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Original Article

Role of Saliva in Diagnosis and Estimation of Disease Severity in Pemphigus Vulgaris using Autoantibody Levels: A Comparative Study between Serum and Saliva

Surya Narayan Das[1], Kirti Jyoti[2], Rachna Rath[3], Dharitri Mohapatra[4], Swapnarani Behera[5], Kalpana Mandal[6], Neha Patyal[7], Sunil Kumar[8]

[1]Professor and Head, Department of Oral Pathology and Microbiology, S.C.B. Dental College and Hospital, Cuttack, Odisha, India, [2]Senior Resident, Department of Oral Pathology and Microbiology, S.C.B. Dental College and Hospital, Cuttack, Odisha, India, [3]Associate Professor, Department of Oral Pathology and Microbiology, S.C.B. Dental College and Hospital, Cuttack, Odisha, India, [4]Associate Professor, Department of Microbiology, S.C.B. Medical College and Hospital, Cuttack, Odisha, India, [5]Assistant Professor, Department of Skin and VD, S.C.B. Medical College and Hospital, Cuttack, Odisha, India, [6]Senior Resident, Department of Oral Pathology and Microbiology, S.C.B. Dental College and Hospital, Cuttack, Odisha, India, [7]Senior Resident, Department of Public Health Dentistry, S.C.B. Dental College and Hospital, Cuttack, Odisha, India, [8]Post Graduate, Department of Oral Pathology and Microbiology, S.C.B. Dental College and Hospital, Cuttack, Odisha, India

Abstract

Introduction: While studies abound on estimation of anti-desmoglein-1,3 autoantibodies in serum of Pemphigus vulgaris patients, there is scarcity of literature investigating their levels in saliva and their correlation with severity of disease.

Purpose: The aim of present study was to estimate and compare the correlations of levels of serum and salivary anti-Dsg-1, anti-Dsg-3 antibodies, and disease severity according to the standardized index of measuring severity of disease, that is, Pemphigus disease area index (PDAI). Furthermore, we attempted to find out correlation, if any, between salivary anti-Dsg-1, anti-Dsg-3 antibodies, and oral mucosal disease severity by including oral mucosal component of PDAI, which is hitherto unexplored in the studies so far.

Materials and Methods: Autoantibodies against desmoglein-1,3 were assayed by Enzyme-linked immunosorbent assays in serum and saliva samples of patients and healthy controls.

Results: Titers of serum and salivary anti-Dsg-1, anti-Dsg-3 significantly correlated with overall disease severity. In addition, the titers of both Abs in saliva showed a statistically significant correlation with oral disease severity, much like its serum counterpart.

Conclusion: Our results indicate that salivary biofluid has tremendous potential to be used for diagnosis and monitoring disease activity in Pemphigus vulgaris patients and merits further studies.

Key words: Pemphigus, Saliva, Serum, Enzyme-linked immunosorbent assays, Autoantibodies

INTRODUCTION

The term Pemphigus derived from the Greek word "Pemphix" (bubble or blister) is used to describe a group of potentially life-threatening autoimmune mucocutaneous diseases characterized by epithelial blistering, on cutaneous and/or mucosal surfaces afflicting skin, scalp, nails, and also the mucosae of the mouth, nose, conjunctivae, genitals, esophagus, pharynx, and larynx.[1]

Of the various forms of pemphigus, pemphigus vulgaris is the most common, potentially fatal tissue specific autoimmune disease primarily afflicting the mucous membranes, predominantly the oral cavity.[2,3] At the cellular level, the defect lies in interruption of the proper intercellular adhesion in the epidermis and mucous

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Corresponding Author: Dr. Kirti Jyoti, Senior Resident, Department of Oral Pathology and Microbiology, S.C.B Dental College and Hospital, Cuttack - 753 007, Odisha, India.

