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Research Note

A Method for Isolation of Exsheathed Microfilariae of *Brugia pahangi*

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Exsheathment is a prerequisite for normal development of sheathed microfilariae of filarial nematodes such as *Wuchereria bancrofti*, *Brugia malayi* and *B. pahangi*. The removal of the microfilarial sheath is, therefore, an obligatory first step in the cultivation *in vitro* of such sheathed microfilariae (Devaney and Howells, 1979). Isolation of microfilariae from blood and removal of their sheaths are also necessary for studying the role of the microfilarial sheath in mosquitoes (Sutherland *et al.*, 1984). Artificial exsheathment of such sheathed microfilariae takes place on agar (Aoki, 1971), in phosphate-free Earle's balanced solution (Devaney and Howells, 1979), or in Hank's balanced salt solution (HBSS) containing endopeptidase or extract of papaya (Devaney and Howells, 1979; Srivastava, 1985). For isolation of microfilariae from blood, on the other hand, an excellent method using agarose gel has been reported by Nogami *et al.* (1982). In this paper, we improved the method for collection of exsheathed microfilariae of *B. pahangi* from small blood samples. In the method described here exsheathed and/or sheathed microfilariae separate from hemolysed blood cells through agarose gel in a column and concentrate in medium in the lower part of the same column.

The separating column for isolation of microfilariae was prepared as follows. The column with a constriction as shown in Fig. 1 was made from glass tube (8.4mm in diameter and 120mm in length). The lower tip of the column was inserted into vinyl tubing which was pinched with forceps. The lower part of the column was filled with autoclavable RPMI 1640 medium (Nissui pharmaceutical, Tokyo) supplemented with

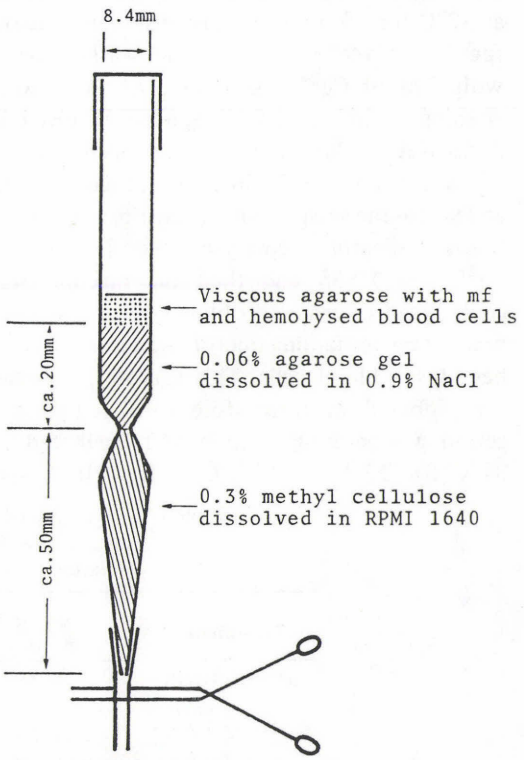

Fig. 1 Separating column

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4000 cP methy cellulose (0.3 g/100 ml). This solution had been held at 5°C for more than 24 hr after autoclaving in order to uniformly dissolve methyl cellulose. Methyl cellulose was added in order to prevent strong convection currents in the column. Hot 0.06% agarose (Sigma, Type II Medium EEO) dissolved in 0.9% NaCl solution was then poured into the middle part of the column where it quickly gelled.

0.2 ml of heparinized blood which had been collected from *Meriones unguiculatus* heavily infected with *B. pahangi* was mixed with 5ml of phosphate buffered NH_4Cl solution (Na_2HPO_4, 1.2 g; KH_2PO_4, 0.7 g; NH_4Cl, 8.3 g; Distilled water, 1000 ml) in order to cause hemolysis. The suspension was centrifuged at 1300 rpm for 5 min and the precipitate was washed twice with 5ml of Ca^{2+} free HBSS by repeated centrifugation under the same conditions. Then the precipitate was mixed with 5ml of Ca^{2+} free HBSS containing Papain 1:350 (Wako chemicals, Osaka) and incubated at 37°C for 30 min. The suspension was centrifuged and the precipitate was washed twice with 5ml of Ca^{2+} free HBSS. At the second washing, 0.5ml of 0.06% agarose dissolved in 0.9% NaCl solution which had been held at 5°C for more than 24 hr after autoclaving was added to the suspension consisting of microfilariae, sheaths, hemolysed blood cells and Ca^{2+} free HBSS and then the mixture was centrifuged in order to obtain a viscous agarose precipitate containing microfilariae, sheaths and hemolysed blood cells. This agarose precipitae was pippeted on to the aforementioned agarose gel in a separating column and incubated at 37°C for 12 hr. Buffer, Ca^{2+} free HBSS and enzyme solution used in this procedure were sterilized by passage through 0.45 μm pore size Sartorius membrane filters.

