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A Simple Method for Isolation of *Plasmodium gallinaceum* from Infected Chicken Red Blood Cells

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Abstract

This study aims to isolate avian malaria parasite (*Plasmodium gallinaceum*) from red blood cell nucleus. Intravenous passage for three days can stimulate the production of a high percentage of schizont stages in malaria-infected chicken blood. Chicken blood was drawn from the jugular vein, washed with PBS solution, the buffy coat layer was removed, frozen at –20 °C overnight, and subjected to 4 steps of differential centrifugation. The pellet of each centrifugal step was prepared into a thin smear on a glass slide, and stained with Giemsa. The examined results had a high concentration of parasites contaminated with a few host red blood cell nuclei at the 4th step while more contamination was found at the 1st-3rd steps. This procedure can be used in parasite purification without using any lysis agents.

Keywords: *Plasmodium gallinaceum*, isolation, red blood cell nucleus

Introduction

Malaria is a disease caused by a protozoan organism belonging to the genus *Plasmodium*. Various living vertebrates can be infected with malaria, such as humans, monkeys, rodents, lizards, and birds [1-3]. Many aspects of malaria research, especially molecular biological studies, need non-contaminated host-cell malarial parasites. No problem was identified with the conduct of malaria research among hosts with non-nucleated red blood cells. By contrast, if a malaria research is to be conducted in hosts having nucleated red blood cell (lizards and birds), a molecular study would be difficult to perform. The avian malarial parasite, *Plasmodium gallinaceum*, is a mosquito-borne malaria species that naturally infects jungle fowl and chickens. Poultry transmission with high mortality rates (10-20%) was reported in 1995 in 17 provinces of Thailand [4].

The common method used to isolate this parasite from nucleated red blood cells is lysing red blood cell membrane with saponin [5], and to apply the hemolysis red cell solution to ficoll-hypaque gradient for parasite purification. The conventional method of chicken erythrocyte lysis was modified to include saponin, a known effective hemolyzing agent. The influence of saponin depends on various lysate pHs and concentrations. The completeness of membrane

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lysis needs sufficient time to destroy the membrane of the red blood cells [6].

This study aimed to isolate the avian malarial parasite (*P. gallinaceum*) from chicken red blood cell nuclei, in order to derive baseline information for further studies, particularly of the parasite's molecular biology, and also to better understand this natural host of malaria and it's pathological effects among birds. This information would be useful when planning for the more effective prevention and control of this disease among poultry in the future.

Materials and Methods

Parasite isolation and maintenance

The parasite used in this study was *P. gallinaceum* isolate NJTH39 (Nong Chok District, Bangkok, Thailand, 1995). Subcutaneous serial passage was continuously maintained through the blood of male 3-week-old chickens (white leghorns). The laboratory procedures were conducted at the Animal Laboratory for Parasitology Unit, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The chickens were maintained under a light:dark cycle of 12:12 hours at 27°C, fed with commercial chick feed (Betagro101; Betagro Group, Thailand) and water *ad libitum*. Parasitemia was monitored daily; each chicken was gently restrained in dorsal recumbency with wings extended. Thin blood films were prepared, stained with 10% Giemsa, and examined microscopically for parasites; those with >50% parasitemia (with a high percentage of mature schizonts) were used for large-volume schizont-production experiments.

Induction of high concentrations of schizont-stage parasites

Two-month-old male chickens were used as recipients for 100 µl blood at 50% parasitemia through the jugular vein, and were heparinized. Thin blood film staining was used to monitor parasites daily. At day 3 post-infection, 6 ml of parasitized blood was collected from each recipient using 22 G × 1" needles and syringes.

WBC removal and red cell lysis

The infected blood was transferred to a collecting tube and aliquoted into each of 4, 1.5 ml centrifuge tubes. Plasma was removed after centrifugation at 4°C (Varifuge 20 RS; Heraeus Sepatech) at 3,214xg for 5 min.

The procedures were repeated 3 times to wash blood samples with PBS (0.005 M Na_2HPO_4, 2M NaCl, pH 7.4). A tube block was used to keep the tubes upright at 4°C in a refrigerator for 1 hour, then the buffy coat layer was removed by micropipette (BIOHIT) before storing the specimens overnight at -20°C.

The remaining WBC was washed by adding PBS solution at room temperature to make up a total volume of 1.5 ml. The tubes were gently inverted, occasionally rotated, and the supernatant solution removed.

