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Original article

Preservation of Wild Isolates of Human Malaria Parasites in Wet Ice and Adaptation Efficacy to In Vitro Culture

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Abstract: Wild isolates of malaria parasites were preserved in wet ice for 2–12 days and cultivated by a candle jar method. In four isolates of *Plasmodium falciparum* collected from Myanmar and preserved for 12 days, all failed to grow. In 31 isolates preserved for 5–10 days, nine were transformed to young gametocytes, but 22 isolates grew well. From Ranong, Thailand, nine isolates preserved for 7 days were examined, and six grew well. On the other hand, all of the 59 isolates collected from eastern Indonesian islands failed to establish as culture-adapted isolates, even most of them were preserved only for 2–3 days: 10 isolates stopped to grow, and 49 isolates were transformed to sexual stages by Day 10. These results indicated that a great difference in adaptation to in vitro culture may exist between wild isolates distributed in continental Southeast Asia and in eastern Indonesia and that gametocytogenesis might be easily switched on in Indonesian isolates. In wild isolates of *P. vivax*, *P. malariae* and *P. ovale* preserved for 2–9 days, ring forms or young trophozoites survived, but adaptation to in vitro culture failed. These results indicate that wild isolates can be preserved in wet ice for 9–10 days.
Key words: *Plasmodium*, human malaria parasite, wild isolate, preservation, wet ice, in vitro culture, gametocytogenesis

INTRODUCTION

In malaria endemic areas, it is difficult to cultivate wild isolates of human malaria parasites, and thus they should be transported to a laboratory in a town with good facilities. Recently, transportation of these parasites in liquid nitrogen by aircraft was prohibited, and carrying of them with dry ice is also regulated, *i.e.*, up to a maximum of 2 kg dry ice. Thus, other suitable preservation and transportation methods, such as the wet ice method, are required for the transportation of wild isolates of malaria parasites to a laboratory. Chen *et al.* [1] and Nguyen-Dinh [2] reported that the longest acceptable period for preservation of *Plasmodium falciparum* in wet ice is only 3 days. However, Nakazawa [3] indicated that culture-adapted *P. falciparum* clones could be preserved in wet ice for 4 days, although older stage parasites such as late trophozoites and schizonts degenerated during preservation, and survival rates of ring forms gradually decreased, depending on the period of preservation.

Previously, we reported a synergistic enhancement for in vitro growth of *P. falciparum* (FCR-3 strain) in G6PD-deficient erythrocytes by the addition of BCS (bathocuproine disulphonate, a copper chelator) and cysteine [4]. We also examined the effects of these chemicals on the in vitro growth of *P. falciparum* wild isolates collected in Myanmar and preserved in wet ice for 4–6 days. During this study, we found that wild isolates of *P. falciparum* could be preserved in wet ice for at least 6 days, with high efficacy (ca. 85%) for adaptation to in vitro culture. In unsuccessful attempts at cultivation, all of the asexual parasites were transformed to sexual stages by Day 6 of culture and stopped asexual growth. Unfortunately, mechanism of transformation to sexual stages remains unclear [5–7].

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These results raised questions as to how long wild isolates of *P. falciparum* and other human malaria parasites can be preserved in wet ice, and how much they can adapt to in vitro culture. In order to answer these questions, we examined in vitro cultivation using wild isolates of the four human malaria parasites preserved in wet ice for 2–12 days.

Many attempts have been made to achieve continuous in vitro cultivation of *P. vivax* [8, 9], *P. malariae* [10, 11] and *P. ovale* [12]. Mons *et al.* [8] and Golenda *et al.* [9] succeeded in cultivating asexual forms of *P. vivax* for 6–8 cycles using RPMI-1640 and McCoy's 5A medium, respectively, supplemented with additional reticulocytes, but other investigations on *P. malariae* and *P. ovale* resulted in cultivation of the parasites for only one asexual cycle, and all these trials failed to establish continuous cultivation of the species. In this study, we also attempted in vitro cultivation of wild isolates of these parasites and examined the effects of BCS and cysteine and their adaptation to the in vitro culture system.

