Objective—To purify neutrophil elastase (NE) from dog blood and develop and validate an ELISA for the measurement of canine NE (cNE) in canine serum as a marker for gastrointestinal tract inflammation.

Sample Population—Neutrophils from 6 dogs immediately after they were euthanatized and serum from 54 healthy dogs.

Procedures—cNE was purified from blood by use of dextran sedimentation, repeated cycles of freezing-thawing and sonication, cation-exchange chromatography, and continuous elution electrophoresis. Antibodies against cNE were generated in rabbits, and an ELISA was developed and validated by determination of sensitivity, dilutional parallelism, spiking recovery, intra-assay variability, and interassay variability. A reference range was established by assaying serum samples from the 54 healthy dogs and by use of the lower 97.5th percentile.

Results—cNE was successfully purified from blood, and antibodies were successfully generated in rabbits. An ELISA was developed with a sensitivity of 1,100 µg/L. The reference range was established as < 2,239 µg/L. Ratios of observed-to-expected results for dilutional parallelism for 4 serum samples ranged from 85.4% to 123.1%. Accuracy, as determined by spiking recovery, ranged from 2.71% to 114.0%. Coefficient of variation for 4 serum samples was 14.2%, 16.0%, 16.8%, and 13.4%, respectively, for intra-assay variability and 15.4%, 15.0%, 10.5%, and 14.8%, respectively, for interassay variability.

Conclusions and Clinical Relevance—The purification protocol used here resulted in rapid and reproducible purification of cNE with a high yield. The novel ELISA yielded linear results and was accurate and precise. Additional studies are needed to evaluate the clinical usefulness of this assay. (Am J Vet Res 2007;68:584–591)

Gastrointestinal tract disease attributable to inflammation of the gastrointestinal mucosa is a common problem in dogs. To make a definitive diagnosis of IBD is challenging and requires invasive and expensive procedures. Also, it can be difficult to make an objective assessment of disease severity and progression. Clinical signs in dogs with IBD are highly variable, and severity of the disease may differ considerably among patients. Because of the diversity of clinical signs, it is difficult to evaluate the effect of treatments. Therefore, it is highly desirable to develop a noninvasive and accurate assay for the objective assessment of the severity of intestinal inflammation. Such an assay may also aid in evaluating the effects of treatment on inflammatory activity.

Neutrophil elastase is a protein released by activated polymorphonuclear granulocytes, and NE plays an important role in endothelial cell injury mediated by neutrophils.1 Neutrophil elastase belongs to the group of serine proteinases and is a major component of azurophil granules within neutrophils.2 Neutrophil elastase has been purified in several species, including humans and dogs.3,11 Ascitic fluid from dogs with experimentally induced peritonitis was used as the starting material for the purification of cNE in some studies.12,13 A less invasive protocol for the purification of cNE is desirable.

In some human patients with IBD, the gastrointestinal mucosa contains a large number of neutrophils.
Therefore, it is not surprising that studies\textsuperscript{13,14} have been conducted to assess various neutrophil-derived markers as a tool for assessment of the severity of intestinal inflammation. The concentration of NE in feces and serum has been used as a marker for intestinal inflammation in human patients with IBD.\textsuperscript{15,16} This marker may also prove to be useful in canine patients with IBD. The objective of the study reported here was to purify cNE from dog blood by use of a novel protocol, develop and validate an immunoassay for measurement of cNE in canine serum, and measure serum concentrations of cNE in a group of healthy dogs to establish a reference range for this marker.

**Materials and Methods**

Sample population—Blood samples were collected from 6 clinically normal dogs immediately after they were euthanatized as part of unrelated research projects, and neutrophils were then extracted and used for purification of cNE. Serum samples obtained from 54 healthy dogs were assayed by use of an ELISA and used to establish a reference range. Dogs used to provide samples for the reference range were owned by students and staff of Texas A&M University. The blood collection procedures were approved by the Clinical Research Review Committee of the Texas Veterinary Medical Center at Texas A&M University.