Differential centrifugation (4 steps)

The white blood cells were removed and the lysed red blood cell samples were added with PBS (at 4°C), vigorously vortexed for 30 sec (G-560E; Scientific Industries) and 4-step differentially centrifugated: at 3,214xg for 5 min, 4,629xg for 5 min, 12,857xg for 5 min, and 21,728xg for 30 min, respectively. The supernatant from each step was introduced to new tubes for further high-spin centrifugation in the next step. The pellet from each step was prepared for thin smear and stained with 10% Giemsa, before examination for parasite cells.

Results

Using conventional serial subcutaneous blood passage, parasites in the blood can be found on day 7 post-infection (Fig 1A). Less than 1% parasitemia present with spot of trophozoite and young schizont-stage, single infection are commonly found. However, when using intravenous passage with 50% parasitemia or higher, parasites can be detected on day 1 post-infection. On day 3 post-infection, parasitemia was around 80%, with high concentrations of mature schizonts and young trophozoites (Fig 1B). Most red blood cells had multiple infections.

The pellet of each centrifugal step was stained with 10% Giemsa and examined under a light microscope. The pellet at the 1st step contained numerous red blood cell nuclei, a large number of young trophozoite and schizont stages with intact

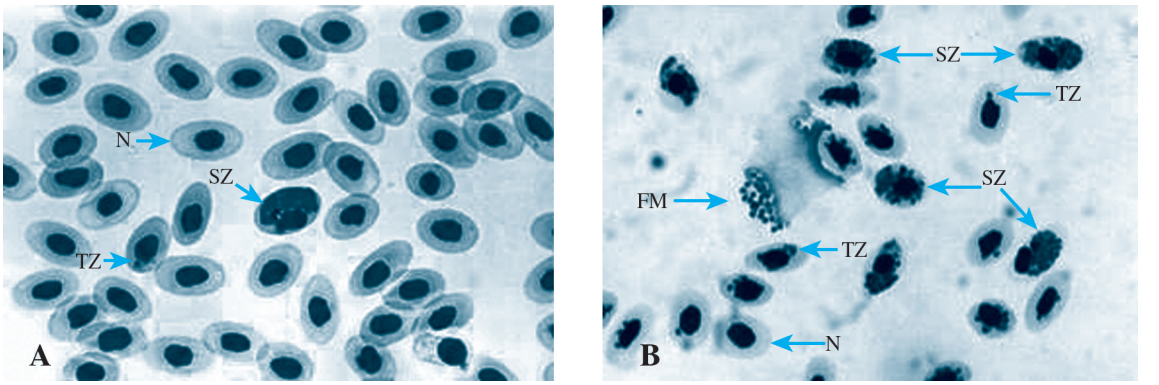

Fig 1 Thin blood film of chicken red blood cells infected with *P. gallinaceum*. A) Commonly found in conventional blood serial passage. B) A high schizont concentration was stimulated by intravenous parasite injection. TZ = Trophozoite, SZ = Schizont, FM= Free merozoite, N = Normal red blood cell with nucleus.

