Original article

Comparison of effectiveness of whole viral, N and N199 proteins by ELISA for the rapid diagnosis of severe acute respiratory syndrome coronavirus

GUO Zhong-min, LU Jia-hai, HAN Wen-yu, LIU Ze-yu, LI Guo-wei, LIAO Jia-wei, WANG Shu-min, WU Ying-song, ZHENG Huan-ying, ZHONG Nan-shan and ZHU Xing-quan

Keywords: *severe acute respiratory syndrome virus; N199; enzyme-linked immunosorbent assay*

Background Although severe acute respiratory syndrome (SARS) has been controlled, the subsequently emerging sporadic cases in 2004 emphasize the necessity of developing a rapid diagnostic method, which would be of great help in clinical diagosis and also wild host screening. This study aims to establish an effective and rapid serological tool for the diagnosis of SARS-CoV by comparison among whole viral, N and N199 proteins by ELISA.

Methods SARS-CoV N and N199 (a truncated nucleocapsid gene) genes were cloned, expressed, identified by Western blotting, and applied in screening of human and swine samples. Sera of SARS convalescent-phase patients, normal human sera, sera of patients with other respiratory diseases, and swine sera were screened by ELISA, with whole SARS-CoV F69, N and N199 proteins as antigens.

Results The sensitivity and specificity of N and N199 proteins in human sera diagnosis were approximate (P=0.743), which was higher than whole viral protein but the difference was not significant (P=0.234). The N199 protein proved to be more specific in swine sera screening than whole viral and N protein (P<0.001).

Conclusion N199 protein is feasible in both clinical diagnosis and SARS-CoV reservoir screening.

Chin Med J 2007;120(24):2195-2199

Severe acute respiratory syndrome (SARS), an epidemic which triggered a worldwide panic in 2003,[1] is currently under control. SARS coronavirus (SARS-CoV), a novel coronavirus phylogenically un-related to previously identified coronaviruses, has been isolated and identified as the causative agent.[2-5] However, since the nosogenesis and immunogenesis of SARS-CoV have not been identified completely, effective treatment for SARS is still unavailable. The subsequently emerging sporadic cases emphasize the necessity of developing a rapid diagnostic method, which would be of great help in clinical diagosis and also wild host screening. Unfortunately, such an effective method has not been established so far, due to the cross reaction between SARS-CoV and other viruses especially in pigs.[3,6,7]

The N protein of coronaviruses is highly conserved in each group, immunogenic, and abundantly expressed during infection. It has been identified as a suitable candidate for diagnostic applications for human and animal coronaviruses.

The sequence of the nucleocapsid gene of SARS coronavirus was found to have 26%−32% homology with nucleocapsid genes of various animal coronaviruses. To eliminate possible cross-reactions between the nucleocapsid protein of the SARS coronavirus and nucleocapsid proteins of various animal coronaviruses, in this study, a fusion protein named N199 was cloned, expressed, purified and applied for the sample screening by ELISA, and was compared with whole viral protein and N protein to evaluate the diagnostic capacity.

METHODS

Virus strains

SARS-CoV F69 (NCBI/Genbank AY313906) was isolated from a SARS patient from Guangdong Province, China in 2003.[8-10]

Experiment Animal Center, Sun Yat-sen University, Guangzhou 510080, China (Guo ZM)
Laboratory for Tropical Disease Control and Prevention, School of Public Health, Sun Yat-sen University, Guangzhou 510080, China (Lu JH, Liu ZY, Li GW and Liao JW)
College of Animal Science and Veterinary Medicine, Jilin University, Changchun 130012, China (Han WY)
School of Life Science, Foshan University, Foshan 528000, China (Wang SM)
School of Biotechnology, Southern Medical University, Guangzhou 510515, China (Wu YS)
Microorganism Institute, Guangdong Center for Disease Control and Prevention, Guangzhou 510300, China (Zheng HY)
Guangzhou Institute of Respiratory Diseases, Guangzhou Medical College, Guangzhou 510120, China (Zhong NS)
College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China (Zhu XQ)
Correspondence to: Dr. LU Jia-hai, School of Public Health, Sun Yat-sen University, Guangzhou 510080, China (Email: jiahailu@yahoo.com.cn); Dr. ZHONG Nan-shan, Guangzhou Institute of Respiratory Diseases, Guangzhou Medical College, Guangzhou 510120, China (Email: nanshan@vip.163.com); Dr. ZHU Xing-quan, College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China (Email: xingquanzh@scau.edu.cn)
GUO Zhong-min, LU Jia-hai, HAN Wen-yu and LIU Ze-yu contributed equally to this study.
This study was supported by a grant from the Science Foundation for SARS of Guangdong Province (No. 2003Z3-E0461).

