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Preparation and characterization of a novel monoclonal antibody specific to severe acute respiratory syndrome-coronavirus nucleocapsid protein

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Abstract

Severe acute respiratory syndrome-coronavirus nucleocapsid (SARS-CoV N) protein has been found to be important to the processes related to viral pathogenesis, such as virus replication, interference of the cell process and modulation of host immune response; detection of the antigen has been used for the early diagnosis of infection. We have used recombinant N protein expressed in insect cells to generate 17 mAbs directed against this protein. We selected five mAbs that could be used in various diagnostic assays, and all of these mAbs recognized linear epitopes. Three IgG_{2b} mAbs were recognized within the N-terminus of N protein, whereas the epitope of two IgG_1 mAbs localized within the C-terminus. These mAbs were found to have significant reactivity with both non-phosphorylated and phosphorylated N proteins, which resulted in high reactivity with native N protein in virus-infected cells; however, they did not show cross-reactivity with human coronavirus. Therefore, these results suggested that these mAbs would be useful in the development of various diagnostic kits and in future studies of SARS-CoV pathology.

Keywords: Severe acute respiratory syndrome; Coronavirus; Nucleocapsid protein; Monoclonal antibody; Diagnosis

1. Introduction

Severe acute respiratory syndrome (SARS), which is caused by the SARS coronavirus (SARS-CoV), is a newly emerging disease. SARS-CoV presented with high virulence and mortality, and affected 30 countries, with more than 8000 cases and over 750 deaths. Indeed, as the clinical symptoms of SARS are non-specific compared to those of other respiratory viruses, diagnosis relies largely on laboratory tests (Ksiazek et al., 2003). Thus, the development of diagnostic laboratory tests for specific and early detection of SARS-CoV infection is of great importance for both rapid treatment of patients and control of SARS outbreaks.

Thus far, virus isolation methods have generally been performed to determine the presence of infectious virus in samples; this process is relatively time-consuming and inefficient (Keyaerts et al., 2005; Yamashita et al., 2005). RT-PCR and real time-PCR for direct detection of SARS-CoV RNA is expensive and labor-intensive, relies on the availability of expertise, and may produce false-positive results due to contamination (Keightley et al., 2005; Huang et al., 2005). Serological methods, such as immunofluorescence assay and enzyme-linked immunosorbent assay (ELISA), are not adequate for the early diagnosis of SARS, because the median time to seroconversion in SARS patients is 17–20 days after the onset of symptoms (Wu et al., 2004). Therefore, the generation of monoclonal antibodies (mAbs) against SARS-CoV antigens may provide possible diagnostic tools for early diagnosis of SARS, because SARS-CoV can be specifically detected in the respiratory specimens, blood and stool much earlier than antibodies can be used for detection (Lau et al., 2004; Di et al., 2005).

The SARS-CoV nucleocapsid (SARS-N) protein is a phosphoprotein of 48 kDa, and performs multiple functions in viral pathogenesis, such as providing a nuclear-import signal, interfering in the cell process, participating in virus replication and packaging RNA (Egloff et al., 2004; Yan et al., 2004; Liao et al., 2005; Surjit et al., 2004, 2005). Thus, this protein may have important roles in the pathogenesis of SARS. Furthermore, this protein is the most abundantly expressed structural protein during infection and is highly detectable in SARS patients (Rota et

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al., 2003; Lau et al., 2004; Di et al., 2005). Therefore, this protein may serve as one of the immunodominant antigens in the early diagnosis of infection. Furthermore, some researchers have suggested that antibody against the N protein could modulate cytokine responses such as IL 11; non-neutralizing antibodies against N protein were found to protect mice against lethal infection (Nakanaga et al., 1986; Cheng et al., 2005). Therefore, the development of mAbs against SARS-N protein may be critical in the development of drugs to treat SARS-CoV infection, and for further study of the pathogenesis of SARS, as well as early diagnosis.

Here, we report the production of 17 mAbs against SARS-N and the properties of the mAbs, which were determined by isotyping, affinity assay, epitope mapping and reactivity with various isoforms of SARS-N protein. We also suggested the applicability of the mAbs in various analytical methods, such as IFA, immunoblot and antigen-capture ELISA, for diagnosis and functional study of SARS-CoV N protein.

2. Materials and methods

2.1. Cell lines and viruses

SP2/0 myeloma cells were kindly provided by Metabolab Inc. (Seoul, Republic of Korea), and MRC-5 cells (ATCC, CCL-171) were obtained from American Type Culture Collection. SARS-CoV stock was provided from the Center for Disease Control and Prevention, and this virus was maintained in biosafety level-3 (BSL-3) containment laboratories in the National Institute of Health, Korea Center for Disease Control and Prevention. The SARS-CoV titer was determined to be 1×10^4 50% tissue culture infectious doses/ml ($TCID_{50}$/ml). The virus culture supernatants were inactivated by heating at 56 °C for 60 min prior to use. Human coronavirus OC43 (ATCC, VR-1558; HCoV OC43), which was tested to determine cross-reactivity with SARS-N mAb, was prepared from MRC-5 cells. Replication of these viruses was confirmed by RT-PCR and immunofluorescence assay using mAb against nucleocapsid protein of human coronavirus OC43 (HCoV mAb). The HCoV OC43 titer was determined by hemagglutination assay (HA) using human red blood cells (RBCs).

2.2. Preparation of recombinant SARS-CoV N protein

The complete coding sequence for the N protein (Urbani strain, GenBank accession No. AY278741, 28,120–29,388 bp) was amplified from the SARS-CoV genomic RNA using PCR. The amplified product was digested with *Eco*RI and *Bam*HI, and then inserted into His-tagged pEntr_BHRNX vector (Neurogenex, Seoul, Republic of Korea) to create pEntr_NP7. Recombinant baculovirus was generated by co-transfection of Sf21 cells with pEntr_NP7 and linearized baculovirus DNA using the BaculoGoldTM system (BD Biosciences, San Jose, CA). Recombinant baculoviruses were harvested from Sf21 cell culture medium 48 h post-transfection and broken by three cycles of freezing–thawing. The 6× histidine-tagged recombinant N protein (BrSARS-N) was purified by metal-chelating affinity chromatography (Merck Bioscience, Darmstadt, Germany). The purified protein was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (Laemmli, 1970). To examine the reactivity of SARS-N mAbs against the phosphorylated protein and non-phosphorylated protein, rSARS-N protein expressed in *Escherichia coli* (ErSARS-N) was purchased as non-phosphorylated protein from Biovendor Laboratory Medicine, Inc. (Heidelberg, Germany).