Preparation of neutrophils—Blood samples were collected from clinically normal dogs, which were used in other research projects, immediately after they were euthanatized. A solution of 3% sodium citrate was used as an anticoagulant. Blood samples were processed by mixing 1 volume of blood with 0.4 volumes of a dextran solution (60 mg of dextran/mL) that contained 9 mg of NaCl/mL. The dextran solution improves sedimentation of RBCs. The mixture was stirred for 5 minutes and incubated at approximately 25°C for 5 hours. After incubation, 2 liquid layers were clearly visible (ie, a dark-red layer on the bottom and a light-colored layer on top). The top layer was carefully aspirated and transferred into 50-mL polypropylene centrifuge tubes. The tubes were centrifuged at 2,000 X g for 20 minutes. Supernatant was carefully discarded, and the pellet was dissolved in RBC lysis solution. The dissolved pellet was incubated on a rocking plate at 25°C for 20 minutes. After incubation, the tubes were centrifuged again at 2,000 X g and 4°C for 20 minutes.

Extraction of NE—After centrifugation, the pellet was suspended in extraction buffer (20 mL of 0.02M Tris, 1M NaCl, and 0.03% Tween 20 [pH, 8.5]), frozen at −20°C for 3 hours, thawed at 25°C, and ultrasonicated for 1.5 minutes at 50% power output. Freezing, thawing, and ultrasonication procedures were repeated 3 times. Subsequently, the solution was centrifuged at 18,000 X g and 4°C for 20 minutes. The pellet was discarded, and supernatant was filtered through a series of filters with decreasing pore size (from 10 to 0.8 µm). The buffer was then exchanged with buffer A (0.05M sodium acetate and 0.05M NaCl [pH, 5.0]) by use of dialysis tubing.

Column chromatography—The filtered and dialyzed solution was loaded onto a 1.3 X 3.5-cm cation exchanger cartridge that had been equilibrated with buffer A. Ten milliliters of sample was loaded onto the column at a flow rate of 1 mL/min. Proteins not bound to the column were washed out with 5 column volumes of buffer A. Proteins that did not bind strongly to the column matrix were eluted by use of a stepwise gradient of 0.3M NaCl in buffer A (20 column volumes). More strongly bound proteins were eluted by use of a linear salt gradient ranging from 0.3 to 0.7M NaCl in buffer A (12 column volumes) at a flow rate of 1 mL/min. Fractions (3 mL/fraction) were collected. Fractions containing proteolytic activity against methoxy-succinyl-alanine-alanine-proline-valine-p-nitroanilide, a synthetic chromogenic substrate, were pooled and concentrated by use of a centrifugal filter device with a molecular mass cutoff of 10 kd.

Continuous elution electrophoresis—The concentrated protein solution was further separated by use of a continuous elution electrophoresis system that was used in accordance with the manufacturer's instructions. The acrylamide gel column was prepared 12 hours before each assay. An aliquot (0.5 mL) of sample was mixed with 0.5 mL of sample buffer (0.06M Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 25% glycerol [wt: vol], and 0.01% bromophenol blue [pH, 6.8]), incubated at 95°C for 4 minutes, and loaded onto the gel column. The buffer reservoir of the continuous-elution electrophoresis system was filled with running buffer (0.025M Tris-HCl, 0.192M glycine, and 0.1% SDS buffer [pH, 8.3]), and a constant voltage of 200 V was applied to the system. Proteins in the solution were separated on the basis of their molecular weights and were discharged into the elution chamber at the bottom of the gel. From there, the proteins were eluted in running buffer with a fraction size of 0.3 mL by use of a peristaltic pump.

Partial characterization—To assess protein purity, SDS-PAGE\textsuperscript{1} in a vertical mini-gel format was used in accordance with the manufacturer's instructions. The gel was stained with silver stain\textsuperscript{2} in accordance with the manufacturer's instructions. Molecular weight of cNE was estimated by use of a chip-based protein assay that was performed on a bioanalyzer\textsuperscript{3} in combination with a protein kit.\textsuperscript{4} Data were analyzed by use of assay software.

