Demonstrating "Abdala" Subunit Vaccine Stability Under Thermal Stress Conditions

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ABSTRACT

From a regulatory standpoint, vaccine stability must be demonstrated, along with the prediction of stability during temperature excursions, before a vaccine can be approved for use in humans.

In this work, Abdala subunit vaccine thermostability was studied under thermal stress conditions (2–8 °C [control], 25 °C, 37 °C, 45 °C, and 60 °C) for 15 days. Molecular integrity of the vaccine active pharmaceutical ingredient was monitored by SDS-PAGE, immunoblotting, RP-HPLC, mass spectrometry, and circular dichroism spectroscopy analysis. While functionality was monitored by immunogenicity assay, inhibition of binding between receptor-binding domain (RBD) and receptor, angiotensin converting enzyme 2 (ACE2), and RBD/ACE2 binding assay.

Results showed that no degradation, loss of disulfide bridges, nor modifications of secondary structure of the RBD molecule were detected at 25 °C and 37 °C. Moreover, high titers (1:48,853 – 1:427,849) of anti-RBD-specific mouse antibodies were detected with the ability to inhibit, to different degrees, the binding between RBD/ACE2.

In conclusion, the Abdala subunit vaccine is stable under thermal stress and storage conditions, which has an advantage over non-subunit vaccines previously approved or currently in development against COVID-19. The demonstrated high stability of this vaccine is a key factor in ensuring vaccine effectiveness, extending immunization coverage with fewer production runs, simplifying immunization logistics, and reducing cold chain-associated costs.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) was first detected in Wuhan, China and is caused by the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2). Respiratory symptoms in humans range from asymptomatic to moderate or severe clinical symptoms and also death.[1]

Since the COVID-19 pandemic is an extraordinary challenge to any healthcare system, scientific institutions have developed several vaccines based on the SARS-CoV-2 spike glycoprotein due to its ability to induce a strong virus-neutralizing immune response.[2, 3] These vaccines are helping to protect millions of lives through the successful application of local and global immunization programs[4, 5], yet countless lives could be lost due to the application of thermally unstable vaccines.

So far, each type of COVID-19 vaccine works differently to introduce the given SARS-CoV-2 antigen-targeting version into the human body, ranging from using unrelated and harmless viral vectors to deliver SARS-CoV-2 genetic material, messenger ribonucleic acid, inactivated or attenuated SARS-CoV-2 virus, and protein subunit vaccines.[6-8] Evidently, each of them have their own stability ranges in response to the light, thermal conditions, radiation, environmental changes, or reactions with containers or other components present in the mixture. Therefore, these features have to be deeply considered during vaccine development stages because even when optimal conditions are maintained, potency may decline gradually as time passes after production.

Scientific biotechnology institutions in Cuba have developed and licensed vaccines against several infectious diseases (*e.g.*, Heberbiovac HB®), which have been produced in a well-established, large-scale biomanufacturing platform using the

Pichia pastoris yeast as the host cell.[9-11] Therefore, the strategy applied to produce the Abdala subunit vaccine as active pharmaceutical ingredient (API) against COVID-19 was also based on the production of the SARS-CoV-2 receptor-binding domain (RBD) in the same *P. pastoris* production and regulatory platforms to speed up the approval process for massive application in humans. Details on the RBD expression are described by Limonta *et al*.[12] A sequence coding for residues 331-530 of the spike protein of SARS-CoV-2 strain 156 Wuhan-Hu-1 (NCBI Acc. No. YP_009724390) with the appropriate N- and C-terminal 157 extensions was expressed in *P. pastoris* under the AOX1 promoter. After the nucleotide sequence verification, a representative clone was used to prepare the yeast seed bank and to inoculate a 3000 L fermenter.

For such purposes, and considering the most updated regulatory issues, the demonstration of vaccine stability is mandatory because API and vaccine stability are critical factors impacting quality, potency, and distribution. Consequently, all immunization campaigns have the challenge of validating and maintaining the cold chain during distribution, delivery, and storage of the vaccines.

The World Health Organization (WHO) and other regulatory agencies have issued guidelines to regulate how stability studies will be conducted under storage and thermal-stress conditions. To fulfill such requirements, the application of physico-chemical and biological assays such as chromatographic and electrophoretic procedures, potency tests, and immunogenicity assays are generally recommended, among others.[13, 14]

Licensed vaccines against diphtheria, tetanus toxoid, pertussis, poliomyelitis, haemophilus influenzae, and hepatitis B have undergone such thermostability studies. Within them, diphtheria, tetanus toxoid, and hepatitis B vaccines have been the most resistant to temperature variations, demonstrating stability for years at 2−8°C, months at 20−28°C, weeks at 37°C, and days at 45°C.[13] The most common aspect of these already-approved vaccines to the Abdala vaccine is that they are all well-characterized conjugated and non-conjugated subunit vaccines.

Therefore, the objective of this paper was to assess Abdala subunit vaccine stability when exposed to thermal stress conditions (25−60°C) for 15 days using established physico-chemical and biological assays. When designing the study, the material tested had to represent routine production batches, and the range of temperatures to assess was chosen because the Abdala subunit vaccine is a liquid suspension consisting of a purified yeast-version of RBD adsorbed onto aluminum salt. As with other vaccines adsorbed on aluminum salts, freezing this vaccine may cause a significant reduction of potency.[14] On the other hand, since other subunit vaccines are stable at 2−8°C for many years, this temperature was chosen as the control throughout the study. Because some yeast-derived recombinant DNA vaccines are also stable at elevated temperatures (*e.g*., 45°C), the range of 25−60°C was decided upon. This justification was based on the degradation mechanism of proteins, previous results of testing under accelerated conditions, batch size, existence of supporting stability data of other subunit vaccines produced in this production platform, and finally, assuming that the same degradation relationship will continue to apply beyond the observed data.

MATERIALS AND METHODS

Vaccine Composition

The Abdala subunit vaccine is composed of a recombinant version of SARS-CoV-2 RBD formulated with an aluminum hydroxide gel (BDC International S.A.) as adjuvant.[12]

Reference Material (RM)

The RM of SARS-CoV-2 RBD (AgRBD-01-0221) was certified and supplied by the Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba.

Source of Biological Materials and Reagents

Eight week-old female mice of the isogenic BALB/c line of haplotype H-2d or H-2K were supplied under controlled conditions by the Center for Laboratory Animal Breeding (CENPALAB), Havana, Cuba. Polyclonal preparation of sheep anti-mouse IgG antibodies (specific for γ-heavy chain) conjugated to horseradish peroxidase (HRP, Sigma-Aldrich), anti-RBD CBSSRBD-S.7 and CBSSRBD-S.8 monoclonal antibodies (mAb), and human-Fc-RBD-HRP conjugate were supplied by the CIGB-Sancti Spíritus, Cuba (CIGB-SS). An anti-RBD antibody-negative mouse serum and anti-RBD antibody-positive control serum were supplied by CIGB, and the humanFc-RBD and mouseFc-ACE2 fusion proteins were supplied by the Center for Molecular Immunology (CIM), Havana, Cuba.

