

## Characterization and application of monoclonal antibodies against N protein of SARS-coronavirus

Bo Shang<sup>a,1</sup>, Xiao-Yi Wang<sup>b,1</sup>, Jian-Wei Yuan<sup>a</sup>, Astrid Vabret<sup>c</sup>, Xiao-Dong Wu<sup>a</sup>, Rui-Fu Yang<sup>b</sup>, Lin Tian<sup>a</sup>, Yong-Yong Ji<sup>a</sup>, Vincent Deubel<sup>d</sup>, Bing Sun<sup>a,d,e,\*</sup>

<sup>a</sup> *Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, China*

<sup>b</sup> *State Key Laboratory of Pathogen and Biosecurity, Institute of Microbiology and Epidemiology, Institute of Basic Medical Sciences, Academy of Military Medical Sciences, Beijing 100071, China*

<sup>c</sup> *Laboratory of Human and Molecular Virology, UPRES EA2128, C.H.U. de Caen, Avenue Georges Clemenceau, 14000 Caen, France*

<sup>d</sup> *Institute Pasteur of Shanghai, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, 225 South Chongqing Road, Shanghai 200025, China*

<sup>e</sup> *E-institutes of Shanghai Universities Immunology Division, China*

Received 15 July 2005

Available online 15 August 2005

### Abstract

Severe acute respiratory syndrome-coronavirus (SARS-CoV) causes an infectious disease through respiratory route. Diagnosing the disease effectively and accurately at early stage is essential for preventing the disease transmission and performing antiviral treatment. In this study, we raised monoclonal antibodies (mAbs) against the nucleocapsid (N) protein of SARS-CoV and mapped epitopes by using different truncated N protein fragments. The mapping of those epitopes was valuable for constructing pair-Abs used in serological diagnosis. The results showed that all of the six raised mAbs were divided into two groups recognizing the region of amino acids 249–317 (A group) or 317–395 (B group). This region spanning amino acids 249–395 contains predominant B cell epitopes located at the C-terminus of N protein. One pair-Abs, consisting of N protein-specific rabbit polyclonal antibody and SARS-CoV N protein-specific mAb, was selected to construct a sandwich ELISA-kit. The kit was able to specifically detect SARS-CoV N proteins in serum samples.

© 2005 Elsevier Inc. All rights reserved.

**Keywords:** Severe acute respiratory syndrome; Nucleocapsid protein; Truncated N protein fragments; Monoclonal antibody; B cell epitope mapping; Human coronaviruses; Diagnosis

A new infectious disease, known as severe acute respiratory syndrome (SARS), broke out in Guangdong province of China in 2002. Based on a WHO report, there were 8437 persons being infected over the world and in which 813 patients died from the disease until July 11, 2003. The overall mortality rate of the disease was about 10.5%. A novel coronavirus was identified

to be the etiologic agent of SARS [1]. The severe acute respiratory syndrome-coronavirus (SARS-CoV) is positive stranded RNA virus of the order of 29,727 nucleotides with 14 open reading frames. The structural proteins of SARS-CoV consist of four proteins: the surface spike protein (S), the nucleocapsid protein (N), the small membrane protein (M), and the envelope protein (E) [2].

Since the infectious disease of SARS spread by the respiratory route and until now there are no effective antiviral drugs and vaccines which have been developed,

\* Corresponding author. Fax: +86 21 54921011.

E-mail address: [bsun@sibs.ac.cn](mailto:bsun@sibs.ac.cn) (B. Sun).

<sup>1</sup> These two authors contributed equally to this work.

it is very important to have a rapid and accurate diagnostic tool to detect SARS-CoV at early stages of the disease.

At present, most of the SARS diagnosis methods mainly focus on detecting viral antigen-specific antibodies in the sera of suspected patients. Generally, the antibody response often develops on 10–14 days following SARS-CoV infection [3], so that the diagnosis based on a viral-specific Ab would miss a good time point for antiviral therapy effectively and quarantine of SARS patients. Therefore, the best option for the disease diagnosis is to detect viral antigens.

N protein reacted with most of SARS patient sera and serum samples from acute phase of SARS patients (5–10 days after SARS-CoV infection). In contrast, the sera samples from acute phase of SARS patients did not respond to S protein, suggesting that antibodies to N protein developed earlier than S protein-specific antibodies. This observation reflected that N protein was released into the blood of SARS-CoV patients during virus replication *in vivo* [4,5]. In addition, coronavirus N protein is highly immunogenic and abundantly expressed *in vivo* after the virus infects human being.

A recent study demonstrated that N protein-specific mAbs were capable of detecting a N protein from sera of SARS patients at early stage on day 5–10 following SARS-CoV infection, indicating that N protein was a good viral antigen for the disease diagnosis [3]. It was noted that N protein was easy to be degraded into small fragments in the lysates of SARS-CoV-infected Vero E6 cells, analyzed by proteomics [6]. Moreover, one study showed that one N protein fragment, N13 (amino acid residues 221–422), can react with 100% of sera from SARS-CoV patients (52/52) [7]. This indicates that the N13 fragment is highly immunogenic and contains predominant B cell epitopes within N protein.

In this study, we generated six mAbs and one polyclonal Ab that were specific to N protein of SARS. Four truncated N protein fragments were used to locate the epitopes recognized by the mAbs within N protein. It was interesting to find that the epitopes recognized by six mAbs were divided into two groups and were located in the C terminus (221–422) of N protein. The selected pair-Abs were able to detect N protein in samples of SARS patients but not in sera of SARS unrelated patients.

