RESEARCH ARTICLE

# N-terminally truncated nucleocapsid protein of SARS-CoV-2 as a better serological marker than whole nucleocapsid protein in evaluating the immunogenicity of inactivated SARS-CoV-2

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## Abstract

The coronavirus disease 2019 pandemic caused by severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) had led to a serious public health crisis, and no specific treatments or vaccines are available yet. A nucleocapsid protein (NP)-based enzyme-linked immunosorbent assay (ELISA) detection method is not only important in disease diagnosis, but is required for the evaluation of vaccine efficacy during the development of an inactivated SARS-CoV-2 vaccine. In this study, we expressed both the NP and N-terminally truncated NP (ΔN-NP) of SARS-CoV-2 in an *Escherichia coli* expression system and described the purification of the soluble recombinant NP and ΔN-NP in details. The identities of the NP and ΔN-NP were confirmed with mass spectrometry. We then used immunoglobulin G detection ELISAs to compare the sensitivity of NP and ΔN-NP in detecting anti-SARS-CoV-2 antibodies. ΔN-NP showed greater sensitivity than NP in the analysis of serially diluted sera from mice and rabbits vaccinated with inactive SARS-CoV-2 and in human sera diluted 1:400. ΔN-NP showed a positive detection rate similar to that of the SARS-CoV-2 S protein in human sera. We conclude that ΔN-NP is a better serological marker than NP for evaluating the immunogenicity of inactivated SARS-CoV-2.

**KEYWORDS**

N-terminally truncated NP, protein expression and purification, SARS-CoV-2, sensitivity

## 1 | INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to threaten global public health. Several vaccines have already completed phase II clinical trials,[1–4] although there are not appropriate therapies for COVID-19.[5] Accurate serological diagnostic strategies are the first step in inhibiting the spread of SARS-CoV-2 and allow the qualitative and quantitative study of the immune responses to SARS-CoV-2.[6,7] The vaccine development process also requires that antibody responses and serological conversion can be determined accurately.

During the SARS-CoV outbreak in 2003, researchers considered the truncated nucleocapsid protein (NP) a good marker for diagnostic purposes.[8] An enzyme-linked immunosorbent assay (ELISA) based on the NP of SARS-CoV-2 has also been used in the serodiagnosis of this novel COVID-19.[9] An antibody directed against NP is more sensitive than one

directed against the spike protein in detecting early infections.[10] However, there are some controversial findings of the nonspecific of NP. Guo et al.[9] showed that recombinant whole NP did not cross-react with antibodies directed against other common coronaviruses. However, Yamaoka et al.[11] reported that full-length (FL)-NP had a higher false-positive rate than N-terminally truncated NP (ΔN-NP) in sera diluted 1:100. They concluded that ΔN-NP is better suited than FL-NP to the development of highly sensitive diagnostic assays for COVID-19. This suggests that an ΔN-NP-based ELISA has potential utility in evaluating the efficacy of inactivated SARS-CoV-2 vaccines. Because the antibody response to NP and its serological conversion in rodents and humans must be detectable during the development of inactivated SARS-CoV-2 vaccines, it is essential to enhance the sensitivity of such an assay to detect low levels of immunoglobulin G (IgG) in mice or humans, especially during the earlier period soon after vaccination.

To address this issue, we used ΔN-NP to develop ELISAs with which to detect anti-SARS-CoV-2 NP antibodies in mouse, rabbit, and human serum. Our results indicated that SARS-CoV-2 ΔN-NP is more sensitive than NP for evaluating the immune effects of an inactivated SARS-CoV-2 vaccine.

## 2 | MATERIALS AND METHODS

### 2.1 | Animal

Balb/c mice and Japanese white rabbits were purchased from ChuShangKeJi Co. Ltd. All animal care and experimental protocols were reviewed and approved by the Yunnan Provincial Experimental Animal Management Association and the Experimental Animal Ethics Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences (CAMS), according to the national guidelines on animal experimentation in China. The animals were housed in specific-pathogen-free facilities and anesthetized with ketamine to minimize their suffering during the relevant procedures.

