The effect of parotid salivary flow rate on the levels of salivary antimicrobial proteins in patients with Sjögren's syndrome

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Objective: The purpose of this study was to examine the effect of salivary flow rate on the levels of antimicrobial salivary proteins in 24 patients with Sjögren's syndrome and 22 age- and race-matched healthy control subjects. Method and materials: Parameters examined included stimulated salivary flow rate, total salivary protein, lactoferrin, lysozyme, amylase, and secretory immunoglobulin A. Results: The mean total salivary protein and the mean salivary amylase were significantly greater in patients than in controls. However, no significant difference was observed in the mean stimulated salivary flow rates or the levels of lactoferrin, lysozyme, or secretory immunoglobulin A of patients and controls. To examine the effect of salivary flow rate on the levels of salivary antimicrobial protein, the levels of these proteins in patients with salivary flow rate of < 0.3 mL/min per gland were compared to those in healthy controls with salivary flow rate > 0.4 mL/min per gland. Analyses showed the levels of lactoferrin to be significantly higher among patients than among controls. Conclusion: The levels of salivary amylase and lactoferrin may be influenced by the levels of salivary output in patients with Sjögren's syndrome. The relationship between salivary flow rate and the levels of amylase and lactoferrin is not clear at the present time. (Quintessence Int 1999;30:700–705)

Key words: antimicrobial salivary protein, salivary flow rate, Sjögren's syndrome

Sjögren's syndrome (SS) is an autoimmune disorder characterized by lymphocytic infiltration of the lacrimal and salivary glands, resulting in xerostomia and xerophthalmia. The presenting oral symptoms of Sjögren's syndrome include xerostomia, recurrent oral infections, impairment of oral functions (such as talking, chewing, and swallowing), and change in taste. Sjögren's syndrome may also affect other exocrine glands, resulting in dry skin, nose, vagina, and/or gastrointestinal and pulmonary problems. It may occur as a primary condition, manifested as oral and ocular dryness only, or as a secondary condition in association with a connective tissue disease, such as rheumatoid arthritis, systemic lupus erythematosus, or progressive systemic sclerosis. Sjögren's syndrome affects primarily middle-aged women, and is the second most common rheumatic disease in the world.

Studies have indicated that people with SS tend to have increased rates of tooth loss, gingival recession, and alveolar bone loss and are at higher risk of having adult periodontitis than are age- and race-matched healthy controls. Patients with SS who have low salivary flow often experience oral infections such as candidiasis and rampant caries because of the change in the oral microflora, salivary flow rate, and salivary composition.
The diagnosis of Sjögren's syndrome requires several clinical and laboratory tests, including a salivary gland biopsy. Currently, salivary gland biopsy is considered the most reliable diagnostic test for Sjögren's syndrome. However, the diagnostic value of the salivary gland biopsy relies on an evaluation of the number of inflammatory cells within the salivary gland. The limitation of this procedure is that it is "disease stage related." In both early and late stages of the disease, the inflammatory cell infiltration is not sufficient to establish a focus score of inflammatory cell infiltration.

Several studies have suggested measurement of salivary output and sialochemistry (chemical analysis of saliva) for the diagnosis of salivary gland diseases. Fluctuation in the levels of antimicrobial proteins such as lysozyme (Lys), lactoferrin (Lf), secretory immunoglobulin A (sIgA), and amylase (Amy) in saliva and tears of patients with SS have been evaluated for potential use in the diagnosis of Sjögren's syndrome.

Salivary antimicrobial proteins play significant roles in preventing oral infections. Both Lys and Lf are present in tears and saliva. They exhibit antimicrobial activity, while the function of lacrimal Amy is unclear at the present time. Lactoferrin is an enzyme that is widely distributed in the exocrine secretions and tissues. Lactoferrin is an iron-binding glycoprotein present in bodily secretions. Secretory immunoglobulin A is the major class of antibodies in external secretions and serves as a first line of defense against bacterial and viral antigens. In saliva, amylase has been shown to possess antibacterial activity; it is also an essential enzyme that facilitates the removal of carbohydrate debris.

Studies have suggested that measurement of the levels of Lf and Lys in tears provides a sensitive method to evaluate lacrimal and salivary gland diseases. However, to date, chemical analyses of saliva and tears are not adequately optimized for clinical application. The purpose of this study was to examine the effect of salivary flow rate (sFR) on the levels of antimicrobial proteins in patients with SS and healthy control (HC) subjects.

