AMJ
ISSN: 0005-2523

ISSN: 0005-2523
Volume 62, Issue 07, September, 2022

Analysis of Pepsin, Tripsyn, and Bile Acid in Saliva of Patients with Laryngopharyngeal Reflux and Non-Laryngopharyngeal Reflux

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Keywords:
Pepsin, Renin, Bile acid, LPR (Laryngopharyngeal Reflux), ELISA (*Enzyme-Linked Immunosorbent Assay*)

ABSTRACT

Laryngopharyngeal reflux (LPR) is the backflow of gastric and or duodenal fluid into the larynx, pharynx, trachea, and bronchi. The prevalence of LPR is difficult to determine due to the limited gold standard and the large variety of LPR symptoms. Damage can occur due to the decrease in pH value and also because of exposure to harmful enzymes in reflux, including pepsin, bile acid, and trypsin. This study is an analytic observational study with a case-control design. The study was conducted in the ORL-HNS Department of Dr. M. Djamil Hospital, Padang, West Sumatra, Indonesia. The total sample size was 44 people. We enrolled 22 healthy subjects as the control group and 22 patients suspected of having LPR. LPR patients are more common in women than in men, with 12 women and 10 men. Pepsin levels in saliva in the LPR group were higher than those in the non-LPR group. For pepsin, the LPR group had a mean of 20.11±9.76 ng/mL and a healthy control with mean of 15.77 ±7.65 ng/mL. Trypsin levels in saliva in the LPR were 103.15±47.69 µg/mL, In the healthy group, the mean was 82.99 ±39.62 µg/mL. For Bile acid in the LPR group, in LPR, the mean of pepsin is 25.08±7.67µM, meanwhile in healthy group, the mean was 18.99±8.26 µM. There is statistically significant in the incidence of LPR with the bile acids ($p = 0.015$) based on ANOVA and Logistic regression test. Our study confirmed that bile acids in saliva play a major role in diagnosing LPR.

1. Introduction

Laryngopharyngeal reflux (LPR) is the backflow of gastric and/or duodenal fluid into the larynx, pharynx, trachea, and bronchi [1]. The fluid would make contact with the upper airway and gastrointestinal (*aerodigestive*) tract, causing symptoms such as hoarseness, cough, *globus* sensation, throat clearing, and

post-nasal drip [2]. LPR is established based on medical history, clinical symptoms, laryngoscopy examination, and determining the presence of gastric backflow fluid in the laryngopharynx. The Reflux Symptom Index (RSI) questionnaire is useful to measure the severity of LPR symptoms and to observe the response toward treatments given to the patient, but it cannot distinguish LPR from any upper respiratory tract symptoms caused by other conditions. The Reflux Finding Score (RFS) indicates the severity of inflammation seen in laryngoscopy findings, but the findings may also occur in other types of chronic laryngeal irritation [3].

The prevalence of LPR is very difficult to determine due to the limited gold standard and the large variety of LPR symptoms. The exact prevalence of LPR is unknown, but it is estimated that 20–30% of patients with laryngeal complaints have LPR [4], *Ambulatory 24-hour double-probe pH-metry* examination is the gold standard to diagnose LPR. However, the sensitivity of pH-metry examination is reported as low as about 50%-80% only. Currently, a combination of *24-hour double-probe pH-metry* with *multichannel intraluminal impedancemetry* (MII) has been developed for the diagnosis of LPR. This combination can identify reflux in the form of liquid, gas, or a mixture of both and can detect both acidic and non-acidic reflux [5].