2.3. Production and purification of SARS-N mAb

Balb/c mice (Samtako Inc., Republic of Korea; 9 weeks) were intraperitoneally injected with a mixture containing 50 μg purified BrSARS-N proteins in 100 μl phosphate buffered saline (PBS) and an equal volume of Freund's complete adjuvant. A boost injection with the same amount of antigen in Freund's incomplete adjuvant was administered at 2-week intervals. Hybridoma fusion was performed using a method similar to that originally described (Kohler and Milstein, 1975), with the following modifications. In brief, the splenocytes were harvested from immunized mice, mixed with SP2/0 cells at a 5:1 ratio, and fusion was carried out with 40% polyethylene glycol-1500 (Roche, Indianapolis, IN). The fused cells were collected by centrifugation at 800 × *g* for 5 min and the cell pellet was resuspended in DMEM (Invitrogen, Carlsbad, CA) containing 20% FBS and HAT supplement (Sigma–Aldrich Korea Co., Seoul, Republic of Korea). The cells were seeded in 96-well plates at 200 μl/well (2×10^5 cells/well) and cultured in a CO_2 incubator. Antibody produced in medium was measured by indirect enzyme-linked immunosorbent assay (indirect ELISA) as described below. A limiting dilution of hybridoma was carried out from putative positive individual wells, and the screening was repeated until hybridoma clones producing a strongly reactive SARS-N mAb were observed. Selected hybridoma clones were maintained in DMEM containing 10% FBS, 1× HT supplement (Sigma–Aldrich Korea) and exchanged with fresh media once every 3 or 4 days. The SARS-N mAb of a selected hybridoma was purified using the ImmunoPure (G) IgG purification kit (Pierce Biotechnology Inc., Rochford, IL) and isotyped with an ImmunoPure Monoclonal Antibody isotyping kit II (Pierce Biotechnology Inc.), used according to the instructions of the manufacturer.

2.4. Indirect ELISA

The indirect ELISA was carried out on a Maxisorp plate (Nalgen Nunc International, Rochester, NY), which had been coated with 1 μg of recombinant SARS-Ns diluted in 50 mM carbonate buffer (pH 9.6) and incubated overnight at room temperature. Non-specific protein binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. Plates were washed with PBS containing 0.05% Tween 20 (PBST). Hybridoma supernatants, SARS-N mAb and anti-SARS serum obtained from mice immunized with heat-inactivated SARS-CoV, or normal mouse serum as negative serum, were then added and incubated for 60 min at 37 °C. After washing with PBST,

a 1:1000 dilution of alkaline phosphatase (AP)-conjugated goat anti-mouse IgG+ IgA+ IgM antibody (Abcam) in PBST containing 1% BSA was added to all wells and incubated for 60 min at 37 °C. After a final wash, *p*-nitrophenyl phosphate disodium salt (pNPP, Pierce Biotechnology Inc.) solution was added, and the plates were further incubated for 30 min at room temperature. The color intensity was measured as the absorbance value at 405 nm (OD_{405}) in an EL340 ELISA reader (Bio-Tek Intstruments Inc., Winooski, VT).

2.5. Isotyping

Isotyping was performed using ImmunoPure Monoclonal Antibody isotyping kit II (Pierce Biotechnology Inc.), used according to the instructions of the manufacturer.

2.6. Immunoblot analysis

To examine whether SARS-N mAb recognizes the linear epitope of SARS-N protein, we performed an immunoblot assay in denaturing conditions of the SARS-N protein. The purified rSARS-N protein were resolved by 10% SDS-PAGE, and were then electrotransferred onto a PVDF membrane (Bio-Rad, Hercules, CA) and blocked with 1% BSA in PBST. Membrane was incubated with SARS-N mAbs (1 μg/ml) or anti-His tag mAb (1:1000 dilution, Abcam), as positive control, for 1 h, and non-specific adsorption was washed away by PBST. The bound antibodies were detected by horseradish peroxidase-conjugated goat anti-mouse IgG+ IgA+ IgM secondary antibody (1:2000 dilution, Abcam), followed by DAB substrate solution (Sigma–Aldrich Korea).

2.7. Peptide-based epitope mapping

A total of 19 linear peptides ranging in size from 11 to 26 amino acid residues were synthesized on the basis of the full-length nucleocapsid protein sequences of SARS-CoV Urbani strain, Genebank AAP13445 (COSMO Co., Seoul, Republic of Korea). The synthesized peptides were characterized by HPLC and mass spectrometry. To identify the epitopes in SARS-CoV N protein that are targeted by SARS-N mAbs, competition ELISA was performed as described previously (Chiang et al., 2005). The SARS-N mAbs (10 μg/ml) were incubated with the competitor peptide or BrSARS-N protein (10 μg/ml) for 1 h at 4 °C. The mAbs were then transferred into the wells of a Maxisorp plate that had been coated with 0.5 μg/well of SARS-N protein. The following steps were then accomplished as described in the indirect ELISA procedure.

2.8. Affinity analysis

Affinity analysis was performed by non-competitive ELISA as described previously (Huang et al., 2005), with the following modifications. In brief, Maxisorp plate was coated overnight with 1 μg/well of BrSARS-N, as described in the indirect ELISA procedure. The plates were blocked with 1% BSA, followed by incubation of SARS-N mAb with serial dilution (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.3 nM). After washing with PBST, AP-conjugated goat anti-mouse IgG+ IgA+ IgM antibody (Abcam) in PBST containing 1% BSA was added to all wells and incubated for 30 min at 37 °C. The following steps were accomplished in the same manner as that used for indirect ELISA, described above. The affinity constants were presented as the concentrations (nM) of mAb at 1.000 of OD_{405}.

2.9. Immunofluorescence assay

Reactivity of SARS-N mAbs with SARS-CoV infected cells was determined by immunofluorescence assay, performed according to the instructions of the manufacturer (Euroimmun, Germany). The SARS-N mAb (10 μg/ml), anti-SARS serum (1:100 dilution) and negative serum were added to each well, and the slides were incubated at 37 °C for 30 min. After washing three times with PBS, fluorescein isothiocyanate-conjugated goat anti-mouse IgG+ IgA+ IgM antibody (ICN biomedical, 1:100 dilution) was added, and the slides were then incubated at 37 °C for 30 min. The slides were then washed three times with PBS and one time with ultrapure water and observed by fluorescence microscopy.

2.10. Antigen-capture ELISA

To prepare detector mAbs, the purified mAbs were labeled with biotin using the EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce Biotechnology Inc.) according to the instructions of the manufacturer. The purified mAb for the antigen-capture was immobilized on the Hi-bind microplate (Costar, Coring Incorporated, NY) by incubating 5 μg/ml antibody in 50 mM carbonate buffer (pH 9.6) at 4 °C overnight. The wells were then washed twice with PBS, followed by blocking with 2% BSA in PBS, pH 7.4, for 4 h at room temperature. After removing the blocking reagent, the wells were dried and stored at 4 °C prior to use. Virus culture supernatant or recombinant N protein diluted in 1% BSA in PBST or virus culture medium was added to the wells (100 μl/well) and incubated for 2 h at 37 °C. After washing, the wells were incubated for 1 h at 37 °C with 100 μl per well of biotin-conjugated detector mAb (diluted 1/200 in PBST with 1% BSA or virus culture medium). After washing, the wells were incubated for 1 h at 37 °C with 100 μl per well of AP-conjugated goat anti-biotin antibody (diluted 1/500 in PBST with 1% BSA or virus culture medium, Abcam). After washing, 100 μl of pNPP was added to each well. The color reaction was stopped after 30 min with 100 μl of 1N NaOH to each well, and the plates were examined at 405 nm in an EL340 ELISA reader (Bio-Tek Instruments Inc.). The experiments involving the use of the inactivated SARS-CoV were performed in a BSL-2 laboratory.