## Materials and methods

**Animals.** New Zealand rabbits and BALB/c mice were purchased from Shanghai Laboratory Animal center, Chinese Academy of Sciences. Animals were kept in conventional conditions and were handled in compliance of Chinese Academy of Sciences Guidelines for Animal Care and Use.

**Expression and purification of SARS-CoV nucleocapsid protein.** The full length and different truncated fragments of SARS nucleocapsid

(N) gene were inserted into vector pETs [7]. After transformation of *Escherichia coli* strain, BL21 (DE3), bacterial cells were induced by 10 mM IPTG at 22 °C for 16 h in tryptone-phosphate medium. Proteins were extracted with buffer A containing 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 300 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 mM imidazole, and 0.5 mg/ml lysozyme. The high-speed supernatant of the extract from 1 liter of culture was loaded onto 8 ml Ni-NTA agarose column, followed by washing with 100 ml of 20 mM imidazole in buffer A. Proteins were then eluted with 50 mM imidazole in buffer A.

**Western blotting.** Recombinant N proteins and inactivated cell lysates from SARS-CoV-infected Vero-E6 cells, HCoV-OC43-infected HRT18 cells, HCoV-229E-infected MRC5H cells, and CoV-NL63-infected LLC-MK2 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels and were then transferred onto a membrane of polyvinylene difluoride (PVDF) as described previously [8]. After blocking with 3% BSA for 1 h, the membrane was incubated with anti-SARS N protein mAbs and stained with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Sigma) for 1 h. The blots were developed using ECL detection reagents (Amersham Pharmacia Biotech).

**Immunization and raising polyclonal antibodies.** Rabbits were immunized subcutaneously at multiple sites on the back of rabbits with 1 mg of *E. coli*-expressed N protein in emulsion 1:1 v/v with complete Freund's adjuvant. The rabbits were boosted three times at 2-week interval with N protein in emulsion with incomplete Freund's adjuvant. The blood was collected after 1 week of last immunization. The titers of the antisera from rabbits were determined by ELISA.

**ELISA assay.** Enzyme-linked immunosorbent assay (ELISA) was performed as described previously [8]. In brief, 96-well microtiter plates were coated with the SARS N protein in 0.1 M carbonate buffer (pH 9.6) (20 µg/ml, 50 µl/well) at 4 °C overnight. After being blocked with phosphate-buffered saline (PBS) containing 10% bovine serum and 0.1% Tween 20, the plates were incubated with diluted antisera at various concentrations at 37 °C for 2 h. Bound antibodies were detected with HRP-coupled goat anti-rabbit IgG antibody (Bio-Rad). Tetramethylbenzidine (TMB) was used as the substrate (Sigma, USA), and the absorbance was measured by microplate autoreader (Thermo) at 450 nm.

**HRP enzyme labeling purified polyclonal antibody.** Anti-SARS N protein antisera from rabbits (pAbs) were initially purified by 33% saturated ammonium sulfate precipitation and then the purified antibodies were labeled with HRP by 1.25% glutaraldehyde (GA). The reactivity was stopped by 0.2 M lysine for 2 h. The enzyme labeled complex was dialyzed with PBS at 4 °C overnight. At last, the HRP-coupled pAbs were precipitated by 33% saturated ammonium sulfate and then dialyzed.

**Preparation of monoclonal antibodies against SARS N protein.** Female BALB/c mice were immunized with 100 µg *E. coli*-expressed N protein in emulsion 1:1 v/v with complete Freund's adjuvant and were boosted three times at 2-week interval in incomplete Freund's adjuvant. The mouse with the highest Ab titer tested by ELISA was boosted intraperitoneally with 100 µg of the N protein in 0.5 ml PBS. On the third day after boosting, the mouse was sacrificed and the spleen cells were harvested. The spleen cells were fused with murine myeloma cells, SP2/0, by 50% PEG and distributed in 96-well culture plates. The cultured supernatants from each well were screened by detecting their reactivity to recombinant N proteins by ELISA. The positive hybridoma clones were selected by limiting dilution method [9]. After three cycles of cloning, the stable hybridoma clones were obtained. The antibody isotypes were identified by mouse sub-isotype panel (Bio-Rad).

In order to acquire abundant mAbs, the pristine-primed BALB/c mice were injected intraperitoneally with  $1 \times 10^6$  hybridoma cells per mouse. The ascites were collected and mAbs were purified by protein G affinity column.

**Measurement of antibody affinity constant.** The antibody affinity constant was determined by non-competitive ELISA as described previously [10]. The ELISA plates were coated with 0.625, 1.25, 2.5, or 5  $\mu\text{g/ml}$  of recombinant SARS N protein, respectively. After saturation with 10% bovine serum in PBS, wells were incubated with different dilutions of mAb. The following steps were performed as mentioned above and the optical absorbance at 450 nm was measured.

**Sandwich ELISA for detection of N protein.** The mAb S-39-2 was coated on microtiter plates at 4 °C overnight. After saturation with 10% bovine serum in PBS, the wells were incubated with human sera or with *E. coli*-expressed N protein. Later on, the HRP-coupled rabbit pAbs were added to detect the bound N protein as described [8].

