The Possible Antigenic Sources of *Brugia pahangi*

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Abstract : **The antigenic sources of adult and the third larval (L3) stages of *Brugia pahangi* were detected by indirect immunofluorescent technique. Six panels of antisera were used, including human antisera against *Brugia malayi* and *Wuchereria bancrofti*, cat antisera against *B.malayi* and *B.pahangi* and jird antisera against *B.malayi* and *B.pahangi* as primary antibodies. All antisera gave the same results, although four of the six were not infected by *B.pahangi*. This indicates non-species specificity, and that *B.pahangi*, *B.malayi* and *W.bancrofti* must share most of the common antigenic molecules. All antisera reacted well with the surface of L3 *B.pahangi* in the whole mount preparation. This indicates non-stage specificity as well as non-species specificity. The most intense fluorescence was located at the epicuticle, the basal lamina lining the body wall, the gut and the reproductive tract, the egg shell *in utero* and the sperm. The hypodermis, the muscle cells, the cuticle beneath the epicuticle, the epithelial cells of the gut and the reproductive tract showed moderate fluorescence. The least fluorescence was observed in the egg interior.**

Key words **:** Filaria, *Brugia pahangi*, *Brugia malayi*, *Wuchereria bancrofti*

เรื่องย่อ : **แหล่งแอนติเจนที่น่าจะเป็นไปได้ของ *Brugia pahangi***

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ได้ศึกษาแหล่งแอนติเจนในพยาธิฟิลาเรีย *Brugia pahangi* ระยะตัวเต็มวัยและตัวอ่อนระยะติดต่อ รู้แสดงด้วยวิธี indirect immunofluorescence โดยใช้แอนติซีรัม 6 ชนิด ได้แก่ แอนติซีรัมของคนต่อ *Brugia malayi* และ *Wuchereria bancrofti* แอนติซีรัมของแมวต่อ *B.malayi* และ *B.pahangi* แอนติซีรัมของ jird ต่อ *B.malayi* และ *B.pahangi* เป็นแอนติบอดีปฐมภูมิ พบว่าการใช้แอนติซีรัมต่างชนิดกันไม่มีความแตกต่างกันในการติดสีฟลูออเรสเซนต์ในพยาธิทั้ง 2 ระยะ ซึ่งแสดงถึงความไม่จำเพาะต่อสปีชีส์ และระยะของการเจริญเติบโต สีฟลูออเรสเซนต์เรืองแสงมากที่สุดที่บริเวณขอบนอกสุดของผนังลำตัว, basal lamina ที่บุผนังตัว, basal lamina ของทางเดินอาหารและระบบสืบพันธุ์ รวมทั้งเปลือกไข่ในมดลูกของเพศเมียและอสุจิในเพศผู้ ส่วนที่เรืองแสงปานกลางได้แก่ hypodermis เซลล์กล้ามเนื้อ ซัยโตพลาสมของเซลล์บุทางเดินอาหารและอวัยวะสืบพันธุ์ ส่วนที่เรืองแสงน้อยที่สุดคือตัวอ่อนภายในไข่

INTRODUCTION

Filariasis is a group of human and animal diseases caused by arthropod-borne nematode parasites of the order Filariidea, commonly called "filariae". Lymphatic filariasis in humans is caused by developing and adult forms of filarial parasites presented in the lymphatic system. The infection is most common in subtropical and tropical regions of the world, but there are no reliable reports on the actual number of people infected. In Thailand, *Wuchereria bancrofti* is found in the western part of the country along the border of Myanmar, while the endemic area of *Brugia malayi* is in the south. It has been reported that the programme to control filariasis in southern Thailand has been successful as the filariasis infection rate was alleged to have decreased. For example, in Chumporn province infection rate decreased from 14.1% in 1964 to 0.30% in 1981.[1] Unfortunately, this optimism was rather short-lived and reflected only the limited information available in 1981. From 1982-1985 according to reports by the Filariasis Division, Ministry of Public Health, including areas previously inaccessible to governmental health personnel, revealed new areas of high endemicity, ranging from 1.16% to 4.6% infection rates. As is often the case, epidemiological data reflect the extent and limitation of the survey carried out and usually represents an underestimation of the problem. In Thailand, this underestimation is also true due to underreporting of the disease, the relative limitation of personnel and the unavailability of proper diagnostic tools used in field surveys. In the field, the diagnostic techniques depend primarily on the detection of microfilariae in the thick blood film. The fact that blood samples need to be taken at night when the microfilariae appear in the circulation is the major hindrance in the detection and in the control programme.

Attempts at serological testing for epidemiological monitoring have met with problems related to specificity and sensitivity since available tests thus far have depended on the use of crude antigen preparation. Furthermore, the available tests are aimed at antibody detection, and this is problematic because the antibodies usually appear late and do not allow detection of early (premicrofilariaemic stage) infection. Chemotherapy can be simpler, more effective and interrupts the transmission. Improved immunodiagnostic tests, particularly those aimed at the detection of the early stages of parasite antigens with high specificity and sensitivity are thus required. Because of the complexity of the antigenic structure of various stages of the parasites previous attempts at immunodiagnostic testing have run into problems. Therefore, careful studies using modern techniques to dissect the antigenic structure of the filarial parasite are necessary.

