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Short communications

Enrichment of *Babesia caballi*-infected erythrocytes from microaerophilous stationary-phase cultures using Percoll gradients

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Abstract. A rapid and simple method for concentrating leucocyte-free *Babesia caballi*-infected erythrocytes from in vitro cultures is described. Infected erythrocytes amounted to at least 95% of all red cells obtained.

Babesia caballi is a pathogen dangerous to equines that occurs in most tropical and subtropical countries of the world. Using the microaerophilous stationary-phase (MASP) culture technique of Levy and Ristic (1980), Müller and Phipps (in preparation) were recently successful in establishing continuous in vitro cultures of *B. caballi*. However, the percentage of parasitized erythrocytes has generally been low, the maximum being 3.85% despite several modifications in culture conditions (Cissoko 1989). The consistently low numbers of parasitized erythrocytes obtained in vitro have prevented biochemical, immunological, and serological studies on this pathogen. Attempts to enrich infected erythrocytes by hypotonic lysis as described by Mahoney (1967) for *B. bovis* have been unsuccessful (Bhushan, unpublished data). Watkins (1962) observed that *B. caballi*-infected erythrocytes formed a layer on top of the erythrocytic sediment after centrifugation. Therefore, it became possible to exploit the difference between the densities of parasitized and non-parasitized erythrocytes to achieve enrichment of parasitized red cells.

Percoll density gradients have been used to enrich erythrocytes infected with *B. bigemina* (Vega et al. 1986), *Plasmodium falciparum* (Kramer et al. 1982), *P. ovale* and *P. vivax* (Andrysiak et al. 1986). This report describes a Percoll density-gradient method for the rapid isolation and enrichment of parasitized erythrocytes from MASP cultures of *B. caballi*.

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Materials and methods

Parasite

The United States Department of Agriculture (USDA) strain of *Babesia caballi* was used in this study.

Culture technique

Blood was collected from healthy ponies and defibrinated by glass beads. The blood was depleted of white blood cells by passing it through a column of dry Whatman CF 11 cellulose (Richards and Williams 1973). Cultures were established as described by Müller and Phipps (in preparation) using RPMI-1640 as the culture medium. The percentage of parasitized erythrocytes (PPE) was calculated from Giemsa-stained thin smears.

Density-gradient medium

An aqueous solution of Percoll (density, 1.130 g/ml) was purchased from Pharmacia, Uppsala, and diluted according to the manufacturer's instructions with 1.5 *M* sodium chloride solution and sterile distilled water to achieve densities of 1.08 (low) and 1.115 g/ml (high).

Gradient centrifugation

Samples (3 ml) of each Percoll dilution were carefully run down the sides of 12-ml test tubes to form a discontinuous gradient (Fig. 1a). Erythrocytes were harvested from cultures (0.4%–1.5% parasitized erythrocytes; Fig. 2a) and centrifuged at 750 *g*, the supernatant was removed, and the packed cell volume (PCV) was adjusted to 70% with fresh culture medium. Then, 2 ml erythrocytes (PCV, 70%) were carefully layered on top of the discontinuous gradient (Fig. 1a) and centrifuged at 500 *g* for 20 min at 20° C. The resultant bands were collected by Pasteur pipette and washed three times in culture medium to remove Percoll. Thin smears were made, stained with Giemsa's stain, and assessed microscopically.

Viability

The isolated parasitized erythrocytes and free merozoites were mixed with negative erythrocytes (dilution, 1:50, v/v), and cultured as described above.

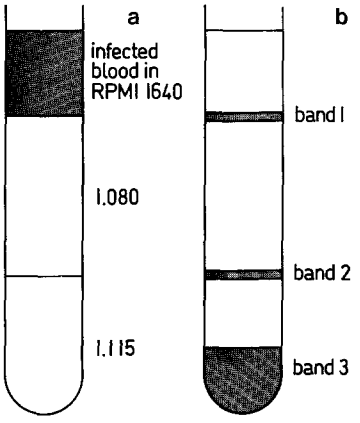

Fig. 1a, b. Density-gradient centrifugation, showing a tube with discontinuous Percoll gradient (densities, 1.08 and 1.115 g/ml) and culture layer **a** before and **b** after centrifugation (20 min at 500 *g* and 20° C) resulting in three different bands

Results

Centrifugation of cultured cells on discontinuous gradients resulted in three distinct bands: one occurred at each interface, and the third passed through these and settled at the bottom of the tube (Fig. 1b). Band one, at the interface between medium- and low-density Percoll, contained erythrocyte ghosts and a few merozoites (Fig. 2b). Band two, at the interface between low- and high-density Percoll, contained a high concentration of infected erythrocytes (95%–98%) and a large number of free merozoites (Fig. 2c). The sediment, which passed through both of these bands to settle at the base of the tube, contained most of the uninfected erythrocytes and a few parasitized red cells (Fig. 2d). Continuous cultures could be re-established using Percoll-isolated parasitized erythrocytes.