Here, we show that ring forms or young trophozoites can be maintained for up to 10 days by preserving wild isolates of the four human malaria parasites in wet ice. In addition, we also report a great difference in adaptation to in vitro culture between wild isolates of *P. falciparum* distributed in continental Southeast Asia (Myanmar and Thailand) and in eastern Indonesian islands.

MATERIALS AND METHODS

1. Collection of wild isolates of the four human malaria parasites

This study was approved by the ethical review committees in the Faculty of Medicine, Nagoya University and Oita University, Japan, the Myanmar Ministry of Health, Ranong Hospital, Thailand, and the health departments of Maluku Province and East Nusa Tenggara Province, Indonesia.

Fresh isolates of the four human malaria parasites were obtained at several villages in Myanmar (Tanintharyi Division, Mandalay Division, Bago Division, Mon State, Rakhine State, Shan State), at a clinic in Ranong, Thailand, and at several villages in eastern Indonesian islands (Buru, Halmahera and Flores; Fig. 1) from March 1998 to December 2006 [4, 13–19]. Informed consent was obtained from all symptomatic volunteers before diagnosis. Thin and thick smears were made from a single finger puncture and examined using the acridine orange (AO) staining method [20, 21]. AO-stained thin smears were examined at a magnification of 400 using a light microscope and a halogen illuminator equipped with an interference filter for excitation of AO dye. If a malaria-positive patient was diagnosed as single-species infection with parasitaemia higher than 0.5%, informed consent was obtained again, and 2–3 ml of venous blood was drawn into an evacuated blood collection tube with sodium heparin powder. Confirmation of single infec-

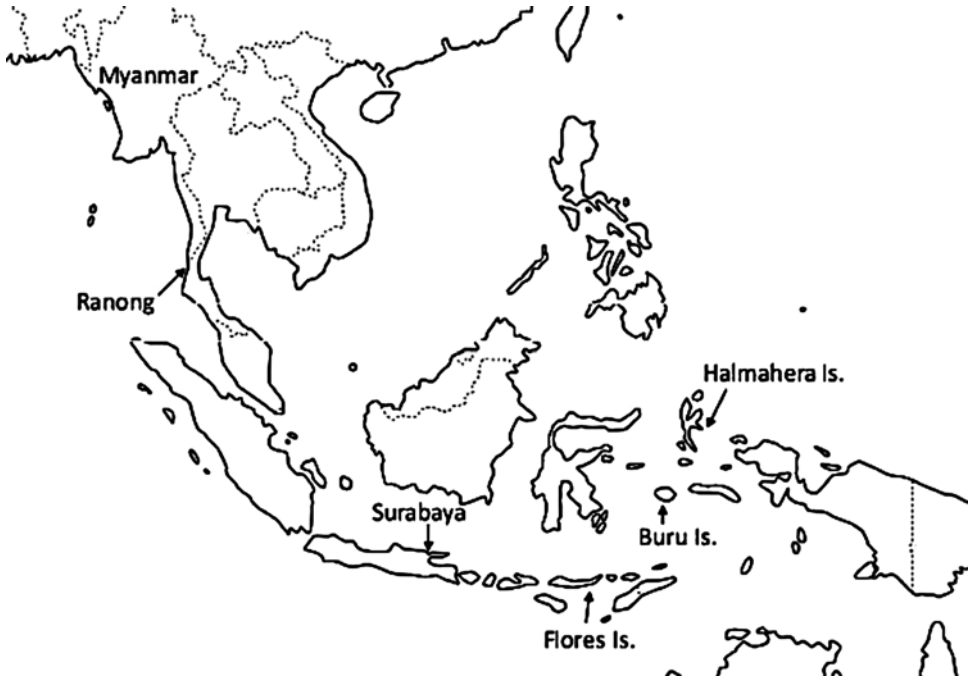

Fig. 1. Map showing the study sites.

tions with *P. falciparum*, *P. vivax*, *P. malariae* or *P. ovale* was performed later at laboratories in Indonesia and Japan by AO- and Giemsa-stained, thin smear microscopy and also by PCR-based diagnosis [16, 18].

