

Differential Sensitivities of Severe Acute Respiratory Syndrome (SARS) Coronavirus Spike Polypeptide Enzyme-Linked Immunosorbent Assay (ELISA) and SARS Coronavirus Nucleocapsid Protein ELISA for Serodiagnosis of SARS Coronavirus Pneumonia

Patrick C. Y. Woo,^{1,2†} Susanna K. P. Lau,^{1,2†} Beatrice H. L. Wong,^{1†} Hoi-wah Tsoi,¹
Ami M. Y. Fung,¹ Richard Y. T. Kao,^{1,2} Kwok-hung Chan,¹ J. S. Malik Peiris,^{1,2}
and Kwok-yung Yuen^{1,2*}

Department of Microbiology¹ and Research Centre of Infection and Immunology,² Faculty of Medicine, The University of Hong Kong, Hong Kong

Received 3 November 2004/Returned for modification 24 February 2005/Accepted 6 April 2005

The use of recombinant severe acute respiratory syndrome-coronavirus (SARS-CoV) nucleocapsid protein (N) enzyme-linked immunosorbent assay (ELISA)-based antibody and antigen tests for diagnosis of SARS-CoV infections have been widely reported. However, no recombinant SARS-CoV spike protein (S)-based ELISA is currently available. In this article, we describe the problems and solutions of setting up the recombinant SARS-CoV S-based ELISA for antibody detection. The SARS-CoV S-based immunoglobulin M (IgM) and IgG ELISAs were evaluated and compared with the corresponding N-based ELISA for serodiagnosis of SARS-CoV pneumonia, using sera from 148 healthy blood donors who donated blood 3 years ago as controls and 95 SARS-CoV pneumonia patients in Hong Kong. Results obtained by the recombinant S (rS)-based IgG ELISA using the regenerated S prepared by dialysis with decreasing concentrations of urea or direct addition of different coating buffers, followed by addition of different regeneration buffer, identified 4 M urea and 1 M sarcosine for plate coating and no regeneration buffer as the most optimal conditions for antibody detection. The specificities of the S-based ELISA for IgG and IgM detection were 98.6% and 93.9%, with corresponding sensitivities of 58.9% and 74.7%, respectively. The sensitivity of the rN IgG ELISA (94.7%) is significantly higher than that of the rS IgG ELISA ($P < 0.001$), whereas the sensitivity of the rS IgM ELISA is significantly higher than that of the rN IgM ELISA (55.2%) ($P < 0.01$). An ELISA for detection of IgM against S and N could be more sensitive than one that detects IgM against N alone for serodiagnosis of SARS-CoV pneumonia.

Severe acute respiratory syndrome (SARS), caused by the SARS coronavirus (SARS-CoV), is a new emerging disease that has affected 30 countries with more than 8,000 cases, causing more than 750 deaths (5, 6, 11, 15–17). For laboratory diagnosis of SARS-CoV pneumonia, isolation of the virus from clinical specimens is insensitive and requires biosafety level 3 laboratory facilities, while detection of viral RNA using reverse transcription-PCR can only achieve a sensitivity of 50 to 79%, depending on the type and number of clinical specimens collected and the protocol used (26). At the moment, the most widely used methods for serodiagnosis of SARS-CoV infection in clinical microbiology laboratories are antibody detection in acute- and convalescent-phase sera by indirect immunofluorescence assay and enzyme-linked immunosorbent assay (ELISA) using cell culture extracts (11, 16). However, antibody detection by indirect immunofluorescence assay using cell culture extracts may be less reproducible, more difficult to standardize, and more labor intensive than ELISA-based antibody detection tests using recombinant antigens. Furthermore, pro-

ducing the infected cell lines for coating the ELISA plates and the slides for indirect immunofluorescence requires cultivation of the SARS-CoV, for which biosafety level 3 laboratory facilities are required. Such facilities are not available in most clinical microbiology laboratories.

