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Evaluation of the accuracy in the mucosal detection of anti-SARS-CoV-2 antibodies in nasal secretions and saliva

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ABSTRACT

COVID-19 vaccination with mRNA vaccines induces immune responses capable of neutralizing SARS-CoV-2. Commercially available serological anti-SARS-CoV-2 quantitative and neutralizing assays are essential for the determination of immune responses to vaccines. Nevertheless, at present there is a lack of validated tests to assess the mucosal response to COVID-19 vaccination and standardized analytic and pre-analytic methods have not yet been defined. The aim of our study was to evaluate the accuracy of two diagnostic immunoassays for COVID-19 (ELISA for IgA-S1 and chemiluminescent assay for IgG-RBD) on serum, saliva, and nasal secretions, by the enrollment of three study populations (healthy controls, vaccinated subjects, and subjects recovered from COVID-19 infection). In order to obtain an appropriate cut-off value for the biological matrices studied, ROC curve analyses were performed. Data demonstrate that the analytical and pre-analytical method we have developed can provide accurate and reliable results for the detection of anti-SARS-CoV-2 mucosal specific antibodies (IgA-S1 and IgG-RBD) on saliva and, as a novelty, on nasal secretions, either after COVID-19 infection or in vaccinated subjects.

1. Introduction

Despite its crucial role in protection against viral infections, mucosal immunity has been largely underestimated in the context of coronavirus disease 2019 (COVID-19). Although most clinical studies focus on antibodies and cellular immunity in peripheral blood, mucosal immune responses in the respiratory tract play a crucial role in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1–4].

Symptomatic SARS-CoV-2 disease causes acute infection with activation of the innate and adaptive immune systems. The first leads to the release of several proinflammatory cytokines, including IL-6. The second induces the activation of B cells and T cells which promotes the creation of specific antibodies against SARS-CoV-2, including IgM, IgA and IgG [5]. The production of coronavirus-specific IgM is transient and leads to the transition of the isotype to IgA and IgG [6]; the latter subtype can persist for a long time in serum, saliva and nasal secretions [7]. Secretory IgA (S-IgA), located on the mucous membranes, plays a key role in mucosal immunity. In fact, this antibody class can neutralize pathogens, particularly in respiratory tract infections caused by viruses, protecting the local mucosa from viral invasion [8].

Since the onset of COVID-19 pandemic, most attention has been given to serum antibodies neutralizing circulating viruses, particularly IgG and IgM [9,10]. IgA, IgM and IgG are all effective in preventing infections and diseases especially if they reach the mucous surfaces where the virus is present. Recent studies show that in the first few weeks after symptom onset, SARS-CoV-2 systemic neutralization correlates more closely with IgA than IgM or IgG [2,3,11].

SARS-CoV-2 mRNA vaccines, in particular BNT162b2 Comirnaty (*Pfizer / BioNTech, New York, NY, USA*), are proven to induce a serum IgG and IgA humoral response predominantly directed against the SARS-CoV-2 RBD region of the S1 Spike protein subunit [12–14]. However, vaccine abilities to elicit mucosal immune is still under study.

In our recently published work [11], we have shown that the BNT162b2 Comirnaty (*Pfizer / BioNTech, New York, NY, USA*) elicited an antigen-specific mucosal immune response: significant levels of IgA against the S1 protein and IgG against the RBD domain of SARS-CoV-2

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have been found in saliva and, in particular, in nasal secretions after the injection of the second COVID-19 mRNA vaccine dose. This mucosal humoral immune response was more prominent in vaccinated subjects than in subjects recovered from natural pauci-symptomatic or mild SARS-CoV-2 infection. Only subjects who recovered from severe COVID-19 infection requiring hospitalization, had higher levels of anti-SARS-CoV-2 IgA at mucosal sites, which were comparable to vaccinated subjects.

Currently, anti-SARS-CoV-2 quantitative and neutralizing assays are crucial in assessing immune responses to vaccines [15–17]. Nevertheless, at present there is a lack of validated tests to assess the mucosal response to COVID-19 vaccination and standardized analytic and pre-analytic methods have not yet been defined. Since most of all commercial kits are validated only on serum or plasma samples, we evaluated if the analytical and pre-analytical procedures used on nasal secretions and saliva [11] could provide consistent data in terms of sensitivity and specificity.

