A Rapid Agglutination Assay for Canine Brucellosis Using Antigen Coated Beads

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ABSTRACT. Brucella canis is the causative agent of canine brucellosis and facultative intracellular pathogen. The diagnosis of canine brucellosis is based on bacteriological examination and serological methods including agglutination and gel diffusion tests. In this study, crude antigens were extracted from B. canis using hot saline, coated on to latex beads and their usefulness in the serological diagnosis of canine brucellosis was examined. Mixing the antigen coated latex beads with the sera of dogs infected with B. canis produced clear agglutination, but this was not so for B. canis free dog sera. N-terminal amino acid sequence analysis of the crude hot saline extracts, showed that they contained copper-zinc superoxide dismutase, ribose ABC transporter and hypothetical protein of Brucella as antigens. A serological survey of canine serum samples conducted by means of an agglutination test using the antigen coated latex beads, showed that this method was more specific than the tube agglutination test using whole bacterial cell antigens. Although these results suggest that our method in which crude hot saline extracted antigens are coated on to latex beads would be useful in the serological diagnosis of canine brucellosis, we need further investigation using more serum samples to confirm the usefulness of our method.

KEY WORDS: agglutination test, brucellosis, canine, diagnosis, latex beads.

Canine brucellosis is widely distributed around the world and this is an important disease due to the economic losses in animal production, and its risks for human health [8]. Reproductive disorders, such as abortions and premature births, are the clinical signals of this bacterial disease in pregnant animals. Diagnosis of the disease is based on bacteriological examination and serological tests [4]. Serological diagnosis is usually performed by the tube agglutination test, rapid slide agglutination test, and gel immunodiffusion test [4, 5, 7]. However, there is the problem that agglutination tests sometimes give false-positive reactions due to cross-reactions with other pathogens, and a general strategy for eliminating cross-reactions is to use purified antigen with unique epitopes in the serological tests. Bacterial cell wall antigens of B. canis can be prepared by hot saline extraction, and have been shown to be useful in serological diagnosis [12]. The characteristics of the several proteins, in such hot saline extracts, however, are still unclear.

This paper describes the characterization of crude hot saline extracts, including identification of the gene encoding the antigens, and provides evidence that for the usefulness of these antigens in the serological diagnosis of canine brucellosis. It also describes a serological investigation of exposure to B. canis in Japan conducted by means of an agglutination test using the latex beads.

MATERIALS AND METHODS

Antigen preparation. Brucella canis QE-13 was cultured in Brucella broth at 37°C, with shaking, and harvested during the stationary phase of growth. The bacterial cells were washed twice with saline and resuspended in saline to a final concentration of 10% (w/v). This suspension was autoclaved at 105°C for 15 min and centrifuged at 10,000 × g for 10 min to eliminate bacterial debris. The supernatant was frozen at –20°C, melted, and centrifuged at 1,000 × g for 10 min to remove the small aggregates. Next, the supernatant was dialyzed against a solution of polyethylene glycol 6000 (15%) to a 10-fold concentration and then against saline. The antigen solution was kept frozen at –20°C.

Coating of antigens on to latex beads: Equal volumes of antigen solution (protein concentration of 300 µg/ml that was measured by Lowry methods) and 1% latex beads (diameter 1 µm, Sekisui Chemical Co.) were mixed for 16 hr at room temperature with stirring. Next, the latex beads were washed three times with washing buffer (phosphate-buffered saline (PBS) containing 1% bovine serum albumin, 0.01% Tween 20, 0.1% polyethylene glycol 6000, 0.2% sucrose) and then blocked with washing buffer for 16 hr at room temperature with stirring. The latex beads were then adjusted to a final concentration of 1% (w/v) with PBS and kept at 4°C.

Agglutination test. Equal volumes (50 µl) of the latex bead solution and canine serum diluted 10-fold with PBS were mixed on a glass slide and incubated at room temperature for 15 min without shaking. Samples showing agglutination were considered to be positive.

The tube agglutination test was performed as follows. Heat-inactivated B. canis QE-13 whole-cell antigens were obtained from Kitasato Laboratories. Equal volumes (0.5
ml) of the whole-cell antigens (optical density of 0.8 at 450 nm) and serum, which had been serially diluted 2-fold with PBS were incubated at 50°C for 24 hr. Agglutination titers were determined from the final dilution of serum showing 50% agglutination. Samples showing a titer higher than 160 were considered to be positive [3].

Canine sera: Serum samples (n=318) were randomly selected from dogs consecutively admitted to animal hospitals in central Japan by hospital staff in 2006. B. canis infected sera were collected from PCR positive dogs, and B. canis free sera were collected from SPF dogs.

SDS-PAGE and Western blotting: The antigen solution was separated using 10% SDS-PAGE and then transferred to Immobilon-P membranes (Millipore). The efficiency of transfer was determined using Coomassie brilliant blue R-250 and then the membranes were tested for reactivity with antibodies in B. canis infected sera that were collected from PCR positive dogs. Protein bands were cut out from the membranes and the N-terminal amino acid sequences were analyzed at TAKARA Biotechnology Co.

