Isoenzyme Patterns of Glucose-6-Phosphate Dehydrogenase, Malate Dehydrogenase and Lactate Dehydrogenase in *Babesia rodhaini* and *Babesia microti*

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ABSTRACT. Glucose-6-phosphate dehydrogenase (G6PD), malate dehydrogenase (MDH), and lactate dehydrogenase (LDH) activities and their isoenzyme patterns in *B. rodhaini* (BR) and *B. microti* (BM), the two major causative species of murine babesiosis, were examined. G6PD and LDH activities were higher in BR than those in BM, whereas MDH activity was lower in BR than that in BM. No differences were observed between BR and BM in the mobility of isoenzyme bands of G6PD and MDH. On LDH isoenzyme pattern, at least 5 bands were detected in BR, while only one band in BM. Since each subunit of LDH is known to be coded by different gene, these results suggests that BR and BM are able to be differentiated genetically.—KEY WORDS: *Babesia microti, Babesia rodhaini*, isoenzyme.

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Babesia rodhaini and *B. microti* are the major causative agents of babesiosis in mice. However, various differences have been observed in their course of infection, immune response, and their hosts' susceptibility [11]. In addition, *B. microti*, as well as *B. equi*, has been reported to proliferate in lymphocytes in its natural infection [15]. Investigations on metabolic characteristics of *Babesia* are essential for its classification as well as development of protectional methods for babesiosis. The classification in various kinds of fungi, bacteria and protozoa has been approached from the pattern of their isoenzymes coded by different genes [3–6, 12]. In this study, the patterns of isoenzymes related to glucose metabolism in *B. rodhaini* and *B. microti* were examined.

Parasites: Babesia rodhaini (Australian strain) and *Babesia microti* (Munich strain) have been maintained in our laboratory by weekly passage to 6 week-old male BALB/c mice obtained from Nippon SLC (Shizuoka, Japan).

Preparation of samples: Parasitized erythrocytes were collected from *Babesia* infected mice by cardiac puncture when percent parasitemia showed approximately 80% and 50% at 6 and 7 days after inoculation of 10^6 infected erythrocytes for *B. rodhaini* and *B. microti*, respectively. Sample preparation was performed as described by Rickard [8]. Briefly, blood collected into syringes, containing 0.1 m*l* of acid citrate dextrose (ACD solution) for each mouse, was pooled in a 15 m*l* tube and centrifuged at 1,600 g for 10 min at 4°C. Pellet was resuspended in PBS (pH 7.4) and passed through the filter (Mini Sepacell, Asahi Medical, Tokyo) to remove white blood cells. After centrifugation, erythrocytes were lysed by 0.75% NH_4Cl in Tris-HCl solution (17 mM, pH 7.65) and centrifuged at 20,000 g for 20 min at 4°C. This process was repeated 3 times. The cell pellet obtained was washed with PBS (pH 7.4, containing 5 mM ethylendiamine tetraacetic acid: EDTA) for 3 times, and mechanically ruptured by sonication with an ultrasonic disrupter (Tomy UD201, Tomy, Tokyo, Japan) at 80 W for 15 min in ice. The supernatant was separated from cell debris by centrifugation at 20,000 g for 30 min at 4°C and stored at −80°C until assay. As a control, blood was collected from clinically healthy mice and processed in the same way mentioned above.

Enzyme assay: The activity of glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49.) was determined by the method of Lohr and Waller [7]. One m*l* of sample was mixed with 1.9 m*l* of 50 mM Tris-HCl buffer (pH 7.5, containing 0.4% EDTA) and 0.05 m*l* of 2.5% β-nicotinamide adenine dinucleotide phosphate (NADP; Sigma, St. Louis, MO. U.S.A.) in 1% $NaHCO_3$, and incubated at 25°C for 5 min. Then, 0.05 m*l* of 1.3% D-glucose 6-phosphate (disodium salt; Sigma) was added and the increase in optical density at 340 nm was measured with a model 200–20 spectrophotometer (Hitachi Ltd., Tokyo, Japan) for 5 min. The malate dehydrogenase (MDH; EC 1.1.1.37.) activity was determined by the method of Bergmeyer and Bernt [2]. Briefly, 2.5 m*l* of 0.1 M phosphate buffer (pH 7.4, containing 41.8 mM aspartate) was mixed with 0.05 m*l* of 66 mM 2-oxoglutarate (Wako Pure Chemical), 0.05 m*l* of 12.1 mM β-nicotinamide adenine dinucleotide (reduced form: NADH; Sigma), and incubated at 20°C for 5 min. The decrease of optical density at 340 nm was measured for 5 min just after the addition of 0.1 m*l* of sample solution. The lactate dehydrogenase (LDH; EC 1.1.1.27.) activity was measured with a commercial kit (LDH Monotest, Boerhinger Mannheim, Mannheim, Germany) using the Video Chemistry System 600 (Nisshin Koki, Tokyo, Japan).

Protein assay: Total protein in the samples was measured by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, U.S.A.) using bovine albumin as the standard.