membranes, by targeting glycoprotein components of desmosomal adhesion molecules, that is, desmoglein-1 (Dsg-1) and desmoglein-3 (Dsg-3) resulting in a loss of cell-to-cell attachment, that is, acantholysis with intraepithelial split formation. This clinically transcends into appearance of very fragile blisters that rupture leaving erosions, which affect either or both the mucosa and the skin resulting in three distinct clinical phenotypes: Mucosal, cutaneous, or mucocutaneous polycythemia vera (PV).[4,5] Oral manifestations constitute an important aspect of PV since often they are the initial presenting features or in some cases the only symptom of the disease.[6,7]

Conventionally, the diagnosis of PV is based on the histopathologic evaluation of skin or mucosal biopsies showing characteristic suprabasal clefting and acantholytic cells, which are confirmed by the demonstration of immunoglobulin G (IgG) and complements deposition on the direct immunofluorescence (DIF) staining of the perilesional normal skin. Beautner and Jordan demonstrated circulating immunoglobulin G (IgG) antibodies reactive against Dsg-3 and Dsg-1 in the serum of PV Patients which can be detected and quantified by Indirect immunofluorescence (IIF).[8] Further studies elucidated that the antibody titer in serum reflect disease activity and could be used to monitor therapeutic response.[5,9] For the past decade, enzyme-linked immunosorbent assays (ELISA) using recombinant desmogleins 1 (Dsg1) and 3 (Dsg3) have emerged as a promising, more sensitive and specific method enabling quantitative measurement of these autoantibody levels and several studies have extensively documented the presence of IgG antibodies against Dsg Ags by serum ELISA assay. At present, there is a growing endeavor to establish non-invasive methods of diagnosis. In this context saliva as a biofluid has shown tremendous promise due to its ease of handling, possibility of repeated sampling, being patient friendly and unlike serum it is devoid of coagulation issues. This has provided much impetus to the use of saliva as a potential diagnostic biological tool in various diseases such as Sjogren's syndrome, cystic fibrosis, diabetes mellitus, and HIV. As such, ELISA using saliva as the biofluid substrate, instead of blood serum, has been proposed as a noninvasive, rapid, and convenient method for the diagnosis of autoimmune disorders, including PV.[3,10-14] However, the use of salivary ELISA for diagnostic purpose in PV has not been extensively studied.[6] Furthermore, there is dearth of literature highlighting the impact of autoantibody titers in saliva on the severity of oral and cutaneous lesions.

The aim of the present study is to evaluate the diagnostic value of Dsg ELISA in PV patients by investigating the correlation between serum and salivary anti-Dsg1, anti-Dsg3 antibodies, and disease severity according to the standardized index of measuring severity of disease, that is, pemphigus disease area index (PDAI).

In addition, we attempted to find out correlation, if any, between saliva anti-Dsg1, anti-Dsg3 antibodies, and oral mucosal disease severity by including oral mucosal component of PDAI (OMPDAI). To the best of our best knowledge, this is the first study to highlight the correlation between anti-Dsg titers and severity of oral disease in PV. Simultaneously assessments were made whether the correlations between salivary antibody levels and disease severity tend to parallel those between serum antibody levels and disease severity, to establish the accuracy of saliva as a diagnostic alternative to serum.

MATERIALS AND METHODS

Thirty untreated patients with Pemphigus Vulgaris presenting to the Department of Skin and V.D., S.C.B. Medical College and Hospital, and SCB Dental College and Hospital, Cuttack, were voluntarily enrolled into the study. The diagnosis of the disease was based on histopathology and direct immunofluorescence findings in favor of PV. Demographic data including gender and age were recorded on a predesigned questionnaire. Clinical characteristics of the disease including the phenotype of disease, namely, cutaneous, mucosal, and mucocutaneous, were recorded as well. Scoring of the disease based on the PDAI was also recorded. Tto compare the patients with an appropriate pemphigus-free control group, 20 age- and sex-matched individuals, clinically free of any autoimmune disease based on their medical history and physical examination were also recruited into the study.

Ethical Standard

The study was conducted with prior approval and clearance from Institutional ethical committee, SCB Dental College and Hospital, Cuttack under the required norms and regulations. (IEC/SCBDCH/011/28/12/2018).

