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Short communication

C-terminal region of 48-kDa rhoptry protein for serological detection of *Babesia caballi* antibodies in horses

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ABSTRACT

A recombinant C-terminal antigen derived from *Babesia caballi* 48-kDa rhoptry protein (rBc48/CT) was made for the development of a serologically diagnostic test. Antiserum raised against the rBc48/CT reacted specifically with the corresponding native protein by Western blotting and the indirect fluorescent antibody test (IFAT). Next, an indirect enzyme-linked immunosorbent assay (Bc48/CT-ELISA) and an immunochromatographic test based on the Bc48/CT (Bc48/CT-ICT) were constructed and employed for the detection of an antibody to *B. caballi* in a variety of equine sera. The results of Bc48/CT-ELISA and Bc48/CT-ICT were highly concordant with those of IFAT and ELISA, with full-length protein of Bc48 used as the reference tests. Our results demonstrate the success of Bc48/CT as antigen for the serological diagnosis of *B. caballi* infection in horses.

Equine piroplasmosis is a febrile tick-borne disease caused by intraerythrocytic protozoa of *Babesia caballi* and *Theileria equi*. The disease is now listed by OIE (World Organization for Animal Health) and is characterized by significant morbidity and mortality worldwide [1]. *B. caballi* causes a disease characterized by fever, inappetence, malaise, persistent anemia, weight loss, and poor exercise tolerance in the susceptible animals [1–3]. Recovered animals often become carriers of the parasite and serve as the reservoirs for transmission to other animals through tick-borne transmission or mechanical transfer by biting insects, needles, or surgical instruments. The organisms are transmitted by at least ten species of the genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus* [4,5]. Economic losses due to the infection are incurred not only from animal mortality and low productivity as well as the high cost of the control measures but also through its impact on the international horse trade and movement. In addition, the interaction of racing and pleasure horses meeting during tournaments poses an important risk for the spread of the infection to free countries and thus concerns quarantine authorities [1]. Therefore, import and export regulations are required for highly specific and sensitive tests for the diagnosis of *B. caballi* infection [6].

Generally, equine piroplasmosis can be diagnosed by identification of the organisms in Giemsa-stained blood or organ smears. However, detecting the parasites from the carrier or chronically infected animals by means of blood smear examination is often difficult, inaccurate, and impractical for large numbers of examined samples. On the other hand, serologically diagnostic tests, including the complement fixation test (CFT), indirect fluorescent antibody test (IFAT), and enzyme-linked immunosorbent assay (ELISA), have been alternatively developed and prescribed for international trade [1,6,7]. Although the CFT and IFAT have been dominantly used for identifying the presence of antibodies to *Babesia* parasites, several drawbacks of these assays have been demonstrated, including their false-positive results and low sensitivity, especially for detecting latent infections [6]. ELISAs using recombinant proteins have shown high sensitivity and specificity for detecting the antibody in both acute and chronic babesial infections. However, their application has been hindered by a limited antigen supply and poor purity and specificity [6–8]. Although a competitive inhibition ELISA (cELISA) has been approved in the OIE, the assay is time-consuming and requires special laboratory equipments and facilities [1,9]. Recently, several immunochromatographic tests (ICTs) have been employed and proven to be reliable for the detection of the antibody to *Babesia* infections [10]. The ICT is simpler and more rapid and practical than the ELISAs. However, high productivity and purity of the target antigens are highly required for the preparation of ICT strips [10]. A rhoptry protein, Bc48, of *B. caballi* was earlier reported as a promising antigen for the serological detection of the *B. caballi* antibody.

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However, the low yield of recombinant Bc48 produced by a bacterial expression system has limited its application in diagnosis [11]. In the present study, the C-terminal region of Bc48 was cloned, expressed to improve the productivity of target antigen, and then validated as a diagnostic antigen in an ELISA and ICT with varieties of equine sera.

A DNA fragment encoding the C-terminal region (269–458 aa) of Bc48 (GenBank ID: BAA83725) was amplified using the gene-specific primers 5′-GGGAATTCTATAAGAAGTGGTACATGAAG-3′ and 5′-CGCTCGAGCTATTTCTTCAATAAAT-3′, subcloned into a pGEX-4T-1 vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using the *Eco*RI and *Xho*I restriction enzymes, and then expressed in *Escherichia coli* as a GST-fusion protein (Amersham Pharmacia Biotech) with a molecular mass estimated by SDS-PAGE to be 47 kDa, including an additional 26 kDa derived from the GST tag (Supplementary Data S1B). The productivity was increased at least 10-fold as compared to the full-length protein, Bc48 (Supplementary Data S1C), possibly due to the hydrophobic nature of the conserved *Babesia*-RAP-1 at the N-termini, which would lead to significant amounts of insoluble forms. An antibody raised against Bc48/CT in mice specifically reacted with *B. caballi* in Western blot analysis and the IFAT performed as previously described [12]. The size of the detected Bc48 was consistent with the expected molecular weight of the authentic protein (Supplementary Data S1D). Confocal microscopic observation of IFAT revealed the apical end localization of the Bc48 in the parasites as well as the cytoplasmic secretion into the infected erythrocytes (iRBCs) in the early stage of intracellular parasites (Supplementary data S1E). These observations were consistent with other *Babesia* rhoptry proteins that had also been found to be secreted into the cytoplasm of iRBCs shortly after/during invasion [13,14].