Table 1. Human sera in ELISA screening

Groups	Samples (*n*)	Origin of serum samples	Date of collection
SARS convalescent-phase patients	36	Chest Hospital of Guangzhou	March, 2003
Healthy controls	50	School of Public Health, Sun Yat-sen University (volunteer blood donors)	September, 2003
Patients with other respiratory diseases	42	First Affiliated Hospital of Sun Yat-sen University	March, 2003

Source of human specimens

The human sera used for this study were collected from various institutions and periods, as listed at Table 1. The SARS patients in our study all satisfied the WHO diagnostic criteria, and all serous specimens were inactivated at 56°C for 30 minutes before application.

Source of swine specimens

The swine sera used for screening were collected from pig farms in Guangdong Province and Xinjiang Uygur Autonomous Region (Table 2).

Table 2. Porcine sera in ELISA screening

Samples (*n*)	Origin of serum samples	Date of collection
14	Foshan, Guangdong	October, 2002
11	Zengcheng, Guangdong	November, 2002
12	Dongguan, Guangdong	October, 2002
11	Zhongshan, Guangdong	November, 2002
12	Ili, Xinjiang	December, 2003

Antigen preparation

The large-scale cultured F69 virus was inactivated with 0.4% formaldehyde for 24−48 hours, verified with the CPE method for security, and centrifuged at 4 000×*g* for 30 minutes to remove the precipitate. The viral supernatant was partially used as whole viral antigen in indirect ELISA assay, and the protein concentration was determined by the Bradford method.

Molecular cloning

The viral RNA was extracted from culture supernatant by QIAamp viral RNA kit (Qiagen), and reversely transcribed to cDNA by SuperScript™ Ⅲ reverse transcriptase (Invitrogen, USA). A standard polymerase chain reaction (PCR) procedure was used to amplify both the full length and the truncated fragment of the nucleocapsid gene (95°C for 1 minute, followed by 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds). The 5' ends of the forward and reverse primers contained digestive loci (EcoRI and SalI for N199 gene, NcoI and SalI for N gene, Table 3).

Table 3. Primers facilitating N and N199 gene amplification

Gene	Primers
N	p1: 5'-GCACCATGGCTTCTGATAATGGACCCCAA-3' (NcoI)
	p2: 5'-CACGTCGACTGCCTGAGTTGAATCA-3' (Sal I)
N199	p1: 5'-GCAGAATTCATGCTAGACAGATTGAACCAGCTT-3' (EcoR I)
	p2: 5'-CACGTCGACTGCCTGAGTTGAATCA-3' (Sal I)

The amplified products were purified with a PCR product purification kit (Watson, Shanghai, China), and digested with the enzymes (NcoI and SalI for N gene, EcoRI and SalI for N199 gene) (TAKARA, Dalian, China). The fragments were ligated into a pET-28a(+) vector (Novagen) expressing His·Tags on N and C termini, and transformed into DH5α *Escherichia coli*. The positive clones were identified by PCR screening, enzymatic digestion, and sequence analysis.

Protein expression

The combined plasmids were extracted by a plasmid extract kit (Watson), and transformed into *E. coli*. strain BL21 (DE3) (Amersham Pharmacia). The transformed bacteria were grown in Luria-Bertani medium with kanamycin (30 μg/ml) until they reached the mid-log phase, a point at which the optical density at 600 nm (OD60) is 0.6. The bacteria were then induced with 1.0 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours at 37°C, and the protein products were visualized by SDS-PAGE.

Western blotting

Before blotting, protein products (20 μl for each well) run in SDS polyacrylamide gel, and then were transferred onto nitrocellulose membranes. Normal equine sera and sera from animals immunized with inactivated SARS-CoV were applied as negative and positive controls at a dilution of 1:100,[11] respectively. The membranes were then incubated with a peroxidase-conjugated secondary antibody (Sino-American Biotech), and visualized with DAB (3,3'-diaminobenzidine tetrahydrochloride), a horseradish peroxidase substrate for membrane color development to confirm the immunological ability of the proteins.

