

Enzyme-Linked Immunosorbent Assay for the Detection of Canine *Leptospira* Antibodies Using Recombinant OmpL1 Protein

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ABSTRACT. OmpL1 is a 31-kDa outer membrane protein characterized in 1993 and known to be expressed only in pathogenic *Leptospira* spp. Recombinant OmpL1 (GST-rOmpL1) was expressed for use as an ELISA antigen for the detection of anti-*Leptospira* antibodies. In immunoblot analysis, the protein reacted with sera of dogs infected with three different serotypes of *Leptospira interrogans*, while did not react with sera of dogs both uninfected negative controls and infected with *Borrelia burgdorferi*, which is closely related to *Leptospira* spp. Moreover, in ELISA using GST-rOmpL1, the optical density (O.D.) values from the positive controls were very high (1.125 ± 0.549). In contrast, the O.D. values from clinically healthy dogs and dogs with diseases other than leptospirosis were very low (0.109 ± 0.046 and 0.089 ± 0.046 , respectively). These data suggest that the detection of anti-*Leptospira* antibodies by ELISA using the GST-rOmpL1 protein can be applied for diagnosis of canine leptospirosis.

KEY WORDS: canine, ELISA, leptospirosis, OmpL1, recombinant.

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Leptospira is a Gram-negative, aerobic bacteria classified into Spirochaetales; family *Leptospira*. *Leptospira* infects many mammals, and is highly pathogenic to dogs [5, 6, 11]. Methods commonly employed to make a definitive diagnosis of leptospirosis include dark-field microscopy, cultivation or polymerase chain reaction (PCR) to detect the pathogen itself from blood or urine of patients, and the latex agglutination test or enzyme-linked immunosorbent assay (ELISA) to detect specific antibodies against the pathogen in the serum [1, 7, 8, 10, 12, 14]. Detection of specific antibodies by the latex agglutination test has been most commonly used for diagnosis of canine leptospirosis. However, this method requires agglutination evaluation that lacks objectivity, and it is difficult to deal with many samples at once. In contrast, the ELISA has merits, such as high sensitivity, objectivity and the possibility of handling many samples at a time. However, the ELISA also involves handling risks because it requires the use of live bacteria for antigen preparation. The antigen is also unstable because it needs to be cultured each time. Therefore, establishment of a safer and more reproducible determination system is necessary. The Glutathione S-transferase (GST) gene fusion system available commercially is an integrated system for the expression and purification of fusion protein, and makes it possible to get stable and reproductive protein [9].

Outer membrane proteins (OMPs) seem to play an important role in pathogenicity of bacteria. OmpL1 is the 31-kDa OMP characterized in 1993 [2] and is known to be a principal target of the host immune response, expressed only in pathogenic *Leptospira* [2–4, 15]. In this study, a recombinant (r) OmpL1 protein was expressed to establish a *Lep-*

protein as the antigen.

MATERIALS AND METHODS

Recombinant plasmid construction: DNA of *Leptospira interrogans* serovar icterohaemorrhagiae (strain RGA) was isolated using the QIAamp DNA Mini kit (Qiagen, Studio City, CA). *OmpL1* DNA was amplified by PCR using forward (5'-GGATCCGTAGCACTATCTTCG-3') and reverse (5'-GAATTAGATTTGCCACCGACAAC-3') primers based on the *L. kirschneri* *OmpL1* sequence (GeneBank accession No. L13284) with *Bam*HI and *Eco*RI restriction sites, respectively. The PCR product was electrophoresed in a 1.5% agarose gel and extracted from the gel. This fragment was digested with *Bam*HI and *Eco*RI, and inserted into *Bam*HI-*Eco*RI sites of the bacterial expression plasmid pGEX6P-1 (Amersham Pharmacia Biotech, Uppsala, Sweden) in which OmpL1 was expressed as a GST fusion protein. *Escherichia coli* thioredoxin (Trx) [13] was transformed with the ligation mixture and plated onto 2xYT agar plates containing ampicillin (50 µg/ml) and chloramphenicol (20 µg/ml). To confirm that the protein-coding DNA sequence could be cloned in the proper translation frame in the vector, direct sequence analysis was performed on both strands using BigDye Terminator Cycle Sequence Ready Reaction kit (Applied Biosystems, Foster City, CA).