2. Preservation and transportation of wild isolates in wet ice and cultivation by the candle jar method

In the field, heparinized blood was collected from malaria-infected volunteers and kept in a cool place. Within 7 hrs after collection, 2 ml of each sample was transferred, using a sterilized syringe, to a pre-cooled, 15-ml conical tube containing 13 ml of the complete RPMI-1640 medium supplemented with glutamine, 25 mM HEPES, 30 μg/ml gentamicin, 15% pooled AB serum and 1.0 mg/ml glucose. This procedure was done within 2–3 seconds under a candle fire in an ordinary room. These tubes were kept in wet ice or with "ice pack" preventing direct contact, and they were mixed gently once every day during preservation. Finally, they were transported to a laboratory in Surabaya, Indonesia.

At the laboratory, they were washed three times in complete RPMI-1640 medium with 15% pooled AB serum and cultivated by the candle jar method [22]. The cell suspensions were dispensed in 25-ml culture bottles with filter caps at 5% hematocrit and cultivated at 37°C in a candle jar. The supernatant was replaced every day with fresh complete medium. Thin smears were routinely stained by the AO method for parasite counts per 10,000 erythrocytes and for morphological examination. *P. falciparum* isolates were cultivated for 7–10 days, and those that grew well were cultured for a month or more to establish culture-adapted isolates. Wild isolates of *P. vivax*, *P. malariae* and *P. ovale* were cultivated using 20% AB serum for 3–4 days.

RESULTS AND DISCUSSION

1. In vitro culture of *P. falciparum* wild isolates

A total of 35 wild isolates collected from Myanmar were examined after preservation for 5–12 days (Tables 1, 2). In four isolates preserved for 12 days (Table 2), almost all parasites died during preservation, and the parasitemias were markedly decreased from the original values (1.2–1.8%). In culture, a few rings, trophozoites and schizonts were seen in an isolate (SA206) up to Day 6. In another isolate (SA205), young rings were also seen up to Day 6, but both disappeared thereafter. In isolates preserved for 7–10 days, on the other hand, many typical rings were seen on Day 0 without a great decline in the original parasitemias,

Table 1. In vitro growth of *Plasmodium falciparum* wild isolates collected in Myanmar and preserved for 5–7 days in wet ice

Sample No.	Preserved days	Parasitemia on Day 0 (%)	Parasite stages observed*						
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
A156	5	3.3	R>T	R>T, S	R, T, S	T, S>YG	**R>T, S>YG**	T>S, R	**R, S>T>YG**#
A327	5	4.0 (8.0)†	R>T	S>T>R	R, S>YG	R, S>T>YG	**R, S>T>YG**	R, S, T, YG	**R, S, T, YG** #
B195	5	2.6	R	S>>T	R>S	T>S>R, YG	YG>>R, T	YG ¶	
O198	5	3.0 (6.0)	R>T	R, T, S	T>R, S>YG	S>YG>T	R, YG	YG ¶	
O206	5	1.8	R	R>>S	R, T, YG	YG>>R	YG ¶		
B115	6	1.0	T>R	S>>T, R	R, T>S>YG	R, S>T, YG	R, S>T, YG	R, T, S>YG	**R, T>S>YG**#
B142	6	5.2	R	S>>R, T	R, T	R, T, S>YG	R, T, S	**R>S>YG**	S>T>R>YG#
A70	7	4.0	R>T	T>S>R	R, T>YG	T>S>R, YG	R, YG	YG ¶	
A72	7	3.5	R	S	R	T>>S>YG	R, T, S	R, T, S>YG	**R, T, S>YG**
A81	7	1.7	R	S>>T	R>T, S	R, S>T>YG	R, T>>S	R, T, S>YG	**R, T, S>YG**#
TN 4	7	4.0 (6.0)	R>T	R, T, S	T>R, S>YG	S>YG>T	R, YG	YG ¶	
TN 20	7	3.3	R>T	R>T, S	R, T, S	T, S>YG	**R>T, S>YG**	T>S, R	**R, S>T>YG**#
TN 64	7	4.0 (18.0)	R>T	T>S>R	R, T>YG	T>S>R, YG	R, YG	YG ¶	
WW220	7	3.5	R	S	R	T>>S>YG	R, T, S	R, T, S>YG	**R, T, S>YG**#
WW228	7	1.9	R	S>>T	R>T, S	R, S>T>YG	R, T>>S	R, T, S>YG	**R, T, S>YG**#
WW257	7	4.0 (8.0)	R>T	S>T>R	R, S>YG	R, S>T>YG	**R, S>T>YG**	R, S, T, YG	**R, S, T, YG**#
WW288	7	2.6	R	S>>T	R>S	T>S>R, YG	R, T>>S	R, T, S>YG	**R>>S>YG**#
WW299	7	1.0	T>R	S>>T, R	R, T>S>YG	R, S>T, YG	YG>R, T	YG ¶	