Protein purification

The expressed recombinant fusion proteins (N and N199) were identified as soluble proteins. The transformed bacteria were harvested by centrifugation. The resulting pellet was suspended in binding buffer (20 mmol/L Tris-HCl pH 7.9, 500 mmol/L NaCl, 5 mmol/L imidazole, 1 mmol/L NaF, and 1 mmol/L PMSF) with bacteriolysin (0.5 mg/ml, Sino-American Biotech), sonicated, and centrifuged at 12 000×*g* for 30 minutes at 4°C. The clear supernatants of both N and N199 proteins were applied respectively to a prepared Ni-NTA affinity column (Qiagen), washed with gradient washing buffers (20 mmol/L Tris-HCl pH 7.9, 500 mmol/L NaCl, 5, 20, 40, and 60 mmol/L imidazole), eluted with elution buffer (20 mmol/L Tris-HCl pH 7.9, 500 mmol/L NaCl, 100 mmol/L imidazole), and concentrated by dialysis. The final protein concentrations were determined by the Bradford method.

Serous specimens screening and specific IgG titer measurement

The human and swine sera screening were performed by ELISA with whole SARS-CoV F69, N and N199 proteins

Table 4. The aminoacid sequence of N199 protein

LDRLNQLESKVSGKGQQQQGQTVTKKSAAEASKKPRQKRTATKQYNVTQAFGRRGPEQTQGNFGDQDLIRQGTDYKHWPQIAQFAPSASAFFGMSRIGM
EVTPSGTWLTYHGAIKLDDKDPQFKDNVILLNKHIDAYKTFPPTEPKKDKKKKTDEAQPLPQRQKKQPTVTLLPAADMDDFSRQLQNSMSGASADSTQA

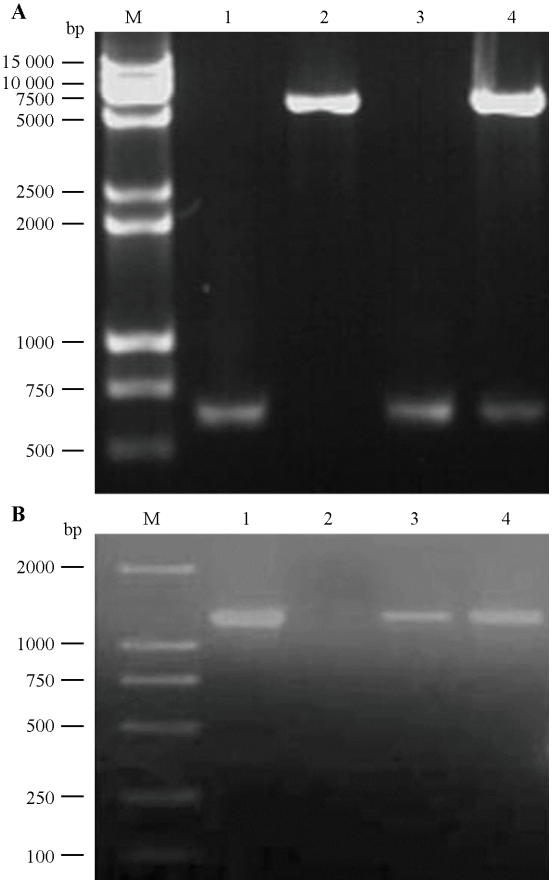

Fig. 1. Identification of N and N199 genes. **A:** N199 gene (Lane M: DNA marker DL2000+15000 (TAKARA); Lane 1: N199 gene PCR; Lane 2: pET empty plasmid bi-enzyme digestion; Lane 3: pET-N199 PCR; Lane 4: pET-N199 bi-enzyme digestion). **B:** N gene (Lane M: DNA marker DL2000 (TAKARA); Lane 1: pET-N bi-enzyme digestion; Lane 2: pET empty plasmid bi-enzyme digestion; Lane 3: pET-N PCR; Lane 4: N gene PCR).

