Meditory
(The Journal of Medical Laboratory)
Volume 13 Issue 2 Page 187–196 December 2025
https://doi.org/10.33992/meditory.v13i2.4315

GROWING *Plasmodium berghei* ANKA *(PbA)* FROM FROZEN AND FRESH DONORS: A COMPARATIVE STUDY

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Article History
Received, 30th June 2025
Revised, 29th August 2025
Reviewed, 16th September 2025
Posted, 25th December 2025

Editor
Zito Viegas Da Cruz

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Keywords
Malaria, Plasmodium berghei, Fresh, Frozen

Abstract

Background: *Plasmodium berghei* is a rodent malaria parasite that infects mice and rats that mimics *Plasmodium falciparum*. It has a problem with standardized growing time and reaching high percentages of parasitemia, which allows optimum conditions in antimalarial research.
Objective: This study aimed to analyze different outcomes from growing *P. berghei* ANKA (PbA) from frozen compared with fresh donors.
Methods: The study involved 20 mice, with 10 infected using fresh and 10 using frozen P. berghei inocula. Blood smears were prepared daily for 7 days, fixed with methanol, stained with Giemsa, and examined every 24 hours at 1000× magnification. Parasitemia was calculated as infected erythrocytes per 1000 cells, recorded from initial parasite detection, and analyzed using a t-test.
Results: Parasite growth in mice inoculated with fresh and frozen P. berghei blood was monitored through parasitemia levels over 7 days. In the fresh blood group, parasites appeared on day 2 (0.8%) and increased steadily to 11.3% by day 7. In contrast, parasites in the frozen blood group were first detected on day 3 at a low level (0.1%) and rose gradually to 5.0% on day 7.
Conclusions: In conclusion, this finding indicates the fresh blood contains more active and viable parasites, enabling them to rapidly replicate within the mice's bodies.

Cite this Article

Apsari PIB, Winianti NW, Laksemi DAAS. Growing Plasmodium berghei ANKA (PbA) From Frozen And Fresh Donors: A Comparative Study. Meditory J Med Lab. 2025;13(2):187–196.

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INTRODUCTION

Malaria is an infectious disease caused by the Plasmodium parasite, which lives and multiplies in human red blood cells (1). It is transmitted by female malaria mosquito species, *Anopheles spp*., and can affect anyone, regardless of gender or age, from infants and children to adults (2). According to the 2023 Basic Health Research by the Indonesian Ministry of Health, the incidence of malaria based on diagnosis was 0.35%, or 3.5 per 1,000 population. In this survey, the three provinces with the highest incidence rates were the same as those reported in routine reports: Papua (6.1%), West Papua (4.5%), and East Nusa Tenggara (2.6%). Meanwhile, the malaria incidence rate based on diagnosis/symptoms was 1.9%, or 19 per 1,000 population (3). The high incidence of malaria in Indonesia has prompted the government to continue developing various research projects in the field of malaria, including treatment, resistance, genetic changes, prevalence, and so on.

PbA is a species of Plasmodium that infects laboratory mice, rats, or hamsters (4). This type of plasmodium is often used as a malaria model that mimics the biological properties of *Plasmodium falciparum,* including similarities in life cycles and clinical symptoms, and most importantly, *P. berghei* can be genetically manipulated more easily than *Plasmodium* species that infect humans, making it highly useful as a model for Plasmodium genetic research (5). PbA is relatively easy to cultivate in vivo, or within the body of mammals. The use of *P. berghei*-infected animal models is frequently employed to test the efficacy of new antimalarial drugs (6), as well as in biomedical research to understand the biological functions of the parasite (7). *P. berghei* is usually stored in -80°C or liquid nitrogen before being used again in malaria research (7).

