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Virulence attenuation of *Babesia gibsoni* by serial passages *in vitro* and assessment of the protection provided by the immunization against the passaged isolate in dogs

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ABSTRACT

The virulence of the *Babesia gibsoni* Oita isolate was attenuated by serial passages *in vitro* by using the microaerophilus stationary phase (MASP) technique. After 400 serial passages, the virulence of the isolate was found to be attenuated. This was evidenced by the response of two dogs inoculated intravenously with 10^9 *B. gibsoni* passaged isolate. Specific antibodies were produced at a titer of 1:20,480, as detected by the fluorescent antibody test (IFAT). These results suggested that the serial passages of *B. gibsoni* reduced its virulence while retaining its antigenicity.

The dogs that were inoculated with the attenuated isolate (1 and 2) and two naïve dogs (3 and 4) were challenged by intravenous inoculation of 2×10^8 infected erythrocytes of the virulent Oita isolate. Protection afforded by exposure to the attenuated isolate was evidenced by a lower parasitemia in dogs 1 and 2 with a rapid decrease to nondetectable levels, accompanied by a slight decrease in the PCV that returned to normal values. Dogs 3 and 4 developed typical acute clinical signs, including severe anemia and hyperthermia. These results suggested that the attenuated isolate was a candidate for live vaccine.

1. Introduction

Babesia gibsoni causes hemolytic anemia, hyperthermia and marked splenomegaly in dogs, which occasionally exhibit as severe clinical signs or prove to be fatal (Boozer and Macintire, 2003; Farwell et al., 1982; Yamane, 1995). While several drugs produced a reduction in parasitemia, they did not eliminate the parasite and drug resistance appeared to develop in dogs (Birkenheuer et al., 2004; Jefferies et al., 2007; Sakuma et al., 2009). A vaccine against *B. gibsoni* is not yet commercially available, although several researchers have attempted to develop this vaccine (Fukumoto et al., 2009; Ohgitani et al., 1990; Sunaga et al., 2002b). Vaccination is considered helpful in controlling the outbreak of clinical disease.

Attenuated isolates of *Babesia bovis* have been used as vaccines against babesiosis in cattle and have met with considerable success in the tropics and subtropics (de Waal and Combrink, 2006). Various ways of reducing the virulence of *Babesia* species have been studied. Rapid passage of *B. bovis* in splenectomized calves has been shown to attenuate virulent isolates (De Vos et al., 1982; de Waal and

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Combrink, 2006), and irradiation of *B. bovis* infected blood was attempted with variable results (Wright et al., 1980). Continuous *in vitro* cultures of *B. bovis* (Levy and Ristic, 1980) and *B. bigemina* (Vega et al., 1985) were developed in the early 1980s and were a great achievement in babesiosis research. Long-term *in vitro* cultivation of *B. bovis* produced an avirulent population of the parasite, and the inoculation in cattle induced no clinical babesiosis but resulted in the production of specific antibodies (Yunker et al., 1987). This was the first proof of *in vitro* attenuation and the protection showed by subsequent challenge. Parasites cultured *in vitro* in Argentina successfully produced the first bivalent *Babesia* vaccine (Mangold et al., 1996). Therefore, continuous *in vitro* cultivation of *B. gibsoni* was considered to be a potential method to attenuate virulence.

This study aimed to assess the virulence attenuation and immunogenicity of the isolate after 400 serial passages using the MASP (Levy and Ristic, 1980) culture technique and to assess the protective efficacy of the attenuated isolate in dogs in response to challenge with virulent Oita isolate.

2. Materials and methods

2.1. Experimental animals

Five female beagles, 1–2 years old, were used in the experiments. Prior to the experiments, indirect fluorescent antibody test and microscopic examination of Giemsa-stained blood smears were carried out to confirm that the dogs were free of natural *B. gibsoni* infection. The animals received a standard amount of food daily, and drinking water was provided *ad libitum*. All the experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Azabu University. Two dogs (1 and 2) were the experimental animals that received the attenuated inoculum and two dogs (3 and 4) served as the controls. The fifth animal served as a donor dog to supply normal blood for parasite cultures.

