

Rapid Salivary Pepsin Test: Blinded Assessment of Test Performance in Gastroesophageal Reflux Disease

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Objective/Hypothesis: Pepsin lateral flow device (LFD) is a rapid noninvasive test to detect salivary pepsin as a surrogate marker for gastroesophageal reflux disease (GERD). We aimed to establish the test sensitivity, specificity, positive and negative predictive values (PPV, NPV) in patients with symptomatic and objective evidence of GERD compared to healthy controls.

Study Design: Prospective, blinded, controlled cohort study.

Methods: A total of 230 samples were analyzed. In vitro bench testing was conducted on 52 gastric juice and 54 sterile water samples to assess test sensitivity and specificity. Saliva was collected from 58 patients with GERD and 51 controls. All patients with GERD underwent esophagogastroduodenoscopy (EGD) and wireless 48-hour pH monitoring off acid suppressive therapy. PPV and NPV were calculated based on disease definition of esophagitis and/or abnormal pH monitoring.

Results: Receiver operating characteristics analysis of in vitro samples found assay sensitivity and a specificity of 87%. There were 6/51 (12%) control subjects and 13/58 (22%) patients with GERD who tested positive for salivary pepsin ($P = .25$). There was a step-wise increase in the prevalence of positive salivary pepsin: esophagitis (55%), abnormal pH monitoring (43%), GERD symptoms only (24%) ($P < .001$). Salivary pepsin test showed a PPV of 81% and NPV of 78% for those with objective evidence of GERD (abnormal pH and/or esophagitis).

Conclusions: Rapid LFD for salivary pepsin has acceptable test characteristics in patients with GERD. A positive salivary pepsin test in this group may obviate the need for more expensive diagnostic testing by EGD or pH monitoring.

Key Words: Gastroesophageal reflux, sensitivity, saliva, pepsin.

Level of Evidence: 2b

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INTRODUCTION

Gastroesophageal reflux disease (GERD) afflicts more than 100 million US adults, significantly impacts quality of life, and imposes more than \$9 billion per year in total cost on the US healthcare system.^{1,2} Yet, today the current diagnostic tests for GERD still have significant limitations. The role of esophagogastroduodenoscopy (EGD) in GERD is limited. Given current recommendations on empiric therapy as the initial treatment option for suspected GERD,² the prevalence of

abnormal findings on EGD is $<30\%$.^{3,4} Reflux measurement employing ambulatory pH and/or impedance monitoring as alternative diagnostic tests for GERD⁵ have acceptable sensitivity (77%–100%) and specificity (85%–100%) in patients with endoscopically proven esophagitis. However, they are less sensitive (0%–71%) in endoscopy negative disease,⁶ which represents a majority of patients for whom the test is currently recommended.² Thus, EGD and ambulatory monitoring devices are suboptimal in GERD diagnosis in addition to being costly and somewhat invasive.

The Montreal Classification defines GERD as “a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications.”⁷ The most common typical presentation of GERD includes heartburn and regurgitation, whereas other extraesophageal manifestations, such as asthma, chronic cough, laryngitis, globus, and throat clearing are increasingly recognized.⁸ Although presentation of heartburn and regurgitation may suggest GERD and initiate an empiric trial of acid suppressive therapy, in extraesophageal manifestations, many patients do not present with concomitant heartburn or regurgitation, thus delaying diagnosis and appropriate treatment. The suboptimal nature of diagnostic tests in GERD is magnified in extraesophageal reflux by poor sensitivity of

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Drs. Strugala and Dettmar are affiliated with RD Biomed Limited, which manufactures the pepsin test described in this study. Their involvement in the study was limited to providing technical support for the test. They had no influence on the study design, conduct, analysis or the final manuscript. There are no financial relationships between any of the other authors and RD Biomed Limited. The authors have no other funding, financial relationships, or conflicts of interest to disclose.

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endoscopy and pH testing⁹ as well as nonspecificity of laryngoscopy¹⁰ in patients with laryngeal symptoms.

