Aus dem Gastrointestinal Labor
der Tierärztlichen Fakultät der Texas A&M Universität, College Station, Texas, USA
Vorstand: Sandee Hartsfield, DVM, MS, Dipl. ACVA

Angefertigt unter der Leitung von
Jörg M. Steiner, Dr.med.vet., PhD, Dipl. ACVIM, Dipl. ECVIM-CA

Vorgelegt über
Prof. Dr. Dr. habil. Johannes Hirschberger
Medizinische Kleintierklinik
der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München
Vorstand: Prof. Dr. Dr. habil. Katrin Hartmann

Reinigung und Teilcharakterisierung von caniner neutrophiler Elastase und die Entwicklung eines Immunoassays zur Messung der Konzentration der neutrophilen Elastase im Serum

Purification and Partial Characterization of Canine Neutrophil Elastase and the Development of an Immunoassay for the Measurement of Neutrophil Elastase Concentration in Serum

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DEDICATION

Dedicated to my parents,

Karin & Fritz Stoll.

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LIST OF ABBREVIATIONS

°C	degree Celsius
α_1-PI	alpha$_1$- proteinase inhibitor
µg	microgram
A	alanine
AGP	alpha acidic glycoprotein
AP3	adaptor protein complex 3
AP3P1	gene encoding the β-subunit of AP3
APS	ammonium persulfate
BCA	bicinchoninic acid
BPI	bactericidal/permeability-inducing protein
BSA	bovine serum albumin
C	cysteine
CIBDAI	canine inflammatory bowel disease activity index
cm	centimeter
CN	cyclic neutropenia
cNE	canine neutrophil elastase
cPLI	canine pancreatic-lipase immunoreactivity
CRP	C-reactive protein
%CV	coefficient of variation; %CV = (SD/mean)*100
D	aspartic acid
EDTA	ethylenediaminetetraacetic acid
ELA2	gene encoding neutrophil elastase
ELISA	enzyme linked immuno assay
F	phenylalanine
FPLC	fast performance liquid chromatography
FRD	food responsive diarrhea
G	glycine
g	gram
H	histidine
HAP	haptoglobin

HCl	hydrochloric acid
I	isoleucine
IBD	inflammatory bowel disease
IEF	isoelectric focusing
IUBMB	International Union of Biochemistry and Molecular Biology
kDa	kilo Dalton
L	leucine
L	liter
M	methionine
M	molar (M=moles of solute/liter of solution)
mg	milligram
ml	milliliter
N	asparagine
N/A	not applicable
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NE	neutrophil elastase
nm	nanometer
O/E	ratio of observed to expected
OmpA	outer membrane protein A
P	proline
p	p-value
pANCA	perinuclear antineutrophilic cytoplasmic antibodies
PBS	phosphate buffer saline
pH	-log 10 concentration of H^+ ions in solution
pI	isoelectric point
Pro	proline
Q	glutamine
R	arginine
s	second
S	serine

SAA	serum amyloid A
SCN	severe congenital neutropenia
SD	standard deviation
SDS	sodium-dodecyl-sulfate
SDS-PAGE	sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis
T	threonine
TEMED	tetra-methyl-ethylenediamine
TLI	trypsin-like immunoreactivity
Tris	2-amino-2-hydroxymethyl-1, 3-propanediol
V	valine
V	Volt
W	tryptophan
x g	centrifugal force, expressed as x gravity

1. INTRODUCTION

Gastrointestinal disease due to inflammation of the gastrointestinal mucosa is a common problem in dogs. A definitive diagnosis of inflammatory bowel disease (IBD) is often challenging and requires invasive and expensive procedures. Also, an objective assessment of disease severity and disease progression can be difficult. Therefore, it is highly desirable to develop non-invasive, inexpensive, yet accurate methods for the objective assessment of severity of intestinal inflammation.

Neutrophil elastase (NE) is a protein released by activated polymorphonuclear granulocytes. This protein plays a significant role in endothelial cell injury mediated by neutrophils (Smedly et al., 1986). Neutrophil elastase belongs to the group of serine proteinases and is a major component of azurophil granules within the neutrophils (Barrett, 1981). Neutrophil elastase has previously been purified in several species including humans and dogs (Boudier et al., 1982; Cotter et al., 1980; Delshammar et al., 1976; Dubin et al., 1994; Geiger et al., 1985; Marossy et al., 1980; Mistry et al., 1999; Ohlsson et al., 1976; Taylor et al., 1975). In some human patients with IBD the gastrointestinal mucosa contains a large number of neutrophils and, therefore, it is not surprising that many studies have been conducted to assess different neutrophil-derived markers as a tool for assessment of intestinal inflammation (Dwarakanath et al., 1997; Poullis et al., 2002). The concentration of neutrophil elastase in feces and serum has been used as a marker for the assessment of human IBD (Adeyemi et al., 1985; Andus et al., 1993). Such a marker might also prove to be useful in canine patients with IBD.

1.1 Hypothesis and objectives

The hypothesis of this study was that NE might be a useful marker for the assessment of canine patients with IBD. In order to prove or disprove this hypothesis, the objectives of this study were: 1. to purify NE from dog blood, 2. to develop and validate an immunoassay for measurement of canine NE (cNE) in serum, and 3. to compare serum concentrations of cNE between healthy dogs, and dogs with inflammatory bowel disease.

2. LITERATURE REVIEW

2.1. Neutrophil Elastase (NE)

Neutrophils are essential for host defense against invading pathogens. They engulf and break down microorganisms using an array of mechanisms, such as reactive oxygen species and antimicrobial peptides and proteases, including NE, cathepsin G and proteinase 3 (Pham, 2006). Neutrophil elastase is located in the azurophil granules of polymorphonuclear leukocytes (Bode et al., 1989). Elastin is one of the many physiological substrates of NE (Bieth et al., 1973). The proteolytic activity of NE is essential for the migration of neutrophils through connective tissue (Bode et al., 1989). If not regulated properly by its natural inhibitors, neutrophil elastase can lead to severe damage by hydrolyzing connective tissue proteins (Travis, 1988).

Neutrophil elastase has been isolated and purified in many species including humans, cows, pigs, sheep, horses, hamsters, rabbits and dogs (Boudier et al., 1982; Cotter et al., 1980; Delshammar et al., 1976; Dubin et al., 1994; Geiger et al., 1985; Marossy et al., 1980; Mistry et al., 1999; Ohlsson et al., 1976; Taylor et al., 1975).

Table 1 summarizes different biochemical characteristics of neutrophil elastase from different species.

2.1.1. Nomenclature

Neutrophil elastase ([E.C. 3.4.21.37]; classification is proposed by the international union of biochemistry and molecular biology) belongs to the family of serine endopeptidases. In the literature many synonyms for neutrophil elastase can be found, such as leukocyte elastase, lysosomal elastase, polymorphonuclear leukocyte elastase, elastase, elaszym, serine elastase, and granulocyte elastase.

The group of elastases, which includes neutrophil and pancreatic elastase, has the ability to cleave the connective tissue protein elastin, which serves as the amorphous component of elastic fibers (Janoff et al., 1968). Elastin is widely distributed in vertebrate tissues, and is especially abundant in lung, skin, arteries, and ligaments. Neutrophil elastase differs from pancreatic elastase in it's specificity towards synthetic substrates and also in inhibitor sensitivity (Bode et al., 1989).

In the literature the term “proteinase” and “protease” are often used synonymously. However, this is incorrect based on the nomenclature proposed by the International Union of Biochemistry and Molecular Biology (IUBMB). Briefly, the term protease comprises two groups of enzymes: the endopeptidases and the exopeptidases. In contrast, the term proteinase only refers to endopeptidases. Four mechanistic classes of proteinases are recognized by the IUBMB: 1) serine proteinases, 2) cysteine proteinases, 3) aspartic proteinases, and 4) metallo proteinases.

Exopeptidases remove amino acids sequentially from either the N- or C-terminus. In contrast, endopeptidases catalyze the hydrolysis of peptide bonds in the interior of proteins (Enzyme Nomenclature, 1984).

Table 1: Differences in biochemical characteristics of neutrophil elastase from different species

Species	Molecular Weight	Isoelectric Point	Optimum pH	Different Isoforms	Starting Material	Literature
Human	22,000 22,000	8.77-9.15 not reported	8.3 not reported	yes (4) yes	blood blood	(Taylor et al., 1975) (Feinstein et al., 1975)
Horse	20,500-24,500	8.8-10	7.0-10.0	yes (2)	blood	(Dubin et al., 1976)
Cow	33,000	not reported	not reported	no	blood	(Marossy et al., 1980)
Sheep	26,000	not reported	8.0	no	blood	(Mistry et al., 1999)
Pig	27,000	> 8	8.0	not reported	blood	(Geiger et al., 1985)
Hamster	not reported	not reported	not reported	not reported	blood	(Boudier et al., 1982)
Rabbit	25,000	5.9	7.5-8.5	no	blood	(Cotter et al., 1980)
Dog	24,800	not reported	8.0-8.5	no	induced peritonitis	(Delshammar et al., 1976)

2.1.2. Structure

Human neutrophil elastase is a glycoprotein that consists of a single polypeptide chain of 218 amino acids residues (Bode et al., 1989). Comparison of the amino acid sequence of neutrophil elastase to other elastolytic serine proteases shows only moderate homology to porcine pancreatic elastase (40% homology) (Bode et al., 1989), human pancreatic elastase I (43% homology) (Tani et al., 1988), or human neutrophil cathepsin G (37% homology) (Sinha et al., 1987).

In contrast to human neutrophil elastase, canine neutrophil elastase molecule lacks carbohydrates (Delshammar et al., 1976). The protein consists of 282 amino acids and does not contain any tyrosine and lysine. The amino acid sequence of canine neutrophil elastase is shown in table 2.

Table 2: Amino acid sequence of canine neutrophil elastase.

This figure shows the complete amino acid sequence of canine neutrophil elastase as published by the canine genome project. The sequence is shown using a one-letter code: A=alanine, C=cysteine, D=aspartic acid, E=glutamic acid, F=phenylalanine, G=glycine, H= histidine, I=isoleucine, L=leucine, M=methionine, N=asparagine, P=proline, Q=glutamine, R=arginine, S=serine, T=threonine, V=valine, W=tryptophan

1 MTARRVPAGP ALGPLLLLAT LLPGPALASE IVGGRPAQPH AWPFMVSLQR RGGHFCGGTL
61 IAPNFVMSAA HCVDGLNFRS VVVVLGAHDL GERESTRQLF AVQRVFENGF DPVRLVNDIV
121 LLQLNGSATI NANVQVARLP AQNQGVGNGV QCLAMGWGQL GTAQPPPRIL QELNVTVVTT
181 LCRRSNVCTL VPRRRAGICF GDSGGPLVCN GLIQGIDSFI RGSCASGFFP DAFAPVAQFV
241 DWINSIIRRP PALPPARPGQ QDPERGAARA PPPAPHRPRP TQ

2.1.3. Function

Stored in azurophil granules, human neutrophil elastase is sequestered in the cell as an active enzyme bound to an inert polysaccharide matrix (Del Mar et al., 1980). Neutrophil elastase plays a part in mediating the defense response to inflammation. During phagocytosis neutrophil elastase is released into the extracellular space.

Neutrophil elastase degrades connective tissue proteins, including elastin, collagens and proteoglycans (Janoff, 1985a). In addition to connective tissue proteins, many plasma proteins, such as immunoglobulins, clotting factors and complement proteins, can be hydrolyzed by neutrophil elastase (Janoff, 1985a). This is accomplished through the cleavage of peptide bonds in the target protein. The specific peptide bonds cleaved by neutrophil elastase are those on the carboxy side of small hydrophobic amino acids, especially valine and to a lesser extent alanine (Barrett, 1981). The proteolytic effect of neutrophil elastase is enhanced by the presence of other neutrophil derived proteins, such as lysozyme and cathepsin G (Boudier et al., 1981). In addition, platelet factor 4, heparin, and high ionic strength in the solution are able to enhance the elastolytic activity of neutrophil elastase (Boudier et al., 1980). Neutrophil elastase activates bactericidal/permeability-inducing protein (BPI) by cleaving the active amino terminal portion of BPI from the intact holoprotein (Edwards, 1994). The active form of BPI is then able to destruct the *Outer membrane protein A* (OmpA) of *E. coli* and other Gram-negative bacteria (Edwards, 1994).

The catalytic function of neutrophil elastase is mediated by the catalytic triad. The catalytic triad refers to the active site of the enzyme, consisting of the amino acids histidine (His-57), serine (Ser-195) (therefore the name serine proteinases), and aspartic acid (Asp-102) (Kraut, 1977). The purpose of this arrangement of amino acids in the active site is presumably to make the serine sufficiently nucleophilic to attack the carbonyl (C=O) carbon of the substrate, thus setting of the catalytic process (Daggett et al., 1991).

2.1.4. Inhibition of neutrophil elastase

During phagocytosis and neutrophil turnover, NE is released into the extracellular space. If NE function is not regulated properly NE can be extremely destructive, due to its capability of degrading many connective tissue proteins, including elastin, collagen, and proteoglycan (Janoff, 1985a). Under physiological conditions NE function is tightly regulated by circulating plasma proteinase inhibitors. The primary physiological inhibitor of NE is α_1-proteinase inhibitor (α_1-PI), a glycoprotein, synthesized in the liver, with a molecular weight of approximately 52,000 (Janoff, 1985a). In normal individuals α_1-PI inactivates any elastase released from neutrophils, either within the circulation or also outside the circulation. This inactivation leads to the formation of a stable, irreversible complex between the inhibitor and the proteinase (Nakajima et al., 1979). Neutrophil elastase binds to the active site of α_1-proteinase inhibitor with subsequent formation of a sodium dodecyl sulfate-stable linkage between the inhibitor and the enzyme (Nakajima et al., 1979). In this reaction a methionyl bond is cleaved, and it has been shown that NE has a high activity against other methionyl substrates (Travis et al., 1979). Oxidized forms of methionine residues in the α_1-PI molecule, which have been converted to a methionine sulfoxide residue, are resistant to proteinases like neutrophil elastase (Travis et al., 1979).

Alpha$_1$-PI deficiency and cigarette smoke are both major risk factors for the development of pulmonary emphysema, a main component of chronic obstructive pulmonary disease in humans (Shapiro et al., 2003). Janoff et al. demonstrated that smoke vapor inactivates α_1-PI, due to the formation of oxidized methionine residues (Janoff et al., 1977).

Alpha$_2$-macroglobulin represents the second most important defense mechanism against NE. It is not as effective as α_1-PI, because due to its molecular mass of 750,000 Daltons its activity is restricted to controlling proteinase activity within the circulation. Besides NE, alpha$_2$-macroglobulin inactivates a variety of other proteinases such as seryl-, cysteinyl-, aspartyl- and metallo-proteinases (Travis, 1988).

2.2. Neutrophil elastase and disease

2.2.1. Alpha$_1$-proteinase inhibitor deficiency

Neutrophil elastase is inhibited by the protein alpha-$_1$-proteinase inhibitor (α_1-PI), which binds neutrophil elastase covalently in a ratio of 1:1. Thus, alpha$_1$-PI deficiency leads to uninhibited destruction of elastic fiber by NE. The deficiency of α_1-PI was first described in 1963 (Laurell et al., 1963). The term α_1-PI deficiency describes the condition that fulfills the following criteria: 1) serum analysis reveals no α_1-band in the electrophoretic strip, 2) on agar gel immunoelectrophoresis only traces of α_1-PI are visible, which also show reduced mobility, and 3) all other serum proteins appear to be normal (Eriksson, 1965).

Alpha$_1$-PI deficiency is one of the most common inherited metabolic disorders in humans with the potential to cause severe injury to at least two organ systems, namely the lung and the liver (Graziadei et al., 1998). Severe α_1-PI deficiency is associated with the development of pulmonary emphysema in early or mid-life (Snider, 1989). Several studies in animal models have shown that an imbalance between proteinases (mostly NE) and anti-proteases in the lung causes the actual emphysema (Janoff, 1985b; Snider et al., 1986).

The gene for α_1-PI deficiency is located on chromosome 14 and is expressed in codominant fashion (Darlington et al., 1982). The phenotype of α_1-PI is determined by two independent expressions of the two parental alleles (Snider, 1989). Over 30 different biochemical variants of α_1-PI exist, and these are classified in the Pi system (Pi stands for “proteinase inhibitor”). Variants are designated by a capital letter corresponding to their mobility on isoelectric focusing (Hutchison, 1988). The most common variant is the M type. Other types are S and Z. In the S and Z types, abnormalities arise from a single amino acid substitution in the primary chain and result in decreased or undetectable α_1-PI concentrations in serum (Hutchison, 1988). Homozygotes of the Z type have been most commonly implicated in the pathogenesis of pulmonary emphysema (Eriksson, 1965).

The pathophysiology of lung injury in α_1-PI deficient patients is due to a deficiency of antiprotease activity and uninhibited proteolytic attack by NE on elastin in the lung (Graziadei et al., 1998). Hepatic injury in α_1-PI deficient patients is linked to

intracellular accumulation of mutant α_1-PI, mainly within the endoplasmatic reticulum (Graziadei et al., 1998).

2.2.2. Hereditary neutropenia

Inherited neutropenia is a rare disease in humans, consisting of two main forms: cyclic neutropenia (CN) and severe congenital neutropenia (SCN) (Haurie et al., 1998).

Human CN is caused by an autosomal dominant defect, and is characterized by fluctuating neutrophil counts over a 21 day period (Haurie et al., 1998). Neutrophil counts can vary from undetectable to almost physiological numbers (Lange, 1983). The periodical fluctuation between high and low neutrophil counts in CN is proposed to be caused by a feedback mechanism, by which mature neutrophils inhibit progenitor cells (Horwitz et al., 2003b). Inhibition of progenitor cells leads to a loss of successive cohorts of maturing cells, eventually depleting the generation of cells producing the inhibitory signal, thus allowing the pattern to repeat (Horwitz et al., 2003a). In turn, patients with low neutrophil counts are predisposed to opportunistic infections.

Severe congenital neutropenia refers to a non-cyclical neutropenia present at birth. Characteristic for SCN is that the bone marrow displays a pro-myelotic arrest (Horwitz et al., 2003b). Myelodysplasia and acute myelogenous leukemia frequently complicate SCN (Horwitz et al., 2004).

In 1999 Horwitz et al. reported that mutations in the ELA2 gene, the gene encoding NE, causes both CN and SCN in humans (Horwitz et al., 1999). A total of 30 different mutations in ELA2 have been identified (Horwitz et al., 2003b). Characterization of the biochemical properties of the mutant NE has been performed by recombinant expression of the mutated ELA2 in rat basophilic leukemia cells (Li et al., 2001). Most of the mutant forms of NE have no consistent effect on proteolytic activity (Li et al., 2001), and no obvious change in substrate specificity and protein stability (Horwitz et al., 2004). The mutant NE differs in its susceptibility to inhibition by α_1-PI (Horwitz et al., 2004). None of these properties were consistent in all mutants.

Recent findings of Benson et al. demonstrate a homozygous mutation of AP3B1, which encodes the β-subunit of the adaptor protein complex 3 (AP3), which has been

suggested as the cause of canine CN and suggests that CN could serve as a model for the molecular basis of hereditary neutropenia (Benson et al., 2003).

Canine cyclic neutropenia is also known as the Gray Collie Syndrome, because it was first described in this breed, and affected dogs have a hypopigmented coat (Lothrop, Jr. et al., 1987).

The canine and human forms of this disease differ in some points: 1) human patients with CN do not show signs of pigment abnormalities, 2) the disease demonstrates an autosomal-recessive transmission in the dog, 3) neutrophil counts cycle every two weeks in the dog versus every three weeks in humans (Horwitz et al., 2004), and 4) proteolytic activity of NE is reduced in humans (Horwitz et al., 2004) and nearly undetectable in dogs (Benson et al., 2003). DNA sequencing and genetic linkage analysis excluded ELA2 mutations as the cause of the Gray Collie Syndrome in dogs (Benson et al., 2003).

