

An immunoassay for canine pancreatic elastase 1 as an indicator for exocrine pancreatic insufficiency in dogs

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Abstract. The detection of pancreatic elastase 1 in stool samples has become the noninvasive gold standard for the diagnosis of pancreatic insufficiency in humans. Accordingly, the development of a sandwich-ELISA specific for canine pancreatic elastase 1, based on monoclonal antibodies, is presented here. The test has a detection range of 4–240 μg canine pancreatic elastase 1/g feces. The intraassay coefficient of variation is 7.4%, and the interassay coefficient of variation is 7.7%. Spiking experiments show that canine elastase 1 is quantitatively detectable in fecal samples. Interestingly, the range of the elastase 1 concentration in canine feces within several days is higher as compared with humans. As the proposed cutoff of 10 $\mu\text{g}/\text{g}$ is below this variation range in 96.1% of the tested samples, the effect on the test specificity is negligible. Because the test detects neither human nor bovine and porcine elastase 1, pancreatic function can be monitored without interrupting an enzyme replacement therapy.

Clinical signs of weight loss, diarrhea, and polyphagia are leading symptoms for malabsorption, usually caused by small intestinal disease/enteritis, and for maldigestion, caused by exocrine pancreatic insufficiency (EPI).²⁰ Thus, differential diagnosis of EPI requires an organ-specific diagnostic method. The most common cause for EPI is pancreatic acinar atrophy, followed by occasionally relapsing pancreatitis in elderly dogs, leading to EPI if more than 90% of the pancreatic tissue is destroyed.^{15,19,20}

Various tests are available for the diagnosis of canine EPI. At present, the most frequently applied test is the detection of canine trypsin-like immunoreactivity (cTLI) in dog's serum with a radioimmunoassay.^{21,22} This test is most reliable and delivers usually accurate results. It was particularly developed for the diagnosis of EPI in dogs.

All other tests are less reliable in terms of sensitivity and specificity,¹¹ like an enzyme-linked immunosorbent assay (ELISA) for the detection of cTLI and a test for chymotrypsin activity in feces.⁷ Similarly, other noninvasive pancreatic function tests (oral fat absorption test, pancreatic proteolytic enzyme activity test, starch tolerance test, BT PABA-test, xylose absorption test) are often misleading because they produce a high proportion of equivocal results.^{1,9,20} Most of these tests (with the exception of the tests for serum canine TLI) were adapted from diagnostic procedures

in human medicine. They did not find their way to a widespread application in general veterinary medical practice.

It was shown in recent years that the detection of pancreatic elastase 1 has the highest sensitivity and specificity among the noninvasive pancreatic function tests for humans.¹³ Pancreatic elastase 1 is a proteolytic enzyme of the acid elastase family. It is produced as zymogen exclusively in the acinar cells of the pancreas and is thus absolutely pancreas specific. The mature enzyme has a molecular weight of 28 kD.¹⁶ It is highly stable during intestinal passage and is resistant to proteolytic degradation in the gut.¹⁷ An ELISA, based on 2 monoclonal antibodies directed specifically against the human enzyme, is available for the detection of elastase 1 in stool specimens.⁸ Numerous studies have shown that the test identifies patients suffering from pancreatic insufficiency with a sensitivity and specificity >90% each.^{2-4,6,14,18} Even mild pancreatic insufficiency can be detected with a sensitivity of up to 63%.⁶ These results, together with the simple test procedure and easy handling of the samples, caused the test to become the most widely used test among the noninvasive human pancreas diagnostic procedures.

The aim of the work presented here is to establish a species-specific test of similar quality for the diagnosis of canine EPI. Quantification of pancreatic elastase 1 in canine feces using the rather crude method of rocket electrophoresis provided the first promising results.¹⁰ As a consequence, monoclonal antibodies specific for canine elastase 1 were generated and an ELISA was established using these antibodies for the quantification of canine pancreatic elastase 1 in fecal samples.