A simple, low-cost, accurate, and minimally invasive test is needed to assess the impact of reflux in both typical and extraesophageal manifestations of GERD. Such a test would greatly improve medical management and quality of life while reducing healthcare costs. To that end, a recently developed noninvasive rapid salivary pepsin lateral flow device (LFD) was advocated as an objective method to diagnose reflux.^{11,12} This test uses two unique monoclonal antibodies to human pepsin-3, one to detect and one to capture pepsin if present in the sample. The primary premise of this test is that pepsin as a constituent of gastric milieu would be present in the salivary fluid only through the retrograde reflux of gastric contents, and its presence would suggest GERD.¹³⁻¹⁶ The LFD is rapid and is capable of detecting pepsin in salivary fluid within 15 minutes. However, the optimal test characteristics of this test are not well studied in patients with objective evidence of GERD.

Thus, in this prospective blinded study we aimed to: 1) determine the sensitivity and the specificity of the pepsin lateral flow device by *in vitro* testing, and 2) assess the prevalence and the test characteristics of the salivary pepsin LFD in healthy volunteer controls and patients with objective evidence of GERD by EGD and pH monitoring.

MATERIALS AND METHODS

The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice and applicable regulatory requirements. The Vanderbilt University institutional review board (IRB) approved this study (IRB# 080329).

Study Design and Patient Population

The study population consisted of those with GERD, defined as symptoms of heartburn and/or regurgitation for more than two episodes per week with history of symptomatic response to acid suppressive therapy, abnormal pH testing off therapy, and/or with endoscopic confirmed esophagitis. Healthy volunteers served as the controls. This group had no history of GERD and no prior diagnosis or therapy for acid reflux related conditions. All participants completed a detailed questionnaire assessing current and past medical history, current medication, information on subject demographics (age, sex, and race), and presence, severity and frequency of GERD (heartburn/regurgitation). Patients with a history of achalasia, esophageal spasm, eosinophilic esophagitis, or dysphagia and predominate complaint of extraesophageal reflux (cough, hoarseness, throat clearing, chest pain, globus, and sore throat) were excluded from the study. Additionally, those with prior surgical procedures involving the stomach or esophagus were not eligible to participate.

All patients with symptomatic GERD underwent endoscopy with wireless pH testing as outlined below to determine esophageal mucosal status as well as measure degree of esophageal acid exposure over a 48-hour period when off acid suppressive therapy. Saliva samples were obtained from control subjects and patients with confirmed GERD (abnormal pH and/or presence of esophagitis). Saliva was collected both on (once daily) and off proton pump inhibitor (PPI) therapy for patients

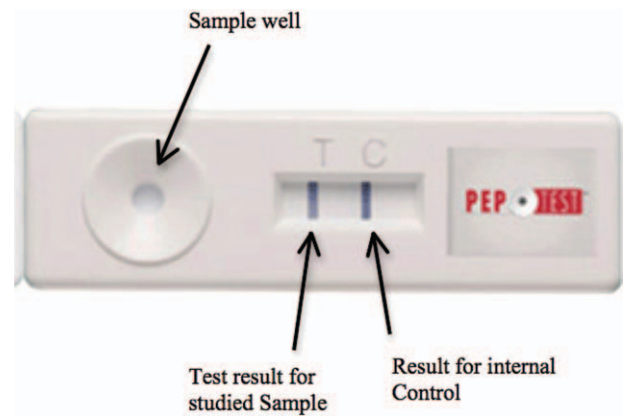


Fig. 1. Pepsin lateral flow device for a gastric juice sample showing a positive pepsin test relative to the control band (C = control band. T = test sample band). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with GERD. Gastric juice samples were also obtained from the patients with GERD for *in vitro* pepsin analysis off therapy. Because pepsin is expected to be present in the stomach and is the source for any potential salivary pepsin, the gastric juice samples served as the positive control, and sterile water was used as the negative control. Pepsin test characteristics of sensitivity, specificity, as well as positive and negative predictive values were then determined.