PCR: Blood samples were collected from tube agglutination test positive dogs, and then DNA specimens prepared using a QIAamp DNA Blood Kit (QIAGEN) for the PCR amplification assay to detect B. canis. Specific primer sequences were designed to detect the virB2 gene for Brucella spp. [6, 13].

RESULTS

Latex bead agglutination test: We coated latex beads with antigens from a hot saline extract of B. canis with the aim of achieving a serodiagnosis method that is faster and easier to perform. B. canis infected serum from dogs, which infection had been confirmed by the tube agglutination test and PCR, was mixed with the antigen coated latex beads and this mixture incubated at room temperature for 15 min. Agglutination was clearly observed (Fig. 1). On the other hand, B. canis free dog serum showed no agglutination (Fig. 1).

When the latex bead agglutination test was conducted for twelve different B. canis infected or non-infected dog sera, we obtained the same results.

Analysis of hot saline extracted antigens: The antigens extracted with hot saline were analyzed by Western blotting with dog serum infected with B. canis. In the separation of proteins by SDS-PAGE, four protein bands reacted with the serum (Fig. 2). Antigen 3 (Ag 3 in Fig. 2) showed high immuno-reactivity and another three antigens showed similar reactivity. Although a further protein band at around 10 kDa reacted with the serum, this band was not separated completely judging by Coomassie blue staining. To identify the genes encoding the antigens, their N-terminal amino acid sequences were analyzed and the homologous proteins searched on a database. The results are shown in Table 1. Three proteins could be analyzed and corresponded with GenBank’s Brucella genome database, but one of the proteins (antigen 2) could not be analyzed.

DISCUSSION

The tube agglutination test with whole B. canis antigens has been used to diagnose brucellosis in dogs in Japan [11]. However, nonspecific reactions occur in the tube agglutination test using whole bacterial cell antigens as well as in the

![Fig. 1. Latex bead agglutination test. B. canis free (A) and infected (B) sera were mixed with beads coated with antigen from a crude hot saline extract. Agglutination is clearly observed in panel B.](image)
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A rapid slide agglutination test [2]. With the aim of developing a serological diagnosis method that is easier to perform, latex beads were coated with antigens extracted by hot saline for use in an agglutination test. Though crude hot saline antigen extracts had been used previously for a gel immunodiffusion test [12], the antigens contained in the extracts had not been clearly identified. In the present study, we were able to detect four antigens and three could be identified. Since Copper-zinc superoxide dismutase (Cu-Zn SOD) is a known antigenic protein of \textit{Brucella abortus} and there is also Cu-Zn SOD in \textit{B. canis} [1], its potential value as a vaccine for brucellosis prevention and diagnostic reagent for the disease has been investigated [9, 10]. This suggests that purified Cu-Zn SOD could be a useful antigen for the specific serological diagnosis of canine brucellosis.

The other antigens, ribose ABC transporter (antigen 1) and hypothetical protein (antigen 4), have not previously been reported to be \textit{B. canis} antigens. Since these proteins show lower reactivity than Cu-Zn SOD against \textit{B. canis} infected dog serum, it is unclear whether, as purified single proteins, they would be suitable for the serological diagnosis of brucellosis or not. Thus the antigenic properties of these newly identified antigens need to be properly characterized. Since the N-terminal of antigen 2 was blocked, its amino acid sequence could not be determined by our method.

The cross-reactivity between \textit{B. canis} and other pathogens in serological tests is still not fully understood [2]. To investigate the specificity of crude hot saline antigen extracts in this respect, an enzyme-linked immunosorbent assay (ELISA) was performed with the above antigens. Unexpectedly, the ELISA reactions with the crude hot saline antigen extracts for both \textit{B. canis} infected and non-infected dog sera were similar (data not shown). This suggests that the crude hot saline extracts were not specific antigens and therefore not useful with a highly sensitive serodiagnosis method such as ELISA. Since the latex bead agglutination test is a less sensitive method than ELISA, we consider that the results obtained with the latex bead agglutination test clearly show the difference between positive and negative.

In surveys of canine brucellosis performed in the same area as that investigated in this study in 1977 and 1989, 2.5 and 1.5\% of the dogs were positive, respectively [11, 12]. Since our results show an increase in the percentage of positive dogs for brucellosis as compared to the previous surveys, we should continue to investigate the seroprevalence of \textit{B. canis} in dogs in order to monitor for epidemics of canine brucellosis in Japan.

Our latex bead agglutination test is user-friendly, needs no special equipment and requires little or no technical skill. The result is obtained within 15 min and the test can be performed under field conditions. Although our results show that the latex bead agglutination test is useful in the serological diagnosis of canine brucellosis, more studies are required in order for it to be approved as a diagnostic tool for canine brucellosis. This will involve testing a large number of serum samples from areas where there are brucellosis epidemics and those that are brucellosis free, as well as a large-scale testing of samples from bacteriologically positive dogs.

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REFERENCES