**Indirect immunofluorescence assay for detecting cross-reactivity of mAbs and pAbs with different human coronaviruses.** In order to assess whether the N protein-specific Abs of SARS-CoV have a cross-reactivity with other coronaviruses, three Abs (N protein-specific S-39-2 and N-17-13 mAbs and rabbit pAbs) were selected and their cross-reactivities to N proteins of other three human coronaviruses were measured by indirect immunofluorescence assay (IFA) as well as Western blot analysis. HCoV-OC43 (ATCC, Rockville, MD, USA) was propagated on HRT18 cells, HCoV-229E (ATCC) on MRC5 cells, and HCoV-NL63 Amsterdam 1 strain (a gift from Dr. Lia van der Hoek, Amsterdam) on LLC-MK2 cells. HCoVs OC43, 229E, and NL63 coronaviruses were propagated on respective cell cultures from the frozen aliquot stock. After 2 days, before extensive cytopathic effect is observed, the cells were removed from the bottle and washed with PBS. The cells were resuspended in a small volume of PBS, deposited on fluoslides until dry, and fixed with acetone in  $-20\text{ }^{\circ}\text{C}$  for 10 min. During staining, 15  $\mu\text{l}$  of first antibody with different dilutions (1/20 and 1/50) was added on the slides and then incubated at 37 °C for 30 min. After washing with 10-fold diluted PBS for 5 min, the slides were treated with suitable diluted secondary antibody fluorescein-labeled anti-mouse or rabbit IgG conjugate (BD, Pharmingen) in 15  $\mu\text{l}$  per spot. The slides were sealed with a cover reagent and observed under a fluorescent microscope. The positive or negative scores were evaluated by two persons.

## Results

### Generated monoclonal antibodies recognizing N protein of SARS-CoV

After fusion and screening, six mAb clones reacting with N protein were selected by ELISA. These hybridoma clones were named as N-17-13, N-30-12, S-39-2, S-125-2, S-144-3, and S-162-2. The isotypes of mAbs secreted by the clones were determined by using mouse sub-isotype panel (Bio-Rad) and all of mAbs corresponded to IgG1k. The results showed that these antibodies highly bound to N protein (Table 1).

### Mapping epitopes of monoclonal antibodies

In order to map epitopes recognized by the raised mAbs, the *E. coli* expressing full-length N protein (1–422) and truncated N protein fragments, N1 (amino acid residues 72–208), N2 (221–422), N3 (249–395), and N4 (317–422), were selected (Fig. 1). The immunoreactivity between the mAbs and proteins was determined by ELISA. The data showed that the six mAbs could be divided into two groups based on their epitope location.

Table 1  
Characteristics of mAbs against N protein

Clone	Ab titer (OD <sub>450</sub> $\pm$ SD)	Ig subclass
N-17-3	<b>1.630 <math>\pm</math> 0.120</b>	IgG1, $\kappa$
N-30-12	1.358 $\pm$ 0.032	IgG1, $\kappa$
S-39-2	<b>1.786 <math>\pm</math> 0.031</b>	IgG1, $\kappa$
S-125-2	1.157 $\pm$ 0.072	IgG1, $\kappa$
S-144-3	1.321 $\pm$ 0.018	IgG1, $\kappa$
S-162-2	1.592 $\pm$ 0.028	IgG1, $\kappa$

Supernatants from N-17-3 and S-39-2 mAbs had higher titers than other clones and they were selected for further experiments.

The supernatants were collected from a 3 day-culture of  $1 \times 10^6$  hybridoma cells in 9 ml medium and were used directly to detect values of OD<sub>450</sub> and isotypes by ELISA.

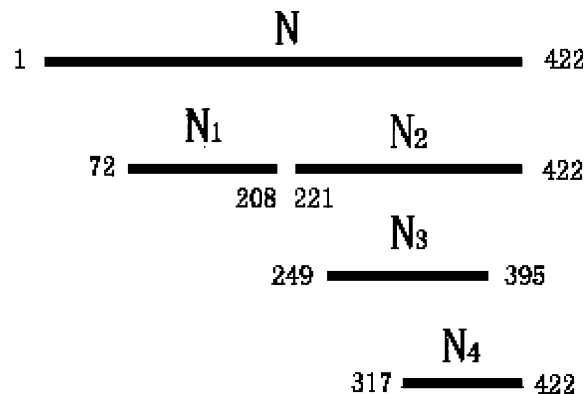


Fig. 1. Structure of truncated N protein fragments. The numbers at both ends of the line indicate the starting and ending amino acid residue position in the fragments.

A group containing N-17-13 and N-30-12 mAbs reacted with N3 fragment, but not N4 fragment, and thus the mAbs recognized the region of amino acids 249–317. B group including S-39-2, S-125-2, S-144-3, and S-162-2 mAbs reacted with N3 and N4 fragments, and recognized the region of amino acids 317–395 (Table 2 and Fig. 2). It was interesting to note that all of the six mAbs did not react with N1 fragment (72–208), but had a response to N2 fragment (221–422), indicating that the dominant epitopes of N protein were located in its C terminus (221–422).