### 2.2 | Human serum samples

This study was approved by the Ethical Review Board of the Institute of Medical Biology, CAMS, Peking Union Medical College. For healthy volunteers vaccinated with a candidate inactivated SARS-CoV-2 vaccine or placebo, written informed consent was obtained from each person before enrollment.

### 2.3 | Candidate inactivated SARS-CoV-2 vaccine

The candidate inactivated SARS-CoV-2 vaccine was provided by Dr. Qihan Li (Institute of Medical Biology, CAMS). All experiments with live SARS-CoV-2 virus were performed in enhanced biosafety level 3 (P3+) facilities at the Institute of Medical Biology, CAMS, which is approved by the National Health Commission of the People's Republic of China.

### 2.4 | NP and ΔN-NP encoded in SARS-CoV-2 expression plasmids

The plasmid expressing NP of SARS-CoV-2 was constructed and kindly provided by Dr. Feng Cong (Guangdong Laboratory Animals Monitoring Institute).

The plasmid expressing SARS-CoV-2 ΔN-NP was constructed in our laboratory. Briefly, open-reading frame 9 of SARS-CoV-2, encoding the N protein, was obtained from SARS-CoV-2 genomic RNA (NC_045512, Wuhan seafood market pneumonia virus isolate Wuhan-Hu-1). The nucleotide sequence encoding the truncated N protein was synthesized after codon optimization by Genewiz® Co. Ltd. The ΔN-NP was then cloned into vector pUC57 (Genewiz). Both this plasmid and the vector pET28a (Novagen) were digested with the EcoRI and XhoI restriction enzymes (NEB) and then ligated with T4 DNA ligase (NEB) to generate the N-terminal His6-tagged N-encoding construct. Escherichia coli DH5α cells (TsingKe) were then transformed with the construct, plated on Luria–Bertani (LB) agar plates containing kanamycin (50 μg/ml), and incubated at 37°C overnight. The plasmid extracted from the clones was doubly digested with EcoRI and XhoI to confirm the presence of the insert. DNA sequencing confirmed that the insert was in-frame and contained no extraneous changes.

### 2.5 | Expression of SARS-CoV-2 NP and ΔN-NP of in an E. coli system

E. coli BL21(DE3) cells (TsingKe) were transformed with the NP or ΔN-NP construct. The cells were plated on LB agar containing kanamycin (50 μg/ml) and grown overnight at 37°C. A single colony was used to inoculate 10 ml of LB starter culture supplemented with kanamycin (50 μg/ml), which was incubated at 37°C overnight. An aliquot (5 ml) of the starter culture was added to 500 ml of LB medium and grown at 37°C with shaking (225 rpm) until the optical density at a wavelength of 600 nm ($OD_{600}$) reached approximately 0.6 (about 3 h). Protein expression was induced with isopropyl β-D-thiogalactoside (IPTG) at 37°C or 24°C. The bacterial pellets were harvested by centrifugation (6500 g for 15 min at 4°C) for 4 or 20 h.

### 2.6 | Solubilization and purification of NP and NP-ΔN

The harvested cells were resuspended in phosphate-buffered saline (PBS; 10 ml/g wet bacteria), incubated on ice for 10 min, and disrupted by sonication (Ningbo Scientz Biotechnology Co., Ltd). The lysates were centrifuged at 12,000 g for 15 min at 4°C to pellet the cell debris. The cell lysates were then filtered through a 0.45-μm filter and loaded onto a 5 ml HisTrap™ high-performance (HP; (GE Healthcare) column for purification in an AKTA Purifier purification system (GE Healthcare). After the lysate was loaded, the column was washed with 25 mM imidazole to remove nonspecifically bound proteins, and then with an imidazole gradient (from 25 to 250 mM)

to elute the 6XHis-tagged protein. The fraction containing the protein was selected for dialysis with a Slide-A-Lyzer™ G2 Dialysis Cassette, γ-irradiated, and 10 kDa molecular-weight cutoff (MWCO; Thermo Fisher Scientific) for 24 h.

