to be reactive ro anrigens of *T. canis, T. cati, A. suum, A. malaysiensis* and *B. malayi*. The positive cases were between the ages of 18-46 years, with 8 males and 3 females. Ethnically, 8 were Malays, and 1 each Javanese, Chinese and Indian.

The results of the Western Blot analysis carried out with *D. immitis, T. canis, T. cati, A. suum, A. malaysiensis*

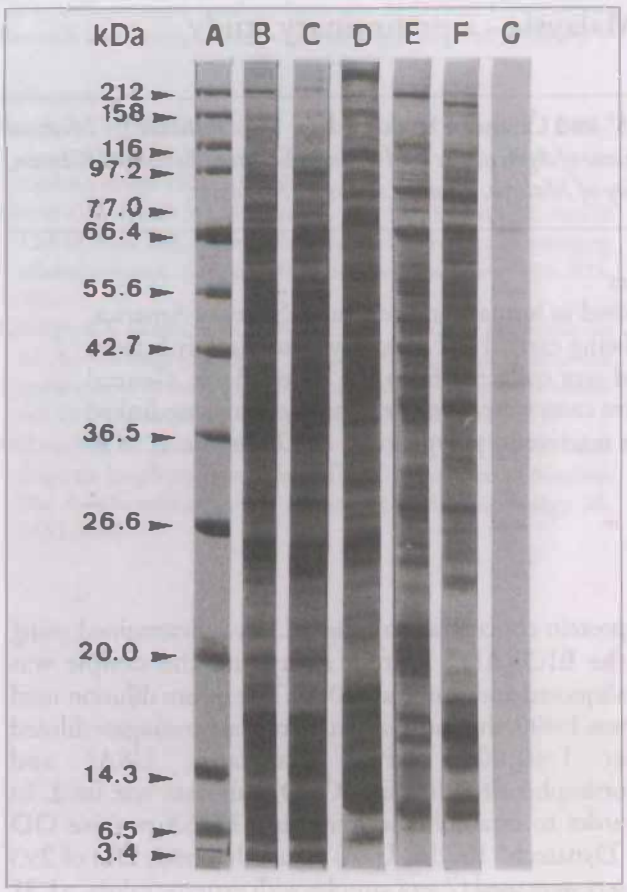


Fig. 1. Protein profile of molecular weight marker (lane A - New England Biolabs), *Toxocara canis* (lane B), *Toxocara cati* (lane C), *Ascaris suum* (lane D), *Dirofilaria immitis* (lane E), *Angiostrongylus malaysiensis* (lane F) and *Brugia malayi* (lane G).

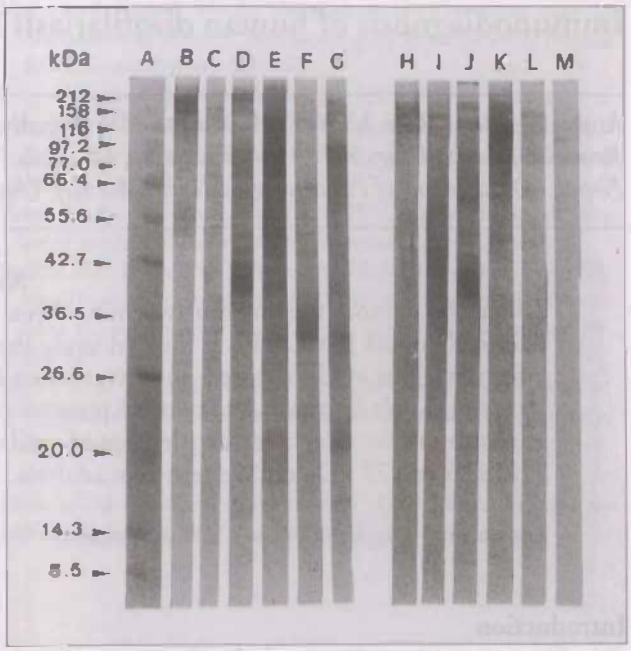


Fig. 2. Western Blot analysis of *D. immitis* ELISA positive sera samples No: 62 (lanes B-G) and No: 75 (lanes H-M) tested with antigens of *Toxocara canis* (lanes B & H), *Toxocara cati* (lanes C & I), *Ascaris suum* (lanes D & J), *Dirofilaria immitis* (lanes E & K), *Angiostrongylus malaysiensis* (lanes F & L) and *Brugia malayi* (lanes G & M); molecular weight marker (lane A).

and *B. malayi* antigens on ELISA (*Dirofilaria*) positive human sera as well as *Toxocara canis*, *B. malayi* and *A. malaysiensis* positive sera are shown on Figs. 1 & 2.