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Materials and methods

Monoclonal antibodies directed against canine pancreatic elastase 1

Balb/c mice were immunized with the purified canine pancreatic elastase 1, and monoclonal antibodies were generated essentially as previously described.⁵ The purified canine pancreatic elastase 1 was derived from dog's pancreas. The antibody-producing hybridomas were screened for a positive reaction with purified elastase 1 by ELISA. Hybridomas yielding a positive signal were subcloned twice and re-screened with canine, bovine, porcine, and human pancreatic elastase 1. Finally, the clones F9 and E2, which react with the canine pancreatic elastase 1 but not with the tested elastases from other species, were selected for use in the sandwich immunoassay. Isotyping was carried out with rabbit antimouse isotype-specific antibodies.^a

Sample preparation

Canine feces (approximately 100 mg) were thoroughly mixed 1:100 (w/v; vortex) with extraction buffer (provided by the manufacturer) at room temperature. A minimum incubation period of 5 min was required to ensure complete extraction of the pancreatic elastase 1. The incubation period may be extended up to 24 hr. Following the extraction, insoluble particles were allowed to settle for 5 min and the supernatant is collected for the elastase 1 quantification. A 1/40 dilution of these extracts in washing buffer was used for the direct quantification of canine pancreatic elastase 1 in the immunoassay. For long-term storage, fecal samples were kept in the freezer at -20°C .

Pankreatin as a substitution drug, obtained from different manufacturers,^{b-d} were ground in a mortar and 1:5 extracted as described above. Different dilutions of these samples (from undiluted to 1:128) were tested in the pancreatic elastase 1-ELISA.

Canine pancreatic elastase 1-ELISA^e

Fifty microliters per well of 1:40 (v/v) diluted fecal extracts, the calibration standards (purified canine elastase 1 corresponding to 4, 20, 80, and 240 μg elastase 1/g feces), a positive control (purified canine elastase 1), and a blank (washing buffer) were incubated on the antibody-coated 96-well plate for 1 hr. Subsequently, the biotinylated secondary antielastase 1 antibody was incubated for 30 min, followed by an incubation with streptavidin-coupled horseradish peroxidase (30 min) and ABTS-solution (2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonate]) (45 min). The steps were separated by a washing procedure with sample buffer (3 \times). All incubations were carried out at room temperature. For analysis, the plates were read with an ELISA reader at 405 nm wavelength at a reference wavelength of 492 nm.

Test parameters

To analyze for potential interference of fecal components with the pancreatic elastase 1 quantification using the ELISA, 9 different samples were spiked with 15 ng of purified canine pancreatic elastase 1 and subsequently subjected to the ELISA. The baseline concentrations of the samples without purified elastase 1 were compared with the concen-

tration determined after addition of elastase 1. The difference between each pair of concentrations was calculated. To demonstrate the species specificity of the ELISA, concentrations from 3.1 to 100.0 ng/ml of purified canine, human, bovine, and porcine pancreatic elastase 1 were tested.

To determine the range of elastase 1 concentration in individual patients, the concentration of fecal pancreatic elastase 1 was tested over a period of several weeks in different fecal samples of 8 individual dogs.

Different concentrations of pancreatic elastase 1 (6, 14, 40, and 67 ng/ml) were stored at 4 and 37 $^{\circ}\text{C}$ over a period of 3 weeks to monitor the long-term stability of pancreatic elastase 1. The elastase 1 concentration was determined at the start, after 1 week, and after 3 weeks of incubation.

Patients

Pancreatic elastase 1 was quantified in fecal samples of 73 dogs of different breeds. Eight of them were healthy. Forty-seven dogs suffered from different diseases that were not primary related to the gastrointestinal system or to the pancreas (heart and renal failure; endocrinopathies; infectious diseases; diseases of the respiratory tract, locomotor system, CNS, and genitals; tumors; and acute poisoning). Acute gastroenteritis was diagnosed in 11 dogs and chronic gastrointestinal disturbances in 7 animals. Due to the results of clinical examination, laboratory tests, and imaging techniques, the individual diagnoses in these dogs were inflammatory bowel disease (IBD, $n = 2$), colitis ($n = 2$), hepatic disease ($n = 2$), and gastric cancer ($n = 1$). In dogs with IBD, exocrine pancreatic insufficiency was excluded by serum cTLI determination.