ELISA-based antibody detection tests using recombinant antigens are well known to offer higher levels of reproducibility, are easy to standardize and less labor intensive than antibody detection by indirect immunofluorescence assay and ELISA using cell culture extract, and do not require cultivation of the SARS-CoV (1, 2, 21, 27). We have reported the use of recombinant SARS-CoV nucleocapsid protein (N) ELISA-based antibody and antigen tests for diagnosis of SARS-CoV infections (4, 12, 22–25). Others have also used similar approaches for serodiagnosis of SARS-CoV pneumonia (13, 18, 20). Recently, one group employed recombinant nucleocapsid-spike fusion protein expressed in insect (Sf9) cells as the antigen in an immunofluorescence assay for detection of SARS-CoV antibodies (8). Although recombinant N (rN) immunoglobulin G (IgG) ELISA achieved a sensitivity of 94.3% for serodiagnosis of SARS-CoV pneumonia, a sensitivity of only 59.4% can be achieved for the IgM ELISA (23). Since the spike protein (S), another immunogenic protein of SARS-CoV virus, is located on the surface of the viral particles and therefore potentially

* Corresponding author. Mailing address: Department of Microbiology, The University of Hong Kong, University Pathology Building, Queen Mary Hospital, Pokfulam, Hong Kong. Phone: (852) 28554892. Fax: (852) 28551241. E-mail: hkumicro@hkucc.hku.hk.

† These authors contributed the same to the manuscript.

more accessible to the immune system, rS-based ELISA may offer higher sensitivities than rN-based ELISA. Paradoxically, in one report, it was noted that the S-based antibody test appeared to have lower sensitivity than the N-based antibody test by Western blot analysis (10). However, the sample size was relatively small, and only N-based ELISA was subsequently developed. In other reports, the authors have used pooled S and N peptide-based ELISA for serosurveillance studies (3, 9). Currently, there is no S-based ELISA available for serodiagnosis of SARS-CoV infections.

In this article, we describe the problems and solutions of setting up the recombinant SARS-CoV S-based ELISA for antibody detection. The SARS-CoV S-based IgM and IgG ELISA were evaluated and compared to the corresponding N-based ELISA for serodiagnosis of SARS-CoV pneumonia.

MATERIALS AND METHODS

rN-based IgM and IgG ELISA for SARS-CoV pneumonia. Cloning and purification of His₆-tagged rN have been reported previously (22, 23). Sera from 148 healthy blood donors who donated blood 3 years ago were used to set up the baseline of the ELISA (22).

Cloning and purification of His₆-tagged rS from *Escherichia coli*. The cloning and purification of His₆-tagged rS have been reported previously (22). Briefly, to produce a plasmid for protein expression, primers LPW742 (5'-CGCGGATCC GAGTGACCTTGACCGGTGC-3') and LPW931 (5'-CGGGGTACCTTAAC GTAATAAGAAACTGTATG-3') were used to amplify the gene encoding amino acid residues 14 to 667 of the S of the SARS-CoV by reverse transcription-PCR. This portion of the S was used because it contains the receptor-binding domain within the S1 domain that is highly immunogenic, whereas the complete S was not expressible in *E. coli*. The PCR product was cloned into the BamHI and KpnI sites of vector pQE-31 (QIAGEN, Hilden, Germany). The resulting clone was digested by PstI, and the PstI fragment, which contained the gene encoding amino acid residues 250 to 667 of the S, was cloned into expression vector pQE-30 (QIAGEN, Hilden, Germany) in frame and downstream of the series of six histidine residues. The His₆-tagged rS was expressed and purified from the insoluble fraction using the Ni²⁺-loaded HiTrap Chelating System (Amersham Pharmacia) according to the manufacturer's instructions.

Development of rS-based IgG ELISA by different methods of S regeneration. The ELISA-based IgG antibody test was performed using the regenerated S prepared by dialysis using decreasing concentrations of urea or direct addition of different coating buffers (1 M *N*-acetylglucosamine, 4 M urea, 4 M urea with 1 M sarcosine, 4 M urea with 2 M sarcosine, 4 M urea with 3 M sarcosine, or 4 M urea with 4 M sarcosine), followed by addition of different regeneration buffers (4 M urea, 0.1 M Tris-HCl, 1 mM EDTA [pH 8] with 10% glycerol, 30% glycerol, 50% glycerol, 1 M sarcosine, 2 M sarcosine, 3 M sarcosine, 4 M sarcosine, 0.5 M ammonium sulfate, 1 M ammonium sulfate, 0.5 M *N*-acetyl-glucosamine, 1 M *N*-acetyl-glucosamine, or 1 M glucose) or no regeneration buffer. Each well of a MaxiSorp Nunc-immuno 96 MicroWell Plate (Nalge Nunc International, Rochester, N.Y.) was coated with 10 ng of purified His₆-tagged rS prepared by dialysis (determined by box titration using different dilutions of His₆-tagged rS as the coating antigen and pooled sera from two SARS-CoV pneumonia patients positive for antibody against the SARS-CoV) or direct addition of different coating buffers and incubated at 4°C for 16 h. The wells coated with regenerated S prepared by dialysis were blocked in phosphate-buffered saline with 5% skim milk, whereas different regeneration buffers were added to the wells coated with S prepared in different coating buffers and incubated at 37°C for 1 h before being blocked in phosphate-buffered saline with 5% skim milk. Diluted (1:20) human sera, pooled from 10 healthy blood donors and two SARS-CoV pneumonia patients positive for antibody against the SARS-CoV by indirect immunofluorescence (16), respectively, were added to the wells of the His₆-tagged rS-coated plates in a total volume of 100 µl and incubated at 37°C for 2 h. After being washed five times with washing buffer, 100 µl of diluted horseradish peroxidase-conjugated goat anti-human IgG (1:4,000) antibodies (Zymed Laboratories, Inc., South San Francisco, CA) was added to the wells and incubated at 37°C for 1 h. After being washed five times with washing buffer, 100 µl diluted 3,3',5,5'-tetramethylbenzidine (Zymed Laboratories, Inc.) was added to each well and incubated at room temperature for 15 min. One hundred microliters of 0.3 M H₂SO₄ was added, and the absorbance at 450 nm of each well was measured.