### Two mAbs recognized immunogenic N2 fragment (221–422) with higher affinity

Based on proteomics analysis of the lysates of SARS-CoV-infected Vero E6 cells and clinical serological analysis of SARS patients, N protein is easy to be degraded into small fragments [6]. One study demonstrated that fragment N8 (amino acid residues 51–422), N11 (133–422), and N13 (221–422) reacted with most of all tested 52 SARS patient sera [11]. The percentages of positive responses were 100%, 96%, and 100%, respectively. The results indicate that the C-terminus of N protein is highly immunogenic and is a good target for

Table 2  
Epitopes recognized by six mAbs within N protein sequence by ELISA

	N(1–422)	N1(72–208)	N2(221–422)	N3(249–395)	N4(317–422)
S-39-2	+	–	+	+	+
S-125-2	+	–	+	+	+
S-144-3	+	–	+	+	+
S-162-2	+	–	+	+	+
N-17-3	+	–	+	+	–
N-30-12	+	–	+	+	–

+, positive reaction; –, negative reaction.

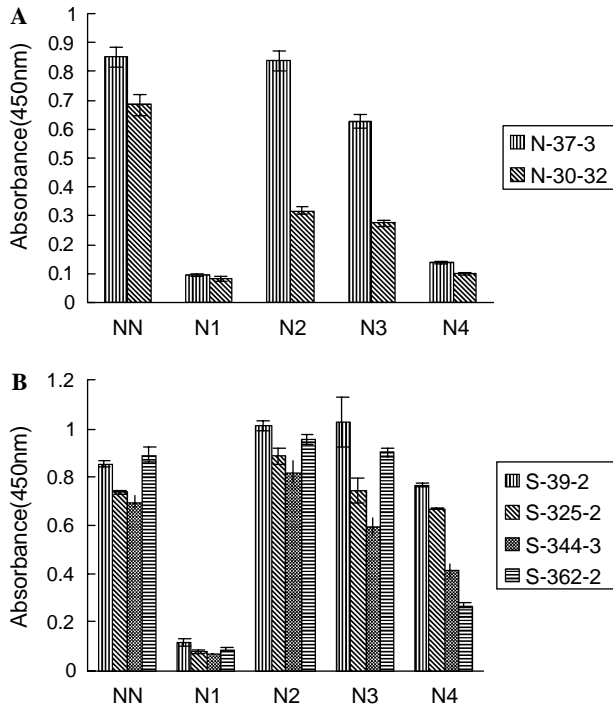


Fig. 2. Characterization of mAbs by ELISA with full-length N protein and truncated N protein fragments (N1–N4). N protein and N1–N4 fragments were coated on a 96-well ELISA plate. After blocking with 10% bovine serum, the plate was incubated with different monoclonal antibodies. Then bound antibodies were detected with HRP-conjugated goat anti-mouse IgG antibody and the OD values were measured at 450 nm. Data points represent means (error bars, standard deviations). (A) Reaction with two mAbs (A group). (B) Reaction with four mAbs (B group).

serological diagnosis. It is assumed that if a mAb can recognize N13 fragment that is similar to our N2 fragment, it will be valuable to be used in serological diagnosis. To achieve this purpose, two mAb clones (N-17-13 and S-39-2) showing the highest affinity to N protein were selected (Table 1). The mAbs recognized two different epitopes (amino acid residues 249–317 and 317–395, respectively) within N2 fragment (221–422) (Table 2).

The affinity for a given mAb is essential in ELISA-kit. To test the affinity of the two selected mAbs (S-39-2 and N-17-3), their affinity constants were measured by non-competitive ELISA as described in Materials

and methods. Their values were  $3.1 \times 10^9$  and  $7.2 \times 10^8$  L/mol, respectively (Fig. 3).

To further assess whether the mAbs recognized the native N protein of SARS-CoV, their reactivity against native N protein from lysates of SARS-CoV-infected Vero E6 cells was analyzed by Western blot. The data showed that the mAbs specifically recognized both of *E. coli*-expressed and native N proteins with apparent molecular weight of 48 and 47 kDa, respectively (Fig. 4). The difference in the molecular weight is due

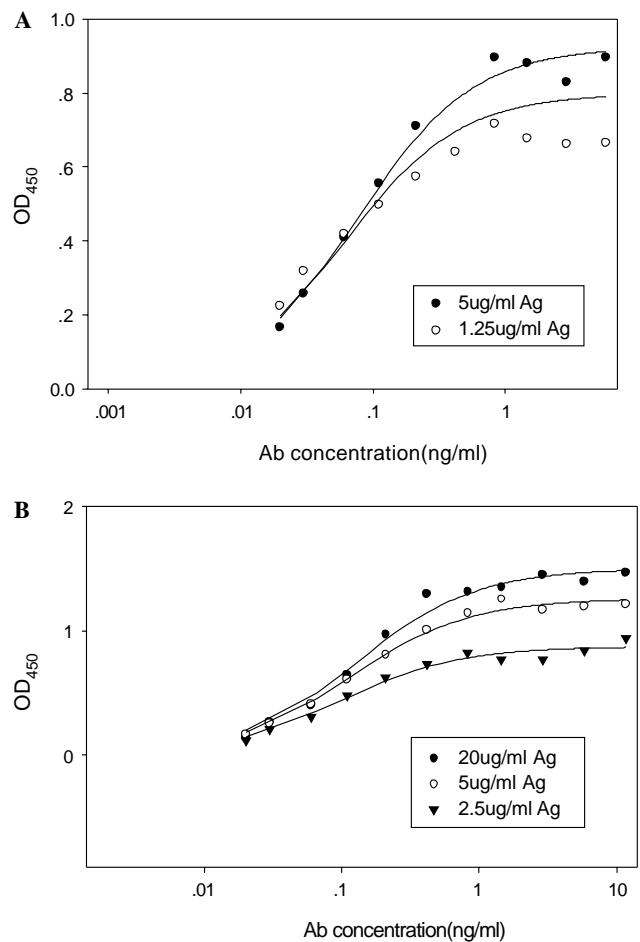


Fig. 3. Affinity of the selected mAbs. Affinity constant was measured as described in Materials and methods. (A) Affinity for S-39-2 mAb ( $3.1 \times 10^9$  L/mol). (B) Affinity for N-17-3 mAb ( $7.2 \times 10^8$  L/mol).

