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Sensitive Pepsin Immunoassay for Detection of Laryngopharyngeal Reflux

John Knight, PhD; Mark O. Lively, PhD; Nikki Johnston, PhD; Peter W. Dettmar, PhD; Jamie A. Koufman, MD

Objectives/Hypothesis: **To determine whether measurement of pepsin in throat sputum by immunoassay could be used as a sensitive and reliable method for detecting laryngopharyngeal reflux (LPR) compared with 24-hour double-probe (esophageal and pharyngeal) pH monitoring.** ***Study Design:*** **Patients with clinical LPR undergoing pH monitoring provided throat sputum samples during the reflux-testing period for pepsin measurement using enzyme-linked immunoadsorbent assay.** ***Results:*** **Pepsin assay results from 63 throat sputum samples obtained from 23 study subjects were compared with their pH monitoring data. Twenty-two percent (14/63) of the sputum samples correlated the presence of pepsin with LPR (pH ≤ 4 at the pharyngeal probe), of which the median concentration of pepsin was 0.18 μg/mL (range 0.003–22 μg/mL). Seventy-eight percent (49/63) of the samples unassociated with (pharyngeal) reflux contained no detectible pepsin. Mean pH values for pepsin-positive samples were significantly lower than negative samples at both esophageal probe (pH 2.2 vs. pH 5.0) ($P < .01$) and the pharyngeal probe (pH 4.4 vs. pH 5.8) ($P < .01$). When the pepsin assay results were compared with the pharyngeal pH data for detecting reflux (events pH ≤ 4), the pepsin immunoassay was 100% sensitive and 89% specific for LPR.** ***Conclusions:*** **Detection of pepsin in throat sputum by immunoassay appears to provide a sensitive, noninvasive method to detect LPR.** ***Key Words:*** **Pepsin, immunoassay, larynx, laryngopharyngeal reflux, gastroesophageal reflux, GERD, pH monitoring, sputum, reflux laryngitis, diagnosis**

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INTRODUCTION

In otolaryngologic practice, recognition of many of the clinical manifestations of laryngopharyngeal reflux (LPR) have gained acceptance;[1–3] however, the prevalence of otolaryngologic and respiratory disorders caused by LPR remains unknown. In part, this appears to be because currently used diagnostics for LPR often rely on testing methods and normative standards that were established for the diagnosis of classic gastroesophageal reflux disease (GERD), which may not be appropriate for use in diagnosing LPR.

Ambulatory 24-hour double-probe (simultaneous esophageal and pharyngeal) pH monitoring (pH-metry) is the current gold standard for diagnosis of LPR,[4] but it is far from being an ideal test. First, the reported sensitivity of pH-metry is only 50% to 80%.[5,6] Second, approximately 12% of otolaryngologic patients cannot tolerate the procedure.[5] Third, dietary modifications (to standardize the test) may lead to false-negative pH studies. And finally, pH-metry is expensive and has limited availability. Thus, there appears to be a need for a sensitive, noninvasive, and inexpensive diagnostic test for LPR.

Pepsinogens belong to a family of aspartic proteinases and are produced primarily by chief cells within the gastric fundus.[7] In the acidic environment of the stomach, pepsinogen is activated by HCL (acid). Pepsin plays a major role in the development of many reflux-related disorders.[5]

Gastroesophageal reflux always contains pepsin, but not all reflux occurs below pH 4.0. Thus, with use of traditional gastroenterology standards for pH-metry, significant LPR may be under diagnosed. Indeed, pepsin exhibits enzymatic activity at pH levels well above 4, and it is only irreversibly inactivated at a pH greater than 6.5.[7–9] Thus, a patient could conceivably have a negative pH study (no reflux events pH ≤ 4) but might still have significant LPR-related disease. We have previously reported that the laryngeal epithelium is far more sensitive to damage by pepsin in the presence of acid than is esophageal epithelium,[8] and that may help explain why the patterns of reflux, reflux mechanisms, and clinical manifestations of LPR and GERD are so different.

We postulated that measurement of pepsin in airway secretions might provide a sensitive diagnostic marker for LPR, and furthermore, because pepsin is a large molecule, that it might be detected in airway secretions long after gastric reflux had occurred, making it a good diagnostic marker (U.S. Patent No. 5,879,897). Our strategy was to

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develop an enzyme-linked immunosorbent assay (ELISA) to detect pepsin and then to test its diagnostic sensitivity and specificity in a clinical setting.

