Purification of a Brucella canis Cell Wall Antigen by Using Immunosorbsorbent Columns and Use of the Antigen in Enzyme-Linked Immunosorbsorbent Assay for Specific Diagnosis of Canine Brucellosis

TADA SERIKAWA,¹ SHUJI IWAKI,² MASAYUKI MORI,¹ TAKEHIKO MURAGUCHI,³ AND JUNZO YAMADA⁴

Institute of Laboratory Animals, Faculty of Medicine, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606,¹ and Aburabi Laboratories, Shionogi Research Laboratories, Shionogi Seiyaku Co. Ltd., Gotanda, Koka-cho, Koka-gun, Shiga 520-34,² Japan

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A cell wall antigen of Brucella canis was purified by immunosorbent columns. The antigen contained two proteins of 30 and 28 kilodaltons and a polysaccharide exhibiting a 12-kilodalton band upon 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Antibody to the purified antigen, which specifically reacted with the polysaccharide, was used as the first coating antibody in an enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of canine brucellosis. Dogs inoculated orally with live B. canis were positive and dogs from B. canis-free colonies were negative in the ELISA. Of 199 dogs from a brucellosis-contaminated area, 116 with negative titers in the tube agglutination test (TAT), using heat-inactivated whole B. canis cells as the antigen, were also negative in the ELISA. Seventy-eight of the dogs with questionable titers in the TAT were divided into two groups: 20 dogs that were positive in the ELISA and 58 that were negative. Of five dogs with positive titers in the TAT, three were positive in the ELISA and the gel immunodiffusion test (GD) with crude B. canis extract as the antigen and were also culture positive for B. canis. One dog was positive in the ELISA and GD but gave a negative culture result. Serum from the remaining dog, which was positive with high titer in the TAT but negative in the ELISA and in culture for B. canis, formed a spur precipitate with a homologous precipitate in the GD. These results indicate that the ELISA is a specific serological test for B. canis infection in dogs.

Brucella canis causes epizootic breeding failure in dogs: abortion in females and epididymitis, prostatitis, and testicular atrophy in males (2). Diagnosis of the disease is based on bacteriological examination and serological tests (2, 7, 12, 13). Serological diagnosis is usually performed by the tube agglutination test (TAT), rapid slide agglutination test, and gel immunodiffusion test (GD) (7, 8, 11). The agglutination tests, however, sometimes give false-positive reactions. Since false-positive have been shown to be associated with 2-mercaptoethanol-sensitive agglutinins, 2-mercaptoethanol has been added to reaction mixtures (1, 7). However, this modified test method did not adequately improve specificity (3, 14), which indicated that the false-positive sera contain some cross-reactive antibodies. Indeed, it has been reported that some organisms, such as Actinobacillus equuli and certain mucoid strains of Pseudomonas aeruginosa and Staphylococcus sp., cross-react with B. canis (3, 4), although most cross-reactive organisms have not yet been identified.

One general strategy for eliminating the cross-reaction would be to use purified antigen with unique epitopes in the serological tests. B. canis is of the nonsmooth (rough) type, and the antigenicity is apparently different from that of smooth-type Brucella species. The rough-specific antigen is known to relate to the composition of lipopolysaccharide complexes that constitute the principal surface antigen (4) and can be extracted with hot saline as a protein complex (6). In fact, when soluble extracts from B. canis were used as an antigen in the GD, a common precipitate was formed with antibodies against a nonsmooth Brucella strain (3, 6, 10, 14). On the other hand, antibody to mucoid-type P. aeruginosa, one of the cross-reactive organisms, formed a spur precipitate with a homologous B. canis antibody (3). This finding suggests that the cell wall antigen of B. canis probably has a unique antigenic determinant that is common in nonsmooth Brucella strains and different from the determinants of other genera. Dogs are usually not infected with Brucella ovis and other nonsmooth brucellae. If an antigen with the specific epitope were separated from B. canis, a specific serological test could be developed. In this study, a cell wall antigen from B. canis was separated by affinity chromatography with the antigen-specific antibody, and an enzyme-linked immunosorbent assay (ELISA) for detecting canine antibodies to the antigen was developed.