SDS-PAGE analysis of *D. immitis* adult worm antigens showed a protein profile of bands ranging from 212kDa to 3.4kDa (Fig. 1). Western Blot analysis of *Dirofilaria* antigen reacted with homologous serum showed a total of 27 reactive bands. *D. immitis* postive sera reacted with the homologous antigen at molecular weights (MW) 185, 158, 93, 64, 52, 47 and 26.6 kDa and also cross-reacted with antigens of *T. canis*, *T. cati*, *A. suum*, and *B. malayi* but not with that of *A. malaysiensis*. Three reactive bands at MW 212, 116, and 77 kDa were only present in *D. immitis* positive sera (Fig. 2). The 77kDa band displayed a higher degree of antigenicity as it reacted with most of the ELISA-positive sera and also with two of the sera with low ELISA positive OD values (1.130 and 1.018; Table 1). The 116kDa band had a similar pattern for positive sera but reacted with only one of the low ELISA-positive sera (1.043; Table 1). The 212kDa band reacted with only four of the positive sera but displayed stronger bands than the other two reactive bands. Other bands that displayed strong antigenicity, were at MW 93, 64, 52, 47 and 26.6 kDa but were found to cross-react with *B. malayi* positive sera. Two other weaker reactive bands, at MW 185 and 158 kDa were found to cross-react with *T. canis* positive sera. There was no cross-reaction against *A. malaysiensis* positive sera (Table 1).

## Discussion

Dirofilariasis in humans is gaining importance due to its remarkable radiologic resemblance to primary bronchogenic carcinoma or metastatic tumour (Leonardi *et al.*, 1977). In many cases of dirofilariasis with lung involvement, open lung biopsy was performed and the parasite found to be located in the tissue section. Therefore, a preoperative diagnosis using non-invasive methods, such as serology would be useful (Kondo & Fujita, 1991).

The minimum prevalence of human dirofilariasis in this study is conservatively estimated to be 3.66%. The seroprevalence of dirofilariasis in the present study is lower than that reported in other countries (Muro *et al.*, 1990; Espinoza *et al.*, 1993; Santamatia *et al.*, 1996). A large number of positive cases in this study was found among the mean age group of 32 years and this conforms with the findings of Simon *et al.*, (1991) in Spain. It is also interesting to note that the majority of infected cases are among the Malays, who for religious reasons do not keep dogs. However, all the blood donors were resident in Kuala Lumpur and Petaling Jaya and these areas have a large number of dogs which have been found

Table 1. Results of ELISA and Western Blot analysis of *Dirofilaria immitis* positive sera using crude *D. immitis* soluble antigen.

| Serum No. | 106 | 167 | 111 | 75 | 201 | 62 | 215 | 259 | 207 | 13 | 191 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Age (yrs) | 29 | 46 | 30 | 35 | 35 | 37 | 23 | 39 | 20 | 18 | 41 |
| Sex (M/F) | M | M | M | M | F | M | M | M | F | F | M |
| Race | Malay | Malay | Malay | Javanese | Chinese | Malay | Malay | Malay | Malay | Indian | Malay |
| ELISA OD | >2.00 | >2.00 | 1.668 | 1.583 | 1.581 | 1.502 | 1.370 | 1.130 | 1.077 | 1.043 | 1.018 |
| Mol. Wt. | kDa | | | | | | | | | | |
| 212 | + | | + | | + | | + | | | | |
| 116 | | + | + | + | + | + | + | | | + | |
| 77 | + | + | + | + | + | + | + | + | | | + |

to be positive for *Dirofilaria* infection (unpublished data).

Villanueva & Rodriguez-Perez (1993) found three reactive polypeptide bands at MW 15, 33 and 69 kDa to be very specific for ELISA-positive *D. immitis* sera and Western Blot analysis showed no cross-reactivity to *T. canis, A. suum* and *Fasciola hepatica* antigens. In our study, positive sera were specifically reactive to three polypeptide bands of *D. immitis* antigen at MW 212, 116 and 77 kDa but nor to similar bands of *T. canis, T. cati, A. suum, B. malayi* and *A. malaysiensis* antigens. Therefore, we conclude that the presence of one or more of these three polypeptide markers may be considered as an indication of *Dirofilaria* infection.

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