Quantification of D-dimer levels in human saliva

**Background:** Plasma D-dimer tests are currently used to exclude deep vein thrombosis and pulmonary embolism. Human saliva has numerous advantages over blood as a diagnostic sample. The aims of our study were to develop a reliable immunoassay to detect D-dimer levels in saliva, and to determine the correlation between salivary and blood D-dimer levels. **Results/methodology:** Saliva and blood samples were collected from 40 healthy volunteers. We developed a AlphaLISA® immunoassay with acceptable analytical performances to quantify D-dimer levels in the samples. The median salivary D-dimer levels were 138.1 ng/ml (morning) and 140.7 ng/ml (afternoon), and the plasma levels were 75.0 ng/ml. Salivary D-dimer levels did not correlate with plasma levels ($p = 0.61$). **Conclusion:** For the first time, we have quantified D-dimer levels in saliva that were twofold higher ($p < 0.05$) than plasma levels. Further studies are required to demonstrate the clinical relevance/utility of salivary D-dimer in patients with confirmed deep vein thrombosis and/or pulmonary embolism.

Venous thromboembolism (VTE) is the third most common cause of death under the broad spectrum of cardiovascular diseases (CVD) [1]. VTE occurs when blood flow stagnates, especially when coupled with either a hypercoagulable state or vascular endothelial damage causing fibrin deposition, thereby trapping platelets, red blood cells and leukocytes to form venous thrombi. When such thrombi form in the deep veins such as in the thigh and calf, this is referred as deep venous thrombosis (DVT). Complications that may result from DVT include local venous insufficiency and leg ulceration, and if the thrombus breaks off, they can migrate to the lung (referred to as pulmonary embolism [PE]), which is a life-threatening condition with over 30% mortality. Plasma D-dimer test plays a critical role in clinical practice, particularly in DVT diagnosis and management since it rises more than 100-fold during acute DVT [2]. Combining it with a scoring system based on a clinical model, plasma D-dimer levels provide a high negative predictive value in the diagnosis of DVT [3] and PE [4]; hence, the need for diagnostic ultrasound imaging is reduced.

Fibrinogen is synthesized by the liver and secreted into the circulation [5]. In the presence of thrombin, fibrinogen can be cleaved into highly self-adhesive fibrin monomers [6]. Plasma D-dimer is a cross-linked fibrin degradation product. The presence of elevated levels of D-dimer in plasma is an indicator of thrombus formation. D-dimer is currently used to diagnose and manage a number of thrombosis-related clinical conditions, including disseminated intravascular coagulation, VTE [7,8], stroke, thrombolytic therapy [9] and PE [10]. However, there are many underlying conditions such as stroke, infection, pregnancy and heart failure that are unrelated to thrombus, but may lead to elevated D-dimer levels in the plasma resulting in a low positive predictive value [11–13]. In addition, plasma D-dimer levels are strongly and positively related to the occurrence of future venous thrombosis [2]. We therefore reasoned that there may be utility in exploring whether D-dimer can be detected in other body fluids that can be easily accessed and sampled in a non-invasive fashion. This could facilitate population screening to identify individuals at a high risk of developing thrombotic events. Indeed, Sivakumaran and Maltoni detected D-dimer levels in urine and raised the question of whether the ratio of plasma to urine levels could be more informative when screening asymptomatic populations to minimize the effect of inter-individual variability [14]. In our study, we explored whether D-dimer could be detected in saliva, and whether there is a correlation between plasma and saliva D-dimer levels taken from the same individuals.

Salivary secretions contain most of the biomolecules that are present in blood or urine [15–19]. Human saliva protein levels reflect our body’s health and well-being, and about 20% of proteins that are present in blood are also found in saliva [20], which highlights the diagnostic potential of saliva. Saliva does not clot
like blood, and its collection is minimally invasive. Saliva samples are relatively easy to collect and handle by people with minimal training, plus it is easy to obtain multiple sample collections with a minimal risk for the collector of contracting blood-borne infectious organisms. Saliva has even been used as a biological fluid for the diagnosis of ischemic heart disease and heart failure. The aims of our study were twofold: to develop a reliable immunoassay to detect D-dimer levels in saliva as a means of providing a preliminary reference range for this biomolecule in saliva and to determine whether the salivary D-dimer levels correlate with blood levels.