Human Serum and Saliva Samples

Collection of serum sample was done by the method recommended by National Institute of Health (NIH-Bethesda, MD, USA, 2009). Five milliliters of blood sample were drawn from the antecubital vein of each participant under aseptic conditions and collected in disposable, non-pyrogenic, and non-endotoxin blood collection tubes. The samples were allowed to remain for 2 h at room temperature to allow sedimentation of cellular fraction of blood. Later, sedimented blood sample was centrifuged for 20 min at approximately 1000× g. The supernatant serum was separated out with the help of micro-pipette in sterile microcentrifuge tubes and stored in −70°C.

For collection of saliva samples, the patients were instructed to collect unstimulated whole saliva in their oral cavity for 5 min without swallowing and then told to spit it into sterile disposable plastic containers. Care was taken to see that the patients did not consume food, drinks or smoke at least 1 h before the saliva collection procedure. Then, the supernatants of the salivary samples were separated by centrifugation at 1000 g for 20 min and collected in sterile microcentrifuge tubes and stored in −70°C. In order to prevent circadian variations, the serum and saliva samples were obtained from 9:00 to 11:00 AM. All the pemphigus patients had the typical clinical oral and skin lesions [Figure 1a and b], histological [Figure 2a] and immunopathological (direct immunofluorescence, Figure 2b) findings, of PV and the serum/saliva samples were collected when these diseases were active.

Anti-Dsg1 and Anti-Dsg3 ELISA

Anti-Dsg1 and anti-Dsg3 ELISA were performed on both the serum and the salivary samples using the EUROIMMUN (Medizinische Labordiagnostika AG, Germany) kit according to previous studies and the manufacturer's instructions for conducting serum ELISA. Anti-Dsg 1 and anti-Dsg 3 ELISA were performed on the serum samples which were diluted to (1/100) in accordance with the manufacturer instructions. For serum anti-Dsg1 and anti-Dsg3 ELISA, the standard manufacturer's recommended cutoff value of 20.0 RU/mL was used. The dilution of salivary samples prepared for ELISA in a previous study was not clearly stated; 15; moreover, we reached acceptable results from non-diluted salivary samples. Therefore, we used non-diluted salivary samples in our study. Since the kit had been specifically designed for serum anti-Dsg1 and anti-Dsg3 measurements, the results of saliva samples were evaluated using the optimized cut-off values based on the previous studies (7.7 RU/mL and13.4 RU/mL for the salivary anti-Dsg1 and anti-Dsg3 ELISA, respectively). According to these studies, different cutoff values were used to provide more reliable results. All procedures were performed according to the manufacturer's recommendation.

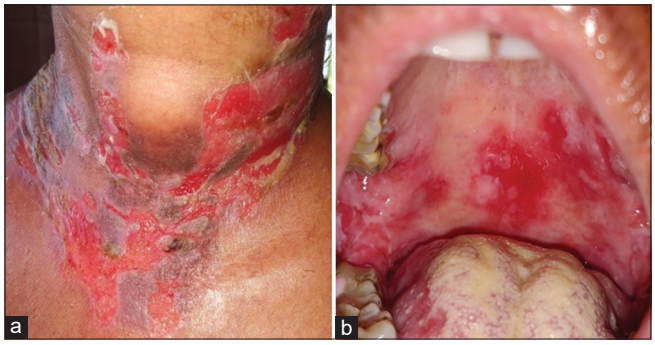

Figure 1: (a) Extensive denuded surface of skin due to detachment of epidermis following rupture of blisters with crust formation. (b) Multiple persistent oral erosions and ulcers in the oral cavity of polycythemia vera patients. These were preceded by blisters that readily ruptured due to oral trauma

