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Development and evaluation of serodiagnostic assays with recombinant BgSA1 of *Babesia gibsoni*

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ABSTRACT

Indirect ELISA, dot-ELISA and double antibody sandwich ELISA (DAS-ELISA) using truncated recombinant BgSA1 (rBgSA1) were developed for detecting *Babesia gibsoni* infection in naturally infected dogs. Truncated BgSA1 gene of 858 bp, encoding 32 kDa protein was cloned in pET-32a(+) expression vector, expressed in *Escherichia coli* and the recombinant protein was purified under native conditions. To evaluate the ability of the truncated rBgSA1as serodiagnostic reagent for *B. gibsoni* infection, a panel of sera/plasma samples from dogs infected with *B. gibsoni* ($n = 13$), uninfected sera ($n = 13$) and sera from dogs infected with other haemoparasites namely, *Babesia canis vogeli* ($n = 3$), *Ehrlichia canis* ($n = 3$), *Hepatozoon canis* ($n = 1$) and *Dirofilaria immitis* ($n = 1$) were used. Besides these, 75 samples collected from dogs suspected for babesiosis were used to evaluate the performance of rBgSA1 based serological assays in comparison to nested PCR. Based on the results, the diagnostic sensitivity of indirect ELISA, dot-ELISA and DAS-ELISA were 97.3%, 91.9% and 100%, respectively, when nested PCR was taken as a reference test, while their specificities were 81.6%, 84.2% and 97.4%, respectively. Further, DAS-ELISA had a quantitation limit of 0.03 μg/ml of the rBgSA1. High kappa values of indirect ELISA, dot-ELISA and DAS-ELISA were recorded, indicating that these assays had substantial to almost perfect agreement at 95% confidence level. There was no cross-reactivity with sera from dogs infected with *B. canis vogeli*, *E. canis*, *H. canis* and *D. immitis*. The results suggest that the indirect ELISA, dot-ELISA and DAS-ELISA with rBgSA1 may be used in large scale epidemiological surveys and clinical diagnosis of *B. gibsoni* infection in dogs. DAS-ELISA has advantages over indirect or dot-ELISA in the detection of current infection as well as monitoring the parasite burden.

1. Introduction

Canine babesiosis caused by *Babesia gibsoni*, a small intra-erythrocytic apicomplexan parasite, is endemic in almost all parts of Asia, Europe, Africa, America and Australia (Irwin, 2010) and may cause hyperacute, acute and more commonly, chronic infection in dogs. The chronic form of infection is mostly asymptomatic and the infected dogs remain as carriers.

Conventionally, *B. gibsoni* infection is diagnosed by demonstration of intra-erythrocytic piroplasms in stained blood smears. But in subclinical or latent infections, when parasitemia is very low, this may be very difficult (Goo et al., 2008). In such circumstances, molecular techniques like polymerase chain reaction (PCR) (Ano et al., 2001; Birkenheuer et al., 2003; Fukumoto et al., 2001) and serological tests using native or recombinant antigens have proved to be useful for diagnosis of the disease (Aboge

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et al., 2007a; Goo et al., 2012; Jia et al., 2007; Zhou et al., 2006).

Various *B. gibsoni* merozoite antigens *viz.* BgP12, BgP22, BgP32, BgP45, BgP47, BgP50, BgP57, BgTRAP, BgAMA1, BgSA1, BgSA3 (Aboge et al., 2007a,b; Fukumoto et al., 2001; Goo et al., 2008, 2009; Jia et al., 2006, 2009; Zhou et al., 2006) have been evaluated for serodiagnosis of *B. gibsoni* infection in dogs. Among these, rBgSA1 is highly specific to *B. gibsoni* and has a proven efficacy in serodiagnostic assays in both acute and chronic stage of infection (Jia et al., 2006). Additionally, secretary nature of this molecule in circulation during invasion of the host cell has prompted its use as an antigenic marker in immunodiagnostic assays. Present study evaluated the diagnostic efficacy of rBgSA1 (truncated) antigen in indirect-ELISA, dot-ELISA and double antibody sandwich ELISA (DAS-ELISA) for the immunodiagnosis of *B. gibsoni* in naturally infected dogs.

2. Materials and methods

2.1. Parasite

A German Shepherd dog naturally infected with *B. gibsoni* and having parasitemia of 13.5% (as determined by examination of Giemsa-stained blood smears under light microscope) served as the source of parasites (Shidharthanagar isolate, India). Blood was collected in EDTA from the dog and stored at −80 °C till further use.

