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Purification of Merozoites from In Vitro Cultured *Babesia caballi*-Infected Equine Erythrocytes

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ABSTRACT

A method for purifying merozoites of *Babesia caballi* was developed. Infected erythrocytes prepared from in vitro culture was enriched to at least 60% by Percoll-gradient centrifugation. Enriched infected erythrocytes were lysed with 0.83% ammonium chloride and then the lysate was subjected to centrifugation in a Percoll density gradient. Free merozoites were recovered from a band formed at the interface of 1.018 and 1.053 g/ml Percoll solutions. These merozoites were revealed to be morphologically intact by the electron microscopic observation. The merozoites were not contaminated with erythrocyte membrane protein as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

Babesia caballi is one of haemoprotozoan parasite that caused equine babesiosis and is distributed world wide. This parasite causes fever, anemia, jaundice and edema, and in some cases, death to horse (Schein 1988), and therefore equine babesiosis is economically important in the horse industry (Friedhoff 1982). The low percentage of parasitized erythrocytes in experimental infection of horse has been prevented the biochemical and immunological studies (Schein 1988). Although continuous in vitro cultures of *B. caballi* was established

by the use of the microaerophilous stationary-phase (MASP) culture techniques (Levy and Ristic 1980), parasitemia has generally been low (Cissoko 1989). Recently, Avarzed et al. (1997) developed the improved method for increase of parasitemia (usually 8-10%) in vitro culture by transferring cultures from a low oxygen to 5% CO_2 in air. However, these parasitemia in vitro was not suitable to prepare enough quantity of materials for biochemical and immunological and serological studies on this pathogen.

Percoll density gradients have been used in order to enrich erythrocytes infected with several haemoprotozoa, such as *B. caballi* (Bhushan et al. 1991), *B. bigemina* (Vega et al. 1986), *Plasmodium falciparum* (Kramer et al. 1982), *P. ovale* and *P. vivax* (Andrysiak et al. 1986). However, the method of purifying of *B. caballi* merozoites has not been established. In present study, we describe a simple and rapid method to obtain pure and morphologically intact *B. caballi* merozoites using combination of ammonium chloride solution and two-step Percoll density-gradient centrifugation.

MATERIALS AND METHODS

Parasite

The *Babesia caballi* (USDA strain) was grown in equine erythrocytes in continuous microaerophilous stationary phase (MASP) cultures as described by Avarzed et al (1997). Percent parasitemia was calculated from on Giemsa-stained thin smears.

Percoll density-gradient solution

An aqueous solution of Percoll (density, 1.130 g/ml) was purchased from Pharmacia (Upsalal, Sweden), and diluted according to the manufacturer's instruction with in 0.15m*M* NaCl to achieve densities of 1.018 and 1.053 g/ml.

Separation of the merozoites by density-gradient centrifugation

Parasitized erythrocytes from in vitro culture were enriched by density gradient centrifugation on the first step of Percoll gradient (Bhushan et al. 1991), and washed once in RPMI-1640 tissue culture medium. The infected erythrocytes were then mixed with nine volume of ammonium chloride solution (0.83% NH_4Cl, 0.17 *M* Tris buffer) and the mixture was incubated at 37°C for 3 min to lyse erythrocytes (Martin et al. 1971). The erythrocyte lysate was centrifuged at 15,000 *g* for 10 min at 4°C, and the resultant sediments were suspended in 1.0 ml of 10 m*M* TRIS-HCl 150 m*M* NaCl 5m*M* EDTA buffer (TRIS-NaCl-EDTA buffer, pH 7.4). One ml of sediments was carefully layered on top of 2 ml the discontinuous density-gradient solution and centrifuged at 3,600 *g* for 20 min at

4°C. The resultant each band was collected with a Pasteur pipet and washed three times with TRIS-NaCl-EDTA buffer to remove Percoll (15,000 *g*, 10 min, 4°C). Thin smears were made, stained with Giemsa solution and examined with light microscope.

Electron microscopy

Samples were fixed with 2.5% (v/v) glutaraldehyde, followed with 2.0% (w/v) osmic acid, dehydrated and embedded in Spurr Resin mixture (TAAB). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (JEM-1010; JEOL, Tokyo, Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were boiled for 5 min in SDS-PAGE sample buffer (Laemmli 1970) and were electrophoresed in 12% SDS-PAGE slab gel with a 4% stacking gel (Laemmli 1970). The gel were stained Coomassie brilliant blue.

