Is pepsin detected in the saliva of patients who experience pharyngeal reflux?

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Abstract

Objectives: To investigate if pepsin is detected, with an activity assay, in the saliva of patients with a clinical diagnosis of laryngopharyngeal reflux (LPR) and can therefore be used as a diagnostic marker of laryngopharyngeal reflux.

Study design: Pilot, prospective study.

Methods: Adult participants with a clinical diagnosis of LPR collected whole saliva samples on regular intervals for a day, and upon experiencing symptoms attributed to LPR. Patients were selected on the basis of presence of severe symptoms and laryngoscopic findings of laryngopharyngeal reflux and symptoms of gastroesophageal reflux. They reported voice disorders, dysphagia, throat clearing, excessive secretions, breathing difficulties, cough, globus sensation and throat pain. Control participants reported the absence of pharyngeal and laryngeal symptoms and of symptoms of gastroesophageal reflux. Saliva samples were assayed with fibrinogen on an agarose gel plate. The detection of pepsin was based on the presence of peptic activity which was qualitatively evaluated.

Results: The control participants had negative assays. No saliva samples from the LPR patients, collected at regular sampling, tested positive for pepsin. All the samples collected at the presence of symptoms and following regurgitation episodes tested negative for pepsin. Saliva samples pH ranged from 7 to 8.

Conclusions: Pepsin was not detected, with an activity assay, in the saliva of patients with a clinical diagnosis of LPR. A concentration method might be more sensitive although saliva and swallowing physiology renders the detection of pepsin in the saliva difficult. Hippokratia 2007; 11 (3): 145-149

Key words: laryngopharyngeal reflux, pepsin, saliva, diagnosis, gastroesophageal reflux, laryngitis

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Laryngopharyngeal reflux (LPR) refers to the backflow of stomach contents into the throat. LPR has been reported to be related with many symptoms and pathologic conditions of the pharynx, the larynx, the upper and the lower airways. The symptoms which have been more frequently associated with LPR are hoarseness, globus pharyngeus, chronic cough, dysphagia, throat pain and throat clearing. Posterior laryngitis is the most common laryngeal condition attributed to LPR. The diagnosis of LPR is currently based on the patients’ symptoms and laryngoscopic findings. In the presence of symptoms and laryngoscopic findings suggestive of LPR, the most common practice among otolaryngologists is the empiric anti-reflux treatment with proton pump inhibitors (PPI). Many studies have shown the limitations of the currently used diagnostic approach. The throat symptoms associated with reflux are very common and can also be due to many other causes: allergy, smoking, asthma, infections, voice abuse or alcohol abuse. The laryngoscopic findings attributed to reflux have been found to be non specific for LPR, with poor inter - rater reliability between otolaryngologists performing the laryngoscopy. Dual-probe 24-hour pH-monitoring is considered to be the current gold standard diagnostic test. It is, however, an invasive test, with poor sensitivity, and not widely available. Normal pH values for the hypopharynx are not well established in the literature.

Gastric refluxed juice contains acid and pepsin both of which are injurious to the laryngeal and pharyngeal mucosa. Pepsins are enzymes that are produced in the stomach in the form of pepsinogens. In the presence of acid, pepsinogen is converted to pepsin and digests protein. Pepsin exhibits enzymatic activity at pH levels well above 4 (the cut-off for diagnosis of a reflux episode with pH monitoring). Pepsin is only irreversibly inactivated at a pH level greater than 6. Several studies have investigated the potential to use pepsin detected in airways as a diagnostic marker for LPR. Pepsin has been reported to be present in the saliva and sputum of patients investigated for reflux, in tracheal aspirates from patients submitted to intubation, in bronchoalveolar lavage fluid from lung allograft recipients and in ear effusions. Polturi et al reported the detection of pepsin in the saliva and sputum with the use of a simple, pepsin specific,
qualitative enzyme plate assay, based on the digestion of fibrinogen by the saliva sample\textsuperscript{25}. Over the last two decades, saliva is being used widely as a diagnostic fluid, because it is easily obtainable and has been proven suitable for many diagnostic tests\textsuperscript{32}. We designed a study to investigate if pepsin is detected in the saliva of patients with a clinical diagnosis of LPR, with the use of a simple, cost-effective enzymatic method, which could be widely available.

