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PROGRESS REPORT NO. 5

EARLY DETECTION OF LYMPHATIC FILARIASIS AND ATTEMPT AT DEFINING PROTECTIVE ANTIGENS

A RESEARCH PROJECT
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PROJECT PROFILE

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INTRODUCTION AND OBJECTIVES

Lymphatic filariasis is a serious disease effecting approximately 90 million people in the tropics with a population at risk of more than 900 millions. It is a mosquito-transmitted disease which is caused by Wuchereria bancrofti, Brugia malayi and B. timori. While W. bancrofti is found throughout the wet tropical world, B. malayi is confined to Asia and B. timori is only found on the islands of Timor, Flores, Alar and Roti of Indonesia.

In Thailand, W. bancrofti is found in the Western part of the country along the border with Burma while the endemic area of B. malayi is in the South. It has been reported that the control programme of filariasis in southern Thailand during 1961-1979 which covered 6 endemic areas of malayan filariasis with a total population of about 1.5 million (people at risk) was successful. However, the Filariasis Division, Ministry of Public Health recently (1982-1985) reported that new areas found to be highly endemic with infection rates of 1.16% to 4.6%. As is often the case epidemiological data reflect the extent and limitation of the survey carried out and usually represent underestimation of the problems. In Thailand and most developing countries, this underestimation is found all the time due to under-reporting and relative limitation of personnels and inavailability of proper diagnostic tools for field surveys. Diagnostic test which has been currently used is the detection of microfilaraemia which occur at night. Therefore, development of a simple, sensitive, specific, accurate and affordable diagnostic systems will make case detection much simpler. Furthermore, a diagnostic system which can detect antigens of early human stages of the parasite in asymptomatic subjects in endemic areas will not only make chemotherapy more effective but will also help to interrupt the life cycle of the parasites. By treating the subjects before they become microfilaraemic, transmission of the parasites will be reduced.

The overall aim of this research is to improve disease control by developing better immunodiagnostic tests for lymphatic filariasis as well as new tests for the detection of early, i.e., premicrofilaraemic stage of filarial infection based on detection of stage specific antigens in the serum and urine.

The specific objectives are to

1. analyse antigenic structure for the development of immunological reagents specific for B. malayi and W. bancrofti and those specific for various stages of the parasites.

2. devise simple, sensitive, specific, accurate and inexpensive immunoassays for the diagnosis of clinical lymphatic filaria as well as for the detection of early asymptomatic infection.

Work done to date

1. Collection of parasite materials
2. Collection of human sera
    - 2.1 Normal human sera from non-endemic area
    - 2.2 Normal human sera from endemic areas of lymphatic filariasis
    - 2.3 Filarial patient sera
3. Production of polyclonal mouse antisera
4. Immunological characterization of human sera
    - 4.1 Detection of serum antibodies in filarial patients in endemic areas of *B.malayi*
    - 4.2 Detection of serum antibodies in filarial patients in endemic areas of *W.bancrofti*
5. Characterization of parasite antigens
    - 5.1 Radioimmunoprecipitation
    - 5.2 Western blot and immunological reaction
6. Production and characterization of monoclonal antibodies
7. Detection of antigenic sources in *B.malayi* and *B.pahangi*

1. Collection of parasite materials

We have collected parasite materials of both filarial and non-filarial species as listed in Table 1 and 2 of progress report no. 1, pages 3,4 and 5 of progress report no. 2, page 1 of progress report no. 3. We also have overcome the problem in the decrease in the production rate of Brugian materials (the problem was mentioned in page 1 of progress report no. 3 and the way we solved the problem was mentioned in page 3 of progress report no. 4).

2. Collection of human sera

Human sera were collected for characterizing the filarial worm antigens. Venous blood was taken from normal people and filarial patients. Sera were obtained and stored at -20°c. Serum collected from the endemic areas were grouped according to their sign and symptom. A portion of serum from individuals within each group was pooled and tested immunologically. As reported in the progress report no. 4, (page 5-7) pools of sera obtained from normal people and filarial patients in the endemic areas were used in the radioimmunoprecipitation of female and male adult *B. malayi* antigens. After submitting that report, we received new lots of better characterized sera from the endemic areas. Therefore, we gave up the previous pools and started again with new pools.

2.1 Normal human sera from non-endemic area

Sera were obtained from 20 ml of venous blood of 17 healthy staffs and graduate students of the Department of Microbiology, Faculty of Science, Mahidol University. These blood donors have never been travelling to any of the known endemic areas of Lymphatic filariasis in Thailand. They have no previous history of any tissue parasite infection and no intestinal parasites were detected in any of their fecal specimens.

2.2 Normal human sera from endemic areas of lymphatic filariasis

Sera were obtained from 5 ml of venous blood of healthy normal people living in endemic areas of *B.malayi* and endemic areas of *W.bancrofti*. These people have never had experience of any sign or symptom of filariasis. Number of cases and their residential areas were as followed:

Number of cases	Endemic areas of	Provinces
45	*B.malayi*	Suratthani
50	*B.malayi*	Nakornsrithammarat
86	*W.bancrofti*	Tak

2.3 Filarial patient sera

Sera were collected from patients suffering from

lymphatic filariasis in endemic areas of B.malayi and endemic areas of W.bancrofti in Thailand by the Filariasis Division, Department of Communicable Disease Control, Ministry of Public Health.

Serum samples were grouped together according to signs and symptoms of the patients as followed:

Group I Sera from asymptomatic microfilaraemic patients

Group II Sera from microfilaraemic patients having lymphadenitis and lymphangitis

Group III Sera from amicrofilaraemic patients following successive treatment with diethylcarbamazine

Group IV Sera from amicrofilaraemic patients following successive treatment with diethylcarbamazine but still having lymphadenitis and lymphangitis

Group V Sera from amicrofilaraemic patients with elephantiasis and still having lymphadenitis and lymphangitis

Group VI Sera from amicrofilaraemic patients having lymphadenitis and lymphangitis

Group VII Sera from amicrofilaraemic patients with elephantiasis

Group VIII Sera from amicrofilaraemic patients with elephantiasis treated with diethylcarbamazine but still having lymphadenitis and lymphangitis

Group IX Sera from amicrofilaraemic patients with elephantiasis treated with diethylcarbamazine and showed no other symptoms

Group X Sera from microfilaraemic patients with elephantiasis

Group XI Sera from a patient who turned to amicrofilaraemic condition spontaneously

3. Production of polyclonal mouse antisera

Polyclonal mouse antisera to antigens of the lymphatic filarial worms as well as antigens of non-filarial nematodes were produced for characterizing the filarial worm antigens. Details have been described in page 4 of progress report no. 4.

4. Immunological characterization of human sera

Since normal human sera and sera from filarial patients were to be used for characterizing filarial worm antigens, we needed to know some of their characteristics such as their specificities and levels of their antibody activities. Therefore in our work all the human sera collected were tested for the presence of antibodies to somatic antigens of adult female *B. malayi* ,adult female *D.immitis* and advanced third-stage larvae of *G.spinigerum* by ELISA technique slightly modified from the one previously described by McLaren *et al*(1). Optimum concentrations of antigens and sera to be used in the assay were titrated by checker board titrations (see Fig.1 and Fig.2). A pool of normal human sera from non-endemic area was used as negative reference serum and a pool of sera from 17 microfilaraemic patients living in endemic areas of *B.malayi* was used as positive reference serum. Results of the checker board titrations indicated that the optimum concentration of the antigens to be used in the ELISA is 5 ug/ml and the optimum concentration of the filarial patient sera to be tested is 1:1600.

4.1 Detection of serum antibodies in filarial patients in endemic areas of B.malayi infection.

As shown in Table 1 100% of normal control sera gave negative reaction while about 40-60% of people in the endemic areas gave positive reaction to the three antigens. Figures 3-6 show that mean OD value obtained from sera of normal people in endemic areas is significantly higher than the mean OD obtained from normal control ($P<0.05$). This result seems to suggest subclinical infections to those people who live in endemic areas of the filarial parasite. When comparing between the reactions of each group of patient sera and the reactions of normal control, all patient sera gave significantly stronger reaction ($P<0.05$),

Table 1 Number of cases from endemic areas of *B.malayi* giving positive ELISA value with antigens of *B.malayi*, *D.immitis* and *G.spinigerum*

Group	Total number of cases	% of cases giving positive ELISA values with		
		B.malayi	*D.immitis*	*G.spinigerum*
Normal control	17	0.00	0.00	0.00
Normal in endemic area	95	49.47	38.95	64.21
I	47	95.74	91.49	70.21
II	5	100.00	100.00	100.00
III	47	59.57	48.94	63.83
IV	27	40.74	62.96	77.78
V	73	60.27	60.01	68.49
VI	31	74.19	58.06	77.42
VII	8	37.50	62.50	100.00
VIII	16	62.50	75.00	43.75
IX	25	68.00	40.00	68.00

while only sera in group I and II reacted significantly stronger to the three antigens when compared to the reaction of sera from normal people in the endemic areas ($P<0.05$). When comparing among groups of the patients those who have microfilaraemia either without (group I) or with symptoms of lymphadenitis and lymphangitis (group II) gave the strongest ELISA reactions to the three antigens (Figure 3). When tested with B.malayi antigen, sera in group I gave the mean OD value at 0.9 and sera in group II gave the value at 0.7 (Figure 3 and 4). While 95% of cases in group I gave positive reaction, 100% of group II gave positive reaction. As can be noticed in our results, sera obtained from normal people and also filarial patients in endemic areas of B. malayi reacted significantly with antigens of G.spinigerum (Figure 3 and 6) when comparing to the reaction obtained with the negative control sera ($P<0.05$). However, the OD values obtained with G.spinigerum are always lower than those obtained with the two filarial antigens. These differences are statistically significant in almost every group ($P<0.05$) except in normal from endemic areas and group IV, V and VII. The antibody reactivities of these sera to G.spinigerum antigen is more likely to be due to cross reactivities among different nematode antigens than to be due to the experience of these people with G.spinigerum infection. This idea is based on the fact that most people in the endemic areas of B.malayi in Southern Thailand do not eat raw flesh. Moreover, the antigen of G.spinigerum advanced third-stage larvae has been proved to be good at detecting the presence of infection with this parasite (2). The ELISA value obtained with this antigen is expected to be much higher (~ 1.00) if those people are carrying the infection. The knowledge that our filarial sera react with antigen of G.spinigerum will be applied to our further study when we make use of these sera in characterizing specific antigens of our lymphatic filarial worms, i.e. B.malayi and W.bancrofti.

4.1 Detection of serum antibodies in filarial patients in endemic areas of W.bancrofti

Since we still do not have enough W.bancrofti antigens to

be used in ELISA, we therefore tested antibodies in sera of patients in W.bancrofti endemic areas with the previously described antigens. Figures 7-10 show the OD values obtained from the ELISA of sera from normal people in endemic areas and sera from different groups of W.bancrofti infected patients tested against antigens of B.malayi ,D.immitis and G.spinigerum. Results are quite similar to those obtained with B.malayi infected patient sera. Sera from normal people in endemic areas of W. bancrofti and also patient sera in group I and III gave higher ELISA values than those given by the negative control ($P<0.05$) when tested with the three antigens. Moreover, sera from microfilaraemic patients gave the highest antibody reaction against B.malayi antigen when comparing among all groups of the filarial sera ($P<0.05$). As summarized in Table 2, 84.78% of sera from microfilariaemic patients gave positive ELISA result with B. malayi antigen. However, 91.30% of group I and 84.61% of group III gave positive results with G.spinigerum antigens. Nevertheless, it is too early to make any conclusion about these filarial patient sera since too small number of cases were tested and antigens of W.bancrofti have not been used. Moreover, the possibility of these "Karen" people in acquiring G.spinigerum infection is quite high. It is therefore remained to be further investigated.