The adaptor protein complex 3 resides on the cytoplasmic surface of membrane-bound vesicles and directs subcellular trafficking of membrane cargo proteins (Boehm et al., 2002). Mutations of the β-subunit of AP3 result in cargo proteins being localized incorrectly in the plasma membrane (Dell'Angelica et al., 1999). Neutrophil elastase is an AP3 cargo protein (Benson et al., 2003). Mutations in either NE or the β-subunit of AP3 result in similar disease phenotypes (Horwitz et al., 2004) as are seen in human CN and SCN as well as in the Gray Collie Syndrome.

2.2.3. Neutrophil elastase as a marker of disease severity of inflammatory bowel disease in humans

Human NE, which is stored in the azurophil granules of polymorphonuclear neutrophils is released into the extracellular space during the process of phagocytosis (Bode et al., 1989). It plays an important role in endothelial cell injury mediated by neutrophils (Smedly et al., 1986).

There are two forms of human IBD, ulcerative colitis and Crohn's disease. Neutrophilic infiltration is a reliable histological finding in patients with ulcerative colitis and is frequently seen in patients with Crohn's disease, suggesting that NE may contribute to the pathogenesis of IBD (Adeyemi et al., 1985). Several studies have been performed to evaluate the utility of NE in the assessment of human patients with

IBD (Adeyemi et al., 1985; Adeyemi et al., 1992; Andus et al., 1993; Fischbach et al., 1987).

Adeyemi et al. measured human NE concentrations in the plasma of 56 patients with Crohn's disease (31 active/25 quiescent disease) and in 38 patients with ulcerative colitis (23 active/15 quiescent disease) (Adeyemi et al., 1985). The mean NE concentration in plasma in patients with active Crohn's disease was significantly greater than in patients with active ulcerative colitis ($p=0.013$) (Adeyemi et al., 1985). Plasma NE concentrations in quiescent IBD were significantly lower, compared to active IBD ($p=0.0001$ for Crohn's disease, and $p=0.0014$ for ulcerative colitis) (Adeyemi et al., 1985). Also, the concentrations of plasma NE correlated with serum C-reactive protein concentrations ($p<0.05$ for ulcerative colitis; $p<0.01$ for Crohn's disease) (Adeyemi et al., 1985). Furthermore, NE concentrations correlated significantly with the Crohn's disease activity index scores ($p<0.005$) (Adeyemi et al., 1985). The conclusions of this study were that the plasma concentration of NE in patients with IBD reflects the disease activity of their intestinal disease and repeated measurements might be useful in the assessment and clinical progression of these patients (Adeyemi et al., 1985).

Andus et al. measured serum and fecal NE concentrations in 70 patients with Crohn's disease and 24 patients with ulcerative colitis with different disease activity indices (Andus et al., 1993). The plasma NE concentrations were significantly higher in patients with active disease than in those that did not have active disease ($p=0.0017$ for Crohn's disease, and $p=0.026$ for ulcerative colitis) (Andus et al., 1993). Plasma and fecal NE concentrations correlated in patients with Crohn's disease $p<0.01$, but not in those with ulcerative colitis ($p=0.05$) (Andus et al., 1993). The correlation of disease activity and fecal NE concentration was significant in patients with ulcerative colitis ($p=0.002$), but not in those with Crohn's disease (Andus et al., 1993). Andus et al. concluded from their study that NE can be measured in plasma as well as in feces of patients with active IBD. Plasma concentrations reflect the disease activity in IBD patients (Andus et al., 1993), but due to the variation of the data and the large overlap between different groups the clinical value of this test in humans is questionable.

2.3. Diagnosis of idiopathic inflammatory bowel disease in dogs

Canine idiopathic IBD is a group of disorders characterized by persistent or recurrent gastrointestinal tract disease associated with histological evidence of inflammation of the lamina propria of the small and/or large intestine without any identifiable cause (Guilford, 1996b). The pathogenesis of IBD is ill-defined, but it is likely immune mediated with environmental and genetic factors contributing to the expression of this disease (Fiocchi, 1998). Inflammatory bowel disease is classified according to the predominant type of inflammatory cell present in the histopathological specimen and the area of the intestine affected (Jergens, 1999). The most common form described is lymphocytic-plasmacytic enteritis (Jergens et al., 1992). Also, frequently occurring are eosinophilic gastroenterocolitis and granulomatous enteritis (Jergens et al., 1992).

The most prominent clinical signs associated with canine idiopathic IBD are chronic vomiting and/or diarrhea (in severe cases hematemesis and/or hematochezia or melena), altered appetite, abdominal discomfort/pain, excessive borborygmus and weight loss (Hall et al., 2005).

Before a diagnosis of idiopathic IBD can be made, a variety of other diseases that may cause intestinal inflammation have to be ruled out. The major differential diagnoses for IBD are: 1) chronic infection (e.g., *Giardia* sp., *Histoplasma* sp., *Toxoplasma* sp., *Mycobacteria* sp., protothecosis, pythiosis, or pathogenic bacteria), 2) food allergy, and 3) bowel inflammation associated with other primary gastrointestinal diseases (e.g., lymphoma and lymphangiectasia) (Hall et al., 2005). Jergens et al. (Jergens et al., 2003) developed a scoring index for disease activity in canine patients with idiopathic IBD, the canine IBD activity index (CIBDAI). The CIBDAI is useful in the management of clinical patients with IBD by helping assessing the response to treatment, as well as long term progress (Jergens et al., 2003).

The gold standard for the diagnosis of idiopathic IBD is endoscopy and histopathologic evaluation of intestinal biopsies (Guilford, 1996b). Because of the disadvantages of these modalities, such as invasiveness of the procedure, high intra- and inter-observer variation on histopathologic evaluation, and the financial aspect, new diagnostic approaches are desirable (Willard et al., 2002).

2.3.1 Serologic markers for canine IBD

Recent studies examined different serologic markers such as perinuclear antineutrophilic cytoplasmic antibodies (pANCA) (Luckschander et al., 2006), and serum acute-phase proteins, such as C-reactive protein (CRP), haptoglobin (HAP), α-acid glycoprotein (AGP) and serum amyloid A (SAA) (Jergens et al., 2003) for their usefulness as a diagnostic tool for IBD.

Luckschander et al. (Luckschander et al., 2006) examined a group of 65 dogs for the presence of pANCA. Of these 65 dogs, 39 dogs had food- responsive diarrhea (FRD) and 26 dogs had idiopathic IBD. In the FRD group the percentage of pANCA positive dogs (62%) was significantly ($p=0.002$) higher compared with the IBD group (23%). There was no difference in pANCA titers between the groups after appropriate treatment. No correlation between pANCA status in FRD or IBD dogs before treatment and scores for CIBDAI, endoscopy, or histopathology before or after treatment was observed (Luckschander et al., 2006).

Jergens et al. compared the serum concentrations of CRP, HAP, AGP, and SAA with the CIBDAI in 58 dogs with IBD (Jergens et al., 2003). Concentrations of SAA were significantly ($p<0.001$) higher in healthy dogs than in dogs with IBD. CRP and AGP concentrations were higher in IBD dogs compared to healthy dogs. The best correlation with the CIBDAI was observed when HAP and histology scores where compared ($p<0.001$) (Jergens et al., 2003). During the 2 to 3 weeks post-treatment period HAP was significantly ($p<0.0001$) increased, whereas CRP and CIBDAI were significantly decreased ($p<0.0001$), compared to their values prior to treatment (Jergens et al., 2003).

In conclusion: although these assays might be useful in some cases, they have not been found to be particularly helpful in diagnosing, monitoring, and predicting the outcome of idiopathic IBD in dogs. As a result, the need for new non-invasive tests for canine idiopathic IBD still remains.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and reagents

3,3',5,5'-tetramethylbenzidine solution	Pierce Chemical CO, Rockford, IL, USA
Agarose immunodiffusion plate	Pierce Chemical CO, Rockford, IL, USA
Ammonium sulfate	Sigma Chemicals, St. Louis, MO, USA
Avidin Immuno Pure HABA assay kit	Pierce Chemical CO, Rockford, IL, USA
Biotin (EZ-Link Sulfo-NHS-LC biotin)	Pierce Chemical CO, Rockford, IL, USA
Bovine hemoglobin	Sigma Chemicals, St. Louis, MO, USA
Bovine serum albumin	Sigma Chemicals, St. Louis, MO, USA
Carbonate-bicarbonate buffer	Pierce Chemical CO, Rockford, IL, USA
Coomassie blue stain	Pierce Chemical CO, Rockford, IL, USA
Dextran 500	Accurate Chemicals & Scientific Corp. Westbury, NY, USA
Dimethyl sulfoxide	Sigma Chemicals, St. Louis, MO, USA
NuPAGE 10% Tris-glycine gel	Invitrogen, Carlsbad, CA, USA
IEF anode buffer	Invitrogen, Carlsbad, CA, USA
IEF calibration markers	Amersham Biosciences, Piscataway, NJ
IEF cathode buffer	Invitrogen, Carlsbad, CA, USA
IEF electrophoresis gel pH 3-10	Invitrogen, Carlsbad, CA, USA

IEF sample buffer	Invitrogen, Carlsbad, CA, USA
Immuno Pure Streptavidin HRP-conjugated	Pierce Chemical CO, Rockford, IL, USA
Molecular weight markers – Mark 12	Invitrogen, Carlsbad, CA, USA
NuPAGE MES SDS running buffer	Invitrogen, Carlsbad, CA, USA
NuPAGE sample reducing agent	Invitrogen, Carlsbad, CA, USA
NuPAGE antioxidant	Invitrogen, Carlsbad, CA, USA
NuPAGE LDS sample buffer	Invitrogen, Carlsbad, CA, USA
Phosphate buffered saline packs	Pierce Chemical CO, Rockford, IL, USA
Polyoxyethylene sorbitan monolaurate (Tween-20)	Pierce Chemical CO, Rockford, IL, USA
Protein 50 LabChip® kit	Agilent Technologies GmbH, Waldbronn, GER
Silver stain kit	Pierce Chemical CO, Rockford, IL, USA
Sodium azide	Sigma Chemicals, St. Louis, MO, USA
Sodium chloride	Sigma Chemicals, St. Louis, MO, USA
Sodium citrate	Sigma Chemicals, St. Louis, MO, USA
Sodium hydroxide	Sigma Chemicals, St. Louis, MO, USA
Sodium metabisulfite	Sigma Chemicals, St. Louis, MO, USA
Sodium phosphate dibasic	Sigma Chemicals, St. Louis, MO, USA
Sodium phosphate monobasic	Sigma Chemicals, St. Louis, MO, USA
Trichloroacetic acid	Sigma Chemicals, St. Louis, MO, USA

Tris-Glycine SDS running buffer	Invitrogen, Carlsbad CA, USA
Tris-Glycine SDS sample buffer	Invitrogen, Carlsbad CA, USA
Urea	Sigma Chemicals, St. Louis, MO, USA
Acrylamide	Sigma Chemicals, St. Louis, MO, USA
Glycerol	Sigma Chemicals, St. Louis, MO, USA
Sodium dodecyl sulfate (SDS)	Sigma Chemicals, St. Louis, MO, USA
Trishydroxymethyl-aminomethane (Tris)	Bio-Rad Laboratories, Hercules, CA, USA
Beta mercaptoethanol	Sigma Chemicals, St. Louis, MO, USA
Bromophenol blue	Sigma-Aldrich, St. Luis, MO, USA
N'N'-bis-methylene-acrylamide (Bis)	Bio-Rad Laboratories, Hercules, CA, USA
N,N,N',N'-Tetra-methyl-ethylenediamine (TEMED)	Bio-Rad Laboratories, Hercules, CA, USA
Ammonium persulfate (APS)	Bio-Rad Laboratories, Hercules, CA, USA
iso-amyl alcohol	EMD Chemicals Inc., Darmstadt, GER
PUREGENE RBC lysis solution	Gentra Systems, Minneapolis, MN, USA
Acetic Acid, glacial	EMD Chemicals Inc., Darmstadt, GER
BCA™ protein assay kit	Pierce Chemical CO, Rockford, IL, USA
Human elastase	Sigma-Aldrich, St. Luis, MO, USA
Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide	Calbiochem, EMD Biosciences, La Jolla, CA, USA

3.1.2. Instruments

Agilent 2100 Bioanalyzer	Agilent Technologies GmbH, Waldbronn, Germany
Analog vortex mixer	VWR, West Chester, PA, USA
Centrifuge 5810R	Eppendorf, Hamburg, Germany
Centrifuge GS6R	Beckman, Palo Alto, CA, USA
Centrifuge rotor F 34-6-38	Eppendorf, Hamburg, Germany
Centrifuge rotor GH 3.8	Beckman, Palo Alto, CA, USA
Chromatography column – HI Trap®	Amersham Biosciences, Piscataway, NJ, USA
Chromatography column PD-10, Sephadex G-25 M	Amersham Biosciences, Piscataway, NJ, USA
Chromatography control system - UNICORN - 2.30	Amersham Biosciences, Piscataway, NJ, USA
Chromatography pump – P-900	Amersham Biosciences, Piscataway, NJ, USA
Chromatography UV-monitor 900	Amersham Biosciences, Piscataway, NJ, USA
Chromatography valve PLV-50	Amersham Biosciences, Piscataway, NJ, USA
Electrophoresis chamber - X-Cell II	Invitrogen, Carlsbad, CA, USA
FPLC - system ÄKTA purifier	Amersham Biosciences, Piscataway, NJ, USA
Fraction collector Frac-900	Amersham Biosciences, Piscataway, NJ, USA
Fraction collector, Model 2110	Bio-Rad Laboratories, Hercules, CA, USA
Mini Prep Cell	Bio-Rad Laboratories, Hercules, CA, USA
Multipipetter Oxford	Fisher Scientific, Pittsburgh, PA, USA

Peristaltic Pump, Mini-pump variable flow	VWR, West Chester, PA, USA
pH-meter – model 8010	VWR, West Chester, PA, USA
Pipettes P-10, P-20, P-100	Rainin, Woburn, MA, USA
Pipettes P-250, P-1000, P-5000	Rainin, Woburn, MA, USA
Plate incubator/shaker Stat Fax©-2200	Awareness Technology Inc., Palm State, FL, USA
Power Pac 3000 Power Supply, 220/240 V	Bio-Rad Laboratories, Hercules, CA, USA
SOFTMAX PRO analysis software package	Molecular Devices, Sunnyvale, CA, USA
Spectrophotometer, Ultrospec 2000	Pharmacia Biotech, Cambridge, UK
Statistical software package Prism 3.0	GraphPad Software Inc., San Diego, CA
Tissue grinder Polytron PT-2100	Kinematica AG, Luzern, Switzerland
Ultrasonic homogenizer, Model 300 V/T	BioLogics, Inc., Manassas, VA, USA
UV MAX ELISA plate reader	Molecular Devices, Sunnyvale, CA, USA

3.1.3. Disposables

Blood collection bottles, evacuated, with anticoagulant ACD solution	The Metrix Company, Dubuque, IA, USA
Centrifugal filter devices - Centripep YM-10	Amicon Bioseparations, Bedford, MA, USA
Centrifugal filter devices - Centripep YM-3	Amicon Bioseparations, Bedford, MA, USA
Centrifugal filter devices -Amicon Ultra - 4 5,000 MWCO	Millipore Corporation, Bedford, MA, USA
Culture tubes 12 x 75 mm	VWR, West Chester, PA, USA
Dialysis cassettes - Slide-A-Lyser 2K	Pierce, Rockford, IL, USA
ELISA plates Combi plates 8	Labsystems, Helsinki, Finland
Membrane filters 3 μm – 0.45 μm	Pall – Gelmann Sciences, Ann Arbor, MI, USA
Pleated dialysis tubing – Snakeskin 10k MW	Pierce, Rockford, IL, USA
Self-Standing centrifugal tubes, polypropylene, 50 ml	Corning Incorporated, Corning, NJ, USA
EZ Flip™ centrifugal tubes, 15 ml	Nalge Nunc International, USA
Blue Max™ Jr., conical polypropylene tube, 15 ml	Falcon, Franklin Lakes, NJ, USA
Protein assay chips, protein 50	Agilent Technologies GmbH, Waldbronn, Germany
ProSorb™, PVDF membrane	Perkin Elmer, Foster City, CA, USA

3.2. Methods

3.2.1. Purification of canine neutrophil elastase

3.2.1.1. Assay for proteolytic activity

Fractions obtained at different stages of the purification procedure were screened for the presence of canine neutrophil elastase (cNE) activity by modification of a published spectrophotometric assay method (Nakajima et al., 1979). The assay was performed in 96-microwell plates. Briefly, 20 µl of a high ionic strength buffer (0.02 M Tris, 1 M NaCl, 0.05% Tween, pH 8.5) were pipetted into each well, 50 µl of test sample were added, and the plate was incubated at room temperature for 5 minutes. After incubation 10 µl of methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide, a synthetic chromogenic substrate, were added to each well. The plate was then placed immediately in an automated plate reader and the increase in absorbance was measured over a period of 15 min at 405 nm.

One unit of enzyme activity was defined as the amount of material, which produced the equivalent activity of 1 µg of human neutrophil elastase under above assay conditions.

3.2.1.2. Preparation of neutrophils

Blood was collected from healthy dogs euthanatized for unrelated research projects. Blood was drawn from the heart immediately following euthanasia. The blood was collected into 500 ml sterile nonpyrogenic evacuated blood collection bottles containing sodium citrate as an anticoagulant. The blood was immediately processed by mixing one volume of blood with 0.4 volume of a 60 mg/ml Dextran 500 solution, containing 9 mg/ml NaCl in a beaker. The mixture was stirred for 5 minutes and incubated at room temperature for 5 hours without stirring. During this period the erythrocytes settled to the bottom of the beaker. After the incubation time, two layers were clearly visible, a dark red layer on the bottom and a lighter layer on top. Preparation was continued with the top layer. The top layer was carefully aspirated and transferred in 50 ml polypropylene centrifugal tubes. The tubes were placed in a fixed angled rotor centrifuge and spun at 2,000 x g at 4ºC for 20 min. The supernatant was carefully discarded and the pellet was dissolved in a commercially available red blood cell lysis solution containing ammonium chloride, ethylenediaminetetraacetic

acid, and sodium bicarbonate (Gentra, Puregene® RBC Lysis solution). The dissolved pellet was incubated at room temperature for 20 minutes on a rocking plate. After the incubation time the tubes were placed into a fixed-angle centrifuge and spun at 2,000 x g and 4ºC for 20 min. The obtained neutrophil rich pellet was stored at -20ºC until further use.

3.2.1.3. Extraction of neutrophil elastase

The pellet was suspended in extraction buffer (20 ml 0.02 M Tris, 1 M NaCl, 0.05% Tween, pH 8.5). For full resuspension of the pellet a tissue grinder was used for 3 minutes and the solution was subsequently vortexed for 2 minutes. The solution was afterwards frozen at -20ºC for 3 hours, thawed at room temperature, and ultra sonicated for 1.5 minutes at 50% power output. The freezing, thawing, sonication procedure was repeated three times. Subsequently the solution was spun at 18,000 x g and 4ºC for 20 min. The pellet was discarded and the supernatant was filtered through a series of filters with decreasing pore size from 10 μm to a 0.8 μm and the buffer was exchanged to buffer A (0.05 M sodium acetate, 0.05 M NaCl, pH 5.0) for 12 hours, using dialysis tubing. After the initial exchange the dialysis buffer was exchanged two more times at 2 hour intervals.

3.2.1.4. Column chromatography - strong cation-exchange chromatography

The filtered and dialyzed solution was loaded onto a 1.3 cm × 3.5 cm Macro-Prep® column. The column matrix consisted of spherical rigid polymers with sulphur ions as functional groups. The column had been previously equilibrated with buffer A. Ten ml 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 using a step gradient of 0.3 M NaCl in buffer A for 20 column volumes. More strongly bound proteins were eluted by a linear salt gradient ranging from 0.3 to 0.7 M NaCl in buffer A over 12 column volumes at a flow rate of 1 ml/min. Fractions of 3 ml each were collected. Fractions containing proteolytic activity against methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide were pooled and concentrated using a centrifugal filter device with a molecular weight cut off of 10 kDa. The filter device was spun at 5,000 x g until the protein solution reached a concentration of approximately 0.6 - 0.7 mg/ml.

