

# Evaluation of Antibody Responses Against SARS Coronaviral Nucleocapsid or Spike Proteins by Immunoblotting or ELISA

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Severe acute respiratory syndrome (SARS)-CoV is a newly emerging virus that causes SARS with high mortality rate in infected people. To study the humoral responses against SARS-CoV, we evaluated nucleocapsid (N) and spike (S) proteins-specific antibodies in patients' sera by Western blotting and enzyme-linked immunosorbent assay (ELISA). Recombinant N and S proteins of SARS-CoV were purified from transformed *E. coli*. Serum specimens from 40 SARS-CoV-infected patients in the convalescent phase were analyzed by Western blotting using the purified antigens. Serial serum specimens from 12 RT-PCR-confirmed SARS patients were assayed by ELISA using the recombinant N protein as coated antigen. By Western blotting, 97.5% of the SARS patients were positive for N protein-specific antibodies whereas only 47.5% of the samples were positive for S protein-specific antibodies. Using N protein-based ELISA, 10 out of the 12 patients were positive for N protein-specific antibodies and 6 of them showed seroconversion at mean of 16 days after onset of fever. Immunoblotting was useful for detecting the humoral immune response after SARS-CoV infection. Antibodies against SARS-CoV N protein appear at the early stage of infection, therefore, N protein-based ELISA could serve as a simple, sensitive, and specific test for diagnosing SARS-CoV infection. **J. Med. Virol. 73:338–346, 2004.** © 2004 Wiley-Liss, Inc.

**KEY WORDS:** SARS; SARS-CoV; ELISA; N protein; S protein

## INTRODUCTION

An outbreak of atypical pneumonia, referred to as severe acute respiratory syndrome (SARS), was first

reported in Guangdong Province of People's Republic of China beginning in November of 2002 and has spread to Hong Kong, Singapore, Vietnam, Canada, America, Taiwan, and several European countries. By July 2003, before it stopped, the epidemic had affected 8,436 individuals and claimed 812 deaths [WHO, 2003]. A novel virus, the SARS-associated coronavirus (SARS-CoV), has been identified as the causal agent [Drosten et al., 2003; Ksiazek et al., 2003; Kuiken et al., 2003; Peiris et al., 2003b].

Coronaviruses can cause severe diseases in many animals such as infectious bronchitis virus (IBV) in chickens, feline infectious peritonitis virus (FIPV) in cats, murine hepatitis virus (MHV) in mice, and transmissible gastroenteritis virus (TGEV) in pigs [Lai and Holmes, 2001]. However, the previously known human coronaviruses, HCoV-229E and HCoV-OC43 are responsible for only ~30% of mild upper respiratory tract illnesses, but not severe ones [Lai and Holmes, 2001]. Since the new SARS-CoV spreads effectively and claims a high case-mortality rate (10%), it is imperative to understand the differences between SARS-CoV and other coronaviruses in the host responses. That is important for both understanding the pathogenesis and establishing early clinical diagnosis.

SARS-CoV is a member of the Coronaviridae family. Its positive-stranded RNA genome encodes 23 putative proteins, including 4 major structural proteins, nucleocapsid (N), spike (S), membrane (M), and small envelope

Grant sponsor: National Science Council, Taiwan; Grant number: NSC92-2751-B-400-002-Y.

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Accepted 15 March 2004

DOI 10.1002/jmv.20096

Published online in Wiley InterScience  
(www.interscience.wiley.com)

(E). Its genomic organization resembles other coronaviruses but the overall similarity between the structural proteins of SARS-CoV and those of other coronaviruses is low [Rota et al., 2003].

Among the viral structural proteins, the N protein appears to be important. It is a phosphoprotein which could bind to viral RNA to form a helical nucleocapsid that participates in viral replication [Baric et al., 1988; Lai and Holmes, 2001]. It is also a major viral antigen stimulating host's immune responses. Previous studies on animal coronaviruses have shown that some antigenic peptides of the N protein can be presented on the surface of infected cells and recognized by T cells [Boots et al., 1991; Wege et al., 1993]. Immunization with DNA vaccine of the N protein of TGEV could elicit both antibody production and T cell response against the N protein in mice [Liu et al., 2001]. It has also been reported that the N protein-specific cellular immune response is essential for protection against FIPV infection [Gerber et al., 1990]. These data suggested that the N protein of coronavirus possesses strong antigenicity and may play an important role in the induction of host's immune responses and even pathogenesis during SARS-CoV infection.

Spike glycoprotein also has several important biological functions. It has been shown that a cellular metalloproteinase, angiotensin-converting enzyme 2 (ACE2) could bind to S1 domain of the SARS-CoV S protein and support viral replication [Li et al., 2003]. Studies on the murine hepatitis virus-4 (MHV-4) showed that monoclonal antibodies against the S protein could neutralize viral infectivity and S protein-specific CTL response could be detected in MHV-4-infected mice [Collins et al., 1982; Fleming et al., 1983; Bergmann et al., 1996].

In patients with SARS, antibodies against SARS-CoV are detected within 2–3 weeks after onset of the disease by immunofluorescence assay (IFA) [Ksiazek et al., 2003; Peiris et al., 2003a]. It has been demonstrated that the convalescent sera from patients with SARS could react strongly with a ~46 kDa protein derived from the supernatant of SARS-CoV-infected Vero E6 cell [Krokhin et al., 2003]. The size of the protein is equivalent to that of nucleocapsid proteins of other coronaviruses. Until now, little was known about the temporal profiles of the antibody responses against N protein or S protein of SARS-CoV. Therefore, we studied the serological responses to SARS-CoV N and S proteins by immunoblotting and an enzyme-linked immunosorbent assay (ELISA).