as antigens. In brief, polystyrene micro-well plates were coated with the antigens (100 μl/well containing 1.0 μg/ml protein). Wells were washed 3 times with PBS and then blocked with 10% bovine serum albumin (BSA) in PBS (200 μl/well) at 4°C overnight. After 3 washes with PBST, human and swine sera (dilution of 1:60) were added to the plates and incubated at 37°C for 1 hour. Following another washing cycle, horseradish peroxidase (HRP)-conjugated goat anti-human or goat anti-pig antibodies (Sigma, USA) were added corresponding to the serous specimens. After incubation at 37°C for 1 hour, the plates were washed as above and the substrate tetramethylbenzidine (TMB) solution (Sigma) was added to the wells. After incubation at 37°C for 15 minutes, the reaction was stopped by adding 2.0 mmol/L sulfuric acid, and the absorbance value was measured with a microplate reader (Model 550, BioRad) at 450 nm (A_{450}). The cutoff value was defined as the mean OD value of control samples plus two standard deviations.

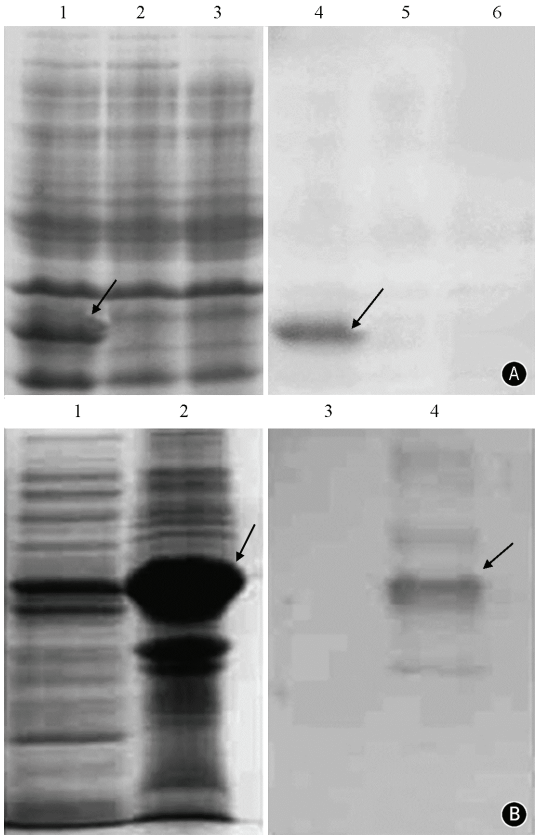

Fig. 2. Identification of N and N199 proteins. **A:** N199 protein (induced pET-N199, un-induced pET-N199, induced pET on SDS-PAGE (Lanes 1, 2, and 3) and Western blot (Lanes 4, 5, and 6)); **B:** N protein (un-induced pET-N, induced pET-N on SDS-PAGE (Lanes 1 and 2) and Western blot (Lanes 3 and 4)).

Statistical analysis

The ratios we obtained were compared according to chi square test by SPSS11 statistical software. *P* value less than 0.05 was considered statistically significant.

RESULTS

Characteristics of recombinant proteins

The full length (N) and truncated fragment (N199) of the nucleocapsid gene were cloned and identified to match the sequences of SARS-CoV by PCR screening, enzymatic digestion and sequence analysis, as shown in Fig. 1. The aminoacid sequence of N199 protein was listed in Table 4.

The target proteins were expressed and visualized by SDS-PAGE, and the immunological capacities of N and N199 proteins against the SARS-CoV anti-sera were confirmed by Western blot as shown in Fig. 2. N and N199 proteins were purified and detected by the Bradford method being 64.2 mg/L and 157 mg/L, respectively.

Results of human sera screening by ELISA

In the test of 36 SARS convalescent-phase human serum

Table 5. Results of indirect ELISA to human serous specimens (*n*(%))

Group	SARS convalescent-phase (*n*=36)		Serum of other respiratory diseases (*n*=42)		Normal sera (*n*=50)	
	Positive	Negative	Positive	Negative	Positive	Negative
Whole virus	27 (75.0)	9 (25.0)	2 (4.8)	40 (95.2)	2 (4.0)	48 (96.0)
N protein	31 (86.1)	5 (13.9)	0 (0)	42 (100)	0 (0)	50 (100)
N199 protein	30 (83.3)	6 (16.7)	0 (0)	42 (100)	0 (0)	50 (100)

Table 6. Results of indirect ELISA to swine sera (*n*(%))