Intestinal stability of canine pancreatic elastase 1

During routine necropsy, pancreatic tissue was taken from the duodenal branch and chyme samples were collected from the duodenum, ileum, jejunum, colon, and rectum of 8 dogs and stored at -20°C until elastase 1 determination with the species-specific ELISA.^e The pancreas of the dogs were macroscopically and histologically normal. The samples for histology were taken from the tissue samples that were used for elastase determination before freezing. For elastase 1 determination, the pancreatic tissue samples were homogenized by a minidounce homogenizer, followed by elastase 1 extraction as described for fecal samples.

Statistical methods

For the measurements of pancreatic elastase in feces samples, a calibration curve was established on the basis of linear regression analysis. Individual points of the calibration curve were calculated as the mean of 2 measurements, with the coefficient of variation indicated as whiskers.

The data of pancreatic elastase 1 determination are expressed as median, quartiles, minimum, and maximum because they were not normally distributed. This was used to describe the data of the range of elastase concentrations in repeated measurements of 8 single dogs over a period of several weeks and the results for the assessment of the intestinal stability of pancreatic elastase 1. Median box and whisker blots were used in the figures.

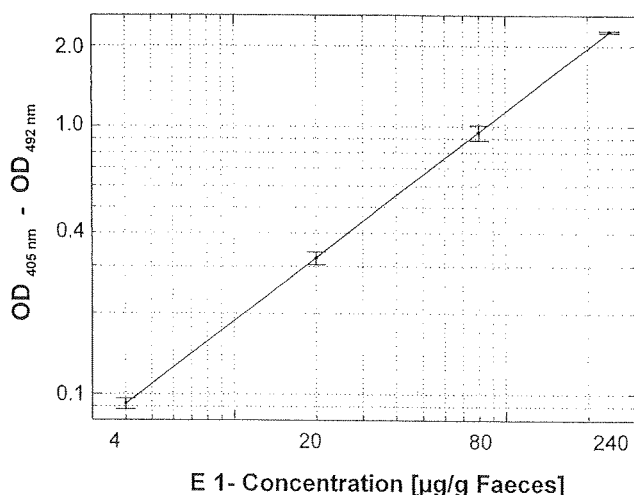


Figure 1. Calibration curve of the canine pancreatic elastase 1 test. The whiskers on the standards indicate the coefficient of variation in percent, based on double measurements. The 4 standards of the calibration curve (1, 5, 20, and 60 ng/ml purified pancreatic elastase 1) result in a detection range of 4–240 µg canine elastase 1/g feces. This is calculated on the basis of a 1:100 dilution in the extraction procedure and a further 1:40 dilution of the samples (= extract), resulting in a total dilution of 1:4,000 (1 ng/ml elastase 1, resulting in 4 µg elastase 1/g feces, and 60 ng/ml elastase 1, resulting in 240 µg elastase 1/g feces).

Comparison of results obtained by rocket electrophoresis and ELISA was carried out by Spearman rank correlation.

Results

Monoclonal antibodies directed against canine pancreatic elastase 1

Immunization of mice and screening of monoclonal antibodies was carried out with purified canine pancreatic elastase 1. The mouse monoclonal antibodies were selected for positively identifying canine pancreatic elastase 1 but not human, porcine, and bovine pancreatic elastase 1. The antibodies chosen for use in the sandwich immunoassay are of the IgG1-subclass.

Development of a sandwich immunoassay for canine pancreatic elastase 1

The immunoassay is designed as a sandwich ELISA with the primary antibody coated on the 96-well plate. The sample incubation is followed by addition of a biotinylated secondary antibody, streptavidin-peroxidase conjugate, ABTS substrate, and measurement of the optical density at 405 nm. The assay requires 3 hours total time. Calculation of the results is carried out by comparing the optical density of the samples with those of the calibration curve, which is based on 4 standards and a blank absorbance.