2.11. Cross-reactivity analysis of mAb against human coronavirus

For the immunofluorescence assay to assess the cross-reactivity of mAbs with human coronavirus, spot slides were prepared by applying a suspension of cells infected with UVC-

irradiated human coronavirus OC43 or a suspension of uninfected MRC-5 cells to the wells of Teflon-coated slides. Slides were allowed to air-dry before they were fixed in methanol. Slides were stored at −70 °C until used for indirect fluorescence assay. The HCoV mAb (1:100 dilution, Chemicon International Inc., Temecula, CA) as positive control, normal mouse serum as negative control, and SARS-N mAbs (100 μg/ml) were added to the slides, and the slides were incubated at 37 °C for 30 min. The following steps were accomplished in the same manner as that described above for SARS-CoV. For antigen-capture ELISA, human coronavirus OC43 (256 HA unit) supernatant that had been cultured from MRC-5 cells was heat-inactivated for 30 min at 56 °C and serially diluted two-fold. The protein samples were then extracted by three cycles of freezing–thawing before centrifugation at 14,000 rpm for 10 min and dilution in PBS with 1% BSA. The preceding steps were performed in the same manner as that used for SARS-CoV, described above.

3. Results

3.1. Generation of SARS-N mAbs

Full-length SARS-N protein was produced in a recombinant baculovirus system and used in the immunization of mice. Two weeks after each antigen boost dose, immunized mice were screened for SARS-N specific antibody response by indirect ELISA. Splenocytes isolated from the mice were fused with SP2/0 myeloma cells, resulting in ∼1000 proliferating hybridomas. Subsequent screening of these hybridomas and single cell cloning yielded 17 positive clones that constitutively secreted mAbs that reacted to SARS-N protein by indirect ELISA (data not shown). Further isotyping of these mAbs were determined in order to facilitate future utilization. For the heavy chain subclasses, most of the mAbs were found to belong to the IgM subtype, two of the mAbs belonged to IgG_1, and three of the mAbs belonged to IgG_{2b}. The light chain of all of these mAbs was of the kappa isotype (Table 1). Five of the 17 mAbs determined to be of the IgG subclass were characterized by further analysis, because these mAbs may be suitable for use as diagnostic mAbs.

3.2. Mapping of the epitope recognized by SARS-N mAbs

Immunoblotting was performed with BrSARS-N protein in order to analyze the reactivity of SARS-N mAbs against SARS-N protein under denaturing conditions and to determine whether these mAbs recognize the conformational epitope of SARS-N protein. The anti-His tag antibody served as a positive control that was capable of recognizing the N-terminal 6× His tag of BrSARS-N protein. As demonstrated in Fig. 1, all SARS-N mAbs and anti-His tag antibody reacted with 48- and 46-kDa isoforms of SARS-N protein. Additionally, anti-His tag antibody and three IgG_{2b} mAbs (22-05-03, 07-19-11 and 07-19-21) bound with a protein of approximately 22-kDa, whereas two IgG_1 mAbs (21-10-06 and 21-10-11) recognized a fragment of about 25 kDa. The isoforms of 48, 46 and 22-kDa could be originally obtained by purification of BrSARS-N protein extracted from baculovirus-infected cells. These isoforms were also observed in immunoblot analysis of total proteins extracted from baculovirus-infected cells and SARS-CoV-infected cells (data not shown). Thus, these isoforms may be fragment cleaved by intracellular protease. The fragment of 25-kDa that was not observed in originally purified BrSARS-N protein could be obtained by freezing and thawing procedures of purified protein. Thus, this result indicated that the epitopes of all of the SARS-N mAbs are linear, and the epitopes of IgG_{2b} and IgG_1 mAbs are located in the N-terminus and C-terminus of SARS-N protein, respectively. To more precisely analyze the epitope of SARS-N protein recognized by these SARS-N mAbs, competitive ELISA was conducted using the 19 synthetic peptides covering the full-length SARS-N protein sequence. Among these synthetic peptides, the N135 peptide (ATEGALNTPKDHIGTR; at position 135-150 of SARS-N protein) and N17 peptide (TFGGPTDSTDNNQNGG; at position 17-32) could effectively compete in the binding of all of the IgG_{2b} mAbs with the SARS-N protein, and the N117 peptide (GPEASLPYGANKEGIV; at position 117-132) seems to have slightly weak activity, while the other peptides did not exhibit any effect in this assay (Fig. 2A). The N17 and N135 peptides contain no apparent common epitopes, which revealed that these mAbs are mixed antibodies. The N215

Table 1
Isotypes of the SARS-N mAbs generated in this study

SARS-N mAbs	Subclass
21-10-06, **21-10-11**	**IgG_1, κ**
22-05-03, **07-19-11**, **07-19-21**	**IgG_{2b}, κ**
21-03-05, 21-24-03, 21-22-16, 21-02-04, 21-26-01, 21-28-15, 21-02-08, 21-03-12, 21-22-03, 21-26-19, 21-28-10, 21-24-15	IgM, κ

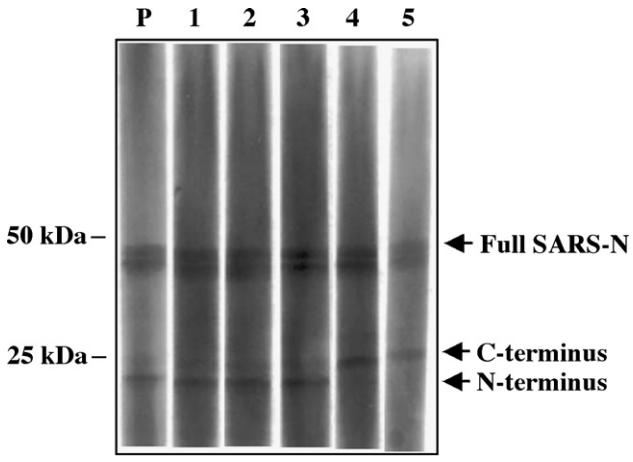

Fig. 1. Reactivity of SARS-N mAbs to denatured SARS-N protein, as determined by immunoblot analysis. The purified BrSARS-N protein was separated by 10% SDS-PAGE under denaturing conditions and electroblotted onto a PVDF membrane as described in Section 2. Lane P, anti-His tag antibody; lane 1, 22-05-03 mAb; lane 2, 07-19-11 mAb; lane 3, 07-19-21 mAb; lane 4, 21-10-06 mAb; lane 5, 21-10-11 mAb. The different fragments of SARS-N were detected with diluted SARS-N mAbs. All five mAbs were reacted with full-length N protein. Three mAbs were reacted with the N-terminus, whereas two mAbs were reacted with the C-terminus.

