Use of Viral Lysate Antigen Combined with Recombinant Protein in Western Immunoblot Assay as Confirmatory Test for Serodiagnosis of Severe Acute Respiratory Syndrome

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Received 27 May 2004/Returned for modification 4 August 2004/Accepted 20 August 2004

A Western immunoblot assay for confirmatory serodiagnosis of severe acute respiratory syndrome (SARS) was developed utilizing viral lysate antigens combined with a recombinant nucleocapsid protein, GST-N (glutathione S-transferase–nucleocapsid) of the SARS coronavirus (SARS-CoV). The viral lysate antigens were separated by electrophoresis and transblotted onto nitrocellulose membranes. The resultant membrane was subsequently added with the GST-N recombinant protein at a specific location. The positions of bands corresponding to some of the structural proteins immobilized on the membrane were then located and verified with mouse or rabbit antisera specific to the respective proteins. The Western immunoblot assay was able to detect antibodies to SARS-CoV in all 40 serum specimens from SARS patients and differentiate the SARS-positive samples from those of the healthy donor or non-SARS patient controls (150 samples) when set criteria were followed. In addition, when the immunoblot was used to test samples considered falsely positive by an in-house-developed SARS-specific enzyme-linked immunosorbent assay, band patterns different from those with samples from SARS patients were obtained.

Severe acute respiratory syndrome (SARS) is a serious respiratory illness with a famous mortality rate and is caused by the novel coronavirus SARS-CoV (2, 4, 9, 14). Affected patients can develop symptoms of atypical pneumonia, and early and accurate differentiation of SARS from atypical pneumonia is critical to successful management of this highly contagious disease. During the outbreak in 2003, diagnosis of the disease relied not only on clinical symptoms but also on travel and contact history (1, 20). However, in a postoutbreak era, travel and contact histories are less significant, as demonstrated by the recently reemerged cases in Guangdong, China. Diagnosis of the disease will inevitably rely more heavily on laboratory evidence. Hence, providing tools for accurate laboratory diagnosis remains a priority and will be critical for future disease management.

Although various approaches and protocols are now available, to date, there are no standardized tests for the laboratory diagnosis of SARS (http://www.who.int/csr/sars/conference/june_2003/materials/presentations/en/laboratorydiagnosis.pdf). In a recent report (21), the reverse transcription-PCR protocols of two World Health Organization SARS network laboratories were evaluated. The findings of this study support a previous suggestion that existing PCR protocols cannot rule out the presence of the SARS virus when a negative result is obtained; neither can the protocols exclude the possibility of a false detection due to laboratory contamination (13). Alternative approaches such as enzyme-linked immunosorbent assay (ELISA) and rapid immunochromatographic tests were also developed and reported to play a role in providing diagnostic information complementary to that provided by PCR (5). However, a confirmatory test to verify results generated by these methods is desirable and may be essential (6).

Western immunoblot assays have been routinely used as confirmatory tests for the last decade and remain important tools for managing viral infections such as those caused by human immunodeficiency virus, human T-cell lymphotropic virus, and hepatitis C virus. This platform may have a similar role in our efforts to diagnose infection with SARS-CoV. In fact, recently, a few attempts have been made to utilize the Western blot platform for detecting antibodies to SARS-CoV in patients (8, 19). However, these efforts mostly centered on applying a single protein marker for detection; thus, the resultant tests gave only limited information very similar to that provided by an ELISA or an immunofluorescent antibody test. In the present study, we extended the above efforts and developed a Western immunoblot utilizing viral lysate antigens combined with a SARS-specific recombinant protein, GST-N (glutathione S-transferase–nucleocapsid). The new test provided not only information on antibody detection but also protein banding patterns that might be useful for diagnostic or prognostic purposes.

MATERIALS AND METHODS

Serum specimens. Forty serum specimens from convalescent-phase patients were collected with consent from SARS patients (age, 17 to 63 years; median age,
34 years) who were admitted to the Tan Tock Seng Hospital or the Singapore General Hospital. Fifty sera from healthy donors, purchased from BioClinical Partners, Inc. (Franklin, Mass.) were also included in the study as controls. For the non-SARS patient controls, archived Genelabs Diagnostics (Singapore) serum samples from previous studies were used; these samples had been obtained prior to the SARS outbreak. These included 50 samples each from patients who had non-SARS-related fever (confirmed to be dengue fever) or who suffered non-SARS-related respiratory illness (confirmed to be tuberculosis). In addition, 18 samples identified as false positives for antibodies to SARS-CoV from screening 1,066 healthy donors in a previous study (6) were included in the present study. These 18 samples were also collected from healthy donors prior to the outbreak of SARS and were purchased from BioClinical Partners, Inc. All serum samples were stored at −20°C until use.