## MATERIALS AND METHODS

### cDNA Cloning and Plasmid Construction

cDNA encoding SARS nucleocapsid or spike proteins were generated by reverse transcription from SARS coronavirus TW1 (accession no. AY291451) using Superscript II (Invitrogen, Carlsbad, CA) followed by amplification using platinum Taq DNA polymerase (Invitrogen). The oligonucleotide primers for nucleocapsid protein were 5'-ATGTCTGATAATGGACCCCA-3'

(forward, nt 28120–nt 28139) and 5'-TTATGCCT-GAGTTGAATCAG-3' (reversed, nt 29369–nt 29388). Primer sets for spike (a.a. 511–1255) were 5'-TCCACT-GACCTTATTAAGAA-3' (forward, nt 23022–nt 23041) and 5'-GTTCGTTTATGTGTAATGTA-3' (reversed, nt 25246–nt 25265). The resulting cDNA fragments were ligated into pGEX-1 (Amersham Pharmacia Biotech., Little Chalfont, England) plasmid for recombinant protein expression.

### Protein Expression and GST-Fusion Protein Purification

GST-nucleocapsid (GST-N) and GST-spike (GST-S) (511–1255) constructs were transformed into *E. coli* DH5 $\alpha$  and BL21 strains, respectively. A single colony was grown overnight in LB medium containing 50  $\mu$ g/ml ampicillin until OD<sub>600</sub> reached 0.2–0.3. Cells were then induced by 0.25 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG) for 3 hr at 30°C.

Cells were collected by centrifugation and then re-suspended in TNE buffer (50 mM Tris, pH 8.0, 0.15 M NaCl, 1 mM EDTA, and 1 mM PMSF), about 1 ml per 25 OD<sub>600</sub> cells. Cells were either lysed by sonication or microfluidizer depending on the volume of culture. After centrifugation, the lysate was separated into supernatant and pellet fractions. The solubility of recombinant proteins was then determined by SDS-PAGE analysis of these two fractions. To purify the recombinant protein, the lysate was incubated with TNE-equilibrated glutathione resin at 4°C for 1 hr. In consequence, the resins were packed and washed with PBS containing 1% Triton X-100 at a flow rate about 1 ml/min. The bound protein was eluted by 10 mM reduced glutathione in 50 mM Tris (pH 8.0) buffer. The purified recombinant N and S proteins were used for antibody generation and also used as the antigens for Western blotting and ELISA.

### Mass Spectrometric Protein Determination

The purified proteins were separated by capillary HPLC (ABI 140D HPLC; PerkinElmer Life Sciences, Inc., Boston, MA) in-line coupled with the LCQ ion trap mass spectrometer (Finnigan). The acquired mass spectra were analyzed by SEQUEST Browser to correlate the MS/MS spectrum with the amino acid sequences of proteins from SARS-CoV genome database.

### Western Blot Analysis of SARS-CoV Nucleocapsid and Spike Proteins From Virus-Infected Cells

Cell lysate of gamma-irradiated SARS-CoV-infected Vero E6, noninfected Vero E6 cells, and recombinant GST-N or GST-S (511–1255) proteins were separated by SDS-PAGE using 10% polyacrylamide gel and transferred to Hybond<sup>TM</sup>-C super nitrocellulose membrane (Amersham Pharmacia Biotech.) by electroblotting [Sambrook and Russell, 2001]. The membrane was blocked for 1 hr with 5% nonfat dry milk at room temperature and then probed with heat-inactivated

serum specimens (1: 200 dilution) from patients with SARS in the convalescent phase, rabbit anti-GST-N sera (1:10,000 dilution), or rabbit anti-GST-S (511–1255) sera (1:1,000 dilution), respectively for 1 hr. The membrane was washed three times with PBS containing 0.1% Tween 20 and then incubated with horseradish peroxidase (HRP)-conjugated sheep anti-human immunoglobulin G (IgG, Amersham Pharmacia Biotech.) or HRP-conjugated goat anti-rabbit immunoglobulins (Igs, DAKO Corporation, Carpinteria), and detected by chemiluminescence (PerkinElmer Life Sciences, Inc.) using Kodak BioMax MR film (Eastman Kodak Company, New York).

### N Protein-Based SARS-CoV ELISA

One hundred and ninety microliters of specimen diluent was added to each well of the purified recombinant GST-N protein-coated plates (coated antigen concentration: 1 µg/ml) and 10 µl of heat-inactivated test sera was added, mixed, and incubated for 60 min at 37°C. The plates were rinsed six times with washing solution and 100 µl of working conjugate (anti-human IgG HRP conjugate) was added and incubated at 37°C for 30 min. The plates were rinsed six times with washing solution and 100 µl of TMB solution was added to each well of the plates and incubated for 30 min at room temperature. The reaction was stopped by adding 100 µl of stop solution 2 N H<sub>2</sub>SO<sub>4</sub> and absorbance at 450 nm was read. The test is co-developed by General Biologicals Corp., Hsinchu, Taiwan and us.

### Serum Specimens

Patient serum specimens were collected from RT-PCR-confirmed SARS patients from National Taiwan University Hospital. For each patient, a series of serum samples were obtained at admission and thereafter. Negative serum specimens were collected from healthy blood donors. Sera used for Western blot analysis were from patients with SARS in the convalescent phase and were provided by Center for Disease Control, Taiwan. Rabbit anti-GST-N and anti-GST-S (511–1255) sera were collected from rabbits immunized three times with purified recombinant GST-N and GST-S (511–1255) proteins, respectively (LTK BioLaboratories, Taipei, Taiwan). For biosafety concern, all patient serum specimens used for ELISA or Western blotting have been inactivated by heating at 56°C for 30 min and all experiments were performed in a P2-level lab.

## RESULTS

### Expression of Recombinant SARS-CoV Nucleocapsid and Spike (511–1255) Proteins

To obtain individual SARS-CoV antigen, we initially selected open reading frames for spike and nucleocapsid proteins from SARS-CoV genome for the expression of their recombinant proteins. Suitable primers covering the complete open reading frame of TW1 strain were used to produce their corresponding cDNAs which were

then inserted into GST fusion expression vector for protein expression [Hsueh et al., 2003; Yeh et al., 2004].