The fraction was then concentrated with an Amicon Ultra-15 Centrifugal Filter Unit, with a 10-kDa MWCO (Merck Millipore). Protein expression and purification were monitored with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10% Coomassie Brilliant Blue R-250 (CBB)-stained gels under reducing conditions induced with β-mercaptoethanol.

## 2.7 | Protein quality test

Protein concentrations were quantified with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The protein sample (20 μg) was loaded onto 10% gel for SDS-PAGE. After concentration at 80 V for 20 min, the sample was separated at 120 V for 90 min. The gel was stained with CBB and decolored until the bands were clearly visible.

## 2.8 | Liquid chromatography–tandem mass spectroscopy analysis

The identities of SARS-CoV-2 NP and ΔN-NP were confirmed with trypsin digestion followed by liquid chromatography–tandem mass spectroscopy (LC–MS/MS) analysis (Novogene). Briefly, a CBB-stained protein band from the immobilized metal affinity chromatography (IMAC) purification was excised from the acrylamide gel in a cube of approximately 1 mm$^3$, and digested with trypsin at 37°C overnight. The lyophilized powder was dissolved. A portion (1 μg) of the sample was injected into a C18 Nano-Trap column. The peptides were separated on an analytical column. The separated peptides were analyzed with a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). The 40 most abundant precursors in the full scan were selected, fragmented with higher energy collisional dissociation, and analyzed with MS/MS. The resulting spectrum for each fraction was searched separately against the Uniport database with the search engine Proteome Discoverer 2.2 (Thermo Fisher Scientific).

## 2.9 | Enzyme-linked immunosorbent assay

To detect IgG, 96-well plates (Corning) were coated with 100 μl per well of spike (S1) protein, NP, or ΔN-NP and incubated at 4°C overnight. The coating solution was removed and 100 μl per well of 2% bovine serum albumin (BSA) prepared in PBS with 0.1% Tween 20 (PBST) was added to the plates at room temperature for 1 h as a blocking solution. Serial dilutions of rabbit serum were prepared in 1% BSA in PBST. The blocking solution was removed and 100 μl of

each serial dilution was added to the plates for 2 h at room temperature followed by washing. A 1:100,000 dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody, HRP-conjugated goat anti-rabbit IgG antibody, or HRP-conjugated goat anti-human IgG secondary antibody (Abcam) was prepared in PBST, and 100 μl of this secondary antibody was added to each well for 1 h followed by washing. Tetramethylbenzidine (Solarbio) solution (100 μl) was added to each well and this substrate was left on the plates for 10 min. The reaction was stopped by the addition of 50 μl per well of 1.5 M sulfuric acid. The $OD_{450}$ was measured with a Synergy 4 (BioTek) plate reader. The end-point titers were calculated as the last dilution before reactivity decreased below an $OD_{450}$ cutoff value. The cutoff value was calculated as 2.1 times the average $OD_{450}$ value of the negative control replicates.

## 2.10 | Statistics

The data are presented as the means ± SEM of the indicated number of experiments. The statistical significance of differences between multiple treatments was determined with a one-way analysis of variance and Tukey's post hoc honest significance test. All statistical analyses were performed with the GraphPad Prism software version 7 (GraphPad Software Inc.).

## 3 | RESULTS

### 3.1 | Expression of NP and ΔN-NP of SARS-CoV-2 in *E. coli*

To produce soluble NP and ΔN-NP, *E. coli* BL21(DE3) cells were transformed with the pET28a-N and pET28a-ΔN vectors, respectively, and expression was induced with 0.1 mM IPTG at 37°C, 28°C, or 24°C for NP and at 37°C or 24°C for ΔN-NP. NP and ΔN-NP were successfully expressed at all temperatures, confirmed by the presence of a band in the induced control that matched the theoretical molecular weight (MW) of 50 (NP) or 36 kDa (ΔN-NP). The soluble NP and ΔN-NP proteins accounted for about 80% of the total expressed protein when we reduced the expression temperature to 24°C (Figure 1).