3.2.1.5. Continuous-elution electrophoresis

The concentrated protein solution obtained from the column chromatography step was further separated using the Bio-Rad Mini Prep Cell following the manufactures instructions. The acrylamide gel column was prepared 12 hours prior to the each run. Briefly, a stock solution of 30% acrylamide and bis (N, N'-bis-methylene-acrylamide) was prepared. From this stock solution 4.33 ml were mixed with 3.15 ml ultra pure water and 2.5 ml of 1.5 M Tris-HCl buffer, pH 8.8, to prepare a 13% resolving gel. Two ml of the resolving gel were blended with 5 µl of a 10% ammonium persulfate solution, 0.5 µl of tetra-methyl-ethylenediamine (TEMED), filled in a glass column and overlaid with iso-amyl alcohol. The acrylamide was allowed to polymerize for 12 hours. One hour prior to each run, a second layer of acrylamide solution, the stacking gel, was applied. The 4% stacking gel consisted of 6.15 ml ddH_20, 2.50 ml 0.5 M Tris-HCl buffer, pH 6.8, and 1.33 ml of 30% acrylamide/bis stock solution. Two ml of the stacking gel were mixed with 10 µl of a 10% ammonium persulfate solution, 2 µl of TEMED, and were carefully layered on top of the resolving gel. After one hour of polymerization time 0.5 ml of sample were mixed with 0.5 ml sample buffer (0.06 M Tris-HCl, 2% SDS, 5% beta mercaptoethanol, 25% glycerol (w/v) and 0.01% bromophenol blue, pH 6.8), incubated at 95°C for 4 min and loaded onto the gel column. The buffer reservoir of the Mini Prep Cell was filled with running buffer (0.025 M Tris-HCl, 0.192 M glycine, 0.1% SDS buffer, pH 8.3) and a constant voltage of 200 V was applied to the system. The proteins in the solution were separated according to their molecular weight and were discharged into the elution chamber at the bottom of the gel. From there the proteins were eluted in running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3) into different 300 µl fractions using a peristaltic pump.

3.2.1.6. Sodium-dodecyl-sulfate gel electrophorese (SDS-PAGE)

The purity of the obtained protein was analyzed by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. A pre-cast, 1 mm thick 10% polyacrylamide gel was used. The gel cassette was tightly looked into the electrophoresis chamber, and the chamber was filled with MES running buffer (1 M 2-(N-morpholino) ethane sulfonic acid, 1 M Tris-base, 70 mM

SDS, 21 mM EDTA, pH 7.3). The samples were prepared by adding 24 µl of sample to 4 µl of reducing agent and 10 µl of NuPAGE® SDS sample buffer (4 g sucrose + 0.68 g Tris-base + 0.67 g Tris-HCl + 0.8 g SDS + 0.006 g EDTA + 0.75 ml Serva blue G250 + 0.25 ml Phenol red + ultra pure water to 10 ml). Samples were incubated at 70°C for 10 min. After incubation 23 µl of each of the samples were loaded into the wells. In an addition to the samples a standard protein solution was loaded into the first lane. The standard protein solution contained 12 different proteins with a known molecular mass, including myosin: 200 kDa, β-galactosidase: 116.3 kDa, phosphorylase: 97.4 kDa, bovine serum albumin: 66.3 kDa, glutamic dehydrogenase: 55.4 kDa, lactate dehydrogenase: 36.5 kDa, carbonic anhydrase: 31 kDa, trypsin inhibitor: 21.5 kDa, lysozyme: 14.4 kDa, aprotinin: 6 kDa, insulin B chain: 3.5 kDa, and insulin A chain: 2.5 kDa. The electrophoresis gel was run at a constant voltage of 200 V for 35 minutes. Immediately after completion the gel was removed from the cassette and stained with a silver stain kit, according to the manufacture's instructions.

3.2.2. Partial characterization of canine neutrophil elastase

3.2.2.1. Estimation of the molecular weight

The molecular weight of canine neutrophil elastase was estimated by a chip based protein assay, performed on the Agilent 2100 bioanalyzer in combination with the Protein 50 Lab-Chip® kit. Data were analyzed with the Protein 50 assay software.

The estimation of the molecular weight was based on the measured electrophoretic migration time of the protein given in seconds. The software automatically performed a sizing based on the alignment with internal makers and an external protein standard (ladder).

In more details: The chip accommodates sample wells, gel wells and a well for an external standard (ladder). Micro-channels are fabricated in glass to create interconnected networks among these wells. During chip preparation, the micro-channels are filled with a sieving polymer and fluorescence dye. Once the wells and channels are filled, the chip becomes an integrated electrical circuit. The 16-pin electrodes of the cartridge are arranged so that they fit into the wells of the chip. Each electrode is connected to an independent power supply. Proteins (charged biomolecules) are electrophoretically driven by a voltage gradient, similar to slab gel electrophoresis. Because of a constant mass-to-charge ratio and the presence of a

sieving polymer matrix, the molecules are separated by size (the smaller the protein the faster it migrates). Proteins are then intercalated with dye molecules and are detected by laser-induced fluorescence. Data is translated into gel-like images (bands) and electropherograms (peaks). A standard curve of migration time versus fragments size is plotted with the help of a ladder (protein standards). From the migration times measured for each fragment in the sample, the size is calculated. Two internal markers are run with each of the samples bracketing the overall sizing range. The "lower" and "upper" markers are internal standards used to align the ladder data with data from the sample wells. This is necessary to compensate for drift effects that may occur during the course of a chip run.

Quantitation is done with the help of the upper marker. The area under the upper marker peak is compared with the sample peak areas. Because the concentration of the upper marker is known, the concentration for each sample can be calculated.

The chip and the sample (purified cNE) were prepared according to the manufacturer's instructions.

3.2.2.2. Estimation of isoelectric point

The isoelectric point was estimated by polyacrylamide gel electrophoresis using a NOVEX® Pre-Cast vertical IEF gel, 5% polyacrylamide, and 2% ampholytes with a pH range of 3 to 10. Special protein markers for isoelectric focusing gels were used to generate a standard curve. The protein marker contained 9 different proteins, ranging from a pI 10.7 (cytochrome C, horse heart) to a pI 3.5 (amyloglucosidase, *Aspergillus niger*).

The sample was prepared by thoroughly mixing one part of sample with one part of NOVEX® IEF sample buffer. The buffer for the cathode was prepared by adding one part NOVEX® IEF cathode buffer to nine parts of deionized water. The buffer was degassed for 20 minutes and then filled into the upper buffer chamber. The anode buffer was prepared by adding 1 part of NOVEX® IEF anode buffer to 49 parts of deionized water and poured into the lower buffer chamber. Samples were loaded into the wells and the gel was run at a constant voltage of 100 V for 1 hour, followed by a constant voltage of 200 V for 1 hour and finally at a constant voltage of 500 V for 30 minutes. The gel was then removed from the cassette and incubated for 30 minutes in

fixing solution (17.3 g sulphosalicylic acid, 57.3 g trichloroacetic acid, in 500 ml deionized water). The gel was stained with a silver stain kit, following the manufacture's instructions. A standard curve was generated by measuring the migration distance from the well to the protein bands of the markers.

3.2.2.3. Determination of the extinction coefficient for neutrophil elastase

The extinction coefficient of cNE was determined based on the bicinchoninic acid (BCA) assay (Smith et al., 1985). The BCA™ protein assay is used for the colorimetric detection and quantification of total protein in a solution. The method is based on the reduction of Cu^{+2} to Cu^{+1} in an alkaline environment. The reduced copper forms a purple colored complex with bicinchoninic acid, which is colorimetrically measured at 542 nm. The absorbance of the complex is nearly proportional with increasing protein concentration.

The assay was performed according to the manufacturer's instructions in a microplate format. Briefly, 8 standards from bovine serum albumin were prepared. The protein concentration of the standards ranged from 2,000 µg/ml to 25 µg/ml. Purified cNE with a known absorbance at 280 nm was diluted 1 in 2, 1 in 4, and 1 in 8 in PBS. The BCA™ working reagent was prepared by mixing 50 parts of BCA™ reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1 M sodium hydroxide) with 1 part of BCA™ reagent B (4% cupric sulfate). A volume of 15 µl of each standard, samples, and a blank was loaded into the microplate wells, and 200 µl of the previously mixed BCA™ working reagent were added to each well. The plate was incubated for 30 min at 37ºC, and the absorbance was measured at 562 nm.

The absorbance of each well was corrected for non-specific binding (blank). A standard curve was prepared by plotting the average of the duplicates for each BSA standard versus its concentration in µg/ml. The protein concentration of each unknown sample was interpolated from the standard curve

The extinction coefficient was calculated by dividing the previously measured absorbance of the pure protein solution at 280 nm by the protein concentration, calculated with the help of the BCA™ protein assay. Each of the 3 cNE dilutions (1 in 2, 1 in 4, and 1 in 8) were calculated and the mean was taken.

3.2.2.4. Material preparation for N-terminal amino acid sequencing

Twenty µg of purified cNE were transferred onto a PVDF membrane using the ProSorb™ sample preparation cartridge. The protein transfer was done according to the manufacture's protocol. The sample was analyzed at the Department of Biotechnology at the Jagiellonian University and Bio Centrum Ltd. facility in Kraków, Poland. The N-terminal amino acid sequence analysis of the first 25 residues of canine neutrophil elastase was obtained by use of the Edman degradation method. During the Edman degradation method phenylisothiocyanate reacts with the amino acid residue at the amino terminus to form a phenylthiocarbamoyl derivative. Trifluoroacetic acid is used to cleave off the first amino acid as its anilinothialinone derivative, followed by the conversion to a phenylthiohydantoin derivative. Bound to phenylthiohydantoin the amino acid is then detected by high-performance liquid chromatography (HPLC). This process was repeated sequentially until the first 25 amino acid residues were determined.

3.2.3. Production of anti-canine neutrophil elastase antiserum

A single rabbit (*Oryctolagus cuniculus*) was vaccinated with 200 µg of cNE mixed with 0.5 ml complete Freund's adjuvant. For reinoculations 150 µg cNE mixed with 0.5 ml incomplete Freund's adjuvant were administered on days 21, 42, and 63. Sera were collected by venipuncture of the ear vein, before each booster vaccination. After a total of 4 vaccinations the rabbit was exsanguinated.

3.2.3.1. Determination of antibody titer

To estimate the antibody concentration in the serum of the rabbit, an indirect ELISA was performed. A 96-microwell plate was coated with 500 ng/well of pure cNE in 100 µl/well carbonate-bicarbonate buffer, pH 9.4, and incubated at 37°C in a plate shaker for 1 hour. The plate was washed 4 times with 200 µl/well of PBS, pH 7.2. Remaining binding sites were blocked with 200 µl/well of 1% bovine serum albumin (BSA) in PBS. The plate was again incubated for 1 hour at 37ºC and washed as described above. The antiserum to be tested was diluted 1 in 100 in PBS +1% BSA +0.05% polyoxy-ethylene sorbitan monolaurate (Tween), followed by a dilution series of 1 in 2, with a final dilution of 1 in 204,800.

A volume of 100 µl/well of the serum dilutions were loaded in duplicates. As a negative control 100 µl of PBS +1% BSA +0.05% Tween (PBS+BSA+Tween) was used. The plate was incubated for 1 hour at 37ºC and washed as described above. A solution containing 100 ng of anti-rabbit antibodies coupled to horseradish peroxidase in 100 µl of PBS+BSA+Tween was added to each well. After incubation for 1 hour at 37ºC and washing as described the retained horseradish peroxidase was visualized by adding 100 µl/well 3,3',5,5'-tetramethylbenzidine solution. The reaction was stopped after 2 minutes by adding 100 µl/well of stopping solution (4 M acetic acid, 1 N sulfuric acid). The absorbance was read at a wavelength of 450 nm in an automated plate reader.

3.2.3.2. Ouchterlony double immunodiffusion assay

The cross reactivity of human neutrophil elastase with cNE antiserum was evaluated by an Ouchterlony double immunodiffusion assay. The assay is based on the principle that macromolecular antigens and antibodies form complexes that become insoluble and precipitate. Canine NE antiserum, produced as described above, purified cNE and commercially available human neutrophil elastase were used for this procedure. A volume of 23 µl of each material, as well as a blank (PBS) were placed in separate wells cut in an agarose plate. The plate was incubated at 37ºC for 12 hours in a moist environment, to prevent the plate from drying out. After the incubation time the plate was examined for precipitation lines.

3.2.4. Purification of anti-canine neutrophil elastase antibodies

3.2.4.1. Preparation of affinity chromatography column

Monospecific polyclonal antibodies were purified by the use of an affinity chromatography column. The column was prepared following the manufacturer's instruction. Briefly, 0.7 mg of cNE was buffer exchanged against a ligand buffer (200 mM $NaHCO_3$, 500 mM NaCl, pH 8.3) using a PD-10 disposable gel filtration column. The PD-10 column was equilibrated with 30 ml of ligand buffer. Two and a half ml of protein solution were applied onto the column and the effluent discarded. Then 3.5 ml of ligand buffer were applied onto the column and the effluent was collected. The ligand solution (0.7 mg of NE in ligand buffer) was concentrated to a volume of 1 ml using a centrifugal filtration device.

Before ligation the affinity column was washed with 3 bed volumes (6 ml) of ice-cold 1 mM HCl at a flow rate of ½ drop per second. The ligand solution was then slowly applied and the column was incubated for 1 hour at room temperature. The column was washed with 6 ml 500 mM ethanolamine, 500 mM NaCl, pH 8.3 (buffer I), followed by 6 ml 100 mM sodium acetate, 500 mM NaCl, pH 4.0 (buffer II), followed by 6 ml of buffer I. The column was incubated for 30 min at room temperature and then washed with 6 ml buffer II, 6 ml buffer I, and 6 ml buffer II. After this the column was equilibrated with 75 mM Tris-HCl, 150 mM NaCl buffer, pH 8.0 and stored until further use.

3.2.4.2. Affinity chromatography of anti-canine neutrophil elastase antibodies

The rabbit antiserum was prepared for purification by lipoprotein precipitation. Briefly, 20 ml of rabbit antiserum were thawed and added to 20 ml of 1 M $CaCl_2$ and 800 µl of 10% dextran sulfate solution. The mixture was gently mixed in a 50 ml centrifuge tube on a plate rocker for 20 minutes at 4ºC and subsequently centrifuged at 10,000 × g and 4ºC for 10 minutes. The precipitate was discarded and the supernatant was filtered through a filter paper. A PD-10 disposable gel filtration column was used for buffer exchange to 75 mM Tris-HCl, 150 mM NaCl, pH 8.0 (buffer III), following the manufacturer's instructions.

The previously prepared affinity column was attached to a fast performance liquid chromatography (FPLC) purification system and equilibrated with buffer III. Two ml of the buffer exchanged antibody solution were applied onto the column. The absorbance of the effluent was measured at a wavelength of 280 nm. The column was washed with buffer III until the absorbance had returned to baseline, then the mobile phase was changed to 100 mM glycine, 500 mM NaCl, pH 2 (buffer IV), and 1 ml fractions of the effluent were collected in test tubes containing 300 µl of 1 M Tris-HCl, pH 8.0. The eluting peak was collected and all fractions belonging to that peak were pooled, concentrated, and buffer exchanged to PBS, pH 7.2 using a centrifugal filter device. The antibody concentration was adjusted to 1 mg/ml, and aliquots of 250 µl were stored frozen at -20ºC until further use.

3.2.4.3. Biotinylation of purified antibodies

For biotinylation, 0.7 mg of the purified monospecific polyclonal cNE-antibody in PBS, pH 7.2, were mixed with a 20-fold molar excess of biotin. After incubation for 45 min at room temperature, the material was immediately buffer exchanged against PBS, pH 7.2. For this procedure a disposable centrifugal filter device was used. The filter was filled with the antibody-biotin mixture, spun at 6,000 x g for 20 min at 4ºC, refilled with PBS and spun another time at 6,000 x g. This procedure war repeated 5 times.

Biotinylation efficiency was determined by use of a 2-4'-hydroxyazonbenzene benzoic acid avidin assay kit. Briefly, 24.2 mg of HABA were mixed with 9.9 ml of ultra pure water and 200 µl of 1 N NaOH were added. The solution was filtered through a 0.45 µm pore-size syringe filter to remove solids. Then 500 µl of a 1 mg/ml avidin solution were mixed with 470 µl PBS and 30 µl HABA solution. The absorbance was measured at a wavelength of 500 nm. Then 50 µl were removed from the avidin/PBS/HABA solution and replaced by 50 µl of biotinylated antibodies. The absorbance was again measured at 500 nm. The procedure was repeated until a biotinylation coefficient of between 3.0 and 4.0 was reached. The biotinylated antibodies were adjusted to a concentration of 1 mg/ml and frozen in aliquots of 100 µl at -20ºC until further use.

3.2.5. Set-up of an enzyme linked immunoassay (ELISA) for the measurement of canine neutrophil elastase concentration in serum

Standard 96-well flat-bottom ELISA plates were coated with 200 ng/well affinity purified anti-cNE antibodies in 100 µl of carbonate-bicarbonate buffer, pH 9.4. Plates were mixed using a plate vortexer and incubated for 1 hour at 37ºC using an automated plate incubator/shaker and then washed 4 times with 200 µl/well of 50 mM Tris-base buffer, containing 0.14 M NaCl, pH 7.2 (TBS). Remaining binding sites were blocked with 200 µl/well of 10% bovine serum albumin (BSA) in PBS. Plates were again incubated for 1 hour at 37ºC and washed as described above. Standard solutions of cNE were prepared by serial dilution of pure canine neutrophil elastase in TBS, 1% BSA, and 0.05% Tween (TBS+BSA+Tween). Standards of 640, 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25 and 0.63 µg/L cNE were prepared and frozen at –80ºC in aliquots of 300 µl each. The standard solutions were thawed immediately prior to

loading the plate. Control and unknown samples were prepared in a 1 in 20 dilution with buffer A. Standards, negative control, and diluted samples were loaded in duplicates (100 µl/well). As a negative control 100 µl of TBS+BSA+Tween were used. Plates were incubated for 3 hours at 37ºC and washed as described above. A solution containing 100 ng of biotinylated anti-cNE antibodies in 100 µl of PBS+BSA+Tween was added to each well. After incubation for 1 hour at 37ºC and washing as described above, 8 ng/well of horseradish peroxidase-labeled streptavidin in 100 µl TBS+BSA+Tween was added to each well. Plates were once again incubated for 1 hour at 37ºC and washed as above. Retained horseradish peroxidase was developed by adding 100 µl/well 3, 3', 5, 5'-tetramethylbenzidine solution. The reaction was stopped after 20 minutes by adding 100 µl/well stopping solution (4 M acetic acid, 1 N sulfuric acid). Absorbance was read at a wavelength of 450 nm using an automated plate reader.

Standard curves were calculated by a 4-parameter curve fit using the mathematical equation: $y=([A-D]/[1+(x/C)^B])+D$, where D is the y-value corresponding to the asymptote of the x-axis at high values, A is the y-value corresponding to the asymptote of the x-axis at low values, C is the x-value corresponding to the midpoint between A and D, and B describes how rapidly the curve makes the transition from the asymptotes in the center of the curve. All four parameters were calculated by use of an algorithm based on the Levenberg-Marquardt equation.

3.2.5.1. Validation of the ELISA

The ELISA was validated by determination of assay sensitivity, upper limit of the working range, dilutional parallelism, spiking recovery, intra-assay variability, and inter-assay variability.

The assay detection limit (sensitivity) was defined as the apparent concentration of cNE that produces an absorbance equal to the mean plus 3 times the standard deviation of the absorbance of 10 duplicates of a negative control solution (PBS+BSA+Tween).

The upper limit of the working range was defined as the apparent concentration of cNE that produces an absorbance equal to the mean minus 3 times the standard deviation of the absorbance of 10 duplicates of a cNE solution of 2,000 µg/L.

Four different 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/L cNE to 4 different serum samples.
Intra-assay variability was determined by evaluating 4 different serum samples 10 times in duplicates within the same assay run.
Inter-assay variability was determined by evaluating 4 different serum samples during 10 consecutive assay runs.

