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Development of recombinant BgP12 based enzyme linked immunosorbent assays for serodiagnosis of *Babesia gibsoni* infection in dogs

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ABSTRACT

Indirect ELISA and dot-ELISA using recombinant BgP12 (rBgP12) were developed for the diagnosis of *Babesia gibsoni* infected dogs. The complete open reading frame of BgP12 gene (378 bp) was cloned in pET-32a(+) expression vector and expressed in *Escherichia coli* as a soluble thioredoxin (Trx) fusion protein. The purified rBgP12 was used for production of anti-rBgP12 rabbit serum, which recognized a native 12-kDa protein in *B. gibsoni* infected erythrocyte by Western blot analysis. To evaluate the potential of rBgP12 for the serodiagnosis of *B. gibsoni*, a panel of serum/plasma samples from dogs infected with *B. gibsoni* ($n = 13$), uninfected sera ($n = 13$) and sera from dogs infected with other haemoparasites *viz.*, *Babesia canis vogeli* ($n = 3$), *Ehrlichia canis* ($n = 3$), *Hepatozoon canis* ($n = 1$) and *Dirofilaria immitis* ($n = 1$) were used in ELISA formats. In addition, the performance of rBgP12 based indirect ELISA and dot-ELISA were evaluated using 75 serum/plasma samples collected from suspected dogs, in respect to the nested PCR as reference test. The diagnostic sensitivities of indirect ELISA and dot-ELISA were 94.59% and 89.18%, respectively, while their specificities were 84.21% and 81.57%, respectively. Moreover, both the assays using rBgP12 showed no cross reaction with sera from dogs infected with other common haemoparasites indicating their high specificity. High kappa values of indirect ELISA and dot-ELISA indicated the potentials of these assays with substantial agreement at 95% confidence level. It is concluded that indirect ELISA and dot ELISA using rBgP12 might be used in large scale epidemiological surveys and clinical diagnosis of *B. gibsoni* infection in dogs.

1. Introduction

Babesia gibsoni, an intra-erythrocytic tick borne apicomplexan protozoon, causes babesiosis in dogs. Apart from transmission through ticks, there is evidence that *B. gibsoni* infection may also be transmitted through dog bites and blood transfusion as well as *via* transplacental route to the developing foetus (Stegeman et al., 2003; Fukumoto et al., 2005). Since the discovery of *B. gibsoni* by Patton (1910) in India, the parasite has been found to be associated with infection of dogs throughout the world (Conrad et al., 1991; Casapulla et al., 1998; Muhlnickel et al., 2002). The acute form of the disease is typically associated with remittent fever, progressive anaemia, lethargy, thrombocytopenia, haemoglobinuria, marked splenomegaly and hepatomegaly (Wozniak et al., 1997; Goo et al., 2008). Apart from the acute form, chronic infection of *B. gibsoni* without apparent clinical manifestation is frequently encountered and diagnosis at this stage is very difficult (Irwin, 2010). Treatment of *B. gibsoni* infection is difficult and a fatal recrudescent infection can also occur even after treatment. All these factors make this protozoon a real threat to canine health.

Diagnosis of *B. gibsoni* is classically made by light microscopic demonstration of intra-erythrocytic parasites in Giemsa-stained blood smears. But in subclinical or latent infection, diagnosis by parasitological examination is difficult due to its poor sensitivity (Goo et al., 2008). Polymerase chain reaction (PCR) has been used with good sensitivity and specificity (Ano et al., 2001; Fukumoto et al., 2001) to detect early or carrier infections (Birkenheuer et al., 2003). But this method is limited by its requirement of precision equipments and ability of screening large number of samples in survey. Alternatively, serological tests like immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) with whole parasites or native antigens have been proven to

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be useful for the diagnosis of chronic infection and in field surveys but they have limited applicability due to poor quality of antigens and cross reactivity (Aboge et al., 2007b).

Of late, various recombinant merozoite antigens of *B. gibsoni viz.* BgP12, BgP22, BgP32, BgP47, BgP50, BgP57, BgTRAP, BgAMA1, BgSA1, BgSA3 have been evaluated for their sero-diagnostic potential using ELISA and immunochromatographic tests (ICT) with promising results (Aboge et al., 2007a,b; Goo et al., 2008, 2009, 2012; Jia et al., 2006, 2007, 2009a; Mandal et al., 2014; Zhou et al., 2006). The use of recombinant antigens rather than crude parasite antigen reduces the chance of cross-reactivity with antibodies raised against other parasites. Among these, BgP12 has been previously reported as a potent immunodiagnostic candidate regarding its uniqueness to *B. gibsoni* and efficacy in serodiagnostic assays without any cross reactivity in both acute and chronic infections (Goo et al., 2009). However, this protein was evaluated for its diagnostic potential using indirect ELISA in experimental set-up only and its ability to detect infection in naturally infected dogs has never been evaluated. Moreover, indirect ELISA lacks the necessary portability for field use. In this context, dot-ELISA plays an important role in diagnosis under field conditions because it is a simpler and cheaper immunoassay readable to the naked eyes. Dot-ELISA has been developed for diagnosis of many important protozoan diseases (Mancianti et al., 1996; Vercammen et al., 1998; Pinheiro et al., 2005). Keeping this in mind the present study was designed to evaluate the serodiagnostic potential of rBgP12 in both indirect ELISA and dot-ELISA formats for diagnosis of *B. gibsoni* in naturally infected dogs.

