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A Nasal Subunit Vaccine Candidate Based on The Viral Antigens RBD and N Combined with C-Di-AMP as an Approach to Enhance Specific Immunity to SARS-Cov-2

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Abstract

Nowadays, immunization through vaccines continues being a best strategy to control the COVID-19 disease and prevent mortality. Recently evidences highlight the need of vaccines for COVID-19 that provides broader protection against new SARS-CoV-2 isolates. In this work we evaluated in BALB/c mice, a nasal vaccine candidate based on recombinant proteins RBD and N from SARS-CoV-2 combining with *c*-di-AMP as adjuvant, as an approach to enhance specific immunity to SARS-CoV-2. Follow the intranasal administration of the bivalent vaccine candidate plus *c*-di-AMP, systemic and mucosal humoral immunity was elicited, in terms of IgG, IgA and neutralizing antibodies against two relevant variants of concern (Delta and Omicron BA1.2). N- and RBD-specific IgG subclasses induced by immunization showed a balanced Th1/Th2 pattern. In addition, an immune-modulator effect in the cell-mediated immunity was detected in mice immunized with the nasal vaccine formulation that included both recombinant antigens plus *c*-di-AMP. Results of this work propose the nasal formulation RBD + N + *c*-di-AMP as a promising option using gentle route for the COVID-19 immunization.

Keywords: SARS-CoV-2; cyclic di-adenosine monophosphate (*c*-di-AMP); intranasal vaccination; cell-mediated immunity; neutralizing antibody; mucosal immunity

Introduction

The coronavirus disease 2019 (COVID-19) outbreak caused by the highly transmissible and pathogenic coronavirus, the SARS-CoV-2 has resulted in 772 million documented infections and 7 million deaths worldwide [1]. According to WHO data, there are even many asymptomatic cases of COVID-19 that can silently amplify these statistics [2, 3]. Nowadays, the specific immunity through vaccination remains the single best strategy to control the COVID-19 disease and prevent mortality. However, less than half of the world's population has access to one dose of COVID-19 vaccines [4]. The Spike-protein (S-protein) and the receptor-binding domain (RBD) of the S-protein of SARS-CoV-2 have been used as the main antigens for vaccine design to elicit mainly neutralizing antibodies [5, 6]. Though, the S protein shows considerable sequence variations among SARS-CoV-2 variants of concern, resulting in these variants being able to evade the neutralizing antibody response generated in vaccinated individuals [7, 8]. While the levels of neutralizing antibodies elicited by vaccination or natural infection are taken as the main predictors of protective immunity [9], recently evidences

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for the function of T cells in mediating immunity to SARS-CoV-2 have accumulated [10]. Hence there is a need for next generation vaccines that are able to protect against multiple variants and encompass additional antigens that provide a broader protection [11-14]. In this sense, the nucleocapsid (N) viral protein is more conserved and stable among of many coronaviruses with 90% amino acid homology and fewer mutations over time [15]. This protein, highly immunogenic and expressed abundantly during natural infection [16], is a representative antigen for the T-cell response in a vaccine setting, inducing SARS-specific T-cell proliferation and cytotoxic activity [15, 17]. For that, N antigen has been tested in several vaccine platforms and formulations with encouraging results against SARS-CoV-2 [14, 18-23].

Parenteral vaccination is the most used method for vaccine administration, leading to elicitation of antigen-specific systemic cellular and humoral immunity. During the COVID-19 pandemic, real world evidence showed that this approach successfully reduced severe forms of disease and death tolls. However, parenteral vaccines have a modest effect in the prevention of infection and subsequent horizontal transmission to susceptible hosts. In contrast, mucosal vaccination effectively stimulates both systemic and mucosal immune responses [24, 25]. This local mucosal response at the portal of entry is critical to provide early protection against viral infection. In addition, it has the advantage of being a non-invasive procedure suitable for immunization of large populations [26, 27]. Hence, the development of nasal vaccines represents a feasible alternative for a more effective immunization [28] against coronavirus, by providing a first-line of defense in the respiratory tract to prevent both viral infection and transmission. However, mucosal vaccination frequently requires specifically tailored delivery systems to ensure efficient breaching and transit a cross the mucosal barrier. Furthermore, to guarantee antigen uptake, processing and presentation, a danger signal is needed (*e.g.* adjuvantations) that promotes stimulation and activation of antigen presenting cells and a local microenvironment conductive towards initiation of an adaptive immune responses without toxicity [26]. At the moment, only five different nasal vaccines have been approved by the regulatory authorities for the markets [27].

In this study we evaluated the cyclic di-nucleotide, bis-(3′,5′)-cyclic dimeric adenosine monophosphate (*c*-di-AMP), as the adjuvant for intranasal vaccination with the RBD and N antigens from SARS-CoV-2. This adjuvant has been recognized as stimulator of IFN genes (STING) pathway, which promote the activation of the immune system, principally to mucosal vaccines against respiratory pathogens [29, 30]. As a result of acting as STING agonist and its subsequent targeting to proteolytic degradation, *c*-di-AMP promotes a self-restricted local immune activation [31]. Our results show that the nasal vaccine candidate elicits efficient systemic immunity, in terms of neutralizing antibodies and cellular immunity, as well as mucosal immunity against the antigens included in the formulation.

Material and Methods

Cell line, antibody and viral antigens

African monkey kidney (Vero E6) cells were obtained from the ATCC (No. CRL-1586) and were used for isolating, passaging and neutralization tests. Cells were grown at 37°C in Eagle's MEM supplemented with 10% heat-inactivated of fetal bovine serum (FBS). Monoclonal antibody #8 that recognizes the RBD region was produced by the CIGB-Sancti Spiritus, Cuba. Variant concerns of SARS-CoV-2, Delta (EPI_ISL_7495138) and Omicron BA1.2 (EPI_ISL_12691753) isolates were used for the neutralization experiments.