* R, ring forms; T, trophozoites; S, schizonts; YG, young gametocytes. † Numerals in parentheses are parasitemias observed after washing in the complete medium, and culture started by diluting with fresh erythrocytes to 3.0–4.0%. Bold letters indicate that parasitemias reached to 4% with many ring forms, and they were sub-cultured and cryopreserved in liquid nitrogen. ¶ All parasites transformed to young gametocytes. # Isolates were cultivated for a month or more and established as culture-adapted isolates.

although it was difficult to determine whether all ring forms seen at the initiation of culture survived or not. On Day 1, however, parasitemias decreased in many isolates, suggesting that some of the ring forms had degenerated or undergone damage during preservation, as reported in *P. falciparum* culture-adapted clones by Nakazawa [3].

In 16 of the 31 isolates preserved for 5–10 days, parasites remained as ring forms on Day 1, similar to those preserved for 4–6 days [4]. From Day 2 onward, all isolates grew well, except for nine isolates (B195, O198, O206, A70, TN4, TN64, WW299, SD105, YNA21), all of which were transformed to sexual stages by Day 6. However, other 22 isolates (71%) grew well for 7 days or more, although young gametocytes appeared in all isolates. Moreover, 14 of the 22 isolates grew particularly well, continuing cultivation for a month or more and reaching establishment as culture-

Table 2. In vitro growth of *Plasmodium falciparum* wild isolates collected in Myanmar and preserved for 8–12 days in wet ice

Sample No.	Preserved days	Parasitemia on Day 0 (%)	Parasite stages observed*						
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
SD101	8	5.0 (8.5)†	R	S>>R, T	R, T	R, T, S>YG	R, T, S	**R>S>YG**	S>T>R>YG
SD105	8	1.6	R	R>>S	R, T, YG	YG>>R	YG ¶		
SD110	8	1.0	R	R>T	S>T>YG	R>T, S>YG	R, T, S>>YG	**R, S>>T**	R, T, S>YG#
BK 34	9	5.0 (12.0)	R>>T, S	S>>T, R	**R>>T>S**	**R>>S>T**	T>>R, S	**R>>T, S, YG**	R, S>T>YG#
BK 36	9	5.1	S>>T, R	R>>T, S	**R>>S>T**	R, T, S	R>T, S	**R, S>T>YG**	R, T>S>YG
BK 38	9	2.2	R	R>>S>T	T>>R, S	R>>T>S	T>S, R>YG	**R>>S, T>YG**	**R>>T>S, YG**
BK 52	9	4.3	T>>S, R	R>>T>S	R>T, S>YG	R>>T, S>YG	T>R, S>YG	**R>>T, S>YG**	R, T>S>YG
KP102	9	2.8	R	S>>T>R	R>S, T>YG	S>R, T>>YG	**R>S>T, YG**	S>>R>T, YG	**R>>T, S>YG**
SBY107	9	5.0 (22.0)	R	T>>S	R>>T, S>YG	S>>R, T>YG	**R>>T, S, YG**	S, R>>T, YG	**R>>T, S>YG**
SBY118	9	1.6	R	R>>T, S	R, T, S	S>R, T	R>S>T, YG	T, S>R, YG	**R>>T, S**#
YNA 5	10	4.0	T>>R	S>>T, R	**R>>T, S**	S>R>T	**R>>T**	T, S>>R	**R>T, S, YG**#
YNA 10	10	2.5	R	S>>T, R	R>>S	S>>T>R	R>>T>YG	**R, S>YG**	R, T>YG
YNA 21	10	1.2	T>R>S	R>T>S	R>>S	R>T>YS	R>YG	YG ¶	
SA205	12	<0.001	(R) §	(R)	(R)	(R)	(R)	(R)	None
SA206	12	<0.001	(R)	(R, T)	(R)	(R)	(R, S)	(R)	None
SA210	12	<0.001	(R)	(R)	(R)	None			
SA228	12	<0.001	(R)	(R)	None				

