

## Detection of Antibodies Against SARS-Coronavirus Using Recombinant Truncated Nucleocapsid Proteins by ELISA

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Severe acute respiratory syndrome (SARS) is a life-threatening emerging respiratory disease caused by the coronavirus, SARS-CoV. The nucleocapsid (N) protein of SARS-CoV is highly antigenic and may be a suitable candidate for diagnostic applications. We constructed truncated recombinant N proteins (N1 [1–422 aa], N2 [1–109 aa], and N3 [110–422 aa]) and determined their antigenicity by Western blotting using convalescent SARS serum. The recombinants containing N1 and N3 reacted with convalescent SARS serum in Western blotting. However, the recombinant with N2 did not. In ELISA using N1 or N3 as the antigens, positive results were observed in 10 of 10 (100%) SARS-CoV-positive human sera. None of 50 healthy sera gave positive results in either assay. These data indicate that the ELISA using N1 or N3 has high sensitivity and specificity. These results suggest that the middle or C-terminal region of the SARS N protein is important for eliciting antibodies against SARS-CoV during the immune response, and ELISA reactions using N1 or N3 may be a valuable tool for SARS diagnosis.

**Keywords:** ELISA, SARS, truncated recombinant nucleocapsid proteins

Severe acute respiratory syndrome (SARS) is a life-threatening, emerging respiratory disease. An outbreak of SARS first occurred in southern China (Guangdong province) in 2002. From there it has spread to several countries: Hong Kong (China), Vietnam, Singapore, Canada, Germany,

Thailand, Taiwan, and beyond [6]. From November 2002 to July 2003, a total of more than 8,098 probable SARS cases with more than 774 deaths were reported in 26 countries [16].

The structural proteins of SARS-CoV are the spike (S) glycoprotein, the membrane (M) protein, the envelope (E) protein, the nucleocapsid (N) protein, and putative uncharacterized proteins [4, 6]. The N protein of SARS-CoV is a basic protein consisting of 422 amino acids. It has strong antigenicity and may play an important role in the induction of the host's immune response and even pathogenesis during SARS-CoV infection [15]. Moreover, this protein is expressed early during the infection period [11]. These data suggest that the SARS-CoV N protein is a suitable candidate for diagnostic applications.

An ELISA is a rapid, sensitive, and specific assay. It was proved in not only serological diagnosis of disease but also quantitative analysis of protein [5, 7, 10].

In this study, truncated recombinant N proteins (N1 [1–422 aa], N2 [1–109 aa], and N3 [110–422 aa]) were cloned, sequenced, expressed in an *E. coli* system, and purified. We developed an ELISA using the purified proteins and evaluated the sensitivity and specificity of the ELISA.

### MATERIALS AND METHODS

#### Serum Samples

Ten serum samples were collected from convalescent SARS-CoV patients in Vietnam (National Institute of Hygiene and Epidemiology). Fifty healthy serum samples were provided by volunteers. This work was approved by the institutional review board of Seoul National University (IRB No. 0705/001-001).

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### Expression and Purification of SARS-CoV Nucleocapsid Protein

The cDNA of SARS-CoV strain Hanoi was used as a template for PCR using Accupower premix (Bioneer, U.S.A.). Different truncated fragments of the SARS-CoV N gene were acquired. The primers were synthesized according to the Urbani Accession No. AY278741; that is N1 forward (5'-CGG GGA TCC ATG TCT GAT AAT GGA CCC CAA-3') and N1 reverse (5'-ACGC GTC GAC TGC CTG AGT TGA ATC AGC-3'), N2 forward (5'-CGG GGA TCC ATG TCT GAT AAT GGA CCC CAA-3') and N2 reverse (5'-ACGC GTC GAC CCA TCT GGG GCT GAG CTC TTT-3'), and N3 forward (5'-CGG GGA TCC ATG TAC TTC TAT TAC CTA GGA ACT GGC-3') and N3 reverse (5'-ACGC GTC GAC TGC CTG AGT TGA ATC AGC-3'). The forward and reverse primers carried BamHI and Sall restriction sites (underlined). Products were amplified under the following conditions: 94°C for 10 min, then 30 cycles of 94°C for 30 sec, 48°C for 30 sec, and 72°C for 1 min, followed by one cycle of 72°C for 5 min, and then examined by 1% agarose gel electrophoresis.