Our ELISA results clearly indicated that microfilaraemic patient sera contain the highest level of antibodies specific to the parasite antigens we used. We compared our results with those previously reported by other groups of investigators and found that those results contradicted ours. Ottesen et al (3) found the lowest antibody activities in sera of microfilaraemic patients when compared with all other groups of filarial patients. They also found that B-lymphocytes of microfilaraemic patients were unable to produce filaria-specific antibody in vitro (4). Moreover, Sim et al (5) reported that serum of microfilaraemic patients failed to promote adherence of human buffy coat cells to intact microfilariae in vitro. Since it is still controversial and immunology of filariasis is still not clearly understood, we cannot make specific conclusion on our results

Table 2 Number of cases from endemic areas of *W.bancrofti* giving positive ELISA value with antigens of *B.malayi*, *D.immitis* and *G.spinigerum*

Group	Total number of cases	% of cases giving positive ELISA values with		
		B.malayi	*D.immitis*	*G.spinigerum*
Normal control	17	0.00	0.00	0.00
Normal in endemic area	86	58.13	32.56	69.76
I	46	84.78	60.87	91.30
III	13	23.07	30.77	84.61
IX	2	50.00	50.00	100.00
X	2	100.00	50.00	100.00
XI	1	0.00	0.00	0.00

obtained at this stage. However,it may indicate the difference in genetic make up of strains existing in different endemic areas.

5. Characterization of parasite antigens

5.1 Radioimmunoprecipitation

It was mentioned in the progress report no. 4 (page 5-7) that the result of our radioimmunoprecipitation of serum pools from different groups of filarial patients to antigens of female and male adult B.malayi suggested the significance of microfilarial antigens in the detection of host immune response. In addition we also mentioned about the possibility of≈15.5 Kd antigen of B.malayi to share the common epitope with antigen of W.bancrofti. Unfortunately further study on this line of experiment is still not carried out. However , this study has to be done again since new lots of better characterized sera from the endemic areas were obtained as mentioned in the paragraph 2.3 (Filarial patient sera).

5.2 Western blot and immunological reaction

Western blot transfer of somatic antigens of female adult B.malayi ,B.pahangi ,D.immitis and advanced third-stage larvae of G.spinigerum from SDS-polyacrylamide gel (50 µg/0.5 cm width) to nitrocellulose filters (0.45 µm, Schleicher and Schuell,Dassel, West Germany) were performed by the technique previously described(6). The filter was cut into 0.4 cm-width strips and immunological reaction was performed by standard technique using 5% skim milk as blocking agent and 2% skim milk as diluent for immune serum (polyclonal mouse antiserum or new pools of filarial patient sera and the peroxidase conjugated second antibodies (HRP-conjugated rabbit antihuman immunoglobulins). 0.5 mg/ml of 4-Chloro-1-napthol containing 0.01% H_2O_2 was used as substrate of the HRP.

As shown in Fig. 11, polyclonal mouse antisera of all specificities reacted strongly with high molecular weight antigens (≈40 to > 200Kd) of female adult B.malayi (strips b,c,d, f,g, and h), while normal mouse serum did not react with any of these antigen components (strip e). It was clearly shown in this experiment that only mouse antisera to adult worms of B.malayi

and B.pahangi strongly reacted with low molecular weight antigen components at 21.5 Kd (strip c) and 14.3 Kd (strip d). When the reference positive filarial patient serum was used, the high molecular weight antigens were also reacted, but the most distinctively created band was found to be the low molecular weight ones at≈25 Kd and≈18 kd (strip j,Fig.11). However, the band 18 Kd was not clearly demonstrated in the photograph. Our further study indicated that the 25 Kd antigen is most likely to be of Brugian microfilariae specificity. As shown in Fig12 A, only the serum from microfilaraemic patients in Narathiwat (strip c) and those in Suratthani (strip e) provinces reacted strongly with the 25 Kd antigen of B.pahangi. Sera from other groups of patient did not recognize this antigen component. Moreover, it was not present in the antigens of D.immitis (Fig. 12 B) or G. spinigerum (Fig.12 C). Corresponding results were obtained when sera of filarial patients in endemic areas of W.bancrofti were used. The 25 Kd antigen was not shown up with any of these patient sera when reacted with antigens of either B.malayi or B. pahangi (Fig.13A and B). This antigen component of B.malayi microfilaria, if it is the same one as previously reported by Maizels (830375,WHO Report of the steering committee of the scientific working group on filariasis, July 1983 to June 1988,p. 14), is most likely to be the surface antigen of a diagnostic value which will be further investigated. The 17-18 Kd antigen component of B.malayi, on the other hand, did not consistently show up when reacted with sera from microfilaraemic patients. We, therefore, still cannot make any specific conclusion about this antigen. The band 14.3 Kd, however, was shown up when antigen of B.pahangi was tested with sera from elephantiasis patients who have been treated with DEC and turned to amicrofilaraemic stage (strip d, Fig.13) and serum from a filarial patient who spontaneously turned to amicrofilaraemic stage (strip f,Fig.13). Nevertheless, the 14.3 Kd antigen of either B.pahangi or B.malayi did not react at all with any pool of the filarial patient serum from endemic areas of B.malayi (Fig.11 and Fig.12A) or any of the polyclonal mouse antisera prepared against antigens of D.immitis, T.cati or G.spinigerum (Fig.11 and Fig.14). We still cannot make

any specific conclusion about the significance of these low molecular weight antigen components of the Brugian parasites until further studies are done.

According to the results of the Western blot and immunological reaction of Brugian antigens, we conclude that the 25 Kd antigen of female adult B.malayi is most likely to be of Brugian specificity. Since it was recognized only by microfilaraemic patient sera, its diagnostic value will only be limited to the cases patently infected with Brugian filaria. Although its value seems to be limited, we believe that by using serodiagnostic tests, the diagnostic problem due to periodicity of the microfilaraemia will be solved. We therefore separated the B.malayi antigens having molecular weight from 14.3-19.0 Kd and from 19.0 to 30 Kd from the SDS-polyacrylamide gel (Fig.15) and immunized into Balb/c mice.

6. Production and characterization of monoclonal antibodies

After Dr. Billing has left, we have started the fusion again since all the 7 stable hybrid colonies gave cross reaction with the panel of testing antigens,(progress report no.4 p.4; the antibodies were tested with all antigens of the panel except the antigen no.3,no.6 and no.9) only clone 28A which gave the OD reading at 0.28,may be our antigen is not a very good immunogen. Thus we have preliminary tried using the polyacrylamide gel fixed with Freund's complete adjuvant as antigen. It was found that some Balb/c/J mice died but some survived within the period of 5 days to 2 months.

Methods and Results

We set the experiment as followed: the female B. malayiextracted antigen from stock solution at the concentration of 6.37 mg/ml was diluted to 60 µg/0.1ml in normal saline solution (NSS) and mixed with Freund's complete adjuvant, (the immunizing data and results were summarized in Table 1) Female Balb/c/J mice 8 week old were immunized three times, the first time with 60 µg/0.1ml B.malayi antigens mixed with Freund's complete adjuvant intraperitoneally (IP), the second time the mouse was injected with 30 ug/0.1ml B.malayi antigen mixed with

Freund's incomplete adjuvant IP 4 weeks after the first inoculation. Finally the prefusion booster was done 3 weeks later with 30 ug/0.1ml antigen in NSS intravenously (IV). Spleen cell from immunized mouse (1.1×10^8 cells) were isolated 3 days after the last injection and fused with X63-Ag8.653 (.653) myeloma cells (2.46×10^7 cells), using polyethylene glycol MW 3700 (PEG, Sigma). Antibody production was assessed between day 14 to 17 by means of enzyme-link immunosorbent assay (ELISA) utilizing B. malayi antigen fixed to ELISA plates (Costar) at concentration5ug /ml. Cultures secreting antibody (OD $\geqslant$ 0.20 up to 1.65 at 490nm by Minireader II) were then assessed for crossreactivities with panel of antigens i.e. extracts of adult male B.malayi , D. immitis (male and female), A.cantonensis (male and female), and third-stage larvae of G.spinigerum . Selected wells were then cloned by limiting dilution. Approximately 14 days after cloning the culture supernatants from each initial well were assessed for antibody production to B.malayi antigens . Selected colonies were then grown in tissue culture, frozen, and used for production of antibodies for immunoblotting later.

The average values obtained in the fusion (F93) experiment were (Table 1): 93% of the total initial wells plated showed growth of hybrid colonies (225/240); 55% of this hybrid-containing wells (133/240) produced antibodies to B.malayi antigens (OD $\geqslant$ 0.20) and 2.25% of hybrid containing wells (3/133) were producing species (♀,♂) specific antibodies. These three hybrids designated F93-3B11, F93 3C7, F93-4E10 from initial well were scaled up in tissue cultures and would be used for productions of ascites fluid in Balb/c mice. The titer of polyclonal antibody from immunized mouse on Day 50 (3-days before fusion) is 1:51,200 (OD = 0.78) and this serum was used for positive control. For the control system we found that "blocking" materials i.e. 5% skim milk, 1% ovalbumin or 2% BSA gave the same results. But in our ELISA system we have used 2% BSA as "blocking" material. For negative control we have used normal Balb/c/J serum and conditioned medium, and also 1% BSA as blank.

Up to date we have separately immunized female B.malayi

antigens 14.3-19.0 Kd and 19.0-30.0 Kd from partially purified 62-100 Kd antigens in Balb/c/J mice at the concentration 40 µg, 20µg and 20µg respectively using same method as before. The first injection was accomplished, the second immunization will be done further. (see paragraph 5.2 ,p. 14)

Table 3

Immunization schedules and their effects on serum titer, on frequency of stimulated spleen cells and on frequency of specific hybridomas

Fusion No.	93
mouse Balb/c/J	1
Immunization (*B.malayi* antigen in µg)	
1 (Day 0,IP)	60
2 (Day 30,IP)	30
3 (Day 50,IV)	30
Polyclonal serum titer	
Titer OD (490 nm; Mineral Reader spectrophotometer)	
Day 0	0
Day 50	1:51,200 (OD= 0.78)
myeloma	X63-Ag 8.653 (.653)
wells with growth	225/240 (93%)
positive wells (OD ⩾ 0.20)	133/240 (55%)
species specific McAb	3/133 (2.25%)

3 Jan. 90	13 Jan. 90	21 Feb.90
F93 3B11 = 0.32	3B11 = 0.39	3B11 = 0.39
3C7 = 0.69	3C7 = 1.08	3C7 = 1.08
4E10 = 0.42	4E10 = 0.44	4E10 = 0.49

References

1. McLaren, M.L., Lilywhite, J.E., Dunne, D.W. and Doenhoff, M.J. (1981). Serodiagnosis of human Schistosoma mansoni infections: enhanced sensitivity and specificity in ELISA using a fraction containing S.mansoni eggs antigens W_1 and α_1. Trans. Roy. Soc. Trop. Med. Hyg. 75:72-79.

2. Dhamkrong-at, A., Migasena, S., Suntharasamai, P., Bunnag, D., Priwan, R. and Sirisinha,S. (1986). Enzyme-linked immunosorbent assay of detection of antibody to Gnathostoma antigen in patients with intermittent cutaneous migratory swelling. J. Clin. Microbiol. 23:847-851.

3. Ottesen, E.A., Weller, P.F., Lunde, M.N. and Hussain, R. (1982). Endemic Filariasis on a Pacific Island. II Immunologic aspects: Immunoglobulin,Complement, and Specific antifilarial IgG, IgM and IgE antibodies. Am. J. Trop. Med. Hyg. 31:953-961.

4. Nutman, T.B., Kumaraswami, V.,Pao, L., Narayanan, P.R. and Ottesen, E.A. (1987). An analysis of in vitro B cell immune responsiveness in human lymphatic filariasis. J. Immunol 138: 3954-3959.

5. Sim, B.K.L., Kwa, B.H. and Mak, J. W. (1982). Immune responses in human Brugia malayi infections: serum dependent cell-mediated destruction of infective larvae in vitro. Trans. Roy. Soc. Trop. Med. Hyg. 76:362-369.

6. Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

Work to be done in the next period

1.Further characterization of B.malayi antigens to work out the specific component of diagnostic value by Western blot and immunological reaction and also radioimmunoprecipitation. Not only the somatic extract of female adult worms and somatic extract of infective third-stage larvae will be studied, excretory-secretory product of the worms will also be used.

2.Production and characterization of monoclonal antibodies

3.Identifying the exact sites of B.malayi and B.pahangi antigens within each tissue source.