Antigens of the vaccine formulation

RBD protein was produced as recombinant protein in *Pichia pastoris* yeast (strain X-33) and obtained as a pyrogen-free product more than 95% pure. The RBD region includes amino acids 331-529 of the Spike protein of SARS-CoV-2 Wuhan-Hu-1 strain (NCBI Acc. No. YP_009724390), it's the pharmaceutical active ingredient of ABDALA Vaccine developed by the Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba). The recombinant full-length N protein of SARS-CoV-2 (Wuhan-Hu-1 strain), 419 amino acids, was obtained as recombinant protein in *Escherichia coli* bacteria, as a pyrogen-free product with more than 95% purity by the Technology Development Department of the CIGB. The *c*-di-AMP (BioLog, Bremen, Germany), provided by the Helmholtz Centre for Infection Research (HZI, Germany), was dissolved in water (Ampuwa; Serumwerk, Bernburg, Germany). All components were prepared in buffer Na_2HPO_4 0.56 mg/mL, NaH_2PO_4 x $2H_2O$ 0.62 mg/mL, NaCl 8.5 mg/mL diluted in water.

Animals and ethics statement

Female BALB/c (Bc, H-2d) mice (aged 6 – 8 week) were purchased from the Center for the Production of Laboratory Animals (CENPALAB, Cuba) and housed in appropriate animal care facilities during the experimental period. Mouse experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the CIGB. The protocol was approved by the Committee on the Ethics of Animal Experiments (CICUAL/CIGB/22034).

Immunization schedule

Groups of BALB/c mice were injected by intranasal route with volume of 50 µL (25 µL in each nasal cavity) on days 0, 14 and 28 (Figure 1). All immunogens were prepared just before the inoculation (Table 1):

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Group 1 (N + *c*-di-AMP): 10 µg of protein N and 5 µg of *c*-di-AMP, n = 12.

Group 2 (N): 10 µg of protein N, n = 12.

Group 3 (N + RBD + *c*-di-AMP): 10 µg of protein N, 50 µg of protein RBD and 5 µg of *c*-di-AMP, n = 12.

Group 4 (N + RBD): 10 µg of protein N, plus 50 µg of protein RBD, n = 12.

Group 5 (RBD + *c*-di-AMP): 50 µg of protein RBD and 5 µg of *c*-di-AMP, n = 12.

Group 6 (Placebo + *c*-di-AMP): 5 µg of *c*-di-AMP, n = 8.

Sera samples from each group were collected at day 49 (n=12 or 8) and day 56 (n=6 or 4) after the last dose for evaluating the systemic humoral immune responses. Nasal washes were taken at day 49 (n=6 or 4) and day 56 (n=6 or 4) using PBS buffer. Finally, in both times animals were splenectomized and collected cells to measure the cellular immune responses (Figure 1).

Table 1: Groups of the immunization schedule in BALB/c mice

Group	Formulations	Immunogens (µg/per mouse)	Adjuvant	No. of animals	Administration route
1	N + *c*-di-AMP	protein N (10 µg)	*c*-di-AMP (5 µg)	12	intranasal
2	N	protein N (10 µg)	-	12	intranasal
3	N + RBD + *c*-di-AMP	protein N (10 µg) + protein RBD (50 µg)	*c*-di-AMP (5 µg)	12	intranasal
4	N + RBD	protein N (10 µg) + protein RBD (50 µg)	-	12	intranasal
5	RBD + *c*-di-AMP	protein RBD (50 µg)	*c*-di-AMP (5 µg)	12	intranasal
6	Placebo + *c*-di-AMP	-	*c*-di-AMP (5 µg)	8	intranasal

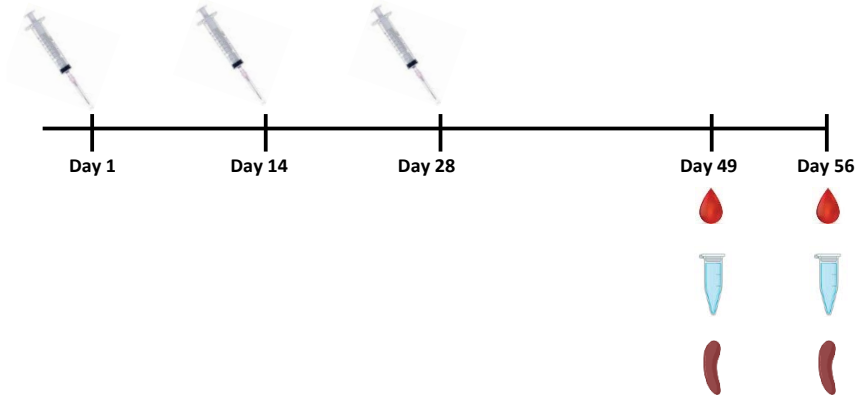

Figure 1: Representation of the immunization schedule. Immunocompetent BALB/c mice were inoculated with three doses by intranasal route, every fourteen days (day 0, 14, 28). After the third dose, samples (sera, nasal washes and spleen cells) were taken to evaluate the humoral and cellular immune responses on days 49 and 56 of the study.

