Measurement of anti SARS-CoV-2 RBD IgG in saliva: validation of a highly sensitive assay and effects of the sampling collection method and correction by protein

Abstract

Objectives: To develop and evaluate a new highly sensitive assay to detect IgG anti-SARS-CoV-2 RBD in saliva samples.

Methods: A two-step sandwich type immunoassay based on the amplified luminescent proximity homogeneous technology was developed and an analytical validation was performed. As a part of this validation, the influence of factors, such as different sampling conditions (stimulated saliva and passive drool) and the correction of values by total protein content, in the ability of saliva to detect increases in antibodies after an immune stimulus and be an alternative to serum, was evaluated. For this purpose, paired samples of saliva and serum at different times after vaccination were used.

Results: Saliva concentrations were lower than serum, but both fluids showed similar kinetics, with higher correlations when saliva was obtained by passive flow and the results were not corrected by protein.

Conclusions: The developed method showed a good analytical performance and can properly measure antibody concentrations in saliva of vaccinated individuals. However, saliva could have a lower sensitivity compared to serum at initial stages of the immune response and also when the antibody response decreased after a stimulus.

Keywords: AlphaLISA; anti SARS-CoV-2 RBD IgG; collection method; saliva.

Introduction

Saliva is a sample easy, cheap and fast to collect without producing pain or stress and with no need of specialized personnel, thus minimizing the exposure of health care workers [1]. In addition, saliva has been described as an alternative diagnostic specimen for SARS-CoV-2 allowing direct detection of the virus, as well as the quantification of the antibody response developed against it [2].

The humoral response following infection or vaccination, could be monitored using serological immunoassays for quantitative measurement of anti-SARS-CoV-2 S antibodies in serum [3, 4]. In this regard there are several assays developed for serum or plasma quantification that can be used to evaluate the immunoglobulin response after infection or vaccination. In addition, there are various reports that have measured antibody response in saliva by using enzyme-linked immunosorbent assays (ELISAs), and other different types of assays such as chemiluminescent immunoassays (CLIA), which can be used on automated analyzers for a high throughput of samples [5–11].

One the main obstacle for the use of saliva for antibody detection is the low concentration of IgGs compared to serum [9–11]. For this reason, highly sensitive methods would be of interest for IgGs measurement. Another limitation for the use of saliva is the lack of knowledge about the optimal conditions for sample collection. In addition, there are other
aspects such as the need to correct the saliva results by total protein concentration, which although it has been recommended by some authors [12, 13], it is still poorly studied. These factors, could influence the values of antibodies detected in saliva and the correlation between saliva and serum, that showed a wide variability between different studies ranging from 0.39 [9, 14] to 0.89 [10, 14]. Therefore, the possible use of saliva as alternative to serum for IgG measurements is still not clarified.

The objective of this study was to develop and evaluate a new highly sensitive assay to detect the IgG anti-SARS-CoV-2 RBD concentrations in saliva samples. As a part of this evaluation, the effect of two different sampling conditions (stimulated saliva and passive drool) and the correction of values by total protein content were studied. Special emphasis was given to evaluate the influence of these factors in the ability of saliva to detect increases in antibodies after an immune stimulus and be an alternative to serum, and for this purpose, paired samples of saliva and serum at different times after vaccination were used.

Materials and methods

Assay description

The assay developed is a two-step sandwich type immunoassay based on the amplified luminescent proximity homogeneous technology (AlphaLISA®), and uses acceptor beads conjugated to a commercially available recombinant SARS-CoV-2 spike glycoprotein receptor binding domain antigen (Rekom Biotech, Spain) (RBD-AB), streptavidin-coated AlphaScreen donor beads (St-Dbeads), and a biotinilated mouse antibody specific for human IgG that showed no cross reactivity with human IgA, IgD, IgE or IgE (MARX1901-1KC, Merck, Germany) (Figure 1). All AlphaLISA® reagents were purchased from Perkin Elmer (MA, USA) and conjugation of the beads was performed following manufacturer protocols.

Briefly, samples and standards (5 μL, pre-diluted 1:4,000 (serum) and 1:4 (saliva) in assay buffer) along with 20 μL of a mix containing 25 μg/mL of RBD-AB and 6 nM biotinylated mouse anti-human IgG in assay buffer 1× (AlphaLISA Assay Buffer 10×; PerkinElmer) were added to 96-well plates. The plates were covered with a lid and incubated at room temperature for 1 h. Then, in a second step, 25 μL of 40 μg/mL Streptavidin Donor beads (PerkinElmer) were added to each well and incubated for 30 min in a final reaction volume of 50 μL per well. After that the AlphaLISA® signal (excitation at 680 nm, emission at 615 nm) was measured on a EnSpireAlpha plate Reader (PerkinElmer, MA, USA).