On this background, the aim of our study was to investigate the reliability and accuracy of the diagnostic tests performed on saliva and nasal secretions, as well as to obtain an appropriate negative cut-off value for the biological matrices studied, by the use of ROC curve analysis.

2. Materials and methods

2.1. E thic al approval

The study was performed according to the local Ethics Committee approval at the Tor Vergata University Hospital in Rome, Italy (protocol number 9962/2021). Written informed consent was obtained from all the participants. The study was performed in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki.

2.2. Study design

As described in detail in our previously published work [11], the study was conducted between April and June 2021. A total of 51 adult subjects were enrolled (26F and 25 M, median age 49 years, range 22–70 years). Subjects were divided into three groups (Table 1).

The first group (*healthy controls*, T0) included 33 subjects (16 M and 17F, mean age 52 years, range 26–70 years) that never contracted natural SARS-CoV-2 infection, enrolled immediately before the first injection of the mRNA BNT162b2 COVID-19 vaccine *Comirnaty* (*Pfizer/BioNTech, New York, NY, USA*).

The second group (*vaccinated subjects*, T1) included 28 subjects (15 M and 13F, mean age 54 years, range 26–70 years) from the first group, tested again 15 days after the injection of the second dose of *Comirnaty*.

The third group (*COVID-19 population*) included 18 subjects (8 M and 10F, mean age 48 years, range 22–65 years) who recovered from a previous SARS-CoV-2 infection diagnosed with a positive quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) <70 days before the enrollment. None of the subjects of this group had residual COVID-19 symptoms nor the injection of any COVID-19 vaccine dose. Fifteen subjects in this group reported mild or pauci-symptomatic infection; three subjects had severe pneumonia that required hospitalization.

Peripheral blood, nasal secretions and saliva samples were collected for each subject of the different groups, for a total of 79 serum, saliva, and nasal secretions samples. At the time of enrollment, none of the subjects had an acute infection, serious illness, or were taking any medication known to alter immune function.

Table 1
Populations studied.

Population	Vaccine	SARS-CoV-2 infection
Healthy controls *T0* *(n = 33)*	Non vaccinated: subjects enrolled immediately before the 1st *m*RNA *BNT126b2b* vaccine injection	Patients with no previous SARS-CoV-2 infection
Vaccinated *T1* *(n = 28)*	Vaccinated: subjects of the *Healthy controls* group, retested 15 days after the 2nd *m*RNA *BNT126b2b* vaccine injection	Patients with no previous SARS-CoV-2 infection
COVID-19 *(n = 18)*	Non vaccinated subjects	Recovered from previous COVID-19 (RT-qPCR + <70 days), no longer reporting symptoms

2.3. Collection of samples

Serum was collected by peripheral venous sampling in dry tubes, then centrifuged for 15 min at 4000*g* and stored in special Eppendorf tubes at a controlled temperature of −80 °C.

The collection of nasal secretions was performed by a cotton wool inserted into both nasal cavities of the subjects under medical supervision. Cotton wools were removed after 10 min, transferred into a syringe and then hydrated for 10 min with 1 mL of 0.9% NaCl solution. Subsequently, cotton wools were squeezed into a test tube and stored in Eppendorf tubes at −80 °C.

A standardized method was selected for saliva collection. Subjects held a cotton roll (*Neutral Salivette®, SARSTEDT, Numbrecht, Germany*) in the mouth for 1 min, prior to replace it into the stopper part of the *Salivette®* tube. The samples were centrifuged at 1500*g* for 5 min at 4 °C and the resulting saliva was stored in Eppendorf tubes at −80 °C.

To evaluate whether the use of cotton could interfere with the detection of anti-SARS-CoV-2 IgG-RBD and IgA-S1 antibodies, we tested whole saliva samples diluted with sample buffer (SAL) and the whole saliva incubated into a *Salivette®* for one minute and then centrifuged at 1500*g* for 5 min at 4 °C diluted with sample buffer (SAL-POST). We obtained a mean reduction of 4.9% for anti-SARS-CoV-2 IgA-S1 and 3.8% for anti-SARS-CoV-2 IgG-RBD. Our results confirmed that the role of cotton was not decisive for the antibody's detection, as anti-SARS-CoV-2 IgA-S1 and IgG-RBD were comparable in whole saliva samples (SAL) and in saliva collected with *Salivette®* (SAL-POST).

