Validation of a commercial 1,2-o-dilauryl-rac-glycero glutaric acid-(6′-methylresorufin) ester lipase assay for diagnosis of canine pancreatitis

Emily L Goodband, Gonçalo Serrano, Fernando Constantino-Casas, Joy Archer, Penny J Watson, Tim L Williams

Department of Veterinary Medicine, University of Cambridge, Cambridge, UK

Correspondence to
Dr Penny J Watson; pjw36@cam.ac.uk

ABSTRACT

The objectives of this study were fourfold: technical validation of a commercial canine 1,2-o-dilauryl-rac-glycero glutaric acid-(6′-methylresorufin) ester (DGGR) lipase assay, to calculate a reference interval for DGGR lipase by the indirect a posteriori method, to establish biological validity of the assay, and to assess agreement between DGGR lipase and specific canine pancreatic lipase (Spec cPL) assays. Dogs with histologically confirmed acute pancreatitis (n=3), chronic pancreatitis (n=8) and normal pancreatic tissue (n=7) with stored (−80°C) serum samples were identified. Relevant controls were selected. Precision, reproducibility and linearity of DGGR lipase, and the effect of sample haemolysis and freezing, were assessed. Sensitivity and specificity of DGGR lipase and Spec cPL were determined. Agreement between these two parameters was calculated using Cohen's kappa coefficient (κ). The DGGR lipase assay demonstrated excellent precision, reproducibility and linearity. Sample haemolysis and storage at −80°C for 12 months did not influence the assay. DGGR lipase (>245IU/l) and Spec cPL (>400μg/l) both showed poor sensitivity but excellent specificity for acute pancreatitis, and poor to moderate sensitivity but excellent specificity for chronic pancreatitis. Substantial agreement (κ=0.679) was found between DGGR lipase and Spec cPL. The validated DGGR lipase assay had similar sensitivity and specificity for the diagnosis of acute and chronic pancreatitis to Spec cPL. DGGR lipase is a reliable alternative to Spec cPL for the diagnosis of pancreatitis.

INTRODUCTION

Pancreatitis is a common condition which can vary widely in presentation. Disease can range from mild and subclinical, to acute, necrotising and fatal. Clinical signs are often non-specific, including vomiting, diarrhoea, lethargy, anorexia and weight loss, making a definitive diagnosis challenging. Histologically, acute cases show peripancreatic fat necrosis, oedema and neutrophil infiltration. Chronic cases show fibrosis with mononuclear cell infiltrate and acinar cell loss.[1] However, acute-on-chronic disease can also occur.

The current gold standard for the diagnosis of pancreatitis is surgical biopsy and histology.[2,3] However, this method is invasive,[4] costly and impractical to perform on all cases with suspected disease. The most commonly used diagnostic method is measurement of circulating markers of pancreatic inflammation in the blood. However, no currently available blood test for pancreatitis demonstrates 100 per cent accuracy. The specific canine pancreatic lipase (Spec cPL) immunoreactivity is regarded as the most sensitive (71 per cent in moderate-severe cases) and specific (86–100 per cent) test available.[5] However it is costly and has a relatively long turnaround time (two to three days). An immediate kennel-side SNAP test is available, but is not quantitative and has low specificity (59 per cent).[6] Catalytic (1,2-diglyceride) lipase assays are quantitative, cheap and quick, but are not organ-specific (with specificity of approximately 43 per cent in moderate to severe cases).[5] The need remains for a sensitive, specific, non-invasive test for pancreatitis which can provide quick diagnostic results.

In 2001 a new catalytic lipase assay, using 1,2-o-dilauryl-rac-glycero glutaric acid-(6′-methylresorufin) ester (DGGR) as substrate, was introduced, and validated in 2005 for use in dogs.[7] The enzyme and substrate interactions are reported to be more selective than previously used lipase assays, so it is suggested that hydrolysis with extrapancreatic lipases and esterases is less likely to occur. The DGGR assay is therefore proposed to be more specific than the traditional lipase assays such as the 1,2-diglyceride assay.

DGGR lipase is cheaper to perform than Spec cPL and the results can be available within one hour of arrival of the sample at many commercial laboratories. Studies have shown good agreement between the Spec cPL

and DGGR lipase assay,[8 9] and fair agreement between the DGGR lipase assay and an ultrasonographic diagnosis of pancreatitis.[10] No studies to date have specifically investigated the agreement of DGGR lipase with a histological diagnosis of pancreatitis in dogs, despite this being the gold standard for the diagnosis of pancreatitis.