The isoelectric point was estimated with PAGE by use of a precast vertical\textsuperscript{5} gel with a pH range of 3 to 10. The extinction coefficient of cNE was determined by use of the bicinchoninic acid assay.\textsuperscript{17} Analysis of the N-terminal amino acid sequence for the first 25 residues of cNE was obtained by use of the Edman degradation method by personnel at another facility.

Assay for proteolytic activity—Fractions obtained during various stages of the purification procedure were screened for cNE activity by use of a modified spectrophotometric assay.\textsuperscript{18} Briefly, 20 µL of a high ionic strength buffer (0.02M Tris, 1M NaCl, and 0.03% Tween 20 [pH, 8.5]) was pipetted into each well of 96-microwell plates. Then, 50 µL of test sample was added to each well, and plates were incubated at 25°C for 5 minutes. After incubation, 10 µL of methoxy-succinyl-alanine-alanine-proline-valine-p-nitroanilide was added...
to each well. Plates were then immediately placed in an automated plate reader, and the increase in absorbance was measured at 405 nm during a period of 15 minutes. One unit of enzyme activity was defined as the amount of material that generated an equivalent change of absorbance over time as was generated by 1 μg of human NE for the same aforementioned assay conditions.

**Anti-cNE antibodies**—Antibodies against cNE were generated in 1 rabbit (*Oryctolagus cuniculus*). A commercial antibody production service was used to purify monospecific polyclonal antibodies from rabbit serum. The column was prepared in accordance with the manufacturer's instructions. As a first step, rabbit antiserum was subjected to lipoprotein precipitation. Briefly, 20 mL of rabbit antiserum was thawed and added to 20 mL of 1M CaCl₂, and 800 μL of 10% dextran sultate solution. The mixture was gently mixed in a 50-mL centrifuge tube on a plate rocker at 4°C for 20 minutes; the mixture was subsequently centrifuged at 10,000 × g and 4°C for 10 minutes. Precipitate was discarded, and supernatant was filtered through filter paper. A disposable gel filtration column was used for buffer exchange with buffer B (75mM Tris-HCl and 150mM NaCl [pH, 8.0]) in accordance with the manufacturer's instructions.

A prepared affinity column was attached to a fast-performance liquid chromatography purification system and equilibrated with buffer B. Two milliliters of the buffer-exchanged antibody solution was applied to the column. Absorbance of the effluent was measured at 280 nm. The column was washed with buffer B until the absorbance returned to baseline values, then the mobile phase was changed to buffer C (100mM glycine and 500mM NaCl [pH, 2]), and 1-mL fractions of effluent were collected in test tubes that contained 300 μL of 1M Tris-HCl (pH, 8.0). The eluting peak was collected, and all fractions for this peak were pooled, concentrated, and buffer exchanged with PBS solution (pH, 7.2) by use of a centrifugal filter device. Antibody concentration was adjusted to achieve a concentration of 1 mg/mL, and aliquots (250 μL) were stored frozen at −20°C until further use.

For biotinylation, 0.7 mg of purified monospecific polyclonal cNE antibody in PBS solution (pH, 7.2) was mixed with a 20-fold molar excess of biotin. After incubation at 25°C for 45 minutes, the material was buffer exchanged with PBS solution (pH, 7.2). For this procedure, a disposable centrifugal filter device was used. The filter was filled with the antibody-biotin mixture, centrifuged at 6,000 × g and 4°C for 20 minutes, refilled with PBS solution, and centrifuged again at 6,000 × g. This procedure was repeated 5 times.

Biotinylation efficiency was determined by use of a 2-4'-hydroxyazobenzene benzoic acid avidin assay kit used in accordance with the manufacturer's instructions. The biotinylation procedure was repeated until a biotinylation coefficient between 3.0 and 4.0 was reached.