Each sample was tested in duplicate, and the mean absorbance for each serum was calculated.

Evaluation of rS-based IgG and IgM ELISA for SARS-CoV pneumonia. Sera from the 148 healthy blood donors who donated blood 3 years ago, all negative for IgG antibodies against the SARS-CoV detected by our indirect immunofluorescence assay, and 95 SARS-CoV pneumonia patients, positive for IgG antibodies against the SARS-CoV detected by our indirect immunofluorescence assay (16), were used for the evaluation of the rS-based IgG and IgM ELISA. Serum samples positive for IgG antibodies against SARS-CoV by indirect immunofluorescence assay from the 95 SARS-CoV pneumonia patients were taken at a median of 25 days (range, 12 to 43 days) from the onset of symptoms. The rS-based IgG ELISA was performed as described above, using 60 ng of S per well in coating buffer with 4 M urea and 1 M sarcosine for plate coating, no regeneration buffer, and serum dilution at 1:80. For the rS-based IgM ELISA, the conditions for the IgG ELISA was used, with 200 ng of rS per well, serum dilution of 1:80, and diluted horseradish peroxidase conjugate goat anti-human IgM (1:10,000) antibodies (Biosource International, CA).

Comparison of rS-based and rN-based IgG and IgM ELISAs for SARS-CoV pneumonia. The same sera of the 95 SARS-CoV pneumonia patients positive for IgG antibodies against the SARS-CoV were tested using the N-based IgG and IgM ELISA for SARS-CoV pneumonia by our previously described method (23). The sensitivities of the rN-based IgG and IgM ELISA and rS-based IgG and IgM ELISA for SARS-CoV pneumonia were compared by the McNemars test.

RESULTS

Development of rS-based IgG ELISA by different methods of S regeneration and evaluation of rS-based IgG and IgM ELISA for SARS-CoV pneumonia. Results obtained by the rS-based IgG ELISA using the regenerated S prepared by dialysis with decreasing concentrations of urea or direct addition of different coating buffers followed by addition of different regeneration buffer identified 4 M urea and 1 M sarcosine for plate coating and no regeneration buffer as the most optimal conditions for subsequent experiments, as determined by examining the maximum optical density values at 450 nm (OD₄₅₀) of titrations of the positive and negative pools of sera.

Box titration was carried out with different dilutions of His₆-tagged rS as the coating antigen and pooled sera from two SARS-CoV pneumonia patients positive for antibody against the SARS-CoV. The results identified 60 ng and 200 ng of purified His₆-tagged rS per ELISA well as the ideal amount for plate coating for IgG and IgM detection, respectively.

To establish the baseline for the IgG and IgM ELISA, serum samples (all tested negative by the indirect immunofluorescence assay) from 148 healthy blood donors who donated blood 3 years ago were tested by the rS-based IgG and IgM ELISA. For the 148 specimens from healthy blood donors, the mean ELISA OD₄₅₀ values for IgM and IgG detection were 0.059 and 0.235, respectively, with standard deviations of 0.041 and 0.122. Absorbance values of 0.141 and 0.479 were selected as the cutoff values (that equaled the sum of the mean values from the healthy control and two times the standard deviations) (Fig. 1). Using these cutoff values, two of the sera obtained from the 148 healthy blood donors had OD₄₅₀ values of >0.479 by the IgG ELISA, and nine had OD₄₅₀ values of >0.141 by the IgM ELISA (Fig. 1). The specificities of the IgG and IgM ELISA were 98.6% and 93.9%, respectively.