Group	*n*	Positive	Negative
Whole virus	60	59 (98.3)	1 (1.7)
N protein	60	58 (96.7)	2 (3.3)
N199 protein	60	5 (8.3)	55 (91.7)

samples, N and N199 proteins appeared to have similar sensitivity, which was 86.1% and 83.3% respectively, while that of whole viral protein was 75.0%. In the test of 50 normal human serum samples and 42 serum samples of other respiratory diseases, N and N199 proteins both showed 100% in specificity, while whole viral protein appeared 95.2% in normal human serum test and 96.0% in serum test of other respiratory diseases, as listed in Table 5. These data indicated that the sensitivity and specificity of N and N199 proteins in human sera screening was similar (P=0.743), which was higher than that of the whole viral protein but the difference was not significant (P=0.234).

Results of swine sera screening by ELISA

In naturally collected swine sera test of 60 specimens, 59 and 58 were detected positive by whole viral and N proteins, respectively, while only 5 samples were identified positive by N199 protein, indicating that the positive rate using whole viral protein and N protein (98.3% and 96.7%) was significantly higher than that using N199 protein (8.3%, P<0.001, Table 6).

DISCUSSION

The pathogen of SARS has been confirmed to be a novel coronavirus, named SARS-CoV.[2,5] Genome sequences of the identified SARS-CoV strains demonstrate the existence of variability.[4,8,9] The threat of SARS is still at large. Moreover, the possibility of SARS-CoV as a biological weapon must be appreciated when one considers the high mortality rate and rapid transmission of SARS.[12] The treatment of SARS is still symptomatic and palliative, since specific medicines or valid vaccines are still unavailable.[13,14]

Furthermore, since the first identification of SARS in 2002, the search for the original ancestor and natural reservoir of SARS-CoV virus has become a major scientific interest and public health necessity.[15-17]

Weingartl et al[18] reported failure to transmit SARS-CoV to six-week-old pigs. But SARS-CoV ribonucleic acid (RNA) in the porcine blood was detected by reverse transcriptase-polymerase chain reaction and the pigs seroconverted with neutralizing antibodies to SARS-CoV was noted in the same experiment, which suggested the susceptibility of pigs to SARS-CoV.

Further more, Chen et al[19] reported SARS-CoV was isolated from a pig during a survey for possible routes of viral transmission after a SARS epidemic, concluding that the pig was infected by a SARS-CoV of human origin.

One of the main barriers of porcine serum screen and antibody titer measurement by ELISA, Western blots, or immunofluorescence etc is the cross-reaction between SARS-CoV and transmissible gastroenteritis virus (TGEV), porcine respiratory CoV (PRCV), both belonging to group I animal coronavirus and widely infected in pigs, which may probably associate with the N protein, found to have 26% to 32% homology with nucleocapsid genes of various animal coronaviruses.[3,6,20] The results using intact SARS-CoV or nucleocapsid (N) protein may be suspect, which hinder definitive analysis of animal reservoirs for SARS. He reported the truncated fragment from the C terminus of the nucleocapsid protein of SARS-CoV was identified to have a strong ability to detect antibodies against SARS-CoV without cross-reaction with antibodies against porcine coronavirus.[21,22]

To eliminate possible cross-reactions between the nucleocapsid protein of SARS-CoV and other animal coronaviruses, a minimal sequence derived from the region encoding the nucleocapsid protein may probably detect the sera effectively. According to this theory, a truncated nucleocapsid protein of the SARS-CoV (N199 protein) expressed by an *E. coli*. system in this study, was identified capable of detecting human and especially porcine antibodies against the SARS-CoV, compared with the whole viral protein and N protein.

In the human sera screening, N and N199 proteins presented similar sensitivity and specificity (86.1% and 100% for N protein, 83.3% and 100% for N199 protein), which was higher than whole viral protein (75.0% and 95.7%), but the difference among those antigens were not significant. In the screening of naturally collected swine sera, the positive rate of whole viral protein and N protein was as high as 98.3% and 96.7%, while N199 protein was only 8.3%, which suggested that N199 protein might be a more specific antigen for reducing false positive in swine sera screening than whole viral and N proteins.

In conclusion, by utilizing molecular cloning techniques, we have cloned a SARS-CoV protein N199, which performed as well as the N protein in human sera screening. However, this N199 protein demonstrated unprecedently higher sensitivity and specificity than whole viral proteins and the N protein in serological

screening in pigs, proving the feasibility of a rapid serological test for the clinical diagnosis and screening of animal reservoirs for SARS.