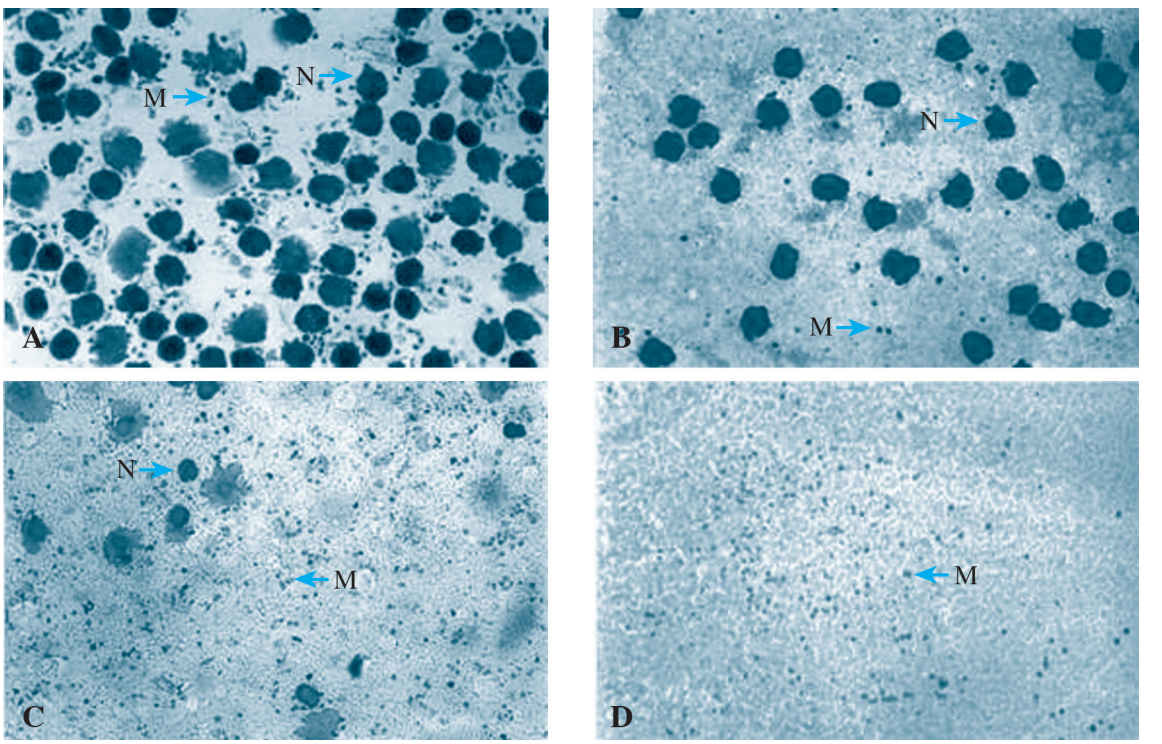

Fig 2 The pellet from each step of differential centrifugation and stain with 10% Giemsa. A) Step I; 3,214xg 5 min, B) Step II; 4,629xg 5 min, C) Step III; 12,857xg 5 min, D) Step IV; 21,728xg 30 min. M = Merozoite, N = Nucleus of red blood cell.

nuclei, small number of merozoite stage, and some white blood cells (Fig 2A). In the 2nd step, the pellet consisted of a fewer number of both red blood cell nuclei and, even fewer, parasite cells than at the 1st step (Fig 2B). In the 3rd step, there were fewer red blood cell nuclei (Fig 2C), and a greater number of parasite cells, when compared to the previous steps. In the 4th final step, many parasite

cells (merozoite stage) were found around the smear area (Fig 2D). Merozoites were obtained and subjected to sonication. Proteins were observed in polyacrelamide gel; phenol-chloroform extraction was performed and followed by DNA analysis in agarose gel. Both protein and DNA can be found at adequate amounts (data not shown).

Discussion

This study describes the pattern of *P. gallinaceum* infections in chickens infected by serial blood passage, using both subcutaneous and intravenous routes. After the initial subcutaneous injection of the chicken with the parasites, an incubation period of 7 days was needed before it was possible to detect parasites in the blood stream. The pre-erythrocytic cells (macrophages) during this period completed several cycles, and merozoite cells were produced in the bloodstream [7]. Alternatively, with intravenous passage of 50% parasitemia or higher, parasites were detected on day 1 post-infection, and the parasites multiplied continuously without any macrophage stage. On day 3 post-infection, the parasites produced many merozoites in the bloodstream, and could infect new red blood cells. The high percentage of mature schizonts results in multiple infections after the red blood cells are broken [8].

Two-month-old chickens were required for challenge with high parasite numbers by injection into the jugular vein. In addition, 3-week-old chickens died after being infected with equal amounts of parasites, indicating a sudden increase in severity, as well as an anemic crisis, prior to the death of the chickens [9]. Many merozoites infected the same red blood cells, which proved that a large number of mature schizonts were present in the prior cycle, which subsequently broke out to become free merozoites in the bloodstream, generally with 16-20 merozoites per red blood cell [10].

The working saponin concentration has been shown to produce 100% hemolysis, with 50% hemolysis being achieved at a concentration of 10 mg/l, but an appropriate pH, 4.7-5.2, is needed [11]. From Fig 2A, no RBC with membrane was present, indicating that an overnight freeze-thaw cycle followed by 30 sec vigorous vortexing was associated with the completeness of hemolysis. In contrast, a single 1 hour freezing-thawing of lysates does not always guarantee complete hemolysis [5]. Some intact mature schizonts can be found; the schizonts rupture rate may increase by using the longer time of vigorous vortexing.

The results demonstrated free merozoites in every isolation step. Figs 2A-2C show both the host's red blood cell nuclei and merozoites, while in Fig 2D, only the merozoite is shown, indicating that the chicken red blood cell and white blood cell membranes were selectively broken by the freeze-thaw cycle and that the avian malaria parasite can be separated from the nuclei of the red and white blood cells [12] by differential centrifugation.

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