Recombinant Protein-Based ELISA and Immuno-Cytochemical Assay for the Diagnosis of SARS

Alessandra Carattoli,[1] Paola Di Bonito,[1] Felicia Grasso,[1] Colomba Giorgi,[1] Francesco Blasi,[2] Matthias Niedrig,[3] and Antonio Cassone[1]*

[1]Department of Infectious, Parasitic and Immuno-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy
[2]Ospedale Maggiore Policlinico, University of Milan, Milan, Italy
[3]Robert Koch Institut, Berlin, Germany

A new Coronavirus (SARS-CoV) is the aetiological agent of the severe acute respiratory syndrome (SARS). Because of the critical role played by serological assays for SARS diagnosis, an in-house ELISA based on SARS-CoV recombinant antigens was developed. The SARS-CoV nucleocapsid protein (N), three N fragments (N1, N2, and N3) and the intraviral domain of the membrane protein (M2) were cloned and expressed in *Escherichia coli* as histidine-tagged proteins. Six reference sera from SARS patients were used to detect virus-specific IgG in an ELISA using each recombinant protein as coating antigen. High-titre positive reactions were detected in all SARS positive sera. The specificity of the assay appears to be high as no positive reaction was detected in the sera of 20 healthy subjects and 73 patients with non-SARS, low-tract respiratory infections. Specific hyper-immune sera to SARS-CoV and the recombinant proteins, N, N1, N2, N3, and M2 were also generated in mice and rabbits. The specificity of these sera was confirmed by an immunocytochemical assay on biochips of SARS-CoV infected and uninfected cells. *J. Med. Virol. 76:137–142, 2005.* © 2005 Wiley-Liss, Inc.

KEY WORDS: SARS; Coronavirus; diagnosis; antibodies; serology

INTRODUCTION

An outbreak of severe acute respiratory syndrome (SARS) swept the world in early 2003, spreading from China to several countries in different continents, and causing more than 8,000 cases with roughly 14%–18% mortality rate [Donnelly et al., 2004]. A new Coronavirus (SARS-CoV) is the etiological agent of this syndrome, accordingly, molecular, virological, and serological tests have been developed for its identification [Chan et al., 2004; Poon et al., 2004b]. Although

retrospective in nature, serological tests are of utmost importance either to confirm or rule out infection by the virus and for seroprevalence studies. In this context, the viral nucleocapsid protein N and the membrane protein M are recognized as useful antigens to monitor antibody response in other coronavirus infections [Ndifuna et al., 1998; Chen et al., 2003; Elia et al., 2003]. The N and M antigen have been also used to detect anti-SARS-CoV antibodies. Despite intense research in this area [Ndifuna et al., 1998; Che et al., 2003; Shi et al., 2003, 2004a,b; Leung et al., 2004; Liu et al., 2004; Woo et al., 2004; Wu et al., 2004], the need for further improvements of SARS serology is widely recognized [Poon et al., 2004a].

Recombinant proteins are potentially inexpensive, safe, and easy to standardize serological reagents. They also represent the safest way to prepare the viral antigens since they can be obtained without propagation of live virus, a fact of considerable importance in view of the recognized capacity of SARS-CoV to infect laboratory workers. Thus, for SARS diagnosis and seroprevalence studies, an in-house ELISA was developed in our laboratory acting as National Reference Center for Infectious Emergences. The assay is based on the use of SARS-CoV recombinant N and M structural proteins as coating antigens. Purified N and M proteins were also used to generate specific polyclonal antibodies for detection of SARS-CoV and its antigens in clinical specimens.

MATERIALS AND METHODS

Recombinant DNA Manipulation

The N and M full-length genes were generated by RT-PCR technology from a 10 µl sample containing heat and gamma-irradiated cell culture supernatant of Vero E6 cells infected with the SARS-CoV isolate Frankfurt 1 [Thiel et al., 2003]. The oligonucleotides used are listed in Table I. The *Bam*HI and *Pst*I sequences were added at the 5′-ends of each forward (FW) and reverse (RV) primers, respectively. The *Bam*HI-*Pst*I restricted cDNAs were cloned in pQE-30 (QIAGEN) and introduced by transformation in the JM109 *Escherichia coli* cells, obtaining the constructs p-N and p-M, respectively. The proteins were expressed as N-terminal MRGS(H)6 tag proteins. The NH2-terminus (1−117 amino acids, aa), the central-domain (110−340 aa), and the COOH-terminus (333−422 aa) of the N protein, and the cytoplasmic-domain (138−222 aa) of the M protein, were also cloned in pQE-30 obtaining the constructs p-N1, p-N2, p-N3, and p-M2, respectively. Numbers refer to the N (AAP33707) and M (AAP33701) EMBL sequences.