2.2. B. gibsoni parasites

The Oita isolate of *B. gibsoni* was isolated from the blood of a naturally infected dog in Oita Prefecture, Japan and has been maintained at our laboratory by passage through dogs, and has retained its virulence for dogs (Sunaga et al., 2002a). The attenuated isolate was gained by 400 passages of Oita isolate *in vitro*.

2.3. Culture

The culture medium was RPMI-1640 (GIBCO) supplemented with 25 mM HEPES (N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)), 2 mM L-glutamine, 1 mM pyruvic acid, 24 mM $NaHCO_4$, penicillin G at 100 units/ml and, streptomycin at 100 μg/ml, include 7.5% dog serum in medium formulation. Blood to supply normal dog erythrocytes was collected from the donor dog with a heparinized syringe. Whole blood was washed in RPMI1640 medium, three times by centrifugation at 700 × *g* for 10 min at 4 °C and buffy layer were removed. The washed erythrocytes was added to 2 volume of culture medium with all components and stored at 4 °C until use. Normal serum was prepared from blood collected from the donor dog and stored at −20 °C until use. Cultures of the Oita isolate of *B. gibsoni* were maintained in the laboratory following previously described protocols (Sunaga et al., 2002a). Every 24 h, 1 ml of the culture supernatant was removed without disturbing the sedimented erythrocyte layer, 10 μl of the sedimented cells was carefully removed to make a smear, and the medium was replaced. The percent of parasitized erythrocytes (PPE) were monitored by microscopic examination of the Giemsa-stained smear at 1000× under oil immersion. The PPE was determined by counting the number of the infected erythrocytes per 1000 erythrocytes on a slide. Subcultures were prepared every 3 days. After 1 ml of the supernatant medium was removed, the sedimented cells were resuspended and 330 μl of the suspension was transferred to a well containing 1.33 ml fresh medium and 330 μl normal dog erythrocyte suspension. The cultures were then incubated in an atmosphere containing 5% CO_2 at 37 °C. We cultivated Oita isolate until 400 passages.

2.4. Inoculation of the attenuated isolate and challenge procedures (Table 1)

The attenuated isolate was tested for virulence and immunogenicity by inoculation of 10^9 infected erythrocytes in 1 ml into dogs 1 and 2. The inoculum was collected from the suspension on day 3 of cultivation at 400 passages. The erythrocytes in the inoculum were washed three times in 3 volume of PBS by centrifugation at 700 × *g* for 10 min at 4 °C. The infected erythrocytes were adjusted to 10^9 ml^{-1} and inoculated intravenously. The dogs were observed for a period of 60 days after inoculation. The rectal temperature was recorded daily. Blood samples were obtained every 2 days to monitor the PPE and PCV. Serum samples were collected before inoculation and every 4 days to monitor the humoral response.

On day 60 after dogs 1 and 2 were inoculated with the attenuated isolate, dogs 1 and 2 and dogs 3 and 4 were each challenged by inoculation with 2×10^8 erythrocytes infected with the virulent Oita isolate intravenously. The dogs were monitored as described above for a period of 40 days after the challenge. Serum samples were collected prior to challenge and every 4 days to monitor the humoral response.

2.5. Indirect fluorescent antibody technique (IFAT)

Antigen slides were prepared from blood samples from dogs with 10%PPE that had been infected experimentally with virulent Oita isolate. The infected blood was centrifuged three times in 3 volume of PBS, at 700 × *g* for 10 min at 4 °C, and the buffy coat was removed. Following the final wash stage the pellet was diluted with 2 volume of PBS. Subsequently, infected erythrocytes were smeared on glass slides, air dried, and fixed with acetone at −20 °C for 15 min, and stored at −20 °C until use. The fixed antigen slides taken from storage at −20 °C were covered by PBS at room temperature for 10 min, dried, and