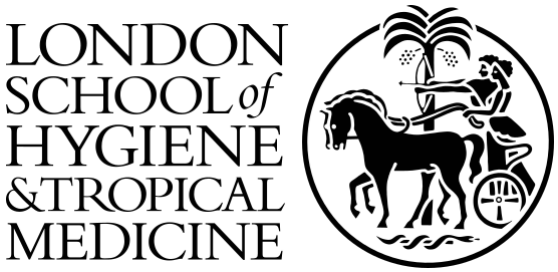

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STUDIES ON SOME IMMUNOLOGICAL ASPECTS OF BRUGIAN AND BANCROFTIAN FILARIASIS

A thesis submitted to the Faculty of Medicine
University of London, for the degree of
Doctor of Philosophy

by

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ABSTRACT

The possibility of developing a satisfactory serological test applicable to seroepidemiological studies of brugian and bancroftian filariasis was investigated. The cat-*Brugia pahangi* model was used. The relative sensitivities of the indirect fluorescent antibody test (IFAT), counter-immunoelectrophoresis (CIEP) and enzyme-linked immunosorbent assay (ELISA), using crude or purified somatic, and metabolic antigens of various developmental stages of six different human and animal filarioids, for the detection of antibodies in sera were compared. Homologous antigens were always better than heterologous antigens in sensitivity. Metabolic antigens seemed to be more species-specific. A rise in antibody level was observed after chemotherapy. IFAT using *B. pahangi* and *Wuchereria bancrofti* infective larvae as antigens were the most sensitive tests for feline (89% positivity), and human brugian (96%) and bancroftian (100%) filariasis respectively. In ELISA, fractions 1 and 2 of the partially purified antigen of *B. pahangi* adults seemed to be more specific for bancroftian and brugian filariasis respectively than the crude antigen. ELISA and IFAT were found useful in the seroepidemiological studies of human filariasis in New Guinea, Trinidad, the Seychelles Islands and Egypt.

ELISA and CIEP were used to detect circulating worm antigen in sera and urine, using rabbit antisera to *B. pahangi* antigen.

Studies with phosphoglucomutase isoenzymes of *B. pahangi* adult worms suggested that these isoenzymes could be the source of specific antigen for serological tests.

The histopathology of the lungs, liver, spleen, kidneys, infected lymph nodes and lymphatics of *B. pahangi*-infected cats was studied. A higher percentage of glomeruli in kidneys from

71 infected cats showed histological lesions when compared to the uninfected controls. Fluorescence studies demonstrated the presence of IgG, worm antigens and complement deposits in the glomeruli of kidneys from infected cats. 40% of infected cats had proteinuria by Albustix test. The presence of circulating immune complexes in sera from infected cats and humans with brugian and bancroftian filariasis was studied by C1q- and conglutinin-binding assays.

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CHAPTER ONE

INTRODUCTION

Filariasis is a group of parasitic diseases of human and animals (amphibians, reptiles, birds and mammals) caused by nematodes of the order Filariidea (Sasa, 1976). The adult (fifth-stage) worms are cylindrical, thin and are several centimetres in length. They live in vessels, tissues or body cavities of vertebrate (definitive) hosts for years. After mating, the female worms produce microfilariae (first-stage larvae, which are snake-like organisms measuring 0.2 - 0.35 mm in length). When the microfilariae are ingested by the specific blood-sucking arthropod intermediate hosts (eg. mosquitoes, black flies, biting midges, mites, ticks, etc.), they invade certain organs or body cavities and moult twice to the infective (third-stage) larvae in 1 - 2 weeks. When the infected intermediate hosts bite the vertebrate hosts, the infective larvae migrate from the skin to the definitive sites where they moult twice to become finally the reproducing adults.

In veterinary medicine, filariasis is often a health hazard to domestic animals, eg. Dirofilaria immitis (Leidy, 1856) Raillet and Henry 1911, and Brugia pahangi (Buckley and Edeson, 1956) Buckley 1958 infecting dogs and cats; Setaria cervi (Rudolphi, 1819) Baylis 1939 of cattle which often causes neurological damage in horses, sheep and goats.

Classification of Human Filariasis

Among the hundreds of filarioid species, eight have become adapted for interhuman transmission. It has been estimated that more than 200 million people world-wide are

affected by filariasis (World Health Organisation, 1974). They can be classified according to the habitat of the adult worms.

(1) Lymphatic Filariasis : Wuchereria bancrofti (Cobbold, 1877), Silva Araujo 1877; Brugia malayi (Brug, 1927) Buckley 1958 and Brugia timori David and Edeson 1964, are transmitted by mosquitoes, causing elephantiasis.
(2) Body Cavity Filariasis : Dipetalonema perstans Manson 1891; and Mansonella ozzardi (Manson, 1897) Faust 1929, are transmitted by midges.
(3) Cutaneous Filariasis : Dipetalonema streptocerca (Macfie and Corson, 1922) Peel and Chardome 1946 is transmitted by midges. Onchocerca volvulus (Leuckart, 1893) Raillet and Henry 1910 is transmitted by black flies, causing river blindness and Loa loa (Guyot, 1778) Castellani and Chalmers 1913, the eye worm causing Calabar swellings, is transmitted by horse-flies.

Their geographical distributions, habitats of adult worms and microfilariae, pathogenicity and vectors are summarized in Table 1.1. The presence of microfilarial periodicity in some species seems to be an adaptation to the behaviour of local vectors (Sasa, 1976). However, Hawking et al (1966) have suggested that the appearance of microfilariae in the blood is controlled by the difference in oxygen tensions between arterial and venous blood in the lungs.

Clinical Manifestations of Lymphatic Filariasis due to W. bancrofti and B. malayi

Patent filariasis is generally not established unless exposure to infective larvae is intense and prolonged. Although exposure to infection occurs throughout childhood in endemic areas, most of the pathology is found in the adult population. The clinical spectrum of the disease has been reviewed and discussed in some details by Sasa (1976) and Ottesen (1980).

(1) Incubation Period -

This is the period between infection and the development of clinical symptoms. It may vary between 3 to 18 months or

Table 1.1 . The geographical distributions, habitats of adult worms and microfilariae, and pathogenicity of and vectors for human filariasis.

FILARIOID SPECIES	GEOGRAPHICAL DISTRIBUTIONS	HABITATS OF ADULTS	HABITATS OF MICROFILARIAE (PERIODICITY)	PATHOGENICITY	VECTORS
LYMPHATIC FILARIASIS					
Wuchereria bancrofti	Asia, Pacific, Tropical Africa.	Lymphatics	Blood (variable)	Lymphangitis Fever, Elephantiasis, Hydrocele, Chyluria.	Mosquitoes: Culex spp. Anopheles spp. Aedes spp.
Brugia malayi	South and East Asia	Lymphatics	Blood (nocturnal or subperiodic)	Lymphangitis, Fever, Elephantiasis.	Mansonia spp. Anopheles spp. Aedes spp.
Brugia timori	Indonesia	Lymphatics	Blood (nocturnal)	Lymphangitis, Fever, Elephantiasis.	Anopheles spp.
BODY CAVITY FILARIASIS					
Dipetalonema perstans	Africa and South America	Peritoneal and Pleural Cavities	Blood (nocturnally subperiodic)	No Definite Pathogenicity	Biting Midges: Culicoides spp.
Mansonella ozzardi	Central and South America	Peritoneal Cavity	Blood, Skin (nonperiodic)	No Definite Pathogenicity	Culicoides spp.
CUTANEOUS FILARIASIS					
Dipetalonema streptocerca	Africa (Ghana, Congo).	Subcutaneous Tissues	Skin (nonperiodic)	Cutaneous Oedema, Elephantiasis.	Culicoides spp.
Onchocerca volvulus	Africa, Central and South America	Subcutaneous Tissues	Skin (nonperiodic)	Subcutaneous Nodules, Pruritic Dermatitis, Sclerosing Keratitis, Blindness.	Black Flies: Simulium spp.
Loa loa	Tropical Africa	Subcutaneous Tissues	Blood (diurnally subperiodic)	Skin Swellings, Allergic Reactions.	Horse Flies: Chrysops spp.

more for W. bancrofti and more than 3½ months for B. malayi infections. Patients in this category are clinically asymptomatic and parasitologically negative.

(2) Asymptomatic Microfilaraemic -

Manson-Bahr (1953) suggested that it required 2 - 7 years for W. bancrofti to mature, become fertilized, and for the microfilariae to appear in the blood. This patent but symptomless period may last several years or even throughout life in the native populations.

(3) Acute Stage -

This is characterized by filarial fevers associated with lymphangitis (spreading centrifugally)and lymphadenitis of legs and hands. In W. bancrofti infection, funiculitis and orchitis may occur. Reversible swellings of distal part of limbs may occur in brugian filariasis. Fevers are accompanied by rigors and general malaise, lasting for about a week and terminate abruptly with profuse sweating. These febrile episodes occur 2 - 6 times a year but become less frequent as the disease becomes more chronic. The patients may be mircofilaraemic.

(4) Chronic Stage -

In bancroftian filariasis, elephantiasis of the arms, breast, scrotum or vulva, etc. occur. Chyluria and hydrocele are common in endemic areas in temperate zone (where elephantiasis is rare). In brugian filariasis, elephantiasis of the legs and less frequently of the arms occur. However, genital and breast involvements are rare or practically absent. Ocular involvements have been reported in both bancroftian and brugian filariasis (Fernando, 1935; Chatterjee, 1954 and Mak et al., 1974). The patients are usually amicrofilaraemic but often still have recurrent filarial fevers.

(5) Tropical Eosinophilia Syndrome -

There appears to be little clinical overlaps between this and other filarial syndromes. Paroxysmal, nocturnal asthma-type symptoms are the presenting complaints. In untreated

cases, chronic interstitial-type lung disease may develop. The patients are amicrofilaraemic although degenerating microfilariae may be found in pulmonary or other tissues.

Recently, Winstead (1978) also described the psychosomatic aspects of filariasis and a case of chronic illness behaviour following an initial episode of filariasis.

Pathology of Lymphatic Filariasis

This has been discussed by Whartman (1944); Nelson (1966); Schacher and Sahyoun (1967); Galindo (1971); Rogers et al (1975); Denham and Nelson (1976); Denham and McGreevy (1977) and Ottesen (1980). Histological reactions to the somatic and metabolic components of different stages of the parasite are observed. Histological changes in the lymph nodes can be linked with the sequential development of the immune response of the host and those in the lymphatics to the infection of the adult worms. It seems that healthy and fully developed microfilariae produce little remarkable lesions but lesions may occur in the lymph nodes and spleen after their disintegration. Living adult worms in the lymphatics cause only moderate dilatation of the lymphatic vessels, which may be accompanied by cell infiltrations. Granulomatous inflammation, proliferation of the reticuloendothelial system and tissue eosinophilia may occur in the lymph nodes. When the destruction of the adult worms begins, thrombolymphangitis with eosinophilic infiltration around the affected lymph vessel develops, proceeding finally to a granulomatous inflammation with partial or complete obliteration of the lymphatic vessel. The worms lose their structures and become calcified. It is suggested that elephantiasis results from the host response which kills the adult worms in the lymphatics. Bacterial superinfection (eg. streptococci) is only a complication but certainly not a necessary determinant of the lymphatic pathology.

Zoonotic Aspects of Filariasis

These have been discussed by Nelson (1965, 1979), Denham and McGreevy (1977) and Dissanaike (1979). Subperiodic *B. malayi*, *B. pahangi* and *Dirofilaria* species are maintained by the transmission between zoophilic vectors and the reservoir vertebrate hosts, eg. monkeys, wild carnivores and domestic animals (Fig. 1.1).

Animal Models for Filariasis

B. malayi (subperiodic) is easily transmissible to animals (eg. cats, dogs, jirds and monkeys) in the laboratory. However, the other human filarioids are not easily transmissible to laboratory animals. *L. loa* can be transmitted to some African monkeys (*Mandrillus*, *Papio*, *Erythrocebus*) and certain strains of *O. volvulus* to chimpanzees (Dissanaike, 1979). It was till only recently that successful transmission of *W. bancrofti* to monkeys (*Macaca cyclopis*, *M. mulatta*, *M. irus*) was reported (Dissanaike and Mak, 1978; Cross *et al.*, 1979). Thus, to study the chemotherapy and immunology of filariasis, laboratory animal models using non-human filarioids have to be used. Among the different animal models commonly used (see Table 1.2), *B. pahangi* in cats seems to be the most attractive. It is because *B. pahangi* is a close relative of *B. malayi* and in fact, it has been shown to infect human experimentally (Edeson *et al.*, 1960b). Furthermore, the course and pathology of the infection are very similar to that of lymphatic filariasis in man.

Chemotherapy of Filariasis

The methods for the control of filariasis are vector control and chemotherapy. Diethylcarbamazine or 1-diethylcarbamyl-4-methylpiperazine (DEC) is the only drug available for mass treatment of brugian and bancroftian filariasis. It is

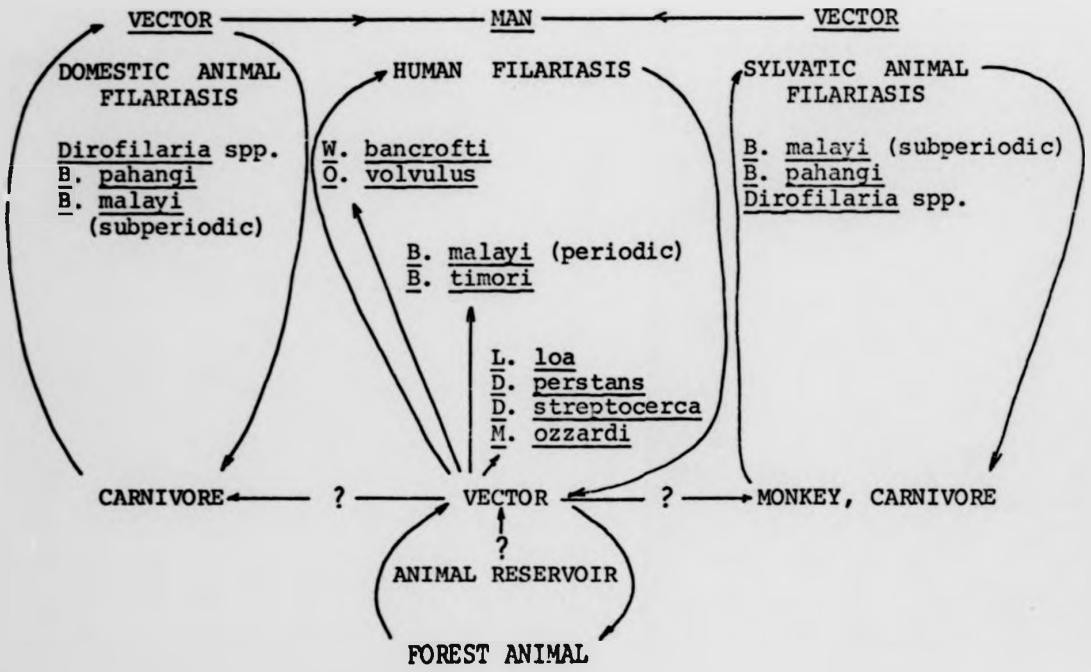

Fig. 1.1 . Chart showing the zoonotic aspects of human filariasis, according to Dissanaike (1979).

Table 1.2 . Some filarioids and their laboratory hosts commonly used for experimental studies

Filariod Species	Intermediate Hosts	Definitive Hosts	References
Litomosoides carinii	Tropical Rat Mite (*Ornithonyssus bacoti*)	Cotton Rat (*Sigmodon* spp.) Jird (*Meriones* spp.)	Scott *et al.* (1947) Bertram (1947)
Dipetalonema viteae	Tick (*Ornithodoros*)	Jird	Baltazard *et al.* (1953) Worms *et al.* (1961)
Dirofilaria immitis	Mosquitoes (*Aedes*, *Armigeres*, *Culex*)	Dog	Wong (1964a,b) Bradley & Pacheco (1972)
Brugia pahangi	Mosquitoes (*Armigeres*, *Aedes*, *Mansonia*)	Cat, Dog, Jird	Edesen *et al.* (1960a) Ewert & Singh (1969) Ash & Riley (1970) Denham *et al.* (1972)
Brugia malayi	Mosquitoes (*Aedes*, *Mansonia*, *Anopheles*)	Cat, Dog, Monkey, Jird	Edesen & Wharton (1957) Ramachandran *et al.* (1960) Laing *et al.* (1961) Macdonald (1962)
Onchocerca gutturosa	Black Flies (*Simulium*)	Cattle	Steward (1937)

a compound of low toxicity (Hawking, 1973; Roychowdhury *et al.*, 1977). Side effects such as fever, general malaise etc. may occur in patients who have never suffered any signs or symptoms of filariasis (Duke, 1980). When used at a dosage of at least 72 mg/kg in total, 80% of *B. malayi* and *W. bancrofti* carriers can be permanently cured (Sasa, 1976). DEC is probably only microfilaricidal against *W. bancrofti*, *B. malayi*, *B. timori*, *O. volvulus* and *L. loa* (Duke, 1980). A macrofilaricidal compound is needed for radical treatment (World Health Organisation, 1974). Denham (1979) has reviewed the current methods for screening compounds for filaricidal activity.

DEC has been suggested to have a direct microfilaricidal action or to modulate the host immune response resulting in phagocytosis of the microfilariae (Kobayashi *et al.*, 1969; Rao *et al.*, 1977; Tanaka *et al.*, 1977; Denham *et al.*, 1978b; Takaoka and Tanaka, 1978; Langham and Kramer, 1980). Recently, Pissens and Beldakas (1979) showed that DEC increased the *in vitro* adherence of leukocytes to *B. malayi* microfilariae mediated by antibodies reacting with the worm surface. Therefore, DEC seems to "unmask" the parasites so that they are recognised as foreign to the host and are then destroyed by the immune system (Duke, 1980).

Immunology of Filariasis

During the past five years, better understanding of the immunology of filariasis has been made (reviewed by Ogilvie and Worms, 1976; Sasa, 1976; Denham and Mcgreevy, 1977; Grove, 1978 and Ottesen, 1980).

Innate Resistance

Natural resistance to filariasis in man is poorly understood, probably because of the lack of parasitological information. The fact that certain filarioids can develop to adults or to infective larvae only in certain hosts

strongly suggests the existence of natural resistance (Ash, 1971; Ash and Schacher, 1971; Siddiqui and Kershaw, 1976; Cross et al., 1979). Attempts have also been made to break down the innate resistance of animals to filarioid infections by splenectomy, neonatal thymectomy and immunosuppression with irradiation or 6-mercaptopurine, resulting in patent infections in some infections (Ahmed, 1967; Subrahmanyam et al., 1974; Vincent and Ash, 1977).

Acquired Immunity

Repeated infections are required before an acquired resistance is developed. In experimental animals, this acquired resistance may be revealed by the retardation of growth and development or death of the infective larvae and adult worms (Scott and MacDonald, 1958; Ewert and Bosworth, 1975; Kowalski and Ash, 1975; Neilson, 1976; Weiner and Soulsby, 1976; Denham and McGreevy, 1977) or the reduction or clearance of microfilariae in the blood (Duke, 1960; Singh and Raghavan, 1962; Bagai and Subrahmanyam, 1970; Pacheo, 1970; Denham and McGreevy, 1977; Neilson, 1978; Tanner and Weiss, 1979). Epidemiological studies on human filariasis have shown that not all members of a family acquire the infection and many patients with elephantiasis are amicrofilaraemic (Subrahmanyam, 1976; Grove, 1978). Microfilariae of W. bancrofti injected into an uninfected volunteer circulated in the blood for 14 days but could survive for only 2½ days in a patient with chronic filariasis (Knott, 1935).

Immunity appears to attack the microfilariae and be stage-specific (Ogilvie and Worms, 1976; Weiss, 1978). Microfilaraemia seems to depend on a delicate balance between the host immune response and the fresh supply of microfilariae by the female worms (Tanner and Weiss, 1979). The spleen does not seem to be involved in the destruction of microfilariae, except for L. loa in monkeys (Duke, 1960; Nooruddin and Ahmed, 1967). The current concept of the mechanism of destruction of microfilariae is a combination of humoral and cellular immune responses. Specific antibodies (probably IgM or IgG) against

microfilariae evoke strong adherence of phagocytic cells (mainly monocytes, but also eosinophils, neutrophils and lymphocytes) to the microfilariae, resulting in the disintegration of the parasite (Bagai and Subrahmanyam, 1970; Nelson et al., 1976; Subrahmanyam, 1976; Subrahmanyam et al., 1976 and 1978; Tanner and Weiss, 1978 and 1979; Weiss and Tanner, 1979; Haque et al., 1980). This process is complement independent and lymphokines may be involved (Kobayakawa et al., 1976). UNDP/World Bank/World Health Organisation (1979a)also suggested the possible participation of superoxide anion and other oxygen derivatives in the actual chemical details of the cell-parasite interaction. In tropical eosinophilia, eosinophils seem to be important effector cells (Webb et al., 1960; Wong and Guest, 1969; Higashi and Chowdhury, 1970).

Antibody Response

The pattern of antibody response to filarioid infections has been studied with different animal models. Depending on the serological test and antigens used, total antibody response can usually be detected early in the infection (Pacheco,1966; Ponnudurai et al., 1974; Benjamin and Soulsby, 1976). The peak antibody response usually occurs some time around patency (Pacheco,1966; Ponnudurai et al., 1974; Weiss, 1978; Grieve et al., 1979). Antibodies during the early phase of the infection are IgM and the response will switch to an IgG class of antibodies at patency (Fujita and Kobayashi, 1969; Weiner and Bradley, 1973; Benjamin and Soulsby, 1976). IgE (homocytotropic) antibody response occurs when the fourth-stage larvae moult to the adults and peaks around patency (Soulsby et al., 1976). Antibodies against the cuticle or sheath of microfilariae occur only after the infected animals have become amicrofilaraemic (Wong, 1964b;Ponnudurai et al., 1974; Weiss, 1978).

In humans, the levels of IgG, IgM or IgE are elevated in bancroftian filariasis (Desowitz et al., 1976; Ottesen et al., 1977 and 1979; Partono et al., 1978; Grove and Forbes, 1979). However, the rise in immunoglobulin levels in brugian filariasis is not significant (Mak et al., 1979). Antibodies against the cuticle or sheath of microfilariae can only be detected in some amicrofilaraemic patients (Jayewardene and Wijayaratnam, 1968; Wong and Guest, 1969; Grove and Davis, 1978).

In tropical eosinophilia syndrome, IgE is markedly elevated, probably as a response to microfilariae and their metabolic products (Guest and Wong, 1965; UNDP/World Bank/ World Health Organisation, 1979a;Ottesen, 1980). Basophils and mast cells are sensitized with the specific IgE (Ottesen et al., 1979). Thus, this syndrome is a form of occult filariasis resulting from the host hypersensitivity to microfilariae.

Cellular Immune Response

The clinical manifestations of lymphatic filariasis seem to result from different types of immune response to antigens associated with different developmental stages of the parasite.

In vitro peripheral blood lymphocyte transformation response to adult and microfilarial antigens is observed in symptomless individuals (Ottesen et al., 1977; Pissens et al., 1980a). It is suggested that this group is probably at the early stage of the infection and the vigorous immune response observed may prevent normal development of the parasite. Lymphocyte response to purified protein derivative (PPD) and streptokinase-streptodornase (SK-SD) is normal.

Studies on animal filariasis give similar results and the cell-mediated immune response develops before the antibody response (Kobayakawa, 1975; Rogers et al., 1975; Weiss 1978; Weller, 1978).

In microfilaraemic patients, there is a marked lymphocyte hypo-responsiveness to adult and microfilarial antigens (Ottesen et al., 1977; Pissens et al., 1980a). Antigen-specific suppressor cells and suppressor factors are present in the blood (Pissens et al., 1980b). In vitro lymphocyte response to PPD and SK-SD are normal. Similar observations have been reported in experimental animals (Rogers et al., 1975; Portaro et al., 1976; Weiss, 1978; Weller, 1978; Nayar, 1979; Kwa and Mak, 1980).

However, lymphocytes from all elephantiasis patients have responded to adult worm antigen and from about half of the amicrofilaraemic patients to microfilarial antigen (Pissens et al., 1980a). It should be noted that elephantiasis has been suggested to result from cell-mediated immune reaction against the adult worms (von Lichtenberg, 1957; Schacher and Sahyoun, 1967; Denham and Nelson, 1976). Lymphocyte reactivities to PPD and SK-SD remain normal (Pissens et al., 1980a). However, there is a suppression of delayed hypersensitivity skin reactions to Candida albicans, mumps and SK-SD and impairment of antibody responsiveness on immunization against tetanus and Salmonella typhi (Grove and Forbes, 1979). This immunosuppression may explain why W. bancrofti patients are prone to cutaneous infections, apart from lymph stasis and skin-folding, etc.. Comparable results have been obtained in animal models but variable responses to non-filarial antigens or mitogens have been reported (D'Alesandro and Klei, 1976; Weiss, 1978; Weller, 1978; Nayar, 1979).

The variety of immune responses observed may indicate the presence of a number of modulating mechanisms, which may extend back to the intra-uterine and prenatal immunological experience of the host (Dutta et al., 1976; Ottesen, 1980). The presence of circulating antigen or immune complexes may also play an important role in modulating the immune response of the host to the infection (Franks, 1946; World Health Organisation, 1977; Lambert et al., 1978; Kaattari et al., 1980).

Epidemiological Survey Methods of Lymphatic Filariasis in the Human Population

Diagnosis of human lymphatic filariasis can be achieved by clinical, parasitological and immunological methods. However, each method has its merits and demerits according to the purposes of the survey programmes.

Presence of Clinical Signs and Symptoms

The spectrum of clinical manifestations has been mentioned above. Features likely to distinguish tropical (filarial) eosinophilia syndrome from the other eosinophilia syndromes with pulmonary involvement have been summarized by Neva and Ottesen (1978). However, the same clinical signs can be caused by other diseases. Hydrocele of chronic, traumatic origin is common among tricycle drivers in tropical countries (Sasa, 1976). Price (1976) and Price and Henderson (1978) have suggested that non-filarial elephantiasis in East Africa is associated with the red clay of volcanic areas, the chemicals (eg. silicates) being absorbed through abrasions in the skin. Lymphoedema and consequent elephantiasis may be due to tuberculosis (Nelson, 1979). Although the standard of clinical diagnosis varies, results of clinical surveys are useful for comparison of the intensity of infection in

different endemic areas or for evaluation of control programmes (Sasa, 1976).

Demonstration of Microfilariae in the Blood

Parasitological demonstration of infection is the ideal way of diagnosing filariasis (World Health Organisation, 1974). Since adult worms can only be recovered on special occasions, the search for microfilariae in the blood is the only practical measure. Several methods have been used and their efficiencies have been discussed by Sasa (1976), Denham and McGreevy (1977) and McMahon *et al* (1979). The three most commonly used methods in decreasing order of sensitivity are membrane filtration, counting chamber and blood smear (thick film) techniques. Differential diagnosis of microfilariae can be made based on their morphology (eg. the presence of sheath, length, tail-shape, caudal nuclei, etc.) or the pattern of distribution of certain enzymes (Yen and Mak, 1978).

This parasitological method is specific but insensitive when the level of microfilaraemia is very low, eg. during the early stages of mass chemotherapy campaign when the overall microfilaria prevalence rate decreases and the number of light infections increases. Furthermore, for periodic species, blood samples have to be taken between 2100 and 0300 hours. Taking blood samples at these unsocial hours could be totally unacceptable to many people. To overcome this problem, a DEC provocative test which can be performed in daytime has been employed (eg. Sasa, 1976; McMahon *et al*., 1979). However, side effects due to DEC may occur, and this method is ineffective in subperiodic *W. bancrofti* infections (Weller and Ottesen, 1978).

Immunological Tests

Immunological (especially serological) tests, because of some of the following reasons, have become preferrable in epidemiological surveys of parasitic diseases.

(1) A small quantity (5 - 200 µl) of serum or plasma, which can be obtained anytime during the day, is usually sufficient. Eluates from blood spots, absorbed on chromatography paper, are also applicable.
(2) Prepatent, patent, latent as well as unisexual infections can be detected. In lymphatic filariasis, microfilariae are absent during early and advance obstructive stages of the disease.
(3) Large number of samples can be tested in a short time, and it is not necessary to do this immediately. Thus, it is more efficient and economical (especially in large scale survey programme) than parasitological methods.
(4) While surveys with parasitological methods can only indicate the point prevalence of the infection (recently acquired), serological antibody tests can indicate period prevalence (past infection) and incidence rates (among different age groups) of the infection at different times (Draper, 1976). These information are valuable to epidemiologists, especially when assessing the success of a control programme.

The advances in the immunodiagnosis of filariasis have been very slow, as compared to other parasitic diseases like malaria and schistosomiasis, etc. chiefly because of the lack of sufficient human parasite materials for specific antigens. Heterologous filarioid antigens obtained from animals have been widely used. During the past 60 years, a large number of studies have been performed, using a variety of tests and antigens. Excellent reviews of this subject have been given by Kagan (1963 and 1974), Denham et al (1971b), Ambroise-Thomas (1974) and Soulsby (1976).

Skin Test - This is a simple, rapid, sensitive and economical tool which has been widely used in epidemiological studies of filariasis. In filariasis, the wheal and flare reactions occurring within 30 minutes (the immediate hypersensitivity reactions) are prominent (Grove, 1930). About 190 reports have appeared so far, using antigens from

about 14 different filarioid species. W. bancrofti microfilarial antigen was reported sensitive and specific (Hunter, 1959). However, due to the difficulty in obtaining adequate amount of Wuchereria antigens, antigens of D. immitis, L. carinii and Setaria species, etc. were widely used. Cross-reactions with other helminths were common and the tests performed by different workers were not standardized at all (Kagan, 1963). Group reactions to filarioids were observed with D. immitis antigen (Cifferri et al., 1965). With the emergence of a purified antigen from D. immitis adult worms, prepared by Sawada et al (1965), there was a tendency to use mainly this antigen in skin tests for filariasis (eg. Desowitz et al., 1966; Sawada et al., 1968 and 1969). This purified antigen could also be obtained from the World Health Organisation (W.H.O.) for field use. However, this antigen was found to be non-specific, cross-reactions might occur up to 40% in non-endemic areas (reviewed by Ambroise-Thomas, 1974). Later, Chandra et al (1974) confirmed the sensitivity (up to 100%) and specificity of W. bancrofti antigen in skin tests and reported that it was superior to the Sawada's D. immitis antigen. Following the successful transmission of B. malayi in laboratory animals, B. malayi antigens were found to be similar to W. bancrofti antigens in skin tests of brugian and bancroftian filariasis (Grove et al., 1977; Chandra et al.; 1978). Thus, the present trend will be the use of B. malayi as antigen, perhaps a purified antigen in future (Grove, 1980).

In Vitro Tests for Cell-mediated Immunity - Leukocyte migration inhibition test, rosette test and lymphocyte transformation test have been used to study the cellular immune response in vitro (Bloch-Michel and Wultzing, 1973; Pinon and Gentilini, 1973; Ottesen et al., 1977; Pissens et al., 1980a). Some appeared to be specific. However, because of the relatively complicated procedures and the need of viable lymphocytes, these tests remain as basic research methods rather than epidemiological tools.

Complement Fixation Test (CFT) - This is one of the first tests used in diagnostic laboratories. More than 10 different filarial antigens have been used, but their sensitivities are not uniform and cross-reactions with non-filarial sera are observed (Kagan, 1963). Using microfilarial antigen of D. immitis, sensitivity rate of 76% was observed in filariasis as a group, as compared to about 10% with Sawada antigen (Ambroise-Thomas, 1974; Hedge and Ridley, 1977). In general, CFT is not a satisfactory test for filariasis.

Agglutination Tests - Agglutinations of D. immitis and B. pahangi microfilariae by immune dog sera have been demonstrated (Franks and Stoll, 1945; Wong, 1964b) Recently, Grove and Davis (1978) employed this technique in detection of W. bancrofti and B. malayi infections giving sensitivity rates of 31 - 40%. In general, inert particles (eg. red cells) coated with soluble filarial antigen are more commonly used.

(a) Indirect Haemagglutination Test (IHA) -

This test is highly reactive but does not demonstrate maximum sensitivity with sera from patients with early acute infections in which the specific antibody is of the IgM class (Kagan, 1979). Dammin and Weller (1945) found this method rather insensitive (13% positive) in the diagnosis of bancroftian filariasis. When used for other filariasis, fairly reliable results were obtained. However, cross-reactions occurred with about 10% of other parasitic infections and 35% of trichinosis, using D. immitis antigen, and with certain feverish conditions and syphilis, using O. volvulus antigen (Kagan, 1963; Kagan et al., 1963; Rosé et al., 1966; Ambroise-Thomas, 1974). A commercial IHA diagnostic kit for filariasis is now available from Italdiagnostic Co., Italy (Kagan, 1979). Recent studies with IHA are mainly concerned with onchocerciasis and little is known about its present status in lymphatic filariasis (Tada and Ikeda, 1976; Weiss and Degrémont, 1976; Ikeda et al., 1978 and 1979).

(b) Bentonite Flocculation Test -

This has been used for detection of filarial infections in man and animals in three occasions (Healy and Kagan, 1961; Adolph et al., 1962; Kagan et al., 1963). The test seemed to be most sensitive for acute infection but less sensitive and specific than the IHA.

Precipitation Tests - Simple precipitation tests in liquid media have been performed. However, the sensitivity and reactivity were very low (reviewed by Kagan, 1963). To date, precipitation tests are performed in media like agar gel.

(a) Ouchterlony Gel Diffusion (OIDT) -

This method has been employed for the detection of bancroftian filariasis, using L. loa, D. viteae or even Ascaris suum as antigens (Dodin et al., 1965; Niel et al., 1972; Petithory et al., 1972 and 1973). Sensitivities vary from 16 to 85% and as many as 7 precipitation bands have been observed. The fact that high concentrations of antigen and serum have to be used makes this technique unattractive for epidemiological use. However, it remains as an analytical tool for identifying common components among sera and antigens.

(b) Immunoelectrophoresis (IEP) -

This test can be used to identify specific antibody against different parasitic species (Kagan, 1974). Using a variety of antigens, O. volvulus, D. viteae, D. immitis, A. suum, etc., IEP has been used to detect human filariasis (Biguet et al., 1964; Capron et al., 1968; Wheeling and Hutchinson, 1971; Weiss and Degrémont, 1976). Characteristic precipitation arcs for loiasis, wuchereriasis and onchocerciasis have been suggested (Capron et al., 1968; Gentilini et al., 1972). This finding is comparable to the diagnostic band #5 in echinococcosis (Capron et al., 1970). Sensitivities from 0 to 63% have been reported in bancroftian filariasis. The test could reflect the level of endemicity of onchocerciasis in Upper Volta (D'Haussy et al., 1972). Concentrated sera and

antigen are required for this method, thus discouraging its common use in sero-epidemiology.

(c) Counter Immunoelectrophoresis (CIEP) -

This is a relatively new technique and its application in filariasis has not been evaluated. Using D. immitis or L. carinii as antigens, the sensitivity rate was 71% for bancroftian filariasis. However, 100% sensitivity was observed in detection of canine and feline filariasis using the homologous antigens (Desowitz and Una, 1976; Desowitz et al., 1978b; Dasgupta et al., 1980). This technique, being sensitive, simple and rapid to perform, may be useful in sero-epidemiological studies. However, it also suffers from the disadvantage that concentrated antigen is required.

Indirect Fluorescent Antibody Tests - This technique has been commonly used for the past 20 years in the detection of antibodies in brugian and bancroftian filariasis. Soluble as well as insoluble filarial antigens have been used.

(a) Soluble Antigen Systems -

In the Soluble Antigen Fluorescent Antibody Test (SAFA), cellulose acetate filter paper discs impregnated with soluble antigen of D. immitis or D. viteae adult worms have been used. The degree of fluorescence was measured objectively by a fluorometer (Duxbury and Sadun, 1967; Garcia et al., 1968; Colwell et al., 1970). The sensitivity rates for bancroftian filariasis vary between 85 to 100%.

In the Defined Antigen Substrate Spheres System (DASS), the soluble antigen is coupled to cyanogen bromide-activated sepharose 4B beads (Deelder et al., 1975). Using antigens of D. immitis or B. malayi, good sensitivity and specificity were observed in the detection of filariasis in jirds, dogs and man (Welch and Dobson, 1978; Singh et al., 1980a).

(b) Insoluble Antigen System (IFAT) -

Intact microfilariae have been used as antigens (Chowdhury and Schiller, 1962; Jayewardene and Wijayaratnam, 1968; Wong and Guest, 1969; Grove and Davis, 1978). Fluorescence occurs at the cuticle or sheath of the microfilariae, mainly with amicrofilaraemic sera. For better reactivities, papain-digested or sonicated microfilariae have been used (Mantovani and Sulzer, 1967; Kaliraj et al., 1979a; Gonzaga Dos Santos et al., 1976; Hedge and Ridley, 1977; Singh et al., 1980a and b). Cryosections of D. immitis and D. viteae adult worms are commonly used for diagnosis of bancroftian filariasis (Coudert et al., 1968; Gentilini et al., 1972; Ambroise-Thomas and Kien Truong, 1972). Glutaraldehyde-fixed adult worms of D. viteae embedded in methacrylate medium have been reported suitable. The egg shells and uterine fluid are found to be the most potent antigen for filariasis as a group (Diesfeld and Kirsten, 1979). In these systems, using heterologous antigens, sensitivity rates vary from 50 to 92%. However, higher sensitivity (up to 100%) and specificity for the detection of W. bancrofti and B. malayi infections have been achieved using cryosections of B. malayi adult worms or infective larvae of W. bancrofti and B. malayi as antigens (Wong and Guest, 1969; Ten Eyck, 1973; Yong, 1973; Grove and Davis, 1978).

IFAT is a sensitive and reliable technique which also permits the detection of the different classes of antibody responsible for the reaction. A few adult worms can provide cryosectioned-antigen for hundreds of tests. However, the requirements of high degree of subjectivity in reading the test (without a fluorometer) and expensive equipments, together with the technical difficulties in testing large numbers of sample (eg. over 400 per day) might mitigate against its wide application in sero-epidemiology of filariasis (Kagan, 1979).

Enzyme-linked Immunosorbent Assay (ELISA) - The principle and potential use of this new technique of the seventies in diagnostic medicine have been discussed by Voller et al (1976b) and W.H.O.(1976). To date, ELISA has already gained its recognised status as a sensitive and rapid sero-epidemiological tool in various parasitic diseases such as leishmaniasis, schistosomiasis, trichinosis and toxocariasis, etc. (Voller et al., 1976a; Ruitenberg et al., 1977; Ambroise-Thomas et al., 1978; de Savigny et al., 1979). Preliminary studies on onchocerciasis have shown that ELISA values of 94% of patient sera are well above those of the European and East Africa control sera (Bartlett et al., 1975). In view of its successful applications in other parasitic diseases, ELISA may be a suitable sero-epidemiological tool for filariasis.

Immunological Tests for IgE -

(a) Prausnitz-Kustner (PK) Test -

Several reports of the use of PK test in the detection of reagin (IgE) antibodies in bancroftian filariasis have been reviewed by Kagan (1963). D. immitis antigen was commonly used.

(b) Radioallergosorbent Test (RAST) -

This is a sensitive technique for measuring the amount of specific IgE antibodies to filarial antigen (Somorin and Heiner, 1976; Somorin et al., 1977).

Since specific IgE antibodies are not found in all individuals, the diagnostic or sero-epidemiological significance of these tests has not been evaluated (Kagan, 1963). However, they may be useful in diagnosing tropical eosinophilia syndrome.

Therefore, for brugian and bancroftian filariasis, skin test still has a significant epidemiological application. IFAT, using worm fragments or cryosections, is at present the most satisfactory and reliable technique. However, the potential applications of CIEP and especially ELISA require further investigations. The fundamental problem in the serology of filariasis is the availability of adequate human filarial materials for antigens. At present, most tests rely on the group reactivity of animal filarial antigens. Cross-reactions with other helminthic infections are observed. However, the degree of this cross-reactivity is unknown since most studies have used sera of people, suffering from the other helminthic infections, living in filariasis endemic areas (eg. Kagan et al., 1963; Duxbury and Sadun, 1967). Also, the use of certain animal filarial species as antigen seems to be unjustified in certain situations where the animal filarioid parasite is highly endemic (Garcia et al., 1968).

Objectives of This Study

In this study, attempts were made to look for a sensitive and specific heterologous antigen for the detection of antibodies to B. malayi and W. bancrofti infections by IFAT and ELISA. Studies were also performed with the B. pahangi-cat model, using CIEP, IFAT and ELISA. In IFAT, the efficiencies of the microfilariae, infective larvae or the adult worms of six different filarial species were compared. Crude or partially purified somatic antigen and metabolic antigens of B. pahangi were used in ELISA. The possible applications of the IFAT and ELISA, using the appropriate antigens, in the sero-epidemiological studies of bancroftian filariasis were studied.

In an attempt to develop a species-specific antibody test, the possibility of using species-specific isoenzymes as antigens was investigated using the B. pahangi-cat model.

Since the presence of circulating antigens would be a good indicator of active infections, ELISA and CIEP were also developed to detect circulating antigens in sera and urine of B. pahangi-infected cats and in sera of humans with B. malayi and W. bancrofti infections.

As preliminary histological examinations of tissues from B. pahangi-infected cats had revealed the localization of microfilariae in the kidneys (Dr. D.A. Denham, personal communications), some lesions in the kidneys were expected. Thus, the remaining part of this project was devoted to the immunopathological study of the renal lesions in B. pahangi-infected cats. Also, the histopathology of the spleen, lungs, livers, the infected lymphatics and lymph nodes was examined. The presence of circulating immune complexes in sera from cats infected with B. pahangi and humans infected with B. malayi and W. bancrofti was studied by radioimmunoassays.

CHAPTER TWO

DETECTION OF ANTIBODIES IN BRUGIA PAHANGI-INFECTED CATS

INTRODUCTION

B. pahangi infection in the cat is a unique laboratory model for human lymphatic filariasis. Studies of the relative efficiencies of different homologous and heterologous antigens and serological tests for the detection of antibodies in B. pahangi-infected cats would give indirect implications of the choice of antigens and serological tests in the human system.

The surface antigen of microfilariae seems to be stage-specific, for antibodies against the cuticle of microfilariae occur only after the infected animals have become amicrofilaraemic (Ponnudurai et al., 1974). However, it has been suggested that the larval stages may share some common antigenic determinants with the adults (Weiss, 1978). In this study, the antigenic relationships between the crude soluble extract (CSE) of microfilariae (mf), infective larvae (L3) and adult worms (L5) of B. pahangi were studied by Ouchterlony immunodiffusion test.

The serological changes during the course of B. pahangi infection in cats have been studied by IFAT, using only mf and L5 as antigens (Ponnudurai, 1974). In this study, the evolution of antibodies to L3, L5 and mf was investigated in 6 cats receiving a single infection with 100 - 200 L3 of B. pahangi by IFAT. ELISA using CSE and metabolic (ES) products of L5 of B. pahangi were also used.

The relative sensitivities of the mf, L3 and L5 antigens for the detection of homologous filarial infections in IFAT have not been reported. In this study, the relative sensitivities of IFAT, using mf, L3 and L5 of *B. pahangi* as antigens, in detecting *B. pahangi* infections in cats were studied. The results were also compared with IFAT using L5 of different heterologous filarioids, *D. immitis*, *D. vitae*, *L. carinii* and *O. gutturosa* as antigens.

CIEP, using CSE of *B. pahangi* L5 as antigens and ELISA, using CSE and ES antigens of *B. pahangi* were also employed for the detection of *B. pahangi* infections in cats. Finally, an overall comparison was made of the relative sensitivities of CIEP, IFAT and ELISA, using different antigens, for the detection of antibodies in *B. pahangi*-infected cats.

MATERIALS AND METHODS

Collection of B. pahangi from Infected Animals for In Vitro Cultures and for Antigens

The mf, L3 and L5 of B. pahangi were obtained from Dr. D.A. Denham, Department of Medical Helminthology. This filarioid was maintained in the laboratory by transmissions between mosquitoes (Aedes aegypti)and jirds and cats. Mosquitoes were infected by allowing to feed on an anesthetized B. pahangi-infected cat resting on the mosquito cage. After about 2 weeks, the infected mosquitoes were killed by insect spray and crushed lightly with a glass test tube on a glass plate. The mosquitoes were then transferred to a Baermann apparatus with 74 μ pores and the L3 were collected from the bottom of the apparatus 2 h later, in medium 199. Healthy L3 were collected with a tuberculin syringe under a stereomicroscope. Cats were then infected by injecting the prescribed number of L3 into the dorsum of the hind foot. Jirds were infected by intraperitoneal injections.

Mf obtained from the peritoneal cavities of infected jirds were transferred from one glass petri dish to another, allowing the peritoneal exudate cells to settle and adhere to the glass for 20 min., for about 12 times. The mf were finally washed thrice in phosphate buffered saline (PBS), pH 7.2 (see appendix for preparation) by centrifugation at 2,000 g for 15 min.

L3 obtained from mosquitoes were transferred to a petri dish. Mosquito tissues were removed by a pasteur pipette under a stereomicroscope. The larvae were then washed thrice in PBS as above.

L5 were obtained from the peritoneal cavities of infected jirds or lymphatics of infected cats. They were placed in medium 199 with a drop of methyridine (ICI), which paralysed the worms, for selection of intact worms and sex. Worms, free of host tissues, were collected and washed thrice in PBS.

In Vitro Culture of B. pahangi for Metabolic Antigens

Three chemically defined low molecular weight culture media were employed, NCTC 135, RPMI 1629 and Dulbecco's MEM (Gibco Biocult). Preliminary experiments were performed to determine the most suitable medium for the *in vitro* culture of mf, L3 and L5. Subsequent cultures were then performed in only one medium. Attempts were also made to improve the culture by supplementation of the medium with glucose and glycyl-L-histydl-L-lysine acetate (MW 444, Sigma) at final concentrations of 10 mg/ml and 500 ng/ml respectively.

12-ml threaded glass tubes (Labco Med. Supplies), sterilized by autoclaving at 15 lbs/sq. in. for 30 min, were used for the culture. The worms were first sterilized by keeping in 5 ml of the relevant culture medium, supplemented with a total of 1500 units of penicillin and 1000 µg of streptomycin, at room temperature for 2 h with 5 changes of the medium. About 2000 mf, 500 L3 and 5 - 10 L5 were then transferred (using sterile pasteur pipettes for mf and L3, and sterile dissecting needles for L5) to tubes containing 8 ml culture medium, supplemented with 150 units penicillin and 100 µg streptomycin per ml medium. The caps for the tubes were screwed tight, after sterilizing the mouth of the tube over a Bunsen flame. Culture of mf and L3 were kept at 28°C and L5 at 37°C. The degree of motility of the worms were examined twice daily (3, vigorous movement; 2, active; 1, slow movement; 0, idle or dead). The percentage survival and the

colour of the medium were noted. The culture medium was changed every 2 days or when the medium had become acidic (yellow). 0.1 mg gentamicin or 100 units mycostatin per ml medium were supplemented when bacterial or fungal contaminations were observed. The worms were removed when the degree of motility was 1 or when the percentage survival was less than 90%. The medium was then centrifuged at 1,000 g for 10 min and the supernatant was kept at -20°C. When about 300 ml had been collected, the medium was thawed and concentrated with simultaneous dialysis against PBS, pH7.2 in an Amicon hollow fibre concentrator (model DC-2) with exclusion limit at MW 10,000. After this process, about 15 ml of the concentrated solution, containing ES products of the worms with molecular weight larger than 10,000 were obtained. The protein content was determined (see below). The solution was then stored at -70°C in 300 µl aliquots.

Preparation of Crude Soluble Extract (CSE) of B. pahangi

Suspensions of mf or L3 in PBS, pH 7.2 contained in 5-ml glass vials were sonicated at 4°C, using an MSE ultrasonicator. Intermittent maximum pulses (30 microns peak to peak) were given until microscopic examination of the suspension showed that the worms had been broken into tiny fragments. About 1000 L5 were homogenised in 5 ml PBS, pH 7.2 in a glass homogenizer in an ice-bath until no clumps of worm remained. Extraction was allowed to proceed for 3 h at room temperature followed by 18 h at 4°C. After centrifugation at 20,000 g for 30 min at 4°C, the supernatant was stored at -70°C in 200 µl aliquots.

Measurement of Protein Concentrations of Antigen Preparations

The Folin-phenol method of Lowry et al (1951) was used. Three stock solutions were prepared : (a) 2% Na_2CO_3 in 0.1N NaOH; (b) 0.5% $CuSO_4.5H_2O$ in 1% Na-K tartrate; (c) Alkaline

copper solution — 50 ml (a) + 1 ml (b) (Prepared fresh). 0.2 ml sample, in duplicate, was added to 1 ml reagent (c) and mixed thoroughly. After standing at room temperature for 10 min, 0.1 ml Folin-phenol reagent (BDH) was added and mixed within 1 - 2 sec. After 30 min, the samples were made up to 3 ml with glass distilled water and read in a UV-spectrophotometer at 500 nm. Glass distilled water was used as the control. Duplicate protein standards prepared from bovine serum albumin (Sigma) at concentrations 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/ml were used for construction of the standardization curve from which the concentration of protein in the sample was determined.

Blood and Serum Samples from B. pahangi-infected Cats

Blood samples were collected from 6 cats, infected with 100 - 200 L3, 1 week before infection by venepuncture and once a week after infection by pricking the marginal ear vein. Microfilarial count was also performed on 10 - 100 mm^3 of blood in a counting chamber (according to Denham *et al.*, 1971a). At necropsy, 5 ml of blood were collected aseptically by cardiac puncture from these 6 cats and also from 76 cats infected with 100 - 200 L3 and 4 normal uninfected cats over a period of 1 year.

Since the presence of immune complexes in the cat sera was to be investigated in another study, the preparation of serum was performed according to the recommendation by W.H.O. (1977). To prevent the loss of immune complexes by cryoprecipitation, the blood was allowed to clot at 37°C in a glass centrifuge tube for 2 h. The serum was then obtained by centrifugation at 1,000 g for 10 min at room temperature.

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Preparation of IgG from Normal Cat Serum

This was performed according to Hudson and Hay (1976). 30 ml distilled water were mixed with 10 ml serum, to lower its ionic strength, and added to 50 g wet weight DEAE cellulose (DE52 Whatman) pre-equilibrated with 0.01M phosphate buffer, pH 8.0 (see appendix for preparation). The suspension was stirred every 10 min for 1 h at 4°C. The slurry was then poured onto a Buchner funnel containing 2 layers of Whatman No. 1 filter paper and sucked through the supernatant containing IgG for 30 sec. The cellulose was washed quickly with 3 volumes of 20 ml 0.01M phosphate buffer, pH 8.0. The combined effluent, containing IgG, was poured into a visking tubing (Scientific Instrument Centre Ltd) and concentrated by dialysis against a concentrated solution of polyethylene glycol, PEG (MW 20,000 Sigma) at 4°C. The concentrated preparation of IgG was then dialysed exhaustively against PBS, pH 7.2 at 4°C.

The purity of IgG was examined by IEP against rabbit antisera to cat IgG and whole serum (Miles Labs). The concentration of IgG preparation (in mg/ml) was estimated by the formula : Absorbence at 280 nm x 0.69.

Preparation of Rabbit Anti-sera

Antisera against cat IgG, CSE of *B. pahangi* mf, L3, L5 males and L5 males and females were prepared by intramuscular injection of 0.5 ml antigen, in an equal volume of complete Freund's adjuvant, into 5 separate sites on the thighs of the rabbits (New Zealand White). This was repeated 3 weeks after the first injection and every 2 weeks afterwards. Blood samples were taken every 3 weeks by venepuncture of the marginal ear vein for determination of the level of precipitating antibodies by OIDT. When a titre of at least 4 had been obtained, the rabbits were anaesthetized with pentobarbitone sodium, i.v. (Squibb and Sons Ltd) and exsanguined by cardiac puncture. The blood was kept at room temperature for 1 h

followed by 18 h at 4°C to allow complete clotting, in glass tubes. Serum was obtained by centrifugation at 1,000 g for 10 min and then stored at -20°C in 500 µl aliquots.

The specificity of the rabbit antiserum against cat IgG was compared with the commercial rabbit antiserum (Miles Labs) by IEP.

Ouchterlony Immunodiffusion Test (OIDT)

1% agarose (BDH) in 0.05M barbitone buffer, pH 8.6 (see appendix for preparation) containing 0.01% merthiolate (Sigma) was used. 2.5 ml heated agarose solution was poured onto a clean 76 x 25 mm microscopic slide to form a gel of 2 mm thick, on a levelling table. After solidification at room temperature for 30 min, the slides were kept in a moist chamber overnight at 4°C before use. Gel cutters and template (Shandon) were used to punch 6 wells (2 mm diameter) around a central well (6 mm diameter). 5 µl of antisera were transferred to the peripheral wells and 25 µl of antigen to the central well. Precipitation bands were allowed to develop for 48 h at 4°C in a moist chamber and were recorded. The gel slides were washed in 0.15M NaCl overnight at 4°C followed by a similar wash in distilled water. A piece of wet Whatman No. 3 filter paper was then placed on the gel slide and dried at 37°C. The precipitation lines were stained with Coomassie brilliant blue (see appendix for preparation) for about 15 min followed by destaining in an acetic acid : ethanol : distilled water (10 : 25 : 65 in volume) solution for 5 - 10 min.

Immunoelectrophoresis (IEP)

This was performed according to Sargent and George (1975) with some modifications. 1% agarose gel slides as for OIDT were used. An electrophoresis tank and a power pack (model SAE 2761) from Shandon were employed. Gel cutter

(Shandon) was used to punch 3 holes (4 mm diameter) in the agar about 1/3 of the way from the cathode, in which 25 µl of the antigen were transferred. Electrophoresis was then performed at room temperature at 8 mA/slide for 1½ h, using 0.05M barbitone buffer, pH 8.6 as electrode solution, Whatman No. 3 chromatography paper as bridges between gel and electrode solution. Two troughs were cut with a scalpel and relevant antisera were transferred. Development of precipitation lines and subsequent washing, drying and staining of the slides were performed as for OIDT.

Counter Immunoelectrophoresis (CIEP)

1% agarose of high electroendosmosis (type III Sigma) was used. Preparation of gel slides was the same as for OIDT. Four pairs of wells (4 mm diameter and 6 mm apart) were punched with a gel cutter and template (Shandon). 25 µl of CSE of *B. pahangi* L5 male and female, at protein concentration of 1 mg/ml, were transferred into the cathode wells and 25 µl of antiserum into wells on the anodic pole. Electrophoresis was performed at 8 mA/ slide for 1½ h. The precipitation bands were noted immediately and again after storage for 48 h at 4°C in a moist chamber. The slides were washed, dried and stained as in OIDT.

Indirect Fluorescent Antibody Test (IFAT)

Preparation of Antigens

(a) Microfilarial Antigen - 10 µl of the suspension containing about 50 - 100 microfilarial fragments in PBS, pH 7.2, prepared by sonication as described above, were transferred to 8 spots, 4 along each slide, on a chromic acid-cleaned microscopic slide (76 x 25 mm). The slides were dried for 1 h under a fan at room temperature. They were then wrapped in tissue paper and stored at -70°C until use.