**METHOD AND MATERIALS**

**Study population**

Patients were selected from the Salivary Dysfunction Clinic at Baylor College of Dentistry. The diagnoses of Sjögren's syndrome were based on the European Community Criteria. Healthy controls were age- and race-matched individuals with no signs or symptoms of dry mouth or dry eyes.

**Saliva collection and protein determination**

Stimulated parotid saliva (HPS) was collected in chilled microfuge tubes with a Carlson-Crittenden cup as previously described. Briefly, 2% citric acid (approximately 200 µL) was applied to the dorsum of the tongue at 30-second intervals. Ten-minute samples were collected in preweighed tubes, immediately placed in a -20°C labtop cooler, and then stored at -50°C until used. Salivary flow rate (output) was expressed as milliliters per minute per gland.

Total salivary protein was determined using the bicinchoninic acid method as previously described.

**Slot blot analyses**

Salivary Amy, sIgA, Lf, and Lys concentrations were measured with a slot-blot immunoassay procedure and the BIO DOT SF Microfiltration apparatus (BIO-RAD). Lyophilized samples were reconstituted with 200 µL deionized water at predetermined protein concentration. Commercial purified proteins (antigens) were used to generate standard curves (7 to 8 points) for Lf (5 to 400 ng), Lys (100 ng to 1 µg), Amy (10 to 400 ng), and sIgA (5 to 400 ng) (Sigma). All samples were applied in triplicate.

Nitrocellulose membrane (Schleicher & Schuell), 0.2 µm, was washed with washing buffer (0.05% Tween 20 in TRIS-buffered saline [TTBS], pH 7.5). The nitrocellulose was then blocked in 5% BSA, with gentle shaking, overnight at 4°C. After 3 washes (10 minutes each) with washing buffer, the nitrocellulose was incubated with appropriate primary antibody (Sigma) for 2 hours at room temperature, under gentle shaking. The antibodies were diluted with 2 mg/mL bovine serum albumin (BSA) in blocking buffer (1:500 for sIgA, Lf, and Lys and 1:1,000 for Amy). The blot was then washed 3 times with 0.05% TTBS followed by incubation with alkaline phosphatase-conjugated secondary antibody, goat anti-Rabbit immunoglobulin G (Calbiochem-Novabiochem), for 2 hours at room temperature. The blot was then washed 3 times (10 minutes each) and then once in TRIS-buffered saline for 5 minutes to wash away the Tween 20.

Antigen-antibody complexes were visualized by incubating the blots with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate-toluidine salt (Gibco-BRL) in AP Buffer (100 mmol of TRIS, 100 mmol of NaCl, 5 mmol of MgCl₂), pH 9.5, until color developed, approximately 5 to 10 minutes. Development of color was stopped by washing the blot with distilled water. Color intensity was measured using the NIH Image 1.5 software (National Institutes of Health) connected to a videocamera, Hi 8 Handy Cam (Sony). The concentration of each specific protein was deter-
TABLE 1  Demographic comparison and salivary parameters in patients and controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SS</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Male</td>
<td>02</td>
<td>04</td>
</tr>
<tr>
<td>Age (y)*</td>
<td>59 ± 2</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>Flow rate (mL/min)*</td>
<td>0.44 ± 0.04</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>Total protein (mg/mL)*</td>
<td>1.63 ± 0.13</td>
<td>1.15 ± 0.09</td>
</tr>
</tbody>
</table>

SS = Sjögren's syndrome; HC = healthy controls.
*Mean ± SEM.
†Unpaired 2-tailed Student’s t test: P = 0.008.

TABLE 2  Levels of each antimicrobial protein examined (mean ± SD) according to different parameters

<table>
<thead>
<tr>
<th></th>
<th>Amylase</th>
<th>Lactoferrin</th>
<th>Lysozyme</th>
<th>Secretory immunoglobulin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sjögren's syndrome patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µg/mL</td>
<td>731 ± 101†</td>
<td>3.0 ± 1.0</td>
<td>17.0 ± 4.0</td>
<td>59.0 ± 20.0</td>
</tr>
<tr>
<td>µg/mg*</td>
<td>409 ± 40†</td>
<td>1.5 ± 0.5</td>
<td>9.4 ± 1.9</td>
<td>21.0 ± 4.6</td>
</tr>
<tr>
<td>µg/min</td>
<td>332 ± 65†</td>
<td>0.5 ± 0.1</td>
<td>5.0 ± 0.7</td>
<td>11.0 ± 2.5</td>
</tr>
<tr>
<td>Healthy control subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µg/mL</td>
<td>283 ± 60†</td>
<td>1.0 ± 0.1</td>
<td>8.0 ± 2.0</td>
<td>14.0 ± 4.0</td>
</tr>
<tr>
<td>µg/mg*</td>
<td>192 ± 36†</td>
<td>0.7 ± 0.2</td>
<td>6.2 ± 1.2</td>
<td>12.0 ± 3.0</td>
</tr>
<tr>
<td>µg/min</td>
<td>130 ± 27†</td>
<td>0.3 ± 0.1</td>
<td>4.4 ± 1.1</td>
<td>8.5 ± 2.3</td>
</tr>
</tbody>
</table>