Protein Purification

The recombinant proteins were purified by affinity chromatography on Ni-NTA resin using a denaturing protocol according to the manufacture instructions (The QIAexpressionist). All proteins were solubilized by 8 M urea in PBS, except the protein N, which was solubilized by 6 M guanidine hydrochloride in PBS. Protein purity and quantity were analyzed by SDS–PAGE followed by staining with Coomassie blue and by standard method (Lowry), respectively. Protein identity was confirmed by Western blotting using the anti poly-histidine antibody (Sigma-Aldrich, St. Louis, MO).

Human Sera

The six SARS-CoV-positive sera were collected at the Robert Koch Institute in Berlin, Italy acting within the European Network for Diagnostics of Imported Viral Diseases (ENVID). They were kindly obtained from Prof. M. Peiris (University of Hong Kong, China), and Dr. M. Zambon (Health Protection Agency, London). All 6 sera were from patients with confirmed SARS according to the WHO criteria, and had documented titres in immunofluorescence, ELISA, and neutralization assay. They were used for quality control of different serological assays performed in different European laboratories acting as National Reference Centers for SARS diagnosis. Sera from 20 healthy donors and 73 patients affected by viral (non-SARS) or bacterial acute respiratory syndromes served as controls.

ELISA

The SARS-CoV recombinant proteins (0.125 µg/well), in denatured form, were adsorbed in carbonate buffer (pH 9.4) into 96-well microtiter plates overnight at 4°C, then incubated with twofold dilutions of each serum, starting from a 1:600 and 1:2,000 dilutions for N1, N2, N3, and M2-based and N-based ELISA, respectively. Incubation with primary and secondary antibody was performed at 37°C for 1 hr. The antigen−antibody complexes were detected by a goat anti-human Ig (IgM, IgG, IgA, H + L) peroxidase-conjugated (Southern Biotechnology Association Inc., Birmingham, AL), using the TMB (Vector Laboratories Inc., Burlinghame, CA) as substrate. Optical density was read at 450 nm.

Mouse and Rabbit Immune Sera

Hyperimmune mouse sera were produced by intraperitoneal immunization in CD1 mice (4−8 weeks old) as previously described in Di Bonito et al. [1999], using 10 µg of purified proteins per mouse. Purified proteins (100 µg per animal) were also inoculated intradermally four times, at 3-weeks interval, in albino New Zealand rabbits. The priming immunization was made with each antigen emulsified with Freund's complete adjuvant (0.5 ml/rabbit). The first and second boosters contained antigen in Freund's incomplete adjuvant (0.5 ml/rabbit) while the final booster consisted in the blotting using the corresponding recombinant protein as antigen.

Immunocytochemical Assay

Slides containing biochips of SARS-CoV infected and uninfected cells (Euroimmun Italia, Padova, Italy) were treated with 10% fetal calf serum in PBS for 30 min to remove aspecific reaction. Rabbit and mouse anti-sera were serially diluted at 1:100 to 1:25,000 and 1:100 to

TABLE I. Primers and Probes Used in RT-PCR Amplification

Primer[a]	DNA sequence	Gene target	Nucleotide positions in EMBL no. AY291315
NQEFW	5′-CGCGGATCCGCGATGTCTGATAATGGACCC-3′	Nucleoprotein N	28120–28137
NQERV	5′-AAAACTGCAGAACTTATGCCTGAGTTGAATCAG-3′	Nucleoprotein N	29388–29369
N1QERV	5′-AAAACTGCAGGCCAGTTCCTAGGTAATAGAAGTA-3′	N1 fragment of N	28471–28447
N2QEFW	5′-CGCGGATCCTACTTCTATTACCTAGGAACTGGC-3′	N2 fragment of N	28447–28470
N2QERV	5′-AAAACTGCAGCAATTTAATGGCTCCATGATAAGT-3′	N2 fragment of N	29139–29116
N3QEFW	5′-CGCGGATCCCACTTATCATGGAGCCATTAAATTG-3′	N3 fragment of N	29116–29139
MQEFW	5′-CGCGGATCCGCGATGGCAGACAACGGTACT-3′	Membrane protein M	26398–26415
MQERV	5′-AAAACTGCAGCCATTACTGTACTAGCAAAGCAAT-3′	Membrane protein M	27063–27043
M2QEFW	5′-CGCGGATCCGTCATTGGTGCTGTGATCATTCGT-3′	M2 fragment of M	26809–26832

[a]*Bam*HI and *Pst*I recognition sequences were added at the 5′-ends of each forward (FW) and reverse (RV) primer, respectively.