MATERIALS AND METHODS

Animals. A colony of 68 and a second colony of 98 beagle dogs free from canine brucellosis were used as negative controls for B. canis infection. Beagle dogs orally inoculated with live B. canis were obtained from a B. canis-free colony. A total of 199 pound dogs for a survey of canine brucellosis were obtained from Gifu prefecture, Japan. Rabbits for immunization were purchased from a commercial breeder.

Bacteria. B. canis K76-620, used for preparation of inoculum and antigens, was isolated from a spontaneously infected dog and stocked in a lyophilized state. The organisms were cultured twice in tryptic soy agar or broth (Eiken Chemical Co., Ltd., Tokyo, Japan) before use. B. ovis 63/290, kindly provided by Y. Isayama, National Institute of Animal Health, Hokkaido, Japan, was cultured in tryptic soy agar or broth containing 5% fetal calf serum in an atmosphere of 10% CO₂.

TAT. Heat-inactivated B. canis QE-13 whole-cell antigens were purchased from Kitasato Laboratories, Tokyo, Japan.
A 0.5-ml amount of serum serially diluted twofold with phosphate-buffered saline (PBS) (pH 7.4) was incubated with the same volume of the B. canis antigen, with an optical density of 0.8 at 450 nm, for 48 h at 50°C. Agglutination titers were determined with the final dilution of serum showing 50% agglutination.

GD. B. canis and B. ovis antigens for the GD were crude hot physiological saline (HS) extracts and crude hot physiological saline extracts subjected to sonication (HS-S extracts). B. canis antigens were also prepared by immunosorbent columns. HS-S extracts were obtained as follows. Brucella cells were dispersed in physiological saline with a concentration of 10% (wt/wt), heated for 15 min at 105°C in an autoclave, and sonicated with an ultrasonic cell disruptor (UR 200 P; Tomy Seiko Co. Ltd., Tokyo, Japan) for 10 min at output control 4. The supernatant obtained after centrifugation at 10,000 × g for 10 min was frozen at −20°C, melted, and centrifuged at 1,000 × g for 10 min to remove the small aggregates. The supernatant was concentrated to 1/10 the original volume by dialysis against a concentrated solution of polyethylene glycol 6000, and the antigen concentrate was dialyzed against PBS (pH 7.4). HS extracts were obtained by the same procedure without sonication. Immunodiffusion was performed in 1.2% purified agar (Difco Laboratories, Detroit, Mich.) in PBS (pH 7.4). The antigens and test sera were placed in a suitable well. The agar plates were incubated at room temperature in a moist environment overnight, washed thoroughly in physiological saline, dried, stained with Coomassie brilliant blue R-250, and destained with acetone.

Isolation of B. canis. A 1-ml sample of blood obtained aseptically was inoculated into tryptic soy broth. Cut surfaces of retropharyngeal lymph nodes obtained aseptically under anesthesia were embedded in tryptic soy agar and cultured for 1 week at 37°C aerobically. B. canis was identified by biochemical and immunological methods previously reported (12).

Antiserum. Rabbits were intradermally injected with antigens emulsified in Freund incomplete adjuvant (Difco) on days 0, 14, and 28. Rabbits were exsanguinated on day 38. Blood was allowed to clot at room temperature, and serum was collected and stored at −80°C.

Affinity chromatography. Cyanogen bromide-activated Sepharose 4B (Pharmacon Fine Chemicals, Piscataway, N.J.) was prepared according to the instructions of the manufacturer. A 20-mg amount of ammonium sulfate-precipitated immunoglobulin G (IgG) from B. canis-infected canine serum or hyperimmunized rabbit serum was used to prepare the column. B. canis extracts were run over the column, and fractions were collected. After the column was sufficiently washed with PBS (pH 7.4), bound fractions were eluted with glycine hydrochloride (pH 2.3) and neutralized with 1 M Tris hydrochloride. Each 3-ml fraction was monitored by A280 for protein and by the GD for antigenicity.