* R, ring forms; T, trophozoites; S, schizonts; YG, young gametocytes. † Numerals in parentheses are parasitemias observed after washing in the complete medium, and culture started by diluting with fresh erythrocytes to 5.0%. Bold letters indicate that parasitemias reached to 4–5% with many ring forms, and they were sub-cultured and cryopreserved in liquid nitrogen. ¶ All parasites transformed to young gametocytes. § Very few parasites were seen in isolates after preservation for 12 days. # Isolates were cultivated for a month and established as culture-adapted isolates.

Table 3. In vitro growth of *Plasmodium falciparum* wild isolates collected at Ranong, Thailand, and preserved for 7 days in wet ice

Sample No.	Preserved days	Parasitemia on Day 0 (%)	Parasite stages observed*						
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
A1	7	3.5	R	S>>R, T	R, T	R, T, S>YG	R, T, S	**R>S>YG**	S>T, R, YG
A2	7	1.6	R	R>>S	R, T, YG	YG>>R	YG ¶		
A7	7	1.0	R	R>T	S>T>YG	R>T, S>YG	R, T, S>>YG	**R, S>>T**	R>T, S, YG#
B21	7	4.5	R>T, S	S>T, R	**R>T>S**	**R>S>T**	T>>R, S	R>T, S, YG	**R>T, S>YG**#
B27	7	5.0	S>T, R	R, T, S	R>>S, T, YG	R, T, YG	YG> R, T	YG¶	
C3	7	2.5	R, S	R>>S>T	T>>R, S	R>>T>S	T>S, R>YG	**R>S, T>YG**	**R>>T, S, YG**#
C10	7	4.4	T>S, R	R>>T>S	R>T, S>YG	R>>T, S>YG	T>R, S>YG	**R>>T, S>YG**	R, T>S>YG#
C12	7	2.8	R	S>>T>R	R>S, T>YG	S>R, T>>YG	**R>S>T, YG**	S>>R>T, YG	**R>T, S>YG**#
C17	7	5.0	R	R, T>S	R>T, S, YG	R, T, S, YG	YG>>R, T	YG¶	

* R, ring forms; T, trophozoites; S, schizonts; YG, young gametocytes. Bold letters indicate that parasitemias reached to 4–5% with many ring forms, and they were sub-cultured and cryopreserved in liquid nitrogen. ¶ All parasites transformed to young gametocytes. # Isolates were cultivated for a month and established as culture-adapted isolates.

Table 4. In vitro growth of *Plasmodium falciparum* wild isolates collected at Buru Island, eastern Indonesia