1:10,000, respectively in PBS 1% non-fat dry milk and incubated with the cell samples for 1 hr at room temperature. Antigen−antibody complexes were revealed by a detection system using the labeled polymer method (DAKO EnVision^{TM}+ System, 3-amino-9-ethilcarbazole, Horseradish Peroxidase, Carpinteria, CA). Samples were observed under the light microscope (Leitz, Germany) at a total magnification indicated in the legend of the Figure 4.

RESULTS AND DISCUSSION

N and M structural SARS-CoV proteins were chosen to develop an ELISA test to detect specific antiviral antibodies. The N and M full-length genes were amplified from heat and gamma-irradiated cell culture supernatant of Vero E6 cells infected with the SARS-CoV isolate Frankfurt 1. The amplified cDNAs were cloned in the expression vector pQE-30 (QIAGEN), obtaining the p-N and p-M constructs, respectively. The C-terminus, extra-membrane tail of the M protein (138−222 amino acids) was cloned and sequenced (Fig. 1, panel a). The deduced amino acid sequences of each insert were compared with the N and M sequences of the SARS-CoV Frankfurt 1 isolate revealing a 100% amino acid identity, thus assuring that the cloning methods had no inserted spurious amino acid variations. While the whole protein M could not be generated, probably for

its toxicity in *E. coli*, the recombinant M2 fragment as well as the full length N protein were successfully expressed in *E. coli* (Fig. 1, panel b). Recombinant proteins were purified and used as coating antigens in ELISA set up to analyze the 6 SARS-positive human sera. Twenty human sera from healthy blood donors and 73 SARS-negative sera from patients affected by acute respiratory syndromes with viral (non-SARS) or bacterial etiology were used as negative controls. All sera from SARS patients showed a strong positive reaction against N and M2 antigen. In contrast, no positive reaction was observed in any serum from non-SARS subjects with the dilution cut-off considered, suggesting a high specificity of the test. Figure 2 particularly shows the titration curves of the 6 SARS serum samples obtained by ELISA with the use of M2 (panel a) and the whole N antigen (panel b). It also shows that the anti-M2 antibody titre was generally lower than the anti-N titre, nonetheless, the anti-M2 response showed particularly low inter-subject variability, as compared to the anti-N response. The mean OD reading values of the six SARS sera tested against either M2 or N antigens, at the dilution of 1: 600 and 1:2,000, respectively, are shown in Figure 2, panel c, together with the values of the 93 non-SARS subjects (OD < 0.2). All high-titre ELISA results were confirmed by Western blotting showing the expected immuno-reactive viral protein band (data not shown).
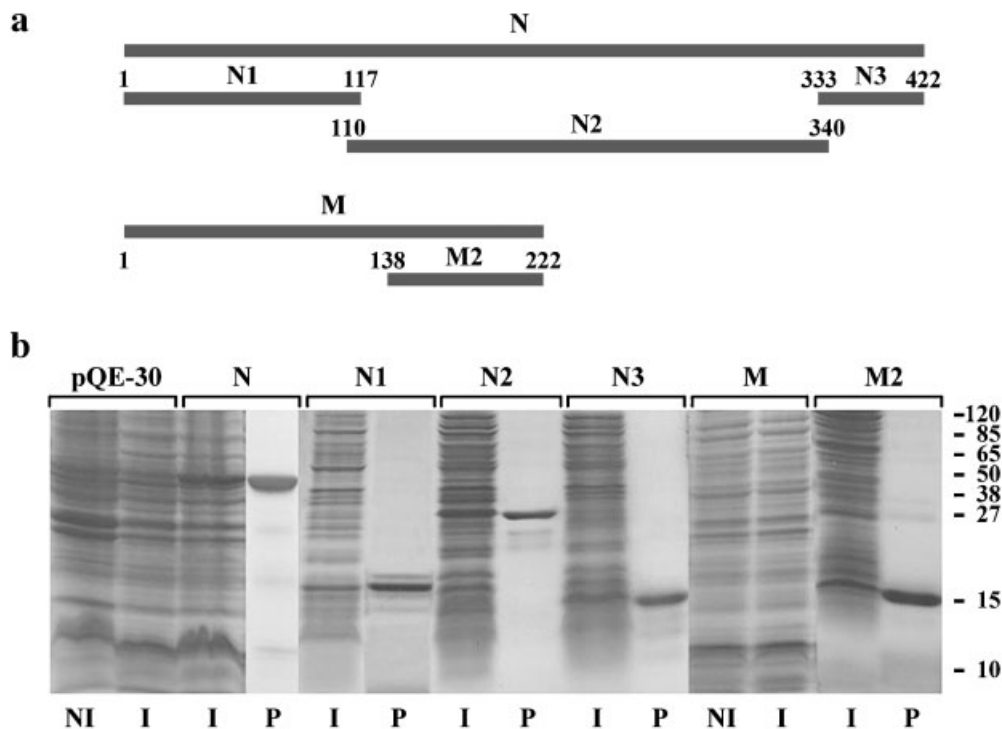