Standardized, reproducible malaria models are foundational for preclinical evaluation of vaccines, antimalarials, and host–pathogen biology. Laboratories worldwide rely on donor-derived blood or cultured material to propagate PbA, but logistics such as shipping, biosafety restrictions, seasonal access to infected donors, frequently make the use of cryopreserved (frozen) material the only practical option (8). Validating whether frozen stocks produce equivalent growth kinetics, infectivity, virulence including cerebral malaria phenotypes, and transmission potential to fresh donor material is therefore critical to ensure results from different labs are comparable and translatable. Evidence supports that various Plasmodium stages (including PbA sporozoites) can retain viability and infectivity after cryopreservation under optimized conditions, but outcomes may depend strongly on method and stage. Several reproducibility and biological concerns arise from reliance on either fresh or frozen donors: loss or alteration of infectivity/virulence after freezing–thawing cycles or prolonged storage; selection or drift of subpopulations during repeated in vivo passages that change growth rate or disease phenotype; variability introduced by donor parasitemia, passage history, or cryoprotectant formulation; and logistical constraints that force different groups to use non-equivalent starting material, confounding cross-study comparisons (9). Historical and contemporary studies show both that cryopreservation methods exist and that outcomes vary with protocol and parasite stage (10).

A head-to-head comparison enables quantification of differences in primary outcomes such as parasitemia growth curves, time to symptomatic disease, incidence or severity of cerebral malaria, gametocyte production, and mosquito infectivity and secondary outcomes: immune response kinetics, drug susceptibility (11). Demonstrating equivalence would validate widespread use of frozen biobanks and facilitate multicentre studies, reduce the number of animals required by enabling centralized quality-controlled

stocks, and simplify regulatory and biosafety logistics. Recent work indicates that short-term cryopreservation can have minimal effects for some Plasmodium species stages, but outcomes are context-dependent, underscoring the need for empirical comparison in PbA (12).

Key precedents include early glycerolization and serum/HEC cryopreservation studies that established feasibility of freezing *Plasmodium spp*., investigations into cryopreserved sporozoite infectivity, and in vitro culture work showing that parasites can remain infectious after freezing when appropriate methods are used (13). However, the literature shows methodological heterogeneity (different stages, cryoprotectants, storage times) and relatively few rigorous side-by-side comparisons of fresh vs. frozen blood-stage PbA donors under identical downstream growth and virulence assays, hence the need for this focused, comparative study. This study aimed to compare the growth outcomes of PbA derived from frozen versus fresh donors.

MATERIALS AND METHODS

Test animals were male *Mus musculus* Balb/c strain mice weighing 25–30 g and aged 6–8 weeks obtained from a sterile breeding facility and pathogen-free. Mouse food consisted of chicken pellets and water in small bottles (14). Before treatment, the mice were adapted to cage conditions for 1 week (15). Twenty mice were divided into two groups: 10 infected by a fresh inoculum and 10 infected by a frozen inoculum containing 106 parasites per 0.2 mL suspension (16). The mice were coded with picric acid on their tails. In the frozen group, before inoculation into healthy mice, the vial containing frozen blood from *Plasmodium berghei*-infected mice is retrieved from the –80°C freezer storage (17). The vial is then immediately thawed in a water bath at 37°C for 1–2 minutes until the contents are completely thawed (18). Once thawed, the vial's contents are immediately transferred to a sterile centrifuge tube containing sterile physiological solution (0.9% NaCl) in a 1:1 ratio (19). This mixture is centrifuged at 1,500 rpm for 10 minutes to separate the erythrocytes from the cryoprotectant supernatant (phosphate-buffered saline). The supernatant is carefully discarded, and the erythrocyte pellet containing the parasites is resuspended in sterile NaCl. This suspension is then immediately inoculated intraperitoneally into healthy mice using a sterile 1 cc syringe, with a volume of 0.2 mL (15). The entire procedure is performed aseptically under a biosafety cabinet to prevent contamination. In the fresh group, the *P. berghei* transfer process was obtained from donor mice infected with a 1-2% parasitemia level collected from the Parasitology laboratory, Brawijaya University. Blood was collected from the tail of one donor mouse, then dropped into a tube pre-treated with PBS and anticoagulant (20). The blood tube was shaken, then inoculated into experimental mice, and subsequently, each mouse was injected with 0.2 mL of blood containing *P. berghei* via intraperitoneal injection (21,22).