3.2.6. Serum canine neutrophil elastase concentration in healthy dogs

A total number of 54 serum samples from clinically healthy dogs were collected. The dogs were owned by students or staff of the Texas A&M University. The drawing of blood was approved by the Clinical Research Review Committee of the Texas Veterinary Medical Center at Texas A&M University. All dogs underwent a short physical examination. Also, trypsin-like immunoreactivity (TLI), canine pancreatic-lipase immunoreactivity (cPLI), cobalamin, folate and C-reactive protein (CRP) were measured and had to be within the normal range for the dog to be included in this healthy control group.
The data were analyzed by determining the mean and standard deviation. The reference range was calculated using the lower 95^{th} percentile.

3.2.7. Serum canine neutrophil elastase concentration in dogs with gastrointestinal disease

Ninety seven dogs were enrolled in this study. Fifty six of these diseased dogs were referred to a veterinary referral center in Denmark, specialized in gastrointestinal diseases. There they underwent endoscopy and intestinal biopsies were taken. Biopsies were evaluated by specialized veterinary pathologists. The diagnosis was based on the histopathologic results. The remaining 41 dogs enrolled in this study were submitted to the GI lab at Texas A&M University and had cPLI concentrations diagnostic for pancreatitis.
The dogs were organized in 4 groups, based on the histopathologic results of their biopsies or their cPLI concentration. Group 1 contained dogs with lymphocytic-

plasmacytic enteritis, group 2 included dogs with eosinophilic enteritis, group 3 consisted of dogs with the diagnosis of gastritis, and group 4 included dogs diagnosed with pancreatitis based on their serum cPLI concentration.

Serum cNE was measured by ELISA in all 97 dogs. Data were analyzed using a statistical software package using the Kruskal-Wallis test for nonparametric comparison with the Dunn's post test for multiple comparisons. Statistical significance was assigned for values of $p < 0.05$.

4. RESULTS

4.1. Purification of Neutrophil Elastase (NE)

For measurement of NE activity a specific substrate for NE was used. Figure 1 shows typical results of this activity assay for solutions containing various NE activities.

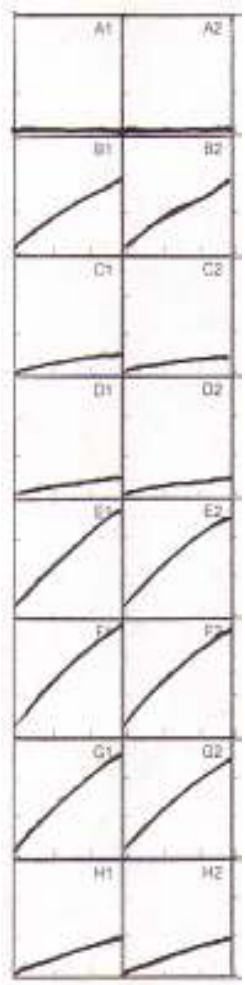
Intracellular proteins were extracted from neutrophils. Briefly, after 5 hours of incubation, the blood-dextran solution formed 2 layers. The erythrocytes settled to the bottom of the beaker forming a dark red layer, while the top layer had a milky appearance. After centrifugation of the top layer, a white opaque pellet with some contaminating erythrocytes was harvested. Following treatment with a hypotonic solution and centrifugation, a pellet free off contaminating red blood cells was obtained.

The extraction of NE from the neutrophil rich pellets resulted in an opaque solution that was further purified by strong cation-exchange chromatography (see Figure 2 for a typical chromatogram). Significant purification of cNE was obtained during this chromatography step.

Figure 3 shows a NuPAGE SDS gel of fractions showing high NE activity during the enzymatic assay after separation by strong cation-exchange chromatography. The two protein bands visible on the gel in figure 3 were successfully separated using continuous-elution electrophoresis on a Mini Prep Cell®. Figure 4 shows pure cNE on a NuPAGE SDS gel after continuous-elution electrophoresis.

Figure 5 shows a test run with prestained molecular weight standards to visualize the separation of a protein mixture into individual bands using continuous-elution electrophoresis. Figure 6 displays the different purity stages of the protein solution during the purification of cNE. Table 3 summarizes the major steps involved in the purification of canine neutrophil elastase from 0.3 liter of blood. Percent of recovery and degree of purification are given for each step. Several different preparative runs gave essentially similar results to those shown in table 3.

A)



B)

A1 0.001	**A2** 0.000
B1 0.155	**B2** 0.154
C1 0.037	**C2** 0.034
D1 0.033	**D2** 0.032
E1 0.231	**E2** 0.231
F1 0.255	**F2** 0.250
G1 0.237	**G2** 0.232
H1 0.082	**H2** 0.079

Figure 1: Activity assay for the measurement of NE activity. The assay was performed using methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide as a NE-specific substrate. A 96-microwell format was used and the increase in absorbance was measured over 15 min at a wavelength of 405 nm using an automated plate reader. Samples were analyzed in duplicate fashion. Panel A graphically displays the increase in absorbance over 15 minutes in 16 different wells arranged in two columns. Panel B shows numerical values for the changes in absorbance displayed in panel A. Wells A1 and A2 are negative controls (blank), B1 and B2 are positive controls, and C1 and C2 to H1 and H2 show the results for unknown samples. The absorbance of the blank is close to zero indicating that no NE is present in the well. The positive control consists of a solution of 5 µg/ml human NE in 0.02 M Tris, 1 M NaCl, 0.05% Tween, pH 8.5 and shows an increase in absorbance over time indicated by the positive number. The samples in this run contain various amounts of NE activity.

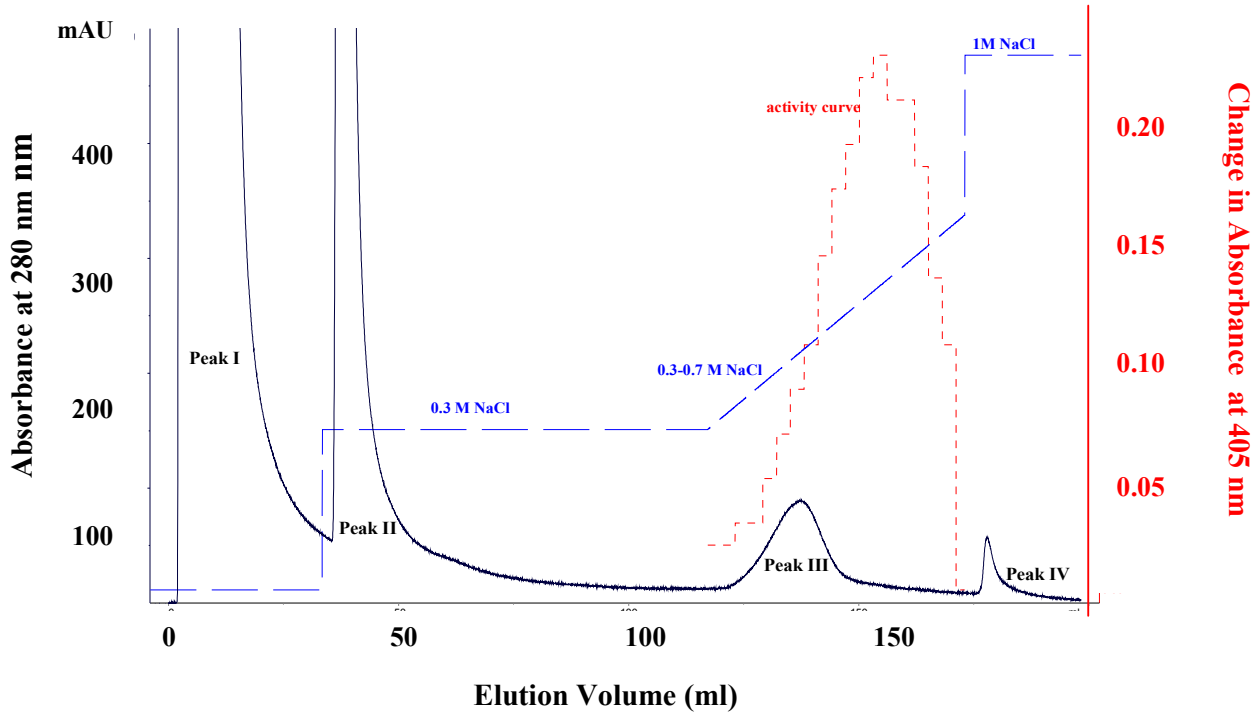


Figure 2: Strong cation-exchange chromatography

This figure shows a typical chromatogram of strong cation-exchange chromatography of a canine neutrophil extract. Absorbance at 280 nm is displayed on the y-axis on the left. The extract separated into four distinct peaks (I-IV). Peak I represents proteins that did not interact with the column matrix and were washed out immediately. Peak II represents proteins that bound only weakly to the column and were washed out with a low ionic strength buffer. Both peaks (I and II) had little enzymatic activity. The red y-axis on the right displays the enzymatic activity of each fraction as measured by the activity assay described. The main NE activity was found in the fractions of the descending slope of peak III. Peak IV consisted of proteins that were strongly bound to the column. Fractions with NE activity were eluted from the column with a salt concentration between 0.4 and 0.7 M NaCl.

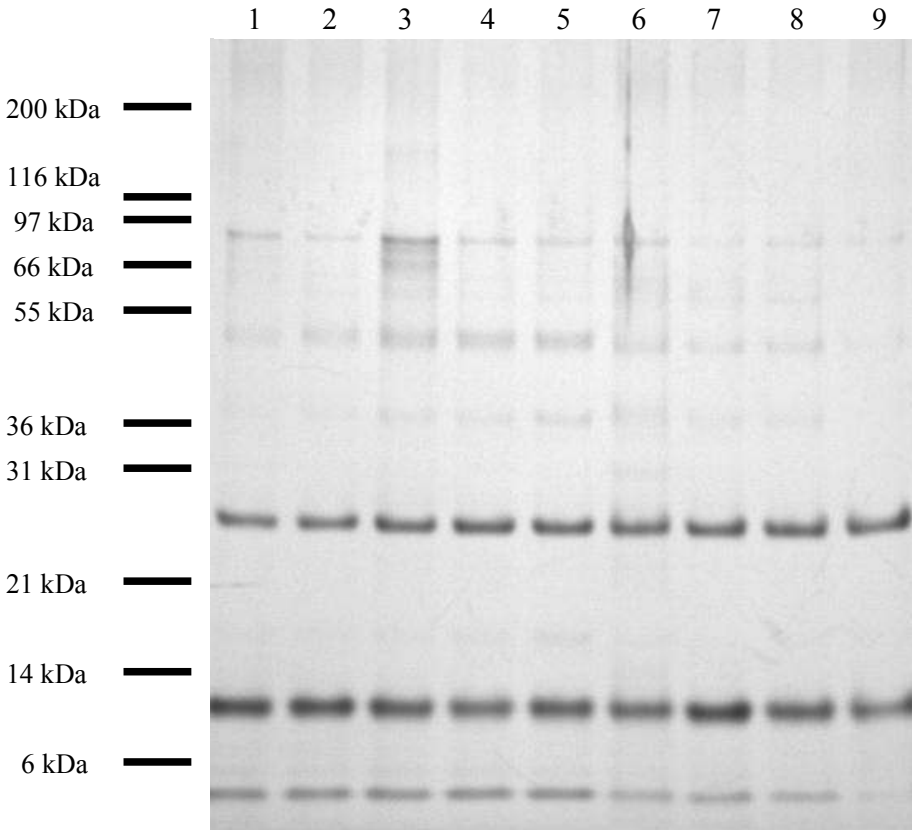


Figure 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after strong-cation exchange chromatography.

SDS-PAGE was performed under reducing conditions on a 1 mm thick, 10% Bis-Tris gel. The numbers and bands on the left of the figure display molecular mass standards. All nine wells of the gel were loaded with various fractions within peak III. All fractions showed a high enzymatic activity. These fractions were concentrated and loaded onto the Mini Prep Cell® for further purification. Clear bands are visible in the molecular mass range reported for NE.

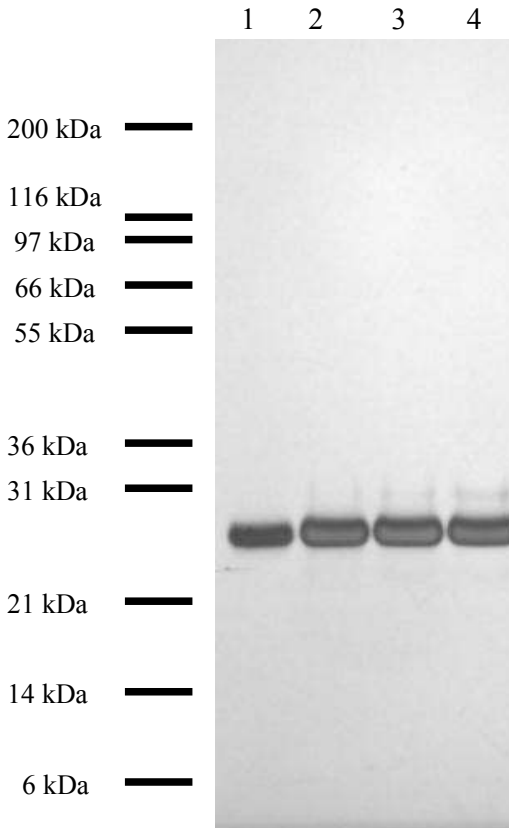


Figure 4: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after continuous elution electrophoresis on the Mini Prep Cell®.

The figure shows a single protein band per lane. These bands represents purified cNE after continuous elution electrophoresis on the Mini Prep Cell®. Four consecutively eluting fractions were loaded onto the gel. SDS-PAGE was run under reducing conditions on a 1 mm thick, 10% Bis-Tris gel. The numbers and bands on the left of the figure, display molecular mass standards.

Figure 5: Continuous-elution electrophoresis on a Mini Prep Cell®.

This picture shows the acrylamide gel column in the center of the Mini Prep Cell® used for continuous gel electrophoresis. In this particular run prestained molecular mass standards were loaded in order to visualize the separation of a protein mixture into individual bands during their migration through the acrylamide gel column. The prestained molecular mass standards consisted of 10 different proteins: myosin (250 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin (30 kDa), lysozyme (16 kDa), aprotinin (6 kDa), and insulin B chain (4 kDa).

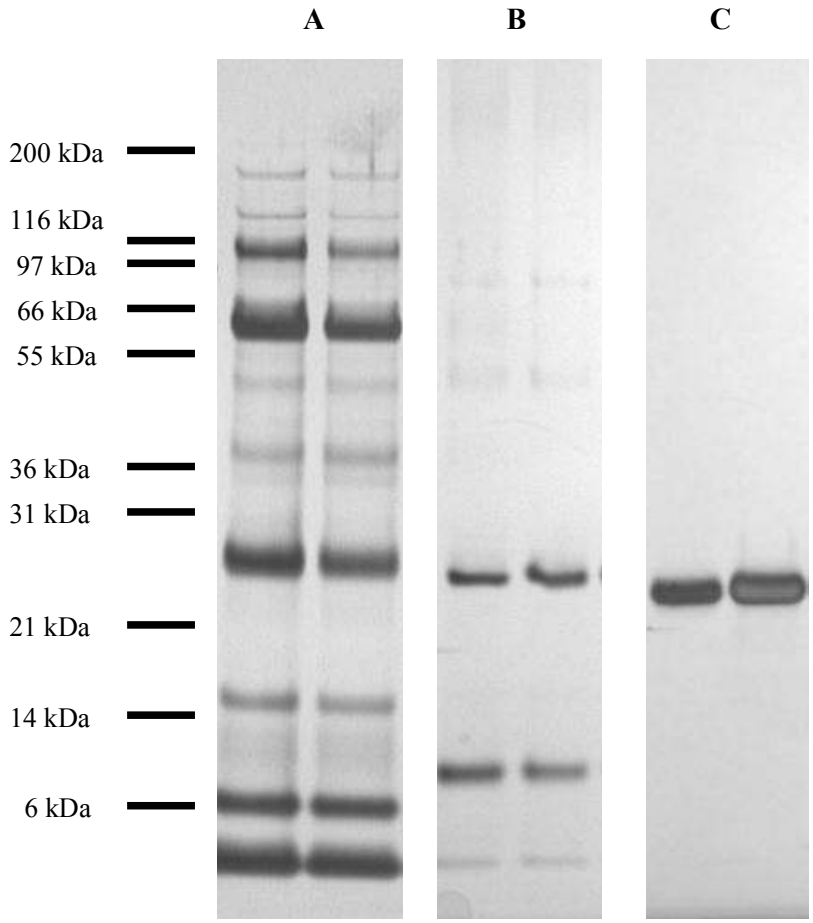


Figure 6: Different purity stages **of cNE visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).**

This figure shows photographs of three different SDS-PAGE gels. Each SDS-PAGE gel was run under reducing conditions on a 1 mm thick 10% Bis-Tris gel. The numbers and bands on the left of the figure, display molecular mass standards. Lanes in panel A show the banding pattern of the neutrophil extract. Lanes in panel B show the banding pattern after cation-exchange chromatography. Lanes in panel C show a single band representing pure canine NE after continuous elution electrophoresis.

Table 3: Sequential purification of cNE from 0.3 L of whole blood.

This table summarizes the total amount of protein in mg, the total elastase activity, the specific elastase activity, and the yield after each purification step starting with 0.3 L of whole blood. The recovery was calculated based on the total activity.

Purification Stage	Total Protein (mg*)	Total Activity (AU**)	Specific Activity (AU/mg of protein)	Yield (%)
Neutrophil preparation	1,100	186	0.17	100.0
Neutrophil extract	220	306	1.39	164.5
Post strong cation-exchange chromatography	1.6	34	21.25	18.3
Post continuous elution electrophoresis	0.6	17	28.33	9.14

* The amount of total protein is based on the absorbance at 280 nm.

** One activity unit (AU) is defined as an increase of absorbance in the methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide activity assay at 405 nm over 15 min multiplied by 1,000.

4.2. Partial characterization of cNE

The molecular mass of cNE as determined by the Agilent 2100 bioanalyzer in combination with the Protein 50 Lab-Chip® kit was found to be 26,500 Dalton (figures 7 and 8, table 4). The IEF gel showed an isoelectric point (pI) for cNE between 8.5 and 9.0 (figure 9).

The extinction coefficient of cNE as determined by bicinchoninic acid (BCA) assay was 0.85 for a 1 mg/ml solution (table 5).

Table 6 shows the first 25 amino acid sequence of NE in different species.

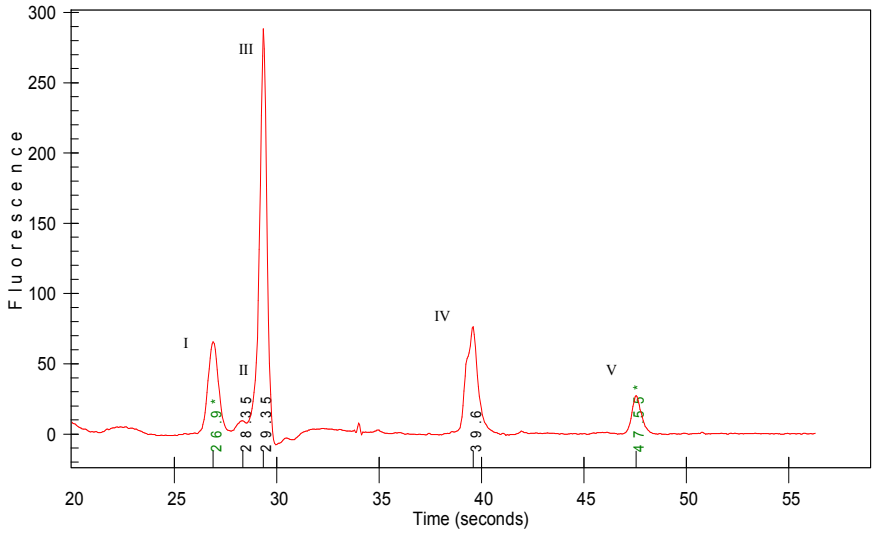


Figure 7: Electropherogram of the analysis of cNE on the Agilent 2100 bioanalyzer.