Detection limit of the assay

The 4 standards of the calibration curve (1, 5, 20, and 60 ng/ml purified pancreatic elastase 1) result in

Table 1. Precision of the canine pancreatic elastase 1 ELISA.

Sample	Intraassay variation		Interassay variation	
	Mean (µg/g)	CV (%)	Mean (µg/g)	CV (%)
A	51.0	7.8	45.8	11.0
B	80.8	7.9	74.1	9.7
C	111.1	6.3	94.3	4.1
D	148.7	6.5	122.5	7.4
E	186.8	11.0	148.4	5.8
F	281.6	7.4	320.6	9.0
G	228.1	5.2	243.8	8.9
H			377.9	5.9
Mean CV (%)		7.4		7.7

a detection range of 4–240 µg canine elastase 1/g feces. This is calculated on the basis of a 1:100 dilution in the extraction procedure and a further 1:40 dilution of the samples (= extract), resulting in a total dilution of 1:4,000 (1 ng/ml elastase 1, resulting in 4 µg elastase 1/g feces, and 60 ng/ml elastase 1, resulting in 240 µg elastase 1/g feces). A typical calibration curve is shown in Fig. 1.

Test parameters

1. *Precision.* Elastase 1 concentration was determined 20 times in each of 7 samples derived from 7 different patients (ranging from 51 to 282 µg elastase 1/g feces) to evaluate the intraassay coefficient of variation (CV; Table 1). The average CV was 7.4% (range: from 5.2 to 11.0%). The interassay CV was evaluated with fecal samples of 8 different patients being tested on 10 consecutive days (Table 1). The average CV was 7.7% (range: 4.1 to 11.0%).

2. *Potential interferences, spiking.* In a spiking experiment with 9 different samples, 15 ng purified canine elastase 1 was added to each of these samples. The baseline concentrations of the samples without purified elastase 1 were compared with the concentration determined after addition of elastase 1. The difference between each pair of concentrations was calculated as 13, 17 (3×), 16, 22, 18 (2×), and 15 ng in samples 1–9 (Table 2). The results indicate that fecal components do not affect the linearity of the assay.

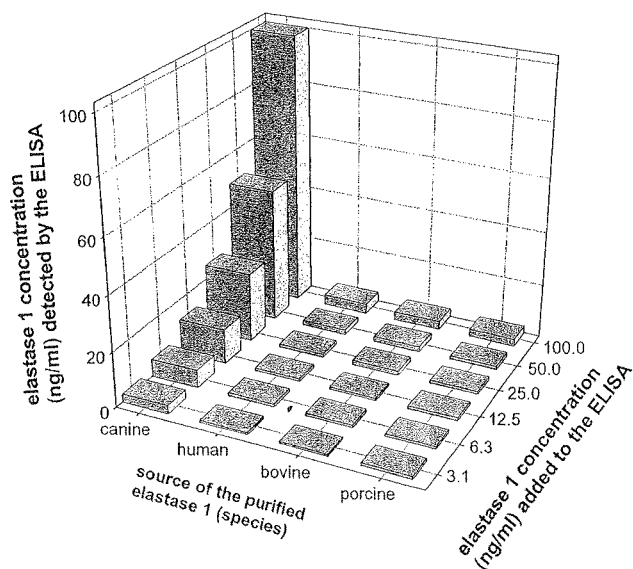
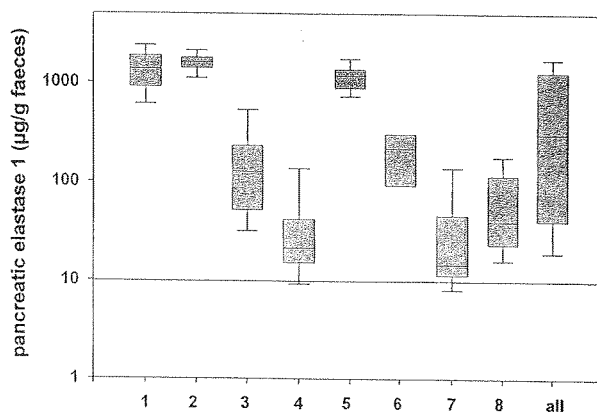
3. *Potential crossreactivity and species specificity.* To demonstrate the species specificity of the ELISA, purified canine, human, bovine, and porcine pancreatic elastase 1 were tested. The concentrations of canine elastase 1 were correctly determined, whereas the elastases derived from other species resulted in background absorbance only (Fig. 2). Similarly, the extracts of the ground substitution drug pankreatin, obtained from different manufacturers,^{b-d} did not result in any detectable signal. Thus, the canine elastase 1 test is species specific and can be carried out while substitution therapy is in progress.