7. DETECTION OF ANTIGENIC SOURCES IN *B. MALAYI* AND *B. PAHANGI*

Tissues that are the potential sources of antigens, that are released from the parasites and elicit antibody production in hosts were detected by indirect immunofluorescence assay. In these experiments frozen sections of adult and whole-mount preparation of L_3 stage of *B. pahangi* and *B. malayi* were stained with immune sera from infected cats, jirds, and human. The cat and jird immune sera were from the animals that were infected with *B. pahangi* and *B. malayi*, and human immune sera were from the hosts that were infected with *B. malayi* and *W. bancrofti*. The tissue sites that bind antibodies were detected by secondary antibodies (goat anti-Ig) against each species that were labelled with FITC. The results were summarized in Tables 4 to 5 and illustrated in Figures 16&17 In summary, the staining pattern of tissues in parasites' bodies and the intensity of fluorescence are quite similar regardless of the source of immune sera. Hence it could be concluded that the tissue sources of antigens in both species of parasites are the same, and consist, in order of fluorescence intensity, of uterine epithelium, egg shell, testicular and vas deferens epithelium and their content, the gut epithelium and its content, and the hypodermis and cuticle, and muscle, respectively. The antigens in each parasite also exhibit strong cross reaction to antisera against another parasite species. And it was also found (not illustrated here) that the tissues of adult of *W. bancrofti* also exhibit similar characteristics when stained with immune sera against either *B. pahangi* or *B. malayi*.

In further experiment we shall try to identify the exact sites of antigens within each tissue source, and compare this to the normal ultra-structure of the tissues.

Table 4 SOURCES OF ANTIGENS IN ADULT *B.PAHANGI*

The tissue sources of antigens were detected at light microscopic level by indirect immunofluorescence assay (IFA). Cat and jird antisera consist of anti *B. pahangi* and *B. malayi* and human antisera consist of anti *B.malayi* and *W. bancrofti*. The pattern of labelling and the intensity of fluorescence are identical regardless of the source of antibodies.

TISSUES	CAT ANTISERA	JIRD ANTISERA	HUMAN ANTISERA
Cuticle	++	++	++
Hypodermis	++	++	++
Muscle	++	++	++
Gut - Content	++	++	++
- Epithelium	+++	+++	+++
Reproductive tracts			
Female - Uterine Epithelium	+++	+++	+++
- Egg Interior	++	++	++
- Egg Shell	+++	+++	+++
Male Vas deferens and Testis			
- Epithelium	+++	+++	+++
- Contents	++	++	++

Remarks
+++ = Intensely fluorescent
++ = Moderately fluorescent
+ = Lightly fluorescent
- = No staining

Table 5 SOURCES OF ANTIGENS IN ADULT *B. MALAYI*

The tissue sources of antigens were detected at light microscopic level by indirect immunofluorescence assay (IFA) Cat and jird antisera consist of anti *B. pahangi* and *B. malayi* and human antisera consist of anti *B. malayi* and *W. bancrofti*. The pattern of labelling and the intensity of fluorescence are identical regardless of the source of antibodies.

TISSUES	CAT ANTISERA	JIRD ANTISERA	HUMAN ANTISERA
Cuticle	++	++	++
Hypodermis	++	++	++
Muscle	++	++	++
Gut - Content	++	++	++
- Epithelium	+++	+++	+++
Reproductive tracts			
Female - Uterine Epithelium	+++	+++	+++
- Egg Interior	++	++	++
- Egg Shell	+++	+++	+++
Male Vas deferens and Testis			
- Epithelium	+++	+++	+++
- Contents	++	++	++

Remarks
+++ = Intensely fluorescent
++ = Moderately fluorescent
+ = Lightly fluorescent
- = No Staining

Table 6 SOURCES OF ANTIGENS IN L_3 STAGE *B. PAHANGI*

The tissue sources of antigens were detected at light microscopic level by indirect immunofluorescence assay (IFA)

Tissue Whole mount of L_3 *B. pahangi* showed intense label of fluorescent dye on the cuticle using 3 kinds of antisera from infection by different species of parasites as stated in tables 1 and 2 as primary antibodies. The labelling patterns were similar regardless of the source of antibodies.

Table 7 SOURCES OF ANTIGENS IN L_3 STAGE *B. MALAYI*

Tissue

Whole mount : The whole mount of L_3 *B. malayi* showed intense label of fluorescent dye on the cuticle using 3 kinds of antisera as stated in tables 1 & 2 as primary antibodies. The labelling patterns were similar regardless of the source of antibodies.

Cryostat section :

TISSUE	CAT ANTISERA
Cuticle	++
hypodermis	++
muscle	++
gut	+++

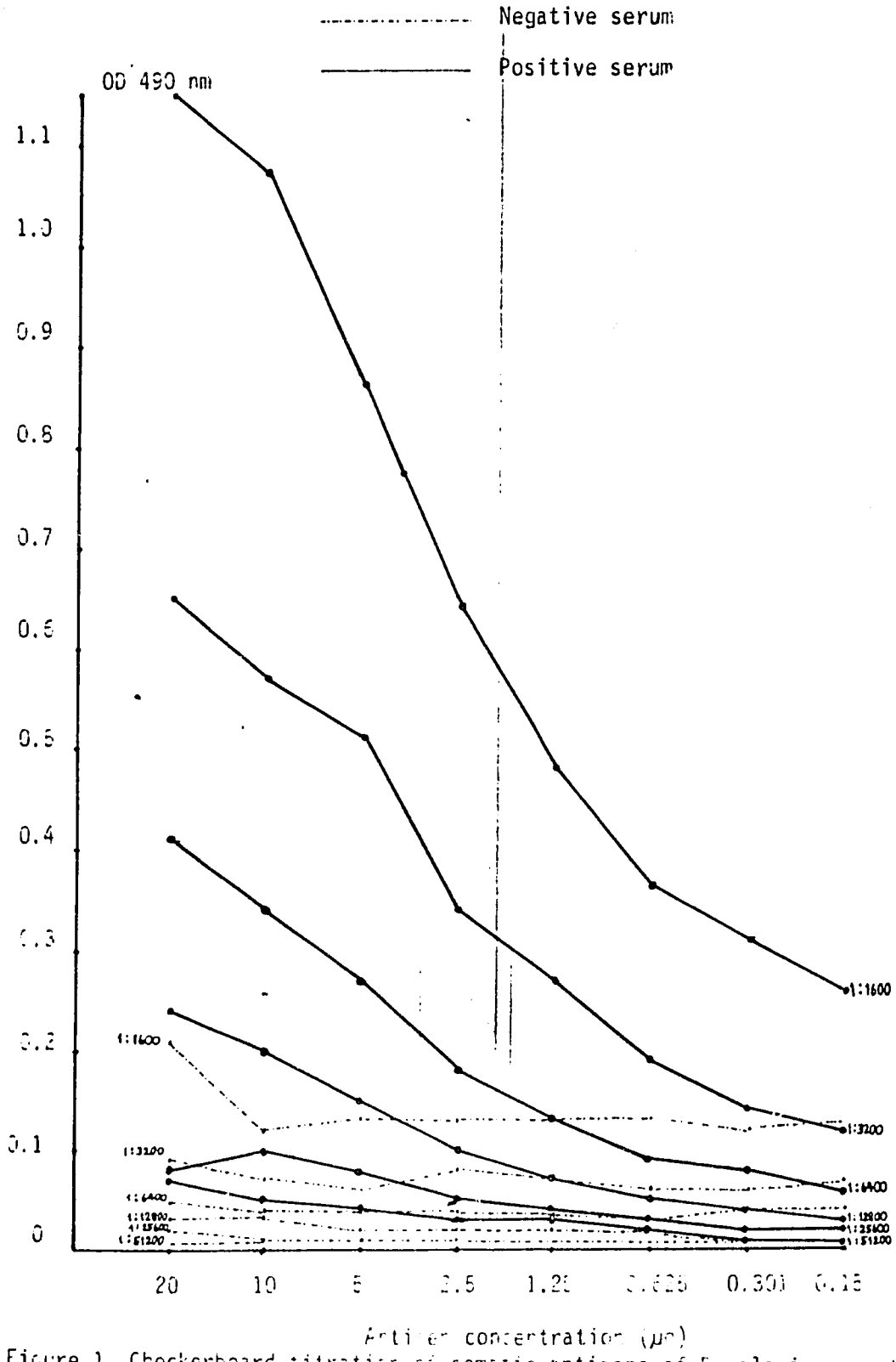


Figure 1. Checkerboard titration of somatic antigens of B.malayi against negative and positive reference sera. The negative serum was a pool of sera obtained from healthy normal people with no history of infection. The positive serum was a pool of sera from microfilaraemic patients in endemic areas of B.malayi.

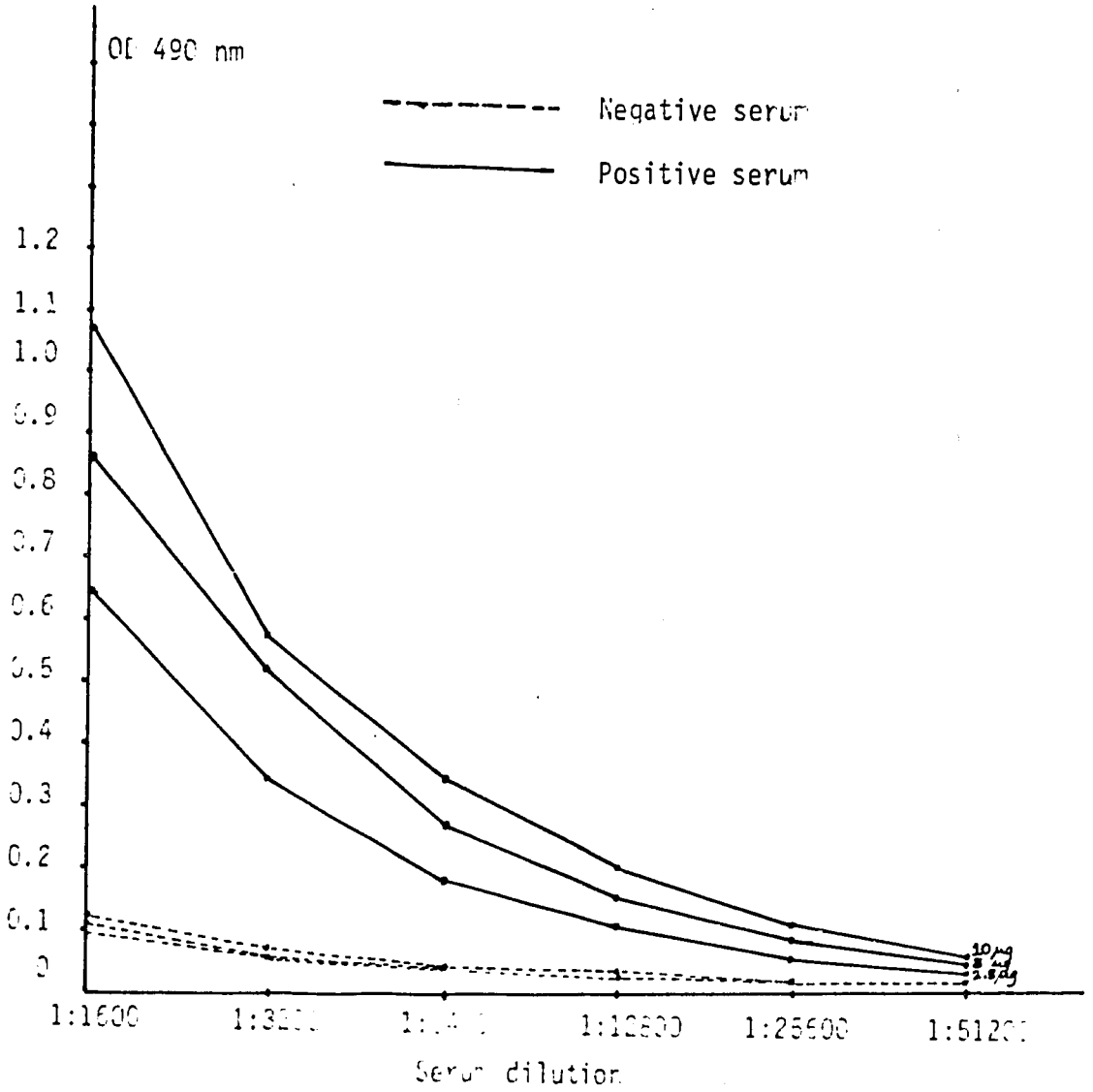
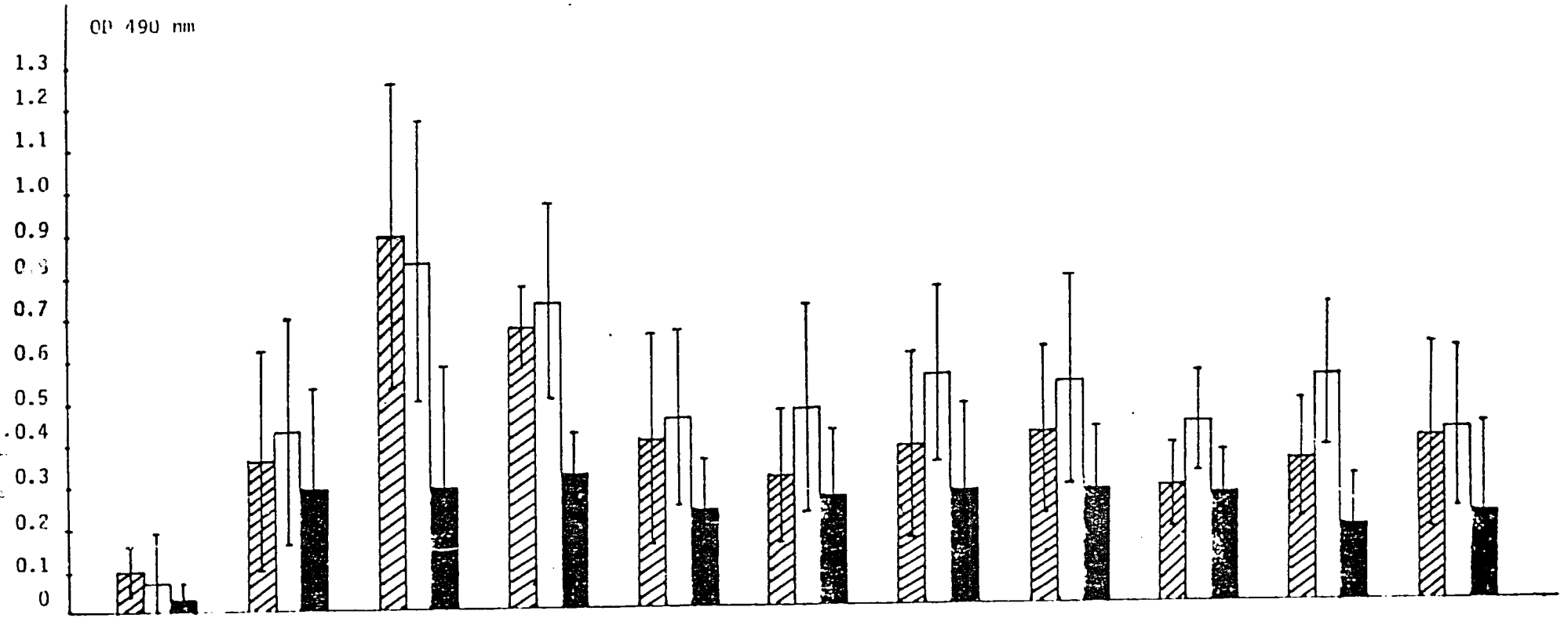