Thermostability Study Conditions

Vaccine samples taken in their primary packing material, 6R vials of first hydrolytic quality borosilicate glass sealed with chlorobutyl stoppers were incubated at five different temperatures, 2−8°C, 25°C, 37°C, 45°C, and 60°C for 15 days with ± 2°C of tolerance and with humidity control. Once the incubation time was over, samples were stored at 2−8°C in a cool chamber until the moment of analysis. The total amount of vials used for assessing each temperature was 150.

Visual Inspection

Sample observation was carried out using an inspection lamp while rotating vaccine samples in all directions on each of the surfaces of the inspection cabinet (white and black backgrounds) for 5 seconds each. Samples were inspected without magnification. As an acceptance criterion, the observation of a slightly opaque, grayish-white suspension was used that, after allowing for sedimentation, separates into two phases, a transparent liquid and a gel. Both phases have to be easily resuspended and free of foreign particles when shaken. The whole study was performed following current European Pharmacopeia guidelines.[15]

RBD Molecular Integrity Characterization Tests

Desorption Procedure

Desorption of RBD was performed by centrifuging a 2 mL sample of the reverse phase-high performance liquid chromatography (RP-HPLC) or a 1 mL sample of SDS-PAGE at 300×*g* in a HIMAC CR150T centrifuge (Hitachi) for 15 min. Subsequently, supernatant was discarded and the pellet (aluminum hydroxide), 400 mM phosphate buffer (400 µL for RP-HPLC sample) or 600 µL (for SDS-PAGE sample), pH 8.0, were mixed, homogenized, and centrifuged again at 1000×*g* for 1 min. The supernatant was then taken and the pellet was discarded.

Adsorption to Aluminum Hydroxide Gel and pH Values Determination

To determine protein concentration and the amount of absorbed RBD, the microcoomassie method was used in a double-wavelength assay.[16] The absorbance was measured at 620 nm and 450 nm to improve sensitivity and linearity of the method at lower protein concentration values. Vaccine sample (0.5 mL) was added to a vial and centrifuged in an Eppendorf model 5424 at 4000×*g* for 10 min. Next, to perform the assay, supernatant was taken, avoiding pipette contact with the vaccine pellet. Subsequently, 60 µL of supernatant were mixed with 240 µL of a coomassie solution in a 96-well plate (Nunc MaxiSorp™, Fisher Scientific) and the absorbance was measured in a kinetic Multiskan GO (Thermo Scientific). Each sample was measured by triplicate. The percentage of RBD absorbed to the gel was calculated using the formula:

$$\text{Adsorption (\%)} = 100 - (\text{concentration of non-adsorbed protein/vaccine nominal concentration value}) \times 100$$

Finally, pH values were measured with a SevenEasy™ pH meter (Mettler Toledo).

RBD Purity and Profile Estimation by Electrophoresis

Desorbed RBD 20 µL (3 µg) was boiled in sample buffer (4% SDS, 20% glycerol, 125 mM Tris, pH 6.8, 0.01% bromophenol blue, 10% 2-mercaptoethanol) for 3 min and separated in a 13.5% SDS-PAGE gel (current of 30 mA and 250 V for 1 h) using a Mini-PROTEAN Tetra Cell (Bio-Rad). Proteins were stained with silver nitrate and images were obtained using a GS-800™ densitometer with Quantity One® 1-D analysis software version 3.2.1 (Bio-Rad).[17]

RBD Immunoblotting Analysis

Immunoblotting experiments were performed to analyze the identity of proteins separated in the SDS-PAGE. For this, the immobilization of separated proteins was carried out on a Hybond-C 0.45 µm pore nitrocellulose membrane (Sartorius) using a Trans-Blot semi-dry transfer cell (Bio-Rad) and transfer buffer (47.9 mM Tris, 38.6 mM glycine, 20% methanol [v/v], 10% SDS, pH 9.2). The transfer process took 1 h at a constant current value of 25 V, calculated by the membrane area × 5.5.

To check the effectiveness of protein transference, the membrane was stained with a Ponceau red solution. Then the membrane was incubated with 50 mL of blocking solution (5% [w/v] non-fat dry milk powder/150 mM PBS, pH 7.2) overnight at room temperature. Later, the membrane was washed with 150 mM PBS, pH 7.2, washing solution and incubated with an anti-RBD polyclonal serum for 1 h at 37°C. Then, the membrane was washed again with the washing solution and incubated with the protein A (Sigma-Aldrich)-HRP conjugate for 1 h at 37°C. Finally, another washing step was done with the same solution. The development of bands in the membrane was carried out with diaminobenzidine and hydrogen peroxide.[18] Precision Plus Protein™ All Blue Prestained Protein Standards 10–250 kDa (Bio-Rad) were used.

RP-HPLC Profiles of the Desorbed RBD

RP-HPLC was performed using a Shimadzu C18 column (250 mm × 4.6 mm, 5 µm) and a volume of 150 µL (38 µg) of desorbed RBD was manually injected. The column was equilibrated with 0.1% trifluoroacetic acid (TFA) solution. Protein was eluted by using a linear gradient of 0.1% TFA solution (solution A) and acetonitrile with 0.05% TFA (solution B) from 10–100% solution B over a 50 min period. Separation was carried out at 35°C at a flow rate of 1 mL/min and the eluted fraction was monitored at 226 nm. A Shimadzu Nexera HPLC instrument was used for the procedure. It was equipped with Shimadzu accessories: an LC-20ADXR binary pump, a CTO-20AC column oven, and an SPD-20A UV-Vis detector.

Trypsin Digestion of RBD

Twenty-five micrograms of RBD dissolved in 150 mM PBS, pH 7.4, were digested with 0.5 µg of a sequencing grade trypsin preparation (Promega) for 2 h at 37 °C. After that, 0.5 µL PNGase F (New England BioLabs) was added and the deglycosylation reaction proceeded for 1.5 h at 37 °C. Then, samples were centrifuged at 2240 ×*g* (Hitachi) and the solution was desalted by a ZipTip C18 (Sigma-Aldrich), washed with 0.2% (v/v) formic acid solution, and eluted with 60% acetonitrile in water containing 0.2% formic acid (v/v).

ESI-MS Analysis of Tryptic Peptides

Tryptic peptides were analyzed in a hybrid orthogonal Q-Tof-2 tandem mass spectrometer (Micromass) by spraying sample material into the ion source using 1200 and 35 V for the capillary and entrance cone, respectively. The ESI-MS spectra were acquired from 200–2000 m/z, and the multiply charged ions were manually fragmented by collision-induced dissociation using appropriate collision energies (20–50 eV). Argon was used as a collision gas and the spectra were processed by using the MassLynx™ v4.1 software (Micromass). The ESI-MS/MS of tryptic peptides with $z \geq 3+$ were deconvoluted by MaxEnt 3.0 (Micromass). The theoretical m/z for tryptic peptides was calculated by using the peptide and protein editor available in the MassLynx v4.1 software. Protein identity was confirmed with four lineal peptides by using the MS/MS ions search option of Mascot v2.5 (Matrix Science) and the SARS-CoV-2 database was used for identification.[19] Asparagine and glutamine deamidation, and methionine oxidation were considered as variable modifications. Up to one missed cleavage with trypsin and an error tolerance of 0.3 Da for precursor and fragment ions were considered. The percentage of peptide coverage was as follows: For the analyzed temperatures 2–8 °C, 25 °C, 37 °C, and 45 °C, the sequence coverage for each case was 99.15%. However, for 60 °C, the obtained sequence coverage was 69.19%.