IgG and IgA antibodies specific to the SARS-CoV-2 RBD and N proteins by ELISA

To evaluate systemic humoral immunity, total IgG, IgG1 and IgG2a antibody titers were performed by ELISA on days 49 and 56 against each recombinant antigen. Briefly, 96-well plates were coated with 5 µg/mL of recombinant proteins RBD or N, and incubated overnight at 4°C in coating buffer (0.16% Na_2CO_3, 0.29% $NaHCO_3$, pH 9.5). The wells were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) after each step of the assay. Subsequently, plates were blocked with 5% (w/v) skim milk, 1 h at 37°C. Then, they were incubated with serum samples diluted in PBS-T and incubated 1 h at 37°C. As previously, three washes with PBS-T were performed after each step of the assay. Finally, anti-mouse IgG (#A0168, Sigma-Aldrich, USA), anti-mouse IgG1 (#A90-105P, ImTec, USA) or anti-mouse IgG2a (#A90-107P, ImTec, USA) antibody-peroxidase conjugated were added and the plates were incubated for 1 h at 37°C. After washing, 0.04% substrate solution (O-phenilendiamine in buffer 2% Na_2HPO_4, 1% citric acid, and 30% H_2O_2, pH 5.0) was added. Plates were kept 10 min at 25°C and the reaction was stopped with 12.5% H_2SO_4. Absorbance at 492 nm was read in a microplate reader (SensIdent Scan; Merck, Germany). For ELISA against antigen RBD, titers were estimated using a standard curve of the monoclonal antibody #8 (CIGB-Sancti Spiritus, Cuba) at know concentrations (0.06 - 4 µg/mL). For

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ELISA against N antigen, titers were defined as the dilution of serum giving twice the absorbance value of the negative control sera (samples from corresponding Placebo group). For both assays, the positive cut-off values were established as twice the average titer calculates for the placebo group. To assess mucosal IgA antibody titers, an ELISA was performed for each recombinant antigen. The methodology for assessing IgA antibody titers was similar to that for the IgG ELISA, replacing serum samples by nasal washes, which were used directly undiluted. Since the positive signals obtained were usually lower, the results were expressed as absorbance units at 492 nm. Positive cut-off values were set as twice the average values calculated for the placebo group.

Inhibition of interaction RBD-ACE2 receptor by ELISA

The quality of the antibody response generated by immunization; was determined as the capacity of sera to inhibit the binding of RBD to its ACE2 receptor. In this assay, flat bottom 96-well ELISA plates (Costar 3590) were coated in coating buffer (0.16% Na_2CO_3, 0.29% $NaHCO_3$, pH 9.5) using 5 µg of the recombinant protein ACE2 coupled to the Fc portion of a murine immunoglobulin (ACE2 mFc) and incubated for 16 - 20 h at 4ºC. Subsequently, the plates were washed with 0.1% Tween 20 in H_2O and blocked with 2% skim milk in PBS 1X, 0.05% Tween 20 for 1 h at 37°C. During the same time, sera (dilution 1:500) and RBD protein coupled to the Fc portion of a human immunoglobulin conjugated to peroxidase enzyme (RBD hFc-HRPO) were pre-incubated in U-bottom culture plates (Costar 3799). The serum and RBD hFc-HRPO reagent were diluted in 0.2% skim milk, 0.05% Tween 20 in PBS 1X. ELISA plates were washed three times and 50 L of the pre-incubation mixture were added to the coated plates. Follow, the plates were incubated during 90 minutes at 37ºC, and washed under the same conditions previously described. Finally, the substrate tetramethyl benzidine was added and the reaction was stopped with 12.5% H_2SO_4, after 10 minutes of incubation. The absorbance at 450 nm was detected in a microplate reader (SensIdent Scan; Merck, Germany). Percentage of inhibition was determined concerning the maximum binding (Umax: absorbance from the binding of RBD hFc-HRPO to the ACE2 in the absence of serum) after subtraction of absorbance values of the background control wells incubated with all the reagents except serum and RBD conjugate. When a negative value arose, it was processed and represented in graphics as zero.

Live virus neutralization assay

Neutralization antibody titers were determined by a traditional virus micro-neutralization assay using SARS-CoV-2 variants under Level 3 Biological Safety Facility (BSL-3) in the Civilian Defense Scientific Research Center, Cuba. Briefly, 96-well culture plates were seeded with $2x10^4$ cells/ well of Vero E6 one day before. Cells were incubated for 24 h at 37˚C and 5% CO_2 to grown until 85-90% confluency. Subsequently, the heat-inactivated sera (by incubation at 56°C for 30 minutes) were serially diluted 1:5 in minimal essential medium (MEM, Gibco, UK) with 2% (v/v) fetal bovine serum (Capricorn, Germany), 25 mM L-glutamine (Sigma, USA), 2 µg/mL sodium bicarbonate (MercK, Germany) and 40 µg/mL of gentamicin (Sigma, USA). Each serum dilution was mix with 100 mean tissue culture infective dose ($TCID_{50}$) of each SARS-CoV-2 variant, and incubated for 1 h at 37˚ C. Serum-virus mixes were added to the semi-confluent cell monolayers and were incubated during 96 h at 37°C, with 5% CO_2. Neutralization was visualized by the colorimetric method, by staining with 0.02 % neutral red and reading the optical density at 540 nm. The virus neutralizing titers (VNT_{50}) were calculated as the highest serum dilution at which 50% of the cells remained intact according to neutral red incorporation in the control wells (no virus added).

Cell culture and viral in vitro stimulation

Spleen cells were obtained under aseptic conditions and erythrocytes were lysed by adding 0.83% NH_4Cl solution. Cells were washed twice with PBS + 2% FBS (PAA Laboratories, Ontario, Canada) and resuspended at $2×10^6$ cells/mL in RPMI-1640 medium (Sigma Aldrich) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco, UK), 2 mM glutamine (Gibco, UK), $5×10^{-5}$ M 2-mercaptoethanol (Sigma St. Louis, MO) and 5% FBS. Finally, $2×10^5$ cells per well were cultured in 96-well round bottomed plates with the antigens (recombinant protein RBD or N, or mock). Concanavalin A (Sigma) was used as a positive control (5 µg/mL). In all experiments, three wells were plated for each antigen. After 96 h of incubation, culture supernatants were collected and stored at -80°C.

IFNγ detection in culture supernatants by ELISA

The culture supernatants of splenocytes previously stimulated with each antigen were analyzed in duplicate for IFNγ concentrations by ELISA using monoclonal antibody pairs. The ELISA protocol recommended by the manufacturers was used with slight modifications (#3321-1H-6, Mabtech, Sweden). The lowest limit of detection of the cytokine was 4 pg/mL.