RESULTS

In order to enrich parasitized erythrocytes from cultured materials, *B. caballi* parasitized erythrocytes were centrifuged on Percoll gradient. Parasitemia of infected cells were increased from about 10% to 60-90% infected cells by first step centrifugation with Percoll-gradient solution (Fig. 1). Enriched infected erythrocytes were then incubated with 0.83% ammonium chloride for 37°C for 3 min to release parasites from erythrocytes and complete hemolysis was occurred with this treatment. Released parasites did not show any alternation in their normal morphology as determined by light microscopy, but they were still contaminated with lysed erythrocyte membrane.

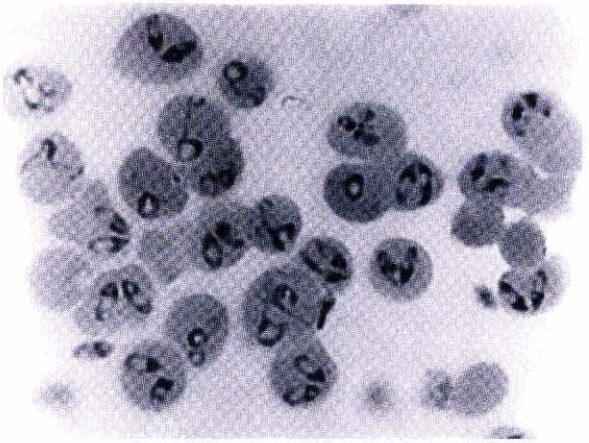

Fig. 1. *Babesia caballi*-infected erythrocytes enriched by density-gradient centrifugation stained by Giemsa solution.

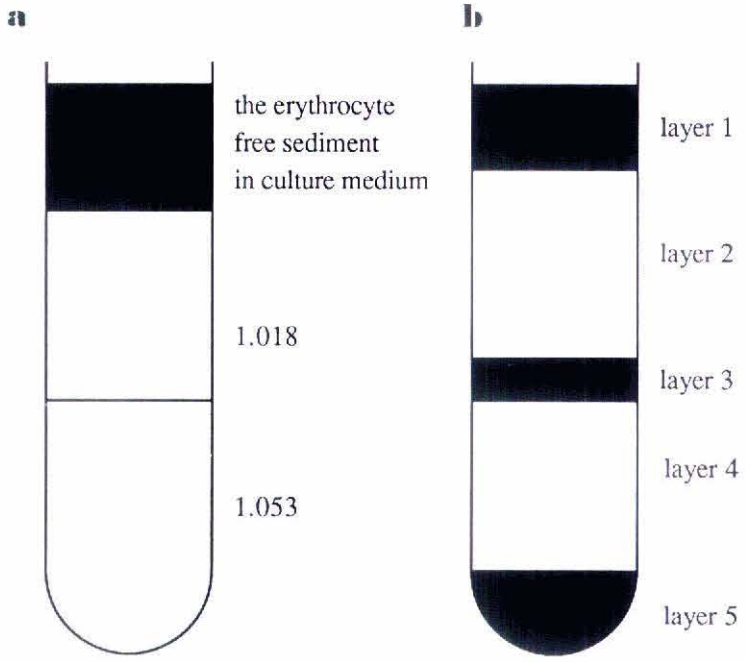

Fig. 2. Density-gradient centrifugation, showing a tube gradient (densities, 1.018 and 1.053 g/ml) and sediments after centrifugation (20 min at 3,600 *g* and 4°C) resulting in five different layer.

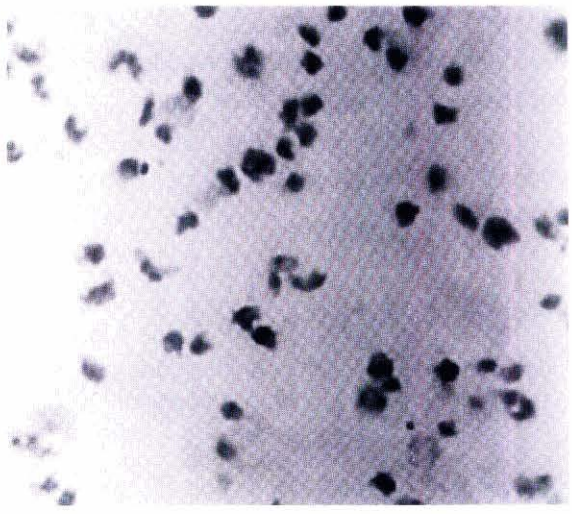

Fig. 3. Micrographs of purified merozoites stained with Giemsa solution.