A standard curve was developed by diluting an inactivated positive serum with alpha buffer up to different dilutions and results were given in arbitrary units (AU) per mL as previously reported [15, 16].

Analytical validation

Within run precision was calculated by measuring patient pools (from serum and saliva) with high and low IgG values 5 times in a single analytical run. The pools were made mixing samples from vaccinated and unvaccinated individuals to create samples with high and low concentration respectively. Between run precision was calculated by measuring the same pools in five different days. For the linearity study two samples were serially diluted (serum: 1:4,000; 1:8,000; 1:16,000; 1:32,000; 1:64,000 dilutions; saliva: 1:4; 1:8; 1:16; 1:32 dilutions) with the buffer used for the assay. The limit of detection was calculated as the lowest concentration of IgG which could be distinguished from a zero sample, and was taken as the mean + 3 standard deviations (SD) of 12 replicates of the blank (buffer used for the assay).

Sample collection and processing methods

To evaluate the effects of different sampling conditions, and correction by protein content, paired saliva and serum samples of 35 vaccinated participants (23 with BNT162b2, Comirnaty from Pfizer/BioNTech, 12 with ChAdOx1-S, Vaxzevria from Astra Zeneca) at three time points post-vaccination were collected: 48–72 h (T1), 3 weeks (T2) and 4 months post second dose (T3). All participant had received two doses of the vaccine separated 21 days in case of Comirnaty and 12 weeks in case of Vaxzevria and were not previously affected by COVID-19. They were 24 females and 11 males; average age (SD)=44 (11) years. Participants were requested to refrain from eating, drinking, and performing basic oral hygiene for 1 h before sample collection. All participants were informed of the purpose and experimental procedures of the study and signed a written informed consent form prior to their participation. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the University of Murcia (protocol code 2952/2020 and date of approval 22/02/2021) for studies involving humans.

To study the possible effect of the collection method on the results obtained, saliva samples were obtained under supervision by two different collection methods: passive drool and stimulated saliva by the use of a Salivette® device (Sarstedt, Germany). In passive drool method, saliva was collected for 2 min by passive flow directly into a 15 mL conical and then centrifuged (4,000×g, 10 min, 4 °C). In stimulated samples participants were instructed to chew the cotton swab of the Salivette® device during 1 min and then the swab was transferred into the Salivette® tube and centrifuged at 4,500 rpm for 10 min.

Figure 1: Schematic illustration of the AlphaLISA® for anti-SARS-CoV-2 RBD IgG detection.

RBD-AB, acceptor bead conjugated to receptor binding domain; DB, donor bead; B-Anti hlgG, anti-human IgG biotinylated antibody.
No samples showed blood contamination as determined by visual inspection. Paired blood samples were obtained immediately after saliva collection using standard procedures and transferred to tubes with a coagulation activator.

Serum was separated from blood by centrifuging at 3,000 rpm for 5 min. Inactivation of potential infectious viruses in serum and saliva was performed by incubation with NP-40 to a final concentration of 0.5% for 30 min and were stored at −80 °C until further analysis. We confirmed in pilot studies (data not shown) that NP-40 and storage at −80 °C up to 6 months had no effect on anti-RBD IgG titers.

**Effect of total protein content of saliva**

Results from anti-RBD IgG in each saliva sample were divided by its total protein content to evaluate the possible influence of protein content of each sample on the results. For this purpose a spectrophotometric assay (protein in urine and CSF, Spinreact, Spain) previously validated for human saliva [17] was used to measure the total protein content (μg/mL) of saliva. The method was adapted to an automatic analyzer (Olympus UA600, Olympus Diagnostica GmbH, Ennis, Ireland) following the manufacturer’s guidelines and showed less than 15% inter and intra-assay imprecision and was linear under serial dilution.