Figure 2. Tritration curves of positive and negative sera diluted from 1:1600 to 1:51200 against somatic antigens of female adult B.malayi at concentrations 10,5 and 2.5 µg/ml.



Group	Normal	Normal in endemic area	I	II	III	IV	V	VI	VII	VIII	IX
N	17	95	47	5	47	27	73	31	8	16	25
$\bar{X}$	.10 .07 .03	.36 .43.29	.89 .83.29	.67.73.32	.40.45.23	.31.47.26	.28.55.27	.41 .53.27	.28.43.26	.34.54.18	.39.41 .21
SD	.06 .12 .04	.26 .27.24	.36 .33.29	.10.23.11	.25.21.13	.16.25.16	.22.21.21	.20 .25.15	.10.12.10	.14.17.12	.22.19 .22

Figure 3 Means and Standard deviations of the ELISA values of total antibodies in the sera from different groups of filarial patients in endemic areas of B.malayi in Thailand and normal controls tested against antigens of B.malayi (▨), D.immitis (▭) and G.spinigerum (■). See materials and methods for details about each group of the filarial patients. Sera were diluted 1:1600.

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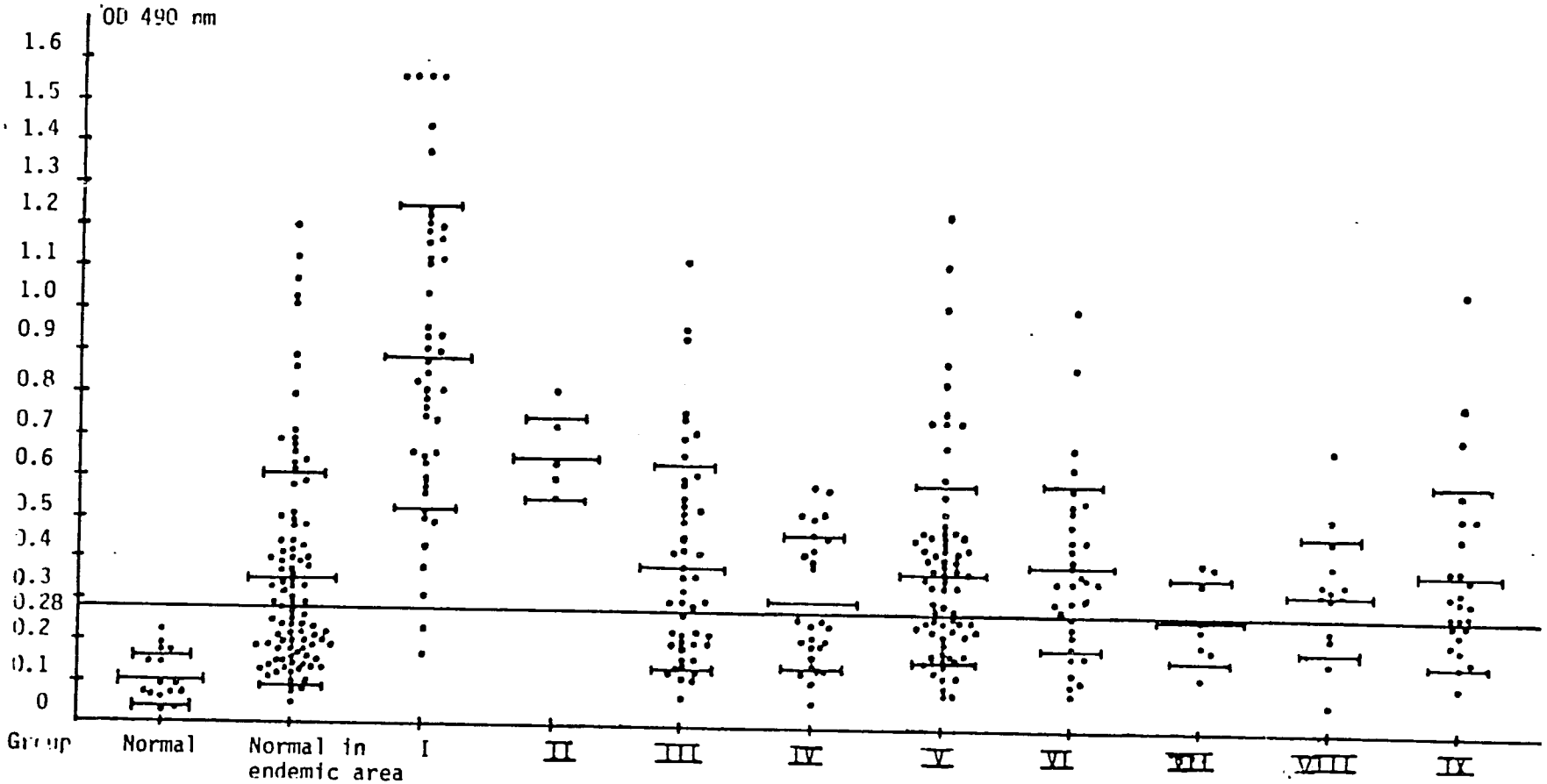

	Normal	Normal in endemic area	I	II	III	IV	V	VI	VII	VIII	IX
N	17	95	47	5	47	27	73	31	8	16	25
$\bar{X}$	0.105	0.357	0.887	0.666	0.390	0.310	0.383	0.407	0.278	0.340	0.392
SD	0.060	0.256	0.364	0.104	0.251	0.163	0.216	0.205	0.101	0.143	0.218

Figure 4 ODs of the ELISA reaction for antibodies to somatic antigen of B.malayi in the sera from different groups of filarial patients in endemic areas of B.malayi in Thailand and normal controls. Sera were diluted 1:1600. See materials and methods for details about each group of the filarial patients.

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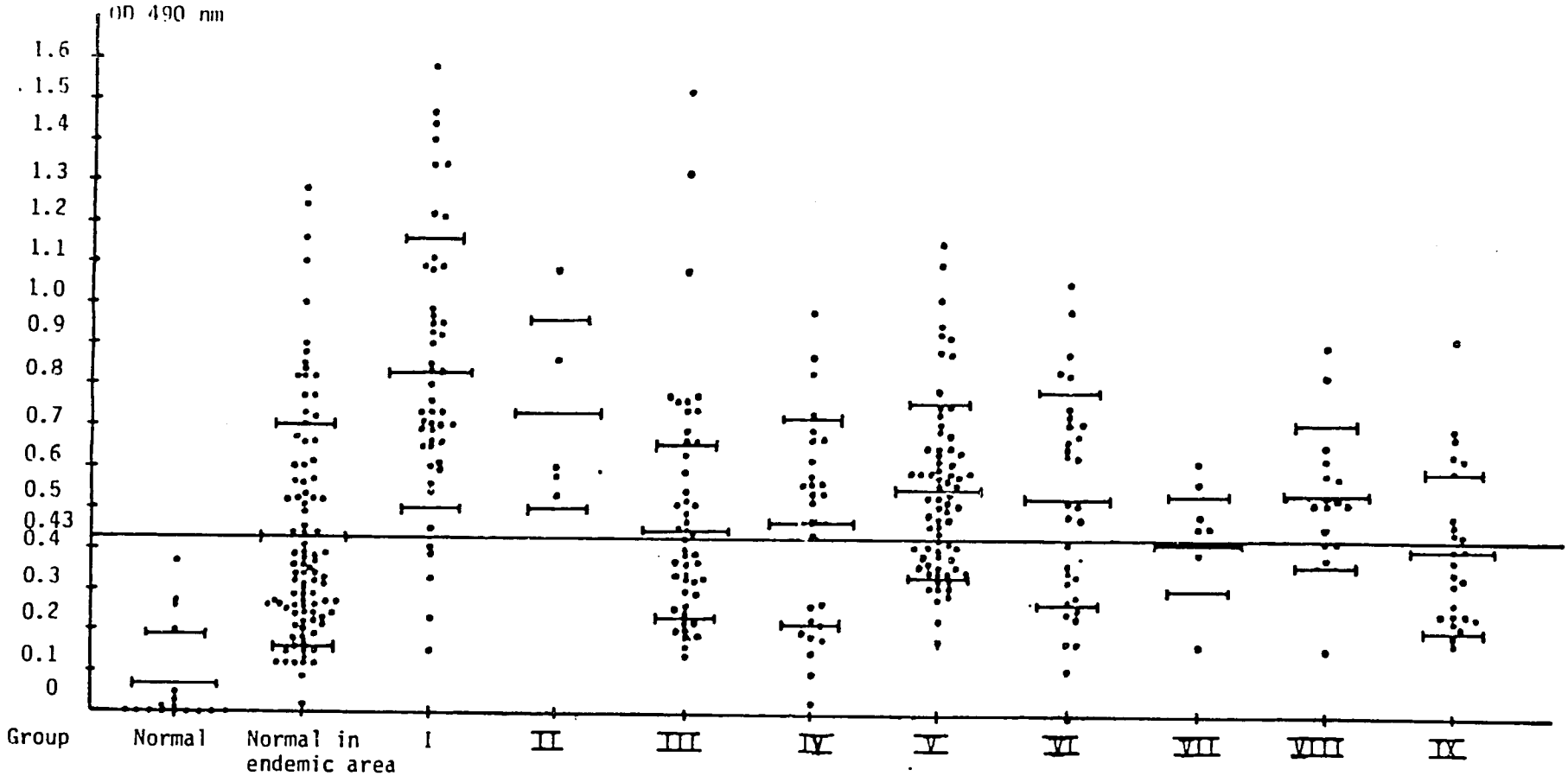

Group	Normal	Normal in endemic area	I	II	III	IV	V	VI	VII	VIII	IX
N	17	95	47	5	47	27	73	31	8	16	25
$\bar{X}$	0.07	0.43	0.833	0.730	0.446	0.469	0.548	0.527	0.426	0.543	0.413
SD	0.12	0.274	0.331	0.234	0.210	0.254	0.211	0.255	0.115	0.171	0.190

Figure 5 ODs of the ELISA reaction for antibodies to somatic antigen of D.inmitis in the sera from different groups of filarial patients in endemic areas of B.malayi in Thailand and normal controls. Sera were diluted 1:1600. See materials and methods for details about each group of the filarial patients.