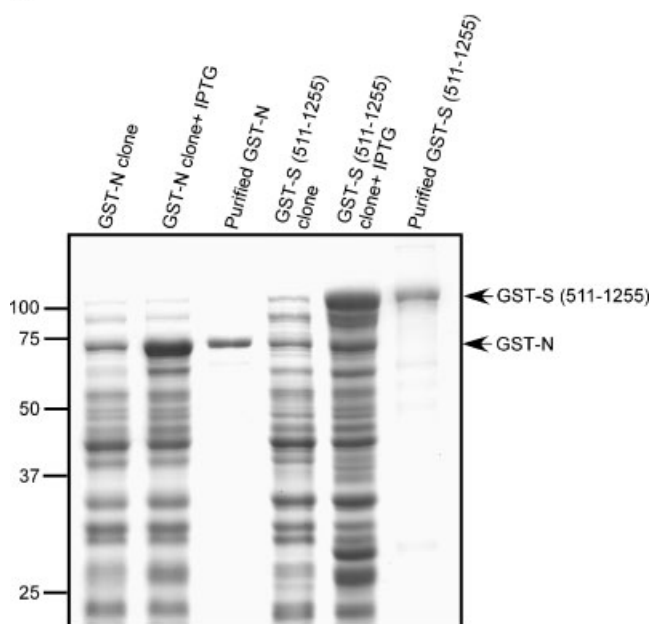
The GST fusion proteins, GST-N and GST-S (511–1255), were expressed in DH5α and BL21 strains respectively which were then lysed and separated into pellet and supernatant fractions. Although the pellet fraction contains residual recombinant N protein, significant portion of recombinant proteins were found in the soluble fraction (data not shown). We could recover the GST-N and GST-S (511–1255) fusion proteins through reduced glutathione agarose chromatography (Fig. 1A, lanes 3, 6). The resultant recombinant protein size was about 72 kDa for GST-N and 108 kDa for GST-S (511–1255). The purified proteins were used as antigens in Western blotting and ELISA. The identity of the purified recombinant proteins was confirmed by ion-trace mass spectrometry. Figure 1B showed a typical result of mass spectrometry.

### Antigenicity of N and S Proteins in SARS-CoV Infection

To determine the antigenicity of the N protein during natural SARS-CoV infection, sera from patients in the convalescent phase (S04, S18, and S45) were reacted with cell lysate of SARS-CoV-infected Vero E6, non-infected Vero E6 cells or the recombinant GST-N protein by immunoblotting. All patients' sera at a 1:200 dilution consistently reacted with a protein of approximately 50 kDa in the cell lysate of SARS-CoV-infected Vero E6 cells (Fig. 2A, lane 2) as well as the recombinant GST-N protein of 72 kDa by immunoblotting (Fig. 2A, lane 3), whereas none of the negative sera had any reactivity at a 1:200 dilution (data not shown). Only one out of the above three sera showed reactivity with a protein of approximately 180 kDa in the cell lysate of SARS-CoV-infected Vero E6 (Fig. 2B, lane 2) as well as the recombinant GST-S (511–1255) protein of 108 kDa (Fig. 2B, lane 3).

We also immunized rabbits with recombinant GST-N and GST-S (511–1255) proteins respectively to produce rabbit anti-N or anti-S antibodies. After three times immunization, sera from immunized rabbits were collected and assayed for reactivity with SARS-CoV-infected Vero E6 cell lysate and recombinant GST-N or GST-S (511–1255) proteins by immunoblotting. The immune sera at a 1:10,000 dilution reacted with the same protein of approximately 50 kDa from the cell lysate of the SARS-CoV-infected Vero E6 and the recombinant GST-N protein (Fig. 2C, lanes 2, 3); and the immune sera from GST-S (511–1255) immunized rabbits could react with the same protein of 180 kDa in the cell lysate of the SARS-CoV-infected Vero E6 cells and the recombinant GST-S (511–1255) protein of 108 kDa at a 1:1,000 dilution (Fig. 2D, lanes 2, 3). These results indicated that the recombinant GST-N and the recombinant GST-S (511–1255) proteins could react to specific antibodies in patients' sera and their antigenicity was similar to their native forms in the cell lysate of SARS-CoV-infected Vero E6 cells.



**A****B**

## SARS coronavirus nucleocapsid protein

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MSDNGPQSNQ  RSAPRITFGG  PTDSTDNNQH  GGRNGARPKQ  RRPQGLPNNT  ASWFTALTQH  GKEELRFPRG  QGVPINTNSG
PDDQIGYYRR  ATRRVRGGDG  KMKELSPRWY  FYYLGTGPEA  SLPYGANKEG  IVWVATEGAL  NTPKDHIGTR  NPNNNAATVL
QLPQGTTLPK  GFYAEGSRGG  SQASSRSSSR  SRGNSRNSTP  GSSRGNSPAR  MASGGGETAL  ALLLLDRLNQ  LESKVSQKQ
QQQGQTVTKK  SAAEASKKPR  QKRTATKQYN  VTQAFGRGPR  EQTQGNFGDQ  DLIRQGTDYK  HWPQIAQFAP  SASAFFGMSR
IGMEVTPSGT  WLYHGAIKL  DDKDPQFKDN  VILLNKHIDA  YKTFPPTEPK  KDKKKKTDEA  QPLPQRQKKQ  PVTLLPAD
MDDFSRQLQN  SMSGASADST  QA

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Fig. 1. Expression and purification of severe acute respiratory syndrome (SARS)-CoV nucleocapsid and spike fusion proteins. **A:** Expression of GST fusion protein in *E. coli*. Transformed cells were induced by isopropyl- $\beta$ -D-thiogalactoside (IPTG) and their lysates were separated by SDS-PAGE followed by Coomassie blue staining. **Lane 1** represents the cell lysate from noninduced SARS-CoV nucleocapsid/pGEX-1 transformed DH5 $\alpha$  cells whereas **lane 2** showed that from IPTG-induced cells. **Lane 3** is the purified recombinant GST-N protein. **Lane 4** shows the cell lysate from noninduced SARS-

CoV GST-S (511-1255)/pGEX-1 transformed BL21 cells whereas **lane 5** shows that from IPTG-induced cells. **Lane 6** is the purified recombinant GST-S (511-1255) protein. The relative protein size is shown in the left side of the panel. The positions of induced products are indicated. **B:** The complete amino acid sequences of SARS-CoV (TW1) N proteins were analyzed by mass spectrometry. The bold sequences are shown as the trypsin digested region identified under LC/MS/MS analysis. About 54% of the sequence for N protein was covered.

