Expression of leptospiral immunoglobulin-like protein by *Leptospira interrogans* and evaluation of its diagnostic potential in a kinetic ELISA

Raghavan U. M. Palaniappan, Yung-Fu Chang, Fahad Hassan, Sean P. McDonough, Margaret Fough, Stephen C. Barr, Kenneth W. Simpson, Hussni O. Mohammed, Sang Shin, Patrick McDonough, Richard L. Zuerner, Jiaxin Qu and Bruce Roe

1 College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA
2 Bacterial Diseases of Livestock Research Unit, National Animal Disease Center, Agricultural Research Service, US Department of Agriculture, Ames, IA 50010, USA
3 Department of Chemistry, University of Oklahoma, Norman, OK, USA

The search for novel antigens suitable for improved vaccines and diagnostic reagents against leptospirosis led to the identification of LigA and LigB. LigA and LigB expression were not detectable at the translation level but were detectable at the transcription level in leptospires grown in vitro. Lig genes were present in pathogenic serovars of *Leptospira*, but not in non-pathogenic *Leptospira biflexa*. The conserved and variable regions of LigA and LigB (Con, VarA and VarB) were cloned, expressed and purified as GST-fusion proteins. Purified recombinant LigA and LigB were evaluated for their diagnostic potential in a kinetic ELISA (KELA) using sera from vaccinated and microscopic agglutination test (MAT)-positive dogs. Sera from vaccinated dogs showed reactivity to whole-cell antigens of leptospires but did not show reactivity in the KELA assay with recombinant antigens, suggesting a lack of antibodies to Lig proteins in the vaccinated animals. The diagnostic potential of recombinant Lig antigens in the KELA assay was evaluated by using 67 serum samples with MAT > 1600, which showed reactivity of 76, 41 and 35 % to rConA, rVarA and rVarB, respectively. These findings suggest that recombinant antigen to the conserved region of LigA and LigB can differentiate between vaccinated and naturally infected animals.

INTRODUCTION

Leptospirosis is caused by spirochaetes belonging to the genus *Leptospira*. Considered the most widespread zoonotic disease in the world (WHO, 1999), leptospirosis affects both humans and a wide variety of animals (Vinetz, 2001). Infection is mainly contracted by exposure to water, food or soil contaminated with urine from infected animals (Levett, 2001). Potential carriers of *Leptospira* include rats, cattle, dogs, horses and pigs (Goldstein & Charon, 1990). Leptospirosis in dogs is recognized as a risk factor for human leptospirosis (Douglin et al., 1997). Increased rainfall is associated with a rise in the prevalence of leptospirosis in dogs (Ward, 2002). Infection can lead to pulmonary haemorrhage, renal and hepatic failure but sometimes it leads to multi-organ failure and even death (Levett, 2001). An infected dog can also act as an asymptomatic carrier and shed infectious organisms in the urine for its entire lifetime (Murray, 1990). Approximately 250 serovars have been identified. Unfortunately, the available leptospiral vaccines elicit only short-term immunity (6–12 months) and do not provide cross-protection against different serovars.

Diagnosis of leptospirosis is complicated by the high degree of cross-reactivity between different serovars. Furthermore, non-pathogenic *Leptospira biflexa* serovar Patoc, which is considered an environmental contaminant, cross-reacts with rabbit sera raised against pathogenic serovars of *Leptospira* (Matsuo et al., 2000; Myers, 1976; Myers & Coltorti, 1978). The currently available microscopic agglutination test (MAT) is laborious. ELISA methods have been developed with a number of modifications (Gussenhoven et al., 1997; Hartman et al., 1984a,b; Levett, 2001; Petchclai et al., 1991; Ribeiro et al., 1995; da Silva et al., 1997), but most of them depend on proteins derived from whole-cell lysates of *Leptospira*. Recombinant antigens such as LipL32, flagellin

Abbreviations: KELA, kinetic ELISA; MAT, microscopic agglutination test. The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of ligB and C are AF534640 and AY327260, respectively.
and heat-shock protein of *Leptospira* have also been recently developed for use as diagnostic reagents (Flannery et al., 2001; Park et al., 1999) but the specificity and sensitivity of these antigens in vaccinated animals have not been determined. The major drawback with MAT and ELISA is that they cannot differentiate between infected and vaccinated animals. Identification of leptospiral antigens expressed only during infection could be useful for the development of new diagnostic reagents that might differentiate between vaccinated and infected animals.

