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Analytical validation of an automated assay for the measurement of adenosine deaminase (ADA) and its isoenzymes in saliva and a pilot evaluation of their changes in patients with SARS-CoV-2 infection

Abstract

Objectives: The aim of the present study was to validate a commercially available automated assay for the measurement of total adenosine deaminase (tADA) and its isoenzymes (ADA1 and ADA2) in saliva in a fast and accurate way, and evaluate the possible changes of these analytes in individuals with SARS-CoV-2 infection.
Methods: The validation, in addition to the evaluation of precision and accuracy, included the analysis of the effects of the main procedures that are currently being used for SARS-CoV-2 inactivation in saliva and a pilot study to evaluate the possible changes in salivary tADA and isoenzymes in individuals infected with SARS-CoV-2.
Results: The automated assay proved to be accurate and precise, with intra- and inter-assay coefficients of variation below 8.2%, linearity under dilution linear regression with R^2 close to 1, and recovery percentage between 80 and 120% in all cases. This assay was affected when the sample is treated with heat or SDS for virus inactivation but tolerated Triton X-100 and NP-40. Individuals with SARS-CoV-2 infection (n=71) and who recovered from infection (n=11) had higher mean values of activity of tADA and its isoenzymes than healthy individuals (n=35).
Conclusions: tADA and its isoenzymes ADA1 and ADA2 can be measured accurately and precisely in saliva samples in a rapid, economical, and reproducible way and can be analyzed after chemical inactivation with Triton X-100 and NP-40. Besides, the changes observed in tADA and isoenzymes in individuals with COVID-19 open the possibility of their potential use as non-invasive biomarkers in this disease.

Keywords: analytical validation; assay; biomarkers; COVID-19; SARS-CoV-2.

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Introduction

Adenosine deaminase (ADA, EC 3.5.4.4) is an enzyme that, among other biological roles, catalyses the conversion of the toxic molecules adenosine and/or deoxyadenosine to inosine

and deoxyinosine, respectively [1]. This enzyme is present in most tissues, especially in those with a lymphoid origin [2]. There are two ADA isoenzymes: ADA1 and ADA2, that can be differentiated by addition to the sample of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA). EHNA is a specific ADA1 isoenzyme inhibitor that, at a proper concentration, is able to completely inhibit ADA1 isoenzyme whereas remains ADA2 unaffected [3]. In humans, ADA1 is mainly present in lymphocytes and macrophages, whereas ADA2 is predominant in plasma [4–8]. In lymphoid tissues, ADA participates in mononuclear cell maturation from monocyte to macrophage, as well as in the differentiation of B and T lymphocytes [9–11]. Therefore, the level of this enzymatic activity in serum reflects the cell-mediated immunity [12], being a biomarker of chronic inflammation [13] and situations in which circulating levels of T lymphocytes are high [14, 15]. Serum levels of ADA were shown to increase in many clinical conditions, including infections [16, 17], inflammation [4, 13], immuno-mediated disorders [6, 18, 19], and malignancies [20–24]. In addition, ADA can also increase in serum due to leakage of enzymes from damaged cells [25, 26].

Saliva is a biofluid with increasing medicine applications since it is easy, safe, pain-free, and stress-free to sampling [27]. This non-invasive specimen can reflect the physiological and physiopathological state not only of the oral cavity but of all the organism [28]. Various biomarkers of the immune system, inflammation, muscle damage, stress, oxidative status, or anaerobic metabolism, among others, have been studied in saliva, showing the potential of this biofluid. In the same line, total ADA (tADA) has been measured in saliva, and higher levels were observed in the presence of oral malignancies [29], Sjögren's syndrome [30], and obesity [3]. However, no reports have been found in which ADA activity of the different isoenzymes had been characterized in human saliva.

The current COVID-19 pandemic caused by SARS-CoV-2 virus has implied two main changes of paradigm in the field of saliva analysis. One is the change in the way of dealing with saliva when used as a specimen for clinical analysis, since there is a need for inactivation of the SARS-CoV-2 virus to minimize the risk of disease transmission. For this purpose, different inactivation protocols have been described, including the inactivation with heat [31, 32] or chemical non-ionic procedures including NP-40 or Triton X-100 [31]. The second one is the consideration of the saliva as a fluid that can be used for diagnosis and monitoring by the direct detection of the virus or antibodies, and also because various biomarkers related to inflammation and immune system, including ADA [33], could be used in this disease. However, to the authors' knowledge, there are no reports about the behaviour of ADA in saliva in COVID-19 patients.