**Statistical analysis**

Arithmetic means, medians, 25th–75th percentile and CVs were calculated using standard descriptive statistical procedures and software (Excel 2013, Microsoft Corp., WA, USA). For post-vaccination samples the D’Agostino & Pearson omnibus normality test was performed to evaluate the normality of distribution giving a nonparametric distribution. Therefore, data were log transformed to get normality and a one-way repeated measures ANOVA followed by uncorrected Fisher LSD test was performed to compare values obtained at different times after vaccine. A p-value<0.05 is considered to be statistically significant in all cases. Correlation coefficients between serum and salivary concentrations and between salivary collection methods were determined using Spearman correlation analysis. The strength of the correlations tested was assessed by the Rule of Thumb [18], according to which an R value between 0.90 and 1 was considered to have very high correlation, 0.70 to 0.90 high correlation, 0.50 to 0.70 moderate correlation, 0.30 to 0.50 low correlation and less than 0.30 little if any correlation. To determine positivity, a cut-off value was generated by measuring 15 prepandemic serum and saliva samples. The cut-off was taken as the mean + 3 SD of this population as previously reported [10].

**Results**

**Analytical validation of the AlphaLISA assay**

Intra-assay CVs for IgG assay were in a range between 6 and 7% for serum and 6 and 13% for saliva. Inter-assay CVs were between 12 and 19% for serum and 13–15% for saliva (Tables 1 and 2). Dilution of two human serum and saliva samples with high IgG concentration resulted in linear regression equations, with determination coefficients close to 1 (Figure 2). The analytical limit of detection of the assay developed was 0.53 AU/mL.

**Effect of collection method**

Significantly higher values of IgG anti-RBD were observed at T2 in saliva obtained by flow and Salivette® (median value: 104.3 AU/mL; 71.32 AU/mL; p<0.0001 for both flow and Salivette respectively) and also in serum (median value: 141,520 AU/mL; p<0.0001) in comparison with T1 (median values: 3.08; 0.84 and 18,960 AU/mL for flow, Salivette® and serum respectively) and T3 (median values: 5.98; 2.08; 18,236 AU/mL for flow, Salivette® and serum respectively) (Figures 3 and 4). In addition, significantly higher IgG values were found in T3 compared with T1 only in case of saliva obtained by both methods, but not in serum. A significant high correlation was observed between saliva obtained by the two different collection methods and serum (r=0.90; p<0.001 for passive flow and serum; and r=0.86; p<0.001 for Salivette® and serum) (Figure 5).

When the results obtained in saliva by the two methods of collection were compared, significantly lower values of

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<th>Table 2: Intra- and inter-assay variation in human saliva samples with different IgG concentrations.</th>
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IgG anti RBD (p<0.0001) were observed in samples obtained with the use of Salivette® device (median: 4.55 AU/mL) compared with those obtained by passive flow (8.7 AU/mL). In addition, a higher number of values under detection limit of the method were found when Salivette device was used (29 by Salivette vs. 10 by passive flow, specially at T1). A significant high correlation (r=0.86 p<0.0001) was found between samples obtained by both collection methods.

With regards to detection of individuals with IgG values higher that the cut-off, in saliva, a higher number of positives was obtained by passive flow compared with Salivette® although the number of positives in saliva was lower than in serum, especially at T1 (Table 3).

Effect of total protein content of saliva

When data were corrected according to their total protein content, significantly higher values of IgG anti RBD per mg of protein were observed in T2 in saliva (p<0.0001) for both flow and Salivette in comparison with T1 and T3 (Figure 6).

The IgG correlation between serum and saliva corrected by protein content was moderate (r=0.70, p<0.0001) when saliva was obtained by flow, showing a higher correlation with serum when saliva was used without correction (r=0.90; p<0.001). However, when Salivette® was used (r=0.86, p<0.001) the correction by protein gave a similar correlation with serum than when no correction was used.

The correlation between values with and without protein content correction in samples obtained by passive flow and Salivette® was high (r=0.85 and 0.88 respectively, p<0.0001).
Discussion

The assay developed in this report allows to measure anti SARS-CoV-2 RBD IgG antibodies in serum and saliva samples with high precision since CVs were lower than the maximum acceptable for immunoassays (CV<20%) [19] and a it showed a good linearity after serial sample dilutions. This method uses AlphaLISA® technology, which offers some advantages compared to ELISA (enzyme-linked immunosorbent assay) kits, such as do not require any wash, have fewer protocol steps, require less sample amount and it has higher analytical sensitivity [20, 21].

