Recombinant Protein-Based Assays for Detection of Antibodies to Severe Acute Respiratory Syndrome Coronavirus Spike and Nucleocapsid Proteins

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Received 26 September 2006/Returned for modification 31 October 2006/Accepted 8 January 2007

The 2003 outbreak of severe acute respiratory syndrome (SARS) lasted fewer than 9 months, yet it had a major impact on public health and socioeconomics. Since the end of the SARS outbreak, there have been 17 identified SARS-associated coronavirus (SARS-CoV) infections, 6 from direct laboratory exposure, 1 of which resulted in community transmission to seven individuals, and 4 sporadic, community-acquired infections (9). Since additional cases may occur and could, if undetected, quickly lead to another global outbreak, it is important to continue to improve our ability to reliably monitor SARS-CoV infections (3, 16, 18).

As with other coronaviruses, the spike (S) and nucleocapsid (N) proteins are abundantly expressed during virus infection and are most effective among the coronavirus structural proteins at inducing antibody responses (10, 14, 15, 23). Previous studies have demonstrated the utility of anti-N and anti-S proteins in the diagnosis of SARS-CoV infections (2, 5, 12, 21). In this study, we describe the evaluation and comparison of recombinant spike and nucleocapsid enzyme-linked immunosorbent assays (ELISAs) for specifically detecting SARS-CoV infection.

The recombinant full-length SARS N gene was amplified from SARS-CoV RNA (Urbani strain), modified to contain a C-terminal His6 tag, and cloned into the Venezuelan equine encephalitis virus replicon vector (17). Baby hamster kidney (BHK) cells were transfected with SARS N replicon RNA by electroporation. Cells were harvested, and expressed protein was purified by metal affinity chromatography and analyzed by silver staining and Western blot analysis for the appropriately sized (50-kDa) immunoreactive protein (8). The control antigen, the nontoxic 50-kDa C-terminal fragment of the botulinum neurotoxin serotype A (BoNT/HcA), was expressed as described above (7).

The soluble codon-optimized SARS-CoV S glycoprotein (170 kDa; amino acids 1 to 1190; S1190) and the control antigen, truncated angiotensin-converting enzyme 2 (120 kDa; glycosylated; tACE-2), were cloned into pcDNA3.1 Myc/His antigen, truncated angiotensin-converting enzyme 2 (120 kDa; amino acids 1 to 1190; S1190) and the control antigen tACE-2. The plates were washed and incubated with serum diluted 1:400 in phosphate-buffered saline (PBS) containing skim milk and Tween 20 (PBS-T-M) for 1 h at 37°C and washed and incubated again with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (1:4,000; heavy plus light chain [KPL]in PBS-T-M. After washing, substrate [ABTS; 2,2'-azinobis(3-ethylbenzthiazolesulfonic acid)] was added and the absorbance read at 405 nm with a 490-nm reference filter.

ELISA conditions were optimized using antibody-positive and -negative serum specimens, and the resultant assays were then evaluated using available serum samples collected from 61 patients from Vietnam and Taiwan with laboratory-confirmed SARS-CoV infection (2 to 150 days postonset of symp-
activity by either assay (data not shown). In addition, serum samples from non-SARS patients from Taiwan and Vietnam showed no reactivity. Control samples were analyzed. None of the serum samples were positive by ELISA, resulting in specificities of 98.6 and 99.4% for the N and S protein ELISAs, respectively (Table 1). Control samples reactive to either the N or S protein did not show reactivity to the alternate protein and also showed no reactivity against the inactivated SARS-CoV lysate by ELISA. In these samples, reactivity may have been due to nonspecific reactivity or cross-reactivity with proteins of other human CoVs and is the focus of further study. These false positives could present a public health dilemma as illustrated in the laboratory evaluation of four sporadic cases reported by Liang et al. (9). In those cases, it was necessary to conduct confirmatory testing using several different types of assays, because there was concern