In this paper, it is hypothesized that tADA, ADA1, and ADA2 might be quantified in saliva by an automated spectrophotometric method and that, due to their relationship with the immune system, they could potentially change in COVID-19 patients. Thus, the objectives of the present study were to validate an automated commercially available spectrophotometric method for the measurement of tADA and its isoenzymes in human saliva and to perform a pilot study to evaluate if salivary tADA, ADA1, and ADA2 change in diseased and convalescent COVID-19 patients compared to healthy controls.

Materials and methods

Participants and procedures

Saliva samples from 13 healthy individuals (adults, six females) were used to characterize the ADA isoforms and establish the optimal concentration of EHNA to be used for ADA1 inhibition. For the calculation of imprecision and accuracy, samples from three healthy individuals and three individuals infected with SARS-CoV-2 showing increases in tADA values were used.

Besides, saliva samples from 10 of the 13 healthy individuals used for ADA characterization (adults, five females) were used for studying the influence of SARS-CoV-2 inactivation protocols in ADA analysis, and saliva from 117 individuals (adults, 64 females) were used for the study of ADA changes in COVID-19.

In all cases, participants were asked not to exercise, eat, drink, smoke, or brush their teeth for at least 1 h before saliva collection. An oral exploration of the subjects was performed to discard the presence of inflammation or bleeding. All the studies were in accordance with the ethical standards of the Institutional and/or National Research Committee (1349/2016) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All participants were informed of the purpose and experimental procedures of the study and signed a written informed consent form prior to their participation. This study was approved by the Ethical Committee of the University of Murcia and the Ethical Committee of the IMIB-Arrixaca.

Saliva was collected under supervision by passive drool using 5 mL polystyrene tubes with round bottoms [34]. Then, immediately after the collection, the saliva was stored on ice until arrival at the laboratory, weighed, centrifuged (4,000×g, 10 min, 4 °C), and the supernatants were stored at −80 °C until further analysis. No samples showed blood contamination as determined by visual inspection.

Analytical validation of the assay for ADA analysis

ADA was measured using a commercially available spectrophotometric assay (Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, California, USA) adapted to an automated analyser. In this method, 50 μL of Reagent 1 containing 2 mM 4-aminoantipyrine (4-AA), 0.1 U/mL purine nucleoside phosphorylase (PNP), 0.2 U/mL xanthine oxidase (XOD), and 0.6 U/mL peroxidase in 50 mM Tris-HCl buffer (pH 8.0) is pipetted to the reaction cuvette with 10 μL of sample. After 180 s, 25 μL of Reagent 2 containing 10 mM adenosine and 50 mM

N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (EHSTP) in 50 mM Tris-HCl buffer (pH 4.0) is added. Adenosine is deaminated by ADA present in the sample to form inosine, which is then converted to hypoxanthine by purine nucleoside phosphorilase. Hypoxanthine is then oxidized by xanthine oxidase appearing hydrogen peroxide as a product of this reaction, which is further reacted with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline and 4-aminoantipyrine in the presence of peroxidase to generate quinine dye which is kinetically monitored at 550 nm wavelength and 37 °C after 486 s [35]. Manufacturer's control solutions of two different values (Diazyme Adenosine Deaminase Control Set, DZ117A-CON 2 Level, Diazyme Laboratories, Poway, California, USA) were used for the quality control analysis throughout the study. According to the data provided by the manufacturer of the kit, the clinical validation of the assay in serum showed <1.47% and <4.9% intra- and inter-assay coefficients of variation (CV), linearity under dilution linear regression equation with $R^2=0.9894$, and lower limit of detection of 0.0333 IU/L.

As a part of this report, the assay was validated in saliva. For isoenzyme determinations, the following procedure was adapted from previous reports [5]. First, the sample was measured with reagents without any treatment giving the value to tADA. Then, EHNA was added to Reagent 1 in an appropriate concentration to inhibit ADA1, and the sample was measured again with the same assay conditions, giving the value of ADA2. Finally, the ADA1 value was determined by the calculation of tADA minus ADA2.