Lymphatic filariasis in humans is caused by *W.bancrofti* and *B.malayi*. There is no satisfactory laboratory mammalian host for *W.bancrofti*. Rodents such as jirds, hamsters and rats can be infected in the lab with *Brugia* spp., however the immune responses can only be described as an artificial infection which is very different from the responses of humans to such parasites. *B.pahangi* occurs naturally as a parasite of cats, and adult form parasitizes the lymphatics and

microfilariae circulate in the blood.[2] Thus the *B.pahangi* infected cat is a good model for various laboratory studies for human filariasis. *B.pahangi*, a common filarial parasite of wild and domestic animals in South-east Asia[3-6] has been experimentally transmitted to humans.[6] Natural human infections have also been reported from Indonesia.[5] It is regrettable that the infections in eight persons reported to have *B.pahangi* microfilariaemia were not possible in animals. Since the animal infection with *B.pahangi* is easier to control and manage than the other two human filarial forms, it is imperative to prove whether *B.pahangi* is antigenically closely related to human filariae. The cross-reactivity of antigens, if proved to be substantial, could provide and convenient comparative means to produce antigens for immunodiagnosis as well as a vaccine. So the objectives of this study were to localize the antigenic sources in the adult and the third stage larvae (L3) of the filarial nematode, *Brugia pahangi*, and test the cross-reaction of these antigens by using the indirect immunofluorescence technique.

MATERIALS AND METHODS

The adult worms were recovered from the peritoneal cavities of jirds (*Meriones unguiculatus*), which had been previously infected by injecting the infective stage larvae into the peritoneal cavities 60 days earlier, by the method of McCall et al.[7] The jirds were sacrificed and the abdominal cavities were exposed by making a small incision line. The adult worms were collected by two pairs of tweezers and then washed several times in PBS.

The third stage larvae were obtained by dissecting *Aedes aegypti* (Liverpool strain) mosquitoes that had been fed on an infected cat 12 days previously.

In the localization of the antigenic sources of the adult worms by indirect immunofluorescene technique, the worms were fixed in 2% paraformaldehyde in 0.1 M sodium cacodylate buffer for 30 min at 4 °C. After washing with the same buffer, they were mounted in the -24 °C cryocut cabinet with the tissue tek O.C.T. embedding medium. The 6 mm sections were transferred onto slides which had been coated with 0.5% gelatin. After drying at room temperature, the sections were incubated with the primary antibodies, which included immune sera from human infected by *B. malayi* and *W. bancrofti*, cat immune sera infected by *B.malyai* and *B.pahangi* and jird immune sera infected by *B.malayi* and *B.pahangi*. The incubations were done in the moist chamber at 37 °C for 30 min. Following extensive washing with 0.1 M PBS, the sections were incubated with corresponding fluorescein-conjugated secondary antibodies at 37 °C for 30 min in the moist chamber. After washing three times with the same buffer, the sections were mounted with 9% buffer glycerol. The slides were examined under Olympus fluorescent microscope, using incident illumination.

The cuticular antigens of the L3 stage were studied by incubating the whole parasites (which had been fixed in 2% paraformaldehyde) in the primary antibodies described above for 30 min at 37 °C. After washing with 0.1 M PBS, they were incubated in the corresponding fluorescein-conjugated secondary antibodies for another 30 min. After washing, the parasites were transferred onto the gelatin coated slides and mounted in buffer glycerol. Examination of the cuticular antigens of L3 was also carried out under the same fluorescent microscope.

In order to clearly identify the tissues that show fluorescence in immunofluorescent assay (IFA), the light microscopic observation of semithin sections were done in parallel. The plastic sections of blue-green interference were collected on glass slides. After drying for one hour on warming plate at 60 °C, the sections were deplasticed by 1 : 1 sodium ethoxide and absolute alcohol for 15 min. The sections were then stained by routine hematoxylin and eosin (H&E), and observed under an Olympus light microscope.

RESULTS

1. Morphology of adult *B.pahangi* at light microscopic level

The H&E staining of semithin sections of adult worms was carried out in parallel with the IFA which was used as a guideline for immunostaining. The HE sections revealed the arrangement of several organs in the worm at the light microscopic level. As shown in Figure 1, 2 and 3, the body wall of the adult

worm is composed of the cuticle, lined by the hypodermis and somatic musculature. The smooth cuticle is about 1-2 μm thick, with the outermost layer appearing most delicate and basophilic. The hypodermis is thickened to form four hypodermal cords which divide the somatic musculature into four quadrants. The lateral cords are the most prominent except when compressed by gravid uteri (Figure 3 A-D). Each occupies about one-fifth of the body circumference and appears basophilic, granular or vacuolated. The somatic musculature appears as large dorsal and ventral bundles, where the dorsal and ventral cords are small. An extensive system of basal laminae segregates all structures from the pseudocoel which is occupied by the gut and reproductive tract. The gravid uterus contains several stages of developing microfilariae within the uterine lumen. The uterine wall has two distinct layers : the thin epithelial cells bound by a thick basal lamina and layers of visceral muscle cells. The testis (Figure 3 E, F) is composed of a thin-wall tube occupied by closely packed immature spermatocytes. The seminal vesicle (Figure 1, 3G) wall is not uniform in thickness and appears distinctly basophilic. Sperm appears as dotlike basophilic granules within the lumen (Figure 3).