Materials & methods

Participants

This research was approved by the University of Queensland Medical Ethical Institutional Board and the Mater Hospital Medical Ethical Review Board. All participants were over 18 years of age and gave written informed consent before donating samples for our study. We recruited 40 healthy controls (20 males [19–36 years] and 20 females [19–25 years]) from the University of Queensland students and staff. The subjects were of European and Asian descent, were in good oral hygiene and had showed no symptoms of fever and/or respiratory tract infection. This information was provided to us via a sample questionnaire. Exclusion criteria obtained via a questionnaire included the existence of any comorbid oral disease (e.g., periodontal disease and gingivitis), autoimmune disease, active infection, malignancy, smoking, undergoing dental treatment, denture wearer, pregnancy and recent operation or trauma.

Samples

Saliva and blood samples from each volunteer were collected during the same day. Blood samples were collected by an experienced phlebotomist (Queensland Medical Laboratory Pathology Services, University of Queensland Collection Centre, Australia) between 0900 and 1600 immediately after either the morning saliva collection or afternoon saliva collection. Blood samples were collected into sodium citrate tubes (Greiner VACUETTE® # 454327, Greiner Bio-one, Graz, Austria) and then immediately centrifuged at 500 × g at room temperature for 15 min. The plasma samples were divided into aliquots, and stored at -80°C until analyzed. Saliva samples were collected in sterile Falcon™ Polypropylene Conical Tubes (50 ml, BD Bioscience, #352070, CA, USA), divided into aliquots and stored at -80°C until further analysis.

Salivary flow rates were calculated for all the study participants as described by Mahvash in order to eliminate any confounding variables due to salivary gland dysfunction. Unstimulated resting saliva was collected by the drool method described by Navazesh and Christensen. All subjects were asked to refrain from eating and drinking for 2 h prior to saliva collection in order to obtain a relatively constant baseline. We collected two saliva samples from each participant in the morning (0800–1200) and in the afternoon (1400–1600) to provide an indication of the diurnal variation of D-dimer levels. The samples were collected and immediately aliquoted and de-identified. Prior to storage, saliva samples were centrifuged at 1500 × g for 15 min at 4°C. The salivary supernatant was then diluted 40 times with AlphalISA® immunoassay Buffer (1X) and stored at -80°C until analysis.

Salivary D-dimer AlphalISA® immunoassay

The D-dimer AlphalISA kit (Product-No: AL290 C/F, Perkin Elmer, MA, USA) was used to determine the concentrations of D-dimer in plasma and saliva samples. It contains a bionylated anti-D-dimer monoclonal antibody, which binds to the streptavidin-coated donor beads, while the anti-D-dimer monoclonal antibody is conjugated to the acceptor beads. In the presence of D-dimer, the beads come into close proximity. After excitation at 680 nm, donor beads will give out singlet oxygen molecules that trigger a cascade of energy transfer in the acceptor beads, resulting in a sharp peak of light emission at 615 nm. AlphalISA immunoassay uses monoclonal antibodies, and as such the specificity of this immunoassay is considerably high, with no cross-reaction with fibrinogen and 34% of crosreactivity with D-monomer (which is also a fibrin degradation product that can indicate fibrin formation). [31] Standards were used to generate a standard curve. The commercially available D-dimer AlphalISA kit is designed and validated for the measurement of human D-dimer levels in citrated plasma or cell culture medium. The kit has not been validated for the use of measuring D-dimer level in human saliva samples. Saliva is a very complex, high viscoelastic biological...
fluid and as such it is important to minimize any influence that saliva might bring to the immunoassay (matrix effects). We have developed and validated an AlphaLISA immunoassay specific for the measurement of salivary D-dimer levels. For salivary measurements, the standards were prepared in pooled saliva (n = 10, 2.5% of pooled saliva) and similarly when measuring the D-dimer levels in plasma, the standards were prepared in pooled plasma (n = 10, 2.5% of pooled plasma) collected from healthy controls to eliminate any matrix effects. The samples were analyzed in 384 wells ProxiPlates™ in triplicate (Perkin Elmer) with an exception of reducing the reaction volumes from 50 to 10 µl. In brief, the immunoassay consisted of 1 µl of sample/analyte, 1 µl of biotinylated antibody and acceptor bead mix (10 nM and 100 µg/ml, respectively), and 8 µl of streptavidin donor beads (50 µg/ml). The final concentration of acceptor beads, biotinylated antibody and streptavidin donor beads was 10 µg/ml, 1 nM and 40 µg/ml, respectively. We used an EnSpire™ plate reader (Perkin Elmer) to read the plates after 1.5 h of incubation at room temperature in the dark.

