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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

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Using paired serum samples obtained from patients with illness associated with increases in anti-human coronavirus OC43 (HCoV-OC43) or anti-HCoV-229E antibodies, we examined the possibility of false-positive results detected in a recombinant severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) nucleocapsid protein immunoglobulin G enzyme-linked immunosorbent assay (ELISA). Three of the 21 and 1 of the 7 convalescent-phase serum samples from persons with increases in antibodies against HCoV-OC43 and HCoV-229E, respectively, tested positive by the recombinant SARS-CoV nucleocapsid protein-based ELISA. None of these samples were found to contain a specific antibody in the recombinant SARS-CoV spike polypeptide-based Western blot assay.

Severe acute respiratory syndrome (SARS), caused by SARS-associated coronavirus (SARS-CoV), has affected 30 countries in five continents, with more than 8,000 cases and 750 deaths (7–11). As for the detection of antibodies against SARS-CoV, at the moment, the most widely used methods are antibody detection in acute- and convalescent-phase sera by indirect immunofluorescence assay and enzyme-linked immunosorbent assay (ELISA) using cell culture extracts (4, 8, 10). However, antibody detection by these methods may be less reproducible, more difficult to standardize, and more labor-intensive than ELISA-based antibody detection tests using recombinant antigens. Furthermore, producing 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 reproducibility and to be easier to standardize and less labor-intensive than antibody detection by indirect immunofluorescence assay and ELISA using cell culture extract, and they do not require cultivation of the SARS-CoV (1, 2, 14, 18). Recently, we have reported the use of recombinant SARS-CoV nucleocapsid protein ELISA-based antibody tests for serodiagnosis of SARS-CoV pneumonia and the study of the seroprevalence of nonpneumonic SARS-CoV infections (15, 16). In addition, others have also reported the use of recombinant-protein-based immunoassays for serodiagnosis of SARS-CoV pneumonia (3, 17). However, in our studies, we have also shown that false-positive reactions were detected if the recombinant SARS-CoV nucleocapsid protein-based ELISA was used alone for antibody detection (15). In this study, using paired serum samples obtained from patients with increases in anti-human CoV OC43 (HCoV-OC43) or anti-HCoV-229E antibodies, we examined the possibility of false-positive results detected by the recombinant SARS-CoV nucleocapsid protein-based ELISA. The importance of using Western blot assays, with the nucleocapsid protein and spike polypeptide of SARS-CoV, for confirmation was also determined.

Paired serum samples collected from 21 and 7 patients with recent infections by HCoV-OC43 and HCoV-229E, respectively, were retrieved from the serum bank of the Respiratory Pathogens Research Unit of the Baylor College of Medicine. The paired serum samples were shown to exhibit significant increases in anti-HCoV-OC43 antibodies by immunoassay or in anti-HCoV-229E antibodies in microneutralization tests similar to those described previously (5, 6).

Cloning and purification of His_6-tagged recombinant nucleocapsid protein and optimization of the ELISA for detection of immunoglobulin G (IgG) against SARS-CoV were as

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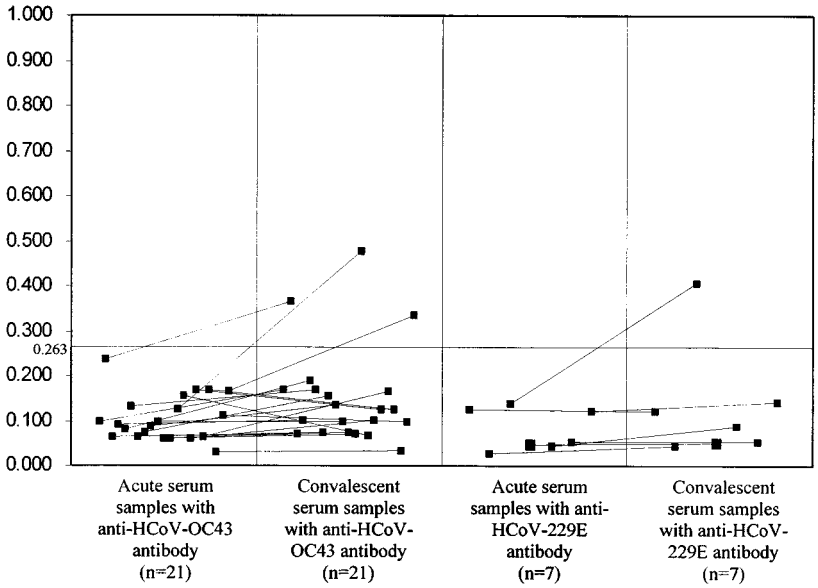

FIG. 1. Recombinant SARS-CoV nucleocapsid protein-based IgG antibody ELISA for serum samples positive for the anti-HCoV-OC43 or anti-HCoV-229E antibody.

reported previously (15). ELISA was performed according to our previous publications (13, 14) using paired serum samples (diluted 1:40) positive for the anti-HCoV-OC43 or anti-HCoV-229E antibody. Cloning and purification of the His_6-tagged recombinant spike polypeptide of SARS-CoV were as reported previously (15). Western blot analysis was performed according to our previous publications (14, 15, 18).

