J Oral Pathol Med (2005) 34: 198–203
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Assessment of SS-A and SS-B in parotid saliva of patients with Sjögren's syndrome

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BACKGROUND: The purpose of this study was to compare the sensitivity of parotid saliva to that of serum in detecting anti-SSA/Ro and anti-SSB/La autoantibodies in patients with Sjögren's syndrome.

METHODS: Forty patients and 20 controls participated in the study; all patients met the 1993 European Community criteria for the diagnosis of Sjögren's syndrome. Healthy controls were age- and sex-matched individuals with no signs or symptoms of Sjögren's syndrome. Serum and saliva samples were evaluated using AffiniTech SSA/Ro and SSB/La antibodies kits (AffiniTech, Ltd. Bentonville, AR, USA). The results were also compared with serological status of SS-A and SS-B as reported by an independent clinical laboratory.

RESULTS: Serum was significantly more sensitive than saliva in detecting SSA/Ro and SSB/La antibodies ($P = 0.001$). There was high agreement between the results with the AffiniTech kits and the independent laboratory (kappa = 0.80; $P < 0.001$). However, there was poor agreement between saliva and serum results (kappa = 0.174; $P = 0.168$).

CONCLUSIONS: The overall results appear to support that serum analysis is effective method for evaluating the presence of SS-A and SS-B autoantibodies.

J Oral Pathol Med (2005) **34**: 198–203

Keywords: autoantibodies; diagnosis; saliva; Sjögren's syndrome; SS-A; SS-B

Introduction

Sjögren's syndrome (SS) is a chronic inflammatory disease of the exocrine glands characterized by dry mouth and dry eyes. It was estimated that SS affects 1–3% of the general population with approximate frequency of 1:5000 (1). Although it has been reported in children, SS is frequently manifested in middle-aged women with female to male ratio of 9:1 (2). The exocrine manifestations of SS may include the eyes, mouth, larynx, nose, trachea, the lungs, pancreas, hepatobiliary system, the gastrointestinal tract, vagina, kidneys and skin (3). SS may also occur with a broad spectrum of systemic manifestations such as inflammatory vascular disease, Raynaud's phenomenon, excessive tiredness, interstitial lung disease, autoimmune endocrinopathies, and renal tubular acidosis (2, 4).

Approximately 60–70% of SS patients may produce autoantibodies in their serum (5). Among these autoantibodies are SSA and SSB autoantibodies. The prevalences of these autoantibodies, SSA/Ro and SSB/La, have been reported to range between 13–88% and 48–73%, respectively, in sera of patients with primary SS. Both SSA and SSB antigens are RNA binding proteins (5). Anti-SSA/Ro antibodies react with a 60-kDa nuclear protein, and sometimes react with a 52-kDa polypeptide (6). The SS-B antigen is a 48-kDa nuclear protein (5). It has been suggested that SS patients with positive SSA and/or SSB may have earlier disease onset and increased disease severity. Manoussakis et al. evaluated the serological profiles for patients with primary SS and compared them to rheumatoid arthritis patients with and without SS (7). They found that anti-SSA/Ro and anti-SSB/La correlated with earlier disease onset, longer disease duration, recurrent parotid gland enlargement, and extraglandular manifestations. Currently, the presence of anti-SSA and/or anti-SSB autoantibodies in patients sera is one of the criteria for the diagnosis of SS (1, 8).

Previous studies have demonstrated the presence of SSA/Ro and/or SSB/La autoantibodies in saliva (9–13). The identification of SSA/Ro and SSB/La autoantibodies in saliva in the presence and absence of circulating antibodies in the serum has suggested that saliva may be useful for evaluating these autoantibodies. However, these studies were performed on whole saliva and the authors did not address possible serum contamination of their samples. The purpose of this study was to compare the sensitivity and specificity of glandular

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Accepted for publication October 18, 2004

(parotid) saliva to that of serum in detecting anti-SSA/Ro and anti-SSB/La autoantibodies in a cross-sectional group of SS patients and healthy controls.

