# Application of Percoll density gradient centrifugation for separation of Babesia ovatainfected erythrocytes

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# Application of Percoll density gradient centrifugation for separation of *Babesia ovata*-infected erythrocytes

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## ABSTRACT

In this study, potential of the Percoll density gradient centrifugation method for separation of bovine red bold cells (RBCs) infected with each merozoite type of *Babesia ovata* was examined. By the Percoll density gradient centrifugation, bovine RBCs infected with active forms of *B. ovata* merozoites were essentially collected from one of the layers. Moreover, this method made it possible to eliminate its crisis forms, a morphologically abnormal intraerythrocytic form, effectively. Moreover, no adverse effect on viability of *B. ovata* merozoites was found. Our results suggest that this method will be applicable to sample preparation for biological analyses which require active forms of *B. ovata* merozoites.

**Keywords:** *Babesia ovata*; *in vitro* culture; Percoll density gradient centrifugation

*Babesia ovata* is an intraerythrocytic protozoan parasite of cattle and transmitted by a tick, *Haemaphysalis longicornis*, and widely distributed in Japan. *B. ovata*-infected cattle do not show highly clinical symptoms, but the immunodeficient or immunosuppressed animals exhibit severe anemia and hemoglobinuria, and sometimes death (Minami and Ishihara, 1980; Shimizu *et al*., 1992).

At least, four morphologically distinguishable merozoites of *B. ovata* are known namely, single pyriform, paired pyriform, budding form, and crisis form (Higuchi *et al*., 1987). However, precise biological features of each merozoite type are not well understood. In addition, the crisis form, a morphologically abnormal intraerythrocytic form, of *B. ovata* merozoites may appear during *in vitro* cultivation. It is important to eliminate crisis form from other three morphologically normal merozoite types in order to carry out downstream analyses by using biologically robust parasites. The methods using Percoll density gradient for concentrating or separating *Babesia* parasites (*B. bovis* and *B. caballi*)-infected red blood cells (iRBCs) have been

developed previously (Bhushan *et al*., 1991; Ikadai *et al*., 1997; Ikadai *et al*., 2000; Ishizaki *et al*., 2016). Moreover, a stable transfection system for *B. ovata* was successfully developed by Hakimi *et al*. (2016). *H. longicornis-B. ovata* experimental infection model revealed the time course of *B. ovata* migration in each organ of *H. longicornis* tick (Maeda *et al*., 2016). These are the powerful experimental tools to improve understanding the biology of *Babesia* parasites and the tick vector; however, efficient purification methods for *B. ovata*-iRBCs are required for promoting further studies on *B. ovata*. Therefore, this study aimed to establish separation method of each merozoite type of *B. ovata* by means of Percoll gradient centrifugation technique.

*B. ovata* (Miyake strain) was cultured using disposable 24-well plates with the medium containing GIT (Nihon Pharmaceutical, Tokyo, Japan) and M199 (Sigma-Aldroch, St. Louis, MO, USA) in the ratio of 2:3 as described by Maeda *et al*. (2016). Fresh bovine RBCs were purchased from Nippon Bio-Test Laboratories (Tokyo, Japan). The culture was maintained at 37°C with 5% $O_2$, 5% $CO_2$ and 90% $N_2$. Culture medium was changed daily with fresh medium. The *B. ovata in vitro* cultivation using bovine RBCs was approved by Animal Care and Use Committee, Obihiro University of Agriculture and Veterinary Medicine (approval no. 28-34).