In order to identify potential antigens that are expressed during leptospiral infection we screened a genomic library of *Leptospira interrogans* with sera from infected animals and obtained several positive clones. One of the clones encodes a gene for leptospiral immunoglobulin-like proteins (LigA) that is only expressed *in vivo* (Palaniappan et al., 2002).

In the present study, we identified another leptospiral immunoglobulin-like protein, LigB, which is identical to LigA at the amino terminus but variable at the carboxy terminus. We expressed truncated forms of the conserved region (Con) and variable regions of LigA (VarA) and LigB (VarB) as GST-fusion proteins in *Escherichia coli*. We used these recombinant antigens in a kinetics-based ELISA (KELA) and evaluated their diagnostic potential using canine sera from either vaccinated dogs or dogs positive by MAT. Our data indicate that these recombinant antigens can serve as useful diagnostic reagents for the detection of leptospiral infection.

**METHODS**

**Sera.** (i) Convalescent sera obtained from five mares that had recently aborted due to leptospiral infection were pooled and used to screen a genomic library of *L. interrogans* serovar Pomona as determined by MAT. (ii) A total of 94 canine sera with MAT titres between 400 and 12,800 were collected from 1999 to 2002 by the New York State Animal Health Diagnostic Laboratory (AHLDL), Cornell University, Ithaca, NY. In the AHLDL our current interpretive criteria for the *Leptospira* MAT test requires either a fourfold rise in titre between the acute and convalescent sera or a single MAT titre of $\geq 1:1600$; this is interpreted as indicative of active infection unless the animal has recently been vaccinated. (iii) Vaccinated sera: seven 8-week-old puppies were divided into three groups consisting of three, two and two animals. Each group was vaccinated with one of the following commercially available vaccines: Grippoprophosa/Pomona (GP), Canicola/Icterohaemorrhagiae (C/I) and Grippotyphosa, Pomona, Canicola and Icterohaemorrhagiae (GPIC). Booster injections were given 3 weeks later. Sera were collected before vaccination and on the 5th and 9th week after vaccination. (iv) Control sera: A total of 20 sera from unvaccinated, healthy, specific pathogen-free (SPF) beagles served as negative controls. Sera from dogs naturally infected with *Leishmania donovani* (2), *Borelia burgdorferi* (2), *Trypanosoma cruzi* (2) and Lyme-vaccinated dogs (2) were also collected and stored at the New York State AHDL (Chang et al., 1995, 2001).

**Bacterial strains and culture conditions.** *L. interrogans* serovar Pomona type kennewicki was isolated from an equine abortion (Palaniappan et al., 2002). Leptospires were maintained on EMJH semi-solid medium, at 30 °C. To isolate low-passage cultures of leptospires, experimentally infected hamster tissues (kidney and liver) were homogenized and inoculated into PLM-5 liquid medium. High-passage cultures were prepared by repeated passage (>15 times) of leptospires in PLM-5 liquid medium. Growth was monitored by dark field microscopy.

**MAT.** MAT was carried out as previously described (Cole et al., 1973) with the whole cell antigens of the following serovars: Pomona, Grippotyphosa, Icterohaemorrhagiae, Hardjo, Canicola, Autumnalis and Bratislava.

**DNA sequencing and analysis.** Positive clones containing the ligB gene (derived from the screening of a genomic library as previously described) were subjected to DNA sequencing (Palaniappan et al., 2002). DNA sequencing was done using an ABI model 377 automated nucleic acid sequencer at the Biosource Center, Cornell University, Ithaca, NY. Homology searches were performed with NCBI BLAST (Altschul et al., 1997).

