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Research Note

An Improved Method for Membrane Feeding of Malaria Parasites

KENYICHI YANO[1], ALBINA KOZLOWSKA AND KARL MARAMOROSCH

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This research note outlines an improved method to make anopheline mosquitoes engorge purified ookinetes in simple chemical solutions via membrane feeders with a phagostimulant instead of blood meal, and to increase infectivity of malaria parasites using lower feeding temperature at 30°C and 25°C, instead of 37°C. The *Plasmodium berghei* (strain ANKA) – *Anopheles stephensi* – golden hamster complex was maintained as described by Vanderberg *et al.*, (1968). For feeding, 40–60 female mosquitoes 3–7 days old, were collected from a stock colony into small mosquito cages, using disposable 350 ml plastic cups as shown in Figure 1. Water-jacketed membrane feeders (2.6 cm and 4.5 cm in diameter) (Rutledge *et al.*, 1964), fitted with a Baudruche membrane from the intestine of oxen (Long and Long, Belleville, NJ) were used for feeding *A. stephensi* at 21°C. Small membrane feeders (2.6 cm) were used for feeding ookinetes.

P. berghei ookinete cultures and purification were carried out by the method of Yano *et al.*, 1989. Feeding solutions were Dulbecco's phosphate buffered saline (Ca^{++} and Mg^{++} free) (PBS) and blood meal; heparinized hamster blood diluted two times with Ham's F-12 medium (GIBCO)or any other medium supplemented with 10% fetal bovine serum, because a small volume of whole blood easily evaporates during feeding, and is too sticky for mosquitoes to engorge. Purified ookinetes were washed with the feeding solution by centrifugation at 175 g for 5 min and resuspended in 0.5 to 1.5 ml of each feeding solu-

Department of Entomology and Economic Zoology, Cook College, Rutgers University, New Brunswick, New Jersey 08903

[1] Present address: Department of Protozoology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan
矢野健一（大阪大学微生物病研究所原虫学部門）

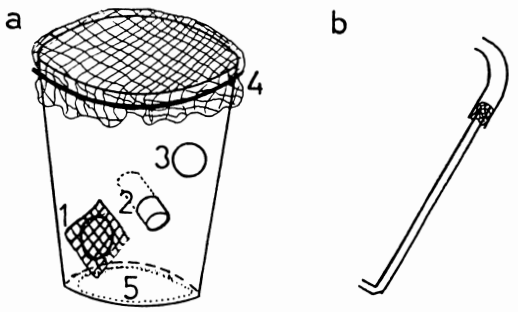

Fig. 1. a) A disposable plastic cup is cut three holes by heated glass tubes. No. 1 hole is a window and covered with nylon mesh (or nylon curtain) sticked on the plastic wall with chloroform solution of acryl plates. No. 2 hole is for dental cotton roll to give mosquitoes sugar solution. No. 3 hole is used for carrying in and out mosquitoes in a cup. The top of cup is sealed with nylon mesh (No. 4) fixed by rubber bands. Two sheets of filter papers (No. 5) is placed on the bottom of the cup to suck up excreted feeding solution by mosquitoes. It is recommended to put a piece of wooden chopsticks in the cup for resting place of mosquitoes.

b) An aspirator is made of a disposable 2 ml-plastic pippet which is cut about 20 cm in length. Its top (about 2 cm) is rectangularly bent and its bottom is sealed with nylon mesh fixed with a rubber tube for sucking. This aspirator is convenient to remove unfed mosquitoes via No. 3 hole and to collect mosquitoes for dissection.

tion, according to the number of ookinetes at the concentration of 1×10^6 ookinetes per ml. Membrane feeders were prewarmed at each set temperature in a 21°C-incubator. Mosquitoes in small cages were fed on ookinetes in solution for 15 min. Infected mosquitoes were maintained at 21°C with 12 hr light-period per day on 5% sucrose provided on dental cotton roll. From day 8–14 post-infection, the dissected midguts were examined with an inverted phase contrast microscope at 360× magnification for the presence of oocysts.

Sodium bicarbonate is a strong phagostimulant for anopheline mosquitoes (Galun *et al.*, 1985), and induced *A. stephensi* engorges even 600 mM of Na bicarbonate in PBS, 740 mOsm/kg (Table 1).

It was difficult to engorge mosquitoes on the *in vitro*-formed purified ookinetes through membrane feeders in such a small suspension volume as 0.5 to 1.0 ml. Even feeding ookinetes to *A. stephensi* in a blood meal of such a small volume was inadequate and the engorgement of ookinetes was unstable. Addition of Na bicarbonate (10 mM) to feeding solution, PBS and blood meal proved effective for increasing the feeding rate of *A. stephensi,* and improved the feeding rate 2.1 times in PBS (from 41.0% to 85.2%), and 1.6 times in blood meal (from 52.6% to 82.9%) (Table 2). It did not cause any significant changes

Table 1 Feeding rate of *A. stephensi* according to different concentration of Na bicarbonate in PBS*

Concentration† of $NaHCO_3$ (mM)	Average osmolarity (mOsm/kg)	% Feeding (No. of examined mosquitoes)	Average weight of fed mosquitoes (mg)‡
0	297	26.0 (336)	2.31
5	304	65.8 (158)	2.18
10	313	87.4 (111)	2.19
20	328	88.4 (346)	2.24
40	347	82.0 (150)	2.36
60	376	92.0 (113)	2.33
120	432	91.0 (167)	2.49
300	597	87.2 (109)	2.42
600	740	88.0 (50)	2.27

* Batches of 50-60 female mosquitoes were allowed to engorge for 30 minutes to acquire solution through a 4.5 cm-membrane feeder at 37°C.

