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ABSTRACT

Simple and non-invasive saliva-based diagnostics may be useful for the identification, understanding, and monitoring of autoimmune and infectious diseases. Previously, Luciferase Immunoprecipitation Systems (LIPS) were used for sensitive detection of patient serum autoantibodies in Sjögren's Syndrome (SjS), a chronic autoimmune disease affecting the salivary and lacrimal glands. Here we explored the ability of LIPS to diagnose SjS based on IgG autoantibodies in patient saliva. From LIPS testing, anti-Ro60 autoantibodies were detected in the saliva of 70% (19/27) of SjS patients with 96% specificity. Positive anti-Ro60 autoantibodies were also found in 70% of the matched serum samples (96% specificity). LIPS detected Ro52 autoantibodies in the saliva and serum of 67% of SjS patients with 100% specificity. Overall, the autoantibody titers in saliva were approximately 4000-fold lower by volume than serum, but still distinguished seropositive patients from controls. These results suggest that LIPS salivary-based testing for SjS autoantibodies is a practical alternative to serum and compatible with point-of-care testing.

KEY WORDS: Sjögren's Syndrome, saliva, autoantigen.

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INTRODUCTION

Sjögren's Syndrome (SjS) is a chronic autoimmune disorder principally affecting the salivary and lacrimal glands (Fox, 2005). While primary SjS is an isolated disease with a prevalence of approximately 0.5%, secondary SjS occurs in association with connective tissue disorders such as systemic lupus erythematosus (SLE) or rheumatoid arthritis. Current classification criteria for SjS include positive minor-salivary-gland biopsy demonstrating lymphocyte infiltration of the gland (focus score >1), oral and ocular dryness, and the presence of specific autoantibodies (Vitali *et al.*, 2002). Extra-glandular disease can also occur, affecting the lungs, kidneys, and gastrointestinal and peripheral nervous systems.

The major autoantibodies in SjS are directed against SSA and SSB, but these autoantibodies are also found in SLE and other rheumatological diseases (Franceschini and Cavazzana, 2005). The SSA antigen is composed of Ro52 and Ro60 proteins. Ro52 is a member of the tripartite motif family of proteins (Chan *et al.*, 1991), while Ro60 is structurally and functionally unrelated to Ro52 and binds small cytoplasmic RNAs (Green *et al.*, 1998). SSB consists of a single 47-kDa protein, also known as La. Clinical ELISAs to detect SSA and SSB autoantibodies typically demonstrate approximately 70% and 40% sensitivity, respectively (Fox, 2005). Luciferase Immunoprecipitation Systems (LIPS) harness light-emitting *Renilla* luciferase recombinant proteins for the efficient detection of patient antibodies (Burbelo *et al.*, 2010a). Previous studies demonstrated that LIPS can sensitively detect patient autoantibodies in SjS (Burbelo *et al.*, 2009a, 2010b). One advantage of LIPS is that autoantibodies to individual Ro52 and Ro60 proteins can be detected separately (Burbelo *et al.*, 2009b), compared with the native SSA ELISA, which detects only autoantibodies to the complex of both proteins. We also observed that the anti-Ro52 and anti-Ro60 autoantibodies detected by LIPS required significant serum dilution to obtain titers in the linear range (Burbelo *et al.*, 2010b). The ability to detect extremely small amounts of autoantibodies will likely be useful for diagnostics with saliva and other bodily fluids.

While serum- or plasma-based antibody diagnostics for SjS are available, saliva offers a unique advantage, in that it can be collected non-invasively (Kaufman and Lamster, 2002). Antibody-based tests of patient saliva have been developed for several diseases, including *Helicobacter pylori* (Loeb *et al.*, 1997), tumor antigens in oral squamous cell carcinoma (Tavassoli *et al.*, 1998), Type I diabetes (Tiberti *et al.*, 2009), and even SjS (Ben-Chetrit *et al.*, 1993; Hammi *et al.*, 2005). The saliva-based tests previously evaluated in SjS diagnosis used solid-phase ELISAs, which did not match the diagnostic sensitivity of serum

(Ben-Chetrit *et al.*, 1993; Hammi *et al.*, 2005). Here, LIPS was used to evaluate autoantibodies in saliva and matching serum in controls and SjS patients.