### 3.2 | Purification of NP and ΔN-NP

Soluble NP and ΔN-NP were purified by IMAC on a 5 ml HisTrap HP column. The NP and ΔN-NP proteins were eluted with a 25–250 mM imidazole gradient (Figures 2A and 2C). After purification, NP and ΔN-NP appeared as single bands (Figures 2B and 2D). The identities of NP and ΔN-NP were confirmed with LC–MS/MS (97.73% and 96.52% abundance, respectively). Together, these data demonstrate that our recombinant NP and ΔN-NP proteins were of high quality
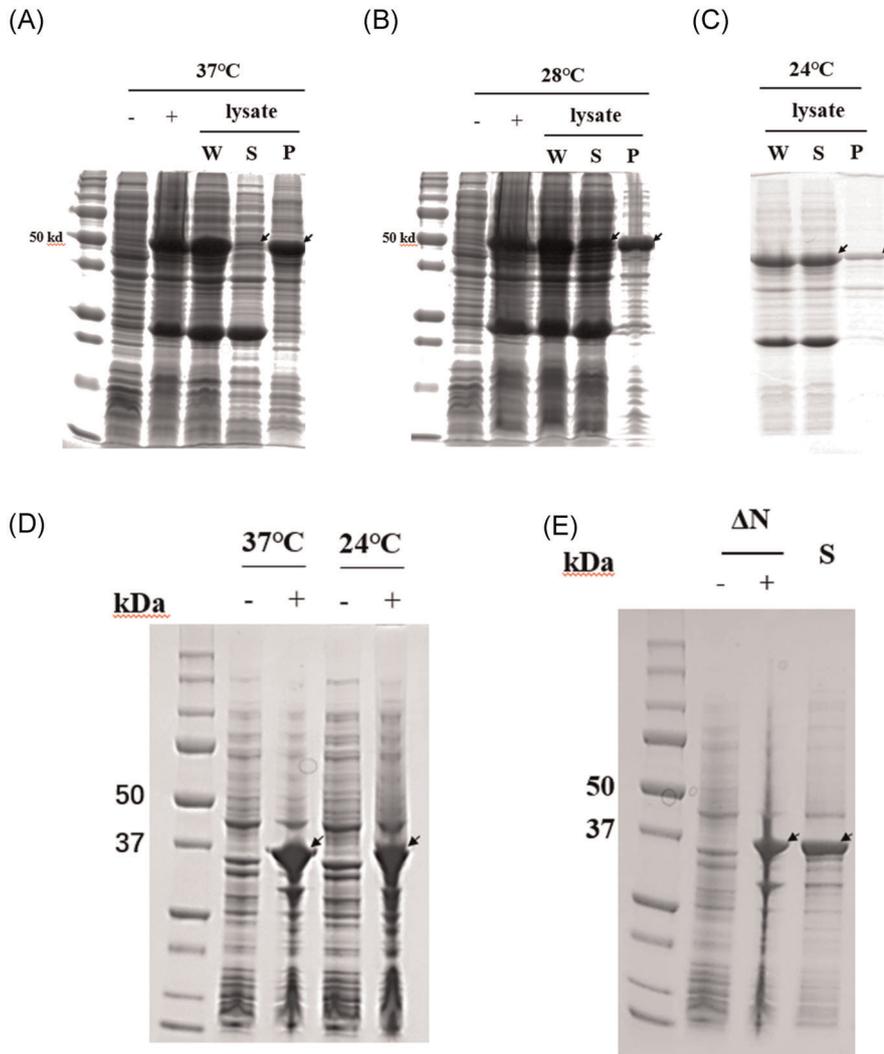
**FIGURE 1** Expression of NP and ΔN-NP of SARS-CoV-2 in *Escherichia coli*. NP expression and solubility induced with 0.1 mM IPTG at 37°C (A), 28°C (B), and 24°C (C) in *E. coli* BL21 (DE3) were detected by SDS-PAGE. (D) ΔN-NP expression was induced with 0.1 mM IPTG at 37°C and 24°C. (E) Solubility tests of ΔN-NP were performed at 24°C. The arrow indicates NP or ΔN-NP migration according to the theoretical MW of 50 or 36 kDa. IPTG, isopropyl β-d-thiogalactoside; MW, molecular weight; NP, nucleocapsid protein; P, pellet; S, soluble; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; W, whole-cell lysate; ΔN-NP, N-terminally truncated NP; −, uninduced, +, induced