2.2. cDNA synthesis

Total RNA was isolated from *B. gibsoni* infected blood using Trizol reagent following manufacturer's (Invitrogen, USA) recommendations and subsequently cDNA was synthesized using oligo dT primer following the standard protocol described in RevertAid H Minus first strand cDNA synthesis kit (Thermo Scientific, USA).

2.3. Cloning, expression and purification of BgSA1 gene

The pET-32a(+) expression vector (Novagen, USA) containing an open reading frame (ORF) encoding His-tag and thioredoxin (Trx)-fusion protein was used for cloning of BgSA1 gene. Oligonucleotide primers, i.e., BgSA1e-F containing *Bam*HI restriction enzyme site (5′-ACTAGGATCCGCAAGTGCAATGCTATTCCTCGC-3′) and BgSA1e-R containing *Hind*III restriction enzyme site (5′-GTACAAGCTTCGCAGAGAATGATGCCTCCTTCG-3′) were designed using published sequence from GenBank (AB246895) and used for amplification of truncated BgSA1 gene of 858 bp (778–1635 nucleotides); encoding 32 kDa truncated protein (243–528 amino acids). The truncated BgSA1 gene was PCR amplified from cDNA in a 25 μl reaction mixture containing 0.2 μl of Dream *Taq* and *Pfu* polymerase blend (15:1), 10 pmol of each primer and 0.2 mM concentration of each dNTPs for 35 cycles (94 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min). The PCR product was gel purified (Qiagen kit), digested with *Bam*HI and *Hind*III restriction enzymes, and cloned into pET-32a(+) and expressed as thioredoxine (Trx) fusion protein in BL21 (DE3). Recombinant protein expression was induced with 1 mM IPTG and the same was purified using Ni-NTA agarose (Qiagen, Germany) following the protocol described in QIAexpressionist™ manual (Qiagen). Similarly, rTrx protein was also expressed and purified.

2.4. Raising of anti-rBgSA1 immune sera

A New Zealand white rabbit (6 weeks old) was immunized subcutaneously with 500 μg of purified rBgSA1 protein emulsified in equal volume of Freund's complete adjuvant (Difco Laboratories) for priming. Two booster doses with 250 μg of same antigen emulsified with equal volume of Freund's incomplete adjuvant (Difco Laboratories) were injected subcutaneously on days 14 and 28. Mice anti-rBgSA1 serum was also raised in two adult Swiss albino mice (4–5 weeks old), immunized subcutaneously with 200 μg of purified rBgSA1 protein emulsified in equal volume of Freund's complete adjuvant for the first injection. For boosting on days 14 and 28, 100 μg of same antigen emulsified with equal volume of Freund's incomplete adjuvant was used. The hyperimmunized rabbits and mice were bled 7 days after the last immunization and serum samples were stored at −20 °C until use. The same procedure was followed for preparation of anti-Trx rabbit and mouse sera.

2.5. Western blot analyses

Immunoreactivity of rBgSA1 was assessed by Western Blot analysis using diluted (1:50) *B. gibsoni* positive and negative dog sera and subsequently probed with goat anti-dog IgG-HRP conjugate (Bethyl, USA). Western blot was also performed with rBgSA1 and mice sera immunized against *B. gibsoni* infected dog erythrocyte lysate and subsequently probed with goat anti-mouse IgG-HRP conjugate (Bethyl, USA) as described previously by Goo et al. (2009).

For identification of native BgSA1 and circulating BgSA1 protein in blood stream, *B. gibsoni* infected and uninfected dog erythrocyte lysate and positive and negative dog plasma samples were analyzed by Western blot as described previously (Terkawi et al., 2011; Zhou et al., 2006). *B. gibsoni* infected and uninfected dog erythrocyte lysate were prepared as previously described by Goo et al. (2009). The membrane was probed with anti-rBgSA1 rabbit serum (1:50) and developed with DAB substrate solution after incubation with goat anti-rabbit IgG-HRP conjugate (1:2000 dilution).

2.6. Sera, plasma and blood samples

Canine serum/plasma samples used in the present study included 13 positive samples from dogs naturally infected with *B. gibsoni*, 13 negative samples from healthy dogs reared in tick free shed of Animal Nutrition Division, Indian Veterinary Research Institute, having no history and clinical signs of canine babesiosis, 3 samples each positive for *Babesia canis vogeli* and *Ehrlichia canis* and 1 sample each of the *Hepatozoon canis* and *Dirofilaria immitis* infected dogs. In addition, 75 blood samples along with their corresponding sera or plasma from dogs suspected for *B. gibsoni* infection and showing signs of fever, lethargy, anaemia and anorexia were also included. All these blood samples were