Table 2. Spiking of individual fecal samples with 15 ng/ml purified canine elastase 1.

Sample	Elastase 1 concentration (ng/ml)		Difference
	Sample concentration	+15 ng/ml	
1	37	50	13
2	14	31	17
3	20	37	17
4	8	25	17
5	16	32	16
6	15	37	22
7	2	20	18
8	13	31	18
9	19	34	15

4. *Range of elastase 1 concentration in individual patients.* The elastase 1 concentration in different fecal samples of one individual ranges from 1- to 13-fold of the mean (Fig. 3). Although an individual's range of elastase 1 concentration in dogs is higher as compared with human pancreatic elastase 1,^{6,14} 96.1% (197/205) of the concentrations presented here are above 10 μg elastase 1/g feces.

5. *Long-term stability of canine elastase 1.* Storage of purified pancreatic elastase 1 at different temperatures showed that 3 weeks storage at 4 C and 1 week storage at 37 C resulted in no significant change of the elastase 1 concentration. Only after 3 weeks incubation at 37 C was a moderate reduction of the concentration

**Figure 2.** Measurement of purified canine, human, bovine, and porcine elastase 1 with the canine elastase 1 ELISA. The source of the purified elastases is indicated on the bottom (y-axis) and the individual concentrations on the right (x-axis). The scale showing the concentrations measured by the canine elastase 1 ELISA is indicated on the left (z-axis).**A****B**

dog no.:	samples:		elastase 1 concentration:	
	no. of samples	sampling period	mean ($\mu\text{g/g}$)	median ($\mu\text{g/g}$)
1	40	47 days	1464	1364
2	30	17 days	1641	1598
3	30	22 days	201	127
4	29	19 days	71	21
5	30	17 days	1185	1178
6	4	13 days	198	218
7	12	11 days	47	15
8	30	35 days	99	39
all	205		760	498

Figure 3. Range of the pancreatic elastase 1 concentration on consecutive days in 8 individual dogs. **A**, Box plot showing the concentration range of pancreatic elastase 1 in individual dogs within several days (the number of days is shown in **B**). The scale indicating the elastase 1 concentration is shown on the left, and the numbers of the individual dogs are shown on the bottom. Whiskers are set at the 5 and 95% percentiles, boxes at the 25 and 75% percentiles, and the lines indicate the median. The straight line on the bottom marks the cutoff of 10 $\mu\text{g/g}$. **B**, Table containing details corresponding to the box plot in **A**.

by an average of 24% observed, indicating high stability of the elastase 1 even under difficult conditions (see Table 3 for details).

Comparison of the canine elastase 1 ELISA with rocket electrophoresis results

The first attempt to detect pancreatic elastase 1 in canine feces was carried out by rocket electrophoresis using polyclonal rabbit antihuman pancreatic elastase 1 antibodies.¹⁰ For comparison, elastase 1 was quantified in canine fecal samples with both methods. Of 73 samples tested, 23 had an elastase 1 concentration below the lower detection limit of the rocket electrophoresis method (<100 $\mu\text{g/g}$ feces). With the ELISA using species-specific monoclonal antibodies, elastase 1 could be quantified precisely in all samples above the detection limit and in all of the 26 samples below

Table 3. Stability of purified canine elastase 1 (concentrations [ng/ml] determined by ELISA) over 3 weeks at 4 and 37 C.