2.4. Biochemical analysis

2.4.1. Quantitative detection of anti SARS-CoV-2 IgG-RBD antibodies

The indirect chemiluminescent immunoassay designed for quantitative detection of IgG anti-RBD levels in serum or plasma samples can be used to characterize the immune response of COVID-19 subjects and individuals who have been vaccinated against the virus, representing an important tool for assessment of the efficacy of *COVID-19* vaccines.

The anti SARS-CoV-2 IgG-RBD were measured using the "MAGLUMI® SARS-CoV-2 S-RBD IgG" (*SNIBE Diagnostics, Shenzhen, China*) kit; detection was performed on the fully automated MAGLUMI 800 analyzer (*SNIBE Diagnostics, Shenzhen, China*).

Serum, saliva and nasal secretions samples were incubated in combination with the magnetic beads of the assay, coated to SARS-CoV-2-RBD antigens, in order to promote immune-complexes. Anti-Human IgG antibodies labeled with ABEI (amino-butyl-ethyl-isoluminol) were added after washing; subsequently to the addition of a starter solution, the emitted light was measured as relative light units (RLUs), directly proportional to the corresponding IgG anti-RBD concentration in the samples. According to the manufacturer's instructions, the serum samples were diluted 1:100 in the sample buffer; nasal secretion samples and saliva were tested without dilution.

Results were reported in AU/mL and the manufacturer's recommended cut-off value in serum is >1.00 AU/mL. To standardize data and to convert the results into Binding Antibody Units (BAU/mL), SNIBE diagnostic provided the following conversion factors: BAU = AU × 4.33 (1 AU/mL is equivalent to 4.33 BAU/mL). Therefore, the cut-off serum value is >4.33 BAU/mL.

The cut-offs obtained in the other biologic fluids for our healthy control study population are 1.19 BAU/mL and 0.63 BAU/mL in saliva and nasal fluids, respectively [11].

All data in the present study are reported in BAU/mL.

2.4.2. Quantitative detection of anti SARS-CoV-2 IgA-S1 antibodies

The "Anti-SARS-CoV-2 ELISA IgA" (*Euroimmun Medizinische Laboradiagnostika, Luebeck, Germany*) kit was used to detect immunoglobulins Class A (IgA) against SARS-CoV-2 spike S1 protein in serum, nasal secretions and saliva. Detections were performed on plates coated with recombinant S1 protein antigens by the automated Euroimmun analyzer instrumentation (*Euroimmun Medizinische Laboradiagnostika, Luebeck, Germany*).

Results were calculated semi-quantitatively by a ratio between the sample OD and a calibrator OD.

According to the manufacturer's instructions, serum samples were diluted 1:101 in sample buffer. By our experimental analysis, the best performance dilutions we tested for nasal secretion samples and saliva were 1:2 and 1:50, respectively.

Positive and negative controls, diluted samples, and a calibrator as a reference for the cut-off value, were transferred in the wells and incubated for 60 min at +37 °C. After washing, 100 μL of enzyme conjugate (anti-human IgA labeled with peroxidase) were added and incubated for 30 min at +37 °C. Finally, 100 μL of the chromogen substrate [TMB (tetra-methyl-benzidine)] solution were added, developing a colorimetric reaction. The optical density was proportional to the quantity of the specific anti-SARS-CoV-2 IgA antibodies present in the samples.

Data were expressed as cut-off index (COI). Serum cut-offs were considered negative for all the values <0.8 COI, equivocal for all the values between 0.8 and 1.1 COI and positive for all the values >1.1 COI, as declared by the manufacturer.

The cut-offs obtained in the other biologic fluids for our healthy control study populations were 10.50 and 0.86 COI in saliva and nasal fluids, respectively [11].

2.5. Data analysis and statistics

Specificity and sensitivity were calculated by Receiver Operating Characteristic (ROC) curves, defining an optimal cut-off for each ROC curve analysis. Statistical analyses were performed with GraphPad Prism 9.3.1 Software (GraphPad Software, San Diego, CA, USA). Non-Gaussian distributions in the study populations were calculated with, Shapiro–Wilk normality test. The categorical data were displayed as numbers and/or percentages and continuous data as median and range. Non-parametric results were analysed by the Mann–Whitney test. For all of the results, $p < 0.05$ was considered statistically significant.