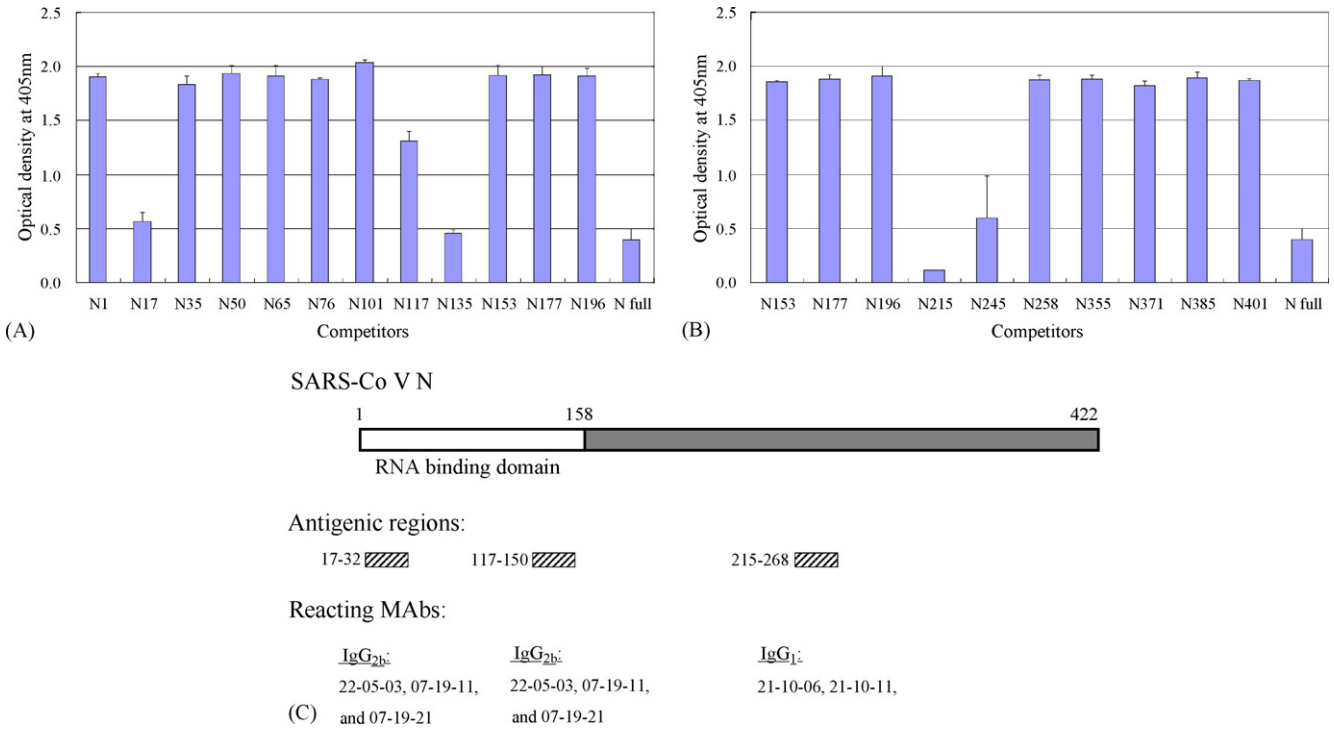

Fig. 2. Epitope mapping of SARS-N mAbs by competition ELISA. The competition ELISA measured the binding of each mAb to SARS-CoV N protein in the presence of peptide competitors as described in Section 2. The IgG_{2b} subclass mAbs recognized two epitopes within the RNA-binding domain; these epitopes were strongly bound in the regions of aa 17–32 and aa 117–150, respectively (A). The IgG_1 subclass mAbs recognized an epitope within aa 215–268 of SARS-CoV N protein (B). The epitope-mapped position of each mAbs is indicated on SARS-N protein (C).

peptide (GGETALALLLLDRLNQLESKVSGKG; at position 215-239) resulted in complete inhibition of the binding activity of all of the IgG_1 mAbs with the SARS-N protein, and the N245 peptide (QTVTKKSAAEASKKPRQKRTATKQ; at position 245-268) seems to have slightly weak activity (Fig. 2B). According to the results of the immunoblot and competitive ELISA, the epitopes of the IgG_{2b} mAbs is located in the N-terminal region at aa 17–32 and 117–150, whereas that of the IgG_1 mAbs is located in the middle region at aa 215–268 (Fig. 2C).

3.3. Reactivity of SARS-N mAbs against SARS-N protein

To examine the reactivity of the SARS-N mAbs against SARS-CoV N protein under non-denaturing conditions, the indirect ELISA was performed with BrSARS-N protein. Four of the selected mAbs (22-05-03, 07-19-11, 07-19-21 and 21-10-06) bound slightly better to SARS-N protein than 21-10-11, as shown in Table 2. To confirm the reactivity of mAbs against SARS-N protein, as estimated by indirect ELISA, the affinity constants of five mAbs were measured by non-competitive ELISA, as described in Section 2. The affinity constants of 22-05-03, 07-19-11, 07-19-21 and 21-10-06 were significantly higher than that of 21-10-11 (Table 2). These results were similar to those of indirect ELISA and indicated that the affinity levels paralleled the reactivity estimated by indirect ELISA, and all SARS-N mAbs, except for 21-10-11 mAb, could be suitable for the development of sensitive methods for SARS-CoV diagnosis.

Since these mAbs were generated using recombinant SARS-N protein expressed in insect cells that contained phosphorylated N protein (data not shown), indirect ELISA was performed to further examine the reactivity of SARS-N mAbs with the ErSARS-N expressed in *E. coli* as non-phosphorylated protein. Although SARS-N mAbs showed slightly higher reactivity with the BrSARS-N protein than with the ErSARS-N protein, all of these mAbs showed significant reactivity with the ErSARS-N protein compared with the reaction of negative serum (Fig. 3). This result revealed that all SARS-N mAbs were effectively bound with both phosphorylated and non-phosphorylated N protein.

Table 2
Affinity constants and ELISA reactivities of the SARS-N mAbs to SARS-CoV N protein

Subclass	Hybridoma clone	Reactivity[a]	Affinity constants[b]
IgG_1	21-10-06	1.950 ± 0.117	4.55
	21-10-11	1.369 ± 0.130	14.54
IgG_{2b}	22-05-03	2.208 ± 0.095	1.19
	07-19-11	2.128 ± 0.200	1.45
	07-19-21	2.310 ± 0.177	2.23

[a] The mAb reactivity as determined by indirect ELISA are presented as the mean ± standard deviation of OD_{405} values obtained from independent test three times.

[b] Affinity constants as determined by non-competitive ELISA are presented as the concentration (nM) of mAbs at 1.000 of OD_{405} value.

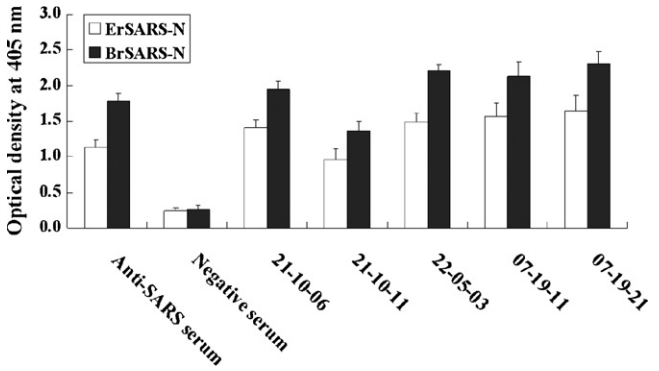

Fig. 3. Reactivity of SARS-N mAbs with non-phosphorylated N protein. The reactivity of SARS-N mAbs with BrSARS-N and ErSARS-N was examined by indirect ELISA. BrSARS-N expressed in insect cells and ErSARS-N obtained in *E. coli* served as phosphorylated and non-phosphorylated N protein, respectively. All of the SARS-N mAbs reacted significantly with non-phosphorylated N protein, as well as phosphorylated N protein.