Concentration (ng/ml)	Incubation (time and temperature)									
	0 weeks		1 week				3 weeks			
	% of 0		% of 0		% of 0		% of 0		% of 0	
7	5.9	100	7.3	124	5.9	100	5.9	100	4.1	69
15	13.9	100	16.0	115	13.7	99	12.9	93	11.1	80
40	39.7	100	43.5	110	38.9	98	42.1	106	30.1	76
75	66.9	100	64.4	96	60.5	90	65.9	99	52.1	78

the detection limit of the rocket electrophoresis. Calculated on the basis of the 73 samples tested, 93% (68/73) had pancreatic elastase 1 concentrations above 10 μg elastase 1/g feces. The lowest detection limit of the ELISA (4 $\mu\text{g}/\text{g}$ feces) is 25-fold below that of the rocket electrophoresis (100 $\mu\text{g}/\text{g}$ feces). Comparison of the elastase 1 concentrations obtained with both tests by Spearman rank correlation resulted in a correlation coefficient of $r = 0.624$.

Intestinal stability of canine pancreatic Elastase 1

An important feature of a valuable diagnostic test is the stability of the analyte in the sample. Thus, the course of pancreatic Elastase 1 concentration during intestinal passage was determined in samples of pancreatic parenchyma and chyme samples from duodenum, ileum, jejunum, colon, and rectum. Samples were taken from 8 dogs of different breeds that died because of nonpancreatic diseases.

Postmortem examinations revealed that pancreatic tissue samples of dogs with histologically normal pancreases contained a median elastase 1 concentration of 3,871.5 $\mu\text{g}/\text{g}$ (217.1–6,324.0 $\mu\text{g}/\text{g}$). In the chyme samples, the median elastase 1 concentration was lowest (48.0 $\mu\text{g}/\text{g}$; range 3.9–169.0 $\mu\text{g}/\text{g}$) in the duodenum, and all dogs showed an individually different increase in elastase 1 concentration during intestinal passage

(Fig. 4). The determination of the elastase 1 concentration in the content of other intestinal parts revealed a median elastase 1 value of 67.5 $\mu\text{g}/\text{g}$ (2.2–1,124.0) in the jejunum, 619.0 $\mu\text{g}/\text{g}$ (0.5–1,543.0) in the ileum, 952.0 $\mu\text{g}/\text{g}$ (405–2,464.0) in the colon, and 1,229.0 $\mu\text{g}/\text{g}$ (403.0–2,204.0 $\mu\text{g}/\text{g}$) in the rectum.

Discussion

The present data describe the development of an ELISA for the quantification of canine pancreatic elastase 1 in feces. Analogous to the established elastase 1 test in human clinical diagnostics, the test can be used for the diagnosis of EPI.

A fecal sample of approximately 100 mg is sufficient to run the test. The sample is 1/100 (w/v) extracted and further diluted 1/40 for direct quantification such that a disturbance by unpleasant odors is negligible. The test is a standard ELISA in a 96-well microtiter plate format. Since the test requires no radioactivity, it can be performed with standard laboratory equipment.

Test parameters are within the range of standard immunoassays. The precision (intra- and interassay coefficient of variation) is well below 10% and is thus acceptable, and the measuring range is from 4 to 240 $\mu\text{g}/\text{g}$. Spiking experiments allow quantitative detection of purified elastase 1 added to fecal samples, indicating that other components in the fecal extract do not interfere with the quantification/linearity of the assay.