To determine the most appropriate EHNA concentration, 13 saliva samples from healthy individuals were separated into four aliquots, and EHNA was added to each of the first three aliquots at increasing concentrations (0.1, 1.0, and 4.0 mM), whereas an equal volume of diluent was added to the fourth aliquot that acted as control.

Once the optimal EHNA concentration for the inhibition was determined, the assay's analytical validation was assessed through precision, accuracy, and limits of detection and quantification, according to protocols described previously [36]. For this, saliva samples with different tADA and ADA2 values were used to determine assay precision (intra- and inter-assay CV), accuracy (linearity under dilution and recovery), and the lower limit of quantification, whereas ultrapure water (Millipore) was used to assess the lower limit of detection [37]. All the assays were made in an automated analyzer (Olympus AU400, Olympus Diagnostica GmbH, Ennis, Ireland).

Influence of SARS-CoV-2 inactivation protocols in salivary ADA

This experiment was designed to investigate the effects of five effective SARS-CoV-2 inactivation protocols [31, 32] in salivary ADA measurements. For this, saliva from 10 healthy subjects (5 women and 5 men, aged between 25 and 35 years old), collected as previously described, were employed. Each saliva sample was homogenized, divided into six aliquots and treated as follows: (1) No inactivation treatment (NT Group); (2) Heated at 65 °C for 30 min (H65 Group); (3) Heated at 92 °C for 15 min (H92 Group); (4) Addition of (10% w/v) sodium dodecyl sulphate (SDS) to a final 0.5% SDS concentration and 30 min incubation at room temperature (SDS Group); (5) Addition of (10% v/v) NP-40 to a final 0.5% NP-40 concentration and 30 min incubation at room temperature (NP Group); (6) Addition of (10% v/v) Triton X-100 to a final 0.5% Triton X-100 concentration and 30 min incubation at room temperature (Triton

Group). To avoid inter-assay imprecision, all samples were measured in the same run.

ADA in saliva in diseased and convalescent COVID-19 patients and healthy controls

Three groups of individuals were used in this pilot study:

– –Individuals that did not have any clinical sign of disease for at least 3 weeks prior to sampling and were negative to SARS-CoV-2 infection (n=35, 21 women, aged between 24 and 51 years old) (healthy control group, HC).
– Individuals that had mild clinical signs of the disease, recovered satisfactorily and were sampled at least 10 days after the cessation of the clinical signs after a positive SARS-CoV-2 PCR (n=11, 7 women, aged between 20 and 22 years old) (convalescent group, CONV).
– Individuals with clinical symptoms of COVID-19 with WHO grade ≥2 in all cases (n=71, 36 women, aged between 32 and 84 years old) (diseased group, DIS), sampled within 24 h of the diagnosis of the disease.

The assay for SARS-CoV-2 detection involved RNA extraction and quantification by a RT-PCR with a commercial kit (FTD SARS-CoV-2, Siemens) from nasopharyngeal swabs (NPS). These NPS had different cycle thresholds (Ct) ranging from <20 to >30. In the diseased group patients, the saliva samples were obtained after confirmation of the diagnosis and within 24 h of the NPS collection and were stored at the Biobank of the Imib Center at Murcia Region. In the convalescent group patients, saliva samples were collected at least 10 days after the cessation of the clinical signs. In addition, saliva samples negative for SARS-CoV-2 by qRT-PCR in NPS made in routine check-ups were used as control group. Saliva was sampled from all individuals and the virus was inactivated with NP40 as described above.

Statistical analysis

Data showed a non-parametical distribution. The effect of the different EHNA concentrations in the salivary ADA activity was assessed by Friedman's followed by Dunn's multiple comparisons tests. Undetectable levels of the analysed biomarkers were changed to its lower limit of detection to examine descriptive statistics and missing for statistical analyses. To determine which values differ significantly ($p<0.05$) between groups and inactivation protocols, Dunn's multiple comparisons test was performed. Statistical analyses were performed with the statistical package GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

Results

ADA characterization and analytical validation

Results observed after incubation with different EHNA concentrations are shown in Figure 1. Statistically significant

inhibition of ADA1 was achieved with 1 mM EHNA (0.12 mM in the reaction mixture). ADA1 was the predominant isoform in saliva, observing values below the lower limit of detection for ADA2 in 2 out of 13 samples. Our results indicated that the EHNA concentration of 1 mM was the most appropriate since it showed similar inhibition of ADA1 than the higher concentrations of 4 mM. Using 1 mM EHNA, the median (25th–75th percentiles) for ADA1 and ADA2 in saliva were 4.82 (3.25–6.50) and 0.49 (0.14–2.12) IU/L, respectively.