**Construction of GST-fusion proteins of LigA and LigB.** LigA and LigB were truncated into conserved (Con), the N-terminal 599 amino acids without the signal sequences) and variable regions (VarA and VarB, the C-terminal 595 and 788 amino acids of LigA and LigB, respectively). The regions were amplified using PCR with the following primers: ligConF (5′-TCCCCCGGCGGCTGCAAAAGA-3′), ligConR (5′-CCCTCCGAATAATCCCGTATTAGA-3′), ligVarF (5′-CCCCGGGGGCTGAAATTACAAAT-3′), ligVarR (5′-CCCCGGGGGCTGGCAAAAGA-3′), VarAIF (5′-CCCCGGGGGCTGGCAAAAGA-3′), VarAIR (5′-CCCCGGGGGCTGGCAAAAGA-3′), VarBIF (5′-CCCCGGGGGCTGGCAAAAGA-3′), VarBIR (5′-CCCCGGGGGCTGGCAAAAGA-3′). The underlined nucleotides indicate the restriction site. PCR was performed with Accuprim Taq polymerase (Invitrogen) and the other reagents were added according to the manufacturer’s instructions (Invitrogen). The reaction mixture was subjected to 35 cycles after initial denaturation at 94 °C for 5 min. Each cycle consisted of 94 °C for 1 min, 50 °C for 2 min and 72 °C for 1 min. PCR products were cloned using a TOPO TA cloning kit (Invitrogen) and were subcloned into pGEX4T-2 plasmids (Amersham Pharmacia). The recombinant plasmids (pLigCon, pLigVarA, pLigVarB) were introduced into *E. coli* BL21(DE3). The resulting transformants were grown at 37 °C overnight on LB agar plates containing 50 µg ampicillin ml$^{-1}$ and the expression of these proteins was induced with 1 mM IPTG.

**Purification of GST-fusion proteins.** IPTG-induced *E. coli* BL21(DE3) containing the recombinant plasmids were harvested by centrifugation at 5000 r.p.m. The cell pellets were washed and suspended in PBS followed by passing through a French pressure cell (American Instrument). The lysates were centrifuged to remove cell debris and the supernatants were subjected to affinity chromatography using glutathione-Sepharose 4B columns. The GST-fusion proteins were eluted according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

**Generation of polyclonal antibodies.** Adult New Zealand white rabbits were immunized intramuscularly with 100 µg of GST-fusion proteins and an equal amount of Freund’s incomplete adjuvant. Rabbits were subcutaneously boosted with the same dosage on the 9th and 35th day. On day 45, the rabbits were bled and the sera were collected for analysis.

**SDS-PAGE and immunoblot analysis.** The recombinant proteins were subjected to SDS-PAGE followed by immunoblotting as previously described by Chang et al. (1993, 1995, 2001).

**RT-PCR.** RNA was isolated from the exponential phase cultures of leptospiras using an RNA mini kit (Qiagen) and treated with RNase-free DNase. One step RT-PCR with gene-specific primers (variable region of ligA and ligB) was performed. The following primers were used for RT-
PCR: VariAF (5’-GAAAATCGATCGTAAAGAC-3’), VariAR (5’-CCCTCGAGGTCCTGTTTAT-3’), VariBF (5’-TAAACAAAAAGGACACCGATGC-3’), VariBR (5’-CCGTCGAGTTGTTTTGTAGCTT-3’). The reactions were carried out according to the manufacturer’s instructions (Qiagen). A reaction that contained all the reagents except reverse transcriptase was used as a negative control. Genomic DNA was used as a positive control.

Southern blot analysis. Genomic DNA isolated from leptospiral strains maintained in our laboratory was digested with EcoRI and subjected to gel electrophoresis. DNA was transferred to Hybond-N+ nitrocellulose membranes (Amersham Pharmacia) and processed as outlined by the ECL Direct nucleic acid labelling and detection system (Amersham Pharmacia). The conserved region of the lig gene was used as a probe for the Southern blot analysis.

Optimization of antigen concentration. Based on MAT titre, canine sera were categorized into high (MAT titre of 12,800 to Pomona, 6400 to Grippotyphosa), low (MAT titre of 6400 to Pomona, 3200 to Grippotyphosa) and negative (SPF serum). A checkerboard titration of recombinant antigens (Con, VarA and VarB), primary antibodies (negative, medium and high) and secondary conjugate (anti-dog conjugated with horseradish peroxidase) was performed to determine the optimum conditions for the KELA.