### N Protein Is a Major Viral Antigen Eliciting Antibody Response After SARS-CoV Infection

To characterize the serological response to N and S proteins during SARS-CoV infection, we tested 40 serum specimens from SARS-CoV-positive patients by Western blotting with recombinant GST-N and GST-S (511-1255) proteins. The results of 15 SARS-CoV-positive samples and 5 SARS-CoV-negative samples from healthy blood donors were shown in Figure 3. Nineteen (47.5%) out of 40 SARS-CoV-positive samples at a 1: 200 dilution showed significant reactivity with the recombinant GST-S (511-1255) protein (Fig. 3, lanes 6-12). Thirty-nine positive sera (97.5%) at a 1: 200 dilution consistently reacted with the GST-N protein by immunoblotting (Fig. 3, lanes 6-20). In addition, most of the samples showed strong reactivity with the GST-N

protein. Only one out of the 40 positive sera showed no reactivity with the recombinant GST-N protein and it also showed no reactivity with the recombinant GST-S (511-1255) protein (data not shown). All the five SARS-CoV-negative samples showed no reactivity with GST-N or GST-S (511-1255) proteins (Fig. 3, lanes 1-5). These data implied that the titer and positive rate for antibodies against the N protein were much higher than those against the S protein during SARS-CoV infection. Therefore, we used the N protein as the coated antigen in ELISA for detection of SARS-CoV infection.

### N Protein-Based ELISA Using Anti-Human IgG mAb HRP Conjugate

We cooperated with General Biologicals Corp. to develop N protein-based ELISA. First, we have performed a