Assay performance characteristics for the in-house modified salivary D-dimer AlphaLISA Recovery
To evaluate the suitability of AlphaLISA immunoassay for measuring salivary D-dimer levels, we spiked three commercial recombinant D-dimer (Product-No: D21012, Perkin Elmer MA, USA), measured the spiked pooled saliva samples using AlphaLISA immunoassay. An unspiked pooled saliva sample was measured at the same time. The percentage recovery of the three spiked saliva samples was calculated in reference to respective un-spiked pooled saliva samples in a single AlphaLISA immunoassay, using the following equation

\[
\text{%CV} = \frac{\text{Mean of SD} \times 100}{\text{mean}}
\]

LOD for the AlphaLISA immunoassay
To determine the LOD of the D-dimer immunoassay, 12 blanks (2.5% pooled saliva diluted in 1X AlphaLISA Immunoassay buffer) were run in triplicate in one immunoassay run. The LOD for the salivary D-dimer immunoassay was read from a sigmoidal-dose response curve based on LOD signal counts derived from the equation

\[
\text{LOD signal count} = \text{(ave of blank signal count)} + 3 \times \text{(SD of blank signal)}
\]

Statistical analysis
We used GraphPad Prism 5 software version 5.03 (GraphPad Software Inc., CA, USA) to perform all the statistical analyses. A D-dimer standard curve was generated by plotting the total raw AlphaLISA counts versus the concentration of D-dimer standards. A four-parameter logistic equation (sigmoidal dose-response curve with variable slope) coupled to a 1/Y² data weighting were used to analyze the data.

We performed a Kolmogorov-Smirnov statistic in order to test for normal distribution of the clinical characteristics (continuous variables) of the volunteers before statistical analysis. Mann-Whitney U test and Wilcoxon test were performed on unpaired and paired data without normal distribution, and Chi-square test for dichotomous variables to compare values from two groups. To investigate the relationship between salivary and plasma D-dimer levels, and between salivary flow rate in the morning and in the afternoon samples, we used nonparametric spearman product moment correlation coefficients.

Results
Participants
In total 40 healthy volunteers were enrolled in the study between 22 August 2012 and 6 December 2012. The details of participants are shown in Table 1.

Salivary flow rate measurements
The salivary flow rates (median and interquartile range [IQR]) for the study participants for morning and afternoon collections were 0.91 g/min (0.470–1.257 g/min) and 1.1 g/min (0.598–1.634 g/min), respectively (Figure 1). There was a significant difference between
morning and afternoon salivary flow rates ($p = 0.0073$), and there was a significant correlation between morning salivary flow rate to afternoon salivary flow rate ($p < 0.001$, correlation coefficient $r = 0.760$).

- **Assay performance characteristics for the in-house modified salivary D-dimer AlphaLISA assay**

  The performance characteristic of the D-dimer immunoassay is summarized in Table 2. Intra-and inter-assay CVs for the salivary D-dimer immunoassays were 5.7 and 7.4%, respectively. The LOD values for the salivary and plasma D-dimer assays were 279 and 748 pg/ml, respectively.

- **Salivary D-dimer concentrations in healthy controls**

  Individual salivary D-dimer concentration is summarized in Figure 2. The D-dimer concentration in the saliva samples collected in the morning ranged from 6.0 to 1523.0 ng/ml with a median value of 138.1 ng/ml (IQR, 42.9 to 287.2 ng/ml) (Figure 3). The D-dimer concentration in the saliva samples collected in the afternoon ranged from 11.2 to 2173.0 ng/ml with a median value of 140.7 ng/ml (IQR, 50.0 to 303.7 ng/ml). There was no significant difference between D-dimer levels in the saliva samples collected in the morning and in the afternoon ($p = 0.4907$).