†µg/mg of total salivary protein.
*Unpaired 2-tailed Student’s t test: P = 0.0003.
†Unpaired 2-tailed Student’s t test: P = 0.0075.

mined by comparing the color intensity of the unknown to a standard curve run parallel to the saliva samples. The concentration of individual protein as expressed as micrograms per milliliter of saliva, micrograms per milligram of total salivary protein, and micrograms per minute.

Statistical analyses

The differences in salivary flow rate, total salivary protein, and antimicrobial proteins between SS patients and HC subjects were evaluated using Student’s t test. The Mann-Whitney U test was used when the population was fewer than 20 individuals.

RESULTS

A total of 46 white subjects participated in the study. Of these, 24 were SS patients and 22 were age- and race-matched controls. All of the SS patients had both oral and ocular dryness, at least 1 positive serum autoantibody (SS-A, SS-B, antinuclear antibody, or rheumatoid factor), and/or a positive minor salivary gland biopsy with a focus score of ≥ 1; a focus score is defined as accumulation of 50 lymphocytes per 4 mm². The age range was 25 to 75 years. Healthy controls were age- and race-matched individuals with no signs or symptoms of dry mouth and dry eyes.

Table 1 shows demographic description and salivary parameters of the study population. Despite the observed difference in the mean salivary flow rate between SS patients (0.44 mL/min per gland) and HC subjects (0.52 mL/min per gland), statistical analyses did not reveal the difference to be significant (P = 0.27; Student’s t test). However, a statistically significant difference (P = 0.008) was observed in the total salivary protein levels of SS patients (1.6 mg/mL) and HC subjects (1.1 mg/mL). Ten SS patients (41%) exhibited high total salivary protein (> 170 mg%).

The levels of Amy, Lys, Lf, and sIgA in the two groups were compared (Table 2). Student’s t test revealed that levels of Amy were significantly higher among SS subjects than among HCs (P = 0.0003). No significant difference between patients and controls was observed in the levels of Lys, Lf, and sIgA.
The levels of salivary Amy, Lys, Lf, and slgA were also evaluated in SS patients with sFR < 0.3 mL/min per gland and HC subjects with sFR ≥ 0.4 mL/min per gland (Table 3). The Mann-Whitney U test did not reveal a significant difference between patients and controls in the levels of Amy, Lys, or slgA. However, the level of Lf was significantly higher among SS patients (1.9 µg/mL) than it was among HC subjects (0.6 µg/mL) \( (P = 0.04) \).

### DISCUSSION

Currently, the diagnosis of Sjögren’s syndrome requires a series of serologic tests and a salivary gland biopsy. Several studies have attempted to use siaiochemical analyses of saliva as an alternative, noninvasive test for the diagnosis of Sjögren’s syndrome. However, to date, the conflicting results of previous studies limit their diagnostic value. The purpose of this study was to examine the effect of salivary flow rate on the levels of antimicrobial salivary proteins in patients with Sjögren’s syndrome and healthy controls. To maximize the reliability of the diagnosis Sjögren’s syndrome, a positive serologic result (at least 1 positive autoantibody) and/or a positive salivary gland biopsy were required as key components of the European Community Criteria.

The results of this study showed that the mean sFR for SS patients was lower than that for HC subjects. However, unlike previous studies, statistical analysis did not reveal a difference in the mean sFR to be statistically significant. Tsiano et al. showed a difference in sFR in patients found to have advanced inflammatory disease (based on a minor salivary gland biopsy) but found no difference between patients with early inflammatory disease and people who had negative results on their salivary gland biopsy. The present finding of no difference in sFR could be due to the level of salivary gland destruction or to a study bias, because SS patients with severely reduced salivary output were not included in the study, because the sample volume that they would have been able to provide for these analyses would have been insufficient.