3.4. Detection of native N protein in SARS-CoV infected cells by immunofluorescence assay

Immunofluorescence assay was performed on SARS-CoV infected Vero cells to further assess whether the SARS-N mAbs recognize the native-form of endogenously synthesized N protein in SARS-CoV infected cells. Both the negative serum and the five mAbs did not show non-specific reactions with uninfected cells. All five SARS-N mAbs strongly reacted with SARS-CoV infected cells, whereas negative serum showed no reaction (Fig. 4). However, 21-10-11 mAb showed a significantly weak reaction in affinity constants, but reacted strongly in the immunofluorescence assay; the reason for this result is unclear. The fluorescence signals of the mAbs were predominantly shown in the cytoplasm of SARS-CoV infected cells. This indicated that all mAbs were able to detect native-form N protein in SARS-CoV infected cells.

3.5. Antigen-capture ELISA for quantification of SARS-N protein

In order to establish a sensitive and less time-consuming antigen-capture ELISA for the SARS-N protein, we tested each of the pairs of mAbs from the five selected mAbs; this allowed us to determine the highest detection sensitivity for recombinant N protein and SARS-CoV culture supernatant. We found that the immobilization of a mixture of 21-10-06 and 07-19-11 mAb as capture antibody on the ELISA plate, followed by the detection with biotin-conjugated 22-05-03 mAb, gave the best result (data not shown). To determine the sensitivity of antigen-capture ELISA, a serial dilution of the recombinant N protein was used to determine a standard curve (Fig. 5). Normal vero cell culture media were used to determine the baseline for antigen-capture ELISA at an optical density of 0.230 at 405 nm (OD_{405}). Therefore, the cut-off value for detection of viruses in cell culture was set to be 0.300, which is equal to the mean + 3S.D. of the OD_{405} for normal cell culture media. According to the cut-off threshold (0.300), a 10^{-3} dilution of recombinant N protein and a 10^{-2} dilution of the virus culture supernatant were considered positive (Fig. 5). Thus, it was deduced that as little as 100 pg of recombinant N protein and 10 $TCID_{50}$ of virus culture supernatant could be detected. This result revealed that the SARS-N mAbs could be useful for detecting the N protein in virus culture supernatant and respiratory specimens from SARS patients.

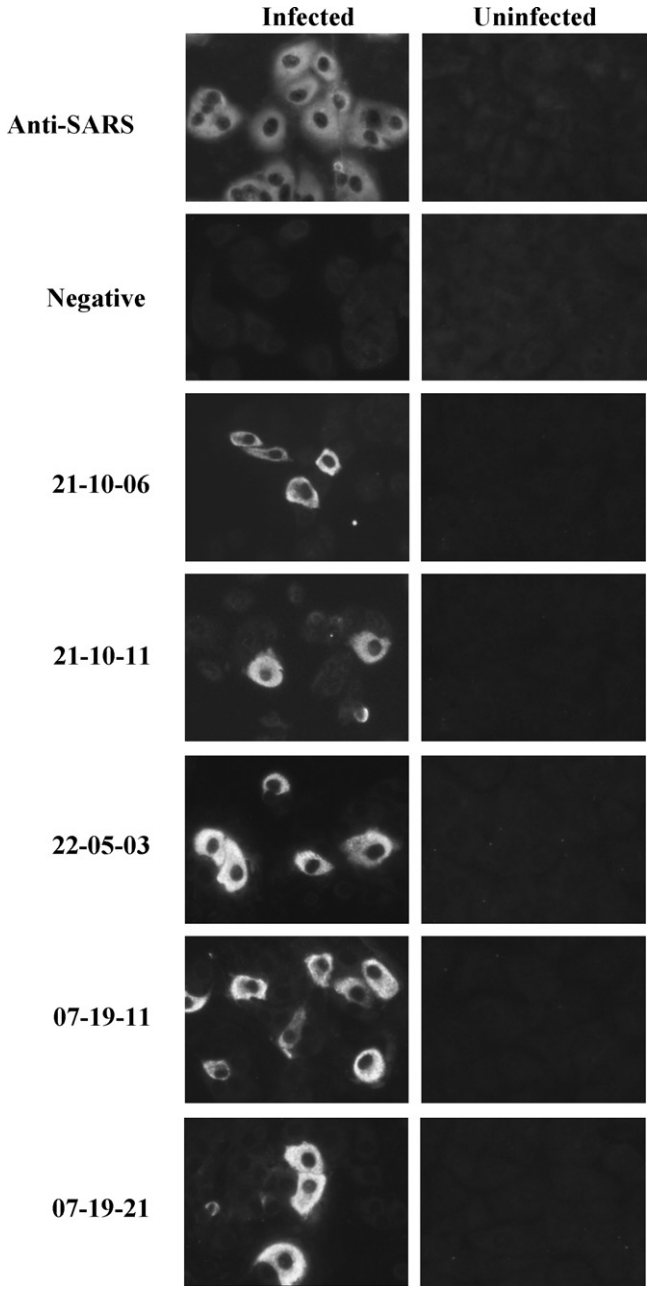

Fig. 4. Detection of SARS-N protein in SARS-CoV-infected cells by immunofluorescence assay. The immunofluorescence assay was performed using the SARS IFA kit according to the instructions of the manufacturer. Anti-SARS indicated positive serum obtained from mice immunized with heat-inactivated SARS-CoV and negative indicated normal mouse serum. These mAbs reacted with SARS-N in virus-infected Vero cells, whereas they did not react with uninfected cells.

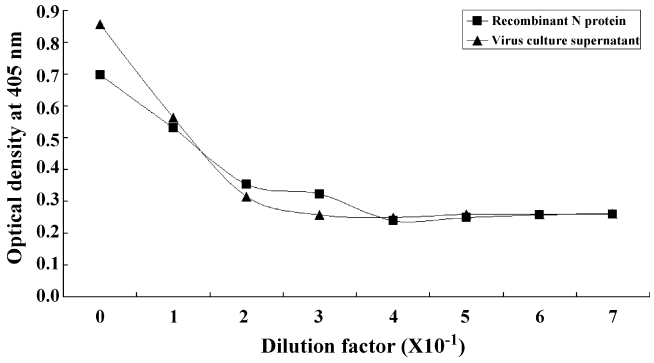

Fig. 5. Sensitivity of antigen-capture ELISA using SARS-N mAbs. Quantitative analysis was performed by using a mixture of 21-10-06 and 07-19-11 mAbs as capture antibody, biotin-conjugated 22-05-03 mAbs as detector antibody and serially diluted BrSARS-N protein as standard protein (■) and virus culture supernatant (▲).

3.6. The specificity of SARS-N mAbs

To determine the specificity of the five SARS-N mAbs, immunofluorescence assays were performed in human coronavirus-infected MRC-5 cells. All five mAbs showed no cross-reactivity with the antigens of the human coronavirus (Fig. 6A); uninfected MRC-5 cells also showed no cross-reactivity (data not shown). To further assess the specificity of the mAbs, antigen-capture ELISA was performed with human coronavirus-infected cell lysates and BrSARS-N protein as positive control (Fig. 6B). All of the mAbs reacted only with SARS-N protein, and did not react with human coronavirus, as shown by the results of immunofluorescence assays. This revealed that all of the SARS-N mAbs could specifically recognize the N protein of SARS-CoV.