For tADA, the intra- and inter-assay CVs were lower than 5.0 and 6.2%, respectively (Table 1). Spiking recovery ranged from 100.20 to 105.74%. Linearity under dilution of the saliva samples in ultrapure water resulted in a linear regression equation with R^2 close to 1 in the three samples analysed (Figure 2). The lower limit of detection of the assay was 0.08 U/L and the lower limit of quantification was 0.19 U/L.

For ADA2, the intra- and inter-assay CVs were below 7.6 and 8.2%, respectively (Table 1). Spiking recovery ranged from 96.67 to 99.60%, and linearity under dilution showed correlation coefficients of $R^2 > 0.98$ in all cases (Figure 2). The lower limit of detection for ADA2 was 0.07 U/L and the lower limit of quantification was 0.11 U/L.

Influence of SARS-CoV-2 inactivation protocols in salivary ADA

Results of the SARS-CoV-2 inactivation protocols on salivary ADA levels are summarized in Table 2. When the effects of the different inactivation protocols were evaluated, three of them (H65, H92 and SDS) showed a marked reduction in tADA and its isoenzymes, with values close to zero. On the other side, the samples inactivated using
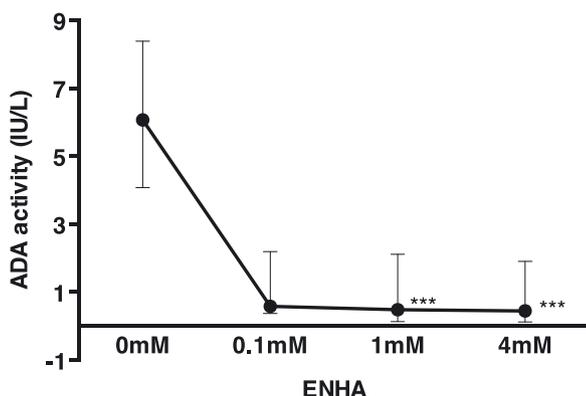
Figure 1: Adenosine deaminase (ADA) activity in saliva from 13 healthy humans before and after adding erythro-9-(2-hydroxy-3-nonyl) adenina (EHNA) to the samples at different concentrations. Asterisks indicate statistically significant results vs. the original samples (***p<0.001).

Table 1: Intra- and inter-assay coefficients of variation (CV) of total adenosine deaminase (tADA) and isoenzyme 2 (ADA2) in three pools of saliva samples with different enzymatic activities.

| Assay	Comparison	Pool	Mean, IU/L	SD, IU/L	CV, %
tADA	Intra	1	2.23	0.11	4.93
		2	16.29	0.15	0.92
		3	57.38	0.58	1.02
	Inter	1	2.29	0.14	6.11
		2	16.42	0.23	1.40
		3	58.97	1.31	2.22
ADA2	Intra	1	0.80	0.06	7.50
		2	5.04	0.15	2.98
		3	17.26	0.30	1.76
	Inter	1	0.74	0.06	8.11
		2	5.36	0.09	1.68
		3	18.28	0.82	4.46

SD, standard deviation.

NP-40 and Triton X-100 treatments did not show significant changes in the measured ADA activities.

ADA in saliva in diseased and convalescent COVID-19 patients and healthy controls

Results of the pilot study with healthy, COVID-19 convalescent, and COVID-19 diseased individuals on salivary ADA levels are summarized in Figure 3. The groups of diseased individuals and convalescents had significantly higher tADA values compared to healthy, with median (25th–75th percentiles) of 10.3 (3.78–18.8), 10.5 (4.75–17.3), and 2.8 (1.75–3) IU/L, respectively. Also, for ADA1, values were higher for diseased and convalescents, with values of 3.7 (1.4–8.8) and 4.03 (2.84–7.56) IU/L, respectively, comparatively to healthy individuals with values of 1.5 (0.8–2) IU/L. For ADA2, values were 6.7 (2.42–10.1), 4.6 (1.73–8.73), and 0.88 (0.38–1.13) IU/L, respectively for diseased, convalescent, and healthy groups. No significant differences were observed between diseased and convalescents.