SDS-polyacrylamide gel electrophoresis. Stacking and separating gels consisted of 5 and 12.5% acrylamide, respectively. Samples were incubated at 100°C for 2 min in 0.05 M Tris buffer (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 1% dithiothreitol, and 10% glycerol. Electrophoresis was carried out with a 12- by 8- cm mini slab gel apparatus (Atto Laboratories, Tokyo, Japan) at a constant current of 10 mA per gel. Gels were resolved with Coomassie brilliant blue R-250 or a silver staining kit (Bio-Rad Laboratories, Richmond, Calif.). Molecular weight was estimated with a low-molecular-weight calibration kit (Bio-Rad) and insulin from bovine pancreas (Sigma Chemical Co., St. Louis, Mo.).

The insulin consisted of two subunits with a molecular weight of about 6,000 (5).

Electroblotting and enzyme immunoassay. A modification of the Western blot (immunoblot) procedure was used for electroblotting. Soluble brucella antigens were electrophoresed on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose on a Bio-Rad transfer blot apparatus according to the instructions of the manufacturer. The efficiency of transfer was determined by silver staining after transfer. The nitrocellulose sheets were tested for reactivity with antibody to B. canis. The sheets were washed with Tris-buffered saline containing 0.05% Tween 20, shaken, incubated with a 1:500 dilution of rabbit anti-B. canis IgG, and shaken for 1 h at room temperature. The sheets were washed three times as described above and shaken for 1 h at room temperature with a 1:3,000 dilution of goat anti-rabbit IgG (heavy and light chains) antibody conjugated to horseradish peroxidase (Organon Teknika, Malvern, Pa.) in Tris-buffered saline plus Tween. The sheets were washed three times for 5 min each in Tris-buffered saline plus Tween and rinsed three times in distilled water. Horseradish peroxidase color development reagent (4-chloro-1-naphthol) in 0.03% H2O2 in Tris-buffered saline was added to the sheets, and they were shaken for up to 15 min. The reaction was stopped by washing the sheets in distilled water, and the sheets were dried.

ELISA. A two-antibody sandwich-type ELISA for detecting brucella antibody in dogs was performed in polyvinyl microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). The first antibody was IgG purified from hyperimmunized rabbits with purified B. canis antigen and coated by incubation with a 100-μl volume containing 1.6 μg overnight at 4°C. After blocking with 5% bovine serum in 0.1 M NaCl and 1 mM MgCl2, a 10% dilution of HS-S extract from B. canis was incubated for coupling specific antigen to the first antibody. The stock HS-S extract had an antigenic titer forming a specific precipitate up to a 1:32 dilution in the GD, using a positive reference serum with a TAT titer of 640. Diluted canine sera were incubated for 1 h at 37°C. Goat antiserum to rabbit IgG (heavy and light chains) conjugated to horseradish peroxidase was purchased from Organon Teknika. A405 was determined after 1 h of incubation with substrate [2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid diammionium salt in 0.03% H2O2] with a dual-wave-length ELISA reader (Colonna Laboratories, Ibaragi, Japan), and A450 was automatically subtracted. All test sera from experimentally infected dogs, dogs in beagle colonies, and field dogs were simultaneously assayed with the 1:40-diluted dual samples, and each ELISA value was shown with the mean optical density.

Analysis for carbohydrate. Carbohydrate was stained by the periodate-Shift reaction on a nitrocellulose membrane sheet blotted with B. canis antigens. 2-Keto-3-deoxyoctonate of lipopolysaccharide was determined by the method by Karkhanis et al. (9).