2. Materials and methods

2.1. Parasites

Whole blood collected in EDTA, from a German Shepherd dog (Kolkata, India) naturally infected with *Babesia gibsoni* (parasitaemia of 12% as determined by examination of Giemsa-stained blood smears under light microscope) served as the source of parasites and was stored at −80 °C without any preservative till further use.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from *B. gibsoni* infected blood using Trizol reagent following the 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 and expression of BgP12 protein

The pET-32a(+) expression vector (Novagen, USA) containing an open reading frame (ORF) encoding the His-tag thioredoxin (Trx)-fusion protein and its *Bam*HI and *Hind*III restriction enzyme sites were used for cloning of the BgP12 gene. The oligonucleotide primers *i.e.* BgP12e-F containing *Bam*HI restriction enzyme site (5′-ATTCGGATCCATGAGGCTCCAGAGATT GCTC-3′) and the BgP12e-R containing *Hind*III restriction enzyme site (5′-GTGCAAGCTTTA TCACGGACGCCAGAATACC-3′) were designed using published sequence information from the nucleotide database in the GenBank (AB378695) and used for amplification of complete open reading frame of the BgP12 gene (378 bp) from cDNA in a 25 μl reaction mixture containing 0.2 μl of the Dream *Taq* and P*fu* polymerase blend (15:1), 10 pmol of each primer and 0.2 mM concentration of each dNTPs for 35 cycles (95 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min). The resulting PCR product was gel purified (Qiagen kit, Germany) and digested with *Bam*HI and *Hind*III restriction enzymes and cloned into the pET-32a(+) expression vector. The recombinant BgP12 protein was expressed as thioredoxin (Trx)-fusion protein in the BL21 (DE3) *Escherichia coli* cells and the expression was induced with 1 mM IPTG. The recombinant protein was purified using Ni-NTA agarose (Qiagen, Germany) following the protocol described in the QIAexpressionist™ manual (Qiagen). Similarly, the recombinant Trx protein was also expressed and purified.

2.4. Preparation of rabbit anti-rBgP12 immune sera

A New Zealand white rabbit (6 weeks old) was immunized subcutaneously with 500 μg of purified rBgP12 protein solution emulsified in equal volume of Freund's complete adjuvant (Difco Laboratories, USA) for the first injection. Two booster doses with 250 μg of same antigen solution emulsified with equal volume of Freund's incomplete adjuvant (Difco Laboratories, USA) were injected subcutaneously into the rabbit on days 14 and 28 after the primary immunization. The hyper immunized rabbit was bled 7 days after the last immunization and serum samples were stored at −20 °C until used. The same procedure was followed for preparation of anti-rTrx rabbit sera.

2.5. Raising of hyperimmune sera against B. gibsoni infected erythrocyte lysate

Two adult Swiss albino mice of either sex weighing approximately 25–30 g each were immunized subcutaneously with 200 μg of *B. gibsoni* infected erythrocyte lysate emulsified in equal volume of Freund's complete adjuvant (Difco Laboratories, USA) for the first injection as described by Goo et al. (2009). Two booster doses with 100 μg of the same lysate emulsified with equal volume of Freund's incomplete adjuvant (Difco) were injected subcutaneously into the mice on days 14 and 28 after first immunization. The sera were separated from the heart blood of mice collected 7 days after the last immunization and stored at −20 °C for further use.

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

For determination of immunoreactivity, the rBgP12 protein was analyzed in 12% acrylamide gel by SDS–PAGE and by Western blotting using diluted (1:50) *B. gibsoni* positive and negative dog sera as previously described (Zhou et al., 2006). This analysis was also performed with rBgP12 and mice sera (1:50 dilution) immunized against *B. gibsoni* infected erythrocyte lysate and subsequently probed with goat anti-mouse IgG HRP conjugate (Bethyl, USA) as described previously (Goo et al., 2009).

For identification of native BgP12 antigen, *B. gibsoni* infected and uninfected dog erythrocyte lysates were prepared as previously described by Goo et al. (2009). *B. gibsoni* infected and uninfected dog erythrocyte lysates were analyzed using SDS–PAGE and Western blot. The membrane was probed with anti-rBgP12 rabbit serum (1:50) and developed with DAB substrate solution after incubation with goat anti-rabbit IgG-HRP conjugate (1:2000).

2.7. Sera, plasma and blood samples of dogs

Canine serum/plasma samples used in the present study included 13 positive samples from dogs naturally infected with *B. gibsoni* as identified by microscopy as well as nested PCR; 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 and were negative for *B. gibsoni* infection as revealed in repeated microscopic examination of stained blood smear and nested PCR; 3 samples each from dogs positive for *B. canis vogeli* and *Ehrlichia canis*; 1 sample each