MATERIALS AND METHODS

Purification of Human Pepsin from Gastric Juice

Purification of pepsin from gastric juice was achieved by ion-exchange chromatography on a Pharmacia MonoQ 5/50 column (Amersham Biosciences, Piscataway, NJ) developed with a gradient of 0.15 to 0.3 mol/L NaCl in 50.0 mmol/L sodium acetate, pH 4.1, as described previously.[10] The major pepsin A isoform, pepsin 3b, was used for antibody production and immunoassay development. Identity and purity of pepsin 3b was confirmed by N-terminal amino acid sequence analysis and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed with a Bio-Rad Ready Gel System (Bio-Rad Laboratories, Hercules, CA) under reducing conditions using a 6% stacking gel and a 12% resolving gel of acrylamide. After electrophoresis, the gels were used for Western blot analysis or were stained with Coomassie Brilliant Blue R 250.

Generation of Polyclonal Antibodies against Human Pepsin

Antibody production was carried out at Lampire Biological Laboratories, Pipersville, PA. Antipepsin antibodies were prepared using two different approaches. Conventional polyclonal antisera were produced by immunizing a goat with highly purified human pepsin 3b. Goat antipepsin antibodies were partially purified by 50% ammonium sulfate precipitation followed by dialysis against phosphate-buffered saline (PBS), pH 7.4. Peptide specific antibodies were also prepared using a synthetic peptide as antigen. The synthetic peptide was designed using the amino acid sequence of human pepsinogen (SwissProt No. P00790; http://www.EXPASY.ch) and the x-ray crystal structure of the enzyme to identify peptide regions that are exposed on the surface of native pepsin.[11]

The Hu-3 peptide was synthesized chemically using standard FastMoc (Applied Biosystems, Foster City, CA) solid phase peptide synthesis chemistry. The synthetic peptide corresponded to amino acid residues 63 to 76 (numbering of SwissProt entry P00790). This sequence corresponds to the N-terminus of active pepsin: Val-Asp-Glu-Gln-Pro-Leu-Glu-Asn-Tyr-Leu-Asp-Met-Glu-Tyr-Cys. The C-terminal cysteine residue was added to provide a chemical attachment site. The synthetic peptide was covalently attached to keyhole limpet hemocyanin (KLH) using m-maleimidobenzoyl-N-hydroxy succimide ester and the KLH-peptide conjugate used to immunize rabbits. The peptide specific antibody (antiHu3) was affinity purified using the synthetic peptide covalently bound to SulfoLink coupling gel (Pierce, Rockford, IL), a chromatography support.

Development of the Pepsin Immunoassay

The immunoassay reported herein is a noncompetitive indirect sandwich ELISA. All incubation steps were carried out at 37° C for 1 hour with shaking in a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA), unless otherwise stated. Blocking buffer contained 1% (w/v) bovine serum albumin, in PBS, pH 7.4. Washing buffer consisted of PBS, pH 7.4, containing 0.1% (v/v) polyoxyethylenesorbitan monolaurate (Tween-20). The wells were decanted and washed three times for 3 minutes after each antibody incubation.

FALCON Pro-Bind polystyrene, 96-well, flat bottom microtiter plates (Becton Dickinson and Co. Lincoln Park, NJ) were coated with affinity purified rabbit antiHu3 antibodies [3 μg/mL] in a volume of 100 μL 0.2 mol/L sodium carbonate buffer, pH 9.6, per well, and incubated at 37° C for 2 hours, with shaking, followed by 4° C for 16 to 18 hours in a humid chamber. After washing, wells were incubated with blocking buffer to saturate potentially remaining sites on the plastic surface. At this stage, the plates were either used immediately or stored in blocking buffer for up to 48 hours at 4° C.

For the assay, wells were washed, and purified pepsin 3b standard and clinical samples were added in duplicate and diluted across the plate. Captured pepsin was detected by incubation with goat antipepsin antibodies (diluted 1:50 in blocking buffer). The peroxidase-conjugated mouse anti-goat/sheep immunoglobulin G (Sigma-Aldrich, St. Louis, MO) was diluted 1:30,000 in blocking buffer. Enzymatic color development was carried out using 3, 3′, 5, 5′-tetramethylbenzidine. Color development was stopped by the addition of an equal volume of 1 mol/L phosphoric acid and the wells read at 450 nm in the microplate reader.