The mean OD₄₅₀ values (IgG and IgM) for the sera obtained from the 95 SARS-CoV pneumonia patients, positive for IgG antibodies against the SARS-CoV detected by our indirect immunofluorescence assay, were 0.690 and 0.339, with standard deviations of 0.494 and 0.347. Fifty-six sera had OD₄₅₀ values of >0.479 by the IgG ELISA and 71 had OD₄₅₀ values

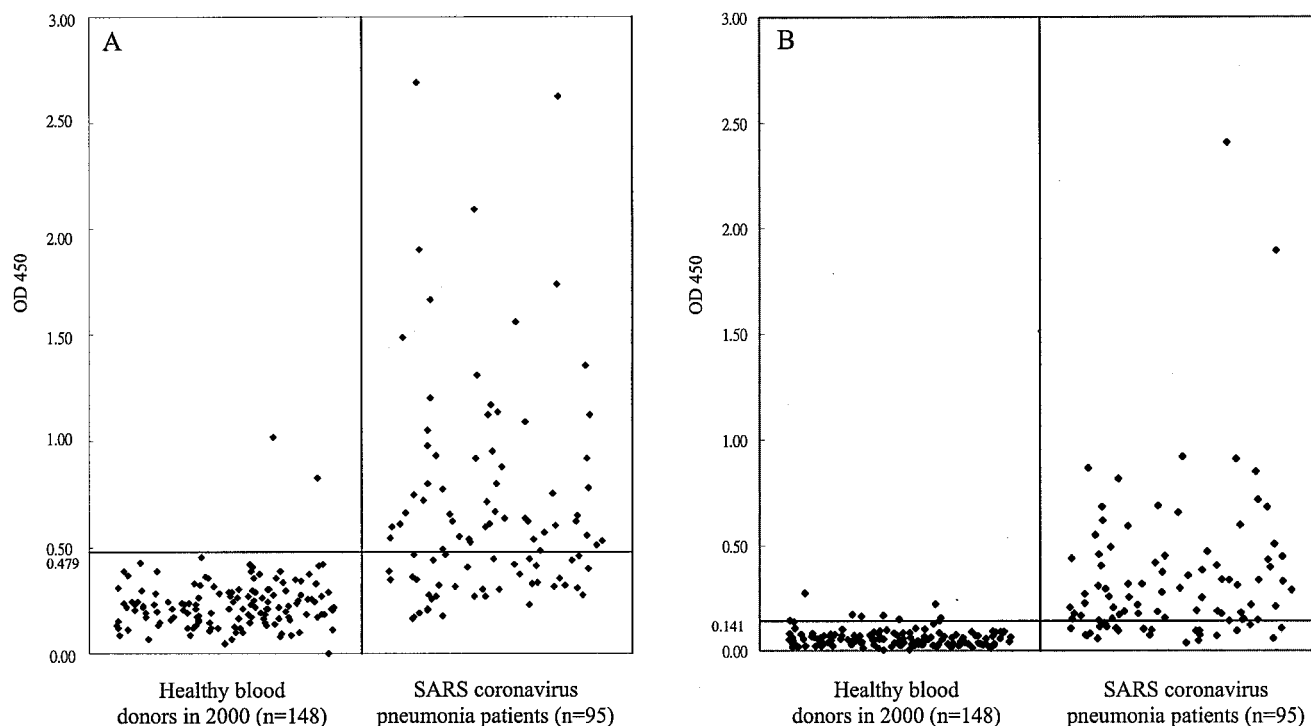


FIG. 1. Evaluation of sensitivity and specificity of rS-based IgG (A) and IgM (B) antibody ELISA for SARS-CoV pneumonia. Serum specimens were obtained from 95 patients with SARS-CoV pneumonia, and control serum specimens were obtained from 148 healthy blood donors. The test results were plotted as OD₄₅₀ values. The cutoff line for positive diagnosis is drawn at a value that equals the sum of the mean value and two times the standard deviation for the healthy blood donors.

of >0.141 by the IgM ELISA (Fig. 1). The sensitivities of the IgG and IgM ELISA, using the indirect immunofluorescence assay as the gold standard, were hence 58.9% and 74.7%, respectively.