Discussion

To the authors' knowledge this is the first report in which ADA isoenzymes are characterized in human saliva samples, and an automated assay for their measurement is described and analytically validated. In addition, it is described that this assay can be performed in saliva samples treated with NP-40 or Triton X-100 for inactivation of SARS-Cov-2, and that can detect increases in tADA and
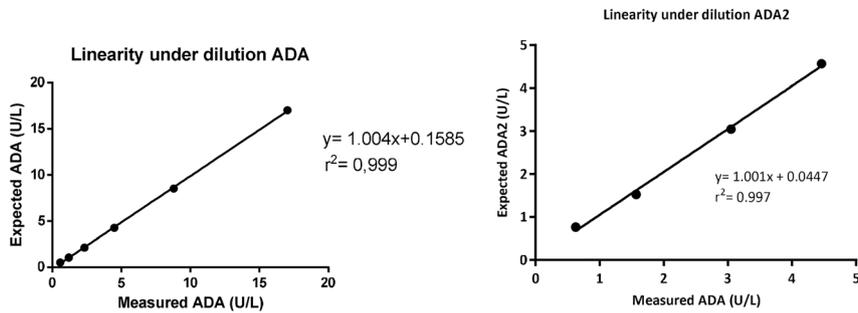
Figure 2: Representative graph of linearity under dilution of total adenosine deaminase (tADA) and ADA2 in saliva.

Table 2: Median (25th–75th percentile) ADA (tADA) and its isoforms (ADA2 and ADA1) in saliva after the different inactivation SARS-CoV-2 treatments.

	NT	H65	H92	SDS	NP	Triton
tADA, IU/L	1.6 (1.05–2.15)	0.1 (0.08[b]–0.15)[a]	0.1 (0.08[b]–0.15)[a]	0.08[b] (0.08[b]–0.15)[a]	1.75 (0.5–2.94)	1.58 (1.37–2.36)
ADA2, IU/L	0.3 (0.2–0.35)	0.07[b] (0.07[b]–0.1)[a]	0.07[b] (0.07[b]–0.09)[a]	0.07[b] (0.07[b]–0.09)[a]	0.25 (0.1–0.44)	0.315 (0.09–0.42)
ADA1, IU/L	1.3 (0.55–1.63)	0 (0–0.125)[a]	0 (0–0.125)[a]	0 (0–0.1)[a]	1.25 (0.7–1.93)	1.4 (0.53–1.83)

NT, no inactivation treatment (control); H65, 65 °C heat; H92, 92 °C heat; SDS, 0.5% SDS; NP, 0.5% NP-40; Triton, 0.5% Triton X-100. [a]Differences of statistical relevance vs. NT measurements. [b]Lower limit of detection has been indicated since no value was obtained.
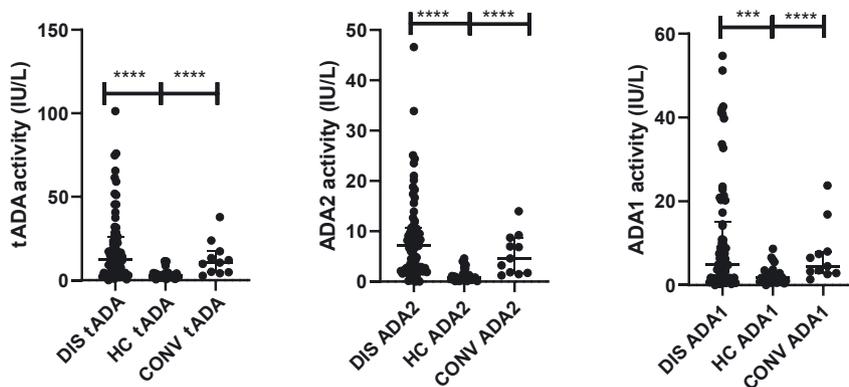
Figure 3: Adenosine deaminase (tADA) and its isoforms (ADA2 and ADA1) activities in saliva in healthy (HC), 10 days after positive SARS-CoV-2 PCR (CONV) and COVID-19 symptomatic individuals (DIS).
Asteriks indicate differences of statistical relevance between the groups (***p<0.001; ****p<0.0001).