4. Discussion

SARS-CoV is an etiological agent that causes severe acute respiratory syndrome, an infectious disease that has only recently emerged (Ksiazek et al., 2003). Therefore, there is an intense need for the development of sensitive and specific detection methods for SARS-CoV infection. Many methods have been employed recently for the detection of SARS-CoV infection (Keyaerts et al., 2005; Yamashita et al., 2005; Keightley et al., 2005; Huang et al., 2005). Of these diagnostic methods, RT-PCR and real time-PCR have been the most widely used. However, these methods possess some general problems, as they are time-consuming and labor-intensive, require sophisticated instruments, and have high rates of false positivity. On

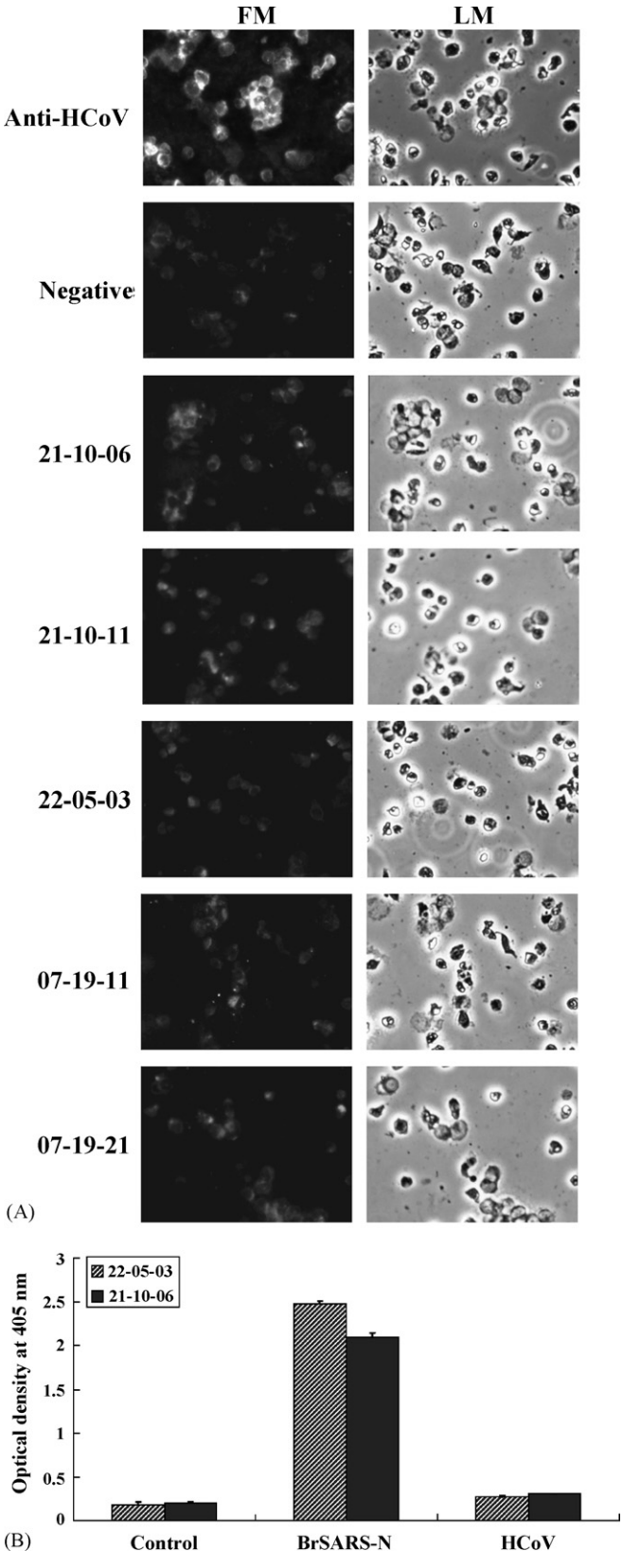

Fig. 6. Cross-reactivity of SARS-N mAbs with human coronavirus antigens. (A) The immunofluorescence assay was performed by using SARS-N mAbs on human coronavirus OC43-infected MRC-5 cells. As a positive control, anti-HCoV was used as the mAb against human coronavirus OC43 N protein, and normal mouse serum was used as the negative control. FM and LM indicate fluorescence and light imagery. None of these mAbs showed cross-reactivity with human coronavirus. (B) Cross-reactivity of SARS-N mAbs was examined by antigen-capture ELISA using human coronavirus OC43 lysates (256 HA unit), BrSARS-N protein (500 ng/well) and PBST buffer with 1% BSA as control.

Table 3
Properties of SARS-N mAbs by different assays

Assay	SARS-N mAb[a]				
	21-10-06	21-10-11	22-05-03	07-19-11	07-19-21
Indirect ELISA	++	+	++	++	++
Immunoblot	++	++	++	++	++
Sandwich ELISA	+	+	++	++	++
Immunofluorescence	+	+	++	++	++

[a] ++, Strong positive; +, positive.

the other hand, laboratory methods detecting viral antigen by mAbs, including antigen-capture ELISA, provide more rapid, less labor-intensive, and more convenient alternatives (Lau et al., 2004; Di et al., 2005). In this study, we generated five positive clones secreting specific and highly reactive antibodies against SARS-CoV N protein in order to develop diagnostic methods. These mAbs were available for use in detecting SARS-CoV N protein by various diagnostic methods, such as immunoblot assay, immunofluorescence assay and antigen-capture ELISA (Table 3). We also revealed the availability of these mAbs in the quantification of SARS-N protein by antigen-capture ELISA. The detection limit of this test is 100 pg of recombinant protein and 10 $TCID_{50}$ of SARS-CoV. This sensitivity is consistent with previous studies of sensitivity in other antigen-capture ELISAs (Che et al., 2004; Di et al., 2005). Therefore, these five mAbs may be employed in the construction of various diagnostic methods for the detection of SARS-CoV and in quantitative analysis of viral antigen and virus titer.

The major antigens of SARS-CoV structure proteins are the spike (S) protein and N protein (Lau et al., 2004; Di et al., 2005; Lu et al., 2004, 2005). However, recent reports have demonstrated that, because the S protein is expressed at very low levels *in vivo* and in cultured cells (Zeng et al., 2004; Wu et al., 2004), it is difficult to directly detect the soluble S protein from SARS patients. Thus, the S protein may not be suitable for use as a practical diagnostic antigen. In contrast, the N protein can be detected at significant levels in patient serum, as well as in respiratory tract samples at early stages of SARS-CoV infection (Lau et al., 2004; Di et al., 2005). These previous reports support that the development of mAbs against SARS-N protein is an adequate approach for the diagnosis of SARS. Therefore, we generated mAbs directed against SARS-CoV N protein and demonstrated that these SARS-N mAbs can successfully detect the N protein in SARS-CoV-infected cells; this is very useful in diagnosing SARS patients.

SARS N proteins exist as phosphorylated forms in mature viral particles, whereas, in host cells, this protein exists in both the dephosphorylated form and the phosphorylated form (Kalicharran and Dales, 1995; Surjit et al., 2005). Therefore, the mAbs available for developing sensitive diagnostic methods have to recognize the non-phosphorylated protein as well as phosphorylated protein. A previous report suggested that, because of conformational differences between proteins, the mAbs recognizing a protein expressed in insect cells cannot recognize the protein of same cDNA constructs expressed in *E. coli* (Vapalahti et al., 1996). Therefore, the SARS-N mAbs obtained in the present study were generated using recombinant SARS-N protein expressed in insect cells; these mAbs may not recognize the N protein expressed in *E. coli*. However, all of the SARS-N mAbs reacted significantly with the ErSARS-N expressed in *E. coli*, as well as the phosphorylated form of the BrSARS-N protein. These results demonstrate that these mAbs can effectively detect the non-phosphorylated N protein that exists in host cells during viral replication, as well as the phosphorylated N protein in host cells and viral particles. All of these mAbs could successfully detect native-form N protein in infected cells, as well as in viral particles. Thus, these mAbs may be useful in the development of sensitive methods used for the diagnosis of SARS.