Circular Dichroism (CD) Spectroscopy Analysis of Desorbed RBD

For the first step, RBD fraction adsorbed to the aluminum hydroxide gel was quantified. Specifically, the soluble fraction was obtained by centrifuging 1 mL of Abdala subunit vaccine solution at 8944 ×*g* in an Eppendorf model 5415D centrifuge for 15 min to extract supernatant. The adsorbed RBD fraction was obtained by resuspending the pellet in 400 µL of 400 mM phosphate, pH 8.0, by vigorous vortexing and incubation at 25 °C for 90 min with gentle agitation. Then, sample was

centrifuged again (as described previously) and supernatant harvested. Protein concentrations of both fractions were determined with an Implen NanoPhotometer® model NP80. Absorbance was measured at 280 nm while using an extinction coefficient of 1.509 (antibody 0.1%, g/L) derived from the amino acid sequence determined with the ProtParam tool of the ExPASy resource portal (Swiss Institute of Bioinformatics).[20] The calculated concentration was further used to quantify percentage of adsorbed RBD (P_{ads}) and recovery of desorption procedure (R_{des}) according to the following equations:

$$P_{ads} = 100 \times \frac{0.1 - P_s}{0.1}$$

$$R_{des} = 100 \times \frac{P_{des} \times 0.4}{(0.1 - P_s)}$$

where P_s is the protein concentration (mg/mL) of soluble protein fraction and P_{des} is the concentration of desorbed fraction. The far-UV (200–250 nm) CD spectra of obtained protein fractions were acquired with the J-1500 Spectropolarimeter (Jasco) at 24 °C. All spectra were recorded after 18 runs using corresponding buffer solutions as baselines for subtracting. A quartz cuvette of 0.1 cm optic pass was used in all measurements.

Quantification of RBD by Enzyme-Linked Immunosorbent Assay (ELISA)

A Nunc MaxiSorp™ ELISA plate (Fisher Scientific) was coated with 100 µL/well (5 µg/mL) of CBSSRBD-S.8 mAb diluted in 0.01 M carbonate-bicarbonate buffer solution, pH 9.6, and incubated for 3 h at 37 °C. Next, the plate was blocked with 3% non-fat dry milk powder for 1 h at 37 °C and washed with 150 mM PBS/0.05% (v/v) Tween-20, pH 7.2. Later, samples and RM were added and incubated for 1 h at 37 °C. Subsequently, the CBSSRBD-S.7 mAb labeled with HRP was added to the wells and incubated for 1 h at 37 °C. Hydrogen peroxide and o-Phenylenediamine (Sigma-Aldrich) were used as the chromogenic substance. The reaction was stopped with 50 µL/well of 2 M sulfuric acid while sample absorbance was measured at 492 nm in a model PR-621 ELISA plate reader (SUMA).

Functionality Tests

BALB/c female mice were intraperitoneally immunized with 50 µg of the Abdala vaccine on a three-dose schedule: 0, 14, and 21 days. Twenty-one and 35 days after the first immunization, blood samples were collected and centrifuged (Zentrifugen D-78532, Hettrich) at 15625 ×*g* for 10 min. Collected sera were evaluated by ELISA to determine titers of antibodies specific for RBD.

Determination of Anti-RBD-Specific Mouse Antibody Titer by ELISA

A 96-well microplate (Corning) was coated with human-Fc-RBD fusion protein in 0.01 M carbonate-bicarbonate buffer overnight and blocked with 2% skim milk at 37℃ for 1 h. Samples were then added and incubated at 37℃ for 2 h. A conjugate of a polyclonal anti-mouse IgG produced in sheep (specific for γ-heavy chain) labeled with HRP was used. The reagent 3,3',5,5'-Tetramethylbenzidine (TMB) was used as reaction chromogen and hydrogen peroxide as substrate. The presence of antibodies was detected at a wavelength of 450 nm in the SUMA PR-621 ELISA plate reader. The plate cut-off value for declaring antibody presence was:

$$\text{Cut-off} = \text{placebo mean} + 2 \times \text{standard deviation}$$

Antibody titers corresponded with the dilution equal to or immediately greater than the plate cut-off value.

Surrogate Virus Neutralization Test (sVNT-ELISA) Performed to Estimate Mouse Serum Inhibition Capacity of RBD Interaction With ACE2

A 96-well microplate (Corning) was coated with 50 µL/well of a 5 µg/mL solution of mouseFc-ACE2 fusion protein in 0.01 M carbonate-bicarbonate buffer, pH 9.6, for 18 h at 4℃. After a washing step with 0.05% (v/v) of Tween-20 in Dulbecco's phosphate-buffered saline (DPBS) (Sigma-Aldrich), the plate was blocked with 2% (w/v) non-fat dry milk powder dissolved in the washing buffer for 1 h at 37℃. In parallel, samples and controls were incubated in a separate non-protein binding plate with humanFc-RBD-HRP diluted in 0.2% (w/v) non-fat dry milk powder in DPBS, for 1 h at 37℃. The CBSSRBD-S.8 mAb that inhibits and neutralizes SARS-CoV-2[21] was used as a positive control in this assay. After the incubation period, 100 µL of the sample-conjugate mixtures were added per ACE2-coated well with two replicates per point, and additionally incubated for another 90 min at 37℃. Unbound reactants were removed via six washes with 0.05% (v/v) Tween-20 in DPBS followed by six washes with ddH₂O. Afterward, 100 µL of a TMB solution in 0.01% phosphate-citrate buffer (200 mM phosphate, 10 mM citrate, pH 5.0) and 0.006% (v/v) hydrogen peroxide were added per well for 15 min. The reaction was stopped by adding 50 µL of 2 M sulfuric acid, and the absorbance was measured at 450 nm in a CLARIOstar microplate reader (BMG Labtech). Inhibition values were determined as:

$$\text{Inhibition (\%)} = 1 - (\text{sample absorbance/negative control absorbance}) \times 100$$

ACE2 Binding Assay

The study of ACE2 binding was conducted using a variation of the described sVNT-ELISA. In this case, the vaccine was used as a competitor instead of mouse sera. Briefly, the assay started with 1:2 dilutions of the vaccine previously mixed and incubated with a constant amount of humanFc-RBD-HRP and immediately added to murineFc-ACE2 to coat the plate. The amount of detectable RBD was then quantified using a standard curve generated with the API (CRBDH6) supplied by the CIGB.

Statistical Analysis

The slopes of variation over time of each investigated parameter, for all samples and temperatures, were calculated. The main effects in analysis of variance (ANOVA) was performed to determine if variation of slopes was due to the difference among parameters in each temperature or sample. To determine if there were differences between initial time and after 15 days, the Student's t-test was applied. In the case of antibody titer estimation by ELISA, a logarithmic transformation of data was previously performed before the analyses, since these data have a log-normal distribution. The values of inhibition assay were plotted in GraphPad Prism 8.0.2 and results were compared using a one-way ANOVA or Kruskall-Wallis test, depending on data distribution properties. In all cases, a significance level of 0.05 was applied.