Isoenzyme electrophoresis: Native polyacrylamide gel electrophoresis was carried out on a computer-controlled electrophoresis unit (Phast-System, Pharmacia Biosystems AB, Uppsala, Sweden) using commercial gels (Native-page homogenous 20, MW 10–250 KD, Pharmacia Biosystems AB). Preelectrophoresis condition was 400 V, 10.0 mA and 2.0 W for 10 Vh. One microliter of sample was applied to each track in the cathodal position on preelectrophoresed gels. The concentrating conditions was 400 V, 1.0 mA and 2.0 W for 2 Vh and the electrophoresis condition was 400 V, 10.0 mA and 2.0 W for 170 Vh.

Staining of gels: The staining solution for G6PD activity

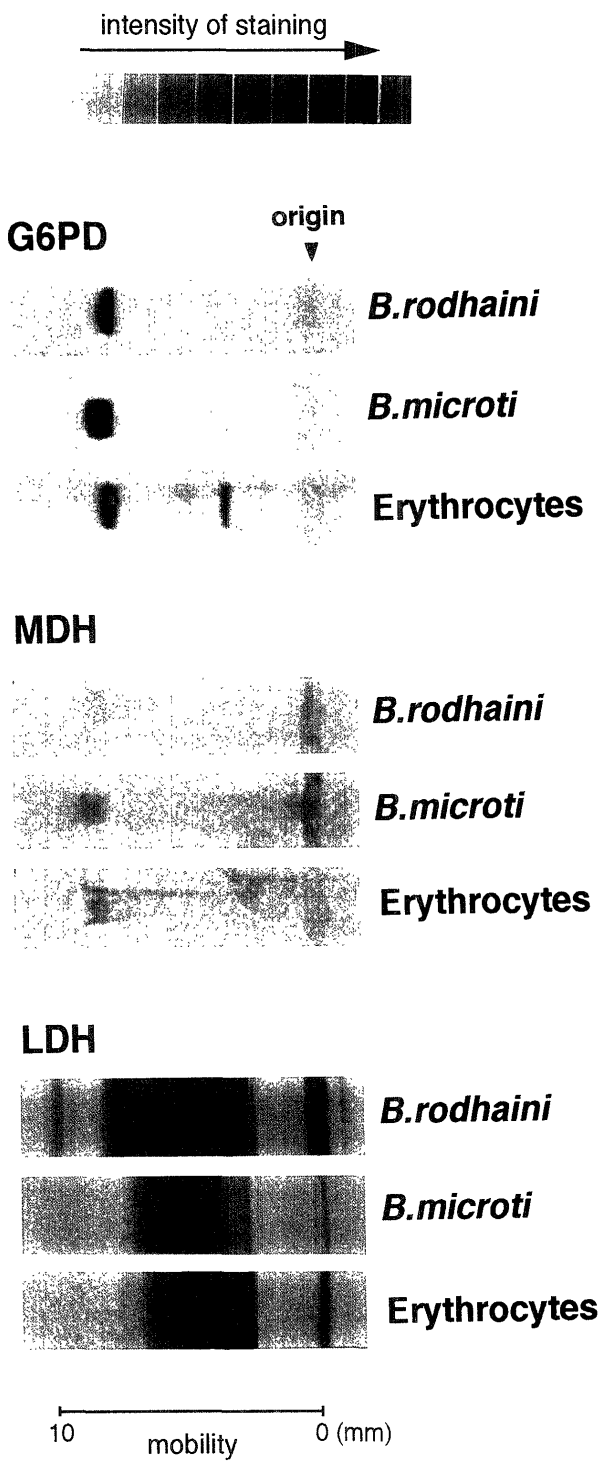

Fig. 1. Isoenzymograms of G6PD, MDH, and LDH in *B. rodhaini* and *B. microti*.

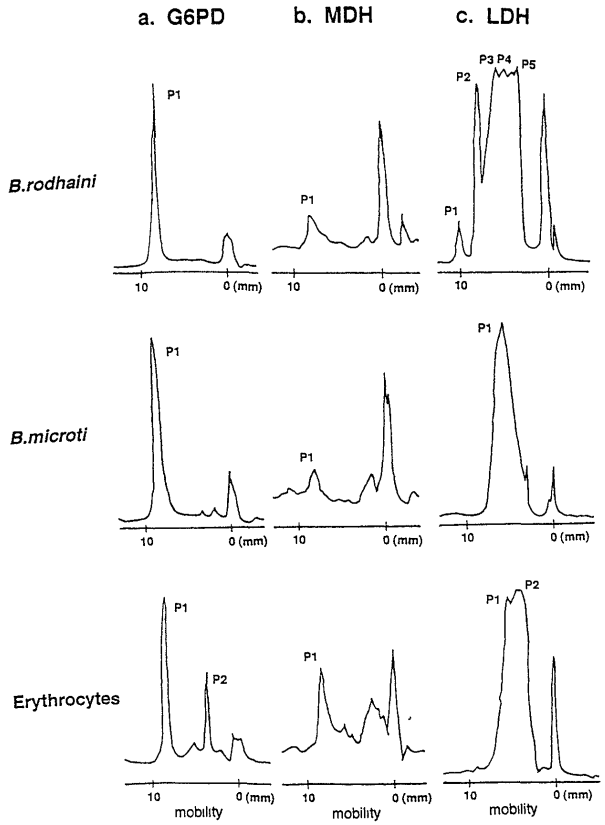

Fig. 2. Isoenzyme patterns of G6PD, MDH, and LDH in *B. rodhaini* and *B. microti*.