Parasitemia examination in mice

Parasitemia counts were performed daily until day 7, using thin blood smears. The procedure involved cutting off the tip of the mouse's tail. Peripheral blood samples of 1.0–1.5 microliters were collected and prepared as thin blood smears using a 10% diluted Giemsa stain, following this step (23). Prepare glass slides by labelling them with the mouse code and date of smear preparation. Cut the mouse tail to obtain peripheral blood, then drop it onto the glass slide and smear it so the blood does not clot and lyse. Fix the specimen

with methanol after it dries (24). Stain with 10% Giemsa solution; apply approximately 3–4 ml of Giemsa solution to one specimen and wait 20 minutes for the specimen to stain. After 20 minutes, rinse the specimen with running water and dry it with a hair dryer. Observation was done under a microscope, Olympus CX21, at 1000x magnification after applying immersion oil. Percent parasitemia calculations were performed manually by counting the percentage of red blood cells infected with *P. berghei* in 1,000 red blood cells, then multiplying by 100% (25).

Statistical data Analyses

The difference of percent parasitemia between group was compared by *t-test* analyses using SPSS version 27 (IBM Corporation). This research was allowed by the Ethical Committee of Health Research Faculty of Medicine and Health Sciences Warmadewa University, by Ethical approval number: 636/Unwar/Ec-Kepk/V/2025, dated 5 May 2025.

RESULTS AND DISCUSSION

The data presented are the results of observations of the growth of *P. berghei* parasites in mice inoculated with fresh and frozen blood, as assessed by the percentage of parasitemia over seven days of observation. Parasitemia dynamics in both groups are summarized in Table 1. In the fresh blood donor group, parasites were first observed on day 2, and parasitemia increased steadily throughout the observation period, reaching its peak on day 7. In contrast, in the frozen blood donor group, parasite emergence was delayed, with the first detection on day 3 at a much lower parasitemia level. Although parasitemia subsequently increased, it remained consistently lower than that of the fresh donor group across all time points. This pattern suggests that cryopreservation may impair early parasite viability and delay the establishment of infection. Morphological examination of infected erythrocytes revealed the sequential development of intraerythrocytic stages from day 2 onward in the fresh group and from day 3 onward in the frozen group. Early trophozoites were visible as ring-shaped forms, which progressively developed into mature trophozoites with dense chromatin and pigmented cytoplasm. By days 5–7, schizonts containing multiple merozoites were observed, indicating active erythrocytic multiplication. The delayed appearance and lower abundance of these developmental stages in the frozen group further support reduced infectivity and slower parasite proliferation compared to the fresh group.

Table 1. Distribution of Infected Erythrocytes by Day Observation

	Fresh	Frozen	P
Day Observation	Mean±SD % Parasitemia	Mean±SD % Parasitemia	
1	0±0	0±0	na
2	0.8±0.07	0±0	0.001*
3	1.1±0.12	0.1±0.1	0.001*
4	3.06±0.2	0.4±0.16	0.001*
5	4.71±0.29	1.2±0.51	0.001*
6	6.67±0.4	3.02±0.40	0.001*
7	11.3±1.34	5.01±0.6	0.017*