Next, the Bc48/CT was used as the antigen for ELISA [12], and its usefulness was then evaluated using non-infected and experimentally *T. equi*- and *B. caballi*-infected equine sera that had been kindly provided by Dr. Yoshinari Katayama (Japan Racing Association). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 50 µl of each recombinant protein at a concentration of 2 µg/ml in a coating buffer (50 mM carbonate–bicarbonate buffer, pH 9.6). The plates were washed once with 0.05% Tween 20-PBS (PBS-T) and then incubated with 100 µl of a blocking solution (3% skim milk in PBS) for 1 h at 37 °C. After washing once with PBS-T, 50 µl of the serum samples diluted to 1:100 with a blocking solution was added and incubated for 1 h at 37 °C. The plates were washed six times with PBS-T and then with 50 µl of the HRP-conjugated rabbit anti-equine IgG antibody (Bethyl Laboratories, Montgomery, TX, USA) diluted to 1:4000 with a blocking solution for 1 h at 37 °C as a secondary antibody. After washing six times, 100 µl of a substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.3 mg/ml of 2,2′-azide-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis, MO, USA), and 0.01% of 30%

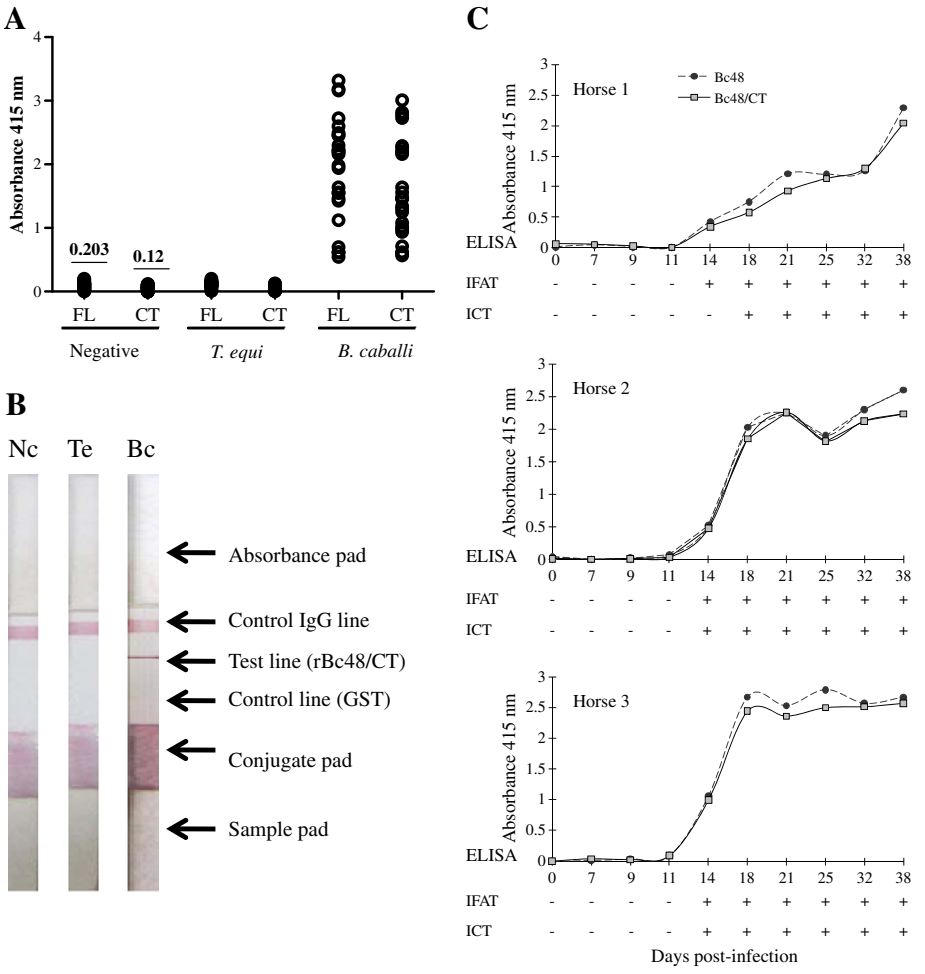

Fig. 1. Evaluation of the diagnostic performance by Bc48/CT antigen. (A) Reactivity of ELISA using recombinant Bc48 (FL) and Bc48/CT (CT) proteins with panels of non-infected, *T. equi*-infected, and *B. caballi*-infected sera. The cut-off of each recombinant protein is indicated. (B) Reactivity with Bc48/CT-ICT with different equine sera. ICT strips before (lane 1) and after (lanes 2 to 4) the test. No reaction with non-infected equine serum (lane Nc) and *T. equi*-infected serum (lane Te) was shown. Positive reaction with *B. caballi*-infected serum (lane Bc). Recombinant Bc48/CT conjugated with gold colloids was dried on the conjugate pad. (C) Comparison of the reactivity of ELISAs with Bc48 and Bc48/CT antigens, IFAT, and Bc48/CT-ICT with *B. caballi*-infected sera collected serially from three horses over a period of 38 days. All experiments were repeated twice to obtain reproducible data. The optimal concentration of the antigen was determined by preliminary experiment using four concentrations of antigen for ELISA (1, 2, 5, 10 µg/ml) and three for ICT (0.25, 0.5 and 1 mg/ml).