KELA. The optimized concentrations of recombinant antigens were diluted in 0.1 M bicarbonate buffer and added to a 96-well microtitre plate (Nunc), rocked for 1 h and then incubated overnight at 4 °C. The plates were washed three times with 0.1 M PBS containing 0.05 % Tween 20 (PBST). Canine sera (primary antibody) in PBST were diluted 1 : 200, 100 µl of 1 : 4000 dilution of goat anti-dog IgG conjugated to horseradish peroxidase (Cappel) for 30 min at room temperature. The plates were washed again three times with PBST and 100 µl TMB (KPL) was added to each well. Each plate was read three times at 650 nm at 1 min intervals (Biotek EL-312, Winoski, VT). The results were calculated by the KELA computer program and expressed as the slope of the reaction between enzyme and substrate to the amount of antibody bound (Chang et al., 1993, 1995).

Statistical analysis. We evaluated the significance of differences between recombinant proteins in relation to KELA units using the analysis of variance (ANOVA) statistical method. The analysis was performed in STATISTIX (Analytical Software). The least square difference between MAT and KELA for the six serovars of Leptospira was evaluated using the Pearson’s Correlation in STATISTIX. This correlation was significant difference of the recombinant proteins. The correlation between MAT and KELA was assessed for each recombinant protein separately. Descriptive statistics were performed to determine the cut-off value for each protein in relation to KELA.

RESULTS

Identification, sequencing and expression of LigB

A leptospiral genomic library was constructed as previously described and screened with convalescent sera from leptospire-infected mares that had aborted (Palaniappan et al., 2002). Several positive clones were identified and one of the recombinant clones contained an open reading frame (ORF) of 5667 bp. The deduced sequence contained 1889 amino acids with an estimated molecular mass of 206 kDa. An N-terminal signal sequence of 31 amino acids was predicted using the signal P program (Nielsen et al., 1997, 1991). Three possible start codons for this protein were identified, and upstream of the start codon of ligB is a potential ribosome-binding site. The recently released genomic sequences of L. interrogans serovar Lai contained LigB but not LigA (GenBank number AE011533) which shows 98 % identity with LigB of L. interrogans serovar Pomona (Ren et al., 2003). NCBI BLAST search revealed identity with the conserved bacterial immunoglobulin-like domain (Pfam Big 2) of intimins from E. coli (AF319597, AF301015, AF116899) and cell adhesion domain from Clostridium acetobutylicum (NC_003030).

Primary structure of LigB and comparison with LigA

LigB consists of 12 repeats (D1–D12) of a 90 amino acid motif, which has homology to bacterial domains with an Ig-like fold (Pfam Big2) (Fig. 1a). We previously reported that LigA contains 12 repeats of a 90 amino acid motif, but according to Pfam LigA actually has 13 repeats of the 90 amino acid motif. Additionally, LigB contains a C-type lectin-like domain, D13 (residues 1014–1165).

Interestingly, the amino-terminal sequence (the first 630 amino acids) of LigB are identical to LigA but the carboxy terminus varies (Fig. 1b). The first five tandem repeats (residues 52–133, 137–222, 226–308, 312–398, 402–487 and 491–576) in the N-terminal region of LigA and LigB are identical. Furthermore, a C-type lectin-like domain, especially the amino acids KEALDLSNY (residues 1150–1158), has 179 and 142, respectively. Regardless, serine and threonine residues in LigB are 224 and 147 whereas LigA has 179 and 142, respectively. Regardless, serine and threonine are the most common amino acids in both of these proteins.

We analysed the hydrophobicity of the deduced amino acid sequence and the potential membrane-spanning region of LigA and LigB. These two proteins are largely hydrophilic with some hydrophobic patches and they consist of β-sheets with a few α-helical regions. The predicted transmembrane region of LigB spans residues 300–319 (IIGSVKLIVTPAALVSI).

lig genes are present in pathogenic serovars

In order to determine the presence of lig genes in different serovars of Leptospira, EcoRI-digested genomic DNA from different serovars of Leptospira was transferred to nitrocellulose membranes and probed with a non-radioactively labelled oligonucleotide from the conserved region of ligA and ligB. Non-pathogenic L. biflexa serovar Patoc did not contain lig genes but the other pathogenic serovars contained two or three copies of the lig genes (Fig. 2).