(b) Infective Larvae and Adult Worm Antigens - L3 of B. pahangi and L5 of B. pahangi, D. immitis (from infected dogs), D. viteae (from infected jirds), L. carinii (from infected cotton rats) and O. gutturosa (from infected cattle) were obtained, frozen at -70°C, from Dr. D.A. Denham, Department of Medical Helminthology. About 1000 L3 and 2 - 5 male and female L5 were transferred into separate aluminium cups containing 0.2 ml Tissue-tek O.C.T. compound medium (Ames) and were frozen slowly in liquid nitrogen. They were kept at -70°C until use. 5 µ-thick cryosections were cut from these blocks with a cryostat (MSE) at -20°C. 8 sections, 4 along each side, were adhered onto a chromic acid-cleaned microscopic slide (76 x 25 mm), precoated with a thin smear of glycerine albumen (Raymond A. Lambs). The antigen slides were dried and stored as above.

Procedures of the Test

The antigen slides stored at -70°C were warmed up to room temperature in a dessicator to avoid condensation of moisture on the surface. The antigens were then fixed by immersing the slides into acetone for about 10 sec. After drying the slides in air, the 8 antigen sites were circled with a water-resistant pen (Marktex Texpen, U.S.A.). This would prevent mixing with adjacent serum samples. 50 - 100 µl of serum samples in 2-fold dilutions (1: 16 - 2048 performed in microtitre plates) were then transferred onto the relevant sites on the antigen-slide and the slides were incubated in a moist chamber at room temperature for 30 min. The slides were then transferred into Coplin jars containing PBS, pH 7.6 (see appendix for preparation). The PBS was changed immediately and washing was performed for 30 min with changes of PBS after 10 and 20 min. The slides were then removed from the Coplin jars and 1 ml of fluorescein-isothiocyanate (FITC) conjugated rabbit anti-cat IgG (Nordic) diluted in 0.1% Evans blue in PBS, pH 7.6 was pipetted onto each slide. After incubation at

room temperature for 30 min in a moist chamber, the slides were washed as before and finally mounted in buffered glycerol, pH 9.0 (see appendix for preparation). Fluorescence was examined at 100x magnification under a Leitz Ortholux fluorescence microscope, with transmitted light from a halogen light source through excitation filters KP500 and KP540 and an interference filter K510. A system of "3+, 2+, + and -" was used to record the intensity of fluorescence, in decreasing order. The highest dilution of serum gaining a 2+ fluorescence was regarded as the end-point. A titre of 16 or above was regarded as positive.

The optimal dilution of FITC-conjugate used in the test was determined by a checker-board titration of 4-fold dilutions (1: 16 - 1024) of a reference positive and a reference negative sera against different dilutions (1: 40, 60, 80, 100 and 120) of the conjugate. The optimal dilution of the conjugate was the one at which the highest titre for the reference positive serum was observed, while the reference negative serum remained negative (-).

Enzyme-linked Immunosorbent Assay (ELISA)

Preparation of Peroxidase-conjugated Rabbit Anti-cat IgG

The γ-globulin fraction of the rabbit antiserum against cat IgG, prepared in the laboratory, was performed according to Voller et al (1976c) with some modifications. 10 ml 36% Na_2SO_4 were added to a mixture of 5 ml antiserum and 5 ml PBS, pH 7.2. The mixture was then stirred gently at room temperature for 30 min. After centrifugation at 3,000 g for 10 min, the supernatant was discarded and the precipitate was washed twice in 18% Na_2SO_4 solution. The precipitate was finally dissolved in 1.5 ml 0.15M NaCl and the solution was dialysed exhaustively against 0.15M NaCl at 4°C. The protein concentration was determined by absorption at 280 nm and the final concentration

was adjusted to 5 mg/ml.

The method of conjugation of peroxidase to the γ-globulin fraction of the antiserum was according to Avrameas and Ternynck (1971). 10 mg of horse-radish peroxidase (Tpye VI Sigma) were dissolved in 0.1M phosphate buffer, pH 6.8 containing 1.25% glutaraldehyde (Grade II Sigma). After incubation at room temperature for 18 h, the solution was passed through a sephadex G-25 column (40 x 1.3 cm) equilibrated with 0.15M NaCl. The brown fractions (containing the activated enzyme) were pooled and concentrated to 1 ml by dialysis against a concentrated solution of PEG at 4°C. After concentration, 5 mg of the γ-globulin fraction of antiserum and 0.1 ml 1M CO_3-HCO_3 buffer, pH 9.5 were added and the mixture was left at 4°C for 24 h. Then, 0.1 ml of 0.2M lysine (Sigma) in 0.15M NaCl was added and the mixture was left at 4°C for 2 h. The mixture was finally dialysed exhaustively against PBS, pH 7.2 and the labelled antibody was precipitated with an equal volume of 36% Na_2SO_4 for 30 min at 4°C. The precipitate was washed twice with 18% Na_2SO_4 at 4°C and then dissolved in a minimal volume of distilled water. After exhaustive dialysis against PBS, pH 7.2 at 4°C, the solution was centrifuged at 20,000 g for 20 min at 4°C. The supernatant was transferred to 1 ml threaded brown bottle. Merthiolate (Sigma) was added to a final concentration of 0.01%. The peroxidase conjugate was then stored at 4°C.

Procedures for ELISA

The method used was similar to that of McLaren *et al* (1978) with some modifications. M129A polystyrene ELISA plates (Dynatech) were used for CSE of *B. pahangi* L5 antigen. However, M29 polyvinyl plates (Dynatech) were used for metabolic antigens of *B. pahangi* as preliminary observations had shown that the use of polyvinyl plates were necessary for better adsorption of ES antigens. Reagents employed were listed in

the appendix. 200 µl of antigen diluted in 0.05M CO_3-HCO_3 buffer, pH 9.6 were added to each well. The plates were incubated at room temperature inside a large sandwich box. After washing the plates 3 times (3 min each) with the washing solution, the plate were tapped dry against a piece of towel and 200 µl serum diluted in PBS-Tween (incubation buffer) were added, in duplicates. In each plate, 2 wells (8 for the first plate) were reserved for the reference positive serum, 2 for the reference negative serum and 4 for PBS-Tween. After incubation at room temperature for 2 h, the plates were washed as before. 200 µl of the peroxidase-conjugated rabbit anti-cat IgG diluted in the incubation buffer were added. The plates were then incubated in a moist-chamber overnight at 4°C. After 3 washings as before, 200 µl of the peroxidase substrate (orthophenylene diamine, Sigma) were added. The enzyme reaction was allowed to proceed at room temperature. The optical density (OD) values of the reference positive serum was measured by a Vitatron spectrophotometer at 492 nm every 5 - 10 min. The enzyme reaction was stopped, by adding 25 µl 2N H_2SO_4, when the OD values of the reference positive serum had reached a pre-determined value. The OD values of samples in each plate were corrected by multiplication with the factor 1/F, where

$$F = \frac{\text{Mean OD Value of Reference Positive Serum in the Plate}}{\text{Predetermined OD Value of Reference Positive Serum}}$$

The mean of the corrected OD values of the duplicate was taken as the final OD value of a particular sample.

Optimization of ELISA

A checker-board titration was performed, using antigen with protein concentrations of 0.4 - 4 µg/ml, reference positive and negative sera at dilutions 1: 200 - 3200 and peroxidase conjugate at dilutions 1: 600 - 10,000. The optimal concentration of antigen and the optimal dilutions of serum and conjugate were the conditions in which the highest ratio

(about 10) of the OD value of the reference positive serum to that of the reference negative serum was obtained, after the enzyme reaction had proceeded for 20 - 30 min at room temperature.

Determination of Cut-off Value

The cut-off value, i.e. OD value above which the sample would be regarded as positive, was taken as $\bar{x} + 3$ S.D., where $\bar{x}$ was the mean of the OD values of 15 normal cat sera obtained at the optimal conditions and S.D. the standard deviation of $\bar{x}$ (according to de Savigny and Voller, 1980). Thus, the cut-off value would be above the 99% confidence limit of the distribution of OD values of the normal sera. When necessary, the OD values of the normal sera were transformed, according to Lutz (1973), in order to obtain a normal distribution for estimation of $\bar{x} + 3$ S.D..

Estimation of Sensitivity and Specificity of Serological Tests

This was performed according to Youden (1950). Sensitivity was determined by the formula a/(a+b), where a was the number of samples which were parasitologically and serologically positive and b the number of samples which were parasitologically positive but serologically negative.

Specificity was estimated by the formula d/(c+d), where c was the number of samples which were parasitologically negative but serologically positive and d the number of samples which were negative parasitologically and serologically.

RESULTS

In Vitro Culture of B. pahangi for Metabolic (ES) Antigens

In vitro culture of mf, L3 and L5 was performed in duplicate tubes, using 3 different culture media : NCTC 135, RPMI 1629 and Dulbecco's MEM. The experiment was repeated 4 times. It was observed that the longest period of survival for mf was 6 days in NCTC 135, 19 days for L3 in RPMI 1629 and 6 days for L5 in RPMI 1629 (Table 2.1). Subsequent cultures of mf, L3 and L5 were then performed in these media respectively. It was also observed in later cultures that mf could survive up to 12 days, L3 up to 23 days and L5 up to 10 days *in vitro*. Supplementation of the culture media with glucose and glycyl-L-histydl-L-lysine acetate at the final concentrations of 10 mg/ml and 500 ng/ml respectively did not prolong the survival of the worms *in vitro*. The female L5 produced mf throughout the period of survival *in vitro* but the quantity of mf produced decreased with time. No development of L3 and mf was observed. The protein concentrations of molecules of molecular weight larger than 10,000 collected from the culture media, presumably containing ES products, were 600 ng/100 mf/ml medium /day, 6 µg/1000 L3/ml medium /day and 5 µg/ L5/ml medium/ day.

Preparation of IgG from Normal Cat Serum and Rabbit Anti-cat IgG

The final concentration of IgG prepared from normal cat serum was 5.5 mg/ml. This preparation was pure IgG for IEP revealed only one precipitation line against anti-cat IgG and anti-cat whole serum (Fig. 2.1a).

The antiserum subsequently raised in the rabbit against cat IgG was specific. It showed only one precipitation line against whole cat serum and was identical to that of the

Table 2.1 The period of survival of the microfilariae, infective larvae and adult worms of *B. pahangi in vitro*.

B. pahangi	Temperature (°C)	Period of Survival (Days) Culture Media Used		
		NCTC 135	RPMI 1629	Dulbecco's MEM
Adult Worms	37	2 - 4	3 - 6*	1
Infective Larvae	28	1 - 4	3 - 19*	1 - 2
Microfilariae	28	6*	2	1

* Media chosen for subsequent cultures

Table 2.1 The period of survival of the microfilariae, infective larvae and adult worms of *B. pahangi in vitro*.

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Microfilariae	28	6*	2	1

* Media chosen for subsequent cultures

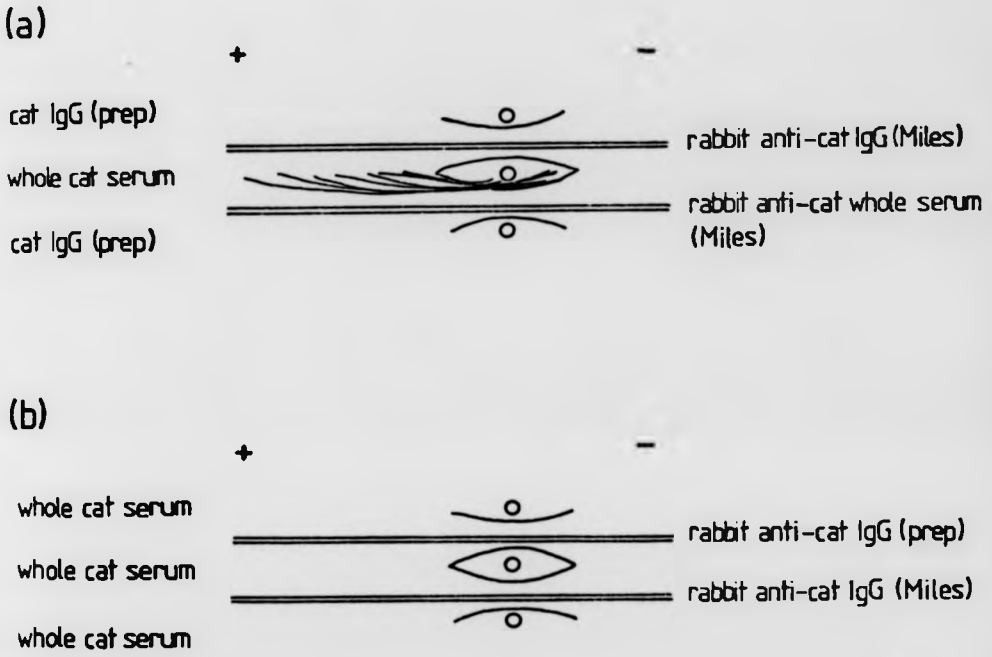


Fig. 2.1 Immunoelectrophoresis -

(a) The purity of the cat IgG preparation (prep) was tested against commercial rabbit anti-cat IgG and rabbit anti-cat whole serum (Miles Labs). Whole cat serum was included for comparison;

(b) Comparison of the specificity of the anti-cat IgG raised in the rabbit (prep) with the commercial antiserum (Miles) by reacting against whole cat serum.

commercial rabbit antiserum against cat IgG (Miles Labs) in IEP (Fig. 2.1b). Its titre in OIDT was 8, as compared to 4 for the commercial antiserum.

Antigenic Relationships between CSE of Different Developmental Stages of B. pahangi

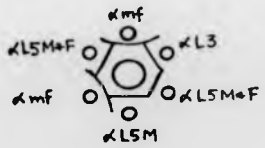
The protein concentrations of the CSE of mf, L3, male L5 and male and female L5 were 0.52, 0.7, 0.25 and 1.4 mg/ml respectively. They were adjusted to 1 mg/ml for the OIDT.

The antigenic relationships between the CSE of mf, L3, L5 male and L5 male and female were studied by reacting them with the rabbit antisera against these antigens (∠mf, ∠L3, ∠L5M and ∠L5M&F respectively) in OIDT (Fig. 2.2). All these developmental stages shared some common antigenic components. The precipitation lines between ∠mf and CSE-mf was partially identical to those between ∠L5M&F, ∠L5M, ∠L3 and CSE-mf (Fig. 2.2a). 3 Antigenic components were observed in CSE-L3, 2 were shared between all 3 developmental stages and no precipitation line specific for L3 was observed (Fig. 2.2b). Two antigenic components were observed in CSE-L5M. The outermost line was shared by all developmental stages except L3 (Fig. 2.2c). However, 3 antigenic components were observed in CSE-L5M&F (Fig. 2.2d). The outermost line seemed to be specific for adult males and the middle line for adult females.

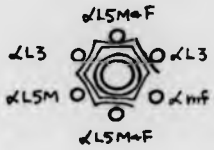
Detection of Antibodies in B. pahangi-infected Cats

Serum samples collected from 82 B. pahangi-infected cats, 31 untreated, 7 treated with DEC, 9 with mebendazole (MBZ), 5 with flubendazole (FBZ) and 30 with other drugs (but sample size of each was too small to be grouped individually) and from 15 normal uninfected cats were tested for antibodies against B. pahangi. 12 cats, 11 of which had received drug treatment, were amicrofilaraemic. Live L5 were not found but masses of dead worms were observed at necropsy.

(a) CSE - mf



(b) CSE - L3



(c) CSE - L5M

(d) CSE - L5M+F

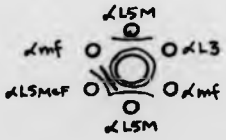


Fig. 2.2 Study of the antigenic relationship between the crude soluble extract (CSE) of B. pahangi (a) microfilariae (mf), (b) infective larvae (L3), (c) adult male (L5M) and adult male and female (L5M&F) by reacting them with the rabbit antisera (∝) against all these CSE, in Ouchterlony immunodiffusion test. Antigen (CSE) was placed in the centre well and antiserum in the peripheral wells.

CIEP

B. pahangi L5 male and female CSE (protein concentration at 1.2 mg/ml) was used as the antigen. A total sensitivity rate of 73% was observed (Table 2.2). Treatment in groups 4 and 5 increased the sensitivity rate by 20 - 30%. In group 1, the test was positive as early as 24 days post-infection and until at least 359 days post-infection. There was no relationship between the positivity of the test and the period post-treatment in groups 2 - 5. Sera from the 15 normal uninfected cats were all negative, 100% specificity.

IFAT

An example of the determination of the optimal dilution of FITC-conjugated antiserum is given in Table 2.3. 1: 80 was taken as the optimal dilution because the highest titre (64) was obtained for the reference positive serum while the reference negative serum did not give any fluorescence.

The optimal dilutions of the FITC-conjugate used in IFAT with B. pahangi L3, L5 and mf; D. immitis L5; D. viteae L5; L. carinii L5 and O. gutturosa L5 antigens were 1: 120, 80, 120, 60, 80, 60 and 60 respectively.

Apple-green fluorescence was observed mainly at the cuticle of cryosections of the L5 but sometimes at the lining of internal organs (Fig. 2.3a). For L3, fluorescence was observed at the cuticle as well as the lining of internal organs (Fig. 2.3c). Intense fluorescence was observed at the broken ends of sonicated mf fragments. The intensity of subcuticular fluorescence was only light or absent (Fig. 2.3e). Fluorescence at the cuticles of L5 and L3 and at the broken ends of mf fragments was taken as positive. In a negative reaction, the worm appeared red, due to Evan's blue (Figs 2.3 b, d and f).

Table 2.2 . The sensitivity and specificity of Counter Immunoelectrophoresis (CIEP) in the detection of antibodies in B. pahangi-infected cats

Groups	Sample Size	Sensitivity(%)	Specificity(%)
1. Infected, untreated	31	61	-
2. Infected, DEC Rx	7	43	-
3. Infected, MBZ Rx	9	67	-
4. Infected, FBZ Rx	5	80	-
5. Infected, Other Rx	30	93	-
Total (1-5)	82	73	-
6. Normal, Uninfected	15	0	100

Rx - Treatment; DEC - Diethylcarbamazine;
MBZ - Mebendazole; FBZ - Flubendazole

Table 2.3. Determination of the optimal dilution of FITC-conjugated rabbit anti-cat IgG (Nordic) used in Indirect Fluorescence Antibody Test (IFAT), with cryosections of B. pahangi adult male and female worms as antigens, for the detection of antibodies in B. pahangi-infected cats.

Conjugate Dilutions	Serum Dilutions 1:								Remarks
	Reference Positive				Reference Negative				
	16	64	256	1024	16	64	256	1024	
1 : 40	3+	3+	3+	2+	2+	+	-	-	
1 : 60	2+	2+	2+	+	2+	-	-	-	
1 : 80	2+	2+	+	-	-	-	-	-	Taken as optimal
1 : 100	2+	+	-	-	-	-	-	-	
1 : 120	+	-	-	-	-	-	-	-	

The intensity of fluorescence was graded from 3+ to + where a 2+ reaction was taken as the end-point. A negative reaction was given a "-" sign.

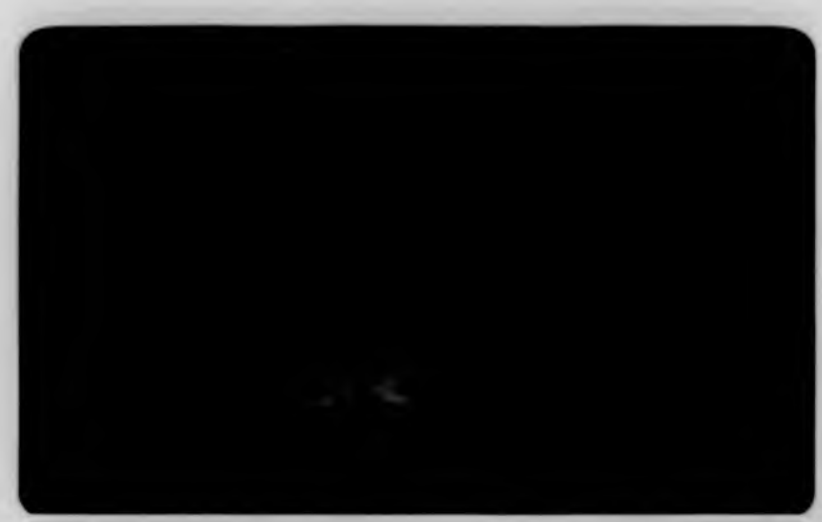


Fig. 2.3a Indirect Fluorescent Antibody Test using cryosection of D. immitis adult worm as antigen. In a positive reaction, apple-green fluorescence was observed at the cuticle and sometimes the lining of internal organs. 250 x magnification.

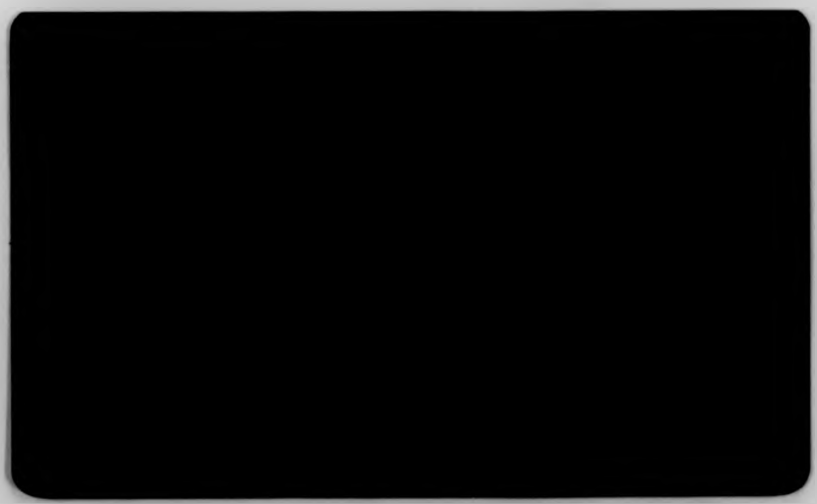


Fig. 2.3b Indirect Fluorescent Antibody Test using cryosection of D. immitis adult worm as antigen. In a negative reaction, the worm appeared red and no fluorescence was observed at the cuticle. 250 x magnification.

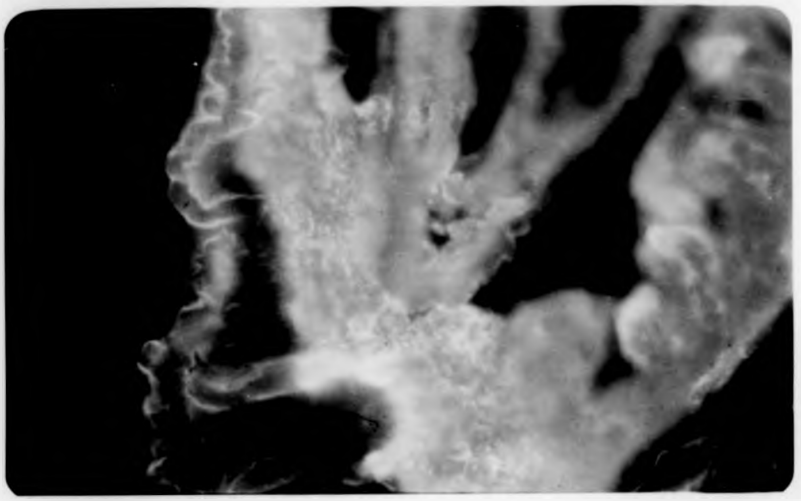


Fig. 2.3a Indirect Fluorescent Antibody Test using cryosection of D. [illegible] adult worm as antigen. In a positive reaction, apple-green fluorescence was observed at the cuticle and underlying the lining of internal organs. 250 x magnification.

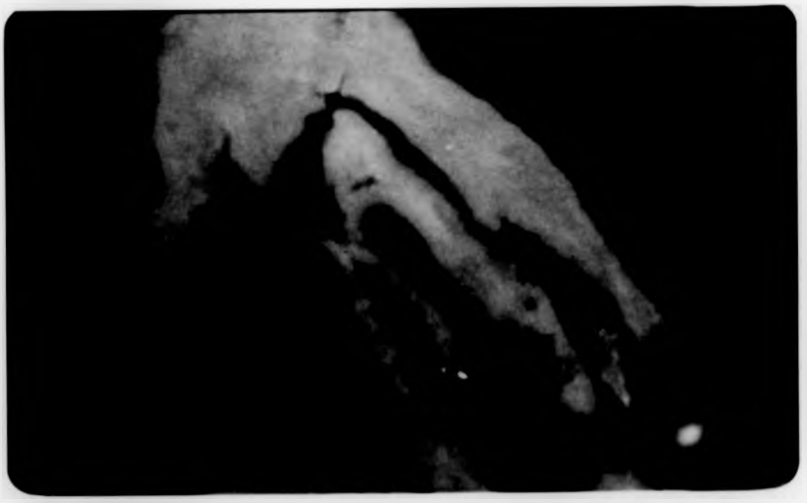


Fig. 2.3b Indirect Fluorescent Antibody Test using cryosection of D. [illegible] adult worm as antigen. In a negative reaction, tissue was counterstained red and no fluorescence was observed at the cuticle. 250 x magnification.

Fig. 2.3c Indirect Fluorescent Antibody Test using cryosection of B. pahangi infective larvae as antigen. In a positive reaction, apple-green fluorescence was observed at the cuticle and also the lining of the internal organs. 250 x magnification.

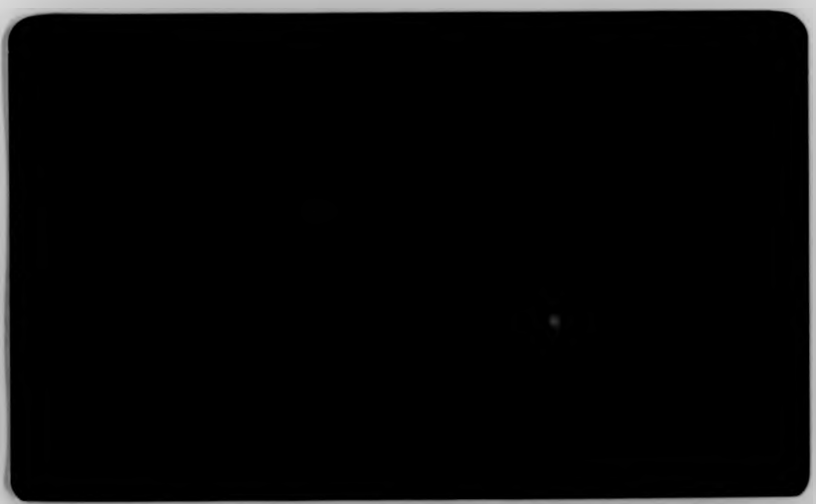


Fig. 2.3d Indirect Fluorescent Antibody Test using cryosections of B. pahangi infective larvae as antigen. In a negative reaction, the worms appeared red. 250 x magnification.

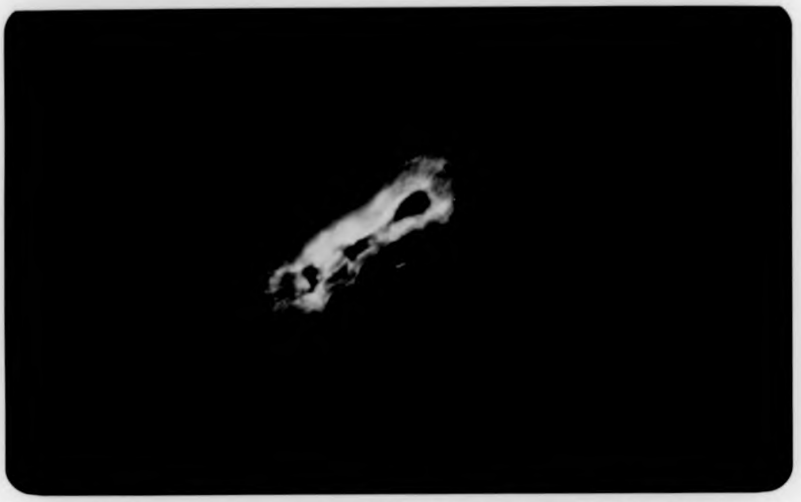


Fig. 2.3c Indirect Fluorescent Antibody Test using cryosection of B. pahangi infective larvae as antigen. In a positive reaction, apple-green fluorescence was observed at the cuticle and also the lining of the internal organs. 250 x magnification.

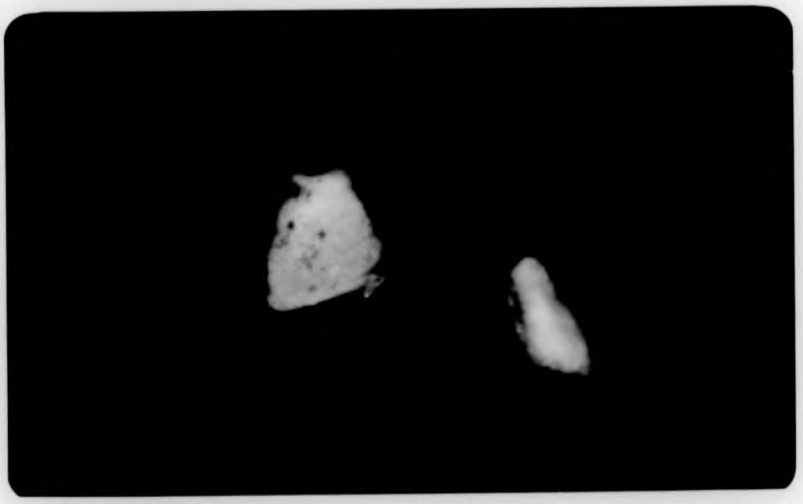


Fig. 2.3d Indirect Fluorescent Antibody Test using cryosections of B. pahangi infective larvae as antigen. In a negative reaction, the worms appeared red. 250 x magnification.

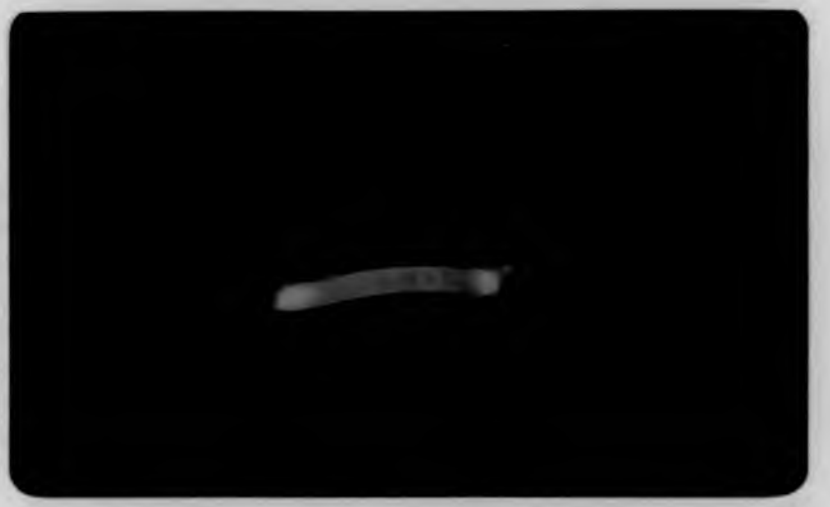


Fig. 2.3e Indirect Fluorescent Antibody Test using sonicated fragments of B. pahangi microfilariae as antigen. Intense fluorescence was observed at the broken ends in a positive reaction. The intensity of subcuticular fluorescence was only light or absent. 250 x magnification.

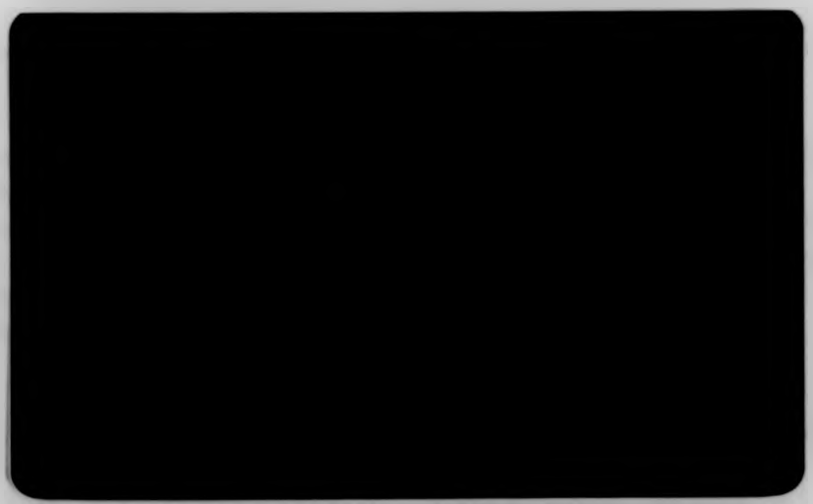


Fig. 2.3f Indirect Fluorescent Antibody Test using sonicated fragments of B. pahangi microfilariae as antigen. In a negative reaction, the whole fragment appeared red. 250 x magnification.

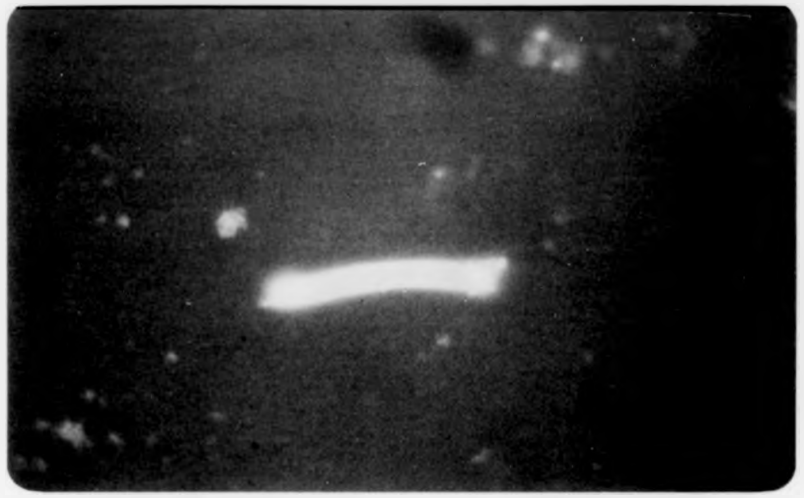


Fig. 2.3e Indirect Fluorescent Antibody test using sonicated fragments of *E. coli* microfilariae as antigen. Intense fluorescence was observed at the broken ends in a positive reaction. The intensity of subsurface fluorescence was only light or absent. 250 x magnification.

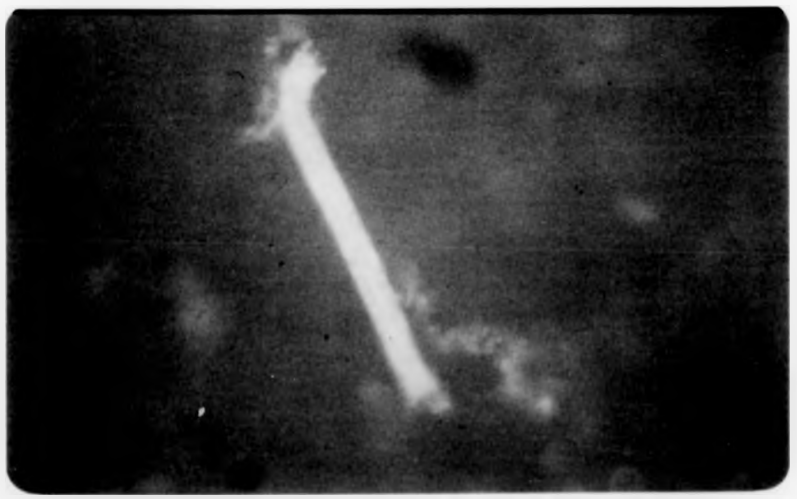


Fig. 2.3f Indirect Fluorescent Antibody Test using sonicated fragments of *E. coli* microfilariae as antigen. In a positive reaction, the whole fragment appeared red. 250 x magnification.

The changes in mean antibody response against the L3, L5 and mf in 6 B. pahangi-infected cats during the course of a single infection are shown in Fig. 2.4. Antibodies against L3 were first observed as early as 1 week post-infection (Fig. 2.4a). By week 8, when some female worms had started producing mf (Fig. 2.4d), all 6 cats had detectable antibodies to L3. The mean peak response (GMT = 74) occurred at week 13. The mean antibody response declined from week 22 and remained at low level till at least week 26.

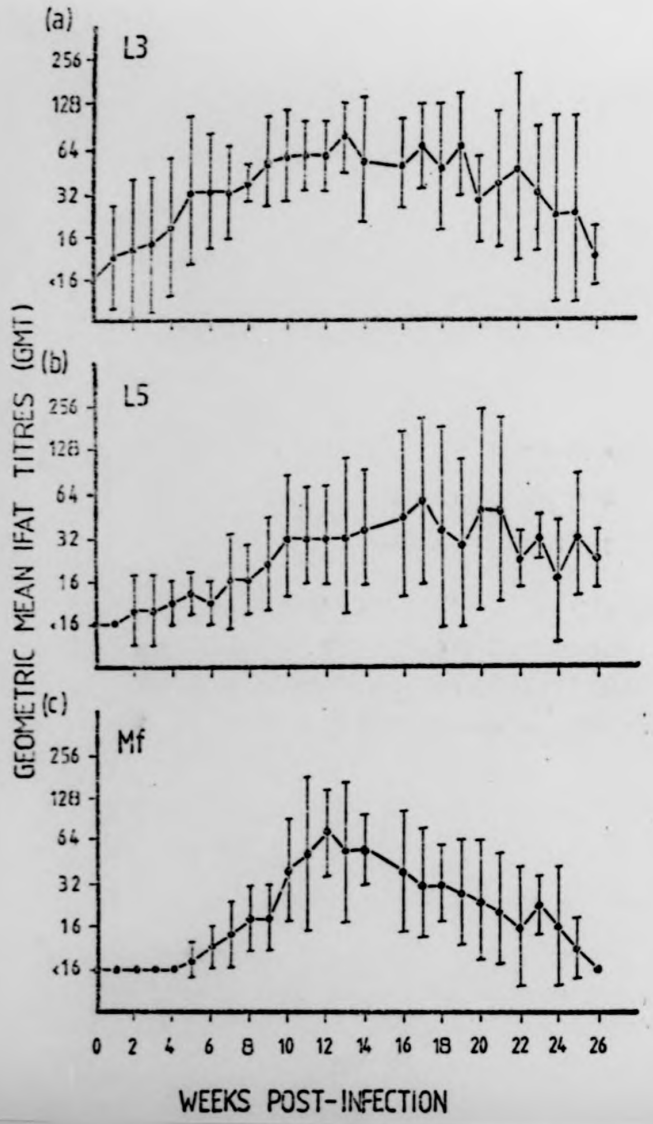
Antibodies against L5 appeared as early as 2 weeks post-infection, when the L3 moulted to the fourth-stage larvae (Fig. 2.4b). By week 11, antibodies to L5 could be detected in all 6 cats. The mean peak response occurred at week 17 (GMT = 56) and levelled off afterwards till at least week 26.

Antibodies to mf first occurred at 5 weeks post-infection, when the adults had migrated to the afferent lymphatics (Fig. 2.4c). By week 10, all cats produced detectable antibodies to mf. At this time, all but one cat were microfilaraemic. The mean peak response (GMT = 74) occurred at 12 weeks and declined gradually afterwards. By week 26, no antibody could be detected in the 2 cats studied. However, the level of microfilaraemia started to increase at week 12 and remained at high levels till week 21 (Fig. 2.4d). Thus, an inverse relationship was observed between the mean level of antibodies against mf and the mean level of microfilaraemia between weeks 12 and 21.

The sensitivities and the geometric mean titres of the IFAT, using seven different homologous and heterologous antigens, in the detection of antibodies in 82 B. pahangi-infected cats are shown in Table 2.4. Among the 3 developmental stages of B. pahangi, the highest sensitivity rate (total 89%) was obtained using the L3 as antigen. The total sensitivity rate of IFAT using L5 as antigen was the same as that using mf as antigen

Fig. 2.4 The changes in mean antibody response against (a) the infective larvae (L3), (b) adult worms (L5) and (c) microfilariae (Mf), measured by the Indirect Fluorescent Antibody Test (IFAT) and (d) in the mean microfilaraemia during the course of a single infection with 100 - 200 L3 in 6 cats.

Error flag or number beside point = 1 S.D.
Sample size between weeks 20 and 21 = 5,
" " " " 22 and 26 = 2.


(a)
L3
256
128
64
32
16
<16
(b)
L5
256
128
64
32
16
<16
(c)
Mf
256
128
64
32
16
<16
GEOMETRIC MEAN IFAT TITRES (GMT)
0 2 4 6 8 10 12 14 16 18 20 22 24 26
WEEKS POST-INFECTION

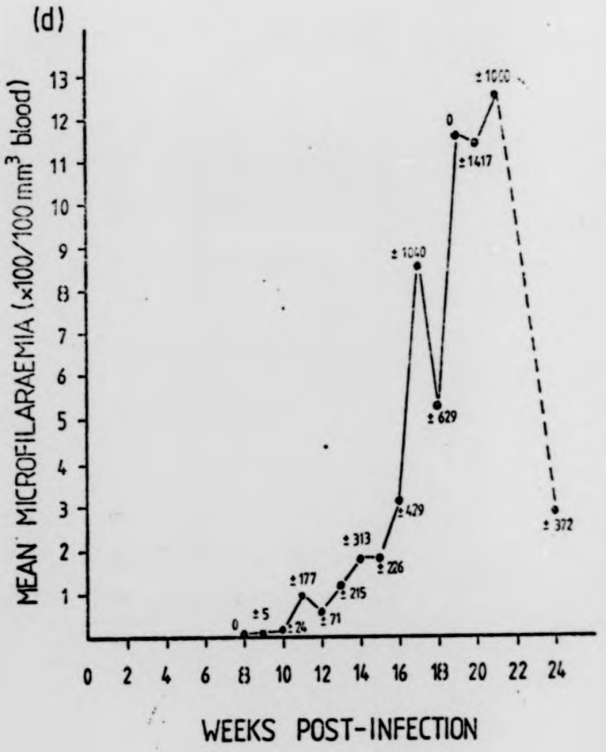

(d)
MEAN MICROFILARAEMIA (×100/100 mm³ blood)
1 2 3 4 5 6 7 8 9 10 11 12 13
0
±5
±24
±177
±71
±215
±313
±226
±429
±1040
±629
0
±1417
±1000
±372
0 2 4 6 8 10 12 14 16 18 20 22 24
WEEKS POST-INFECTION

Table 2.4 . The sensitivities and geometric mean titres (GMT) of Indirect Immunofluorescence Antibody Tests (IFAT), using seven different homologous and heterologous filarial antigens, for the detection of antibodies in B. pahangi-infected cats.

		SENSITIVITY (GMT±S.D.) ANTIGENS						
		B. pahangi			D. immitis	D. viteae	L. carinii	O. gutturosa
Groups	N	L5	L3	Mf	L5	L5	L5	L5
1. Infected, untreated	31	77 (36±2.9)	81 (61±3.8)	71 (45±3.5)	39 (30±2.2)	45 (32±2.9)	42 (29±2.5)	45 (32±3.3)
2. Infected, DEC Rx	7	57 (91±3.8)	71 (74±4.9)	71 (111±3.8)	57 (27±1.4)	43 (81±2.2)	43 (64±2.0)	43 (51±2.9)
3. Infected, MBZ Rx	9	100 (43±3.0)	100 (75±3.6)	100 (220±3.8)	78 (48±3.3)	89 (117±3.3)	100 (40±2.5)	89 (54±2.0)
4. Infected, FBZ Rx	5	80 (108±2.8)	100 (338±2.2)	100 (97±3.5)	60 (81±2.9)	80 (54±5.1)	60 (161±4.2)	80 (76±6.2)
5. Infected, other Rx	30	93 (82±3.5)	97 (98±2.9)	93 (105±3.4)	87 (49±2.8)	87 (55±2.9)	93 (43±2.9)	87 (52±2.9)
Total (1-5)	82	84 (58±5.9)	89 (86±3.5)	84 (88±3.7)	63 (43±2.9)	67 (54±3.2)	68 (46±2.9)	67 (47±3.0)
Days p.i. +ve as early as		24	7	35	24	24	81	24
+ve till at least		510	510	510	375	375	375	375
Normal	15	0	0	0	0	0	0	0

N: Sample size ; Rx: treatment ; p.i. : post-infection

(84%). There were significant ($p < 0.001$) positive correlations between the IFAT titres using L5 and L3 ($r = 0.66$), L5 and mf ($r = 0.62$), and L3 and mf ($r = 0.62$) as antigens. The sensitivity rates of IFAT using homologous antigens (84 - 89%) were also higher than those (63 - 68%) using heterologous filarial antigens (Table 2.4). Higher mean titres were also observed with homologous antigens (58 - 88 as compared to 43 - 54). In fact, the IFAT titres were never above 512 when heterologous antigens were used (Fig. 2.5). The best antigen for IFAT, in the detection of antibodies in B. pahangi-infected cats seemed to be the cryosections of B. pahangi L3. Apart from the highest sensitivity rate observed, the test could detect an infection as early as 7 days post-infection and remained positive till at least 510 days post-infection (Table 2.4). All the 15 sera from normal uninfected cats were negative in the IFAT using different antigens, i.e. 100% specificity. There were no correlations between the IFAT titres against the different antigens and the number of live L5 recovered at necropsy, the last mf count, the period post-infection and drug treatment.

After chemotherapy with DEC, MBZ and FBZ, the geometric mean titres were in general higher, though not statistically significant, as compared to the untreated cats which had been infected for similar periods of time (Fig. 2.6).

No cuticular fluorescence of mf fragments was observed with sera from 12 cats which were amicrofilaraemic.

ELISA

An example of the determination of optimal conditions for ELISA is given in Fig. 2.7. The OD values decreased with dilutions of antigens and sera (Fig. 2.7a). The highest ratio of the OD value of the reference positive serum to that of the

Fig. 2.5 The distribution of antibody titres for sera from 82 *B. pahangi*-infected cats against 7 different filarial antigens in the Indirect Fluorescent Antibody Test (IFAT).

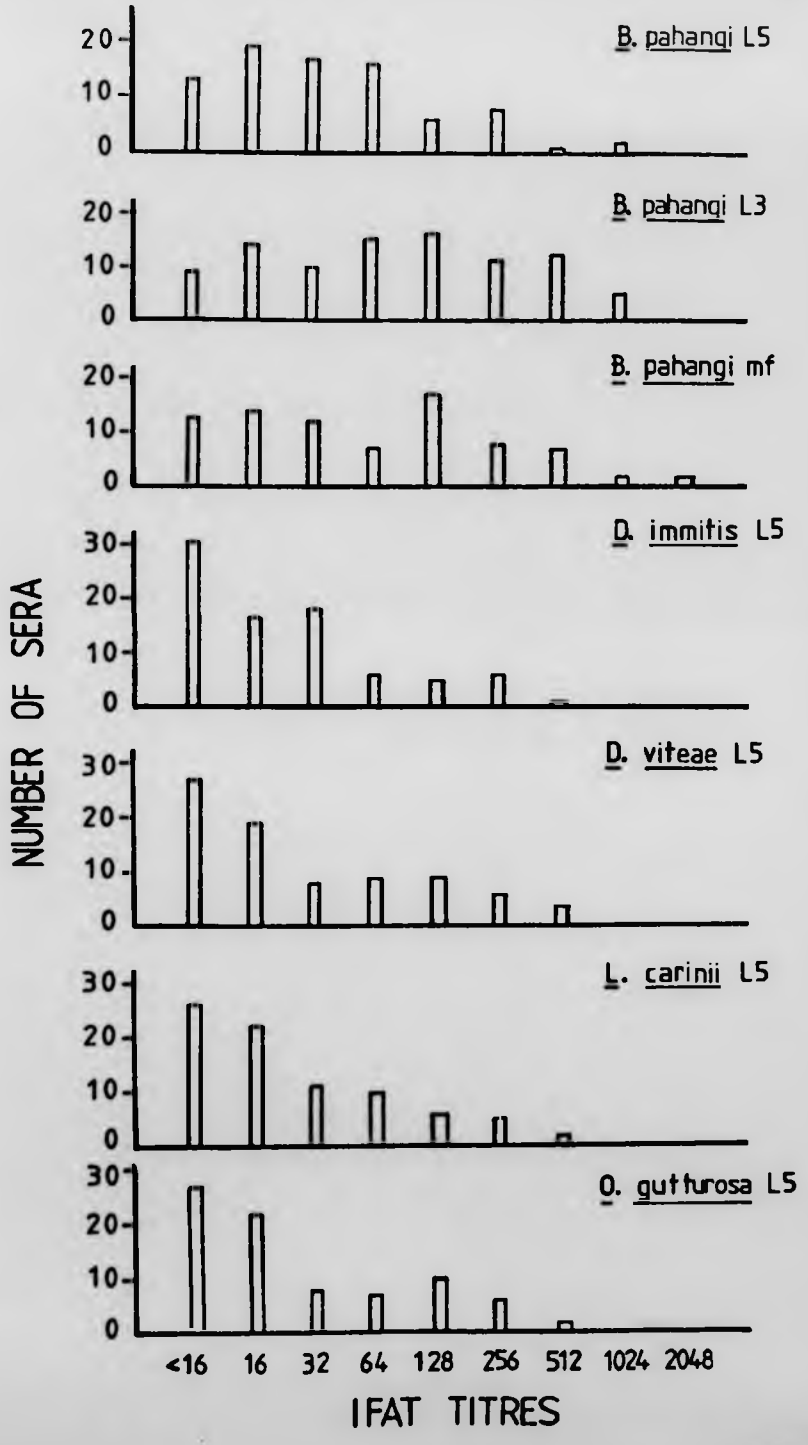

B. pahangi L5
B. pahangi L3
B. pahangi mf
D. immitis L5
D. viteae L5
L. carinii L5
O. gutturosa L5
NUMBER OF SERA
<16 16 32 64 128 256 512 1024 2048
IFAT TITRES

Fig. 2.6 The antibody titres against B. pahangi (a) adult (L5), (b) infective larvae (L3) and (c) microfilariae (Mf) antigens in the Indirect Fluorescent Antibody Test (IFAT) in sera from B. pahangi-infected cats, which were untreated or treated with diethylcarbamazine (DEC), mebendazole (MBZ) and flubendazole (FBZ).

All the cats were 90 - 200 days post-infection with 100 - 200 L3.

—— denotes the geometric mean titre.

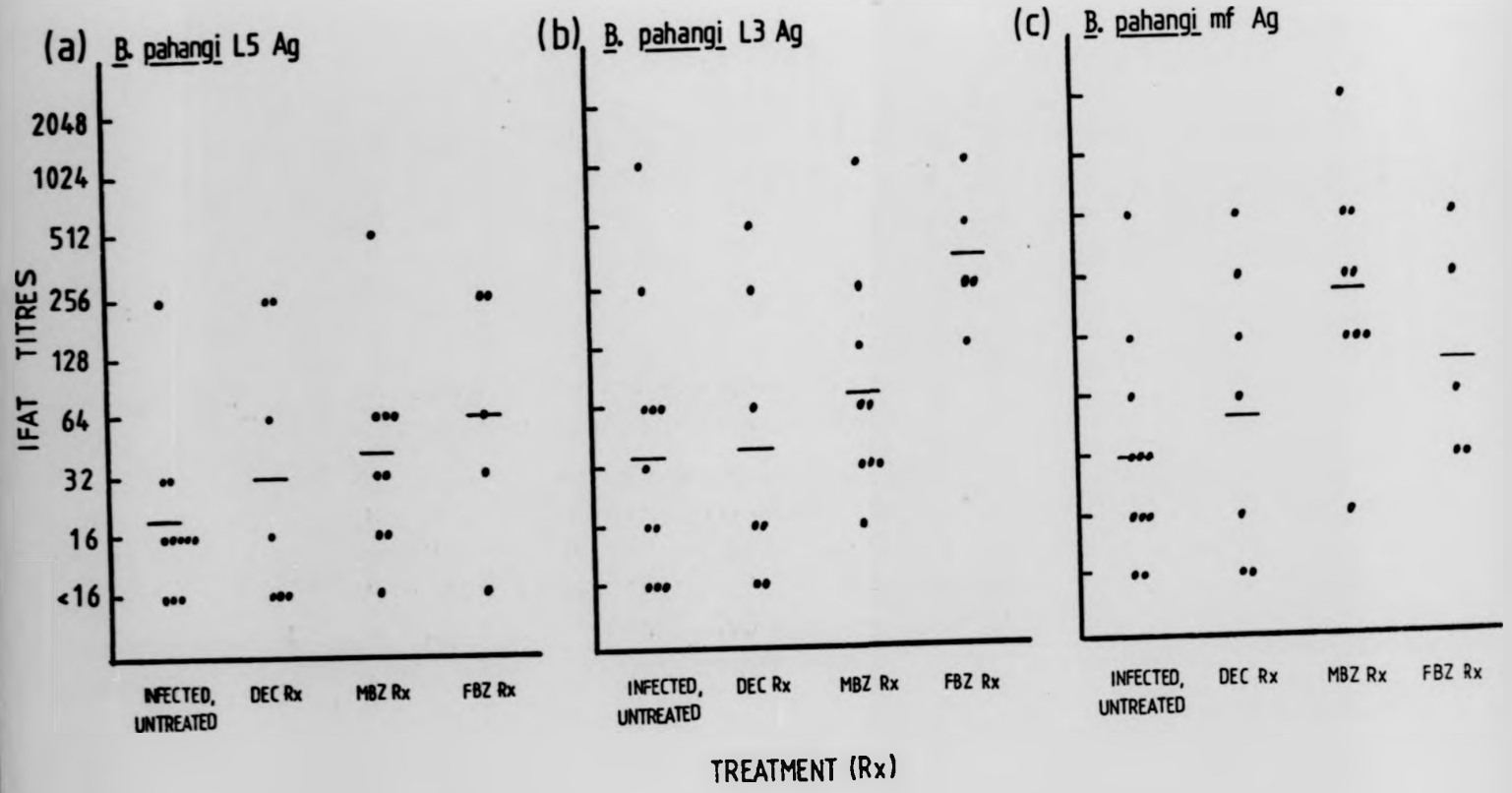
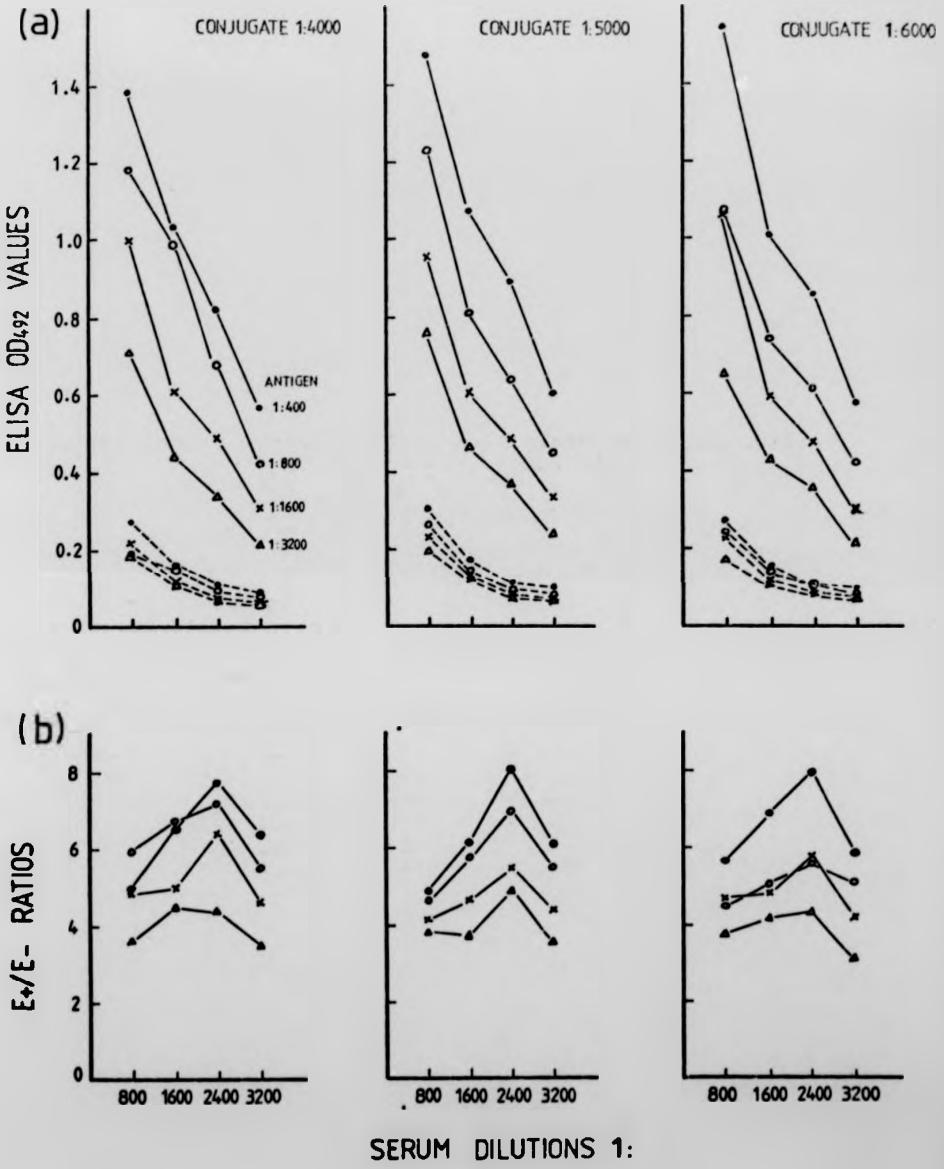

(a) B. pahangi L5 Ag
(b) B. pahangi L3 Ag
(c) B. pahangi mf Ag
IFAT TITRES
2048
1024
512
256
128
64
32
16
<16
INFECTED, UNTREATED
DEC Rx
MBZ Rx
FBZ Rx
TREATMENT (Rx)

Fig. 2.7 Determination of the optimal concentration of antigen (B. pahangi adult worm extract) and dilutions of serum and conjugate in the enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies in B. pahangi-infected cats.