2. Detection of the tissue sources of antigens in adult *B.pahangi*

The indirect immunofluorescence technique was carried out on 50 samples of both sexes for one type of primary antibody. The sections showed satisfactory fluorescence at 1:2 to 1:4 dilution of primary antibodies together with 1:20 dilution of the fluorescein-conjugated secondary antibody. Nonspecific fluorescence appeared as pale green while the specific one as bright green. The intensities of the fluorescence in cuticles and internal organs when stained with different antibodies were compared and the result shown in Table 1.

The cryostat sections processed by IFA were compared to H&E sections for identification of structural organization and approximation of the thickness of various layers, and the basal laminae. The fluorescence was present on the cuticle, hypodermis, somatic musculature, the gut wall, the uterine wall, the egg shell and the male reproductive tract. The most intense fluorescence appeared as bright green zones and was confined along the basal lamina lining the body wall (Figure 4,5,6), the basal lamina and the luminal aspect of gut epithelium (Figure 4 A,B ; Figure 5A,B,D), the basal lamina of the testis and seminal vesicle (Figure 5 D,E) and the basal lamina and luminal aspect of the uterine epithelium. The continuous and intensely green lines appeared on the egg shells enveloping the developing microfilariae *in utero* (Figure 4A,B ; Figure 5A,B ; Figure 6A,B). In some preparations (Figure 4D,E ; Figure 5D,E ; Figure 6E), the outermost layer of the cuticle also showed an intense bright green line.

The hypodermis and the musculature showed uniformly homogenous fluorescence with moderate intensity. Microfilariae within the bright line of the egg shell and the gut content showed moderately to light fluorescence, while the content of the male reproductive tract showed varying degrees of fluorescence. The highest intensity appeared at the spermatogonia within the testis (Figure 6E) and the sperms within the seminal vesicle (Figure 4D,E ; Figure 5D).

3. Detection of the cuticular antigens of L3 *B.pahangi*

The whole mount of L3 was processed by IFA, using the same dilution of primary antibodies as indicated above. The intense fluorescence was along the entire cuticle of L3 which appeared as a continuous bright green line in all samples and in all kinds of antibodies (Figure 7, A-F). In the control sections, using PBS instead of the primary antibody, there was no fluorescence (Figure 7G).

DISCUSSION

Tissue sources of adult filarial antigens

The immunofluorescence method was selected to localize the tissue sources of antigen in cryocut sections of adult *B. pahangi*. The morphological details which could not be easily observed in the cryocut sections were compared with the H&E stained specimens of semithin sections of the worms. Six types of antisera were used as primary antibodies; they are cat and jird antisera against *B.pahangi* and *B.malayi*, and human antisera against *W.bancrofti* and *B.malayi*. All antisera reacted in exactly the same manner with *B.pahangi* cryocut sections (Table 1).

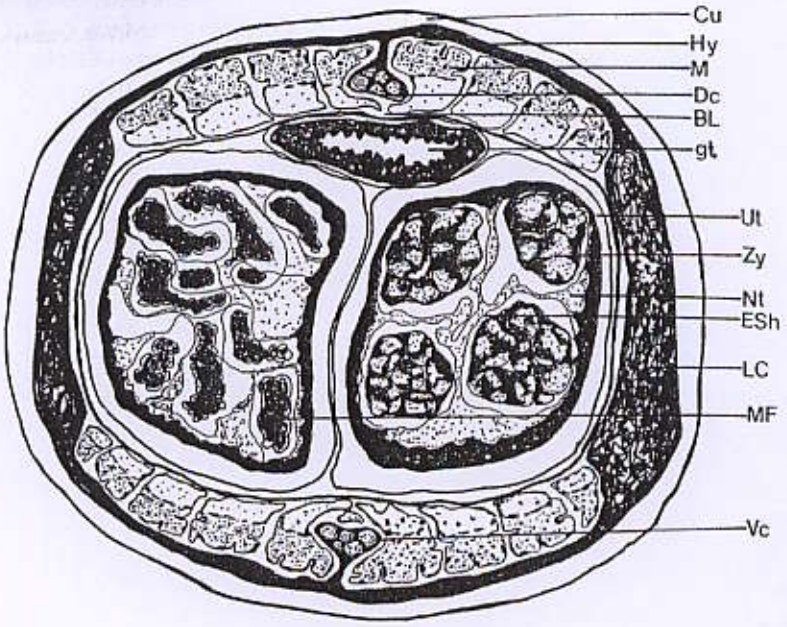

Figure 1. A schematic drawing of the midtransverse section of an adult female of *B. pahangi* showing the cuticle (Cu) covering the hypodermis (Hy) and somatic muscle (M) ; these components of the body wall were separated from the pseudocoel by a continuous layer of basal lamina (BL). The hypodermis is thickened at four hypodermal cords : two broad lateral cords (LC), one dorsal (Dc) and one ventral cord (Vc). The body cavity or pseudocoel is occupied by the uterus (Ut) and the gut (gt) which are also surrounded by their own basal laminae. Depending on the level of the section, the uterus is filled with developing microfilariae (MF) or the zygotes (Zy) which are covered by the egg shell (ESh). These developing stages are bathed in the uterine fluid which is colloidal in nature and may contain nutritive material (Nt).