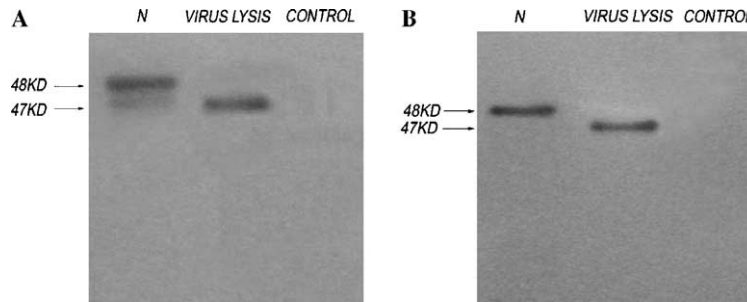


Fig. 4. Western blotting to detect N protein. (A) with N-17-3 monoclonal antibody; (B) with S-39-2 monoclonal antibody. The *E. coli*-expressed N protein, the lysates of SARS-CoV-infected Vero E6 cells and uninfected Vero E6 cells (control) were subjected to SDS-PAGE and then transferred to PVDF membrane. The blots were probed with different monoclonal antibodies and detected with HRP-conjugated goat anti-mouse IgG antibody.

to an additional six histidine tag at the C-terminus of the recombinant N protein.

#### Specificity of immunoreactivity of anti-SARS-CoV N protein antibodies

To confirm whether N-17-13, S-39-2 mAbs, and N protein pAbs had a cross-reactivity with other human coronaviruses (HCoV), the three HCoV-OC43, 229E, and NL63 classic human coronaviruses were selected and the N proteins from the virus-infected cells were detected by IFA and Western blot. Before the cell lysates were prepared, the corresponding Abs to each virus were used to check in infected host cells and make sure the cells were infected with the viruses (data not shown). The data showed that the Abs had no cross-re-

activity with the N proteins from other HCoV by IFA (Table 3). Similarly, a Western blot analysis revealed the same patterns, the Abs only recognized N protein of SARS-CoV, but did not react with the N proteins from other coronaviruses (Fig. 5).

#### Selected mAbs and N protein-specific pAbs were used as pair-Abs for the detection of N protein

To consider further a specific and sensitive pair-Abs to detect SARS-CoV N protein, N protein-specific pAbs and N-17-13 or S-39-2 mAbs were selected. Since N-17-13 and S-39-2 mAbs were capable of capturing the major immunogenic fragment N2 of N protein, both of them could be used as coating Ab. Our previous work demonstrated that purified IgG from antisera of

Table 3

Cross-reactivity of SARS N protein-specific Abs to other three coronaviruses N proteins (HCoV-OC43, 229E, and NL63) were detected by indirect immunofluorescence assay

Strains of coronaviruses	HCoV-OC43	HCoV-229E	HCoV-NL63	SARS-CoV
N protein of BCoV mAb	+++	–	–	NT
Positive human serum	+++	+++	+++	NT
Anti-DNP mAb	–	–	–	–
mAb (S-39-2)	–	–	–	+++
mAb (N-17-13)	–	–	–	+++
Rabbit pAbs	–	–	–	+++
Normal rabbit sera	–	–	–	–

To control the experimental system, Human serum from coronaviruses infected patients served as positive control, and anti-DNP mAb and rabbit normal sera served as negative control. NT, not tested.

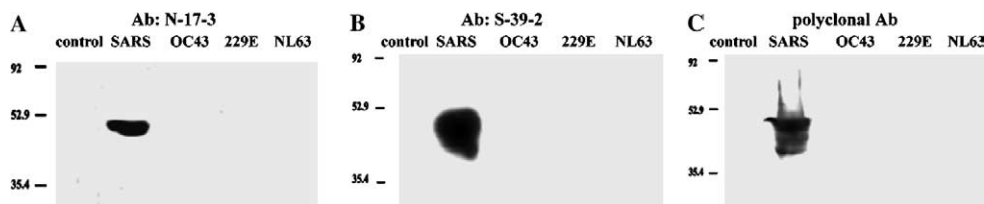


Fig. 5. Immunoreactivity of anti-N protein antibodies with four human coronaviruses. Reactivity to other three coronavirus N proteins (HCoV-OC43, 229E, and NL63) was detected by SARS-CoV N protein-specific Abs by Western blot. (A) Detected with N-17-3 mAb (1:1000). (B) Detected with S-39-2 mAb (1:1000) and (C) detected with N protein pAb (1:80,000). In each panel, five samples were tested. Control means normal Vero E6 cell lysates. SARS means SARS-CoV-infected Vero E6 cell lysates. Other three are from OC43, 229E, and NL63-infected host cell lysates.