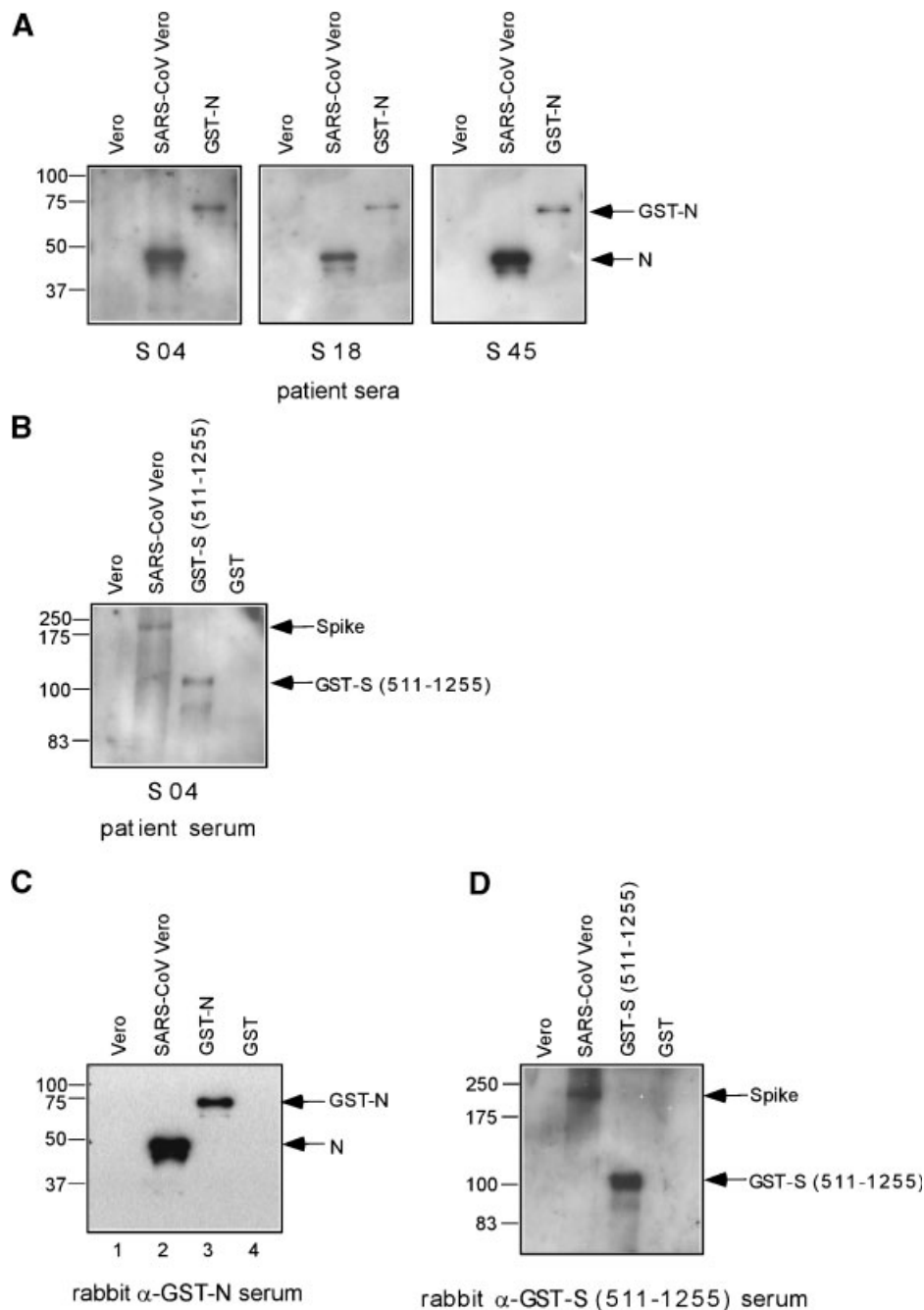


Fig. 2. Western blot analysis of SARS patients' sera, rabbit anti-GST-N serum, and rabbit anti-GST-S (511–1255) serum with cell lysate of SARS-CoV-infected Vero E6 cells, purified recombinant GST-N or purified recombinant GST-S proteins. The cell lysates and recombinant proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Each membrane was incubated with diluted sera followed by horseradish peroxidase (HRP)-conjugated anti-human IgG (1:3,000 dilution) or anti-rabbit Igs (1:2,000 dilution) and detected by chemiluminescence. **A**: Reactivity of the patients' sera with the native N protein in cell lysate (**lane 2**) and the purified recombinant GST-N protein (**lane 3**). Each membrane was incubated with a 1:200 dilution of patient sera. **Lane 1** (Vero) was a preparation of cell lysate of Vero E6 cells and was served as a negative control. **B**: Reactivity of the patients' sera with the native S protein in cell lysate

(**lane 2**) and the purified recombinant GST-S (511–1255) protein (**lane 3**). Each membrane was incubated with a 1:200 dilution of patients' sera. **Lane 1** (Vero) was a preparation of cell lysate of Vero E6 cells, **lane 4** was a preparation of the purified GST protein and both were served as a negative control. **C** and **D**: Reactivity of the rabbit anti-GST-N or rabbit anti-GST-S (511–1255) sera with the native N protein or S protein in cell lysate (**lane 2**) and the purified recombinant GST-N or GST-S (511–1255) proteins (**lane 3**). The membrane was incubated with a 1:10,000 dilution of anti-GST-N serum (**panel C**) and a 1:1,000 dilution of anti-GST-S (511–1255) serum (**panel D**), respectively. **Lane 1** was a preparation of cell lysate of Vero E6 cells and **lane 4** was a preparation of the purified GST protein and both were served as a negative control.

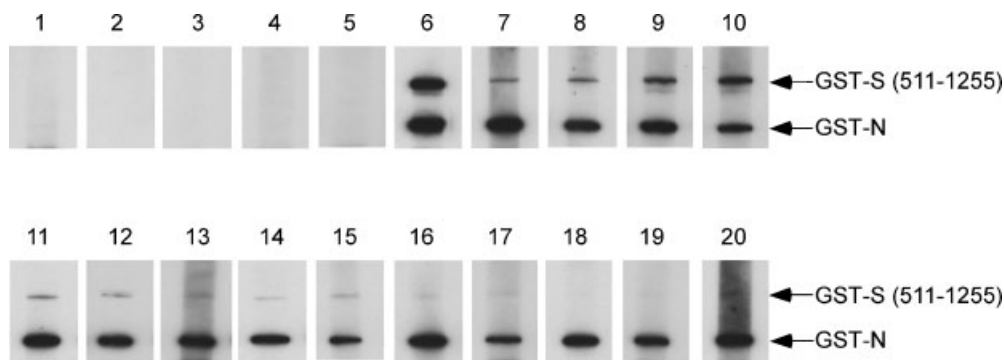


Fig. 3. Western blot analysis of SARS-CoV-positive and -negative human sera with the recombinant GST-N and GST-S (511–1255) proteins. The recombinant GST-N and GST-S (511–1255) proteins were mixed and separated by SDS–PAGE using 10% polyacrylamide gel and transferred to nitrocellulose membrane. Each lane of the

membrane was incubated with a 1:200 dilution of human sera followed by HRP-conjugated anti-human IgG (1:3,000 dilution) and detected by chemiluminescence. **Lanes 1–5** showed the reactivity with negative serum specimens. **Lanes 6–20** showed the reactivity with SARS-CoV-positive serum specimens.

checkerboard titration of antigen coating on the micro-well plates by using 1  $\mu\text{g}/\text{ml}$ , 2.5  $\mu\text{g}/\text{ml}$ , 5  $\mu\text{g}/\text{ml}$ , and 10  $\mu\text{g}/\text{ml}$  of antigen, respectively. The absorbance at 450 nm of the control sera increased when more coated antigen was used. Therefore, we used 1  $\mu\text{g}/\text{ml}$  antigen for coating plates. The mean  $A_{450}$  of normal control sera in this N-protein ELISA was quite low (0.06–0.07) at each dilution of sera ( $n = 5$ ) (Fig. 4A). The number of normal control sera has been expanded to 192 and the mean  $A_{450}$  ( $\pm$ standard deviation, SD) at a 1:20 dilution was 0.0456 ( $\pm 0.054$ ). The mean  $\pm 4$  SD (0.262) was set as the cutoff value and the false positive rate of the negative sera was 1.04% when using this cutoff value. ELISA performed on serially diluted positive sera produced typical ELISA curve and had an end point ( $A_{450}$  below 0.262) between 1:640 and 1:1280 dilutions (Fig. 4A). However, an obvious variation of titers of N protein-specific antibodies in different patients' sera was noted as reflected by the huge error bars. Since all positive and negative sera gave distinct results at a 1:20 dilution, we used this dilution to assay serial serum specimens collected from twelve PCR-confirmed SARS patients (Fig. 4B–D).

Serum samples from 10 of the 12 patients were positive for N protein-specific IgG (Fig. 4B,C). As shown in Figure 4B, The IgG seroconversion to N proteins shown by six patients was at mean of 16 days. There were four patients whose N protein-specific IgG was positive in their first serum specimens (patients 2, 5, 7, 9). This may be due to a later serum collection after onset of fever in the cases of patients 2, 5, 7 (Fig. 4C). For unknown reasons, the first serum specimen from patient 9 was positive for N protein-specific antibodies although it was collected at day 2 after onset of fever (Fig. 4B, patient 9). High titer of N protein-specific antibodies in SARS patients could last for 70 days after infection (Fig. 4C). N protein-specific IgG were undetectable in the serial serum specimens of patient 1 and patient 8 (Fig. 4D). This may be due to low titers of antibodies in their serum specimens. According to Figures 4B, the ascending  $A_{450}$  of serum samples from four patients implied that the

titers of N protein-specific IgG increased after the onset of SARS.