3. Results

3.1. ROC curve analysis for anti-SARS-CoV-2 IgA-S1

The ROC curves obtained for anti-SARS-CoV-2 IgA-S1 in the group of *vaccinated* subjects were highly accurate on serum and nasal secretions and perfect on saliva: in serum samples an area under curve (AUC) value of 0.9957, with a sensitivity of 100%, a specificity of 96,77% and a cut-off value of 178.8 COI was obtained; in saliva an area under curve (AUC) value of 1.000, with a sensitivity of 100%, a specificity of 70.97% and a cut-off value of 12.75 COI was obtained; in nasal secretions an area under curve (AUC) value of 0.9830, with a sensitivity of 100%, a specificity of 60.65% and a cut-off value of 1.150 COI was obtained (Table 2; Fig. 1A).

In subjects recovered from previous *COVID-19* infection, the ROC curves showed that the anti-SARS-CoV-2 IgA-S1 test is moderately accurate on serum and saliva, while it is perfect on nasal secretions: in serum samples an area under curve (AUC) value of 0.8990, with a sensitivity of 94.44%, a specificity of 78.79% and a cut-off value of 41.92 COI was obtained; in saliva samples an area under curve (AUC) value of 0.7482, with a sensitivity of 61.11%, a specificity of 61.29% and a cut-off value of 11.25 COI was obtained; in nasal secretions an area under curve (AUC) value of 0.9840, with a sensitivity of 100% and a specificity of 63.64% a cut-off value of 1.210 COI was obtained (Table 2; Fig. 1B).

3.2. ROC curve analysis for anti-SARS-CoV-2 IgG-RBD

Regarding the anti-SARS-CoV-2 IgG-RBD detection, the ROC curves in *vaccinated* group were perfect on serum, moderately accurate on saliva and highly accurate on nasal fluid: in serum samples an area under curve (AUC) value of 1.000, with a sensitivity of 100%, a specificity of 93.94% and a cut-off value of 3.821 BAU/ml was obtained; in the saliva samples of vaccinated group an area under curve (AUC) value of 0.8721, with a sensitivity of 96.30%, a specificity of 51.52% and a cut-off value of 1.199 BAU/ml was obtained; in the nasal fluid of vaccinated cohort an area under curve (AUC) value of 0.9615, with a sensitivity of 100%, a specificity of 63.64% and a cut-off value of 0.650 BAU/ml was obtained (Table 2; Fig. 2A).

The ROC curve in the *COVID-19* population showed that the anti-SARS-CoV-2 IgG-RBD test is highly accurate on serum and saliva, while it is moderately accurate on nasal secretions: in serum samples an area under curve (AUC) value of 0.9916, with a sensitivity of 94.44%, a specificity of 93.94% and a cut-off value of 4.707 BAU/ml was obtained; in saliva samples an area under curve (AUC) value of 0.9108, with a sensitivity of 94.44%, a specificity of 51.52% and a cut-off value of 1.189 BAU/ml was obtained; in nasal secretions an area under curve (AUC) value of 0.7500, with a sensitivity of 72.22%, a specificity of 63.64% and a cut-off value of 0.650 BAU/ml was obtained (Table 2; Fig. 2B).

Table 2
ROC curve characteristics of anti-SARS-CoV-2 IgA-S1 and IgG RBD antibodies in the *vaccinated* population compared to *COVID-19* group.