Reflux can cause a significant drop in the pH value of the larynx. Damage can occur due to the decrease in pH value and also because of exposure to harmful enzymes in reflux, including pepsin, bile acid, and trypsin. Clinical evidence suggests that the reflux of gastric fluid and its contents into the laryngopharynx contribute to the pathophysiology of nonspecific inflammatory and neoplastic disorders [6]. The measurement of gastric pepsin in the oropharynx has been studied as a non-invasive method to detect oropharyngeal reflux in adults and children [7]. Pepsin is not synthesized by upper aerodigestive tract cells and is only produced in gastric, so pepsin can be used as a marker of LPR because it is easily detected in upper aerodigestive secretions. The method for examining pepsin is *Enzyme-Linked Immunosorbent Assay* (ELISA) and also *peptest* to detect the presence of pepsin in saliva semi-quantitatively [8], [9]. The presence of pepsin in the upper aerodigestive tract indicates a backflow of gastric fluid contents. However, the previous study showed that there were fluctuations in the levels of pepsin detected in saliva, depending on the time the sample got collected to be examined and the diet before the test, this result was also found in 20% of the normal population [10].

Other enzymes such as bile acids and trypsin have been reported to have the potential to be the diagnostic markers of LPR [11]. Bile acid levels were found to be up to three times higher in LPR patients than in the normal group [11]. Bile acid examination in saliva was proven to be a useful diagnostic value. Bile acid content > 1 mmol/L is considered the most suitable to describe the severity of LPR with a sensitivity of 86% [13].

With the existing diagnostic modalities, the diagnosis of LPR is often based on the signs and symptoms found in patients, which is highly subjective. Future diagnostic approaches should address the clear relationship between clinical signs and symptoms, such as the MIIpH examination, as an easy and reliable biomarker examination to improve the accuracy for diagnosing LPR

The reported study of the examination of reflux markers in saliva was unable to answer the question of whether there is a consistent association between reflux component levels and the diagnosis of LPR. There has never been a study that compared all reflux components as diagnostic markers for LPR. Therefore, this study was conducted to determine the value ranges of pepsin, trypsin, and bile acids separately or in combination as markers of laryngeal pharyngeal reflux so that diagnosis can be established simply by saliva

ISSN: 0005-2523
Volume 62, Issue 07, September, 2022

examination.

2. MATERIALS AND METHODS

2.1 Patients and study design

This study is an observational study with a case-control design. The study was conducted in the ORL-HNS Department of Dr. M. Djamil Hospital, Padang, West Sumatra, Indonesia. The sample size was determined by the estimation formula. The total sample size was 44 people. We enrolled 22 healthy subjects as the control group and 22 patients suspected of having LPR. The subjects of this study were LPR patients with symptoms of laryngopharyngeal reflux with RSI > 13 and RFS > 7. The participants who were not diagnosed with LPR were subjects with RSI values 13 and RFS 7. The inclusion criteria were patients who were willing to be included in the study by signing informed consent and also patients with LPR who did not have any history of diseases such as asthma, pulmonary tuberculosis, chronic obstructive pulmonary disease, or laryngeal diseases, including polyps, nodules, vocal cord paralysis, and laryngeal carcinoma. The exclusion criteria is saliva that cannot be continued for examination by the immunoassay method (ELISA) because they are damaged are based on considerations from Biomedical laboratory installation. The sample for this study and is 22 for LPR patient and 22 non-LPR (healthy controls). Informed consent was obtained from all subjects and the protocol of the study was approved by the Ethics Committee of the Faculty of Medicine, Andalas University with Number 315/UN.16.2/KEP-FK/2021

2.2 Examination of Pepsin in Saliva

The patient's saliva was collected in sterile tubes, then centrifuged for 2 minutes at the speed of 1000 g, and other materials in the saliva were separated. Saliva (which was not yet to be examined) is then stored by mixing 0.5 mL of 0.01 M citric acid (pH 2.5) as a pepsin-stabilizing agent, frozen at -20°C, and examined for no more than 2 months after the sample is collected. At the time of examination, the samples were centrifuged at 1000g at the temperature of 4°C and the precipitate was removed. Samples were dropped into the well, reagent A was added and incubated for 1 hour at 37°C. Then the well was washed and reagent B was added for 30 minutes at 37°C. Then the well was washed again and incubated with TMB substrate solution for 15 minutes at 37°C. Then stop solution was added, and then each well was examined using a microplate reader at 450 nm.