Three of the 21 convalescent-phase serum samples, but none of the acute-phase serum samples, from patients with recent HCoV-OC43 infections were positive by the recombinant SARS-CoV nucleocapsid protein-based ELISA for IgG antibody detection, with optical density at 450 nm (OD_{450}) values of 0.337, 0.365, and 0.478 (Fig. 1). Although two serum samples produced very faint bands in the recombinant SARS-CoV nucleocapsid protein-based Western blot assay, none of them were found to contain the specific antibody by the recombinant SARS-CoV spike polypeptide-based Western blot assay (Fig. 2). There was no relationship between the convalescent-phase sample titer or magnitude of the increase in the HCoV-OC43 ELISA and a positive result in the recombinant SARS-CoV nucleocapsid protein-based ELISA.

One of the seven convalescent-phase serum samples, but none of the acute-phase serum samples, from patients with recent HCoV-229E infection was positive by the recombinant SARS-CoV nucleocapsid protein-based ELISA for IgG detection, with an OD_{450} value of 0.405 (Fig. 1). Although the serum sample produced a very faint band in the recombinant SARS-CoV nucleocapsid protein-based Western blot assay, it did not contain the specific antibody according to the recombinant SARS-CoV spike polypeptide-based Western blot assay (Fig. 2). The convalescent-phase serum positive in the recombinant SARS-CoV nucleocapsid protein-based ELISA was from the serum pair with the greatest rise in titer (eightfold) and had the highest neutralizing-antibody titer.

The present study showed evidence that cross-reactivity in the recombinant SARS-CoV nucleocapsid protein-based ELISA between the SARS-CoV and serum samples positive for antibodies against HCoV-229E or HCoV-OC43 is possible. In our previous study on the optimization of the SARS-CoV recombinant nucleocapsid protein-based ELISA for IgG antibody detection, seven of the serum samples obtained from 149 healthy blood donors who donated blood in 2000 had OD_{450}s greater than the cutoff value (15). However, none of them were found to contain the specific antibody by both the nucleocapsid protein- and spike polypeptide-based Western blot assays. Furthermore, when this ELISA was used to screen healthy blood donors who donated blood during the SARS outbreak in Hong Kong (March to May 2003), nonpneumonic hospitalized patients, and asymptomatic health care workers, 33 (4%) of the 828 serum samples screened were positive for IgG antibodies (15). However, only 4 (12%) of the 33 serum samples were confirmed to contain specific SARS-CoV antibodies by both nucleocapsid protein- and spike polypeptide-based Western blot assays. We speculated that the false-positive results could be due to cross-reactivity between the nucleocapsid protein of SARS-CoV and serum samples positive for antibodies against HCoV-229E, HCoV-OC43, or other undiscovered human coronaviruses, as there is significant homology between the nucleocapsid protein of SARS-CoV and those of HCoV-OC43 and HCoV-229E. In the present study, we showed that cross-reactivity between the nucleocapsid protein of SARS-CoV and serum samples positive for antibodies against HCoV-229E or HCoV-OC43 is indeed possible. For about 14% of the HCoV-OC43 and HCoV-229E infections detected by serum antibody increases, an antibody increase was detected by the SARS-CoV nucleocapsid protein-based ELISA. The cross-reactive serum from an HCoV-229E infection had the highest convalescent-phase sample titer and greatest increase in titer, but no relationship between the HCoV-OC43 ELISA titers and detection of antibody in the SARS-CoV nucleocapsid protein-