RESULTS

Purification of specific cell wall antigen. Infected dog serum confirmed to contain antibody to specific B. canis cell wall antigen was used to prepare the first affinity column. The canine serum used formed a common precipitate with the HS and HS-S extracts of B. canis and B. ovis in GD (Fig. 1) but not after preincubation of the serum with live B. canis. After B. canis HS-S extract (antigen A) was applied into the affinity column, adsorbed materials were eluted with acid of
VOL. 27, Bovis antigens and B. canis antibodies in the GD. Wells: 1, B. canis pH 2.3. cated either that cell wall rabbit with the GD was applied was pH (for 3R 4, antigen; antiserum against antigen A); 2, B. canis HS antigen; 3, B. HS-S B. (pH 7.4) applied was completely and heated, to remove protein was prepared. Antigen A was applied to the second column coupled with hyperimmunized antibody to antigen B was prepared. Antigen A was applied to the second column to remove other antigens. Unbound fractions were monitored by A_{280} for protein and assayed by the GD, using antiserum to antigen A for the specific antigen. Fractions 12 to 14 (antigen C) formed only the specific precipitate (Fig. 2). Rabbit serum hyperimmunized with antigen C formed only the specific precipitate in the GD with antigen A and also formed precipitates with B. ovis HS and HS-S extracts (Fig. 1). The third affinity column was prepared with the specific antibody, and antigen A was applied to the column. Bound antigens were eluted with acid and neutralized. The neutralized eluates (antigen D) did not form a precipitate with antigen A in the GD.

**Electrophoretic, chemical, and immunological analyses of specific cell wall antigens.** B. canis HS-S extracts (antigen A) and the other three antigens (B, C, and D) prepared by affinity chromatography were electrophoretically analyzed by 12.5% SDS-polyacrylamide gel electrophoresis. Two sets of four samples each were stained with Coomassie blue and silver, respectively; the third set was electrotransferred to a nitrocellulose sheet and then visualized by enzyme immunoassay, using antibody to antigen C. Antigens C and D each

**FIG. 1.** Comparison of precipitates formed between B. canis or B. ovis antigens and B. canis antibodies in the GD. Wells: 1, B. canis HS-S antigen (antigen A); 2, B. canis HS antigen; 3, B. ovis HS antigen; 4, B. ovis HS-S antigen; 5, positive reference canine serum (for 3R 4, antigen; antiserum against antigen A); 2, B. canis HS antigen; 3, B. HS-S B. (pH 7.4) applied was completely and heated, to remove protein was prepared. Antigen A was applied to the second column coupled with hyperimmunized antibody to antigen B was prepared. Antigen A was applied to the second column to remove other antigens. Unbound fractions were monitored by A_{280} for protein and assayed by the GD, using antiserum to antigen A for the specific antigen. Fractions 12 to 14 (antigen C) formed only the specific precipitate (Fig. 2). Rabbit serum hyperimmunized with antigen C formed only the specific precipitate in the GD with antigen A and also formed precipitates with B. ovis HS and HS-S extracts (Fig. 1). The third affinity column was prepared with the specific antibody, and antigen A was applied to the column. Bound antigens were eluted with acid and neutralized. The neutralized eluates (antigen D) did not form a precipitate with antigen A in the GD.

**FIG. 2.** Affinity chromatography of crude B. canis antigen (antigen A), using a Sepharose 4B column coupled with rabbit IgG against nonspecific B. canis antigens (antigen B). Results show no precipitate (O), specific precipitate (●), and other precipitates (▲) in the GD.

**FIG. 3.** Silver staining (panel 1) and Western blot analysis (panel 2) after SDS-polyacrylamide gel electrophoresis of B. canis antigens. Lanes A, B, C, D are antigens A, B, C, and D, respectively. Molecular size standards are shown by the numbers (in kilodaltons) on the left.
produced three bands after silver staining (Fig. 3). Although bands a and b were also very slightly stained with Coomassie blue, band c was not detected by the staining method. In addition, antigens A, C, and D dot blotted on a nitrocellulose membrane in a 5-μl volume were positively stained by the periodic acid-Schiff stain, but B was negative even in a 20-μl volume. 2-Keto-3-deoxyoctonate was detectable in all four antigens, although the concentration was not determined because the antigens were too small to be weighed. From these results, the three bands in antigens C and D were estimated to represent two proteins with molecular weights of about 30 and 26 kilodaltons and a polysaccharide (12-kilodalton band), respectively. In the enzyme immunoassay using antibody to antigen C, the polysaccharide (band c in Fig. 2) in lanes of antigens A, C, and D was specifically stained.