Characteristics of Assay

The minimal detection limit (concentration of pepsin at which the mean sample signal was greater than or equal to the mean blank signal by 3 SD) was 0.1 ng/mL. The standard curve ranged from 0.1 to 10 ng/mL (Fig. 1A). Serial dilution of human gastric juice samples, collected from subjects after an overnight fast, showed a dose response curve parallel to the standard curve. The median pepsin concentration of eight human gastric juice samples was 0.4 mg/mL (range 0.09–0.8 mg/mL). These values are within the expected pepsin concentration range of gastric juice.[10]

The pepsin A isoforms, pepsin 3a, 3b, and 3c, purified from human gastric juice, were equally reactive with anti Hu-3. Cross reactivity with other acid proteases was measured by comparing the concentration of purified human pepsinogen 1 (Sigma-Aldrich, St. Louis, MO) and gastricsin, purified from human gastric juice, to pepsin at half maximal reactivity. Gastricsin and pepsinogen 1 showed less than 3% and less than 0.03% cross reactivity, respectively (Fig. 1B). Serum did not contain any pepsin immunoreactivity (n = 3).

The precision of the ELISA was determined by calculating the intra- and intertest coefficients of variation (CV). The intra- and interassay CV were less than 10%, demonstrating the suitability of the ELISA to quantify pepsin (Table I).

The ability of the ELISA to accurately determine the presence of known amounts of pepsin in throat sputum samples was measured (n = 7). In all cases, addition of 1 μg/mL pepsin to throat sputum samples resulted in greater than 89% recovery of the added pepsin. Throat sputum samples with 100 ng/mL and less added pepsin showed variable recovery (Table II).

Clinical Study Design

Patients 18 years or older with clinical LPR undergoing pH-metry at the Center for Voice and Swallowing Disorders were recruited to participate in the study. Before participation, each study subject signed an informed, written consent that was approved by the Wake Forest University School of Medicine Institutional Review Board.

Subjects were given six 30 mL tubes each containing 0.5 mL 0.1 mol/L citric acid, pH 2.5. Citric acid was used to maintain the sample at acidic pH after collection to stabilize the pepsin and to act as a simple antibacterial agent for the sample.

At the time of LPR-related symptoms (e.g., throat burning, bad taste in the mouth, sensation of lump in the throat, heartburn, cough, regurgitation, or eructation), subjects were instructed to cough and clear the sputum from back of their throat and spit it into one of the tubes provided. They were asked to record the time and symptoms on a diary sheet. In addition, study