SARS-CoV viral lysate and recombinant proteins. The SARS-CoV viral lysate was purchased from ZeptoMetrix Corporation (Buffalo, N.Y.), and they were obtained from SARS-CoV-infected Vero cells after sucrose gradient purification and treatment with a disruption buffer (0.6 M KCl) containing 0.5% Triton X-100.

Four recombinant proteins were used for the purpose of generating protein-specific antisera: glutathione S-transferase (GST) fusion proteins of spike (GST-S; amino acids [aa] 460 to 820), nucleocapsid (GST-N; aa 121 to 422), matrix (GST-M; aa 98 to 221) and envelope (GST-E; aa 38 to 76) proteins. In addition, the GST-N protein was used in producing the Western immunoblot; it has a deletion of a highly conserved motif (aa 111 to 118) found in all CoVs (15). The materials and methods used for obtaining these recombinant proteins have been described in detail previously (19; S. Shen et al., unpublished data). Briefly, all the proteins were expressed as GST fusion proteins in Escherichia coli, but only GST-N was purchased from BioClinical Partners, Inc. (Franklin, Mass.) at a dilution of 1:1,000 in the blocking buffer. After the incubation, the conjugate was removed again by aspiration, and the membrane strips were washed another three times. This was followed by a 15-min incubation of the membrane strips with a substrate solution of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium. The resultant protein bands were analyzed subjectively by the intensity of the bands on the strips.

For assays with the mouse or rabbit antisera, a specific dilution was used for each antiserum, due to the differences in their titers. In particular, a 1:1,000 dilution was used for the mouse anti-S antiserum and the rabbit anti-E antiserum, whereas a 1:500 dilution was used for the mouse anti-M antiserum and a 1:100,000 dilution for the mouse anti-N antiserum. However, the conjugates of either anti-mouse or anti-rabbit antibodies labeled with alkaline phosphatase employed for the detection of the respective antisera were used at the same dilution, i.e., 1:5,000.

RESULTS

Identification of immunoreactive proteins of SARS-CoV by Western immunoblot analysis. The apparent molecular masses of the immunoreactive proteins were estimated by extrapolating a plot of the logarithm of the molecular masses versus the electrophoretic mobilities of standard proteins. The immunoreactive proteins seen as bands on the immunoblots when assayed with two strong SARS-positive control samples (P2 and P8) included discreet, diffused, and clustered proteins of approximately 150 kDa, 97 kDa (triplet), 60 kDa, 45 kDa (doublet), 28 kDa (triplet), and 24 kDa (diffused). When the immunoblot was tested with the mouse or rabbit antisera, a specific dilution was used for each antiserum, due to the differences in their titers. In particular, a 1:1,000 dilution was used for the mouse anti-S antiserum and the rabbit anti-E antiserum, whereas a 1:500 dilution was used for the mouse anti-M antiserum and a 1:100,000 dilution for the mouse anti-N antiserum. However, the conjugates of either anti-mouse or anti-rabbit antibodies labeled with alkaline phosphatase employed for the detection of the respective antisera were used at the same dilution, i.e., 1:5,000.

Performance of various protein markers and the Western immunoblot. When the immunoblot was tested with different sets of specimens from SARS patients or the healthy donor or non-SARS patient controls, distinctive banding patterns specific to samples from the SARS patients or to those from the controls were observed (Fig. 2). An analysis of the band patterns showed that two protein bands (designated Nhigh and Nlow) at approximately 45 kDa associated with the N protein of SARS-CoV occurred most often, reacting to all the samples from SARS patients (Table 1). However, although the band intensities of the two proteins were much higher with those samples from the SARS patients, both Nhigh and Nlow were rather nonspecific and also reacted to samples from the healthy or disease controls (Fig. 2 and Table 1). For example, the Nhigh protein cross-reacted to 64, 68, and 52% of the samples from healthy donors, the respiratory illness patients, and the fever patient controls, respectively (Table 1). In fact, these N-related
proteins reacted to 15 of 18 samples (83%) previously found to be falsely positive by a SARS-specific ELISA (Table 1). In contrast, the S, M, and the N recombinant proteins reacted to 78, 75, and 100% of the 40 samples from the SARS patients without any cross-reactivity with any of the controls, including those identified as false positive by ELISA (Table 1). Other highly immunoreactive proteins included the 97- and 28-kDa proteins reacting, respectively, to 78 and 75% of the samples from the SARS patients but only to a few of the controls, with a cross-reactivity rate of 2 to 6% (Table 1). Furthermore, the 60-kDa protein was reactive to a portion of all the tested samples (11 to 34%) but rather nondiscriminatory, regardless of whether the samples were from SARS patients or the healthy or disease controls (Table 1). The E protein as identified by the rabbit antisera, on the other hand, showed no immunoreactivity to any of the human serum samples tested in the study (Table 1).