The aims of this study were first to perform technical validation of a novel DGGR lipase assay for use in canine serum, secondly to calculate a reference interval for DGGR lipase by the indirect a posteriori method, thirdly to perform biological validation of this assay by assessing the sensitivity and specificity of DGGR lipase for the diagnosis of acute and chronic pancreatitis, and fourthly to verify the agreement of this DGGR lipase assay with Spec cPL.

MATERIALS AND METHODS
Measurement and technical validation of DGGR lipase
DGGR lipase activity was measured using a commercially available assay (DiaSys Lipase DC FS, Holzheim, Germany). For technical validation purposes, samples with DGGR lipase greater than 300IU/l were excluded as these exceeded the upper end of the linear range of the assay. Precision and repeatability were assessed by evaluating intra-assay and interassay coefficients of variation (CV) for serum samples with low and medium serum DGGR lipase activities (approximately 100IU/l and 250IU/l). For intra-assay precision, three replicates of each sample were evaluated within the same run. For assessment of interassay variability, pooled canine serum samples were evaluated in duplicate on three consecutive working days. Linearity was evaluated using canine serum pools of medium and low DGGR lipase activities (250IU/l and 100IU/l), with dilution samples prepared by mixing the medium and low pooled samples in different proportions. The linearity was determined by comparing the observed DGGR lipase activity following dilution with the expected (calculated) DGGR lipase activity. Linearity following dilution of a sample with medium DGGR lipase activity (approximately 250UI/l) with saline was also determined. Stability of the photometrically determined DGGR lipase after 12 months of storage at −80°C was also assessed. Interference by haemolysis was determined by addition of canine blood haemolysate to canine serum samples. The haemolysate was prepared by washing of canine erythrocytes three times in saline, before lysis of the erythrocytes by addition of double distilled water (diH$_2$O). The haemolysate was sequentially added to the serum samples, with the final haemoglobin concentration of the serum determined grossly and photometrically. Four samples with different haemoglobin concentrations (0g/l, 1g/l, 2g/l and 4g/l) were obtained. Expected DGGR lipase activity was calculated and compared with the observed DGGR lipase activity in order to evaluate the influence of the haemolysis on the DGGR lipase measurement.

Construction of a reference interval for DGGR lipase by the a posteriori method
In order to calculate a reference interval for DGGR lipase by the indirect a posteriori method,[11] all dogs that had DGGR lipase activity measured by the Central Diagnostic Services between January 1, 2015 and December 31, 2015 were retrospectively evaluated. Cases with a clinical history of pancreatitis, gastrointestinal disease or thromboembolic disease, those with abnormal cardiac auscultation or congestive heart failure, those with documented azotaemia (elevated serum urea and/or creatinine concentrations), and cases treated with corticosteroids were excluded. The remaining cases were then used to construct a reference interval using computerised software (Reference Value Advisor V.2.1; http://www.biostat.envt.fr/spip/spip.php?article63),[12] which calculated the lower and upper limits of the DGGR lipase reference interval by the robust method using Box-Cox transformed data.

Biological validation of DGGR lipase in dogs with acute and chronic pancreatitis and controls
For the biological validation, the pathology database of the authors' institution was searched to identify dogs between 2008 and 2015 with a histological diagnosis of acute pancreatitis, chronic pancreatitis and normal pancreatic tissue. Normal cases were selected based on documentation of clinical signs for which pancreatitis was a differential diagnosis (eg, vomiting, diarrhoea, abdominal pain). Histological slides of either postmortem sections or surgical biopsies from these cases were reviewed by one pathologist to confirm disease status (acute or chronic pancreatitis, or normal). Histological samples were categorised as acute pancreatitis given the predominance of neutrophils, fat necrosis and oedema, whereas samples were categorised as chronic pancreatitis if there were fibrosis and lymphocytic infiltrate. Serum samples (stored at −80°C) taken within 24 days of the time of tissue sampling were used for measurement of DGGR lipase and Spec cPL. Cases were excluded if biochemistry results indicated azotaemia, because reduced glomerular filtration rate may falsely elevate serum lipase measurements due to reduced renal clearance of the enzyme.[13 14] DGGR lipase activity was measured using the validated DGGR lipase assay in the authors' laboratory. Spec cPL was measured by IDEXX Laboratories (Wetherby, UK).

Statistical analysis
Statistical analysis was performed using commercial software (SPSS V.21, IBM). Data are presented as median (25th and 75th percentile). Sensitivity and specificity (plus 95 per cent confidence intervals (CI)) of both the Spec cPL and DGGR lipase for a diagnosis of either acute or chronic pancreatitis were calculated. Correlations were assessed using Spearman's correlation coefficient. Agreement between the two assays was assessed using Cohen's kappa coefficient (κ). κ values indicate the following agreement: <0=no agreement; 0–0.20=slight agreement;

0.21–0.4=fair agreement; 0.41–0.60 moderate agreement; 0.61–0.8=substantial agreement; and 0.81–1.0=almost perfect agreement.