RESULTS AND DISCUSSION

Subunit vaccines are prepared by extracting and purifying components of pathogens that can elicit an immune response. Compared to classical vaccines, subunit vaccines are safer, as they circumvent stimulation of the immune system, and reduce risks of subsequent reversion caused by traditional attenuated microorganism vaccines, and are less thermally sensitive.[16] However, these vaccines must be administered to patients in several doses at designated time intervals to ensure their long-term stability and efficiency.[22, 23]

With regard to vaccine stability tests, one of the procedures used for measuring stability includes accelerated degradation procedures. Vaccines are subjected to temperatures at which API degradation is induced in a relatively short period of time. Then, the rate at which it occurs can be extrapolated to lower temperatures than vaccines should be stored[24], which is why the Abdala subunit vaccine thermostability study was the main subject of this paper.

Initial results revealed that vaccine samples met the established vaccine organoleptic specifications since they remained the same, slightly opaque, white-gray

TABLE 1. Data from RBD quantification and adsorption to the aluminum hydroxide gel in three samples of Abdala subunit vaccine exposed to thermal stress conditions. Organoleptic characteristics include two phases: (1) a transparent liquid; and (2) in gel form. Both were easily resuspended and free of external particles when shaken together.

Sample	Test	Temperature and Time			
		2–8 °C 0 days	T = 37 °C 15 days	T = 45 °C 15 days	T = 60 °C 15 days
1	Unabsorbed RBD concentration (mg/mL)	0.030	0.031	0.022	0.005
	RBD adsorption (%)	70	69	78	95
	pH value	6.73	6.84	6.90	6.96
	RBD concentration by ELISA (µg/mL)	64.49	54.56	16.67	0.31
2	Unabsorbed RBD concentration (mg/mL)	0.030	0.030	0.021	0.005
	RBD adsorption (%)	70	70	79	95
	pH value	6.73	6.84	6.87	6.96
	RBD concentration by ELISA (µg/mL)	42.56	40.25	18.46	0.31
3	Unabsorbed RBD concentration (mg/mL)	0.030	0.029	0.018	0.006
	RBD adsorption (%)	70	70	79	95
	pH value	6.70	6.85	6.89	6.98
	RBD concentration by ELISA (µg/mL)	44.77	53.40	23.14	0.31

suspension. Even when vaccine samples were separated into two phases after a settling time: (1) a transparent liquid; and (2) in gel form; both easily resuspended and without foreign particles (**Table 1**). This finding coincides with results found in other subunit vaccines where organoleptic properties do not change under storage conditions.[9]

The analysis of the Abdala vaccine API adsorption percentage to the aluminum hydroxide gel also evidenced the lack of temperature effects (≥50%), (2–8 °C [70 ± 0.00%], 37 °C [69.6 ± 5.7%], 45 °C [78.6 ± 0.57%], and 60 °C [95 ± 0.00%]) on the binding of the API to the gel with respect to the storage condition (2–8 °C), as shown in **Table 1**. Nevertheless, a significant increase in the API adsorption to the gel was observed from 45 °C ($p = 1.35 \times 10^{-5}$) and 60 °C ($p = 1.17 \times 10^{-10}$), respectively.

This phenomenon might be explained by the effect of the temperature on the gel properties, which allows a higher binding of API. This corresponds with the lowest RBD values detected in the adsorption reaction supernatant by using a total protein determination test. In contrast, values of API absorbed in the vaccine, measured directly by ELISA, decreased to around 50% in samples stored at 45 °C, and 100% at 60 °C with respect to the 2–8 °C storage conditions. Hence, authors hypothesize this decrease was produced by the loss of the recognition capacity of the CBSSRBD-S.8 mAb[21] specific for a RBD conformational epitope, as observed in samples stored at

the highest temperatures (**Table 1**).

The electrophoresis analysis allowed the detection of a 42 kDA band in samples exposed to different temperatures of 2–8 °C, 25 °C, 37 °C, 45 °C, and 60 °C. A decrease in the signal intensity was observed in samples exposed to thermal stress, in comparison to the API used as RM. Since this finding was observed in samples exposed to 2–8 °C, this behavior could likely be due to inefficiency of the API desorption process. The reason is that not all API molecules bound to the aluminum hydroxide gel can be desorbed (**Figure 1A**) but not because of temperature effects on samples. As illustrated in **Figure 1B**, the presence of a 42 kDa band in the desorbed API was confirmed by immunoblotting in all samples, as well as in the RM. To summarize, due to all samples having similar electrophoretic and immunoblotting profiles, it can be stated that studied temperatures and time do not have significant impact on the vaccine API molecule integrity (purity).