MATERIALS & METHODS

Patient Samples

SjS patients used in this study fulfilled the American-European Consensus Group criteria and showed 3 of the 4 objective criteria (Vitali *et al.*, 2002). Participants were recruited from the University of Rochester Medical Center (Salivary Gland Dysfunction Center and from the Sjögren's Clinic of the Rheumatology Division) under IRB-approved protocols. This exploratory study with LIPS was designed to evaluate the possibility of detecting autoantibodies in saliva and was powered based on the assumption that we could detect autoantibodies in the saliva in at least 50% of the serum-autoantibody-positive patients. In total, 27 control individuals and 27 SjS patients were evaluated. Sera were collected at baseline from participants following IRB approval. Whole saliva was collected following IRB approval of the salivary protocol. According to standardized protocols (Navazesh, 1993; Burlage *et al.*, 2005; Denny *et al.*, 2008; Yan *et al.*, 2009), participants were asked to refrain from eating, drinking, smoking, and performing any dental hygiene at least 2 hrs prior to collection, which was performed between 9:00 and 11:00 a.m. Unstimulated whole saliva was collected by participants' spitting into pre-chilled conical tubes over a period of 15 min; saliva was then mixed with a protease inhibitor cocktail in 0.1 M Tris-HCl, pH 7.4, 0.1 M ϵ -aminocaproic acid, 0.05 M Na-EDTA, while parotid and submandibular/sublingual saliva samples were collected under stimulation with a Lashley cup-like device (Lashley, 1916) or a Block and Brottman collector, respectively (Block and Brottman, 1962), and 0.4% citric acid for a period of 30-120 min. Samples were stored at -80°C .

LIPS Analysis

LIPS was performed in a 96-well plate format as described (Burbelo *et al.*, 2010a). For each test, a 1- μL equivalent of serum or a 5- μL quantity of patient saliva was used. Additional sera dilutions were required for anti-Ro60 assays. Plates were washed on a Tecan Hydroflex (Tecan Systems, San Jose, CA, USA), and light units (LU) were measured in a Berthold LB 960 Centro luminometer (Berthold Technologies, Bad Wildbad, Germany) with coelenterazine mix (Promega, Madison, WI, USA). LU data were the average of at least two independent experiments.

Statistical Analysis

GraphPad Prism software (San Diego, CA, USA) was used for statistical analysis. Mann-Whitney *U* tests were used to compare antibody titers among the different groups. Cut-offs for sensitivity and specificity were determined by optimal separation based on receiver operator characteristics (ROC).

RESULTS

LIPS Detection of anti-Ro60 Autoantibodies in SjS Patient Saliva and Serum

Evaluation of a pilot set of saliva samples for anti-Ro60 autoantibodies by LIPS showed that 5 μL was sufficient to generate robust autoantibody titers (data not shown). Next, serum and saliva from a cohort of SjS patients ($N = 27$) and healthy control individuals ($N = 27$) were evaluated. While the geometric mean titer (GMT) of the saliva from healthy control individuals for Ro60 was 10,600 light units (LU) [95% confidence interval (CI): 8,150-13,800], the SjS cohort had a 10-fold higher GMT of 144,300 LU (95% CI: 68,120-306,000) (Fig. 1A). A Mann-Whitney *U* test showed a marked difference in autoantibody titers between SjS and control groups ($P < 0.0001$). With a cut-off based on optimum separation *via* ROC (63,570 LU), LIPS displayed 70% (95% CI: 50%-86%) sensitivity and 96% specificity (95% CI: 81%-100%) for the diagnosis of SjS with whole saliva (Fig. 1B). To rule out the possibility of blood contamination as a source of autoantibodies, we examined saliva taken directly from the submandibular/sublingual and parotid glands in a small number of samples ($N = 5$). While the anti-Ro60 autoantibody titers in these pure salivary gland secretions were lower than in whole saliva, four of the five SjS patients still showed highly detectable autoantibodies (data not shown). These results suggest that at least some of the autoantibodies detected in saliva are likely not derived from blood.