Materials and Methods

Participants

Participants to this study were recruited from the ENT Outpatient Clinic and the Voice Clinic of a Tertiary General Hospital. Normal control participants were recruited from the hospital staff. Patients, 18 years or older, with a clinical diagnosis of LPR, based on the symptoms and the laryngoscopic findings, were invited to participate in the study. Exclusion criteria included diseases known to impair salivation, a history of radiotherapy in the head and neck, active oral or dental problems, reduced gastric acid secretion and medication known to affect saliva flow. Patients were asked to abstain from medication affecting gastric acid secretion for a week prior to sample collection. Smoking was recorded in the history, but was not a reason for exclusion from the study. The study was approved by Papa-georgiou Hospital Scientific Ethics Committee. Before participation each participant signed written informed consent.

Over a three months period, 9 patients and two normal control participants were recruited providing, 93 and 50 samples respectively. The clinical diagnosis of LPR was based on the presence of a combination of symptoms and laryngoscopic findings: voice disorders, throat clearing, cough related or unrelated to meals and lying down, dysphagia, globus pharyngeus, excessive throat secretions, breathing difficulties and throat pain, in the presence or not of symptoms of gastroesophageal reflux (GERD). The laryngoscopic findings which were evaluated for the diagnosis of LPR were erythema and oedema of the posterior or whole larynx, posterior commissure hypertrophy, ventricular obliteration, granuloma formation, vocal fold oedema, pharyngeal wall cobe stoning.

The sample collection

Patients were instructed to collect samples of saliva by spitting into a separate plastic specimen container for each sample, every 30 minutes while awake and whenever experiencing symptoms such as cough, breathing difficulties, regurgitation, and heartburn. Sample collection lasted a day. Patients were asked to record the time of symptoms’ occurrence, and the time periods of meals and sleep. In two cases of reported regurgitations up to the mouth the patients were asked to collect samples every three minutes for an hour following such a regurgitation episode. The normal control participants were asked to collect saliva samples every 30 minutes for a day. All participants collected the samples at home and were instructed to store each sample in the refrigerator (4°C) immediately after collection and deliver all samples at the hospital the morning after the day of collection. Each sample was then numbered sequentially and divided to several aliquots of 0.1ml to 0.3ml which were stored at -80°C. All further handling of the samples was anonymous.

The assay

Saliva samples were assayed for pepsin with bovine fibrinogen. The detection of pepsin is based on its ability to digest fibrinogen. The pepsin assay which was used for this study is the one reported recently by Potluri et al\textsuperscript{25} and Ufberg et al\textsuperscript{28}. The plates were placed in a humid chamber overnight and the assays were read qualitatively after 12 hours by an examiner blinded to the history. Clearing of the agarose gel around the sample was considered a positive assay. Porcine pepsin (by Sigma-Aldrich, St. Louis, MO) and human gastric juice were used as positive controls and saline as negative control on every plate.

Results

Six of the nine patients who participated in this study were women. The age of the patients ranged from 24 to 77 with a median age of 38. Both normal subjects were women, 32 and 39 years old. An overview of the patients’ symptoms at the time when they enrolled in the study appears in Table 1.