## DISCUSSION

Our data clearly demonstrated that both N and S proteins of the SARS-CoV are antigenic during the natural course of infection. Antibodies to the N protein were detected consistently among the sera from patients with SARS in the convalescent phase. Furthermore, the recombinant N protein expressed in *E. coli* was antigenically similar to the native one. Most importantly, the recombinant N protein-based ELISA was effective for detecting specific antibodies in sera from patients with SARS.

Most of the serial serum specimens showed seroconversion or increasing antibody titers to the N protein. These data were similar to those obtained from SARS-CoV-based ELISA or IFA [Ksiazek et al., 2003; Peiris et al., 2003a]. Until now, there are still no satisfactory diagnostic methods for SARS-CoV infection. Real-time polymerase chain reaction (RT-PCR), SARS-CoV-based ELISA, and IFA are three commonly used methods for detecting either viral RNA or virus specific antibodies [Ksiazek et al., 2003]. RT-PCR technique is a relatively sensitive assay comparing with the others and it could detect as low as 10 copies of SARS-CoV viral transcripts per reaction, corresponding to 830 RNA molecules per milliliter of specimen [Drosten et al., 2003]. However, the false positive rate may be higher than the other methods because of the cross-contamination in laboratories. A serological examination will provide another independent evidence of infection. Both SARS-CoV-based ELISA and IFA need the preparation of viruses as a source of antigen, which may reduce their accessibility for the general laboratories without biosafety containment facilities. The N protein-based ELISA can be performed in laboratories without containment facilities, due to the use of a single immunogenic viral protein instead of highly infectious SARS-CoV as coated antigen. In contrast to the IFA, which is not suitable for

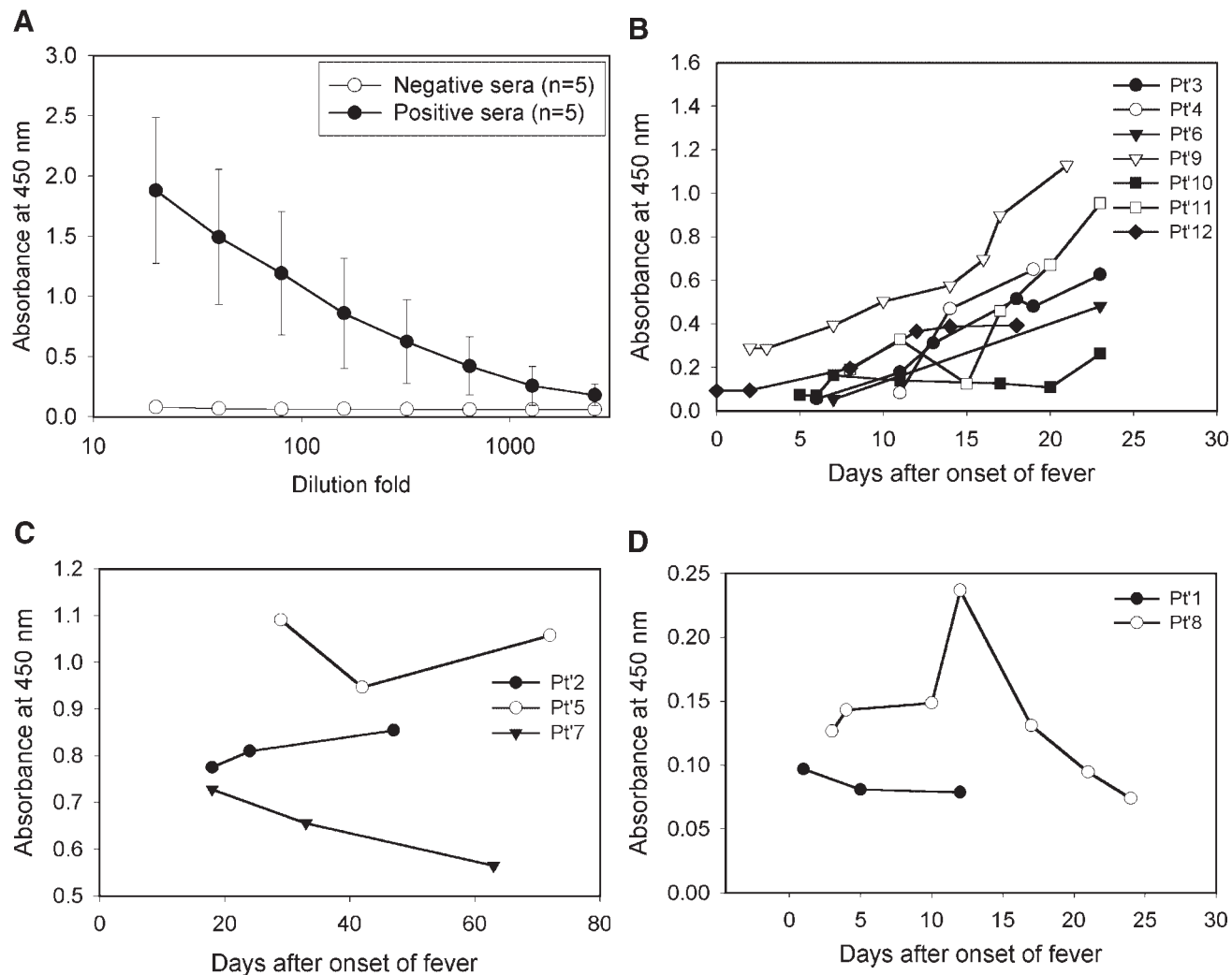


Fig. 4. N protein-based ELISA by anti-human IgG mAb HRP conjugate. **A:** Presence of N protein-specific IgG in sera of SARS patients detected by ELISA. Twofold serial dilutions of known positive serum specimens ( $n=5$ ) were tested in plates coated with the recombinant GST-N protein. Serum specimens from healthy blood donors ( $n=5$ ) were used as negative control. The results were expressed as mean  $A_{450}$  and standard error. **B:** Serum samples from seven PCR-confirmed SARS patients were assayed. First serum

specimens from the seven SARS patients were collected within 2 weeks after onset of fever. **C:** Serum samples from three PCR-confirmed SARS patients were assayed. These patients represented the group whose first serum specimens were collected after 14 days after onset of fever. **D:** Serum samples from two PCR-confirmed SARS patients were assayed. This group represented the non-responders who did not show seroconversion after SARS-CoV infection.

large-scale surveys, an ELISA based on a recombinant protein as coated antigen is easier to be produced and performed.