This figure shows the fluorescence intensity plotted versus size/migration time. Peak I (26.9 s) represents the lower molecular mass marker with a size of 3.5 kDa, peaks II and III (28.35 s and 29.35 s) are system peaks (used for internal calibration), and peak V (47.55 s) represents the upper molecular mass marker with a size of 53.0 kDa. The lower and upper molecular mass markers are internal standards used to align the ladder data with the data from the sample wells. Peak IV represents cNE at a migration time of 39.6 s.

Table 4: Molecular mass of cNE.

The molecular mass of each protein was calculated by interpolation from a standard curve, which was generated by plotting migration time of the protein versus the molecular mass from data generated with the Agilent 2100 bioanalyzer. The molecular mass of cNE was determined to be 26.5 kDa.

The concentration of the protein was calculated by comparison of the area under the curve of the upper molecular mass marker with the area under the curve of the sample. The concentration for cNE in the sample analyzed is 414.5 µg/L.

(N/A= not applicable)

Peak	Migration Time (in s)	Corrected Area	Size (kDa)	Relative Conc. (µg/ml)	Total %	Observations Marker
1	26.90	147.01	3.5	N/A	N/A	Lower marker
2	28.35	16.67	4.1	N/A	N/A	System peak
3	29.35	408.10	4.6	N/A	N/A	System peak
4	39.60	128.01	26.5	414.5	100.0	N/A
5	47.55	30.88	53.0	100.0	N/A	Upper marker

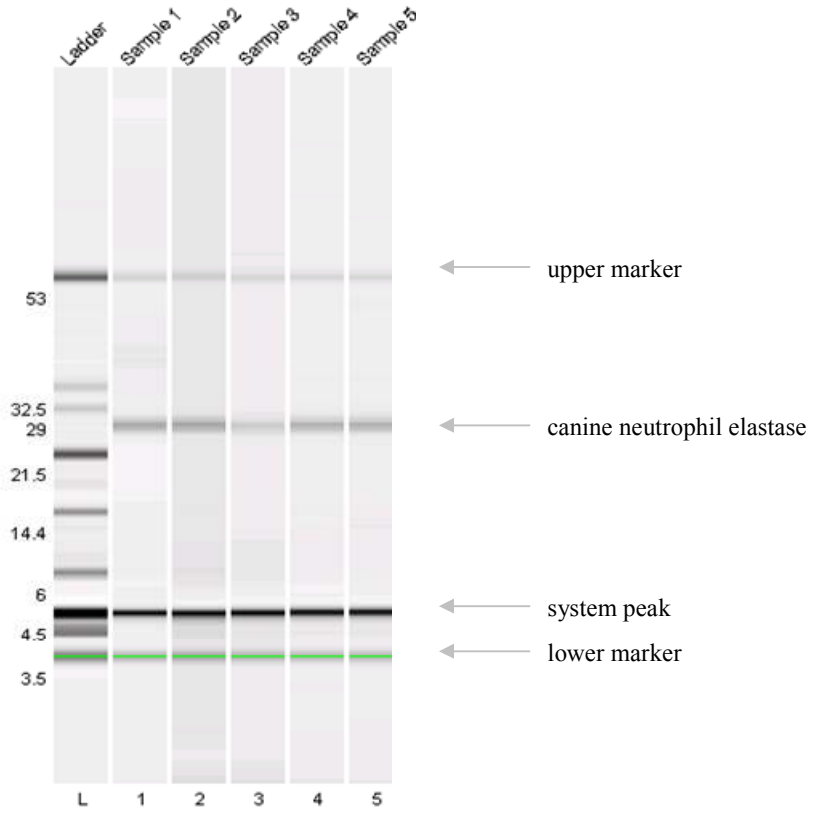


Figure 8: Molecular mass of cNE.

This figure shows and SDS gel-like image obtained from the Agilent 2100 bioanalyzer. The ladder on the left side of the gel contains markers of known size. Lanes 1-5 contain different samples of pure cNE. In each lane, cNE, the upper and lower markers, as well as the system peak are visible.

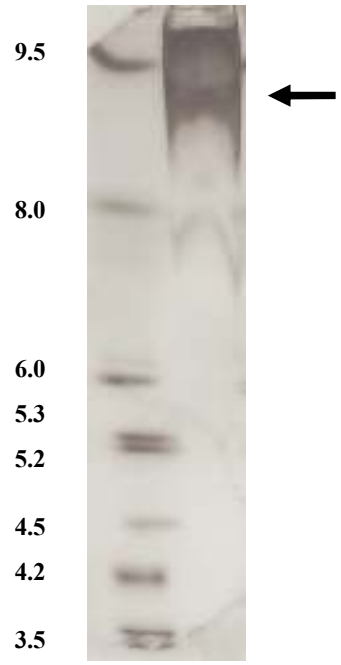


Figure 9: Isoelectric focusing of cNE.

The left side of this figure displays the isoelectric points of the marker bands. The marker bands are proteins with known isoelectric points, including ribonuclease A (pI 9.5), lectin (pI 8.0), carbonic anhydrase (pI 6.0), β-lactoglobulin (pI 5.3 and 5.2), trypsin inhibitor (pI 4.5), glucose oxidase (pI 4.2), and amyloglucosidase (pI 3.5). On the right side, cNE results in a band in the pI range of pI 8.5-9.0.

Table 5: BCA assay.

This table shows the results of a BCA assay. The absorption of pure cNE in PBS, pH 7.2 at a wavelength of 280 nm (A_{280}) was 0.225. The concentration of this solution was measured in the BCA assay at dilutions of 1 in 2, 1 in 4, and 1 in 8. PBS, pH 7.2 was used as diluent. The mean of the three dilutions was calculated, and the specific absorbance of cNE at 280 nm was determined to be 0.85 for a 1 mg/ml solution.

Dilution factor	A_{280}	Protein concentration (µg/ml)	Extinction coefficient
2	0.1125	126.95	0.89
4	0.0563	66.04	0.85
8	0.0281	34.62	0.81
Mean			**0.85**

Table 6: Amino acid sequence of the first 25 N-terminal amino acids of cNE compared to the amino acid sequence of NE from 3 other species.

This table shows the amino acid sequence of the first 25 N-terminal amino acids of dog, rat, mouse, and human NE. Compared to cNE, rat NE shows 100% homology, mouse NE shows 96% homology, and human NE shows 92% homology. The amino acids in the rat, mouse, and human sequence that are different from the one in the canine sequence are bold and in red.

Dog	I	V	G	G	R	P	A	Q	P	H	A	W	P	F	M	V	S	L	Q	R	R	G	G	H	F	
Rat	I	V	G	G	R	P	A	Q	P	H	A	W	P	F	M	V	S	L	Q	R	R	G	G	H	F	**100%**
Mouse	I	V	G	G	R	P	A	**R**	P	H	A	W	P	F	M	V	S	L	Q	R	R	G	G	H	F	**96%**
Human	I	V	G	G	R	**R**	A	**R**	P	H	A	W	P	F	M	V	S	L	Q	**L**	R	G	G	H	F	**92%**

4.3. Production of anti-canine neutrophil elastase antibodies

A single rabbit (*Oryctolagus cuniculus*) was vaccinated with an initial dose of 200 µg of cNE on day 1, followed by 150 µg of cNE on days 21, 42, and 63. The antibody titer was evaluated by an indirect ELISA (Figure 10). An increase in binding over a period of the 4 vaccinations with cNE was observed.

There was no immuno cross-reactivity between human NE and the cNE antiserum, as tested by an Ouchterlony double immunodiffusion assay.

For the development and validation of the cNE-specific ELISA, monospecific polyclonal anti-cNE antibodies were purified by affinity chromatography. Figure 11 shows a typical chromatogram of the affinity purification of anti-cNE antibodies. The specific yield for antibody purification was 0.1 mg of anti-cNE IgG per 1 ml antiserum. A total of 3.2 mg of anti-cNE antibody was purified. A total of 0.8 mg of anti-cNE antibodies were biotinylated for use as the reporter antibody in the ELISA. Use of a 20-fold molar excess of biotin and incubation for 1 hour and 20 minutes at room temperature led to a biotinylation coefficient of 2.7.

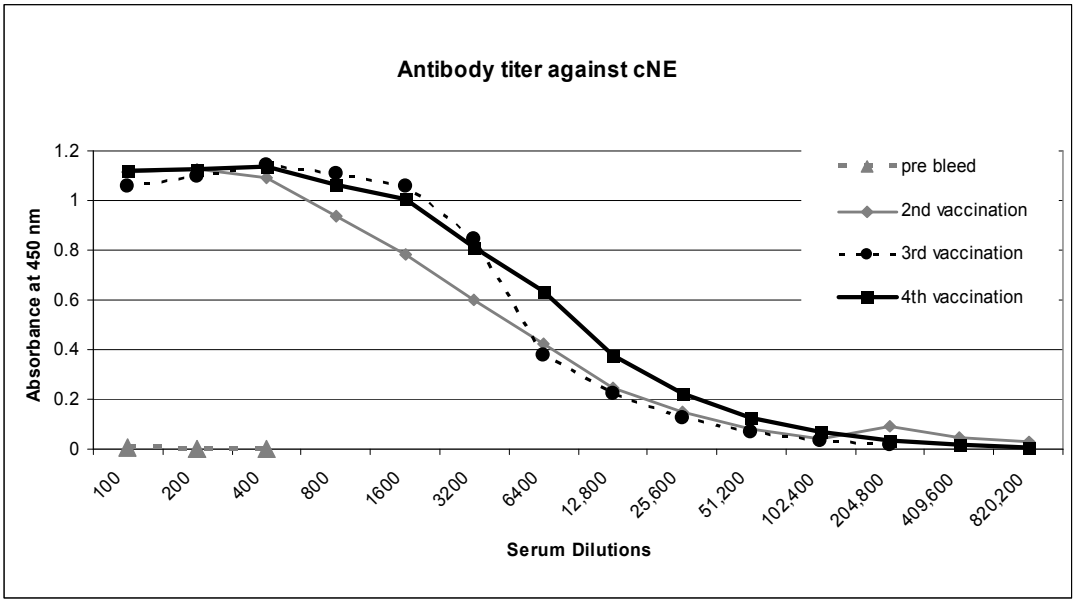


Figure 10: Antibody titer against cNE.

This figure shows the serum anti-cNE antibody titer in a rabbit after the 2nd, 3rd, and 4th vaccination measured by indirect ELISA. The rabbit was initially vaccinated with 200 µg cNE in PBS (1st vaccination) and subsequently with 150 µg cNE in PBS (2nd - 4th vaccinations). A volume of 0.5 ml of incomplete Freund's adjuvant was added to each vaccination. An increase in binding from the 2nd vaccination (gray solid line) to the 4th vaccination (black solid line) is apparent. The serum was also tested for anti-cNE antibodies prior to the first vaccination and showed no binding (pre-bleed, gray dashed line).

The figure displays the absorbance at 450 nm, as measured by an indirect ELISA, at different antiserum dilutions starting at 1/100 (labeled 100) down to 1/820,200 (labeled 820,200).

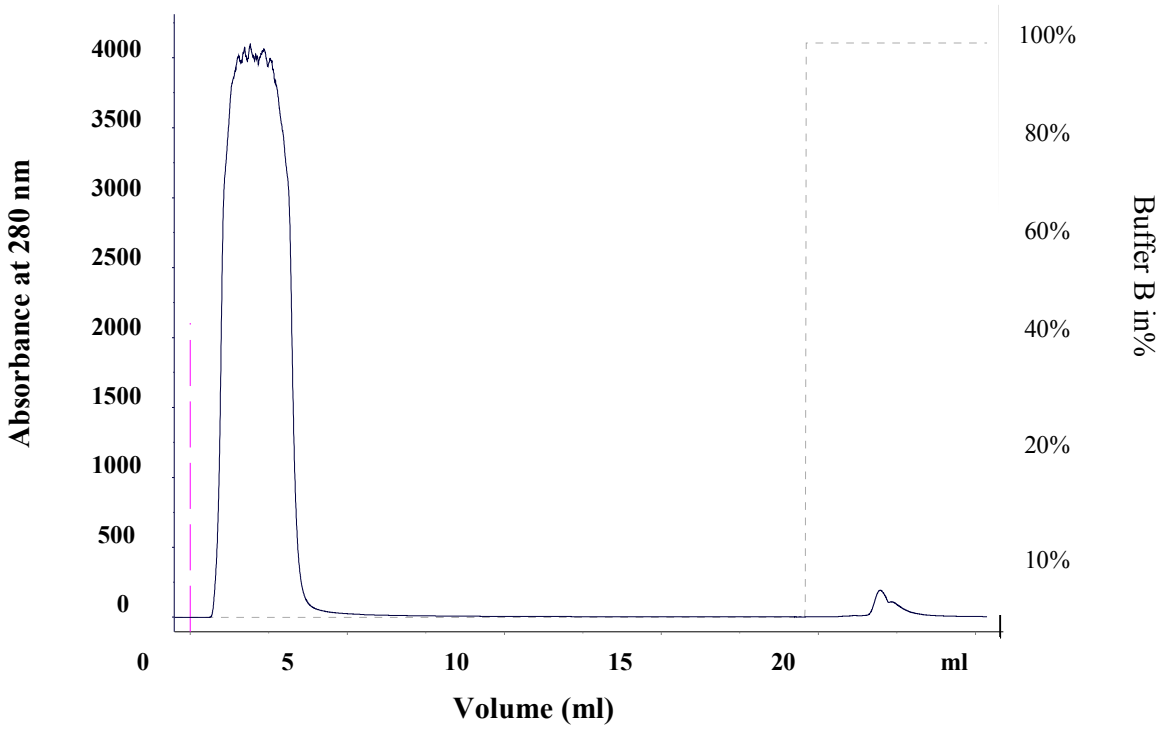


Figure 11: Affinity chromatography of anti-cNE antiserum.

Purification of anti-cNE monospecific polyclonal antibodies by affinity chromatography. The column was equilibrated with 75 mM Tris HCl, 150 mM NaCl, pH 8.0 and 2 ml of rabbit serum was applied onto the column. Absorbance at 280 nm is shown on the y-axis on the left side. There are two distinct peaks (solid black line). Peak I represents proteins that did not bind to the column and were washed out immediately. Peak II represents anti-cNE monospecific polyclonal antibodies that bound to the affinity column and were then eluted with 100% buffer B (100 mM glycine, 500 mM NaCl, pH 2.0; dotted gray line).

4.4. Development and validation of an ELISA for the measurement of cNE concentration in serum

The ELISA protocol described resulted in reproducible standard curves, with standards ranging from 10 µg/L to 1,280 µg/L (Figure 12).

The mean (±SD) absorbance at 450 nm of 10 duplicate samples of a negative control sample (blank: 50 mM TBS, 1% BSA, 0.05% Tween-20, pH 8.0) was 0.156 (±0.0154). Extrapolation from the standard curve for an absorbance of the mean plus three times the SD was equivalent to a cNE concentration of 55 µg/L. Because serum samples were diluted 1:20 prior to running 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 per liter was 1.181 (±0.050). Extrapolation from the standard curve for an absorbance of the mean minus three times the SD was equivalent to a cNE concentration of 2075 µg/L. As for the sensitivity, since serum samples were diluted 1:20 prior to running the assay the corresponding maximum in serum was 41,500 µg/L.

Linearity of the assay was determined by evaluating dilutional parallelism. Ratios of observed to expected results for 4 different serum samples and 4 dilutions ranged from 85.4 to 123.1% with a mean (±SD) of 101.7% (±14.9%; Table 7).

Accuracy of the assay was evaluated by determining spiking recovery. Ratios of observed to expected results for 4 different serum samples and 7 different spiking concentrations ranged from 27.1 to 114.0% with a mean (±SD) of 93.0% (±15.45%; Table 8).

Intra-assay variability for 4 different serum samples ranged from 13.4 to 16.8% with a mean (±SD) of 15.1% (±1.6%; Table 9).

Inter-assay variability for 4 different serum samples ranged from 10.5 to 15.4% with a mean (±SD) of 13.9% (±2.3%; Table 10).

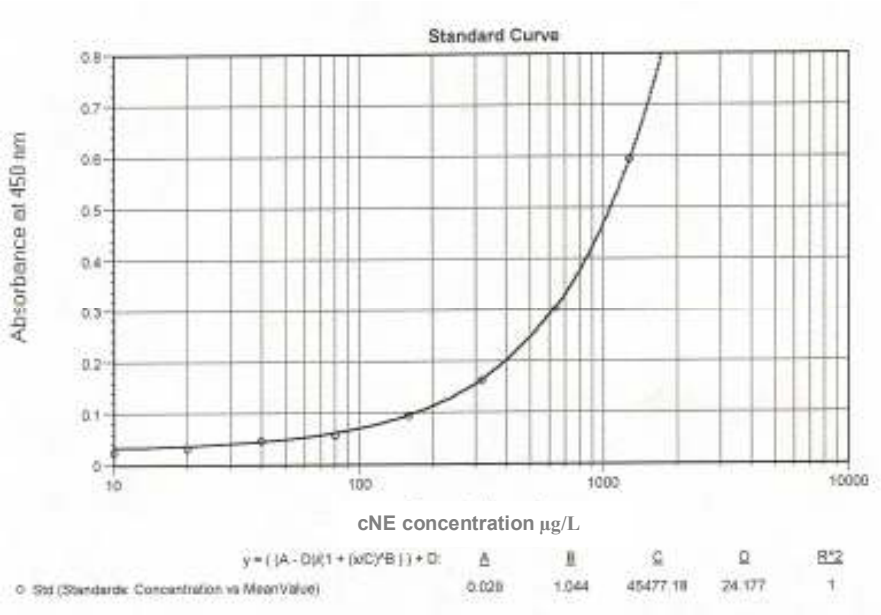


Figure 12: Standard curve for the cNE ELISA.

This figure shows a typical standard curve for the cNE ELISA. Standard concentrations ranged from 10 to 1280 µg/L. The curve was calculated by the use of a computer software package using a 4-parameter curve fit. All four parameters were calculated by use of an algorithm based on the Levenberg-Marquardt equation.

Table 7: Dilutional parallelism for the cNE ELISA.

Dilutional parallelism for 4 different serum samples. Samples were diluted in TBS, 1% BSA, 0.05% Tween-20, pH 8.0. All samples were evaluated in a duplicate fashion. (O/E = (observed / expected)*100; N/A = not applicable).

Sample	Dilution	Observed (µg/L)	Expected (µg/L)	O/E (%)
1	**1 in 10**	3458.8	N/A	N/A
	1 in 20	2114.2	1729.4	122.3
	1 in 40	756.4	864.7	87.5
	1 in 80	370.7	432.4	85.7
2	**1 in 10**	7788.5	N/A	N/A
	1 in 20	4794.8	3894.3	123.1
	1 in 40	2350.9	1947.1	120.7
	1 in 80	963.8	973.6	99.0
3	**1 in 10**	29883.9	N/A	N/A
	1 in 20	12758.3	14941.9	85.4
	1 in 40	7176.5	7470.9	96.1
	1 in 80	3233.3	3735.5	86.6
4	**1 in 10**	4203.5	N/A	N/A
	1 in 20	2426.0	2101.8	115.4
	1 in 40	1059.7	1050.9	100.8
	1 in 80	513.2	525.4	97.7

Table 8: Spiking recovery for cNE ELISA.

Spiking recovery was determined by adding 10, 20, 40, 80, 160, 320, and 640 µg/L cNE to aliquots of 4 different serum samples. The recovery of the spiked cNE ranged from 27.1% to 114.0% (O/E = (observed / expected)*100; N/A = not applicable).