**Evaluation of ELISA in dogs orally inoculated with *B. canis***

The purified antigen was difficult to collect in large quantity and was relatively unstable; that is, aggregates were easily formed in the stock. The two-antibody sandwich system was therefore used in the ELISA for detecting the canine antibody. Concentrations of rabbit anti-purified *B. canis* antigen IgG, *B. canis* crude antigen, and horseradish peroxidase-conjugated goat anti-rabbit IgG (heavy and light chains) antibody were preliminarily determined by ordinal box titrations. ELISA was performed as described in Materials and Methods.

Beagle dogs from a *B. canis*-free colony were inoculated orally with 3 × 10⁶ live *B. canis* cells. Serum antibodies detected by the TAT, GD, ELISA, and blood culture were monitored weekly. Titters obtained by the TAT changed to 40 from less than 20 at week 3 after inoculation and increased to 1,280 at week 7 (Fig. 4). *B. canis* cells were detected in blood samples from week 3. ELISA values rose to more than 0.45 in week 3 and to 1.9 in week 8.

**TAT and ELISA in *B. canis*-free dogs.** Results of the TAT in beagle dogs from two colonies free of canine brucellosis are summarized in Fig. 5. Although all 68 dogs in colony A showed titters of 40 or less, 3 and 1 of 98 dogs in colony B showed questionable titters of 80 and 160, respectively. Although the titters of dogs in colony B were checked seasonally, similar results were obtained, and no *B. canis* was detected in any of the blood samples examined. In addition, there were no prominent cases of breeding failure.

In the ELISA, all of the 68 dogs in colony A and 94 dogs in colony B had values of less than 0.32. The remaining four dogs, with titters of 20 to 80 in the TAT, showed values of between 0.32 and 0.45. One sample with a titer of 160 in that TAT had a low value of 0.18.

**Survey of pound dogs obtained from an area contaminated with canine brucellosis.** The results of assays for serum antibodies to *B. canis* by TAT, GD, ELISA, and cultures of blood and lymph nodes are summarized in Fig. 6 and Table 1. All of the 116 dogs with negative titters of 20 or less in the TAT showed values of less than 0.45 in the ELISA. We determined, therefore, that the ELISA values of higher than 0.45 were positive and that values of 0.45 or lower were negative. The 78 dogs with questionable titters of 40 to 160 were divided into two groups of 58 dogs with values lower (negative) and 20 dogs with values higher (positive) than 0.45 in the ELISA. All dogs with titters of 160 or lower in the TAT showed negative results in the GD (Fig. 7) and also in cultures of blood and lymph nodes for *B. canis*. One of two dogs with titters of 320 in the TAT formed a specific precipitate in the GD (wells 1 and 3 in Fig. 8) and showed positive results in the ELISA and culture. The other dog was positive in the GD, with appearance of the same specific precipitate, and in the ELISA but not in cultures. Two of three dogs with titters of 1,280 were positive in the three tests. In the GD, one of the two dogs formed the specific precipitate and the other
demonstrated another precipitate as well (wells 2 and 5 in Fig. 7). The remaining dog was negative in the ELISA and cultures, and a spur precipitate was formed with a homologous precipitate in the GD (Fig. 8).

**DISCUSSION**

It is not yet fully understood why false-positive reactions in serological tests for *B. canis* infection appear, although some cross-reactive organisms have been proposed as the cause (3, 4). Carmichael et al. (3, 14) reported the difference in the GD between positive and false-positive sera as follows: the HS extract of *B. canis* formed one or two precipitates with the positive sera (as shown in Fig. 7), and the antigens related to the precipitates were designated 3R (the common precipitate) and 2R (the additional precipitate formed adjacent to the antigen well). Although the 2R antigen showed no false-positive reactions, positive reactions were found in some dogs infected with *B. canis*. On the other hand, the 3R precipitate was detected in all of the *B. canis*-infected dogs and not in specific-pathogen-free dogs, whereas nonspecific precipitates were detected in field dogs. When the false-positive sera were compared with the positive reference serum, the precipitate was diffuse in comparison with the homologous precipitate.