GST-OmpL1 fusion protein preparation: The expression plasmid was transformed into *E. coli* (Trx). As a control, pGEX6P-1 plasmid without the insert was also transformed into *E. coli* (Trx). Transformants were isolated and grown overnight at 37°C. Expression of recombinant fusion protein was induced in the culture with 0.1 mM isopropyl β-D-

thiogalactoside (IPTG) for 6 hr at 37°C. Cultures of *E. coli* (Trx) transformed were centrifuged at $4,000 \times g$ for 15 min at 4°C. Cell pellets were resuspended in 1/20 of starting volume in phosphate buffered saline (PBS, 1.37 M NaCl, 27 mM KCl and 15 mM $\text{KH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$, pH 7.2) containing 2 mM benzamidine. Lysates were sonicated on ice (4×30 sec), and centrifuged at $4,000 \times g$ for 20 min at 4°C. Then 1% (final concentration) of Triton X-100 was added and mixed gently for 30 min at 4°C. Glutathione Sepharose 4B (Amersham Pharmacia Biotech) was equilibrated with PBS three times. The bacterial sonicate was applied three times to bind the protein to the matrix. The matrix was washed with PBS three times. The GST-OmpL1 fusion protein and the control GST protein were eluted using Glutathione Elution Buffer (Amersham Pharmacia Biotech) for 10 min. The proteins were collected and stored at -80°C until use.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Samples were analyzed using a 15% polyacrylamide gel with a 5% polyacrylamide stacking gel containing 0.1% (w/v) SDS. Prior to electrophoresis, the samples were boiled for 5 min with 5% (v/v) 2-mercaptoethanol. The gels were stained with 0.5% Coomassie brilliant blue and destained in 10% methanol and 7% acetic acid.

Serum samples: Serum samples used in this study was summarized in Table 1. Positive controls were serum samples ($n=9$) from dogs with leptospirosis diagnosed based on clinical signs and the latex agglutination test (DENKA SEIKEN, Tokyo, Japan), then dark-field microscopy or necropsy, including *L. interrogans* serovar icterohaemorrhagiae infection ($n=1$), *L. interrogans* serovar autumnalis infection ($n=5$) and *L. interrogans* serovar hebdomadis infection ($n=3$). Negative controls were obtained from dogs under appropriate management without vaccination and without the influence of transitional antibodies (SPF, Fuji Animal Farm, Tokyo, $n=3$), and from 131 dogs with recorded vaccination dates brought to various animal hospitals (Sigeeda Veterinary Hospital, Hashimoto Animal Hospital, Yamamoto Animal Hospital and Leo Animal Clinic) in Yamaguchi prefecture as well as Yamaguchi University Veterinary hospital, including clinically healthy dogs ($n=72$) and patients with liver, renal, hemolytic or icteric diseases other than leptospirosis ($n=59$). To examine specificity, canine Lyme disease positive control serum (1:2,000 in TBSTM, VMRD, U.S.A.) was also used as a negative control. Furthermore, to determine the influence of *lep-*

toospira vaccination, *L. interrogans* serogroup icterohaemorrhagiae serovar copenhageni and *L. interrogans* serovar canicola (Hond Utrecht IV) vaccinations (Duramune 8, Kyoritsu Seiyaku, Japan) were carried out on experimental dogs kept in our laboratories ($n=3$) at days 1 and 29, and their sera were collected at 0-92 days after vaccination.