2.3 Saliva Trypsin Analysis

The examination was performed at pH 7.4 using a modified colorimetric assay based on the production of a new n-terminal amino group that reacts with trinitrobenzenesulfonate (TNBS) to produce a trinitrophenyl derivative that can be measured spectrophotometrically. A total of 500 l of succinyl albumin (SA) at a concentration of 8 mg/ml was added to test tubes each containing 200 l of the sample or standard trypsin (0–2.5 g/ml) and the tubes were incubated at 37oC for 30 minutes. Proteolysis was stopped by the addition of 500l of 4% NaHCO3 followed by 500l of 0.05% TNBS and incubated at 50oC for 10 minutes. 500l of 10% sodium dodecyl sulfate (SDS) was then added to 500l of 1 M HCl acid. Then the result was read at 340 nm. Negative control was achieved by adding substrate and immediate addition of NaHCO3.

2.4 Examination of Bile Acid in Saliva

A total of 2 ml of saliva was collected from the patient and stored (frozen at below -20°C). In the presence of thio-NAD and 3--hydroxy steroid dehydrogenase (3-HSD) enzymes, bile acids were converted to 3-keto-steroids and thio-NADH. Then, the concentration of bile acids was measured.

2.5 Statistical analysis

The data analysis in this study is presented in the form of tables. Analysis of differences in the mean levels of pepsin, trypsin, and bile acids measured in pg/ml between the LPR group and the comparison group (non-LPR) use independent t-test if the data were normally distributed, and use Mann Whitney test if the data were not normally distributed. Multivariate analysis is using ANOVA test dan logistic Regression test. The results were analyzed with SPSS software with a P value of 0.05 being considered statistically significant.

3. RESULTS

3.1 Research Subject Characteristics

The LPR group had the most females, with up to 12 people, mean age of 43.7±12.07 years, a mean RSI of 19±7.11, and a mean RFS of 10.3±2.98. Meanwhile, in the non-LPR group, the most common gender was also female; as many as 16 people; with a mean age of 24.6±3.01 years; mean RSI of 1.45±2.67; and RFS of 0.23±0.70.

Table 1. Characteristic of LPR and non LPR based on gender

Characteristic	LPR		Non LPR		Total
	n	%	n	%	
Gender					
Male	10	45	7	31,82	17 (38,64)
Female	12	54	15	68,18	27 (61,36%)
Total	22	100	22	100	44 (100%)

Table 2. Characteristic of LPR and non LPR based on age and diagnostic score

Characteristic	LPR		Non LPR	
		SD		SD
Age	43,7	12,07	24,6	3,01
Diagnosis				
RSI	19	7,11	1,45	2,67
RFS	10,3	2,98	0,23	0,70

3.2 Pepsin levels in the saliva of patients with LPR and non-LPR

Pepsin levels in saliva in the LPR group were higher than those in the non-LPR group. The LPR group had a mean of 20.11±9.76 ng/mL. While in the non-LPR group, with a mean of 15.77 ±7.65 ng/mL. After being analyzed using the T-independent test, no significant difference was found (P = 0.108).

Table 3. Characteristic of pepsin in saliva LPR's patient and non LPR

Group	Pepsin (ng/ml)		p
	Mean ± SD	(Min – Max)	
LPR	20,11± 9.76	(7,856– 47,436)	0,108
Non LPR	15,77± 7.65	(1,286– 29,409)	

3.3 Trypsin levels in the saliva of non-LPR LPR patients.

Trypsin levels in saliva in the LPR group were higher than those in the non-LPR group. The LPR group had a mean of 103.15±47.69 µg/mL. In the non-LPR group, the mean was 82.99 ±39.62 µg/mL. The T-

ISSN: 0005-2523
Volume 62, Issue 07, September, 2022

independent test showed no significant difference (P = 0.135).