The SARS-CoV N genes were cloned into the pGEM-T easy vector (Promega, U.K.) by standard techniques and then transformed into *E. coli* strain DH5 $\alpha$ . The constructed pGEMT-N genes were digested with BamHI and Sall (Roche, Switzerland) and then inserted into the expression vector pET21a (Novagen, Germany), thereby introducing a small hexahistidine tag at the C-terminus of the protein to facilitate subsequent purification. The recombinant plasmids were transformed into *E. coli* strain DH5 $\alpha$ . The constructed pET21a-N genes were sequenced.

The pET21a-N genes were transformed into *E. coli* strain Origami B (DE3) pLysS Competent Cells. Bacterial cells were treated with 1 mM IPTG (Invitrogen, U.S.A.) at 37°C for 4 h in LB broth containing selective antibiotics. Cells were harvested by centrifugation, and the pellets were extracted with BugBuster Protein Extraction Reagent (Novagen, Germany). The supernatants of the extracts were filtered with a 4.5- $\mu$ m syringe filter, and the proteins were purified from the filtered supernatants using His-Bind kits. The filtered supernatants were loaded onto an Ni-IDA resin column, followed by washing with binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9), and then washing buffer (0.5 M NaCl, 20 mM Tris-HCl, 60 mM imidazole, pH 7.9). The proteins were eluted with elution buffer (0.5 M NaCl, 20 mM Tris-HCl, 1 M imidazole, pH 7.9).

### Western Blotting

The recombinant N proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gels and were then transferred onto cellulose nitrite membranes. After blocking with 5% skim milk for 2 h, the membranes were incubated with either a mouse anti-SARS-CoV N protein monoclonal IgG<sub>2a</sub> (Zymed, U.S.A.) or a serum from a convalescent SARS patient or a healthy volunteer. After washing, the membranes were stained with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG<sub>2a</sub> Ab (Zymed, U.S.A.) or goat anti-human IgG Ab (Sigma, U.S.A.) for 2 h. The blots were developed using ECL detection reagents (Amersham Pharmacia Biotech, Sweden).

### ELISA

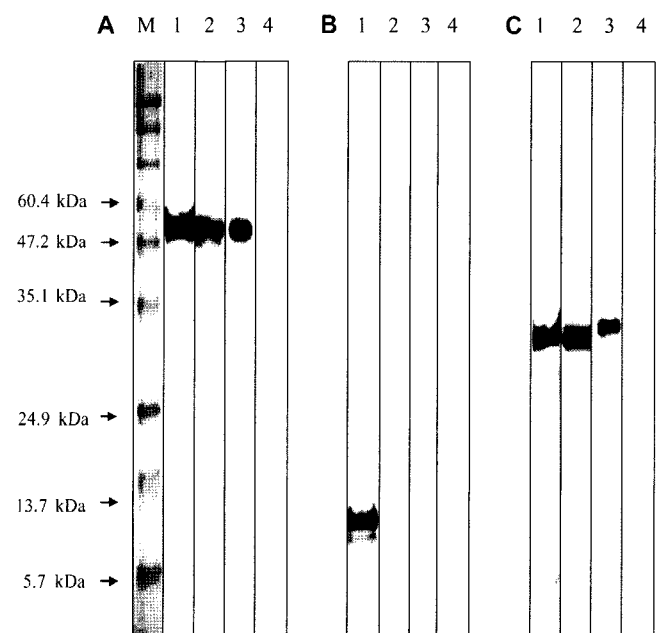
Purified N protein (250 ng) dissolved in coating buffer (0.016 M Na<sub>2</sub>CO<sub>3</sub>, 0.034 M NaHCO<sub>3</sub>, pH 9.6) was added to a 96-well microplate