An aqueous solution of Percoll (GE Healthcare, Little Chalfont, UK) was diluted with sterilized phosphate buffered saline (PBS) to prepare the solution with densities ranging from 1.09 to 1.12 g/ml according to the manufacturer's instruction. One ml of each Percoll dilution was carefully poured to form layers in a 15 ml-centrifuge tube (Fig. 1A). Two separate experiments were performed where the RBC cultured with *B. ovata* (2.98% of parasitemia in Experiment No.1 and 1.98% in Experiment No.2) was mixed with the fresh medium at a ratio of 7:3. Seven-hundred-fifteen $\mu$l of the RBC solution was layered on top of the Percoll solution (Fig. 1A) and then centrifuged at 500 × *g* for 20 min at room temperature. After centrifugation, each layer (L1 to L6) as shown in Fig. 1B were collected and transferred to new tubes and then washed with 10 ml of sterilized PBS. Giemsa-stained smears of each layer were made and examined under a microscope. Merozoites of *B. ovata* observed as single pyriform, budding form, paired pyriform and crisis form (Higuchi *et al*., 1987) were counted and the ratio of each form to more than 2,000 RBCs was calculated.

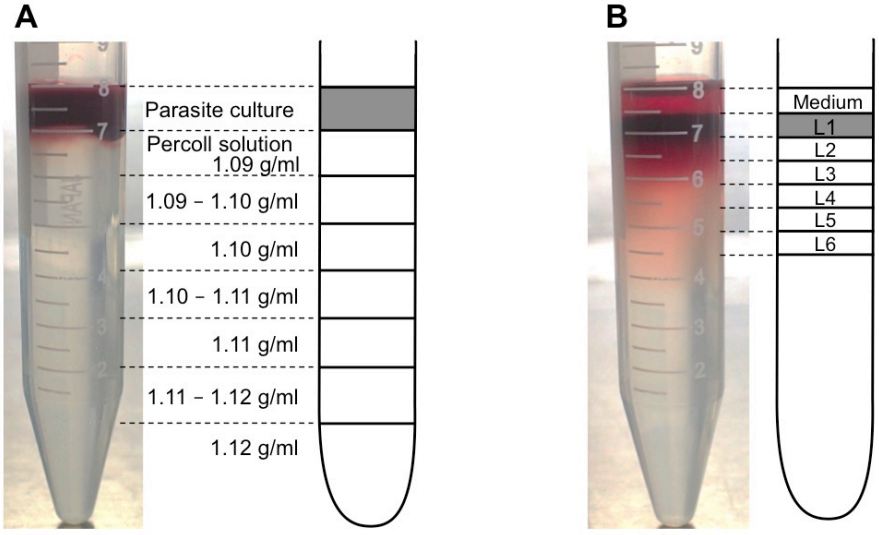


**Fig. 1.** Percoll density gradient. (A) Parasite culture and Percoll solution having densities from 1.09 to 1.12 g/ml before centrifugation. (B) After centrifugation, resulting in six different layers.

*B. ovata*-iRBC suspention on Percoll dilution became separated into six layers (L1-L6) showing color gradation after centrifugation (Fig. 1B). In both Experiment No. 1 and No. 2, the single pyriforms were collected in L1 and/or L2 (Table 1). The ratio of budding form to *B. ovata*-iRBC in L1 was increased after centrifugation compared to before centrifugation. Notably, the paired pyriform was separated in L1 and the crisis form almost in L2-L6 having lower densities of Percoll. These data indicate that mitotic phase of parasites were separated in L1.

**Table 1.** Separation of four morphologically different merozoites by Percoll density gradient centrifugation.

| Experiment No. | | Four types of merozoite | | | |
|---|---|---|---|---|---|
| | | Single pyriform (%) | Budding form (%) | Paired pyriform (%) | Crisis form (%) |
| 1 | BC | 14.5 | 47.3 | 27.3 | 10.9 |
| | AC | | | | |
| | L1 | 20.0 | 62.0 | 12.0 | 6.0 |
| | L2 | 11.8 | 17.6 | 0.0 | 70.6 |
| | L3 | 0.0 | 0.0 | 0.0 | 100.0 |
| | L4 | 0.0 | 0.0 | 0.0 | 100.0 |
| | L5 | 0.0 | 0.0 | 0.0 | 100.0 |
| | L6 | 0.0 | 0.0 | 0.0 | 100.0 |
| 2 | BC | 28.2 | 28.2 | 23.1 | 20.5 |
| | AC | | | | |
| | L1 | 19.2 | 65.4 | 15.4 | 0.0 |
| | L2 | 0.0 | 20.0 | 0.0 | 80.0 |
| | L3 | 0.0 | 57.1 | 0.0 | 42.9 |
| | L4 | 0.0 | 0.0 | 0.0 | 100.0 |
| | L5 | 0.0 | 0.0 | 0.0 | 100.0 |
| | L6 | 0.0 | 0.0 | 0.0 | 100.0 |