Materials and methods

Study population

The study population consisted of three groups. Group 1, consisted of SS patients with a history of positive SS-A/Ro and/or SS-B/La antibodies (based on serological testing by a commercial clinical laboratory). Group 2, consisted of SS patients with history of negative anti-SSA/Ro and anti-SSB/La antibodies. Group 3, consisted of age- and sex-matched healthy individuals with no signs or symptoms of SS. Patients with SS were selected randomly from the Salivary Dysfunction Clinic at Baylor College of Dentistry. The diagnosis of SS was based on the European Community Criteria (1). Individuals with a history of radiation therapy to the head and neck region, chemotherapy, corticosteroids therapy, acquired immunodeficiency syndrome, pre-existing lymphoma, primary biliary cirrhosis, amyloidosis, sarcoidosis, and graft-vs.-host disease were excluded from the study.

All study participants signed an informed consent (which was approved by the Baylor College of Dentistry Institutional Review Board), and completed a questionnaire addressing symptoms of generalized exocrinopathy and other systemic illnesses (4).

Saliva collection

To standardize saliva collection, patients were instructed to cease eating, smoking, and drinking (except water) for 2 h prior to saliva collection. All collections were performed between 9:00 AM and 12:00 PM or 1:00 and 2:30 PM. Stimulated parotid saliva was collected with a Carlson-Crittenden cup (14). Briefly, 2% citric acid (200 μl) was applied to the dorsum of the tongue at 30-s intervals. Ten-minute samples were collected into chilled, pre-weighed microfuge tubes and stored at −50°C, until used. Salivary flow rate was expressed as millilitre per minute per gland.

Protein determination

Total salivary protein was determined using the Bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) (14). Analyses were performed on 10 μl aliquots of saliva, in duplicate, using bovine serum albumin for a standard. Total salivary protein was expressed as percent milligram.

Serum collection

Serum collection was performed within 15 min of saliva collection. Blood samples were collected into Vacutainer® containing SST® gel and clot activator (Becton Dickinson, NJ, USA). The samples were allowed to stand at room temperature for at least 30 min before separating the serum by centrifugation at $2000 \times \boldsymbol{g}$, using a bench centrifuge, for 15 min. The samples were then treated with 0.1% sodium azide (Sigma, St Louis, MO, USA) and stored at 4°C, until used.

Antibody assay

Anti-SSA/Ro and anti-SSB/La antibodies were examined using AffiniTech kits, according to manufacturer's instructions (AffiniTech, Ltd. Bentonville, AR, USA). The kit contains purified SSA/Ro or SSB/La antigens that were immobilized on nitrocellulose strips by Western blot. Using this kit, antigen/antibody complexes appeared as bands at approximately 60 kDa for SSA, and 43 kDa for SSB. To ensure validity of the comparison, both serum and saliva samples were examined simultaneously, in parallel, for each assay.

Statistical analysis

Salivary parameters (flow rates and total protein) were compared between the three groups using a one-way ANOVA. Student–Newman–Keuls test of multiple comparisons was used to assess pairwise differences in salivary parameters among the three groups.

The reliability of the AffiniTech kit in accurately detecting anti-SSA/Ro and/or anti-SSB/La antibodies was evaluated using the following equation:

$$\text{Sensitivity} = \frac{a}{b} \times 100\%$$

where a represents SS patients who were positive for SSA/SSB using the AffiniTech kit and b represents SS patients who were positive for SSA/SSB based on clinical laboratory results (assuming that the results of the clinical laboratory were the 'true' results). The specificity was calculated using the following equation:

$$\text{Specificity} = \frac{a}{b} \times 100\%$$

where a represents healthy controls who were negative for both autoantibodies tested using the AffiniTech kit and b represents the total number of healthy controls.

The agreement between results of the clinical laboratory and the AffiniTech kit, and the agreement between salivary and serum results were examined using McNemar's test. Agreement was also quantified with the kappa statistic.