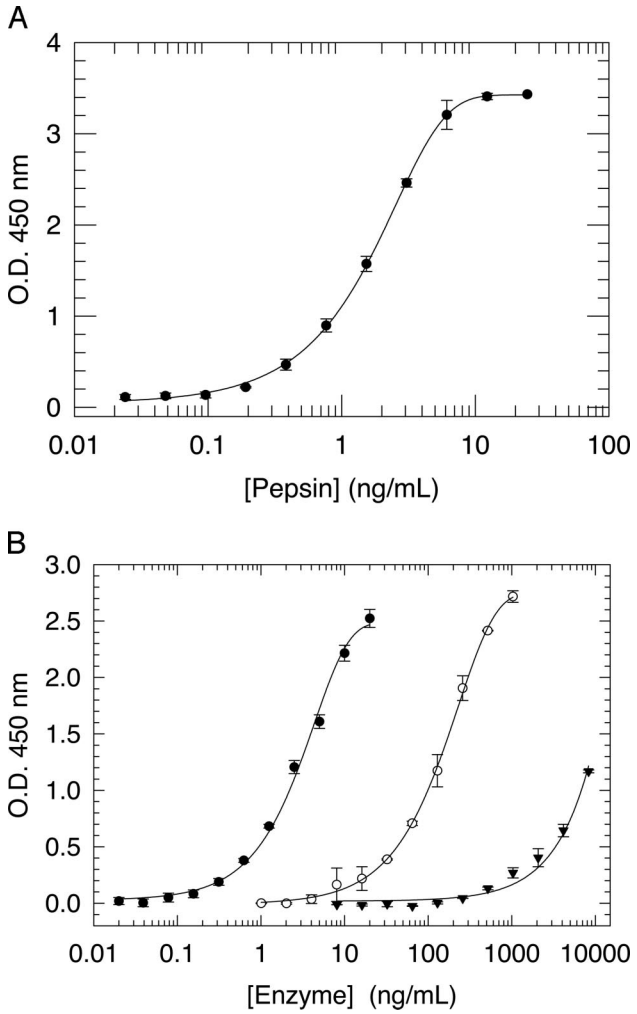

Fig. 1. Standard titration curve for pepsin enzyme-linked immunoadsorbent assay (ELISA). (**A**) Standard titration curve for the noncompetitive, indirect, sandwich ELISA for purified human pepsin 3b. Pepsin concentration was determined by absorbance at 280 nm where 1 mg/mL pepsin has an absorbance of 1.43. (**B**) Dose response curves for the indirect sandwich ELISA showing a comparison of pepsin 3b (●), gastricsin (○), and pepsinogen 1 (▼).

subjects were asked to provide a sputum sample 30 minutes after completion of each meal.

On receipt of throat sputum samples in the laboratory (usually within 24 hours of collection), they were gently vortexed for 1 minute and then centrifuged for 20 minutes, 4° C, 21,000*g*, in a bench top Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany). The supernatants and pellets were stored separately at -20° C. For the ELISA, sample supernatants were diluted 1:5 in blocking buffer to restore neutral pH and reduce the viscosity of sample. After ELISA, only samples with dose response curves parallel to the purified pepsin standard curve were used to calculate pepsin amounts.

TABLE I.
Intra- and Interassay Coefficients of Variation of Indirect Sandwich Immunoassay Determination of Human Pepsin.

Pepsin (ng/mL)	Intraassay (n = 6) CV	Interassay (n = 10) CV
2.5	5.5	6.4
1.25	8.0	6.9
0.63	8.3	8.1
0.31	9.9	8.0

CV = percent coefficient of variation.

Technique of pH-Metry

Our techniques and standards for pH-metry have been previously described.[4] A Mark III Digitrapper pH monitor (Medtronic Inc., Shoreview, MN) was connected to a dual sensor, single silicone catheter pH probe (Medtronic Inc.). A four channel Castell-type solid state esophageal manometry catheter was used for sphincter finding for accurate pH probe placement. All subjects on proton pump inhibitors had their medications discontinued 5 to 7 days before pH study. Histamine-2-receptor antagonists (H_2-blockers) and gastric motility drugs were stopped at least 4 days before the study. No antacids were taken during or within 6 hours of the start of pH-metry.

At the completion of the study, recorded data were analyzed on the EsopHogram software package (SWS-10000, Medtronic Inc.). Manual reviews of the computer evaluated data were performed to ensure reporting of true reflux events. Events occurring during and within 5 minutes of feeding were excluded from the count of reflux events. A LPR event was defined as a recorded pH decrease to below pH 4.0 for any length of time recorded by the esophageal sensor and followed by a pH drop of similar or larger magnitude at the pharyngeal sensor within 20 seconds.

Comparison of pH-Metry and Pepsin Data

Esophageal and pharyngeal pH values were recorded continuously during the 24-hour period of the study, so the pH was known at the time of sample collection. The pepsin assays were read by a single investigator (J. Knight) who was blinded to subjects' clinical status and to the pH-metry results. The results of the pepsin assay were compared with pH data.

Statistical Analysis

A mixed model analysis of variance (ANOVA) was used to assess differences in esophageal and pharyngeal pH at the time of sampling between subjects with positive and negative pepsin levels while accounting for multiple observations for each individual. A P value of less than .05 was regarded as significant. Unless otherwise stated, data are expressed as the mean ± SD.

RESULTS

Seventeen female and six male subjects, median age 51 (range 35–77) years, were enrolled in this study.

TABLE II.
Recovery of Different Amounts of Purified Human Pepsin Added to Seven Different Throat Sputum Samples.

Pepsin Level (ng/mL)	Median Recovery* (%)	Range (%)
1,000	94	89–95
100	92	50–93
10	68	27–82
5	60	20–75
2	60	8–73
1	53	4–72

*Recovery was calculated as observed sample concentration/expected concentration of purified pepsin standard.