Sample	Spiking conc. added	Observed (µg/L)	Expected (µg/L)	% O/E
1	0.0	54.8	N/A	N/A
	10.0	52.9	64.8	81.7
	20.0	73.6	74.8	98.4
	40.0	78.1	94.8	82.5
	80.0	130.2	134.8	96.6
	160.0	230.5	214.8	107.3
	320.0	390.5	374.8	104.2
	640.0	639.4	694.8	92.0
2	0.0	197.2	N/A	N/A
	10.0	182.2	207.2	87.9
	20.0	190.4	217.2	87.6
	40.0	209.8	237.2	88.4
	80.0	236.9	277.2	85.4
	160.0	311.9	357.2	87.3
	320.0	497.5	517.2	96.2
	640.0	702.3	837.2	83.9
3	0.0	307.4	N/A	N/A
	10.0	328.4	317.4	103.5
	20.0	373.1	327.4	114.0
	40.0	325.1	347.4	93.6
	80.0	378.2	387.4	97.6
	160.0	467.5	467.4	100.0
	320.0	601.9	627.4	95.9
	640.0	992.2	947.4	104.7
4	0.0	60.2	N/A	N/A
	10.0	72.1	70.2	102.7
	20.0	21.8	80.2	27.1
	40.0	110.1	100.2	109.9
	80.0	138.2	140.2	98.6
	160.0	203.7	220.2	92.5
	320.0	328.1	380.2	86.3
	640.0	589.9	700.2	84.3

Table 9: Intra-assay variability for the cNE ELISA.

This table shows the intra-assay variability for the cNE ELISA. Four different serum samples were assayed 10 times during the same assay run. All samples were evaluated in duplicates. The results displayed have been multiplied by 20 to reflect true serum concentrations. The variability ranged from 13.4 to 16.8% with a mean (±SD) of 15.1% (±1.6%).

(SD = standard deviation; %CV = coefficient of variation; %CV = (SD/mean)*100).

sample	Mean (µg/L)		SD (µg/L)		%CV
1	5568.1		791.0		14.2
2	5312.2		891.7		16.0
3	19171.0		3073.7		16.8
4	3363.8		451.6		13.4

Table 10: Inter-assay variation for cNE ELISA.

This table shows the inter-assay variability for the cNE ELISA. Four different serum samples were assayed 10 times during consecutive assay runs. All samples were evaluated in duplicate fashion. The results displayed have been multiplied by 20 to reflect true serum concentrations. The inter-assay variability ranged from 10.5 to 15.4% with a mean (±SD) of 13.9% (±2.3%).
(SD = standard deviation; CV = coefficient of variation; CV = (SD/mean)*100).

sample	Mean (µg/L)		SD (µg/L)		CV (%)
1	4882.2		753.9		15.4
2	8764.4		1315.4		15.0
3	25073.2		2641.5		10.5
4	4056.8		592.9		14.6

4.5. Serum canine neutrophil elastase concentrations in healthy dogs

Serum cNE concentrations were measured in 54 healthy dogs. The dogs were owned by staff or students at Texas A&M University. All 54 dogs were judged to be healthy on physical examination and owners did not report any clinical signs on the provided questionnaire. Serum concentrations of canine trypsin-like immunoreactivity, canine pancreatic lipase immunoreactivity, canine C-reactive protein, cobalamin, and folate were measured in all samples at the Gastrointestinal Laboratory at Texas A&M University and the results were all within the reference ranges. Serum cNE was within the working range of the assay (1,100 to 41,500 µg/L) in 5 of the 54 dogs (9.3%), with concentrations ranging from 1,420.6 to 3,400 µg/L, and a median of 1,099 µg/L (Table 11).

The reference range determined by the lower 95th percentile in these 54 clinically healthy dogs was < 1,631 µg/L (Figure 13).

Table 11: Serum cNE concentrations in 54 healthy dogs.

This table shows the serum cNE concentrations in 54 healthy dogs. Of the 54 dogs, 49 had undetectable serum cNE concentrations (<1100 µg/L) of the ELISA. In the remaining 5 dogs with measurable cNE concentrations, serum concentrations ranged from 1,420.6 µg/L to 3,400 µg/L (displayed in bold numbers). The median serum cNE concentration was 1099 µg/L.

Healthy dogs sample #	Serum cNE µg/L		Healthy dogs sample #	Serum cNE µg/L
	working range 1100 µg/L-41500 µg/L			working range 1100 µg/L-41500 µg/L
1	1099		**28**	**2344.0**
2	1099		**29**	**1420.6**
3	1099		**30**	1099
4	1099		**31**	1099
5	1099		**32**	**1241.3**
6	1099		**33**	**3400.3**
7	1099		**34**	1099
8	1099		**35**	1099
9	1099		**36**	1099
10	1099		**37**	1099
11	1099		**38**	1099
12	1099		**39**	1099
13	1099		**40**	1099
14	1099		**41**	1099
15	1099		**42**	1099
16	1099		**43**	1099
17	1099		**44**	1099
18	1099		**45**	1099
19	1099		**46**	1099
20	1099		**47**	1099
21	1099		**48**	1099
22	1099		**49**	1099
23	1099		**50**	1099
24	**2022.0**		**51**	1099
25	1099		**52**	1099
26	1099		**53**	1099
27	1099		**54**	1099

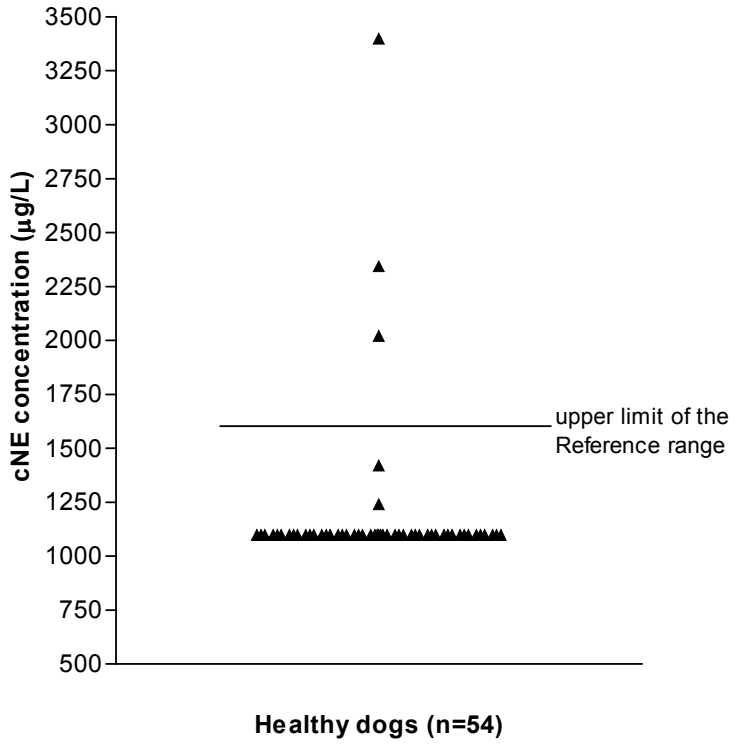


Figure 13: Serum cNE concentrations in 54 healthy dogs.

This graph shows the serum cNE concentrations in 54 healthy dogs. In 49 of the 54 dogs serum cNE were below the working range of the ELISA, while only 5 dogs had serum cNE concentrations ranging from 1,420.6 µg/L to 3,400 µg/L. The reference range, determined by the lower 95th percentile in these 54 healthy dogs, was < 1,631 µg/L.

4.6. Serum canine neutrophil elastase concentrations in dogs with gastrointestinal disease or pancreatitis

Serum cNE concentrations were measured by cNE ELISA in 97 dogs with gastrointestinal disease (n=56) or pancreatitis (n=41). The dogs were divided into 4 groups based on their diagnosis. Of the 97 dogs, 28 were diagnosed with lymphocytic-plasmacytic enteritis (group 1), 10 with eosinophilic enteritis (group 2), and 18 with lymphocytic-plasmacytic gastritis (group 3). The diagnosis was based on histopathologic evaluation of biopsy samples. Group 4 consisted of 41 dogs with pancreatitis. Diagnosis of pancreatitis was based on an increased serum cPLI concentration (> 200 µg/L). Table 12 shows the cNE concentration for each dog in the four disease groups, as well as the median, the range and the percentage of dogs with measurable cNE concentrations. Canine NE was detectable in 17.9% (5 out of 28) of the dogs with lymphocytic-plasmacytic enteritis (group 1), with a median serum cNE of 1,099 µg/L, ranging from 1,099 µg/L to 10,396 µg/L. Eighty percent (8 out of 10) of the dogs with eosinophilic enteritis (group 2) had detectable cNE concentrations, with a median of 2,200 µg/L, and a range from 1,099 µg/L to 7,246 µg/L. Of the 18 dogs with lymphocytic-plasmacytic gastritis (group 3) 11.1% (2 out of 18) had detectable cNE concentrations, with a median of 1,099 µg/L, ranging from 1,099 µg/L to 1,543.8 µg/L. Finally, of the 41 dogs with pancreatitis (group 4) 53.7% (22 out of 41) had detectable cNE concentrations, with a median of 1,719 µg/L, and a range from 1,099 µg/L to 24,240 µg/L. The percentage of dogs with a detectable serum cNE concentration in each group is graphically displayed in Figure 14. Figure 15 shows the percentage of dogs with serum cNE concentrations above the reference range. Figure 16 shows the 4 different groups compared with each other

Statistical analysis using the Kruskal-Wallis test for nonparametric comparison showed a significant difference between the mean serum cNE concentrations and the disease group each animal was assigned to ($p<0.0001$). The Dunn's post test for multiple comparisons showed that the median serum cNE concentration was significantly higher in dogs with eosinophilic enteritis compared to healthy dogs ($p<0.05$). In addition, dogs with pancreatitis had a significantly higher median serum cNE concentration compared to healthy dogs ($p<0.01$), dogs with eosinophilic enteritis had a significantly higher median serum cNE concentration compared to dogs with lymphocytic-plasmacytic enteritis ($p<0.05$), dogs with pancreatitis had a

significantly higher median serum cNE concentration compared to dogs with lymphocytic-plasmacytic enteritis ($p<0.05$), and dogs with gastritis had a significantly higher median serum cNE concentration compared to dogs with eosinophilic enteritis ($p<0.05$). Finally dogs with pancreatitis had a significantly higher median serum cNE concentration than dogs with gastritis ($p<0.05$; Table 13).

Table 12: Serum cNE concentrations in 68 dogs with gastrointestinal disease or pancreatitis. This table shows serum cNE concentrations for 68 dogs with gastrointestinal disease or pancreatitis. The dogs were divided into 4 groups based on their final diagnosis. Values in bold represent values above the reference range (reference range: <1,630 µg/L). The total number of dogs in each group, the median, the range, and the percentage of dogs with measurable cNE concentrations are presented in this table. Note the working range of the assay: 1,099 µg/L to 41,500 µg/L.

Dog #	Lymphocytic-plasmacytic enteritis		Eosinophilic enteritis		Lymphocytic-plasmacytic gastritis		Pancreatitis	Pancreatitis
	cNE µg/L		cNE µg/L		cNE µg/L		cNE µg/L	cNE µg/L
1	1099		1099		1543.8		***22910.7***	***1719.4***
2	1099		***2190.0***		1099		***2454.2***	1099
3	1099		***2886.0***		1099		1099	***4695.6***
4	***10396.0***		***2210.0***		1099		***3125.3***	***24242.3***
5	***2948.5***		***7148.0***		1099		1099	1099
6	1099		***7246.0***		1099		1099	***12711.3***
7	1099		1099		1099		***16559.7***	***12700.9***
8	***1827.3***		***1643.0***		1099		1193.8	***9125.1***
9	1099		***7144.0***		1099		1099	***16977***
10	1099		***2116.0***		1363.8		1099	1099
11	1099				1099		1099	1099
12	1099				1099		***5510.7***	***11802.7***
13	1099				1099		***5290.8***	1099
14	1099				1099		***4383.8***	
15	1099				1099		1099	
16	1099				1099		1099	
17	***2976.0***				1099		1099	
18	1099				1099		***12108.8***	
19	1099						1099	
20	1099						***17174.8***	
21	1099						1099	
22	1110.0						***8271.1***	
23	1099						1099	
24	1099						***8045.9***	
25	1099						1099	
26	1099						1099	
27	1099						***5487.8***	
28	1099						***16111.3***	
Number of dogs	**28**		**10**		**18**			**41**
Median	**1099**		**2200.0**		**1099**			**1719.4**
Min	**1099**		**1099**		**1099**			**1099**
Max	**10396.0**		**7246.0**		**1543.8**			**24242.3**
% of total	**17.9**		**80.0**		**11.1**			**53.7**

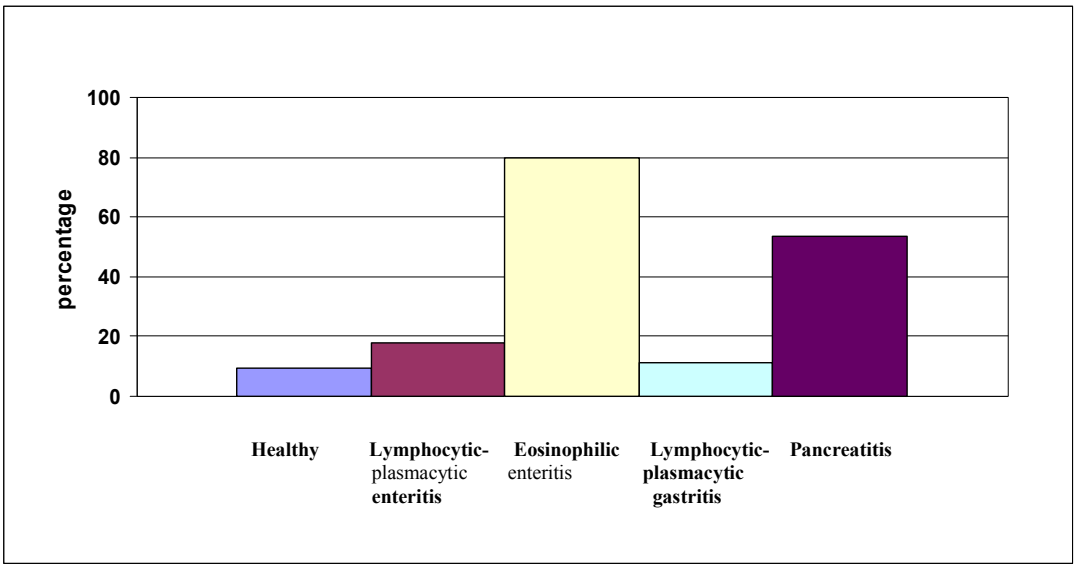


Figure 14: Percentage of dogs with serum cNE concentrations within the working range of the assay in 4 disease groups and a group of healthy dogs.

This figure shows the percentage of dogs with detectable serum cNE concentrations in each group. Serum cNE concentrations were detected in 9.3% (5 out of 54) of healthy dogs, 17.9% (5 out of 28) of dogs with lymphocytic-plasmacytic enteritis, 80.0% (8 out of 10) of dogs with eosinophilic enteritis, 11.1% (2 out of 18) of dogs with lymphocytic-plasmacytic gastritis, and 53.7% (22 out of 41) of dogs with pancreatitis.

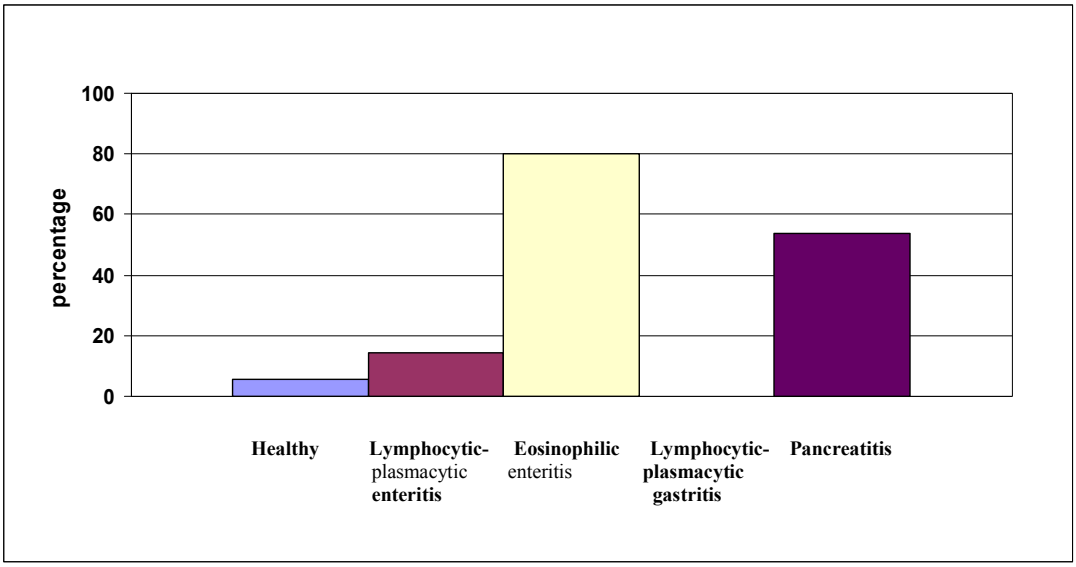


Figure 15: Percentage of dogs with serum cNE concentrations above the reference range in 4 disease groups and a group of healthy dogs.

This figure shows the percentage of dogs in each group with cNE concentrations above the upper limit of the reference range. Serum cNE concentrations were above the upper limit of the reference range in 5.7% (3 out of 54) healthy dogs, 14.3% (4 out of 28) dogs with lymphocytic-plasmacytic enteritis, 80.0% (8 out of 10) dogs with eosinophilic enteritis, 0% (0 out of 18) dogs with lymphocytic-plasmacytic gastritis, and 53.7% (22 out of 41) of dogs with pancreatitis.

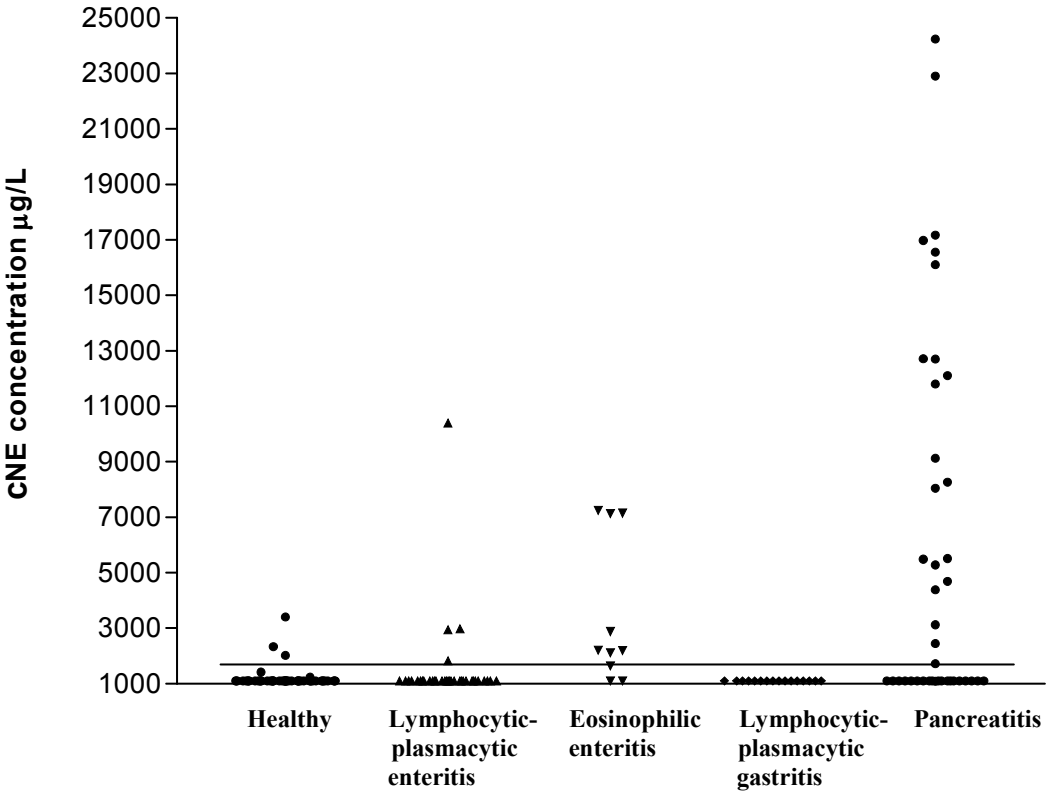


Figure 16: Comparison of serum cNE concentrations between healthy dogs and diseased dogs.