Anti-Ro60 autoantibody titers were also evaluated in parallel in serum samples from the same 27 SjS patients and 27 healthy control individuals. With a 1:200 serum dilution, the GMT of the control group was 18,400 LU (95% CI: 12,200-27,700), while the GMT of the SjS group was 398,900 LU (95% CI: 159,600-997,000) (Fig. 1C). From LIPS testing of both saliva and serum, a single healthy control outlier was detected. Nevertheless, identical to the saliva studies, with a cut-off of 292,400 LU, LIPS analysis of serum anti-Ro60 autoantibodies demonstrated 70% sensitivity (95% CI: 50%-86%) and 96% specificity (95% CI: 81%-100%) for diagnosis of SjS. Although the saliva anti-Ro60 titers did not correlate quantitatively with the titers measured in serum ($r_s = 0.23$), every anti-Ro60 autoantibody-seropositive patient was also positive based on saliva, and both sources showed the identical diagnostic performance (Fig. 2). These results suggest that the LIPS saliva anti-Ro60 autoantibody test provides diagnostic information as valuable as that provided by serum.

LIPS Detection of anti-Ro52 Autoantibodies in SjS Patient Saliva and Serum

From Ro52 autoantibody testing in the same cohort, the GMT of the healthy controls was 56,330 LU (95% CI: 45,470-69,790), while the GMT of the SjS patients was approximately 10-fold higher at 497,600 LU (95% CI: 295,200-838,900) (Fig. 3A). With a cut-off based on optimum separation of control individuals *vs.* SjS patients of 317,900 LU, the LIPS Ro52 saliva test yielded a sensitivity of 67% (18/27, 95% CI: 46%-83%)

(Fig. 3A). Although the sensitivity of the Ro52 test was lower than that of the Ro60 test, no false-positives were detected (100% specificity, 95% CI: 87%-100%). We also examined Ro52 autoantibody titers in submandibular/sublingual and parotid gland secretions from five SjS patients in the cohort and found that three of the five patients had autoantibody titers above the cut-off for whole saliva, suggesting again that at least a portion of autoantibodies detected in whole saliva may be derived from these pure salivary gland secretions (data not shown).

LIPS testing of the corresponding serum samples for anti-Ro52 autoantibodies utilizing an N-terminal Ro52 deletion fragment (Ro52- Δ 1) that did not require serum dilution (Burbelo *et al.*, 2010b) revealed a GMT of 4,450 LU in the control group and 151,000 LU in the SjS patient group (Fig. 3B). A cut-off of 252,100 LU, derived by optimum separation by ROC analysis, revealed 67% sensitivity (18/27, 95% CI: 46%-83%) with the LIPS test for Ro52 autoantibodies. Similar to the Ro60 results in saliva and serum tests, there was no quantitative correlation between serum and saliva autoantibody titers ($P = 0.2$, $r_s = 0.3$). These results demonstrate that the saliva anti-Ro52 autoantibodies are also highly informative for the diagnosis of SjS.