Twenty-two of the subjects were white and one was black. A total of 63 samples of throat sputum were collected for pepsin immunoassay, of which 22% (14/63) tested positive for pepsin. By ELISA, the median pepsin concentration was 0.18 μg/mL

Seventy-eight percent (49/63) of the samples contained no measurable pepsin and were collected at a time when no decrease in pH at either the esophageal or pharyngeal probe was observed. Five of the subjects provided at least one throat sputum sample positive for pepsin; four of these also had negative pepsin samples.

A mixed model ANOVA was used to assess differences in esophageal and pharyngeal pH at time of sampling between those subjects with positive and negative pepsin levels while accounting for multiple observations for each individual. Estimated means for esophageal and pharyngeal pH for each pepsin group and their differences are shown in Table III. Those subjects with positive pepsin samples had significantly decreased esophageal and pharyngeal pH ($P = .01$, both outcomes). When comparing the pepsin immunoassay results with pH data, the pepsin immunoassay was 100% sensitive and 89% specific for LPR at the pharyngeal probe.

Representative pH tracings from one study subject are shown in Figures 2 and 3. This subject had five LPR events over the course of the 24-hour study, all occurring between 20:05 hours and 20:27 hours. The patient collected throat sputum samples at 20:37 hours, 22:00 hours, and 09:00 hours (A, B, and C, respectively, Fig. 2). Samples A and B tested positive for pepsin, and sample C tested negative.

Sample A, which tested positive for pepsin, was collected 10 minutes after the last of the five LPR events and correlated with an esophageal event (pH 3.7) but no pharyngeal event. Sample B, which also tested positive for pepsin, correlated with an esophageal event (pH 1.5) but again not with a pharyngeal event. The data from this subject shows that pepsin remained in the throat 90 minutes after the five previous LPR events.

Samples were collected when the subjects were experiencing symptoms: cough (38%), eructation (35%), throat burning (34%), globus (18%), throat clearing (10%), regurgitation (9%), and heartburn (7%). All samples associated with regurgitation, and 60% associated with heartburn

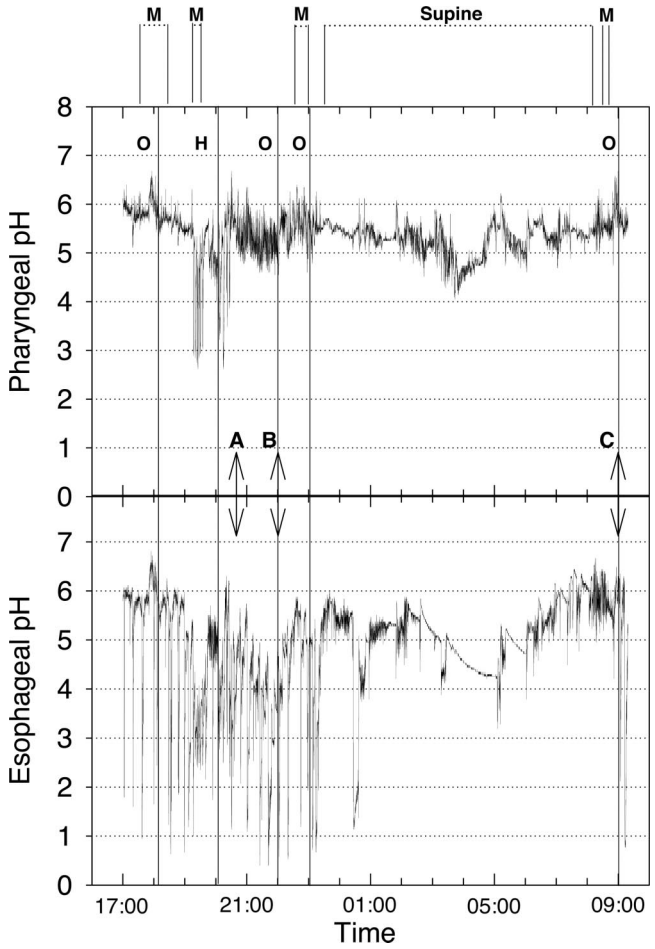

Fig. 2. Representative 24 hour dual probe pH study. 24-hour pharyngeal and esophageal pH tracings of study subject 4 who had five laryngopharyngeal events between 20:05 hours and 20:27 hours. Sample A (20:37 hours) and sample B (22:00 hours) were positive for pepsin, containing 6.5 $\pm$ 0.15 ng/mL and 3.5 $\pm$ 0 ng/mL pepsin, respectively. Sample C (09:00 hours) tested negative for pepsin. H = heartburn; O = other symptom; M = meal.