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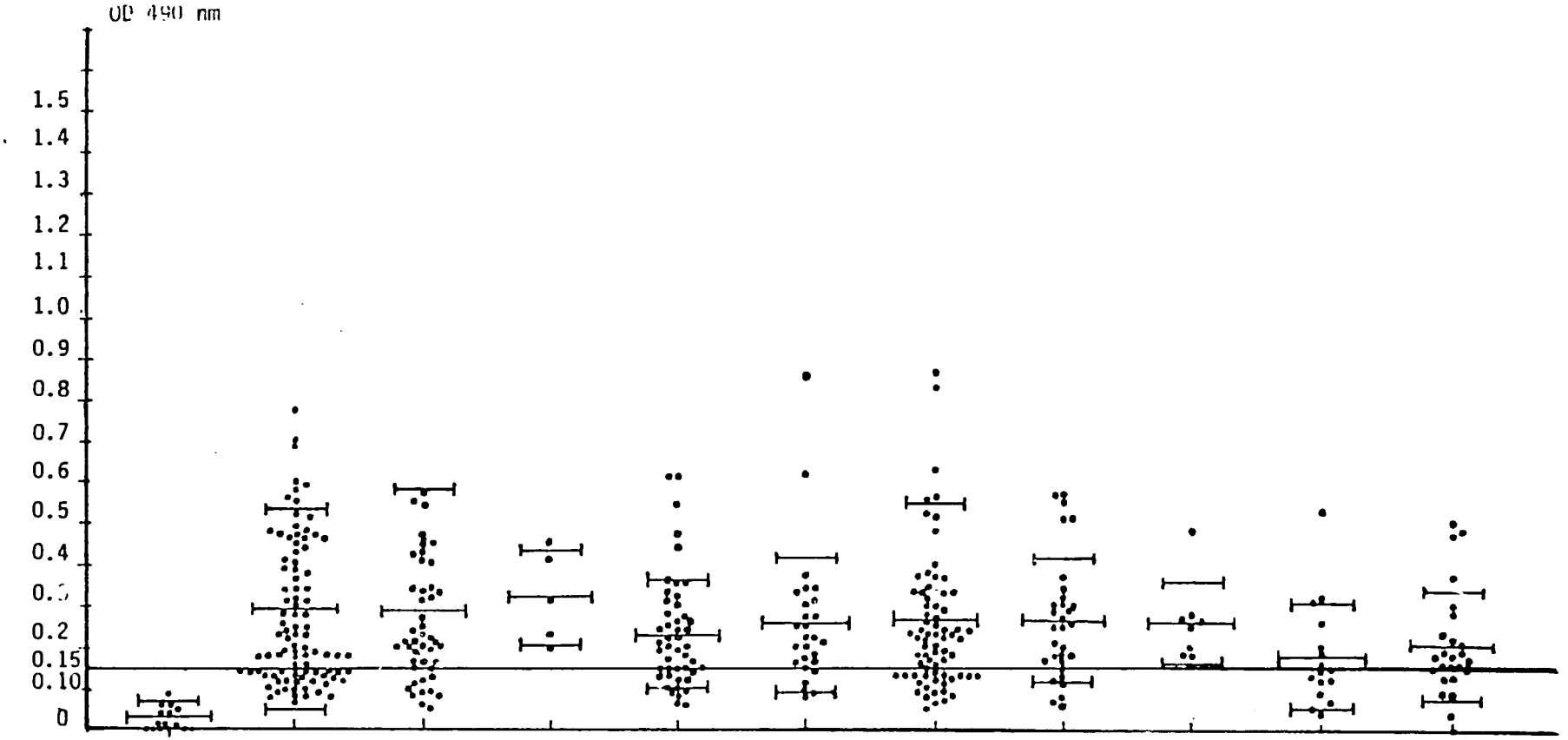

	Normal	Normal in endemic area	I	II	III	IV	V	VI	VII	VIII	IX
N	15	95	45	5	47	27	73	31	8	16	25
$\bar{X}$	0.03	0.290	0.281	0.320	0.231	0.256	0.266	0.263	0.263	0.180	0.209
SD	0.04	0.244	0.391	0.109	0.134	0.165	0.208	0.147	0.097	0.125	0.129

Figure 6 ODs of the ELISA reaction for antibodies to somatic antigen of G.spinigerum in the sera from different groups of filarial patients in endemic areas of B.malayi in Thailand and normal controls. Sera were diluted 1:1600. See materials and methods for details about each group of the filarial patients.

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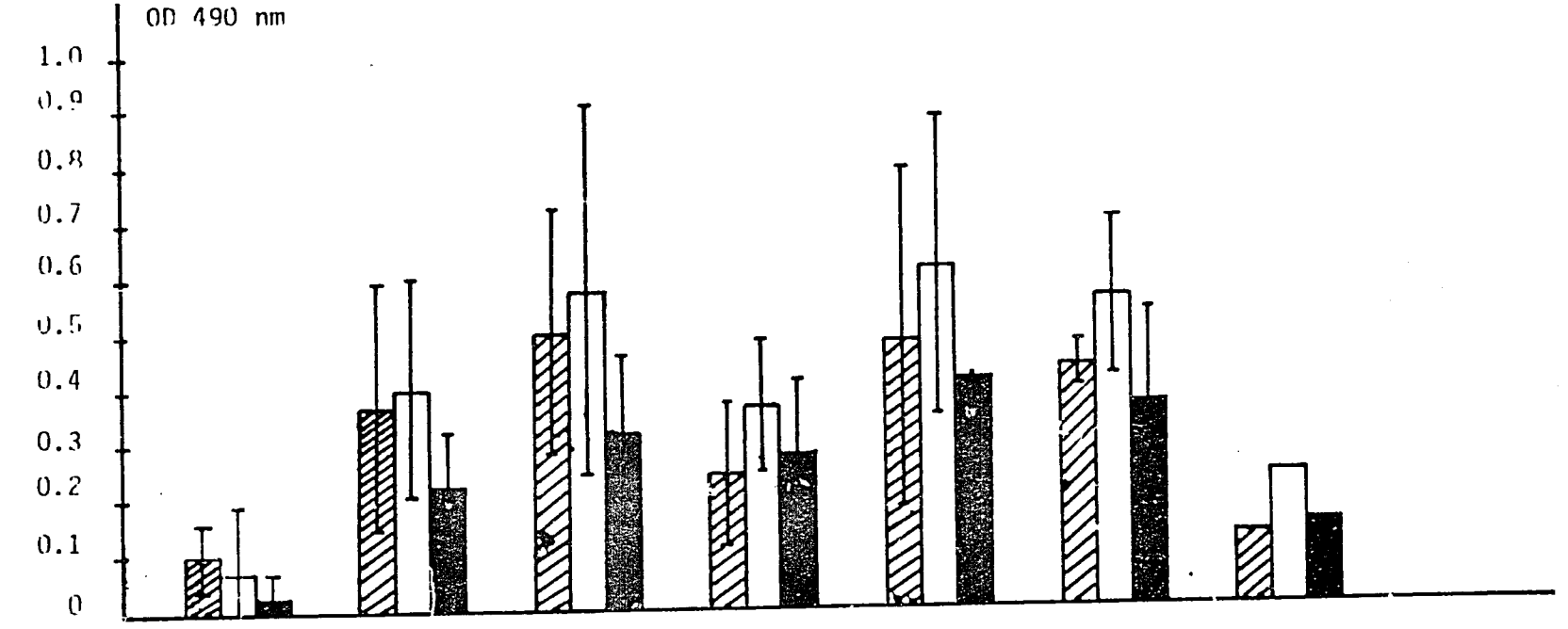

Group	Normal	Normal in endemic area	I	III	IX	X	XI
N	17	86	46	13	2	2	1
$\bar{X}$	.10.07.03	.37.40.22	.50.57.32	.24.36.28	.48.61.41	.44.56.37	.13.24.15
SD	.06.12.04	.22.19.10	.22.33.14	.13.12.14			

Figure 7 Means and Standard deviations of the ELISA values of total antibodies in the sera from different groups of filarial patients in endemic areas of W.bancrofti in Thailand and normal controls tested against antigens of B.malayi (▨), D.inmitis (□) and G.spinigerum (■). See materials and methods for details about each group of the filarial patients. Sera were diluted 1:1600.