DISCUSSION

Although analysis of biomarkers in saliva could represent a valuable approach to the diagnosis and monitoring of disease (Garcia and Tabak, 2009), few studies and technologies exploit this non-invasively obtained fluid as a source of diagnostically informative biomarkers. Here, the utility of saliva in LIPS testing was demonstrated in the detection of IgG salivary autoantibodies for the diagnosis of SjS. Our attention focused only on detecting salivary anti-Ro52 and anti-Ro60 autoantibodies by LIPS because of our previous work demonstrating extraordinarily high levels of serum autoantibodies to these two antigens (Burbelo *et al.*, 2010b). From testing either Ro60 or Ro52 autoantibodies in saliva, LIPS showed approximately 70% sensitivity and nearly 100% specificity. Nevertheless, 35% of the SjS patients tested had undetectable autoantibodies to Ro52 and Ro60, which is consistent with results of other studies showing that a significant subset of SjS patients lack detectable SSA autoantibodies (Fox, 2005). The lack of even low levels of autoantibodies in the saliva of seronegative SjS patients further confirms that these

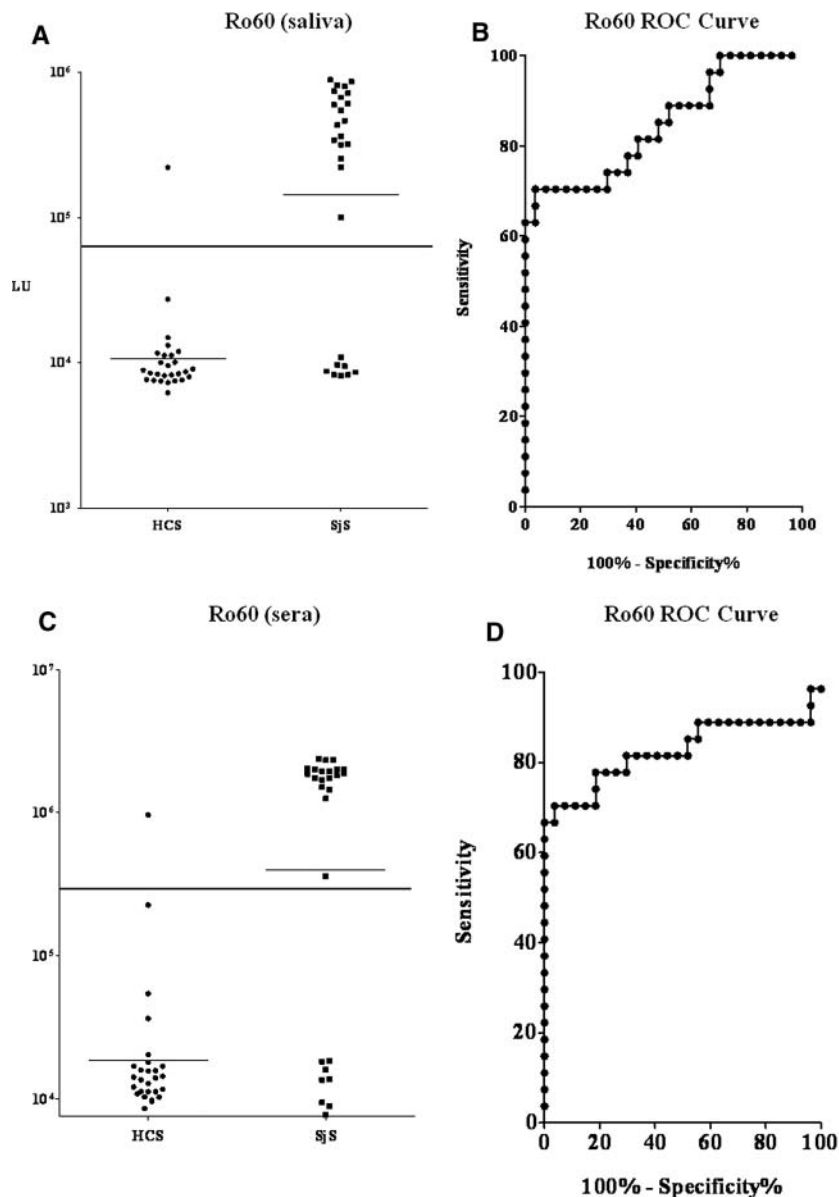


Figure 1. LIPS detection of anti-Ro60 autoantibodies in saliva and sera. SjS patients' (N = 27) and healthy control individuals' (N = 27) saliva (A) and sera (C) were evaluated for anti-Ro60 autoantibodies by LIPS. Each circle or square symbol represents an individual healthy control or SjS patient sample, respectively. A cut-off, shown by the long solid line (A and C), was calculated by ROC analysis for saliva (B) and sera (D). The short solid lines indicate the geometric mean titer of each group.