Simultaneously, chromatographic profiles of the desorbed RBD from vaccine exposed to thermal stress for 15 days were also verified and compared with the control samples stored at 2–8 °C (**Figure 2**). The relevant signal in all samples is the broad peak with a retention time of about 27 min, which corresponded to those expected in RBD used as control. With regard to this broad peak, it appears to be a doublet, probably due to the heterogeneity produced by the microheterogeneity provoked by glycosylation of the RBD molecule. The same phenomenon was
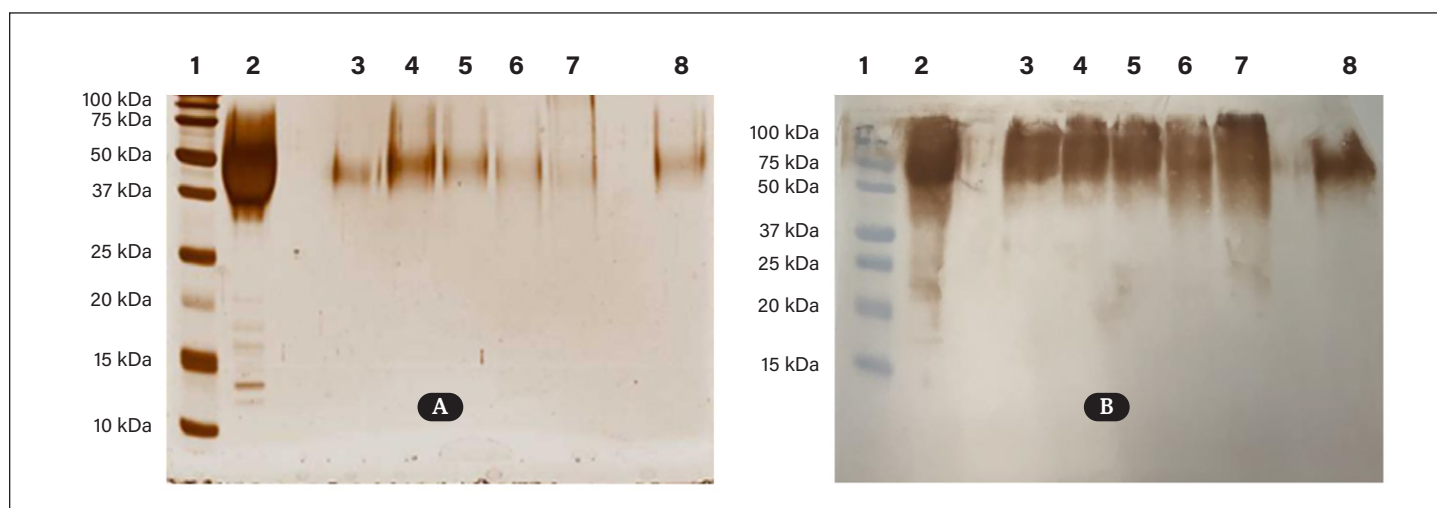
FIGURE 1. API purity measured by SDS-PAGE combined with a densitometry analysis. **(A) Lane 1:** Molecular weight marker; **Lane 2:** API used as SDS-PAGE inner control; **Lane 3:** Vaccine RM; **Lane 4:** Sample at 2−8°C (0 days); **Lane 5:** Sample at 37°C (15 days); **Lane 6:** Sample at 45°C (15 days); **Lane 7:** Sample at 60°C (15 days); **Lane 8:** Sample at 25°C (15 days). **(B)** Immunoblotting of (A) SDS-PAGE using antibodies directed against SARS-CoV-2 RBD.
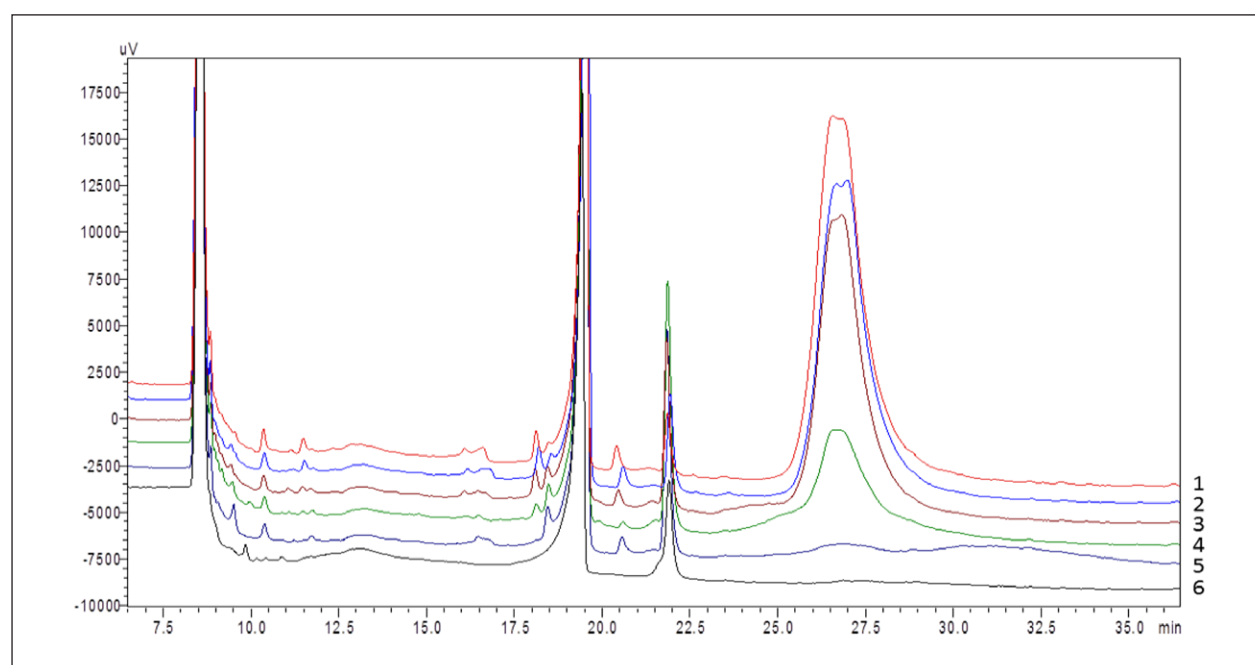

FIGURE 2. RP-HPLC chromatograms of the desorbed RBD from the vaccine material exposed to thermal stress conditions for 15 days: **(1)** 2−8°C, control; **(2)** 25°C; **(3)** 37°C; **(4)** 45°C; **(5)** 60°C; and **(6)** placebo (without API) exposed to 2−8°C.

observed in RP-HPLC analysis (data not shown).

The signal of RBD peaks decreased drastically (more than a half) in samples at 45°C, and in samples exposed to higher temperatures, this peak almost disappeared in samples at 60°C. In addition, two small peaks appeared at 18 and 20.5 min, respectively, which were not present in the placebo samples. Nevertheless, the purity percentage of these two peaks was insignificant (<1%).

To delve into the level of molecular characterization,

tryptic peptides derived from vaccine API samples exposed to thermal stress conditions were then sequenced. As shown in **Figure 3**, disulfide-containing peptides were detected in samples exposed to 2−8°C, 25°C, 37°C, and 45°C, respectively, which also allowed confirming the high stability of the vaccine API. Conversely, the analysis of samples exposed to 60°C for 15 days failed to show enough intensity of all peptide signals, giving a unique signal at m/z 765.84 (4+) corresponding to the
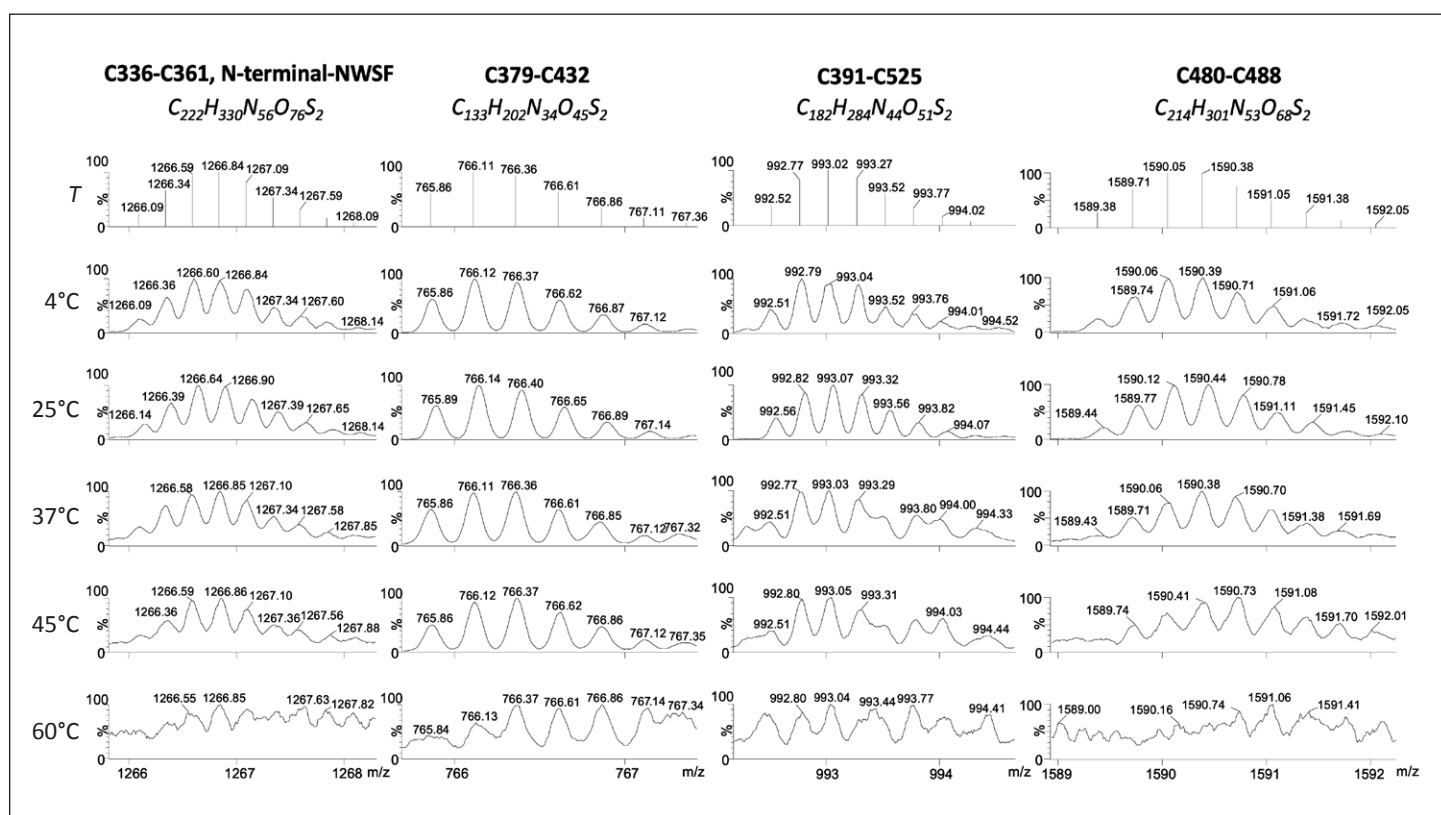
FIGURE 3. Theoretical (T) and experimental isotopic distribution of disulfide-containing peptides among cysteines C336−C361, C379−C432, C391−C525, and C480−C488 in vaccine samples exposed to thermal stress conditions (2−8 °C, 25 °C, 37 °C, 45 °C, and 60 °C) for 15 days.