Results

Three groups (1–3) of subjects participated in the study. Group 1 and 2 were patients with SS, group 3 were healthy controls. All SS patients met the European Community Criteria for the diagnosis of SS; they all had dry mouth, dry eyes, at least one positive autoantibody (antinuclear antibody, rheumatoid factor, SS-A or SS-B) and/or a positive minor salivary gland biopsy (1). Twenty-nine patients had positive ANA, 20 patients had positive SS-A, 17 patients had positive RF, 12 patients had positive SS-B, and 28 patients had positive salivary gland biopsy. The majority of the patients, 37 of 40 (92.5%), were primary SS and only three (7.5%) patients had secondary SS; of these, one had scleroderma, one had rheumatoid arthritis, and one had CREST (*calcinosis*, *Raynauds*, *esophageal motility*

Table 1 Demographics of the study population

Group	*Age (years) (mean ± SD)*	*Gender (female/ male)*	*Disease status (primary/ secondary)*	*Total*
1 – SSA/SSB (+)	52.2 ± 10.2	19/1	19/1	20
2 – SSA/SSB (–)	64.2 ± 9.98	17/3	18/2	20
3 – Healthy controls	58.0 ± 7.67	18/2	NA	20

NA, not determined.

disorders, *sclerodactyly*, and *telangiectasia*). The two patient groups consisted of 36 females (90%) and four males (10%), the healthy control (group 3) had 18 females (90%) and two males (10%) (see Table 1). The average age of group 1 was 52.2 years of age (95% CI: 47.4–57.0; range: 36–72). The average age of subjects in group 2 was 64.2 years of age (95% CI: 59.5–68.9; range: 42–82). The average age of subjects in group 3 (healthy controls) was 58.0 years of age (95% CI: 54.4–61.6; range: 43–73). None of the study participants was tobacco smoker.

Figure 1 shows various systemic manifestations of the study population. Both patient groups show similar distributions of hypothyroidism, Raynaud's, migraine, and fibromyalgia. However, the patient group that was SSA/SSB− had a significantly greater proportion with hypertension (40%) compared with the patient group that was SSA and/or SSB+ (5%). This is probably a reflection of the older age of those patients in the SSA/SSB− group compared with the patients in the SSA and/or SSB+ group. Twenty-five percent of healthy controls also had hypertension, although no healthy controls were positive for any of the other systemic manifestations of SS presented in Fig. 1.

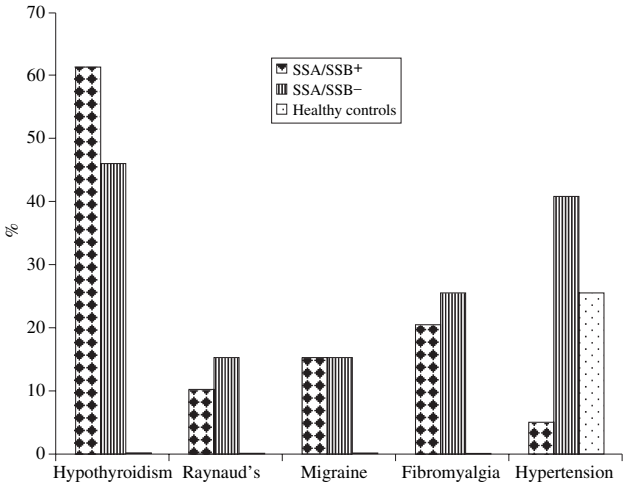

Figure 1 Systemic manifestations. Fisher's exact test. Hypothyroidism – significant difference between patients and controls, $P < 0.001$; Raynaud's – no difference between patients and controls, $P = 0.159$; Migraine – no difference between patients and controls, $P = 0.165$; Fibromyalgia – significant difference between patients and controls, $P = 0.023$; Hypertenson – no difference between patients and controls, $P = 1.000$.