Epitope mapping studies of the SARS-N mAbs demonstrated that one of the three epitopes that were originally reported to be located in the highly immunodominant region (Chen et al., 2004; He et al., 2004) is located at the middle region of SARS-N protein (aa 215–239; IgG_1 subclass mAbs). The others were newly identified at the N-terminus, which is shared with an RNA binding domain (aa 17–32 and 135–150; IgG_{2b} subclass mAbs) that is the minor B cell epitope (He et al., 2004). Furthermore, all of these SARS-N mAbs were reactive in immunoblotting, which suggests that they recognized linear epitopes in the N protein. A recent report demonstrates that the N protein is easily degraded into various isoforms in the lysates of SARS-CoV-infected cells (Zeng et al., 2004). We can also suggest, as previously described, that various isoforms existed in SARS-N protein expressed in insect cells and could be detected by immunoblot assay using these mAbs. Therefore, the blend of two mAbs against the different epitopes can be used to detect various fragments from SARS-N protein and enhance the sensitivity of diagnostic tools.

Although the SARS-N protein shares low homology (approximately 20–30%) with N proteins of other HCoVs, a previous report has described that the SARS-N protein has strong cross-reactivity with sera against HCoVs (Sun and Meng, 2004). Hence, anti-sera against SARS-CoV may be cross-reactive with other HCoVs. However, previous reports support the idea that the SARS-N mAbs did not recognize the N proteins of other HCoVs (Che et al., 2004). Therefore, the issues of cross-reactivity during the detection of SARS-N protein with polyclonal anti-sera can potentially be overcome by the use of a specific mAb against SARS-CoV. In the present study, the SARS-N mAbs did not show cross-reactivity with N proteins of HCoV, as determined by immunofluorescence assay and antigen-capture ELISA. Therefore, all of these SARS-N mAbs will be useful as mAbs for the development of specific diagnostic methods to discriminate SARS-CoV infection from HCoVs infection.

SARS-N protein is currently assumed to play an important role in the pathogenesis of SARS, as well as in viral transcription and replication. For example, the N protein can modulate numerous intracellular mechanisms involved in apoptotic signal transduction pathways, cell cycle regulatory pathways and cellular immune response and inflammation (Egloff et al., 2004; Yan et al., 2004, 2006; Liao et al., 2005; Surjit et al., 2004, 2006, 2005; Chang et al., 2006). Furthermore, SARS-N protein is released as a soluble antigen in infected cells, and as high levels of N protein circulating in the blood vessels (Che et

al., 2004; Di et al., 2005). Thus, SARS-N protein in its soluble form may play an important role in extracellular pathogenesis of SARS. However, it is totally unclear which of the intracellular and extracellular mechanisms are involved in viral replication and pathogenesis, and how the intracellular mechanisms are regulated by the N protein. Therefore, these SARS-N mAbs may be extremely useful in providing further insight into the mechanisms of the N protein involved in the pathogenesis of SARS.

In conclusion, the SARS-N mAbs generated in the present study will be useful in providing reliable, sensitive, specific and convenient diagnostic kits for SARS-CoV detection; these diagnostic methods may include the immunoblot assay, immunofluorescence assay and antigen-capture ELISA. Furthermore, these mAbs will be very useful in future studies concerning the contribution of SARS-N protein in SARS-CoV pathology.

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References

Chang, C.K., Sue, S.C., Yu, T.H., Hsieh, C.M., Tsai, C.K., Chiang, Y.C., Lee, S.J., Hsiao, H.H., Wu, W.J., Chang, W.L., Lin, C.H., Huang, T.H., 2006. Modular organization of SARS coronavirus nucleocapsid protein. J. Biomed. Sci. 13, 59–72.

Che, X.Y., Hao, W., Wang, Y., Di, B., Yin, K., Xu, Y.C., Feng, C.S., Wan, Z.Y., Cheng, V.C., Yuen, K.Y., 2004. Nucleocapsid protein as early diagnostic marker for SARS. Emerg. Infect. Dis. 10, 1947–1949.

Chen, Z., Pei, D., Jiang, L., Song, Y., Wang, J., Wang, H., Zhou, D., Zhai, J., Du, Z., Li, D., Qiu, M., Han, Y., Guo, Z., Yang, R., 2004. Antigenicity analysis of different regions of the severe acute respiratory syndrome coronavirus nucleocapsid protein. Clin. Chem. 50, 988–995.

Cheng, M., Chan, C.W., Cheung, R.C., Bikkavilli, R.K., Zhao, Q., Au, S.W., Chan, P.K., Lee, S.S., Cheng, G., Ho, W.K., Cheung, W.T., 2005. Cross-reactivity of antibody against SARS-coronavirus nucleocapsid protein with IL-11. Biochem. Biophys. Res. Commun. 338, 1654–1660.

Chiang, C.Y., Chang, J.T., Lin, M.S., Wang, S.R., Chang, H.Y., 2005. Characterization of a monoclonal antibody specific to the Gag protein of porcine endogenous retrovirus and its application in detecting the virus infection. Virus Res. 108, 139–148.

Di, B., Hao, W., Gao, Y., Wang, M., Wang, Y.D., Qiu, L.W., Wen, K., Zhou, D.H., Wu, X.W., Lu, E.J., Liao, Z.Y., Mei, Y.B., Zheng, B.J., Che, X.Y., 2005. Monoclonal antibody-based antigen capture enzyme-linked immunosorbent assay reveals high sensitivity of the nucleocapsid protein in acute-phase sera of severe respiratory syndrome patients. Clin. Diagn. Lab. Immunol. 12, 135–140.

Egloff, M.P., Ferron, F., Campanacci, V., Longhi, S., Rancurel, C., Dutartre, H., Snijder, E.J., Gorbalenya, A.E., Cambillau, C., Canard, B., 2004. The severe acute respiratory syndrome-coronavirus replicative protein nsp9 is a single-stranded RNA-binding subunit unique in the RNA virus world. Proc. Natl. Acad. Sci. U.S.A. 101, 3792–3796.

He, Y., Zhou, Y., Wu, H., Kou, Z., Liu, S., Jiang, S., 2004. Mapping of antigenic sites on the nucleocapsid protein of the severe acute respiratory syndrome coronavirus. J. Clin. Microb. 42, 5309–5314.

Huang, J.L., Lin, H.T., Wang, Y.M., Yeh, Y.C., Peck, K., Lin, B.L., Liu, H.W., Chen, A., Lin, C.S., 2005a. Rapid and sensitive detection of multiple genes from the SARS-coronavirus using quantitative RT-PCR with dual systems. J. Med. Virol. 77, 151–158.

Huang, Z., Zhu, W., Szekeres, G., Xia, H., 2005b. Development of new rabbit monoclonal antibody to estrogen receptor: immunohistochemical assessment on formalin-fixed, paraffin-embedded tissue sections. Appl. Immunohistochem. Mol. Morphol. 13, 91–95.