BC: Before centrifugation, AC: After centrifugation

Subsequently, to examine the effects of Percoll density gradient centrifugation on growth of *B. ovata* in L1, 5 $\mu$l, 10 $\mu$l and 20 $\mu$l of L1 were cultured with 100 $\mu$l of fresh bovine RBCs at 37ºC with 5% $CO_2$, 5% $O_2$ and 90% $N_2$ for 4 days. Culture medium was changed daily and growth of *B. ovata* was observed in Gimsa-strained blood smears. Parasitemia increased from day 1 to day 4 (Fig. 2) as reported by Igarashi *et al*. (1994), suggesting that viability of merozoites in L1 was not inhibited by the procedure unlike other report (Cursino-santos *et al*., 2016).

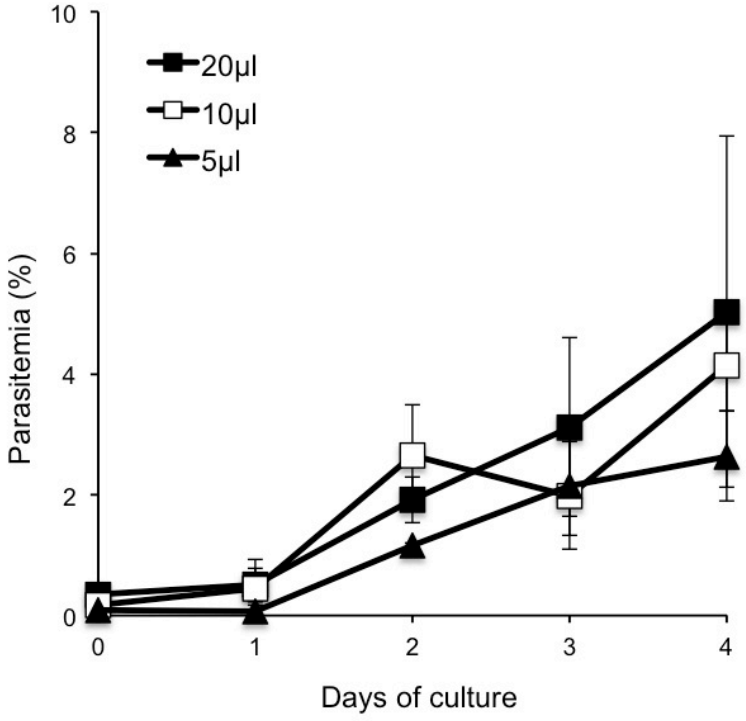


**Fig. 2.** Growth of *B. ovata* collected by Percoll gradient density centrifugation method. Five µl (▲), 10 µl (□), and 20 µl (■) from L1 were cultured with fresh bovine RBCs for 4 days and the parasitemia was measured daily. Each legend symbol and error bar shows the average value of triplicate cultures and the standard deviation, respectively.

In our study, bovine RBCs infected with active forms of *B. ovata* merozoites were collected from one of the layers by the Percoll density gradient centrifugation, making it possible to eliminate its crisis forms effectively. This method will be applicable to sample preparation for various experiments, such as bioactivity assays, omics analyses as well as artificial feeding system using tick vector, which require active forms of *B. ovata* merozoites.

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## CONFLICT OF INTEREST

All authors declare no actual or potential conflicts of interest.

## SUBMISSION DECLARATION AND VERIFICATION

This manuscript is approved by all authors, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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