TABLE III.
Estimated Means and Standard Errors for Esophageal and Pharyngeal pH for Each Pepsin Group and their Difference.

Pepsin	N*	Mean	Standard Error	95% CI
Esophageal pH				
+	5	2.19	0.39	0.93–3.44
−	22	5.04	0.21	4.37–5.71
Difference		2.85	0.42	1.50–4.20
Pharyngeal pH				
+	5	4.44	0.23	3.73–5.16
−	22	5.82	0.12	5.44–6.20
Difference		1.38	0.24	0.61–2.15

*Unique patients.

tested positive for pepsin. Eructation was associated with positive pepsin samples 62% of the time. These results are consistent with the pH data.

At the level of the esophageal probe, regurgitation and heartburn were associated with a mean of pH 2.3 $\pm$ 0.9 (range 1–3) and pH 2.3 $\pm$ 1.3 (range 0.5–3.7), respectively. At the level of the pharyngeal probe, regurgitation and heartburn were associated with a mean of pH 4.2 $\pm$ 1.3 (range 3–6) and pH 4.4 $\pm$ 1.0 (range 3.4–6.0), respectively. Eructation was associated with a low mean pH at both the esophageal, pH 3.0 $\pm$ 1.8 (range 1–6) and pharyngeal, pH 4.8 $\pm$ 1.2 (range 3–6) probes.

Cough and throat burning were the most common symptoms associated with negative pepsin samples; 85% of all samples associated with cough and 83% of all samples associated with throat burning were negative for pepsin. Interestingly, 40% of samples associated with the symptom of throat burning correlated with esophageal events but not pharyngeal events. For example, one subject had produced three samples, negative for pepsin, which correlated with severe esophageal events, mean pH

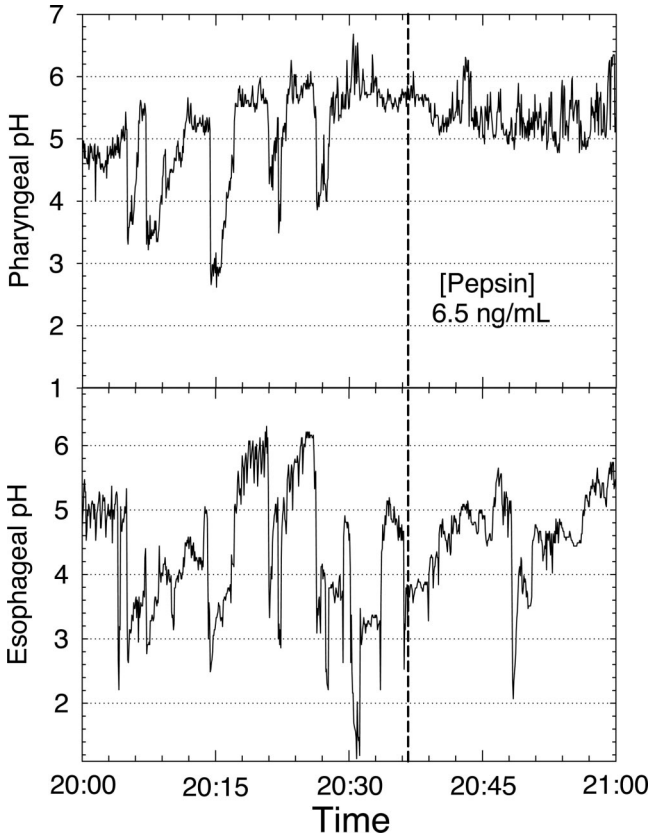

Fig. 3. Expanded pH tracing showing laryngopharyngeal reflux (LPR) events. Pharyngeal and esophageal pH tracings between 20:00 hours and 21:00 hours of representative patient shown in Figure 2. The expanded time scale shows five LPR events that were recorded during that interval. The dashed line at 20:37 hours indicates the time at which the subject collected the throat sputum sample A that contained 6.5 ± 0.15 ng/mL pepsin, as determined by the pepsin immunoassay.