Immunoblot analysis of anti-OmpL1 antibodies: Purified GST-OmpL1 and GST proteins were resolved by a 15% SDS-PAGE gel, and the fractionated proteins were transferred onto Clear Blot nitrocellulose membranes (ATTO). The filters containing GST-OmpL1 and GST were immersed in Tris buffered saline (TBS, 0.05 M Tris and 0.74 M NaCl, pH 7.2) with 0.1% Tween 20 (TBST) and 3% nonfat dry milk (TBSTM) to block unoccupied binding sites, and incubated with rabbit sera against *L. interrogans* serovar icterohaemorrhagiae, *L. interrogans* serovar canicola, *L. interrogans* serovar autumnalis, *L. interrogans* serovar hebdomadis or *L. interrogans* serovar australis (1:200 in TBSTM, DENKA SEIKEN, Tokyo, Japan) overnight at 4°C in TBSTM. After three washes with TBST, final detection was obtained using alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:30,000 in TBSTM, EY LABORATORIES) as the secondary antibody and AP conjugate substrate kit (Bio-Rad Laboratories). Serum samples from confirmed *leptospira* cases ($n=9$, 1:200 in TBSTM) and alkaline phosphatase-conjugated rabbit anti-dog immunoglobulin antibody (1:30,000 in TBSTM, Kirkegaard & Perry Laboratories) were used as the positive controls, and as the negative controls, SPF dogs ($n=3$, 1:200 in TBSTM) and alkaline phosphatase-conjugated rabbit anti-dog immunoglobulin antibody (1:30,000 in TBSTM, Kirkegaard & Perry Laboratories) were used. To determine specificity, canine Lyme disease positive control serum (1:200 in TBSTM, VMRD, INC. U.S.A) and alkaline phosphatase-conjugated rabbit anti-dog immunoglobulin antibody (1:30,000 in TBSTM, Kirkegaard & Perry Laboratories) were also used.

ELISA using recombinant-antigen: Flat-bottomed polystyrene microtiter plates (Nalge Nunc International, U.S.A.) were coated at 4°C overnight with 100 μl of GST-OmpL1 fusion protein containing 0.39, 0.78, 1.6, 3.1, 6.3, 13 or 25 nM, suspended in 1 M sodium carbonate (pH 9.6) for antigen concentration determination. GST protein, as the negative control, was also coated at the same concentrations. For the measurement of serum samples, 25.0 nM of GST-OmpL1 or GST was used as the antigen. The plates were washed twice with distilled water and three times with PBS-0.05% (v/v) Tween 20 (PBST). Plates were incubated with blocking solution (PBST with 3% nonfat milk) for 1 hr at 37°C. After three washes with PBST, the wells were incubated with 50 μl of sera, diluted 50-fold in blocking solution, for 1 hr at 37°C on a plate shaker. After three washes with PBST, the wells were incubated with 50 μl of a 100-fold dilution of horseradish peroxidase-conjugated goat affinity purified antibody to dog IgG (ICN/CAPPEL) for 1 hr at 37°C. Then, plates were washed three times with PBS and incubated with 100 μl /well of ABTS Peroxidase Sub-

Table 1. Serum samples

| | n | Purpose* |
|-----------------------------------|----|----------|
| Positive controls Leptospirosis | 9 | 1, 2, 3 |
| Negative controls SPF | 3 | 1, 2 |
| Lyme disease | 1 | 1, 2 |
| Clinically healthy | 72 | 3 |
| Diseases other than leptospirosis | 59 | 3 |

*1, Confirmation of the antigenicity of GST-rOmpL1; 2, Determination of the antigen concentration for ELISA; 3, Detection of antibodies from various patients.