This suggests the highly common nature that *B.pahangi* antigens share with *B.malayi* and *W.bancrofti*. The sources of antigens in adult *B.pahangi* were identified in several tissues of the cryocut sections. The strongest fluorescence which appeared as a bright green line, indicated by +++ in Table 1, is confined to the epicuticle, the basal lamina lining the body wall, the basal laminae of the gut, uterus and male reproductive tract, the egg shell and sperm.The moderately intense labeling, indicated by ++ in Table 1, appeared as homogenous green zones in the hypodermis, the cuticle beneath the epicuticle, the muscle cells, the epithelial cells of the gut, reproductive tract and uterus. The least intense labeling, indicated by + in Table 1, was confined to the microfilariae *in utero* and the egg interior. The contents of the gut and pseudocoelomic cavity were not labeled.

The cuticular antigen of L3 *B.pahangi*

Using six similar categories of antisera with the whole mount of L3 *B.pahangi*, the fluorescent label along the entire surface of L3 showed no difference among all antisera being used, indicating that all the antisera were neither species nor stage specific, at least among the adult and the L3 stages. The cross-reactivity of antisera among different species of filarial worms further suggests that the three parasites may share common antigenic determinants among antigens from each positively labeled tissue. *B.pahangi*, an animal parasite, is closely related to human lymphatic filariae. It has been

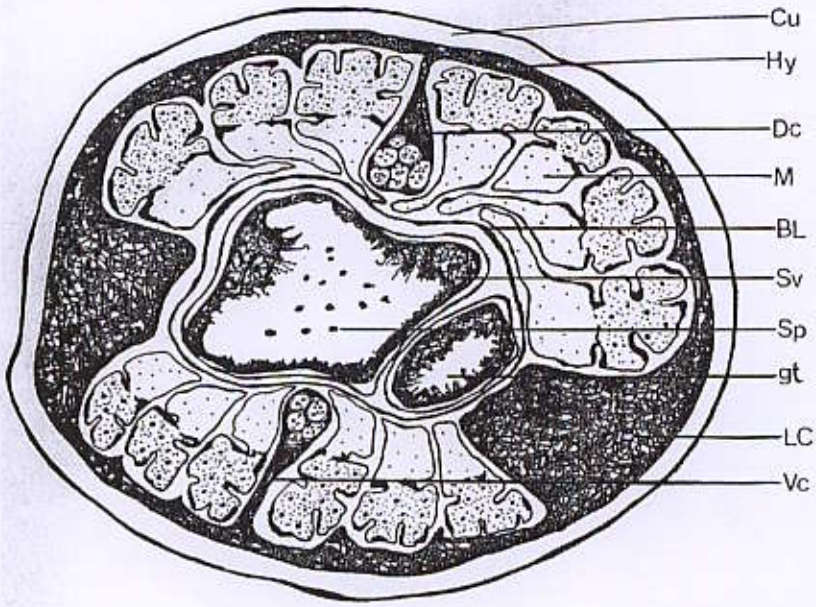

Figure 2. A schematic drawing of the midtransverse section of an adult male *B. pahangi*, the body wall consists of similar components as in the female shown in Fig. 6. The pseudocoel is, instead, occupied by the male genital tract comprising the seminal vesicle (Sv) which is filled with sperms (Sp) and the gut (gt). Each of these two organs is surrounded and therefore separated from the pseudocoel by its own basal lamina.

previously illustrated to have antigenic homology with human *Brugia* species.[8] Meizel et al[9] studied the cross-reactive surface antigens on three stages of *B.malayi*, *B.pahangi* and *B.timori* by radioiodination and immunoprecipitation. They found that the surface antigens had characteristic patterns in each stage, and the adult and L3 had relatively more complex patterns than the microfilariae. Furthermore, the surface antigens of the three stages of these three species were all closely homologous. Immunoprecipitation revealed that antibodies raised in mice against one stage or species reacted with surface antigens from other stages and species. They also showed the cross-reaction with stage-specific antisera which suggested that there must be shared epitopes on *Brugia* surface antigens from each stage. Moreover, Meizels[10] showed that the surface antigens of adult *B.pahangi* were also recognized by antibodies from patients with *W.bancrofti* and *Loa loa*. Our immunofluorescence study clearly confirmed the commonness of antigens from various tissue sources among *B.malayi*, *B.pahangi* and *W.bancrofti*. At molecular level, only few defined antigens of selected interest, such as the surface protein of MW 29,000 appeared to be recoginized in all developmental stages of the parasite. Another dominant cross-reacting antigenic determinant was phosphorylcholine as reported by Gualzata et al.[11] This haptenic group was present in many different components, including a protoglycan-like polymer found in the circulation of *Onchocerca-Wuchereria* - and *Brugia*- infected people.[12] This hapten was so immunodominant among filarial antigens that it was difficult to resist the conclusion that an antiphosphorylcholine antibody could be protective to the host.[12-15]

Because of the shared antigenicity among *B.pahangi* and human filarial species, it seems possible that the antigens released by these parasites

