Validation of Fecal Elastase-1 Determination Using Immunoenzymatic Assay in HIV-Infected Patients

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Pancreatic elastase-1 is a proteolytic enzyme exclusively produced in the pancreas, is stable when passing through the bowel, and its determination is associated with chronic pancreatitis. The clinical diagnosis of pancreatitis is based on anamnesis, physical examination, radiological, sonographic, endoscopic, and laboratory findings. Nowadays, there is a test for the determination of fecal elastase-1, by enzymatic reaction (enzyme-linked immunosorbent assay, ELISA), which specifically determines human elastase-1, promoting the pancreatic function evaluation. Parameters such as linearity, calibration curve, sensitivity, specificity, precision, accuracy, recovery, and receiver operator characteristic (ROC) curve are used to evaluate the

test. The aim of this study was the validation of the immunoenzymatic assay (ELISA) and the use of its results in patients with HIV, alcoholics, and under antiretroviral therapy. The study involved 157 patients, 95 of them were HIV-infected, and 62 were completely healthy. The elastase-1 ELISA kit from Bioserv was used, and we noted that the obtained results were linear, sensitive, precise, and accurate. Moreover, our results suggest that this test can be a laboratory evaluation to determine the relationship of pancreatitis with alcohol use, but not its association with antiretroviral use in HIV patients ($P = 0.424$). This test is useful to diagnose pathologies related to pancreatic insufficiency. *J. Clin. Lab. Anal.* 22:286–290, 2008. © 2008 Wiley-Liss, Inc.

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INTRODUCTION

The pancreatic secretion contains enzymes to digest every kind of food: proteins, carbohydrates, and fats. It also presents great amount of bicarbonate ions, which plays an important role in the neutralization of the chyme that goes from the stomach to the duodenum. Among the proteolytic enzymes, the most important are trypsin, chymotrypsin, and carboxypolypeptidase. The most abundant is trypsin that, with chymotrypsin, splits the integral and, also, the partially digested proteins, resulting in peptides of different sizes, but not in the release of isolated aminoacids. On the other hand, carboxypolypeptidase breaks down the peptides in isolated aminoacids, bringing the proteins digestion into an end (1).

When pancreas undergoes a severe lesion or even when a duct is blocked, a great amount of pancreatic secretion accumulates in the damaged areas, and this

condition will overcome the trypsin inhibitory effect, quickly activating the pancreatic secretion. The digestion of this gland can cause what we call acute pancreatitis, which is generally lethal owing to shock. Even when it is not deadly, the condition can cause long-term pancreatic insufficiency. There is another debilitating variation of the problem, the chronic pancreatic inflammation, characterized by severe and recurrent abdominal pain and progressive fibrosis, leading to pancreatic insufficiency (1–3).

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Chronic pancreatitis is, usually, consequence of acute pancreatitis recurrence, which brings about the acinar cells progressive destruction followed by healing or fibrosis; this scenario culminates in endocrine and exocrine functions. Similar to the acute one, chronic pancreatitis is associated with alcohol use and, less commonly, to biliary tract disorders, hypercalcemia, and hyperlipidemia. The typical chronic pancreatitis is pancreatic calcification, which is strongly related to alcohol use (4).

The clinical diagnosis of pancreatitis is based on anamnesis, physical examination, radiological, sonographic, and endoscopic findings, as well as exocrine pancreatic function test (5,6). Owing to the vast number of analytical methods, we can obtain evidences on the severity of the morphological damages and on the endocrine and exocrine function disorders. It is possible to establish the exact diagnosis after a 5-year follow-up from the first clinical symptoms diagnosis (5).

Laboratory trials such as lipase, amylase, trypsin, gamma-GT, alkaline phosphatase, bilirubin, calcium, lipidogram, glucose metabolism, prothrombin time, DD-xylose absorption test, fecal chymotrypsin activity, PABA test, vitamin A tolerance test, and fecal weight, as well as imaging tests like abdominal ultrasound, radiography exams, and upper GI endoscopy are useful to the etiological classification of chronic pancreatitis (hypercalcemia and hyperlipidemia) (7).

One of the consequences of chronic pancreatitis is the endocrine pancreatic insufficiency, which is related to diabetes. On the other hand, we can note its clinical signs, as steatorrhea. Recently, some authors related HIV virus with exocrine pancreatic insufficiency. This is a relevant cause of malabsorption in HIV patients (8,9).

Pancreatic elastase-1, which is a proteolytic enzyme exclusively produced by pancreas, is stable when passing through the bowel. We can find higher levels of pancreatic elastase in feces than in duodenal juice. Chronic pancreatitis, cystic fibrosis (mucoviscidosis), pancreas cancer, type 1 diabetes mellitus (insulin-dependent diabetes), Shwachman-Diamond syndrome, and other pancreatic insufficiency-related pathologies are correlated to this enzyme determination (10).

