

## Research

## Analysis of bile acid in saliva of patients with laryngopharyngeal reflux and non-laryngopharyngeal reflux

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### ABSTRACT

**Background:** 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 (potential of Hydrogen) value, also because of exposure to harmful enzymes in reflux, including bile acid. **Purpose:** To analyze bile acid levels in the saliva of LPR patient and non-LPR subject. **Method:** An observational study with a case-control design. The study was conducted in the Department of Otorhinolaryngology Head and Neck Surgery, Dr. M. Djamil Hospital, Padang, West Sumatra, Indonesia. The total sample size was 44 subjects, consisted of 22 healthy subjects as the control group, and 22 patients suspected of having LPR. **Result:** LPR patients were more common in female than in male, with 12 women and 10 men. Bile acid in the LPR group mean was  $25.08 \pm 7.67 \mu\text{M}$ , meanwhile, in the healthy group, the mean was  $18.99 \pm 8.26 \mu\text{M}$ . There was a statistically significance in the incidence of LPR with bile acids ( $p=0.015$ ) based on t- independent test. **Conclusion:** This study confirmed that bile acids in saliva played a major role in diagnosing LPR.

**Keywords:** bile acid, laryngopharyngeal reflux, ELISA

### ABSTRAK

**Latar belakang:** Laryngopharyngeal reflux (LPR) adalah aliran balik cairan lambung dan/ atau duodenum ke dalam laring, faring, trakea, dan bronkus. Prevalensi LPR sulit ditentukan karena baku emas yang terbatas, dan dikarenakan gejala LPR sangat bervariasi. Kerusakan dapat terjadi karena penurunan nilai pH (potential of Hydrogen), yang berarti peningkatan keasaman, dan juga karena paparan enzim berbahaya dalam refluks, termasuk asam empedu. **Tujuan:** Untuk menganalisis kadar asam empedu pada saliva pasien LPR dan subjek non-LPR. **Metode:** Penelitian observasional dengan desain case control. Penelitian dilakukan di Bagian THT-KL RSUP Dr. M. Djamil, Padang, Sumatera Barat, Indonesia. Jumlah sampel sebanyak 44 orang, terdiri dari 22 subjek sehat sebagai kelompok kontrol dan 22 pasien yang diduga menderita LPR. **Hasil:** Penderita LPR lebih banyak terjadi pada wanita dibandingkan pria, dengan 12 wanita dan 10 pria. Rerata kadar asam empedu pada kelompok LPR adalah  $25,08 \pm 7,67 \mu\text{M}$ , sedangkan pada kelompok non-LPR rerata  $18,99 \pm 8,26 \mu\text{M}$ . Terdapat hasil yang signifikan secara statistik antara LPR dengan kadar asam empedu ( $p \text{ value} = 0,015$ ) berdasarkan uji t-independen. **Kesimpulan:** Studi kami mengkonfirmasi bahwa asam empedu dalam saliva memiliki peran penting dalam mendiagnosis LPR.

**Kata kunci:** asam empedu, laryngopharyngeal reflux, ELISA

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## INTRODUCTION

Laryngopharyngeal reflux (LPR) is the backflow of gastric and/or duodenal fluid into the larynx, pharynx, trachea, and bronchi.<sup>1</sup> The fluid would contact the mucous membrane of the upper airway and aerodigestive tract, causing symptoms such as hoarseness, cough, globus sensation, throat clearing, and post-nasal drip.<sup>2</sup> 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.<sup>3</sup>

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.<sup>4</sup> It is important to know the pH (potential of Hydrogen) of the reflux fluid. *Ambulatory 24-hour double-probe pH-metry* examination is the gold standard to diagnose LPR. However, the sensitivity of pH-metry examination was reported only about 50%-80%. 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.<sup>5</sup>

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 suggested that the reflux of gastric fluid and its contents into the laryngopharynx contributed to the pathophysiology of nonspecific inflammatory, and neoplastic disorders.<sup>6</sup> The bile acids had been reported to have the potential to be the diagnostic markers of LPR.<sup>7</sup> Bile acid levels were found to be up to three times higher in LPR patients than in the normal group.<sup>8</sup> Bile acid examination in saliva was proven to be a useful diagnostic value. Bile acid content >1 mmol/L was considered the most suitable to describe the severity of LPR with a sensitivity of 86%. The method for examining bile acid was *Enzyme-Linked Immunosorbent Assay* (ELISA).<sup>9</sup>

With the existing diagnostic modalities, the diagnosis of LPR was often based on the signs and symptoms found in patients, which was highly subjective. Future diagnostic approaches should address the clear relationship between clinical signs and symptoms, such as the MII pH 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 was a consistent association between reflux component levels and the diagnosis of LPR.<sup>8,9</sup>

This study was conducted to analyze bile acid levels in the saliva of LPR patients and non-LPR subjects, so that diagnosis of LPR could be established simply by saliva examination.