Table 4. Characteristic of tripsin in saliva LPR's patient and non LPR

Group	Tripsin (µg/ml)		p
	Mean ± SD	(Min – Max)	
LPR	103,15± 47,69	(7,856– 47,436)	0,135
Non LPR	82,99± 39,62	(1,286– 29,409)	

3.4 Bile acid levels in the saliva of LPR and non-LPR patients.

In the LPR group, bile acid levels were higher in saliva compared to the non-LPR group. LPR group, with a mean of 25.08±7.67µM. In the non-LPR group, the mean was 18.99±8.26 µM. A significant difference was discovered after the *T-independent* test was used (P = 0.015).

Table 5. Characteristic of bile acid in saliva LPR's patient and non LPR

Group	Bile acid (µM)		p
	Mean ± SD	(Min – Max)	
LPR	25,08± 7,67	(7,856– 47,436)	0,015
Non LPR	18,99± 8,26	(1,286– 29,409)	

3.5 Pepsin, trypsin, and bile acid levels in saliva are measured to diagnose LPR.

In the LPR group, the ANOVA analysis value of pepsin enzyme was 0.108, trypsin enzyme 0.135, and bile acid 0.015. After the logistic regression test, the p-value for pepsin is 0.092 and for tripsin is 0.200, meanwhile, the value for bile acids is 0.015. This value was smaller than 0.05, so bile acid was the reflux component that had the most significant effect on the incidence of LPR. From both ANOVA and logistic regression tests, it that appears bile acid plays the biggest role in diagnosing LPR.

Table 6. Test of ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Pepsin	Between Groups	207.856	1	207.856	2.704	.108
	Within Groups	3227.954	42	76.856		
	Total	3435.810	43			
Tripsin	Between Groups	4469.230	1	4469.230	2.325	.135
	Within Groups	80737.776	42	1922.328		
	Total	85207.006	43			
Empedu	Between Groups	408.865	1	408.865	6.436	.015
	Within Groups	2668.106	42	63.526		
	Total	3076.971	43			

Table 7. Test of Regression Logistic

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	H.Pylori	1.584	.936	2.865	1	.091	4.873
	Tripsin	-.059	.046	1.642	1	.200	.942
	Pepsin	.416	.247	2.835	1	.092	1.516
	Empedu	-.153	.063	5.960	1	.015	.858
	Constant	-.552	1.903	.084	1	.772	.576

a. Variable(s) entered on step 1: H.Pylori, Tripsin, Pepsin, Empedu.

4. DISCUSSION AND CONCLUSION

In this study, it was found that LPR patients were more common in women than in men, with 12 women and 10 men. From this data, the percentage ratio between women and men is 54% and 45%. This result is similar to a previous study conducted by Junaid et al., which found that the incidence of LPR is higher in women, which is 56.9%. Junaid et al. conclude that gender differences are not significantly related to the development of LPR disease [11]. The mean age of LPR patients in this study was 43.7 years (range: 23-66 years). This number is slightly higher than the research of Montasir J et al, who found the average age of LPR patients was 41.8 10.1 years. Another study conducted by Silva et al., found a higher average age in LPR patients, which was 47.2 years, from the age of 29 years to 73 years. All of these studies show that the average age of LPR patients globally is above 40 years [12], [13]. Analysis of the characteristics of respondents in this study explained that there was no significant difference between gender in the incidence of LPR. From the age characteristics, the incidence of LPR varies greatly, starting in the second decade, with the average LPR sufferer over 40 years of age.

Abnormal secretion and activation of pepsin play an important role in the pathogenesis of LPR. Pepsin is converted from pepsinogen produced by gastric main cells and causes proteolysis and cell damage. Pepsin is activated at pH 2.0–6.0, but at pH 4.0, it may cause mucosal injury. Pepsin levels in saliva were observed to be higher in the LPR group than in the non-LPR group in this study. In healthy people, pepsin cannot be detected in the laryngeal mucosa. Pepsin is only produced in the stomach, so the presence of pepsin in saliva indicates that there is a pathological reflux. Pepsin can downregulate E-cadherin and reduce cell adhesion, these can cause the release and accumulation of catenin from the cell membrane into the cytoplasm, which will likely increase the possibility of tumor cell infiltration and metastasis. Pepsin is reactivated by re-exposure to an acidic atmosphere and can cause mitochondrial damage and increase the risk of malignancy [14]. The pepsin levels with varying results in previous studies were due to differences in the number of samples, the number of saliva samples collected, the time of saliva collection, and the method of pepsin detection, as well as the criteria for diagnosing LPR [15]. Saliva pepsin levels were observed to be higher during awake hours than at other times. Fasting saliva pepsin levels are more suggestive of LPR than non-fasting saliva pepsin levels [16].