**Development of an ELISA for the measurement of cNE**—Standard 96-well flat-bottom ELISA plates were coated with 200 ng of affinity-purified anti-cNE antibodies/well in 100 μL of carbonate-bicarbonate buffer (pH, 9.4). Solutions in the wells were mixed, and plates were incubated at 37°C for 1 hour by use of an automated plate incubator-shaker and then washed 4 times with 200 μL of PBS/well. Remaining binding sites were blocked with 200 μL of 10% BSA in PBS solution/well. Plates were again incubated at 37°C for 1 hour and washed as described previously.

Standard solutions of cNE were prepared by serial dilution of pure cNE in TBS+BSA+Tween. Standards of 1,280, 640, 320, 160, 80, 40, 20, and 10 μg of cNE/L were prepared and frozen at −20°C in aliquots (300 μL/ aliquot). Standard solutions were thawed immediately before being loaded onto a plate. One hundred microliters of TBS+BSA+Tween was used as a negative control (blank) sample. Serum samples were prepared in a 1 in 20 dilution by use of TBS+BSA+Tween.

Standards, a negative control sample, and diluted serum samples were loaded in duplicate wells (100 μL/well). Plates were incubated at 37°C for 3 hours and then washed as described previously. A solution that contained 100 ng of biotinylated anti-cNE antibodies in 100 μL of TBS+BSA+Tween was added to each well. After incubation at 37°C for 1 hour, which was followed by washes as described previously, 8 ng of horse-radish peroxidase–labeled streptavidin in 100 μL of TBS+BSA+Tween was added to each well. Plates were once again incubated at 37°C for 1 hour and then washed as described previously. Retained horse-radish peroxidase was developed by adding 100 μL of 3,3′,5,5′-tetramethylbenzidine solution to each well. The reaction was stopped after 20 minutes by adding 100 μL of stop solution (4M acetic acid and 1N sulfuric acid) to each well. Absorbance was measured at 450 nm by use of an automated plate reader.

Standard curves were calculated by a 4-variable curve fit by use of the following equation:

$$y = \frac{(A - D)/(1 + (x/C)^n)} + D$$

where A is the y-value corresponding to the asymptote of the x-axis at low values, D is the y-value corresponding to the asymptote of the x-axis at high values, C is the x-value corresponding to the midpoint between A and D, and B describes the rapidity with which the curve makes the transition from the asymptotes in the center of the curve. All 4 variables were calculated by use of an algorithm based on the Levenberg-Marquardt equation.

**Validation of the ELISA for cNE**—The ELISA was validated by determining the assay sensitivity, upper limit of the working range, dilutional parallelism, spiking recovery, intra-assay variability, and interassay variability. Assay detection limit (sensitivity) was defined as the apparent concentration of cNE that yielded an absorbance equal to the mean plus 3 times the SD of the absorbance of 10 duplicates of a negative control sample (ie, TBS+BSA+Tween). Upper limit of the working range was defined as the apparent concentration of cNE that yielded an absorbance equal to the mean minus 3 times the SD of the absorbance of 10 duplicates of a solution of 2,000 μg of cNE/L. Four serum samples were
used to determine dilutional parallelism. The samples were evaluated at dilutions of 1 in 10, 1 in 20, 1 in 40, and 1 in 80. Spiking recovery was determined by adding 640, 320, 160, 80, 40, 20, and 10 µg of cNE/L to 4 serum samples. Intra-assay variability was determined by evaluating 4 serum samples 10 times in duplicates within the same assay, whereas interassay variability was determined by evaluating 4 serum samples during 10 consecutive assays.

Reference range for cNE—The reference range for cNE in serum was determined by evaluating serum samples from 54 clinically healthy dogs and calculating the lower 97.5th percentile. Dogs used to provide samples for the reference range differed with regard to age and breed.