and homogeneous and that the estimated masses matched those of the mature native N protein of SARS-CoV-2.

## 3.3 | ΔN-NP is better than NP as a serological marker for evaluating the immunogenicity of inactivated SARS-CoV-2

To assess the sensitivity of NP- and ΔN-NP-based ELISA, we performed an IgG detection ELISA to analyze inactive SARS-CoV-2 vaccinated sera. The ΔN-NP ELISA showed higher IgG titers than NP in serial diluted mouse and rabbit sera (Figure 3). We then tested IgG in 30 volunteer sera collected on Day 28 after vaccination with a candidate inactive vaccine or placebo. ΔN-NP showed a similar positive rate similar to that achieved with the SARS-CoV-2 S protein in human sera diluted 1:400 (Figure 4). In contrast, the NP-based ELISA exhibited lower IgG titers and had a lower positive rate than both the ΔN-NP- and S-protein-based ELISAs.

## 4 | DISCUSSION

In the development of an inactivated SARS-CoV-2 vaccine, the antibody response to the candidate vaccine and its serological conversion must be determined. However, some findings of the sensitivity of NP-based ELISAs have been controversial. In this study, we confirmed that ΔN-NP is better than NP as a serological marker for evaluating the immunogenicity of inactivated SARS-CoV-2.

SARS-CoV-2 detection with an NP-based ELISA is widely used to screen individuals for COVID-19. It has been reported that the specificity of NP detection is good in clinical samples.[9] However, several studies have also shown that the specificity and sensitivity of FL-NP-based ELISAs in the diagnosis of COVID-19 are lower than those of the ΔN-NP-based ELISAs.[11] ΔN-NP has also been used by Yu et al.[12] to reduce the high rates of false-positive results when testing healthy donor sera for SARS-CoV. These findings suggest that ΔN-NP could be used for ELISA-based immunogenicity assay. Consistent with patient serum, our results demonstrate that ΔN-NP is more sensitive in evaluating the immunogenicity of inactivated SARS-CoV-2 than is NP.