This figure shows serum cNE concentrations in 54 healthy dogs, 28 dogs with lymphocytic-plasmacytic enteritis (median cNE concentration: 1,099 µg/L), 10 dogs with eosinophilic enteritis (median cNE concentration: 2,200 µg/L), 18 dogs with lymphocytic-plasmacytic gastritis (median cNE concentration: 1,099 µg/L), and 41 dogs with pancreatitis (median cNE concentration: 1,719.4 µg/L). The solid line indicates the upper limit of the reference range for this assay (1,631 µg/L).

Statistical analysis using the Kruskal-Wallis test for nonparametric comparison was significant ($p<0.0001$).

Table 13: Results for the Dunn's post test, multiple comparisons.

This table shows the results for the statistical comparison of the 5 different groups: healthy, lymphocytic-plasmacytic enteritis, eosinophilic enteritis, lymphocytic-plasmacytic gastritis, and pancreatitis. The Dunn's post test for multiple comparisons was used for this statistical analysis.

Dunn's Multiple Comparison Test for:		P value:
healthy dogs versus dogs with **lymphocytic-plasmacytic enteritis**		not significant
healthy dogs versus dogs with **eosinophilic enteritis**		P<0.05
healthy dogs versus dogs with lymphocytic-plasmacytic **gastritis**		not significant
healthy dogs versus dogs with **pancreatitis**		P<0.01
dogs with **lymphocytic-plasmacytic enteritis** versus dogs with **eosinophilic enteritis**		P<0.05
dogs with **lymphocytic-plasmacytic enteritis** versus dogs with lymphocytic-plasmacytic **gastritis**		not significant
dogs with **lymphocytic-plasmacytic enteritis** versus dogs with **pancreatitis**		P<0.05
dogs with **eosinophilic enteritis** versus dogs with lymphocytic-plasmacytic **gastritis**		P<0.05
dogs with **eosinophilic enteritis** versus dogs with **pancreatitis**		not significant
dogs with lymphocytic-plasmacytic **gastritis** versus dogs with **pancreatitis**		P<0.05

5. DISCUSSION

5.1. Purification of canine neutrophil elastase

Canine neutrophil elastase (cNE) was successfully purified from dog blood. The described purification protocol reproducibly resulted in cNE of high purity as shown by SDS PAGE (Figure 4), as well as by Agilent 2100 bioanalysis in combination with the Protein 50 Lab-Chip® kit (Figure 7).

The purification of cNE from ascitic fluid of dogs with experimentally induced peritonitis has previously been reported (Berlov et al., 2001; Delshammar et al., 1976). Due to the invasiveness of the described procedure to induce peritonitis, blood was chosen as a neutrophil source in this study. Different protocols for the isolation of neutrophils from blood have previously been described. These include density gradient centrifugation using various media, such as Percoll™ (Gruber et al., 1990), Polymorphprep™ (Ferrante et al., 1980), and Dextran T (Boudier et al., 1982). In this study these different protocols were evaluated for their yield and purity, as well as their cost efficiency and time factor, and it was concluded that Dextran-T was the most useful protocol. Berlov et al., as well as Delshammar et al. used cation-exchange chromatography and gel filtration chromatography for the purification of cNE (Berlov et al., 2001; Delshammar et al., 1976). The results, as described by these investigators, could not be reproduced in our laboratory. In order to achieve a purification protocol with a high yield and high purity of the end product, multiple attempts using various chromatographic columns were made. The combined use of cation-exchange and size exclusion chromatography, as used by Berlov et al. and Delshammar et al., did not lead to pure cNE. By replacing the size exclusion column with a continuous-elution electrophoresis system (Bio-Rad Mini Prep Cell), which also separates proteins according to their size, we were able to separate cNE from all contaminating proteins. This protocol resulted in a purification yield of 9% (Table 3). Berlov et al. reported a purification yield of cNE from ascitic fluid of 43% (Berlov et al., 2001), while the yield reported by Delshammar et al. was 55% (Delshammar et al., 1976). Both investigators had a higher yield for the purification of cNE than was achieved by our protocol, which could be due to a difference in starting material for the purification (blood versus ascitic fluid). Our purification yield was similar to the yield reported for the purification of human NE from blood (6.4%) (Heck et al., 1985).

5.2. Partial characterization of canine neutrophil elastase

The molecular mass of cNE as determined by Agilent 2100 bioanalysis in combination with the Protein 50 Lab-Chip® kit was found to be 26,500 Dalton (Figures 7 and 8, Table 4). These results were slightly different from the results reported by Delshammar et al. and Berlov et al., who reported a molecular mass of 24,800 Dalton (Delshammar et al., 1976) and 24,500-26,000 Dalton (Berlov et al., 2001), respectively. This discrepancy could be explained by the fact that different methods were used to estimate the molecular mass of cNE in each study. Berlov et al. and Delshammar et al. used SDS gel electrophoresis for the determination of the molecular mass. When the molecular mass was calculated based on NuPAGE SDS gels (which are similar to the gels used by Delshammar et al. and Berlov et al.), our results were comparable to those of the previous studies.

The isoelectric point of cNE was found to be between 8.5 and 9.0 (Figure 9), and was similar to that reported for porcine NE (>8.0) (Geiger et al., 1985). The isoelectric point of canine NE has not previously been reported.

The approximate specific absorbance of cNE at 280 nm was determined to be 0.85 for a 1 mg/ml solution (Table 5) and has previously not been reported.

The analysis of the sequence of the first 25 amino acids of the N-terminal end of cNE showed 100% homology to the amino acid sequence predicted by the nucleotide sequence available through the canine genome project. Comparison of the sequence of the first 25 amino acids of the N-terminal end of NE among different species revealed that cNE shows 100% homology to rat NE, 96% homology to mouse NE, and 92% to human NE (Table 6). However, it should be pointed out that only a portion of the amino acid sequence (the first 25 amino acids from the N-terminal end of a total of 282 amino acids) of NE was compared.

5.3. Production of anti-canine neutrophil elastase antibodies

Antibodies are fundamental reagents for any ELISA, and in fact any immunoassay. For antibody production, a specific protein (in this case cNE) is injected into a host (in this case rabbits) stimulating the production of antibodies.

Both polyclonal or monoclonal antibodies could be used in an ELISA. Because the production of monoclonal antibodies is associated with significant expense and expertise, and because monospecific polyclonal antibodies have been used successfully for immunoassays for the measurement of other analytes previously (Steiner et al., 2000), monospecific polyclonal antibodies were used in this project.

The rabbit was chosen as a host species for this project, because the Gastrointestinal Laboratory at Texas A&M University has vast experience in raising antisera against different proteins using the rabbit as host for monospecific polyclonal antibody production. Also the production of antibodies in this species is less expensive compared to the production of antibodies in other species (e.g., sheep).

The rabbit in this project showed a sufficient antibody titer after 4 vaccinations with pure cNE mixed with Freund's complete and incomplete adjuvant (Figure 10). The amount of antibodies that were harvested after affinity chromatography of the antiserum was sufficient for the development and validation of the cNE ELISA.

5.4. Development and validation of an ELISA for the measurement of canine neutrophil elastase

A direct sandwich ELISA for the measurement of NE in canine serum samples was successfully developed and validated. In a direct sandwich ELISA, an antibody is attached to the solid phase to capture the antigen (in this case cNE). The bound antigen is then detected using an enzyme labeled antibody (Crowther, 1995). The capture antibody and the enzyme labeled antibody can be from the same antiserum. However, the antigen must have at least two epitopes (Crowther, 1995). Also, the polyclonal antibody must contain at least 2 clones of antibodies that are directed against a different epitope of the antigen.

Validation of the ELISA was performed by determination of sensitivity, linearity, accuracy, and reproducibility. Specific goals for these performance characteristics of the assay were outlined and included: observed to expected ratios for dilutional parallelism and spiking recovery between 80 to 120% and coefficients of variation for intra- and inter-assay variability between 10 to 15% (Steiner et al., 2000). In literature, no rationales are given for these target values (Steiner et al., 2000). While there is little evidence to suggest the validity of these targeted performance indices, many immunoassays that have been proved to be clinically relevant have shown performance indices meeting these criteria (Melgarejo et al., 1993; Steiner et al., 2000; Steiner et al., 2001).

The assay sensitivity was determined to be 1,100 µg/L cNE. Evaluation of dilutional parallelism of the ELISA indicated acceptable linearity for clinical use. All 4 samples had observed to expected ratios between 85.4% and 122.3% (Table 7), which is considered acceptable for clinical application, as mentioned above. Results of spiking recovery showed acceptable accuracy of the assay (Table 8). In a single sample spiked with 20 µg/L of cNE, a low recovery (27.1%) was observed. An explanation for this result is the low concentration of cNE in this specific sample, which was below the sensitivity range of the assay and therefore resulted in an inaccurate result. The intra-assay variability for 4 different serum samples was between 13.4 to 16.8% with a mean (±SD) of 15.1% (±1.6%; Table 9). The inter-assay variability for 4 different serum samples ranged from 10.5 to 15.4% with a mean (±SD) of 13.9% (±2.3%; Table 10). The results for the intra- and inter-assay variability's were considered acceptable for clinical application, by the guidelines mentioned above.

5.5. Serum cNE concentrations in clinically healthy dogs

A reference range of <1,631 µg/L for serum cNE was established based on results from 54 clinically healthy dogs using the lower 95th percentile. Serum cNE concentrations were only detectable in 9.3% of healthy control dogs (Table 11).

The established reference range for the cNE ELISA is higher than what has been reported for human NE, which has be determined to be between 20 and 180 µg/L (Neumann et al., 1984). It has to be pointed out, however, that the assay for human NE measures the NE/α_1-proteinase inhibitor complex (Neumann et al., 1984) and, therefore, it is not possible to compare the reference ranges for these two different species.

5.6. Serum cNE concentrations in diseased dogs

In human patients with IBD, neutrophilic infiltration of the gastrointestinal mucosa is a common histological finding (Andus et al., 1993; Dwarakanath et al., 1997). It is reliably seen in patients with ulcerative colitis and also frequently observed in patients with Crohn's disease (Adeyemi et al., 1985). Serum NE concentrations correlated significantly with Crohn's disease activity index scores in human IBD patients (Adeyemi et al., 1985). In order to determine the usefulness of serum cNE concentrations in dogs with IBD, we evaluated serum samples from 38 dogs with histopathological evidence of IBD. In addition, and because neutrophilic infiltration and subsequent cNE production can take place in many organs other than the intestine, cNE concentrations were measured in the serum of 41 dogs with cPLI concentrations diagnostic of pancreatitis and 18 dogs with lymphocytic-plasmacytic gastritis. Inflammatory bowel disease (lymphocytic-plasmacytic enteritis and eosinophilic enteritis), as well as lymphocytic-plasmacytic gastritis, were diagnosed based on the combination of clinical signs and histopathological evaluation of endoscopically collected biopsies.

Statistical analysis of the data was performed with a statistical software package using the Kruskal-Wallis test for nonparametric comparison with the Dunn's post test for multiple comparisons.

The median concentration of serum cNE in dogs with lymphocytic-plasmacytic enteritis did not differ significantly from that of clinically healthy dogs. Only 2 of the 28 dogs with lymphocytic-plasmacytic enteritis had detectable serum cNE concentrations. The concentrations of serum cNE in these 2 dogs were within the reference range for the assay. This finding can be explained by the fact that, by definition, the predominant inflammatory cell types in lymphocytic-plasmacytic enteritis are lymphocytes and plasmacytes, which are not capable of producing NE.

Dogs with eosinophilic enteritis had significantly higher median concentration of serum cNE compared with healthy control dogs. In 8 of 10 dogs with eosinophilic enteritis, serum cNE concentrations were above the established reference range for the assay. One explanation for this finding is that the pathologists, who evaluated the intestinal biopsies from these dogs, frequently mentioned neutrophilic infiltration in combination with eosinophilic infiltration. Another possible explanation is that eosinophils are capable of producing NE or a protein that is identical with the chemical properties of NE. Additional studies are necessary to further evaluate this issue.

Median serum cNE concentrations were significantly higher in dogs with pancreatitis, compared to healthy control dogs. In 21 out of 41 dogs diagnosed with pancreatitis based on an elevated serum cPLI concentration, serum cNE concentrations were above the reference range. This finding can be explained by the fact that the pancreas is frequently infiltrated with neutrophils during pancreatitis (Gross et al., 1990), and suggests that serum cNE concentrations cannot be used as a specific marker for IBD.

Median serum cNE concentrations in dogs with eosinophilic enteritis were significantly higher than those in dogs with lymphocytic-plasmacytic enteritis. This suggests that the measurement of serum cNE concentration may be helpful to differentiate between eosinophilic and lymphocytic-plasmacytic enteritis. However, a larger number of dogs need to be evaluated before reaching a conclusive answer to the question whether or not serum cNE could be clinically useful in the diagnosis of eosinophilic enteritis.

5.6. Conclusion

In conclusion, canine neutrophil elastase has been successfully purified from dog blood. An ELISA for the measurement of cNE in dog serum was established and validated. Canine neutrophil elastase concentration of 56 serum samples from diseased dogs were measured and statistically analyzed. These data demonstrate that there is activation of neutrophils, resulting in the release of NE in most dogs with eosinophilic enteritis as well as in many dogs with pancreatitis. Also, the median cNE concentration in serum is significantly higher in dogs with eosinophilic enteritis and pancreatitis, when compared to healthy control dogs, and also when compared to dogs with lymphocytic-plasmacytic enteritis. However, due to the overlap between patients with eosinophilic enteritis and other inflammatory conditions (e.g. pancreatitis), serum cNE concentration is not specific for intestinal neutrophilic inflammation.

Further studies are needed to evaluate the usefulness of this assay for clinical applications, such as monitoring the disease severity and progression in patients with known inflammatory conditions of the intestinal mucosa.

6. SUMMARY

Purification and Partial Characterization of Canine Neutrophil Elastase and the Development of an Immunoassay for the Measurement of Canine Neutrophil Elastase in Serum

Canine neutrophil elastase (cNE) belongs to a group of serine proteinases and is a constituent of azurophil granules of neutrophils. It has previously been reported that the concentration of neutrophil-derived proteins in serum and feces can be used as a marker for intestinal inflammation in humans. The aim of this study was to develop a rapid and reproducible protocol for the purification of cNE from dog blood, to partially characterize cNE, to produce anti-cNE antibodies, to develop and validate an ELISA for the measurement of neutrophil elastase, and to compare serum cNE concentrations between healthy dogs and dogs with either gastrointestinal disease or pancreatitis.

An efficient and reproducible protocol for the isolation of neutrophils, the extraction of neutrophil elastase from the neutrophilic granules, and further purification of the protein was established. Some physiological properties of the protein were evaluated. An ELISA for cNE was developed and validated and a reference range was established. Finally, the serum concentrations of canine neutrophil elastase were compared between healthy dogs and those with gastrointestinal disease or pancreatitis.

The molecular mass of cNE was estimated to be 26,500 Dalton. Isoelectric focusing showed an isoelectric point between 8.5 and 9.0. The N-terminal amino acid sequence of the first 25 residues was Ile-Val-Gly-Gly-Arg-Pro-Ala-Gln-Pro-His-Ala-Trp-Pro-Phe-Met-Val-Ser-Leu-Gln-Arg-Arg-Gly-Gly-His-Phe. The sequence showed 100% homology with the N-terminal amino acid sequence predicted by the nucleotide sequence available through the canine genome project. The approximate specific absorbance of cNE at 280 nm was determined to be 0.85 for a 1 mg/ml solution. The

ELISA for measuring the concentration of cNE in canine serum was sufficiently sensitive, linear, accurate, precise, and reproducible for clinical use. Canine NE could be detected in 17.9% of the dogs with lymphocytic-plasmacytic enteritis, 80% of the dogs with eosinophilic enteritis, 11.1% of the dogs with lymphocytic-plasmacytic gastritis and in 53.7% of the dogs with pancreatitis. The mean serum cNE concentration was significantly higher in dogs with eosinophilic enteritis and pancreatitis, when compared to healthy control dogs or dogs with lymphocytic-plasmacytic enteritis.

In conclusion serum cNE concentration can serve as a marker for neutrophilic infiltration. Further studies are needed to evaluate the clinical usefulness of this assay.

7. ZUSAMMENFASSUNG

Reinigung und Teilcharakterisierung von caniner neutrophiler Elastase und die Entwicklung eines Immunoassays zur Messung der Konzentration der neutrophilen Elastase im Serum

Canine neutrophile Elastase (cNE) gehört zu der Gruppe der serinen Proteinasen und ist in den azurophilen Granula der neutrophilen Granulozyten eingelagert. Es gab Berichte wonach die Konzentration von Proteinen, die aus den neutrophilen Granulozyten stammen, in Serum und Kot gemessen werden können und als Marker für eine Infiltration der Darmmukosa mit Entzündungszellen beim Menschen verwendet werden können. Der Zweck dieser Studie war es, ein schnelles und reproduzierbares Protokoll für die Reinigung von cNE aus Hundeblut zu erstellen, die Teilcharakterisierung von cNE, die Produktion von Antikörpern gegen cNE, die Entwicklung und Validierung eines Immunoassays, sowie die Messung und den Vergleich der Konzentration von cNE im Serum von gesunden Hunden und Hunden mit gastrointestinalen Erkrankungen oder Pankreatitis.

Ein effizientes und wiederholbares Protokoll für die Trennung von neutrophilen Granulozyten, die Extraktion von neutrophiler Elastase aus den azurophilen Granula, sowie die Reinigung des Proteins wurden erstellt. Einige physiologische Eigenschaften von cNE wurden ebenfalls ermittelt.

Ein ELISA für die Messung von cNE wurde entwickelt und validiert, sowie ein Referenzbereich ermittelt. Schließlich wurde die Konzentration von cNE im Serum von gesunden Hunden mit der von Hunden mit gastrointestinalen Erkrankungen oder Pankreatitis verglichen.

Das molekulare Gewicht von cNE wurde auf 26.500 Dalton geschätzt. Die isoelektrische Fokussierung ergab einen isoelektrischen Punkt zwischen 8,5 und 9,0. Die N-terminale Aminosäuresequenz der ersten 25 Aminosäuren war Ile-Val-Gly-

Gly-Arg-Pro-Ala-Gln-Pro-His-Ala-Trp-Pro-Phe-Met-Val-Ser-Leu-Gln-Arg-Arg-Gly-Gly-His-Phe. Diese Sequenz zeigte 100% Übereinstimmung mit der vom Hunde-Genom-Programm veröffentlichten Nukleotidsequenz abgeleiteten N-terminalen Sequenz. Der spezifische Absorptionskoeffizient von cNE bei 280 nm wurde als 0,85 für eine 1 mg/ml-Lösung ermittelt. Der entwickelte ELISA zur Messung der Konzentration von cNE in Hundeserum ist ausreichend sensitiv, linear, akkurat, sowie genau und reproduzierbar. Canine NE konnte bei 17,9 % der Hunde mit lymphoplasmazellulärer Enteritis, bei 80 % der Hunde mit eosinophiler Enteritis, 1,1 % der Hunde mit lymphoplasmazellulärer Gastritis und 53,7 % der Hunde mit Pankreatitis gemessen werden. Der Mittelwert der cNE-Konzentration im Serum war signifikant höher bei Hunden mit eosinophiler Enteritis und Hunden mit Pankreatitis als bei gesunden Hunden oder Hunden mit lymphoplasmazellulärer Enteritis.

Abschließend kann festgestellt werden, dass die cNE Konzentration als Marker für Erkrankungen eingesetzt werden kann, die mit einer Infiltration mit Entzündungszellen einhergehen. Weitere Studien sind notwendig um den klinischen Nutzen dieses Assays zu prüfen.

8. REFERENCES

Adeyemi, E. O. and H.J.Hodgson. 1992. Faecal elastase reflects disease activity in active ulcerative colitis. Scand.J.Gastroenterol. 27(2):139-142.

Adeyemi, E. O., S.Neumann, V.S.Chadwick, H.J.Hodgson, and M.B.Pepys. 1985. Circulating human leucocyte elastase in patients with inflammatory bowel disease. Gut. 26(12):1306-1311.