The mean total salivary protein among SS patients was significantly greater than that of healthy controls. This finding is in disagreement with previous studies, which did not show a significant difference in the total salivary protein between SS patients and healthy controls. Differences may be due to the technique used for saliva collection and/or differences in study populations. An increase in total salivary protein was also reported in patients undergoing radiation therapy, which also results in xerostomia.

Slot-blot immunoassay, a modification of dot-blot immunoassay, was used to examine the levels of salivary amylase, lactoferrin, lysozyme, and slgA in saliva of patients with Sjögren’s syndrome and healthy controls. Unlike Western blot analysis, this technique does not require pre-electrophoresis and electrophoretic transfer of the sample, which could result in loss of sample from the additional steps required for Western blotting technique. Furthermore, incomplete transfer of the protein is not uncommon during electrophoretic transfer; such a potential imposes an additional source for loss of sample and may limit the reliability of quantitative estimate of the amount of protein in the sample. Protein transfer is not required in the slot-blot technique because the sample is applied directly to the membrane support.

Further, the slot-blot technique allows several samples to be assayed simultaneously; this allows a direct comparison between different samples on the same blot. The consistency of this technique was verified by the reproducibility of the standard curve that was run with each experiment. Jahn et al. compared the dot immunoblotting technique with the radioimmunoassay. They found that the dot assay offers sensitivity and range of linearity comparable to that of radioimmunoassay. Moreover, Yiannaki et al. found the dot-blot immunoassay to be more sensitive than the enzyme-
linked immunosorbent assay. For these reasons, the slot-blot immunoblot was used to evaluate antimicrobial salivary proteins.

A significant increase in the levels of salivary Amy was observed among SS patients compared to healthy controls. However, no significant difference was observed in the levels of Lf or sIgA. This result is in agreement with that reported by Tsianos et al., who also found no significant difference in sIgA levels. In contrast, several other studies did not find a significant difference in the levels of salivary amylase but did observe a significant increase in the levels of Lf and sIgA among SS patients compared to healthy controls. Other studies have also suggested that the level of salivary Lf is negatively correlated with the total protein and that the level of sIgA is inversely related to sFR. This disagreement could be due, in part, to the fact that total protein concentration was significantly higher among the present SS population than it was among HC subjects. In addition, unlike previous studies, the present study found that the levels of sFR of SS patients were not significantly lower than those of the HCs. Rudney et al. found that comparisons of sIgA concentrations might yield different results, depending on the time of collection, which may offer an additional explanation for the differences observed in various studies. In this study, saliva collection was performed between 10 AM and 12 PM and 2 and 4 PM.

The levels of Lys in SS patients and HC subjects were not found to be significantly different. This finding is in agreement with results of some studies but differs from results of others. These differences could be due to differences in the study population, the selection criteria, or the technique used to measure the concentration of Lys. For example, Moutsopoulos et al. selected SS patients who also had enlarged parotid glands, but such criteria were not used for the current study population.

When the effect of salivary output on the levels of salivary antimicrobial proteins was examined, no significant difference was observed between patients and controls in the levels of Amy, Lys, or sIgA. However, a significant increase in the levels of Lf was observed among SS patients compared to healthy control subjects. This result is in agreement with findings of previous studies. The relationship between reduced salivary output and the level of LF is not clear at the present time. It is possible that the increased level of Lf is a reflection of tissue inflammation or damage. An increased level of Lf was also reported in patients undergoing a course of radiation therapy for head and neck cancer. Lacrimal Lf and Lys have been shown to be independent of tear flow rate, whereas lacrimal sIgA appears to be dependent on the level of tear flow rate.  

Although an increase in the levels of Amy, Lys, and sIgA was observed among SS subjects, analyses did not reveal a statistically significant difference between groups. However, the difference in the levels of Amy approached significance; it is possible that a significant difference would have been observed with a larger study population.

**CONCLUSION**

The levels of antimicrobial salivary proteins (amylase, lactoferrin, lysozyme, and sIgA) in patients with Sjögren's syndrome and healthy controls were compared. Results suggested that the levels of amylase and lactoferrin are affected not only by the disease process but also by the level of salivary output. These findings suggest that salivary flow rate may be an important factor that should be considered when salivary proteins are evaluated in patients with Sjögren's syndrome. The results of this study may explain the inconsistency among previous studies concerning the evaluation of salivary antimicrobial proteins in patients with salivary gland disease.

**REFERENCES**