Five of the patients had been previously diagnosed endoscopically to suffer from hiatus hernia. Another one had a hiatus hernia diagnosed after her participation to the study. Regarding the laryngoscopic findings of the participants at the time of sample collection, erythema

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Participants</th>
</tr>
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<tbody>
<tr>
<td>Voice disorders</td>
<td>+</td>
</tr>
<tr>
<td>Cough</td>
<td>+</td>
</tr>
<tr>
<td>Throat.clearing</td>
<td>+</td>
</tr>
<tr>
<td>Throat.secretions</td>
<td>+</td>
</tr>
<tr>
<td>Hoarse</td>
<td>+</td>
</tr>
<tr>
<td>Gastroesophageal reflux</td>
<td>+</td>
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<tr>
<td>Breathing.difficulties</td>
<td>+</td>
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Table 1. An overview of the symptoms of the patients at the time of participation

P1-P9: Participant 1-Participant 9. GERD: Gastroesophageal reflux
and posterior commissure hypertrophy were noted to all patients’ larynges. Oedema of the posterior larynx was noted to five patients, ventricular obliteration to four and vocal fold oedema to two.

Five patients collected 11 to 22 samples each, whereas four patients collected only a few samples each, sampling at regular intervals and at symptoms. Besides the two patients who collected samples following a regurgitation episode another seven samples were collected from the other 7 patients while they experienced symptoms. The remaining samples were collected at regular intervals sampling. The control participants collected 22 and 28 samples. For seven samples collected at regular intervals and five samples collected after regurgitation, pH values were recorded. They ranged from 7 to 8 with a median pH value of 7.8.

The pepsin assays of the normal control samples were as expected all negative. The samples obtained from patients with a clinical diagnosis of LPR were also all uniformly negative for the presence of pepsin (Figure 1). Interestingly, the samples collected after a regurgitation episode to the mouth also tested negative for the presence of pepsin.

Discussion

The current diagnostic approach of laryngopharyngeal reflux is based on the use of diagnostic modalities with well described low sensitivity and specificity for LPR. This is the case for the symptoms and laryngoscopic findings and also for the empirical PPI treatment and the dual probe pH-monitoring. Pepsin has been shown to cause damage to the laryngeal mucosa and the combination of pepsin and acid is considered the main injurious factor in the gastric refluxate reaching the pharynx and larynx. Pepsin is not produced anywhere in the respiratory tract. Therefore, pepsin detected in the airways would be a specific marker of LPR. The use of saliva as a diagnostic fluid is expanding over the last 10 years due to technological advances and the unique properties of saliva as a test fluid. Saliva is readily available and easily obtainable by many patient groups (adults, children, neurologically impaired). Many methods have been reported for the detection of pepsin activity and concentration. This study was designed to examine if pepsin is detected in the saliva of patients with a clinical diagnosis of LPR, with the use of a simple and inexpensive enzyme activity assay, easily applicable in any hospital laboratory. This is the first reported study investigating the presence of pepsin in saliva samples obtained with a methodology distinguishing them from sputum.

The saliva contains water, electrolytes, mucous, enzymes and antibacterial agents. We collected saliva samples from normal control individuals in order to examine the possibility of presence of the saliva of pepsin of other than gastric origin. Although this is a small number of control participants, regular sampling produced no positive samples.

We chose the spitting saliva collection method because this method does not result to stimulation of saliva secretion. Stimulation can increase the saliva flow rate significantly resulting in wash out, dilution, or inactivation of pepsin that might have reached the mouth. We acknowledge that saliva flow stimulation can be caused by reflux itself. Some patients suffering from oesophagitis experience sudden filling of the mouth with saliva that accompanies heartburn. We did not ask our patients specifically for occurrence of this symptom.