**Results**

Results for the purification procedure of cNE from 300 mL of blood were summarized (Table 1). Progression of the purification process was characterized by use of SDS-PAGE (Figure 1). The protein solution was separated during cation-exchange chromatography (Figure 2). The cNE bound strongly to the cation-exchange column and was eluted from the column with a concentration between 0.4 and 0.7M NaCl. Proteolytic activity was detected at the descending slope of the third peak. Fractions from this peak area were pooled, concentrated, and loaded onto the continuous elution electrophoresis system. Continuous elution electrophoresis achieved removal of contaminating proteins, as indicated in results of SDS-PAGE.

The overall yield of the purification protocol was 9.14% (Table 1). There was a 167-fold increase in proteolytic activity. Molecular mass of cNE, as estimated by use of the bioanalyzer in combination with the protein kit, was 26.5 kd (Figure 3). The isoelectric point for cNE was 9.5 (Figure 4). Extinction coefficient of cNE as determined by use of bicinchoninic acid was 0.85 for a solution of 1 mg/mL. The N-terminal amino acid sequence of the first 25 residues of cNE was determined (Figure 5). Homology of the N-terminal amino acid sequence for the first 25 amino acids of the active form of cNE, compared with the sequence of rats, mice, and humans, was 100%, 96%, and 92%, respectively.

The yield for antibody purification was 0.1 mg of anti-cNE IgG/mL of antiserum. A total of 3.2 mg of anti-cNE antibody was purified, and 0.8 mg of anti-cNE antibody was biotinylated for use as the reporter antibody in the ELISA. Use of a 20-fold molar excess of biotin and incubation at 25°C for 80 minutes led to a biotinylation coefficient of 2.7.

Reproducible standard curves were generated by use of the ELISA (Figure 6). The mean ± SD absorbance at 450 nm of 10 duplicate samples of a negative control sample (ie, TBS+BSA+Tween) was 0.156 ± 0.015. Extrapolation from the standard curve for an absorbance of the mean plus 3 times the SD was equivalent to a cNE concentration of 55 µg/L. Because serum samples were diluted 1:20 before inclusion in the assay, this resulted in a calculated assay sensitivity of 1,100 µg/L for serum samples. Mean ± SD absorbance at 450 nm of 10 duplicate samples containing 2,000 µg of cNE/L was 1.181 ± 0.050. Extrapolation from the standard curve for an absorbance of the mean minus 3 times the SD was equivalent to a cNE concentration of 2,075 µg/L.

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Total protein (mg)</th>
<th>Total activity (AU)*</th>
<th>Specific activity (AU/mg of protein)</th>
<th>Recovery (%)</th>
<th>Increase in purity†</th>
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</thead>
<tbody>
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<td>Neutrophil preparation</td>
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<td>0.17</td>
<td>100</td>
<td>NA</td>
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<tr>
<td>Extract of NE</td>
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<td>306</td>
<td>1.39</td>
<td>164.5</td>
<td>8</td>
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<td>Cation-exchange chromatography</td>
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<td>21.25</td>
<td>18.3</td>
<td>125</td>
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<tr>
<td>Continuous elution electrophoresis</td>
<td>0.6</td>
<td>17</td>
<td>26.33</td>
<td>9.1</td>
<td>167</td>
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*One activity unit (AU) was defined as an increase of the absorbance of the methoxysuccinyl-alanine-proline-valine-p-nitroanilide activity assay multiplied by 1,000. †Represents the fold increase in purity; NA = Not applicable.

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**Table 1—Values for various stages during sequential purification of cNE from 300 mL of canine blood.**
Similar to the procedure for the sensitivity, serum samples were diluted 1:20 before inclusion in the assay, and the corresponding maximum concentration in serum was 41,500 μg/L.

Figure 2—Representative chromatogram of cation-exchange chromatography of a canine neutrophil extract. Enzymatic activity of each fraction is represented on the y-axis on the right. The extract was separated into 4 distinct peaks (1 to 4, respectively). Peak 1 represents proteins that did not interact with the column matrix and were washed out immediately, and peak 2 represents proteins that bound weakly to the column and were washed out with a low ionic strength buffer; peaks 1 and 2 had little enzymatic activity. The predominant cNE activity was found in the fractions of the descending slope of peak 3. Peak 4 consisted of proteins that were strongly bound to the column. Notice that fractions with the predominant cNE activity were eluted from the column by solutions that had concentrations between 0.4 and 0.7 M NaCl. AU = Activity unit.