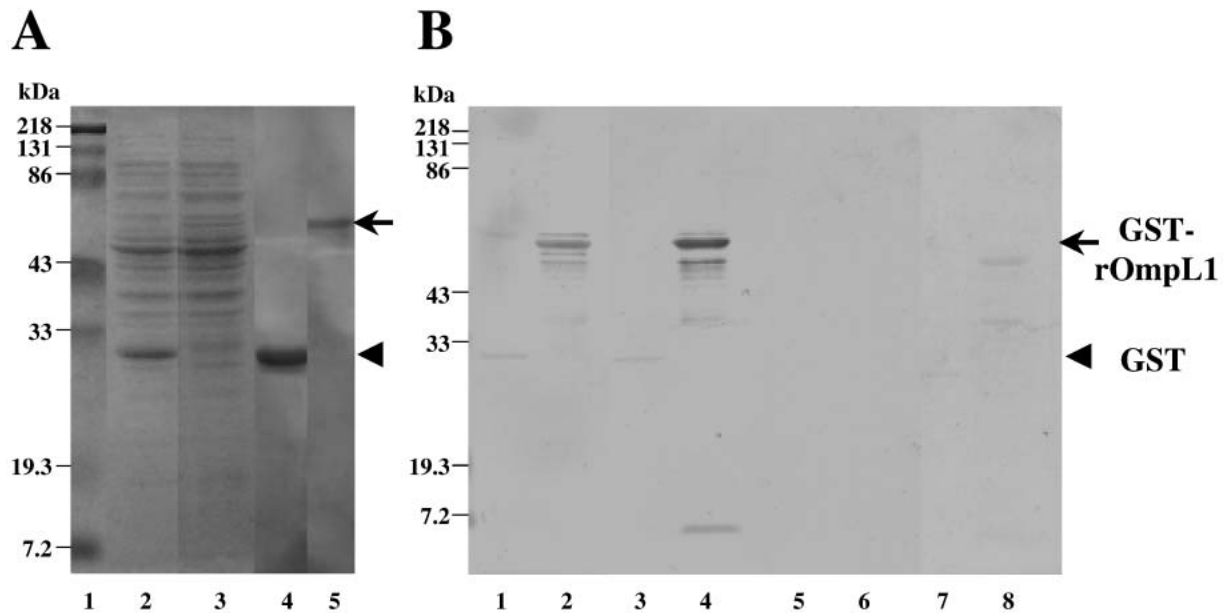


Fig. 1. Expression of GST-recombinant (r) OmpL1 protein. A, SDS-PAGE analysis. Whole cell lysates containing pGEX vector (lane 1) or pGEX-OmpL1 (lane 2), and GST (lane 3) or GST-rOmpL1 (lane 4) purified with Glutathione Sepharose 4B were electrophoresed by SDS-PAGE and the gel was stained with Coomassie brilliant blue. B, Immunoblot analysis of the recombinant protein. Affinity-purified GST (lanes 1, 3, 5 and 7) or GST-OmpL1 (lanes 2, 4, 6 and 8) was separated through 15% SDS-PAGE and immunodetected with *L. interrogans* serovar canicola antiserum (lanes 1 and 2), *L. interrogans* serovar icterohaemorrhagiae positive dog serum (lanes 3 and 4), SPF dog serum (lanes 5 and 6) or *B. burgdorferi* positive dog serum (lanes 7 and 8). Arrow heads and arrows show GST and GST-rOmpL1, respectively.

strate (Kirkegaard & Perry Laboratories, U.S.A.) for 50 min in the dark at 37°C. The optical density (O.D.) was measured using an ELISA reader (MULTISCAN Bicromatick, Lavosystem, U.S.A.) at 405 nm. The means of three wells were calculated for each analysis. For the analyses of the antigen concentrations and the influence of *leptospira* vaccination, three independent studies were performed for each experiment.

Statistics: For the determination of the antigen concentration, the Mann-Whitney *U*-test was used to compare O.D. values between GST-rOmpL1 and GST. Statistical differences in O.D. values among healthy dogs, dogs with leptospirosis, and dogs with diseases other than leptospirosis were assessed by the Kruskal-Wallis test and Dunn test. Statistical significance was defined as $p < 0.05$.

RESULTS

OmpL1 expression and purification: OmpL1 DNA (*L. interrogans* serovar icterohaemorrhagiae) amplified by PCR was ligated into the pGEX6P-1 plasmid (Amersham Pharmacia Biotech). Sequence analysis showed that the OmpL1 protein-coding sequence was in the proper translation frame in the pGEX6P-1 vector, and 299 of 320 (93.4%) amino-acid residues could be fused with GST. *E. coli* (Trx) was transformed with the recombinant vector. Under the control of IPTG, a 57-kDa protein, which was approximately the same size as the calculated GST-recombinant OmpL1

(GST-rOmpL1), was produced (Fig. 1A, lane 2 & 4). When this protein was cleaved by PreScission protease in the presence of Triton X-100, a faint band with a molecular weight of approximately 31-kDa protein was observed, but almost all the protein was found in the post-sonication pellet (data not shown).