- **Plasma D-dimer concentrations in healthy controls**

  The D-dimer concentration in the plasma samples ranged between 1.3 to 393.2 ng/ml with a median of 75.0 ng/ml (IQR, 42.8 to 176.7 ng/ml) (Figure 3). There was a significant difference between morning saliva D-dimer levels and plasma D-dimer levels ($p < 0.05$) as well as the afternoon’s salivary D-dimer levels and plasma levels ($p < 0.05$) (Figure 3). However, by comparing individual samples using Wilcoxon matched-pairs signed rank test, we found no significant correlation between plasma and salivary D-dimer measurements taken from the same individual ($p = 0.605$ for morning saliva vs plasma and $p = 0.955$ for afternoon saliva vs plasma) (Figure 4).

**Discussion**

To our knowledge, this is the first time that the D-dimer levels in saliva has been investigated in healthy young adults. We observed that the D-dimer level in saliva was significantly higher than in the plasma from the same individuals and that there was no correlation between salivary and plasma D-dimer levels. Furthermore, salivary D-dimer levels did not significantly differ between morning and afternoon samples. The lack of correlation between the salivary and plasma D-dimer levels may suggest that the D-dimer levels found in saliva could be produced in situ in the oral cavity. Saliva contains a thromboplastin-like substance and it has been identified as a tissue factor that is associated with

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**Table 1. Characteristics of healthy controls.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy controls (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>24 (19–36)</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>20:20</td>
</tr>
<tr>
<td>Ethnicity (Caucasian: Asian)</td>
<td>19:21</td>
</tr>
<tr>
<td>Body mass index</td>
<td>22.7 (18.0–29.2)</td>
</tr>
</tbody>
</table>

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**Figure 1.** Comparison of salivary flow rates for samples collected in the morning and in the afternoon. (A) The Whisker plots for morning and afternoon salivary flow rates. (B) Correlations between morning and afternoon salivary flow rate (spearman correlation coefficient $r = 0.760$, $p < 0.001$).
circulating cell-derived microparticles and these can become coagulant. Recently Berckmans et al. demonstrated that salivary tissue factor is associated with exosomes and microparticles that promote the clotting of blood. Their study also showed that salivary tissue factor level was much higher than plasma tissue factor level (~2000 vs ~300 pg/ml, respectively). Therefore, we hypothesize that the salivary tissue factor can promote blood clotting in capillaries in oral cavity, and to prevent these capillaries being blocked by the blood clot, fibrinolysis activities must also be present. Therefore, the D-dimer production is increased in the capillaries surrounding the oral cavity leading to high D-dimer levels in saliva. However, more research is warranted to determine the exact mechanism leading to elevated salivary D-dimer levels and its role in saliva, and also to determine whether D-dimer levels differ between men and women.

In this study, we recruited 40 volunteers to collect their blood and saliva samples. These volunteers were all young healthy nonsmoking adults (ages <35 years) with no history of medical conditions, that is, lipemia. In addition, we also performed visual inspection of the citrate plasma samples and they showed no visible lipemia. Blood and saliva samples were generally collected at about the same time, which should provide a mirror image of pathological changes. In our study, since healthy people have functioning salivary gland functions, we collected only resting mouth whole saliva samples from our study participants. Saliva can also be stimulated by either oral or mechanical stimulations which is ideal in a clinical setting when dealing with patients with impaired salivary gland functions. It will be interesting to find out whether the D-dimer levels are affected by stimulation.

In order to detect D-dimer in human saliva samples, we developed an existing commercially available immunoassay from Perkin Elmer (AlphaLISA D-dimer immunoassay). Due to the high concentrations of D-dimer in saliva, after diluting the samples 40-times, the samples were still in the measuring range of the D-dimer assay. In order to minimize the matrix effects, the standards for the assay needed to be prepared in the same matrix as the samples. We tried different dilutions of pooled healthy saliva as the diluent for the standards (data not shown), and 40 times diluted pool saliva was the best diluent to prepare the standards in and gave recoveries of 84.3%. The performance characteristic of this assay indicated that the assay performance was reliable. The total incubation time for this

Table 2. Performance characteristics of the in-house developed salivary D-dimer AlphaLISA® immunoassay.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>% Recovery</th>
<th>% Intra-assay variation (± std error)</th>
<th>% Inter-assay variation (± std error)</th>
<th>LOD (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-dimer in saliva</td>
<td>84.3</td>
<td>5.7 (2.7)</td>
<td>7.4 (7.2)</td>
<td>279</td>
</tr>
<tr>
<td>D-dimer in plasma</td>
<td>85.5</td>
<td>6.7 (4.3)</td>
<td>7.5 (3.1)</td>
<td>748</td>
</tr>
</tbody>
</table>