**FIGURE 2** purification of NP and ΔN-NP. Soluble NP and ΔN-NP were purified by immobilized metal affinity chromatography on a 5 ml HisTrap™ HP column (A, C). NP and ΔN-NP purity were detected by SDS-PAGE (B, D). The arrow indicates NP or ΔN-NP migration according to the theoretical MW of 50 and 36 kDa. HP, high performance; MW, molecular weight; NP, nucleocapsid protein; ΔN-NP, N-terminally truncated NP; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
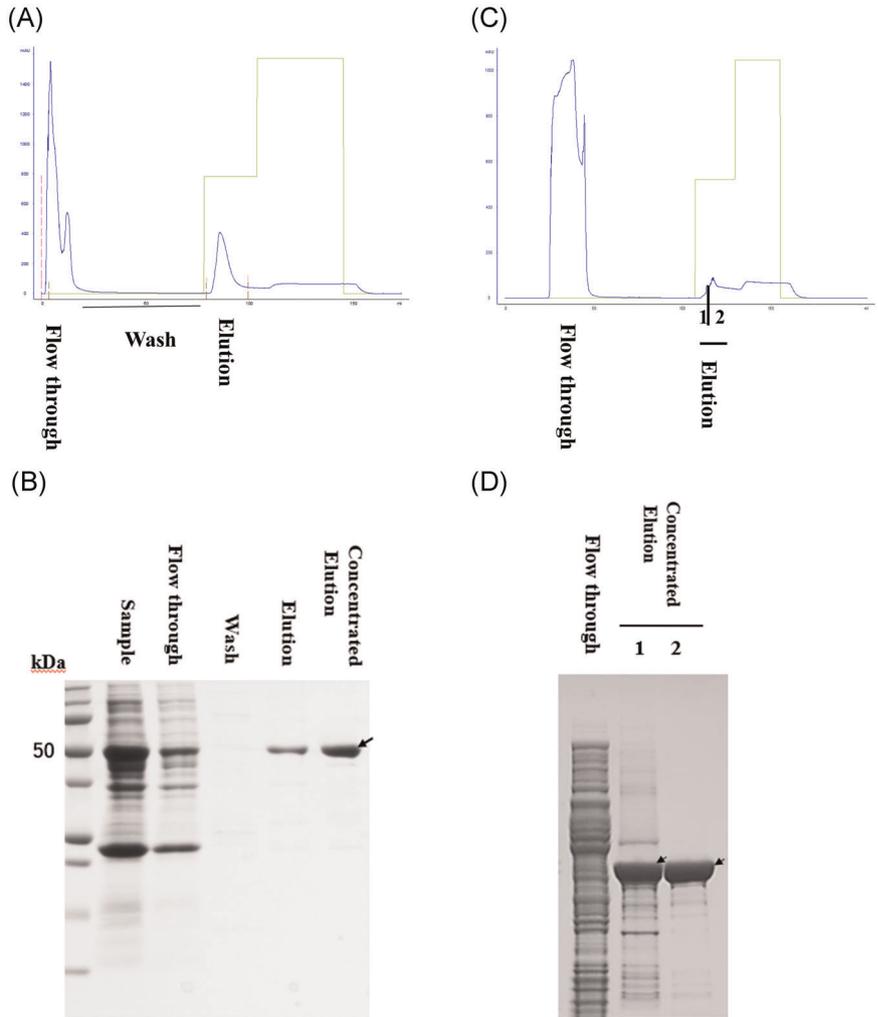


As Yamaoka et al.[11] pointed out, although the amino acid sequences of the entire NP of other coronaviruses are dissimilar to that of SARS-CoV-2, the conserved residues at the N-terminal domain of NP show a high degree of similarity. This may lead to high cross-reactivity of NP with antibodies to other human coronaviruses. ΔN-NP, which was devoid of the homogenous conserved residues at the N-terminal region, is confirmed better suited than NP to develop high-sensitivity diagnostic assays for both SARS-CoV[8] and SARC CoV-2.[11]
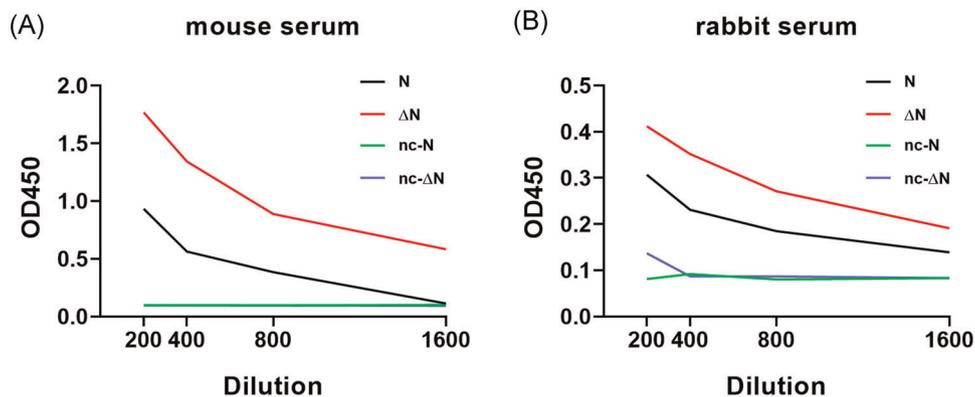