Expression and purification of LigA and LigB

In order to over-express LigA and LigB, the truncated forms of the conserved and variable regions of LigA and LigB were
Fig. 1. (a) Alignment of 12 repeats of 90 amino acid sequences of LigB and its identity with the bacterial Ig-like domain from Pfam (Ig1 and Ig2). Gaps have been introduced to optimize alignment among the polypeptides. (b) Alignment of variable regions of LigA and LigB.
cloned and expressed as GST-fusion proteins. The expressed recombinant proteins of the conserved and variable regions of LigA and LigB had molecular masses of 92, 93 and 120 kDa, respectively. GST-fusion proteins were purified using affinity column chromatography and thrombin-cleaved proteins migrated at 66, 63 and 82 kDa, respectively (Fig. 3a–c).

**Lack of LigA and LigB expression at the translation level in leptospires grown in vitro**

To examine LigA and LigB expression in leptospires, immunoblots of whole-cell proteins from low- and high-passage cultures of leptospires were probed with polyclonal antibodies to Con, VarA and VarB. LigA and LigB expression were not detectable in leptospires grown in vitro (Fig. 4a and b). In contrast, E. coli containing the recombinant plasmids showed strong reactivity. The negative control, E. coli without the insert in the vector, showed no reactivity.

**Detection of ligA and ligB at the transcription level in leptospires grown in vitro**

RT-PCR with RNA from low- and high-passage cultures detected LigA and LigB expression at the transcript level (Fig. 5). Genomic DNA of leptospires was used as a positive control. The negative control that lacked reverse transcriptase did not show any amplification. This indicates that LigA and LigB may either be expressed at extremely low levels under in vitro conditions or that the proteins are very unstable.

**Optimization of recombinant antigen concentrations**

LigA and LigB were truncated into the conserved region of LigA and LigB, the variable region of LigA and the variable region of LigB, and expressed as GST-fusion proteins. A checkerboard titration technique was used to determine the
optimal concentrations of reagents for ELISA. Based on this, 1μg of recombinant antigen was chosen and the optimum dilution of primary and secondary conjugated antibodies was assessed as 1:200 and 1:4000, respectively. Since these recombinant antigens were expressed as GST-fusion proteins, GST was used as a control and the reactivity rate of GST was subtracted for the analysis of samples.

Determination of cut-off value

A total of 20 sera collected from unvaccinated, healthy dogs were analysed by KELA with GST, rCon, rVarA and rVarB. The KELA value for the reactivity of unvaccinated sera to the recombinant antigens was obtained by subtracting the reactivity of GST. The cut-off value was determined from the unvaccinated dog sera using descriptive statistical analysis. The maximum KELA unit of sera from unvaccinated, healthy dogs was considered as the cut-off value. The cut-off KELA values of rCon, rVarA and rVarB were 7, 42 and 42 units, respectively. Sera from dogs infected with leishmaniasis, trypanosomosis and borreliosis did not show reactivity to the recombinant antigens indicating that there is no cross-reactivity of Lig antigens with these agents.

Lack of antibodies in the vaccinated sera to recombinant antigens of LigA and LigB

Serial samples from dogs vaccinated with commercially available vaccines showed MAT titres of less than 400. Analysis of these vaccinated sera showed no reactivity to recombinant antigens Con, VarA and VarB (rCon, rVarA and rVarB) by KELA and Western blot analysis (data not shown), but showed reactivity to the whole-cell proteins of *L. interrogans* in Western blot analysis (Fig. 6) and in ELISA (our unpublished data). In the Western blot analysis with whole-cell proteins, most of the vaccinated sera from dogs showed reactivity with leptospiral antigens. For example, Grippo/Pomona combined vaccinated sera reacted with whole cell antigens at 66, 50 and 42 kDa, whereas sera from naturally infected dogs showed reactivity with leptospiral antigens at 66, 42, 33, 32, 27 and 21 kDa (Fig. 6). The descriptive statistical analysis of KELA with sera from the vaccinated dogs was below the cut-off value previously established using SPF dogs. These results suggest that the vaccinated animals lacked antibodies to rCon, rVarA and rVarB, which make these recombinant antigens suitable for the serodiagnosis of leptospirosis.