(a) a reference positive serum (——) and a reference negative serum (---) were titrated (from 1: 800 - 3200) against different concentrations of antigen (● , ○ , × , △) and dilutions of conjugate (1: 4000 - 6000). The antigen concentration at 1: 400 dilution was 3.5 μg/ml.

(b) the ratios of the OD values of the reference positive (E+) to that of the reference negative (E-) were then plotted against different dilutions of serum. The highest E+/E- ratio indicated the optimal conditions for the system.


(a)
CONJUGATE 1:4000
CONJUGATE 1:5000
CONJUGATE 1:6000
ELISA OD492 VALUES
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ANTIGEN
1:400
1:800
1:1600
1:3200
(b)
E+/E- RATIOS
8
6
4
2
0
800 1600 2400 3200
800 1600 2400 3200
800 1600 2400 3200
SERUM DILUTIONS 1:

reference negative serum was obtained at antigen dilution 1: 400 (i.e. 3.5 µg/ml), serum dilution 1: 2400 and conjugate dilution 1: 5000 (Fig. 2.7b).

Attempts to develop ELISA, using ES products of L3 and mf, for the detection of antibodies in B. pahangi-infected cats were unsuccessful. The highest ratios of the OD values of the infected sera to those of the normal sera were only between 2 to 4.

The optimal conditions for ELISA, using CSE and ES antigens of B. pahangi L5 as antigens, for the detection of antibodies in B. pahangi-infected cats are shown in Table 2.5. Preliminary observations showed that better results could be obtained by incubating the conjugate for 18 h at 4°C instead of 3 h at room temperature. The cut-off values were found to be 0.26 and 0.17 in ELISA using CSE and ES antigens respectively (Table 2.5).

The patterns of mean antibody response against the CSE and ES antigens of B. pahangi L5 during the course of a single infection in 6 B. pahangi-infected cats were quite similar to each other (Fig. 2.8). Antibody response to both antigens were detected by 2 weeks post-infection. By week 6, all cats had detectable antibodies to the CSE antigen. However, antibodies against the ES antigen could only be detected in every cat by week 10 when all cats had become microfilaraemic. The first peak mean antibody response to both antigens occurred at weeks 11 - 12 when the mean level of microfilaraemia began to rise (Fig. 2.4d). The second peak (which was the highest mean response, mean OD values = 0.65 against CSE and 0.54 against ES antigens) occurred at week 21 which coincided with the maximum mean level of microfilaraemia in these cats. The decrease in the antibody response also paralleled the fall in the mean level of microfilaraemia.

Table 2.5. The optimal conditions of Enzyme-linked Immunosorbent Assay (ELISA), using somatic (CSE) and metabolic(ES) antigens of B. pahangi adult worms,and the respective cut-off values,for the detection of antibodies in B. pahangi-infected cats.

Conditions	Incubation Time, Temperature	CES Antigen	ES Antigen
Antigen concentration	18 hr, room temperature	3.5 µg/ml	4.7 µg/ml
Serum dilution	2 hr, room temperature	1: 2400	1: 1600
Conjugate dilution	18 hr, 4°C	1: 5000	1: 3000
Test stopped when E+ reaches		0.9	1.0
Cut-off value		0.26	0.17

E+ - the OD492 value of the reference positive serum

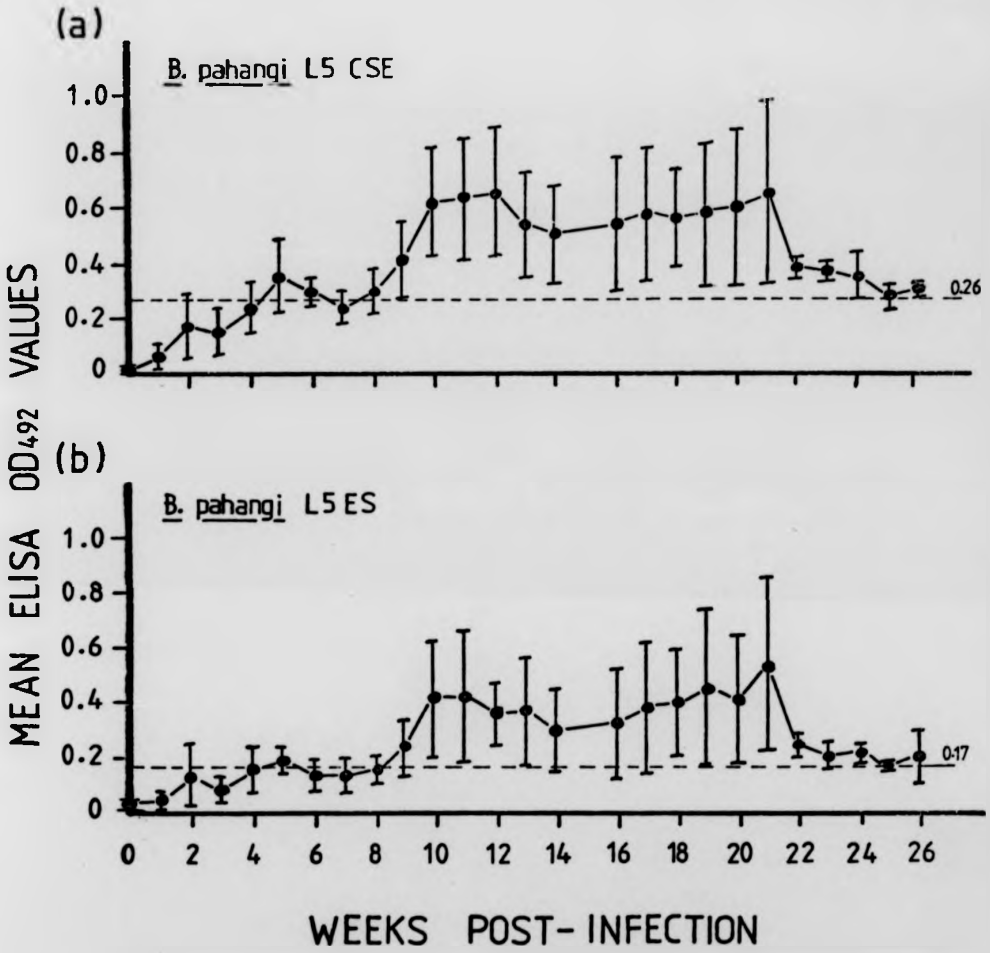


Fig. 2.8 The changes in mean antibodies against B. pahangi adult worm (L5) crude soluble extract (CSE) and metabolic (ES) antigens in the Enzyme-linked Immunosorbent Assay (ELISA) during the course of a single infection of 100 - 200 infective larvae of B. pahangi in 6 cats.

Error flag : 1 S.D.
--- cut-off value
Sample size between weeks 20 and 21 = 5,
" " " " 22 and 26 = 2.

For the detection of antibodies in 82 B. pahangi-infected cats, the sensitivities of ELISA using CSE and ES antigens were almost the same in every group of cats (Table 2.6). A significant ($p < 0.001$) positive correlation ($r = 0.94$) was observed between the OD values of sera measured by these two assays. Both were 100% sensitive in detecting antibodies in infected cats treated with DEC, MBZ and FBZ.

The distribution of ELISA values in both assays are shown in Fig. 2.9. Some sera were strongly positive, 3 had OD values larger than 2.0.

Higher mean OD values, although not statistically significant, were observed in cats treated with DEC, MBZ and FBZ as compared to those untreated cats infected for similar periods of time (Fig. 2.10).

There were no correlations between the ELISA values of these two assays and the number of live L5 recovered at necropsy, the last mf count and the periods post-infection and post-treatment.

Table 2.6. The sensitivities and the mean OD492 values of Enzyme-linked Immunosorbent Assays (ELISA), using somatic (CSE) and metabolic (ES) antigens of B. pahangi adult male and female worms, for the detection of antibodies in B. pahangi-infected cats.

Groups	Sample Size	Sensitivity % (mean OD values ± S.D.) CSE Antigen	ES Antigen
1. Infected, untreated	31	52 (0.37±0.4)	45 (0.28±0.3)
2. Infected, DEC treated	7	100 (0.79±0.5)	100 (0.69±0.5)
3. Infected, MBZ treated	9	100 (1.29±0.5)	100 (1.08±0.5)
4. Infected, FBZ treated	5	100 (1.11±0.6)	100 (0.89±0.6)
5. Infected, other treatment	30	90 (0.86±0.5)	90 (0.68±0.5)
Total (1 - 5)	82	77 (0.73±0.6)	76 (0.59±0.5)

DEC - Diethylcarbamazine, MBZ - Mebendazole, FBZ - Flubendazole.

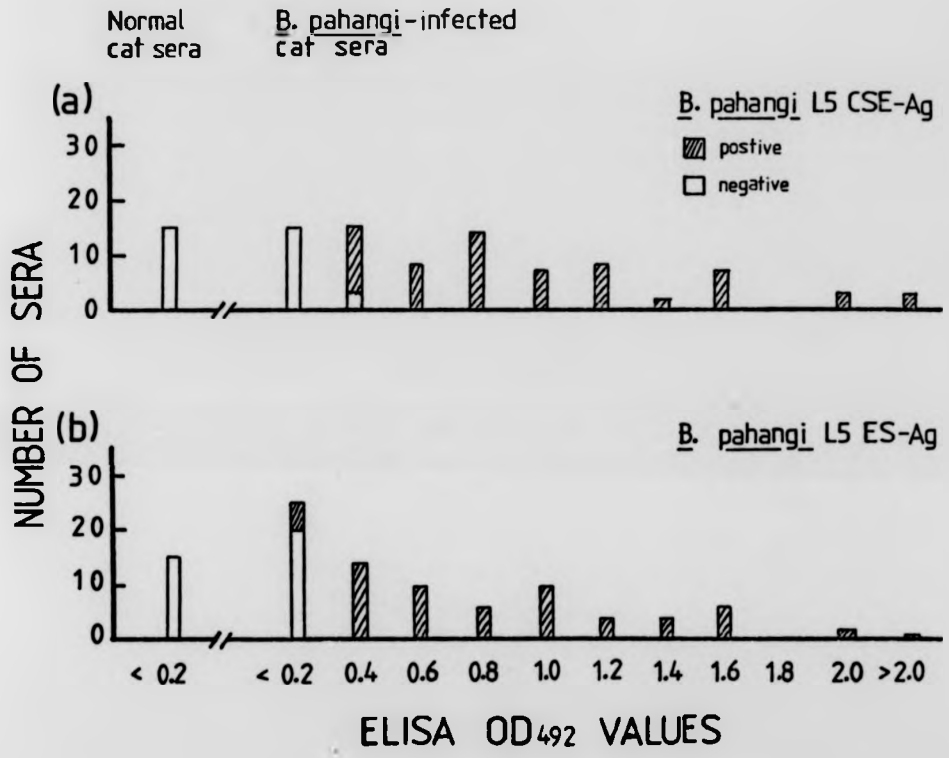
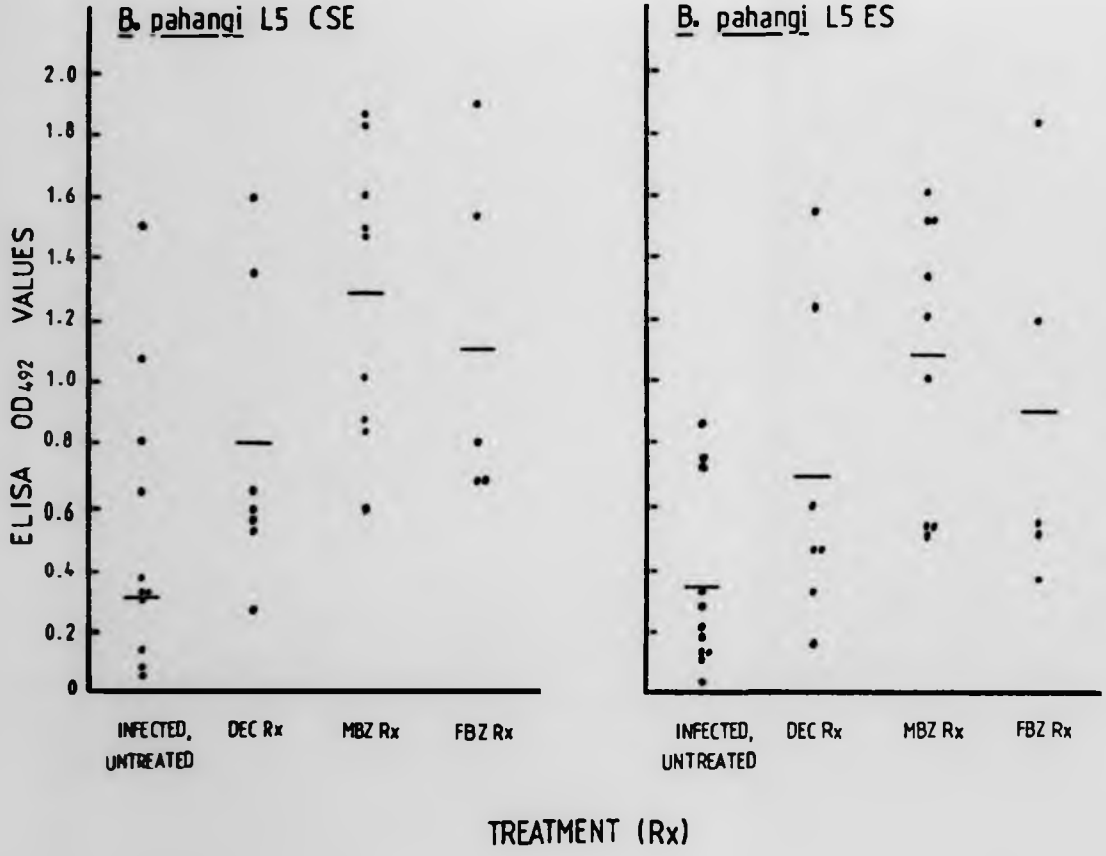


Fig. 2.9 The distribution of OD values of sera from 82 *B. pahangi*-infected cats when tested against the crude soluble extract (CSE) and metabolic (ES) antigens (Ag) of *B. pahangi* adult worms (L5) in the Enzyme-linked Immunosorbent Assay (ELISA). 15 normal sera from uninfected cats were also tested.

Fig. 2.10 The antibody levels (OD values) against the crude soluble extract (CSE) and metabolic (ES) antigens of B. pahangi adults (LS) in the Enzyme-linked Immunosorbent Assays (ELISA) in sera from B. pahangi-infected cats, which were untreated or treated with diethylcarbamazine (DEC), mebendazole (MBZ) and flubendazole (FBZ).

All the cats were 90 - 200 days post-infection with 100 - 200 infective larvae.

— denotes the mean OD value.


B. pahangi L5 CSE
B. pahangi L5 ES
ELISA OD492 VALUES
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
INFECTED, UNTREATED
DEC Rx
MBZ Rx
FBZ Rx
INFECTED, UNTREATED
DEC Rx
MBZ Rx
FBZ Rx
TREATMENT (Rx)

DISCUSSION

The longer the parasites could be maintained *in vitro*, the more ES products would be released into the culture medium. Supplementation of the culture medium with serum would prolong the survival of parasites *in vitro* (Weinstein and Sawyer, 1961). However, the coexistence of serum factors of high molecular weight with the ES products of the parasites would interfere with the sensitivity of the serological tests. Thus, cultures were performed in chemically defined low molecular weight media only. Supplementation of the culture media with some low molecular weight substances like glucose and glycyl-L-histydl-L-lysine acetate (according to Stromberg *et al.*, 1977), which would pass through the Amicon filter, did not improve the survival of parasites *in vitro*. Similar supplementation with cysteine, sodium caseinate and yeast extract have been reported to have no effect on *B. pahangi* L3 *in vitro* (Chen and Howells, 1979). The maximum periods of survival of *B. pahangi* mf and L5 *in vitro* were similar to those of *L. loa* mf, *W. bancrofti* mf, *D. immitis* mf and L5 (Taylor, 1960; Weinstein and Sawyer, 1961). Surprisingly, *B. pahangi* L3 could survive up to 23 days *in vitro* in this study as compared to a maximum of 20 days as reported by Chen and Howells (1979), who employed various serum supplements and tissue cell lines. No moulting of L3 was observed *in vitro*. Chen and Howells (1979) demonstrated that moulting of L3 *in vitro* required stimuli *in vivo*. Development of mf was not observed in this study. It seems the presence of insect cells or mammalian cells is necessary for the development of mf *in vitro* (Dhar *et al.*, 1967; Weinstein, 1970; Devaney and Howells, 1979). It is believed that antigenic substances are present in the moulting fluid of nematodes (Stromberg and Soulsby, 1977). The absence of development of L3 and mf *in vitro* might explain why ELISA could not be established for the detection of antibodies to *B. pahangi* in this study, using ES products of L3 and mf which might not be antigenic.

A parallel study by Dr. D. de Savigny (Department of Clinical Tropical Medicine) obtained the same results. However, the ES products of mf of W. bancrofti, B. malayi and D. immitis have been used for the study of histamine release from basophils in filariasis patients (Ottesen et al., 1979).

Polystyrene ELISA plates are suitable for most crude antigen preparations and they are commonly used. For better adsorption of immunological reagents, eg. a pure preparation of antigen or Y-globulins, polyvinyl plates are preferred (Voller et al., 1979). In this study, it was found that the use of polyvinyl plates was necessary in order to obtain uniform adsorption of B. pahangi L5 ES antigens. Perhaps, ES antigens may be purer than CSE antigens. However, antigens seem to adhere to the solid-phase by physical adsorption (eg. hydrophilic bonds) and it is still not known what part of the antigen is preferentially bound (W.H.O., 1976; Kagan, 1979).

Common antigens were shared between the L5, L3 and mf of B. pahangi. One stage-specific antigenic component seemed to be present in L5 male and in L5 female (Fig. 2.2d). The antigens of mf seemed to be only partially identical to those of L3 and L5 (Fig. 2.2a). Since the L5 female had mf in utero, it also suggested that uterus mf and blood mf might not be antigenically identical. In fact, the abilities of uterus mf and blood mf of L. carinii to induce acquired resistance to mf in cotton rats seemed to be different too (Wegerhof and Wenk, 1979). In this study, L3 did not seem to have any stage-specific soluble antigen (Fig. 2.2b). However, further studies are required to confirm this.

Studies on the changes of mean antibody (IgG) responce to B. pahangi in 6 infected cats by IFAT showed that antibodies first appeared against L3, then L5 and mf accordingly (Fig. 2.4). The highest antibody titres against these antigens were 128,

256 and 128 respectively. The pattern of mean antibody response to L5 was essentially similar to that reported by Ponnudurai *et al* (1974) except that the peak mean antibody response occurred at week 17 in this study rather than at day 60. There seemed to be an inverse relationship between the mean level of microfilaraemia and the mean antibody response to the mf fragments between weeks 12 and 21 (Figs 2.4c and d). When the antibody response against mf started to decline at week 12, the mean mf level began to increase, reaching a high level (mean count = 1253 mf/ 100 mm^3 blood) at week 21. This may be explained by the suggestions that living mf are antigenically inert, or large amounts of antibody are present but they are either absorbed by the mf or combine with the soluble antigen of mf (Franks, 1946; Guest and Wong, 1965; Guest *et al*., 1967; Capron *et al*., 1968 and Smithers, 1968). High antibody titres were obtained only in cats which did not develop microfilaraemia (Ponnudurai *et al*., 1974).

The evolution of the antibody response to CSE and ES antigens of L5 in ELISA in the 6 *B. pahangi*-infected cats seemed to be correlated to the production of mf by the L5. The first peak mean antibody response at week 11 - 12 coincided with the time when all cats had become microfilaraemic (Figs 2.4d and 2.8). The second peak at week 21 coincided with the peak mean microfilaraemia. This suggests that the antibody response may be against antigens released by the mf-producing females.

The sensitivities of ELISA, using CSE and ES antigens of *B. pahangi* L5, were similar in the detection of antibodies in *B. pahangi*-infected cats (Fig. 2.8 and Table 2.6). Both assays seemed to be particularly sensitive (100%) for the detection of antibodies in infected cats which had been treated with DEC, MBZ and FBZ, which were effective filaricides in cats (Denham *et al*., 1978a&b, 1979). Perhaps, a larger amount of

soluble antigens might have released upon the death of the worms. The OD values of sera measured by both assays were significantly correlated too ($p < 0.001$, $r = 0.94$). Similar sensitivities of serological tests using CSE and ES antigens have been reported (Sadun and Norman, 1957; Takaoka and Tanaka, 1975). However, ES antigens may be better in some cases (Desowitz *et al.*, 1978a). This similarity in sensitivity may be due to the presence of common antigenic components between ES and CSE antigens (Ishii, 1970). ES products are most probably present within the L5, but somatic components like the surface antigens which are shed by the L5 may be present in the ES products collected *in vitro* (Dr. M. Philipp, personal communications).

Studies on the relative sensitivities of CIEP, IFAT and ELISA, using various homologous and heterologous antigens for the detection of antibodies in 82 *B. pahangi*-infected cats have shown that homologous antigens are always better than heterologous antigens (Tables 2.2, 2.4 and 2.6). IFAT was more sensitive than ELISA, which in turn was slightly more sensitive than CIEP. IFAT, using *B. pahangi* L3 as antigen, was the most sensitive test — 81% sensitivity for the infected but untreated cats in group 1 and an overall sensitivity of 89% (Table 2.4). It detected antibodies as early as 1 week post-infection and remained positive till at least 510 days post-infection.

Although ELISA had been reported more sensitive than IFAT in the detection of antibodies to other parasitic infections in man like leishmaniasis (Edrissian and Darabian, 1979), it was found to be less sensitive than IFAT in the *B. pahangi*-cat system. CIEP was the least sensitive and it detected antibodies between 24 to only 359 days post-infection (Table 2.2). The results of CIEP in this study agreed with that of Desowitz *et al* (1978b) in that one infected cat which had become amicrofilaraemic naturally did not react with the L5 antigen.

The mean antibody levels, detected by IFAT and ELISA, increased after chemotherapy with DEC, MBZ and FBZ (Figs 2.6 and 2.10). An overall increase in IFAT titres in loiasis patients after treatment with DEC has been observed by Ambroise-Thomas and Kien Truong (1972).

CHAPTER THREE

DETECTION OF ANTIBODIES IN HUMAN BRUGIAN AND BANCROFTIAN FILARIASIS

INTRODUCTION

From the studies in the *B. pahangi*-cat system (Chapter 2), IFAT seemed to be the most sensitive serological test. Although ELISA had been found to be less sensitive, it would still be worthwhile to investigate for its applications in human filariasis because soluble antigen, which seemed to be the form of specific antigen, in minute quantities were adequate to test a large number of sera in ELISA. In Chapter 2, it was demonstrated that homologous antigens were better than heterologous antigens. Unfortunately, for human filariasis, perhaps with the only exception of *B. malayi*, it is difficult to obtain adequate quantity of human parasite materials for antigens in serological tests. The use of other heterologous filarial antigens seems to be the only alternative (Ambroise-Thomas, 1974). However, heterologous antigens have imposed problems of being less sensitive apart from the broad cross-reactivity with other helminthic infections. Attempts to compare the relative sensitivities of a variety of heterologous filarial antigens in the detection of antibodies in human filariasis have not been reported. Therefore, in this study, the sensitivities of antigens from 6 different filarial species in the detection of antibodies in filariasis due to *B. malayi* and *W. bancrofti* were compared by IFAT.

B. pahangi, being similar to *B. malayi* and *W. bancrofti* in life history and pathology, may be the source of sensitive antigens for detecting antibodies in brugian and bancroftian filariasis. In fact, *B. pahangi* antigen has been reported to give the best results for detection of filariasis in IFAT

(Hedge and Ridley, 1977). In this study, the efficiencies of crude soluble extract (CSE) of L5, partially purified CSE of L5 (ppCSE) and metabolic (ES) antigens of L5 and L3 of B. pahangi in ELISA for the detection of antibodies in brugian and bancroftian filariasis were investigated.

Cross-reactivities of IFAT and ELISA, using different filarial antigens, with non-filariasis sera : schistosomiasis, cysticercosis and trichinosis, were also studied.

MATERIALS AND METHODS

Indirect Fluorescent Antibody Test (IFAT)

The mf of *B. pahangi*; L3 of *B. pahangi* and *W. bancrofti* and L5 of *B. pahangi*, *D. immitis*, *D. viteae*, *L. carinii* and *O. gutturosa* were used as antigens in IFAT. L3 of *W. bancrofti* were obtained by dissection of infected mosquitoes (*Culex* spp., kindly brought back by Dr. C. Curtis, Ross Institute, from Tanzania and Sri Lanka). The other filarial antigens were obtained from Dr. D.A. Denham, Department of Medical Helminthology.

FITC-conjugated sheep anti-human immunoglobulins (Wellcome) was used in the test. The preparation of antigen-slides, the determination of the optimal dilution of the FITC-conjugate and the procedures of IFAT were the same as those described in Chapter 2. A titre of 16 or above was regarded as positive.

Enzyme-linked Immunosorbent Assay

CSE of L5 and ES products of *B. pahangi* were used as antigens in ELISA. Their preparation was mentioned in Chapter 2. Partially purified CSE of *B. pahangi* L5 was also used. It was prepared according to Sawada *et al* (1965) with some modifications. 25 mg of CSE of *B. pahangi* L5 in 3 ml of PBS, pH 7.2 were applied to a sephadex G-200 column (12 x 1.5 cm) pre-equilibrated with PBS, pH 7.2. Elution was performed at a flow rate of 30 ml/h with PBS, pH 7.2. Fractions of 1.2 ml were collected and the absorbence at 280 nm measured by a UV-spectrophotometer. Fractions of the same peak (with absorbence larger than 0.1) were pooled and concentrated by dialysis against a concentrated solution of PEG (MW 20,000) at 4°C. The concentrated solution was then dialysed exhaustively against 0.005M acetate buffer, pH 4.6 (see appendix for preparation)

at 4°C. The soluble antigen in 2 ml of acetate buffer was applied to a carboxymethyl (CM) cellulose (CM52 Whatman) column (14.5 x 1.5 cm) pre-equilibrated with 0.005M acetate buffer, pH4.6. Elution was performed by a step-wise gradient with 30-ml quantities of 0.005M acetate buffer, pH 4.6 followed by 0.005M phosphate buffer, pH 6.0; 0.1M NaCl, 0.2M NaCl, 0.4M NaCl, 0.8M NaCl and 2M NaCl in 0.005M phosphate buffer, pH 7.0. The column was finally eluted with 0.1N NaOH to wash down all residual proteins. The flow rate was 17 ml/h and 3 ml fractions were collected. The absorbence of the fractions were measured by a UV-spectrophotometer at 280 nm. Fractions of the same peak were pooled and finally concentrated against PEG and dialysed exhaustively against PBS, pH 7.2 at 4°C. These partially purified fractions of CSE were stored at -70°C until use.

Peroxidase-conjugated goat anti-human IgG (Miles Labs) was used for the study. M129A polystyrene plates (Dynatech) were used for CSE antigens and M29 polyvinyl plates (Dynatech) for ES antigens. The optimization, procedures of the assays and the determination of cut-off values (using 39 normal European sera) were the same as those mentioned in Chapter 2, except that the incubation time for the conjugate was 3 h at room temperature.

Filariasis Sera

Sera from patients with W. bancrofti infections and from residents without clinical and parasitological evidence of filariasis in W. bancrofti endemic areas were obtained from Dr. C.K. Rao (National Institute of Communicable Diseases, Delhi, India) and Dr. M.M. Ismail (Medical Research Institute, Colombo, Sri Lanka). Sera from residents in areas non-endemic

for W. bancrofti in Sri Lanka were also obtained from Dr. M.M. Ismail. Sera from patients with B. malayi infections and from residents, without clinical and parasitological evidence of filariasis, in B. malayi endemic areas were obtained from Dr. C.K. Rao and Dr. J.W. Mak (Institute for Medical Research, Kuala Lumpur, Malaysia). Sera from residents in areas non-endemic for B. malayi in Malaysia were also obtained from Dr. J.W. Mak. A few sera from patients with O. volvulus and L. loa infections were obtained from Dr. D.S. Ridley, Hospital for Tropical Diseases, London. M. ozzardi sera were obtained from Dr. M. Nathan, Caribbean Epidemiology Centre, Port of Spain, Trinidad.

Non-filariasis Sera

Sera from patients with trichinosis, cysticercosis and schistosomiasis due to S. mansoni and S. haematobium were obtained from Miss J.E. Lillywhite, Drs. M.L. McLaren and M.M. Ismail, Ross Institute.

Human Control Sera

Sera collected from 39 healthy Europeans, who had not been to the tropics, at a general hospital at Northampton were used as negative control sera.

RESULTS

Fractionation of B. pahangi L5 CSE by Column Chromatography

One peak was observed after elution of B. pahangi L5 CSE through a sephadex G-200 column (Fig. 3.1). Subsequent dialysis of the fraction against 0.005M acetate buffer, pH 4.6 had resulted in precipitation of a considerable quantity of protein (about 18 mg). Further separation of the soluble fraction (6.6 mg) was performed in a CM cellulose column. After a step-wise elution with an increasing NaCl gradient and finally 0.1N NaOH, 7 fractions were obtained (Fig. 3.2). Because of the small amount of protein collected at each peak, fractions with absorbence (at 280 nm) greater than 0.05 were pooled. The two peaks in fraction 5 were pooled together by mistake.

IFAT for the Detection of Antibodies in Brugian and Bancroftian Filariasis

The optimal dilutions of FITC-conjugated sheep anti-human immunoglobulins (Wellcome) in IFAT, using different filarial antigens were shown in Table 3.1. Loiasis, onchocerciasis, trichinosis and schistosomiasis sera were tested against different antigens with conjugate dilutions used for testing W. bancrofti infections.

B. malayi Infections

The positivity rates of IFAT, using different filarial antigens, for the detection of antibodies in humans with B. malayi infections are summarized in Table 3.2. Highest positivity rates were observed with W. bancrofti L3 antigen (96% for both clinical and microfilaraemic cases). B. pahangi L3 and mf gave similar overall positivity rates (about 90%)

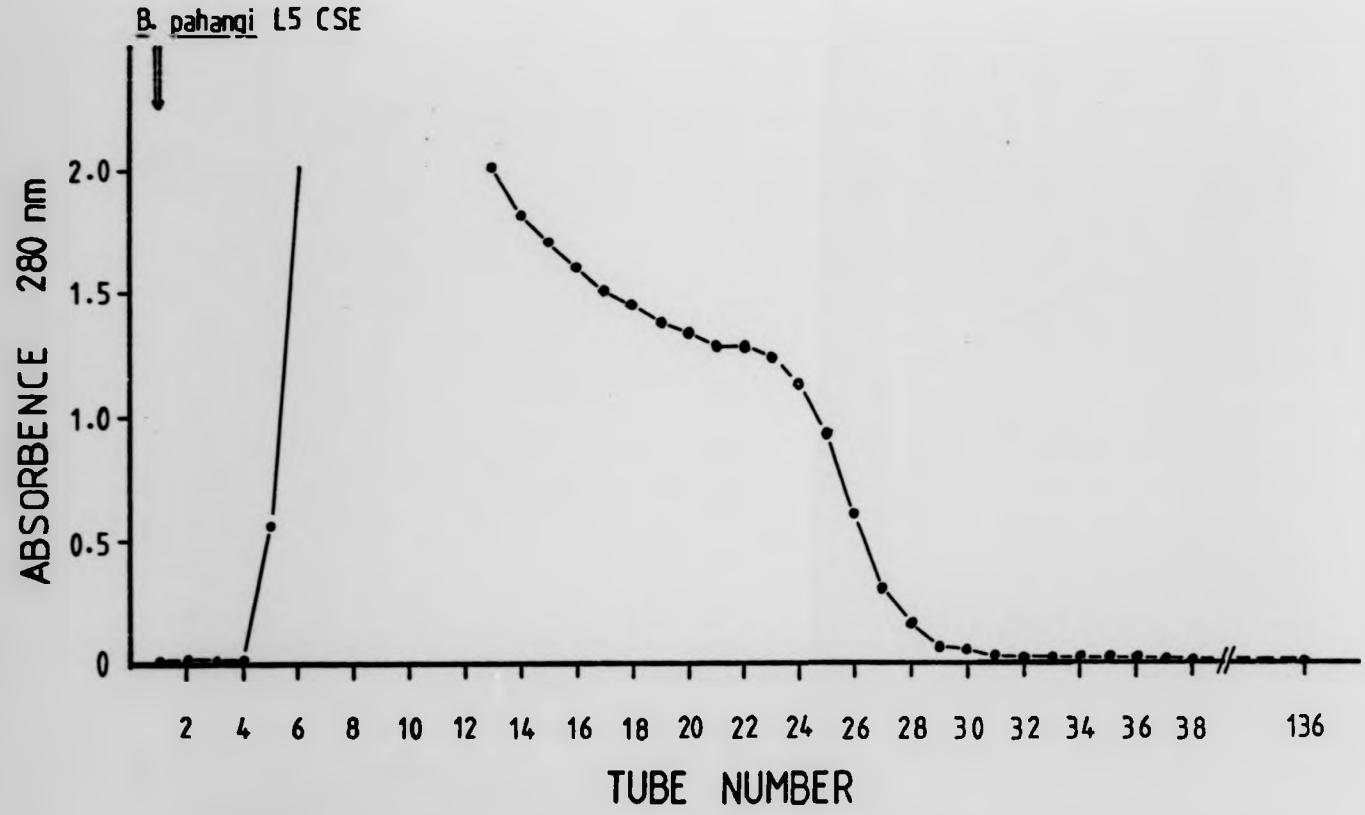


Fig. 3.1 Fractionation of the crude soluble extract (CSE) of B. pahangi adult worms (L5) in a sephadex G-200 column, eluted with PBS, pH 7.2.

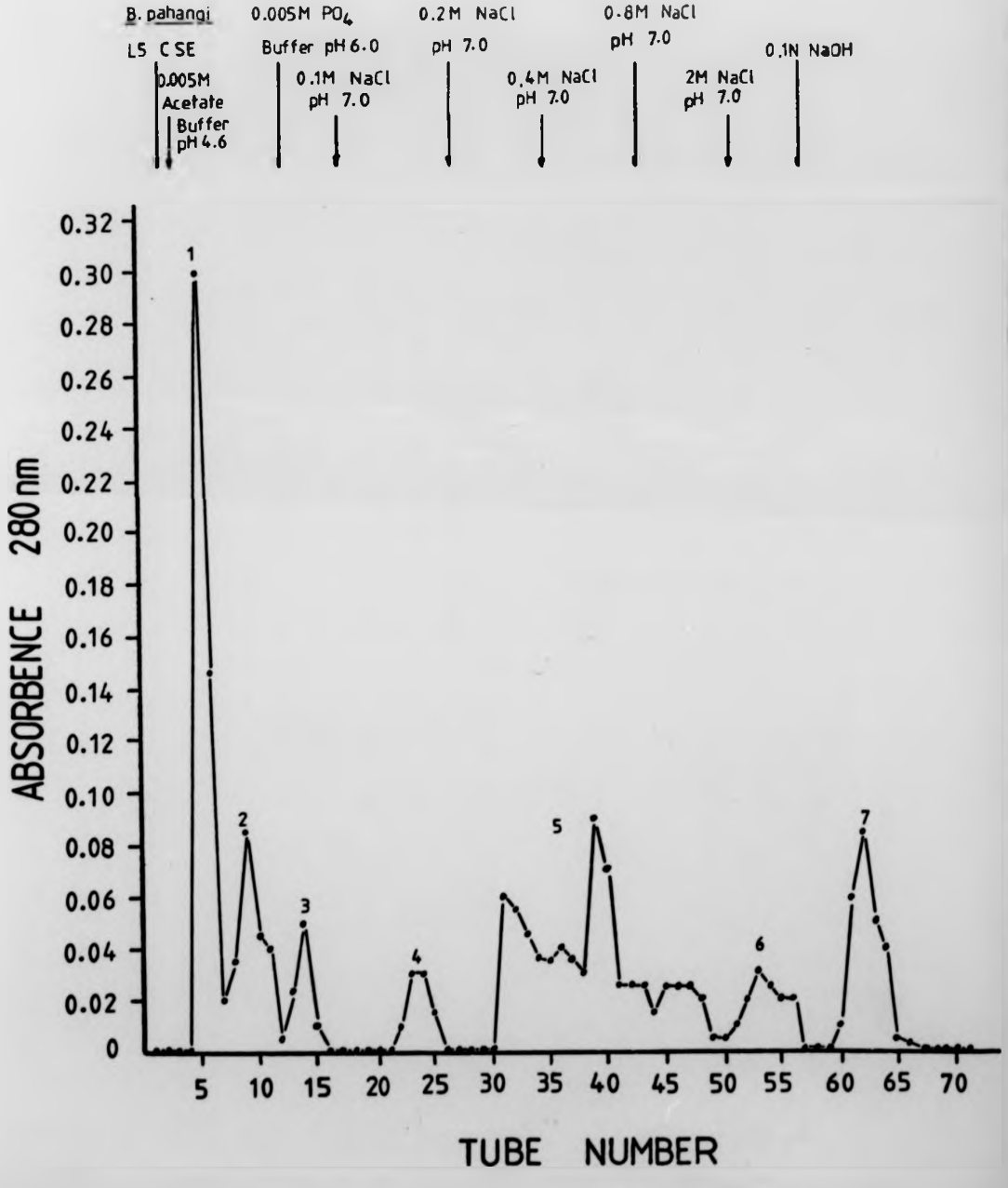


Fig. 3.2 Elution profile of the fraction of B. pahangi adult (L5) crude soluble extract (CSE), obtained from the sephadex G200 column, in a carboxymethyl cellulose column. Elution was performed by a step-wise increasing NaCl gradient as indicated (↓). Fractions 1 - 7 were obtained. The 2 peaks in fraction 5 were pooled by mistake.

Table 3.1. The optimal dilutions of FITC-conjugated sheep anti-human immunoglobulins (Wellcome) for the detection of antibodies in human B. malayi and W. bancrofti infections, in Indirect Immunofluorescence Antibody Tests (IFAT) using different filarial antigens.

	Dilutions of Conjugate 1:							
	Antigens							
	B. pahangi			W. bancrofti	D. immitis	D. viteae	L. carinii	O. gutturosa
Sera	L5	L3	Mf	L3	L5	L5	L5	L5
B. malayi	100	120	60	120	120	80	40	100
W. bancrofti	40	60	120	60	40	60	40	60

L5 - cryosections of adult male and female worms; L3 - infective larvae (cryosections); Mf - sonicated fragments of microfilariae.

Table 3.2 . Indirect Immunofluorescence Antibody Test (IFAT) for the detection of antibodies in human B. malayi infections, using different filarial antigens.

Groups	Sample Size	% POSITIVITY Antigens							
		B.p.L5	B.p.L3	B.p.Mf	W.b.L3	D.i.L5	D.v.L5	L.c.L5	O.g.L5
B. malayi clinical cases	25	84	96	88	96	60	56	84	84
B. malayi microfilaraemic cases	26	85	88	92	96	73	85	69	77
Sera from endemic area	27	85	78	89	70	41	56	52	85
Sera from nonendemic area, Malaysia	12	17	17	8	0	0	0	8	0
Normal European sera	39	0	0	0	0	0	0	0	0

B.p. - B. pahangi; W.b. - W. bancrofti; D.i. - D. immitis; D.v. - D. viteae;
L.c. - L. carinii; O.g. - O. gutturosa;
L5 - cryosections of adult male and female worms; L3 - cryosections of infective larvae;
Mf - sonicated fragments of microfilariae.

with B. malayi infections. B. pahangi L5 was slightly less sensitive (about 85%) than L3 and mf. D. viteae, L. carinii and O. gutturosa L5 antigens gave about 85% positivity rates with either clinical or microfilaraemic cases. D. immitis L5 antigen was the least sensitive. In general, W. bancrofti and B. pahangi (lymphatic filarioids) antigens were more superior. Higher positivity rates (78 - 89%) were obtained with sera from residents in B. malayi endemic areas, using B. pahangi antigens. IFAT, using different filarial antigens were not reactive with the normal European sera. 8 - 17% positivities were observed with sera from people in non-endemic areas in Malaysia, using B. pahangi and L. carinii as antigens.

The distributions of IFAT titres of sera from different groups against different filarial antigens are shown in Table 3.3. Titres as high as 512 and 1024 were observed with W. bancrofti and B. pahangi antigens respectively. However, the most frequent titre was 16 with all antigens. Sera from endemic areas with titres as high as 256 and 512 were observed. Those positive sera from non-endemic areas had titres of 16 and 32.

W. bancrofti Infections

100% positivity rates were obtained with clinical and microfilaraemic cases, using W. bancrofti L3 as antigen (Table 3.4). IFAT using B. pahangi L3 and mf antigens had positivity rates of 92 - 100% while that using B. pahangi L5 antigen was less sensitive (87 - 92%). IFAT using D. viteae L5 antigen was quite sensitive too (85 - 100%). IFAT using D. immitis, L. carinii and O. gutturosa antigens had positivity rates ranging from 74 - 92%. 29 - 81% positivity rates were obtained with sera from residents in W. bancrofti endemic areas, in IFAT using different filarial antigens. 20 - 70% positivity rates were also obtained with sera from residents in non-endemic areas in Sri Lanka. However, all normal European sera were negative.

Table 13 . The distribution of IFAT titres in sera from B. malayi clinical and microfilaraemic patients and from people living in B. malayi endemic and nonendemic areas against different filarial antigens in IFAT.

	Number of Sera Giving Indicated IFAT Titres																											
	B. malayi Clinical Sera (N=25)								B. malayi Microfilaraemic Sera (N=26)								Endemic Sera (N=27)							Nonendemic Sera (N=12)			
Antigen Used	<16	16	32	64	128	256	512	1024	<16	16	32	64	128	256	512	1024	<16	16	32	64	128	256	512	<16	16	32	64
W. bancrofti L3	1	14	2	5	1	1	1	0	1	16	6	1	2	0	0	0	8	14	1	2	1	1	0	12	0	0	0
B. pahangi L5	4	15	3	3	0	0	0	0	4	15	2	3	1	1	0	0	4	12	5	3	2	0	1	10	1	1	0
B. pahangi L3	1	11	3	6	2	1	0	1	3	11	4	4	3	1	0	0	6	17	2	2	0	0	0	10	2	0	0
B. pahangi Mf	3	14	2	3	2	1	0	0	2	8	5	6	4	0	0	1	3	11	7	5	0	1	0	11	1	0	0
D. immitis L5	10	12	2	1	0	0	0	0	7	14	2	3	0	0	0	0	16	8	3	0	0	0	0	12	0	0	0
D. viteae L5	11	12	1	1	0	0	0	0	4	18	3	1	0	0	0	0	12	13	0	2	0	0	0	12	0	0	0
L. carinii L5	4	16	3	1	1	0	0	0	8	15	1	2	0	0	0	0	13	12	0	1	0	1	0	11	1	0	0
C. gutturosa L5	4	18	2	0	1	0	0	0	6	14	3	3	0	0	0	0	4	16	4	3	0	0	0	12	0	0	0

N - Sample Size; L3 - Cryosections of infective larvae; L5 - Cryosections of adult male and female worms; Mf - Sonicated fragments of microfilariae.

Table 3.4 . Indirect Immunofluorescence Antibody Test (IFAT) for the detection of antibodies in human W. bancrofti infections, using different filarial antigens.

Groups	Sample Size	% POSITIVITY Antigens B.p.L5	B.p.L3	B.p.Mf	W.b.L3	D.i.L5	D.v.L5	L.c.L5	O.g.L5
W. bancrofti Clinical cases	39	87	92	95	100	74	100	79	77
W. bancrofti microfilaraemic cases	13	92	100	92	100	85	85	77	92
Sera from endemic area	21	67	81	52	81	29	81	62	48
Sera from nonendemic area	10	40	20	30	50	50	70	40	20
Normal European Sera	39	0	0	0	0	0	0	0	0

B.p. - B. pahangi; W.b. - W. bancrofti; D.i. - D. immitis; D.v. - D. viteae;
L.c. - L. carinii; O.g. - O. gutturosa;
L5 - cryosections of adult male and female worms; L3 - cryosections of infective larvae
Mf - sonicated fragments of microfilariae.

The distribution of titres of sera from different groups in IFAT using different filarial antigens for the detection of W. bancrofti infections are shown in Table 3.5. An IFAT titre as high as 1024 was observed with W. bancrofti L3 and B. pahangi mf antigens. Among the clinical sera, the most frequent titre was 64 with W. bancrofti L3 and B. pahangi mf antigens and 16 with other filarial antigens. Titres as high as 256 were observed with sera from endemic areas. Those positive sera from non-endemic areas had titres only as high as 32.

Other Filarial and Helminthic Infections

B. pahangi antigens were reactive with sera from patients with M. ozzardi, L. loa and O. volvulus infections (Table 3.6). W. bancrofti L3 antigen was also reactive with M. ozzardi sera from Trinidad, where W. bancrofti was also endemic.

0 - 86% reactivities were observed with schistosomiasis sera from Egypt, St. Lucia and Sudan, where filariasis were also endemic. However, all antigens did not react with the 11 trichinosis sera. The S. haematobium sera were positive only at titre 16 and S. mansoni sera at titres 16 to 64 (Table 3.7).

ELISA for the Detection of Antibodies in Brugian and Bancroftian Filariasis

Attempts to develop ELISA, using ES products of B. pahangi L5 and L3, for the detection of antibodies in human lymphatic filariasis were unsuccessful. The highest ratios of the OD values of the positive sera to those of the negative sera were only 2 - 3. The optimal conditions for ELISA, using CSE of B. pahangi L5 and its partially purified fractions (ppCSE),

Table 3.5 . The distribution of IFAT titres in sera from W. bancrofti clinical and microfilaraemic patients and from people living in W. bancrofti endemic and non-endemic areas against different filarial antigens in IFAT.

	Number of Sera Giving Indicated IFAT Titres																											
	W. bancrofti Clinical Sera (N=39)									W. bancrofti Microfilaraemic Sera (N=13)									Endemic Sera (N=21)							Non-endemic Sera (N=10)			
Antigen Used	<16	16	32	64	128	256	512	1024		<16	16	32	64	128	256	512	1024		<16	16	32	64	128	256		<16	16	32	
W. bancrofti L3	0	4	4	17	3	11	0	0		0	1	1	5	1	4	0	1		4	6	1	7	2	1		5	4	1	
B. pahangi L5	5	17	7	7	2	1	0	0		1	3	3	4	1	1	0	0		7	8	5	1	0	0		6	4	0	
B. pahangi L3	3	7	7	15	5	2	0	0		0	3	4	3	3	0	0	0		4	10	5	2	0	0		8	2	0	
B. pahangi Mf	2	15	3	6	6	6	1	0		1	2	0	3	2	4	0	1		10	6	0	3	1	1		7	1	2	
D. immitis L5	10	25	3	1	0	0	0	0		2	8	1	1	1	0	0	0		15	6	0	0	0	0		5	5	0	
D. viteae L5	0	34	5	0	0	0	0	0		2	5	4	2	0	0	0	0		4	16	1	0	0	0		3	7	0	
L. carinii L5	8	22	2	7	0	0	0	0		3	9	0	0	1	0	0	0		8	13	0	0	0	0		6	4	0	
O. gutturosa L5	9	20	5	4	1	0	0	0		1	5	0	6	0	1	0	0		11	9	0	1	0	0		8	2	0	

N - sample size; L3 - cryosections of infective larvae; L5 - cryosections of adult male and female worms; Mf - sonicated fragments of microfilariae.

Table 3.6 . Indirect Immunofluorescence Antibody Test (IFAT) - Reactivities of different filarioid antigens with non-lymphatic filariasis and some other helminthiasis sera.

Sera	Sample Size	% POSITIVITY							
		Antigens							
		B.p.L5	B.p.L3	B.p.Mf	W.b.L3	D.i.L5	D.v.L5	L.c.L5	O.g.L5
M. ozzardi (Trinidad)	6	67	50	100	100	-	-	-	-
L. loa	1	-	-	100	-	-	-	-	-
O. volvulus	3	67	100	100	-	-	-	-	-
T. spiralis	11	0	0	0	0	0	0	0	0
S. haematobium (Egypt)	15	0	0	33	-	13	-	-	-
S. mansoni (St. Lucia)	15	47	20	53	-	7	-	-	-
S. mansoni (Sudan)	21	71	86	10	-	43	76	5	38

B.p. : B. pahangi; W.b. : W. bancrofti; D.i.: D. immitis; D.v.: D. viteae;
L.c.: L. carinii; O.g.: O. gutturosa;
L5: cryosections of adult male and female worms; L3: cryosections of infective larvae;
Mf: sonicated fragments of microfilariae; - : not done.

Table 3.7. The distribution of IFAT titres in schistosomiasis sera against different filarial antigens

Antigen Used	Number of Sera Giving Indicated IFAT Titres											
	S.h. Sera (Egypt) (N 15)				S.m. Sera (St. Lucia) (N 15)				S.m. Sera (Sudan) (N 21)			
	16	16	32	64	16	16	32	64	16	16	32	64
B. pahangi L5	15	0	0	0	8	5	2	0	6	11	4	0
B. pahangi L3	15	0	0	0	12	2	1	0	3	17	1	0
B. pahangi Mf	10	5	0	0	7	6	2	0	19	2	0	0
D. immitis L5	13	2	0	0	14	1	0	0	12	6	3	0
D. viteae L5	-				-				5	16	0	0
L. carinii L5	-				-				20	1	0	0
O. gutturosa L5	-				-				13	5	2	1

N: sample size; L5: cryosections of male and female adult worms;
L3: cryosections of infective larvae; Mf: sonicated fragments of microfilariae;
- : not done

for the detection of antibodies in B. malayi and W. bancrofti infections are shown in Table 3.8. Less antigen (880 ng/ml) and serum (1: 1600) were required for the detection of the former than for the latter infections (antigen at 1.8 µg/ml, serum dilution at 1: 800). Because of the small quantities of ppCSE obtained, which were inadequate for optimization of the systems, conditions for detection of B. malayi infections with CSE antigen were used.

B. pahangi L5 CSE as Antigen

The enzyme reactions were stopped when the OD492 values of the reference positive serum had reached 1.1 for ELISA optimized to detect B. malayi infections and 1.0 for that optimized to detect W. bancrofti infections. The cut-off values for B. malayi infections was 0.27 and W. bancrofti infections 0.24. The positivity rates of ELISA in detecting B. malayi and W. bancrofti infections, with different groups of sera are shown in Table 3.9.

B. malayi Infections - When the system was optimized for the detection of B. malayi infections, 92 and 85% of the clinical and microfilaraemic cases of B. malayi infections respectively were positive (Table 3.9). The most frequent OD values were between 0.4 - 0.8 but 8 had OD values larger than 1.0 (Fig. 3.3). 85% positivity was obtained with sera from B. malayi endemic areas (Table 3.9). 5 sera had OD values higher than 1.0 too (Fig. 3.3). However, no serum from the non-endemic area in Malaysia was reactive. In this system, quite high reactivities (67 - 100%) with other filariasis were observed (Table 3.9). There were low reactivities (10 - 33%) with schistosomiasis sera, all except one had OD values less than 0.4, from Egypt, St. Lucia and Sudan where filariasis were also endemic (Table 3.9 and Fig. 3.3). Trichinosis sera were all negative.

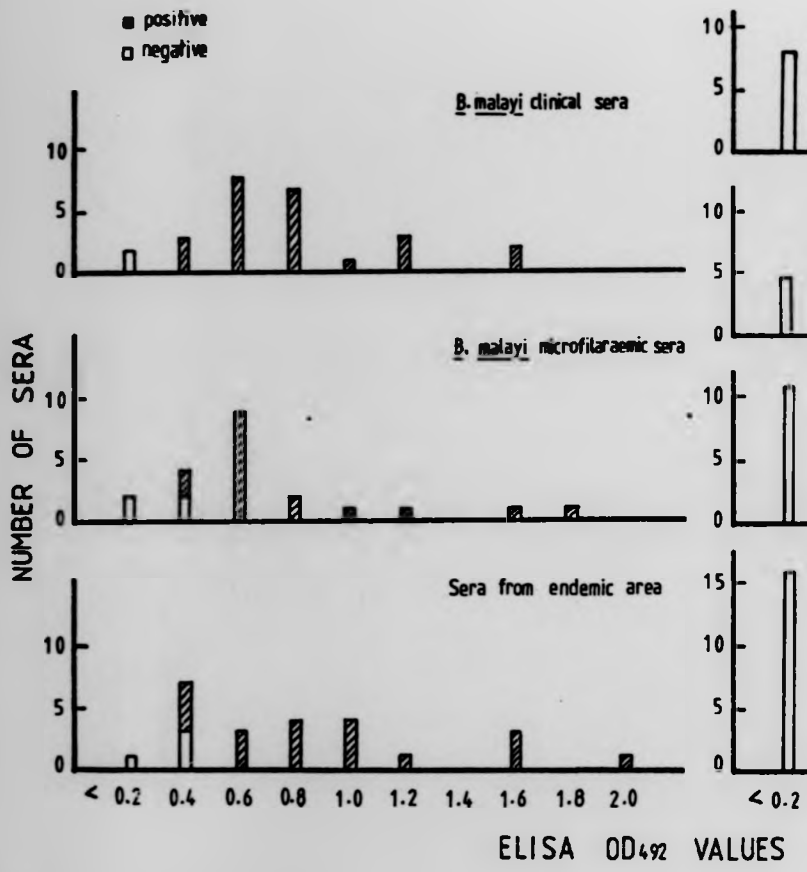
Table 3.8 . Optimal conditions for Enzyme-linked Immunosorbent Assays (ELISA), using crude soluble extract (CSE) and its partially purified fractions (ppCSE) as antigens, in the detection of antibodies in human B. malayi and W. bancrofti infections.