Sample No.	Preserved days	Parasitemia on Day 0 (%)	Parasite stages observed*									
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
A44O	2	4.2	R, S	R, T	R, T, S, YG	R, T, YG	R, S, YG	YG>>R	YG¶			
440O	2	3.5	R	R, S	R, YG	YG>>R	YG¶					
621B	2	2.7	R	R, S	R, T, YG	YG>>R	YG¶					
626B	2	3.3	R	R, T, S	R, T, YG	YG>>R	YG¶					
627B	2	4.0	R	R, T, S	R, YG	YG>>R	YG¶					
630B	2	1.6	R	R, T	R, S	R, T, S	R, T, S	R, T, YG	YG>R	YG¶		
632A	2	1.6	R	R, T, S	R, S, YG	R, T, YG	YG>>R	YG¶				
644B	2	3.8	R, S	R, T	R, YG	YG>>R	YG¶					
650A	2	5.2	R, T, S	R, T	R, T, YG	YG>R	YG¶					
700A	2	2.0	R, S	R, T, S	T, S, YG	R, S, YG	R, T, YG	YG¶				
712B	2	1.0	R, T	R, S	R, T, S, YG	YG>R	YG¶					
716O	2	1.5	R	R, S	R, T, S, YG	T, YG	R, T, YG	R, YG	YG>>R	YG¶		
900A	2	4.2	T, S	R, T, S	T, S, YG	R, YG	R, S, YG	YG>>R	YG¶			
901B	2	2.0	R, T, S	R, T, S	R, S, YG	R, T	S, YG	YG>>R	YG¶			
936O	2	2.2	R	R, S	R, T, S	R, T	R, T, S	R, T, S, YG	YG>>R, T	YG¶		
A2A	3	4.6	S	R, T	R, YG	YG>>R	YG¶					
A12A	3	3.0	R	R, T	R, T, S, YG	R, T, S, YG	YG¶					
A23B	3	1.8	S>R	R, T, S	R, T, YG	R, T, S	R, T, S	R, T, S, YG	YG>>R	YG¶		
B70A	3	2.5	R, S	R, T, S	R, T, YG	R, T, S, YG	R, S, YG	R, S, YG	R, S, YG	R, T, S, YG	YG>>R, T	YG¶
446B	3	2.9	R	R, T, S	R, T, YG	R, T, S, YG	R, T, S, YG	YG>R, T	YG>>R	YG¶		

* R, ring forms; T, trophozoites; S, schizonts; YG, young gametocytes.¶ All parasites transformed to young gametocytes.

adapted isolates. These results clearly indicated that *P. falciparum* wild isolates could be preserved in wet ice for up to 10 days.

Nine wild isolates collected from Ranong, Thailand were examined in vitro after 7 days of preservation (Table 3). Three isolates were transformed to young gametocytes, but other six isolates (66.7%) grew well, and five of them were established as culture adapted isolates.

In the Indonesian isolates, a total of 59 wild isolates collected from eastern Indonesian islands (Buru 20, Halmahera 11 and Flores 28) were applied to in vitro culture (Tables 4–6) by the same culture system used for Myanmar and Ranong isolates. However, we could not establish any culture-adapted isolate from eastern Indonesian islands, even though most of the isolates were preserved for only 2–3 days, a much shorter period than Myanmar and Ranong samples. Eight isolates collected from Halmahera and Flores islands did not develop beyond ring forms and disappeared by Day 4 (Tables 4, 5). All of these parasites were probably damaged during preservation. In two isolates from Flores island, hemolysis occurred for some unknown reasons (Table 6). In the other 49 isolates, all parasites were transformed to sexual stages and disappeared from cultures by Day 5–10. It should be emphasized that parasitemias never reached 4–5% in the isolates collected from Indonesia. These results suggest that a great difference in the mechanism of adaptation to in vitro culture exists between wild isolates in continental Southeast Asia (Myanmar and Thailand) and in eastern Indonesian islands. Indonesian wild isolates of *P. falciparum* seem to be very sensitive to stress, and gametocytogenesis might be easily switched on after new ring forms appear. To clarify the transformation mechanism to sexual stages, further comparative studies should be conducted on genomes, particularly at telomere regions [23], between wild isolates from continental Southeast Asia and eastern Indonesia.

2. In vitro culture of wild isolates of *P. vivax*, *P. malariae* and *P. ovale*

All wild isolates of the other three malaria species collected from Myanmar and Indonesia were preserved for 2–9 days as shown in Table 7. In all these isolates, late trophozoites, schizonts and/or gametocytes were observed before the washing-out of heparin. As reported by Nakazawa [3] in *P. falciparum*, these older stage parasites disappeared during washing in the complete medium three times, probably because of membrane damage to both parasites and infected erythrocytes during preservation. In any cryo-preservation method, it is also quite common that parasites older than ring form degenerate during the freeze-thaw process. Therefore, only ring forms or young trophozoites remained, and parasitemias at the initiation of culture were markedly decreased as compared to the original state (Table 7).