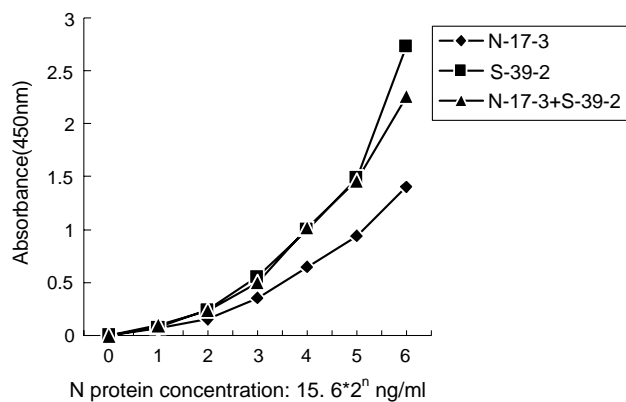


Fig. 6. Reactivity of pair-Abs with N protein. N-17-3 mAb or S-39-2 mAb or the mixture of N-17-3 mAb and S-39-2 mAb was selected as a coating Ab on the 96-well plate, and then different concentrations of the N protein were added into the plate. HRP-pAb (1:500) was used to detect the N protein.

recombinant N protein immunized rabbits detected the native N protein of SARS-CoV [12]. In this study, the rabbit pAbs were conjugated with HRP to be a revealing Ab (HRP-pAb). In the ELISA test, optimal conditions were obtained from primary experiments. The concentration of HRP-pAb (1:500) was selected. For determination of suitable coating mAb, either N-17-13 or S-39-2 mAb or the mixture of N-17-13 and S-39-2 (1:1) was used as coating mAb, but their detecting curves with N protein were similar (Fig. 6). Our results suggested that both of the selected mAbs were suitable to be the coating Ab and that the pair-Abs (mAb and HRP-pAb) had a potential in detecting N protein.

#### Specificity and sensitivity of the sandwich ELISA-kit to detect N protein

To further assess whether the pair-Abs was suitable for detecting soluble N protein in serum specimens, only S-39-2 mAb (with the affinity  $3.1 \times 10^9$  L/mol) and HRP-pAbs (pair-Abs) were selected to construct a sandwich ELISA-kit. The detection curve for N protein and the cross-reactivity to sera from different patients with irrelevant disease were tested by the kit. The results showed that the kit was able to detect N protein with a linear curve (from 1 to 1000 ng/ml) (Fig. 7). It was also observed that all of selected patient sera, including tumor, systemic lupus erythematosus (SLE), syphilis, hepatitis B virus (HBV), and hepatitis C virus (HCV) did not react with the kit (data not shown). To confirm whether the kit was suitable in detecting N protein in the sera, total 186 SARS-CoV patients were selected in Beijing area. All the patients were diagnosed to be SARS cases based on their clinical syndromes and tested positive for SARS-CoV S protein-specific IgG in the sera. In addition, the sera of 103 health individuals were collected in the same area to serve as negative control.

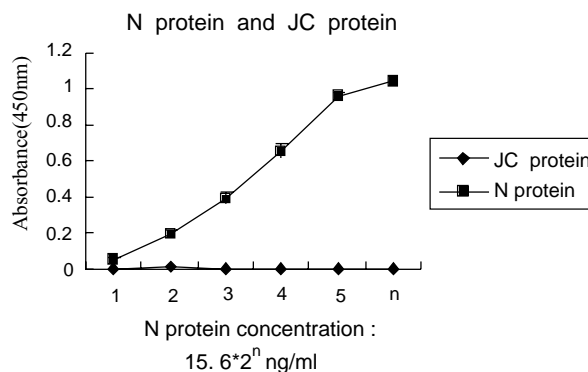


Fig. 7. Detection curve for N protein and JC protein by sandwich ELISA. Monoclonal antibody S-39-2 (5  $\mu$ g/ml) was coated on an ELISA plate. Serial dilutions of N protein and JC protein were detected by HRP-pAb (1:500).

Table 4

N protein detection in the sera of SARS-CoV patients by ELISA-kit

Days after onset of symptoms	No. of positive patients/ total tested patients (%)
SARS-CoV patients	
Day 5–10	3/4 (75)
Day 15–21	0/36 (0)
Day 21–90	0/146 (0)
Health individuals	0/103 (0)

Total 186 SARS-CoV patients with different stages of the disease and 103 health individuals were selected and N protein in the sera was tested by ELISA-kit.

The data showed that three out of four patients at early stage of SARS infection were positive in N protein detection, while the other patients including health individuals showed negative responses (Table 4). The results suggested that the developed kit has a potential in SARS-CoV diagnosis by N antigen capture.

#### Discussion

SARS-CoV has relatively high transmissibility and mortality. In order to block the disease transmission and perform antiviral therapy efficiently, a rapid and accurate diagnostic method to detect SARS-CoV at the early stage of the disease is needed. Three methods are currently used for SARS diagnosis. The first is a real-time PCR method that has a poor sensitivity due to false positive results. The second is to detect viral antigen-specific antibodies, but the Abs are detected only after 10–14 days following SARS-CoV infection. The third is to isolate the virus from cell culture, which needs more time and a biosafety P3 laboratory. Therefore, a sandwich ELISA to detect viral antigen can be helpful for the disease diagnosis.