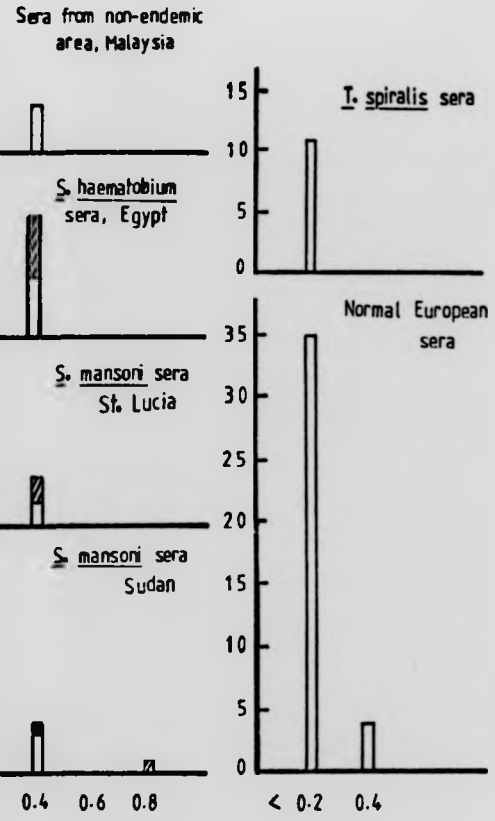
Conditions	ANTIGEN USED		
	CSE B.pahangi L5		ppCSE B. pahangi L5
	B. malayi Infections	W. bancrofti Infections	
Antigen Concentration	880 ng/ml	1.8 µg /ml	880 ng /ml
Serum Dilution	1: 1600	1: 800	1: 1600
Conjugate Dilution	1: 4000	1: 4000	1: 4000

Table 3.9. Enzyme-linked Immunosorbent Assays (ELISA), optimized for detection of *B. malayi* and *W. bancrofti* infections, in the detection of antibodies in different groups of sera.

Groups of sera	Sample Size	% Positivity: ELISA Optimized for *B. malayi* infections	% Positivity: ELISA Optimized for *W. bancrofti* infections
B. malayi clinical cases	25	92	100
B. malayi microfilaraemic cases	26	85	92
B. malayi endemic area	27	85	100
Non-endemic area, Malaysia	12	0	8
W. bancrofti clinical cases	40	95	100
W. bancrofti microfilaraemic cases	12	83	100
W. bancrofti endemic area	32	47	94
Non-endemic area, Sri Lanka	30	30	37
M. ozzardi cases (Trinidad)	6	67	67
L. loa cases	1	100	100
O. volvulus cases	3	100	100
T. spiralis cases	11	0	0
S. haematobium cases (Egypt)	15	33	93
S. mansoni cases (St. Lucia)	15	13	60
S. mansoni cases (Sudan)	21	10	43
Cysticercosis cases (New Guina)	15	-	0

Fig. 3.3 The distribution of the OD values in different groups of sera in the Enzyme-linked Immunosorbent Assay (ELISA) optimized for the detection of *B. malayi* infection using *B. pahangi* adult worm soluble extract as antigen.


positive
negative
B. malayi clinical sera
B. malayi microfilaraemic sera
Sera from endemic area
NUMBER OF SERA
< 0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
ELISA OD492 VALUES


Sera from non-endemic area, Malaysia
S. haematobium sera, Egypt
S. mansoni sera St. Lucia
S. mansoni sera Sudan
0.4
0.6
0.8
T. spiralis sera
15
10
5
0
Normal European sera
35
30
25
20
15
10
5
0
< 0.2
0.4

W. bancrofti Infections - When the system was optimized for the detection of W. bancrofti infections, 100% of the clinical and microfilaraemic cases of W. bancrofti were positive (Table 3.9). The most frequent OD values in these 2 groups were between 0.6 - 0.8 and 0.4 - 0.6 respectively (Fig. 3.4). 18 sera had OD values larger than 1.0. 94 and 37% of the sera from endemic and non-endemic areas respectively were positive too. 4 sera in the endemic area had OD values larger than 1.0 but those positive sera from non-endemic area were all less than 0.6 (Fig. 3.4). With the use of more antigen and sera in this system (Table 3.8), higher positivity rates with B. malayi sera were observed (Table 3.9). Even 8% of the sera from areas non-endemic for B. malayi became positive. Again, 67 - 100 positivities with M. ozzardi, L. loa and O. volvulus sera were observed. 43 - 93% of schistosomiasis sera were positive, all except one had OD values less than 0.7 (Table 3.9 and Fig. 3.4). However, all trichinosis, and cysticercosis (from New Guinea where filariasis was endemic) were negative.

B. pahangi L5 ppCSE as Antigens

Reactivities to B. malayi and W. bancrofti sera seemed to decrease from fractions 3 to 7 (Table 3.10). However, fraction 1 seemed to be slightly more sensitive (92 - 100% positivity) than the whole CSE (78 - 89%) in the detection of W. bancrofti infections. Furthermore, there were decreased reactivities to M. ozzardi and S. haematobium but not to L. loa and O. volvulus. Fraction 2 seemed to have retained the same reactivity to B. malayi as for the whole CSE (81 - 93%) but its reactivities to W. bancrofti, M. ozzardi, S. mansoni and S. haematobium were less. However, it was still as reactive as the whole CSE with L. loa and O. volvulus sera. Therefore, fraction 1 seemed to be more specific to W. bancrofti and fraction 2 to B. malayi as compared to the whole CSE.

Fig. 3.4 The distribution of OD values in different groups of sera when reacted against *B. pahangi* adult worm crude soluble extract in the Enzyme-linked Immunosorbent Assay (ELISA), optimized for the detection of *W. bancrofti* infections.

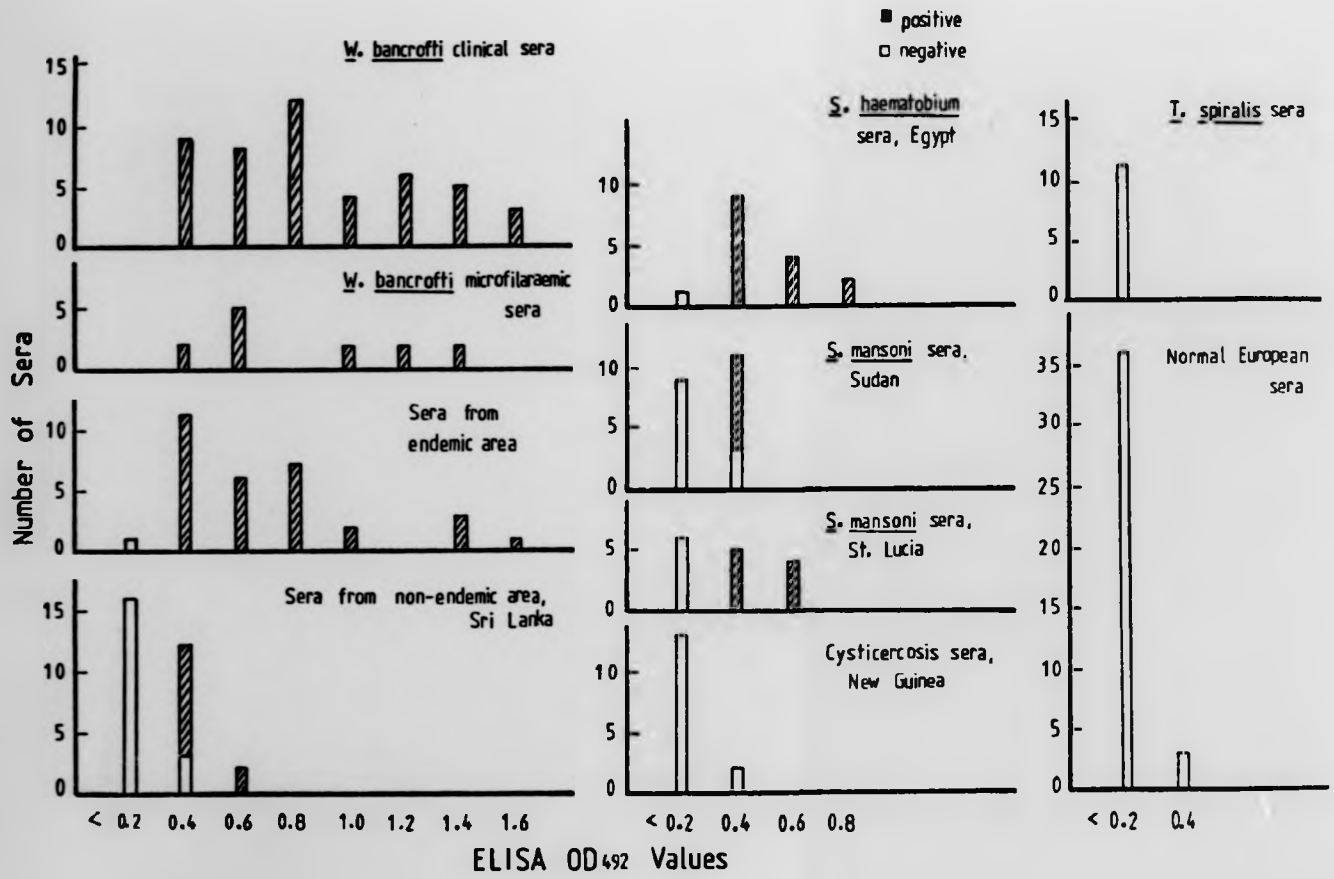

■ positive
□ negative
W. bancrofti clinical sera
W. bancrofti microfilaraemic sera
Sera from endemic area
Sera from non-endemic area, Sri Lanka
S. haematobium sera, Egypt
S. mansoni sera, Sudan
S. mansoni sera, St. Lucia
Cysticercosis sera, New Guinea
T. spiralis sera
Normal European sera
Number of Sera
< 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6
< 0.2 0.4 0.6 0.8
< 0.2 0.4
ELISA OD492 Values

Table 3.10 . Enzyme-linked Immunosorbent Assay (ELISA), using partially purified fractions of *B. pahangi* L5 CSE as antigens, for the detection of antibodies in different groups of sera.

Groups of Sera	% POSITIVITY (Sample Size)							
	Whole CSE	Fractions of CSE 1	2	3	4	5	6	7
B. malayi microfilaraemic cases	93(21)	89(9)	93(21)	56(9)	100(3)	56(9)	67(6)	33(9)
B. malayi clinical cases	81(21)	83(6)	81(21)	83(6)	50(2)	33(6)	20(5)	17(6)
W. bancrofti microfilaraemics	78(9)	100(3)	78(9)	100(3)	100(1)	67(3)	100(1)	67(3)
W. bancrofti clinical cases	89(18)	92(12)	72(18)	58(12)	75(4)	17(12)	18(11)	8(12)
M. ozzardi cases (Trinidad)	67(6)	50(6)	50(6)	50(6)	33(3)	33(6)	0(3)	0(6)
L. loa serum	All Positive (1)							
O. volvulus cases	All Positive (3)							
S. mansoni cases (Sudan)	67(3)	67(3)	33(3)	33(3)	100(1)	33(3)	33(3)	0(3)
S. haematobium cases (Egypt)	100(4)	75(4)	0(4)	75(3)	-	0(4)	0(3)	0(4)
Normal European sera	0(16)	0(16)	0(16)	0(16)	0(4)	0(16)	0(16)	0(16)
Cut-off value	0.29	0.10	0.11	0.14	0.05	0.11	0.11	0.10

The cut-off value was taken as $\bar{x}$ + 3S.D., where $\bar{x}$ was the mean OD values of the normal European sera tested and S.D. the standard deviation of $\bar{x}$.

Some fractions were tested with a smaller number of sera because inadequate amount was obtained from column chromatography fractionation.

DISCUSSION

Although B. pahangi is phylogenetically related to B. malayi, B. pahangi antigens in IFAT have been found less sensitive than W. bancrofti antigen in the detection of B. malayi infections (Table 3.2). However, it is possible that B. malayi patients in India and Malaysia may have been exposed to W. bancrofti too. The use of human filarioids seems to be better than animal filarioids as antigens in IFAT for the detection of human filariasis. Grove and Davis (1978), using B. malayi L5 as antigen in IFAT, obtained 100% positivities with sera from W. bancrofti and B. malayi patients. In this study, B. pahangi L5 antigen reacted only with 84 - 92% of sera from W. bancrofti and B. malayi patients while W. bancrofti L3 antigen gave highest positivity rates (96%) with B. malayi and (100%) W. bancrofti infections (Tables 3.2 and 3.4). Results in the latter further confirm the finding in the B. pahangi-cat system that homologous antigens are better than heterolgous antigens in IFAT (Chapter 2). In this study, results of IFAT using W. bancrofti L3 antigen agree with those (94 - 100% positivites) reported by Ambroise-Thomas and Kien Truong (1972) and Yong (1973) and seem to be better than that of Ten Eyck (1973), who reported 79% positivity with clinical cases.

Using B. pahangi L3 as antigen, 88 - 96% positivity rates with B. malayi infections were observed (Table 3.2). However, Wong and Guest (1969) reported positivity rates of 57 - 86% with B. malayi infections, even using B. malayi L3 as antigen in IFAT. It was probably because whole L3 rather than cryo-sections (as in this study) were used.

For detection of B. malayi infections, D. immitis, D. vitae, L. carinii and O. gutturosa antigens were found to be inferior to B. pahangi and W. bancrofti antigens. Lower

positivity rates and titres were also observed (Tables 3.2 and 3.3). Using different filarial antigens, 41 - 89% positivity rates were observed with sera from residents without clinical or parasitological evidence of filariasis in endemic areas. An IFAT titre as high as 512 was observed (Table 3.3). Most probably, some of them may be in the incubation period of the infection or have microfilaraemic levels so low that the microfilariae could not be detected by counting chamber technique employed. However, cross-reactions with other intestinal helminthic infections, which are common in the tropics, are also possible.

B. pahangi is known to be common among dogs and cats in Malaysia (Dr. J.W. Mak, personal communications). This may explain why 8 - 17% positivity rates have been observed with sera from Malaysians living in areas non-endemic for *B. malayi* when *B. pahangi* rather than *W. bancrofti* (0% positivity) antigens were used. Interestingly, using *B. pahangi* antigens, the positivity rates with sera from people without clinical and parasitological evidence of filariasis in *B. malayi* endemic areas were 8 - 19% higher than that (70%) when *W. bancrofti* antigen was used. This difference is similar to the positivity rates (8 - 17%) with sera from non-endemic areas (Table 3.2). Therefore, the use of *B. pahangi* as antigens for the detection of filariasis in areas where *B. pahangi* are common in animals seems to be less justified. Similar problems have been noted by Garcia *et al* (1968) and Yong (1973). It should also be noted that in the tropics, humans may also be exposed to other animal filarioids eg. *Onchocerca* spp., *Dirofilaria* spp. and *Dipetalonema* spp., etc.. This somehow suggests that a sensitive immunological test has to be specific for the parasitic species studied.

Among the 3 *B. pahangi* antigens used for the detection of bancroftian filariasis, L3 seemed to be the most sensitive (Table 3.4). This again agrees with the earlier finding in

the homologous system in Chapter 2. However, B. pahangi mf seemed to be only slightly less sensitive than L3 and both were superior to other heterologous filarioid antigens used, i.e. higher positivity rates and IFAT titres were observed (Tables 3.4 and 3.5). Since an adequate amount of W. bancrofti antigens is difficult to obtain, until a common laboratory host for this filarioid is available, this study suggests that B. pahangi L3 or mf, with positivity rates of 92 - 100%, can be used as antigens in serological tests for bancroftian filariasis. IFAT, using B. pahangi mf as antigens, gave an overall positivity rate of 88 - 100% with filariasis as a group (Tables 3.2 and 3.4). Hedge and Ridley (1977) reported a positivity rate of 95% with the same antigen in IFAT.

D. viteae L5 antigen gave 85 - 100% positivities with sera from bancroftian filariasis in this study (Table 3.4). This result agrees with that (83 - 92%) reported by Ambroise-Thomas and Kien Truong (1972) but is better than those (24 - 58%) reported by Diesfeld and Braun-Munzinger (1972) and Dutta and Diesfeld (1978). Furthermore, the positivity with D. immitis L5 antigen (74 - 85%) in this study is similar to that (77%) reported by Gentilini et al (1972).

Using different filarial antigens in IFAT, 29 - 81% positivity rates were observed with sera from residents without any clinical or parasitological evidence of filariasis in areas endemic for W. bancrofti. The IFAT titre was as high as 256 (Table 3.5). All these people have been exposed to W. bancrofti and probably some of them might be in the incubation period of the infection or had very low microfilaraemia. In fact, those sera from India in this group were from families in which at least one member had clinical or microfilaraemic evidence of bancroftian filariasis.

20 - 70% positivities were observed with sera from residents in non-endemic areas in Sri Lanka, using different filarial antigens in IFAT. As some of these people always travelled to the endemic areas and some had been living in endemic areas for a long time (Dr. M.M. Ismail, personal communications), it was possible that they had been exposed to W. bancrofti. Also, cross-reactions with other intestinal parasitic infections were possible.

Using worm sections or fragments in IFAT, a whole "mosaic of antigens" of the parasite is present (Ambroise-Thomas, 1976). Therefore, cross-reactivities with other filarioids and also with other helminths are expected (Table 3.2, 3.4 and 3.6). In this study, all filarial antigens did not react with trichinosis sera. However, positivity up to 86% was observed with schistosomiasis sera. They may be true cross-reactions. Nevertheless, the fact that these schistosomiasis sera have been collected from patients in areas where filariasis are endemic makes interpretation of the results difficult. Similar problems have been encountered by most of the workers (Kagan et al., 1963; Duxbury and Sadun, 1967). Therefore, to evaluate the specificity of serological tests, efforts should be taken to collect heterologous sera from areas where the disease under study are non-endemic. Using B. pahangi as antigens in IFAT, the highest titre with schistosomiasis sera was 32 (Table 3.7). It would then seem reasonable to take a titre of 64 or above as specific for filariasis.

In ELISA optimized for B. malayi, less B. pahangi antigen (880 ng/ml) and serum (1: 1600 dilution) were required as compared to that for W. bancrofti (1.8 µg/ml antigen, serum at 1: 800 dilution). It seems that soluble antigens of B. pahangi are more related to B. malayi than to W. bancrofti.

Using B. pahangi L5 CSE as antigen, 85 - 92% positivities were observed with B. malayi infections (Table 3.9). These positivities and that (85%) with sera from people living in endemic areas were similar to those obtained by IFAT, using different B. pahangi antigens (Table 3.2). However, all sera from people living in nonendemic areas in Malaysia were negative in ELISA, as compared to 8 - 17% positivities in IFAT (Tables 3.2 and 3.9). Furthermore, less reactivities with schistosomiasis sera in ELISA (10 - 33%) were observed, as compared to (10 - 86%) in IFAT (Tables 3.6 and 3.9). Therefore, ELISA seems to be a better serological test than IFAT for detecting B. malayi infections, using B. pahangi as antigens. From Fig. 3.3, all schistosomiasis sera, except one, had OD values less than 0.4. Therefore, in an ELISA using B. pahangi L5 CSE as antigen, it seems reasonable to take OD values of 0.4 or above as specific for brugian filariasis.

When ELISA was optimized for the detection of W. bancrofti infections, 100% positivities were observed (Table 3.9). Therefore, ELISA seems to be more sensitive than IFAT (87 - 100% positivities) in detecting antibodies in W. bancrofti infections, using B. pahangi as antigens (Tables 3.4 and 3.9). However, the positivity rates with sera from people in W. bancrofti endemic areas (94%) and schistosomiasis sera (43 - 93%) also increased as compared to those obtained with IFAT, 52 - 81% and 10 - 86% respectively. The positivity rate in ELISA with sera from non-endemic areas in Sri Lanka (37%) remained similar to those (20 - 40%) obtained in IFAT (Tables 3.4 and 3.9). All trichinosis and cysticercosis sera were negative. The latter were from New Guinea where filariasis was endemic. So, it seems that cross-reactivities of ELISA, using B. pahangi antigens, with other helminthic infections may not be very extensive. The OD values of all reactive schistosomiasis sera, except one, were less than 0.7 in an ELISA optimized for detection of W. bancrofti infections (Fig. 3.4). Therefore, it seems feasible to take OD values of

0.7 or above as specific for bancroftian filariasis.

In an earlier study (Chapter 2), ELISA was found to be less sensitive than IFAT in the B. pahangi-cat system. However, in the present study with the human system using B. pahangi as antigen, ELISA has been found to be better than or at least as good as IFAT for the detection of antibodies in brugian and bancroftian filariasis. It seems that different tests are suitable for different host-parasite systems. Preliminary studies with O. gutturosa antigens for the detection of antibodies in infected cattle in ELISA have shown that bovine sera give very non-specific and high background (Dr. D. de Savigny, personal communications). However, in the human system, good distinction between onchocerciasis patient sera and human control sera have been observed in ELISA, using crude or purified Onchocerca antigens (Bartlett et al., 1975; Marcoullis et al., 1978).

Using B. pahangi L5 CSE as antigens in ELISA optimized for the detection of antibodies in brugian and bancroftian filariasis, cross-reactions with schistosomiasis sera and extensive reactivities with other filariasis (M. ozzardi. L. loa. O. volvulus) sera were observed (Table 3.9). To improve the specificity of ELISA, using B. pahangi as antigen, efforts were made to establish ELISA with ES antigens of L5 and L3 and also with ppCSE of L5 of B. pahangi. Attempts to develop ELISA, using ES products of B. pahangi L5 and L3 for detecting brugian and bancroftian filariasis were unsuccessful. This has been confirmed by a later study by Dr. D. de Savigny, Department of Clinical Tropical Medicine. However, in an earlier study (Chapter 2), B. pahangi L3 ES products were not reactive but L5 ES antigens were as good as L5CSE antigens in ELISA for the detection of B. pahangi infections in cats. In this study, ELISA using the L5 CSE antigen has been found to be very sensitive. Therefore, the

low reactivities of B. pahangi L5 ES antigens with human filariasis sera suggest that the ES antigens would probably be more species-specific and ELISA using these ES antigens may be a specific test for infections by a particular species. Further investigations are needed to prove this.

Some somatic antigens are shared between different filarioids and other helminths (Bagai et al., 1968; Capron et al., 1968). The other approach to develop a more specific serological test is to fractionate the CSE by chromatographic means for the specific antigenic components. In this study, the elution profile of B. pahangi L5 CSE in sephadex G-200 column chromatography has revealed one peak (Fig. 3.1) suggesting that soluble antigens of B. pahangi L5 are quite homogeneous in molecular weight. Similar observation has been reported with D. immitis antigen (Sawada et al., 1965). Subsequent separation of the antigens eluted from the sephadex G-200 column by CM-cellulose chromatography resulted in 7 fractions (Fig. 3.2). Fraction 1 seemed to be more specific to W. bancrofti and fraction 2 to B. malayi as compared to the whole CSE in ELISA (Table 3.10). Because of the inadequate amounts of the fractions obtained, no further attempts were made to fractionate fractions 1 and 2, for example by DEAE cellulose chromatography, etc.. Tanaka et al (1968) estimated a recovery rate of 25 - 50% for L. carinii antigens separated by column chromatography. However, results of this study suggest the possibility of obtaining a more specific antigen for serological tests by chromatographical techniques. In fact, high reactivities and sensitivities have been reported in serological tests with purified antigens (Yamashita et al., 1976; Welch and Dobson, 1978).

CHAPTER FOUR

SEROEPIDEMIOLOGICAL STUDIES OF BANCROFTIAN FILARIASIS

INTRODUCTION

Studies on the sensitivities of IFAT and ELISA, using different heterologous filarial antigens, for the detection of antibodies in bancroftian filariasis in Chapter 3 showed that IFAT, using B. pahangi L3 and mf antigens, and ELISA, using B. pahangi L5 CSE antigen, were the most sensitive techniques, having positivity rates of 92 - 100%. The possible applications of these tests in the seroepidemiological studies of bancroftian filariasis were investigated. Four different areas were studied, Papua New Guinea and Trinidad where bancroftian filariasis were still endemic, the Seychelles Islands where bancroftian filariasis had been endemic but nothing was known about the current status of endemicity and the Mechanised Farm at Beheira, Egypt, where studies of the existence of bancroftian filariasis had not been made.

MATERIALS AND METHODS

Indirect Fluorescent Antibody Test (IFAT)

IFAT was used for the study in Papua New Guinea. Since it was laborious to prepare B. pahangi L3 cryosections for antigens, B. pahangi mf fragments were used as antigens. The test was performed in the same way as in Chapter 3.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA, using B. pahangi L5 CSE as antigen, was used for the studies in New Guinea, Trinidad, the Seychelles Islands and Egypt. It was performed in the same way as in Chapter 3. The changes in antibody levels 2 weeks after DEC treatment were also studied in 15 patients in Trinidad.

Blood Spot and Serum Sample

Documented serum samples from 208 residents in Papua New Guinea were collected by Dr. R. Knight, Liverpool School of Tropical Medicine, in 1973.

397 documented blood spots on Whatman No. 3 chromatography paper collected from residents in Blanchisseuse, Trinidad, in 1979 were obtained from Dr. M. Nathan, Caribbean Epidemiology Centre, Port of Spain, Trinidad. Serum samples were also collected from 15 patients before and 2 weeks after chemotherapy with DEC. Serum was eluted from the 50 µl blood spot in 400 µl PBS, pH 7.2 (containing 0.01% merthiolate) to give a dilution of 1: 16, overnight at 4°C.

Documented serum samples were collected from 413 outpatients attending the General Hospital of Victoria, Seychelles, by Prof. M. Nuti, Department of Tropical and Infectious Diseases, University of Rome, in 1979.

129 documented blood spots were collected from residents in the Mechanised Farm at Beheira, Egypt, by Mr. P. Hawkins, Ross Institute, in February, 1980.

RESULTS

Seroepidemiological Study in New Guinea

Filariasis in Papua New Guinea was solely due to nocturnally periodic *W. bancrofti*. A total microfilaraemic (Mf) rate of 50% was observed among the sample population. The Mf rates increased with age after age 20 (Fig. 4.1). The mean mf counts in microfilaraemic individuals of different age groups are shown in Fig. 4.2. Individuals at 10 - 20 years old had the lowest mean mf counts. However, the mean mf counts in individuals of more than 20 years old seemed to stabilize between 325 - 430/ 50 mm^3 blood.

IFAT

154 serum samples were tested by IFAT, using *B. pahangi* mf fragments as antigens. All samples were positive (titre $\geq$ 16) and a unimodal distribution of the IFAT titres was observed, with the peak frequency at titre 256 (Fig. 4.3). 15 sera with titres as high as 1024 were observed but only 3 sera had titres below 64.

The geometric mean IFAT titres in individuals in different age groups are shown in Fig. 4.4. The mean antibody levels were between titres 128 and 512 among individuals of all age groups (regardless of sex). Quite high mean antibody level (mean titre = 256) was already observed in children less than 10 years old.

ELISA

205 serum samples were tested by ELISA, using *B. pahangi* L5 CSE as antigens. Again, all samples were positive (OD values $\geq$ 0.24). The distribution of OD values in the

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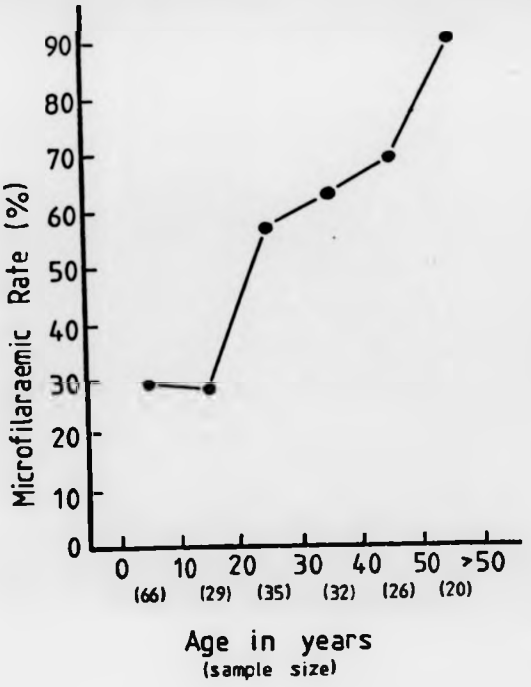
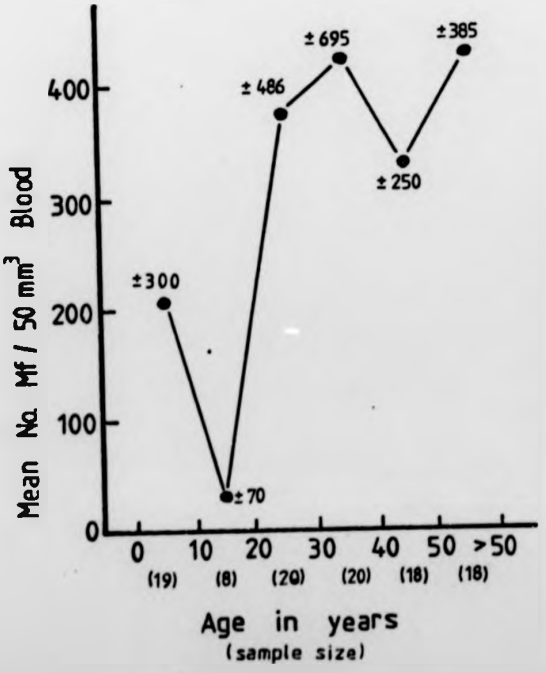
Fig. 4.1 The *W. bancrofti* microfilaraemic rate among different age groups in the sample population in New Guinea. (Data from Dr. R. Knight).

Fig. 4.2 The mean level of microfilaraemia in the microfilaraemic individuals among different age groups in the sample population in New Guinea (Data from Dr. R. Knight).

Number beside point = 1 S.D.


Microfilaraemic Rate (%)
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 >50
(66) (29) (35) (32) (26) (20)
Age in years
(sample size)


Mean No. Mf / 50 mm³ Blood
400
300
200
100
0
±300
±70
±486
±695
±250
±385
0 10 20 30 40 50 >50
(19) (8) (20) (20) (18) (18)
Age in years
(sample size)

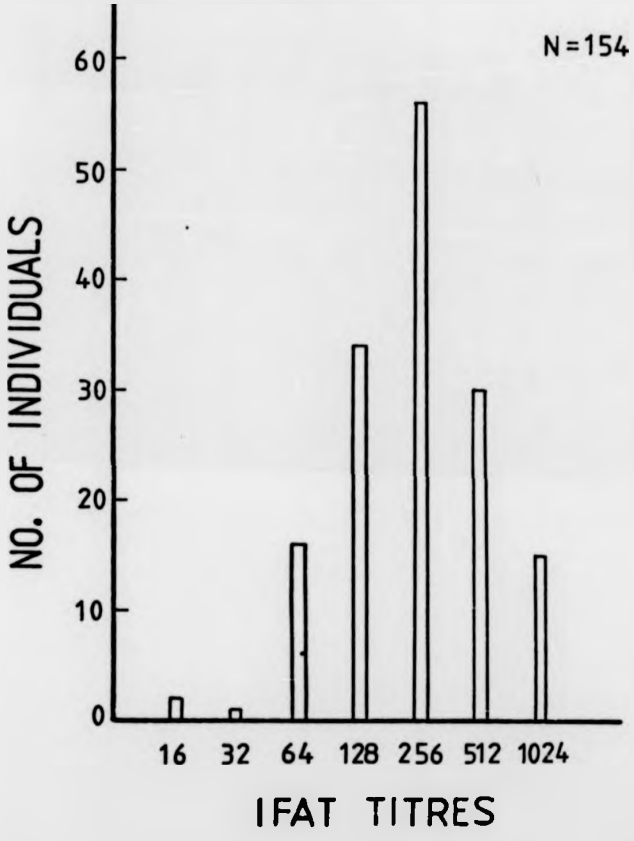


Fig. 4.3 The distribution of the levels of antibodies against B. pahangi microfilariae, measured by the Indirect Fluorescent Antibody Test (IFAT), in the sample population (sample size N = 154) in New Guinea.

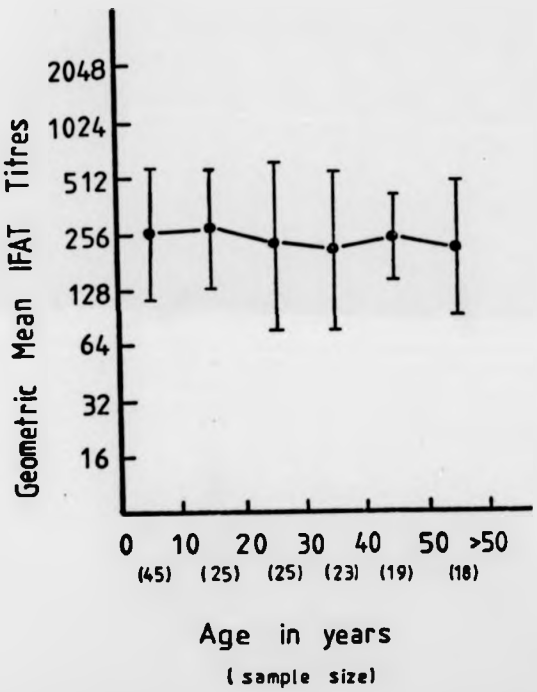


Fig. 4.4 The geometric mean levels of IFAT antibodies to B. pahangi microfilariae among the different age groups in the sample population in New Guinea. Error Flag = 1 S.D.

sample population was unimodal with the peak frequency at 0.8 - 1.0 (Fig. 4.5). 6 sera had high OD values between 1.8 - 2.0.

The mean antibody levels in individuals in the different age groups are shown in Fig. 4.6. The patterns in males and females were similar. A peak mean antibody level was observed among individuals of 10 - 20 years old. The mean antibody levels in individuals of 20 years or more were similar (mean OD values between 0.78 and 0.82) and they were slightly lower than those in people less than 20 years old.

Seroepidemiological Study in Trinidad

In Blanchisseuse, Trinidad, W. bancrofti and M. ozzardi were co-endemic. The Mf rates were 7% for W. bancrofti only, 22% for M. ozzardi only and 9% for both filarioids. Therefore, total Mf rates of 16% for W. bancrofti and 38% for W. bancrofti or M. ozzardi were observed.

The Mf rates among different age groups are shown in Fig. 4.7. The Mf rates for W. bancrofti alone were quite low (less than 12%) among different age groups up to 70 years, above which the Mf rate was zero. The Mf rate for W. bancrofti and M. ozzardi fluctuated between 4 - 24%. The Mf rate for M. ozzardi alone seemed to increase with age. For people above 50 years old, the Mf rate was more than 40%. The total Mf rates also seemed to increase with age up to 40 years, above which fluctuations between 43 - 65% were observed (Fig. 4.8). The maximum total Mf rate was in the 60 - 70 age group.

The mean level of M. ozzardi microfilaraemia increased with age up to a maximum in the 60 - 70 age group (Fig. 4.9a). Fluctuations of the mean level of W. bancrofti microfilaraemia were observed (Fig. 4.9b). The lowest level occurred in the 20 - 30 age group followed by a rise till 60 year-old.

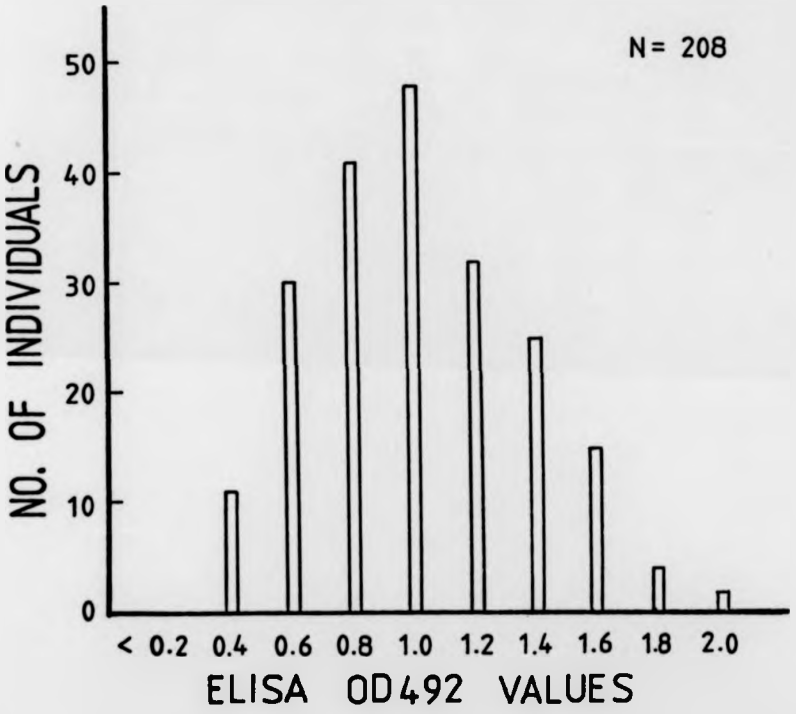


Fig. 4.5 The distribution of the levels of antibodies to B. pahangi adult worm antigen (OD492 values) as measured by the Enzyme-linked Immunosorbent Assay (ELISA) in the sample population (sample size N = 208) in New Guinea.

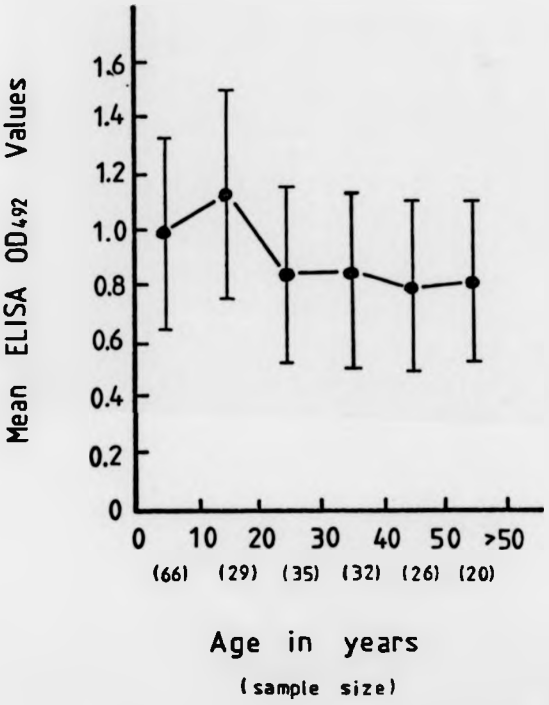


Fig. 4.6 The mean level of antibodies to B. pahangi adult worm antigen (OD492 values) as measured by the Enzyme-linked Immunosorbent Assay (ELISA) among the different age groups in the sample population in New Guinea.

Fig. 4.7 The microfilaraemic (Mf) rate for W. bancrofti, W. bancrofti and M. ozzardi, and M. ozzardi among different age groups in the sample population in Blanchisseuse, Trinidad.
(Data from Dr. M. Nathan).

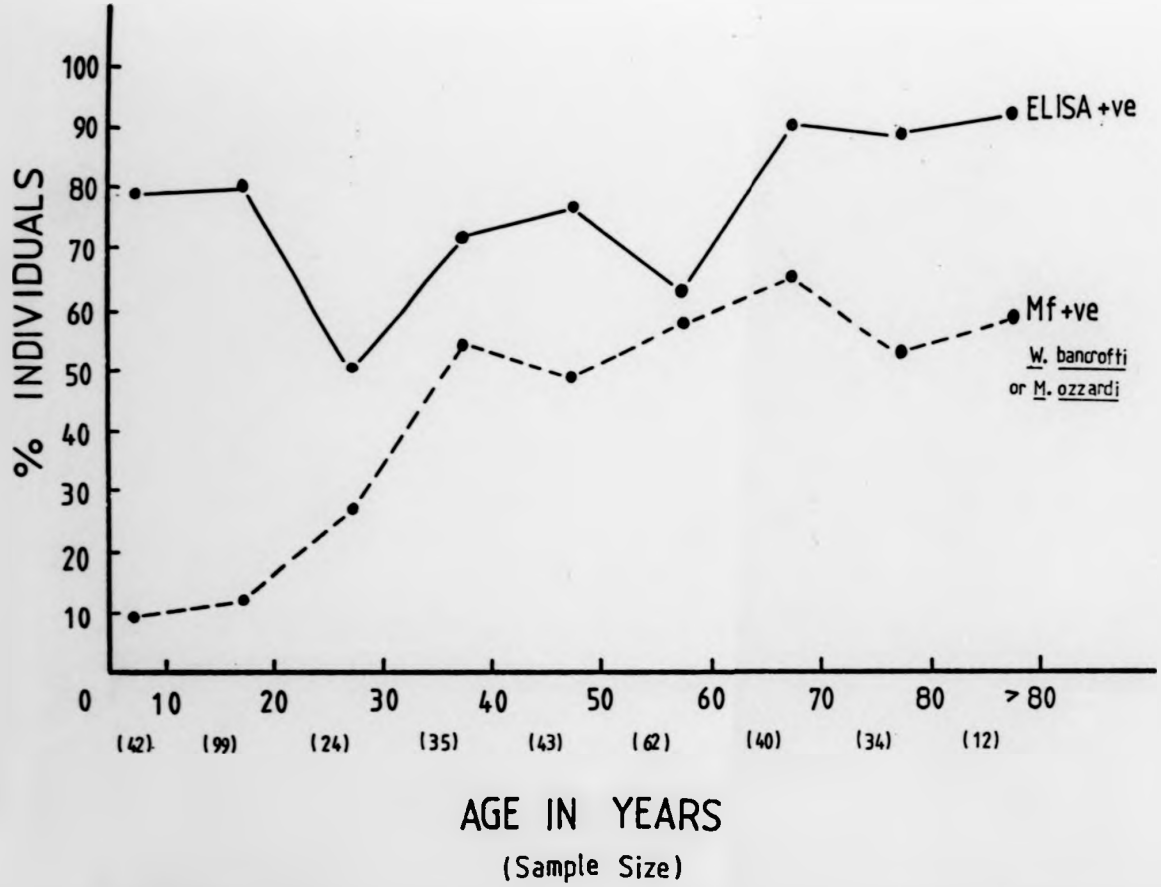
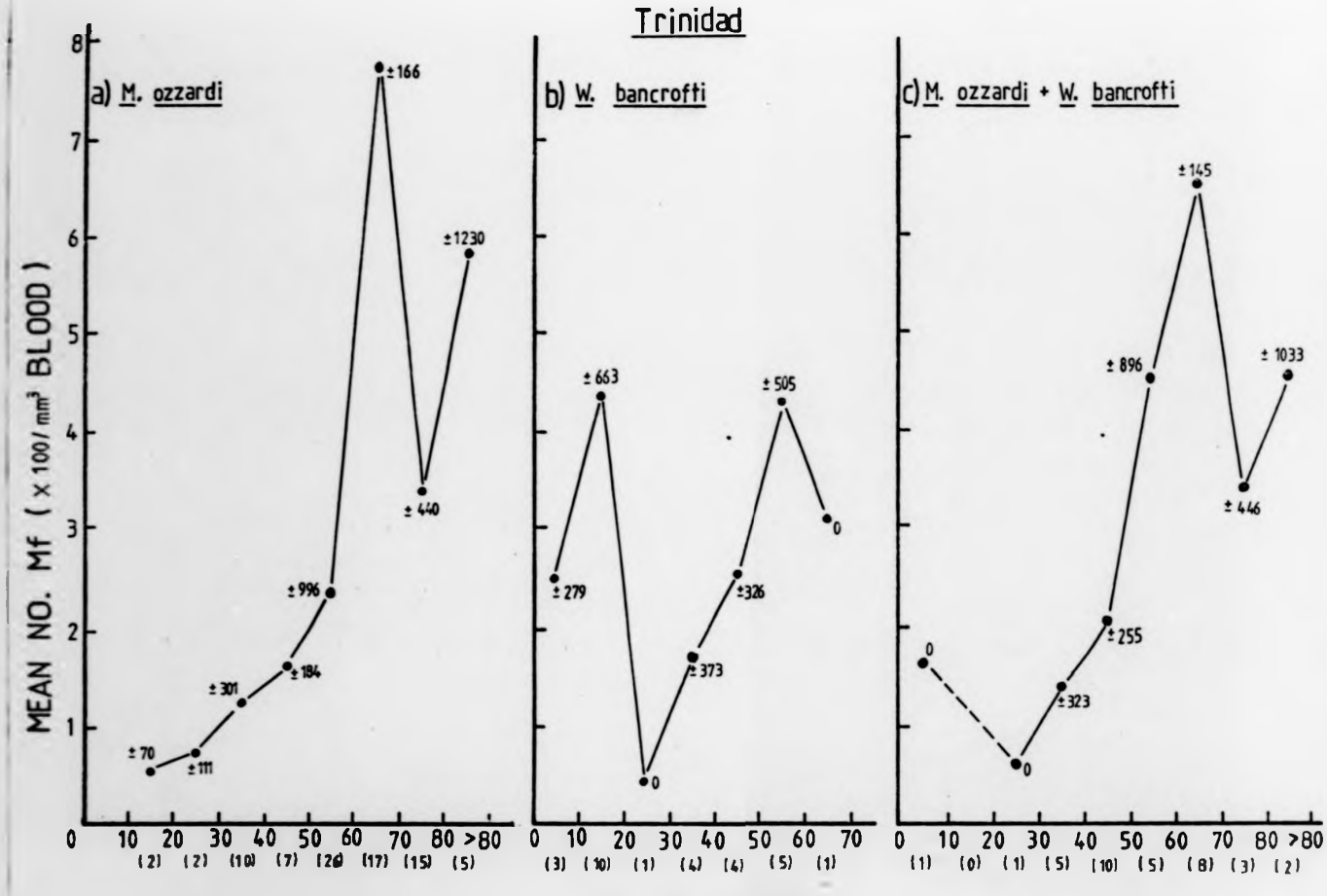


Fig. 4.8 The relationship between the ELISA positivity rate (OD $\geq$ 0.24) and the total microfilaraemic (Mf) rate among different age groups in the sample population in Trinidad.
(Data of Mf rate obtained from Dr. M. Nathan)

Fig. 4.9 The mean level of microfilaraemia among the microfilaraemic individuals, with (a) *M. ozzardi*, (b) *W. bancrofti* and (c) *M. ozzardi* and *W. bancrofti*, in different age groups in the sample population in Trinidad.
Number beside point = 1 S.D.


Trinidad
a) M. ozzardi
b) W. bancrofti
c) M. ozzardi + W. bancrofti
MEAN NO. Mf (x 100/ mm³ BLOOD)
± 70
± 111
± 301
± 184
± 996
± 166
± 440
± 1230
± 279
± 663
0
± 373
± 326
± 505
0
0
0
± 323
± 255
± 896
± 145
± 446
± 1033
0 10 20 30 40 50 60 70 80 >80
(2) (2) (10) (7) (20) (17) (15) (5)
0 10 20 30 40 50 60 70
(3) (10) (1) (4) (4) (5) (1)
0 10 20 30 40 50 60 70 80 >80
(1) (0) (1) (5) (10) (5) (8) (3) (2)
AGE IN YEARS
(Sample Size)

A rise in the mean level from the 20 - 30 age group to the maximum in the 60 - 70 age group was observed in *M. ozzardi* and *W. bancrofti* concurrent infections (Fig. 4.9c).

An ELISA, optimized with a reference bancroftian filariasis serum from India (where *M. ozzardi* was absent), as in Chapter 3 was used for this study. 79% of the samples were positive (OD values $\geq$ 0.24). Similar positivity rates were observed in individuals having circulating *W. bancrofti* mf only (84%), *M. ozzardi* mf only (85%) and both mf (86%). About 80% of individuals less than 21 years old were positive in ELISA (Fig. 4.8). The lowest ELISA positivity rate was observed in the 20 - 30 age group. The positivity rates fluctuated among the 30 - 60 age groups. About 90% of individuals more than 60 years old were ELISA positive. The overall changes of ELISA positivity rates seemed to correlate with the changes of total Mf rates in most of the age groups.

The distribution of ELISA OD values among the sample population are shown in Fig. 4.10. A bimodal distribution with peaks at OD values 0.2 - 0.3 and 0.7 - 0.8 were observed. 6 sera had OD values higher than 1.5.

The mean ELISA OD values fluctuated between 0.35 - 0.6 (Fig. 4.11). Children under 10 years old had a mean OD value of 0.5. The lowest mean OD value (0.38) was observed in the 20 - 30 age group. The mean OD values stayed between 0.55 - 0.6 in people more than 50 years old.

A rise in antibody level, as revealed by an increase of OD values (by 0.2 - 0.3) in ELISA, was observed in 80% of the 15 patients 2 weeks post-treatment with DEC (Table 4.1). 9 of them had concurrent *M. ozzardi* and *W. bancrofti* infections.

Fig. 4.10 The distribution of the levels of antibodies (OD492 values) to *B. pahangi* adult worm antigens, as measured by the Enzyme-linked Immunosorbent Assay (ELISA) in the sample population in Trinidad. N = sample size.

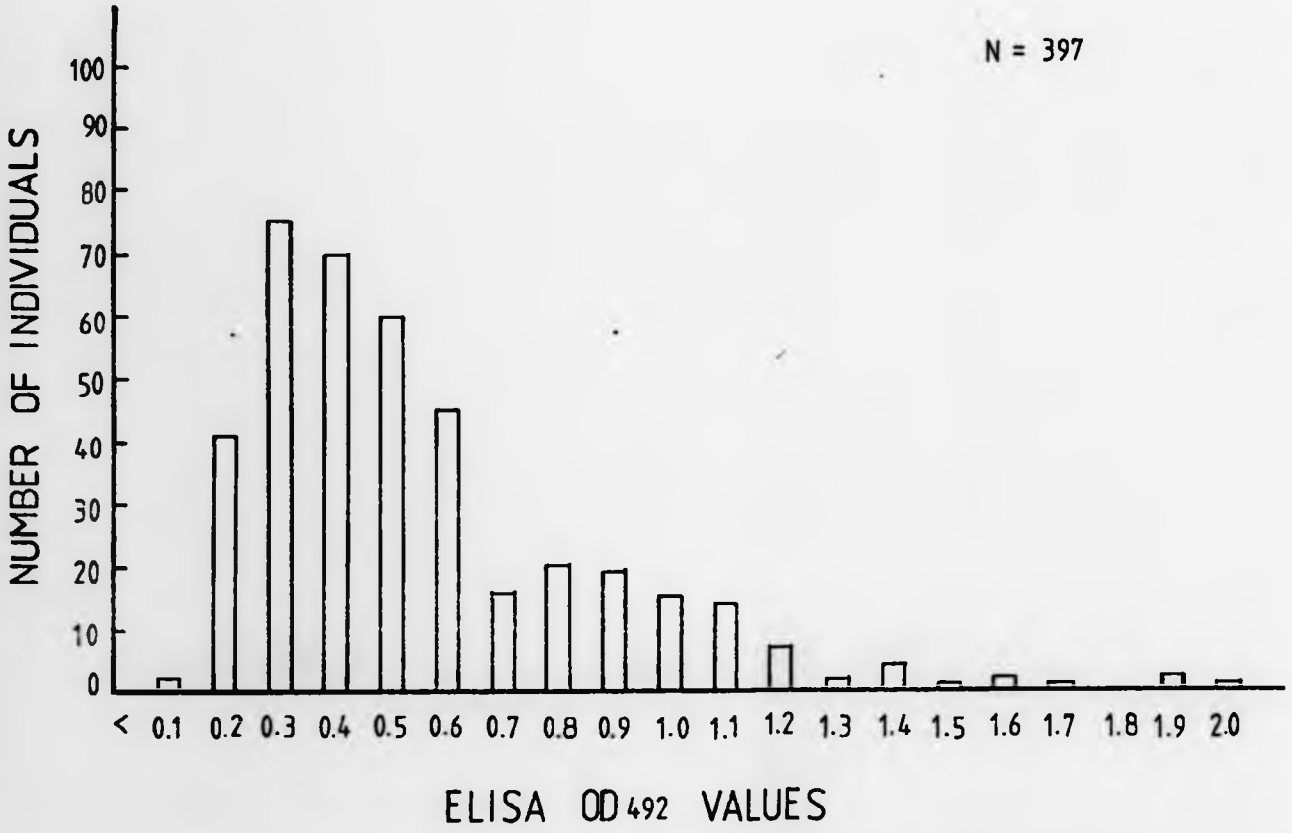

N = 397
NUMBER OF INDIVIDUALS
100
90
80
70
60
50
40
30
20
10
0
< 0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
ELISA OD 492 VALUES

Fig. 4.11 The mean levels of antibodies against B. pahangi adult worm antigens in the Enzyme-linked Immunosorbent Assay (ELISA) among different age groups in the sample population in Trinidad.
Error flag - 1 S.D.

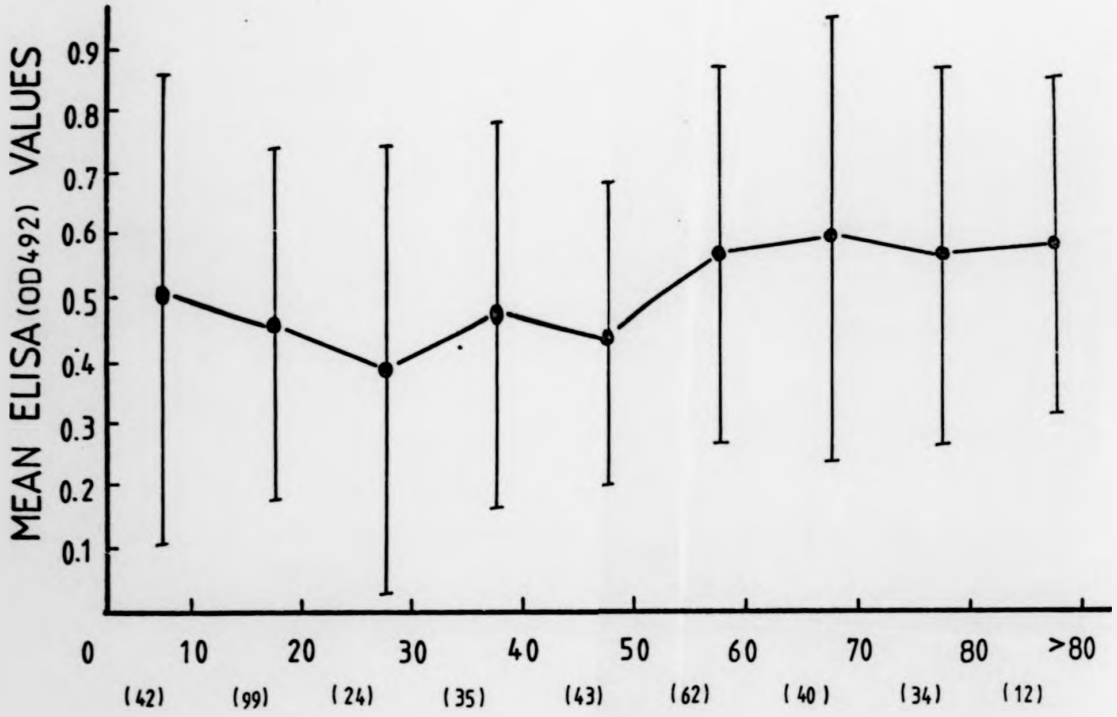

MEAN ELISA (OD492) VALUES
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
>80
(42)
(99)
(24)
(35)
(43)
(62)
(40)
(34)
(12)
AGE IN YEARS
(Sample Size)

Table 4.1 The changes of antibody levels, as measured by ELISA, after DEC treatment in 15 filariasis patients, Trinidad.