Attachment: The sequences reported in this paper have been deposited in the GenBankTM database (accession No. AY313906).

REFERENCES

1. Tsang KW, Ho PL, Ooi GC, Yee WK, Wang T, Chan YM, et al. A cluster of cases of severe acute respiratory syndrome in Hong Kong. N Engl J Med 2003; 348: 1977-1985.
2. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Eng J Med 2003; 348: 1967-1976.
3. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 2003; 348: 1953-1966.
4. Marra MA, Jones SJ, Astell CR, Holt RA, Brooks-Wilson A, Butterfield YSN, et al. The genome sequence of the SARS-associated coronavirus. Science 2003; 300: 1399-1404.
5. Peiris JSM, Lai ST, Poon LLM, Guan Y, Yam LYC, Lim W, et al. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 2003; 361: 1319-1325.
6. Sun ZF, Meng XJ. 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 Microbiol 2004; 42: 2351-2352.
7. Holmes KV. SARS coronavirus: a new challenge for prevention and therapy. J Clin Investig 2003; 111: 1605-1609.
8. Lu JH, Guo ZM, Han WY, Wang GL, Zhang DM, Wang YF, et al. Preparation and development of equine hyperimmune globulin F(ab')2 against severe acute respiratory syndrome coronavirus. Acta Pharmacologica Sinica 2005; 26: 1479-1484.
9. Zhang CH, Guo ZM, Zheng HY, Lu JH, Wang YF, Yan XG, et al. Humoral immune responses in rabbits induced by an experimental inactivated severe acute respiratory syndrome coronavirus vaccine prepared from F69 strain. Chin Med J 2004; 117: 1625-1629.
10. Liao JW, Lu JH, Guo ZM, Wang GL, Zhang DM, Chen LJ, et al. A retrospective serological study of severe acute respiratory syndrome cases in Guangdong Province, China. Chin Med J 2007; 120: 718-720.
11. Chinese SARS Molecular Epidemiology Consortium. Molecular evolution of the SARS coronavirus during the course of the SARS epidemic in China. Science 2004; 303: 1666-1669.
12. WHO. Cumulative number of reported probable cases of SARS. (Accessed on July 6, 2003 at *http://www.who.int/csr/sars/country/2003_07_11/en/*)
13. Zhang DM, Wang GL, Lu JH. Severe acute respiratory syndrome: vaccine on the way. Chin Med J 2005; 118: 1468-1476.
14. Zhang CH, Wang YF, Liu XJ, Lu JH, Qian CW, Wan ZY, et al. Antiviral activity of cepharanthine against severe acute respiratory syndrome coronavirus *in vitro*. Chin Med J 2005; 118: 493-496.
15. Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, et al. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science 2003; 302: 276-278.
16. Song HD, Tu CC, Zhang GW, Wang SY, Zheng K, Lei LC, et al. Cross-host evolution of severe acute respiratory syndrome coronavirus in palm civet and human. Proc Natl Acad Sci U S A 2005; 102: 2430-2435.
17. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, et al. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc Natl Acad Sci U S A 2005; 102: 14040-14045.
18. Weingartl HM, Copps J, Drebot MA, Marszal P, Smith G, Gren J, et al. Susceptibility of pigs and chickens to SARS coronavirus. Emerg Infect Dis 2004; 10: 179-184.
19. Chen WJ, Yan MH, Yang L, Ding BL, He B, Wang YZ, et al. SARS-associated coronavirus transmitted from human to pig. Emerg Infecti Dis 2005; 11: 446-448.
20. Saif LJ. Animal coronaviruses: what can they teach us about the severe acute respiratory syndrome? Rev Sci Tech Off Int Epiz 2004; 23: 643-660.
21. He QG, Chong KH, Chng HH, Leung B, Ling AE, Wei SW, et al. Development of a Western blot assay for detection of antibodies against coronavirus causing severe acute respiratory syndrome. Clin Diagn Lab Immunol 2004; 417-422.
22. He QG, Du QY, Lau S, Manopo I, Lu LQ, Chan SW, et al. Characterization of monoclonal antibody against SARS coronavirus nucleocapsid antigen and development of an antigen capture ELISA. J Virolog Methods 2005; 127: 46-53.

(Received May 10, 2007)
Edited by SUN Jing