We initially considered the spike protein as a good candidate for antigen capture. Unexpectedly, our data and those from other authors suggested that the S protein was expressed at very low levels in vivo and in cultured cells [6,12]. It was difficult to directly detect the soluble S protein from the patient's blood. The S protein is exposed on the surface of SARS-CoV and distributed mainly in infected tissues, such as the lungs [13,14]. Based on our observation, it was suggested that the S protein-specific Abs from patient sera had a potential in serological diagnosis, but at later stage of SARS infection [8]. In contrast, SARS N protein is important for the viral RNA transcription and provides nuclear-import signal during SARS-CoV replication [15,16]. In addition, N protein is located inside the SARS-CoV particle, but is abundantly released in patient's blood during the virus infection. Thus, its potential in clinical diagnosis has been investigated [3].

In this study, we generated specific mAbs against recombinant SARS-CoV N protein expressed in *E. coli* and analyzed the epitopes recognized by the mAbs within N protein. It was interesting to observe that all of the mAbs recognized a spanning region of C-terminus of N protein (amino acid residues 249–395), but did not react with the N-terminus. These data indicate that the C-terminus of N protein contains predominant B cell epitopes. Our observation is consistent with the other report that the N protein fragment, N13 (amino acid residues 221–422), can react with 100% of sera from SARS patients [11]. Our further experiments demonstrated that two mAbs (A group) recognized epitopes located at the region of amino acids 249–317, while other four mAbs (B group) reacted with the amino acids residues 317–395.

One report demonstrated that N protein was degraded into different fragments [6]. It is likely that a mAb recognizing the predominant N2 protein fragment (221–422) will be critical in detecting N protein in vivo. In this study, two mAbs (N-17-13 from A group and S-39-2 from B group) with high affinity to N2 fragment (221–422) were identified. The mAbs were confirmed to be capable of specifically binding to native N protein of SARS-CoV. In addition, the mAbs produced against N protein were reactive both in immunofluorescence and Western blot, suggesting that they recognized linear epitopes in N protein.

To evaluate whether anti-N mAbs were suitable for detecting N protein, a sandwich ELISA-kit was developed using the S-39-2 mAb and N protein-specific pAbs. The kit was calibrated using the *E. coli*-expressed recombinant N protein. In our initial tests, sera from 100 normal health individuals and 400 patients with different diseases, including tumor, SLE, syphilis, HBV, and HCV, showed negative responses with the kit. This kit needed to be validated for its capacity to detect N protein. Second, in all tested SARS patients, it is interesting

to note that the N protein was detectable with the kit only at early stage (5–10 days) after SARS-CoV infection. A possible explanation is that N protein of SARS-CoV could be released into the peripheral blood during the stage of virus replication and later on would be neutralized and eliminated by the patient's high titers of Abs raised against N protein. Our results suggested that the kit has a potential in the diagnosis of SARS infection.

In summary, six mAbs and one pAb against N protein of SARS-CoV were generated. All of six mAbs recognized the C-terminus of N protein (249–395) that contains predominant B cell epitopes and represents a good target of the viral antigen for serological diagnosis. The sandwich ELISA-kit, which was developed with mAb and pAb, has a potential in detecting specifically N protein of SARS-CoV.

### Acknowledgments

The authors thank Professor Francois Freymuth for his support and Dr. Lia von der Heck for providing NL63 coronavirus. This work was supported by the grant of Technology Commission of Shanghai Municipality (03DZ19113, 04DZ19108, and 04DZ14902), National Key Basic Research Program of China (2001CB510006), National Natural Science Foundations of China (30421005 and 30340032), SARS 973 Grant (2003CB514117), grant of Sino-Germany center on SARS project (GZ238(202/11)), the Outstanding Young Scientist Fund by The National Natural Science Foundation of China (30228016 and 30325018), and grant from E-institutes of Shanghai Universities Immunology Division.

### References

- [1] P.A. Rota, M.S. Oberste, S.S. Monroe, W.A. Nix, R. Campagnoli, J.P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M.H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J.L. DeRisi, Q. Chen, D. Wang, D.D. Erdman, T.C. Peret, C. Burns, T.G. Ksiazek, P.E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A.D. Osterhaus, C. Drosten, M.A. Pallansch, L.J. Anderson, W.J. Bellini, Characterization of a novel coronavirus associated with Severe Acute Respiratory Syndrome, *Science* 300 (5624) (2003) 1394–1399.
- [2] M.A. Marra, S.J. Jones, C.R. Astell, R.A. Holt, A. Brooks-Wilson, Y.S. Butterfield, J. Khattra, J.K. Asano, S.A. Barber, S.Y. Chan, A. Cloutier, S.M. Coughlin, D. Freeman, N. Girm, O.L. Griffith, S.R. Leach, M. Mayo, H. McDonald, S.B. Montgomery, P.K. Pandoh, A.S. Petrescu, A.G. Robertson, J.E. Schein, A. Siddiqui, D.E. Smailus, J.M. Stott, G.S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T.F. Booth, D. Bowness, M. Czub, M. Drebot, L. Fernando, R. Flick, M. Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G.A. Tipples,