Sample Collection

Gastric juice was collected during endoscopy and placed in a 15-mL sterile plastic tube containing 0.5 mL of 0.01 mol/L citric acid at pH 2.5. Expecterated saliva samples were collected from patients with GERD and the healthy volunteers into a 15-mL sterile plastic tube containing 0.5 mL of 0.01 mol/L citric acid at pH 2.5. Citric acid was used to slow down the degradation of any pepsin in the sample and to act as a simple antibacterial agent.¹⁷ The samples were then stored on ice and promptly transferred to the refrigerator at 4°C.

Pepsin Analysis

Pepsin was detected using the Peptest lateral flow device (RD Biomed Ltd, Hull, UK; www.rdbiomed.com). In this clinical evaluation study, a preproduction prototype was used (B.N. 080107 and 080729). This pepsin LFD was developed with two unique monoclonal antibodies specific to human pepsin.

Within a week of collection, samples were gently vortexed for 1 minute and then centrifuged for 5 minutes at 4,000 rpm, in a bench top centrifuge, and the supernatants were then collected. A total of 80 μ L of the supernatants layer was then mixed with 240 μ L of migration buffer solution, and 80 μ L of the mixture was added to the well of the LFD (Fig. 1). After a few minutes a line appeared under the letter C on the LFD, confirming that the test was working correctly. If pepsin was present in the saliva sample tested, then a second line appeared under the letter T (test) between 5 and 15 minutes after applying the sample. The test has the ability to detect pepsin down to 16 ng/mL. For this study, semiquantitative assessment of pepsin in the samples was carried out with the scale 0, +0.5, +1, +2, +3, +4, corresponding to approximately 0, 25, 50, 100, 500, and 1,000 ng/mL of pepsin, respectively. This semiquantitative measurement was based on the intensity of the test sample line compared to that of the control line (Fig. 1). The samples were batch tested and interpreted for presence of pepsin

employing the rapid pepsin LFD by an individual who was blinded to sample source (gastric vs. sterile water) and subject classification (GERD vs. healthy volunteers).

Esophageal pH Testing and Endoscopic Evaluation

Ambulatory pH monitoring was performed for 48 hours using a wireless pH monitoring device (Given Imaging Inc., Duluth, GA). Study patients were instructed to stop taking all proton pump inhibitors for seven days and H₂ receptor antagonists (H₂RA)s for 4 days before undergoing evaluation. The details for the conduct of the methods were previously described.¹⁸ Acid exposure time (% total time pH <4) >4.2% per day was considered abnormal.¹⁹ The presence of esophagitis was noted and graded based on Los Angeles classification.²⁰

Statistical Analysis

Data were collected and stored at the secure web-based Vanderbilt Digestive Disease Center REDCap (Research Electronic Data Capture) (1 UL1 RR024975 NCCRR/NIH). There was strict control and supervision of the data entry and access to this study.

Subject characteristics were described using medians and interquartile ranges (continuous variables) or proportions (categorical variables). Two group comparisons were made using the Wilcoxon rank sum test or Pearson χ^2 test. Receiver operating characteristic (ROC) analysis was performed on gastric juice and water samples to determine the test measurement cutoff with the optimal sensitivity and specificity. These were the only samples where the presence or absence of pepsin is known with certainty. The probability of test positivity at each cutoff was then determined in healthy volunteers, subjects with GERD, and subgroups of subjects based on esophagitis and pH parameters. A test score of +1.0 or greater determined pepsin positivity by an ROC analysis. Using this definition, we calculated the sensitivity and specificity of the pepsin test to predict GERD or healthy status.

The primary utility of the pepsin test is to predict true disease in a clinically relevant population of subjects who might undergo pH and/or endoscopy. To that end, we used GERD subjects to evaluate the PPV and NPV of the test at a cutoff of +1.0. True disease was defined as a subject who had positive pH findings (total % time < pH4 > 4.2%) or esophagitis. Analyses were performed using the R statistics program version 2.13.2 (R Foundation, www.r-project.org).