ADA isoenzymes in saliva from COVID-19 diseased and convalescent patients.

The assay used in our study offers several advantages compared to other methods because it is easy to perform and set-up, economical, rapid, and allows differentiating between the different ADA isoenzymes. For ADA2 determination, EHNA was added directly to the commercial reagent to achieve a final concentration in the reaction mixture of 0.12 mM, which was the concentration that in our conditions completely inhibits ADA1 isoenzyme in human saliva, in agreement with previous reports [5]. The whole procedure for ADA2 determination can be fully automated, reducing the required time for the assay and the risk of error due to human manipulation. The results for intra- and inter-assay precision CVs were within the acceptable limits proposed by the Guidance for Industry Bioanalytical Method Validation [36]. Recovery and linearity assays showed good accuracy,

and lower limits of detection and quantification, indicating that the method has enough analytical sensitivity to measure tADA and ADA2 isoenzyme in saliva samples. Overall, the automated assay used for the measurement of tADA and ADA2 in saliva showed an adequate analytical performance according to the requirements for good method functionality [36, 38] and give in the validation procedure similar results to those obtained by the manufacturer in serum samples. Lastly, the present assay requires a low sample volume (10 μL), which may be an advantage when this volume is limited, such as in some oral diseases, dehydration, or elderly or neonate patients.

When the effects on ADA measurements of different SARS-CoV-2 inactivation protocols in saliva were evaluated, both heating treatments (65 and 92 °C for 30 min) and 0.5% SDS inhibits ADA activity to almost zero values. This could be explained by the effects of heat on proteins, which

usually leads to denaturation and/or aggregation and the denaturing effect of SDS [39, 40]. On the other side, NP-40 and Triton X-100 inactivation protocols did not cause alterations in ADA or its isoenzymes in saliva and could be used in routine settings.

The increases found in tADA and their isoenzymes in patients with COVID-19 could be related to the presence of T lymphocyte activation and increased monocytes/macrophage activity, since this enzyme has been previously associated with these cells [41]. Increases of ADA in serum have been reported for tuberculosis [42, 43]. As well, serum increases of the isoenzyme ADA2 has been described in other viral diseases such as immunodeficiency virus infection [44]. It was also interesting to observe that ADA levels remain elevated, comparatively to control, even during convalescence, suggesting that assessment of ADA in saliva can be useful in COVID-19 management in different phases of the disease. It would be of interest in the future to perform studies involving a larger number of patients in which it could be explored the behaviour of ADA in individuals with different severity of clinical signs and also to evaluate if there could be a relationship between this enzyme and the prognosis of the disease. In addition, the relation of ADA with other biomarkers of inflammation and changes in lymphocyte subsets population would be of interest to be explored. If the results of these trials were satisfactory, the measurements of ADA in saliva could be used as marker of lymphocyte T activation. This could complement the assays that are currently used in this disease for the pathogen detection and the quantification of antibodies produced, with the advantage that all these measurements could be performed in saliva [33]. The present study presents some limitations. First, although no saliva sample showed blood contamination by visual inspection and none of the participants showed apparent oral diseases, an objective criterion such as haemoglobin or transferrin measurement was not employed. Second, the results of tADA and its isoenzymes evaluation in patients with COVID-19 should be considered as preliminary due to the limited number of samples and confirmed in a larger number of individuals.

Three conclusions can be summarized from the present study. First, tADA and its isoenzymes ADA1 and ADA2 can be measured accurately and precisely in saliva samples in a rapid, economical, and reproducible way. Second, ADA was not affected by NP-40 and Triton X100 SARS-CoV-2 inactivation protocols, opposing 65 and 92 °C heating and SDS treatments that reduced ADA activity. Last, higher values in tADA and its isoenzymes were found in diseased and convalescent COVID-19 patients. Overall, the automated assay presented in this report can contribute to a broader use and application of the measurement of tADA and its isoenzymes in human saliva and to the performance of additional studies to gain knowledge about the possible application of these enzymes in different clinical situations.

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

Ethical approval: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by Ethical Committee of the University of Murcia and the Ethical Committee of the IMIB-Arrixaca.

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