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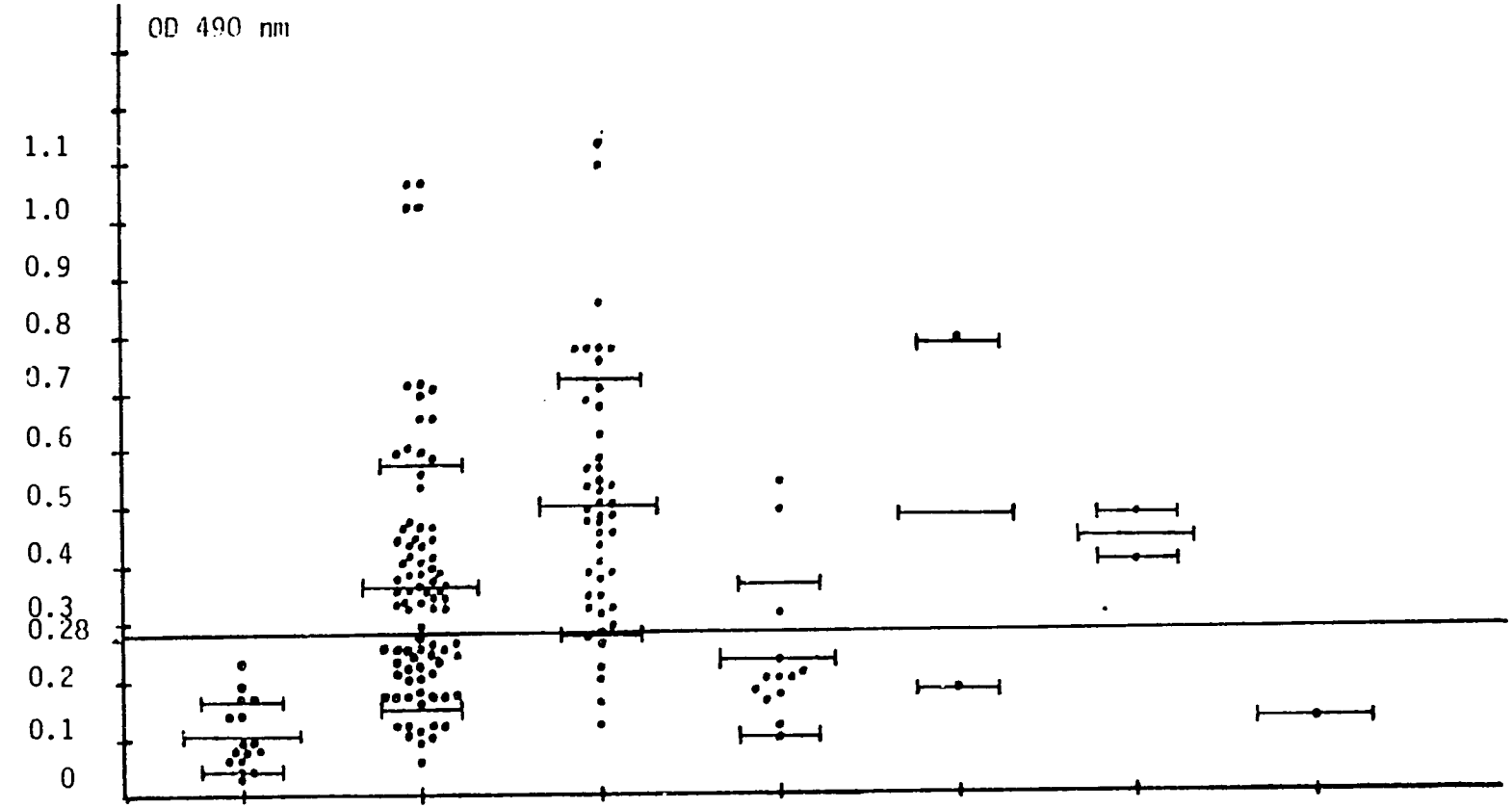
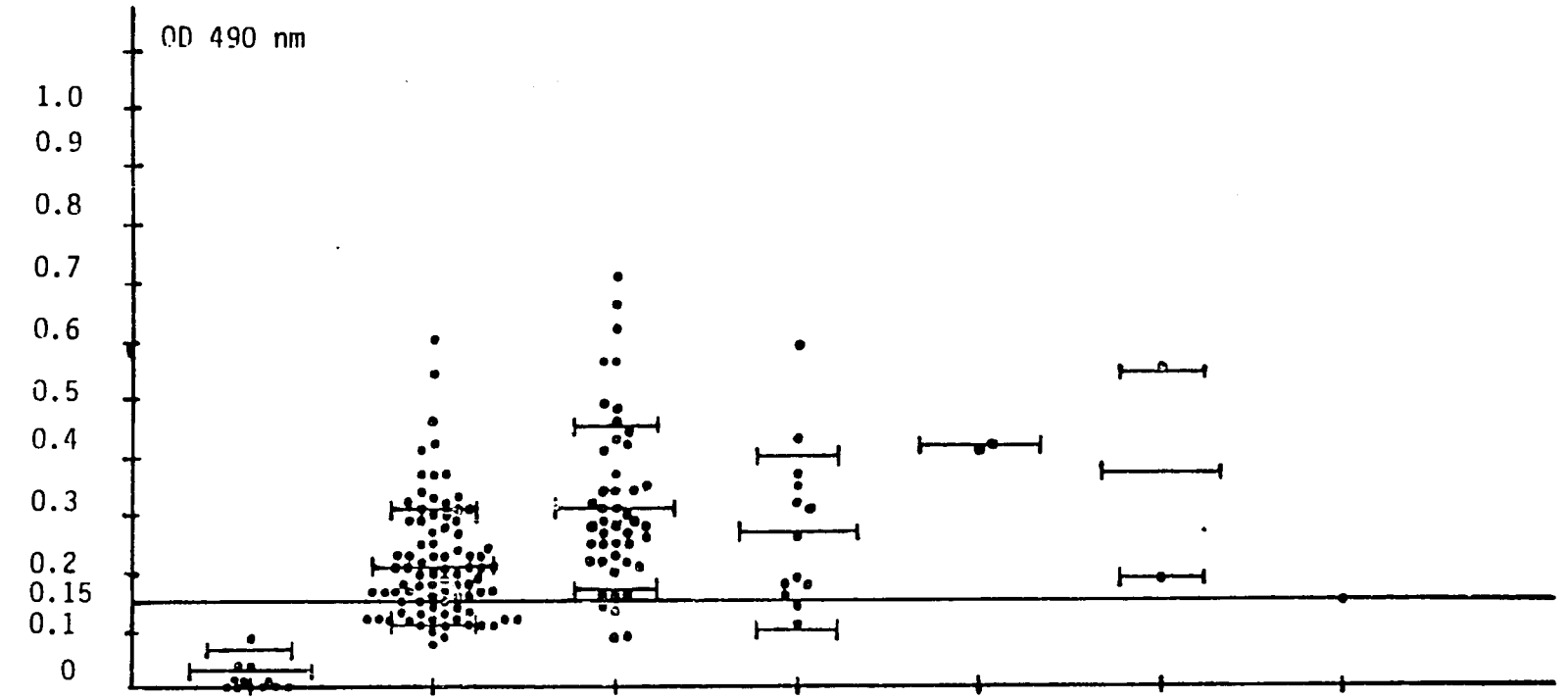


	Normal	Normal in endemic area	I	III	IV	V	VI
N	16	86	46	13	2	2	1
$\bar{X}$	0.105	0.366	0.50	0.239	0.485	0.44	0.13
SD	0.05	0.218	0.22	0.133			

Figure 8 ODs of the ELISA reaction for antibodies to somatic antigen of B.malayi in the sera from different groups of filarial patients in endemic areas of W.bancrofti in Thailand and normal controls. Sere were diluted 1:1600. See materials and methods for details about each group of the filarial patients.



Group	Normal	Normal in endemic area	I	III	IX	X	XI
N	15	86	46	13	2	2	1
$\bar{X}$	0.03	0.218	0.318	0.276	0.415	0.37	0.15
SD	0.04	0.100	0.145	0.137			

Figure 9 ODs of the ELISA reaction for antibodies to somatic antigen of G.spinigerum in the sera from different groups of filarial patients in endemic areas of W.bancrofti in Thailand and normal controls. Sera were diluted 1:1600. See materials and methods for details about each group of the filarial patients.

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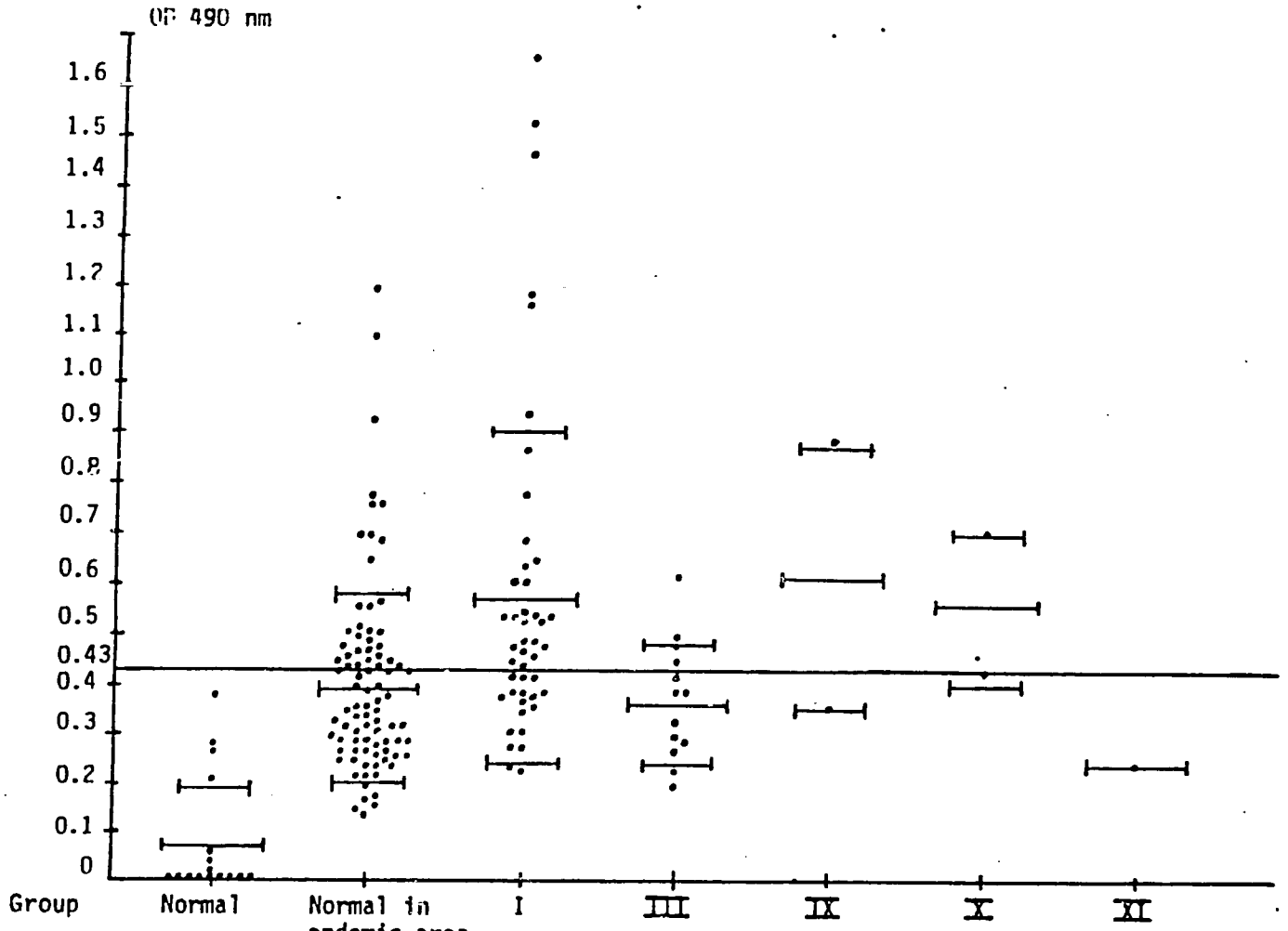

Group	Normal	Normal in endemic area	I	III	IV	V	VI
N	17	86	46	13	2	2	1
$\bar{X}$	0.07	0.396	0.572	0.365	0.615	0.56	0.24
SD	0.12	0.194	0.331	0.120			

Figure 10 ODs of the ELISA reaction for antibodies to somatic antigen of D.immitis in the sera from different groups of filarial patients in endemic areas of W.bancrofti in Thailand and normal controls. Sere were diluted 1:1600. See materials and methods for details about each group of the filarial patients.

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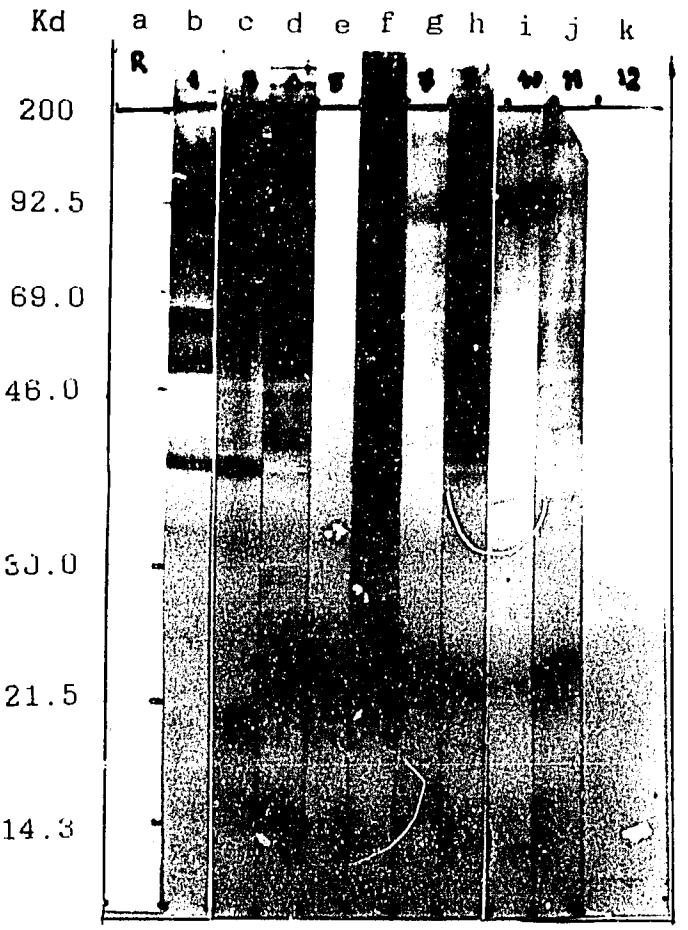

Figure 11 Antigen components of female adult B.malayi somatic extract transferred to nitrocellulose filter by Western blot technique were analysed by allowing to react with polyclonal antisera obtained from mice immunized with somatic extract of male adult B.malayi (b), male and female adult B.malayi (c), male and female adult B.pahangi (d), male and female adult T.cati (f) male adult D.immitis (g), female adult D.immitis (h) and advanced third-stage larvae of G.spinigerum (i). Normal mouse serum(e), reference positive (j) and reference negative (k) filarial patient sera were also used. (a) Rainbow marker (Amersham,U.K.).