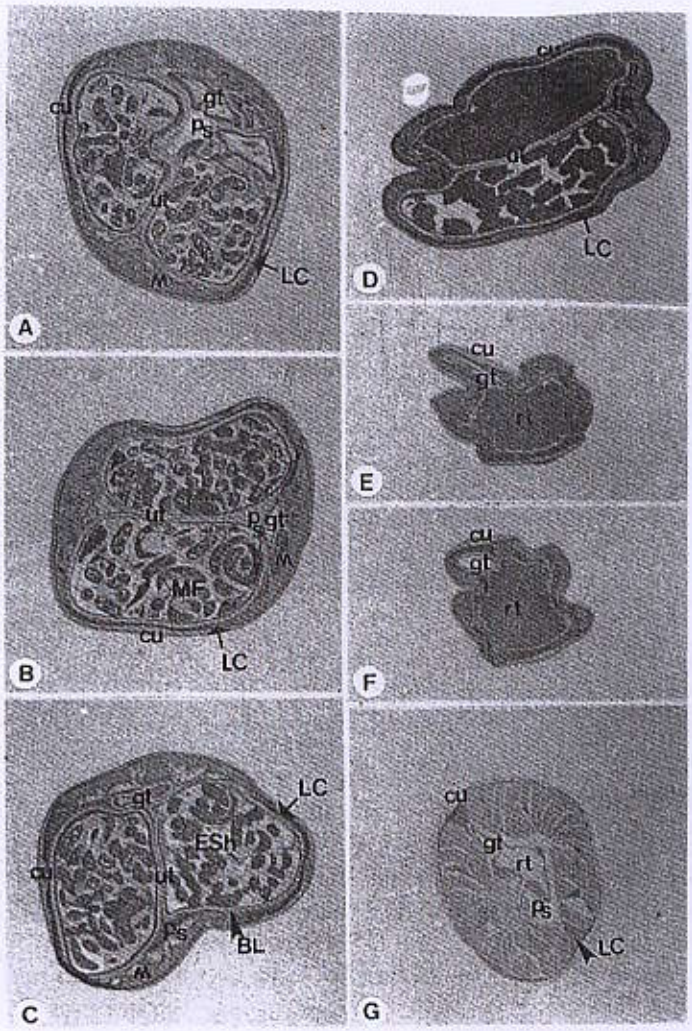

Figure 3. Hematoxylin-Eosin staining of semithin sections of adult *B.pahangi* : A-D are females, E-G are males. A-G reveals the body wall of the adult worms consisting of the cuticle (CU), lined by hypodermis and somatic musculature (M). The cuticle is about 1-2 μm thick, smooth throughout with the outermost layer appearing more basophilic. The hypodermis is thickened to form four hypodermal cords and divides the muscle into four quadrants. The lateral cord (LC) is prominent, except when compressed by gravid uteri (Ut). The somatic muscle (M) occupies about one-fifth of the body circumference and appears basophilic. The dorsal and ventral cords are not prominent in H&E sections. An extensive system of basal laminae (BL) segregates all structures from the pseudocoel (Ps). X 600

A-D The pseudocoel (Ps) of female worm is occupied by the uteri (Ut) and gut (gt). The gravid uteri contain several stages of developing microfilariae (MF) in the lumen, each surrounded by a loosely stretched egg shell (ESh).

E-F Germinal testis (rt = reproductive tract) consisting of a thin wall occupied by closely packed immature spermatocytes.

G Seminal vesicle wall is not uniform in thickness and appears distinctly basophilic.