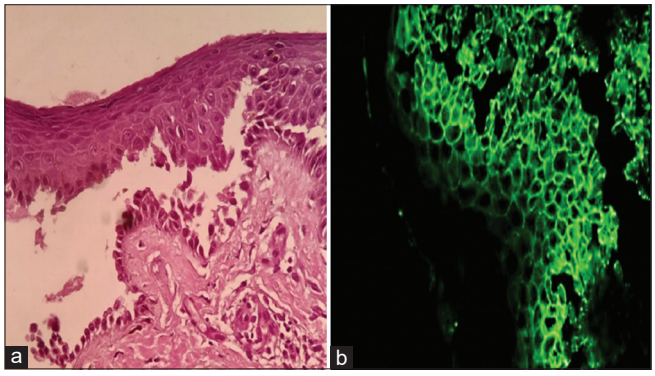

Figure 2: (a) Histopathologic examination of mucosal lesion biopsy revealed supra-basilar split with separation of overlying epithelium and residual basal keratinocytes at the basement membrane zone, producing a "tombstone effect." (b) Direct immunofluorescence perilesional area revealed an intercellular staining of IgG antibodies giving rise to a "chicken wire pattern."

Statistical Analysis

Statistical analysis of the data was performed using the IBM SPSS Statistics software (version 21.0; IBM Corp., Armonk, NY, USA) for Microsoft Windows. The Spearman's (rs) correlation coefficient was used to investigate correlation between anti-Dsg 1and anti-Dsg 3 in serum and saliva and their correlation with the disease severity. $P < 0.01$ was considered statistically significant.

RESULTS

The study involved a total of 50 subjects, out of which 30 were Pemphigus Vulgaris patients and 20 were healthy controls. Among the 30 diseased cases, 13 were males and 17 females while the control group comprised 7 males and 13 females. The mean age in PV patients was 48.3 ± 10.21 while in healthy controls it was 43.95 ± 10.45.

Raised Serum and Salivary Anti-Dsgs in PV Patients

The findings of this study showed that mean titers of anti-Dsg-1 and 3 Abs in both serum and saliva of PV patients were significantly greater than those found in healthy controls using unpaired t test [Table 1].

For Anti-Dsg 1 93.33%, patients were positive for serum antibodies while the same Abs in saliva were found to be present in 83.33% patients. As regard Anti-Dsg-3 96.66%, PV patients were positive for Abs in serum, in contrast 86.66% patients had anti-Dsg-3 Abs in saliva. In our study, we have found mean levels of Anti-Dsg-3 to be greater than mean levels anti-Dsg-1 in both serum and saliva.

Table 1: Summary of data for Dsg-1 and Dsg-3 ELISA in serum and saliva of PV patients and healthy controls

Group	Anti-Dsg-1 Abs		Anti-Dsg-3 Abs	
	Serum (mean±SD*)	Saliva (mean±SD*)	Serum (mean±SD*)	Saliva (mean±SD*)
Cases (*n*=30)	155.66±87.23	17.21±10.27	181.76±102.95	25.12±13.10
Controls (*n*=20)	1.5±0.94	1.42±0.94	1.49±0.80	1.11±0.65

*Standard deviation

Correlation of Serum and Salivary Anti-Dsg Antibodies

There was statistically significant correlation between serum anti-Dsg titers with their salivary levels. Good correlation was found between serum and salivary anti-Dsg-1 levels (r = 0.897, *P* = 0.000) while a stronger positive correlation was obtained between serum and salivary anti-Dsg-3 levels (r = 0.964, *P* = 0.000) [Table 2].

Correlation of Serum and Salivary Anti-Dsg Values with Pemphigus Score (PDAI)

Total and oral disease severity in PV patients was studied using Total PDAI score and OMPDAI score, respectively. PDAI was calculated for each patient by two independent observers. The results were analyzed using "kappa" statistics as a measure of inter observer agreement and excellent agreement values of 0.896 and 0.893 were obtained for both total PDAI and OMPDAI, respectively.

Individual correlations of mean titers of anti-Dsg-1, Anti-Dsg-3 Abs in saliva with each of the two severity scores, that is, total PDAI and OMPDAI were made using Spearman's correlation coefficient and these were compared with similar correlations using mean Ab titers in serum [Table 3].

As per our findings, salivary Anti-Dsg-1 levels showed statistically significant correlation with total PDAI as well as OMPDAI scores (*r* = 0.831, 0.725 respectively; *P* = 0.001 for each) [Figure 3c and d]. Drawing comparison with the serum counterpart, serum Anti-Dsg-1 titers also showed statistically significant but stronger correlation with total PDAI and OMPDAI (*r* = 0.933, 0.756 respectively; *P* = 0.001 for each) [Figure 3a and b].