We must address the development and analysis of indirect tests of pancreatic function that quantify serum enzymes, such as pancreatic isoamylase and immunoreactive trypsin, or that determine serum degradation products like NBT-PABA (bentiromide). In the same manner, we can mention the pancreolauryl test, the fecal enzyme assay (chymotrypsin), both for assessing exocrine pancreatic insufficiency. The gold standard is the secretin-pancreozymin test, which must be carried out in a qualified gastroenterology center, as it is time consuming, invasive, and expensive (6). The ideal test

must meet all the following requirements: simplicity of handling, low cost, reproducibility, and high sensitivity, not only for diagnosing chronic pancreatic insufficiency but also for mild and moderate cases (11).

Enzyme-linked immunosorbent assay (ELISA) method is quite feasible to analyze elastase-1. The regular concentration of elastase-1 in pancreatic secretion varies from 170 to 360 $\mu\text{g}/\text{ml}$. In feces, the reference value for patients free of pancreatic conditions is greater than 200 $\mu\text{g}/\text{g}$ (12). ELISA is an enzymatic assay that employs two monoclonal antibodies, which are specific to the different epitopes of human elastase-1 and present two major advantages. The first one is that elastase-1 is highly stable during its passage through the digestive tract, presenting resistance to the proteolytic degradation. The second is that ELISA's immunoenzymatic reaction specifically determines human elastase-1, promoting an evaluation of the pancreatic function. Recent studies demonstrated that fecal elastase determination is more valuable than fecal chymotrypsin or pancreolauryl tests (11).

The analytical kit used in this study takes as reference the following concentrations of elastase-1 in feces: less than 100 $\mu\text{g}/\text{g}$ is indicative of severe exocrine pancreatic insufficiency; from 100 to 200 $\mu\text{g}/\text{g}$ is indicative of moderate pancreatic insufficiency, and greater than 200 $\mu\text{g}/\text{g}$ is indicative of normal exocrine pancreatic function (12).

This study aims to use the results of pancreatic elastase-1 concentration as a diagnostic method for pathologies related to pancreatic insufficiency and to validate the immunoenzymatic method (ELISA) to quantify elastase-1 in fecal samples.

MATERIALS AND METHODS

Hundred and fifty-seven patients were eligible for the study, including 95 HIV-positive, whereas 62 completely healthy patients constituted the control group. The HIV-positive participants were recruited for the study during clinical visits at Primary Care Clinics near their place of residence and were referred to our Gastroenterology Outpatient Clinic for further testing. Control participants were recruited during regular testing in the same outpatient service.

The mean age of the 95 HIV-positive patients was 39.25 years (± 9.7 years), and 61 (64.4%) of them were men. The control group presented a mean age of 39.06 (± 13.7), and 54.8% of participants were male.

Among HIV-positive participants, 32 presented CD4 count higher than 500 cells per cubic millimeter of blood. In 33 participants, CD4 results were between 500 and 250. Finally, 30 patients showed CD4 values less than 250. Patients showed few clinical symptoms, such

as fever (11% of the total), weight loss (25%), and chronic diarrhea (28.4%). The mean body mass index was 23.65. Blood count results for these patients, including hemoglobin, hematocrit, AST, ALT, Na^+ , and K^+ were normal.

Clean, dry, wide-mouthed containers were provided to study participants. They were asked to collect stools, not directly from the toilet bowl and in an amount the same as a thumb size, during the morning and bring them immediately to the laboratory. The samples were frozen at -20°C until they could be analyzed.

We used the elastase-1 ELISA kit (enzyme-linked immunosorbent assay) from Bioserv Analytics and Medical Devices Ltd (Rostock, Germany). The procedure included transferring of 0.7 g of feces into a Falcon® (BD Biosciences, San Jose, CA) conical tube followed by addition of 7.0 ml of a sodium phosphate buffer solution (1:10 dilution). This mixture was homogenized by means of a Fisher-Scientific® (Schwerte, Germany) vortex for 2 min. After that, the sample was maintained under a temperature of $2-8^\circ\text{C}$ overnight, allowing its precipitation and, further, the complete extraction of the enzyme. Briefly, fecal elastase-1 determination has been accomplished according to the instructions provided by the kit manufacturer.

For the validation of the method, we used the mathematical equations that Chasin et al. (13) have proposed. Four standard samples from the kit have been used to conduct the calibration curve and linearity of the method. Standards with the following concentrations: 50, 100, 200, and $500\text{ }\mu\text{g/g}$ were used (12). After carrying out the calibration curve, we confirmed the linearity through the coefficient of determination R^2 .