Kalicharran, K., Dales, S., 1995. Dephosphorylation of the nucleocapsid protein of inoculum JHMV may be essential for initiating replication. Adv. Exp. Med. Biol. 380, 485–489.

Keightley, M.C., Sillekens, P., Schippers, W., Rinaldo, C., George, K.S., 2005. Real-time NASBA detection of SARS-associated coronavirus and comparison with real-time reverse transcription-PCR. J. Med. Virol. 77, 602–608.

Keyaerts, E., Vijgen, L., Maes, P., Neyts, J., Van Ranst, M., 2005. Growth kinetics of SARS-coronavirus in Vero E6 cells. Biochem. Biophys. Res. Commun. 329, 1147–1151.

Kohler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495–497.

Ksiazek, T.G., Erdman, D., Goldsmith, C.S., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A., Lim, W., Rollin, P.E., Dowell, S.F., Ling, A.E., Humphrey, C.D., Shieh, W.J., Guarner, J., Paddock, C.D., Rota, P., Fields, B., DeRisi, J., Yang, J.Y., Cox, N., Hughes, J.M., LeDuc, J.W., Bellini, W.J., Anderson, L.J., The SARS Working Group, 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. 348, 1953–1966.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lau, S.K., Woo, P.C., Wong, B.H., Tsoi, H.W., Woo, G.K., Poon, R.W., Chan, K.H., Wei, W.I., Peiris, J.S., Yuen, K.Y., 2004. Detection of severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein in SARS patients by enzyme-linked immunosorbent assay. J. Clin. Microbiol. 42, 2884–2889.

Liao, Q.J., Ye, L.B., Timani, K.A., Zeng, Y.C., She, Y.L., Ye, L., Wu, Z.H., 2005. Activation of NF-kappaB by the full-length nucleocapsid protein of the SARS coronavirus. Acta Biochim. Biophys. Sin. 37, 607–612.

Lu, L., Manopo, I., Leung, B.P., Chng, H.H., Ling, A.E., Chee, L.L., Ooi, E.E., Chan, S.W., Kwang, J., 2004. Immunological characterization of the spike protein of the severe acute respiratory syndrome coronavirus. J. Clin. Microb. 42, 1570–1576.

Lu, W., Wu, X.D., Shi, M.D., Yang, R.F., He, Y.Y., Bian, C., Shi, T.L., Yang, S., Zhu, X.L., Jiang, W.H., Li, Y.X., Yan, L.C., Ji, Y.Y., Lin, Y., Lin, G.M., Tian, L., Wang, J., Wang, H.X., Xie, Y.H., Pei, G., Wu, J.R., Sun, B., 2005. Synthetic peptides derived from SARS coronavirus S protein with diagnostic and therapeutic potential. FEBS Lett. 579, 2130–2136.

Nakanaga, K., Yamanouchi, K., Fujiwara, K., 1986. Protective effect of monoclonal antibodies on lethal mouse hepatitis virus infection in mice. J. Virol. 59, 168–171.

Rota, P.A., Oberste, M.S., Monroe, S.S., Nix, W.A., Campagnoli, R., Icenogle, J.P., Penaranda, S., Bankamp, B., Maher, K., Chen, M.H., Tong, S., Tamin, A., Lowe, L., Frace, M., DeRisi, J.L., Chen, Q., Wang, D., Erdman, D.D., Peret, T.C., Burns, C., Ksiazek, T.G., Rollin, P.E., Sanchez, A., Liffick, S., Holloway, B., Limor, J., McCaustland, K., Olsen-Rasmussen, M., Fouchier, R., Gunther, S., Osterhaus, A.D., Drosten, C., Pallansch, M.A., Anderson, L.J., Bellini, W.J., 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science 300, 1394–1399.

Sun, Z.F., Meng, X.J., 2004. Antigenic cross-reactivity between the nucleocapsid protein of severe acute respiratory syndrome (SARS) coronavirus and polyclonal antisera of antigenic group I animal coronaviruses: implication for SARS diagnosis. J. Clin. Microb. 42, 2351–2352.

Surjit, M., Liu, B., Jameel, S., Chow, V.T., Lal, S.K., 2004. The SARS coronavirus nucleocapsid protein induces actin reorganization and apoptosis in COS-1 cells in the absence of growth factors. Biochem. J. 383, 13–18.

Surjit, M., Kumar, R., Mishra, R.N., Reddy, M.K., Chow, V.T., Lal, S.K., 2005. The severe acute respiratory syndrome coronavirus nucleocapsid protein is phosphorylated and localizes in the cytoplasm by 14-3-3-mediated translocation. J. Virol. 79, 1147–11486.

Surjit, M., Liu, B., Chow, V.T., Lal, S.K., 2006. The nucleocapsid protein of severe acute respiratory syndrome-coronavirus inhibits the activity of cyclin-cyclin-dependent kinase complex and blocks S phase progression in mammalian cells. J. Biol. Chem. 281, 10669–10681.

Vapalahti, O., Lundkvist, A., Kallio-Kokko, H., Paukku, K., Julkunen, I., Lankinen, H., Vaheri, A, 1996. Antigenic properties and diagnostic potential of puumala virus nucleocapsid protein expressed in insect cells. J. Clin. Microbiol. 34, 119–125.

Wu, H.S., Hsieh, Y.C., Su, I.J., Lin, T.H., Chiu, S.C., Hsu, Y.F., Lin, J.H., Wang, M.C., Chen, J.Y., Hsiao, P.W., Chang, G.D., Wang, A.H., Ting, H.W., Chou, C.M., Huang, C.J., 2004a. Early detection of antibodies against various structural proteins of the SARS-associated coronavirus in SARS patients. J. Biomed. Sci. 11, 117–126.

Wu, X.D., Shang, B., Yang, R.F., Yu, H., Ma, Z.H., Shen, X., Ji, Y.Y., Lin, Y., Wu, Y.D., Lin, G.M., Tian, L., Gan, X.Q., Yang, S., Jiang, W.H., Dai, E.H., Wang, X.Y., Jiang, H.L., Xie, Y.H., Zhu, X.L., Pei, G., Li, L., Wu, J.R., Sun, B., 2004b. The spike protein of severe acute respiratory syndrome (SARS) is cleaved in virus infected Vero-E6 cells. Cell Res. 14, 400–406.

Yamashita, M., Yamate, M., Li, G.M., Ikuta, K, 2005. Susceptibility of human and rat neural cell lines to infection by SARS-coronavirus. Biochem. Biophys. Res. Commun. 334, 79–85.

Yan, H., Xiao, G., Zhang, J., Hu, Y., Yuan, F., Cole, D.K., Zheng, C., Gao, G.F., 2004. SARS coronavirus induces apoptosis in Vero E6 cells. J. Med. Virol. 73, 323–331.

Yan, X., Hao, Q., Mu, Y., Timani, K.A., Ye, L., Zhu, Y., Wu, J., 2006. Nucleocapsid protein of SARS-CoV activates the expression of cyclooxygenase-2 by binding directly to regulatory elements for nuclear factor-kappa B and CCAAT/enhancer binding protein. Int. J. Biochem. Cell Biol. 38, 1417–1428.

Zeng, R., Ruan, H.Q., Jiang, X.S., Zhou, H., Shi, L., Zhang, L., Sheng, Q.H., Tu, Q., Xia, Q.C., Wu, J.R., 2004. 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, 549–555.