In this study only a small number of participants provided regular saliva sampling. As a total a significant number of samples were assayed and found negative for the presence of pepsin. The question of when to sample LPR patients to optimise the detection of pepsin if it is present in the saliva is not solved. Polturi et al. reported sampling every two hours and at the presence of symptoms. They obtained 11.25 samples per patient. The actual sampling frequency for saliva in their study is not known. That study reports that 10.6% of their samples (sputum and saliva) were positive for pepsin. Knight et al. reported sampling sputum upon the presence of symptoms and at 30 minutes after completion of a meal. They obtained 2.7 samples per participant and 22% of the samples tested positive for pepsin. In studies that used varying methods for the detection of pepsin, one sample per patient was performed for middle ear effusion, one sample per day was obtained for tracheal aspirates and one bronchoalveolar lavage per patient was performed. These studies yielded positive samples in rates that range from 9% to 100% of the samples. We opted to sample every 30 minutes, as we anticipated that patient compliance would not be satisfactory for more frequent sampling for a whole day, due to resulting restrictions to professional activities and family life. Compliance was not great: only three out of 7 patients collected more than 10 samples (11, 17 and 22).
Two participants who reported regurgitations up to the mouth were asked to collect samples every 3 min following an episode in an effort to record “a time chart” of the presence of pepsin in saliva. Comparing our results with those of other studies we conclude that sampling may not be the main factor that determined the absence of any positive assays in this study. We postulate that different restrictions apply to the detection of pepsin in the saliva from those concerning other airway fluids.

We addressed the possibility of washing out of the pepsin from the mouth. In humans saliva flow is continuous in the awake state with a resting flow of 0.5 ml/min36. Resting flow saliva is carried into the oesophagus by spontaneous swallows that occur about once a minute36. Oesophageal clearance studies have shown that clearing of the refluxed material from the oesophagus is accomplished with one or two peristaltic waves that reduce a bolus of 2ml, 5ml or 15ml to a minimal residual amount followed by acid neutralization by swallowed saliva39. Oesophageal clearance is accomplished within minutes36. One would expect that clearance of pepsin containing refluxate from the mouth would occur in a few minutes in subjects with normal saliva secretion and unaffected swallowing.

In the mouth, saliva functions as a buffer to maintain a relatively alkaline pH40. The ability of saliva to buffer acid within the range of 5-7 has been well described38. In our study all the samples tested had pH values 7 and greater. Costa et al reported the pH values of saliva samples obtained during 24hour dual probe pH monitoring41. Only one patient had a pH value lower than 6.5, which corresponds to the upper value of pH at which pepsin activity can be expected22,23. For the pH values recorded in our study and in Costa’s study all pepsin that might have reached the mouth is expected to be irreversibly inactivated.

Storing conditions can affect the activity of gastric juice pepsin. Freezing and thawing of gastric juice with a pH lower than 2 has been shown to affect the activity of pepsin44. Our saliva samples had much greater pH values. Michishige reported the storing of whole saliva for proximal gastric juice pepsin. Freezing and thawing of gastric juice with a pH lower than 2 has been shown to affect the activity of pepsin irreversibly. Krishnan et al report no pepsin activity changes in vitro when tracheal samples where stored at 37°C for three hours and at -80°C for up to 6 months37. Travelling distances were calculated and we estimate that our samples were not left at room temperatures for longer than an hour. We therefore assume that storing did not affect the determination of pepsin activity in our study.

The participants to this study were selected on the basis of severe symptoms and findings suggestive of LPR, the majority suffered from GERD and 6 of the 9 patients had a hiatus hernia. Attempting to interpret the absence of any positive assays we conclude that storing conditions are unlikely to have affected the results. Sampling was more frequent than in other reported studies. Even with that frequent sampling clearance of the mouth from most of the refluxed material happens apparently well before sampling, due to normal physiology of saliva secretion and swallowing. We postulate that the ability of human saliva to act as a buffer in the mouth inactivates irreversibly any pepsin that reaches the mouth with the refluxate and an activity method cannot therefore detect pepsin in the saliva samples. At the only published study reporting the detection of pepsin with an activity method it is unclear from the methods description if saliva and sputum samples have been distinguished from each other35. It appears that once collected by the patients the samples were coded and tested without identification of their origin and positive results concern the sputum and saliva samples indiscriminately35.

Conclusions

We consider the determination of pepsin concentration a more appropriate method for the detection of pepsin in the mouth. ELISA methods have been developed that allow the detection of pepsin concentrations down to 1ng/ml50 and 0.1ng/ml52. Further study of our samples with ELISA testing has been planned to allow us to better evaluate if saliva could be an appropriate diagnostic fluid for the diagnosis of LPR.

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