Vaccines BNT162b2 from Pfizer/BioNTech and ChAdOx1-S from AstraZeneca (which are the most commonly administered in Spain) were used in our study. Both induce antibodies to the SARS-CoV-2 S-protein, including neutralizing antibodies (Nabs) [22, 23], leading in our report to a significant increase of IgGs at T2 in serum in all vaccinated individuals followed by a significant decrease in T3. In our experimental conditions, Pfizer vaccine resulted in higher antibody values than the AstraZeneca (Supplementary material). These results are in agreement with others previously reported [23, 24]. Overall this would indicate that our experimental model was adequate to get samples with different anti IgGs concentrations and that the developed assay can properly detect changes in antibody concentrations after vaccination.

In our report saliva results were 1,200 – 1,900 times lower than serum, but the assay developed was sensitive enough to detect changes after vaccination and both fluids showed a similar dynamic over time. However, the method of collection and the way the data were expressed affected the results obtained in saliva and its correlation with serum. Saliva collected by Salivette® gave significantly lower antibody values and a high number of samples below the detection limit. In addition, the correlation with serum was weaker for Salivette® and the percentage of positive cases detected was lower at the three times tested. Therefore, based on these results, it would be advisable for the assay developed in this report, to collect the sample by flow rather than by Salivette®. In any case, if Salivette® is used,
a different reference range and cut-offs points should be established.

When saliva results were corrected by protein, a weaker correlation with serum was observed, especially in samples obtained by passive flow. Similar results were found by Lapic et al. 2021 who found a moderate correlation (r=0.60) between antibody titers in serum and saliva with saliva values corrected by protein content. Hence, it would be not recommended to correct the results for protein content, being this in line with previous reports for other salivary analytes [17, 25].

It must be pointed out that a high correlation between serum and saliva was found in our study indicating that the IgG found in saliva is most probably derived from plasma through filtration [11, 26]. Therefore, saliva could potentially be an useful sample to evaluate antibody levels in the population in a non-invasive way and by a simple and easy collection method. However, a lower number of positives were detected in saliva in comparison to serum, especially at T1. All the cases that were negative in saliva but positive in serum had serum antibody levels lower than 31,000 AU/mL. These results could indicate a possible threshold for the passing of anti-RBD IgG from serum to saliva, and could represent a limitation of saliva for detecting situations in which there is a low amount of this antibody in serum or at initial stages of the immune stimulation. Another hypothesis could be that, at this early stage, IgA could inhibit the binding of IgG to the RBD-AB or compete for the binding and decrease the ability of IgG quantification. Studies to elucidate if IgA could produce some interference in IgG measurements should be performed in the future.

Furthermore, it should be noted that at T3 the median IgG values in serum decreased to reach similar values to those found at T1, but this was not the same for saliva, where the values were still significantly higher at T3 vs. T1, with some cases being positive in saliva but negative in serum. The appearance of these positives in saliva could indicate a delay in the kinetics of decrease of saliva compared to serum. Therefore, saliva could have a lower sensitivity compared to serum at initial stages of the immune response and also when the immune response is decreasing after an stimulus.

The assay developed in this report was designed to detect antibodies against RBD, since previous studies have shown a high correlation between anti RBD antibodies and neutralizing antibodies [27, 28]. Therefore, the method described in this report could serve to predict neutralization activity in COVID-19 vaccinated individuals or even recovered patients, as has been previously suggested for other anti RBD IgG methods in saliva [5].

Although the number of samples used is valid according to the statistical power calculated, it would be of interest to perform a study with a larger population to better assess the correlation between saliva and serum and even to establish the cut-off point in saliva from which it can be considered that there is an adequate antibody response to the vaccine.

The use of two different vaccines in our experimental model could be considered a limitation, however, given that in some countries such as Spain, vaccines with different mechanisms of action (mRNA and vector based) have been administered, the use of samples from vaccinated individuals with different vaccines could more closely represent the real characteristics of the population and allow to obtain a more complete spectra of antibody levels.

In summary, a sensitive method to detect and quantify IgG anti-SARS-CoV-2 RBD in saliva has been developed. The method can properly measure antibody concentrations in saliva of vaccinated individuals, although it can show false negative results compared to serum, especially in cases of low serum levels at the onset of the immune response triggered by the vaccine and also false positive results when the decreases in serum have started. It is recommended to use saliva obtained by passive flow and not correct the results for protein, and in any case, specific cut-off points for each different saliva collection method should be used.

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Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the University of Murcia (protocol code 2952/2020 and date of approval 22/02/2021) for studies involving humans.
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