Comparison of rS-based and rN-based IgG and IgM ELISAs for SARS-CoV pneumonia. The same 95 serum samples of the SARS-CoV pneumonia patients positive for IgG antibodies against the SARS-CoV were tested using the N-based IgG and IgM ELISA for SARS-CoV pneumonia. Ninety (94.7%) and 53 (55.8%) of the 95 serum samples were positive by the rN-based IgG and IgM ELISA, respectively. The sensitivity of the rN IgG ELISA was significantly higher than that of the rS IgG ELISA ($P < 0.001$), whereas the sensitivity of the rS IgM ELISA was significantly higher than that of the rN IgM ELISA ($P < 0.01$).

The sensitivities of the rS-based ELISA and rN-based ELISA for detection of IgG and IgM in serum samples obtained from patients at different periods after disease onset are shown in Table 1. For IgG detection, the sensitivity of the rN ELISA was significantly higher than the rS-based ELISA for serum samples obtained from patients at 16 to 20, 21 to 25, and 26 to 30 days after disease onset ($P < 0.005$, <0.001 , and < 0.05 , respectively). For IgM detection, the sensitivity of the rS-based ELISA was significantly higher than the rN-based ELISA at 21 to 25 days after disease onset ($P < 0.05$).

Performance of combination of rN-based and rS-based ELISAs. The results for IgG and IgM detection in the 95 serum samples from patients with SARS-CoV pneumonia when the rN-based ELISA and the rS-based ELISA were combined is

shown in Table 2. For IgG detection, there was no significant difference between the sensitivity of the two ELISAs combined (97%) and that of the rN-based ELISA (95%), but the sensitivity of the two ELISAs combined and that of the rN-based ELISA were significantly higher than that of the rS-based ELISA (59%; $P < 0.001$ in both comparisons). For IgM detection, the sensitivity of the two ELISAs combined (84%) and that of the rS-based ELISA (75%) were significantly higher than that of the rN-based ELISA (55%; $P < 0.001$ and $P < 0.01$, respectively), but there was no significant difference between the sensitivity of the two ELISAs combined and that of the rS-based ELISA.

TABLE 1. Differential sensitivities of rS- and rN-based ELISA for detection of IgG and IgM in serum samples obtained at different periods after disease onset

Days after disease onset	No. of serum samples	No. (%) positive for IgG by ELISA		No. (%) positive for IgM by ELISA	
		N based	S based	N based	S based
11–15	2	2 (100)	0 (0)	1 (50)	1 (50)
16–20	17	15 (88)	7 (41)	11 (65)	13 (76)
21–25	31	31 (100)	18 (58)	15 (48)	24 (77)
26–30	27	24 (89)	16 (59)	16 (59)	21 (78)
31–35	7	7 (100)	6 (86)	3 (43)	4 (57)
36–40	5	5 (100)	4 (80)	4 (80)	4 (80)
41–45	6	6 (100)	5 (83)	3 (50)	4 (67)

TABLE 2. Summary of results for the 95 serum samples from patients with SARS-CoV pneumonia

ELISA	Results (no. [%] of serum samples)			
	Positive by both N- and S-based ELISA	Positive by N-based but negative by S-based ELISA	Positive by S-based but negative by N-based ELISA	Negative by both N- and S-based ELISA
IgG	54 (57)	36 (38)	2 (2)	3 (3)
IgM	44 (46)	9 (9)	27 (29)	15 (16)

DISCUSSION

In this study, we report the development of the rS-based ELISA for serodiagnosis of SARS-CoV pneumonia. Although previous studies have been able to detect S-specific antibodies in patients with SARS by Western blot analysis or immunofluorescence assay (10, 19), there are no published data on the availability of S-based ELISA for high-throughput analysis of antibodies against SARS-CoV S. This is likely due to the difficulties in stably expressing and purifying the S, which is glycosylated with high mannose and/or hybrid oligosaccharides. The detection of S by immunoassays using human convalescent-phase sera was found to be difficult by some researchers, who even suggested that the protein may not be strongly immunogenic (7). When we first performed the ELISA for IgG detection using regenerated S prepared by the traditional method of dialysis with decreasing concentrations of urea, a significant amount of S was lost during dialysis. Moreover, 600 ng of rS was required for coating the ELISA plate to obtain a reasonable absorbance value for sera obtained from patients with SARS-CoV pneumonia (data not shown). When 10 ng of rS was used for coating the ELISA plates, only a very low absorbance value could be achieved. Since it was very time consuming, labor intensive, and expensive to produce such a large amount of rS for coating ELISA plates, various other methods that were reported in the literature for regeneration of resolubilized denatured proteins were examined for their usefulness in the regeneration of the S. In a previous study, 4 M urea with 4 M sarcosine was observed to be useful for regeneration of lysozyme, an enzyme well known to be difficult to regenerate after denaturation (14). In the present study, we found that 4 M urea with 1 M sarcosine for plate coating and no regeneration buffer were the most optimal conditions for the ELISA (ratio of OD₄₅₀ for positive control to OD₄₅₀ for negative control = 3.85), better than using 4 M urea alone or with 2, 3, or 4 M sarcosine (ratios of OD₄₅₀ for positive control to OD₄₅₀ for negative control = 2.15, 3.25, 3.13, and 2.76, respectively).

