Development and validation of a sandwich ELISA for use in measuring concentrations of canine surfactant protein A in serum of dogs

Katsuhito Sone, DVM; Hideo Akiyoshi, DVM, PhD; Mika Aoki, DVM, PhD; Shunji Sugii, DVM, PhD; Fumihito Ohashi, DVM, PhD

**Objective**—To develop and evaluate a sandwich ELISA incorporating rabbit antiserum specific for canine surfactant protein A (SP-A) for use in measuring concentrations of SP-A in serum of dogs.

**Sample**—Serum samples obtained from 6 healthy dogs and 3 dogs with pulmonary disease.

**Procedures**—Rabbit antiserum was prepared against purified canine SP-A. The IgG fraction was isolated via protein G affinity chromatography and was then biotinylated. The sandwich ELISA was performed by use of anti–SP-A antibody (IgG) preabsorbed with sera from healthy dogs. Validity of the ELISA was confirmed by determination of the detection limit, precision, reproducibility, and accuracy. Serum SP-A concentrations were measured in 6 healthy dogs and 3 dogs with pulmonary disease.

**Results**—Detection limit of the ELISA was 2.0 ng/mL. Within- and between-assay coefficients of variation ranged from 3.8% to 14.1% and from 15.5% to 35.6%, respectively. The observed-to-expected recovery ratio ranged from 77.1% to 89.9%. Serum SP-A concentrations measured by use of the ELISA were ≤2.3 ng/mL in the 6 healthy dogs, 25.6 ng/mL in a dog with severe cardiac pulmonary edema, 8.3 ng/mL in a dog with pneumonia, and 10.1 ng/mL in a dog with lung lobe torsion.

**Conclusions and Clinical Relevance**—The sandwich ELISA was found to be useful for measuring purified canine SP-A concentrations and canine SP-A concentrations in serum samples. The ELISA was precise, reproducible, and accurate. The ELISA may be beneficial in assessing serum concentrations of canine SP-A as a potential biomarker of pulmonary diseases in dogs. (Am J Vet Res 2011;72:833–837)

Pulmonary surfactant is a lipid-protein complex that lines the alveolar surface. It prevents the collapse of small alveoli and the enlargement of large alveoli by decreasing the surface tension of the air-alveolar surface and plays an important role in gaseous exchange by increasing the surface area in the lungs.1,2 This surfactant, which is synthesized by alveolar type II cells and secreted into the alveolar space, is a mixture of lipids (approx 90% [wt/wt]) and proteins (approx 10% [wt/wt]).

Surfactant proteins are classified into types A to D; SP-A is the major protein.3 Surfactant protein A belongs to the collectin (collagen-lectin) family, is a water-soluble oligomeric apolipoprotein, and is involved in various host defense mechanisms.4 Surfactant protein in the alveolar spaces permeates into the bloodstream in rabbits with experimentally induced lung injury,5,6 and the SP-A concentration in blood increases in humans with pulmonary diseases, such as idiopathic pulmonary fibrosis,7,8 pulmonary alveolar proteinosis,7 acute respiratory distress syndrome,10 CPE,10 and bacterial pneumonia.9 On the basis of results of these studies, the measurement of SP-A concentrations in blood has been used as a biomarker for pulmonary diseases in humans.9,11,12 In veterinary medicine, it has been suggested13 that the measurement of SP-A concentrations may be useful for understanding lung conditions and in monitoring the progress of pulmonary diseases in horses because of fluctuations in serum concentrations of SP-A during prolonged transportation.

Dogs are afflicted with various pulmonary diseases; however, to our knowledge, there is no blood marker specific for the lungs in dogs. In veterinary medicine, it is typically necessary to anesthetize an animal when
performing a lung biopsy, CT imaging, bronchoscopy, or BAL to obtain information to provide an accurate diagnosis. Thus, diagnoses in clinical settings may be made on the basis of the experience of the attending veterinarian, results of physical examinations and hematologic analysis, and evaluation of thoracic radiographs. There is a continuing need for noninvasive diagnostic approaches for dogs.

Therefore, the purpose of the study reported here was to develop and validate a sandwich ELISA for use in measuring concentrations of canine SP-A in serum and to assess whether the measurement of serum concentrations of canine SP-A may be applicable as a biomarker for pulmonary disease in dogs.