Fig. 1. **Panel a**: schematic representation of the coding region of SARS-CoV nucleocapsid (N) and membrane (M) proteins. The N1, N2, N3, and M2 fragments of the N and M proteins are represented. Numbers on the bars indicate the first and last amino acids present in the constructs. **Panel b**: SDS−PAGE analysis of lysates from induced (I) and not induced (NI) *E. coli* transformed by the expression plasmids containing N, N1, N2, N3, and M2 coding sequences. The gel was stained by Coomassie blue. **Lanes** P show the purified proteins. The molecular mass size markers (in kDa) are reported on the right of the Figure.
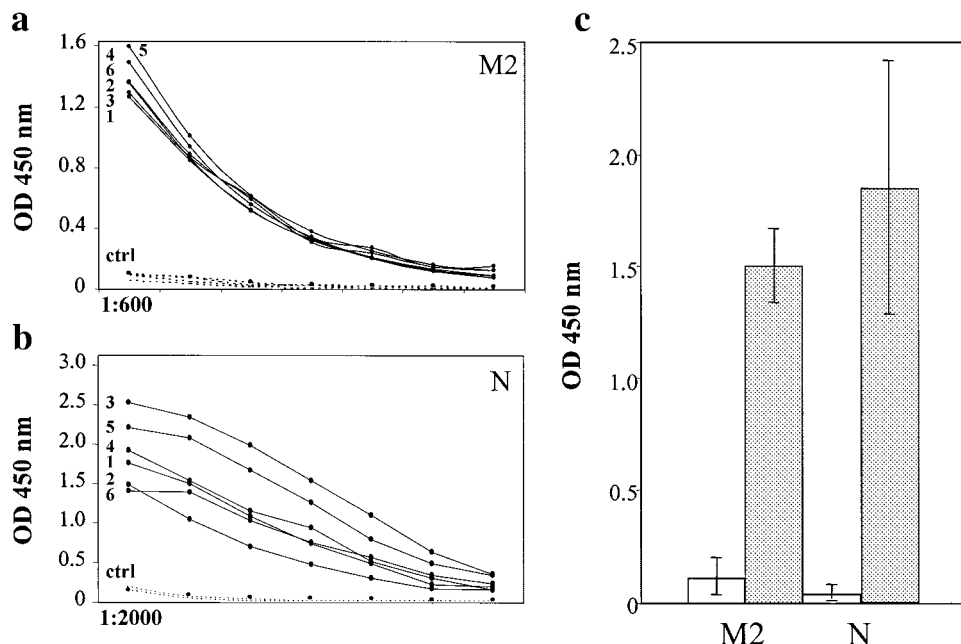
Fig. 2. Detection of specific immunoglobulins in SARS-CoV positive human sera using M2 (**panel a**) and N (**panel b**) based ELISA. Optical density values are reported in ordinate, dilutions of the sera are reported in abscissa. In N based ELISA, sera dilutions are from 1:2,000 to 1:128,000. In M2 based ELISA, sera dilutions are from 1:600 to 1:22,400. The serum of each SARS patient is indicated by a number (from 1 to 6). Dotted lines indicate SARS-CoV negative human sera. **Panel c**: comparison between the average OD values of positive and negative SARS-CoV sera obtained from the recombinant based ELISA. The dilutions of sera are of 1:600 and 1:2,000 for M2 and N, respectively. White bars indicate the mean values ± SD of the SARS-CoV negative sera, grey bars indicate the values ± SD of the SARS-CoV positive sera.
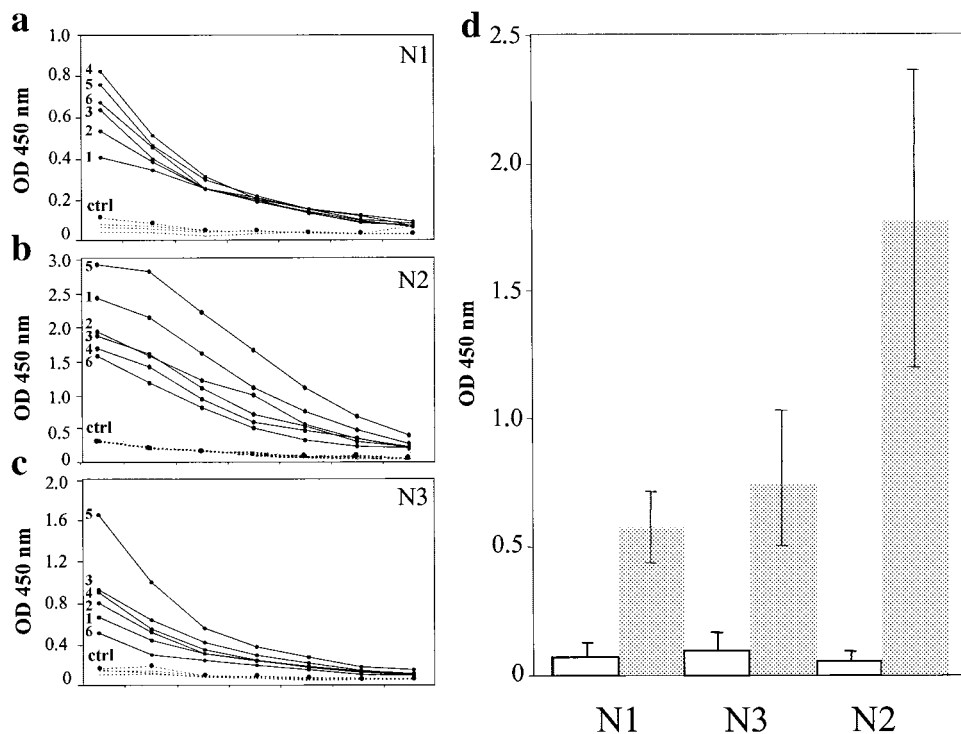