Serum No.	No. Mf/25mm^3 Blood *W. bancrofti*/*M. ozzardi*	Antibody Levels: Pre-treatment OD Values	Antibody Levels: 2 weeks post-DEC OD Values
785797	< 1 / 1	0.917	1.120*
785800	4 / 0	0.543	0.762*
787090	105 / 59	0.544	0.839*
785979	2 / 39	0.353	0.487*
787612	11 / 143	0.494	0.736*
787616	70 / 146	0.710	1.091*
788671	< 1 / 163	0.507	0.457
787930	7 / 12	0.375	0.424*
788967	11 / 93	0.236	0.520*
789704	2 / 1	0.529	0.773*
789709	124 / 0	0.369	0.240
789816	7 / 0	0.323	0.400*
789967	15 / 0	0.323	0.293
790300	5 / 0	0.244	0.370*
789966	1283 / 0	0.377	0.445*

Mf - microfilaariae; * an increase in ELISA OD values.

Seroepidemiological Study in the Seychelles Islands

86 blood samples were examined by filtration technique, among the 413 outpatients studied, no microfilariae were found. There were 2 cases of elephantiasis but they were amicrofilaraemic too. 20% of the sample population had intestinal infections including amoebiasis, balantidiasis, giardiasis, strongylosis, ancylstomiasis, trichuriasis and ascariasis, by stool examinations. In view of this high incidence of intestinal infections, it was decided that the cut-off values of ELISA should be estimated not only from the 39 normal European sera (as in Chapter 3) but 42 sera from residents without clinical and parasitological evidence of filariasis in areas non-endemic for filariasis in Sri Lanka and Malaysia were also included. As a result, the cut-off value was estimated to be 0.44 at the 99% confidence level.

Screening of the 413 sera with ELISA optimized for bancroftian filariasis revealed a total positivity rate of 24%. 18% of people without intestinal parasitic infections were positive and 14% for those with intestinal infections. The positivity rates among different age groups are shown in Fig. 4.12. One of the 3 samples in the 1 - 10 age group was positive. Total positivity rates between 20 - 27% were observed in the 10 - 50 years age groups. The lowest positivity rate was observed in the 50 - 60 age group and the highest in the >60 age group.

The mean OD values in ELISA among different age groups are shown in Table 4.2. They were less than the cut-off value with a range of 0.276 - 0.411. The lowest mean OD value was observed in the 50 - 60 age group and the highest in the >60 age group. 3 - 8% had OD values larger than 0.7 in the 10 - 50 age groups and 18% in the >60 age group. A serum sample was obtained from one of the 2 elephantiasis cases, it gave a negative reaction in ELISA (OD value = 0.33).

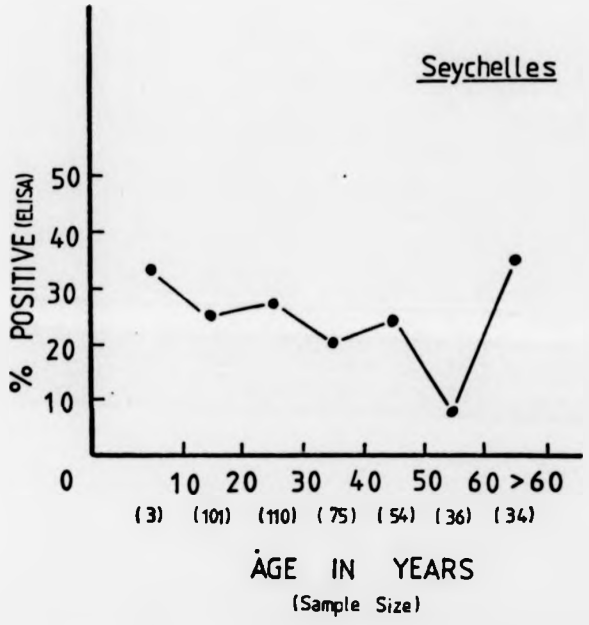


Fig. 4.12 The ELISA positivity rates (for antibodies to B. pahangi adult antigen) among the different age groups in the sample population in the Seychelles Islands.

Table 4.2 The mean OD values and percentage individuals with OD values larger than 0.7 among the different age groups in the sample population in the Seychelles Islands.

Age Groups	N	Mean OD ± S.D.	% with O.D. > 0.7
1 - 10	3	0.309 ± 0.21	0
10 - 20	100	0.350 ± 0.23	7
20 - 30	110	0.343 ± 0.23	8
30 - 40	75	0.293 ± 0.18	3
40 - 50	54	0.341 ± 0.21	7
50 - 60	36	0.276 ± 0.13	0
> 60	34	0.411 ± 0.22	18

N - sample size

A bimodal distribution of the OD values in the sample population was observed with peak frequencies at 0.2 - 0.3 and 0.7 - 0.8 (Fig. 4.13). 4 sera had OD values greater than 1.0.

Seroepidemiological Study in the Mechanised Farm, Beheira, Egypt

The possible existence of bancroftian filariasis in this area was completely unknown although mosquitoes like Culex spp. were present, especially during summer months. No elephantiasis cases were observed. 39% of the sample population, mainly in the younger age group of less than 20 years old, had helminth infections due to S. haematobium, S. mansoni and Ascaris lumbricoides by stool examinations. An ELISA cut-off value was taken as 0.44 as in the preceding study in the Seychelles.

The positivity rates among the different age groups are shown in Table 4.3. 3 - 16% were positive in the age groups of less than 20 years old. 5 sera in the $\geq$21 age groups were positive.

The mean OD values in ELISA among the different age groups ranged from 0.24 - 0.34 and they were less than the cut-off value, 0.44 (Table 4.3). 4 of the 11 (36%) ELISA positive individuals had other helminth infections. A unimodal distribution of the OD values in the sample population with the peak at OD values 0.2 - 0.3 was observed (Fig. 4.14). One sample, from an 8-year old boy without detectable intestinal infections had an OD value of 0.744.

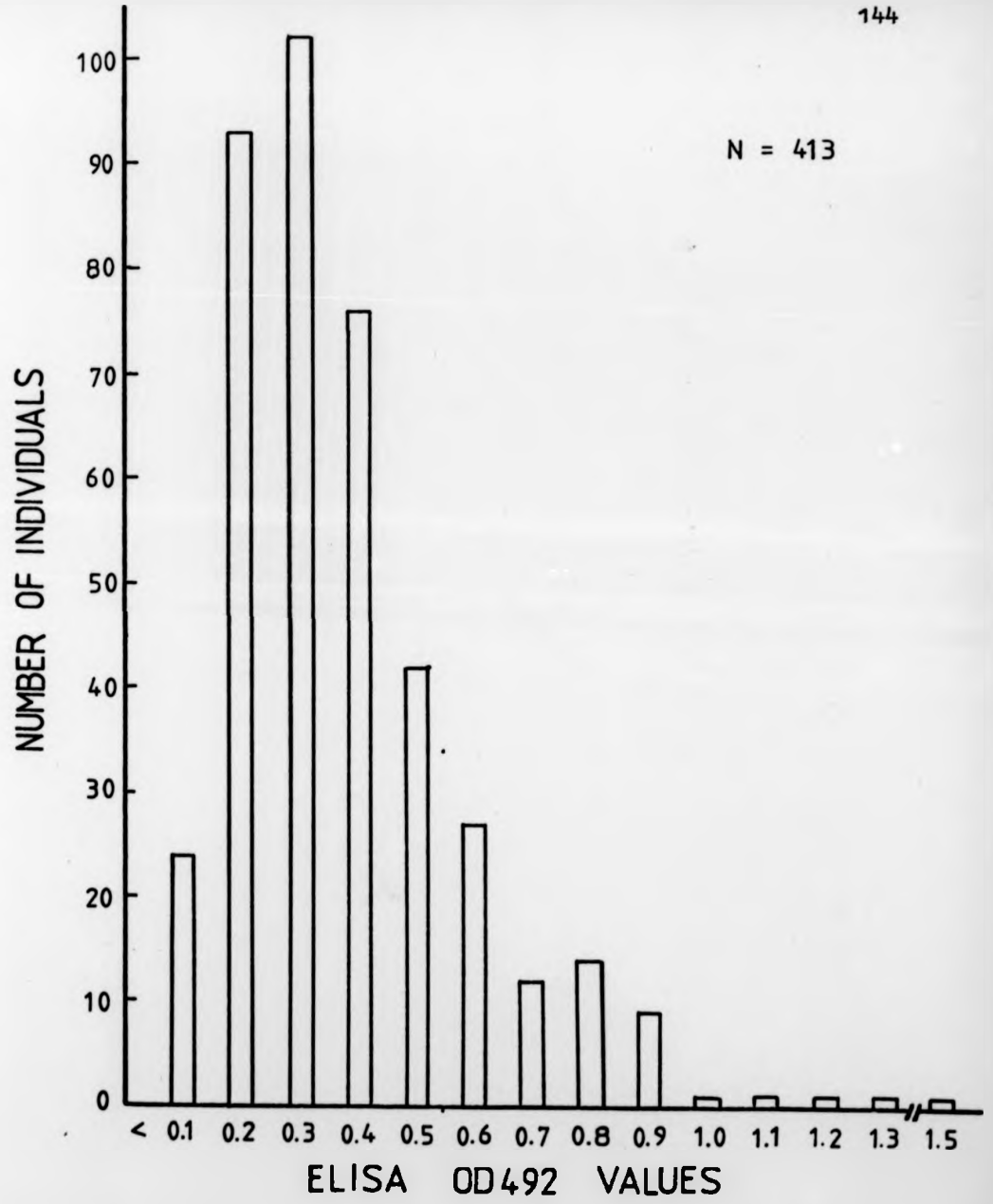


Fig. 4.13 The distribution of the levels of antibodies to B. pahangi adult antigens in the Enzyme-linked Immunosorbent Assay (ELISA) among the sample population in the Seychelles Islands. N = sample size.

Table 4.3 ELISA positivity rates and mean ELISA OD492 values for filariasis antibodies among the different age groups in the sample population at the Mechanised Farm, Beheira, Egypt.

Age Groups	Sample Size	Positivity Rates (%)	Mean ELISA OD492 Values ± S.D.
1 - 10	37	16	0.28 ± 0.17
10 - 20	75	3	0.24 ± 0.10
20 - 30	9	11	0.26 ± 0.16
30 - 40	5	0	0.24 ± 0.10
> 40	3	33	0.34 ± 0.19

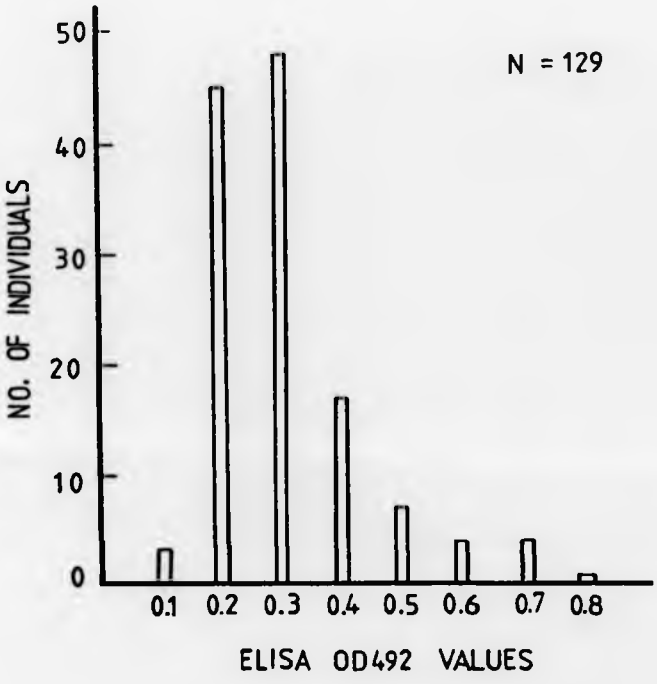


Fig. 4.14 The distribution of the levels of antibodies to B. pahangi adult antigen (OD492 values) in the Enzyme-linked Immunosorbent Assay (ELISA) among the sample population in the Mechanised Farm, Beheira, Egypt.
N - sample size.

DISCUSSION

Bancroftian Filariasis in Papua New Guniea

Bancroftian filariasis is considered to be widely endemic in Papua New Guinea (Sasa, 1976). A total Mf rate of 50% was observed in the sample population, suggesting bancroftian filariasis was highly endemic in the area studied. A unimodal distribution of IFAT titres and ELISA OD values was observed, with peak frequencies at IFAT titres 256 and OD values 0.8 - 1.0 indicating that most individuals had high antibody levels (Figs. 4.3 and 4.5). This pattern is typical for a highly endemic situation (Kagan, 1973). Therefore, the results from IFAT and ELISA were parallel to the high level of endemicity of bancroftian filariasis in New Guinea. All samples were positive by IFAT and ELISA (100%), positivity rate being twice the Mf rate. The total serological positivity rates were also observed to be 2 - 3 fold higher than the total Mf rate by Desowitz *et al* (1966) and Grove *et al* (1978). A higher serological positivity rate is expected since there always exists a number of individuals among the microfilaria negative group who are actually infected but whose microfilaraemic levels are so low that no mf show up in the blood samples examined at one time (Sasa, 1976). However, cross-reactions with other parasitic infections are possible (Ambroise-Thomas *et al*., 1975).

Filariasis in Blanchisseuse, Trinidad

The seroepidemiological study of bancroftian filariasis in Blanchisseuse, Trinidad, were complicated by the co-existence of *M. ozzardi* infection, the prevalence and distribution of which had been reported by Nathan *et al* (1979). Therefore, people in this area were exposed to concurrent infections by

W. bancrofti and *M. ozzardi*. The latter infection seems to be unaffected by DEC treatment (Bartholomew *et al.*, 1978). It also seems to suppress the host response to *W. bancrofti* infections such that there are less elephantiasis cases than expected (Prof. G.S. Nelson, personal communications). Earlier studies in Chapter 3 have shown that *M. ozzardi* sera from Trinidad reacted with the various filarial antigens in IFAT and ELISA. Since no reference sera for pure *M. ozzardi* infections were available, it was also difficult to estimate the relative sensitivity of ELISA used in this study, optimized for the detection of bancroftian filariasis with *B. pahangi* L5 CSE antigens, in detecting *M. ozzardi* infections. A specific serological test was unavailable, therefore, the seroepidemiological study of filariasis in Blanchisseuse was for both *W. bancrofti* and *M. ozzardi* infections.

There was an overall Mf rate of 38% (7% for *W. bancrofti* only, 22% for *M. ozzardi* only and 9% for both infections). A bimodal distribution with peaks at OD values 0.2 - 0.3 and 0.7 - 0.8 was observed (Fig. 4.10). According to Kagan (1973), the first mode in the low-value range may include non-specific reactions with sera from noninfected individuals or from members in the population with low antibody levels. The second mode may represent specific antibody levels with the antigen employed. This pattern of distribution is typical in an endemic area. Hence, ELISA used in this study is able to reflect the pattern of endemicity of filariasis in Blanchisseuse. It is interesting to note that in Chapter 3, an OD value of 0.7 or more has been suggested to represent specific antibody level to filariasis. In this study, the second mode, probably representing specific antibodies, also falls into the same range of OD values.

79% of the samples were positive, about twice the Mf rate. A higher serological rate was expected because the examinations

of blood samples for mf were performed by the blood smear technique, a rather insensitive method. However, cross-reactions with other parasitic infections are also possible. In the earlier study (Chapter 3), ELISA used in this study gave 100% positivity rates with bancroftian filariasis. In this study, only 84 - 86% of the W. bancrofti microfilaraemic individuals were positive in ELISA. It seems to support the observation by Prof. G.S. Nelson (personal communications) that the host response to W. bancrofti seems to be suppressed by M. ozzardi concurrent infections.

The overall changes of ELISA positivity rate among the individuals more than 20 years old seem to correlate with those of the Mf rates (Fig. 4.8). The high ELISA positivity rate in the youngest age group, which has a low Mf rate, may have been partly contributed by the transplacental maternal IgG antibodies and also possibly circulating antigens, at least in early postnatal life (Dutta et al., 1977; Bloomfield et al., 1978; Dissanayake et al., 1980).

A rise in antibody levels 2 weeks after DEC chemotherapy was observed in 80% of the 15 patients studied (Table 4.1). The remaining 20% showed a decrease instead. Ambroise-Thomas and Kien Truong (1972) showed that an overall increase in antibody level was followed by a gradual decline in loiasis patients treated with DEC. A decrease in antibody levels in bancroftian filariasis patients and D. immitis-infected dogs post-treatment with DEC has been reported by Kaeuffer et al (1976) and Desowitz et al (1978a). However, for evaluation of the success of filariasis control programmes with serological tests, it is essential to study the changes of antibody levels over a period of time post-treatment, eg. how long it would take for antibodies to decline and how long will antibodies remain at low levels after successful chemotherapy with and without subsequent continuous exposure to the infections. Murthy et al (1978) reported that skin test reactions gradually

increased to near pretreatment level in 12 months' time, in the presence of continuous exposure to the infection, after the decline upon DEC treatment.

Bancroftian Filariasis in the Seychelles Islands

The epidemiology of filariasis in the Seychelles Islands has been discussed by Sasa (1976). W. bancrofti is the only human filarioid present. After the wide-scale DEC treatment in 1969 - 1970, 27 out of 1270 patients (2%) hospitalized in the General Hospital of Victoria were found to be microfilaraemic in 1972 (Prof. M. Nuti, personal communications). 9% of the Culex pipiens fatigans were found to be infected with L3 in 1975. Blood surveys in 1976 and 1977 on 372 people revealed an Mf rate of 2 - 7% (Garcin-Gibert, 1978). Recent studies have shown that 73% of the population have intestinal infections, trichiuriasis, giardiasis and amoebiasis being the most common (prof. M. Nuti, personal communications). However, it is not known whether W. bancrofti is still endemic in the Seychelles Islands.

24% of the samples were positive in ELISA. With the adjusted cut-off value to 0.44, cross-reaction with intestinal parasitic infections seems to be less likely since 18% of the people without intestinal infections were ELISA positive but only 14% for those with intestinal infections. The observations that lowest ELISA positivity rate and mean ELISA values were in the 51 - 60 age group but the highest in the >60 age group could not be explained (Fig. 4.12 and Table 4.2). Probably, these samples were highly selected since they belonged to outpatients attending the General Hospital of Victoria. The mean OD values among different age groups were less than the out-off value (Table 4.2). However, a bimodal distribution of the OD values was observed, with peak frequencies at OD values of 0.2 - 0.3 and 0.7 - 0.8 (Fig. 4.13). This pattern is similar

to that observed in Trinidad (Fig. 4.10) and it is typical for an endemic area (Kagan, 1973). From Table 4.2, 3 - 18% of individuals in the 11 - >60 age group had OD values larger than 0.7, which had been suggested to represent specific antibody levels to filariasis in the earlier study in Trinidad. The highest percentage of sera (18%) with OD values larger than 0.7 was observed in the >60 age group. It is to be expected since older people will have been exposed to the infection for a longer period of time. Two elephantiasis cases were observed. The serum from one of them was ELISA negative, probably as the result of treatment. Therefore, all these evidence suggest that W. bancrofti is still endemic in the Seychelles Islands but probably at a low level (only 7% of the samples had OD values larger than 0.7). The question of whether recent transmissions are evidenced cannot be determined because only 3 samples were obtained from the 1 - 10 age group.

Bancroftian Filariasis in the Mechanised Farm, Beheira, Egypt

The mechanised Farm at Beheira was opened for settlement in 1968 - 1972. So, residents there of the age of 8 and above came from various parts of Egypt where bancroftian filariasis might be endemic. The possible endemicity of W. bancrofti in this area is completely unknown although suitable mosquito vectors are present. The distribution of the ELISA OD values in the sample population is a unimodal type with the peak at low OD values, 0.2 - 0.3. The highest OD value was below 0.8. According to Kagan (1973), this pattern is typical for situations in which the particular parasitic disease is absent or transmission does not occur or prevalence of infection is very low. From earlier studies in Chapter 3 and in Trinidad, an OD value of 0.7 or above seems to be specific for filariasis. In this study, only one serum sample, from an 8-year old boy, who did not have any detectable intestinal parasitic infections, had an OD value of 0.744. It is possible that this boy may have been exposed to filariasis before moving into this area.

However, the possibility that he may be exposed to filariasis in his family, which consists of the older generations with filariasis acquired elsewhere, is unknown because very few samples were obtained from the older age group. Basing on the facts that all the samples, except one, mainly from the younger age groups, have OD values less than 0.7 and from the pattern of distribution of OD values, it seems very likely that recent transmissions of W. bancrofti in this area are absent. However, to confirm this and the non-existence of the disease in this area, blood samples especially from the older people should be examined for the presence of mf since they would be the "reservoirs" of infection for the younger generations in the presence of suitable mosquito vectors. Also, mosquito catches should be performed for search of W. bancrofti L3.

In general, the above studies have suggested that IFAT, using B. pahangi mf as antigen and ELISA, using B. pahangi L5 CSE as antigen, can be applied to the seroepidemiological studies of bancroftian filariasis. Both IFAT and ELISA could reflect the high level of endemicity of W. bancrofti in New Guinea. ELISA could also reflect the pattern of endemicity of filariasis in Trinidad and it was useful in revealing the level of endemicity of bancroftian filariasis in the Seychelles Islands and the Mechanised Farm at Beheira, Egypt. ELISA using small quantities of soluble antigen (possibly the form of specific antigen) can be used to screen a large number of samples in a short time. As compared to blood surveys for mf, it is more efficient and economical when an overall idea of the level of endemicity is required. It seems that ELISA will have an important role to play in the seroepidemiology of filariasis in future. Its useful applications in other parasitic infections has already been discussed by Voller et al (1976a).

However, this study has also revealed the greatest drawback of serology in filariasis — the existence of cross-reactions with other parasitic species, which sometimes makes interpretation of the results difficult (Ambroise-Thomas, 1974; Kagan, 1974). Thus, the development of a more specific test is needed for filariasis.

CHAPTER FIVE

DETECTION OF ANTIBODIES TO ENZYMES OF PARASITIC ORIGIN IN B. PAHANGI-INFECTED CATS

INTRODUCTION

During the course of parasitic infections, the host is invariably exposed to the numerous enzymes present in the secretion, excretion or moulting fluids of the parasites. Also, enzymes which are normally confined within the cytoplamic matrix will be released upon the death and subsequent disintegration of the parasites. Some of these enzymes are likely to be antigenic. In fact, the presence of antibody response to acetylcholinesterase (AchE) of Nippostrongylus brasiliensis, Oesophagostomum radiatum, Trichostrongylus colubriformis and Hyostrongylus rubidus has been reported in infected rats, cattle, sheep and pigs respectively (Jones and Ogilvie, 1972; Bremner et al., 1973; Rothwell et al., 1973; Masaba and Herbert, 1978).

It is generally accepted that the patterns of migration of certain isoenzymes of parasites in electrophoresis are species-specific and hence are of taxonomic significance (Taylor and Muller, 1979). Furthermore, antibodies from infected animals or artificially immunized rabbits have been shown to react only with the specific isoenzymes of the homologous species or to a lesser extent with the isoenzymes of a closely related species (Rothwell et al., 1973; Shirley and Rollinson, 1979).

To date, species-specific antigens are not available for the serodiagnosis of most helminthic infections, especially filariasis. The serological tests using heterologous antigens

can only reflect the level of endemicity of filariasis in a certain region but the cross-reactions with other helminths often make interpretation of the seroepidemiological results difficult (see Chapters 3 and 4). Much work remains to be accomplished for the immunodiagnosis of helminthic infections (Kagan, 1979). It seems highly possible that species-specific isoenzymes of the parasite may serve as sources of specific antigens and the demonstration of this specific isoenzyme-antibody reaction would help in diagnosing specific parasitic infections in man and animals. Therefore, this study was designed to detect antibodies against enzymes of parasitic origin in B. pahangi-infected cats, using polyacrylamide gel electrofocusing and spectrophotometric techniques. The specificity of the isoenzyme-antibody reaction was studied by reacting the B. pahangi isoenzymes with immune sera against various heterologous parasitic infections.

MATERIALS AND METHODS

Soluble Antigens of Parasites

Soluble extract (CSE) of adult worms (L5) of B. pahangi, D. immitis, L. carinii and D. viteae; infective larvae (L3) of B. pahangi and T. spiralis and of microfilariae (mf) of B. pahangi were prepared according to the Materials and Methods described in Chapter 2. CSE of O. gutturosa L5 was obtained from Dr. H.A. Flockhart, Winches Farm Field Station.

Metabolic products of B. pahangi and the different purified antigen of B. pahangi L5 CSE were prepared as described in Chapters 2 and 3.

Immune Sera

Sera from B. pahangi-infected cats were obtained as described in Chapter 2. Sera from T. spiralis-infected rats and a rabbit antiserum against O. gutturosa L5 were obtained from Dr. C.D. Mackenzie, Department of Medical Helminthology. Sera from human with hydatidosis and schistosomiasis (due to S. mansoni) and a rabbit antiserum against S. mansoni egg antigen were obtained from Dr. M.L. McLaren, Ross Institute. Sera from human infected with B. malayi, W. bancrofti, O. volvulus, L. loa, M. ozzardi and Dracunculus medinensis were provided by Drs C.K. Rao, J.W. Mak, N.M. Ismail, D.S. Ridley and M. Nathan. Rabbit antisera against B. pahangi L5 males (M), male and female (M&F), L3 and mf were prepared according to those described in Chapter 2.

The γ-globulin fractions of immune sera were prepared as described in Chapter 2.

Immune Serum and Worm Antigen Reactions

Sera were inactivated at 56°C for 30 min to destroy endogenous enzymes. 6 µl were then mixed with an equal volume of worm extract and incubated at 37°C for 15 min.

Polyacrylamide Gel Isoelectric Focusing (PGIF)

The LKB ampholine polyacrylamide gel plates(PAGplates) and the LKB electrofocusing kit were used. The gel template was placed on the multiphor cooling plate with a thin layer of liquid paraffin oil (as an insulating layer) smeared in between. The PAGplate was then placed on the template with a thin smear of liquid paraffin oil in between. Care was taken to avoid trapping air bubbles in between. The cathode-electrode strip

was soaked with 1M NaOH and the anode-electrode strip with 1M H_3PO_4. They were then laid onto the gel plate at the proper positions marked on the template. The parts of the strips extruding beyond the gel were cut-off. 10 µl of the sample, worm extract alone or a mixture of worm extract and serum, were applied onto a small application piece. 22 - 24 samples were tested simultaneously and the application pieces were placed along the cathodic side of the plate according to the areas marked on the template. The pair of forceps used to transfer the application pieces were rinsed in distilled water every time to avoid contamination of the subsequent sample. The lid was then fixed in position and connected to the power pack (LKB 2103). Electrofocusing was performed across the width of the PAGplate in a pH gradient of 3.5 to 9.5 at 30W and 1500V. After 30 min, the sample application pieces were removed and electrofocusing was performed for another hour. At the end of the run, the gel plate was cut into 2 halves. Each half was placed onto a plexi glass template resting on a cooling plate. 50 ml of the 1% agar gel solution containing the respective enzyme substrates (see appendix) was transferred onto the PAGplate. After the agar gel had solidified, the template was covered with a glass plate and was then incubated at 37°C. The enzyme reaction, indicated by the appearance of colour bands in the gel, was observed after 30 min, 1h and then 2h. Photographs were taken for records.

Since some enzymes might not be antigenic to the host (Edwards *et al.*, 1971), the following 6 enzymes were studied in the preliminary investigation : phosphoglucomutase (PGM), glucose phosphate isomerase (GPI), aldolase (ALD), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and hexokinase (HK) in the extract of *B. pahangi* L5 M&F.

Measurement of Enzyme Activity by Ultra-violet Spectrophotometry

The activity of PGM present in the CSE of *B. pahangi* L5 M&F was also measured by UV-spectrophotometry. 5 µl of CSE or the CSE-serum mixture were transferred into duplicate sample cuvettes, containing 1 ml of the enzyme substrate solution (see appendix) and mixed. 5 µl of distilled water or distilled water-serum mixture were transferred into the blank cuvette containing 1 ml enzyme substrate. The enzyme activity in the sample cuvettes was then measured against the blank for 20 min at 30°C by a UNICAM SP1700 UV-spectrophotometer at 340 nm. The changes in absorbence were monitored automatically by a UNICAM AR25 linear recorder at 1 min intervals and range 0.2. 2 samples, in duplicates, could be measured simultaneously. A straight line was then drawn through at least 5 points on the recording paper and the enzyme activity (in units per ml) was estimated by the formula:

$$\Delta E \times \frac{\text{Range}}{10} \times \frac{\text{Total Volume in Cuvette}}{\text{Volume CSE Used}} \times \text{Dilution} \times \frac{1}{6.3}$$

where ΔE was the number of units (on recording paper) per min.

RESULTS

Detection of Anti-enzyme Activity in Immune Sera by PGIF

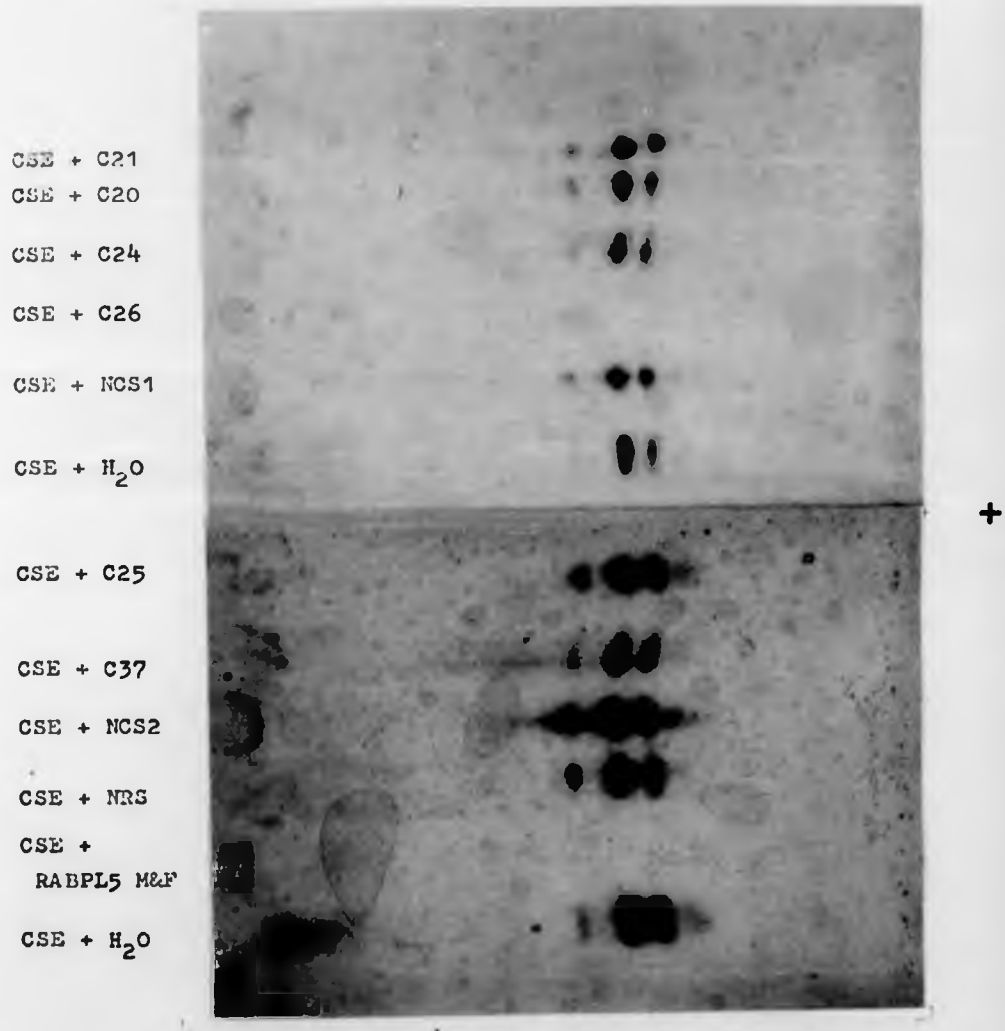
In the preliminary study, the CSE of *B. pahangi* L5 M&F, with protein concentration of 1.5 mg/ml, was reacted with a rabbit antiserum against *B. pahangi* L5 M&F and sera from 6 *B. pahangi*-infected cats. The activity of only one enzyme, PGM, in the CSE was inhibited by the rabbit antiserum and one immune cat serum (C26), as revealed by the disappearance of the characteristic bands in the PAGplate (Fig. 5.1). The normal rabbit and cat sera did not inhibit the PGM activity. Upon further screening of another 21 sera from *B. pahangi*-infected cats, 3 more sera (C18, C34 and C58) were found to have anti-PGM activities (Fig. 5.2). The enzyme did not seem to be retained at the origin nor its migration in PGIF retarded by the reactive sera. For cat serum C64, a weaker staining of PGM activity was observed, suggesting a partial inhibition of PGM activity (Fig. 5.2). The γ-globulin fraction of the rabbit antiserum and cat serum (C26) also exhibited anti-PGM activity but not that of normal rabbit and cat sera.

Investigation of the Nature of Anti-PGM Activity in the Immune Sera

The PGM activity in the CSE of *B. pahangi* L5 M&F was measured by UV-spectrophotometry. Preliminary studies showed that at least 7 µg of CSE had to be used in order to detect significant PGM activity. From Table 5.1, 50 - 68% of the PGM activity in *B. pahangi* L5 M&F CSE was inhibited by the immune cat sera C26 and C64 and the γ-globulin fraction of serum C26. Very little inhibition (6%) was observed with serum C47, which did not exhibit any anti-PGM activity in PGIF (Table 5.1 and Fig. 5.2). The PGM activity was enhanced by the normal cat serum by 27%. PGM activity was not detected in the inactivated sera and the γ-globulin fraction of serum C26.

Fig. 5.1 Polyacrylamide Gel Electrofocusing -
The detection of neutralization activity of immune sera, from cats infected with *B. pahangi* (C21, C20, C24, C26, C25, C37) and from a rabbit immunized with the crude soluble extract (CSE) of *B. pahangi* male and female worms (RABPL5 M&F), on the isoenzymes of phosphoglucomutase (PGM) from the CSE.

NCS1 and 2: sera from 2 normal uninfected cats;
NRS : normal rabbit serum;
The PGM neutralization activity of the immune serum was revealed by the disappearance of the characteristic isoenzyme bands in the gel plate after staining with the specific substrate for 30 min - 2 h at 37°C.
Sera C26 and RABPL5 M&F showed neutralization activities.
H_2O : Distilled water.


CSE + C21
CSE + C20
CSE + C24
CSE + C26
CSE + NCS1
CSE + H_2O
CSE + C25
CSE + C37
CSE + NCS2
CSE + NRS
CSE + RABPL5 M&F
CSE + H_2O

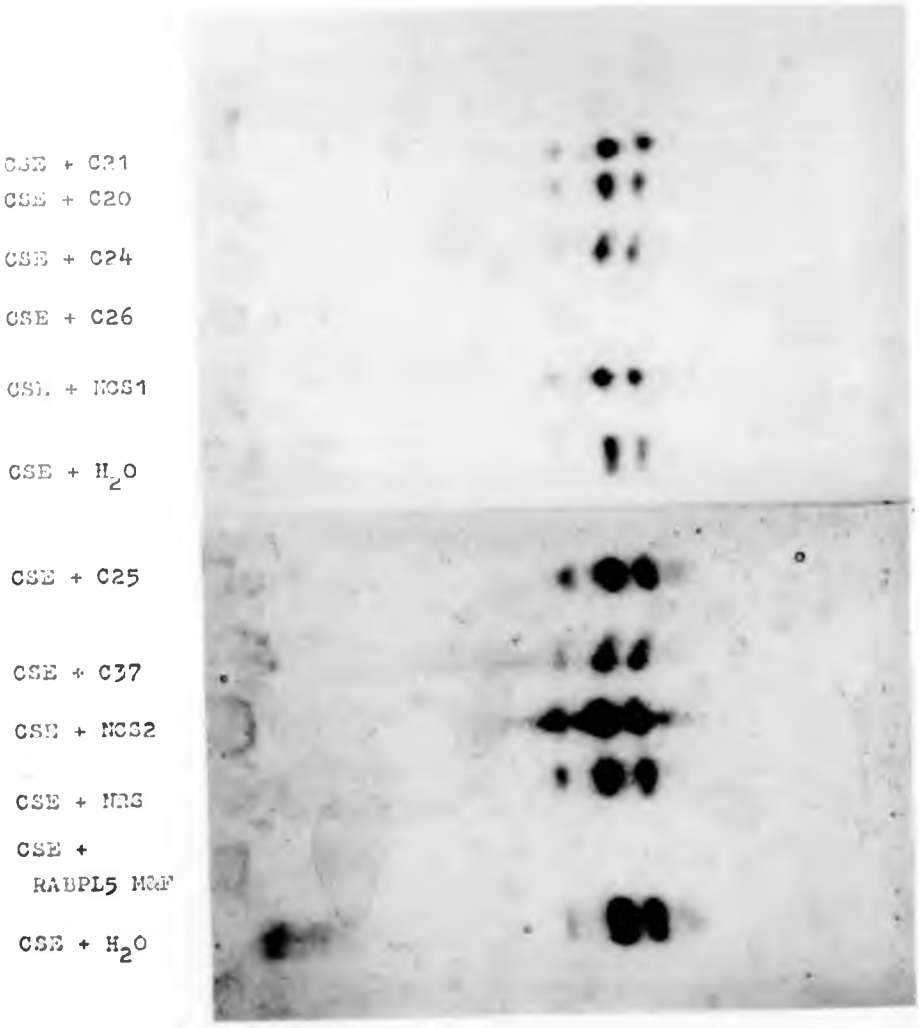

CSE + C21
CSE + C20
CSE + C24
CSE + C26
CSE + MCS1
CSE + H_2O
CSE + C25
CSE + C37
CSE + MCS2
CSE + MRS
CSE + RABPL5 MCF
CSE + H_2O

Fig. 5.2 Polyacrylamide Gel Electrofocusing -
The detection of neutralization activity in sera from cats infected with B. pahangi (those numbered with a prefix C) against phosphoglucomutase from B. pahangi male and female worm extract (CSE).

NCS3 : normal uninfected cat serum

H_2O : distilled water

Sera from cats C18, C34, C58 and C26 showed neutralization activities, as revealed by the disappearance of the characteristic bands. Serum from cat C64 only showed partial neutralization activity.

CSE + C6

CSE + C3

CSE + C18

CSE + C64

CSE + C16

CSE + C34

CSE + C47

CSE + C48

CSE + C39

CSE + C55

CSE + C7

CSE + NCS3

CSE + H_2O

CSE + C19

CSE + C53

CSE + C44

CSE + C58

CSE + C60

CSE + C26

CSE + C59

CSE + C15

CSE + C5

CSE + C4

CSE + C22

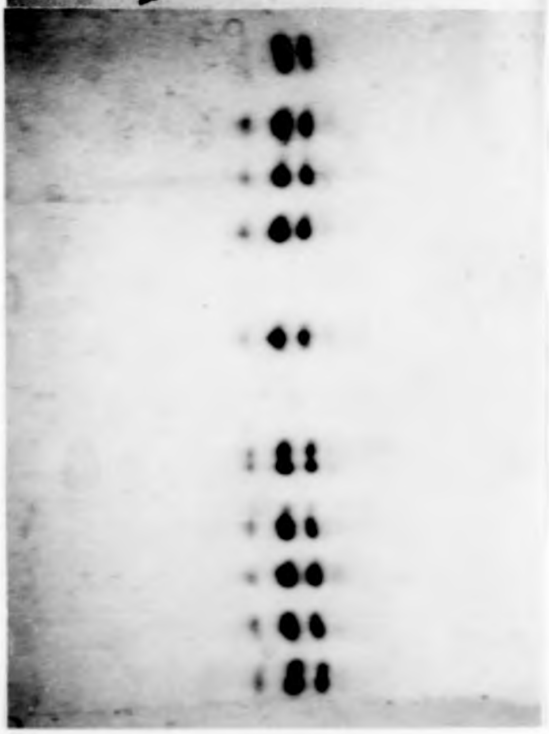


Table 5.1 The percentage inhibition of phosphoglucomutase (PGM) activity in the crude soluble extract (CSE) of male and female adult worms of B. pahangi by immune sera, measured by UV-spectrophotometry.

Reaction Mixtures	Mean PGM Activity (U/ml)	% Inhibition
(1) CSE + Distilled H_2O	2.14	-
(2) CSE + Immune cat serum C26	0.79	63
(3) CSE + γ-globulin fraction of serum C26	1.06	50
(4) CSE + Immune cat serum C64	0.69	68
(5) CSE + Immune cat serum C47	2.02	6
(6) CSE + Normal cat serum	2.71	-27

$$\% \text{ inhibition} = \frac{\text{Mean PGM Activity in (1) - Mean Activity in test}}{\text{Mean PGM Activity in (1)}} \times 100$$

Protein concentration of CSE = 3 mg/ml

6 µl CSE were incubated with 6 µl serum at 37°C for 15 min and 5 µl of the mixture were used for the assay.

Specificity of PGM of B. pahangi Adult Male and Female Worms in PGIF

The pattern of PGM isoenzymes of B. pahangi L5 M&F in PGIF was different from those of O. gutturosa and L. carinii (Fig. 5.3). The patterns of PGM isoenzymes from L5, L3 and mf of B. pahangi were also different from each other. No PGM activities could be detected in the extract of D. immitis, T. spiralis and S. mansoni. Attempts to demonstrate PGM activities in the metabolic products of L5, L3 and mf of B. pahangi and in the various purified fractions of the CSE of B. pahangi L5 M&F were unsuccessful.

Detection of Cross-reactions between PGM of B. pahangi and Immune Sera Against Heterologous Parasitic Species

The PGM activity of B. pahangi L5 M&F was found to be inhibited only by all 4 rabbit antisera against B. pahangi L5 M&F and the one against L5 M in PGIF (Fig. 5.4). Rabbit antisera against the L3 and mf of B. pahangi, L5 of O. gutturosa and against the egg antigen of S. mansoni did not show any anti-PGM activities. The pooled immune sera against heterologous species, T. spiralis, L. loa, W. bancrofti, B. malayi, O. volvulus, M. ozzardi, D. medinensis, S. mansoni, hydatid and normal sera from rabbit, rat and human did not neutralize the PGM activity of B. pahangi L5 M&F in PGIF.

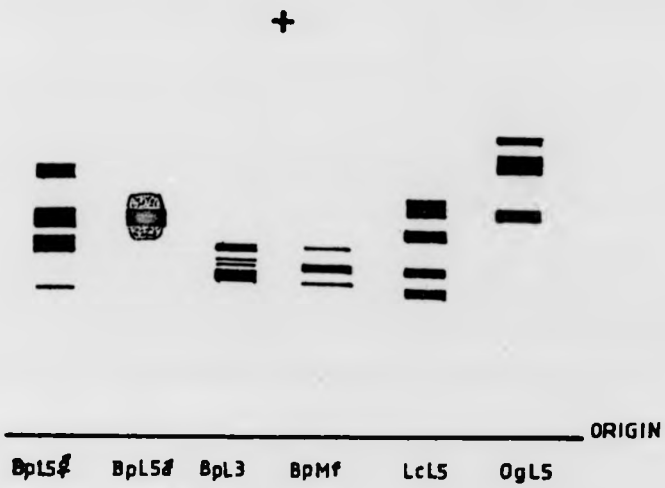


Fig. 5.3 Zymogram - the pattern of migration of phosphoglucomutase isoenzymes (PGM) of B. pahangi adult (L5) male and female worms (♀), L5 male worms (♂), infective larvae (L3) and microfilariae (Mf), L. carinii adult worms (LcL5) and O. gutturosa adult worms (OgL5) in polyacrylamide gel electrofocusing.

Fig. 5.4 Polyacrylamide Gel Electrofocusing - The detection of neutralization activities in a variety of immune sera to the homologous and heterologous parasitic species on the phosphoglucomutase isoenzymes in the crude soluble extract (CSE) of *B. pahangi* adult male and female worms.

H_2O - Distilled water

R1, 2, 3, 4 - Rabbit antisera against the CSE of *B. pahangi* adult male and female worms.

RAL5M, RAL3, RAMf: Rabbit antisera against the adult male worms, infective larvae and microfilariae of *B. pahangi*, respectively.

RAOgL5 - Rabbit antiserum against *O. gutturosa* adult worms

RASEA - Rabbit antiserum against *S. mansoni* egg antigen

NRS - Normal rabbit serum

RaATs - A pool of 2 rat immune sera to *T. spiralis*

NRaS - Normal rat serum

AL.l. - A pool of 3 human immune sera to *L. loa*

AW.b. - A pool of 12 human immune sera to *W. bancrofti*

AB.m. - A pool of 12 human immune sera to *B. malayi*

AO.v. - A pool of 3 human immune sera to *O. volvulus*

AM.o. - A pool of 6 human immune sera to *M. ozzardi*

AD.m. - A pool of 3 human immune sera to *D. medinensis*

AS.m. - A pool of 10 human immune sera to *S. mansoni*

AHyd - A pool of 10 human immune sera to hydatid

NHS - A pool of 10 normal European sera

Note: a) The pattern of migration of the isoenzymes in the top row was distorted. It often occurred when electrofocusing was performed along the margin of the gel plate.

b) The 3 bands nearest to the cathode in the run with rabbit sera were residual enzyme activities of the inactivated rabbit sera.

c) Neutralization activities were shown in R1, R2, R3, R4 and RAL5M, as revealed by the disappearance of the characteristic bands for PGM isoenzymes.

CSE + H_2O

CSE + R1

CSE + R2

CSE + R3

CSE + R4

CSE + RAL5M

CSE + RAL3

CSE + RAMf

CSE + RAOgL5

CSE + RASEA

CSE + NRS

CSE + RaATs

CSE + NRaS

CSE + AL.l.

CSE + AW.b.

CSE + AB.m.

CSE + AO.v.

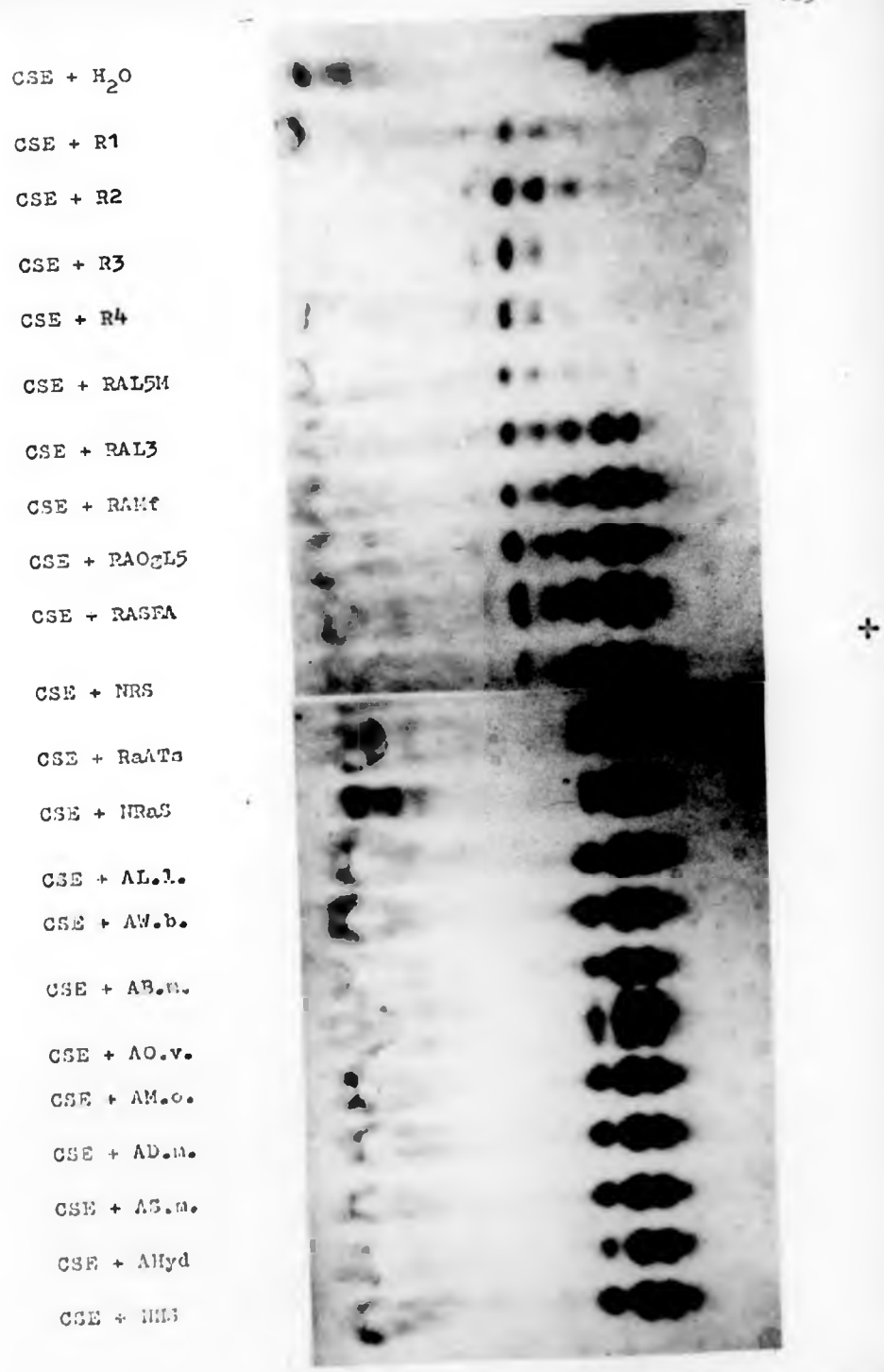
CSE + AM.o.

CSE + AD.m.

CSE + AS.m.

CSE + AHyd

CSE + NH3


CSE + H_2O
CSE + R1
CSE + R2
CSE + R3
CSE + R4
CSE + RAL5M
CSE + RAL3
CSE + RAMf
CSE + RAOcL5
CSE + RASFA
CSE + NRS
CSE + RaATs
CSE + NRaS
CSE + AL.l.
CSE + AW.b.
CSE + AB.m.
CSE + AO.v.
CSE + AM.o.
CSE + AD.m.
CSE + AS.m.
CSE + AHyd
CSE + NHS

DISCUSSION

Phosphoglucomutase (PGM) is the enzyme catalysing the reversible conversion between glucose-6-phosphate and glucose-1-phosphate in the carbohydrate metabolic pathway. In *B. pahangi*, glycolysis is the sole source of energy (Barret, 1976). However, the exact distribution of PGM in this filarioid is unknown. It is probably present in the cytoplasm. It may be released only upon the death and disintegration of the filarioid.

In general, the enzyme activities are probably affected by the antibodies in one of the following ways -

(1) If the antigenic determinants and the active site of the enzyme molecule are close to each other, the biochemical activity of the enzyme may be inhibited, i.e. a neutralization effect.

(2) The combination of the enzyme molecules with the antibody molecules may result in a change in the overall electrical charges such that the migration of the complexed molecule in electrophoresis may be retarded as compared to that of the enzyme molecules alone.

(3) The enzyme, complexed with antibodies, may be stabilized against heat inactivation, at 60°C.

In this study, PGM activity of *B. pahangi* adult worms seemed to be neutralized by antibodies (the γ-globulin fraction) of the rabbit antiserum and the cat immune serum C26 (Table 5.1). The anti-AchE activities in sera of rats and sheep infected with *N. brasiliensis* and *O. columbianum* have been reported to be associated with IgG1 and IgM classes of antibodies respectively (Jones and Ogilvie, 1972; Rothwell *et al.*, 1976). In Table 5.1, a less degree of inhibition of PGM activities was observed with the γ-globulin fraction of serum C26 than with the whole serum itself. This was probably due to the fact that the γ-globulin preparation was more dilute than the whole serum. An enhancement of PGM activity was observed

with inactivated normal cat serum, probably due to the presence of some heat-stable enhancing factors in the cat serum.

The isoenzymes of PGM in B. pahangi L5 M&F were different from those of O. gutturosa and L. carinii in PGIF (Fig. 5.3). They were also different from those of O. lienalis, O. gibsoni and B. malayi but similar to those of B. patei, a very close relative of B. pahangi phylogenetically (Dr. H.A. Flockhart, personal communications). The PGM isoenzymes of B. pahangi L5 M&F did not cross-react with a variety of heterologous immune sera (Fig. 5.4) although most of these heterologous sera cross-reacted with the CSE and cryosections of B. pahangi L5 M&F in ELISA and IFAT respectively (Chapter 3). Whether antibodies in sera against heterologous species would still bind to PGM of B. pahangi but without affecting the enzyme activity was not known. Yeates and Ogilvie (1976) showed that antibodies in rat antisera to N. brasiliensis and human antisera to Necator americanus were able to bind to AchE secreted by both species, hence a cross-reaction. However, whether such a cross-reaction would affect the enzyme activity of the heterologous AchE isoenzymes was not mentioned. The PGM isoenzymes of B. pahangi L5 seemed to be stage-specific too, for they were different from those of the L3 and mf (Fig. 5.3). Furthermore, the PGM activities of L5 M&F were neutralized by rabbit antisera against L5 but not by those against L3 and mf (Fig. 5.4).

Although the specificity of PGM isoenzymes of B. pahangi L5 needs further proofs (eg. testing for cross-reactions of immune sera against B. pahangi with the PGM isoenzymes of a variety of heterologous species), the experimental evidence so far obtained have strongly suggested that PGM (perhaps some other enzymes too) of B. pahangi L5 could be the source of species-specific antigen for detection of antibodies in B. pahangi-infected cats.

The use of PGIF for serodiagnosis seems to have a number of advantages. The whole test procedures only takes 3 - 4 h. CSE is applicable and the specific enzyme activity can be detected by the specific substrate. However, the sensitivity as shown in this study was rather low. Only 5 out of 27 (19%) immune cat sera showed neutralization activities (Fig. 5.1 and 5.2). It might be due to the high concentration of CSE used (at least 1 - 1.5 mg/ml) in order to detect significant PGM activities in PGIF and UV-spectrophotometry. In fact, most of the antigen preparations in which no PGM activity was detected in PGIF were less than 0.5 mg/ml in protein concentration. With the use of concentrated CSE, only strong immune sera would show isoenzyme neutralization activities. A weaker serum would only show a partial neutralization effect, as in the case of serum C64 (Fig. 5.2). Other more sensitive techniques should be used for further development of the isoenzyme neutralization assay. For examples, using purified isoenzymes as antigens in ordinary ELISA or detecting enzyme neutralization activities of sera in ELISA plates coated with the CSE or purified enzyme preparation of the homologous parasitic species could be the possible avenues.