Statistical analysis

Direct or transformed (Log10) data normality was verified with the Kolmogorov-Smirnov (n = 6 or 4) or D'Agostino & Pearson (n = 12 or 8) normality test, as well as the homogeneity of variance using the Bartlett´s test, and after were analyzed by ANOVA parametric tests (One-way analysis of variance with Tukey's post-test). Data that do not fulfill both conditions, even after transformations,

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were analyzed by a nonparametric test (Kruskal–Wallis non-parametric test with Dunn's multiple comparison post-test). In all cases, the GraphPad Prism version 9.0 for Windows, GraphPad Software, San Diego California USA, was employed.

Results

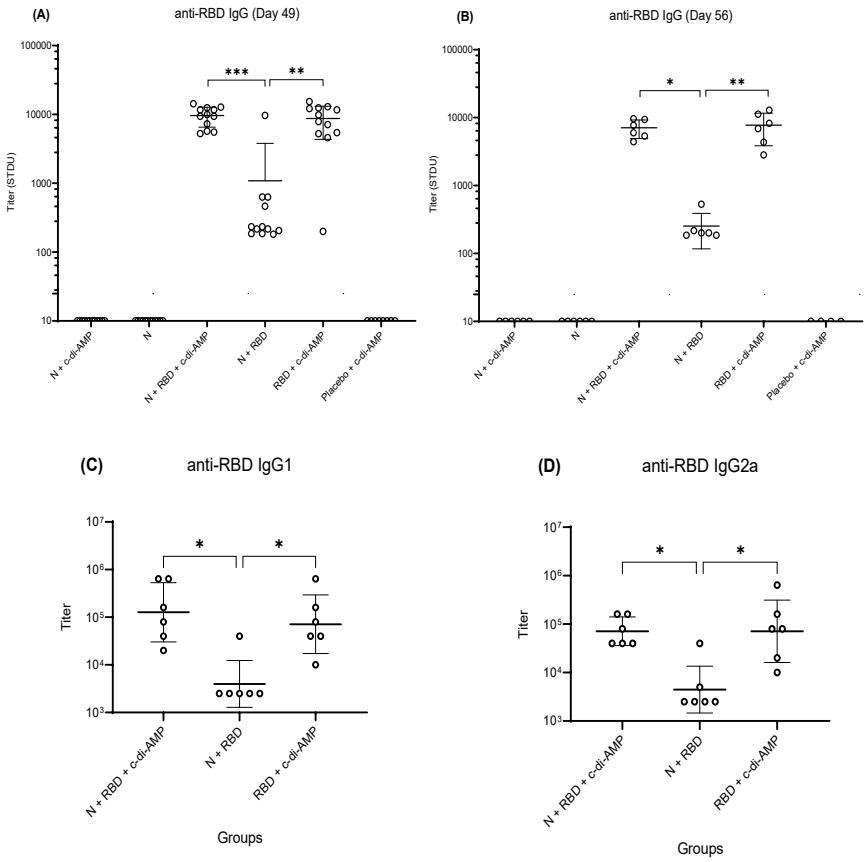
Immunological evaluation in mice of a nasal formulation based on RBD and N antigens combined with c-di-AMP as immune-modulator

The immunogenicity of a nasal formulation comprising RBD and N antigens of SARS-CoV-2 combined with *c*-di-AMP was tested in BALB/c mice. As control groups were included animals immunized with Placebo (Placebo + *c*-di-AMP) or immunized with formulations contains each independent antigen (N, N + *c*-di-AMP, N + RBD, RBD + *c*-di-AMP). Animals received three doses of the formulations by intranasal route, every fourteen days, containing 50 µg of protein RBD and/or 10 µg of protein N as viral antigens (Table 1). After the third dose, the humoral immune and cellular immune responses were evaluated on day 49 and day 56 of the study (Figure 1).

Systemic and mucosal humoral immune response

Systemic humoral immune responses elicited by the formulations were firstly evaluated by ELISA systems, in terms of total IgG and IgG subclasses (IgG1 and IgG2a) in sera against the RBD or N recombinant antigens (Figure 2A - 2H). As shown in Figure 2, after immunizations with the nasal formulations containing RBD and/or N recombinant proteins, all animals seroconverted and showed high IgG antibody titers against each viral antigen contained in the formulation. Furthermore, formulations including viral antigens combined with the immune-modulator *c*-di-AMP showed higher antibody titers compared with their mono or bivalent equivalents without adjuvant at both time points studied ($P<0.05$), (Figure 2).

To broad characterization of the humoral immune response induced, sera from animals immunized were tested by antibody subclasses IgG1 and IgG2a at day 56. As shown in Figure 2C – 2D, animals inoculated with N + RBD + *c*-di-AMP and RBD + *c*-di-AMP formulations exhibited higher antibody titers against the RBD recombinant antigen, compared with response observed in animals inoculated with the formulation that included alone both viral antigens (N + RBD), for both subclasses ($P<0.05$). A similar behavior was observed in the recognition of the N-specific antibody titers. Vaccinated animals with formulations including the immune-modulator *c*-di-AMP (N + *c*-di-AMP and N + RBD + *c*-di-AMP) generated the highest IgG1 and IgG2a antibody titers ($P<0.05$), indicating the induction of a mixed Th1/Th2 pattern (Figure 2G – 2H).