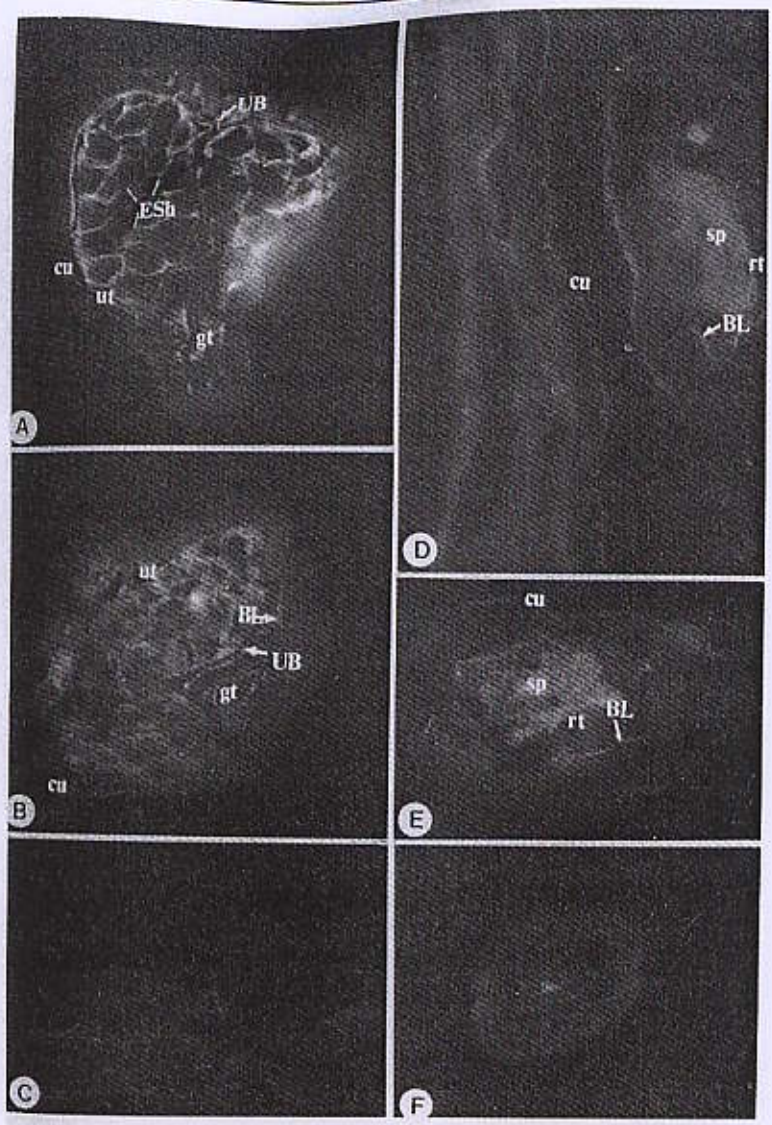

Figure 4. Indirect immunofluorescence assay to localize antigen sources in the cryocut sections of female (A-C) and male (D-F) *B.pahangi*, using human antisera against *W.bancrofti* (A,D) and against *B.malayi* (B,E) as primary antibodies. C = control of female, F = control of male. x 600.

A, B Cross-sections of a female worm showing equal fluorescence despite using different types of antibody. The fluorescence was intense along the outermost layer of the cuticle, the basal lamina of the body wall, the uterine and gut basal lamina and the egg shell covering the *in utero* microfilariae. The labeling was moderate at the deeper part of the cuticle, hypodermis, musculature, epithelial cells of the uterus and gut. Weak fluorescence was confined to the egg interior.

D, E Cross-sections of a male worm showing equal fluorescence on the body wall as seen in the female specimens. The male reproductive tract luminal content and spermatocytes were also intensely fluorescent.

C, F In the control experiment using PBS instead of primary antibodies, there was no fluorescence except a weak autofluorescence in the gut.

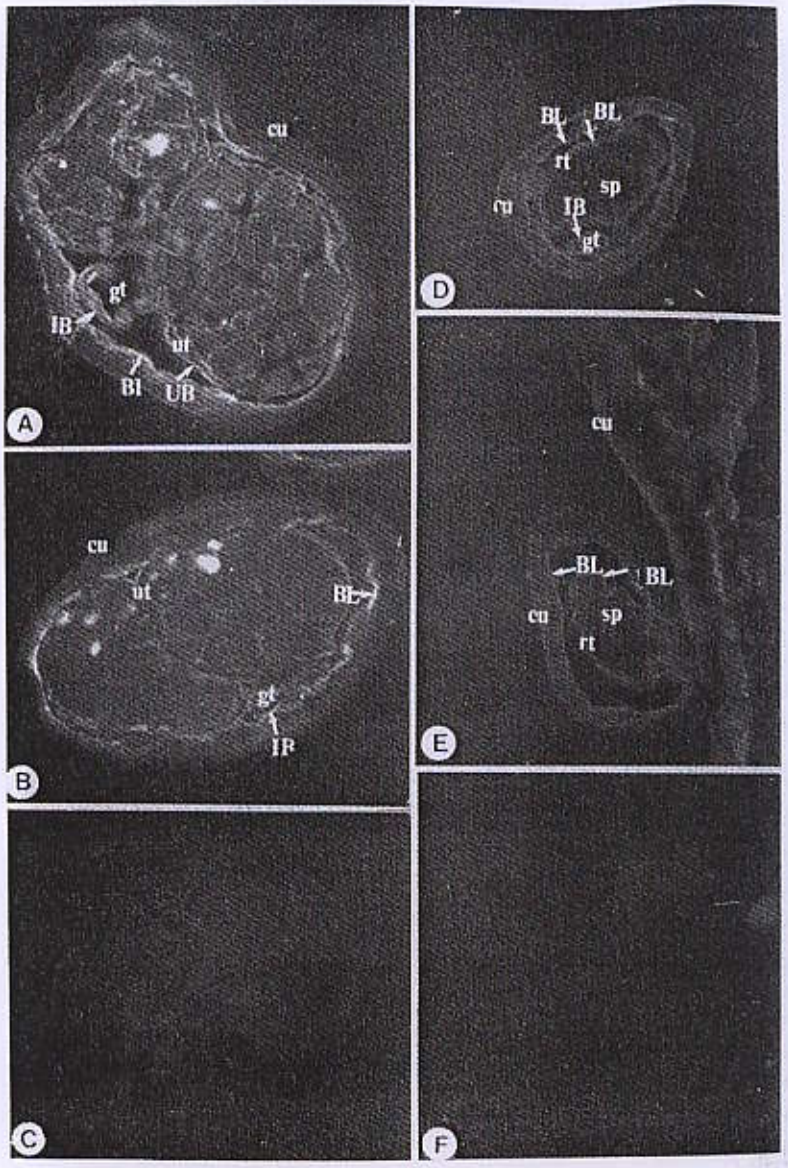

Figure 5. Indirect immunofluorescence assay to localize antigen sources in cryocut sections of female (A-C) and male (D-F) *B.pahangi* by using cat antisera against *B.malayi* (A,D) and against *B.pahangi* (B,E) as primary antibodies. C = control of female, F = control of male tissue. x 600.