**Materials and Methods**

**Isolation of canine SP-A** — We collected BAL fluid from 8 lungs of 4 dogs by use of procedures described elsewhere. The lavage solution was 5mM Tris-HCl and 100mM saline (0.9% NaCl) solution (pH, 7.4). Pooled BAL fluid was centrifuged (650 × g for 10 minutes) to remove cellular debris. Surfactant protein A then was purified in accordance with the method described in another study. Briefly, calcium chloride (final concentration, 5mM) was added to the supernatant of the BAL fluid, and the resulting solution was centrifuged (43,000 × g for 3 hours). Lipid was removed from the precipitate by extraction with 1-butanol (98 mL of butanol/2 mL of pellet). The butanol-insoluble protein precipitate was suspended in 5mM Tris-HCl buffer (pH, 7.4) and dialyzed against the same buffer. The suspension was centrifuged (43,000 × g for 3 hours), and the supernatant was harvested. Calcium chloride (final concentration, 5mM) was added to the supernatant, and the resulting solution was applied to a column. Bound proteins were eluted with 5mM Tris buffer (pH, 7.4) containing 5mM EDTA. The solution containing the proteins was dialyzed against 5mM Tris buffer (pH, 7.4) and concentrated.

All procedures were performed at 4°C, and samples were stored at 4°C or −80°C until analyzed. Approximately 5.26 mg of purified SP-A was obtained from the 8 lungs of the 4 dogs.

**Antibody against SP-A** — Antiserum directed against purified canine SP-A was obtained from a male Japanese White rabbit. Purified canine SP-A (100 µg) mixed with Freund complete adjuvant was injected SC in the neck of the rabbit once each week for 4 weeks. Serum was obtained from the rabbit 2 weeks after the last injection. All procedures were conducted in accordance with the Osaka Prefecture University Policy on the Use of Animals.

The IgG fraction was isolated by use of protein G affinity chromatography performed in accordance with the manufacturer’s protocol; the IgG fraction was then biotinylated as described elsewhere. The purified IgG was absorbed for 60 minutes at 37°C by mixing 10 µL of pooled canine serum with 1 mL of the purified IgG. The resulting precipitate was removed by centrifugation (2,000 × g for 20 minutes), and absorption was repeated by mixing 10 µL of canine serum (diluted 1:10 [vol/vol] in saline solution) with 1 mL of purified IgG, which was followed by incubation at 37°C for 60 minutes to separate the precipitate. Absorption was repeated with canine serum (diluted 1:10 [vol/vol] in saline solution) until no precipitate was visible. Finally, the absorbed IgG fraction was passed through a purifying filter (pore size, 0.2 µm).

**Electrophoresis and western blotting** — We performed SDS-PAGE and western blotting as described elsewhere. The SDS-PAGE for the analysis of purified canine SP-A was performed by use of 12.5% polyacrylamide gels under reducing conditions; proteins were detected by use of Coomassie brilliant blue R-250. For western blot analysis, electrophoresis was performed, and proteins in the gel then were transferred to a nitrocellulose membrane. The membrane was blocked by incubation with 1% BSA solution containing 0.1% Triton X-100 (blocking buffer) at 37°C for 1 hour. The membrane was washed with PBS solution containing 0.1% Triton X-100 (pH, 7.4) and incubated with absorbed IgG (0.5 µg/mL in blocking buffer) at 37°C for 1 hour. The membrane was washed with PBS solution containing 0.1% Triton X-100 and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:2,300 in blocking buffer) at 37°C for 1 hour. Finally, the membrane was washed again, and the color was developed by use of 3,3′-diaminobenzidine.

**Amino acid sequencing** — The purified 32-kDa canine protein separated via SDS-PAGE was electrophoretically transferred onto a polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was stained with Coomassie brilliant blue R-250, and the transferred 32-kDa band was excised and subjected to NH₂-terminal amino acid sequencing.

**Serum samples** — Serum samples were obtained at Okayama Animal Medical Center Hospital between April 2008 and August 2009 from 6 healthy dogs and 3 dogs with pulmonary disease. Owner consent was obtained for each dog. Healthy dogs were recruited by the hospital staff. The 6 dogs (3 males, 2 females, and 1 spayed female) ranged from 2 to 4 years of age; breeds represented were Golden Retriever, Dalmatian, Cavalier King Charles Spaniel, Yorkshire Terrier, French Bulldog, and mixed. The dogs with pulmonary disease included a 14-year-old male Miniature Dachshund with severe CPE, a 9-year-old male Miniature Pinscher with pneumonia, and a 5-year-old female Pug with lung lobe torsion. The diagnosis of CPE was made on the basis of clinical examination findings, medical history, and evaluation of thoracic radiographs; the dog died 2 days after it was admitted to the hospital. The diagnosis of lung lobe torsion was made on the basis of evaluation of radiographs, ultrasonograms, and CT images and results of specimens obtained via fine-needle aspiration of the affected lung; the dog underwent corrective surgery. The diagnosis of pneumonia was made on the basis of physical examination findings, medical history, results of routine hematologic tests (CBC, measurement of C-reactive protein concentration, and biochemical analyses), evaluation of radiographs and CT images, and results of specimens obtained via fine-needle as-
piration of the lungs. Serum was prepared immediately after collection and stored at −80°C until analysis.