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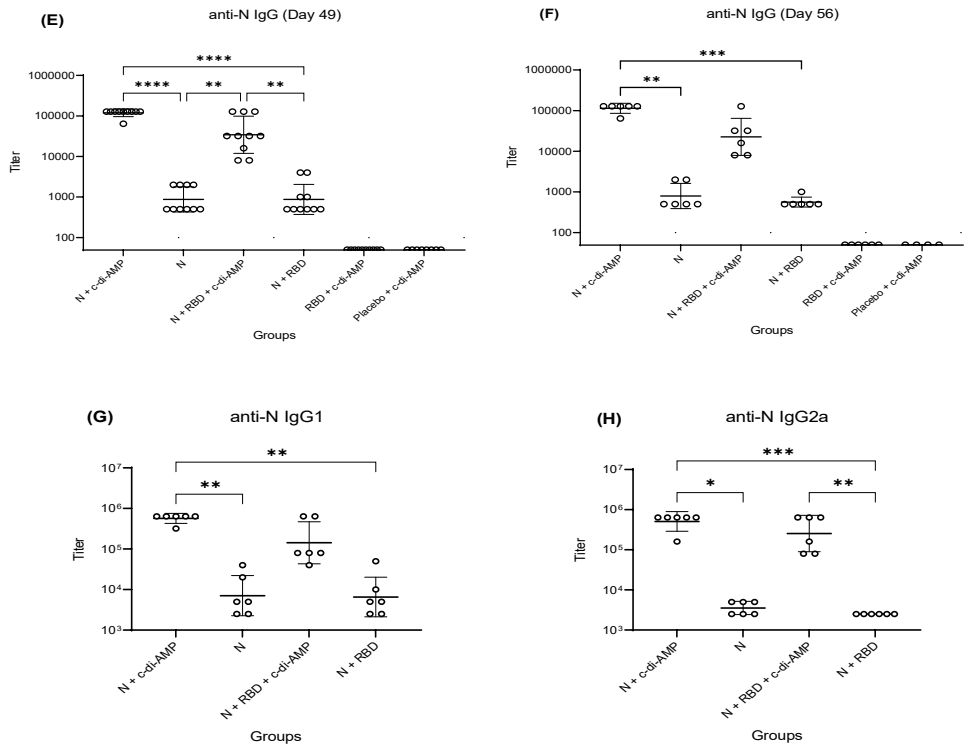

Figure 2: Systemic humoral response measured by ELISA in serum from immunized mice. (A) anti-RBD IgG antibody titers at day 49 (n = 12 or 8). (B) anti-RBD IgG antibody titers at day 56 (n = 6 or 4). (C) anti-RBD IgG1 antibody titers at day 56 (n = 6). (D) anti-RBD IgG2a antibody titers at day 56 (n = 6). (E) anti-N IgG antibody titers at day 49 (n = 10). (F) anti-N IgG antibody titers at day 56 (n = 6 or 4). (G) anti-N IgG1 antibody titers at day 56 (n = 6). (H) anti-N IgG2a antibody titers at day 56 (n = 6). In all cases, data represent the mean ± SD. The analysis of data was performed using a Kruskal–Wallis non-parametric test with Dunn's multiple comparison tests, (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$). The dashed lines represent the cut-off value defined as twice the average titer measured in the placebo group.

Nasal washes from animals vaccinated were used to evaluate by ELISA the local mucosal immune response induced, in terms of IgA antibody titers that recognize the RBD or N antigens in both times (Figure 3). As results, only mice from groups immunized with formulations including *c*-di-AMP were positives for IgA (N + *c*-di-AMP, N + RBD + *c*-di-AMP, and RBD + *c*-di-AMP) ($P<0.05$) (Figure 3A – 3D).

To evaluate the functionality of antibodies two methods were exploited. Firstly, the inhibition capacities to block the interaction between the RBD with ACE2 receptor were tested using sera collected at day 49 and 56. For these assays, only were used sera from animals inoculated with formulations including RBD antigen (N + RBD + *c*-di-AMP, N + RBD and RBD + *c*-di-AMP groups). Figure 4 shows that in almost all mice receiving N + RBD + *c*-di-AMP or RBD + *c*-di-AMP formulations, more than 50% inhibition was observed at both time points of the study, but only the group N + RBD + *c*-di-AMP was statistical different ($P<0.01$) (Figure 4A - B).

In addition, sera collected from vaccinated animals were used to evaluate the neutralizing activity against two viral variants of concern by VNT_{50}. In Figure 4 were represented neutralizing antibody titers to Delta and Omicron BA1.2 isolates. Only neutralizing titers were detected in sera from mice vaccinated with the nasal formulations N + RBD + *c*-di-AMP and RBD + *c*-di-AMP, ($P<0.05$) (Figure 4C - D). However, a discrete increase in the titers were detected in animals immunized with the formulation including N and RBD combined with *c*-di-AMP, with titer means of 220.8 and 126.2 to Delta and Omicron BA1.2 variants, respectively ($P<0.05$); compared with the titer means from animals that received RBD + *c*-di-AMP formulation (68.5 and 62.0 to Delta and Omicron BA1.2 variants) (Figure 4C - D). All sera from the rest of groups did not neutralize.

Cellular immune response

To measure the cell-mediated immunity, IFNγ concentration was detected by ELISA in the supernatant collected from spleen cells harvested on day 49 and day 56, after *in vitro* stimulation with the recombinant antigens (Figure 5A - 5B). Figure 5, shows the results of the two cell-mediated immunity assays performed. In the first experiment, done at day 49, only after stimulation with the recombinant antigen N was detected concentration of IFNγ in two groups, N + *c*-di-AMP and N + RBD + *c*-di-AMP, with statistical