About 10% of the total microfilariae were lost when they were transferred into the column. About 85% of the total microfilariae treated with low doses of Papain passed through the agarose gel to the RPMI 1640. However, in the case of microfilariae treated with 2% Papain only 5.6% were recovered. Percentages of exsheathment in microfilariae recovered from RPMI 1640 were 87.5 and 100% when sheathed microfilariae had been treated with 1 and 2% Papain, respectively (Table 1).

Artificially exsheathed microfilariae which had been isolated after 1% Papain treatment were resuspended in heparinized rat blood and fed to *Aedes aegypti* (Liverpool strain) orally by the method of Chuang *et al.* (1979). Sheathed microfilariae isolated through the column were also fed to *Ae. aegypti* as a control. Mosquitoes were dissected at 0 and 10 days after inoculation and the number of microfilariae ingested or number of infective larvae was counted. The recovery of infective larvae as a percentage of the number of ingested microfilariae was 23.5 and 30.9% when mosquitoes had ingested microfilariae treated with 1% Papain or sheathed microfilariae, respectively (Table 2). This shows that 1% Papain treatment is not seriously harmful to filarial development and our method for collection of exsheathed microfilariae of *B. pahangi* can therefore be used for studying filarial development *in vitro* and the role of the microfilarial sheath in mosquitoes. This method, however, cannot be applied to *B. malayi* (Che-ju strain) because many microfilariae remained in agarose gel

Table 1 Recovery and exsheathment of *Brugia pahangi* microfilariae following Papain treatment and isolation

Treatment		No. of mf used	No. of mf recovered (%)	Exsheathment (%) in recovered mf
Ca^{2+} free HBSS		8550	7400 (86.5%)	3.2%
Papain	0.5%	7240	6300 (87.0%)	3.8%
	1.0%	11800	9900 (83.9%)	87.5%
	2.0%	5720	320 (5.6%)	100%

Table 2 Recovery of infective larvae from *Aedes aegypti* which were fed with artificially exsheathed or sheathed microfilariae of *Brugia pahangi*

	Mean no. of mf ingested per mosquito (Average ± SD)	Mean no. of infective larvae recovered per mosquito (Average ± SD)	% recovery of infective larvae
Exsheathed mf*	16.2 ± 6.2 (10)	3.8 ± 2.5 (20)	23.5%
Sheathed mf	8.1 ± 2.7 (10)	2.5 ± 2.5 (20)	30.9%

*Exsheathed mf: mf isolated after 1% Papain treatment Parentheses show the number of mosquitoes dissected.

in the column even after 24 hrs' incubation (Ogura, unpublished observation).

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短　報

Brugia pahangi 脱鞘ミクロフィラリアの分離回収法

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酵素処理により脱鞘させた *Brugia pahangi* ミクロフィラリア（mf）を血液材料からカラムを通して分離する方法を考案した．分離カラムは下端から約50mm上方にくびれのある内径8.4 mm長さ 120 mmのガラス管で，くびれの部分までメチルセルロース（3 mg/mℓ）を溶かしたRPMI 1640 を入れ，くびれより上方約20mmまでの部分にアガロース（0.6 mg/mℓ）を溶かした0.9％NaCl を入れてゲル化させた．血液材料は高mf 血症を示すスナネズミの血液0.2 mℓで，NH_4Cl（8.3mg/mℓ）を溶かしたリン酸緩衝液と混合して溶血させた後に遠心した．得られた沈渣をCa^{2+} を除いたハンクス液（$-Ca^{2+}$HBSS）で2回洗浄した後にパパインを溶かした$-Ca^{2+}$HBSSに入れて37℃下で30分間反応させ，再び$-Ca^{2+}$HBSSで2回洗浄した．最終の洗浄の際に，アガロース（0.6mg/mℓ）を加熱して溶かした後5℃下で24時間以上保存した 0.9%NaCl 0.5mℓを加えて遠心し，mf・鞘および壊れた血球のまじったアガロース沈殿を得た．この沈殿を分離カラムのアガロースゲル上に添加した．37℃下で12時間放置後に分離カラム下層のRPMI 1640から mf が回収できた．1％パパインと反応させた後カラムを通して分離した mf と処理を加えないで分離した mf をそれぞれ *Aedes aegypti* に人工的に摂取させたところ，双方の *Ae. aegypti* から感染幼虫が得られた．1％パパインによる脱鞘誘起とその後のカラムによる mf 分離は*Brugia pahangi*の発育に著しい悪影響を与えないと思われる．