![Figure 2. The D-dimer levels in saliva and in plasma of 40 healthy individuals.](image-url)
immunoassay was 1.5 h. The total time to perform D-dimer AlphaLISA assay for 40 samples (including samples preparation, reagents preparation and the addition of the samples onto the assay plate) would be around 3 h. However, the assay time can be further reduced when one is implementing robotics automation and these assays can be run on the JANUS® automation work station. Furthermore, AlphaLISA technology is amenable for decentralized point-of-care testing and could either be placed at the doctor’s office or could be used at a reference laboratory.

The medium level of salivary D-dimer was 150.9 ng/ml (IQR 56.9–87.2 ng/ml) and this information is likely to be useful when determining saliva reference ranges for D-dimer levels within a healthy population. In addition, there were no significant differences in the levels of D-dimer between morning and afternoon samples (p = 0.5). Our findings are in line with Iversen et al. who showed that there was no D-dimer diurnal variation in plasma. In contrast, a study by Rudnicka et al. using large number of controls (n = 7667), found that the plasma D-dimer levels peaked at 14:00 h, but there was not a significant diurnal variation for plasma D-dimer level (p = 0.17). Therefore, a wide range of blood collection times should not contribute significantly to the inaccuracies of the results.

**Conclusion**

In summary, we have demonstrated that D-dimer is detectable in saliva in a group of healthy individuals and that the levels are approximately twofold higher in saliva than in plasma. Furthermore, there was no significant correlation between salivary and plasma D-dimer levels. These findings suggest that salivary D-dimer may be produced in situ in the oral cavity. The potential for salivary D-dimer testing needs to be explored once pre-analytical variables are optimized and standardized. Future studies should then investigate its clinical utility in both the diagnosis and screening for thromboembolic diseases. Measuring salivary D-dimer levels at an early stage of DVT and PE patients (without any treatment) could provide a reference range for salivary D-dimer level in thromboembolic diseases. By comparing the salivary D-dimer levels of healthy controls and DVT/PE patients, a cut-off value can be obtained for clinical diagnosis of these diseases.
Future perspective

CVD is becoming prevalent due to an aging and a growing population. Early diagnosis of CVD can greatly reduce morbidity and mortality. But most importantly early detection improves the chances of recovery for the patients, decreasing the burden on healthcare providers. Simple and cost-effective diagnostic fluids and methods are pivotal in reducing the growing burden of CVD. Saliva as a diagnostic/screening fluid has enormous potential over blood-based analysis. Data presented in this manuscript suggests that salivary D-dimer levels have great potential as a biomarker for the detection of DVT and PE. The long-term aim should be to develop a point-of-care testing device to facilitate large population based screening of DVT and PE. To date, we only have the salivary D-dimer levels in 40 healthy controls, further and more comprehensive studies with sufficient number of VTE/PE patients may reveal its diagnostic utility. This we plan to perform in the near future. In our study, the variations observed between individuals are larger in saliva than those observed in plasma, which may increase the number of false positives. Further and more comprehensive studies on confounding variables that may influence concentration changes may be warranted in the future.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

**Assay performance characteristics for the in house developed salivary D-dimer AlphaLISA® assay**

- We used AlphaLISA sandwich immunoassay technology to detect D-dimer levels in saliva. We accounted for immunoassay matrix effects by using a 2.5% pooled saliva to prepare standard curve to determine D-dimer concentrations. Intra- and inter-assay CVs for the salivary D-dimer immunoassays were at acceptable level (5.7 and 7.4%, respectively).

**Salivary & plasma D-dimer concentrations in healthy controls**

- In a healthy population (n = 40), the salivary D-dimer level ranged from 6.0 to 1523.0 ng/ml with a median value of 138.1 ng/ml, which was approximately twofold higher than in plasma (ranged between 1.3 and 393.2 ng/ml with a median of 75.0 ng/ml)

**Future perspective**

- Further studies are required to understand the biological role of D-dimers in saliva.

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- of interest
- of considerable interest


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