Level of significance at p<0.05

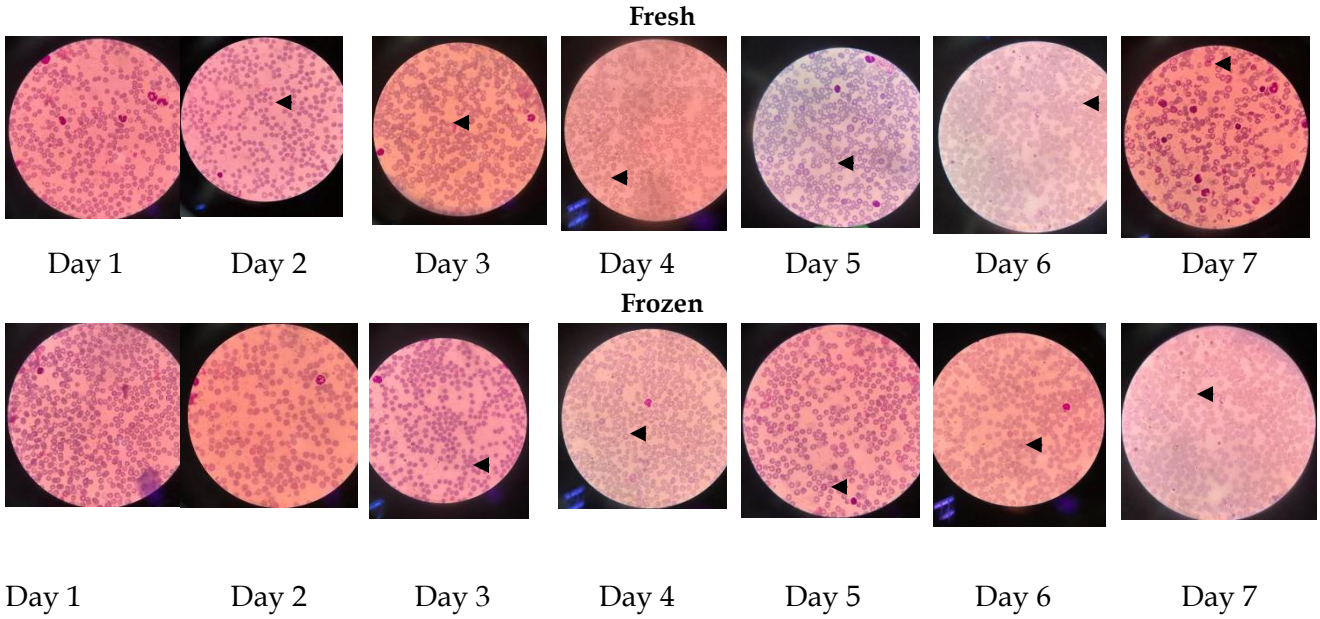

Figure 1. The development of *PbA* infection in mouse erythrocytes from two treatment groups: fresh and frozen blood over a 7-day observation period. The first row shows the observation results for the fresh blood group from day 1 to day 7, while the second row shows the frozen blood group for the same period. Parasites are indicate by black arrow.

The images shown in Figure 1 are microscopic documentation of the development of *P. berghei* infection in mouse erythrocytes from two treatment groups: fresh blood and frozen blood over a 7-day observation period. The first row shows the observation results for the fresh blood group from day 1 to day 7, while the second row shows the frozen blood group for the same period. No infected erythrocytes were observed in the fresh group on the first day. However, from day 2 to day 7, the number of erythrocytes containing Plasmodium gradually increased, marked by ring-shaped or dark purple structures within the red blood cells, indicating the early and late stages of trophozoites. From day 5 to day 7, the number of infected erythrocytes becomes increasingly dense and distinct, reflecting a significant increase in parasitemia, as indicated by previous quantitative data. In contrast, no clear parasites were detected in the frozen group on days 1 and 2. Parasites began appearing on day 3, but in much smaller numbers than in the fresh group. From day 4 to

day 7, parasites gradually increased, but the infection density remained lower than in the fresh blood group. The appearance of the parasites also indicated viability. However, their numbers were limited, supporting the finding that parasite viability post-thawing was lower and required more time to reach a significant infection level.

CLINICAL IMPLICATION

Inoculation of *PbA*from fresh and frozen donors resulted in significant differences in terms of parasite viability, infection rate, and the level of parasitemia achieved (25). Scientifically, fresh blood contains parasites in an active physiological state and is not subjected to extreme environmental stress, so when inoculated into healthy mice, the parasites can immediately invade host erythrocytes and replicate without experiencing adaptation delays (26). In contrast, frozen blood—although stored in cryoprotectants such as DMSO to maintain cellular integrity—undergoes stress due to freezing and thawing, which can damage erythrocyte membranes and parasites, reduce viability, and cause the death of most parasites or delay the reactivation of their life cycle after inoculation.