patients do not have antibodies to these antigens and may represent a distinct subset of SjS. Since it is formally possible that autoantibodies to unique targets in the salivary gland might be relatively enriched in saliva compared with serum, future studies directed at using saliva for autoantigen discovery are needed.

A major advantage of the highly quantitative LIPS assay is its ability to generate highly robust signals using saliva. For example, anti-Ro60 autoantibody titers detected by LIPS in seropositive patients were over 400-fold higher than the healthy control individuals. Although SjS patients show a decreased rate

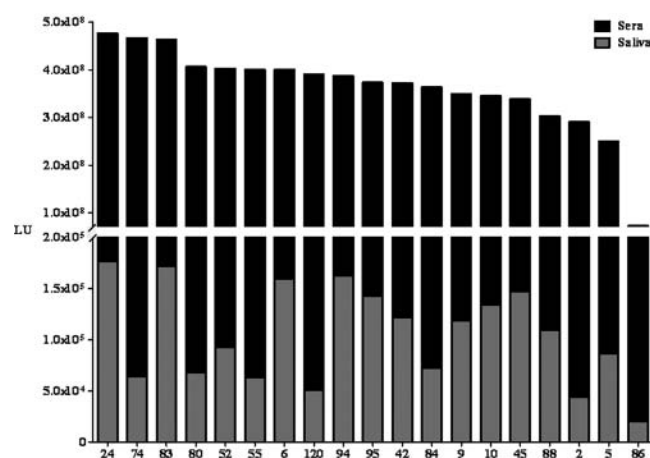


Figure 2. Comparison of anti-Ro60 autoantibody titers in sera and saliva in SjS patients. Titers for 19 SjS patients with significant anti-Ro60 autoantibody titers in both sera (black bars) and saliva (grey bars) are shown. Patients are shown in rank order by serum anti-Ro60 autoantibody titer. Patient numbers are shown on the x-axis.

of salivary flow, here we show that diagnostically useful autoantibodies can be detected by LIPS with 5 μ L of whole saliva. Previous studies attempting to use salivary diagnostics in SjS have typically required a larger volume of saliva. For example, using a commercial ELISA, Ben-Chetrit and co-workers detected antibodies directed at the SSA complex in the saliva of 53% of SjS patients, but reported only qualitative results (Ben-Chetrit *et al.*, 1993). Another group attempted to detect autoantibodies in SjS saliva taken directly from the parotid gland, but found that the matched serum was markedly superior with greater sensitivity (Hammi *et al.*, 2005). In contrast, our results with LIPS demonstrate that saliva testing is promising, yielding diagnostically useful autoantibody titers in saliva. Despite the need for more LIPS testing, validation, and standardization, the question remains whether salivary diagnostics using LIPS or other technologies might have a practical advantage over using serum. Because autoantibody titers can be rapidly and easily obtained using LIPS with saliva, one potential useful application would be point-of-care diagnostics as part of routine oral examination. Although collection of whole, unstimulated saliva from xerostomic patients would take more time than performing a blood draw, this non-invasive approach, coupled with the ability to use saliva in the QLIPS format (Burbelo *et al.*, 2009a), could make it practical for testing in under 20 min for either Ro52 and Ro60. Since Ro52 and Ro60 are not specific for SjS, diagnosis of SLE and other rheumatological illnesses might also be possible. Another interesting application besides diagnosis might be to use salivary autoantibody titers to evaluate the efficacy of novel local therapies for SjS. For example, reproducible saliva autoantibody sampling might be useful to determine if orally acting therapeutic agents might decrease inflammation within the salivary glands of patients with SjS. One might expect that saliva might more accurately detect titer changes compared with measuring serum autoantibodies found in the systemic circulation.