**FIGURE 3** The ΔN-NP ELISA showed higher IgG titers than NP in mouse and rabbit sera. A total of 96-well plates were coated with NP or ΔN-NP protein for IgG titer test of mouse (A) and rabbit (B) serum with serial dilutions (1:200, 1:400, 1:800, and 1:1600). End-point titers were calculated. The cutoff value was calculated by 2.1-fold the average A450 of negative-control replicates. ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; NP, nucleocapsid protein; OD$_{450}$, optical density at a wavelength of 450 nm; ΔN-NP, N-terminally truncated NP
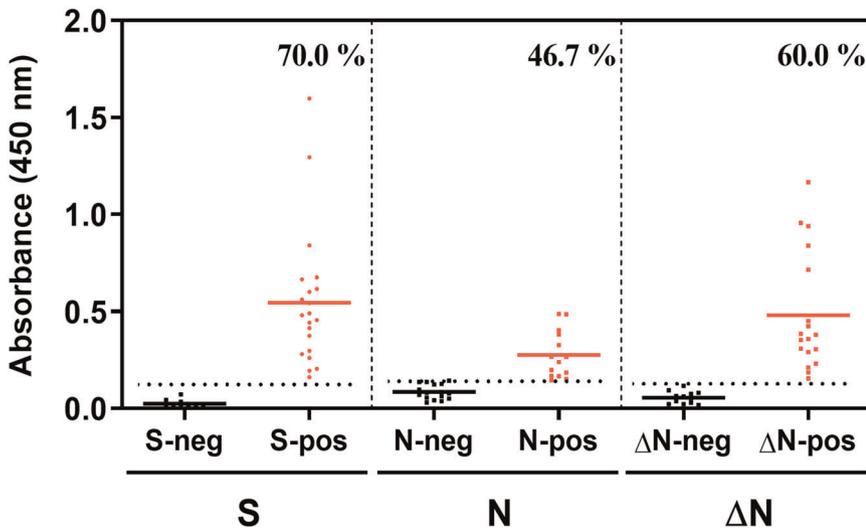
**FIGURE 4** ΔN-NP showed a similar positive rate similar to that achieved with the SARS-CoV-2 S protein in human sera. A total of 96-well plates were coated with S protein, NP, or ΔN-NP protein for IgG titer test of human serum with 1:400. End-point titers were calculated. The cutoff value was calculated by 2.1-fold the average A450 of negative-control replicates. IgG, immunoglobulin G; neg, negative; NP, nucleocapsid protein; pos, positive; SARS-CoV-2, severe acute respiratory syndrome-coronavirus 2; ΔN-NP, N-terminally truncated NP

According to these studies, truncated NP is very likely to be a good marker in other coronaviruses except SARS-CoV and SARS-CoV-2. In addition, in our study, we concluded that ΔN-NP is also a better serological marker than NP for evaluating the immunogenicity of inactivated SARS-CoV-2. From the above, ΔN-NP could be widely used for evaluating the immunogenicity of other inactivated coronaviruses.

Although Zeng et al.[13] and Guo et al.[9] expressed NP in *E. coli* from vectors pET28a and pET30a, respectively, its expression and purification were not described in detail. In contrast, the expression of ΔN-NP has only been reported in the wheat germ cell-free protein production system.[11] In the present paper, we have described in detail the expression and purification of both recombinant soluble NP and ΔN-NP of SARS-CoV-2 in an *E. coli* expression system. A low-temperature induction strategy greatly increased the solubility of the NP and ΔN-NP expressed in *E. coli*.

Zeng et al.[13] characterized the biochemical properties of SARS-CoV-2 NP with static light scattering, circular dichroic spectra, and a fluorescence polarization assay. That study detected no significant difference in the soluble expression and affinity purification of NP and ΔN-NP in an *E. coli* expression system. However, the differences in their biochemical characteristics must be determined in future work.

## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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