We have assessed precision by means of a nine-fold repetition of the kit's positive control analysis, using analogue conditions, namely the analyst, the reader, the pipettes, the extraction solution, and the ELISA plaque. Accuracy has been achieved through the "inaccuracy equation" (13). We have also verified recovery to evaluate the sampling method efficiency, i.e., fecal sample enzyme extraction.

Besides the receive operator characteristic (ROC) curve, applied to deem positive predictive value, negative predictive value, specificity, and sensitivity, we have evaluated the correlation among the categorical variables through χ^2 and Fisher tests. The software used was SPSS version 15.0.

RESULTS

The spectrophotometric assay was performed with the standards provided within the kit. We have examined the linearity of the immunoenzymatic reaction through the calibration curve, as shown in Figure 1.

Fig. 1. Calibration curve for fecal elastase-1 determination.

TABLE 1. Concentrations From the Positive Control Analysis, With Reference Values Varying From 160.0 to $240.0\text{ }\mu\text{g/g}$ in feces

Date	Concentration of elastase in $\mu\text{g/g}$
December 18, 2003	185.52
February 11, 2004	159.25
March 10, 2004	185.79
March 24, 2004	163.00
March 31, 2004	220.51
April 14, 2004	218.48
July 07, 2004	201.60
July 14, 2004	154.80
July 21, 2004	208.40
Average = >	191.79
Standard deviation = >	25.10
Coefficient of variation = >	13.09

Regarding the repetition of the positive control analysis, the kit is accurate as can be noted in Table 1.

According to Chasin (13), the estimation of accuracy requires assessing the test inaccuracy, as follows:

$$\text{Inaccuracy (\%)} = \frac{\text{Obtained concentration} - \text{Estimated concentration} \times 100}{\text{Estimated concentration}}$$

$$\text{Inaccuracy (\%)} = \frac{191.79 - 200 \times 100}{200}$$

$$\text{Inaccuracy (\%)} = 4.1\%.$$

When evaluating the sampling method efficiency using the recovery equation above, we ascertained that only 4% of the sample was not used.

$$\text{Recovery \%} = \frac{\text{Average of the obtained values} \times 100}{\text{Estimated concentration}}$$

$$\text{Recovery} = \frac{191.79 \times 100}{200}$$

$$\text{Recovery} = 96\%.$$

Fig. 2. ROC curve.

According to Sackett (14), the ROC curve is the best way to estimate the threshold, favoring sensitivity and specificity of the diagnosis. The ideal test must reach the left upper area of the curve. The studied test presented an area above the curve of 0.707.

We demonstrated that elastase is a suitable HIV-marker, as the area above the curve is near 0.75. The method showed a sensitivity of 19% and a specificity of 96.8%, as well as positive and negative predictive values of 90 and 43.8%, respectively. The accuracy of the elastase kit was 49.7%.

When associating alcohol use with altered elastase concentration ($<200 \mu\text{g/g}$), we could notice a positive correlation ($P = 0.020$). The group presenting altered elastase concentration had a higher rate of alcohol use than the group with normal elastase concentration. In the meantime, the association between altered elastase concentration and antiretroviral use in HIV-patients demonstrated no positive correlation ($P = 0.424$).

DISCUSSION

Our results demonstrated a significant association ($P = 0.020$) between alcohol use and altered fecal elastase-1 concentration ($<200 \mu\text{g/g}$), explained by the fact that alcohol is the major cause of 70–90% of chronic pancreatitis. It is difficult to evaluate the risk of chronic pancreatitis as a result of alcohol use. In most patients, the intake of at least 150 g/day can be associated with the appearance of this disease (15).

Interestingly, the results of this study suggest that elastase determination is a proper laboratory test to

validate the link between alcohol use and pancreatitis, as well as to early diagnosing it. We also observed that HIV-patients in use of retroviral drugs are not associated with the alteration of fecal elastase-1 concentration. The diarrhea presented by these patients can be, in most cases, owing to opportunistic infections (8). Nevertheless, patients presenting diarrhea, which is an unknown etiologic agent condition, also shows evidences of fat malabsorption and enteropathy.

Carroccio et al. (16) described the association between pancreatic dysfunction and fat malabsorption in HIV-infected children. Fecal elastase-1 is able to act as an indicator of this link in HIV-patients using retroviral drugs. However, the patients assigned for this study did not show this association. This can be related to the small number of patients who were under retroviral therapy, or even to the multifactorial association between HIV and pancreatic disease.

According to Löser et al. (17) and Katschinski et al. (11), other methods for measuring elastase concentration present some practical disadvantages, such as being time-consuming, expensive, uncomfortable, and invasive. Elastase-1 determination is an adequate alternative, as it only uses fecal samples.