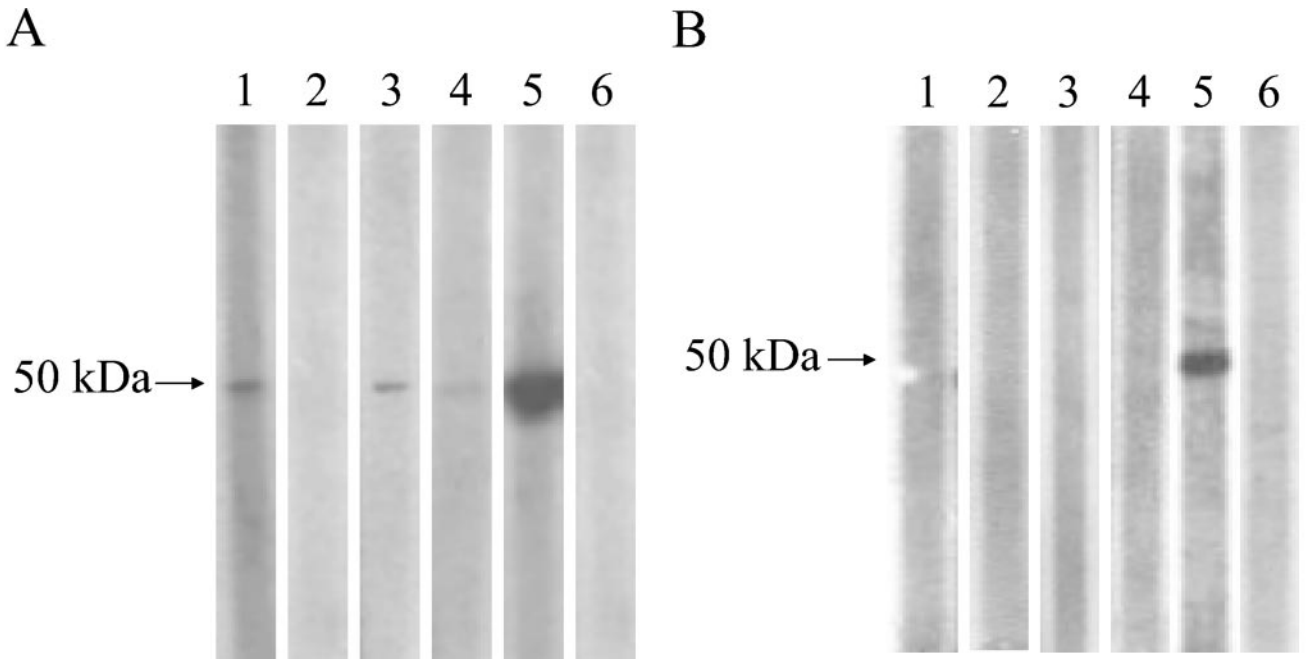

FIG. 2. Western blot analysis of purified recombinant SARS-CoV nucleocapsid protein (A) and spike polypeptide (B) using human sera that tested positive by the recombinant SARS-CoV nucleocapsid protein-based IgG antibody ELISA. Two of the three serum samples with the anti-HCoV-OC43 antibody (lanes 1 to 3) and the serum sample with the anti-HCoV-229E antibody (lanes 4) produced very faint bands in the recombinant SARS-CoV nucleocapsid protein-based Western blot assay, but not the recombinant SARS-CoV spike polypeptide-based Western blot assays. Also shown are a positive control using serum of a patient with SARS-CoV pneumonia (lanes 5) and a negative control using serum of a healthy blood donor (lanes 6).

based ELISA was observed. Patients with a positive SARS-CoV nucleocapsid protein-based ELISA result tended to be younger than those with negative tests, but clinical characteristics of the illnesses for these two groups were the same (data not shown). For the two HCoV-OC43 antibody-positive serum samples that produced bands when they were tested by the nucleocapsid protein-based Western blot assay, the bands were stronger than that produced by the HCoV-229E antibody-positive serum sample that tested positive by the nucleocapsid protein-based ELISA (Fig. 2). This is probably because the percentage of amino acid identity between the nucleocapsid protein of HCoV-OC43 and that of SARS-CoV (33%) is higher than the amino acid identity between the nucleocapsid protein of HCoV-OC43 and that of SARS-CoV (21%). Cross-reactivity with HCoV-OC43, HCoV-229E, and other coronaviruses remains an important issue for future studies on SARS-CoV serology. The use of synthetic peptides, instead of the whole protein, for antibody detection could be a solution to cross-reactivity with proteins of other CoVs (13).

The present study confirmed the high specificity of the recombinant SARS-CoV nucleocapsid protein-based ELISA, with positive results confirmed by Western blot assays using the recombinant nucleocapsid protein and recombinant spike polypeptide of SARS-CoV. It is well known that, in the presence of possible cross-reactions, the positive predictive values of serological assays depend on the prevalence of the infection in a particular locality at a particular moment. In the present study the four false-positive results were rectified by considering Western blot positivity for both the recombinant nucleocapsid protein and spike polypeptide a genuinely positive result. As the present ELISA produces a small proportion of false-positive reactions, all positive results, especially those obtained in the context of a low disease prevalence or from clinically incompatible cases, need confirmation by Western blot analysis using the recombinant nucleocapsid protein and spike polypeptide of SARS-CoV. Tan et al. recently demonstrated the high sensitivity and specificity of recombinant nucleocapsid protein-based Western blot analysis and recombinant spike protein-based immunofluorescence assay for the serodiagnosis of SARS-CoV infection (12). As ELISA is less labor-intensive and tedious to perform than Western blot assays, Western blot confirmation of positive ELISA results is probably a better choice for routine use in clinical microbiology laboratories. The Western blot assay for the spike polypeptide may appear more specific than that for the nucleocapsid protein because of possibly lower antibody titer of and/or avidity for recombinant nonglycosylated protein. Further studies could be performed to see whether Western blot analysis using only the spike polypeptide, instead of using both the nucleocapsid protein and spike polypeptide, would be able to screen out all the false-positive results.

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