Role of Industry

The protocol was an independent investigator initiated study by the principal investigator (M.F.V.). RD Biomed Limited (United Kingdom) provided the pepsin test kit (Peptest) and the technical support for the conduct of the test. They had no role in the study design, conduct, statistical analysis, manuscript preparation, interpretation, or decision to submit the manuscript for publication.

RESULTS

Demographics

One hundred nine subjects were studied: 51 controls and 58 patients with GERD. Both groups were similar with respect to demographic characteristics of gender, age, race distribution, as well as smoking and alcohol status (Table I). PPI use was common (53%) in patients with GERD, whereas per study definition none

TABLE I.
Demographic Data of the Study Population.

	Control, n = 51	GERD, n = 58	P
Gender (% male)	31	38	.76*
Median age (IQR), yr	46 (32–56)	50 (43–60)	.10 [†]
Race, % Caucasian	90	93	.88*
PPI use, %	0	53	<.001*
Current smoking, %	8	31	.37*
Current alcohol, %	59	41	.17*

*Pearson test.

[†]Kruskal-Wallis test.

IQR = interquartile range, PPI = proton pump inhibitor.

of the controls were on PPIs. The remaining 47% of patients with GERD were either on H₂RA (33%) or intermittent acid suppressive therapy (14%). There were 47/58 (81%) patients with GERD who underwent EGD and pH monitoring. There were 11/47 (23%) patients with GERD who had erosive esophagitis (grade A = 5/11 [45%]; grade B = 3/11 [27%]; grade C = 2/11 [18%], and grade D = 1/11 [9%]). A total of 14/47 (30%) patients had abnormal pH testing, with three patients in this group also having esophagitis. Thus, 22 patients with GERD had either esophagitis and/or abnormal pH results. Median (interquartile range) percent of total upright and supine time of pH <4 was 6.4% (3.1%–11.0%), 8.4% (3.7%–14.2%), 1.30% (0.0%–7.1%); respectively.

Optimal In Vitro Pepsin Test Characteristics

ROC analysis²¹ was performed based on test results from 52 gastric juice and 54 sterile water samples (the former representing true positive and the latter true negative samples). Based on possible semiquantitatively measured values of +0.5, +1, +2, +3, or +4 (Fig. 1), a score of +0.5 was established to be negative/zero, and thus the first discernible level of pepsin by this semiquantitative scale was +1.0, which equates to approximately 50 ng/mL pepsin. Therefore, the optimal pepsin test cutoff for positivity was determined to be +1.0 or greater (Fig. 2). The sensitivity of the pepsin rapid LFD was at 87% (45/52) and the specificity was 87% (47/54) at the measured optimal cutoff value. Subsequent salivary measurements in patients with GERD and the controls employed the cutoff of +1.0 (~50 ng/mL) or greater to define presence of pepsin.

Salivary Pepsin Test in GERD

Overall, 19/109 (17%) of the saliva samples tested positive for pepsin: 6/51 (12%) in control samples and 13/58 (22%) in patients with GERD ($P = .25$). There was a stepwise increase in the prevalence of positive pepsin among the GERD subjects (Fig. 3): highest in those with endoscopic esophagitis (6/11 = 55%), followed by patients with abnormal pH findings (6/14 = 43%), and lowest in those with a symptom report of heartburn only (7/28 = 24%). The prevalence of pepsin in those with objective GERD (positive pH or esophagitis) was significantly