The other possible reason for the low sensitivity of the PGIF technique for the detection of isoenzyme neutralization antibodies in immune sera might be that only a proportion of the B. pahangi-infected cats had been exposed to PGM of adult worms during the course of infections. Further investigations for other enzymes will be necessary, probably one which is excreted or secreted by the parasite. AchE has been shown to be secreted by a number of parasitic nematodes of the gastrointestinal tract in vitro (Ogilvie et al., 1973). Collagenase-like enzymes are secreted by a number of tissue penetrating helminths (Lewert and Lee, 1954). Furthermore, antibodies to AchE could be detected in most animals infected with N. brasiliensis and T. colubriformis, at certain stage of the infection, by polyacrylamide gel electrophoresis (Jones and Ogilvie, 1972; Rothwell et al., 1973).

CHAPTER SIX

DETECTION OF FILARIAL ANTIGENS IN SERUM AND URINE IN FELINE AND HUMAN LYMPHATIC FILARIASIS

INTRODUCTION

Although a large number of studies have been reported on the detection of antibodies against filarial antigens in filariasis, relatively little have been performed on the detection of filarial antigens. It was Franks (1946) who first demonstrated the presence of circulating filarial antigens in patients with W. bancrofti infections. He also suggested that the circulating antigens could be utilized for diagnostic tests in filariasis. Later studies in bancroftian filariasis also demonstrated the presence of circulating filarial antigens in the serum or urine (Tanabe, 1959; Dasgupta and Bala, 1978; Kaliraj et al., 1979b). Similar observations have also been reported in D. immitis-infected dogs (Yamanouchi, 1972; Desowitz and Una, 1976; Takei et al., 1977). The presence of circulating antigens in excess of available antibodies has been suggested to account for a proportion of "false negative" antibody tests (Franks, 1946; Berggren and Weller, 1967). While antibody tests detect infections acquired recently as well as previously, the detection of circulating antigens may be a good indicator of an active infection (Kaliraj et al., 1979b). In this study, the presence of filarial antigens in sera from B. pahangi-infected cats and from patients with brugian and bancroftian filariasis was investigated by CIEP and ELISA. The presence of filarial antigens in the urine of B. pahangi-infected cats was also studied by CIEP and ELISA. The sensitivities of these antigen tests in the detection of filarial infections were then compared with those of the antibody tests performed in Chapters 2 and 3.

MATERIALS AND METHODS

Serum and Urine Samples

Sera from 79 *B. pahangi*-infected cats, 15 normal uninfected cats, patients with brugian and bancroftian filariasis and from healthy Europeans were obtained as described in Chapters 2 and 3. At necropsy of the *B. pahangi*-infected cats and 4 normal uninfected cats, 5 ml of urine were obtained by bladder-puncture. The samples were then stored at -70°C in 0.5 ml aliquots.

Rabbit Antiserum against Male and Female Worms of B. pahangi (RABPL5)

This was prepared by immunization of a rabbit with the crude soluble extract of *B. pahangi* adult worms (BPL5CSE) according to that described in Chapter 2.

Counter Immunoelectrophoresis (CIEP)

This was performed as described in Chapter 2 but the serum samples were placed in the cathodic wells and RABPL5 in the anodic wells. The normal rabbit serum was included for every sample tested as the control.

Enzyme-linked Immunosorbent Assay (ELISA)

A double antibody sandwich method according to Voller *et al* (1976a) and Ferreira *et al* (1979) with some modifications was employed, i.e. the worm antigens in test sera were bound to the γ-globulins of the anti-worm serum (γ-RABPL5) coated

on ELISA plates and then detected by adding peroxidase-labelled γ-RABPL5 as the indicator reagent. γ-RABPL5 and the peroxidase conjugate were prepared according to the methods described for peroxidase-conjugated rabbit anti-cat IgG in Chapter 2. The basic reagents for and the procedures of the assay were similar to those described in Chapter 2, for detection of antibodies. In this study, M29 polyvinyl plates were coated with γ-RABPL5 overnight at 4°C. Cat sera, in duplicates, were tested in 4-fold dilutions (1: 2 - 2048) and human sera in duplicates at dilution 1: 2. The cat urine samples were tested neat. In each plate, 2 wells (8 for the first plate) were reserved for the reference positive (100 µg BPL5CSE), 2 for the reference negative serum and 4 for PBS-Tween. Preliminary experiments showed that uniform and reproducible results could be obtained only if the serum samples were incubated at 37°C for 2 h rather than 2 h at room temperature. After addition of the conjugate, the plates were incubated at room temperature for 3 h. The OD values of 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, 200, 600 and 1000 µg of BPL5CSE were also determined to construct a calibration curve from which the amount of filarial antigens in the sera could be quantitated. The enzyme reaction was stopped when the OD values of the reference positive had reached a pre-determined value (0.56). The correction of ELISA values for serum samples were then performed according to that described in Chapter 2.

The highest OD value, which was within the range of OD values of the different concentrations of BPL5CSE used for constructing the calibration curve, was chosen for estimation of the level of circulating worm antigens. It was given by - (Corresponding value from calibration curve x serum dilution x 5) µg/ml. The factor 5 was included because there was only 0.2 ml of the diluted serum sample in each well and the concentration was expressed in terms of 1 ml of serum.

The optimal concentration of the γ-RABPL5 used to coat the plate was determined by a checker-board titration of 7 and 14 μg of BPL5CSE against different concentrations of the γ-RABPL5 (5, 10, 20 and 40 μg/ml) at conjugate dilution 1: 2000. The concentration of γ-RABPL5 at which the highest OD values for the two concentrations of BPL5CSE could be obtained was considered as optimal. Preliminary experiments showed that a prozone occurred in some cat sera with the peak OD values at 1: 40 serum dilution. The optimal dilution of the conjugate was then determined by titration of a positive cat serum and a reference negative normal cat serum (determined by CIEP) at 1: 40 dilution against different dilutions of the conjugate (1: 2000, 4000 and 8000). The ratios of the OD values of the positive serum (E+) to that of the negative serum (E-) were determined. The conjugate dilution at which the highest E+/E- ratio could be obtained was taken as the optimal. For the detection of antigen in cat urine and human sera, the same optimal conditions as for the cat sera were used.

Detection of Antiglobulins in Sera

The latex agglutination test kit (Rheuma-Wellcotest, Wellcome) for detection of human rheumatoid factors was employed. A 1: 20 dilution of the serum was prepared in glycine saline buffer, pH 8.2 in a U-bottomed polystyrene microtitre plate (Cooke Engineering). A drop of the diluted serum was then transferred to the circle on the glass test slide, using the pipettes provided. A drop of human IgG-coated polystyrene latex suspension in glycine saline buffer, pH 8.2 was then added to the circle. The contents were mixed with a disposable mixing rod, spreading the mixture to cover the area within the circle. The slide was rocked gently for a maximum of 2 min while examining for macroscopic agglutination. A positive reaction was revealed by the clearly visible clumping of the latex particles with simultaneous clearing of the milky back-

ground opalescence. A negative result was shown by the persistence of the milky appearance of the reagents on the slide throughout the 2 min period of the test. Controls of the test included a reference positive serum for rheumatoid factor, a reference negative serum and the glycine saline buffer.

RESULTS

Detection of B. pahangi Antigens in the Infected Cat Sera by CIEP

The sera from 15 normal uninfected cats were all negative. All serum samples did not form any precipitation lines with the normal rabbit serum in the controls. 81% of the infected but untreated cats were positive and lower positivity rates (14 - 67%) were obtained in groups 2 - 4 which had been treated with DEC, MBZ and FBZ (Table 6.1). An overall positivity rate of 76% was observed. The test was positive as early as 24 days post-infection when the fourth-stage larvae moulted to L5. However, sera from cats infected for more than 243 days were all negative. 3 out of 11 cats which did not have an active infection but still harboured some dead worm fragments in the lymphatics were also positive.

Detection of B. pahangi Antigens in the Infected Cat Sera by ELISA

The optimal concentration of Y-RABPL5 for coating the ELISA plates was 20 µg/ml. Conjugate at dilution 1: 8000 was found to be optimal.

On titration of the sera, 2 basic patterns were observed. The OD values decreased on dilution of the serum (eg. serum C6 in Fig. 6.1a). This pattern was similar to that obtained with the titration of BPL5CSE (Fig. 6.1b). However, a prozone was observed in some sera below dilutions 1: 8 - 128 (eg. sera C66, C24 and C1 in Fig. 6.1a).

Table 6.1 Counter Immunoelectrophoresis (CIEP) and Enzyme-linked Immunosorbent Assay (ELISA) for the detection of worm antigens in sera from *B. pahangi*-infected cats.

Groups	N	% POSITIVITY		
		CIEP	ELISA (Mean [Ag] ± 1S.D.)	CIEP + ELISA
1. Infected, Untreated	31	81	58 (179 ± 443)	45
2. Infected, DEC Rx	7	14	100 (170 ± 214)	14
3. Infected, MBZ Rx	9	67	100 (280 ± 312)	67
4. Infected, FBZ Rx	5	60	100 (403 ± 545)	60
5. Infected, Other Rx	27	90	70 (303 ± 520)	50
Total	79	76	73 (246 ± 448)	48
Test positive (days post-infection) as early as		24	24	-
till at least		243	510	-

[Ag] - concentration of worm antigens in serum (µg/ml);
N - sample size; Rx - treatment; DEC - diethylcarbamzaine;
MBZ - mebendazole; FBZ - flubendazole;
In group 5, cats were treated by different drugs but the sample size was too small to be grouped individually.

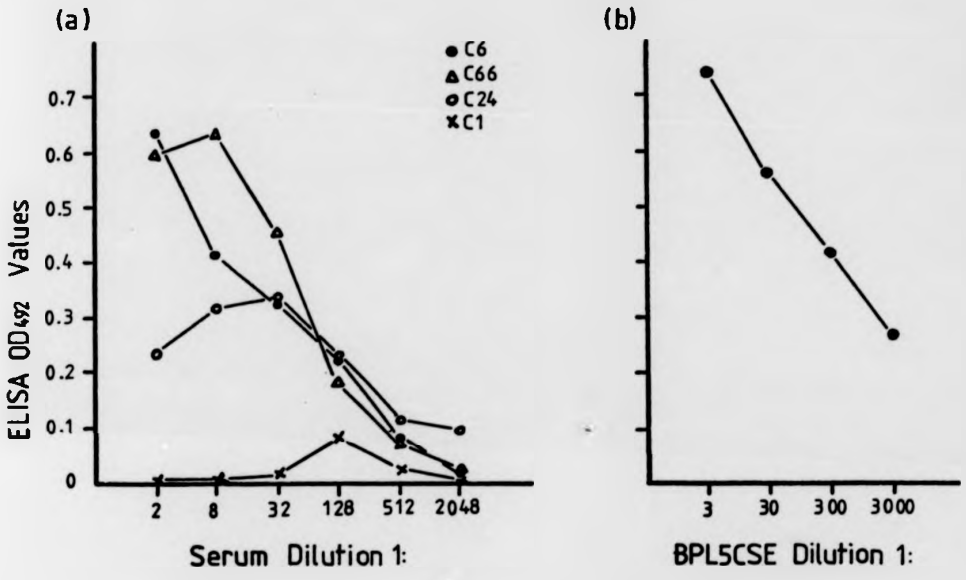


Fig. 6.1 Enzyme-linked Immunosorbent Assay (ELISA) for detection of filarial antigens in sera.

(a) The variation of OD values in sera from 4 B. pahangi-infected cats (C6, C66, C24, C1) at different dilutions.

(b) The variation of OD values in the crude soluble extract of adult male and female worms of B. pahangi (BPL5CSE) at different dilutions.

The OD values for the 15 normal uninfected cat sera (range = 0 - 0.08, mean = 0.01, S.D. = 0.015) were obtained. The cut-off value was chosen at 0.1, which was above the upper limit of the range of OD values for the normal sera.

In the infected but untreated group, only 58% were positive (Table 6.1). However, 100% positivities were obtained in groups 2 - 4, which had been treated with DEC, MBZ and FBZ. A total positivity rate of 73% was obtained, similar to that in CIEP. The assay was positive as early as 24 days post-infection and remained so till at least 510 days post-infection. A total of 48% of the sera were positive by both CIEP and ELISA. Sera from the 11 infected cats which did not have an active infection but still had dead worm fragments in the lymphatics were all positive.

The calibration curve for estimation of the level of circulating antigen is shown in Fig. 6.2. The mean concentration of antigen in the sera in group 1, infected but untreated, was 179 $\pm$ 443 µg/ml (Table 6.1). Higher mean concentrations were observed in groups 3 - 5 (280 - 403 µg/ml) which were treated by various means. However, the mean concentration of antigen (170 $\pm$ 214 µg/ml) in group 2 which was treated with DEC, was similar to that in the untreated group 1.

Detection of Antiglobulins in B. pahangi-infected Cats

32 infected and 3 uninfected cat sera were screened for antiglobulin activities, using the latex agglutination test (Wellcome). Two sera, one treated with FBZ (C55) and the other with niridazole (C66), were positive for antiglobulin activities against human IgG. Serum C55 was negative in CIEP but positive in ELISA. C66 was positive in both CIEP and ELISA.

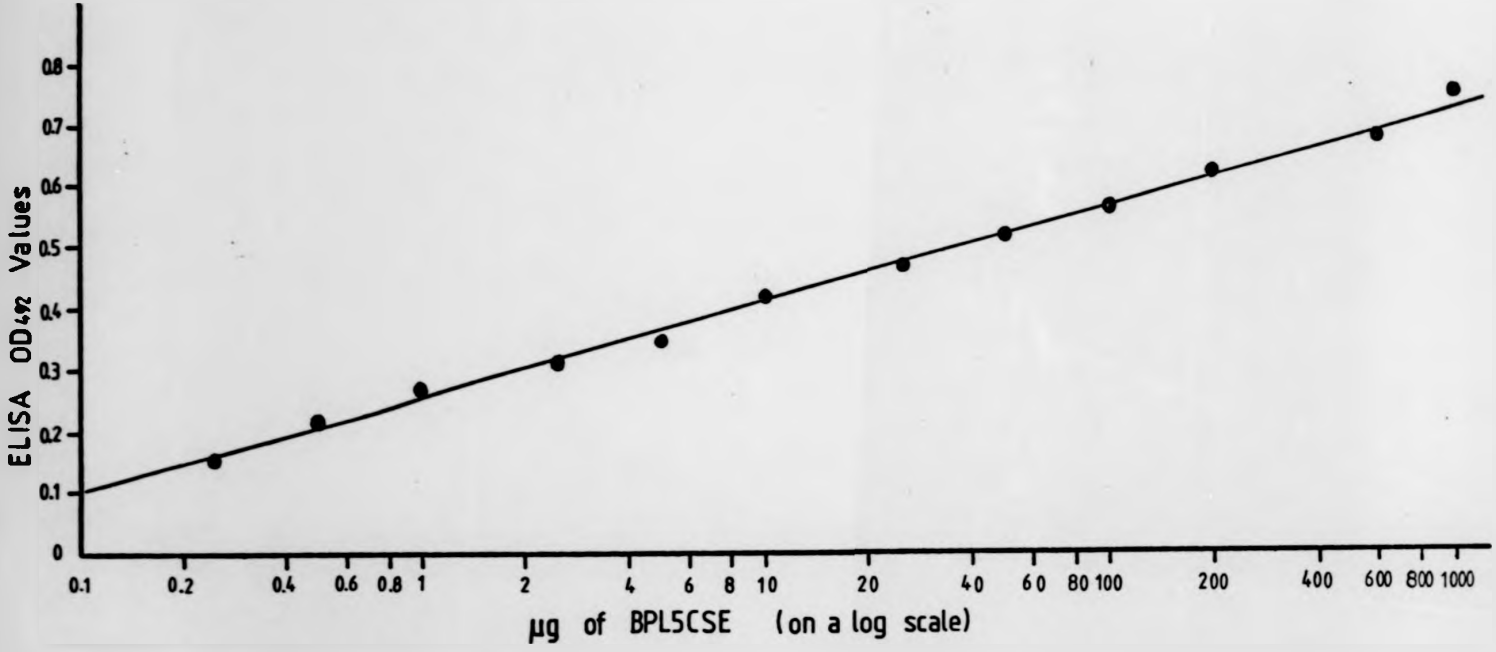


Fig. 6.2 The calibration curve for determination of the level of circulating filarial worm antigens in sera .
BPL5CSE: crude soluble extract of adult worms of *B. pahangi*
ELISA: enzyme-linked immunosorbent assay

Detection of Worm Antigens in Urine from B. pahangi-infected Cats

Using CIEP and ELISA, urine samples collected from 72 of the infected and 4 normal uninfected cats were all negative for worm antigens.

Detection of Filarial Antigens in Sera from Patients with Brugian and Bancroftian Filariasis

CIEP

Because of the inadequate quantity of RABPL5 available, an antiserum raised in another rabbit was used for this study. All normal Malaysian and European sera were negative. All sera did not form a precipitation line with the normal rabbit serum in the controls. 29 - 43% positivity rates were obtained with the brugian filariasis sera (Table 6.2). Only 25% of the W. bancrofti microfilaraemic sera were positive.

ELISA

10 sera were first tested at dilutions 1: 2 to 2048. The OD values decreased with the dilutions of sera and no prozone was observed. All sera were then tested at dilution 1: 2. The OD values of 15 normal European sera were determined (range = 0 - 0.095). The cut-off value was then taken as 0.1, which was above the upper limit of the range of OD values for normal sera.

89 - 93% of brugian filariasis sera were positive (Table 6.2). These positivity rates were higher than those (50 - 53%) with bancroftian filariasis sera. Also, the mean ELISA values were higher in the brugian filariasis sera. Two of the 12 (17%) normal Malaysian sera were positive too.

Table 6.2 Detection of filarial antigens in sera from patients with brugian and bancroftian filariasis by Counter Immunoelectrophoresis (CIEP) and Enzyme-linked Immunosorbent Assay (ELISA), using rabbit antiserum against *B. pahangi* adult worms as the indicator reagent. The presence of rheumatoid factors (RF) in these sera were tested by latex agglutination test (LAT).

Groups	N	% POSITIVITY			
		CIEP	ELISA (Mean OD ± 1S.D.)	CIEP + ELISA	LAT
B. malayi clinical sera	18	29	89 (0.45 ± 0.38)	22	28
B. malayi mf positivie sera	14	43	93 (0.53 ± 0.31)	36	0
B. malayi endemic sera	16	19	69 (0.34 ± 0.34)	13	6
W. bancrofti clinical sera	17	0	53 (0.16 ± 0.21)	0	24
W. bancrofti mf positive sera	12	25	50 (0.20 ± 0.34)	17	25
W. bancrofti endemic sera	16	0	56 (0.33 ± 0.39)	0	0
Normal Malaysian sera from non-endemic area	12	0	17 (0.10 ± 0.29)	0	17
Normal European sera	15	0	0 (0.02 ± 0.03)	0	0

N - sample size;
mf - microfilariae.

with OD values of 0.24 and 0.99. Higher positivity rates were observed as compared with those in CIEP. 22 - 36% of the brugian filariasis sera and 17% of the bancroftian filariasis sera were positive by both CIEP and ELISA.

Detection of Rheumatoid Factors in Human Filariasis Sera

The presence of rheumatoid factors in these sera were tested by the latex agglutination test (Wellcome). 13 worm antigen positive and 1 negative sera (as shown in ELISA) were positive for rheumatoid factors. The 2 normal Malaysian sera positive for worm antigen in ELISA were also positive for rheumatoid factors.(Table 6.2).

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DISCUSSION

The significance of ELISA in the detection of antigen in the body fluids of patients with parasitic diseases has been noted by Kagan (1979). This technique can probably detect free antigens as well as immune complexes with high antigen to antibody molar ratios. However, Voller et al (1976a) and Madwar and Voller (1977) pointed out that with the unknown spectrum of reactivity of the indicator antiserum and the possible presence of antiglobulins in the test sera, results of ELISA for detection of antigens should be analysed with cautions. So far, no detailed study has been made on the problems underlying ELISA for detection of antigens. In the present study, a prozone was observed in some cat sera (Fig. 6.1). In ELISA for detection of IgG antibodies, a similar prozone due to the interference of IgM was observed (de Savigny and Voller, 1980). Therefore, in ELISA for detection of antigens, such a prozone would probably be due to some other serum factors related to the immune system. One of these factors may be immune complexes with some antigenic determinants being unsaturated by antibodies. The antigenic determinants on the antigen molecule in these complexes may be saturated after binding to the Y-RABPL5 coated on the plate. Thus, subsequent addition of enzyme-labelled Y-RABPL5 cannot detect the antigen in the complex. Immune complexes bound to the well may also prevent binding of free antigen molecules to the Y-RABPL5 on the plate by steric hindrance. Antiglobulins (like rheumatoid factors) are found to react with homologous as well as heterologous IgG (Turner, 1977). According to Dr. M.W. Steward (personal communications), antiglobulins seem to bind better to IgG of another species. Therefore, competitions between IgG-antiglobulins and worm antigens in the test serum for Y-RABPL5 coated on the plate may be responsible for the prozone observed. These IgG-antiglobulins may be responsible for "false negative" reactions in some sera with low concentrations of circulating worm antigens too.

However, IgM-antiglobulins, having 10 antigen binding sites, will bind to the δ-RABPL5 coated on the plate as well as those in the enzyme conjugate resulting in a "false positive" reaction. This view may be supported by the observations that 2 normal Malaysian sera, which were negative for antibodies to filarial antigens in IFAT and ELISA (Chapter 3) and for worm antigens in CIEP but positive for rheumatoid factors, gave "positive" reactions in ELISA for detection of antigens. The presence of rheumatoid factors have been reported in sera from patients with infectious diseases like toxoplasmosis and Epstein-Barr virus infections, etc., contributing to "false positive" serological tests for IgM (Remington, 1978; Henle *et al.*, 1979; Chantler, 1980). Therefore, all positive sera in ELISA which were positive for rheumatoid factors should be retested after treatment, for example, with heat aggregated IgG or IgG-coated inert particles to remove the antiglobulins. However, due to the lack of sufficient quantities of sera, further investigations could not be made on the possible contribution of rheumatoid factors present in the test sera to the "positive" reactions in ELISA in this study.

From Table 6.2, 93% positivity (all negative for rheumatoid factors) was observed with the *B. malayi* microfilaraemic sera. This is more sensitive than IFAT (85 - 92%) and ELISA (85%) for detection of antibodies (Tables 3.2 and 3.9). This suggests that with better understanding and control of the interfering serum factors especially antiglobulins, ELISA can probably be developed into a sensitive and reliable diagnostic tool for filariasis. Some antigens will exist in form of immune complexes. The study of the immune complexes present in the infected sera (eg. avidity of antibodies involved) would also help developing a sensitive ELISA for detection of worm antigens. The formation of immune complexes also follows the Law of Mass Action (Steward, 1974), i.e.

$K = \frac{(AbAg)}{(Ab) \times (Ag)}$, where K is the equilibrium constant, (AbAg), (Ab) and (Ag) the concentrations of immune complexes, antibody and antigen respectively. When antigens are present in large excess of antibodies, most of the antigens would be in form of immune complexes. Thus, dissociation of immune complexes at low pH (less than 4.0) may be required before antigen could be detected (Theofilopoulos and Dixon, 1979).

The possible presence of antiglobulins in the cat sera was less understood since the sera were tested by the latex agglutination test optimized for the detection of human rheumatoid factors, and 2 out of 35 tested were positive. Further experiments are required to study the presence of antiglobulins to cat and rabbit IgG in these sera.

In view of the presence of various serum factors which may affect the results of ELISA for quantitation of antigens in sera, the amounts of antigen estimated in the test sera by ELISA in this study are by no means the absolute quantities actually present in the sera. No attempts can therefore be made to correlate the level of circulating antigens with the stage of infection, etc..

This study also supports the suggestion that ELISA would be potentially useful in quantitation of antigens. With the cut-off value as 0.1, ELISA in this study could detect as little as 0.1 µg of BPL5CSE (Fig. 6.2). Since all sera were initially diluted twice and there were 0.2 ml of diluted serum in each well (i.e. 0.1 ml neat serum in each well), therefore, the lower limit of sensitivity of this assay is 1.0 µg/ml (0.1 x 10).

For detection of antigens, CIEP seems to be better than ELISA in that it is not likely to be affected by the presence

of antiglobulins in test sera. Under the experimental conditions at pH 8.6, the antiglobulins present in the test sera will migrate towards the cathode and the worm antigen towards the anode. The latter then forms a precipitation line with the immunoglobulins of anti-worm serum from the anodic well. Even if some antiglobulins do migrate towards the anode, as a result of the failure of the agarose gel and electrode buffer to maintain a constant pH, they will not form any precipitation with the native (non-denatured) IgG in the anti-worm serum (Maini, 1977).

The overall sensitivities (73 - 76%) of CIEP and ELISA for detection of circulating worm antigens in B. pahangi-infected cats were less than those of IFAT (84 - 89%) but similar to those of CIEP and ELISA (73 - 77%) for detection of antibodies (Tables 2.2, 2.4, 2.6 and 6.1). However, in the untreated group 1, 81% positivity was obtained with CIEP for the detection of antigen. This was the same as the highest positivity rate obtained in antibody tests (IFAT using B. pahangi L3 as antigens, Table 2.4). This suggests that an antigen test like CIEP can be as sensitive as an antibody test like IFAT. Nevertheless, this study does not support the view of Kaliraj et al (1979b)that antigen test appears to be more promising in diagnosis of active parasitic infections as 3 out of 11 cats which did not have an active infection but still harboured dead worm fragments in the lymphatics were positive for circulating antigens in CIEP. In ELISA, all these 11 sera were positive. Perhaps, detection of metabolic antigens rather than somatic antigens of the parasite would be a better indicator of active infections.

In the human system, antigen tests using RABPL5 had higher positivity rates with brugian filariasis sera than with the bancroftian filariasis sera (Table 6.2). This may suggest that brugian filariasis patients are more likely to

have circulating antigens or, more probably, that B. pahangi is antigenically closer to B. malayi than to W. bancrofti.

In the B. pahangi-cat system, CIEP and ELISA had similar total positivity rates (Table 6.1). However, in the human system, ELISA had higher positivity rates than CIEP even when those with rheumatoid factors were regarded as negative for worm antigens in the former test. In the latter technique, a different and weaker RABPL5 was used. This anti-worm serum was found to give negative reactions with a few cat sera which were positive when tested against the original antiserum. This seems to indicate the importance of standardization of antisera used in antigen tests if results from different laboratories are to be compared.

Using RABPL5, circulating worm antigen was detected in 25% of the W. bancrofti microfilaraemic sera by CIEP (Table 6.2). This was similar to that (23%) reported by Kaliraj et al (1979b)using an antiserum to W. bancrofti mf in CIEP. However, in their study, 8 and 3% positivities were obtained with sera from clinical cases and from apparently healthy individuals in endemic areas as compared to 0% in this study. 19 - 69% of sera from people living in B. malayi endemic areas were positive for worm antigens in this study (Table 6.2). Probably, they were in the incubation period of the infection or having occult filariasis (Beaver, 1970). However, cross-reactions with other helminth antigens may be possible.

Worm antigen could not be detected in the urine of B. pahangi-infected cats in this study. Therefore, worm antigen may not appear in infected cat urine at all or other forms of worm antigens may be present. Saito (1966) showed that the antigen present in the urine of D. immitis-infected dogs was probably a metabolite of the parasite.

The spectrum of reactivity of the anti-worm sera (RABPL5) used as the indicator reagent for the presence of worm antigens in test sera is unknown. Since the rabbits were not immunized with a purified and specific antigen, the antisera raised from them would certainly cross-react with some other helminth antigens. Therefore, antigen tests also have the same fundamental problem as antibody tests — a specific antigen is required. For detection of specific circulating antigens, purification of homologous worm antigen seems to be a pre-requisite. For future studies, one should use antisera to purified somatic antigens of L5 or mf as these 2 stages are present in active infections, and especially to metabolic antigens, the presence of which may be a good indicator for active infections.

CHAPTER SEVEN

SOME IMMUNOPATHOLOGICAL STUDIES IN FILARIASIS ESPECIALLY IN THE *B. PAHANGI*-INFECTED CATS

INTRODUCTION

Glomerulonephritis was reported to be associated with filariasis in dogs (Klei *et al.*, 1974; Simpson *et al.*, 1974; Casey and Splitter, 1975), hamsters (Simpson and Neilson, 1976) and man (Bariety *et al.*, 1967; Zuidema, 1971; Couzineau *et al.*, 1973; Chugh *et al.*, 1978). Some authors suggested that immune complexes or direct mechanical damage by microfilariae were the possible pathogenic factors involved in the renal lesions. In the light microscopic examinations of organs from *B. pahangi*-infected cats, localization of microfilariae were often observed in the kidneys. However, no detailed histological study was performed (Dr. D.A. Denham, personal communications). Therefore, in this study, the histopathology of the kidneys from *B. pahangi*-infected cats was studied by light and electron microscopy. Urine samples from these cats were tested for proteinuria by the Albustix test. The possible involvement of immune complexes in the renal lesions was investigated by fluorescence microscopy and detecting the presence of circulating immune complexes by C1q- and conglutinin-binding assays. The histology of the lungs, liver and spleen from *B. pahangi*-infected cats was studied. A comparative study was also made on lesions in the *B. pahangi*-infected popliteal lymph nodes and lymphatics from mebendazole-treated and untreated cats.

The presence of circulating immune complexes in sera from humans infected with *B. malayi* and *W. bancrofti* was also studied by C1q- and conglutinin-binding assays.

MATERIALS AND METHODS

Preparation of Tissues for Light Microscopy

The kidneys, popliteal lymph nodes and associated lymphatics and portions of the lungs, liver and spleen were removed from the cat immediately at necropsy. They were cut into small blocks by a scalpel and fixed in formol-saline. After storage in formol-saline for at least 10 days, the tissue blocks with the respective paper-labels were placed in individual metal baking pans. The samples were then processed in a Histokinnette (Hendrey Automation) for 2 h in each of the following reagents: -

1. 70% alcohol;
2. 95% alcohol;
3. Absolute alcohol;
4. Absolute alcohol;
5. Absolute alcohol;
6. Toluene;
7. Toluene;
8. Molten paraffin wax;
9. Molten paraffin wax.

Wax infiltration *in vacuo* was finally performed for 1 h. The tissue blocks were then removed from the metal baking pans. Molten paraffin wax at 60°C was dispensed into a metal template, into which 8 - 10 tissue blocks were placed. The end of the paper-label was embedded beside the respective tissue block for identification. Air bubbles present at the surface of the wax were removed by a Bunsen flame. After the wax had solidified at room temperature, the whole template was immersed in water for about 15 min. The whole block was removed from the template by banging against the floor. The individual tissue blocks were separated from each other by a knife and trimmed. Each tissue block was then mounted onto a wooden object holder (2 x 2 x 3.5 cm) by melting one side of the block with a hot spatula and pressing the melted side against the

wooden holder. The paper-label was stuck onto one side of the holder.

Using a microtome, a ribbon of sections (5 μ thick) was cut and transferred into a paraffin mounting bath at 45°C. The ribbon of sections floated on top of water and folding was prevented by surface tension. The good and thin sections were separated from the poor ones by a pair of forceps. 3 sections were mounted on a chromic acid cleaned microscopic slide (76 x 25 mm) and were dried on a hot-plate at 37°C. The sections were stained by haematoxylin and eosin (H&E) according to Thompson and Hunt (1966) and BDH Chemicals Ltd (1975) with some modifications. The procedures were as follows : -

1. 5 min. in xylene (to remove paraffin wax);
2. 5 min. in xylene;
3. 2 min. in absolute alcohol;
4. 2 min. in 95% ethanol;
5. 10 min. in Ehrlich's haematoxylin (see appendix for preparation);
6. 2 min. in running water;
7. 10 swirls in 1% HCl in 70% ethanol;
8. 5 - 10 min. in running water (since Thames' water was alkaline) until the sections were blue in colour. (If the sections were adequately differentiated, the nuclei were distinctly blue and other tissue components were nearly colourless when examined under a low power microscope).
9. 5 min. in 1% water soluble eosin in distilled water;
10. The slides were rinsed in tap water and then shaken;
11. 1 min. in 95% ethanol;
12. 1 min. in 95% ethanol;
13. 2 min. in absolute ethanol;
14. 2 min. in absolute ethanol;
15. 5 min. in xylene;
16. 5 min. in xylene.

The slides were dried in air. The sections were finally mounted in D.P.X. mounting medium (Raymond A. Lambs) and dried overnight at room temperature.

Some kidney sections stained by periodic acid Schiff (PAS) technique was kindly performed by Mr. H. Furse, Department of Medical Helminthology.

Preparation of Kidney Tissues for Electron Microscopy

Before the last breath of the cat at necropsy, the left kidney was removed. A few small pieces were cut by a scalpel and placed in a petri dish containing cold 3% glutaraldehyde in 0.1M cacodylate solution (see appendix for preparation). Tiny tissue blocks of about 1 mm^3 were cut and kept in 3% glutaraldehyde in 0.1M cacodylate solution overnight at 4°C. The tissue blocks were then washed with 0.1M cacodylate/0.2M sucrose solution, pH 7.4, 3 - 4 times with 3 min in between. The tissue blocks were left in the same solution overnight at 4°C. The following morning, the tissue blocks were washed again in the same solution and then kept in osmium solution (see appendix for preparation) for 2 h. The tissue blocks were washed in 0.1M cacodylate/0.2M sucrose solution for 10 min followed by distilled water for at least 1 h with changes in between. They were then dehydrated at room temperature in 30% methanol for 5 min followed by 3% uranyl acetate in 30% methanol for 30 min. The tissue blocks were washed once with 30% methanol before dehydrated up through the series of methanols (30, 60, 90, 2 x 100%) by keeping in each solution for 5 min. After dehydration, the tissue blocks were placed in 100% propylene oxide for 30 min in the fume cupboard followed by 33% propylene oxide/araldite (see appendix for preparation) for 30 min and then 66% propylene oxide/araldite for 30 min. The tissue blocks were finally kept in 100% araldite overnight at room temperature. The next morning,

each tissue block was embedded in fresh araldite in a small plastic pan and kept at 37°C for 3 days. The tissue blocks were then ready for cutting. The cutting, mounting of sections on copper grids and staining with lead were kindly performed by Miss S. Stamford, Department of Protozoology. Preliminary examination of the kidney sections was performed by Dr. D. Ellis, Department of Medical Protozoology. Detailed examination was then performed under the supervision of Dr. D. Ellis.

Preparation of CSE of B. pahangi Adult Worms and Microfilariae (CSEBPL5/Mf), Cat IgG and Rabbit Antisera against CSEBPL5/Mf (RABPL5/Mf) and cat IgG

These were performed according to the materials and methods described in Chapter 2. For preparation of RABPL5/Mf, the rabbit was immunized with CSE of B. pahangi mf in complete Freund's adjuvant for 2 months followed by the CSE of L5 in complete Freund's adjuvant for 3 months.

Preparation of Complement (C3) from Normal Cat serum and Rabbit Antiserum against C3 (RACC')

This was performed according to Hudson and Hay (1976). Fresh serum from a normal cat was prepared according to the methods described in Chapter 2. The clotting of blood was allowed at 4°C in glass centrifuge tubes. 500 mg zymosan (Koch-Light Labs) were boiled for 30 min in 50 ml 0.14M normal saline. After centrifugation at 1,000 g for 10 min, the supernatant was discarded. When cool, the precipitate was mixed with 11 ml fresh cat serum and incubated for 1 h at 37°C with mixing. The suspension was then centrifuged at 2,000 g for 10 min and the supernatant discarded. The precipitate, which was the zymosan-C3 complex, was washed

6 times with barbitone buffered saline (BBS), 0.15M, pH 7.6 (see appendix for preparation). The complex was finally suspended in 12 ml BBS and stored at -20°C in 0.5 ml aliquots (containing about 20 mg zymosan-C3 complex).

A rabbit was immunized every week for 4 weeks with 20 mg zymosan-C3 complex in Freund's incomplete adjuvant. Four days after the last immunization, the rabbit was exanguined by cardiac puncture and RACC' was collected according to the method described in Chapter 2 for rabbit antisera. Zymosan bound C3 preferentially but not specifically. The antiserum would show other specificities if immunization was prolonged.

Complement Fixation Inhibition Test for Activity of RACC'

Serum samples from 4 cats immunized against sheep red blood cells (sRBC) were kindly provided by Dr. G. Dean, Department of Immunology, Middlesex Hospital Medical School. They were pooled and inactivated at 56°C for 30 min.

Sensitization of sRBC with Cat Anti-sRBC Antibodies

This was performed according to Hudson and Hay (1976). 12 ml 25% sRBC in Alsever's solution (Oxoid) were washed 3 times in 0.15M BBS by centrifugation at 200 g for 10 min. The sRBC were resuspended to make up a 6% suspension in BBS in a graduated conical centrifuge tube. 5 ml of inactivated pooled cat anti-sRBC serum at dilutions 1: 5, 25, 50, 100, 200, 400, 800 and 1600 in BBS were mixed with 5 ml 6% sRBC and incubated at 37°C for 30 min. 5 ml BBS were also mixed and incubated with 5 ml 6% sRBC as the control. The sensitized sheep cells (EA) were used within 24 h.

Source of Complement

Preliminary experiments showed that normal cat sera had heterophile agglutinins for sRBC. Therefore, these agglutinins were removed by adsorption with 3% sRBC suspension, giving a final dilution of 1: 10, 20 and 40, for 30 min at room temperature. The sRBC were then removed by centrifugation at 200 g for 10 min. It was found that haemolysis occurred at dilutions 1: 10 and 20. Thus, cat serum at 1: 40, after adsorption with 3% sRBC, was used as the source of complement.

Optimal Dilutions of Complement and EA

Preliminary experiments showed that EA prepared at antiserum dilutions of 1: 50 and above did not show any haemolysis. Therefore, only EA prepared at antiserum dilutions of 1: 5 EA(1:5) and 25 EA(1:25) were used. Reagents were added into a U-bottomed microtitre plate (Cooke Engineering) as shown in Table 7.1. Cat complement was diluted in BBS at 2-fold dilutions from 1: 10 to 160. 3% sRBC was included as the control. The suspension in each well was mixed and then incubated at 37°C for 1 h followed by 4°C for 30 min. The degree of haemolysis in each well was noted, by comparing the size of the button of red cells at the bottom of the well with those formed by 10 µl of 3, 2.4, 1.8, 1.5, 1.2 and 0.6% suspension of sRBS in 60 µl of BBS in row D.

Detection of Anti-complement Activity of RACC'

Reagents were added into a microtitre plate as shown in Table 7.2. RACC' was first inactivated at 56°C for 30 min. and diluted 4-fold from 1: 2 to 128 in BBS. Inactivated normal rabbit serum was included as the control. The suspension in each well was mixed and then incubated at 37°C for 15 min. 10 µl of 3% EA sensitized with the optimal dilution of inactivated cat anti-sRBC serum were then added into rows A and B.

Table 7.1 Determination of the optimal dilutions of cat complement (C') and EA used in the complement fixation test.

Reagents		Well Number 1-2	3-4	5-6	7-8	9-10	11-12
A.	BBS	20µl	20µl	20µl	20µl	20µl	60µl
	40 µl cat C'	1:10	1:20	1:40	1:80	1:160	-
	3% EA(1:5)	10µl	10µl	10µl	10µl	10µl	10µl
B.	Repeat as in A. except that 3% EA(1:25) was used instead of 3% EA(1:5)						
C.	Repeat as in A. except that 3% sRBC was used instead of 3% EA						
D.	BBS	60µl	60µl	60µl	60µl	60µl	60µl
	sRBC(10 µl)	3%	2.4%	1.8%	1.5%	1.2%	0.6%

EA(1:5) and (1:25) - sheep red blood cells (sRBC) sensitized with inactivated cat anti-sRBC serum at dilutions 1:5 and 1:25.
BBS - barbitone buffered saline, 0.15M, pH7.6

Table 7.2 Detection of the anti-complement activity of rabbit antiserum against cat complement (RACC')

Reagents		Well Number 1-2	3-4	5-6	7-8	9-10	11-12
A.	BBS	-	-	-	-	20µl	20µl
	cat complement	40µl	40µl	40µl	40µl	40µl	-
	20µl inactivated RACC'	1:2	1:8	1:32	1:128	-	-
B.	As in A except that inactivated normal rabbit serum was used instead of RACC'						
C.	As in A						
D.	As in B						

1. sheep red blood cells (sRBC) sensitized with the optimal dilution of inactivated cat anti-sRBC serum were added into row A and B after incubation of the plate at 37°C for 15 min.
2. sRBC were added to rows C and D as the control.

BBS - barbitone buffered saline, 0.15M, pH7.6.

10 µl of 3% SRBC were added into rows C and D as the control. After mixing the contents in the wells, the plate was incubated at 37°C for 1 h followed by 4°C for 30 min. The degree of inhibition of complement fixation by RACC' was noted.

Reaction between RACC' and Whole Cat Serum in IEP

The normal cat serum was first electrophoresed and then reacted with RACC', rabbit anti-whole cat serum and normal rabbit serum as described in Chapter 2. Cat IgG was also electrophoresed for comparison. The precipitation lines were examined after incubating the slides at 4°C for 48 h in a moist chamber.

Flucrescent Antibody Tests for Immune Deposits in Kidneys

These were performed according to Natali and Cioli (1976), Nairn (1976) and Wilson (1976) with some modifications. Small blocks of kidney samples were collected from 71 B. pahangi-infected cats and 3 normal uninfected cats at necropsy. They were embedded in Tissue-Tek O.C.T. Compound medium (Ames) in small aluminium cups and frozen in liquid nitrogen. They were kept at -70°C.

6 µ thick cryosections of the kidney samples were prepared at -20°C, using a cryostat (MSE) and adhered onto 2 chromic acid-cleaned microscopic slides which had a thin smear of glycerin albumen (R.A. Lambs). One slide was used as the test slide and the other as the control. Four areas were marked on the slide onto which cryosections from 4 different samples were adhered. At least 5 cryosections from each sample were adhered onto each area. The sections were then checked under a compound microscope to ensure the presence of glomeruli in the sections. The sections were then dried in a desiccator for 1 h at room temperature before being tested.

Detection of Antibody Deposits

The slides were washed in PBS, pH 7.6, for 3 min in a Coplin jar to remove any non-specifically bound proteins. 1 ml FITC-conjugated rabbit anti-cat IgG (Nordic) at 1: 20 dilution in PBS, pH 7.6 was transferred onto the test slide and incubated at room temperature for 30 min in a moist chamber. After 3 washes in PBS, pH 7.6 for 30 min, the sections were mounted in 0.01M Tris-buffered glycerol [1 part tris(hydroxymethyl)aminomethane, 9 parts glycerol], pH 9.6 and examined under a fluorescence microscope as described in Chapter 2. For each control slide, a blocking test was done. The sections were treated with 1 ml of rabbit antiserum against cat IgG at 1: 2 dilution in PBS, pH 7.6 for 30 min followed by 3 x 10 min washes in PBS, pH 7.6 before being treated with FITC-conjugated rabbit anti-cat IgG as for the test slides.

Detection of Worm Antigen Deposits

The sections were first treated with 0.02M sodium citrate buffer, pH 3.2 (see appendix for preparation) in a Coplin jar at 37°C for 2 h to remove immunoglobulins which might have bound onto the worm antigens in the immune deposits. After 3 x 3 min washings in PBS, pH 7.6, the sections were fixed with ether-95% ethanol (1 : 1 in volume) for 10 min followed by 95% ethanol for 20 min. The slides were then washed 3 times with PBS, pH 7.6 for 9 min. 1 ml of RABPL5/Mf at 1: 2 dilution in PBS, pH 7.6 was transferred onto each test slide and 1 ml of normal rabbit serum at 1: 2 dilution in PBS, pH 7.6 onto each control slide. After inqubation in a moist chamber at room temperature for 30 min, the slides were washed 3 times with PBS, pH 7.6 for 30 min. 1 ml FITC-conjugated swine anti-rabbit IgG (Nordic) at 1: 40 dilution in PBS, pH 7.6 was then transferred onto each slide and incubated in a moist chamber at room temperature for 30 min. After PBS washings as before, the sections were mounted and examined as described above.

Detection of Complement Deposits

The sections were first washed with PBS, pH 7.6 for 3 min to remove any non-specifically bound proteins. 1 ml of RACC' at 1: 2 dilution in PBS, pH 7.6 was transferred onto each test slide and 1 ml of normal rabbit serum at the same dilution onto each control slide. After incubation at room temperature for 30 min in a moist chamber, the slides were washed 3 times in PBS, pH 7.6 for 30 min. 1 ml FITC-conjugated swine anti-rabbit IgG (Nordic) at 1: 40 dilution in PBS, pH 7.6 was then transferred onto each slide. The slides were incubated for 30 min as before. After 3 washings with PBS, pH 7.6 for 30 min, the sections were mounted and examined as described above.

Albustix Test for Proteinuria

Albustix reagent (tetrabromphenol blue) strips (Ames) were used for testing proteinuria in 66 B. pahangi-infected and 3 uninfected cats. The test area of the strip was dipped in the fresh urine sample and removed immediately. After removing excess urine by tapping the edge of the strip against the container, the colour of the test area of the strip was immediately compared closely with the colour chart provided. The degree of proteinuria was graded from -, ±, +, 2+, 3+ to 4+. A '+' reaction or above was regarded as positive.

Preparation of IgG from Normal Rabbit Serum and Rabbit Antiserum against B. pahangi Adult Worm Antigen

Rabbit antiserum against B. pahangi adult worms (RABPL5) was prepared according to the method described in Chapter 2. The IgG fractions of normal rabbit serum (NRS-IgG) and RABPL5 (RABPL5-IgG) were prepared according to Hudson and Hay (1976)

as described in Chapter 2 for preparation of cat IgG. The IgG preparation was finally dialysed exhaustively against 0.1M phosphate buffer, pH 7.0 (see appendix for preparation). The purity of the IgG preparation was examined by IEP as described in Chapter 2 for normal cat IgG, using goat antisera to rabbit IgG and whole serum (Miles Labs).

Preparation of Fab fragments of NRS-IgG and RABPL5-IgG

This was performed according to Stanworth and Turner (1973) and Hudson and Hay (1976). The concentration of IgG was adjusted to 20 mg/ml in 0.1M phosphate buffer, pH 7.0. Cysteine (Sigma) and EDTA were added to a final concentration of 0.01M and 0.002M respectively. 1 mg mercuripapain (Sigma) was added for every 100 mg IgG used. The mixture was incubated at 37°C overnight. The digest was then dialysed exhaustively against 0.01M acetate buffer, pH 5.5. This process removed cysteine and EDTA and inactivated the enzyme. Crystals formed were removed by centrifugation at 800 g for 10 min. The suspension was then applied to a CM-cellulose column (20 x 2.4 cm for 150 mg protein). The column was first washed with 200 ml 0.01M acetate buffer, pH 5.5. Fractionation was then performed with a gradient of increasing ionic strength, with 0.01M acetate buffer, pH 5.5 and 0.9M acetate buffer, pH 5.5 as the starting and limiting buffers respectively. The flow rate was 1.7 ml/min. 4.2 ml fractions were collected and the absorbence at 280 nm. Fractions with absorbence at 280 nm being greater than 0.1 in the first 2 peaks, containing the Fab fragments, were concentrated separately by dialysis against a concentrated solution of PEG (MW 20,000). The concentrated solution was then dialysed exhaustively against PBS, pH 7.2 at 4°C. The absorbence at 280 nm was measured. The preparations, Fab fragments of NRS-IgG [NRS-IgG(Fab)] and RABPL5-IgG [RABPL5-IgG(Fab)], were then kept at -20°C in 0.5 ml aliquots.

Test of Antibody Activities of RABPL5-IgG(Fab) and NRS-IgG(Fab)

An ELISA was performed similarly to that described in Chapter 3. A polystyrene ELISA plate (Dynatech) was coated with 200 µl of CSE of *B. pahangi* adult worms at 1.8 µg protein per ml 0.05M CO_3-HCO_3 buffer overnight at 4°C in a moist chamber. RABPL5-IgG(Fab) and NRS-IgG(Fab) were tested at dilutions 1: 300. RABPL5 and NRS at 1: 300 dilution were also tested. The serum incubation was carried out at room temperature for 2 h. Peroxidase-conjugated goat anti-rabbit IgG (Miles Labs) at dilution 1: 2000 was used. The conjugate incubation was carried out at room temperature for 3 h. The OD values of the samples were read at 492 nm after the enzyme reaction had proceeded for 20 min.

Conjugation of RABPL5-IgG(Fab) and NRS-IgG(Fab) with ^{125}Iodine by the Chloramine T method

2 mg IgG(Fab) in 200 µl PBS, pH 7.2, were mixed with 300 µCi ^{125}Iodine, in 5 µl, in a polystyrene tube (LP3, Luckham). 120 µl chloramine T (1 mg/ml PBS) were added and mixed. After 2 min at room temperature, 120 µl of sodium metabisulphate (2 mg/ml PBS) were added to stop the reaction. 50 µl of KI (0.5 mg/ml) were then added to mop up any free ^{125}Iodine. The solution was then transferred into a visking tubing and dialysed overnight against 5 l PBS, pH 7.2 to remove free iodine. The ^{125}I-labelled proteins were stored at -70°C in 50 µl aliquots.

Test of ^{125}I-IgG(Fab) by Trichloracetic Acid (TCA) Precipitation

Duplicate samples of 10 µl ^{125}I-RABPL5-IgG(Fab) and ^{125}I-NRS-IgG(Fab) at 1: 10 dilutions in PBS-Tween were mixed separately with 90 µl PBS-Tween and 100 µl 20% TCA in 0.4 ml-

microcentrifuge tubes (Beckman). Other duplicate samples of each were also mixed separately with 90 µl PBS-Tween and 100 µl PBS, pH 7.2. After incubation at 4°C for 30 min, they were centrifuged at 10,000 g for 5 min at 4°C. 100 µl of the supernatant (i.e. one half) were removed from each tube with a syringe and transferred into a clean tube. The radioactivity in these tubes was counted automatically by a LKB 1280 Ultrogramma counter at preset count 100,000 and preset time 60 sec. The percentage radioactivity bound was calculated from the formula: -

$$\frac{p - s/n}{p + s/n} \times 100$$

, where p was the number of counts from the tube containing half of the supernatant and the precipitate, and s/n the number of counts from tube containing half of the supernatant only. For satisfactory conjugation, at least 95% of the counts were TCS precipitable. Repeated dialysis to remove free iodine would be necessary if less than 95% of the counts were precipitated.

Comparison of Antibody Activities of RABPL5-IgG(Fab) and NRS-IgG(Fab) before and after Labelling with ^{125}Iodine

An ELISA was performed as described above. The OD values of 5 and 10 µg RABPL5-IgG(Fab) and NRS-IgG(Fab) were compared with those of the same quantities of ^{125}I-RABPL5-IgG(Fab) and ^{125}I-NRS-IgG(Fab) respectively. This was to check whether the binding activity of Fab had been destroyed after conjugation with ^{125}Iodine by the chloramine T method.

Detection of Binding of ^{125}I-RABPL5-IgG(Fab) and ^{125}I-NRS-IgG(Fab) to B. pahangi Antigens in Solution

6 µg B. pahangi adult worm soluble antigen (CSE) in 50 µl PBS, pH 7.2 were mixed with an equal volume of 6 µg

^{125}I-RABPL5-IgG(Fab) in 6 microcentrifuge tubes. 6 µg ^{125}I-NRS-IgG(Fab) were also included in other 6 control tubes. They were incubated at 37°C for 1 h and then at 4°C for 3 h. 100 µl of 5, 10 and 16% PEG (MW 6,000 Sigma) in PBS, pH 7.2 were added into duplicate tubes and incubated overnight at 4°C. After centrifugation at 10,000 g for 5 min, 100 µl supernatant were transferred into a clean tube. The percentage radioactivity precipitated was calculated as described above.

Antigen-specific Test for Immune Complexes

^{125}I-RABPL5-IgG(Fab) was used to label the worm antigen component of circulating immune complexes in B. pahangi-infected cats. Since Fab fragments were used, the immune complexes would not be precipitated. ^{125}I-NRS-IgG(Fab) was used as the control. The immune complexes in serum were then precipitated by PEG or rabbit antiserum cat IgG, which would bind to the antibody component of the immune complex. The radioactivity present in the precipitate would represent the amount of immune complexes with B. pahangi worm antigen as the component.

Determination of the Precipitation Curve for Immune Complexes Formed between B. pahangi Adult Worm Antigens and RABPL5 in vitro

This was performed according to Hudson and Hay (1976) with some modifications. 5, 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240 and 260 µl of RABPL5 were mixed with 12 µg of CSE of B. pahangi L5, in 5 µl PBS, in duplicate polystyrene LP3 tubes. The final volume was adjusted to 350 µl with PBS, pH 7.2. 5 µl worm antigen were also mixed with 345 µl PBS, pH 7.2 as the control. The tubes were stoppered and incubated at 37°C for 1 h followed by 4°C overnight. The tubes were then centrifuged at 10,000 g for 5 min. The super-

natant was removed. The precipitate was washed twice with cold PBS, pH 7.2. The precipitate was finally dissolved in 1 ml 0.1N NaOH and the absorbence was measured at 280 nm.

Determination of Optimal Concentration of PEG to Precipitate Immune Complexes

From the preceding study, the volume of reagents used to form immune complexes at equivalence or in antibody excess would exceed the capacity of the 0.4 ml-microcentrifuge tube. Thus, immune complexes formed in antigen excess were employed in this study. Immune complexes were prepared from 12 µg antigen and 20 µl RABPL5 in 16 microcentrifuge tubes as described above. 12 µg ^{125}I-RABPL5-IgG(Fab) were added to 8 tubes and 12 µg ^{125}I-NRS-IgG(Fab) to the other 8 tubes as the controls. After mixing by shaking the tubes, they were incubated at 37°C for 1 h and then at 4°C for 3 h. 100 µl of 5, 10, 16 and 20% PEG were added into duplicate experimental and control tubes. After mixing, they were incubated at 4°C overnight. After centrifugation at 10,000 g for 5 min, 100 µl of the supernatant was transferred to a clean tube. The percentage of radioactivity precipitated was estimated as described above.

Determination of the Optimal Quantity of Rabbit Antiserum against Cat IgG (RACIgG) Required to precipitate IgG Present in 10 µl Cat Serum

20, 40, 60, 80, 100, 120, 140, 160 and 180 µl of RAC IgG were added to duplicate polystyrene LP3 tubes containing 10 µl pooled normal cat serum. The final volume was adjusted to 200 µl with PBS, pH 7.2. In the 2 control tubes, 190 µl of PBS, pH 7.2 were mixed with 10 µl pooled normal cat serum. The tubes were stoppered and mixed. They were then incubated at 37°C for 1 h and then at 4°C overnight. After centrifugation at 10,000 g for 5 min, the supernatant was discarded. The precipitate was washed twice with cold PBS, pH 7.2. The

precipitate was finally dissolved in 1 ml 0.1N NaOH and the absorbence at 280 nm measured. The quantity of RACIgG giving the largest amount of precipitate was taken as the optimal.

Test Using PEG to Precipitate the Immune Complexes

10 µl test serum were mixed with 80 µl PBS, pH 7.2 and 10 µl ^{125}I-RABPL5-IgG(Fab) in duplicate microcentrifuge tubes. 10 µl ^{125}I-NRS-IgG(Fab) were used in the duplicate control tubes. A normal cat serum was also included as the serum control. They were incubated at 37°C for 1 h and then at 4°C for 3 h. 100 µl of PEG at optimal concentration were added and mixed. The tubes were incubated at 4°C overnight. After centrifugation at 10,000 g for 5 min, 100 µl supernatant was transferred into a clean tube. The percentage radioactivity in the precipitate was estimated as described above.