As compared to salivary anti-Dsg-1 levels, the anti-Dsg-3 Ab levels in saliva showed a stronger statistically significant correlation with total disease severity (PDAI; *r* = 0.903) [Figure 4c] as well as oral mucosal disease severity (OMPDAI; *r* = 0.754) [Figure 4d] with p value=0.001 for both correlations. Interestingly, this stronger correlation of anti-Dsg 3 Abs compared to anti-Dsg-1 as obtained in saliva, was also closely simulated by the serum titers of anti-Dsg-3 (*r* = 0.948 for total PDAI; *r* = 0.762 for OMPDAI; *P* = 0.001 for each) [Figure 4a and b] showing a stronger positive correlation than anti-Dsg-1 in serum.

Table 2: Correlation coefficients between serum and salivary anti-Dsg antibodies

Parameters		*r**	*P***
Serum anti-Dsg-1	Salivary anti-Dsg-1	0.897	0.000
Serum anti-Dsg-3	Salivary anti-Dsg-3	0.964	0.000

*Spearman correlation coefficient, ***P* value significant at <0.01.

Table 3: Correlations of serum and salivary anti-Dsg 1 and 3 with PDAI scores

Disease severity index	Serum anti-Dsg-1	Salivary anti-Dsg-1	Serum anti-Dsg-3	Salivary anti-Dsg-3
Total PDAI	0.931*	0.831*	0.948*	0.903*
OMPDAI	0.756*	0.725*	0.762*	0.745*

*Spearman's correlation coefficient, *P*=0.000 for all correlations; *P* value significant at <0.01. PDAI: Pemphigus disease area index, OMPDAI: Oral mucosal component of pemphigus disease area index

DISCUSSION

The present study aimed at evaluation of serum and salivary anti-Dsg1 and anti-Dsg3 IgG autoantibodies by ELISA and correlation of antibody levels with disease severity as measured by PDAI for each patient. In addition, we aimed to investigate the correlation of anti-Dsg1 and anti-Dsg3 autoantibodies in serum and saliva with the severity of oral mucosal involvement by incorporating the oral mucosal component of PDAI. Serum and salivary anti-Dsg1 and anti-Dsg3 ELISA were also performed on samples from 20 healthy control subjects.

First, in case of serum, the higher levels of serum anti-Dsg-3 IgG Abs as compared to mean serum anti-Dsg-1Abs obtained in the present study are in agreement with findings of Hallaji *et al.* who reported the mean serum anti-Dsg3 antibody titers of 179.35 ± 68.78 RU/mL with lower positive serum anti-Dsg1levels 78.61 ± 67.09 RU/mL. In their study also the control values were below the cut off.[10] Similarly, De *et al.* found serum anti-Dsg1 levels of 88.08 ± 77.75 RU/mL with higher mean serum anti-Dsg3 antibody titers of 134.52 ± 55.43 RU/mL.[15] The increased titers of serum Dsg-3 IgG autoantibodies as compared to serum anti-Dsg1 levels obtained in our study may be explained by the pivotal role of anti-Dsg3 in both the cutaneous and mucosal forms of PV as Dsg-3 has

