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# GROWING *Plasmodium berghei anka* FROM FROZEN AND FRESH DONORS: A COMPARATIVE STUDY

**Article History**

**Editor**

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**Keywords**

**Abstract**

**Background:** *Plasmodium berghei* is a plasmodium-infected rat 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 compare growing Plasmodium berghei anka from frozen and fresh donors

**Methods:** The experimental method used 10 mice infected by a fresh inoculum and 10 infected by a frozen inoculum of *P.berghei*. Parasite identified by observation under microscope 1000x magnificent. Time first parasite detected and number of parasites per 1000 erythrocytes, then tabulated for 7 days.

**Results:**

**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.

**Cite this Article**

## 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 mosquitoes 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. 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 (3).

*Plasmodium berghei* 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, 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). Plasmodium berghei 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 use again in malaria research (7). This study aimed to analyse different outcomes from growing P. berghei anka from frozen compared with fresh donors.

## MATERIALS AND METHODS

Test animals were male Mus musculus Balb/c strain mice weighing 25–30 g and aged 6–8 weeks. Mouse food consisted of chicken pellets and water in small bottles (8). Before treatment, the mice were adapted to cage conditions for 1 week (9). 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 cc suspension (10). 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 (11). The vial is then immediately thawed in a water bath at 37°C for 1–2 minutes until the contents are completely thawed (12). 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 (13). 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 1cc syringe, with a volume of 0.2 mL (9). 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, Universitas Brawijaya. Blood was collected from the tail of one donor mice, then dropped into a tube pre-treated with PBS and anticoagulant (14). 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 (15,16).

*Parasitemia examination in mice*

Parasitaemia 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 (17). 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 (18). 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 done under a microscope, Olympus CX21 at 1000x magnification after applying immersion oil. Percent parasitaemia 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%(19).

This research was allowed by the Ethical Committee of Health Research Faculty of Medicine and Health Sciences Warmadewa University, by Ethic Number: 636/Unwar/Ec-Kepk/V/2025 Date 5 May 2025

**RESULTS AND DISCUSSIONS**

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 parasitaemia over seven days of observation. In the fresh blood group, infection was first detected on day 2 with a parasitaemia of 0.8%, then increased progressively to 1.1% (day 3), 3.0% (day 4), 4.7% (day 5), 6.6% (day 6), and reached 11.3% on day 7. This indicates that fresh blood contains more active and viable parasites, enabling them to rapidly replicate within the mice's bodies. Conversely, no parasites were detected in the frozen blood group on days 1 and 2. Parasites began to be detected on day 3 with a very low parasitaemia percentage (0.1%), then gradually increased to 0.4% (day 4), 1.2% (day 5), 3.0% (day 6), and 5.0% on day 7.

**Table 1. Distribution of Infected Erythrocytes by Day Observation**

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

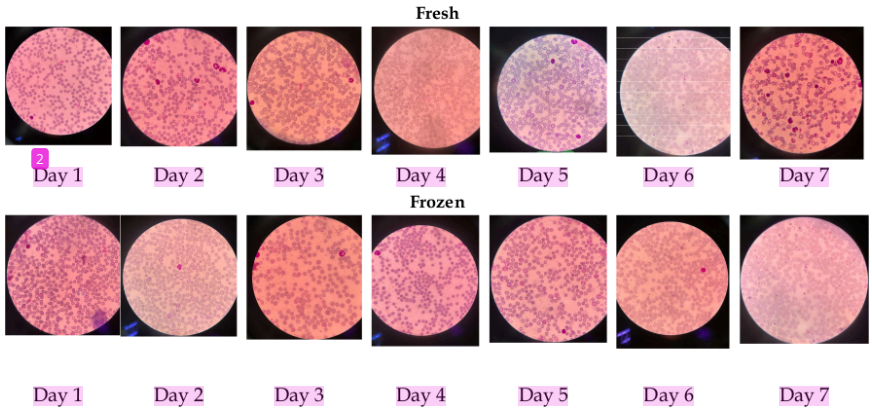


**Figure 1**. The development of *Plasmodium berghei* infection in mouse erythrocytes from two treatment groups: fresh and frozen blood over a 7-day observation period. The first row shows the observation results for the fresh blood group from day 1 to day 7, while the second row shows the frozen blood group for the same time period.

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 time 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 parasitaemia, 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 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 *Plasmodium berghei* from fresh and frozen donors resulted in significant differences in terms of parasite viability, infection rate, and the level of parasitaemia achieved (19). 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(20). 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(17). The viability of *P. berghei* decreased drastically after storage for more than a few months, with lower infection rates when inoculated into mice (21). Murine malaria parasites stored in liquid nitrogen remained infectious but required more time to reach peak parasitaemia 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 (20). 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. 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 (22). 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 defence 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 P. berghei 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.

## AUTOR CONTRIBUTIONS

PIB concept the research, doing experimental study, write manuscript, NWW validate parasitemia, DAASL validate the study, supervising and validating parasitemia, revised manuscript.


## ACKNOWLEDGMENTS

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