Detection of anti-OmpL1 antibodies in canine serum: In order to confirm the antigenicity of the purified fusion protein, GST-rOmpL1, affinity-purified GST-rOmpL1 or GST protein (same molar ratio) was resolved by a 15% SDS-PAGE gel, and immunoblot analysis was performed. Figure 1B shows the representative result of the immunoblot analysis. The rabbit *leptospira* antiserum against five different serotypes and nine positive dog sera reacted with the GST-rOmpL1 fusion protein but not with GST (Fig. 1B and data not shown). In contrast, three SPF sera and canine Lyme disease positive control serum did not react with either GST-rOmpL1 or GST.

Determination of the antigen concentration for ELISA: To determine GST-rOmpL1 concentration for ELISA, an assay was performed using various concentrations of GST-rOmpL1 as the antigen, or GST protein as the negative-control antigen. Figure 2 is a comparison of ELISA assay results using positive serum obtained from a confirmed case of *L. interrogans* serovar icterohaemorrhagiae infection, negative serum obtained from a SPF dog or Lyme disease positive serum as primary antibodies. Significant differences ($p < 0.001$) between GST-rOmpL1 and the GST con-

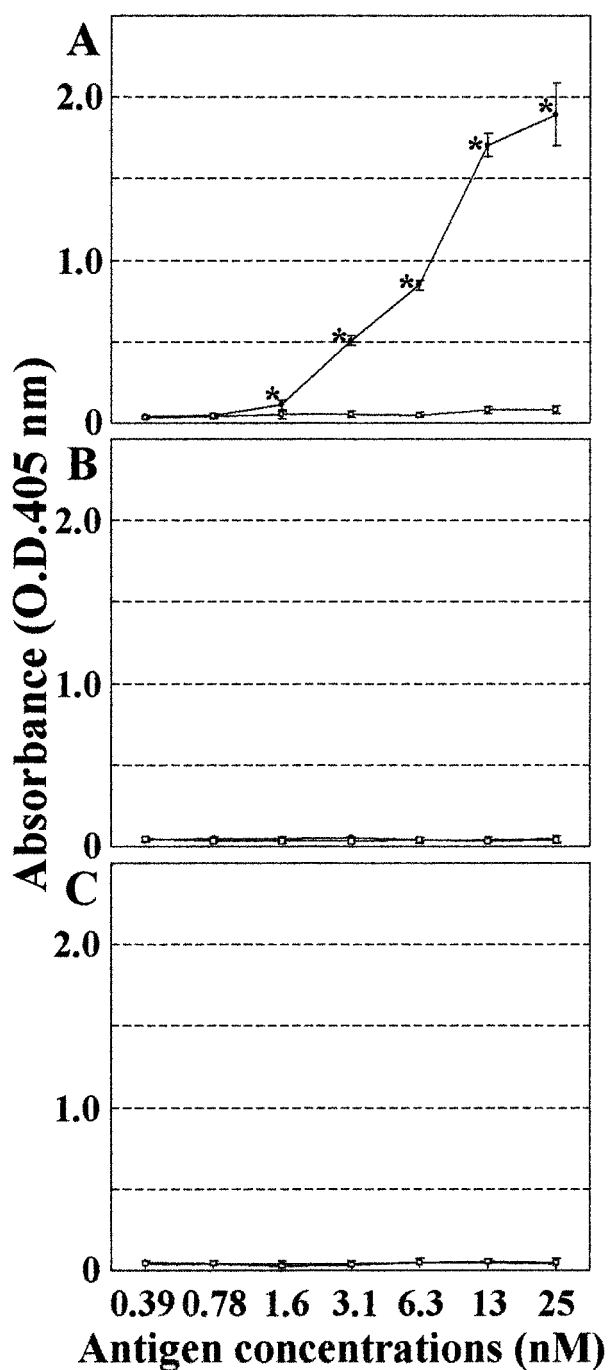


Fig. 2. ELISA using GST-rOmpL1 or GST at different antigen concentrations. *L. interrogans* serovar icterohaemorrhagiae positive dog serum (A), SPF dog serum (B) or *B. burgdorferi* positive dog serum (C) was used as the primary antibody. For each point, an absorbance value (O.D.405 nm) was plotted for GST-rOmpL1 (closed circle) or GST (open square). Asterisks (*) show significant differences ($p < 0.001$) between GST-rOmpL1 and GST control. Short vertical bars represent standard errors ($n=9$).