**Sandwich ELISA**—Serum samples were diluted 1:10 in 0.67% BSA solution containing 3% Triton X-100. Purified SP-A was diluted with 0.67% BSA solution containing 3% Triton X-100 to create standard solutions (concentrations ranged from 0.05 to 50 ng/mL). To free the SP-A from any associated components, we used a method described in another study. There are reports of the use of SDS and Triton X-100 or SDS, Triton X-100, and EDTA to treat serum when measuring SP-A concentrations. We compared the methods, and the absorbance was highest for pretreatment with 3% Triton X-100 (data not shown).

A sandwich ELISA was performed by use of microtiter ELISA plates. Wells were coated by incubation with absorbed IgG (diluted 1:1,000 in 0.67% BSA solution containing 0.1% Triton X-100). An aliquot (50 µL) of a standard solution or serum sample was added to each well; plates were incubated at 37°C for 1 hour. Wells then were washed 3 times with PBS solution containing 0.1% Triton X-100. An aliquot (50 µL) of a standard solution or serum sample was added to each well; plates were incubated at 37°C for 1 hour. Plates were washed 4 times, and 50 µL of biotin-labeled absorbed IgG (diluted 1:1,000 in 0.67% BSA solution containing 0.1% Triton X-100) was added to each well; plates were incubated at 37°C for 1 hour. Plates were washed 4 times, and 50 µL of horseradish peroxidase–conjugated streptavidin solution (diluted 1:4,000 in 0.67% BSA solution) was added to each well; plates were incubated at 37°C for 30 minutes. Plates were washed 4 times, and 50 µL of 3,3',5,5'-tetramethylbenzidine solution was added to the wells. The reactions were stopped after 15 minutes by the addition of 50 µL of 1N HCl to each well. Absorbance was measured at 450 nm.

The ELISA was validated by determination of the detection limit, precision, reproducibility, and accuracy. The detection limit was defined as the lowest mean value minus 2 times the SD of the minimum absorbance that did not overlap with the mean plus 2 times the SD of the negative control sample on the standard curve. To ascertain the precision of the ELISA, 3 serum samples that contained low, medium, and high concentrations of SP-A were measured 5 times in 1 session to determine the within-assay variation. Reproducibility was determined by measurement of 3 serum samples 5 times on different days to determine the between-assay variation. Accuracy was determined by use of a recovery test that measured concentrations in 3 serum samples after the addition of 10, 25, and 50 ng of purified SP-A/mL to each of the 3 samples. The line of best fit for the ELISA was determined for concentrations in the range of 2.0 to 25.6 ng/mL.

**Additional protein analysis**—Protein concentrations were measured in purified canine SP-A and antibodies. Concentrations were determined on the basis of absorbance at 280 nm or by use of the Bradford method with BSA as the standard.

**Results**

**Characterization of the anti–canine SP-A polyclonal antibody**—Analysis of results for SDS-PAGE of purified canine SP-A under reducing conditions revealed a major band at 32 kDa and 2 minor bands at 28 and 36 kDa (Figure 1). To confirm whether these bands were canine SP-A, the NH2-terminal amino acids of the 32-kDa protein were sequenced. The first 5 amino acids at the NH2-terminal were Ile-Glu-Asn-Asn-Thr. Western blot analysis for absorbed IgG revealed a weak minor band at 64 kDa in addition to the 3 bands at 28, 32, and 36 kDa.

**Canine SP-A ELISA**—A sandwich ELISA was developed to measure purified canine SP-A. The minimum detection concentration was 0.2 ng/mL from a standard curve created with purified SP-A (Figure 2). Because samples were diluted 1:10 prior to assay, the detection limit was 2.0 ng/mL. Within- and between-assay CV ranged from 3.8% to 14.1% and from 15.5% to 35.6%, respectively (Table 1). The accuracy of the...
ELISA was determined by means of a recovery test; the observed-to-expected recovery ranged from 77.1% to 89.8% (Table 2).