contained 100 mg of D-glucose 6-phosphate (Sigma), 20 mg of NADP, 20 mg of nitroblue tetrazolium (NBT), and 4 mg of phenazine methosulfate (PMS), in a total volume of 100 m*l* of 50 mM Tris-HCl buffer (pH 7.4). The staining solution for MDH activity contained 1,755 mg of malic acid (Sigma), 225 mg of β-nicotinamide adenine dinucleotide (NAD), 6 mg of PMS, and 50 mg of NBT in a total volume of 110 m*l* of 50 mM Tris-HCl buffer (pH 7.3). The staining solution for LDH activity contained 50 mg of L(+) lactic acid sodium salt (Sigma), 20 mg of NBT, 50 mg NAD, and 4 mg PMS in 100 m*l* of 50 mM Tris-HCl buffer (pH 8.0). Gels were incubated in these staining solutions protected from light at 37°C until the bands of enzyme activity fully developed. Gels were scanned on a laser densitometer (Phast Image, Pharmacia Biosystems AB). The relative intensity of the staining of the bands were represented as different colors which was shown in Fig. 1. Isoenzyme peaks were described according to the intensity and the mobility of the bands (Fig. 2).

Enzyme activities: The activities of G6PD and LDH in *B. rodhaini* (4.38×10^4 and 5.10×10^5 U/mg protein, respectively) were higher than those in *B. microti* (2.43×10^4 and 3.43×10^5 U/mg protein, respectively). On the other hand, MDH activity in *B. microti* (25.8×10^5 U/mg protein) was 3 times higher compared with *B. rodhaini* (7.82×10^5 U/mg protein) (Table 1). Various enzyme activities related to glucose metabolism such as hexokinase, LDH, and MDH have been examined in *B. rodhaini*. Rickard [8] suggested that energy production of *B. rodhaini* depends mainly on Embden-Meyerhoff pathway, the anaerobic glucose metabolism, according to the difference in utilization rate of various carbohydrate

Table 1. Enzyme activities (IU/mg protein) in *B. rodhaini* and *B. microti*

	B. rodhaini	*B. microti*	Erythrocytes
Glucose-6-phosphate-dehydrogenase	4.38×10^4	2.43×10^4	3.08×10^4
Malate dehydrogenase	7.82×10^5	25.8×10^5	1.35×10^5
Lactate dehydrogenase	5.10×10^5	3.43×10^5	2.15×10^5

substrates. On the other hand, Schmatz [10] suggested that the major energy source of *Eimeria tenella*, a protozoon belonging to Sporozoea like *Babesia,* was mannitol cycle according to the utilization of mannitol and high activity of mannitol-1-phosphatase. Our results showed that MDH activity in *B. microti* was remarkably higher than that in *B. rodhaini*. In addition, LDH and MDH activities in *B. rodhaini* showed similar levels as those observed by Rickard [9], whereas in *B. microti* MDH activity was 8 times higher than LDH activity. Since lipid metabolism is not considered to be essential in *Babesia* because of the lack of lipid droplet in this organism under electron microscopy [13, 14], *B. microti* might depend on TCA cycle indicating aerobic pathways for its glucose metabolism.

Isoenzyme patterns: Isoenzyme patterns of G6PD, MDH and LDH in each samples on a homogenous gel are shown in Figs 1 and 2. The major bands (P1) of G6PD revealed almost same mobilities in *B. rodhaini, B. microti* and control erythrocytes. The activities of MDH were also detected as a single band (P1) in both *Babesia* species at similar mobilities. No differences were observed between *B. rodhaini* and *B. microti* in the mobility of the isoenzyme bands of both G6PD and MDH. However, a remarkable difference was observed in LDH isoenzyme patterns between the two species. At least five bands were detected in *B. rodhaini* at 10.19 mm, 7.78 mm, 5.63 mm, 4.77 mm and 3.22 mm in mobilities, whereas only a single major band (P1) was observed in *B. microti* at 5.54 mm. Erythrocytes showed 2 bands at 4.89 mm and 3.63 mm. In addition, one (P2) of these 5 bands in *B. rodhaini* exhibited approximately a double in molecular weight of another band (P1) in estimation by means of molecular markers, suggesting the presence of a dimer that has been observed in other mammalian tissues [1]. Since each of the subunits that comprise LDH isoenzymes is known to be coded by different gene, the difference of LDH isoenzyme patterns observed between *B. rodhaini* and *B. microti* suggested that these species were able to be differentiated genetically.

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