Test Using RACIgG to Precipitate the Immune Complexes

10 µl test serum were mixed with 20 µl PBS, pH 7.2 and 10 µl (containing 5 µg) ^{125}I-RABPL5-IgG(Fab) in duplicate microcentrifuge tubes. 10 µl ^{125}I-NRS-IgG(Fab) were used in the duplicate control tubes. A normal cat serum was also included as the serum control. The tubes were incubated at 37°C for 1 h and then at 4°C for 3 h. An optimal quantity of RACIgG was added and mixed. The tubes were incubated at 37°C for 1 h and then at 4°C overnight. After centrifugation at 10,000 g for 5 min, 100 µl supernatant were transferred into a clean tube. The percentage radioactivity in the precipitate was estimated as described above.

C1q-Binding Assay (C1q-BA) for Immune Complexes

This was performed according to Devey et al (1980). Complement-fixing immune complexes were bound to C1q coated on the solid phase. The immune complexes were then detected by ^{125}I-Protein A. C1q isolated from normal human serum was kindly provided by Dr. M.W. Steward, Immunology Unit, Department of Medical Microbiology. Polystyrene LP3 tubes were coated with 10 µg C1q/ ml PBS, pH 7.2 at 4°C for 3 - 4 days. 60 µl test sera were pre-incubated with 120 µl 0.2M EDTA, pH 7.0 at 37°C for 30 min in order to prevent fixation of endogenous complement. They were then kept in an ice-bath. After washing the C1q-coated tubes 3 times with PBS-0.05% Tween 20 (PBS-T), 175 µl PBS-T were added. 75 µl (1/3 dilution) of serum samples were added into duplicate tubes and mixed. After incubation at 37°C for 1 h and 4°C for 30 min, the tubes were washed 3 times with PBS-T. 1 µg of ^{125}I-Protein A in 250 µl PBS-T (kindly prepared by Miss B. Sisley, Immunology Unit), containing 1 µCi of radioactivity, were added. The tubes were incubated at 37°C for 1 h and then at 4°C for 30 min. The tubes were finally washed with PBS-T (washings collected by suction into a 1 l flask). The tubes were stoppered, wiped dry and counted as described above. The results were expressed as the percentage increase of test serum mean counts per min (CPM) over the mean cpm of the pooled control serum from normal individuals,

$$\text{i.e. } T\% = \frac{\text{test mean cpm} - \text{mean control cpm}}{\text{mean control cpm}} \times 100 .$$

A test sample was regarded as positive when the T% value was larger than $\bar{x}$ + 3S.D., where $\bar{x}$ was the mean T% values for the normal control sera (14 normal cat sera and 16 normal human sera) and S.D. the standard deviation of $\bar{x}$.

Conglutinin-Binding Assay (KBA) for Immune Complexes

This was performed according to Devey *et al* (1980) with some modifications. Immune complexes with fixed C3bi were bound to conglutinin (K) coated on the solid phase. The complexes were then detected by ^{125}I-Protein A. K isolated from bovine serum was kindly provided by Dr. M.W. Steward. Polystyrene LP3 tubes were coated with 5 µg K/ml PBS, pH 7.2 at 37°C for 3 h and then at 4°C overnight. After 3 washes with veronal buffered saline-0.05% Tween 20 (VBS-T), 225 µl VBS-T were added. 25 µl test serum were then added into duplicate tubes and mixed. The tubes were incubated at room temperature for 1 h. After 3 washes with VBS-T, 1 µg of ^{125}I-Protein A in 250 µl VBS-T (containing 1 µCi of radioactivity) were added. The tubes were incubated at room temperature for 4 h. The tubes were finally washed with VBS-T and counted as in C1q-BA. Expression of results was the same as described for C1q-BA.

Statistical Analysis

The Standard Normal Deviation Test was used to determine the level of significance of difference between 2 sample proportions. A p value of less than 0.02 was regarded as significance.

RESULTS

Histopathology of Kidneys from B. pahangi-infected Cats in Light Microscopy

Kidney samples obtained from 71 cats 24 - 510 days post-infection with 100 - 200 infective larvae of B. pahangi were studied. Microfilariae were frequently found in the capillaries in the glomerular tuft and the interstitium. The capillaries in the glomeruli were often obliterated and congested (Fig. 7.1). Lobulations of the glomerular tuft were also observed. Other glomerular lesions including necrosis, moncytic and/or polymorphonuclear cell infiltrations, thickening of the epithelium of the Bowman's capsule and pericapsular fibrosis (Figs 7.2 - 7.5) were observed in 19%, 45%, 64% and 23%, respectively, of the kidney samples. The percentage of glomeruli showing these lesions increased with the duration of infection. By 180 days post-infection, more than 50% of the glomeruli showed histopathological lesions (Fig. 7.6). No correlation was found between the degree of glomerular lesions and the number of adult worms recovered at necropsy and the last microfilaraemic count.

Staining of kidney samples from 7 infected cats with PAS did not show any marked thickening of the basement membrane of the capillaries in the glomerular tuft as compared to those from 2 normal uninfected cats.

Protein casts (calcified or not) were commonly found in the tubules and collecting ducts. Vacuolations of the tubular epithelium were commonly observed.

Studies of the kidney samples from 2 of the 3 normal uninfected cats showed that similar lesions were present but only in 24 and 26% of the glomeruli. In the remaining uninfected cat CN1 which was 5 years old, 80% of the glomeruli showed histological lesions, similar to those described above.

Fig. 7.1 Kidney specimen from cat C20, showing the obliteration of glomerular capillaries. The whole glomerular tuft appears smaller in size, leaving a wider urinary space (S). Lobulation (L) of the glomerulus is also observed. H&E (400x)
The cat was 153 days post-infection with 172 infective larvae of B. pahangi

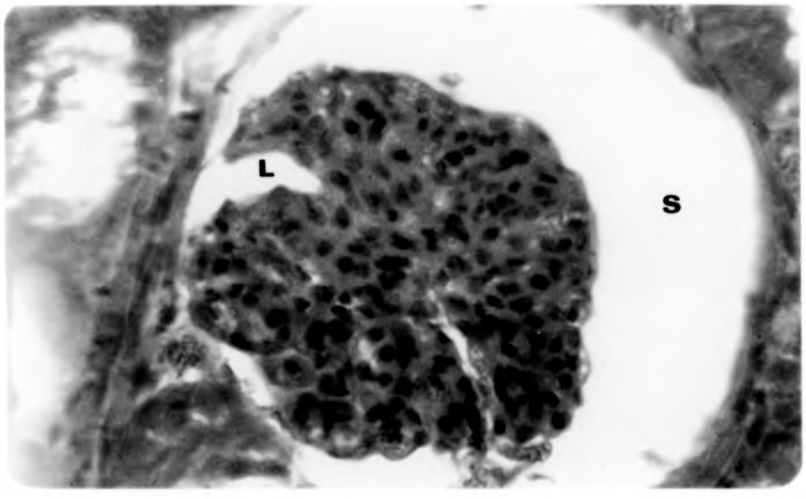
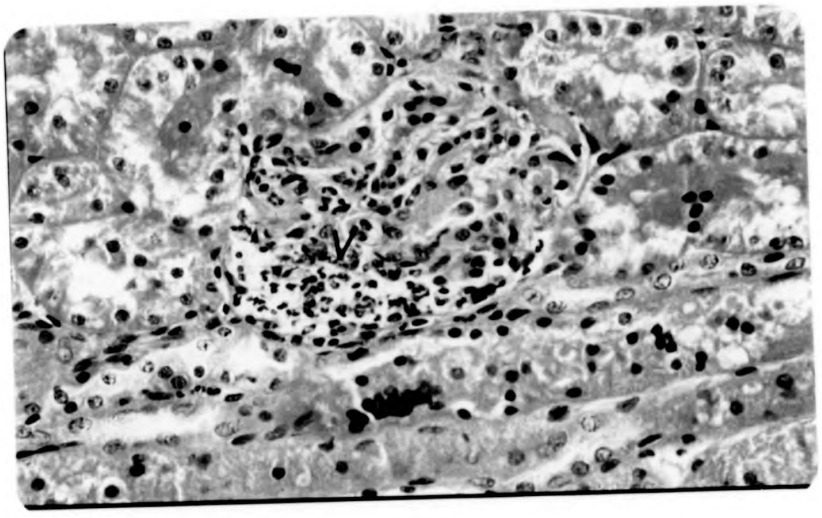


Fig. 7.1 Kidney specimen from cat C20, showing the obliteration of glomerular capillaries. The whole glomerular tuft appears smaller in size, leaving a wider urinary space (S). Lobulation (L) of the glomerulus is also observed. H&E (400x)

The cat was 15½ days post-infection with 172 infective larvae of B. pahangi

Fig. 7.2 Kidney specimen from cat C7, showing necrosis of the glomerulus with predominant polymorphonuclear cell infiltrations. A microfilaria is present in the capillary (arrow). H&E (250x)
The cat was 364 days post-infection with 199 infective larvae of *B. pahangi*.

Fig. 7.3 Kidney specimen from cat C7, showing cellular infiltration (predominantly monocytes) around the glomerular tuft. A microfilaria is present in the glomerulus (arrow). Necrosis (N) of the tubules, with cellular infiltration, is also observed. H&E (250x)


V

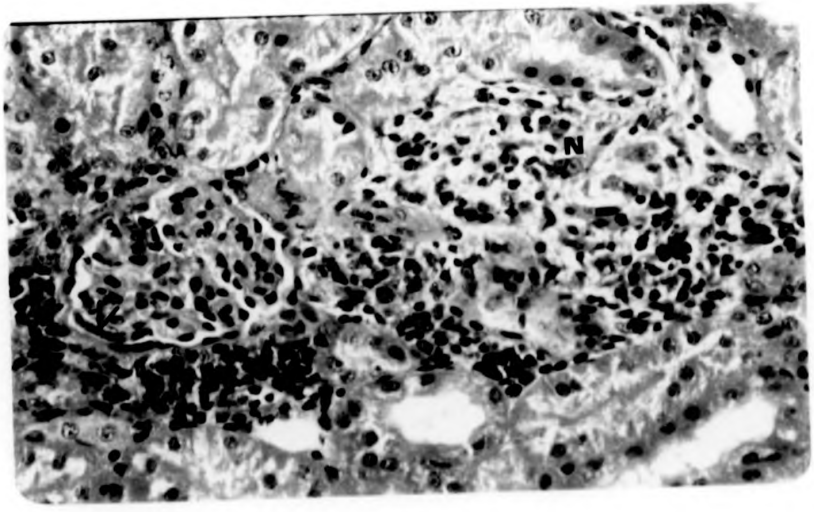
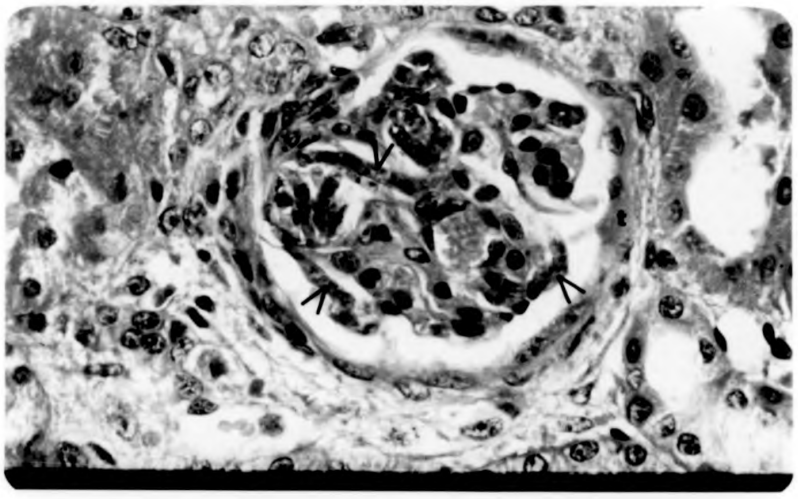
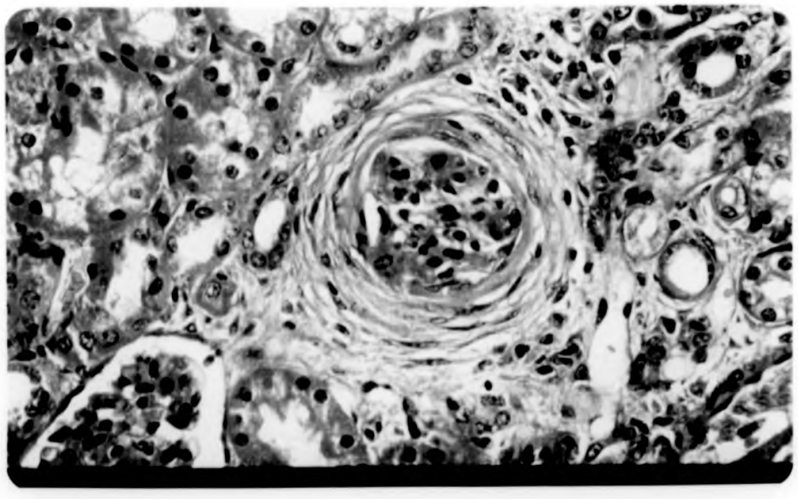

N

Fig. 7.4 Kidney specimen from cat C5, showing thickening of the epithelium of the Bowman's capsule (t). Microfilariae (arrows) are present in the glomerulus, the structure of which is distorted. H&E (250x)
The cat was 188 days post-infection with 199 infective larvae of *B. pahangi*

Fig. 7.5 Kidney specimen from cat C5, showing pericapsular fibrosis of the glomerulus. H&E (250x)





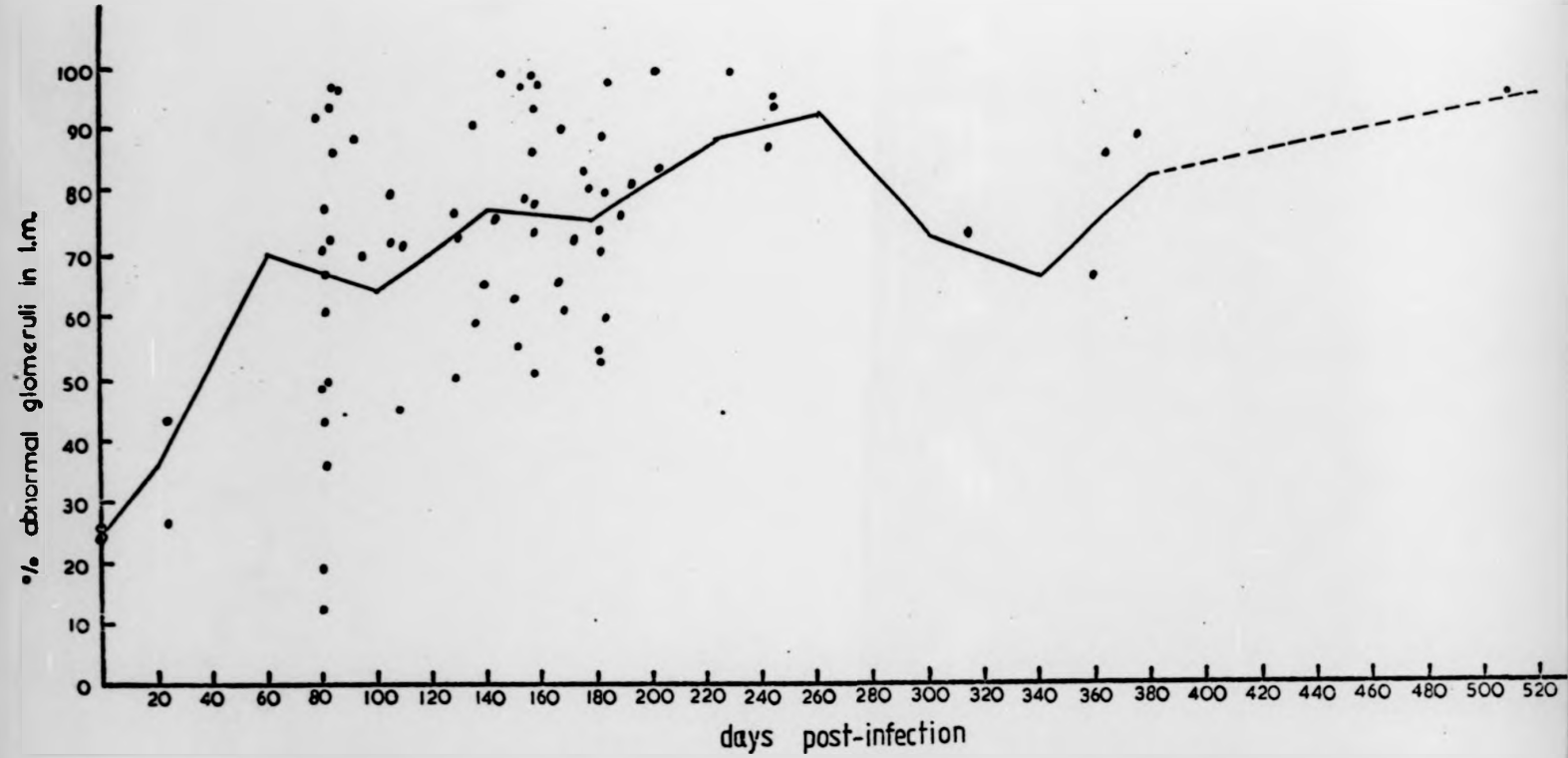


Fig. 7.6 Scatter diagram showing the relationship between the percentage of glomeruli showing histological lesions in light microscopy (l.m.) in B. pahangi-infected cats (●) and the period post-infection. 24 and 26% of glomeruli in kidneys from 2 normal uninfected cats (○) showed histological lesions. The line represents mean percentage.

Histological Studies of the Lungs, Liver, Spleen, Infected Popliteal Lymph Nodes and Lymphatics in B. pahangi-infected Cats

Paraffin sections of the lungs, liver and spleen from 8 B. pahangi-infected and 2 normal uninfected cats were studied. In the lungs of infected cats, microfilariae were occasionally found in the capillaries. Little eosinophils were present. In the liver, there were vacuolations of some perilobular parenchymal cells. Eosinophils and microfilariae were sometimes found in the sinusoids. Focal monocytic cell infiltrations especially around the portal triads were observed. These lesions were also observed in liver samples from 2 normal uninfected cats. Although microfilariae could sometimes be found in the spleens of infected cats, no histological lesions were observed. Therefore, in the lungs, liver and spleen of B. pahangi-infected cats, there were no obvious histological lesions associated with the infections.

The infected popliteal lymph nodes and lymphatics from 1 untreated cat, which had been infected with 200 infective larvae for 157 days, and 3 MBZ-treated cats, which had been infected with 100 - 200 infective larvae for 143 - 181 days, were studied. In the untreated cat, many germinal centres were present in the popliteal lymph nodes. However, there was little proliferation at the paracortical region. The cell density in the medullary region remained low. Fibrotic and granulomatous masses of dead worms were present in the lymphatics. Giant cells were formed round the worm fragments. There were heavy polymorphonuclear cell infiltrations. The wall of the lymphatic vessel around these masses was very much thickened and fibrotic. There were cell infiltrations too (plasma cells, eosinophils, monocytes and some neutrophils). Some live adult worms were also present in some parts of the lymphatic vessel, which were thickened and had some mixed cell infiltrations. The worms were, however, free of any cellular

infiltrations. The female worms appeared to be producing microfilariae, which were found in the lymph and the popliteal lymph node.

On day 28 post-treatment with MBZ, the histology of the lymphatic vessel was similar to that of the untreated cat except that the cellular infiltrations in the lymphatic wall were predominantly monocytic cells, with only a few plasma cells and neutrophils. No active proliferation of cells in the paracortical region in the popliteal lymph node was observed.

On day 42 post-treatment with MBZ, no live adult worms were seen in the lymphatic, which had turned into granulomatous masses. The lymphatic vessel wall was very thickened and fibrotic. There were infiltrations of eosinophils, neutrophils, monocytes and plasma cells. Again, no active proliferation of the paracortical region in the popliteal lymph node was observed.

On day 72 post-treatment with MBZ, masses of amorphous dead worm fragments were still present in the thickened and fibrotic lymphatic vessel. There were monocytes and neutrophils infiltrating the lymphatic vessel wall. Very little proliferation was observed in the paracortical region in the popliteal lymph node.

Histology of Kidneys from B. pahangi-infected Cats by Electron Microscopy

1 - 2 glomeruli in the kidney specimens from 3 uninfected and 7 B. pahangi-infected cats were studied. In the specimens from 2 uninfected cats, which had 24 and 26% of the glomeruli showing lesions in light microscopy (LM), no abnormality was observed. However, in the specimens from the old uninfected cat CN1, which had 80% of the glomeruli showing histological lesions in LM, a layer of subendothelial electron dense deposits

was found in some capillaries of the glomerulus (Fig. 7.7). Furthermore, electron dense deposits were observed in the mesangial matrix which seemed to have expanded (Fig. 7.8).

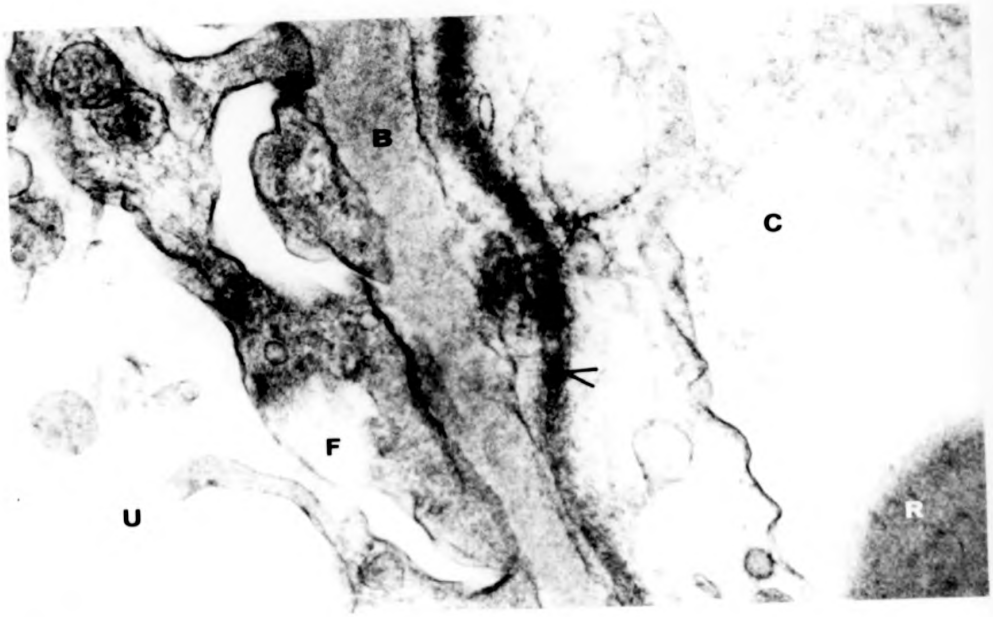
In the kidney specimens from the infected cats, the most common feature observed was diffuse or focal thickening of the epithelial aspect of the basement membrane of capillaries in the glomerular tuft, forming a "hump" (Fig. 7.9). Lacunae were observed in the glomerular basement membrane. Some electron dense materials seemed to be present in specimens from 2 cats (Fig. 7.10). In one cat, 115, these deposits seemed to be present in the desmosomes between endothelial cells of the capillaries. Extensive collapse of a segment of the glomerulus with wrinkled basement membrane was also observed (Fig. 7.11). This represented a focal sclerosis. Within the capillary lumen, some electron dense materials were present. They appeared to be blood clot or thrombi (Fig. 7.12). Eosinophils which appeared to be stimulated, were observed but there was no sign of degranulations (Fig. 7.13). In the specimen from cat C7 which showed cellular infiltrations of glomeruli in LM, a monocyte (probably a macrophage) was found engulfing some materials from the basement membrane of the capillary (Fig. 7.14). In fact, the surface of this monocyte was partly fused with the basement membrane of the capillary. In the specimen of cat 115, a microfilaria, which appeared to be alive, was found in the capillary lumen. The surface of the sheath had a layer of electron dense granules (Fig. 7.15). This layer of electron dense granules seemed to adhere the parasite to the glomerular basement membrane, the fine structure of which seemed to have distorted. No viral particles were observed in all specimens studied.

Fig. 7.7 Electron micrograph showing a layer of sub-endoepithelial electron dense deposits (arrow) in the glomerular capillary of an uninfected cat, CN1. 50,000x

R - red blood cell; C - capillary lumen;
B - glomerular basement membrane;
F - foot process of epithelium;
U - urinary space.

Fig. 7.8 Electron micrograph showing electron dense deposits (arrows) in the mesangial matrix, which seems to have expanded, in the glomerulus of an uninfected cat, CN1. 12,600x

N - nucleus of mesangial cell;
M - mesangium; U - urinary space;
En - endothelial cell;
R - red blood cell.


B
C
F
U
R

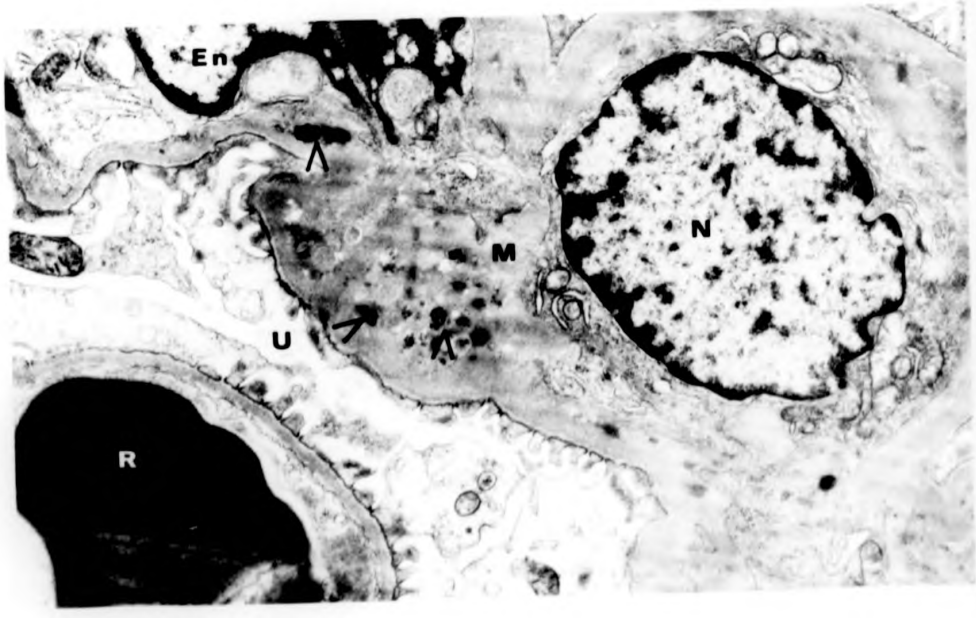

En
M
N
U
R

Fig. 7.9 Electron micrograph showing focal thickening of the epithelial aspect of the glomerular basement membrane forming a "hump" (H). Lacunae (L) are present in the basement membrane. There is a mitochondrion immediately above the "hump".

80,000x

The specimen was from cat C22 which was 314 days post-infection with 193 infective larvae of B. pahangi.

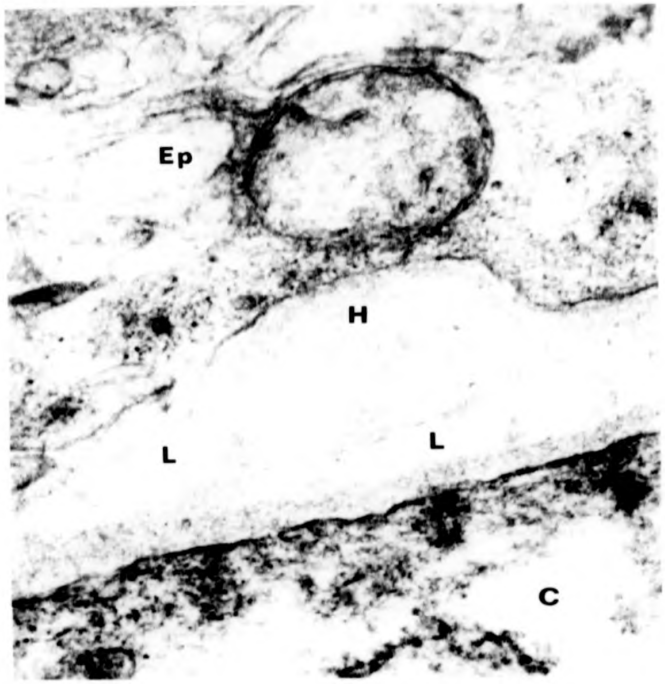
C - capillary lumen; Ep - epithelial cell.

Fig. 7.10 Electron micrograph showing electron dense deposits (arrows) in the glomerular basement membrane.

126,000x

The specimen was obtained from cat 115, which was 183 days post-infection with 99 infective larvae of B. pahangi and 29 days post-treatment with a drug 94A (which was found to be an ineffective filaricide).

F - foot process; Ep - epithelial cell


Ep
H
L
L
C

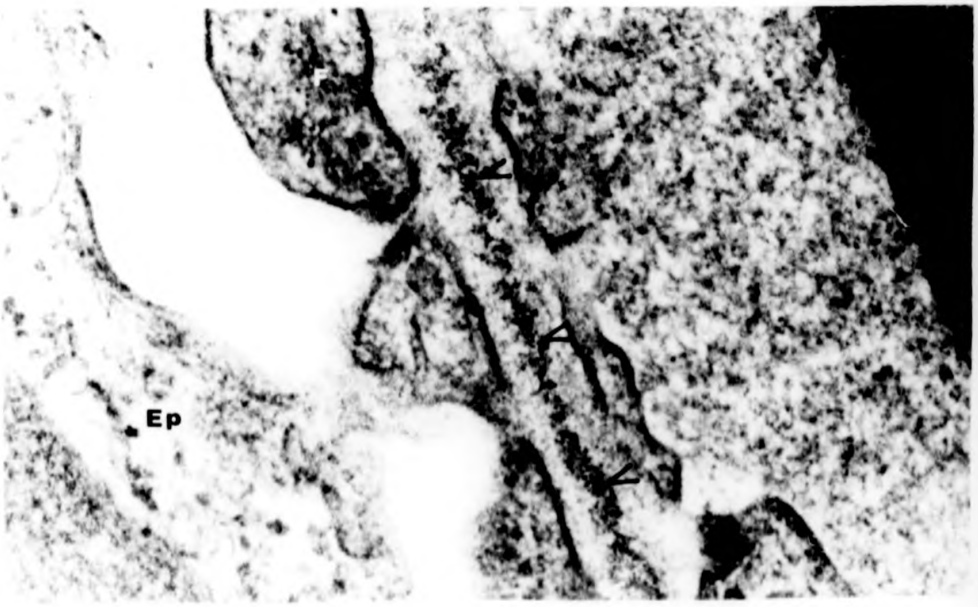
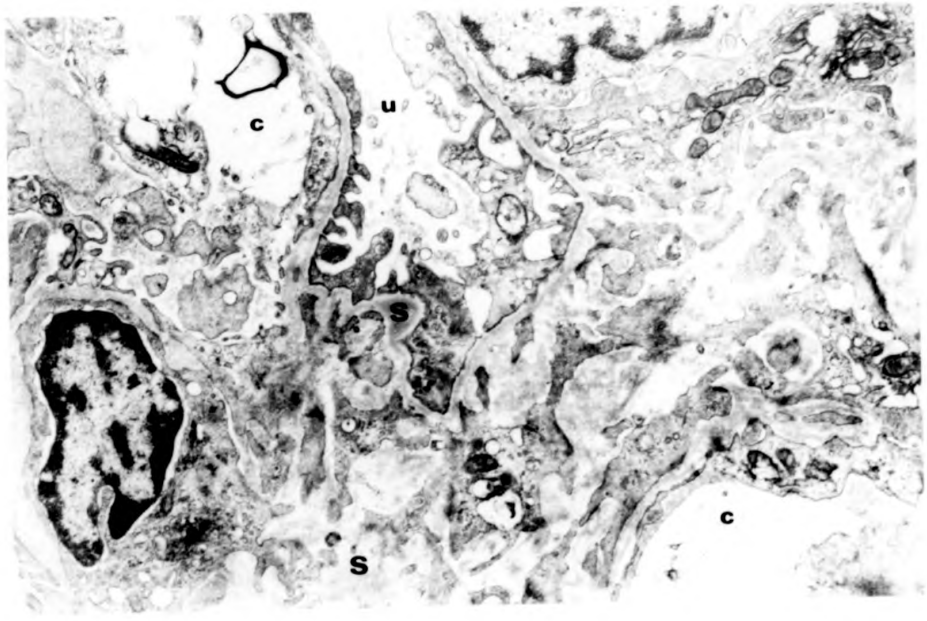

Ep

Fig. 7.11 Electron micrograph showing extensive collapse of a segment of the glomerulus with wrinkled basement membrane, representing a focal sclerosis (S). 12,600x

The specimen was obtained from cat 115
c - capillary lumen;
u - urinary space

Fig. 7.12 Electron micrograph showing electron dense materials, thrombus (T), within the glomerular capillary lumen. 20,000x

The specimen was obtained from cat C7.
U - urinary space.


c
u
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S
c

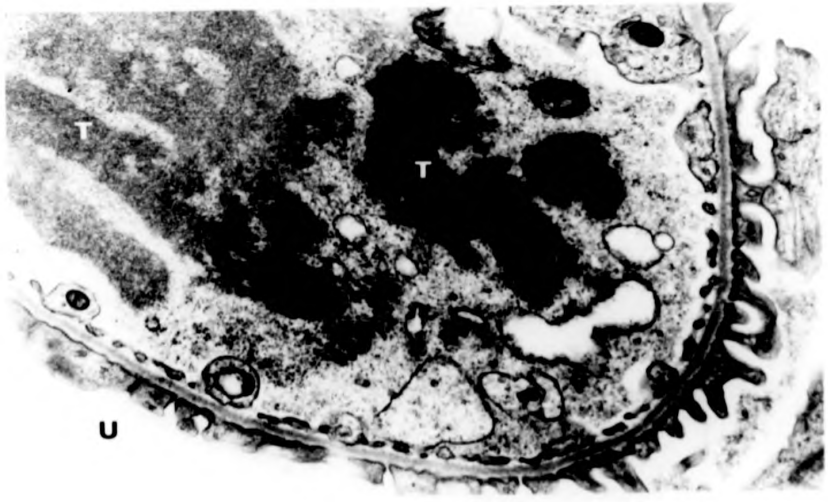

T
T
U

Fig. 7.13 Electron micrograph showing an eosinophil (Eo) within the glomerular capillary lumen. It appears to be stimulated, because most of the granules are swollen (s), but there is no sign of degranulation. A few resting granules, elongated in shape (e), are also present. Part of the glomerular basement membrane is thickened (t). 12,600x

The specimen was obtained from cat C7.
R - red blood cell; U - urinary space

Fig. 7.14 Electron micrograph showing a monocyte (mo), probably a macrophage, engulfing some materials (o) in the glomerular capillary lumen. The surface of the monocyte is partly fused with the glomerular basement membrane (x). 12,600x

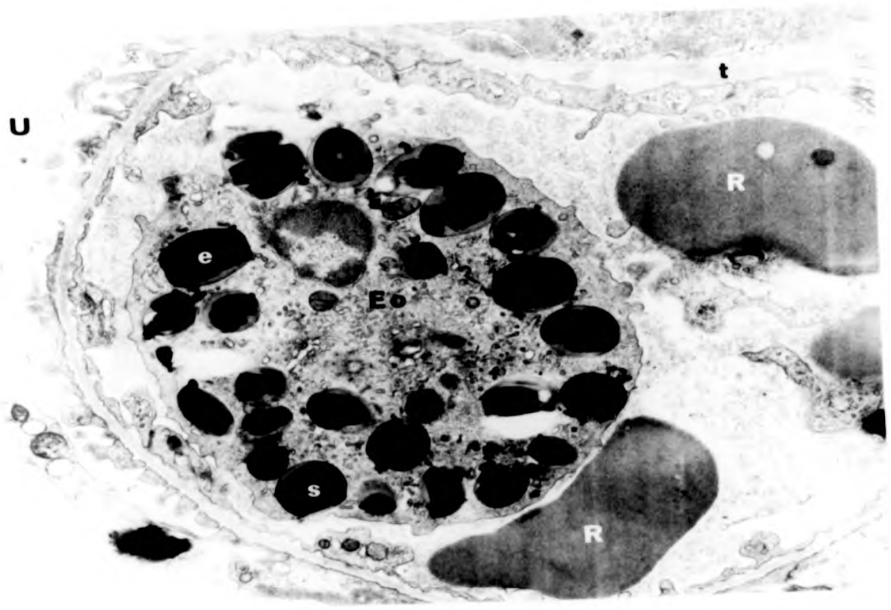
The specimen was obtained from cat C7.
U - urinary space;
(cell infiltrations were observed by light microscopy, Figs 7.2 and 7.3).

Fig. 7.13 Electron micrograph showing an eosinophil (Eo) within the glomerular capillary lumen. It appears to be stimulated, because most of the granules are swollen (s), but there is no sign of degranulation. A few resting granules, elongated in shape (e), are also present. Part of the glomerular basement membrane is thickened (t). 12,600x

The specimen was obtained from cat C7.
R - red blood cell; U - urinary space

Fig. 7.14 Electron micrograph showing a monocyte (mo), probably a macrophage, engulfing some materials (o) in the glomerular capillary lumen. The surface of the monocyte is partly fused with the glomerular basement membrane (x). 12,600x

The specimen was obtained from cat C7.
U - urinary space;
(cell infiltrations were observed by light microscopy, Figs 7.2 and 7.3).


t
U
R
e
Eo
s
R

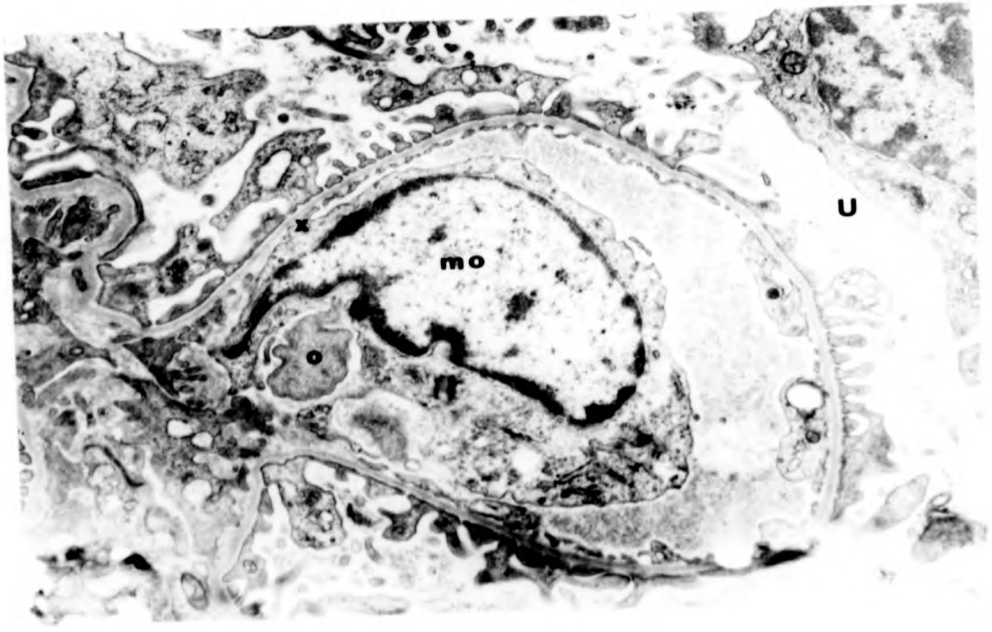

U
mo
o

Fig. 7.15 Electron micrograph showing a microfilaria (Mf) within the glomerular capillary lumen. The surface of the sheath (s) has a layer of electron dense granules (g). This layer of electron dense granules adheres (adh) the parasite to the glomerular basement membrane (bm), the ultrastructure of which is distorted. 42,000x
The specimen was from cat 115.
Ep - epithelial cell.

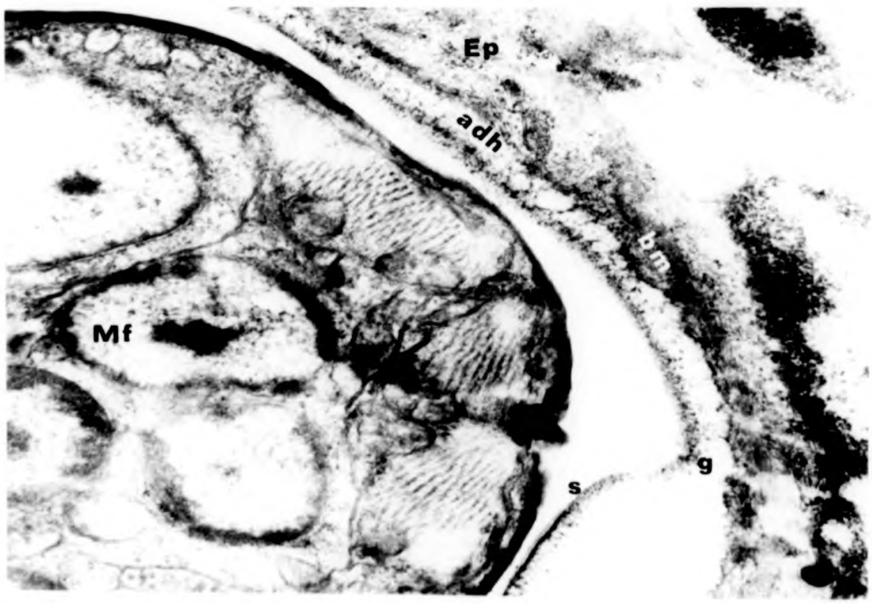


Fig. 7.15 Electron micrograph showing a microfilaria (Mf) within the glomerular capillary lumen. The surface of the sheath (s) has a layer of electron dense granules (g). This layer of electron dense granular adherent material (adh) is apposed to the glomerular basement membrane (bm), the ultrastructure of which is apparent. X8,000
The specimen was from cat 113.
Ep = epithelial cell.

Activity of RACC'

Cat complement (normal cat serum at 1: 40 dilution after adsorption with 3% sRBC) diluted at 1: 10 and 3% EA(1:5) were found to result in complete haemolysis after incubation at 37°C for 1 h and at 4°C for 30 min. These preparations were then used in subsequent tests. In the complement fixation inhibition test, RACC' was found to give 50% inhibition of complement fixation at 1: 2 dilution (i.e. final dilution 1: 6) and 20% inhibition at 1: 8 dilution (i.e. final dilution 1: 24). At dilution 1: 32, no inhibitory activity was found. No haemolysis was observed in the BBS and sRBC controls.

In IEP, RACC' did not form a line with cat IgG but a precipitation line was formed (probably of β-electromobility) with the whole cat serum (Fig. 7.16). No precipitation line was observed using normal rabbit serum as the control.

This RACC' was also shown to have anti-complement activity in the complement-dependent eosinophil adherence reaction to B. pahangi mf by Dr. C.D. Mackenzie, Department of Medical Helminthology.

Fluorescent Antibody Tests for Immune Deposits in Kidneys

In the uninfected cat CN1, which showed abnormal histology in light and electron microscopies, 89% of the glomeruli showed linear IgG deposits along the capillary walls. In the specimens from the other 2 normal uninfected cats, no immune deposits were found.

Among the 71 kidney samples from B. pahangi-infected cats studied, 89% had cat IgG deposits along the capillary walls, in 2 - 99% of the glomeruli present in a section. These deposits were lumpy or granular (Fig. 7.17). They were

Activity of RACC'

Cat complement (normal cat serum at 1: 40 dilution after adsorption with 3% sRBC) diluted at 1: 10 and 3% EA(1:5) were found to result in complete haemolysis after incubation at 37°C for 1 h and at 4°C for 30 min. These preparations were then used in subsequent tests. In the complement fixation inhibition test, RACC' was found to give 50% inhibition of complement fixation at 1: 2 dilution (i.e. final dilution 1: 6) and 20% inhibition at 1: 8 dilution (i.e. final dilution 1: 24). At dilution 1: 32, no inhibitory activity was found. No haemolysis was observed in the BBS and sRBC controls.

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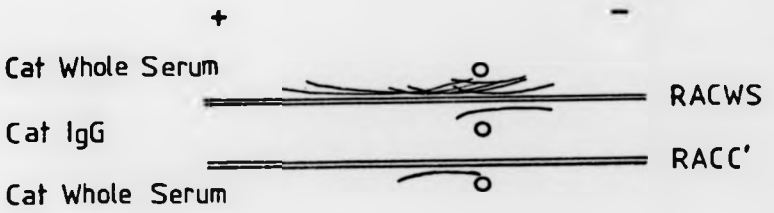


Fig. 7.16 Immunoelectrophoresis - Test of the activity of the rabbit antiserum against cat complement (RACC') prepared in the laboratory by reacting against cat IgG and cat whole serum. Rabbit antiserum against cat whole serum (RACWS) was included for comparison. RACC' did not form a precipitation line with cat IgG but a line was formed with cat whole serum, probably of β-electromobility.

Fig. 7.17 Fluorescence micrograph showing granular deposits of IgG along the glomerular capillary walls. The specimen was from cat C4, 241 days post-infection with 100 infective larvae of *B. pahangi*. 250x

Fig. 7.17 Fluorescence micrograph showing granular deposits of IgG along the glomerular capillary walls. The specimen was from cat C4, 241 days post-infection with 100 infective larvae of *B. pahangi*. 250x

Fig. 7.17 Fluorescence micrograph showing granular deposits of IgG along the glomerular capillary walls. The specimen was from cat C4, 241 days post-infection with 100 infective larvae of *D. mahaffeyi*. 250x

also found to be deposited on the microfilariae present in the glomeruli (Fig. 7.18).

Worm antigen deposits were found to occur in 54% of the samples which also had IgG deposits. The proportion of glomeruli present in a section having such deposits varied from 1 to 87%. These deposits were in granular forms along the capillary walls (Fig. 7.19). However, some were actually microfilariae present in the glomeruli (Fig. 7.20).

Fine granular deposits of complement were observed on capillary walls in the glomeruli (Fig. 7.21). They occurred in 23% of the samples, which also had both IgG and worm antigen deposits. These deposits were found in 1 - 27% of the glomeruli in a section.

11% of the samples from B. pahangi-infected cats did not have any immune deposits at all.

7 infected cats had been treated with DEC and 5 with FBZ. Higher percentage of glomeruli without immune deposits were observed in these cats as compared to 24 untreated cats infected for similar periods of time (Table 7.3). However, this was not observed in the kidneys from 9 MBZ-treated cats. The percentage of glomeruli with histological lesions in kidneys of all these treated cats remained high.

No correlation was found between the percentage of glomeruli with immune deposits and period post-infection, the number of live adult worms recovered at necropsy, the last microfilarial count and the antibody level as measured by IFAT and ELISA (in Chapter 2). No correlation was observed between the percentage of glomeruli with immune deposits and that showing histological lesions too. In 91% of the samples, there was a higher percentage of glomeruli showing histological lesions than those having immune deposits.

Fig. 7.18 Fluorescence micrograph showing IgG deposits on microfilariae in the glomerulus. The specimen was obtained from cat C39, 152 days post-infection with 191 infective larvae of B. pahangi. 250x

Fig. 7.19 Fluorescence micrograph showing focal granular worm antigen deposits along the glomerular capillary walls. The specimen was obtained from cat C5. 250x

Fig. 7.20 Fluorescence micrograph showing microfilariae in the glomerulus. The specimen was obtained from cat C39. 250x

Fig. 7.21 Fluorescence micrograph showing focal fine granular deposits of complement in the glomerulus. The specimen was obtained from cat C6, 359 days post-infection with 190 infective larvae of *B. pahangi*. 250x

Table 7.3 The percentage of glomeruli with antibody (Ab), worm antigen (Ag) and complement (C') deposits in the kidneys from drug-treated and untreated *B. pahangi*-infected cats.

Deposits	% Glomeruli			
	Untreated cats	Treated cats		
		DEC	MBZ	FBZ
No deposits	8	50	11	20
Ab only	42	33	56	20
Ag only	0	0	0	0
Ab + Ag	25	17	33	40
Ab + Ag + C'	25	0	0	20
Sample Siz	24	7	9	5

DEC - diethylcarbamazine; MBZ - mebendazole;
FBZ - flubendazole.
The cats were 91 - 202 days post-infection with 100 - 200 infective larvae of *B. pahangi*.

Proteinuria

40% of urine samples from 66 B. pahangi-infected cats were positive for proteinuria by Albustix test. Proteinuria seemed to have a closer relationship with histological lesions than with the presence of immune deposits. For those having a reaction of 2+ or higher, more than 40% of the glomeruli in the kidneys showed histological lesions.

A 3+ reaction was observed with the urine from the old uninfected cat CN1 which had extensive glomerular lesions. No proteinuria was observed in the urine from the other 2 normal uninfected cats.

Preparation of Fab Fragments from NRS-IgG and RABPL5-IgG

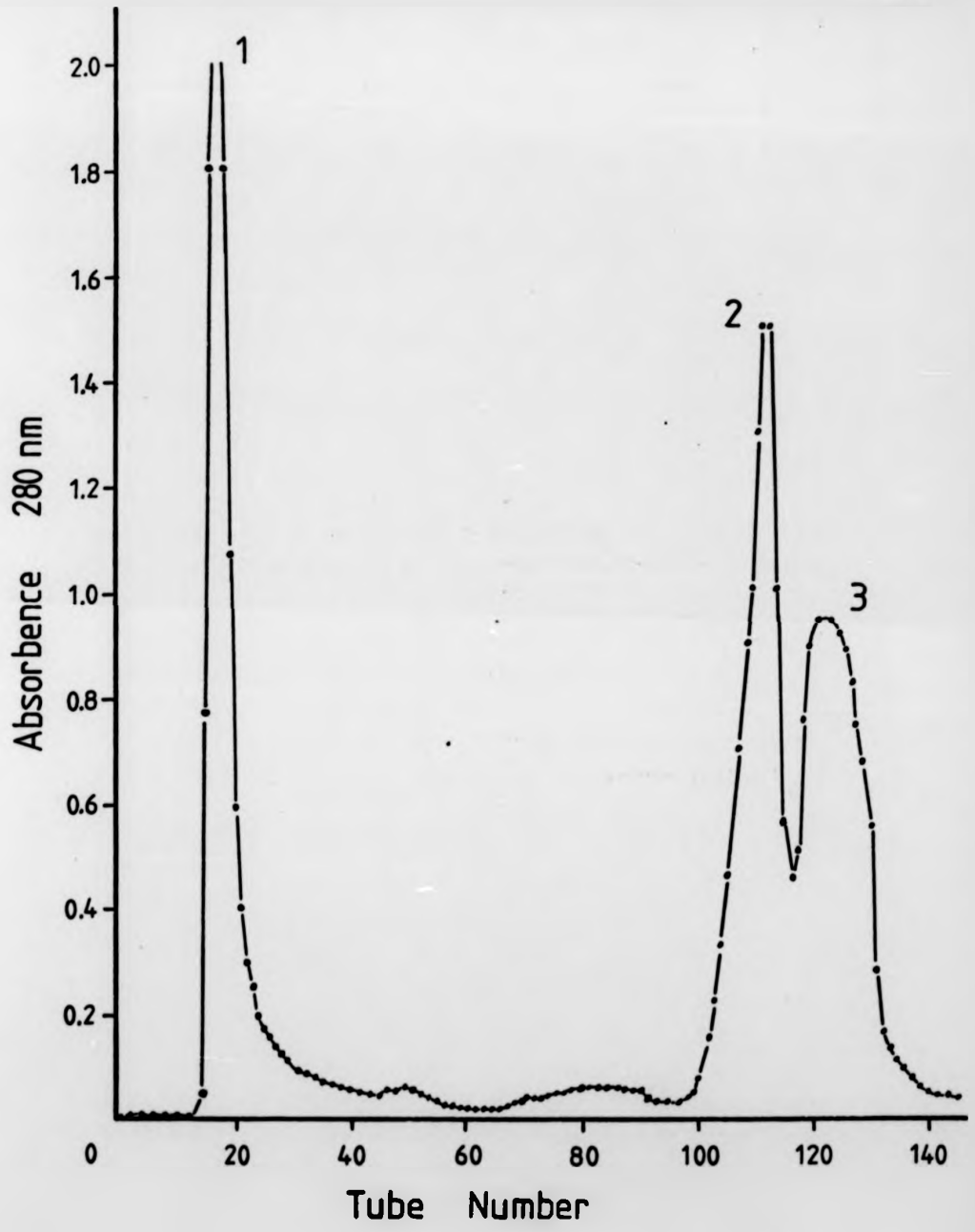
The IgG preparation from NRS and RABPL5 were pure. In IEP, they gave comparable results to that of cat IgG (cf. Fig. 2.2a) when reacted against goat antisera against rabbit whole serum and IgG.

An example of the elution profile of papain-digested IgG from normal rabbit serum in CM-cellulose was shown in Fig. 7.22. The first 2 peaks contained Fab fragments of IgG while the third contained Fc fragments.

Four batches of NRS-IgG(Fab) and 5 batches of RABPL5-IgG(Fab) were prepared. When tested in ELISA, NRS-IgG(Fab) from the 4 preparations all gave very low OD values (0.01 - 0.05) while RABPL5-IgG(Fab) gave very high OD values (1.36 - 2.24). The whole NRS and 3 RABPL5 sera gave OD values of 0.1 and 2.48 - 2.60 respectively.

Fig. 7.22 Elution profile of 200 mg papain-digested rabbit IgG in CM-cellulose chromatography by 0.01M - 0.9M acetate buffer, pH 5.5.

Column size = 27 x 2.6 cm
Flow rate = 1.7 ml/min
Fraction volume = 4.2 ml
Peaks 1 and 2 contained Fab fragments while peak 3 contained Fc fragments.


1
2
3
Absorbence 280 nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
140
Tube Number

^{125}I-NRS-IgG(Fab) and ^{125}I-RABPL5-IgG(Fab)

The ^{125}Iodine conjugation was satisfactory because 95 - 96% of the radioactivity was TCA precipitable. There were about 1 µCi per 10 µg of protein. The antibody activities of NRS-IgG(Fab) and RABPL5-IgG(Fab) were similar to those of the same quantities of ^{125}I-NRS-IgG(Fab) and ^{125}I-RABPL5-IgG(Fab) respectively for the OD values in ELISA were similar (Table 7.4). ^{125}I-RABPL5-IgG(Fab) also combined to the worm antigen in solution for the complex could be precipitated using PEG of concentration of more than 10% (i.e. 16%). No precipitable radioactivity was detected in the ^{125}I-NRS-IgG(Fab)-worm antigen mixture.

Precipitation Curve for Immune Complexes Formed between B. pahangi Adult Worm Antigen and RABPL5 in vitro

From Fig. 7.23, 160 µl of RABPL5 were required to precipitate 12 µg CSE of B. pahangi L5 at equivalence. In the subsequent study, immune complexes formed in antigen excess, i.e. 12 µg worm antigen in 5 µl + 20 µl RABPL5 were used.

Optimal Concentration of PEG for Precipitation of Immune Complexes

From Fig. 7.24, precipitation of ^{125}I-NRS-IgG(Fab) was observed when 16 and 20% PEG were used. In the study mentioned above, complexes formed between ^{125}I-RABPL5-IgG(Fab) and worm antigen alone could be precipitated using 16% PEG. Thus, 10% PEG was taken as the optimal to precipitate immune complexes formed between worm antigen, to be labelled with ^{125}I-RABPL5-IgG(Fab), and antibodies.