- S. Tyler, R. Vogrig, D. Ward, B. Watson, R.C. Brunham, M. Kraiden, M. Petric, D.M. Skowronski, C. Upton, R.L. Roper, The Genome sequence of the SARS-associated coronavirus, *Science* 300 (5624) (2003) 1399–1404.
- [3] H.S. Wu, Y.C. Hsieh, I.J. Su, T.H. Lin, S.C. Chiu, Y.F. Hsu, J.H. Lin, M.C. Wang, J.Y. Chen, P.W. Hsiao, G.D. Chang, A.H. Wang, H.W. Ting, C.M. Chou, C.J. Huang, Early detection of antibodies against various structural proteins of the SARS-associated coronavirus in SARS patients, *J. Biomed. Sci.* 11 (1) (2004) 117–126.
- [4] Y.J. Tan, P.Y. Goh, B.C. Fielding, S. Shen, C.F. Chou, J.L. Fu, H.N. Leong, Y.S. Leo, E.E. Ooi, A.E. Ling, S.G. Lim, W. Hong, Profiles of antibody responses against severe acute respiratory syndrome coronavirus recombinant proteins and their potential use as diagnostic markers, *Clin. Diagn. Lab. Immunol.* 11 (2) (2004) 362–371.
- [5] X.Y. Che, Nucleocapsid protein as early diagnostic marker for SARS, *Emerg. Infect. Dis.* 10 (11) (2004) 1947–1949.
- [6] R. Zeng, H.Q. Ruan, X.S. Jiang, H. Zhou, L. Shi, L. Zhang, Q.H. Sheng, Q. Tu, Q.C. Xia, J.R. Wu, Proteomic analysis of SARS associated coronavirus using two-dimensional liquid chromatography mass spectrometry and one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by mass spectrometric analysis, *J. Proteome Res.* 3 (3) (2004) 549–555.
- [7] X.Y. Che, L.W. Qiu, Y.X. Pan, K. Wen, W. Hao, L.Y. Zhang, Y.D. Wang, Z.Y. Liao, X. Hua, V.C. Cheng, K.Y. Yuen, Sensitive and specific monoclonal antibody-based capture enzyme immunoassay for detection of nucleocapsid antigen in sera from patients with severe acute respiratory syndrome, *J. Clin. Microbiol.* 42 (6) (2004) 2629–2635.
- [8] Wei Lu, Xiao-Dong Wu, Mu De Shi, Rui Fu Yang, You Yu He, Chao Bian, Tie Liu Shi, Sheng Yang, Xue-Liang Zhu, Wei-Hong Jiang, Yi Xue Li, Lin-Chen Yan, Yong Yong Ji, Ying Lin, Guo-Mei Lin, Lin Tian, Jin Wang, Hong Xia Wang, You Hua Xie, Gang Pei, Jia Rui Wu, Bing Sun, Synthetic peptides derived from SARS coronavirus S protein with diagnostic and therapeutic potential, *FEBS Lett.* 579 (2005) 2130–2136.
- [9] Ying Lin, Zhiduo Liu, Jianmin Jiang, Ziqing Jiang, Yongyong Ji, Bing Sun, Expression of intracellular domain of epidermal growth factor receptor and generation of its monoclonal antibody, *Cell. Mol. Immunol.* 1 (2) (2004) 137–141.
- [10] J.D. Beatty, B.G. Beatty, W.G. Vlahos, Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay, *J. Immunol. Methods* 100 (1–2) (1987) 173–179.
- [11] Z. Chen, D. Pei, L. Jiang, Y. Song, J. Wang, H. Wang, D. Zhou, J. Zhai, Z. Du, B. Li, M. Qiu, Y. Han, Z. Guo, R. Yang, Antigenicity analysis of different regions of the severe acute respiratory syndrome coronavirus nucleocapsid protein, *Clin. Chem.* 50 (6) (2004) 988–995 (Epub 2004 Mar 30).
- [12] Xiao-Dong Wu, Bo Shang, Rui Fu Yang, Hao Yu, Zhi-Hai Ma, Xu Shen, Yong Yong Ji, Ying Lin, Ya-Di Wu, Guo-Mei Lin, Lin Tian, Xiao-Qing Gan, Sheng Yang, Wei-Hong Jiang, Er-Hei Dai, Xiao-Yi Wang, Hua liang Jiang, You Hua Xie, Xue-Liang Zhu, Gang Pei, Lin Li, Jia Rui Wu, Bing Sun, The spike protein of severe acute respiratory syndrome (SARS) is cleaved in virus infected vero-E6 cells, *Cell Res.* 14 (205) (2004) 400–406.
- [13] G. Simmons, J.D. Reeves, A.J. Rennekamp, S.M. Amberg, A.J. Piefer, P. Bates, Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry, *Proc. Natl. Acad. Sci. USA* 101 (2004) 4240–4245.
- [14] Y.L. Lau, J.M. Peiris, Pathogenesis of severe acute respiratory syndrome, *Curr. Opin. Immunol.* 17 (4) (2005) 404–410.
- [15] R. He, A. Leeson, A. Andonov, Y. Li, N. Bastien, J. Cao, C. Osiowy, F. Dobie, T. Cutts, M. Ballantine, X. Li, Activation of AP-1 signal transduction pathway by SARS coronavirus nucleocapsid protein, *Biochem. Biophys. Res. Commun.* 11 (2003) 870–876.
- [16] B. King, D.A. Brian, Bovine coronavirus structural proteins, *J. Virol.* 2 (1982) 700–707.