The evaluation of the calibration curve indicates that the ELISA method used to quantify fecal elastase-1 is linear. Thus, when evaluating real samples we can obtain known concentrations, which would support the diagnosis.

Regarding the precision of the method, it presented repeatability when we analyzed known concentrations in different days. The standard deviation was 25.10, with an average of 191.79, ranging from 160 to $240 \mu\text{g/g}$. Considering the method's accuracy, assessed by Chasin's (13) inaccuracy test, we could observe that the deviation of the results was of 4%. Hence, this methodology is accurate as the inaccuracy test result is near zero, and the theoretical value from the kit is 4.3%.

During the assessment of the recovery, we have attained 96%. This result reveals that despite the fact that biological matrix (feces) is extremely complex and contains many interference factors, such as bacteria, protozoa, and helminths, it slightly interferes with the assay and makes the material collection easier.

The validation of an enzymatic assay with pre-analytical extraction is relevant, as it allows the accomplishment of the analytical test within defined parameters, leading to a better acceptance of the obtained data.

This study strongly indicates that the commercially available ELISA Kit used to test pancreatic function is linear, sensitive, precise, and accurate, according to our results. Therefore, studied assay is valid and qualified to be used as a diagnostic method for pathologies associated with pancreatic insufficiency.

REFERENCES

1. Ghyton MD, Arthur C, Hall JE, editors. 1998. *Fisiologia Humana e Mecanismos das Doenças*. Rio de Janeiro: Ed. Guanabara Koogan.
2. Jop J. 2001. Trypsin activation peptide (TAP) in acute pancreatitis. In: deGryter W, editor. *Pathophysiology to Clinical Usefulness*. Geneva, Switzerland: Jean Louis Frossard Division of Gastroenterology, Geneva University Hospital.
3. Stein J, Caspary WF. Fecal tests in the diagnosis of exocrine pancreatic insufficiency. *Clin Lab* 1997;43:361–368.
4. Rubin E, Farber JL, Scarpelli DG, editors. 1997. *Pathology*. Philadelphia: JB Lippincott Co.
5. Layer P, Yamamoto H, Kalthoff L, et al. The different courses of early- and late-onset idiopathic and alcoholic chronic pancreatitis. *Gastroenterology* 1994;107:1481–1487.
6. Lankisch PG. Now that fecal elastase is available in the United States, should clinicians start using it? *Curr Gastroenterol Rep* 2004;6:126.
7. Morgenroth K, Kozushek W. 1991. Pancreatitis. In: Frossard JL, editor. *Cooperation With Jurgen Hotz*. New York: Gruyter Berlin.
8. Carroccio A, Di Prima L, Di Grigoli C, et al. Exocrine pancreatic function and fat malabsorption in human immunodeficiency virus-infected patients. *Scand J Gastroenterol* 1999;34:729–734.
9. Price D, Schmid ML, Ong ELC, Adjukiewicz KMB, Peaston B, Snow MH. Pancretic exocrine insufficiency in HIV-positive patients. *Br HIV Assoc* 2005;6:33–36.
10. Garcia B, Carlos AR, Thomas M, et al. Quantification of fecal elastase-1 using either polyclonal or monoclonal antibodies. *Gastroenterology* 2002;122:A-510.
11. Katschinski M, Schirra J, Bross A, Göke B, Arnold R. Duodenal secretion and fecal excretion of pancreatic elastase-1 in healthy humans and patients with chronic pancreatitis. *Pancreas* 1997;15:191–200.
12. Bioserv Analytics and Medical Devices Ltd. Elastase-1 ELISA. Cat No BS-86-01. Germany, 2003.
13. Chasin AM, Nascimento ES, Siqueira LMR, et al. Validação de Métodos em Análises Toxicológicas: Uma Abordagem Geral. *Rev Bras Toxicol* 1998;11:1–6.
14. Sackett DL, Haynes RB, Tugwell P, editors. 1991. *Clinical Epidemiology: A Basic Science for Clinical Medicine*, second edition. Boston: Little, Brown and Company.
15. Feldman M, Friedman LS, Brandt LJ. 2006. *Gastrointestinal and Liver Disease (Pathophysiology/Diagnosis/Management)*, eighth edition, Vol. 1. Philadelphia: Saunders Elsevier.
16. Carroccio A, Guarino A, Zuin G, et al. Efficacy of oral pancreatic enzyme therapy for the treatment of fat malabsorption in HIV-infected patients. *Aliment Pharmacol Ther* 2001;15:1619–1625.
17. Löser C, Möllgaard A, Fölsch UR. Faecal elastase 1: A novel, highly sensitive, and specific tubeless pancreatic function test. *GUT Int J Gastroenterol Hepatol* 1996;39:580–586.