In contrast with many veterinary diagnostic tests, which were adapted from diagnostic procedures in human medicine, this test is specifically developed for the quantification of pancreatic elastase 1 in dogs, like the serum cTLI determination by RIA.²¹ The elastase 1 ELISA is species specific for dogs, so monitoring of the pancreatic function is possible without interrupting an enzyme replacement therapy, which usually requires drugs delivering porcine enzymes (Pankreatin). In contrast with this, determination of fecal chymotrypsin activity leads to false normal test results in dogs with EPI when they are under treatment with enzyme supplements.⁷

Similar to its human counterpart, the canine pancreatic elastase 1 appears to be highly resistant to proteo-

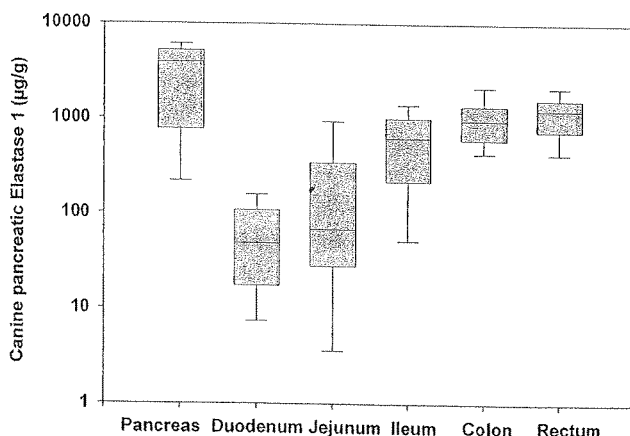


Figure 4. Median concentration of canine pancreatic elastase 1 in pancreatic tissue in different parts of the intestine in 8 dogs without pancreatic diseases.

lytic degradation in the gut.¹⁷ Concentrations of human pancreatic elastase 1 were found to be 5–6 times higher in feces than in pancreatic juice. Similarly, canine fecal elastase 1 concentrations in the colon and rectum are 10–20-fold higher than in the duodenum. The increase of the pancreatic elastase 1 concentration could be due to dehydration of the feces during intestinal passage. Because the test is not based on enzymatic activity but quantifies the elastase immunologically, a quantification is still possible if the enzymatic activity of the protein would be affected during intestinal passage.

An interesting observation in the course of this study was that the range of the pancreatic elastase 1 concentration in feces of healthy dogs was more extensive than in humans^{6,14} when monitored over a period of several days. If this physiological difference is due to, e.g., variations in the output of pancreatic juice, it should affect the concentration of all pancreatic enzymes. Irregular meals and a short intestinal passage time might be other reasons for this phenomenon. However, 96.1% of these samples show pancreatic elastase 1 concentrations above 10 µg/g feces. This concentration limit was shown to result in a sensitivity of 95%¹² and can thus be considered as a preliminary cutoff. Thus, the observed extended concentration range is irrelevant when it comes to clinical management and diagnosis of exocrine pancreatic insufficiency.

Initial experiments using polyclonal antielastase 1 antibodies in rocket electrophoresis have shown that the enzyme is detectable in canine feces.⁹ The test had very high sensitivity (100%). However, because all samples below the (inadequate) lower detection limit had to be considered pathological, the specificity was rather low (56.5%) and had to be optimized. The comparison of rocket electrophoresis and ELISA shows that the results of both tests correlate reasonably well in terms of relative quantification. The lower detection limit of the ELISA is, however, 25-fold below the limit of the rocket electrophoresis. With this decreased detection limit, the quantification of pancreatic elastase 1 in canine feces becomes highly sensitive and specific.

The canine pancreatic elastase 1 ELISA, which is now commercially available as a ready-to-use test kit,⁶ appears to be of a quality that is equal to the test for human pancreatic elastase 1. It is easy to perform without using radioactivity, which makes the test available for standard clinical laboratories. A single fecal sample can be mailed to the laboratory instead of having the veterinarian drawing blood for laboratory investigations.

More studies will follow to further evaluate the clinical relevance of this test. A strong current interest is

focused on the question of whether the test could detect a certain percentage of subclinical EPIs, i.e., EPIs in an early stage of the disease, as described for the quantification of pancreatic elastase 1 in human.^{2,3,6}

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- Kreon®, Solvay, Kali-Chemie Pharma GmbH, Hannover, Germany.
- ScheBo® • Biotech AG, Giessen, Germany.

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