A,B,D,E Cross-section of the adult worm of both sexes showed equally strong fluorescence at the same sites as indicated in Figure 2, although different antisera were used as primary antibodies.

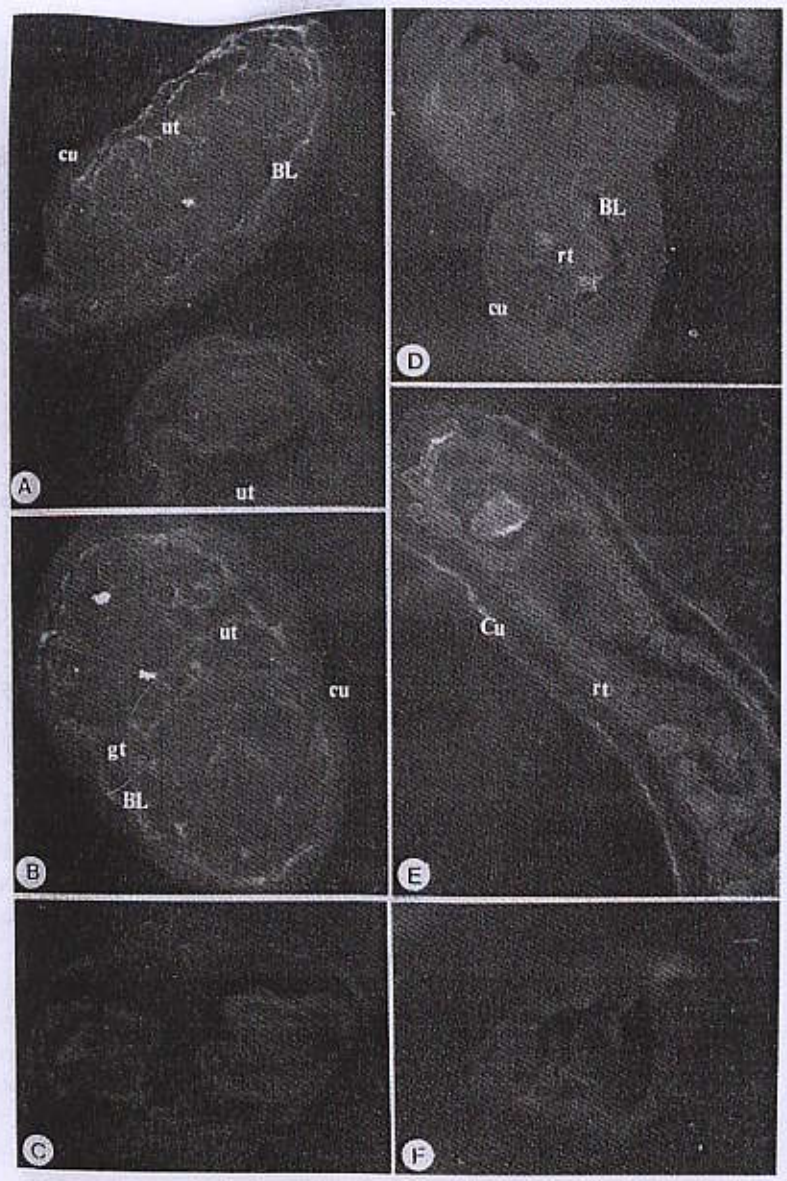

Figure 6. Indirect immunofluorescence assay to localize antigenic sources in cryocut sections of female (A-C) and male (D-F) *B.pahangi* by using jird antisera against *B.malayi* (A,D) and against *B.pahangi* (B,E) as primary antibodies. x 600.

A,B,D,E Different antisera showed equally strong fluorescence at the same sites in the adult worm tissues as shown in Figure 2,3.

C,F Control sections stained with PBS instead of primary antibodies showed no fluorescence.