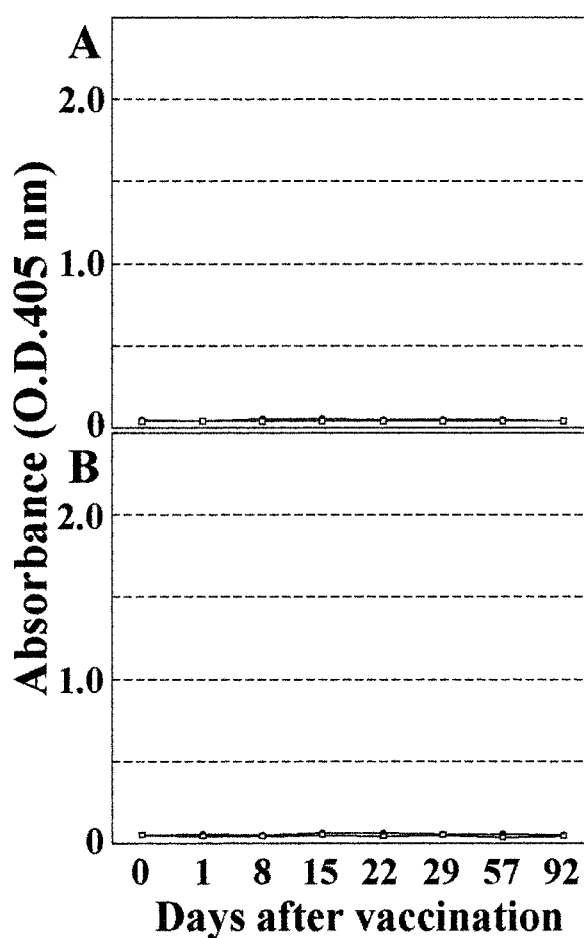


Fig. 3. Influence of *Leptospira* vaccination. ELISA was performed using 3.1 nM (A) or 25 nM (B) of GST-rOmpL1 (closed circles). GST was used as the negative control antigen (open squares). For each point, a mean absorbance value was plotted for serum samples from a dog kept in our laboratory vaccinated at days 1 and 29. Sera obtained from the other two dogs in our laboratory showed similar results (data not shown).

control were observed in the ELISA using *L. interrogans* serovar icterohaemorrhagiae positive serum with antigen concentrations of 1.6 nM or more (Fig. 2A). In contrast, there were no differences at any of the antigen concentrations in the ELISA using the negative and Lyme disease antiserum (Fig. 2B & C).

Influence of *Leptospira* vaccination: To explore the influence of vaccination, *L. interrogans* serogroup icterohaemorrhagiae serovar copenhageni and *L. interrogans* serovar canicola vaccinations were carried out on three experimental dogs on days 1 and 29, and the sera were analyzed using 3.1 nM (low) or 25 nM (high) of the GST-OmpL1 fusion protein as the antigen. GST protein was used as the negative control. Figure 3 shows representative O.D. values of dog serum from 0 to 92 days after vaccination. Throughout the 92 days, low O.D. values were obtained for the GST-