Table 2 Salivary parameters

Group	*n*	*Salivary output (ml/min/gland)*[a] *[mean ± SD (median)]*	*Total protein (mg%)*[a] *[mean ± SD (median)]*
1 – SSA/SSB (+)	20	0.291 ± 0.308 (0.143)	141.0 ± 48.2 (136.0)
2 – SSA/SSB (–)	20	0.360 ± 0.243 (0.318)	215.6 ± 210.3 (113.0)
3 – Healthy controls	20	0.672 ± 0.355 (0.539)	92.3 ± 32.7 (102.5)

[a]One-way ANOVA revealed significant difference between patients and controls, $P < 0.001$. *Post hoc* Student–Newman–Keuls multiple range test indicated no significant difference between the two patient groups (group 1 and group 2).

Table 2 shows the salivary flow rate and protein levels by group. Based on one-way ANOVA's, both salivary flow rate and protein levels varied significantly by group ($P < 0.001$). In addition, based on Student–Newman–Keuls multiple range test, there were significant differences in both salivary flow rate and total protein between patients and control groups. However, no significant difference was observed between the two patient groups (group 1 and group 2) for either salivary flow rate or for total protein.

Table 3 shows the results of serum and salivary antibody testing. Seventeen of the 20 patients (85%) who had previously tested positive by a commercial laboratory for anti-SSA and/or anti-SSB antibody, also had serum that tested positive with the AffiniTech kit, but only seven of them (35%) were positive for salivary antibodies. One group 2 patient and one group 3 healthy control had a positive serum SSB antibody with the AffiniTech kit. However, these were considered false positives because one of them tested negative on a subsequent test by an independent commercial clinical laboratory.

The sensitivity of the AffiniTech kit was 85% for serum, assuming the results of the clinical laboratory were accurate, in contrast, the sensitivity of the kit was only 35% for saliva. The specificity of the AffiniTech kit was 95% for serum and 100% for saliva (none of those testing negative for SSA and SSB previously in group 2 or healthy controls in group 3 tested positive for saliva using the AffiniTech kit). The agreement between the clinical laboratory for serum and the AffiniTech kit was very high with a kappa of 0.80 ($P < 0.001$).

Results of McNemar's analysis indicated that the serum had significantly greater sensitivity than the saliva ($P = 0.002$). The agreement between the saliva and serum in detecting antibodies was poor with a kappa of 0.174 ($P = 0.168$).

Discussion

The sensitivity and specificity of parotid saliva in detecting SSA and SSB autoantibodies was examined in two groups of SS patients and a group of healthy controls. Group 1 consisted of SS patients who had positive serum antibody to SSA/or SSB and/or a positive salivary gland biopsy. Group 2 patients met

Table 3 Comparison of serum and salivary antibody

		AffiniTech kit				*Clinical lab*
		Serum		*Saliva*		
Group	*n*	*SSA+/SSB+*	*SSA+ or SSB+*	*SSA+/SSB+*	*SSA+ or SSB+*	*SSA+/SSB+*
1 – SSA/SSB (+)	20	15/15	17	3/7	7	20/12
2 – SSA/SSB (−)	20	0/1	1	0/0	0	0/0
3 – Healthy controls	20	0/1[a]	1	0/0	0	NA

McNemar's analysis indicated that the serum had significantly greater sensitivity than the saliva ($P = 0.002$). The agreement between the saliva and serum in detecting antibodies was poor, kappa = 0.174 ($P = 0.168$).
[a]One patient who tested positive with the test strips was later tested for serology with a commercial laboratory. The commercial laboratory results were negative. NA, not determined.