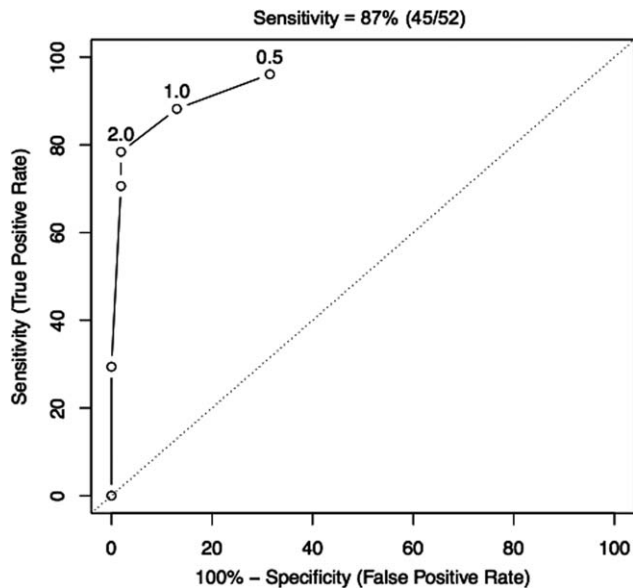


Fig. 2. Receiver operating characteristics curve for the pepsin lateral flow device.²¹ Using the positive cutoff of +1.0 (equivalent to 50 ng pepsin/mL of sample), the sensitivity and specificity of the test were determined to be both at 87%.

higher ($P < .001$) than the controls. There was no significant difference in the prevalence of salivary pepsin in GERD subjects whether on or off PPI therapy (22% vs. 24%, respectively; $P = .78$).

The pepsin LFD had the following test characteristics for patients with objective GERD (abnormal pH and/or esophagitis): sensitivity = 50%, specificity = 92%, PPV = 85%, and NPV = 68% (Table II). With true disease prevalence in this case of 47% (22/47), a positive salivary pepsin would increase the likelihood of GERD to 85% (PPV).

DISCUSSION

This is the first large-scale, prospective, blinded study in which we assessed test characteristics of salivary pepsin LFD (Peptest) both in vitro as well as in patients with typical GERD and healthy controls. Our study demonstrated that the rapid pepsin LFD has acceptable sensitivity and specificity at 87% with optimized test cutoff of +1.0 (equivalent to pepsin concentration of approximately 50 ng/mL). The prevalence of positive salivary pepsin was low in all tested subjects: 12% in controls and 22% in GERD patients. In our population of patients with GERD, with esophagitis plus pH abnormality prevalence of 47%, the PPV of salivary pepsin was 85%. Thus, a positive salivary pepsin test in a patient suspected of having GERD increases the likelihood that the patient has esophagitis and/or abnormal pH findings from 47% to 85%. This would possibly obviate the need for expensive testing with EGD and/or pH monitoring. Additionally, we showed that the presence of pepsin was independent of PPI use.

The role of the pepsin LFD in diagnosing GERD is intriguing in that it provides a convenient, office-based, noninvasive, quick, and inexpensive technique different

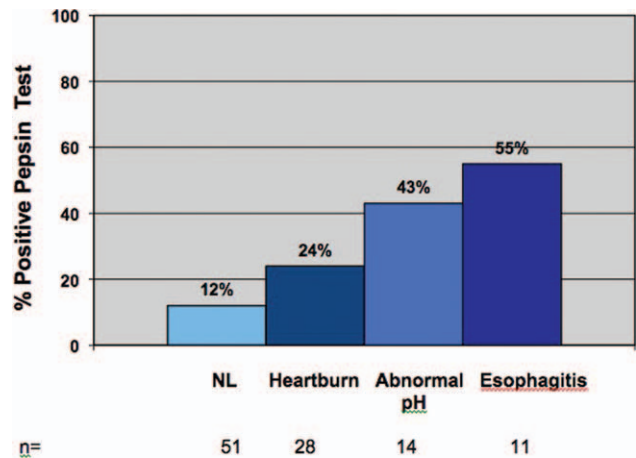


Fig. 3. Step-wise increase in the prevalence of positive salivary pepsin test in gastroesophageal reflux disease (GERD) subjects compared to controls. GERD subjects with objective evidence of GERD (abnormal pH or esophagitis) had a higher likelihood of positive salivary pepsin than those with only a symptom complaint of heartburn. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