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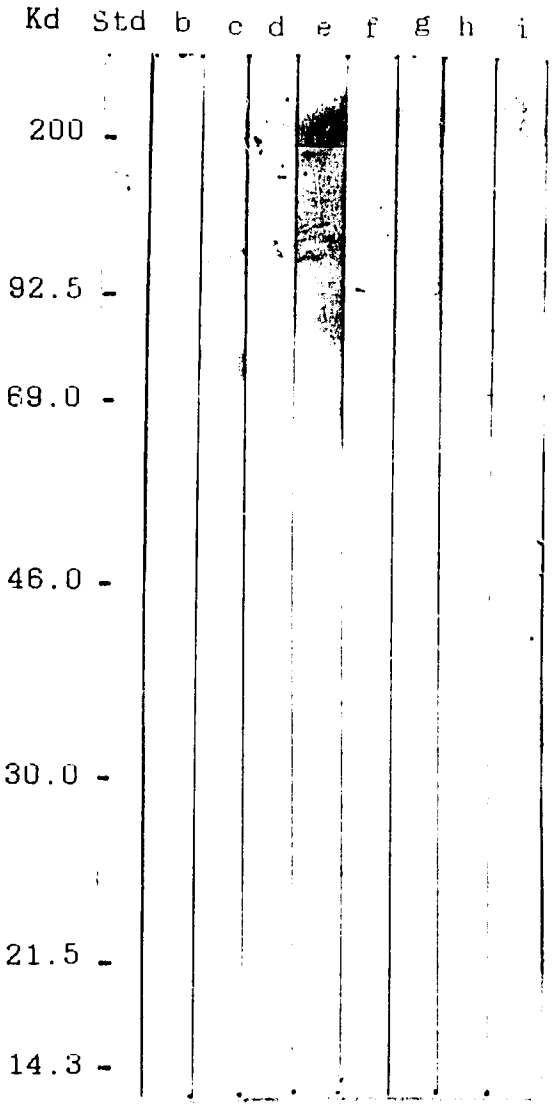

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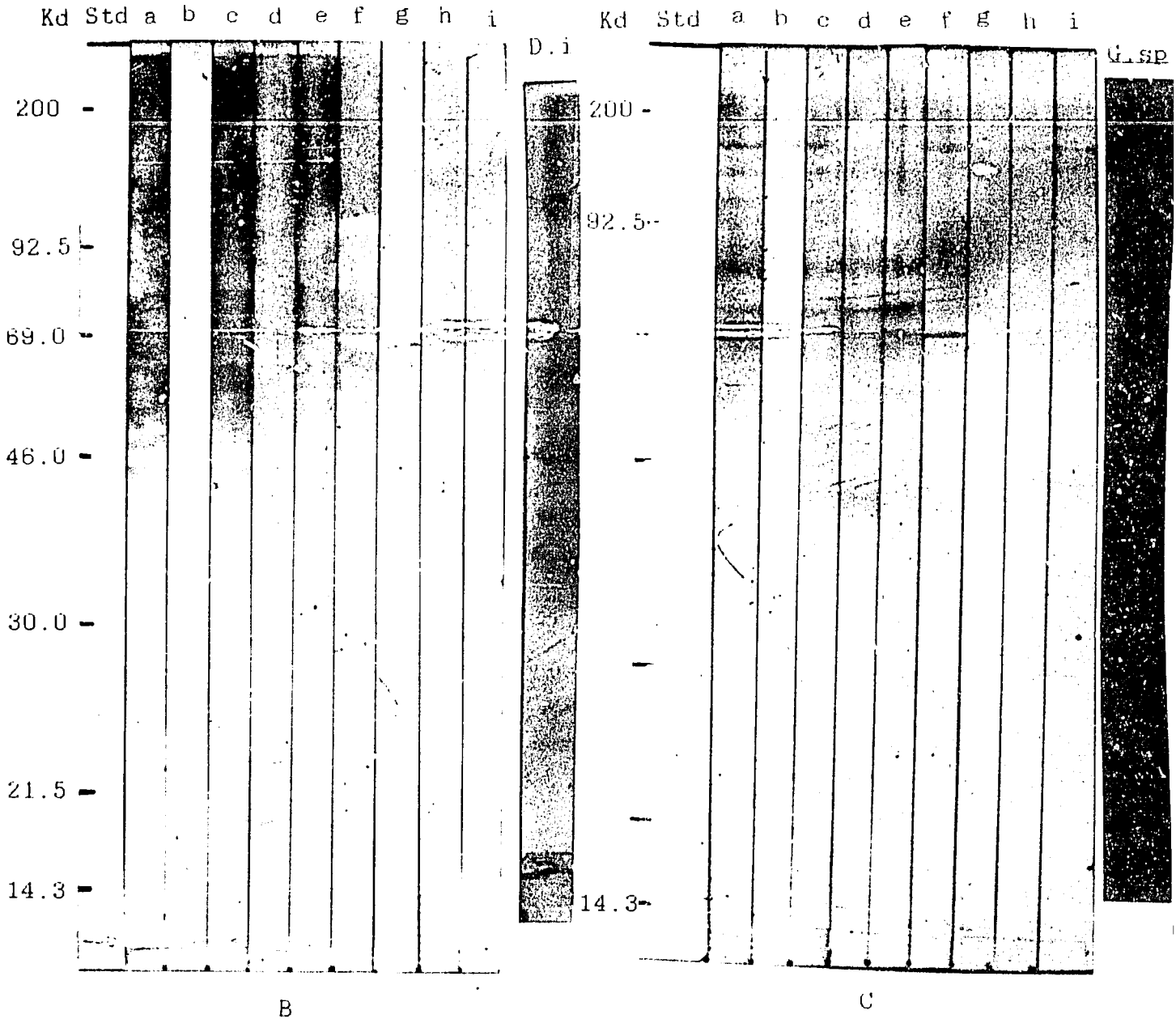

Figure 12 Western blot and immunological reactions of somatic extracts of female adult B.pahangi (A), female adult D.immitis (B) and advanced third-stage larvae of G.spinigerum (C) tested against reference positive filarial patient serum (a), reference negative human serum (b), and pools of filarial patient serum from endemic areas of B.malayi (c) pools of patient sera from group I,(e) group II,(f) group V, (g) group VII, (h) group IV and (i) group VI. Serum pool of normal people in endemic areas of B. malayi (d) was also used. (see paragragh 2.3 for full detail about filarial patient sera)

(D_1)and (G.sp): Amido black staining of female adult D.immitis and advanced third-stage larvae of G.spinigerum somatic extract on nitrocellulose filter.

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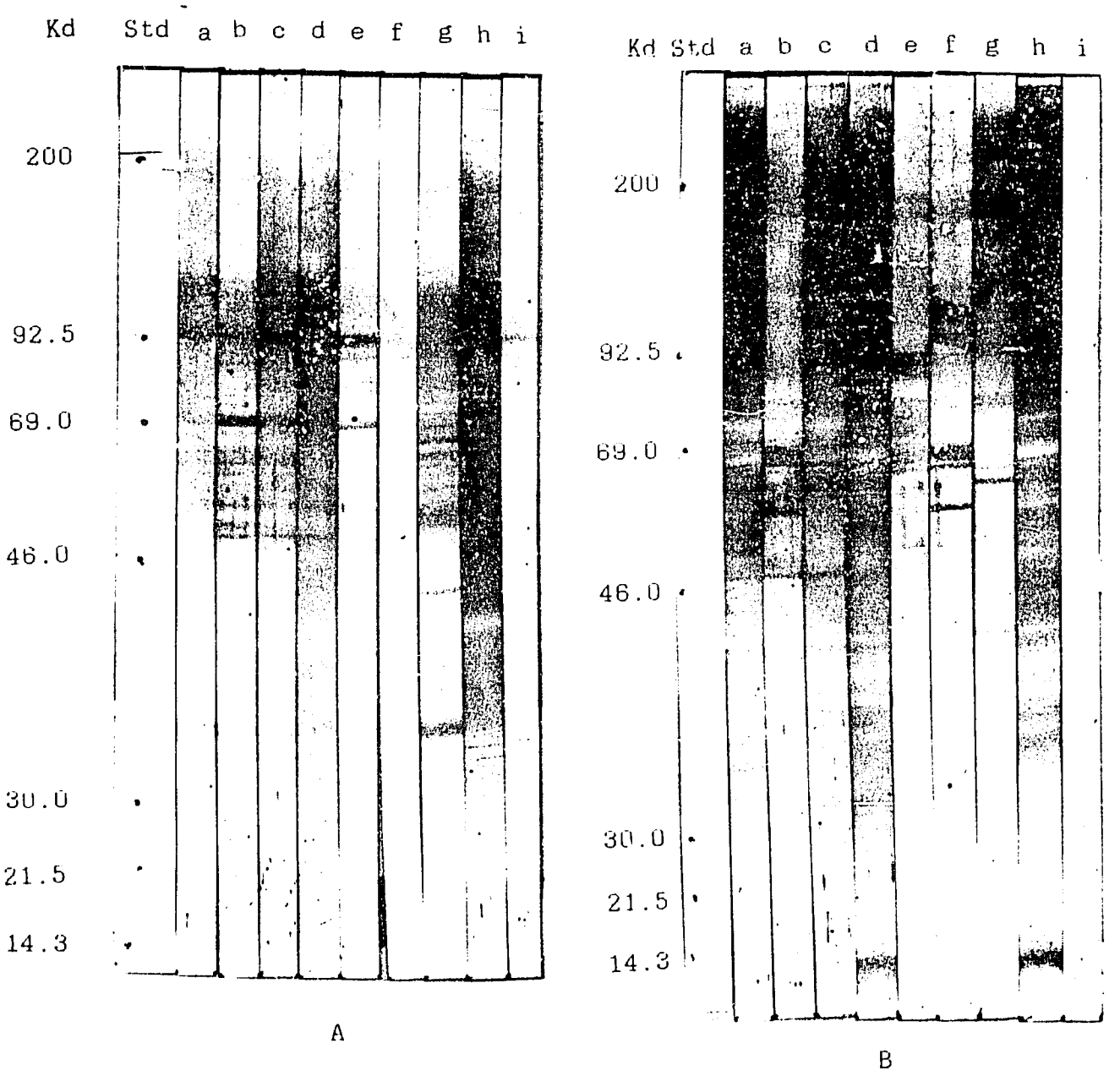

Figure 13 Western blot and immunological reactions of somatic extract of female adult B.malayi (A) and female adult B.pahangi (B) tested against filarial patient sera from endemic area of W. bancrofti, (a) pool of sera from patients in group I, (b) group III,(d) group IX, (e) group X and (f) group XI. Sera of normal people in endemic area (c), sera of a microfilaraemic patient who, later on, turned to amicrofilaraemic stage spontaneously (g), reference positive (h) and reference negative (i). B.malayi patient sera were also used.

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(a) pool of sera from patients in group I, (b) group III, (d) group IX, (e) group X and (f) group XI. Sera of normal people in endemic area (c), sera [illegible] patient who, later on, turned to amicrofilaraemic group spontaneously (g), reference positive (h) and reference negative (i). B.malayi patient sera were also used.

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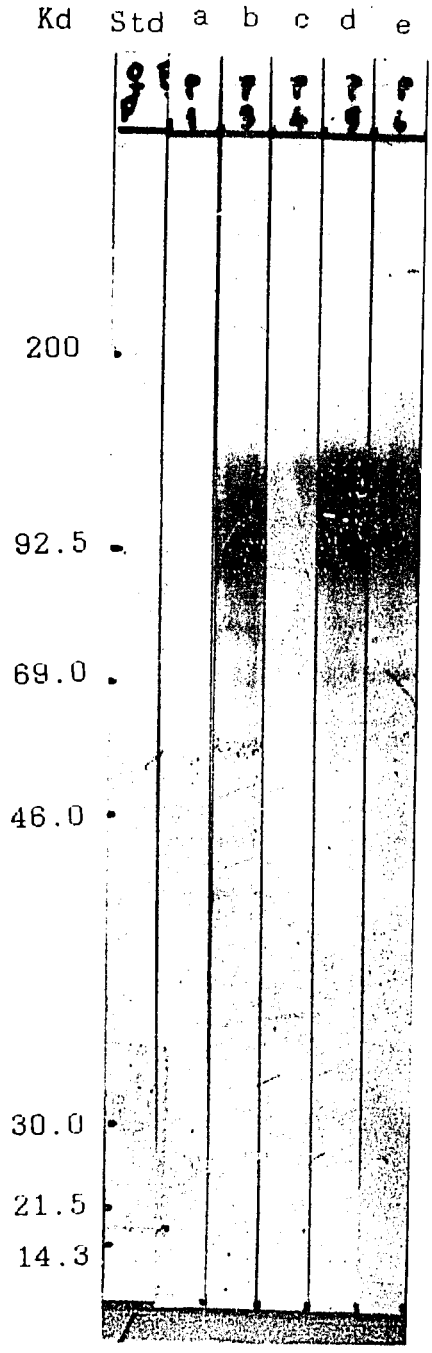

Figure 14 Western blot and immunological reactions of female adult B.pahangi somatic extract tested against polyclonal antisera from mice immunized with somatic extracts of female adult T.cati (b), male adult D.immitis (c), female adult D. immitis (d) and advanced third-stage larvae of G.spinigerum (e). Normal mouse serum was used as the negative control (a).