the European Community Criteria for the diagnosis of SS, but their serology was negative for both SSA and SSB antibodies. None of the healthy controls had signs or symptoms of SS. The patients also exhibited other clinical manifestations that are commonly reported in association with SS, such as hypothyroidism, Raynaud's phenomenon, fibromyalgia, and migraines (Fig. 1). Sixty percent of group 1 patients and 45% of group 2 patients were using medication for hypothyroidism. These findings are consistent with Perez-E et al. (15), where 45% of their 33 patients with primary SS had thyroid dysfunction. Of these patients, 24% had autoimmune thyroiditis, 6% had autoimmune hypothyroidism and 15% had reversible iodine-induced hypothryoidism. Warfvinge et al. (16) reported a 10-fold increase of primary SS in their patients with autoimmune thyroiditis. They found that 32% of the patients with autoimmune thyroiditis had primary SS. Both authors suggested that thyroid and salivary gland disease might share a common mechanism for pathogenesis.

Raynaud's phenomenon was also reported in 10% of group 1 and 15% of group 2 patients in the current study. These percentages are lower than previous reports, Skopouli et al. (17) found that this non-inflammatory vascular disease was a more common manifestation (33%) in patients with primary SS than was found in our study. Similarly, Kraus et al. (18) also found a high occurrence (29%) of Raynaud's phenomenon in 104 primary SS patients. These patients who had Raynaud's phenomenon, also had other manifestations like arthritis, vasculitis, and pulmonary fibrosis significantly more frequently than those without Raynaud's phenomenon. Both studies found no difference in the autoantibody profiles of patients with primary SS and Raynaud's phenomenon and those SS patients without Raynaud's phenomenon.

Approximately 15% of our study population of SS patients suffered from frequent migraine headaches. Central nervous systems manifestations are common in SS (2). Pal et al. (19) noted that 46% of patients with primary SS suffered from migraines. They also found that a significant association exists between occurrence of Raynaud's phenomenon and migraines, possibly related to small vessel pathology. Escudero et al. (20) also reported that the central nervous system manifestation of migraines was noted in over half of their 48 patients with primary SS. They also found that their primary SS patients also had 29% neuropsychiatric disease, and 23% had a history of focal acute neurological deficits.

In our study, approximately 23% of the SS patients also had fibromyalgia. Vitali et al. (21) report that in their patient group, fibromyalgia was a common occurrence (47%) in primary SS patients. They also noted that the presence of fibryomyalgia in primary SS was related to psychological changes, such as depression. The prevalence of primary SS in fibromyalgia patients has been estimated to be as high as seven times the expected occurrence of SS in the general population (22).

As expected, the stimulated parotid salivary flow rates were significantly lower for patients (group 1 and 2) than healthy controls (group 3) (Table 2). The healthy controls had an average stimulated parotid salivary flow rate of 0.672 ml/min/gland, which is similar to that of other studies (23). The average stimulated parotid salivary flow for group 1 and 2 patients were 0.291 and 0.360 ml/min/gland, respectively. This marked reduction in salivary flow was expected for SS patients. Mason et al. (24) found that stimulated parotid saliva flow rates < 0.50 ml/min/gland were abnormal and suggested that it indicated salivary gland hypofunction.

The protein content in the saliva samples was also significantly different between various study groups (Table 2). Group 2 SS patients had the highest average total salivary protein with 215.6 mg%, which was significantly greater than that of the healthy controls (92.3 mg%), but was not significantly larger than that of group 1 SS patients (141.0 mg%). These results are in agreement with pervious studies. Van der Reijden et al. (25) found similar increase in salivary protein in their study population. They noted that absolute concentrations of total protein were increased significantly in both primary and secondary SS, but they suggested that the observed increase in salivary protein was because of diminished salivary output rather than because of absolute increase in total salivary proteins. Increased total salivary protein levels can also be attributed to the lymphocytic infiltration of the salivary glands in SS (26). Thus, the protein content results of this study is in agreement with findings in the literature.