Fig. 3. Detection by ELISA of immunoglobulins specific to the N1 (**panel a**), N2 (**b**), and N3 (**c**) fragments of the N protein in SARS-CoV positive human sera. Optical density values are reported in ordinate, dilutions of the sera are reported in abscissa. In N1 and N3 based ELISA, sera dilutions are from 1:600 to 1:22,400. In N2 based ELISA, sera dilutions are from 1:2,000 to 1:128,000. Each human serum is indicate by a number (1–6) on the correspondent line. Dotted lines indicate SARS-CoV negative human sera. **Panel d**: comparison between the average of the OD values of positive and negative SARS-CoV sera obtained with the N fragments based ELISAs. The dilutions of sera are 1:600 for N1 and N3, and 1:2,000 for N2. White bars indicate the mean values ± SD of 90 SARS-CoV negative sera, grey bars indicate the mean values ± SD of the 6 SARS-CoV referent sera.
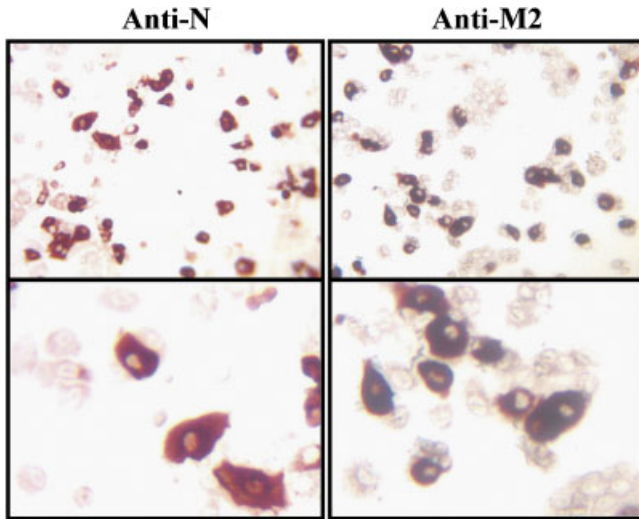
Fig. 4. Immunocytochemical staining of SARS-CoV infected and uninfected Vero cells by using rabbit anti-N and anti-M2 polyclonal antibodies at dilution of 1:1,600. Samples were magnified 12.5×16 (**top panels**) or 12.5×40 (**bottom panels**) times under light microscope.