Despite the lack of quantitative correlation between autoantibody titers measured in saliva vs. sera, and the lower levels of

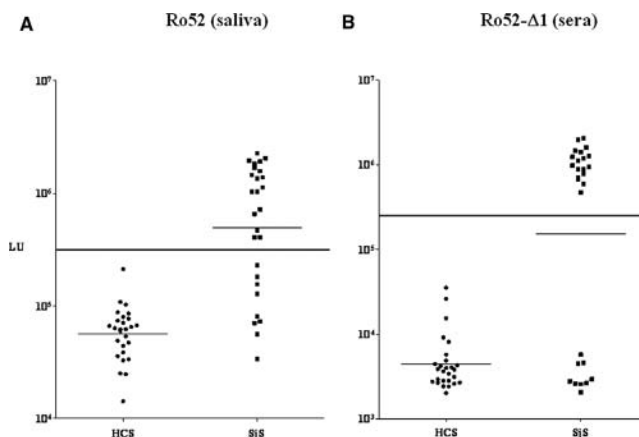


Figure 3. LIPS detection of anti-Ro52 autoantibodies in saliva and sera. SjS patients' (N = 27) and healthy control individuals' (N = 27) saliva (A) and sera (B) were evaluated for anti-Ro52 autoantibodies by LIPS. For autoantibody evaluation in saliva, the full-length Ro52 protein was used. For autoantibody evaluation in sera, an N-terminal fragment of Ro52, designated Ro52- Δ 1, was used. Each circle or square symbol represents an individual healthy control or SjS patient sample, respectively. A cut-off, indicated by the long solid line, was calculated by ROC analysis. The short solid lines indicate the geometric mean titer of each group.

autoantibodies in saliva compared with sera, the same seronegative and seropositive samples were detected in both bodily fluids. One possible reason for lack of quantitative correlation between the two fluids is that the immunoglobulins in saliva represent local production of autoantibodies and are relatively distinct compared with those found in the systemic circulation (Tengner *et al.*, 1998). Based on the lower level of autoantibody titers detected in saliva, some autoantibodies might also be undetectable in saliva. For example, autoantibodies directed against several extraglandular targets, including AQP-4 and thyroid peroxidase in some SjS patients (Burbelo *et al.*, 2009b), are likely to be below the threshold of detection with saliva. Despite our findings, we cannot discount that some of the autoantibodies detected in patient whole saliva are derived from blood contamination. Several studies have shown that SjS patients have worse periodontal condition compared with healthy control individuals, and have particularly higher bleeding on probing (Helenius *et al.*, 2005; Antoniazzi *et al.*, 2009), which could account for the origin of autoantibodies detected in saliva. Our finding of significant, albeit lower, autoantibody titers to both Ro52 and Ro60 in pure salivary secretions compared with whole saliva suggests that at least some of the autoantibodies detected in whole saliva are derived from salivary secretions.

In the current format of LIPS, the protein A/G beads mainly detect IgG autoantibodies, but modification of the assay with other immunoprecipitation capture reagents could be incorporated to evaluate other immunoglobulin isotypes, such as IgA and IgM. Detection of IgA might provide information as to mucosal secretions of antibodies in SjS. Last, it is likely that antibody responses to several infectious agents, including HIV, HTLV, and HCV (Sasaki *et al.*, 2000; Vassilopoulos and Calabrese, 2005; Shanti and Aziz, 2009), could also be easily detectable in saliva with this same approach. Since many of these infectious agents

have also been linked to sialadenitis, it may be of interest to compare antibody titers in saliva vs. serum in infected patients with and without salivary disease. The ability to examine these antibodies in saliva along with SjS-related autoantigens might yield new insights in the cause of infection-related SjS symptoms.

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