## METHOD

This was an observational study with a case-control design, 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 subjects, consisted of 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; and participants with RSI values ≤13 and RFS ≤7 were not diagnosed as LPR subjects.

The inclusion criteria were patients who were willing to be included in the study by signing informed consent, and 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 were saliva that could not be further examined by the immunoassay method (ELISA) because they were damaged, based on considerations from Biomedical laboratory installation. The sample for this study was 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. Number 315/UN.16.2/KEP-FK/2021.

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.<sup>10</sup>

The data analysis in this study was presented in the form of tables. Analysis of differences in the mean levels of bile acids measured in μM between the LPR group and the comparison group (non-LPR) using independent T-test if the data were normally distributed, and using Mann Whitney test if the data were not normally distributed. The results were analyzed with SPSS software with a p value of 0.05 being considered statistically significant.

## RESULT

The LPR group had the most females, with up to 12 subjects, 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 subjects; with a mean age of 24.6±3.01 years; mean RSI of 1.45±2.67; and RFS of 0.23±0.70.

In the LPR group, bile acid levels in saliva were higher 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 1. Characteristic of LPR and non-LPR based on gender**

Characteristic	LPR		Non-LPR		Total
	n	%	n	%	
<b>Gender</b>					
Male	10	45	7	31.82	17 (38.64%)
Female	12	54	15	68.18	27 (61.36%)
<b>Total</b>	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

**Table 3. Characteristic of bile acid in saliva of LPR patient and non-LPR**

Group	Bile acid ( $\mu\text{M}$ )		p
	Mean $\pm$ SD	(Min – Max)	
LPR	25.08 $\pm$ 7.67	(7.856–47.436)	0.015
Non-LPR	18.99 $\pm$ 8.26	(1.286–29.409)	

## DISCUSSION

In this study, it was found that LPR patients were more common in female than male, with 12 women and 10 men. From this data, the percentage ratio between female and male was 54% and 45%. This result was similar with a previous study conducted by Junaid et al.<sup>11</sup>, which found that the incidence of LPR was higher in women, which was 56.9%. They concluded that gender differences were not significantly related to the development of LPR disorder. The mean age of LPR patients in our study was 43.7 years (range: 23-66 years). This number was slightly higher than the research of Junaid et al.,<sup>11</sup> who found the average age of LPR patients was 41.8 $\pm$ 10.1 years. Another study conducted by Silva et al.<sup>12</sup>, 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 showed that the average age of LPR patients globally was above 40 years.<sup>11,12</sup> Analysis of the characteristics of respondents in our 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.

Reflux from the duodenum and stomach contains bile acids and pancreatic secretions, and 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 our 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.<sup>13</sup> Conjugated bile acids could cause mucosal damage at low pH (1.2 to 1.5). The bile acid, primarily contained 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 were more harmful to the mucosa, while chenodeoxycholic acid is active at pH 5 to 8.<sup>13</sup> In the study of Sereg-Bahar et al.,<sup>14</sup> three control subjects (6.3%) had bile acid levels in saliva above 2.1 mol/L, which was the average bile acid value in RLF patients. Previous studies from De Corso et al.<sup>9</sup> had shown that conjugated bile salts could

reduce the proteolytic activity of pepsin at pH 2. When gastric reflux reaches the pharyngolaryngeal tract, it mixes with saliva, and its pH is increased by bicarbonate. An experimental study by Ali et al.<sup>15</sup> found that bile acid did not impair pepsin activity at pH >2.

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 our study supported the hypothesis that bile acids could cause pharyngeal-laryngeal mucosal damage independently and synergistically with pepsin and HCl. The salivary bile acid test was found clinically useful in the management of LPR to identify patients with more aggressive reflux disorder. High salivary bile acids (>1 mol/L) had a high risk of having a history of upper airway malignancy with an odds ratio of 2.8.<sup>8,9</sup> Duodenal and gastric components could be mixed effortlessly in patients who had undergone biliary-enteric anastomosis. The concentration of bile acid in the duodenum ranged between 10 mM and 22 mM. Conjugated bile acids were detected in gastric reflux while unconjugated ones were rarely found.<sup>15</sup>

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

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