The rN-based IgG ELISA is more sensitive than the rS-based IgG ELISA, but the rS-based IgM ELISA is more sensitive than the rN-based IgM ELISA for SARS-CoV pneumonia. We have reported that the rN-based IgG ELISA was useful for serodiagnosis of SARS-CoV pneumonia, especially during an epidemic of SARS. As the prevalence of background seropositivity in the general population increases due to SARS-CoV pneumonia and nonpneumonic SARS-CoV infections, single IgG readings may not be useful for serodiagnosis of SARS-CoV pneumonia. In such circumstances, detection of

IgM antibody or a rise in IgG titers in serial samples may be the method of choice for serodiagnosis of SARS-CoV pneumonia. However, a sensitivity of only 60% was achieved when the rN-based ELISA for IgM detection was used for serodiagnosis of SARS-CoV pneumonia. Therefore, alternative targets must be sought to achieve higher sensitivity in IgM detection. The differential sensitivity of the rS-based and rN-based IgG ELISA demonstrated in the present report is in line with the results of a recent study, which showed that 89% of patients with SARS-CoV pneumonia had a highly restricted IgG dominated antibody response directed at the N, but only 63% of patients had such an antibody response directed at the S (13). As the difference in sensitivities of the two IgG ELISA decreases with time after disease onset (Table 1), we speculate that this difference is due to a relatively late IgG response to the S compared to the IgG response to the N in patients with SARS-CoV pneumonia, which is in line with results from a previous study using an immunofluorescence method for S expression (19). On the other hand, the difference in sensitivities of the two IgM ELISAs occurred evenly throughout the whole course of the illness, although in some patient categories, such as 11 to 15 days after disease onset, the number of serum samples was too small for statistical comparison (Table 1). This difference could be due to an intrinsic difference in the IgM response to the two antigens. An ELISA for detection of IgM against S and N could be more sensitive than one that detects IgM against N alone for serodiagnosis of SARS-CoV pneumonia, as 84% of the sera in this study contained IgM against S and/or N, but only 55% of them contained IgM against N (Table 2). On the other hand, an ELISA for detection of IgG against S and N would not be more sensitive than one that detected IgG against N alone for serodiagnosis of SARS-CoV pneumonia, as the ELISA for detecting IgG against N is already able to catch 95% of the positive sera (Table 2). Further studies could be performed using ELISAs using a cocktail of S and N.

ACKNOWLEDGMENTS

This study was supported by the Research Fund for the Control of Infectious Diseases of the Health, Welfare, and Food Bureau of the Hong Kong SAR Government (01030282); a Research Grant Council grant, SARS Research Fund; and the University SARS Donation Fund, The University of Hong Kong.

REFERENCES

1. Briese, T., C. G. Hatalski, S. Kliche, Y. S. Park, and W. I. Lipkin. 1995. Enzyme-linked immunosorbent assay for detecting antibodies to Borne disease virus-specific proteins. *J. Clin. Microbiol.* **33**:348–351.
2. Chan, C. M., P. C. Y. Woo, A. S. P. Leung, S. K. P. Lau, X. Y. Che, L. Cao, and K. Y. Yuen. 2002. Detection of specific antibodies to an antigenic cell wall galactomannoprotein for serodiagnosis of *Aspergillus fumigatus* aspergillosis. *J. Clin. Microbiol.* **40**:2041–2045.
3. Chan, P. K., W. K. To, E. Y. Liu, T. K. Ng, J. S. Tam, J. J. Sung, J. M. Lacroix, and M. Houde. 2004. Evaluation of a peptide-based enzyme immunoassay for anti-SARS coronavirus IgG antibody. *J. Med. Virol.* **74**:517–520.
4. Che, X. Y., L. W. Qiu, Y. X. Pan, K. Wen, W. Hao, L. Y. Zhang, Y. D. Wang, Z. Y. Liao, X. Hua, V. C. Cheng, and K. Y. Yuen. 2004. Sensitive and specific monoclonal antibody-based capture enzyme immunoassay for detection of nucleocapsid antigen in sera from patients with severe acute respiratory syndrome. *J. Clin. Microbiol.* **42**:2629–2635.
5. Drosten, C., S. Günther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. M. Fouchier, A. Berger, A. M. Burguière, J. Cinatl, M. Eickmann, N. Escρίου, K. Grywna, S. Kramme, J. C. Manuguerra, S. Müller, V. Rickerts, M. Stürmer, S. Vieth, H. D. Klenk, A. D. M. E. Osterhaus, H. Schmitz, and H. W. Doerr. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N. Engl. J. Med.* **348**:1967–1976.