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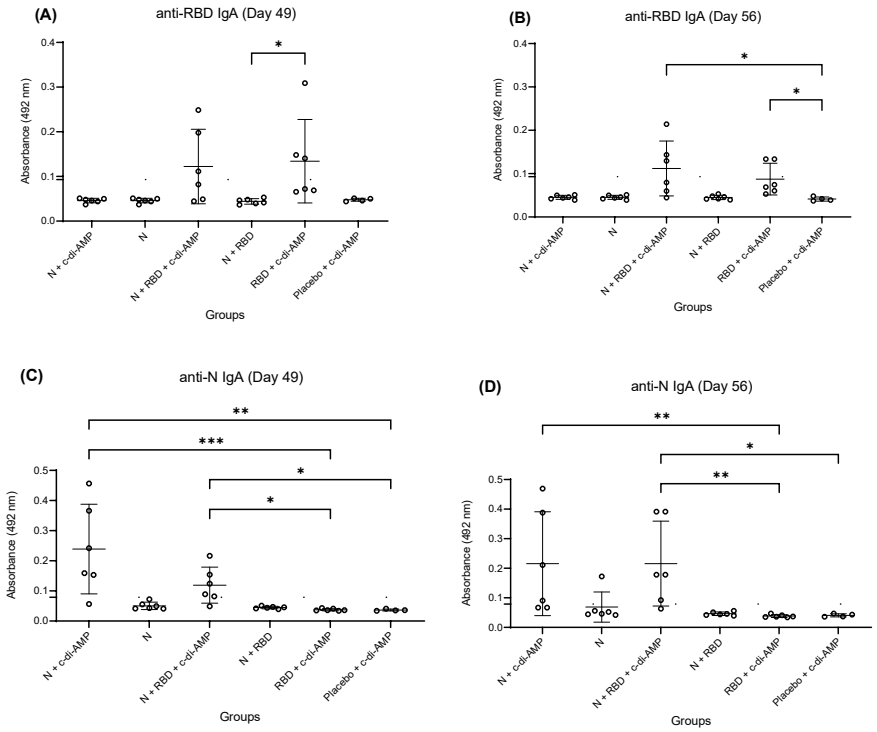

Figure 3: Mucosal humoral response measured by ELISA from immunized mice. (A) anti-RBD IgA in nasal washes (Day 49, n = 6 or 4). (B) anti-RBD IgA in nasal washes (Day 56, n = 6 or 4). (C) anti-N IgA in nasal washes (Day 49, n = 6 or 4). (D) anti-N IgA in nasal washes (Day 56, n = 6 or 4). In all cases, data were represented as absorbance units at 492 nm. Graphics represent the mean ± SD. The analysis of data was performed using a Kruskal–Wallis non-parametric test with Dunn's multiple comparison tests, (*: $P<0.05$; **: $P<0.01$). The dashed lines represent the cut-off value defined as twice the absorbance unit average measured in the placebo group.

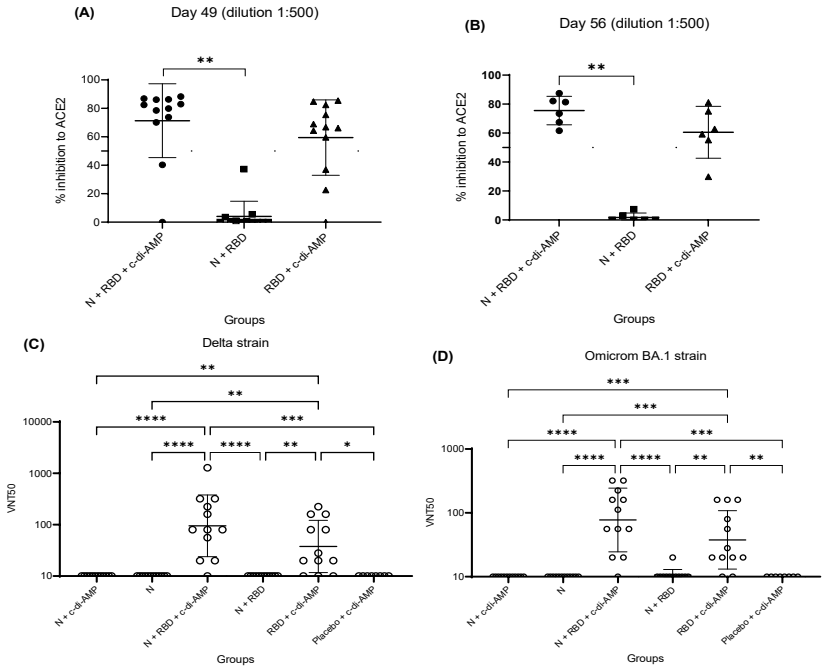

Figure 4: Functionality of antibodies in serum from vaccinated mice. (A) Percentage of inhibition to the interaction of RBD-ACE2 receptor at day 49 (n = 12). (B) Percentage of inhibition to the interaction of RBD-ACE2 receptor at day 56 (n = 6). (C) Neutralizing antibody titers against Delta measured strain at day 49 (n = 12 or 8). (D) Neutralizing antibody titers against Omicron strain at day 49 (n = 12 or 8). In all cases, data represent the mean ± SD. The analysis of data was performed using a Kruskal–Wallis non-parametric test with Dunn's multiple comparison tests, (*: $P0.05$; **: $P<0.01$; ***: $P<0.001$). The dashed lines represent the cut-off value defined as 50% of inhibition to the interaction of RBD-ACE2 receptor.

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significant differences compared to the rest of groups (P<0.05) (Figure 5A). A second experiment was achieved at day 56, 28 days after the last dose; once again the spleen cells were *in vitro* stimulated with the recombinant proteins RBD and N. As results, in the supernatants stimulated with N antigen, IFNγ concentrations were detected in animals that received N + *c*-di-AMP and N + RBD + *c*-di-AMP intranasal formulations (P<0.05) (Figure 5B). In addition, in this assay a tendency to promote the secretion of this antiviral cytokine was observed in animals from N + RBD + *c*-di-AMP and RBD + *c*-di-AMP groups, upon *in vitro* stimulation with the RBD antigen (Figure 5B). As expected, in both assays, spleen cells from animals immunized with placebo formulation did not produce the antiviral cytokine.