disulfide-containing peptide between C379−C432, which might be a consequence of a decrease in protein amount. Also, signals produced by the incorrect disulfide bonds were not detected.

In addition, a secondary structure analysis done by means of a CD tool was also used to study effects of temperature on vaccine samples. This is a powerful tool used to identify changes in the secondary structure of protein molecules.[25] As a result, the CD spectra of the non-formulated (**Figure 4A**) and adsorbed (**Figure 4B**) fractions acquired in the far ultraviolet range was compared with the spectra of API dissolved in the
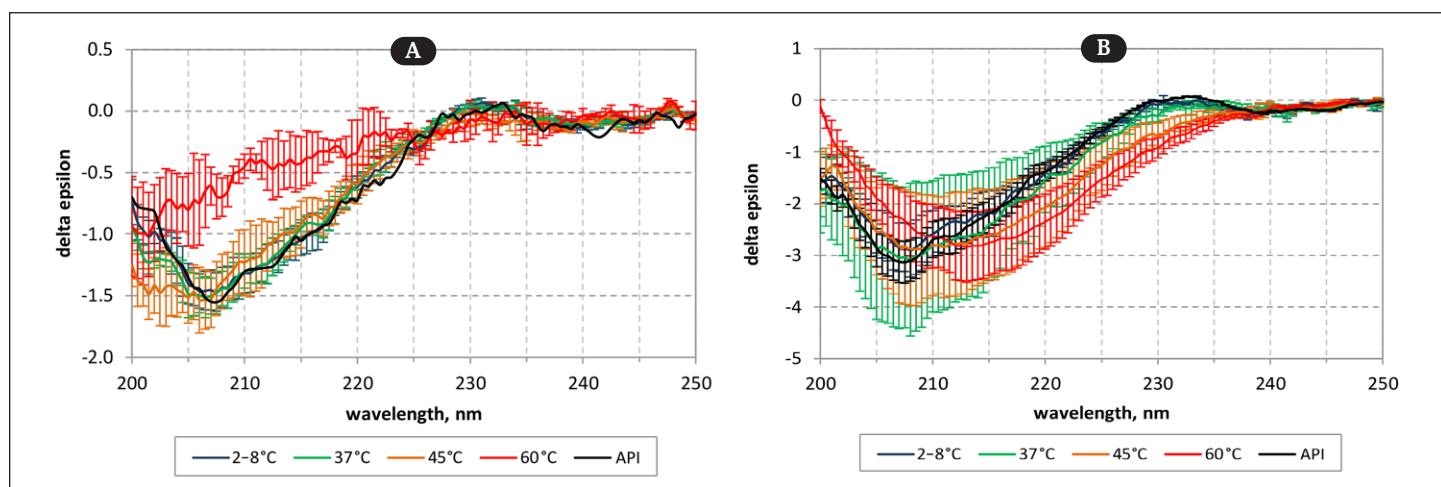

FIGURE 4. CD spectra of the: **(A)** non-formulated; and **(B)** formulated fractions acquired in the far ultraviolet range and compared with the spectra of the vaccine API dissolved in corresponding buffer solutions. Spectra corresponded with vaccine samples exposed to thermal stress conditions (2−8 °C, 25 °C, 37 °C, 45 °C, and 60 °C) for 15 days.

corresponding buffer solutions. As observed in formulated fractions incubated at 37°C, soluble fractions showed CD spectra very similar to each other and to the spectrum of the API acquired at 2–8°C. The spectrum corresponding to the incubation at 45°C was also similar, but displayed some differences at wavelengths below 207 nm, and the incubation of samples at 60°C induced irreversible structural changes in the protein leading to likely protein denaturation. The analysis of the adsorbed fractions showed a similar trend, with minimal structural changes observed at 2–8°C and 37°C, and irreversible conformational modifications taking place at 45°C and 60°C. The observed changes corresponding to the incubation at 45°C were more pronounced in the adsorbed fraction, suggesting a potential role of the aluminum hydroxide gel contributing to structural modifications and protein denaturation.

Finally, three additional assays (immunogenicity in mice, sVNT-ELISA, and RBD/ACE2 binding) were also performed to characterize the relative functionality of vaccine samples exposed to thermal stress conditions.

In the immunogenicity assay, high antibody titers were estimated 35 days after mouse immunization with vaccine samples exposed to thermal stress conditions for 15 days (1:48,853–1:427,849 [2–8°C]; 1:54,868–1:109,763 [25°C]; 1:64,052–1:356,132 [37°C]; 1:99,217–1:160,188 [45°C]; 1:177,033–376,090 [60°C]). The antibody titers detected in all mice immunized with vaccine samples were characterized by a great dispersion of data, but statistically similar to those detected in samples of vaccines exposed to 2–8°C (**Figure 5**). In future studies, more mice should be included at each temperature to narrow the variability. Interestingly, this behavior did not correspond with data obtained in the analysis of vaccine API adsorption percentage where high temperatures increased the binding of the vaccine API molecule to the aluminum hydroxide gel.