and incubated at 4°C overnight. The wells were then blocked with 1% BSA (Sigma, U.S.A.) in phosphate buffer saline (PBS) for 1 h at 37°C. For detection of antibodies (Abs), sera were diluted in PBS (1:40) and then incubated at 37°C for 2 h. Each well was washed three times with PBS containing 0.05% Tween-20 and then incubated with a HRP-conjugated anti-human IgG Ab for 2 h at 37°C. After washing, the peroxidase reaction was visualized using OPD (*O*-phenylenediamine) as a substrate (Sigma, U.S.A.) for 10 min at room temperature. The reaction was stopped by adding 100  $\mu$ l/well of 1 N H<sub>2</sub>SO<sub>4</sub>, and the absorbance was read at 492 nm with a 692 nm reference filter. The serum samples were run in triplicate. The cutoff value was defined as the mean optical density (OD) of the control samples plus three standard deviations (SD).

## RESULTS

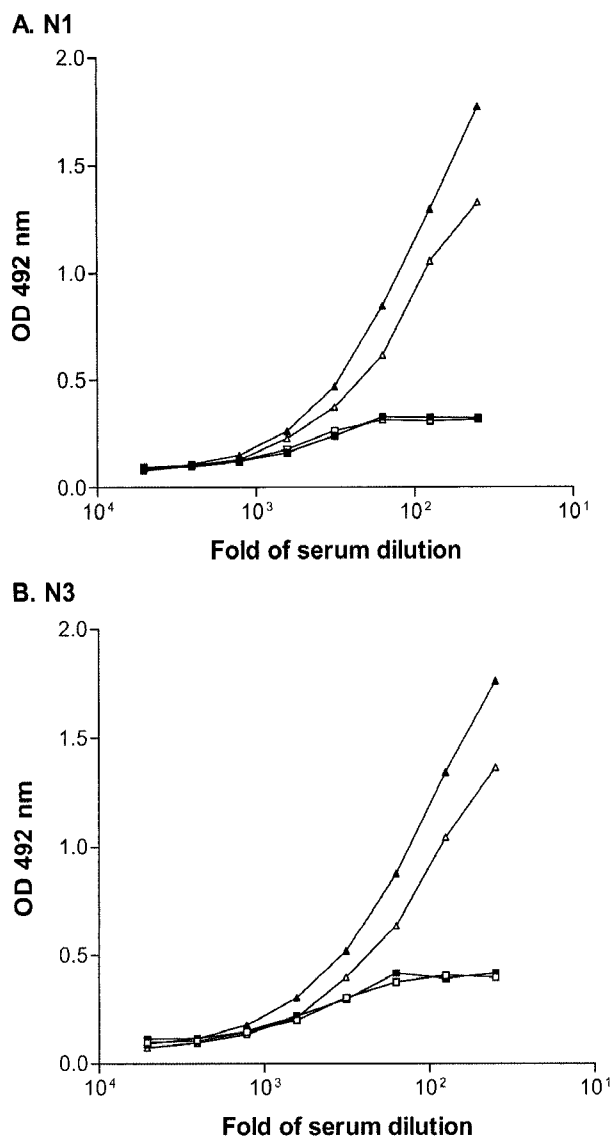
### Expression and Purification of SARS-CoV Nucleocapsid Protein

The truncated recombinant N proteins (N1 [1–422 aa], N2 [1–109 aa], and N3 [110–422 aa]) were designed for an ELISA to detect Abs against the SARS-CoV N protein. The truncated recombinant N proteins were efficiently expressed in the *E. coli* strain Origami B (DE3) pLysS Competent Cells with a hexahistidine tag, and the purified truncated recombinant N proteins were visually detected by SDS-PAGE analysis (Fig. 1).