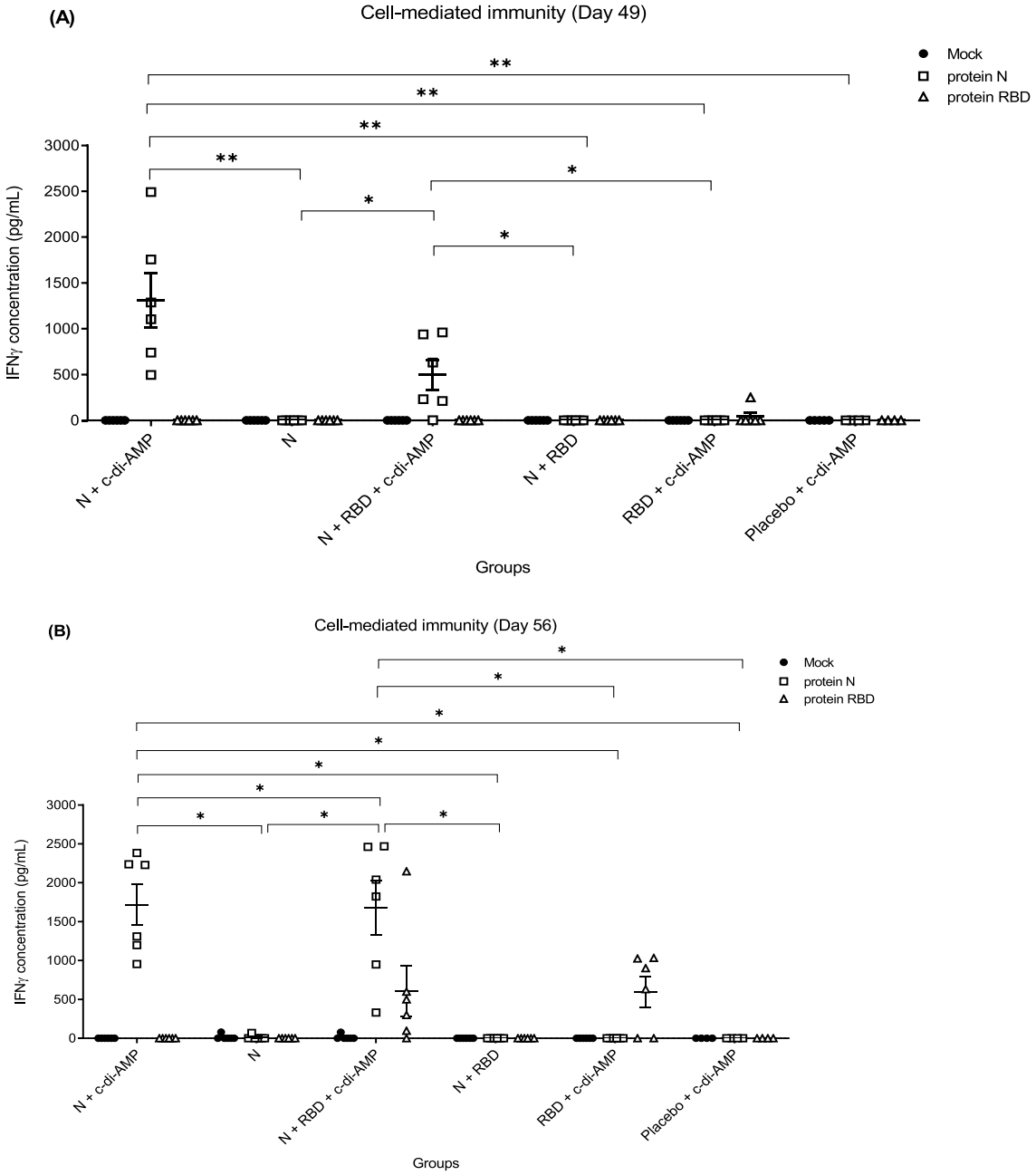

Figure 5: Cell-mediated immune responses induced in BALB/c mice immunized with nasal formulations of RBD and N antigens from SARS-CoV-2. IFNγ secretion measured by ELISA in the culture supernatants of splenocytes after *in vitro* stimulation with mock, protein N or protein RBD. (A) IFNγ concentration in the culture supernatants of splenocytes at day 49. (B) IFNγ concentration in the culture supernatants of splenocytes at day 56. The graph represents mean ± SEM for each group. The analysis of data was performed using a Kruskal–Wallis non-parametric test with Dunn's multiple comparison tests (n = 4 or 6 per group), (*: P<0.05; **: P<0.01). The dashed lines represent the cut-off values defined as twice the average of IFNγ concentrations measured from Placebo groups after *in vitro* stimulation with the specific antigen.

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Discussion

After the COVID-19 pandemic, an unprecedented global effort was triggered to develop safe and effective vaccines against the variants of concern of SARS-CoV-2. COVID-19 vaccines mostly contain the Spike antigen or fragments thereof, and are designed to generate robust neutralizing antibodies to prevent not only SARS-CoV-2 infection, but also severe outcomes of the disease, hospitalizations and deaths [5, 6]. To date, the WHO has approved several vaccines against COVID-19, which are based on nucleic acid, protein subunits, viral vectors and inactivated viruses platforms [4]. However, the effectiveness of these vaccines is significantly reduced in relation to newer strains [32, 33]. Data shows that current COVID-19 vaccines confer around 70% protection against intensive care unit admission some months after immunization, despite marked reductions in protection from symptomatic infection [34]. These observations suggest that vaccines continue to provide protection against severe disease despite the emergence of progressively more antibody-evasive and increasingly transmissible variants [34].