Trypsin is secreted by pancreatic cells in the form of zymogen. Trypsin has an optimal pH range of 7.5–8.5, so the use of Proton Pump Inhibitors (PPIs) as a treatment for LPR, which causes an increase in pH, can increase trypsin activity. The presence of trypsin outside the stomach indicates reflux from the duodenum, as trypsin is not present in the upper aerodigestive tract. To be able to cause damage to the upper aerodigestive tract, trypsin must pass through the stomach without being activated [17]. In this study, it was found that the LPR group had higher trypsin levels in their saliva compared to the non-LPR group. Trypsin

ISSN: 0005-2523
Volume 62, Issue 07, September, 2022

is the most effective activator of proteinase-activated receptor-2 (PAR-2). PAR-2 is involved in intestinal inflammation and neurogenic inflammatory epithelial responses. It is expressed in esophageal epithelial cells, odontoblasts, sinus epithelial ciliated cells, and others. Activation of PAR-2 by trypsin affects the regulation of the lower esophageal sphincter (LES). LES dysfunction underlies the pathogenesis of LPR [14]. An analysis of trypsin in saliva is detected together with the examination of pepsin in saliva.

Reflux from the duodenum and stomach contains bile acids and pancreatic secretions, this reflux can reach and make contacts with the larynx. The cause of unsuccessful reflux treatment in patients with LPR is that biliary reflux can also reach the upper aerodigestive tract. In this study, it was found that the LPR group had higher bile acid levels in their saliva compared to the non-LPR group. Bile acid levels were positively correlated with symptom severity, risk of LPR, and risk of laryngeal cancer in patients with LPR. Bile reflux is a major cause of inflammation and increases the risk of laryngotracheal stenosis, tracheal fibrosis, and laryngotracheal malignancy [14]. Conjugated bile acids can cause mucosal damage at low pH (1.2 to 1.5). The bile acid, chenodeoxycholic acid, is activated at the pH of 7 and inactive at the pH of 2. An experimental study showed that, at an acidic pH, conjugated bile acids are more harmful to the mucosa, while chenodeoxycholic acid is active at pH 5 to 8 [19]. When gastric reflux reaches the pharyngeal-laryngeal tract, it mixes with saliva, and its pH is increased by bicarbonate. An experimental study by Ali et al. found that bile acid does not impair pepsin activity at pH > 2. [13].

The majority of patients (58.6%) had reflux that was predominantly a mixture of acidic and basic substances. It was observed that patients with mixed reflux and alkaline LPR significantly had higher RSI and RFS scores than those with pure acid. The results of this study support the hypothesis that bile acids can cause pharyngeal-laryngeal mucosal damage independently and synergistically with pepsin and HCl. The salivary bile acid test is found clinically useful in the management of LPR to identify patients with more aggressive reflux disease. High salivary bile acids (> 1 mol/L) have a high risk of having a history of upper airway malignancy with an odds ratio of 2.8 [13]. Duodenal and gastric components can be mixed effortlessly in patients who had undergo biliary-enteric anastomosis. The concentration of bile acid in the duodenum ranges between 10 mM and 22 mM. Conjugated bile acids are detected in gastric reflux while unconjugated ones are rarely found [20].

In conclusion, we reported that there were elevated amount levels of pepsin, trypsin, and bile acids in the saliva of LPR patients compared to the control group. However, after statistical analysis, only bile acids in saliva were proven to be meaningful for the diagnosis of LPR. Further research is needed to find out the associations between these enzymes to the incidence of LPR using other methods of sampling.

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