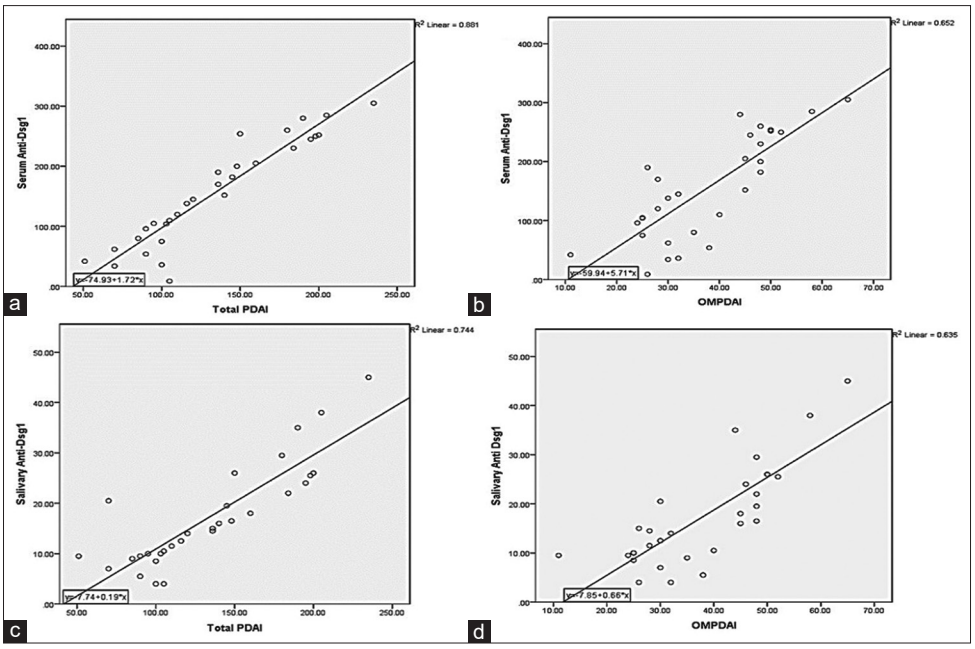

Figure 3: (a) The overall severity of disease (total pemphigus disease area index [PDAI]) positively correlated with serum anti-Dsg1 antibodies. (b) Positive correlation between serum anti-Dsg-1 values and oral mucosal disease severity (OMPDAI). (c) The overall severity of disease (total PDAI) positively correlated with salivary levels of anti-Dsg1 antibodies. (d) The oral mucosal disease severity (OMPDAI) positively correlated with salivary levels of anti-Dsg1 antibodies

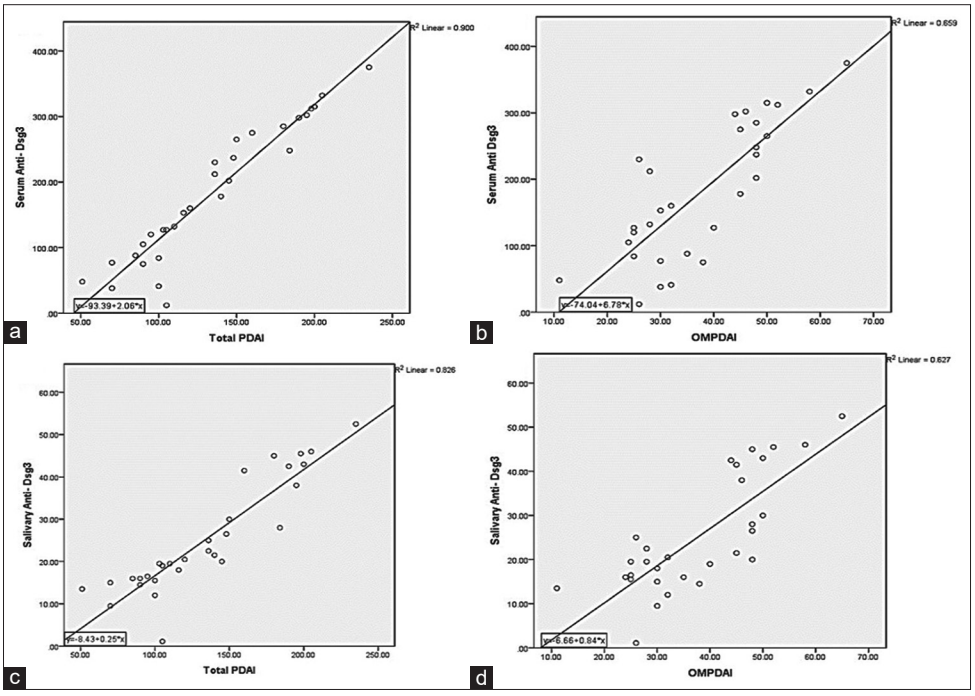

Figure 4: (a) The overall severity of disease (total pemphigus disease area index [PDAI]) positively correlated with serum anti-Dsg3 antibodies. (b) Positive correlation between serum anti-Dsg-3 values and oral mucosal disease severity (OMPDAI). (c) The overall severity of disease (total PDAI) positively correlated with salivary levels of anti-Dsg3 antibodies. (d) The oral mucosal disease severity (OMPDAI) positively correlated with salivary levels of anti-Dsg3 antibodies