In Myanmar, more than 10 wild isolates of *P. vivax* single infection were collected and preserved for 3–9 days. As the majority of them contained later stage parasites, almost all parasites disappeared during washing-out of heparin, but two isolates (MC701 and SBY115) remained preserved for 6 and 9 days, respectively. Both had ring forms and young trophozoites, and they were used for this study. In both samples, many reticulocytes were seen in AO stained thin smears, and so additional reticulocytes were not supplemented into cultures. In *P. malariae* (Type A variant form [18]) and *P. ovale* (variant form [16, 17, 24]), small numbers of ring forms or young trophozoites were detected in six isolates collected from Myanmar and Indonesia (Table 7).

Table 5. In vitro growth of *Plasmodium falciparum* wild isolates collected from Halmahera Island, eastern Indonesia

Sample No.	Preserved days	Parasitemia on Day 0 (%)	Parasite stages observed*						
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
615	2	2.7	R	S, T>R	R, T, YG	YG>R	YG>>R	YG¶	
629	2	1	S	R, T	R, YG	YG>>R	YG¶		
631	2	3.3	R, S	R	R, YG	YG>R	YG¶		
638	2	4.8	R	R	None #				
665	2	2.5	R, T, S	R, T	S, YG	R, T	R, S, YG	YG>>R	YG¶
705	2	3.2	R	R	R	None #			
755	2	2	R	R, S	R, T, YG	Y G>>R	YG¶		
785	2	5.2	R	R	R	None #			
517	3	3	R	R, T	R, T, YG	YG>>R	YG¶		
518	3	5.2	R	R, T	T, S, YG	R, S, YG	YG¶		
550	3	1.4	R, T, S	R, T	R, YG	YG>>R	YG¶		

* R, ring forms; T, trophozoites; S, schizonts; YG, young gametocytes. ¶ All parasites transformed to young gametocytes. # Isolates might be damaged during preservation.

Table 6. In vitro growth of *Plasmodium falciparum* wild isolates collected at Flores Island, eastern Indonesia

Sample No.	Preserved days	Parasitemia on Day 0 (%)	Parasite stages observed*						
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
SIT417	2	5.2	R, T	S>>T	R, T, YG	R, T, S, YG	YG>>R	YG¶	
SIT441	2	1.0	R	R, T, S	R, T, YG	YG>>R, T	YG¶		
SDK530	2	3.3	R	T, S	T, S, YG	YG>>R, T	YG¶		
SDK534	2	4.5	R	R	None #				
SDK536	2	2.0	R	R, S	R, T, S	T, S, YG	YG>>R	YG¶	
SDK551	2	2.6	R, T	S, T>R	R, T, YG	YG>>R, S	YG¶		
PSB513	2	1.2	YS, R, T	R, T, S	R, T	T	Hemolysis		
SDN248	3	4.2	R	S, T>R	R, T, S	YG, R, T	YG>>R, S	YG¶	
SDN252	3	3.1	R	R, T, S	R, T, S, YG	R, T, S, YG	YG>R, T	YG>>R	YG¶
PAT225	3	0.5	R, S	R, T	R, T, YG	YG>>R	YG¶		
PAT230	3	1.0	R	R, T	R, T, YG	YG>>R	YG¶		
SDN242	3	5.2	R	R	None #				
SDN244	3	2.0	R	R, T, S	R, T, YG	YG>>R, T	YG¶		
SDN301	3	5.0 (10.0) †	R	R, T	R	None #			
SDN310	3	1.5	R, T	R, T, S	R, T	Hemolysis			
SDN312	3	1.6	R, T	R, T, S	R, YG	YG>>R	YG¶		
SDN318	3	1.4	R	S	R, T, S, YG	YG>>R, T	YG¶		
SDP207	3	6.3	YS, T, R	R>S	R, T, S	R, T, YG	YG>>R, T	YG¶	
SDP227	3	1.0	R	R	R	None #			
NGS81	4	4.7	R>T	S, T	R, T, S	R, T, S, YG	R, T, YG	YG>>R	YG¶
NGS93	4	4.0 (8.0)	R	R, T, S	T, S>YG	YG>>R, T	YG>>R, T	YG¶	
SIT118	4	4.5	YS, R, T	R, S	R, T, YG	R, T, YG	YG>>R, S	YG¶	
SIT120	4	0.5	R, T	YS>R	YS, T, YG	YG>>R	YG¶		
SIT123	4	1.2	R	R	None #				
SIT130	4	2.2	R, T	S	R	R, T, YG	R, T, YG	YG¶	
PSB20	5	4.5	S, R	T, S, R	T, S, R, YG	YG>>T, R	YG¶		
PSB21	5	4.0 (8.0)	S>>T	R, T, YG	R, T, S, YG	YG>>R, S	YG>>R	YG¶	
PSB22	5	0.5	R	S	YG, R	YG>>R	YG¶		