Serum samples—To assess whether the sandwich ELISA was applicable for use in measuring unpurified canine SP-A concentrations in biological samples, we determined the concentrations of canine SP-A in serum obtained from dogs. The serum SP-A concentration measured with the ELISA was ≤ 2.3 ng/mL in the 6 healthy dogs, 25.6 ng/mL in the dog with CPE, 10.1 ng/mL in the dog with lung lobe torsion, and 8.3 ng/mL in healthy dogs, 25.6 ng/mL in the dog with CPE, 10.1 ng/mL in healthy dogs, and 8.3 ng/mL in the dog with lung lobe torsion.

Concentrations were measured in 3 serum samples 5 times in 1 session on the same day for the within-assay assessment and in 3 serum samples 5 times on different days for the between-assay assessment. All samples were evaluated in triplicate.

Within-assay

<table>
<thead>
<tr>
<th>Variable</th>
<th>Serum sample</th>
<th>n</th>
<th>Mean ± SD (ng/mL)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-assay</td>
<td>1</td>
<td>5</td>
<td>3.1 ± 0.4</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>7.5 ± 0.3</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>9.9 ± 0.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Between-assay</td>
<td>1</td>
<td>5</td>
<td>2.1 ± 0.7</td>
<td>35.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>8.3 ± 0.8</td>
<td>16.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>10.1 ± 1.6</td>
<td>15.5</td>
</tr>
</tbody>
</table>

Concentrations were measured in 3 serum samples 5 times in 1 session on the same day for the within-assay assessment and in 3 serum samples 5 times on different days for the between-assay assessment. All samples were evaluated in triplicate.

n = Number of times each serum sample was assayed.

Table 1—Precision and reproducibility for measurement of canine SP-A concentrations with a sandwich ELISA.

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>SP-A added (ng/mL)</th>
<th>SP-A concentration (ng/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>2.0</td>
<td>NA NA</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.8</td>
<td>78.4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>23.6</td>
<td>86.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>45.2</td>
<td>86.5</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>8.5</td>
<td>NA NA</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>16.4</td>
<td>79.1</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>30.7</td>
<td>88.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>52.4</td>
<td>89.8</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>25.6</td>
<td>NA NA</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>33.5</td>
<td>77.7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>45.8</td>
<td>81.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>64.9</td>
<td>78.5</td>
</tr>
</tbody>
</table>

All samples were evaluated in triplicate. NA = Not applicable.

Table 2—Recovery for measurement of canine SP-A concentrations with a sandwich ELISA.

Discussion

Measuring SP-A concentrations in blood is a non-invasive and useful clinical tool for monitoring disease activity and for determining the prognosis in humans with pulmonary disease.2,8,11,12 However, there currently is no lung-specific marker in blood tests for dogs. Therefore, we prepared a rabbit antiserum against canine SP-A and developed a sandwich ELISA for use in measuring the SP-A concentration in serum samples obtained from dogs.

Results for SDS-PAGE of purified canine SP-A under reducing conditions revealed protein bands at 28, 32, and 36 kDa. Similar findings have been reported for SDS-PAGE with canine SP-A.21 The first 5 amino acids at the NH2-terminal of the 32-kDa protein were identical to those of canine SP-A reported in another study,23 which indicated that the purified protein was canine SP-A. Analysis via western blotting revealed a weak band at 64 kDa in addition to the bands at 28, 32, and 36 kDa, which suggested that the 64-kDa band may be equivalent to dimeric canine SP-A.24

The detection limit of the ELISA was 2.0 ng/mL. The within- and between-assay CVs ranged from 3.8% to 14.1% and from 15.5% to 36.6%, respectively. The CVs of the serum from 1 healthy dog were higher than those for 2 other sera, possibly because the SP-A concentration in that serum sample was near the detection limit. Accuracy was determined via a recovery test; accuracy was 77.1% to 89.9%, which was considered low. This may be increased by additional optimization with more serum samples.

We were able to measure serum concentrations of canine SP-A by use of the ELISA, which indicated that the ELISA was applicable for use in measuring concentrations of unpurified canine SP-A. Results for the sera from the 6 healthy dogs and the 3 dogs with pulmonary disease revealed that the SP-A concentrations in sera from dogs with pulmonary disease were higher than those from healthy dogs. However, sera from only 3 dogs with pulmonary disease were used in the present study. Additional studies will be needed to assess whether measurement of the SP-A concentration in serum is useful as a biomarker of pulmonary disease in dogs.

In the study reported here, we prepared a rabbit antiserum against canine SP-A and developed a sandwich ELISA for use in measurement of the SP-A concentration in serum. The ELISA was precise, reproducible, and accurate. Analysis of the results suggests that the ELISA may be applicable for use in assessing serum concentrations of SP-A as a potential biomarker for pulmonary disease in dogs.

References