Nevertheless, the statistical similarity demonstrated in antibody titers raised by vaccines exposed to different temperatures will not necessarily mean that these vaccines induce antibodies with the same quality and target epitope recognition capacity. It is entirely possible that conformational epitopes destroyed in the vaccine samples after exposure to the highest temperatures will modify the recognition capacity of the antibodies produced by mice, but not vaccine immunogenicity and antibody titers. Another explanation can be related to the sensitivity and type of the immunoassays because conformational epitopes can also be destroyed in the interaction of proteins with the ELISA plate surface. Therefore, further
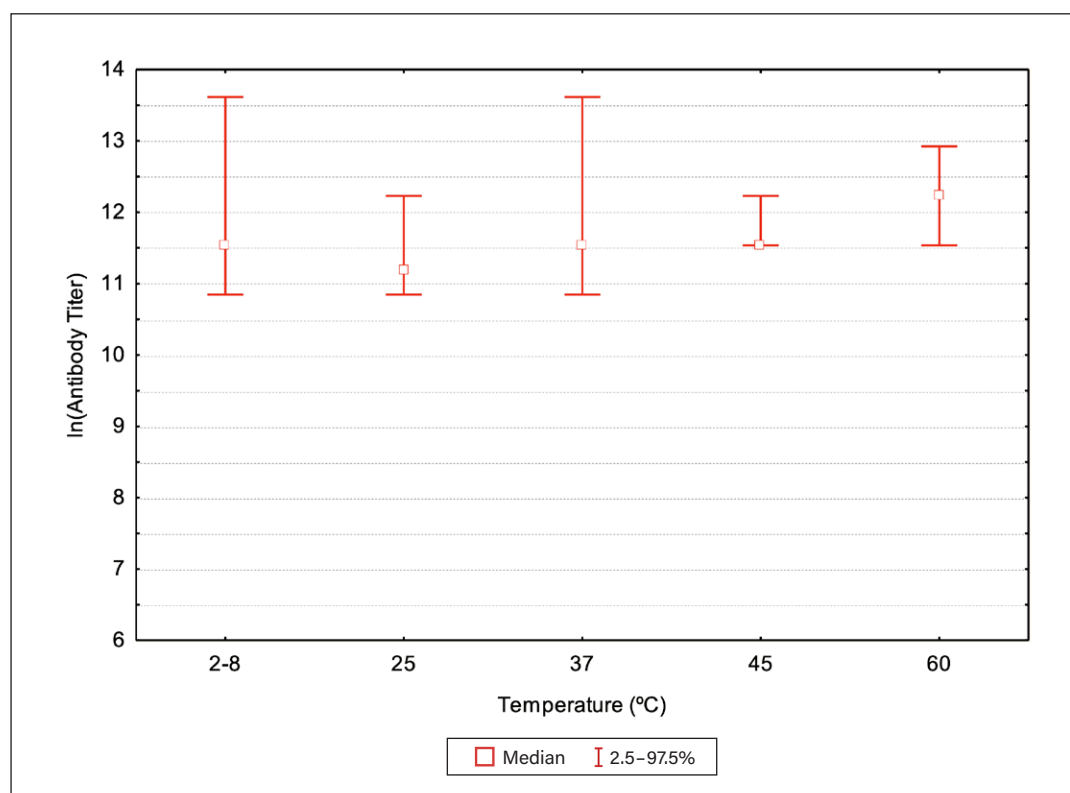

FIGURE 5. Titers of anti-RBD-specific antibodies measured 35 days after the 15-day vaccine sample exposure to thermal stress conditions (2–8°C, 25°C, 37°C, 45°C, and 60°C). Results measured at 2–8°C were used as the control (initial day).

experiments such as sVNT-ELISA can be performed to confirm these hypotheses.

With regard to the animal seroconversion analysis performed by sVNT-ELISA, the inhibition of ACE2 binding by humanFc-RBD by specific antibodies contained in mouse sera was not affected after incubation of samples at 25°C and 37°C for 15 days (with reverse titers of 709 ± 440.2 [2–8°C], 967 ± 460 [25°C], and 718.5 ± 585 [37°C]), respectively ($p > 0.05$). Conversely, the levels of specific RBD antibodies neutralizing the antigen interaction with the ACE2 dropped significantly ($p < 0.0001$) after incubation at 45°C and 60°C (**Figure 6**).

The fact that antibody titers increased with the incubation temperatures could not necessarily correlate with the functionality of the target antibodies in terms of the induction of relevant neutralizing antibodies with the ability to hamper RBD/ACE2 interaction. As a matter of fact, no competition was detected with sera of mice

immunized with vaccine samples exposed to 45°C and 60°C. These results point out that the loss of ACE2 binding site integrity is a potential cause for the significant reduction or total lack of the inhibited RBD/ACE2 interaction in the sera of animals receiving the vaccine incubated at 45°C and 60°C, respectively.

The RBD of the SARS-CoV-2 spike protein binds to ACE2 with high affinity to initiate viral attachment and pathogenesis. The spike protein is a trimer where protomers undergo conformational changes essential for viral infectivity. Some RBD mutations are predicted to affect the antibody neutralization, either through their role in the spike protein conformational changes, or through changing its surface charge distribution.

The effects of temperature on the binding of the ACE2 and the RBD used as API in the vaccine was also analyzed in this study. The percentage of inhibition measured at 25 μg/mL was 85% (2–8°C), 89% (25°C), 86% (37°C), 66%
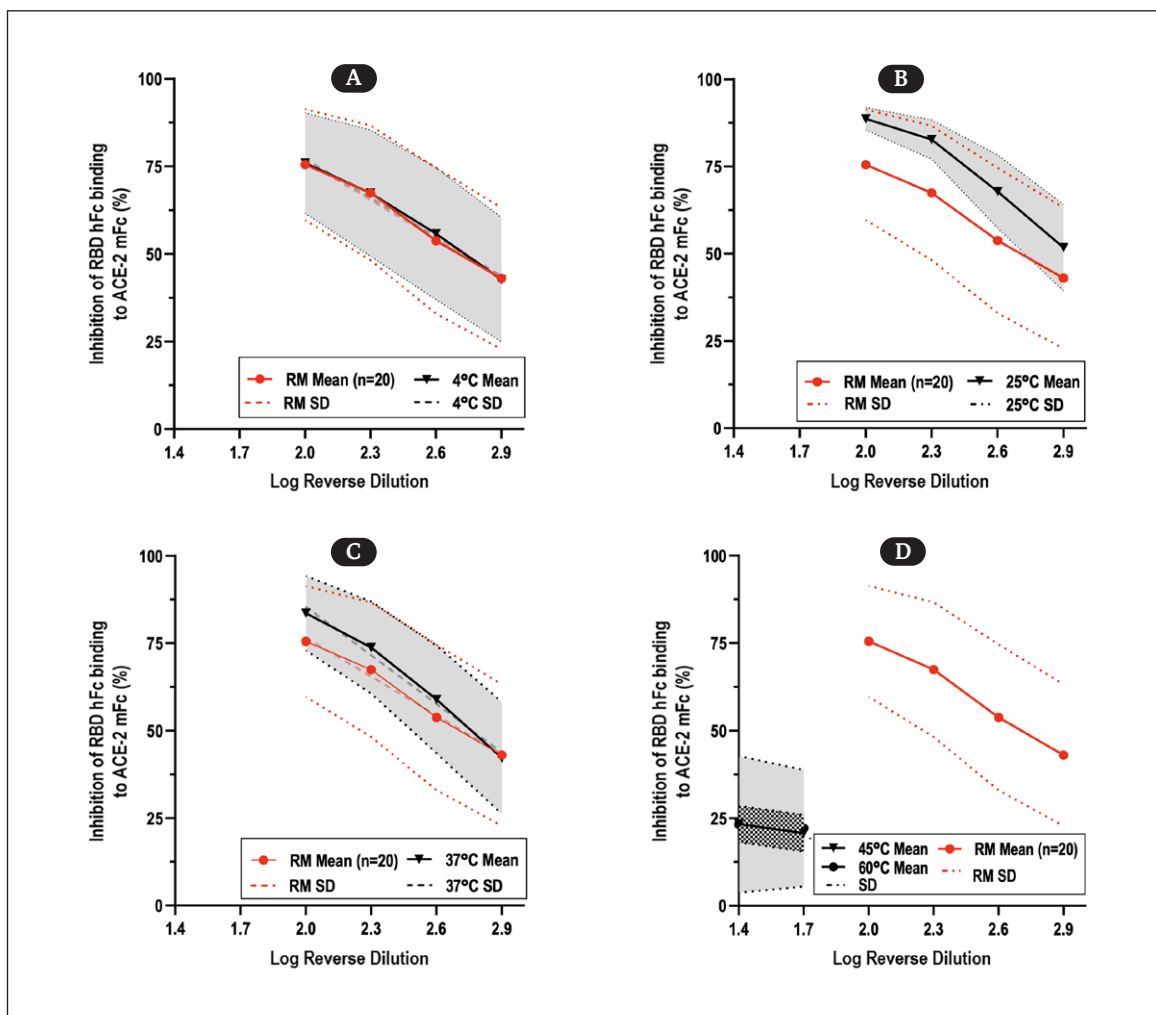