6. Guan, Y., B. J. Zheng, Y. Q. He, X. L. Liu, Z. X. Zhuang, C. L. Cheung, S. W. Luo, P. H. Li, L. J. Zhang, Y. J. Guan, K. M. Butt, K. L. Wong, K. W. Chan, W. Lim, K. F. Shortridge, K. Y. Yuen, J. S. Peiris, and L. L. Poon. 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science* **302**:276–278.
7. Han, D. P., H. G. Kim, Y. B. Kim, L. L. Poon, and M. W. Cho. 2004. Development of a safe neutralization assay for SARS-CoV and characterization of S-glycoprotein. *Virology* **326**:140–149.
8. He, Q., I. Manopo, L. Lu, B. P. Leung, H. H. Chng, A. E. Ling, L. L. Chee, S. W. Chan, E. E. Ooi, Y. L. Sin, B. Ang, and J. Kwang. 2005. Novel immunofluorescence assay using recombinant nucleocapsid-spike fusion protein as antigen to detect antibodies against severe acute respiratory syndrome coronavirus. *Clin. Diagn. Lab. Immunol.* **12**:321–328.
9. Hsueh, P. R., C. L. Kao, C. N. Lee, L. K. Chen, M. S. Ho, C. Sia, X. D. Fang, S. Lynn, T. Y. Chang, S. K. Liu, A. M. Walfield, and C. Y. Wang. 2004. SARS antibody test for serosurveillance. *Emerg. Infect. Dis.* **10**:1558–1562.
10. Huang, L. R., C. M. Chiu, S. H. Yeh, W. H. Huang, P. R. Hsueh, W. Z. Yang, J. Y. Yang, I. J. Su, S. C. Chang, and P. J. Chen. 2004. Evaluation of antibody responses against SARS coronavirus nucleocapsid or spike proteins by immunoblotting of ELISA. *J. Med. Virol.* **73**:338–346.
11. Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, M. S. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, L. J. Anderson, and the SARS Working Group. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* **348**:1953–1966.
12. Lau, S. K. P., P. C. Y. Woo, B. H. L. Wong, G. K. S. Woo, R. W. S. Poon, H. W. Tsoi, K. H. Chan, W. I. Wei, J. S. M. Peiris, and K. Y. Yuen. 2004. Detection of SARS-CoV nucleocapsid protein in SARS patients by enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **42**:2884–2889.
13. Leung, D. T., F. C. Tam, C. H. Ma, P. K. Chan, J. L. Cheung, H. Niu, J. S. Tam, and P. L. Lim. 2004. Antibody response of patients with severe acute respiratory syndrome (SARS) targets the viral nucleocapsid. *J. Infect. Dis.* **190**:379–386.
14. Maeda, Y., H. Yamada, T. Ueda, and T. Imoto. 1996. Effect of additives on the renaturation of reduced lysozyme in the presence of 4 M urea. *Protein Eng.* **9**:461–465.
15. Peiris, J. S. M., C. M. Chu, V. C. C. Cheng, K. S. Chan, I. F. N. Hung, L. L. M. Poon, K. I. Law, B. S. F. Tang, T. Y. W. Hon, C. S. Chan, K. H. Chan, J. S. C. Ng, B. J. Zheng, W. L. Ng, R. W. M. Lai, Y. Guan, K. Y. Yuen, and the HKU/UCH SARS Study Group. 2003. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia—a prospective study. *Lancet* **361**:1767–1772.
16. Peiris, J. S. M., S. T. Lai, L. L. M. Poon, Y. Guan, L. Y. C. Yam, W. Lim, J. Nicholls, W. K. S. Yee, W. W. Yan, M. T. Cheung, V. C. C. Cheng, K. H. Chan, D. N. C. Tsang, R. W. H. Yung, T. K. Ng, K. Y. Yuen, and the SARS Study Group. 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* **361**:1319–1325.