		VACCINATED			COVID-19		
		Serum	Saliva	Nasal Secretions	Serum	Saliva	Nasal Secretions
IgA	Sensitivity	100%	100%	100%	94,44%	61,11%	100%
	Specificity	96,77%	70,97%	60,65%	78,79%	61,29%	63,64%
	Cut-off	178,8 COI	12,75 COI	1,15 COI	41,92 COI	11,25 COI	1,21 COI
	Area under ROC curve (AUC)	0,9957	1,000	0,9830	0,8990	0,7482	0,9840
	95% confidence interval	0,9856 to 1,000	1,000 to 1,000	0,9585 to 1,000	0,8025 to 0,9955	0,6096 to 0,8868	0,9572 to 1,000
IgG	Sensitivity	100%	96,30%	100%	94,44%	94,44%	72,22%
	Specificity	93,94%	51,52%	63,64%	93,94%	51,52%	63,64%
	Cut-off	3,821 BAU/ml	1,199 BAU/ml	0,650 BAU/ml	4,707 BAU/ml	1,189 BAU/ml	0,650 BAU/ml
	Area under ROC curve (AUC)	1,000	0,8721	0,9615	0,9916	0,9108	0,7500
	95% confidence interval	1,000 to 1,000	0,782 to 0,961	0,919 to 1,000	0,9748 to 1,000	0,8214 to 1,000	0,6053 to 0,8947

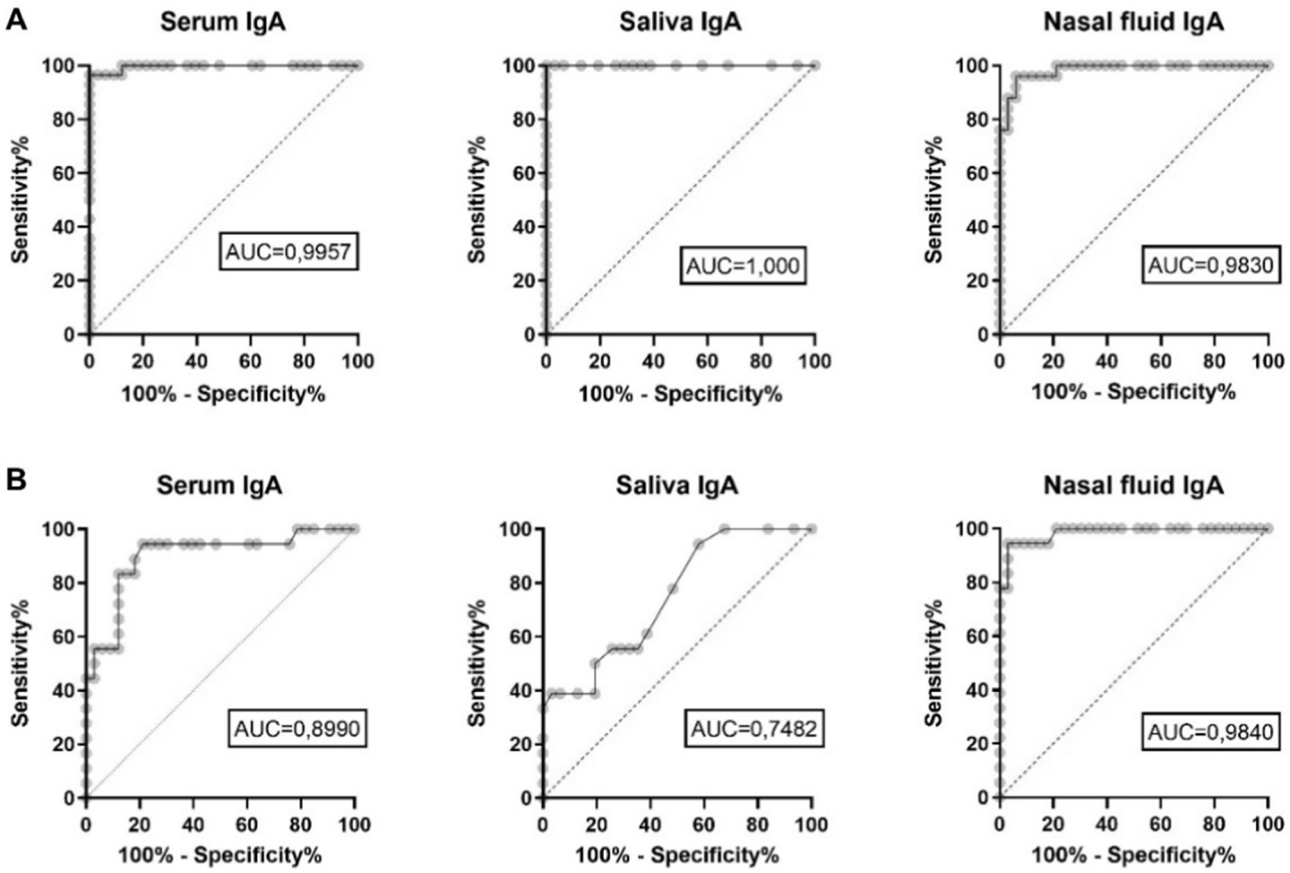

Fig. 1. Anti-SARS-CoV-2 IgA-S1 antibodies ROC curve of serum, saliva and nasal secretions from vaccinated subjects (A) and COVID-19 population (B).