† After adding $NaHCO_3$, the solution was adjusted to pH 7.3 by 1N NaOH.

‡ Average weight of 186 unfed mosquitoes is 1.62 mg.

Table 2 Effect of Na bicarbonate on the infectivity of cultured *P. berghei* ookinetes fed in PBS and blood meal to *A. stephensi**

Feeding solution	Mean of % feeding ± SD (No. of experiments)	% Infectivity† (No. examined)	No. of oocysts/ mosquito ± SD
PBS	41.0 ± 22.1 (7)	54.0 (87)	6.4 ± 6.9
PBS + $NaHCO_3$	85.2 ± 11.2 (9)	42.7 (150)	4.5 ± 6.4
Blood meal	52.6 ± 20.3 (9)	87.7 (106)	31.6 ± 35.1
Blood meal + $NaHCO_3$	82.9 ± 14.8 (3)	86.5 (37)	31.7 ± 34.0

* Batches of 40-50 female mosquitoes were allowed to engorge ookinetes in each feeding solution for 15 minutes at 37°C through a 2.6 cm-membrane feeder in a 21°C-incubator.

† Infected mosquitoes were dissected on 8-14 days post-infection and their midguts were examined for the presence of oocysts with a phase contrast microscope at 360× magnification.

in infectivity of purified ookinetes and their transformation to oocysts (Table 2).

The phagostimulant effect of Na bicarbonate was profound specially at lower temperatures. The feeding rate of blood meal supplemented with 10 mM $NaHCO_3$ resembled that without the phagostimulant at 37°C, but increased 1.3 times at 30°C and 2.5 times at 25°C (Table 3). Sodium bicarbonate was essential to make anopheline mosquitoes engorge PBS. *A. stephensi* engorged 3.9 times more PBS supplemented with 10 mM $NaHCO_3$ at 37°C, than did mosquitoes without the phagostimulant. At 30°C and at 25°C the increases were 4.9 and 3.5 times, respectively (Table 3).

Depending on the temperature of the feeding solution, the infectivity of ookinetes and numbers of oocysts in *A. stephensi* that engorged purified ookinetes in PBS with 10 mM of Na bicarbonate through membrane feeders varied from 43.2% and 2.6±2.0 at 37°C, 65.7% and 5.8±7.9 at 30°C to 85.7% and 5.4±5.2 at 25°C, respectively (Table 4). When the temperature of the feeding solution in membrane feeders was lower than the usual temperature of 37°C, infectivity at 30°C and 25°C improved but the feeding rates decreased. The oocyst index per mosquito was 2.1 at 30°C, the highest as compared to 0.8 at 37°C and 2.0 at 25°C, respectively (Table 4). It is recommended to use 30°C instead of the usual 37°C for membrane feeding of ookinetes. This result is explained by very sensitiveness of the sporogony of *P. berghei* to high temperature (Vanderberg and Yoeli, 1966; Yano *et al.*, 1989).

Table 3 Feeding rates of *A. stephensi* at 25°C, 30°C and 37°C (temperature of water-jacketed membrane feeders, 2.6 cm in diameter) in a 21°C-incubator*

Feeding solution	Temperature of feeders	Means of % feeding rate with and without $NaHCO_3$ (10 mM)	
		$NaHCO_3$ (−) (total mosquitoes used)	$NaHCO_3$ (+) (total mosquitoes used)
PBS	25	12.3± 9.3 (202)	43.1±14.8 (193)
	30	13.6± 5.0 (197)	66.2±20.9 (195)
	37	22.3±25.2 (198)	87.3± 9.2 (182)
Blood meal	25	16.8±12.7 (144)	41.6± 2.2 (144)
	30	53.4±15.1 (152)	68.8±13.9 (143)
	37	53.7±41.4 (151)	54.6±29.6 (149)

* 50 female mosquitoes in each experiment were allowed to engorge for 15 minutes.

Table 4 Infectivity of purified ookinetes in PBS supplemented with 10 mM Na bicarbonate and their transformation to oocysts in *A. stephensi* at 25°C, 30°C and 37°C (temperature of water-jacketed membrane feeders) in a 21°C-incubator*

Temperature	% Feeding (total No.)	% Infectivity (No. examined)	No. of oocysts/ mosquito ± SD	Oocyst index†
25	42.7 (96)	85.7 (35)	5.4±5.2	2.0
30	55.6 (99)	65.9 (44)	5.8±7.9	2.1
37	75.0 (96)	43.2 (37)	2.6±2.0	0.8

* Mosquitoes in batches of 50 females were allowed to engorge ookinetes, 1.5×10^6 per 1.5 ml of PBS with $NaHCO_3$ (10 mM), in a 2.6 cm-feeder for 15 minutes and dissected to examine for the presence of oocysts on 8-10 day post-infection.

† Oocyst index was calculated as the sum of % Feeding rate/100 × % Infectivity/100 × No. of oocysts per mosquito.

In conclusion, by using 10 mM Na bicarbonate as phagostimulant, better results were obtained by feeding purified ookinetes in PBS via membrane feeders at lower temperature, 25°C and 30°C than at 37°C in a 21°C-incubator. This procedure can be recommended for feeding malaria parasites via membrane-feeders because it is more convenient for standardizing experimental conditions of parasitized red blood cells than direct bites of mosquitoes on animals carrying gametocytes. The defined system of feeding purified ookinetes in simple chemical solution, PBS, assists qualitatively to a study on the environmental conditions of transformation from ookinetes to oocysts.

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