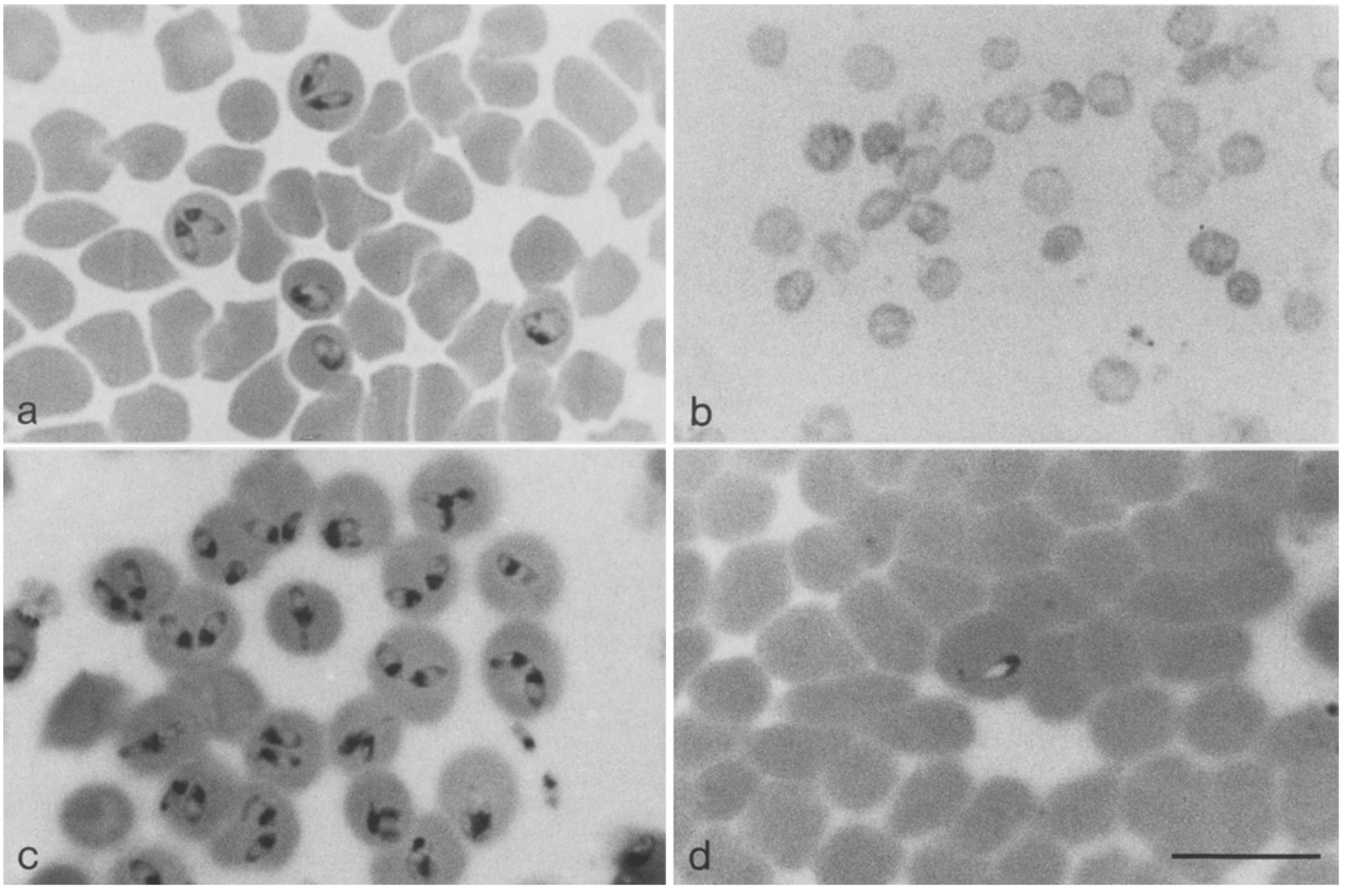

Fig. 2a–d. Enrichment of *Babesia caballi*-infected erythrocytes, showing Giemsa-stained thin smears of culture **a** before and **b–d** after density-gradient centrifugation. **b** *Band 1:* interface, medium- to low-density Percoll containing ghosts. **c** *Band 2:* interface, low- to high-density Percoll containing most of the parasitized red cells and free merozoites. **d** *Band 3:* sediment with few parasites. Bar = 10 μm

Discussion

Instead of Ficoll, Percoll was selected as the density-gradient medium because of its low osmolality (10 mOsm/kg H_2O at 1.13 g/ml for Percoll vs 130 mOsm/kg H_2O at 1.1 g/ml for Ficoll). Ficoll's high osmolality might cause osmotically induced cell damage (Pertoft et al. 1977). The hypotonic lysis described by Mahoney (1967) for concentration of *Babesia bovis* is based on differences between the osmotic fragility of parasitized and nonparasitized red cells. We were unsuccessful in enriching parasitized red cells by this method because there was little, if any, difference in their osmotic fragility. Vega et al. (1986) also could not concentrate *B. bigemina*-parasitized red cells by hypotonic salt lysis (Mahoney 1967). The technique described in the present report provides a quick and simple method of enriching parasitized cells. This separation procedure may be useful for biochemical, immunological, and serological studies on *B. caballi*.

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