from our currently available tools. Furthermore, as the definition of GERD expands beyond typical heartburn and regurgitation, there is increasing demand on improved methods for GERD diagnosis. There is a growing recognition that reflux may present silently as extraesophageal symptoms unaccompanied by typically recognized symptoms. GERD is implicated as a contributing factor to pulmonary diseases and conditions, including asthma, posterior laryngitis, chronic cough, recurrent pneumonitis, hoarseness, pharyngitis. However, given the lack of poor sensitivity of EGD and pH monitoring in this group^{9,10} and poor specificity of laryngeal evaluation,¹⁰ extraesophageal symptoms represent a challenge for GERD diagnosis. Recently, Kim et al.²² studied patients with suspected extraesophageal symptoms (throat pain, globus, chest pain, cough, and belching) and reported salivary pepsin Western blot analysis sensitivity of 89%, specificity of 68%, PPV of 44%, and NPV of 95%. However, they used pH monitoring data alone as the basis for test characteristics, which is known to have very low sensitivity for patients with suspected extraesophageal GERD.^{9,10} Thus, the salivary pepsin LFD has potential as an alternative diagnostic test for this group of patients.

TABLE II.
Salivary Pepsin Test Characteristics in Gastroesophageal Reflux Disease (Defined as Abnormal pH and/or Esophagitis).

Test Result	True Positive, n = 22	True Negative, n = 25	
Positive pepsin test	11	2	PPV = 11/13 (85%)
Negative pepsin test	11	23	NPV = 23/34 (68%)
	Sensitivity = 11/22 (50%)		Specificity = 23/25 (92%)

PPV = positive predictive value; NPV = negative predictive value.

Prior studies with a range of pepsin tests have used the salivary pepsin detection in the most difficult population of patients, namely suspected extraesophageal reflux patients for whom definitive contribution for reflux is problematic at best. We chose to first understand the LFD response in vitro as well as in patients with endoscopic and pH confirmed GERD. This group should serve as the template for better understanding of the test response characteristics for the pepsin test. Our data on the prevalence of 22% for positive salivary pepsin in GERD patients are similar to those by Knight et al.¹⁷ showing positive salivary pepsin by enzyme-linked immunosorbent assay in 14/63 (22%) of patients with laryngopharyngeal reflux. However, the prevalence of positive salivary pepsin in our study is higher than those previously reported by Potluri et al.,¹¹ reporting only 19/180 (11%) positive samples in sputum and saliva collected from 16 patients with symptomatic GERD. The difference in the findings may be due to a smaller group of patients studied by Potluri et al. (16 patients with GERD, no control group) compared to our population of 53 patients with GERD, and the limitations of the digestion assay used that relies on active pepsin. Additionally, their findings may be diluted by the combined sputum and salivary samples in their report versus only salivary samples in our study.

The strengths of our study include: 1) large patient population and controls; 2) in vitro assessment for test sensitivity and specificity; 3) blinded evaluation of salivary samples, which has not previously been reported; and 4) use of a rapid easy-to-use pepsin test. One limitation of our study is the observed prevalence of salivary pepsin (only 22%) in patients with confirmed GERD. However, it is important to note that for salivary pepsin to be present in the saliva, the reflux of gastric contents must travel into the oropharynx, which is not a highly common occurrence in GERD. Another potential reason for the low prevalence of pepsin may be due to the timing of saliva collection. Our patients provided saliva samples randomly during the course of the day. In a pilot study, Strugala et al.²³ reported that the detection of pepsin may depend on if the samples were collected during a symptomatic episode (82%) or asymptomatic episode (35%). In the published study by Potluri et al.,¹¹ where 49% of the saliva samples were collected during the symptomatic phase, they only found pepsin positive prevalence of 11%. The issue of salivary sample collection time is one that deserves further attention as we explore the relative importance of salivary pepsin test in patients with extraesophageal GERD.

CONCLUSION

Our prospective blinded study showed that the salivary pepsin test employing the rapid LFD has high sensitivity, specificity, PPV, and NPV. The value of this test may be in obviating the need for EGD and pH testing if salivary samples are positive, and possibly reserving the more invasive tests for those with negative

salivary pepsin. Based on data from this study, a more robust pepsin LFD, which is quantifiable in conjunction with a spectrophotometric reader, was recently developed to further improve on the test characteristics and the minimum detection level. New clinical studies will shortly be underway to evaluate this next generation pepsin LFD.

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