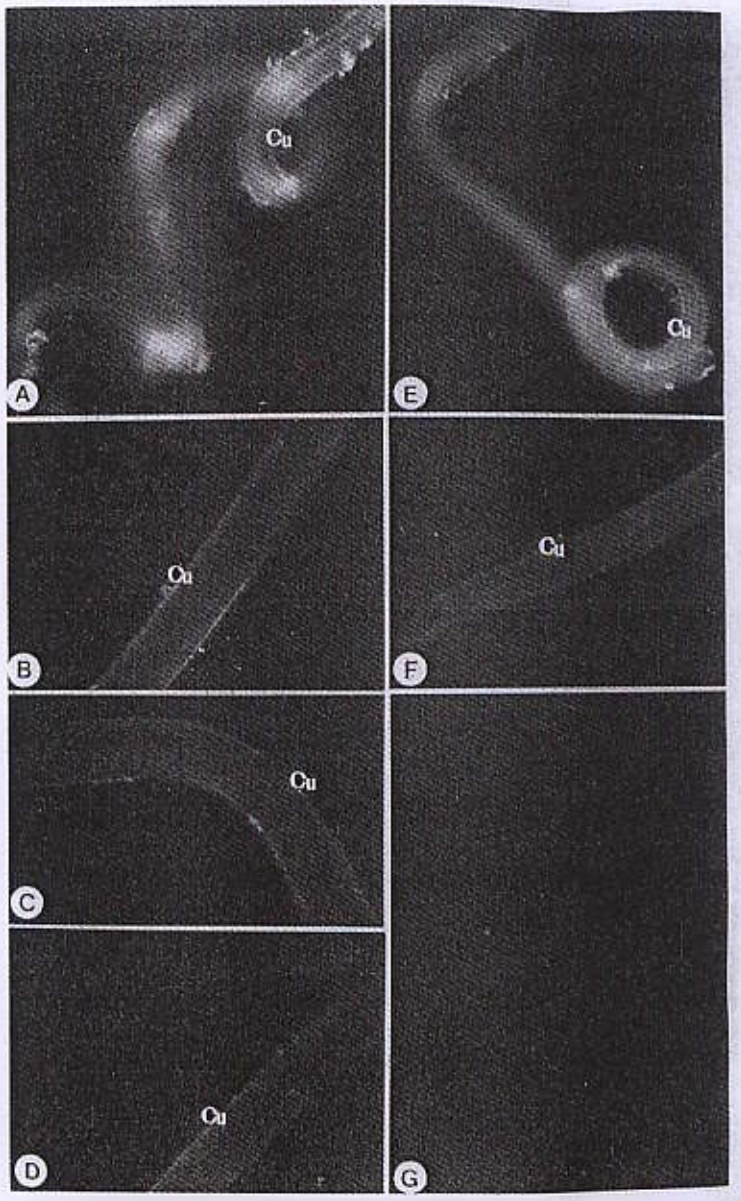

Figure 7. Indirect immunofluoresecence assay to localize the cuticular antigens on the whole mount of L3 *B.pahangi*, using six categories of antisera as primary antibodies. x 600.

A Human antiserum against *W.bancrofti*.
B Human antiserum against *B.malayi*.
C Cat antiserum against *B.malayi*.
D Cat antiserum against *B.pahangi*.
E Jird antiserum against *B.malayi*.
F Jird antiserum against *B.pahangi*.
The fluorescence was equal along the entire cuticle of L3 *B.pahangi*, despite the fact that different types of antisera were being employed.
G Control parasites stained with PBS instead of primary antibodies showed no fluorescence.

Table 1. Comparison of the fluorescence intensities in various tissues of adult *B.pahangi* as detected by indirect immunofluorescence assay.

TISSUE	Human antisera		Cat antisera		Jird antisera	
	B.m	*W.b*	*B.m*	*B.p*	*B.m*	*B.p*
BODY WALL						
Cuticle	++	++	++	++	++	++
Epicuticle	+++	+++	+++	+++	+++	+++
Hypodermis	++	++	++	++	++	++
Muscle	++	++	++	++	++	++
Basal lamina	+++	+++	+++	+++	+++	+++
GUT						
Epithelial cell	++	++	++	++	++	++
Basal lamina	+++	+++	+++	+++	+++	+++
Intestinal content	++	++	++	++	++	++
UTERUS						
Epithelial cell	++	++	++	++	++	++
Basal lamina	+++	+++	+++	+++	+++	+++
Egg shell	+++	+++	+++	+++	+++	+++
Egg interior	+	+	+	+	+	+
TESTIS AND SEMINAL VESICLE						
Epithelial cell	++	++	++	++	++	++
Basal lamina	+++	+++	+++	+++	+++	+++
Sperm	+++	+++	+++	+++	+++	+++

Bm = *Brugia malayi*
Wb = *Wuchereria bancrofti*
Bp = *Brugia pahangi*

+++ = Intense fluorescence
++ = Moderate fluorescence
+ = Light fluorescence

may be mostly similar and originated from the same tissue sources. Therefore, the research on antigens of immunodiagnostic potential that has been carried out in *B.pahangi* may be readily applied to other human filarial species. If this is the case, the work to define useful antigens for immunodiagnosis and for the development of vaccine can be greatly simplified since *B.pahangi* can be kept in cycle more easily than human filarial species. Further work at the molecular level is obviously needed to identify the antigens from various tissue sources before any realistic application can be contemplated. The details of all the structures of the antigenic sources of all stages of *B.pahangi* should also be worked out at the transmission electron microscopic level.

CONCLUSION

The indirect immunofluorescent technique was employed to identify and localize tissue sources of antigens in adult and L3 of *Brugia pahangi*. Six types of antiserum were used as primary antibodies, i.e., cat and jird antisera against *B.pahangi* and *B.malayi*, and human antisera against *B.malayi* and *W.bancrofti*. All antisera reacted in exactly the same manner as *B.malayi* cryocut tissues. This indicated the strong cross-reaction of the antisera against different filarial species and the common antigens shared among these three species of filarial worms. The antigenic sources of adult *B.pahangi* were composed of several types of tissue. The most intense labeling was confined to the epicuticle, the basal

lamina lining the body wall, the basal laminae covering the gut and reproductive tracts, the egg shell *in utero* and the sperm surface. The moderate fluorescence occurred in the hypodermis, the cuticle, the muscle cells, the epithelial cells lining the gut, the uterus and male reproductive tract. The least fluorescence labeling was seen at the egg interior.

When the same set of antisera was used against the whole mount of L3 *B.pahangi*, the strong fluorescence occurred along the entire surface of L3. There was no difference among the different types of antibodies being used. The results again indicated non-species specificity as well as non-stage specificity of the antisera. The commonness of antigens was shared among all filarial species.

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