* R, ring forms; T, trophozoites; S, schizonts; YG, young gametocytes. † Numerals in parentheses are parasitemias observed after washing in the complete medium, and culture started by diluting with fresh erythrocytes to 4–5%.
¶ All parasites transformed to young gametocytes. # Isolates might be damaged during preservation.

Using these isolates, we examined the effects of 10 μM BCS and 100 μM cysteine on in vitro growth.

In *P. vivax*, one of the two isolates (SBY115) showed development, and maturing schizonts were seen on Day 2–3, but their morphology was abnormal in AO stain. In Giemsa stain, it was found that their nuclei were pycknotic (data not shown). No new ring was observed in erythrocytes with or without BCS and cysteine. On Day 4, all parasites disappeared from the culture. In *P. malariae* and *P. ovale*, young and maturing schizonts were observed on Day 1–2 and Day 1, respectively, but they were also pycknotic. In both, no new ring was seen in erythrocytes with or without BCS and cysteine, and parasites disappeared from all cultures by Day 2 or 3. Overall, there was no significant stimulating effect of either chemical on the in vitro growth of these three human parasites.

Although we failed to obtain culture-adapted isolates in species other than *P. falciparum*, we found that the young stages of wild isolates of all four species can be preserved in wet ice at least for 9–10 days after collection. Preservation in wet ice in the field is simpler than other cryo-preservation methods using liquid nitrogen or a deep freezer with facilities such as centrifugator, clean bench, and sterile pipettes. In spite of the limited term of preservation, the wet ice method may be useful for shipping wild isolates of human malaria parasites, particularly *P. falciparum*, from malaria endemic areas to laboratories with good facilities. If many ring forms or young trophozoites are included in collected

Table 7. Effects of BCS and cysteine on the in vitro growth of *Plasmodium vivax*, *P. malariae* and *P. ovale* wild isolates

Species	Sample No.#	Parasitemia (%) when collected	Preserved days	BCS + cysteine †	Parasitemia on Day 0 (%)	Parasite stages observed*				
						Day 0	Day 1	Day 2	Day 3	Day 4
P. vivax	MY-MC701	0.5	6	–	0.03	R, YT	R, YT	None		
				+		R, YT	R, YT	None		
	MY-SBY115	1.8	9	–	0.08	R, YT	R, YT	S, T	S	None
				+		R, YT	R, T, YS	S, T	S	None
P. malariae	MY-KG458	0.6	2	–	0.05	YT	YS	S	None	
				+		YT	YS	S	None	
	MY-KG164	1.2	5	–	0.01	YT	T	YS	None	
				+		YT	T, YS	YS	None	
	MY-VS230	0.5	5	–	0.01	YT	None			
				+		YT	None			
	IND-NR452	0.5	6	–	0.02	YT	T	YS	None	
				+		YT	T, YS	S	None	
P. ovale	IND-NR474	1.3	3	–	0.02	R, YT	T, S	None		
				+		R, YT	T, S	None		
	MY-VS243	1.2	5	–	0.01	YT	S	None		
				+		YT	S	None		

MY, Myanmar isolates; IND, Indonesian isolates. † Presence or absence of 10 μM BCS + 100 μM cysteine
* R, ring; YT, young trophozoite; T, trophozoite; YS, young schizont; S, schizont

wild isolates, all human malaria parasites can be used for further experiments in laboratories after preservation in wet ice.

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