Reactivity of MAT-positive canine sera to recombinant antigens in KELA

A total of 94 canine serum samples with MAT titres between 400 and 12,800 were used to evaluate the diagnostic potential of rCon, rVarA and rVarB in the KELA assay (Fig. 7a–c). Sixty-seven samples had MAT titre >1:1600 and 76, 41 and 35% reacted to rCon, rVarA and rVarB, respectively, in the KELA assay (Table 1). The other 27 serum samples with MAT
titre $\leq 800$ were also evaluated. Of these, three were reactive in the KELA assay. Post-hoc tests showed that VarB had significantly lower KELA units (19.4) in comparison to rCon (53.6) and rVarA (41.4).

**DISCUSSION**

On first exposure of a host to pathogenic bacteria, the immune system will generate antibodies directed against cell surface or membrane antigens. Since the antibodies directed against a cell surface antigen may prevent colonization, recombinant antigens from cell surface or membrane proteins may serve as ideal candidates for development of novel vaccines and improved diagnostic tests. In this study, we identified LigB by immuno-screening a genomic library of *L. interrogans* serovar Pomona and studied its expression by leptospires grown in *vitro*. We also expressed the conserved and variable regions of LigA and LigB as GST-fusion proteins and developed a KELA test for the detection of leptospiral infection using these novel reagents.

We previously demonstrated the lack of expression of LigA in *vitro* using high-passage cultures of leptospires (Palaniappan et al., 2002). Similarly, LigB expression in *vitro* is also not detectable in high- and low-passage cultures of leptospires. However, both LigA and LigB are detectable at the transcript level. The lack of detection of LigA and LigB in *in vitro*-grown leptospires suggests that these proteins either are poorly expressed in *vitro* or are unstable after expression. Recently, LigA and LigB were reported to be expressed very weakly in low-passage *Leptospira kirschneri* RM52 (Matsumaga et al., 2003). However, we cannot detect the expression of LigA or LigB at the translational level in a low-passage strain of *L. interrogans*. This may be due to differences between the
serovars and/or the media used for culture. The re-establishment of LigA and LigB expression upon infection and the absence of these genes in non-pathogenic leptospires suggest that they are an important leptospiral virulence factor.

Currently available serological tests cannot discriminate between vaccine-induced leptospiral antibodies and those due to infection. Therefore, the development of diagnostic reagents based on antigens that are only expressed during infection would be a valuable tool to identify animals that contract leptospirosis despite vaccination. To our knowledge, only a few investigators have attempted to differentiate between vaccinated and infected animals (Gitton et al., 1994; Goddard et al., 1991). Some of the commercially available bovine leptospiral vaccines can raise the MAT titre into the range of 3200 to 12 800 (Bolin & Alt, 2001; Brown et al., 1995; Prescott et al., 1994; Brihuega & Hutter, 1995; Prescott et al., 1991), and in the case of serovar Bratislava MAT, titres of 12 800 have been reported (Prescott scott et al., 1991). Based on our results, the vaccine that combined Grippotyphosa/Pomona induced MAT titres between 1 : 100 and 1 : 400 within 7 weeks of administration, and showed reactivity to the whole-cell proteins of leptospires by Western blot analysis. These vaccinated sera showed high absorbance values in ELISA with whole-cell proteins of leptospires (our unpublished data) but did not react in the KELA with recombinant antigens of LigA and LigB (rLigA and rLigB), suggesting a lack of antibodies to rLigA and rLigB in the vaccinated sera. Furthermore, none of the vaccinated sera showed reactivity to rLigA and rLigB by Western blot analysis (data not shown).