**Fig. 1.** SDS-PAGE and Western blotting analysis of the recombinant truncated N proteins (A: N1, B: N2, C: N3).

Purified recombinant truncated N proteins were run in SDS PAGE 12% gels with molecular mass markers in Lane M. One of the gels was stained with Coomassie blue (Lanes M and 1). Protein bands in the other gel were transferred onto cellulose nitrite membranes for Western blotting with a monoclonal serum (Lane 2), a serum from a convalescent SARS patient (Lane 3), and a serum from a healthy volunteer (Lane 4). This figure was edited from our published data [2].



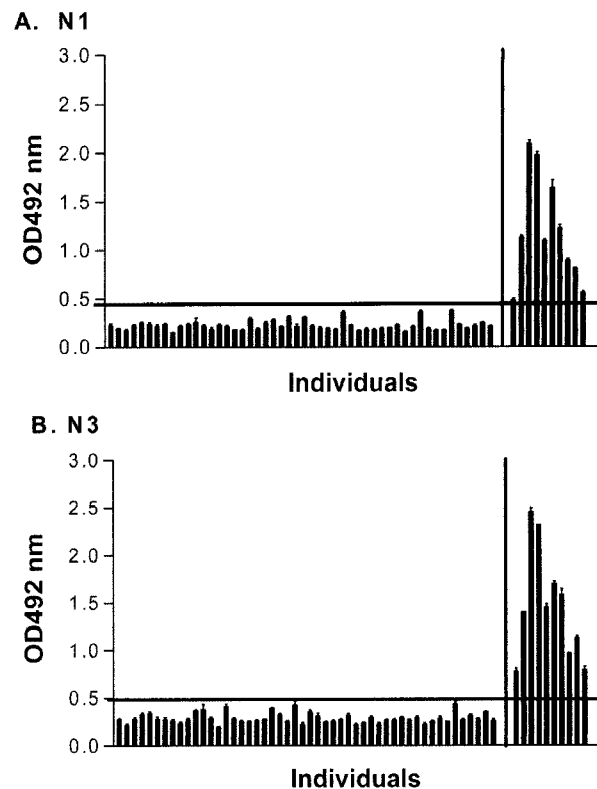
**Fig. 2.** Sensitivity of the ELISA using N1 and N3. ELISA plates were coated with recombinant N1 and N3. Serum samples from two convalescent SARS patients ( $\blacktriangle$ ,  $\triangle$ ) and two healthy individuals ( $\blacksquare$ ,  $\square$ ) were serially diluted and dispensed into the wells. HRP-conjugated goat anti-human IgG Ab was used as the secondary antibody with OPD as the substrate. The results are expressed as an absorbance reading at a wavelength of 492 nm with a 692 nm reference filter.

### Western Blotting

The truncated recombinant N proteins were identified by Western blotting with a mouse anti-SARS-CoV N protein monoclonal IgG<sub>2a</sub> and a convalescent SARS serum. N1 and N3 reacted with the mouse anti-SARS-CoV N protein monoclonal IgG<sub>2a</sub> and the convalescent SARS serum, but N2 did not (Fig. 1).

### ELISA

Sera from two convalescent SARS patients and two healthy individuals were serially diluted and tested in an ELISA using the N1 or N3. When the antigens were coated at a



**Fig. 3.** Comparison of the ELISA using N1 and N3. Ten convalescent SARS sera (right side of vertical line) and fifty healthy sera (left side of vertical line) were screened with the ELISA using N1 (A) and N3 (B). The serum samples were diluted 40-fold. The results are expressed as absorbance readings at a wavelength of 492 nm with a 692 nm reference filter. The horizontal line shows the cutoff value (N1: 0.373; N3: 0.456).

concentration of 250 ng and the sera were diluted 1:40, the ELISA detected Abs against the SARS-CoV in a specific and sensitive manner (Fig. 2).