Figure 3—Illustration of an SDS gel-like image obtained by use of a bioanalyzer depicting the molecular mass of cNE. Numbers on the left side represent number of kilodaltons. Lanes consisted of a ladder of markers of known molecular weight (lane L) and 5 samples of pure cNE (lanes 1 through 5, respectively). In each lane, cNE markers with the highest (high) and lowest (low) molecular weight, as well as the system peak, are indicated.

Weights of the cNE samples were 9.5, 8.0, 6.0, 5.3, 5.2, 4.5, and 3.5 kilodaltons. Lanes consisted of a ladder of markers of known molecular weight (lane L) and 5 samples of pure cNE (lanes 1 through 5, respectively). In each lane, cNE markers with the highest (high) and lowest (low) molecular weight, as well as the system peak, are indicated.

Linearity of the assay was determined by evaluating dilutional parallelism. Ratios of observed-to-expected results for 4 serum samples and 4 dilutions ranged from 85.4% to 123.1% (mean ± SD, 101.7 ± 14.9%; Table 2).

Accuracy of the assay was evaluated by determining spiking recovery. Ratios of observed-to-expected results for 4 serum samples and 7 spiking concentrations ranged from 27.1% to 114.0% (mean ± SD, 93.0 ± 15.4%; Table 3).

Intra-assay variability for 4 serum samples ranged from 13.4% to 16.8% (mean ± SD, 15.1 ± 1.6%; Table 4). Interassay variability for 4 serum samples ranged from 10.5% to 15.4% (mean ± SD, 13.9 ± 2.3%).
The reference range for serum concentrations of cNE was determined for 54 dogs. The reference range for those dogs was < 2,239 µg/L.

**Discussion**

We successfully purified cNE from dog blood. The described purification protocol reproducibly yielded cNE of high purity, as indicated by results for SDS-PAGE and analysis with the bioanalyzer in combination with the protein kit. Purification of cNE from ascitic fluid of dogs with experimentally induced peritonitis has been reported elsewhere.\(^ {11,12} \) Because of the invasiveness of the procedure used to induce peritonitis, blood was chosen as a neutrophil source in the study reported here. In those other studies, investigators used cation-exchange chromatography and size-exclusion chromatography for the purification of cNE. Results described by those investigators could not be reproduced in our laboratory. To achieve a purification protocol with a high yield and high purity of the end product, multiple attempts with various chromatographic columns were made. The combined use of cation-exchange and size-exclusion chromatography, similar to techniques used by those other investigators,\(^ {11,12} \) did not lead to pure cNE. By replacing the size-exclusion column with a continuous elution electrophoresis system, which also separated proteins on the basis of their molecular weight, we were able to separate cNE from all contaminating proteins. This protocol resulted in a purification yield of 9.14%. A purification yield of cNE from ascitic fluid of 43% was reported in 1 study,\(^ {12} \) whereas the yield reported in another study\(^ {11} \) was 55%. Investigators in

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino acid sequence</th>
<th>Homology (%)</th>
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<tr>
<td>cNE</td>
<td>I V G G R P A Q P H A W P F M V S L Q R R G H F</td>
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<td>Mice</td>
<td>I V G G R P A Q P H A W P F M V S L Q R R G H F</td>
<td>96</td>
</tr>
<tr>
<td>Humans</td>
<td>I V G G R P A Q P H A W P F M V S L Q R R G H F</td>
<td>92</td>
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Figure 5—Schematic depicting the sequence and homology of the first 25 N-terminal amino acids of cNE, compared with the amino acid sequence of NE in rats, mice, and humans. Amino acids in the sequence for rats, mice, and humans that differ from those in the sequence for cNE are underlined. NA = Not applicable.