Previous research showed that frozen malaria parasites have varying levels of viability depending on storage duration, temperature, type of cryoprotectant, and thawing rate (23). Parasite survival decreases with longer storage times, as prolonged exposure to suboptimal cryogenic conditions may lead to membrane rupture, metabolic damage, or loss of infectivity. Some studies report acceptable viability up to several years when stored in liquid nitrogen, but rapid decline is observed at higher subzero temperatures (e.g., –80 °C). Optimal long-term preservation requires liquid nitrogen (–196 °C), where metabolic activity is essentially halted. Storage at –80 °C can maintain parasites for shorter periods but often results in reduced recovery rates and impaired infectivity after thawing. The formulation of the cryoprotective medium (commonly glycerol, DMSO, or combinations with serum or HEPES-buffered media) strongly influences parasite survival. Glycerolized preparations have historically been most common, but alternative formulations show different efficiencies depending on parasite species and stage. Controlled, rapid thawing is crucial to prevent ice crystal formation and osmotic stress. Suboptimal thawing often leads to hemolysis of host erythrocytes, loss of parasite integrity, and lower infectivity. Conversely, standardized rapid-thaw protocols can restore viability comparable to freshly obtained parasites in some cases. The viability of *P. berghei* decreased drastically after storage for more than a few months, with lower infection rates when inoculated into mice (27). Murine malaria parasites stored in liquid nitrogen remained infectious but required more time to reach peak parasitemia than fresh donors. Suboptimal thawing processes were also reported to cause erythrocyte lysis and the release of parasites in non-infectious forms. This slower growth suggests that the freezing and thawing process may cause some parasites to lose viability or require more time to re-adapt within the host (26). Nevertheless, parasites from frozen blood can still infect and proliferate, albeit at a lower rate than those from fresh blood. These data emphasize the importance of proper storage and thawing techniques to maintain infection efficacy in animal models. Thus, standardized and optimized storage conditions (preferably in liquid nitrogen with validated cryoprotectants) and rapid, controlled thawing protocols are essential to preserve parasite infectivity and ensure that animal models accurately mimic the natural course of infection. This methodological rigor ultimately safeguards the reliability of conclusions drawn from PbA models and supports the validity of translational malaria research (12). This difference has major implications for

experimental research and disease modelling. The use of fresh donors is recommended when rapid and consistent infection is required, particularly in immunology studies or drug efficacy testing (28). However, frozen blood remains useful for maintaining long-term parasite stocks, provided its use is preceded by revitalization and standardization of infection to ensure reproducible results.

LIMITATIONS

The limitations of the study were that the immunity status of mice was difficult to control, which implied that the ability of parasite growth against immune defense varied. Still, by multiplying the number of mice per group, we can control parasitemia by statistical analysis. Despite these limitations, our findings suggest that the time to grow *PbA* must be longer than the time for the fresh inoculum. Future studies should address these limitations by excluding extreme parasitemia or negative parasite growth in all groups under the same conditions.

CONCLUSIONS

Frozen inoculum has a longer waiting time to grow and reach a higher percent parasitemia than fresh inoculum. Fresh inoculum produces a fast response of parasitemia and a higher percentage of parasitemia at the same time of observation.

CONFLICT OF INTEREST

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

PIB conceptualized the study, conducted the experimental work, and drafted the manuscript, NWW validate parasitemia, DAASL validate the study, supervising and validating parasitemia, and revised manuscript.

ACKNOWLEDGMENTS

We thank the Parasitology laboratory, Universitas Brawijaya, for giving us the PbAisolate in mice. We also thank the laboratory staff who helped us maintain the experimental mice infected with *PbA*

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

DECLARATION OF ARTIFICIAL INTELLIGENCE USE

The authors used Chat GPT-5 for language editing. The authors reviewed and verified the content for accuracy.

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