Table 7.4 Comparison of antibody activities of RABPL5-IgG(Fab) and NRS-IgG(Fab) before and after conjugation with ^{125}Iodine by the chloramine T method.

Fab Fragments	Mean ELISA OD492 Values
5µg RABPL5-IgG(Fab)	1.61
5µg ^{125}I-RABPL5-IgG(Fab)	1.68
10µg RABPL5-IgG(Fab)	2.07
10µg ^{125}I-RABPL5-IgG(Fab)	2.05
5µg NRS-IgG(Fab)	0.03
5µg ^{125}I-NRS-IgG(Fab)	0.05
10µg NRS-IgG(Fab)	0.05
10µg ^{125}I-NRS-IgG(Fab)	0.09

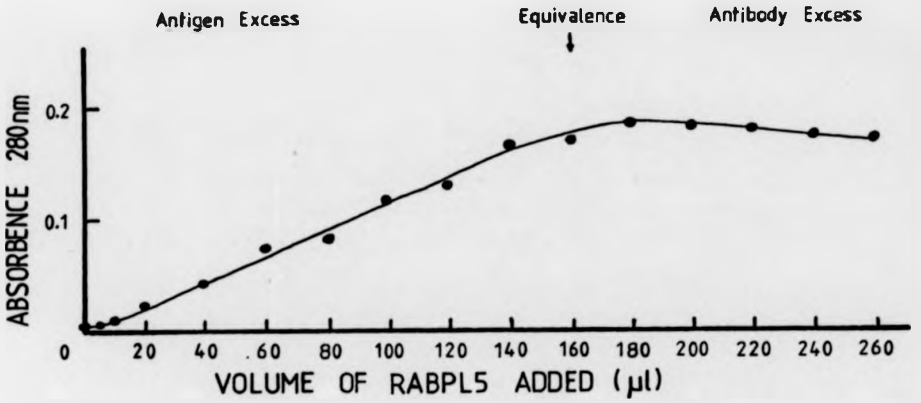
RABPL5-IgG(Fab) : Fab fragments of IgG of rabbit antiserum against *B. pahangi* adult worm antigens.

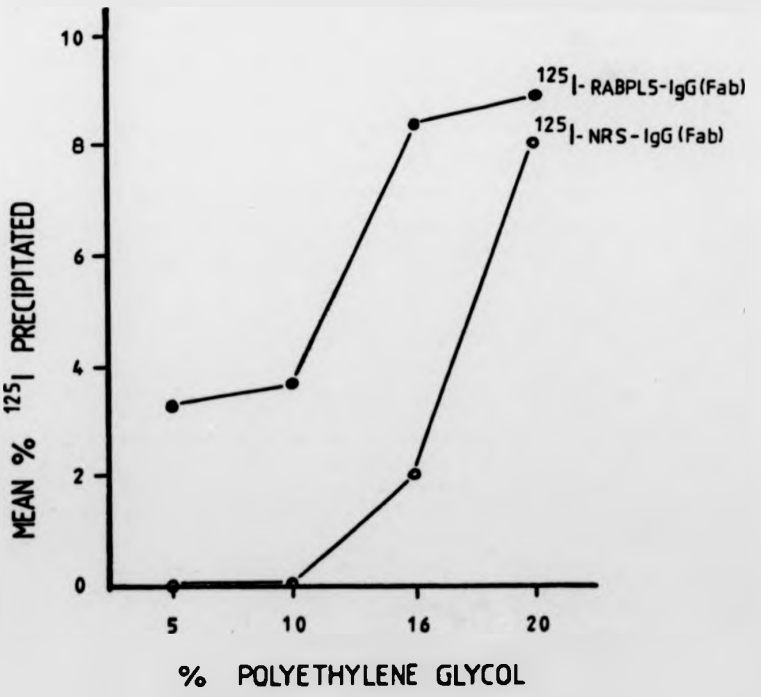
NRS-IgG(Fab) : Fab fragments of IgG from normal rabbit serum.

^{125}I- : ^{125}Iodine conjugated.

Fig. 7.23 Precipitation of immune complexes formed between 12 µg *B. pahangi* adult worm antigen and increasing quantities of rabbit antiserum against this worm antigen (RABPL5).

Fig. 7.24 Precipitation of immune complexes formed in antigen excess (12 µg *B. pahangi* adult worm antigen + 20 µl rabbit antiserum against this worm antigen, RABPL5) with different concentrations of polyethylene glycol (MW 6,000). The antigen component in the complex was labelled with 12 µg of ^{125}I-conjugated Fab fragments of IgG from RABPL5 [^{125}I-RABPL5-IgG(Fab)]. 12 µg of ^{125}I-conjugated Fab fragments of IgG from normal rabbit serum [^{125}I-NRS-IgG(Fab)] were used as the control.


Antigen Excess
Equivalence
Antibody Excess
ABSORBENCE 280nm
0.2
0.1
0
20
40
60
80
100
120
140
160
180
200
220
240
260
VOLUME OF RABPL5 ADDED (µl)


MEAN % 125I PRECIPITATED
10
8
6
4
2
0
125I- RABPL5-IgG(Fab)
125I- NRS-IgG(Fab)
5
10
16
20
% POLYETHYLENE GLYCOL

Optimal Quantity of RACIgG for Precipitation of IgG in 10 µl of Cat Serum

From Fig. 7.25, maximum precipitation of IgG in 10 µl pooled normal cat serum was observed when using 160 µl RACIgG. Therefore, in subsequent studies, 160 µl of RACIgG were used to precipitate immune complexes with IgG antibodies as the component.

Antigen Specific Test for Immune Complexes in Sera from B. pahangi-infected Cats

Sera from 4 B. pahangi-infected cats which were positive for circulating worm antigen by CIEP and ELISA in Chapter 6 were used in the preliminary studies. A pooled serum (PNS) from 10 normal uninfected cats was used as the control. Using 10% PEG or 160 µl RACIgG to precipitate immune complexes after labelling the worm antigen component with ^{125}I-RABPL5-IgG(Fab), higher percentage of radioactivity was precipitated in sera G5 and C54 than in PNS (procedures 1a and 2a in Table 7.5). However, an even higher percentage of radioactivity was precipitated in sera G5 and C54 in the control using ^{125}I-NRS-IgG(Fab) (procedures 1b and 2b in Table 7.5). Attempts were made to modify the experimental procedures by precipitating the immune complexes with RACIgG before labelling the worm antigen component in the complex with ^{125}I-RABPL5-IgG(Fab). Again, higher percentages of radioactivity were precipitated in the control (procedure 3 in Table 7.5). Attempts to include Tween-20 in PBS to prevent non-specific binding of ^{125}Iodine-labelled protein on the microcentrifuge tubes did not improve the results (procedure 4 in Table 7.5). In a later study using latex agglutination test for human rheumatoid factors described in Chapter 6, antiglobulin activities were observed in the normal rabbit serum, from which NRS-IgG(Fab) were prepared, with a titre of 20 (see discussion). When RABPL5, PNS and

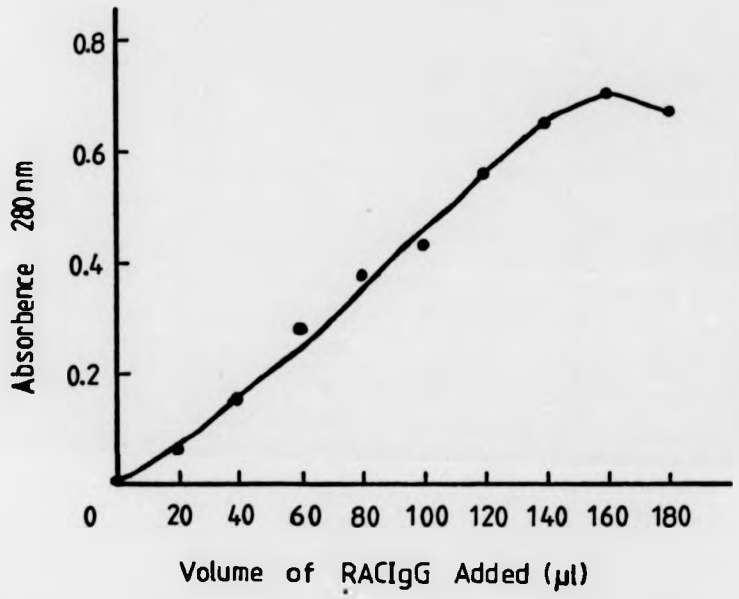


Fig. 7.25 Precipitation of IgG in 10 µl pooled normal cat serum with increasing quantities of rabbit anti-cat IgG (RACIgG).

Table 75 Results of the preliminary trials of the antigen specific test using ^{125}Iodine-labelled Fab fragments of IgG isolated from rabbit antiserum against B. pahangi adult worm antigens [^{125}I-RABPL5-IgG(Fab)] and ^{125}Iodine-labelled Fab fragments of IgG isolated from normal rabbit serum [^{125}I-NRS-IgG(Fab)] as the control.

Experimental Procedures	% Radioactivity in Precipitate				
	Serum Number				
	C45	C12	G5	C54	PNS
1(a) 10 µl serum + 80 µl PBS + 5 µg ^{125}I-RABPL5-IgG(Fab) in 10 µl PBS (37°C 1h, 4°C 3h) + 100 µl 10% PEG (4°C, overnight)	0.6	0.5	1.9	5.8	-0.9
(b) Control for (a) using 5 µg ^{125}I-NRS-IgG(Fab) in 10 µl PBS	-0.4	-0.2	10.1	8.5	-6.4
2(a) 10 µl serum + 20 µl PBS + 5 µg ^{125}I-RABPL5-IgG(Fab) in 10 µl PBS (37°C 1h, 4°C 3h) + 160 µl RACIgG (37°C 1h, 4°C overnight)	-2.7	-5.3	19.5	23.7	-1.1
(b) Control for (a) using 5 µg ^{125}I-NRS-IgG(Fab) in 10 µl PBS	-3.3	-1.3	36.9	41.8	-0.3
3(a) 10 µl serum + 30 µl PBS + 160 µl RACIgG (37°C 1h, 4°C overnight), centrifuge, precipitate washed once with PBS + 5 µg ^{125}I-RABPL5-IgG(Fab) in 100 µl PBS (37°C 1h, 4°C 3h)	6.0	5.8	17.5	17.8	5.9
(b) Control for (a) using 5 µg ^{125}I-NRS-IgG(Fab) in 100 µl PBS	10.9	8.3	27.7	31.9	8.7
4(a) Same as 1(a) except PBS-0.05%Tween was used instead of PBS	-	2.2	34.2	-	2.0
(b) Control for (a) using ^{125}I-NRS-IgG(Fab)	-	2.8	50.9	-	6.0
(c) Precipitate in (a) washed once with PBS-Tween and counted*	-	1.5	31.8	-	11.7
(d) Precipitate in (b) washed once with PBS-Tween and counted*	-	2.3	54.5	-	14.4

* Results expressed in thousand counts/min.

PEG - polyethylene glycol (MW 6,000)

RACIgG - rabbit antiserum against cat IgG

C45, C12, G5 and C54 were sera from B. pahangi-infected cats positive for circulating worm antigens

PNS - a pooled serum from 10 normal uninfected cats.

sera from the infected cats were tested at 1: 20 dilutions, no antiglobulin activities were observed.

Detection of Circulating Immune Complexes in Sera from B. pahangi-infected Cats by C1q-BA and KBA

Sera from 82 B. pahangi-infected and 14 normal uninfected cats were tested. $\bar{x}$ + 3S.D. for the T% values of the normal sera was estimated. Increase of test serum mean cpm over the mean cpm of the pooled control serum by 126% or more in C1q-BA and 117% or more in KBA were regarded as positive.

Among the sera from 31 infected but untreated cats, 10% were positive by KBA (Table 7.6). Two were 81 days and one being 359 days post-infection with 100 - 200 infective larvae. 14 and 22% of sera from DEC and MBZ-treated cats but none from FBZ-treated cats were positive. An overall positivity rate of 12% was observed.

Only 1 serum from a cat treated with anti-lymphocyte serum was positive in C1q-BA (Table 7.6). No serum was positive in both C1q-BA and KBA. A total positivity rate of 13% was observed for circulating complement-fixing immune complexes.

Using KBA, the percentage positivity in the different groups having no immune deposits, antibody deposits only, antibody and worm antigen deposits, and antibody, worm antigen and complement deposits in the glomeruli of kidneys were similar (Table 7.6). The cat which was positive in C1q-BA had antibody, worm antigen and complement deposits in the glomeruli of the kidney. Serum from the uninfected cat CN1 which had extensive histological lesions and linear IgG deposits in the glomeruli of the kidney was positive for circulating complement-fixing immune complexes by KBA.

Table 7.6 Detection of circulating immune complexes in sera from *B. pahangi*-infected cats.

Groups	N	% Positive (No.) KBA	C1q-BA	KBA or C1q-BA
Infected, untreated	31	10%(3)	0	10%(3)
Infected, DEC Rx	7	14%(1)	0	14%(1)
Infected, MBZ Rx	9	22%(2)	0	22%(2)
Infected FBZ Rx	5	0	0	0
Infected, Other Rx	30	13%(4)	3%(1)	17%(5)
Total	82	12%(10)	1%(1)	13%(11)
No immune deposits in kidneys	8	13%(1)	0	13%(1)
Ab deposits only	24	17%(4)	0	17%(4)
Ab + Ag deposits	22	14%(3)	0	14%(3)
Ab + Ag + C' deposits	16	13%(2)	6%(1)	19%(3)

KBA - Conglutinin Binding Assay; C1q-BA - C1q Binding Assay;
N - sample size; Rx - treatment; DEC- diethylcarbamazine;
MBZ - mebendazole; FBZ - flubendazole; Ab - cat IgG;
Ag - *B. pahangi* antigens; C' - complement.

Detection of Circulating Immune Complexes in Sera from Humans Infected with B. malayi and W. bancrofti by C1q-BA and KBA

Sera from 32 patients with brugian filariasis, 16 apparently healthy individuals living in areas endemic for B. malayi, 33 patients with bancroftian filariasis, 14 apparently healthy individuals living in areas endemic for W. bancrofti and from 12 Malaysians living in areas non-endemic for filariasis were tested for complement-fixing circulating immune complexes. Sera from 16 healthy Europeans who had never been to the tropics were used as the controls. Increases of test serum mean cpm over the mean cpm of the pooled serum from the 16 control samples by 182% ($\bar{x}$ + 3S.D.) or more in C1q-BA and 8% ($\bar{x}$ + 3S.D.) or more in KBA were regarded as positive.

Only 28% of B. malayi clinical sera, 9% W. bancrofti clinical sera and 9% W. bancrofti microfilaraemic sera were positive in C1q-BA (Table 7.7).

The highest positivity rate (33%) in KBA was observed in the B. malayi clinical sera (Table 7.7). 1 of these sera was also positive in C1q-BA. 7% of B. malayi microfilaraemic and 5% W. bancrofti clinical sera were positive. None of the 11 W. bancrofti microfilaraemic sera was positive. 6% and 14% of sera from people in areas endemic for B. malayi and W. bancrofti, respectively, were positive. All sera from Malaysians living in non-endemic areas were negative.

56% of the B. malayi clinical sera were positive for circulating immune complexes by either C1q-BA or KBA (Table 7.7). This was significantly ($p < 0.02$) higher than those in the other groups of sera tested.

Table 7.7 Detection of immune complexes in sera from humans with brugian and bancroftian filariasis by Conglutinin Binding Assay (KBA) and C1q-Binding Assay (C1q-BA).

Groups	N	% Positive (NO.) KBA	C1q-BA	KBA or C1q-BA
B. malayi clinical	18	33%(6)	28%(5)	56%(10)
B. malayi mf	14	7%(1)	0	7%(1)
Sera from *B. malayi* endemic areas	16	6%(1)	0	6%(1)
W. bancrofti clinical	22	5%(1)	9%(2)	14%(3)
W. bancrofti mf	11	0	9%(1)	9%(1)
Sera from areas endemic for *W. bancrofti*	14	14%(2)	0	14%(2)
Sera from areas non-endemic for filariasis in Malaysia	12	0	0	0

mf - microfilaraemic; N - sample size.

DISCUSSION

Histological studies of kidneys from 3 normal uninfected cats showed some degree of abnormalities. In cat CN1, which was 5 years old, 80% of the glomeruli showed histological lesions in LM. Under electron microscopy (EM), sub-endothelial and intramesangial electron dense deposits were found (Figs 7.7 and 7.8). This suggests that immune complexes are present. In fact, circulating complement-fixing immune complexes were demonstrated in this cat by KBA. The nature of immune complexes involved is unknown. Naturally occurring immune complex glomerulonephritis of unknown aetiology has been reported in cats by Lucke (1968), Slauson *et al* (1971) and Slauson and Lewis (1979). Fluorescence microscopy revealed linear IgG deposits along the capillary walls, the pattern of which was similar to that in Goodpasture's syndrome of humans. This strongly suggests that anti-glomerular basement membrane antibodies were also present in this old cat. According to Dr. V. Lucke (personal communication), this seemed to be the first report in cats. Severe proteinuria was also observed in this cat, indicating some functional damage of the kidneys. In the other 2 uninfected cats, 24 and 26% of the glomeruli showed histological lesions in LM but no abnormalities could be observed in EM. However, it should be noted that only one glomerulus was examined in EM. No proteinuria was observed in these 2 cats. Vacuolation of the renal tubular epithelium was frequently observed. Slauson *et al* (1971) regarded this as a normal change in cats.

In the kidneys from *B. pahangi*-infected cats, histological lesions typical of glomerulonephritis were observed in LM (Figs 7.1 - 7.5). These are probably of a mild membranous type but not proliferative (confirmed by Dr. V. Lucke, Department of Pathology, School of Veterinary Medicine, University of Bristol). Similar lesions have also been reported

in D. immitis-infected dogs and D. viteae-infected hamsters (Klei et al., 1974; Simpson et al., 1974; Simpson and Neilson, 1976). The severity of renal damage has also suggested by these authors to correlate with the degree of microfilaraemia or the load of infection. However, such relationships were not observed in this study. In EM, thickening of the glomerular basement membrane with some degree of vacuolation was observed (Fig. 7.9). This was also reported in D. immitis-infected dogs and D. viteae-infected hamsters (Klei et al., 1974; Simpson et al., 1974).

In fluorescence microscopy, granular deposits of IgG, worm antigen and complement were present in 89, 54 and 23% of the kidney samples from B. pahangi-infected cats. Immune deposits present in different proportions of glomeruli have also been reported in Nigerian patients infected with Plasmodium malariae (Albini et al., 1979). Worm antigens were associated with the presence of IgG deposits. Complement was associated with the presence of both IgG and worm antigen deposits. Some electron dense deposits, which might suggest the presence of immune complexes, were found in the glomerular basement membrane of kidney specimens from cats C22 and 115 (Fig. 7.10). The former cat had granular IgG and worm antigen deposits while the latter cat had IgG, worm antigen and complement deposits. Heavy electron dense deposits have been reported in D. viteae-infected hamsters (Klei et al., 1974). In the specimen from an infected cat having fine granular immune deposits, no electron dense materials could be found in the glomerular basement membrane in EM. Houba (1979) also reported that no typical electron dense materials could be found in the glomerular basement membrane in EM studies on biopsy specimens from malaial patients although finely granular immune deposits could be detected in fluorescence microscopy.

In Fig. 7.14, a monocyte was observed engulfing some electron dense materials from the glomerular basement membrane in EM. Since monocytes have been reported to be involved in the removal of immune complexes from renal glomeruli (Striker *et al.*, 1979), this also suggests that immune complexes may be involved in the pathogenesis of the glomerular lesions observed.

Circulating soluble worm antigen could be detected by CIEP and ELISA (in Chapter 6) in all infected cats having granular antigen deposits in the glomeruli. Circulating complement-fixing immune complexes could also be demonstrated by KBA and C1q-BA in 16% of the infected cats having immune deposits in the glomeruli (Table 7.6). These seem to suggest that circulating immune complexes may be involved. However, 13% of the infected cats without immune deposits in the kidneys were also positive for circulating immune complexes. This suggests that local formation of immune complexes may also be possible (Houba, 1979; Couser and Salant, 1980).

Observations in EM seemed to suggest that obliteration of glomerular capillaries would result from thrombi and microfilariae (Figs 7.12 and 7.15). In the latter, a layer of electron dense granules present on the sheath was found adhering the parasite to the glomerular basement membrane, the fine structure of which was distorted (Fig. 7.15). This layer of electron dense granules is absent or only rarely found on the sheath of microfilariae of *B. pahangi in utero* and in the circulation (Laurence and Simpson, 1974; Rogers *et al.*, 1976). However, this layer is present on the sheath of other blood microfilariae, *L. loa* and *L. carinii*, and has been suggested to represent materials acquired from the host (McLaren, 1972). Microfilariae of *L. loa* and *W. bancrofti* have been observed to adsorb human blood group antigen on the sheath but not for microfilariae of *B. pahangi* and other unsheath human microfilariae (Ridley and Hedge, 1977). The layer of electron

dense granules is probably composed of IgG for the latter can be demonstrated on microfilariae in the glomeruli (Fig. 7.18). Whether this layer of electron dense granules is present on the sheath of all microfilariae in the glomeruli requires confirmation. Also, whether coating of microfilariae with host antibodies is a prerequisite for their subsequent localization in the glomeruli is another interesting point worth studying. However, this study seems to suggest the possibility that glomerular lesions may result from the local tissue reactions to microfilariae coated with the host antibodies. Direct mechanical damage of the glomeruli by motile microfilariae is also possible (Simpson et al., 1974).

Some degree of functional damage seemed to occur in the kidneys from B. pahangi-infected cats. Proteinuria was present in 40% of these cats. For those having a reaction of 2+ or higher in Albustix test, more than 40% of the glomeruli showed histological lesions in LM. Proteinuria has also been reported in L. loa nephropathy and correlated to D. immitis infections in dogs (Pillay et al., 1973; Klei et al., 1974).

Immune deposits seemed to be cleared by DEC and FBZ therapy for higher percentages of glomeruli without immune deposits were observed in DEC- and FBZ-treated cats as compared to the untreated cats infected for similar periods of time (Table 7.3). This may result from a disturbance of the balance between antigen and antibody production. Nevertheless, the percentage of glomeruli showing histological lesions remained high. In P. falciparum infection of man, the glomerular lesions resolve within 4 - 6 weeks after anti-malarial treatment (Boonpucknavig and Sitprija, 1979). However, this does not occur in P. malariae infections (Houba, 1979). Since some of the cats in this study had been treated with some drugs of unknown toxicity, the possibility that some glomerular lesions resulted from drug toxicity should not be neglected.

Because of the chronic nature of parasitic infections, the host is exposed to parasite antigens throughout the course of infection. Immune complexes are then continuously formed between the host antibodies and the parasite antigens. According to Theofilopoulos and Dixon (1979), whether these immune complexes are of great pathological potential is determined by a number of factors: - the size, valence and chemical composition of antigen; amount of antigen and its rate of production; class, subclass, valence, amount and affinity of antibody involved; ratio of antigen and antibody and the state of the complement and phagocytic system. The most important factor is the ratio of antigen to antibody. Immune complexes formed in slight antigen excess are of intermediate size and soluble but are not rapidly phagocytosed. They are widely disseminated and are large enough to fix complement. Thus, they can exert their pathogenic effects widely. Since the glomerular capillaries are subject to a blood pressure approximately 4 times that of the other capillary beds, this favours the deposition of immune complexes (Albini et al., 1979). In fact, glomerulonephritis occurring in human with malaria and schistosomiasis has been well documented to be associated with the deposition of immune complexes in the glomeruli (see review article by Hutt, 1979). Some immune complexes may not fix complement (Theofilopoulos and Dixon, 1979). This may explain why only 23% of the kidney samples were shown to have complement deposits as compared to 89 and 54% for IgG and worm antigen deposits in this study. Immune complexes deposited in the glomeruli are removed by the mesangial cells (Albini et al., 1979; Striker et al., 1979). Thus, lesions will result only when the amount of immune complexes deposited exceed the phagocytic capacity of the mesangial cells. This may partly account for the observation that in 9% of the samples, there were higher percentages of glomeruli having immune deposits than those with histological lesions.

Glomerulonephritis has been reported in a few cases of filariasis in humans and the involvement of immune complexes has been suggested or demonstrated (Bariety et al., 1967; Zuidema, 1971; Couzineau et al., 1973; Pillay et al., 1973; Chugh et al., 1978; Date et al., 1979a and b). In the studies of animal filariasis, glomerulonephritis has been demonstrated. Antibody deposits have been shown in some cases. No attempts have been made to demonstrate the presence of worm antigen and complement deposits (Klei et al., 1974; Simpson et al., 1974; Casey and Splitter, 1975; Simpson and Neilson, 1976). In this study, lesions typical of glomerulonephritis have been observed. Although histological lesions were found in the 2 normal uninfected cats, 94% of the samples from B. pahangi-infected cats had higher percentage of glomeruli showing lesions, which seemed to increase with the duration of infection (Fig. 7.5). With the demonstration of immune deposits in the glomeruli in the infected cats and the evidence that microfilariae may be involved in the pathology, it seems that the non-progressive glomerular lesions in the kidneys of B. pahangi-infected cats may be linked with immune complexes and microfilariae "trapped" in the glomeruli. However, feline leukaemia virus (FLV) infections of cats have been reported to cause glomerulonephritis (Thornburg et al., 1979; Francis et al., 1980). In view of the high prevalence of feline leukaemia virus infection of cats in the United Kingdom (Dr. V. Lucke, personal communications), it seems difficult to correlate the histological findings with the fluorescence studies in the present investigation. Glomerular lesions resulting from other factors like FLV might account for the observation that in 91% of the samples, the percentage of glomeruli with histological lesions were higher than those showing immune deposits. Therefore, further investigations are required in order to demonstrate the relationship between glomerulonephritis and immune complexes in B. pahangi-infected cats. Since the formation of immune complexes and their removal is a dynamic process which varies between individuals, biopsy specimens obtained from individual cats before and at different stages of B. pahangi-infection should

be studied by LM (using H&E, PAS and also methenamine silver, which specifically stains the basement membrane), EM and fluorescence microscopy. Furthermore, a larger sample size of normal uninfected cats should be employed.

Clearance of microfilariae presumably takes place constantly in the lungs, liver and spleen but this clearance appears to be unassociated with any definable clinical symptoms in humans (Ottesen, 1980). Histological studies of the lungs, spleen and liver from B. pahangi-infected cats did not reveal any lesions associated with the parasite. Pulmonary lesions have been reported in jirds infected with Brugia species and dogs with D. immitis (Winter, 1959; Vincent et al., 1976). In the former, it was considered indicative of an abnormal host-parasite relationship while in the latter, it was related to the definitive site (heart) of the adult worms. In human lymphatic filariasis, pulmonary lesions occur in tropical eosinophilia syndrome which is regarded as a form of "occult filariasis" (Ottesen, 1980). Microfilarial granulomata have been reported in human infected with W. bancrofti and jirds with B. pahangi (Piyaratn and Pradatsundarasar, 1961; Vincent and Ash, 1978). In human, the splenic granuloma is rare and suggested to be a reversible process.

Results of the histological studies of infected popliteal lymph nodes and lymphatics essentially agree with those reported by Rogers and Denham (1974) and Rogers et al (1975). In chronic infections, germinal centres in the popliteal lymph nodes were still active but there were very little proliferation in the paracortical region. Extensive fibrosis and hyalinisation of infected lymph nodes had been reported in B. pahangi-infected cats (Schacher and Sahyoun, 1967). However, no such changes were observed in this study. Rogers et al (1975) could not find such fibrotic changes in the infected lymph nodes

too. Histologically, live and dead worms induced pathological changes in the lymphatics, the latter being to a greater extent. On day 28 after MBZ treatment, the most obvious feature in the lymphatics was the predominance of monocytic cell infiltration of the vessel wall, as compared to mixed cellular infiltrations in the untreated but infected control. Although only one specimens was studied, the possibility that it represents a type IV (cell-mediated) hypersensitivity reaction to the increased release of antigens from the worms being killed by drug treatment needs further investigation. By day 42 post-treatment with MBZ, there were mixed cellular infiltration of the lymphatic vessel wall. No live worms could be observed and the lymphatics had turned into granulomatous masses. This agrees with the observations in the lymphatics of B. pahangi-infected cats after DEC treatment (Denham and Nelson, 1976). However, they reported that the infected lymphatics had returned to normal size 7 weeks after treatment with DEC, while in this study, masses of dead worm fragments were still present in the thickened and fibrotic lymphatic vessel 10 weeks after treatment with MBZ.

C1q-BA has been suggested to be a sensitive test (1 - 10 µg/ml) for immune complexes (Theofilopoulos and Dixon, 1979). It appears to be more effective for the detection of complexes of a higher molecular weight range (Devey et al., 1980). K is an unusual plasma protein which occurs only in bovidae. It reacts by way of calcium ions with a predominantly carbohydrate determinant found in the cell wall of yeasts and a number of other fungi as well as in cell bound C3bi from the complement of all mammalian species so far tested (Macanovic and Lachmann, 1979). KBA is therefore specific for the inactivated form C3bi of C3b. This assay potentially binds complexes of lower molecular weight range and detects complexes that have activated complement via the classical or the alternative pathways (Devey et al., 1980). The sensitivity of this assay is in the order of 5 - 10 µg/ml. However, it suffers

from the disadvantages that only a portion of the complement-fixing immune complexes are detected owing to the specificity of K for probably short-lived intermediate fragments of C3b (Theofilopoulos and Dixon, 1979). Since certain immune complexes preferentially react with one or several reagents, it is recommended that several assays are used for detection of immune complexes (Lambert et al., 1978; Theofilopoulos and Dixon, 1979).

Using C1q-BA and KBA, a total of 13% of sera from 82 B. pahangi-infected cats were positive for complement-fixing immune complexes. 12% were positive in KBA and 1% in C1q-BA. Therefore, it seems that the complement-fixing immune complexes present were in the lower molecular range. Since C1q-BA and KBA only detect complement-fixing immune complexes, some non-complement fixing immune complexes may be present in the sera too. Furthermore, some immune complexes detected may contain antigens other than those derived from the parasite although all the positive sera were positive for circulating antigens by CIEP or ELISA (in Chapter 6). The significance of the presence of circulating immune complexes in these positive sera is not understood although 91% of these cats had immune deposits in the kidneys. Using C1q-BA, 41% of sera collected from jirds 8 months post-infection with B. pahangi were positive for immune complexes. No correlation between the immune complex levels and the numbers of circulating microfilariae was observed (Karavodin and Ash, 1980). To study the pathological and immunological significance of immune complexes in B. pahangi-infected cats, further studies on the evolution of immune complexes during the course of infection by several assays are required.

Using C1q-BA and KBA, more than half (56%) of the sera from B. malayi clinical cases were positive for complement-fixing immune complexes. This was significantly ($p < 0.02$)

higher than those (6 - 14%) in the other groups. Increased positivities of sera from filariasis patients with a variety of immune complex assays have been reported by Lambert et al (1978) and Verroust et al (1979). In infectious diseases like Dengue fever and malaria, the acute haemorrhagic shock in the former and the development of renal and cerebral complications in the latter have been related to the formation of immune complexes in massive amounts (reviewed by Zubler and Lambert, 1977). Elephantiasis has been suggested to result from a host response which kills the parasite (Denham and Nelson, 1976). Therefore, whether the presence of immune complexes in the circulation is correlated with the clinical manifestations (lymphoedema, elephantiasis, etc.) of B. malayi infection in man deserves further investigations.

Attempts to develop an antigen specific test for immune complexes in B. pahangi-infected cats have been unsuccessful. The ^{125}I-RABPL5-IgG(Fab) was found to retain antibody activity to B. pahangi antigen, which was similar to that of the same quantity of RABPL5-IgG(Fab) (Table 7.4). It also bound to worm antigen in solution and could then be precipitated at PEG concentration more than 10% (i.e. 16%). ^{125}I-NRS-IgG(Fab) did not show any binding activity to the worm antigen. Using different test procedures, a higher percentage of radioactivity was precipitated in the control using ^{125}I-NRS-IgG(Fab) (Table 7.5). Basing on the same principle but without using ^{125}I-NRS-IgG(Fab) as the control, this method has been reported satisfactory in detecting immune complexes with hepatitis B antigen (Madalinski and Bragiel, 1979). The possible interference by antiglobulins in the test sera was eliminated because similar results were obtained when the complexes were precipitated and washed before labelling with ^{125}I-RABPL5-IgG(Fab) (procedure 3 in Table 7.5). The presence of non-specific binding of ^{125}I-labelled protein to the plastic microcentrifuge tubes seemed to be unlikely since similar

results were obtained when Tween-20 was included in the reaction mixture (procedure 4 in Table 7.5). However, anti-globulin activities could be demonstrated in the normal rabbit serum, rather unexpectedly. Therefore, the higher percentage of radioactivity precipitated in the control using ^{125}I-NRS-IgG(Fab) could be attributed to the non-specific binding of this conjugate, with antiglobulin activities, to the antibody molecules in the immune complex. These antiglobulins may also bind to other aggregated or denatured form of cat IgG in the test serum. The resultant complexes are then precipitated by PEG and RACIgG in the assay. Therefore, the antiglobulin activity in NRS-IgG(Fab) should be removed by passing through an IgG-sepharose 4B column according to the method described by Hudson and Hay (1976) before being used in the assay.

CHAPTER EIGHT

GENERAL DISCUSSION

A specific immunological test for brugian and bancroftian filariasis in humans is still unavailable chiefly because of the lack of specific antigen from homologous species. The use of animal filarioids as antigens has been unsatisfactory because of the low sensitivity and poor specificity (Ambroise-Thomas, 1974; Kagan, 1974). In search for a better heterologous filarial antigen from species more closely related to human filarioids, B. pahangi microfilarial fragments have been reported the best for detection of antibodies in filariasis as a group (Hedge and Ridley, 1977). In the present study, B. pahangi infective larvae and microfilarial fragments were found to be the best heterologous antigens among the other animal filarioids (D. immitis, D. viteae, L. carinii and O. gutturosa) in IFAT for detection of antibodies in human lymphatic filariasis (Tables 3.2 and 3.4). Furthermore, IFAT using B. pahangi microfilarial fragments was able to reveal the high level of endemicity of bancroftian filariasis in New Guinea (Fig. 4.3). Crude soluble extract of B. pahangi adult worms (BPL5CSE) was also found applicable in ELISA, which was able to reflect the level of endemicity of filariasis in New Guinea, Trinidad, the Seychelles Islands and Egypt (Figs 4.5, 4.10, 4.13 and 4.14). However, cross-reactions with other non-filariasis sera were observed. This poor specificity thus made the interpretation of serological results difficult. Therefore, IFAT and ELISA using crude B. pahangi antigens cannot be used for diagnostic purposes.

Attempts were made to look for a possible source of specific antigen, using mainly B. pahangi infection of the cat as a model. Antigens obtained from the infective larvae,

adult worms and microfilariae of B. pahangi were studied by IFAT. B. pahangi-infected cats were found to produce antibodies first to infective larvae, then adult worms and microfilariae accordingly (Fig. 2.4). Thus, the use of infective larvae as antigens in serological test may be useful for detection of early infections. However, for a population living in an area endemic for filariasis, it is expected that every individual will be exposed to infecitve larvae from infected mosquitoes. Thus, everyone would produce antibodies against infective larvae. However, the disease does not develop in everybody. Therefore, the use of infective larvae as antigens in serological tests seems unjustified. According to the results obtained in this study using OIDT, infective larvae do not seem to possess a stage-specific antigen (Fig. 2.2). Hence further investigations are required to confirm this finding using purified antigens of the different developmental stages.

Attempts were also made to purify BPL5CSE by ion-exchange chromatography for use in ELISA. This resulted into 2 fractions, after CM-cellulose chromatography, one retained high reactivity to B. malayi sera and the other to W. bancrofti sera with a 50 - 100% reduction of reactivities with schistosomiasis sera (Table 3.10). There were still high levels of reactivities with filariasis sera as a group. Perhaps further purification may result in a more group-specific antigen. Purified antigens of Setaria digitata which had first been thought as specific for bancroftian filariasis have now been found to cross-react with other helminths too (Dr. M.M. Ismail, personal communications). Therefore, attempts to purify animal filarial antigens for a group-specific antigen will likely to follow the fate of the Sawada antigen prepared from D. immitis, as reviewed in Chapter 1 and by Ambroise-Thomas (1974). O. volvulus antigen purified by chromatographic techniques has been reported satisfactory in differentiating onchocerciasis sera from control normal sera in a preliminary study using

ELISA (Marcoullis *et al*., 1978). Chromatographic purification of homologous filarial antigens seems logical but not practical since a large amount of worm material is required and the yield is very low, as shown in the present study and by Tanaka *et al* (1968) and Doenhoff *et al* (in press). Perhaps it is only applicable to *B. malayi*, which can be maintained easily in a number of laboratory animals (Table 1.2).

The metabolic antigen of *B. pahangi* adult worms seemed to be more species-specific for it reacted with homologous sera from infected cats (positivity rate of 76%) and only slightly or not at all with sera from individuals with *B. malayi*, *W. bancrofti*, *L. loa* and *O. volvulus* infections (Chapter 2 and 3). Therefore, this study suggests that the metabolic products of filarioids may be the source of specific antigens. IgE antibodies of tropical eosinophilia patients have been shown to exhibit significant species specificity to metabolic antigens of *W. bancrofti* and *B. malayi* microfilariae (Ottesen *et al*., 1979). In fact, de Savigny *et al* (1979) have reported successful application of metabolic antigens of *Toxocara canis* larvae for specific detection of antibodies in toxocariasis of humans.

PGM isoenzymes obtained from *B. pahangi* adult worms seemed to be species-specific in polyacrylamide gel electrophoresis (Flockhart, personal communications). In this study, this enzyme was found to be antigenic to the infected cats. This PGM-antibody reaction seemed to be species as well as stage-specific (Fig. 5.4). Therefore, this study also suggests the possibility of using species-specific isoenzymes obtained from the filarioid as antigens in serological tests. PGM does not seem to induce antibody response in a large number of infected cats. Neutralization antibodies to PGM could be detected in only 19% of the cat sera tested. However, in view of the observation that antibodies to acetylcholinesterase could be detected in most animals infected with *M. brasiliensis*

and T. colubriformis at certain stage of the infections (Jones and Ogilvie, 1972; Rothwell et al., 1973), it seems worthwhile to look for another enzyme, which would induce antibody response in most of the infected individuals, in B. pahangi adult worms and perhaps in human filarioids too.

In general, results of the antibody tests obtained in this study have suggested that a species-specific antigen can be obtained presumably from the homologous species only. However, among the different species of human filarioids, only B. malayi can be maintained easily in common laboratory animals. W. bancrofti and L. loa can be maintained only in some monkeys (see Chapter 1). Therefore, efforts should be made to search for suitable common laboratory animal hosts and in vitro cultivation methods for human filarioids so that adequate worm materials can be obtained for preparation of specific antigens (UNDP/World Bank/W.H.O., 1979b). In view of the difficulty of obtaining large amounts of parasite materials, Mitchell et al (1979) have recently proposed the use of anti-idiotype antibodies to a parasite specific hybridoma antibody. These could be employed in place of worm antigens in serodiagnostic tests. Although preliminary trials only met with a limited success in the Mesocestoides corti-mouse system, it suggests that the same prinicple can be applied to human filariasis.

Attempts were also made to detect circulating filarial antigens, instead of antibodies, in sera from cats infected with B. pahangi and from humans infected with B. malayi and W. bancrofti by CIEP and ELISA. Results of this study suggests that antigen tests in certain instances can be as sensitive as antibody tests (see Chapter 6). Although ELISA for detecting antigens has the potential application in quantitation of circulating antigen in test sera, this assay seems to be interfered with by the presence of rheumatoid factors and

perhaps some other serum factors too. Thus, further investigations of the interfering serum factors and their effects on ELISA are required. An antigen test also has the same fundamental problem as antibody test —— the requirement of a specific antigen. Some sera from cats without an active infection, but which still harboured dead worm fragments in the lymphatics, were still positive in the antigen tests. Thus, this suggested that the use of antisera against the somatic antigens of the parasite might not be a satisfactory indicator of active infections. Therefore, the use of antisera against the metabolic antigens of the adult worms or microfilariae may be a logical approach. Furthermore, metabolic antigens of B. pahangi adult worms have been shown to be more species-specific in this study. This study also showed that the activities of different antiworm sera varied. Thus, standardization of anti-worm serum is necessary if results of antigen tests performed in different laboratories are to be compared. However, the spectrum of reactivities and potency of antisera raised in different rabbits would be different becuase of the difference in individual immune response. The volume is also limited. With the recent development of hybridoma technology, monoclonal specific antibodies can be produced, in potentially unlimited amounts. This would serve as a source of reference specific antibodies which can be distributed for general use. In fact, the potential usefulness of hybridoma monoclonal antibodies in parasitic diseases, especially for specific detection of infections, has been discussed by Mitchell et al (1979), UNDP/World Bank/W.H.O. (1979a) and Pearson et al (1980).

Brugian and bancroftian filariasis, as well as the other forms of filariasis, mainly occur in developing countries in the tropical and subtropical regions where technology is usually less advanced. Attempts to develop an immunological

test for these diseases should take the following into considerations: -

1. Performance of the test - This should be simple so that ordinary technicians can handle the procedures. The test should be cheap to perform so that a small laboratory can afford the cost of purchase and maintenance of equipment. It should also have the capacity of screening a large number of samples in a short time.

2. Sensitivity of the test - The test should be sensitive enough to detect acute as well as chronic infections and especially those with low infections. According to Ekins (1980), radioimmunoassay (RIA) is at present the most sensitive test available although chemiluminescent and time-resolved fluorescence techniques are potentially capable of revolutionizing the current immunoassay methodology. However, the need of expensive equipment and the potential health hazards of radioactivity have discouraged the common use of RIA in developing countries.

ELISA, a new immunoassay technique of the 70's, has been shown to give sensitive, reproducible and comparable results with RIA (Voller et al., 1977). Its useful applications in many parasitic deseases have been reported by a number of workers (Voller et al., 1976a; Ambroise-Thomas et al., 1978; McLaren et al., 1978). This technique has recently been shown suitable for a simple laboratory in St. Lucia for the sero-epidemiological survey of schistosomiasis (McLaren et al., 1979). In the present study, using even crude antigen, ELISA was found to be useful in seroepidemiological studies of human filariasis (Chapter 4).

Immunofluorescence microscopy is another sensitive technique. However, the subjectivity of reading the test and the technical difficulties in evaluating large numbers of sera have mitigated against its

wide application (Kagan, 1979). The development of a reliable fluorometer seems to have solved the problem of subjectivity of reading the test. Nevertheless, the total cost of the equipment is expensive.

In this study, CIEP was shown to be less sensitive than IFAT and ELISA (Tables 2.2, 2.4 and 2.6). It seemed to be a useful technique in detection of worm antigens in body fluids. However, the requirements of concentrated reagents and test sera seem to discourage its wide application in the field of seroepidemiology.

3. Specificity of the test - The test should be specific to the disease studied. This seems to rely solely on the quality of the worm antigen used. It is unlikely that a group specific antigen can be obtained from animal filarioids and perhaps from human filarioids too. Thus, a species-specific antigen is required. This study has suggested that immunogenic enzymes present in the crude extract and metabolic products of the filarioid may be possible sources of a more species-specific antigen. Further studies using human filarioids especially B. malayi are required to elucidate this. In fact, using metabolic products from one adult O. volvulus, a recent preliminary small-scale study with ELISA for detection of antibodies in onchocerciasis sera seems to be satisfactory (Ambroise-Thomas, unpublished observations).

4. Detection of antibodies or antigens in body fluids - Antibody tests are useful to epidemiologists for they can provide information on period prevalence and incidence rates among different age groups at different times (Draper, 1976). When a brief idea about the level of endemicity of filariasis in a certain area is required, antibody tests using even crude antigen (as in Chapter 4)

seem to be applicable. However, antibody tests detect exposure not resulting in infections, past infections as well as active infections. Since antigens are shed or secreted almost continuously by the parasite (Ottesen, 1980), antigen tests may be promising for detection of active infections (Kaliraj et al., 1979b). Perhaps, detection of the presence of metabolic rather than somatic antigens may be a logical approach.

In this study, naturally occurring glomerulonephritis which might be related to immune complexes or antiglomerular basement membrane antibodies was observed in an uninfected cat CN1. Abnormal histology was found in LM and EM (Figs 7.7 and 7.8). Linear IgG deposits were observed along the capillary walls in the glomeruli in fluorescence microscopy. Circulating complement-fixing immune complexes were detected by KBA. Severe proteinuria, suggesting renal damage, was also observed.

In the 71 cats infected with B. pahangi, 94% had a higher percentage of glomeruli showing histological lesions in LM as compared to 2 normal uninfected cats. The percentage of glomeruli showing these lesions increased with the period post-infection (Fig. 7.6). However, glomerular lesions did not seem to be progressive. Although only a few specimens were available, the observations by EM showed some abnormal histology in the glomeruli of kidneys from infected cats (Figs 7.9 - 7.14). This study also suggested that local tissue reactions to microfilariae in the glomeruli could occur (Figs 7.15 and 7.18). IgG, worm antigens and complement deposits were observed in the glomeruli of kidneys from infected cats by fluorescence microscopy (Figs 7.17 - 7.21). This suggests immune complexes may be involved. The immune deposits seemed to be cleared after DEC and FBZ treatment (Table 7.3). Some degree of renal

damage was possible since proteinuria was detected in 40% of the infected cats and could be related to the high percentage (> 40%) of glomeruli showing histological lesions. Therefore, although abnormal histology is present in kidneys from 2 normal uninfected cats, a higher percentage of glomeruli showing histological lesions can be shown in the kidneys from B. pahangi-infected cats. It seems that the histological lesions can probably be linked with the presence of microfilariae and immune deposits in the glomeruli. However, since naturally occurring glomerulonephritis was present in an uninfected cat, kidney samples from more uninfected cats are required for the control. As FLV infections of cats are common in the United Kingdom, the histological lesions observed may be partly due to FLV too. Therefore, with the results obtained so far, one cannot draw a conclusion that the histological lesions observed in the kidneys from B. pahangi-infected cats are associated with the filarioid infection.

Although it is difficult to envisage the significance of the presence of circulating complement-fixing immune complexes in sera from B. pahangi-infected cats, however, circulating immune complexes are present in a significantly higher percentage of sera from B. malayi clinical cases. The possible role played by immune complexes in the pathogenesis of the clinical manifestations of this disease eg. lymphoedema and elephantiasis, deserves further investigations.

In this study, 7 - 56% of lymphatic filariasis sera were shown to have circulating immune complexes. Immune complexes have also been shown in a large proportion of patients with filariasis due to O. volvulus, W. bancrofti, L. loa and D. perstans (Lambert et al., 1978; Verroust et al., 1979; Ottesen, 1980). These immune complexes may also be responsible for other pathological lesions like glomerulonephritis in filariasis (Pillay, 1973; Chugh et al., 1978;

Date *et al.*, 1979a and b). Moreover, immune complexes are known to exert enhanced or inhibitory effects on the immune system (W.H.O. 1977; Theofilopoulos and Dixon, 1979). As mentioned in Chapter 1, different immunological responses are associated with different clinical manifestations of lymphatic filariasis. These also occur in animal filariasis. Therefore, immune complexes may play an important role in initiating or modulating numerous aspects of the overall host immune response to the filarioid (Kasarodin and Ash, 1980; Ottesen, 1980). Recently, the depression of delayed hypersensitivity reactions to the parasite in patients with generalized onchocerciasis has been suggested to be due to IgM immune complexes exerting an immunoregulatory role on T cell function (Paganelli *et al.*, 1980). Better understanding of the immunological profile and pathology of brugian and bancroftian filariasis could be achieved by studies of immune complexes at different stages of the infections.

APPENDICES

APPENDIX FOR CHAPTER TWO

Phosphate Buffered Saline (PBS), pH 7.2, 0.15M

8.0 g NaCl + 0.2 g KCl + 1.15 g Na_2HPO_4 + 0.2 g KH_2PO_4 in 1.0 litre of distilled water.

0.01M Phosphate Buffer, pH 8.0

$NaH_2PO_4.H_2O$: 1.38 g l^{-1};

Na_2HPO_4 : 1.45 g l^{-1}.

The 2 solutions were mixed to obtain the required pH using a pH meter.

0.05 M Barbitone Buffer, pH 8.6

10.0 g barbitone sodium and 6.5 g sodium acetate were dissolved in 64.4 ml HCl, 0.1 N and diluted to 1 litre with distilled water.

Coomassie Blue Staining Solution

1.0 g Coomassie blue + 100 ml acetic acid + 450 ml absolute ethanol + 450 ml distilled water.

0.01M PBS, pH 7.6 for IFAT

Stock solution : 13.36 g Na_2HPO_4 + 1.8 g $NaH_2PO_4.H_2O$ + 85 g NaCl dissolved in 1 litre distilled water.

Working solution : 100 ml stock solution diluted with 900 ml distilled water.

Buffered Glycerol for IFAT

1 volume of 0.2M Na_2HPO_4 was mixed with 9 volumes of glycerol.

Reagents for ELISA

Coating Buffer:- 1.59 g Na_2CO_3 + 2.93 g $NaHCO_3$ in 1 litre distilled water.

PBS, pH 7.6:- 170 g NaCl, 25.6 g Na_2HPO_4, 3.12 g $NaHPO_4.2H_2O$ dissolved in 20 litres of distilled water.

Incubation Buffer (PBS-Tween):- 1 litre PBS + 0.5 ml Tween-20

Washing Solution:- 45 g NaCl + 2.5 ml Tween 20 dissolved in 5 litres distilled water.

Peroxidase Substrate:- Stock OPD - 100 mg orthophenylene diamine (OPD) dissolved in 10 ml absolute methanol (stored at 4°C and discarded after 1 week).

Working Solution - 50 ml distilled water + 25.3 ml Na_2HPO_4, 0.2M + 24.7 ml citric acid, 0.1M + 50 µl H_2O_2 + 1 ml Stock OPD (prepared fresh).

APPENDIX FOR CHAPTER THREE

0.005M Acetate Buffer. pH 4.6

0.005M acetic acid was mixed with 0.005M sodium acetate to obtain pH 4.6, with a pH meter.

APPENDIX FOR CHAPTER FIVE

Substrate for PGIF

PGM - 8.5 ml 0.3M Tris/HCl, pH 7.4 + 0.2 ml NADP (10 mg/ml) + 0.25 ml 1M $MgCl_2$ + 1.25 ml G1P/G1,6DP (20 mg/ml) + 75 µl G6PD (100 IU/ml) + 1.25 ml distilled water + 1 ml MTT (3-(4,5-Dimethyl-Thiazolyl-2)-2,5-diphenyl Tetrazolium bromide) (5 mg/ml) + 1 ml PMS (phenazine methosulphate) (1 mg/ml) + 12.5 ml Agar.

GPI - 8.5 ml 0.3M Tris/HCl, pH 8.0 + 0.2 ml NADP (10 mg/ml) + 0.5 ml F6P (10 mg/ml) + 0.25 ml 1M $MgCl_2$ + 75 µl G6PD (100 IU/ml) + 0.675 ml distilled water + 1 ml MTT (5 mg/ml) + 1 ml PMS (1 mg/ml) + 12.5 ml agar.

LDH - 10 ml 0.05M Tris/HCl, pH 8.0 + 80 mg lactic acid (Li salt) + 0.5 ml NAD (10 mg/ml) + 0.5 ml MTT (5 mg/ml) + 0.5 ml PMS (1 mg/ml) + 10 ml agar.

ALD - 12.5 ml 0.1M Tris/HCl, pH 8.0 + 80 mg F16DP + 1 ml NAD (10 mg/ml) + 3 ml NaArs (10 mg/ml) + 25 µl G3PDH (1300 IU/ml) + 1 ml MTT (5 mg/ml) + 1 ml PMS (1 mg/ml) + 12.5 ml agar.

MDH - 2.25 ml distilled water + 5.35 ml 0.3M Tris/HCl, pH 7.4 + 3.5 ml 1M malate + 1.8 ml NAD (10 mg/ml) + 1 ml PMS (1 mg/ml) + 1 ml MTT (5 mg/ml) + 10 ml agar.

HK - 6.65 ml 0.3M Tris/HCl, pH 7.4 + 2 ml glucose (100 mM) + 2 ml ATP (10 mM) + 0.375 ml NADP (10 mg/ml) + 25 µl G6PD (100 IU/ml) + 0.1 ml 1M $MgCl_2$ + 0.4 ml distilled water + 1 ml MTT (5 mg/ml) + 1 ml PMS (1 mg/ml) + 10 ml agar.

Substrate for UV-Spectrophotometry

PGM - 2.5 ml Tris/HCl 0.1M, pH 7.4 + 0.1 ml EDTA (10 mg/ml) + 0.05 ml $MgCl_2$, 0.1M + 0.05 ml NADP (10 mg/ml) + 0.01 ml G6PD (100 IU/ml) + 0.25 ml G1P/G1,6DP (20 mg/ml).

Abbreviations:- F16DP: Fructose-1,6-diphosphate; F6P: Fructose-6-phosphate; G1P: Glucose-1-phosphate; G1,6DP: Glucose-1,6-diphosphate; G3PDH: Glucose-3-phosphate dehydrogenase; G6PD: glucose-6-phosphate dehydrogenase; NAD: Nicotinamide adenine dinucleotide; NADP: Nicotinamide adenine dinucleotide phosphate.

APPENDIX FOR CHAPTER SEVEN

Ehrlich's Haematoxylin

Solution A - 2 g haematoxylin + 100 ml absolute ethanol

Solution B - 100 ml glycerol + 100 ml distilled water
+ 10 ml glacial acetic acid
+ 3 g aluminium potassium sulphate

The two solutions were mixed and allowed to ripen (unstoppered in bright daylight) for several weeks.

3% Glutataldehyde in 0.1M Cacodylate

20 ml distilled water + 15 ml 0.2M cacodylate + 5 ml 25% glutaraldehyde + 0.5 ml 1% $CaCl_2$ (prepared fresh)

Osmium Solution

13.4 ml distilled water + 6.6 ml 0.2M cacodylate + 0.2 g OsO_4 + 0.2 ml 1% $CaCl_2$

Araldite (Extra Hard)

80 ml resin (CY212) + 100 ml hardener (D.O.S.A.) + 2 ml plastizer + 4 ml accelerator. They were mixed with stirrer for 20 min and then degassed for no longer than 15-20 min.

Barbitone Buffered Saline, pH 7.6, 0.15M

Stock solution A - 85 g NaCl + 3.75 g sodium diethylbarbiturate in 1400 ml distilled water

Stock solution B - 5.75 g diethylbarbituric acid in 500 ml hot distilled water

Stock solution C - 20.3 g $MgCl_2$(2.0M) dissolved in 50 ml distilled water + 30 ml 1.0M $CaCl_2$. Adjust to 100 ml with distilled water.

Solutions A and B were mixed and cooled to room temperature. 5 ml solution C were added and the final volume adjusted to 2 litres with distilled water. This was 5 x in concentration.

0.2M Sodium Citrate Buffer, pH 3.2

0.2M sodium citrate was mixed with 0.2M citric acid to obtain pH 3.2, with a pH meter.

0.1M Phosphate Buffer, pH 7.0

0.1M Na_2HPO_4 was mixed with 0.1M NaH_2PO_4 to obtain pH 7.0, measured by a pH meter.

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