Anti-SSA/Ro and anti-SSB/La antibodies have been reported in sera of SS patients (27–29). Harley et al. (30) suggested that all SS patients had anti-SSA/Ro and/or anti-SSB/La antibodies in their sera. Utilizing

enzyme-linked immunosorbent assay (ELISA), 71% of patients with primary SS had serum IgG anti-SSA/Ro antibodies and 67% had anti-SSB/La antibodies (31). Using double immunodiffusion technique, Yamagata et al. (32) found that in their Japanese population of 75 SS patients, the frequencies of antibodies to SSA/Ro and SSB/La antigen in sera were significantly higher in SS patients than in patients with connective tissue diseases. SSA/Ro antibodies have been reported to be a consistent indicator of subclinical SS (33). It was suggested that SSB/La antibodies were highly predictive of primary SS. Hansen and Manthorpe (31) reported that the predictive value of primary type of SS in patients with increased levels of sera SSB antibodies was 78%. Manoussakis et al. (7) evaluated the serological profiles for patients with primary SS and compared them to rheumatoid arthritis patients with and without SS. Pease et al. (34) reported that primary SS patients with autoantibodies to SSA/Ro and/or SSB/La antigens have elevated serum IgG levels when compared with those SS patients without these autoantibodies. Other studies suggested that the levels of SSA and SSB correlated with earlier disease onset, longer disease duration, recurrent parotid gland enlargement, and extraglandular manifestations (7) and the level of SS-A/SS-B and smoking (35). In this study none of the patients was a smoker, and only two SS-A/SS-B positive patients and one SS-A/SS-B negative patient had salivary gland swelling.

The SSA and SSB autoantibodies were also reported in saliva of SS patients (9–13). However, these studies were performed on whole saliva and the authors did not address the possibility of serum leakage. Manoussakis et al. (36) demonstrated that the presence of autoantibodies in whole saliva was because of protein leakage from blood to saliva and not from local synthesis of salivary glands. Thus, the results of studies that utilized whole saliva may be because of possible contamination. The use of parotid saliva rather than whole saliva would decrease the opportunities of contamination in the salivary results. The current study utilized a well-characterized population of SS patients. The study examined parotid saliva, not whole saliva, to eliminate possible serum contamination. Both saliva and serum samples were evaluated in duplicate simultaneously.

The results of this study suggest that serum was significantly more sensitive than parotid saliva in detecting SSA and SSB antibodies (Table 3). The result of AffiniTech kit for serum had a significant agreement with the independent clinical laboratory results (kappa of 0.80, $P < 0.001$). All patients who had positive salivary autoantibodies also tested positive for serum autoantibodies. Therefore, our findings do not confirm previous studies which suggested that saliva might be more sensitive than serum in detecting SSA and SSB antibodies. Such a finding does not seem to support either the concept of local synthesis of these autoantibodies or their increased concentrations in the salivary glands (9–13).

Based on our results, serum contamination in whole saliva seems to be the most likely explanation for the detection of these autoantibodies in saliva. Horsfall et al. (13) acknowledged this possibility and attempted to compensate for it by assuming that only a ratio of saliva to serum IgA SSB antibody >1.0 was evidence of salivary enrichment. If the saliva to serum IgA anti-La antibody ratio was >1.0, then they deemed the likelihood of contamination of saliva by serum to be low. However, Manoussakis et al. (36) reported that leakage of SSA and SSB autoantibodies from blood into saliva was likely by examining IgG index and the proportion of albumin in serum and saliva. Considering the difference in total salivary and serum proteins and because equal volumes and dilutions of saliva and serum were used in the test, the amount of salivary protein might not be sufficient for detecting these antibodies.

Finally, the result of AffiniTech kit for serum had a significant agreement with the results of the independent clinical laboratory. While salivary results were not in agreement with the independent laboratory (Table 3). These results confirm the reliability of the AffiniTech kit in detecting SSA and SSB autoantibodies.

Summary

The overall results of this study suggest that serum was significantly more sensitive than parotid saliva in detecting SSA/Ro and SSB/La antibodies. The AffiniTech kit provides a reliable diagnostic test for SS. The salivary parameters (flow rates and total salivary protein) were significantly different between SS patients and healthy controls.

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