FIGURE 6. The ability of mouse sera to inhibit RBD/ACE2 binding measured by the sVNT-ELISA. Vaccine samples were exposed to: **(A)** 2–8°C; **(B)** 25°C; **(C)** 37°C; and **(D)** 45°C and 60°C thermal stress conditions.
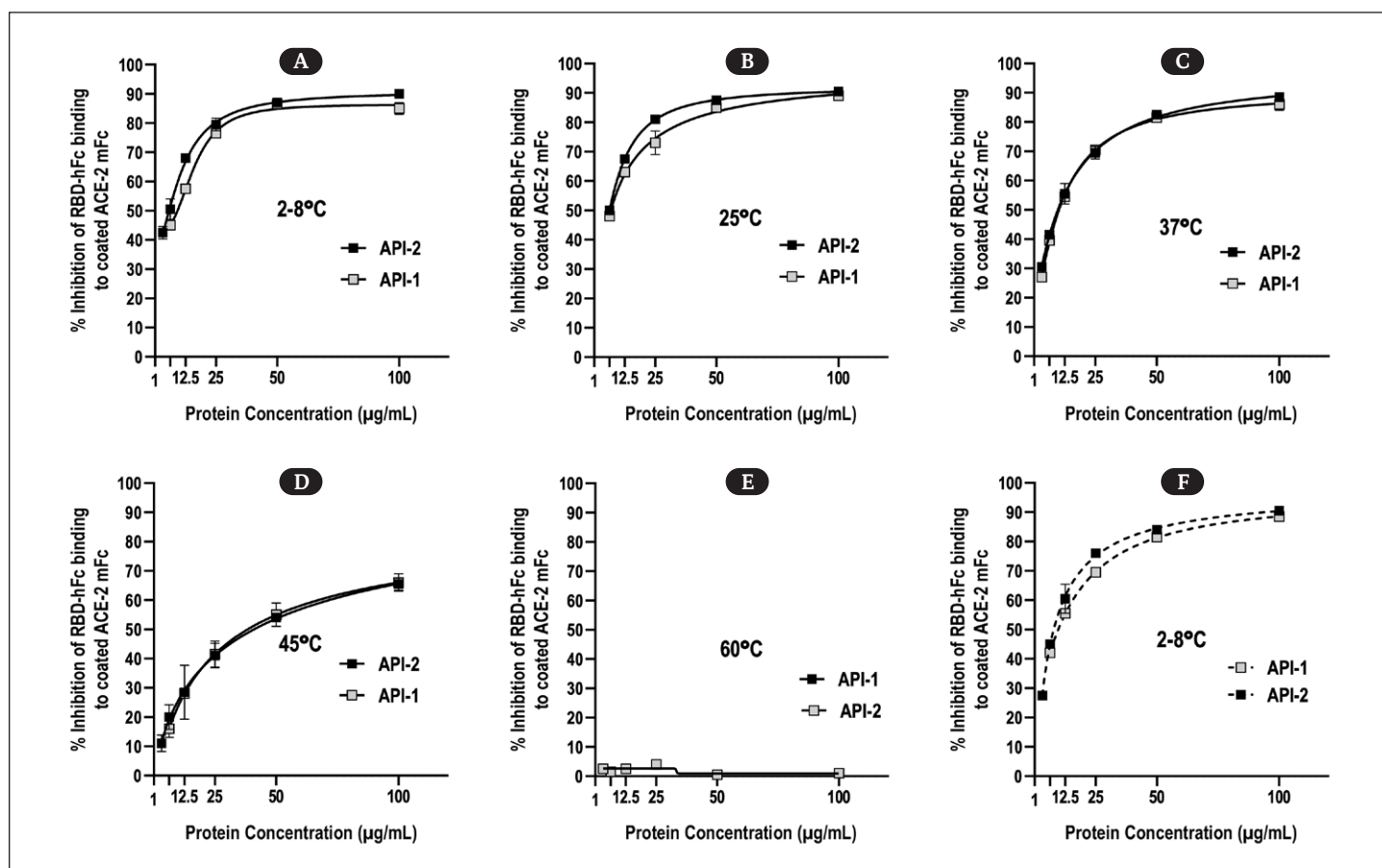
FIGURE 7. RBD/ACE2 binding analysis results of vaccine samples exposed to thermal stress conditions for 15 days.

(45°C), and 1.5% (60°C) (**Figure 7**). These findings are in agreement with other results that evidenced a drastic inhibition of RBD/ACE2 binding in samples incubated at high temperatures. The explanation of this phenomenon could be related to a decrease in the dielectric constant of water provoked by the increase of temperature, which affects the strong electrostatic interactions that characterize the RBD/ACE2 binding.

After the molecular integrity and functional characterization, a stability prediction based on the Kirkwood method[26] was carried out to predict vaccine stability when incubated at 2–8°C. Higher temperatures (25–60°C) were not used in this prediction study since conformational epitopes in the vaccine API were destroyed in that temperature range. This prediction method is commonly used to predict stability of a biological standard product during long-term storage at low temperatures. Usually this kind of prediction involves the analysis of several parameters. However, in this case, the stability prediction analysis was done by considering only the adsorbed amount of RBD in the vaccine samples. Analysis revealed that the amount of adsorbed RBD decreased 4.60% (2–8°C) in 12 months of storage, predicting a very high vaccine stability at low

temperature. These results will be confirmed in future shelf-life stability studies. The results would increase immunization coverage without extra vaccine bioproduction runs while simplifying immunization logistics and reducing cold chain-associated costs.

CONCLUSIONS

The Abdala subunit vaccine is stable at 37°C for at least 15 days since there is no effect of the temperature on the most important physico-chemical and functional properties of the vaccine. Results allow predicting a very high vaccine stability at 2–8°C temperature. Therefore, we foresee the use of this vaccine in places with inadequate infrastructure to keep a validated cold chain.

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CONFLICTS OF INTEREST

All authors were assessed for conflicts of interest, and none were declared.

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