All serum samples from patient 1 and 8 were negative for N protein-specific antibodies (Fig. 4D). The same serum specimens of patient 1 have been assayed by IFA and the antibodies were also undetectable at a 1:25 dilution (data not shown). This may be due to that the antibody titer of patient 1 was not high enough to be detected by ELISA and IFA at day 12 after onset of fever. Patient 8 was immunocompromised and this may explain why the antibodies against the N protein remained below the cutoff value at day 24 after onset of fever.

According to our data, antibodies against the S protein could not be detected in the sera of some SARS-CoV-infected individuals although they have already pro-

duced anti-N protein antibodies. This may be due to the use of truncated spike protein that does not cover the major antigenic N-terminal to be recognized by antibodies generated in SARS-CoV-infected patients. It has been found in pigs upon TGEV infection that in order to achieve a protective immune response equivalent to that induced by full S protein, the intact globular N-terminal half of the protein was essential [Tuboly et al., 1995]. The globular N-terminal part of SARS-CoV S protein was almost not included in the GST-S (511–1255) construct. Therefore, it needs further studies to elucidate whether SARS-CoV globular N-terminal-spike-specific antibodies are generated in most of the SARS patients.

N proteins were proved to be the most abundant proteins found in purified SARS-CoV virions [Rota et al.,



2003]. A lot of examples showed that in other coronavirus infections, immune response against the N protein could be generated. The N protein of the MHV could elicit both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses which contributed to the protection against coronavirus-induced encephalomyelitis [Stohlman et al., 1992; Wege et al., 1993]. Chickens primed with the recombinant nucleocapsid protein of the IBV showed increased tracheal protection against challenge with live IBV [Boots et al., 1992]. Increased antibody titers against the nucleocapsid protein of human coronavirus could be detected in some paired sera of volunteers experimentally infected with human coronavirus 229E group viruses [Macnaughton et al., 1981]. The evidence implied that large amount of N protein may be produced in infected cells and the antigenicity of the N protein is very strong in vivo. Consistently, we found the N protein could elicit strong immune response (antibody production) at the early stage of SARS infection. Further studies are needed to understand whether the immune response against the N protein is associated with the pathogenesis of SARS-CoV.

Because SARS-CoV is a newly emerging virus and there is still no available reliable method for detection of N protein-specific antibodies other than ours, therefore it is hard to evaluate the sensitivity of this N protein-based ELISA. Since the false positive rate of the N-protein based ELISA was 1.04% among healthy blood donors, the specificity of this ELISA was about 99%. It has been reported that antibodies against human 229E and OC43-like coronaviruses are widespread in the human population [McIntosh et al., 1970; Bradburne and Somerset, 1972]. According to the observation that there is no serological reactivity against SARS-CoV among healthy blood donors and patients with respiratory or other diseases in Hong Kong [Peiris et al., 2003b], it suggests that there is no cross reactivity between the antibodies against previously known coronaviruses and SARS-CoV. It still needs further studies to elucidate whether the false positive cases were caused by cross-reactivity of the antibodies against pre-existing human coronaviruses and the recombinant N protein of SARS-CoV. We also suggest to use immunoblotting to confirm those false positive samples as in the cases of detecting the anti-HIV or anti-HTLV antibodies.

In conclusion, the N protein-based ELISA is a useful recombinant protein-based ELISA for detection of antibodies against SARS-CoV. It could serve as a simple, sensitive, and specific test for conducting the large-scale seroepidemiology studies to help to understand SARS-CoV epidemic.

#### ACKNOWLEDGMENTS

We would like to thank Dr. Lih-Hwa Hwang, Dr. Hui-Lin Wu, and Dr. Chun-Jen Liu for critically reading the article and constructive comments on this article; General Biologicals Corp. for providing all the reagents in ELISA.