Table 2—Representative results* for dilutional parallelism of serum samples for the cNE ELISA.

<table>
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<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Observed (µg/L)</th>
<th>Expected (µg/L)</th>
<th>Observed/expected (%)</th>
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<td>1</td>
<td>1 in 10</td>
<td>3,458.8</td>
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<td>1 in 20</td>
<td>2,114.2</td>
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<td></td>
<td>1 in 80</td>
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<td></td>
<td>1 in 40</td>
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<td></td>
<td>1 in 80</td>
<td>963.8</td>
<td>973.8</td>
<td>99.0</td>
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*Results were obtained for 4 serum samples; results for the other 2 samples were similar to those for the 2 samples reported here. See Table 1 for remainder of key.

Table 3—Spiking recovery for the cNE ELISA determined by adding various concentrations of cNE to aliquots of 4 serum samples. See Table 1 for key.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spiking concentration</th>
<th>Observed (µg/L)</th>
<th>Expected (µg/L)</th>
<th>Observed/expected (%)</th>
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<td>640</td>
<td>589.9</td>
<td>700.2</td>
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both of those studies achieved a higher yield for the purification of cNE than was achieved by use of our protocol, which may have been attributable to a difference in starting material (blood vs ascitic fluid) used for the purification process. It can be speculated that ascitic fluid, especially after experimental induction of peritonitis, contains more neutrophils than blood samples obtained from healthy dogs. Our purification yield was similar to the yield reported for the purification of NE from blood obtained from humans (6.4%).

The molecular mass of cNE, as determined by use of the bioanalyzer in combination with the protein kit, was 26.5 kd. These results differed slightly from results reported by other investigators (24.8 kd\(^{11}\) and 24.9 to 26.0 kd\(^{12}\) respectively). This discrepancy could be explained by the fact that differing methods were used to estimate the molecular mass of cNE in each study. In those other studies,\(^{11,12}\) investigators used SDS gel electrophoresis for determination of the molecular mass. When the molecular mass in the study reported here was calculated on the basis of SDS-PAGE (similar to the gels used by those other investigators), our results were comparable to their results.

The isoelectric point of cNE was 9.5 and was similar to that reported\(^{8}\) for porcine NE (pH 8.0). To our knowledge, the isoelectric point of cNE has not been reported elsewhere.

The approximate specific absorbance of cNE at 280 nm was 0.85 for a solution of 1 mg/mL. Again, to our knowledge, this information has not been reported elsewhere.

Analysis of the sequence of the first 25 amino acids of the N-terminal end of cNE revealed 100% homology with the amino acid sequence predicted by the nucleotide sequence available through the canine genome project, which confirmed the identity of the purified protein. Initial comparison of the sequence of the first 25 amino acids of the N-terminal end of NE among species revealed that cNE had 100% homology with rat NE, 96% homology with mouse NE, and 92% homology with human NE. However, when the predicted amino acid sequence for the entire protein was deduced from the canine and human genome projects and aligned against each other, the degree of sequence homology between human NE and cNE decreased to only 68%.

In the study reported here, a direct sandwich ELISA for the measurement of cNE in canine serum samples was successfully developed and validated. Validation of the ELISA was performed by determination of sensitivity, linearity, accuracy, and reproducibility. Evaluation of dilutional parallelism for the ELISA indicated acceptable linearity for clinical use. Results of spiking recovery revealed acceptable accuracy of the ELISA. In a single sample spiked with 20 µg of cNE/L, low recovery (27.1%) was observed. An explanation for this result is the low concentration of cNE in this specific sample, which was less than the sensitivity range of the assay and therefore resulted in an inaccurate result. Another possible explanation for this outlier is laboratory error. Values for the intra-assay and interassay variability indicated that the ELISA was precise and yielded reproducible results.

A reference range of < 2,239 µg/L for serum concentrations of cNE was established on the basis of results for samples obtained from 54 healthy dogs; this range was determined by use of the lower 97.5th percentile. Serum cNE concentrations were detectable in only 9.3% of healthy dogs.