To evaluate the diagnostic potential of rCon, rVarA and rVarB, a KELA assay was performed using MAT-positive canine sera. The conserved regions of LigA and LigB (rCon) showed stronger reactivity than rVarA and rVarB to MAT-positive canine sera. The sensitivity of KELA with rCon was 85 % when evaluated using high MAT-titre sera (MAT ≥6400). The overall sensitivity of recombinant antigens rCon, rVarA and rVarB were 76, 41 and 35 %, respectively, to MAT-positive canine sera (MAT ≥1600). Interestingly, of 67 MAT-positive sera, 16 had no reactivity in the KELA assay. MAT is a reliable test for diagnosis of leptospirosis but it cannot differentiate between vaccine-induced and infection-induced antibodies. Since we did not know the vaccination history of these dogs, it was not possible to know whether the MAT titres of these 16 serum samples were due to vaccination or infection. Three sera that failed to react in the KELA assay had MAT titres of 12 800 towards serovar Bratislava. Vaccination of animals sensitized either by prior infection or vaccination may result in higher antibody levels than is achieved during initial vaccination but this has not been studied yet in dogs (Smith et al., 1994). In addition, all sera tested to date from naturally infected mares and from experimentally infected hamsters contain antibodies to Lig proteins (Palaniappan et al., 2002). Taken together, the lack of antibodies to Lig proteins in these 16 MAT-positive serum samples strongly suggest that the MAT titres are due either to prior vaccination or non-specific reactivity. The sera used in this study are defined as positive or negative to particular Leptospira serovars only on the basis of the MAT results. Further experimental studies using sera from dogs with both leptospiral vaccination and natural/experimental infection are pivotal to answer this question.

Sera from naturally infected mares contained antibodies to Lig proteins (Palaniappan et al., 2002), but Lig antibodies are not present in rats infected or vaccinated with killed low-passage leptospires (Matsunaga et al., 2003). Our study also

### Table 1. Comparison of efficiency of recombinant antigens in KELA to MAT-positive canine sera

The sensitivity of recombinant antigens in KELA was determined from MAT-positive canine sera, which did not have a case history for vaccination. Based on the cut-off value, the percentage of reactivity of recombinant antigens to MAT titre value is represented.

<table>
<thead>
<tr>
<th>No. MAT-positive canine sera</th>
<th>MAT titre</th>
<th>Reactivity of recombinant antigens in KELA (%)</th>
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<tr>
<td></td>
<td>Con</td>
<td>VarA</td>
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<tr>
<td>26</td>
<td>12 800</td>
<td>88* (23/26)</td>
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<tr>
<td>11</td>
<td>6400</td>
<td>81 (9/11)</td>
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<td>12</td>
<td>3200</td>
<td>67 (8/12)</td>
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<td>18</td>
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<tr>
<td>67†</td>
<td>76‡ (51/67)</td>
<td>46‡ (31/67)</td>
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* Out of three dog’s sera which failed to react in our KELA assay, two of them were towards Bratislava and one towards both Bratislava and Grippotyphosa.

† Total number of animals.

‡ Mean of KELA reactivity.
indicates the lack of antibodies to the recombinant antigens of LigA and LigB in vaccinated dogs, which suggests that Lig proteins are upregulated during infection. Therefore, antibody titres to Lig proteins indicate infection and not a response to vaccination. KELA was performed with recombinant antigens on 27 dogs’ sera with MAT titre \( \leq 800 \). Of these, two with MAT titres of 800 and one with a MAT titre of 400 reacted in the KELA assay, indicating that MAT alone cannot discriminate between infection and vaccination. Sera from dogs infected with \textit{Leishmania donovani}, \textit{T. cruzi} and \textit{B. burgdorferi} showed no reactivity in our KELA with recombinant antigens. This implies that the recombinant antigens used in this KELA detected only leptospirosis infection. Thus, KELA with rConA is a potential diagnostic tool to distinguish between infection- and vaccination-induced leptospirosis titres. However, serial samples from leptospirosis-infected dogs are essential to determine the sensitivity of rLigA and rLigB in KELA.

In conclusion, KELA with rConA appears to be specific for serodiagnosis of leptospirosis infection, with rConA showing stronger reactivity than rVarA and rVarB. The lack of antibodies to rConA in the vaccinated sera suggests that these recombinant antigens can be used to identify leptosomal infection despite vaccination. It appears that rConA could play an important role in differential diagnosis of animals with vaccinal and/or infected antibodies. However, this is only a preliminary study and further analysis using more field positive serum samples with known history of vaccination and experimental infection are needed. Also, it is necessary to study the kinetics of antibody responses against rLigA/rLigB during infection. This analysis will help us to assess the utility of rLigA/rLigB for the early serological diagnosis of the leptospirosis infection. Further evaluation of this antigen in diagnosis of equine and bovine leptospirosis is in progress in our laboratory.

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