In this pipeline, a new generation of vaccines is emerging, aimed to solve the limitations of actual approved vaccines. In this regard, the goal is to induce broader immune responses, stimulate longer-lasting immunity, and activate the mucosal immune response [35]. To achieve these outcomes, various approaches have been pursued, including the incorporation of more viral antigens, the use of adjuvants, alternatives administration routes, and more effective delivery systems, among others [36]. Recombinant subunit proteins provide advantages regarding safety, costs, and speed of vaccine production; making those very attractive platforms for the development of vaccines for emerging viruses [37]. In correspondence, until last year near to 32% of vaccine candidates to COVID-19 diseases were based on protein subunits [4]. In our center was develop the ABDALA Vaccine, based on RBD region of the surface antigen of SARS-CoV-2 obtained as a recombinant protein in the yeast *Pichia pastoris* [38]. The ABDALA Vaccine has demonstrated that is safe, well tolerated, and protect humans against the COVID-19 disease [39]. The ABDALA Vaccine has successfully delivered as part of the initial vaccination or booster scheme of the population in several countries [40, 41]. On the other hand, the N antigen from SARS-CoV-2 have been used in several vaccine approaches [11, 17-19, 42, 43], as this protein serves as a representative antigen for eliciting T-cell response in a vaccine setting. It induces SARS-specific T-cell proliferation and cytotoxic activity [15] and cross-reactive responses to other relevant coronavirus [17]. Various studies have evaluated in preclinical models the ability to induce the immune response of vaccine candidates that combine the RBD and N antigens as subunit proteins [13, 43-48].

In the present study, we explore the immunogenicity in BALB/c mice of a nasal vaccine candidate based on recombinant proteins RBD and N in combination with *c*-di-AMP as immune-modulator. In our strategy was used the nasal vaccination to promote mucosal and systemic immunity. To this path has an important role to prevent seeding of the initial reservoir and control viral transmission [49]. This immunization path could be an advantage as non-invasive procedure suitable for immunization of large populations [26]. Furthermore, the inclusion of *c*-di-AMP as an adjuvant seeks to increase the mucosal immunogenicity generated by both viral antigens. Prior studies have been evaluated the adjuvant properties of cyclic di-nucleotides in respiratory diseases [29, 30, 50, 51], these molecules are bacterial second messengers which are consider as PAMP, rapidly sense by the immune system as a danger signal, allowing their utilization as activators of the immune response [52]. Cyclic di-nucleotides are able to boost antibody titers, increase Th1/Th2-associated responses, and expanded germinal center B cells and memory T cells [51, 53, 54].

First, our results demonstrated that RBD and N recombinant proteins combined with *c*-di-AMP, in a nasal formulation, induced systemic and mucosal humoral immune responses. High IgG and IgA antibody titers against both recombinant antigens were detected, which were significantly boosted when *c*-di-AMP was incorporated in the nasal formulation. Neutralizing antibodies have been well established as a key correlate of protection against COVID-19 [55, 56]. In the study was demonstrated that these humoral responses elicited against ancestral strain were functional in terms of induction neutralizing antibodies against two relevant SARS-CoV-2 isolates (Delta and Omicron BA1.2), primarily due to the presence of the recombinant RBD subunit. In this sense, clinical data show that current COVID-19 vaccines continue to provide good protection against severe disease, despite the generation of low neutralizing antibody titers against new variants of concern [34]. Nevertheless, the addition of N antigen to nasal formulation N + RBD + *c*-di-AMP suggests a modulatory effect of this component, since a slight increase in neutralizing titers was observed. N antigen might stimulate high-affinity antibody clones to the ACE2 cellular receptor. Further studies should be performed to confirm this statement. Although, in the present study was not evaluated the functional role of antibodies generated to N subunit, several reports have shown that N-specific IgG antibodies could mediate antibody-dependent cell-mediated cytotoxicity (ADCC) [57-59].

Likewise, these results are in agreement with previous studies in mice using the molecule *c*-di-AMP as adjuvant in a subunit SARS-CoV-2 vaccine based on the RBD, but administered by intramuscularly, which showed that *c*-di-AMP improves the specific immune response to SARS-

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CoV-2 [29]. However, the clinical applicability of mucosal vaccine strategies remains promising, it is evident that the induction of upper respiratory tract immune responses by these vaccines could significantly enhance protection from transmission and improve durability against emerging variants [24]. Afterward, we demonstrate that incorporation of *c*-di-AMP to the formulations including N antigen, stimulated functional cell-mediated immune responses that secreted IFNγ after the *in vitro* stimulation with this recombinant protein. Thus, an immune-modulator effect in the cell-mediated immune response was detected in mice immunized with the nasal bivalent formulation that included the two recombinant antigens, RBD and N, plus *c*-di-AMP. These results corroborate the capacity of N antigen as a distinctive antigen for the T-cell response induction [15, 17]. In addition, these results are relevant in the context of infection with antibody-evasive variants; where T-cells with antigen specificity for conserved SARS-CoV-2 epitopes could control viral replication to prevent progression to more severe disease [34, 60]. A prior study showed that recombinant N protein mixed with a specific oligonucleotide (ODN-39M), promoted the aggregate formation of the nucleocapsid [18]. Although in the present study this effect not was assessed, we do not discard that the cyclic di-nucleotide can also induce the same behavior in the nasal formulation RBD + N + *c*-di-AMP. In this regards, next experiments could be focused on confirming the induction of self-assembling particles based on RBD and N proteins after combination with the *c*-di-AMP, which offer advantages over other antigen presentation forms [61]. In summary, this study establishes that an intranasal vaccine formulation based on RBD and N recombinant subunits plus *c*-di-AMP as adjuvant elicits comprehensive specific immunity against SARS-CoV-2.

Statements and Declarations:

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CRediT authorship contribution statement:

Iris Valdes: Writing–review & editing, Writing–original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Lazo**: Methodology, Investigation, Formal analysis, Conceptualization. **Edith Suzarte**: Methodology, Investigation, Formal analysis, Conceptualization. **Karem Cobas**: Methodology, Investigation. **Yusleidi Pérez**: Methodology. **Rocío Garateix**: Methodology. **Enrique Noa**: Methodology. **Rubén Amaya**: Methodology. **Dionne Casillas**: Methodology. **Elias Nelson**: Methodology. **Yinet Cartaya**: Methodology. **Monica Bequet**: Methodology, Formal analysis. **Julio C. Aguilar**: Methodology, Conceptualization. **Carlos A. Guzmán**: Conceptualization, Writing–review & editing. **Gerardo Guillen**: Writing–review & editing, Conceptualization, Resources, Funding acquisition, Project administration.

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