4. Discussion

A key aspect in controlling the COVID-19 outbreak is the availability of diagnostic methods that ensure accurate monitoring of the immunity developed following the viral infection and/or the vaccination [15,18,19]. Many serological tests are currently available for the determination of systemic humoral immunity generated by SARS-CoV-2 infection and vaccine doses. Much attention has been paid to the systemic detection of IgG-RBD antibodies, but recently the focus has shifted to the study of anti SARS-CoV-2 IgA-S1 antibodies in mucous membranes [1,4,20].

Studies have shown that specific IgA are detectable in saliva samples during acute SARS-CoV-2 infection [21–23], but are also detectable in saliva samples from mRNA vaccinated subjects.

Currently, validated serum tests are employed to analyse the humoral immunity produced by the mRNA COVID-19 vaccine and by SARS-CoV-2 infection. Nevertheless, there are still no validated tests and standardized procedures to evaluate the immune response localized at the mucosal level.

As reported in our previous publication [11], after the second dose of the BNT162b2 COVID-19 mRNA vaccine (*Pfizer/BioNTech, New York, NY, USA*) significant levels of IgA directed against the SARS-CoV-2 S1 protein and IgG directed against its receptor binding domain (RBD) could be detected in saliva and above all, in nasal secretions. The level of mucosal anti-SARS-CoV-2 IgA-S1 and IgG-RBD was higher in vaccinated subjects in comparison to subjects recovered from previous mild COVID-19 infection. In particular, regarding saliva our data were consistent with other studies in literature [24–27], reporting significant levels of specific antibodies against SARS-CoV-2, mostly IgG, in saliva after two doses of mRNA vaccine injection. Since antibodies levels were higher in serum than in the salivary compartment, some authors [28,29] concluded that oral mucosal immunity was poorly activated by this vaccination. For what concerns nasal secretions, to the best of our knowledge, our study was the first to assess mucosal immunity elicited by BNT162b2 COVID-19 mRNA vaccine directly from a rhinological point of view. Subsequently, other authors confirmed the presence of specific anti-SARS-CoV-2 antibodies in nasal fluids [30].

Since commercial diagnostic kits are validated only on serum or plasma, to confirm saliva and nasal secretion results, we assessed a clear negative threshold for anti-SARS-CoV-2 IgG-RBD and IgA-S1 through the enrollment of a control group consisting of healthy controls, never infected by SARS-CoV-2 and who never received COVID-19 vaccination [11].

Based on these previous data, our consequent purpose was to evaluate the reliability and accuracy of the diagnostic tests for the matrices studied (saliva and nasal secretions) by the ROC curve analysis, in order to obtain an appropriate negative cut-off value. ROC curve plays a central role in evaluating diagnostic ability of tests to discriminate the true state of subject and in finding the optimal cut off values [31]. Our findings, showing higher sensitivities values respect to specificities, confirm our aim to discriminate, in a pandemic context, patients true positive for the presence of anti-SARS-CoV-2 immune response in the matrices studied. Specificities values may appear less satisfactory, but it should be taken into account that usually class IgG antibodies are not detectable, or at least they have low concentrations, in saliva and nasal secretions.