17. Poutanen, S. M., D. E. Low, B. Henry, S. Finkelstein, D. Rose, K. Green, R. Tellier, R. Draker, D. Adachi, M. Ayers, A. K. Chan, D. M. Skowronski, I. Salit, A. E. Simor, A. S. Slutsky, P. W. Doyle, M. Krajden, M. Petric, R. C. Brunham, A. J. McGeer, the National Microbiology Laboratory, Canada, and the Canadian Severe Acute Respiratory Syndrome Study Team. 2003. Identification of severe acute respiratory syndrome in Canada. *N. Engl. J. Med.* **348**:1995–2005.
18. Shi, Y., Y. Yi, P. Li, T. Kuang, L. Li, M. Dong, Q. Ma, and C. Cao. 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.
19. Tan, Y. J., P. Y. Goh, B. C. Fielding, S. Shen, C. F. Chou, J. L. Fu, H. N. Leong, Y. S. Leo, E. E. Ooi, A. E. Ling, S. G. Lim, and W. Hong. 2004. Profiles of antibody responses against severe acute respiratory syndrome coronavirus recombinant proteins and their potential use as diagnostic markers. *Clin. Diagn. Lab. Immunol.* **11**:362–371.
20. Timani, K. A., L. Ye, L. Ye, Y. Zhu, Z. Wu, and Z. Gong. 2004. Cloning, sequencing, expression, and purification of SARS-associated coronavirus nucleocapsid protein for serodiagnosis of SARS. **30**:309–312.
21. Woo, P. C. Y., K. T. K. Chong, A. S. P. Leung, S. S. Y. Wong, S. K. P. Lau, and K. Y. Yuen. 2003. *AFLMPL1* encodes an antigenic cell wall protein in *Aspergillus flavus*. *J. Clin. Microbiol.* **41**:845–850.
22. Woo, P. C. Y., S. K. P. Lau, H. W. Tsoi, K. H. Chan, B. H. L. Wong, X. Y. Che, V. K. P. Tam, S. C. F. Tam, V. C. C. Cheng, I. F. N. Hung, S. S. Y. Wong, B. J. Zheng, Y. Guan, and K. Y. Yuen. 2004. Relative rates of non-pneumonic SARS coronavirus infection and SARS coronavirus pneumonia. *Lancet* **363**:841–845.
23. Woo, P. C. Y., S. K. P. Lau, B. H. L. Wong, H. W. Tsoi, A. M. Y. Fung, K. H. Chan, V. K. P. Tam, J. S. M. Peiris, and K. Y. Yuen. 2004. Detection of specific antibodies to SARS coronavirus nucleocapsid protein for serodiagnosis of SARS coronavirus pneumonia. *J. Clin. Microbiol.* **42**:2306–2309.
24. Woo, P. C. Y., S. K. P. Lau, B. H. L. Wong, K. H. Chan, C. M. Chu, H. W. Tsoi, Y. Huang, J. S. M. Peiris, and K. Y. Yuen. 2004. Longitudinal profile of immunoglobulin G (IgG), IgM, and IgA antibodies against the severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein in patients with pneumonia due to the SARS coronavirus. *Clin. Diagn. Lab. Immunol.* **11**:665–668.
25. Woo, P. C. Y., S. K. P. Lau, B. H. L. Wong, K.-H. Chan, W.-T. Hui, G. S. W. Kwan, J. S. M. Peiris, R. B. Couch, and K.-Y. Yuen. 2004. False-positive results in a recombinant severe acute respiratory syndrome-associated coronavirus (SARS-CoV) nucleocapsid enzyme-linked immunosorbent assay due to HCoV-OC43 and HCoV-229E rectified by Western blotting with recombinant SARS-CoV spike polypeptide. *J. Clin. Microbiol.* **42**:5885–5888.
26. Yam, W. C., K. H. Chan, L. L. M. Poon, Y. Guan, K. Y. Yuen, W. H. Seto, and J. S. M. Peiris. 2003. Evaluation of reverse transcription-PCR assays for rapid diagnosis of severe acute respiratory syndrome associated with a novel coronavirus. *J. Clin. Microbiol.* **41**:4521–4524.
27. Yuen, K. Y., C. M. Chan, K. M. Chan, P. C. Y. Woo, X. Y. Che, A. S. P. Leung, and L. Cao. 2001. Characterization of *AFMPL1*: a novel target for serodiagnosis of aspergillosis. *J. Clin. Microbiol.* **39**:3830–3837.