Three N fragments (N1, N2, N3) were also expressed as described in Methods (Fig. 1, panel a). The deduced protein sequences of each N fragment were compared with the N sequences of the SARS-CoV Frankfurt 1 isolate revealing a 100% amino acid identity with the exception of the p-N2 construct where the Tyr at position 248 was replaced by Ala. The three N fragments were purified and used as coating antigen in ELISA to analyze the six SARS-CoV reference sera (Fig. 3). In all cases, clear-cut positive reactions of the six SARS-positive human sera were detected. In particular, the central N portion (N2) (panel b), demonstrated high levels of positive reaction, with an OD average value (at the dilution of 1:2,000) of 1.8 ± 0.6, with respect to the negative sera 0.05 ± 0.04. The N1 and N3 proteins generated a lower positive reaction, reaching at the 1:600 dilution the OD average values of 0.6 ± 0.1 (negative sera mean values: 0.07 ± 0.05) and 0.7 ± 0.3 (negative sera mean values: 0.09 ± 0.07), respectively. The specificity of the this ELISA assay (Fig. 2, panel e; Fig. 3, panel d) would appear to be rather high as no positive reaction was observed in the N- and N2-based ELISA at the 1:2,000 dilution and only two out of the 93 SARS-negative sera showed a weak positive reaction against all antigens at the 1:600 dilution, reading however twofold lower than the mean SARS-positive sera values. No positive reaction with any non-SARS sera was however observed at 1:1,200 dilution.

The results described above, showing high immunogenicity of the N and M proteins in SARS patients, prompted us to produce polyclonal antibodies against the recombinant proteins, to be used for the detection of viral antigens. Thus, all the five SARS-CoV recombinant proteins were purified and inoculated either in rabbit or in mice in order to generate specific hyperimmune sera. All viral proteins showed high immunogenicity in both animal species, inducing specific antibodies, reactive

both in ELISA and Western blotting (data not shown). The specificity of the sera was examined further by an immunocytochemical assay on slides containing a mixing of mock and SARS-CoV-infected cell. All sera showed strong reactivity against the infected cells, with a comparable pattern of cellular staining. In Figure 4 the immunocytochemical assays obtained with the anti-N and anti-M2 rabbit sera at the dilution of 1:1,600, are shown. In particular, the anti-N and anti-M2 rabbit sera could still detect viral antigen at the 1:25,000 dilution (data not shown). The anti-SARS-CoV murine sera generally showed a lower titre, irrespective of the antigen used (1:10,000). Both animal sera showed no cross-reactivity with other human Coronavirus (data not shown).

The low number of available SARS-positive sera would preclude a sound assessment of the sensitivity and diagnostic efficiency of the described in-house ELISA test. While our studies clearly warrant further, extended investigation, we would like to emphasize here the effectiveness of recombinant antigen-based ELISA tests, in particular with the use of the novel N2 and M2 antigen for the detection SARS-CoV-antibodies. In addition, our recombinant proteins have been found to be useful for the generation of hyperimmune sera to use for the detection of viral antigens in clinical specimens. Overall, our results strongly suggest the effectiveness of recombinant antigens M and N in laboratory diagnosis of SARS and invite to further, more extensive investigations to validate our methods.

REFERENCES

Chan PK, To WK, Ng KC, Lam RK, Ng TK, Chan RC, Wu A, Yu WC, Lee N, Hui DS, Lai ST, Hon EK, Li CK, Sung JJ, Tam JS. 2004. Laboratory diagnosis of SARS. Emerg Infect Dis 10:825–831.