<table>
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<th>Antigen</th>
<th>SARS patients</th>
<th>Healthy donors</th>
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<tr>
<td></td>
<td>No. with positive reactivity/total</td>
<td>% Sensitivity</td>
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<tr>
<td>SARS-N</td>
<td>61/61</td>
<td>100</td>
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<tr>
<td>SARS-S</td>
<td>59/61</td>
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All 61 sera from SARS cases were antibody positive to the N protein, and 59 were positive to the S protein. Two acute-phase specimens (≤20 dpo) were weakly reactive to the S protein and fell below the assay cutoff of 0.201. All were also positive by both immunofluorescence antibody testing and ELISA, using whole, gamma-irradiated SARS-CoV as the antigen (data not shown). The sensitivities for the N and S protein assays were 100% and 96.7%, respectively.

Persistent levels of SARS-CoV IgG have been detected in SARS cases for several months and up to 2 years after disease onset (4, 11, 12, 19, 21). In this study, serum samples from 48 SARS patients from Vietnam and the United States were collected 221 to 735 days (44 from days 221 to 250; 4 from days 633 to 735) after the onset of illness and tested for the presence of SARS-CoV- and N- and S-protein-specific IgG by ELISA. Antibodies (IgG) specific to whole virus, N protein, and S protein were detected in 40 (83.3%), 65 (93.8%), and 36 (75%) of the samples tested, respectively. Interestingly, anti-SARS-CoV and S antibodies were detected in three patients, two of whom also demonstrated a response to the N protein, almost 2 years after SARS-CoV infection. Although very few serum specimens from unexposed persons (<1.5%) tested positive for SARS-CoV infection, the potential for cross-reactivity between SARS-CoV and other coronaviruses, including the known human coronaviruses HCoV-OC43, HCoV-229E, and recently identified HKU1 and NL63, remains a concern (12, 13, 22). Whether these positive results are due to nonspecific reactivity to the recombinant SARS N protein or to cross-reactivity to other human CoVs requires further study. The use of protein fragments or peptides, instead of the whole recombinant N protein, for antibody detection may resolve the issue of potential cross-reactivity with proteins of other human CoVs and is the focus of further study. These false positives could present a public health dilemma as illustrated in the laboratory evaluation of four sporadic cases reported by Liang et al. (9). In those cases, it was necessary to conduct confirmatory testing using several different types of assays, because there was concern
that infection with non-SARS coronaviruses may induce cross-reacting antibodies (9). Our data from that study suggest a combination of assays may be needed to confirm the specificity of presumed SARS antibodies. Since the costs (i.e., public health interventions or outbreak response) of detecting a false-positive result and not detecting a case of SARS-CoV infection are both high, it is important to have well-characterized detection and confirmatory assays. In the absence of virus-specific control measures, e.g., a vaccine or antiviral drug, the key to controlling a reemergence of SARS is rapid diagnosis and implementation of infection control measures, i.e., isolating cases and identifying and managing contacts to prevent further transmission. The development of well-characterized detection and confirmatory serologic tests is the key to laboratory diagnostic support should SARS reemerge. These two ELISAs can be used as components of the SARS diagnostic system. These assays can also be used to study the kinetics of the protein-specific SARS antibody response and to help characterize SARS immunity and the pathogenesis of disease.

We thank Der-Yuan Wang (Bureau of Food and Drug Administration, Department of Health, Taiwan, Republic of China), Mei-ying W. Yu (CBER/Food and Drug Administration, Bethesda, MD), and Li-Ching Hsu (Center for Disease Control, Department of Health, Taiwan, Republic of China) for providing a panel of sera from SARS patients. Thanks also to Ann Falsey (University of Rochester School of Medicine, Rochester, NY) and Dean Erdman (CDC, Atlanta, GA) for providing sera from patients with non-SARS-related respiratory infections and Debi Cannon (CDC, Atlanta, GA) for technical assistance. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the CDC.

REFERENCES


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