An antigen common to *B. canis* and *B. ovis*, both rough-type strains, is known to be on the cell surface, and this antigenicity determines rough specificity of the genus *Brucella* (3, 4, 6, 10, 14). *B. canis* organisms are mucoid and therefore easily autoagglutinate (8). *B. ovis*, however, is nonmucoid and is therefore used as the antigen for the rapid slide agglutination test in serological assays for *B. canis* infection (1, 8). Nonspecific reactions appeared both in the TAT, using *B. canis* as the antigen, and in the rapid slide agglutination test. Since the 3R antigen is a surface antigen common to *B. canis* and *B. ovis* and forms cross-reactive precipitates, it was thought to be the cause of the nonspecific

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*+, Positive; -, negative.

† Positive defined as optical density of greater than 0.45 at 405 nm.

‡ 3R precipitate.

§ 2R and 3R precipitates.

∥ Spur precipitate formed with a homologous precipitate.

**FIG. 7.** Immunoprecipitates detected in dogs infected with *B. canis*. Wells: 1 and 4, positive reference canine serum (for 3R precipitate); 2 and 5, positive serum (for 2R and 3R precipitates); 3 and 6, negative serum.

**FIG. 8.** Spur precipitate formed with a reference homologous precipitate in the GD. Wells: 1 and 3, positive serum of dog infected with *B. canis*; 2 and 5 (same sample), positive reference canine serum; 4 and 6 (same sample), canine serum with a cross-reactive antibody found in the survey.
reaction in the serological tests. Carmichael et al. therefore proposed that the four internal antigens are more suitable for obtaining specificity in the GD (3, 14). The positive reaction by the antigens continued for more than 10 months after recovery from the bacteremic state, but the change from a negative to a positive reaction appeared 14 to 16 weeks after oral inoculation with live cells. On the other hand, the bacteremic state started 4 weeks after the inoculation, and a positive reaction in the GD with the 3R antigen appeared at 4 to 8 weeks and continued until at least 4 months after recovery from the bacteremic state. Although the internal antigen may be considered excellent for specificity, practical use seems to be limited since the antibodies cannot be detected in the early stages of infection.

Although large quantities of purified antigen could not be obtained in this study and the antigen structure may have changed so as to be undetectable by the GD with acid treatment, two proteins and a polysaccharide containing 2-keto-3-deoxyoctonate were detected in the purified preparations, and the antibody raised in a rabbit formed the 3R precipitate in the GD. This antigen was therefore assumed to be a lipopolysaccharide-protein complex or a part of the complex containing specific epitopes of 3R antigen.

In the ELISA, the specific antibody was coated on the well and then B. canis crude antigen was applied. As a result, only antigens with the specific epitopes bound on the well. The ELISA was considered to give a specific reaction, as judged from the results with specific-pathogen-free dog colonies, experimentally infected dogs, and a survey of field dogs. One dog was positive with high titer in the TAT but negative in culture and the ELISA, and a spur precipitate formed with the homologous 3R precipitate in the GD. This result indicated that the cross-reactive antibody to the 3R antigen was not detected in the ELISA. Since the ELISA is generally more sensitive than the GD and ELISA values correlated with TAT titers in experimentally infected dogs and in the positive group surveyed, the ELISA may detect even low-positive antibodies in chronic states for which there are equivocal results in the GD. Although all samples in this study were assayed simultaneously for direct comparisons of ELISA values in experimentally infected dogs, dogs in B. canis-free colonies, and field dogs, in practical use the ELISA should be performed with positive and negative control sera; also, an optical density of 0.45 is not an absolute value discriminating between positive and negative.

Ten years ago, from April 1976 to March 1977, we surveyed the area used in this study for canine brucellosis; at that time, B. canis was isolated from 21 of 847 dogs (2.5%). In the study described here, B. canis was isolated from 3 of 199 dogs (1.5%). These findings indicate that the disease persists in field dogs if no eradication programs are used.

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