widespread distribution in skin as well as mucosa while Dsg-1 is largely restricted to epidermis. As such, in our study all patients had extensive oral mucosal involvement besides cutaneous involvement.

However, even with lower titers the mean values of serum anti-Dsg1 Abs (154.46+/-88.91 RU/ml) obtained in our study as well as the sensitivity (93.33%) are much higher than most of the previous studies reported in the

literature.[10,16] This could be attributed to racial differences in serum levels of these antibodies as revealed by Harman *et al.* and Sharma *et al.*[9,17] The values obtained by us are higher than the serum anti-Dsg1 values of PV patients from northern Europe (46%) and Japan (53% and 55.6%) as reported by various studies.[9,18,19]

Anand *et al.* also conducted anti-Dsg1 and anti-Dsg3 ELISA on serum samples from 63 active PV patients and obtained sensitivity of about 74% in serum anti-Dsg-1. Similar to our results they found increased levels of both anti-Dsg3 and anti-Dsg1 in PV with the predominant distribution of anti-Dsg3 antibody. This could be explained by crosstalk between Dsg1 and Dsg 3 antigens and epitope spreading, as previously mentioned by Khandpur *et al.* and Chan *et al.*[17,19,20] Simply, Epitope spreading refers to a phenomenon in which that a primary autoimmune or inflammatory process may produce tissue damage in such a manner that certain protein components that are immunologically "hidden" from the immune system become "revealed" and subsequently evoke a secondary autoimmune response.[20] All our cases had extensive oral and cutaneous involvement and in almost all cases, the disease initiated with oral lesions subsequently spreading to skin. Several studies have emphasized that in mucocutaneous PV autoAbs recognize a secondary distinct epitope on Dsg-3 which cross-react with Dsg-1, inducing blister on mucosa and subsequently skin as explained by "epitope spreading".[20,21] However, the original response is directed against an epitope on Dsg-3 which is more accessible in mucosa.[21]

As regard saliva, in our study, we have obtained higher concentration of salivary anti-Dsg-3 antibodies as compared to anti-Dsg-1 antibodies in saliva. Studies in untreated patients have also reported higher IgG antibodies to Dsg3 in saliva in up to 70–94% of patients with PV.[8] In our results the serum IgG anti-Dsg-3 assay revealed 29 of 30 patients as positive and the salivary assay was positive for 27 of 30 patients. IgG antibodies present in saliva are derived mainly as a serum transudate from gingival crevicular fluid, mucosal inflammation, or through an ulcer along with the possibility of some direct passage of IgG across mucosa.[22] The difference may reflect healed mucosal lesions and reduced access to saliva of serum IgG. In addition, serum components, such as antibodies, could be transferred to saliva through capillary walls in the salivary glands.[10] Therefore, in patients with PV, serum anti-Dsg1 and anti-Dsg3 antibodies can be transmitted through the intercellular spaces between the cells to the lumen of salivary ducts through injured epithelial mucosa. Hence, its amounts mainly depend on the integrity of the epithelial barrier and tend to reflect serum levels.[23]

Importantly, there was statistically significant strong positive correlation between serum anti-Dsg titers with their salivary levels. Our results concur with the findings of previous studies. Hallaji *et al.* also obtained statistically significant correlation between serum and salivary anti-Dsg-1 and anti-Dsg-3 titers with $P < 0.001$ and 0.001, respectively.[10] Andrealis *et al.* found statistically significant correlation between serum anti-Dsg titers with their salivary levels ($P < 0.05$ for both associations).[16] In our study, the sensitivity of salivary Dsg3 ELISA was 86.66% and of Dsg1 ELISA was 83.33%. We found an association between salivary and serum values for both Dsg antibodies Therefore, salivary as well as serum samples, can be used for the assessment of anti-Dsg antibodies.