Andus, T., V.Gross, I.Caesar, D.Krumm, J.Hosp, W.Gerok, and J.Scholmerich. 1993. PMN-elastase in assessment of patients with inflammatory bowel disease. Dig.Dis.Sci. 38(9):1638-1644.

Barrett, A. J. 1981. Leukocyte elastase. Methods Enzymol. 80(C):581-588.

Benson, K. F., F.Q.Li, R.E.Person, D.Albani, Z.Duan, J.Wechsler, K.Meade-White, K.Williams, G.M.Acland, G.Niemeyer, C.D.Lothrop, and M.Horwitz. 2003. Mutations associated with neutropenia in dogs and humans disrupt intracellular transport of neutrophil elastase. Nat.Genet. 35(1):90-96.

Berlov, M. N., P.A.Lodygin, Y.V.Andreeva, and V.N.Kokryakov. 2001. Isolation and some physical and chemical properties of elastase and cathepsin G from dog neutrophils. Biochemistry (Mosc.). 66(9):1008-1013.

Bieth, J. and C.G.Wermuth. 1973. The action of elastase on p-nitroanilide substrates. Biochem.Biophys.Res.Commun. 53(2):383-390.

Bode, W., Meyer E Jr, and J.C.Powers. 1989. Human leukocyte and porcine pancreatic elastase: X-ray crystal structures, mechanism, substrate specificity, and mechanism-based inhibitors. Biochemistry. 28(5):1951-1963.

Boehm, M. and J.S.Bonifacino. 2002. Genetic analyses of adaptin function from yeast to mammals. Gene. 286(2):175-186.

Boudier, C., K.K.Andersson, C.Balny, and J.G.Bieth. 1980. Enhancement of human leukocyte elastase activity by ionic strength. Biochem.Med. 23(2):219-222.

Boudier, C. and J.G.Bieth. 1982. Estimation and characterization of hamster leukocyte elastase. Biochem.Med. 28(1):41-50.

Boudier, C., C.Holle, and J.G.Bieth. 1981. Stimulation of the elastolytic activity of leukocyte elastase by leukocyte cathepsin G. J.Biol.Chem. 256(20):10256-10258.

Cotter, T. G. and G.B.Robinson. 1980. Purification and characterization of an 'elastase-like' enzyme from rabbit polymorphonuclear leucocytes. Biochim.Biophys.Acta. 615(2):414-425.

Crowther, J. R. 1995. ELISA Theory and Practice. Methods in Molecular Biology. J.M.Walker. Humana Press; Volume 42.

Daggett, V., S.Schroeder, and P.Kollmann. 1991. Catalytic Pathway of Serine Proteases: Classical and Quantum Mechanical Calculation. J.Am.Chem.Soc. 113:8926-8935.

Darlington, G. J., K.H.Astrin, S.P.Muirhead, R.J.Desnick, and M.Smith. 1982. Assignment of human alpha 1-antitrypsin to chromosome 14 by somatic cell hybrid analysis. Proc.Natl.Acad.Sci.U.S.A. 79(3):870-873.

Del Mar, E. G., C.Largman, J.W.Brodrick, M.Fassett, and M.C.Geokas. 1980. Substrate specificity of human pancreatic elastase 2. Biochemistry. 19(3):468-472.

Dell'Angelica, E. C., V.Shotelersuk, R.C.Aguilar, W.A.Gahl, and J.S.Bonifacino. 1999. Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. Mol.Cell. 3(1):11-21.

Delshammar, M. and K.Ohlsson. 1976. Isolation and Partial Characterization of Elastase from Dog Granulocytes. Eur.J.of Biochem. 69:125-131.

Dubin, A., A.Koj, and J.Chudzik. 1976. Isolation and some molecular parameters of elastase-like normal proteinases from horse blood leucocytes. Biochem.J. 153(2):389-396.

Dubin, A., J.Potempa, and J.Travis. 1994. Structural and functional characterization of elastase from horse neutrophils. Biochem.J. 300 (Pt 2):401-406.

Dwarakanath, A. D., I.A.Finnie, C.M.Beesley, G.M.O'Dowd, J.Nash, H.H.Tsai, N.Parker, and J.M.Rhodes. 1997. Differential excretion of leucocyte granule components in inflammatory bowel disease: implications for pathogenesis. Clin.Sci.(Lond). 92(3):307-313.

Edwards, S. W. 1994. The antimicrobial granule enzymes. Biochemistry and physiology of the neutrophil. Cambridge University Press.1st edition; chapter 2.6:54-74.

Eriksson, S. 1965. Studies in α_1 antitrypsin deficiency. Acta Med.Scand. 177:1-27.

Feinstein, G. and A.Janoff. 1975. A rapid method for purification of human granulocyte cationic neutral proteases: purification and characterization of human granulocyte chymotrypsin-like enzyme. Biochim.Biophys.Acta. 403(2):477-492.

Ferrante, A. and Y.H.Thong. 1980. Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leucocytes from human blood by the Hypaque-Ficoll method. J.Immunol.Methods. 36(2):109-117.

Fiocchi, C. 1998. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology. 115(1):182-205.

Fischbach, W., W.Becker, J.Mossner, H.Ohlemuller, W.Koch, and W.Borner. 1987. Leucocyte elastase in chronic inflammatory bowel diseases: a marker of inflammatory activity? Digestion. 37(2):88-95.

Geiger, R., A.Junk, and M.Jochum. 1985. Isolation and characterization of porcine leukocyte elastase. Leukocyte elastase-inhibitor complexes in porcine blood, II. J.Clin.Chem.Clin.Biochem. 23(12):821-828.

Graziadei, I. W., J.J.Joseph, R.H.Wiesner, T.M.Therneau, K.P.Batts, and M.K.Porayko. 1998. Increased risk of chronic liver failure in adults with heterozygous alpha1-antitrypsin deficiency. Hepatology. 28(4):1058-1063.

Gross, V., J.Scholmerich, H.G.Leser, R.Salm, M.Lausen, K.Ruckauer, U.Schoffel, L.Lay, A.Heinisch, E.H.Farthmann, and . 1990. Granulocyte elastase in assessment of severity of acute pancreatitis. Comparison with acute-phase proteins C-reactive protein, alpha 1-antitrypsin, and protease inhibitor alpha 2-macroglobulin. Dig.Dis.Sci. 35(1):97-105.

Gruber, D. F., A.B.Laws, and K.P.O'Halloran. 1990. Biochemical and physiological alterations in canine neutrophils separated by lysis or Percoll gradient isolation technologies. Immunopharmacol.Immunotoxicol. 12(1):93-104.

Guilford, W. G. 1996b. Idiopathic Inflammatory Bowel Disease. Strombeck's Small Animal Gastroenterology. W.G.Guilford, S.Center. W.B. Saunders Company: 3rd edition, chapter 24:451-486.

Hall, E. J. and A.J.German. 2005. Disease of the Small Intestine. Textbook of Veterinary Internal Medicine. S.J.Ettinger and E.C.Feldman. Elsevier Saunders; 6th edition, chapter 222:1332-1378.

Haurie, C., D.C.Dale, and M.C.Mackey. 1998. Cyclical neutropenia and other periodic hematological disorders: a review of mechanisms and mathematical models. Blood. 92(8):2629-2640.

Heck, L. W., W.L.Darby, F.A.Hunter, A.Bhown, E.J.Miller, and J.C.Bennett. 1985. Isolation, characterization, and amino-terminal amino acid sequence analysis of human neutrophil elastase from normal donors. Anal.Biochem. 149(1):153-162.

Horwitz, M., K.F.Benson, Z.Duan, F.Q.Li, and R.E.Person. 2004. Hereditary neutropenia: dogs explain human neutrophil elastase mutations. Trends Mol.Med. 10(4):163-170.

Horwitz, M., K.F.Benson, Z.Duan, R.E.Person, J.Wechsler, K.Williams, D.Albani, and F.Q.Li. 2003a. Role of neutrophil elastase in bone marrow failure syndromes: molecular genetic revival of the chalone hypothesis. Curr.Opin.Hematol. 10(1):49-54.

Horwitz, M., K.F.Benson, R.E.Person, A.G.Aprikyan, and D.C.Dale. 1999. Mutations in ELA2, encoding neutrophil elastase, define a 21-day biological clock in cyclic hematopoiesis. Nat.Genet. 23(4):433-436.

Horwitz, M., F.Q.Li, D.Albani, Z.Duan, R.E.Person, K.Meade-White, and K.F.Benson. 2003b. Leukemia in severe congenital neutropenia: defective proteolysis suggests new pathways to malignancy and opportunities for therapy. Cancer Invest. 21(4):579-587.

Hutchison, D. C. S. 1988. Natural history of alpha-1-protease inhibitor deficiency. Am.J.Med. 84 (Suppl 6A):3-12.

Janoff, A. 1985a. Elastase in tissue injury. Annu.Rev.Med. 36:207-216.

Janoff, A. 1985b. Elastases and emphysema. Current assessment of the protease-antiprotease hypothesis. Am.Rev.Respir.Dis. 132(2):417-433.

Janoff, A. and H.Carp. 1977. Possible mechanisms of emphysema in smokers: cigarette smoke condensate suppresses protease inhibition in vitro. Am. Rev.Respir.Dis. 116(1):65-72.

Janoff, A. and J.Scherer. 1968. Mediators of inflammation in leukocyte lysosomes. IX. Elastinolytic activity in granules of human polymorphonuclear leukocytes. J.Exp.Med. 128(5):1137-1155.

Jergens, A. E. 1999. Inflammatory bowel disease. Current perspectives. Vet.Clin. North Am.Small Anim.Pract. 29(2):501-21, vii.

Jergens, A. E., F.M.Moore, J.S.Haynes, and K.G.Miles. 1992. Idiopathic inflammatory bowel disease in dogs and cats: 84 cases (1987-1990). J.Am.Vet.Med.Assoc. 201(10):1603-1608.

Jergens, A. E., C.A.Schreiner, D.E.Frank, Y.Niyo, F.E.Ahrens, P.D.Eckersall, T.J.Benson, and R.Evans. 2003. A scoring index for disease activity in canine inflammatory bowel disease. J.Vet.Intern.Med. 17(3):291-297.

Kraut, J. 1977. Serine proteases: structure and mechanism of catalysis. Annu.Rev.Biochem. 46:331-358.

Lange, R. D. 1983. Cyclic hematopoiesis: human cyclic neutropenia. Exp.Hematol. 11(6):435-451.

Laurell, C. B. and S.Erikkson. 1963. The electrophoretic alpha1-globulin pattern of serum in alpha1-antitrypsin deficiency. Scan.J.Clin.Lab.Invest. 15:132-140.

Li, F. Q. and M.Horwitz. 2001. Characterization of mutant neutrophil elastase in severe congenital neutropenia. J.Biol.Chem. 276(17):14230-14241.

Lothrop, C. D., Jr., P.A.Coulson, H.L.Nolan, B.Cole, J.B.Jones, and W.L.Sanders. 1987. Cyclic hormonogenesis in gray collie dogs: interactions of hematopoietic and endocrine systems. Endocrinology. 120(3):1027-1032.

Luckschander, N., K.Allenspach, J.Hall, F.Seibold, A.Grone, M.G.Doherr, and F.Gaschen. 2006. Perinuclear antineutrophilic cytoplasmic antibody and response to treatment in diarrheic dogs with food responsive disease or inflammatory bowel disease. J.Vet.Intern.Med. 20(2):221-227.

Marossy, K., M.Hauck, and P.Elodi. 1980. Purification and characterization of the elastase-like enzyme of the bovine granulocyte. Biochim.Biophys.Acta. 615(1):237-245.

Melgarejo, T., E.K.Asem, and D.A.Williams. 1993. Enzyme-linked immunoadsorbent assay for canine alpha$_1$-protease inhibitor (α_1-PI). J.Vet.Int.Med. 7133-133.

Mistry, R., P.D.Snashall, N.Totty, A.Guz, and T.D.Tetley. 1999. Purification and N-terminal amino acid sequence of sheep neutrophil cathepsin G and elastase. Arch.Biochem.Biophys. 368(1):7-13.

Nakajima, K., J.C.Powers, B.M.Ashe, and M.Zimmerman. 1979. Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. Studies with peptide substrates related to the alpha 1-protease inhibitor reactive site. J.Biol.Chem. 254(10):4027-4032.

Neumann, S., G.Gunzer, N.Hennrich, and H.Lang. 1984. "PMN-elastase assay": enzyme immunoassay for human polymorphonuclear elastase complexed with alpha 1-proteinase inhibitor. J.Clin.Chem.Clin.Biochem. 22(10):693-697.

Ohlsson, K., I.Olsson, M.Delshammar, and H.Schiessler. 1976. Elastases from human and canine granulocytes, I. Some proteolytic and esterolytic properties. Hoppe Seylers.Z.Physiol.Chem. 357(9):1245-1250.

Pham, C. T. 2006. Neutrophil serine proteases: specific regulators of inflammation. Nat.Rev.Immunol. 6(7):541-550.

Poullis, A., R.Foster, T.C.Northfield, and M.A.Mendall. 2002. Review article: faecal markers in the assessment of activity in inflammatory bowel disease. Aliment.Pharmacol.Ther. 16(4):675-681.

Shapiro, S. D., N.M.Goldstein, A.M.Houghton, D.K.Kobayashi, D.Kelley, and A.Belaaouaj. 2003. Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice. Am.J.Pathol. 163(6):2329-2335.

Sinha, S., W.Watorek, S.Karr, J.Giles, W.Bode, and J.Travis. 1987. Primary structure of human neutrophil elastase. Proc.Natl.Acad.Sci.U.S.A. 84(8):2228-2232.

Smedly, L. A., M.G.Tonnesen, R.A.Sandhaus, C.Haslett, L.A.Guthrie, R.B.Johnston, Jr., P.M.Henson, and G.S.Worthen. 1986. Neutrophil-mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase. J.Clin.Invest. 77(4):1233-1243.

Smith, P. K., R.I.Krohn, G.T.Hermanson, A.K.Mallia, F.H.Gartner, M.D.Provenzano, E.K.Fujimoto, N.M.Goeke, B.J.Olson, and D.C.Klenk. 1985. Measurement of protein using bicinchoninic acid. Anal.Biochem. 150(1):76-85.

Snider, G. L. 1989. Pulmonary disease in α1-antitrypsin deficiency. Annals of Internal Med. 111(12):957-959.

Snider, G. L., E.C.Lucey, and P.J.Stone. 1986. Animal models of emphysema. Am.Rev.Respir.Dis. 133(1):149-169.

Steiner, J. M., S.R.Gumminger, and D.A.Williams. 2001. Development and validation of an enzyme-linked immunosorbent assay (ELISA) for the measurement of canine pancreatic lipase immunoreactivity (cPLI) in serum. J.Vet.Int.Med. 15311-311.

Steiner, J. M., D.A.Williams, E.M.Moeller, and T.L.Melgarejo. 2000. Development and validation of an enzyme-linked immuno sorbent assay (ELISA) for feline trypsin-like immunoreactivity (f TLI). Am.J.Vet.Res. 61:620-623.

Tani, T., J.Ohsumi, K.Mita, and Y.Takiguchi. 1988. Identification of a novel class of elastase isozyme, human pancreatic elastase III, by cDNA and genomic gene cloning. J.Biol.Chem. 263(3):1231-1239.

Taylor, J. C. and I.P.Crawford. 1975. Purification and preliminary characterization of human leukocyte elastase. Arch.Biochem.Biophys. 169(1):91-101.

Travis, J. 1988. Structure, function, and control of neutrophil proteinases. Am.J.Med. 84(6A):37-42.

Travis, J., P.J.Giles, L.Porcelli, C.F.Reilly, R.Baugh, and J.Powers. 1979. Human leucocyte elastase and cathepsin G: structural and functional characteristics. Ciba.Found.Symp. (75):51-68.

Willard, M.D., A.E.Jergens, R.B.Duncan, M.S.Leib, M.D.McCracken, R.C.DeNovo, R.G.Helman, M.R.Slater, and J.L.Harbison. 2002. Interobserver variation among histopathologic evaluations of intestinal tissues from dogs and cats. J. Am.Vet.Med.Assoc. 220(8):1177-1182.

APPENDIX

A.1. Legends for Figures

A.2. Legends for Tables

A.3. List of Buffer

Buffers used during purification of cNE:

- **Extraction buffer:**

0.02 M Tris, 1 M NaCl, 0.005% Tween, pH 8.5

- **Solution for blood sedimentation:**

60 mg/ml Dextran 500, 9 mg/ml NaCl

- **Buffer A for cation-exchange chromatography:**

0.05 M sodium acetate, 0.05 M NaCl, pH 5.0

Buffer for continuous elution-electrophoresis:

- **Resolving gel buffer**

1.5 M Tris-HCl, pH 8.8

- **Stacking gel buffer**

0.5 M Tris-HCl, pH 6.8

- **Gel casting Buffer**

0.375 M Tris-HCl, pH 8.8

- **Running/Elution buffer**

0.025 M Tris-HCl, 0.192 M glycine, 0.1% SDS, pH 8.3

- **Sample buffer**

0.06 M Tris-HCl, 2% SDS, 5% beta-mercaptoethanol, 25% glycerol (w/v) and 0.01% bromophenol blue, pH 6.8

- **Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis buffer:**

MES running buffer

1 M 2-(N-morpholino) ethane sulfonic acid, 1 M Tris-base, 70 mM SDS, 21 mM EDTA, pH 7.3

- **NuPAGE® SDS sample buffer**

4 g sucrose + 0.68 g Tris-base + 0.67 g Tris-HCl + 0.8 g SDS + 0.006 g EDTA + 0.75 ml Serva blue G250 + 0.25 ml Phenol red + ultra pure water to 10 ml

Buffers used for cNE ELISA:

- **Sample buffer, TBS+BSA+Tween**

50 mM Tris-base, 0.14 M NaCl, 1% BSA, 0.05% Tween, pH 8.0

- **ELISA wash buffer, TBS**

50 mM Tris-base, 0.14 M NaCl; pH 8.0

- **Binding buffer, sodium carbonate-bicarbonate buffer**

0.2 M sodium carbonate-bicarbonate, pH 9.4

- **ELISA stopping solution**

17.4 M acetic acid, 2 N sulfuric acid

CURRICULUM VITAE

Name:	Anja Stoll
Date of birth:	July 17th 1978
Place of birth:	Hamburg, Germany
Parents:	Fritz Ullrich Stoll
	Karin Stoll (maiden name: Boettger)

Education and occupation:

1984-1988	Elementary school in Hamburg
1988-1997	Gymnasium Christianeum in Hamburg
1997-2003	Study of veterinary medicine,
	Ludwig-Maximilians University in Munich
August 2003	Approbation
November 2003	Start dissertation
11/03 – 08/06	Research assistant at the Gastrointestinal Laboratory at
	Texas A&M University, College Station, Texas, USA

Publications:

Stoll A, Ruaux CG, Suchodolski JS, Williams DA, and Steiner JM: Purification and partial characterization of canine neutrophil elastase. JVIM 20(3), 789, 2006

Stoll A, Suchodolski JS, Ruaux CG, and Steiner JM: Purification and partial characterization of canine neutrophil elastase (cNE) and the development of an immunoassay for the measurement of cNE in serum. *Submitted to Journal for review*

Steiner JM, Suchodolski JS, Ruaux CG, Berghoff N, Fetz K, Tress U, **Stoll A**, Rodriguez FH, Xenoulis P, Williams DA: Combined test for gastric emptying and oro-cecal transit time in healthy dogs before and after tylosin administration. JVIM 19(3), 475, 2005

Ruaux CG, Suchodolski JS, Berghoff N, Fetz K, **Stoll A**, Tress U, Steiner JM, Williams DA: Alterations in markers assessing the canine small intestinal microflora in response to altered housing and tylosin administration. JVIM 19(3), 441, 2005

Berghoff N, Ruaux CG, Steiner JM, **Stoll A**, Shah B, Williams DA. Determination of intestinal permeability and mucosal absorptive capacity in Norwegian Lundehunds using a five-sugar blood test. *In preparation*