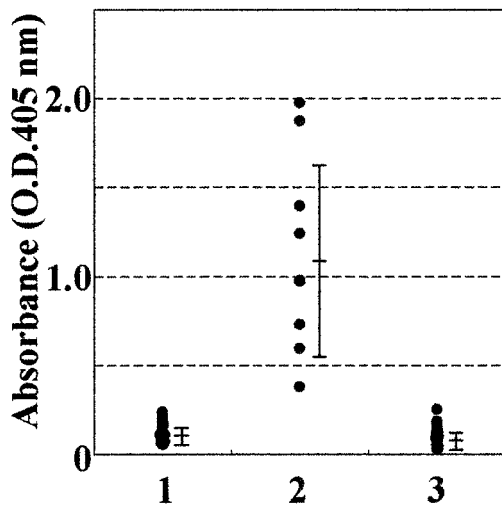


Fig. 4. Distributions of O.D. values in healthy dogs, dogs with leptospirosis, and dogs with diseases other than leptospirosis. ELISA was performed with 25 nM GST-rOmpL1 as the antigen. Column 1, sera of clinically healthy dogs ($n=72$); Column 2, sera from dogs infected with *L. interrogans* serovar icterohaemorrhagiae, *L. interrogans* serovar autumnalis or *L. interrogans* serovar hebdomadis ($n=9$); Column 3, sera from dogs with liver, renal, hemolytic or icteric diseases other than leptospirosis ($n=59$). Plotted points show each O.D. value and bars show average \pm S.D.

OmpL1 fusion protein as well as the GST control at both low and high concentrations of the antigens. Sera obtained from the other dogs showed similar results.

Detection of antibodies from various patients: To compare O.D. values of various serum samples, ELISA was performed using 25 nM GST-OmpL1 fusion protein as the antigen (Fig. 4). The mean \pm standard deviation (SD) of O.D. values of sera from clinically healthy dogs (negative controls, $n=72$) was 0.109 ± 0.046 . Those of the serum samples from nine dogs with leptospirosis (positive controls) was 1.125 ± 0.549 (range: 0.377–1.974). A significant difference ($p<0.001$) between these two groups was found. A positive cut-off value was provisionally estimated as 0.339, calculated using the following formula: mean + 5SD, based on the O.D. values of the negative controls, because none of the negative controls revealed O.D. values above the cut-off value. In contrast, all the positive control values were above the cut-off value. Moreover, O.D. values of sera from dogs with diseases other than leptospirosis ($n=59$) were much lower than the cut-off value (0.089 ± 0.046).

DISCUSSION

In this study, *OmpL1* gene of *L. interrogans* serovar icterohaemorrhagiae (strain RGA) was cloned into the pGEX 6P-1 vector, producing GST-rOmpL1 protein. In the immunoblot analysis, this antigen reacted with not only *L. interro-*

gans serovar icterohaemorrhagiae antiserum but also antiserum against four other serotypes examined in this study. Moreover, in the ELISA, this antigen also reacted with sera obtained from naturally occurring canine *leptospira* cases of *L. interrogans* serovar icterohaemorrhagiae, *L. interrogans* serovar autumnalis or *L. interrogans* serovar hebdomadis infection. These data suggest that ELISA using this antigen can detect antibodies against different serotypes.

Although OmpL1 is known to be expressed only in pathogenic *Leptospira* spp. [2], we examined serum against *Borrelia burgdorferi*, which is closely related to *Leptospira* spp. As expected, GST-rOmpL1 protein did not react with *B. burgdorferi* anti-serum both in the immunoblot analysis and in the ELISA. This finding confirms the specificity of the GST-rOmpL1 protein for the detection of *leptospira* antibodies.

Nowadays *leptospira* inactivated vaccines to prevent canine leptospirosis are widely used. Since many *leptospira* vaccines used in Japan contain *L. interrogans* serovar icterohaemorrhagiae, *L. interrogans* serovar canicola and/or *L. interrogans* serovar hebdomadis as their antigen, the influence of vaccines can not be disregarded in the process of diagnosis. Therefore, the influence of *leptospira* vaccinations on O.D. values was investigated in this study. No serum showed high O.D. values throughout the course of this study (0 to 92 days). One possible explanation for this phenomenon is that the secondary antibody used in this study could not detect IgM. It is unlikely, because elevation of O.D. values were observed from 5 (to > 100) days after the vaccinations in an ELISA using the same secondary antibody and crude outer membrane proteins as an antigen (Okuda, M., unpublished observation), suggesting that the secondary antibody could detect not only canine IgG, but also canine IgM. Moreover, no sera from clinically healthy dogs and dogs other than leptospirosis showed high O.D. values. These data suggest that vaccination does not influence the ELISA using GST-rOmpL1 developed in this study, although we could not elucidate the reason(s).

In the present study, we established the ELISA for the detection of canine *leptospira* antibodies using GST-rOmpL1. We believe that the ELISA system established in this study is useful for the diagnosis of leptospirosis.

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