The area under ROC curve (AUC) is an effective and combined measure of sensitivity and specificity that describes the accuracy and the inherent validity of diagnostic tests [31]. Our optimal AUC results suggest that mucosal immunity against SARS-CoV-2 can be assessed directly at the virus entry site, providing accurate and reliable data. In fact, in our studies we propose an analytical and pre-analytical

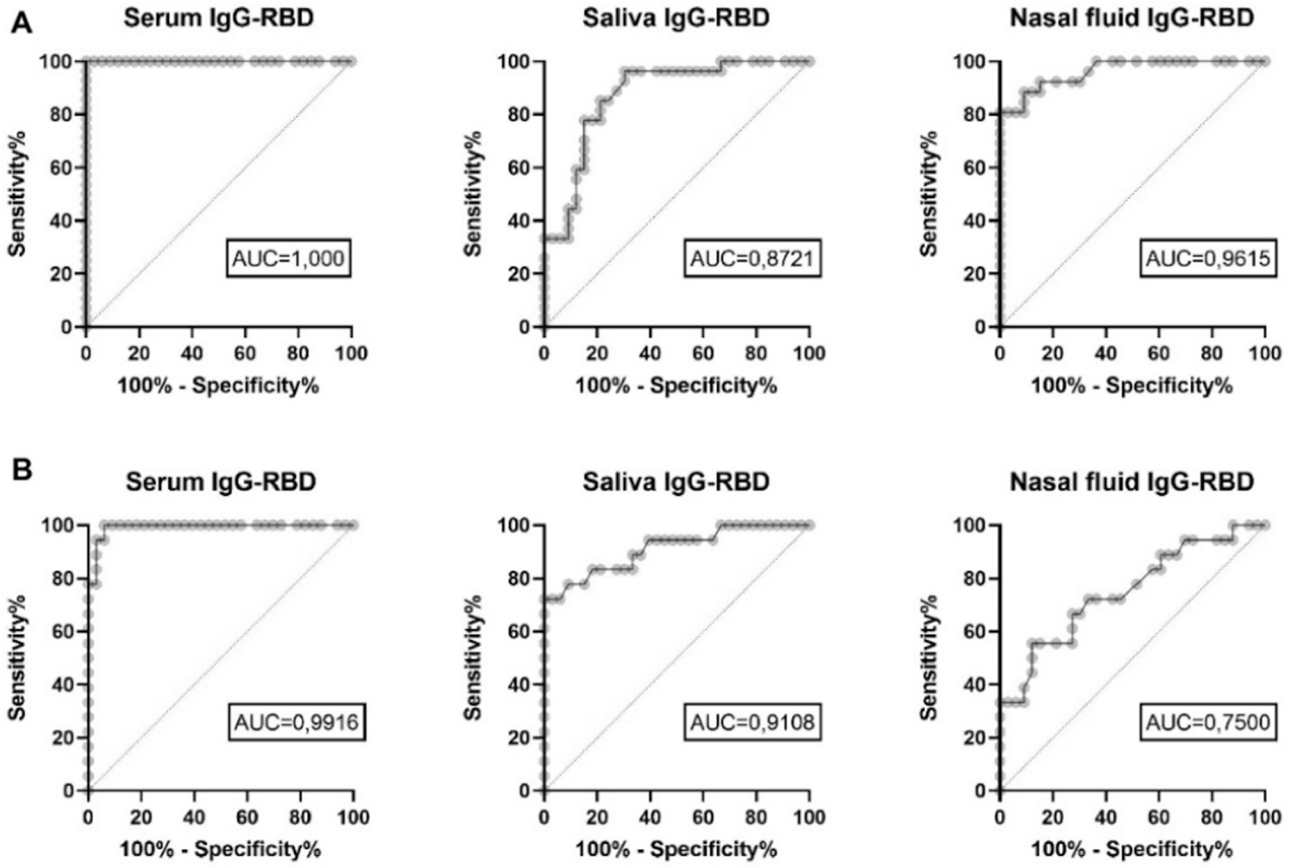

Fig. 2. Anti-SARS-CoV-2 IgG-RBD antibodies ROC curve of serum, saliva and nasal secretions from vaccinated subjects (A) and COVID-19 population (B).

procedure that can give consistent data to evaluate specific IgA and IgG localized at a mucosal level directed against SARS-CoV-2 S1-protein and its RBD, respectively. These procedures could be an effective non-invasive way to assess vaccine-induced immune responses, the individual timing of antibody decay and the effective individual protection against COVID-19.

In conclusion, mucosal immunity could provide additional data, complementary to serological analysis, useful to deepen our understanding of SARS-CoV-2 immune response induced both by the natural infection and by vaccination. Further studies are needed to determine kinetics of mucosal antibodies against SARS-CoV-2 directly on the mucosal surfaces.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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