The cutoff value was calibrated from the ELISA results using 50 healthy sera. All serum samples from the 50 healthy volunteers were negative (Fig. 3). When serum samples (diluted 1:40) from 10 convalescent SARS patients were analyzed sequentially, all of them were positive, suggesting a high sensitivity for the ELISA using the N1 or N3 constructs (Fig. 3).

### DISCUSSION

SARS-CoV N protein is known as a useful antigen for the diagnosis of SARS, and the antigenic site of the SARS-CoV N protein has been investigated by many researchers [3, 9, 15]. Studies of ELISA systems using the SARS-CoV N protein have also reported [8, 14, 18].

In this study, we constructed N1 (1–422 aa: full protein sequence), N2 (1–109 aa: N-terminal region known to have weak antigenicity), and N3 (110–422 aa: middle and C-terminal regions known to have strong antigenicity) to

compare their antigenicities. We confirmed the antigenicity of the recombinant truncated N proteins by Western blotting using a serum from a convalescent SARS patient. When ELISA reactions using N1 or N3, which had been determined to have antigenicity in the Western blot, were performed with sera from 10 convalescent SARS-CoV patients, the results were all positive. Furthermore, none of the healthy sera reacted positively with N1 or N3 in the ELISA. On the other hand, N2 did not react positively with serum from convalescent SARS-CoV patients in the Western blot.

These data show that the middle or C-terminal regions of SARS-CoV N protein have strong antigenicity, but the N-terminal region of the SARS-CoV N protein has weak antigenicity, as has been reported previously [3, 9, 15]. The sensitivity and specificity of N1 and N3 were each high in ELISA.

A highly conserved motif is present in the N-terminal half of all coronavirus N proteins [6]. Human coronaviruses (HCoVs) are responsible for 10–35% of the upper respiratory tract infections known to cause the common cold, but these infections are not severe [12, 13]. We can distinguish SARS-CoV patients from patients with HCoV infections by the severity of the symptoms. However, a false-positive result is possible in ELISA systems using recombinant N proteins because of cross-reactivity with HCoVs [6, 17, 18]. Yu *et al.* [18] reported that ELISA using NΔ121 protein (122–422 aa) showed a reduced nonspecific reaction compared with ELISA using N protein (5–422 aa), because the N proteins of coronaviruses have a highly conserved motif in the N-terminal half. However, other authors have reported that the possibility of a false-positive result was low, although a full sequenced N protein was used for SARS diagnosis [1, 4, 15]. In this study, although N1 and N3 contain the conserved motif, the ELISA using them did not show false-positive results. However, sera with high titers against HCoVs can react with recombinant N proteins [17].

Therefore, we tried to confirm the specificity of the ELISA that we developed. HCoV-229E- or OC43-infected cell lysate was analyzed by Western blotting using sera from convalescent SARS patients. None of them reacted with HCoV-229E or OC43 (data not shown). In fact, we should have investigated the reactivity of the ELISA with HCoV-infected sera. However, unfortunately, we could not obtain those sera. Instead, we checked the cross-reactivity between HCoV-229E or OC43 and sera from convalescent SARS patients. Abs in convalescent SARS patients did not react with HCoV-229E or OC43. The epitope of N1 or N3 and HCoV-229E or OC43 may be different, because the results of the reactivity with the same Abs (sera from convalescent SARS patients) were different. Therefore, we guess that ELISA using N1 or N3 will not react with sera of HCoV-229E- or OC43-infected patients. However, the

cross-reactivity of this diagnostic method in patients with recent HCoV infections (229E, OC43, and NL63) must be studied further.

In this study, ELISA using N1 or N3 showed high sensitivity and specificity in SARS-CoV antibody testing. These results not only showed that recombinant N protein and its specific monoclonal antibodies may be used as diagnostic reagents for SARS, but also offered a potential target site for the design of an epitope-based vaccine against SARS.

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