#### REFERENCES

- Baric RS, Nelson GW, Fleming JO, Deans RJ, Keck JG, Casteel N, Stohlman SA. 1988. Interactions between coronavirus nucleocapsid protein and viral RNAs: Implications for viral transcription. *J Virol* 62:4280–4287.
- Bergmann CC, Yao Q, Lin M, Stohlman SA. 1996. The JHM strain of mouse hepatitis virus induces a spike protein-specific Db-restricted cytotoxic T cell response. *J Gen Virol* 77:315–325.
- Boots AM, Van Lierop MJ, Kusters JG, Van Kooten PJ, Van der Zeijst BA, Hensen EJ. 1991. MHC class II-restricted T-cell hybridomas recognizing the nucleocapsid protein of avian coronavirus IBV. *Immunology* 72:10–14.
- Boots AM, Benaissa-Trouw BJ, Hesselink W, Rijke E, Schrier C, Hensen EJ. 1992. Induction of anti-viral immune responses by immunization with recombinant-DNA encoded avian coronavirus nucleocapsid protein. *Vaccine* 10:119–124.
- Bradburne AF, Somerset BA. 1972. Coronaviric antibody titres in sera of healthy adults and experimentally infected volunteers. *J Hyg (Lond)* 70:235–244.
- Collins AR, Knobler RL, Powell H, Buchmeier MJ. 1982. Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell–cell fusion. *Virology* 119:358–371.
- Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RA, Berger A, Burguiere AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Muller S, Rickerts V, Sturmer M, Vieth S, Klenk HD, Osterhaus AD, Schmitz H, Doerr HW. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 348:1967–1976.
- Fleming JO, Stohlman SA, Harmon RC, Lai MM, Frelinger JA, Weiner LP. 1983. Antigenic relationships of murine coronaviruses: Analysis using monoclonal antibodies to JHM (MHV-4) virus. *Virology* 131:296–307.
- Gerber JD, Ingersoll JD, Gast AM, Christianson KK, Selzer NL, Landon RM, Pfeiffer NE, Sharpee RL, Beckenhauer WH. 1990. Protection against feline infectious peritonitis by intranasal inoculation of a temperature-sensitive FIPV vaccine. *Vaccine* 8: 536–542.
- Hsueh PR, Hsiao CH, Yeh SH, Wang WK, Chen PJ, Wang JT, Chang SC, Kao CL, Yang PC. 2003. Microbiologic characteristics, serologic responses, and clinical manifestations in severe acute respiratory syndrome, Taiwan. *Emerg Infect Dis* 9:1163–1167.
- Krokhin O, Li Y, Andonov A, Feldmann H, Flick R, Jones S, Stroehrer U, Bastien N, Dasuri KV, Cheng K, Simonsen JN, Perreault H, Wilkins J, Ens W, Plummer F, Standing KG. 2003. Mass spectrometric characterization of proteins from the SARS virus: A preliminary report. *Mol Cell Proteomics* 2:346–356.
- Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ, Anderson LJ. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* 348:1953–1966.
- Kuiken T, Fouchier RA, Schutten M, Rimmelzwaan GF, van Amerongen G, van Riel D, Laman JD, de Jong T, van Doornum G, Lim W, Ling AE, Chan PK, Tam JS, Zambon MC, Gopal R, Drosten C, van der Werf S, Escriou N, Manuguerra JC, Stohr K, Peiris JS, Osterhaus AD. 2003. Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* 362:263–270.
- Lai MMC, Holmes KV. 2001. Coronaviridae: The viruses and their replication. In: Knipe D, editor. *Fields Virology*. Philadelphia, Pennsylvania, USA: Lippincott Williams & Wilkins. pp 1163–1185.
- Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426:450–454.
- Liu C, Kokuho T, Kubota T, Watanabe S, Inumaru S, Yokomizo Y, Onodera T. 2001. DNA mediated immunization with encoding the nucleoprotein gene of porcine transmissible gastroenteritis virus. *Virus Res* 80:75–82.
- Macnaughton MR, Hasony HJ, Madge MH, Reed SE. 1981. Antibody to virus components in volunteers experimentally infected with human coronavirus 229E group viruses. *Infect Immun* 31:845–849.



- McIntosh K, Kapikian AZ, Turner HC, Hartley JW, Parrott RH, Ahanock RM. 1970. Seroepidemiologic studies of coronavirus infection in adults and children. *Am J Epidemiol* 91:585–592.
- Peiris JS, Chu CM, Cheng VC, Chan KS, Hung IF, Poon LL, Law KI, Tang BS, Hon TY, Chan CS, Chan KH, Ng JS, Zheng BJ, Ng WL, Lai RW, Guan Y, Yuen KY. 2003a. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: A prospective study. *Lancet* 361:1767–1772.
- Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, Nicholls J, Yee WK, Yan WW, Cheung MT, Cheng VC, Chan KH, Tsang DN, Yung RW, Ng TK, Yuen KY. 2003b. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 361:1319–1325.
- Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, Penaranda S, Bankamp B, Maher K, Chen MH, Tong S, Tamin A, Lowe L, Frace M, DeRisi JL, Chen Q, Wang D, Erdman DD, Peret TC, Burns C, Ksiazek TG, Rollin PE, Sanchez A, Liffick S, Holloway B, Limor J, McCaustland K, Olsen-Rasmussen M, Fouchier R, Gunther S, Osterhaus AD, Drosten C, Pallansch MA, Anderson LJ, Bellini WJ. 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 300:1394–1399.
- Sambrook J, Russell DW. 2001. Commonly used techniques in molecular cloning. *Molecular cloning*, 3rd edn. New York: Cold Spring Harbor Laboratory Press. pp A8.40–A48.54.
- Stohman SA, Kyuwa S, Cohen M, Bergmann C, Polo JM, Yeh J, Anthony R, Keck JG. 1992. Mouse hepatitis virus nucleocapsid protein-specific cytotoxic T lymphocytes are Ld restricted and specific for the carboxy terminus. *Virology* 189:217–224.
- Tuboly T, Nagy E, Derbyshire JB. 1995. Passive protection of piglets by recombinant baculovirus induced transmissible gastroenteritis virus specific antibodies. *Can J Vet Res* 59:70–72.
- Wege H, Schliephake A, Korner H, Flory E. 1993. An immunodominant CD4+ T cell site on the nucleocapsid protein of murine coronavirus contributes to protection against encephalomyelitis. *J Gen Virol* 74:1287–1294.
- WHO. 2003. Cumulative number of reported probable cases of severe acute respiratory syndrome. [www.who.int/csr/sars/country/2003\\_07\\_09/en/](http://www.who.int/csr/sars/country/2003_07_09/en/) accessed July 10, 2003.
- Yeh SH, Wang HY, Tsai CY, Kao CL, Yang JY, Liu HW, Su IJ, Tsai SF, Chen DS, Chen PJ, Lee YT, Teng CM, Yang PC, Ho HN, Chang MF, Wang JT, Chang SC, Wang WK, Hsiao CH, Hsueh PR. 2004. Characterization of severe acute respiratory syndrome coronavirus genomes in Taiwan: Molecular epidemiology and genome evolution. *Proc Natl Acad Sci USA* 101:2542–2547.