Che XY, Hao W, Qiu LW, Pan YX, Liao ZY, Xu H, Chen JJ, Hou JL, Woo PC, Lau SK, Kwok YY, Huang Z. 2003. Antibody response of patients with severe acute respiratory syndrome (SARS) to nucleocapsid antigen of SARS-associated coronavirus. Di Yi Jun Yi Da Xue Xue Bao 23:637–639.

Chen H, Coote B, Attree S, Hiscox JA. 2003. Evaluation of a nucleoprotein-based enzyme-linked immunosorbent assay for the detection of antibodies against infectious bronchitis virus. Avian Pathol 32:519–526.

Di Bonito P, Nicoletti L, Mochi S, Accardi L, Marchi A, Giorgi C. 1999. Immunological characterization of Toscana virus proteins. Arch Virol 144:1947–1960.

Donnelly CA, Fisher MC, Fraser C, Ghani AC, Riley S, Ferguson NM, Anderson RM. 2004. Epidemiological and genetic analysis of severe acute respiratory syndrome. Lancet Infect Dis 4:672–683.

Elia G, Fiermonte G, Pratelli A, Martella V, Camero M, Cirone F, Buonavoglia C. 2003. Recombinant M protein-based ELISA test for detection of antibodies to canine coronavirus. J Virol Methods 109:139–142.

Leung DT, Tam FC, Ma CH, Chan PK, Cheung JL, Niu H, Tam JS, Lim PL. 2004. Antibody response of patients with severe acute

respiratory syndrome (SARS) targets the viral nucleocapsid. J Infect Dis 190:379–386.

Liu X, Shi Y, Li P, Li L, Yi Y, Ma Q, Cao C. 2004. Profile of antibodies to the nucleocapsid protein of the severe acute respiratory syndrome (SARS)-associated coronavirus in probable SARS patients. Clin Diagn Lab Immunol 11:227–228.

Ndifuna A, Waters AK, Zhou M, Collisson EW. 1998. Recombinant nucleocapsid protein is potentially an inexpensive, effective serodiagnostic reagent for infectious bronchitis virus. J Virol Methods 70:37–44.

Poon LL, Guan Y, Nicholls JM, Yuen KY, Peiris JS. 2004a. The aetiology, origins, and diagnosis of severe acute respiratory syndrome. Lancet Infect Dis 4:663–671.

Poon LL, Chan KH, Peiris JS. 2004b. Crouching tiger, hidden dragon: The laboratory diagnosis of acute respiratory syndrome. Clin Infect Dis 38:297–299.

Shi Y, Yi Y, Li P, Kuang T, Li L, Dong M, Ma Q, Cao C. 2003. Diagnosis of severe acute respiratory syndrome (SARS) by detection of SARS coronavirus nucleocapsid antibodies in an antigen-capturing enzyme-linked immunosorbent assay. J Clin Microbiol 41:5781–5782.

Shi Y, Wan Z, Li L, Li P, Li C, Ma Q, Cao C. 2004a. Antibody responses against SARS-coronavirus and its nucleocaspid in SARS patients. J Clin Virol 31:66–68.

Shi Y, Yi Y, Li P, Kuang T, Li L, Dong M, Ma Q, Cao C. 2004b. Detection of severe acute respiratory syndrome (SARS) coronavirus nucleo-capsid protein in SARS patients by enzyme-linked immunosorbent assay. J Clin Microbiol 42:2884–2889.

Thiel V, Ivanov KA, Putics A, Hertzig T, Schelle B, Bayer S, Weissbrich B, Snijder EJ, Rabenau H, Doerr HW, Gorbalenya AE, Ziebuhr J. 2003. Mechanisms and enzymes involved in SARS coronavirus genome expression. J Gen Virol 84:2305–2315.

Woo PC, Lau SK, Wong BH, Tsoi HW, Fung AM, Chan KH, Tam VK, Peiris JS, Yuen KY. 2004. Detection of specific antibodies to severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein for serodiagnosis of SARS coronavirus pneumonia. J Clin Microbiol 42:2306–2309.

Wu HS, Hsieh YC, Su IJ, Lin TH, Chiu SC, Hsu YF, Lin JH, Wang MC, Chen JY, Hsiao PW, Chang GD, Wang AH, Ting HW, Chou CM, Huang CJ. 2004. Early detection of antibodies against various structural proteins of the SARS-associated coronavirus in SARS patients. J Biomed Sci 11:117–126.