RESULTS
Technical validation
Interassay precision for samples containing medium and low lipase activities (CV=0.3 per cent and 1.1 per cent, respectively) and intra-assay precision for samples containing medium and low lipase activities (CV=0.7 per cent and 0.9 per cent, respectively) were excellent. Following dilution of serum of medium lipase activity with serum of low lipase activity, the assay was linear in the range of 51–246nmol/l (r^2=0.998) with acceptable analyte recovery (<5.3 per cent deviation from calculated value at any point). Dilution of serum with medium lipase activity with saline was linear (r^2=0.999); however, a matrix effect was evident. Storage of serum at −80°C for 12 months did not result in a significant change in DGGR lipase activity (61 (82, 102) IU/l (median (25th, 75th percentile)) versus 86 (107, 132) IU/l, n=12; P=0.209). Haemolysis of the sample up to 8g/l (consistent with 3+ haemolysis grossly) did not result in significant changes to the lipase activity.

DGGR reference interval
Thirty-seven dogs were eligible for inclusion in the population of dogs used to construct the DGGR reference interval. Sixteen dogs had neurological disease, six cases had orthopaedic disease, five had inflammatory diseases (non-gastrointestinal), four had neoplastic diseases, four had respiratory disease, one had urolithiasis and one had dermatological disease. The calculated reference interval for DGGR lipase was 23–245IU/l.

Biological validation
Eighteen dogs were included in the biological validation study. Three dogs had histologically confirmed acute pancreatitis, eight had histologically confirmed chronic pancreatitis and seven control dogs (with clinical signs compatible with pancreatitis) had normal pancreatic tissue (six at postmortem examination and one on pancreatic biopsy). Signalment and the time between serum and tissue sampling in these cases are shown in Table 1. The maximum time between blood and tissue sampling was 24 days; however, the DGGR lipase and Spec cPL values for this case were 380IU/l and 374µg/l, respectively.

DGGR lipase for dogs with histologically normal pancreatic tissue was 61 (43, 138IU/l), and for cases diagnosed with chronic pancreatitis on histology was 173 (62, 435IU/l). DGGR lipase values in cases with acute pancreatitis were 106IU/l, 195IU/l and 233IU/L. The DGGR lipase activities in all three acute cases were within the calculated DGGR lipase reference interval. Spec cPL for dogs with histologically normal pancreatic tissue was 53µg/l (36, 169µg/l); for dogs with chronic pancreatitis was 217µg/l (42, 509µg/l), and in the three dogs with acute pancreatitis Spec cPL concentrations were 98µg/l, 375µg/l and 642µg/l.

DGGR lipase greater than 245IU/l was 0 per cent (95 per cent CI 0–69 per cent) sensitive and 100 per cent (95 per cent CI 56–100 per cent) specific for acute pancreatitis. Spec cPL concentration greater than 400µg/l was 33 per cent (95 per cent CI 18–87 per cent) sensitive and 100 per cent (95 per cent CI 56–100 per cent) specific for acute pancreatitis.

DGGR lipase greater than 245IU/l was 57 per cent (95 per cent CI 20–88 per cent) sensitive and 100 per cent (95 per cent CI 56–100 per cent) specific for chronic pancreatitis. Spec cPL concentration greater than 400µg/l was 42 per cent (95 per cent CI 12–80 per cent) sensitive and 100 per cent (95 per cent CI 56–100 per cent) specific for chronic pancreatitis. DGGR lipase and Spec cPL were highly correlated (r_s=0.925, n=18; P<0.001). Agreement analysis indicated that DGGR lipase greater than 245IU/l and Spec cPL greater than 400µg/l had substantial agreement (κ=0.679).

DISCUSSION
The results of this study indicate that the validated DGGR lipase assay demonstrates excellent precision and reproducibility. The DGGR lipase assay appeared to be unaffected by sample haemolysis, and DGGR lipase activity was not significantly different after 12 months of storage at −80°C. DGGR lipase and Spec cPL showed excellent agreement and had similar sensitivity and specificity for diagnoses of histologically confirmed acute and chronic pancreatitis.

The control dogs used were a relevant population because they presented with similar clinical signs to the cases of histologically confirmed pancreatitis, resulting in a more representative comparison that is faced by practitioners than if normal healthy animals had been used as the control group.