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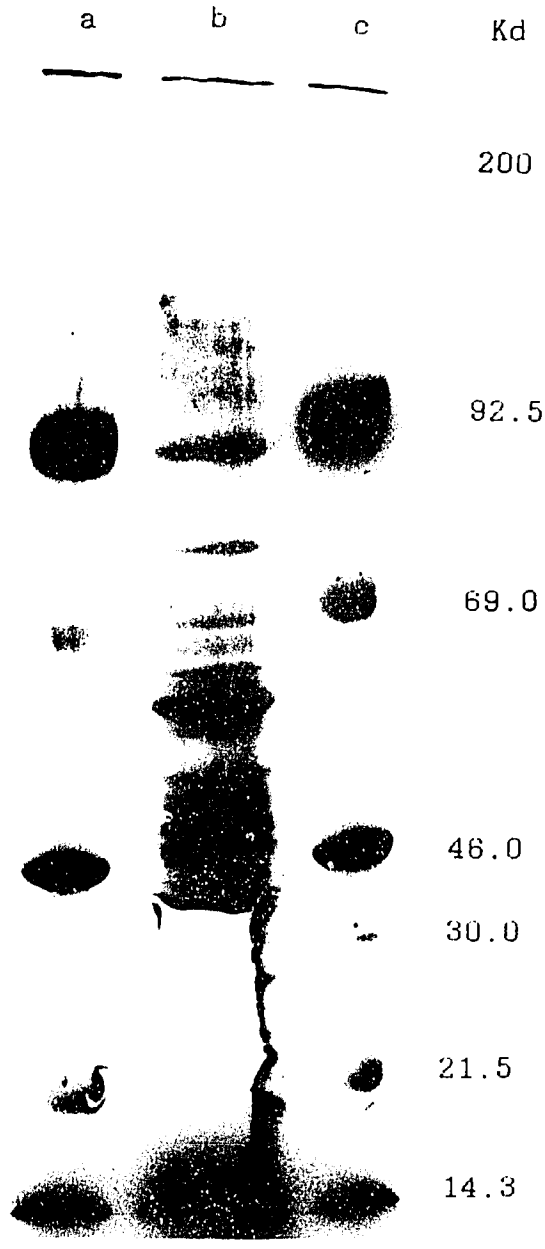

Figure 15 SDS-polyacrylamide gel containing somatic extract of female adult B.malayi (b). The gel was cut and antigen components at 25 Kd, 18.0 Kd and 14.3 Kd were injected to Balb/c mice. After the antigens were removed, the gel was stained with Silver nitrate. (a) and (c) are standard proteins.

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FIGURE 16 INDIRECT IMMUNOFLUORESCENCE ASSAY FOR TISSUE-ANTIGEN SOURCES

A & B Cryosections of adult female *B. malayi* stained with cat antiserum (A - control, B - experiment) showing light fluorescence over cuticle (c), moderate fluorescence on hypodermal-muscle layers (h, m) and strong fluorescence on uterine epithelium (u) and egg (e).

C & D Cryosections of adult male *B. pahangi* stained with jird antiserum (C - control, D - expe iment), showing moderate fluorescence on the hypodermal-muscle layer (h, m) and strong fluorescence on the testicular epithelium (t).

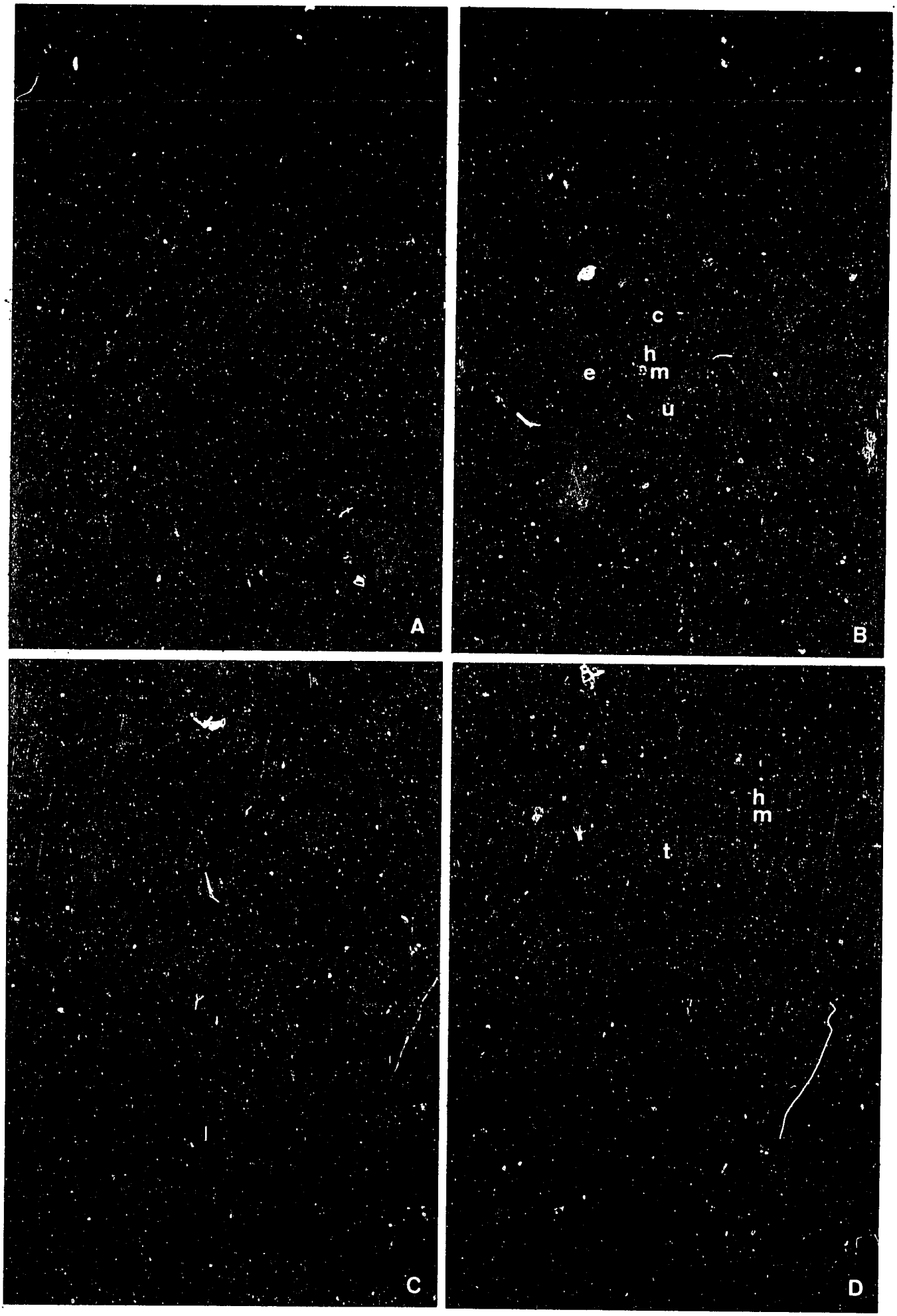

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FIGURE 17 INDIRECT IMMUNOFLUORESCENCE ASSAY FOR TISSUE-ANTIGEN SOURCES

A & B Cryosections of adult female *B. pahangi* stained with human antiserum (A - control, B - experiment), showing moderate fluorescence over cuticle (c), hypodermal-muscle layer (h, m) and strong fluorescence on egg shell (e) and secretory materials between eggs (arrow)

C & D Whole mount of L_3 stage *B. pahangi* stained with human antiserum (C - control, D - experiment) show generally strong fluorescence on the entire cuticular covering of the parasite.

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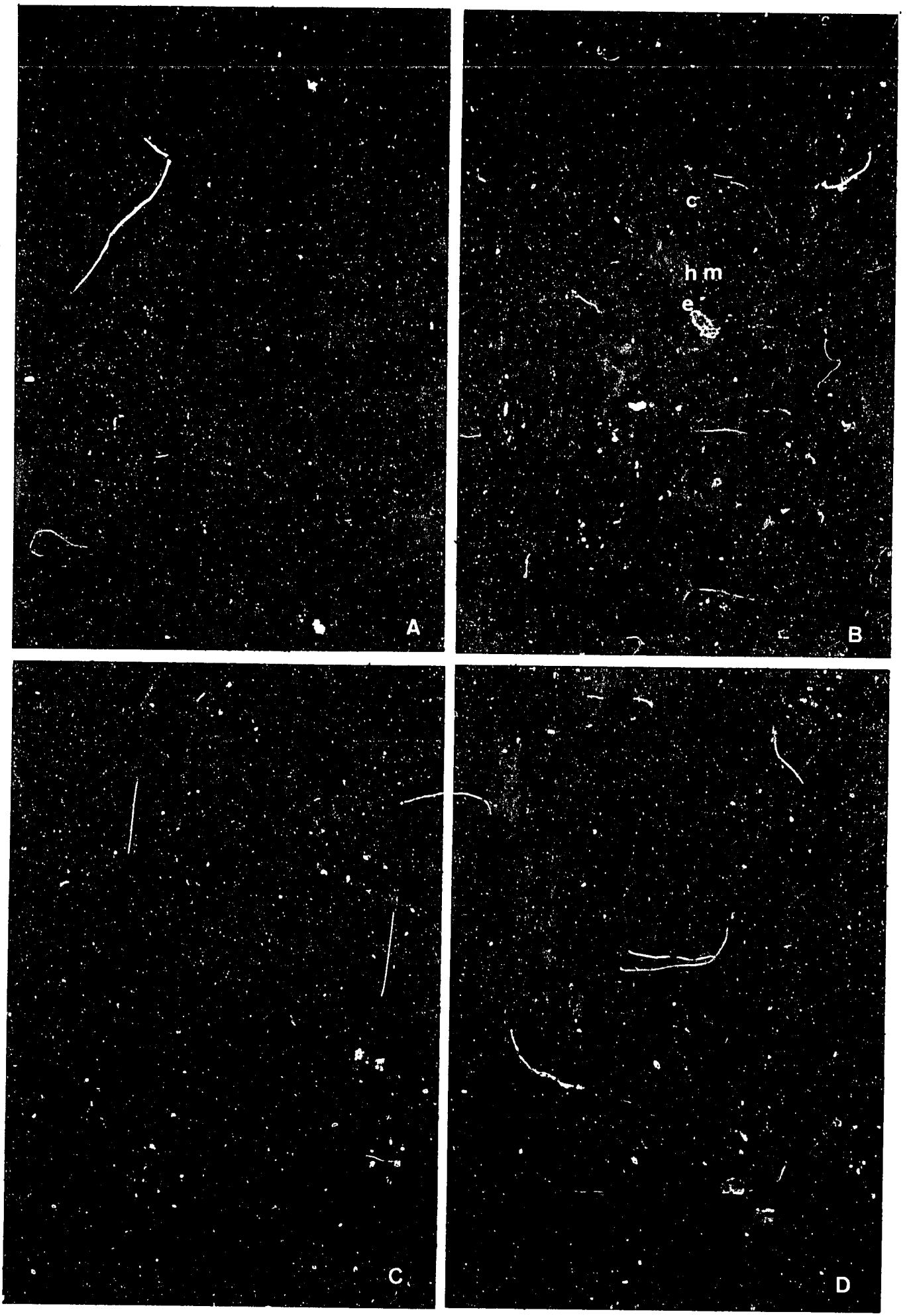
Figure 17



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