2.0 ± 0.95, with no simultaneous pharyngeal reflux episodes. It is interesting that in these cases, throat burning may be associated with heartburn. Cough was always associated with a pH 5.5 or greater at the pharyngeal probe, mean pH 6.1 ± 0.3, and at the esophageal probe associated with the highest mean pH of all symptoms, pH 5.1 ± 1.6, which may explain why throat sputum samples that correlated with cough were largely negative.

DISCUSSION

This study evaluated a sensitive immunoassay method for the detection of pepsin in throat sputum and compared it to 24-hour double-probe pH monitoring. The results suggest that detection of pepsin in throat sputum provides a sensitive and noninvasive diagnostic for LPR. When comparing the pepsin immunoassay results with the pH data, positive pepsin samples had a mean pH 4.44 and did not always correlate with pharyngeal events pH 4 or greater (89% specific). Interestingly, 78% of the sputum samples that tested negative for pepsin were associated with no pH drop in either the esophageal or pharyngeal probes, and yet the subjects collected samples because they experienced symptoms. These data suggest that LPR symptoms may occur in patients experiencing reflux at above pH 4.0. We have previously shown experimentally that laryngeal damage occurs at pH 5.0 or greater.[8] Thus, the findings of this study support the concept that the threshold of pH 4.0 for defining LPR probably should be raised to 5.0.[4]

An advantage of our immunoassay is that it is equally sensitive for detecting active and inactive forms of pepsin. Furthermore, the assay methods described here distinguishes between pepsinogen and pepsin; it has very low cross-reactivity with pepsinogen (<0.03%). This study supports previous results, which have used pepsin determinations in airway secretions to incriminate LPR.[12–15]

How valuable is this test (assay) likely to be in clinical practice? The finding of pepsin in the sputum of a patient with clinical manifestations of LPR does not prove causality. Likewise, a negative sputum test does not rule out LPR. Nevertheless, as we go forward with clinical testing, the authors believe that a ″diagnostic pepsin profile″ will emerge. In the meanwhile, the finding of pepsin in the airway (or in sputum) is essentially as diagnostic of LPR as is abnormal pH-metry.

Pepsin Detection: Clinical Relevance and Future Studies

This report provides very encouraging initial clinical data. We have begun to study normals, and although the numbers are small (n = 12), none of the normals have pepsin in their sputum samples. The question of when to sample LPR patients is not fully resolved; however, we believe that first-in-the-morning and before-bed samples are likely to be positive in LPR patients. In addition, when a patient has discrete reflux-related symptoms, such as cough or laryngospasm, then sampling at the time that the symptom is experienced also appears to have a high positive yield.

The most significant findings of this study are that the assay was 100% sensitive—when the sputum contained measurable pepsin, the patient always experienced LPR by pH monitoring criteria—and 89% specific, that is, when a pharyngeal reflux event occurred, measurable pepsin was found 89% of the time. The latter observation, that we found pepsin in some patients without a preceding pH-documented LPR event, suggests that pepsin is a better marker for LPR than acid detected by pH monitoring. Thus, from the clinician's point of view, a single positive pepsin assay result is diagnostic of LPR, although in and of itself, severity cannot be determined.

Not reported herein is our experience with pepsin immunohistochemistry. This method of LPR diagnosis requires a tissue sample; however, with the transnasal esophagoscope, we are now obtaining pharyngeal tissue samples in LPR patients for analysis. The finding of pepsin embedded in tissue or within inflammatory cells is virtually diagnostic of LPR-related disease. In the future, we will sample sputum and tissue in both normals and LPR patients; a large prospective study is anticipated. Inevitably, there may be a matrix or pattern that is not only diagnostic of LPR but also of its severity and it role in causing disease. We believe that the pepsin assay as a spit-in-a-cup test will be found to be the best available

screening test for LPR and that tissue sampling tested for markers such as pepsin, carbonic anyhydrase, and E-cadherin will be diagnostic.[8–15]

CONCLUSIONS

Assessment of throat sputum samples for pepsin by immunoassay provides a simple noninvasive test for detection of LPR. Because pepsin plays a primary role in causing laryngeal and airway tissue damage and because it is a relatively large molecule, pepsin is an excellent clinical marker for LPR.

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