Two acute cases in this study had DGGR lipase activity and Spec cPL values within reference intervals. An explanation for the observed low sensitivity of DGGR lipase (and Spec cPL) for the diagnosis of chronic pancreatitis could be that the inflammation was mild in the cases that the authors included. It would have been useful, and a consideration for future studies, to utilise a histological grading system to further classify cases into severe, moderate or mildly affected. Another explanation could be the delay between histological confirmation of disease and sampling. The sensitivities of DGGR lipase and Spec cPL were higher for chronic pancreatitis where persistent elevations of enzymes might be expected.

DGGR lipase and Spec cPL were measured using serum samples taken as close to the time of pancreatic biopsy or postmortem examination as possible in order to obtain representative serum lipase activities while the patient was still clinically affected. One of the chronic cases had the blood sample taken 24 days before the postmortem examination took place, and an acute case

TABLE 1: Raw data of the results of DGGR lipase and Spec cPL assays for cases of histologically confirmed acute and chronic pancreatitis, and control animals, including signalment and time difference between blood sampling and biopsy or postmortem examination

	Age (years)	Sex	Breed	DGGR lipase (IU/l)	Spec cPL (µg/l)	Time of blood sample relative to tissue sample (days)
Acute						
	10	MN	Crossbreed	195.4	642	16 before postmortem examination
	10	FN	Crossbreed	233.3	375	1 after biopsy
	5	MN	Springer Spaniel	106	98	3 before biopsy
Chronic						
	12	FN	Border terrier	73.1	42	1 after biopsy
	3.25	MN	Labradoodle	61.2	30	1 after biopsy
	11	FN	Chihuahua	61.3	59	7 after biopsy
	9	FN	Great dane x whippet	273.2	411	7 before postmortem examination
	4	FN	Crossbreed	63.2	43	2 after biopsy
	11	FN	Cocker Spaniel	454.2	541	15 after biopsy
	12.8	FN	Jack Russell terrier	379.5	374	24 before postmortem examination
	7	MN	Cocker Spaniel	1119	939	1 before biopsy
Control						
	12	FE	Cavalier King Charles Spaniel	84.4	62	11 days before biopsy
	7.5	ME	Hungarian Vizsla	156.7	169	2 days before postmortem examination
	12	MN	English pointer	60.2	36	Same day as postmortem examination
	0.25	FE	Labrador retriever	42.3	38	6 days before postmortem examination
	8	FN	Maltese terrier	61	53	1 day before postmortem examination
	6	MN	Golden retriever	138.2	190	Same day as postmortem examination
	6	MN	Labrador retriever	43	30	6 days before postmortem examination

DGGR, 1,2-o-dilauryl-rac-glycero glutaric acid-(6'-methylresorufin) ester; FE, female entire; FN, female neutered; ME, male entire; MN, male neutered; Spec cPL, specific canine pancreatic lipase.

had a 16-day period between blood sample and postmortem examination. Considering the serum half-life of canine lipase is thought to be relatively short (approximately two hours), it is likely that in some cases the serum lipase activity may not have correlated as well with the lesions observed on histology. However, blood samples were taken at, or around, the time that the animal presented with clinical signs. It is therefore likely that the lipase activity was representative of the presence or absence of pancreatic inflammation at the time of clinical presentation. One would expect histological changes associated with pancreatitis to be present for some time after the initial disease insult, particularly in chronic cases where histological changes are permanent. Inclusion of these cases was therefore deemed justifiable.

DGGR has been considered to be more pancreas-specific than previously used lipase assays. However, a recent preliminary study found normal DGGR lipase activities in dogs with exocrine pancreatic insufficiency.[15] This suggests DGGR may not be exclusively hydrolysed by pancreatic lipase; however, despite this, DGGR lipase was highly specific for pancreatitis in the present study. More studies are required to investigate these findings further, and DGGR is still likely to be more specific than the previously used 1,2-diglyceride assay.

The main limitation of the biological validation study was the small number of cases that were included, which was a result of the criteria required for case selection. Although the number of cases included in this study was low, the control dogs used were a relevant population

because they presented with similar clinical signs to the cases of histologically confirmed pancreatitis, resulting in a more representative comparison that is faced by practitioners than if normal healthy animals had been used as the control group. However, further studies with higher number of cases are warranted to verify and strengthen these findings.

In conclusion, the results of this small, preliminary study indicate that the DGGR lipase assay may be used as a reliable alternative to Spec cPL in the diagnostic work-up of cases with suspected pancreatitis. This is suggested by the similar sensitivity and specificity found for the two assays for a diagnosis of acute and chronic pancreatitis, alongside the substantial agreement demonstrated.

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