Centrifugation of released parasites on Percoll density-gradients resulted in five distinct layers (Fig. 2). The first layer was on the top gradient solution, consisting mainly medium, and the second layer of consisted of 1.018 g/ml Percoll solution. The third layer was formed at the interface between 1.018 and 1.053 g/ml density and contained free merozoites without contamination of lysed

erythrocytes membrane (Fig. 3). The sediment at the base of the tube contained most of uninfected erythrocytes and debris of erythrocytes membrane. Electron microscopic study showed that the merozoites retained their intact ultrastructure (Fig. 4).

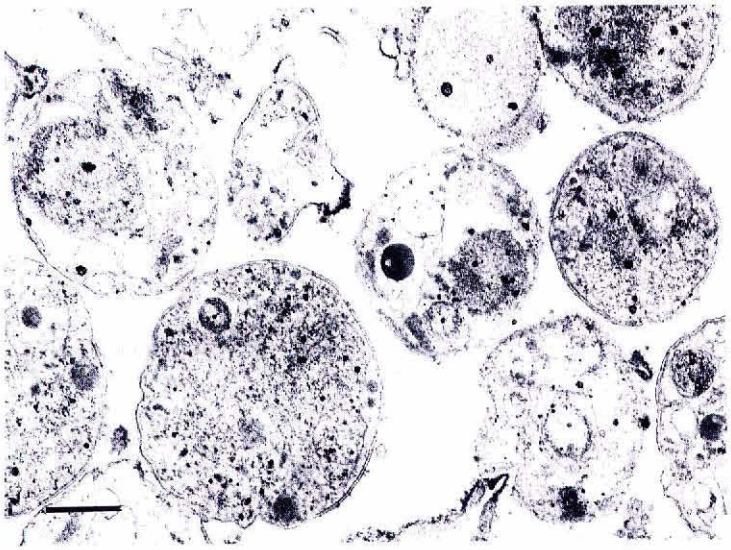

Fig. 4. Electromicrographs of purified merozoites. Merozoites retain their intact ultrastructure. Bar= 500 nm.

The analysis of the purified merozoites by SDS-PAGE is shown in Fig. 6. In the protein pattern of the purified merozoites (Fig. 5, lane 1), no obvious bands corresponding to the protein bands of erythrocyte membrane (Fig. 5, lane 2) were detected.

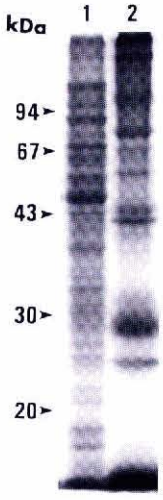

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining of proteins of purified merozoites and erythrocyte membranes. Lane 1, merozoites purified from ammonium chloride solution-treated erythrocytes by Percoll density-gradient centrifugation. Lane 2, equine erythrocyte membrane. Approx. 5 μg protein was applied to each lane. Molecular mass markers are indicated on the left in kilodalton (kDa).

DISCUSSION

In the present study, the combination of two centrifugation on Percoll

gradient and the hemolysis with ammonium chloride was developed for the isolation of merozoites without contamination of erythrocyte membrane. Enrichment of *B. caballi*-infected erythrocytes using Percoll gradient was reported by Bhushan et al. (1991) and they obtained more than 95% infected erythrocytes. We also got similar enrichment of *B. caballi*-infected erythrocytes in the present study. Centrifugation of parasites on Percoll gradient was repeated to remove debris of erythrocyte membrane from merozoites after hemolysis with ammonium chloride. Resultant merozoites were free of erythrocyte membrane and kept normal ultrastructure, confirmed by light microscope and SDS-PAGE analysis, and electron microscopy, respectively. Centrifugation of infected erythrocytes on Percoll gradient did not affect infectivity of Percoll-isolated parasitized erythrocytes for in vitro culture, perhaps due to it's low osmolality (Bhushan et al. 1991).

The treatment of infected erythrocytes with ammonium chloride for release of merozoites was also known to give less damage to merozoites and easier to carry out compared to the treatment with hemolysin (Sugimoto et al. 1991), or other methods such as using French press (Martin et al. 1971; Hamburger and Kreier 1980). Therefore, this isolation method of merozoite may not impair the biological activity of the parasites as suggested for *Theileria sergenti* by Sugimoto et al. (1991), although we did not examine the viability of the merozoites collected in this study. In conclusion, the purified merozoites by this method may be useful and applicable for the further studies on *B. caballi* in biological, biochemical and immunological aspects, including parasite invasion to erythrocytes, production of monoclonal antibodies, antigen preparation of serological tests, protein and gene analysis of the parasites.

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