Assay sensitivity was 1,100 µg/L of cNE. Additional studies will be necessary to determine whether the sensitivity of the assay is sufficient for clinical use. It has been reported\(^{10}\) that the mean concentration of NE in serum of human patients with IBD is significantly higher than the concentration in healthy individuals. This may also prove to be true for dogs with gastrointestinal tract inflammation, compared with values for healthy dogs.

### Table 4—Mean ± SD intra-assay and interassay variability and the coefficient of variation (CV) for the cNE ELISA determined by use of 4 serum samples assayed in (in duplicates) 10 times within the same assay (intra-assay variability) or in 10 consecutive assays (interassay variability).

<table>
<thead>
<tr>
<th>Variability</th>
<th>Sample</th>
<th>Mean ± SD (µg/L)</th>
<th>CV (%)</th>
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<tr>
<td>Intra-assay</td>
<td>1</td>
<td>5,568 ± 791</td>
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<tr>
<td></td>
<td>2</td>
<td>5,312 ± 592</td>
<td>16.0</td>
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<tr>
<td></td>
<td>3</td>
<td>19,171 ± 5,074</td>
<td>16.8</td>
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<tr>
<td></td>
<td>4</td>
<td>3,364 ± 452</td>
<td>13.4</td>
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<tr>
<td></td>
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<td>NA</td>
<td>15.1 ± 1.6</td>
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<tr>
<td>Interassay</td>
<td>1</td>
<td>4,882 ± 754</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8,764 ± 1,315</td>
<td>15.0</td>
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<tr>
<td></td>
<td>3</td>
<td>25,073 ± 2,641</td>
<td>10.5</td>
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<tr>
<td></td>
<td>4</td>
<td>4,057 ± 583</td>
<td>14.8</td>
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<td>All</td>
<td>NA</td>
<td>13.9 ± 2.3</td>
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</table>

See Table 1 for remainder of key.

a. Dextran 500, Accurate Chemicals, Westbury, NY.
b. Polypropylene cenifuge tubing, 50 mL, Corning Inc, Corning, NY.
d. PD-10, Sephadex G-25 M column, Amersham Biosciences, Piscataway, NY.
g. Snakeskin, 10 kMW, Pierce, Rockford, Ill.
h. Econo-Pac High-S cartridge, Bio-Rad Laboratories, Hercules, Calif.
i. PD-10, Sephadex G-25 M column, Amersham Biosciences, Piscataway, NY.
k. Filters, Pall-Gelman Sciences, Ann Arbor, Mich.
m. Mini Prep Cell, Bio-Rad Laboratories, Hercules, Calif.

Agilent 2100 Bioanalyzer, Agilent Technologies GmbH, Waldbronn, Germany.

o. Protein 50 Lab-Chip, Agilent Technologies GmbH, Waldbronn, Germany.
p. Protein 50 assay software, Agilent Technologies GmbH, Waldbronn, Germany.

q. NOVEX precast vertical isoelectric focusing (IEF) gel, Invitrogen, Carlsbad, Calif.
r. Bicinchoninic acid assay, Pierce, Rockford, Ill.
s. The Department of Biotechnology, Jagiellonian University, and Bio Centrum Ltd Facility, Krakow, Poland.
t. UV max ELISA plate reader, Molecular Devices, Sunnyvale, Calif.
u. SOFTMAX PRO analysis software package, Molecular Devices, Sunnyvale, Calif.
w. Lampire Biological Laboratories, Pipersville, Pa.
x. HI Trap, Amersham Biosciences, Piscataway, NY.
y. EZ-LinkSulfo-NHS-LC biotin, Pierce, Rockford, Ill.
z. ImmunoPure HABA assay kit, Pierce, Rockford, Ill.
aa. ImmunoPure strepavidin HRP-conjugated, Pierce, Rockford, Ill.

bb. TMB substrate kit, No. 34021, Pierce, Rockford, Ill.
References