Another important aspect of the present study was correlation of Ab titers with disease severity. Our results indicate a significant positive correlation between serum and salivary levels of both Anti-Dsg 1 and 3 antibodies and overall disease severity (total PDAI). Further, we also attempted to explore whether or not there is correlation between salivary Desmogleins-1,3 and severity of oral mucosal involvement in PV patients by including oral mucosal component of PDAI for each patient (OMPDAI). Interestingly, levels of anti-Dsg 1 and 3 antibodies present in saliva positively correlated with severity of oral mucosal involvement (OMPDAI), with the strength of this correlation simulating the correlation of serum levels of these antibodies and severity of oral mucosal disease. On comparing salivary Dsg-1 and Dsg-3 Abs, the levels of salivary Dsg-3 autoantibodies better correlated with severity of oral mucosal disease much like its serum counterpart.

Mortazavi *et al.* also demonstrated that serum anti-Dsg 1 and anti-Dsg 3 values significantly correlated with total score of the disease. Further they showed that salivary anti-Dsg-1 and anti-Dsg-3 positively correlated with severity of mucosal disease. They concluded that salivary anti-Dsg 1, and anti-Dsg 3 ELISA, could be used as safe and noninvasive methods for the diagnosis of PV, when obtaining a blood sample is difficult (in certain circumstances, e.g., pediatric and senile patients).[11] Similarly Hallaji *et al.* concluded that serum Dsg-3 and salivary Dsg-1 antibody levels correlated with severity of mucosal lesions in PV patients.[10] However, another study was conducted to assess the relationship between salivary and serum Dsg1 and Dsg3 levels, and whether salivary Dsg1 and Dsg3 levels correlate with clinical disease severity of oral mucosal lesions in PV using objective component of the oral mucosal Autoimmune Bullous Skin Disorder Intensity Score (ABSIS).In this study no significant correlation of salivary titers with either mucosal or cutaneous disease severity was found.[15]

A recent systematic review and meta-analysis have shown that anti-Dsg ELISA is a valuable laboratory

diagnostic method for the initial diagnosis of autoimmune bullous diseases including PV and could be used in daily practice.[11,24]

ELISA is a quantitative method that has been extensively used to determine titers of Dsg1 and Dsg3 in sera of PV patients, and in this study these serum titers have been demonstrated to correlate with disease severity. However, there is little information on salivary Dsg1/Dsg3 levels using ELISA, or on the correlation between Dsg1/Dsg3 salivary levels and disease severity. In the present study, salivary anti-Dsg1 and anti-Dsg3 levels were found to correlate with their serum counterpart. In addition, our findings revealed a significant correlation of salivary Ab titers with total and oral mucosal disease severity closely simulating serum levels.

CONCLUSION

This study supports reports by previous literature indicating that saliva as well as serum could be used for detection of anti-Dsg1 and 3 antibodies by employing ELISA test. Serum anti-Dsg1 and anti-Dsg3 antibodies are reflective of disease severity in PV patients. Surprisingly, salivary anti-Dsg1 and anti-Dsg-3 antibodies significantly correlated with oral mucosal and overall disease severity and paralleled the correlations of same parameters with their corresponding serum counterparts. Therefore, Dsg ELISA using both serum and saliva is not only a sensitive tool for diagnosis of PV, it can also serve as a predictive means of its severity as well. Recently, there has been a surge in projects to develop miniaturized salivary diagnostic techniques which facilitate use of minute amounts of oral fluid to yield critical information regarding disease status. As such, easy and painless collection, possibility of repeated sampling along with ease of handling and storage makes saliva an attractive matrix for ELISA. According to the results of this study, the use of saliva Dsg ELISA for diagnosis of PV is comparable to its serum counterpart and may be a useful diagnostic screening tool for PV. However, further studies with larger sample sizes are recommended for demonstrating usefulness of saliva Dsg ELISA for diagnosis of PV.

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