A further analysis of the band patterns of the immunoreactive proteins revealed useful criteria for differentiating samples from the SARS patients and those from the controls. When simultaneous detection of the N proteins (both N\textsubscript{high} and N\textsubscript{low}) and the S protein was set as a prerequisite for a test result to be interpreted as positive for SARS, the immunoblot assay was found to have a sensitivity of 78% with a specificity of 100% to all four control groups (Table 2). Both the 60-kDa and E protein, however, were less useful in any combination, due to the lack of sensitivity (Table 2). A combined interpretation criterion utilizing the N proteins and at least one of the specific proteins, including the S, 97-kDa, 28-kDa, and M proteins, provided a kit sensitivity of 95% and specificity of 100% (Table 3). Further addition of the GST-N recombinant protein to the above interpretative criteria improved the sensitivity to 100% without altering the specificity (Table 3).

**DISCUSSION**

Serological tests for SARS, whether ELISA or immunochromatographic tests as previously reported, all had either sensitivity or specificity issues (11, 16, 6). In addition, although a good correlation existed between new serological tests with the immunofluorescence test, discrepancies among the different approaches were also observed in a previous study (6). Hence, it is critical to resolve those discrepancies, identify those potentially false-positive results, and confirm the true results for an effective management of SARS. With different protein markers presented separately, a Western immunoblot can thus provide an array of information that the other serological ap-

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**FIG. 1.** Western immunoblot patterns and locations of the immunoreactive proteins of SARS-CoV. (A) The apparent molecular masses of the proteins identified were estimated by extrapolation from a plot of the logarithm of the molecular masses versus the electrophoretic mobilities ($R_f$) of standard proteins. Lanes 1 and 2, SARS-CoV blot with SARS-positive serum samples P2 and P8; lane 3, rainbow molecular mass markers (14 to 200 kDa). (B) Locations of the immunoreactive proteins of SARS-CoV as identified with antibodies raised against specific recombinant proteins. From left to right, strip 1, SARS-positive control; strip 2, mouse anti-spike protein antiserum; strip 3, mouse anti-nucleocapsid protein antiserum; strip 4, mouse anti-matrix protein antiserum; strip 5, rabbit anti-envelope protein antiserum. SAC, sample addition control.
approaches lack but need for precise serological differentiation and confirmation.

The present study revealed the utilities of several proteins as indicators for the purpose of confirmatory serodiagnosis of SARS; these included the immunoreactive proteins of 150, 97, 45, 28, and 24 kDa. A comparison study of these proteins suggested that the 150-kDa, 45-kDa, and 24-kDa proteins were associated, respectively, with the S, N, and M protein because their molecular masses were similar to those predicted by the genomic sequences of the novel SARS-CoV (12). The identities of these proteins were verified by their specific reactivities to the respective mouse or rabbit antisera. However, the triplet presented at approximately 97 kDa on the immunoblot appeared to be novel proteins not predicted by the genomic sequence (12). On the other hand, the appearance of another triplet at approximately 28 kDa is consistent with the finding of a previous report of a unique protein (18). Using an anti-U274 specific rabbit antiserum, this previous study reported three protein bands designated U274 on Western blots at a similar region of 28 to 32 kDa (18).

The N protein of the SARS-CoV was reported to be a useful marker for serodiagnosis, and several kits are reported to have utilized this protein either in its lysate form or a recombinant counterpart as their antigen (10, 11, 16, 19). However, our study, while confirming its utility as an immunoreactive marker, also revealed the nonspecific nature of this protein in its lysate form. In particular, two protein bands presented on

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**TABLE 1. Reactivity patterns of immunoreactive proteins on Western immunoblots**

| Marker     | % Reactive samples<sup>a</sup> | SARS | Healthy donors | Non-SARS (TB) | Non-SARS (fever) | ELISA (FP) |
|------------|--------------------------------|------|----------------|---------------|------------------|------------|
| S          | 78                             | 0    | 0              | 0             | 0                | 0          |
| 97-kDa     | 78                             | 4    | 2              | 2             | 0                | 0          |
| 60-kDa     | 23                             | 22   | 34             | 22            | 11               |
| N<sub>high</sub> | 100                            | 64   | 68             | 52            | 83               |
| N<sub>low</sub> | 100                            | 42   | 34             | 34            | 72               |
| 28-kDa     | 75                             | 0    | 6              | 0             | 0                |
| M          | 75                             | 0    | 0              | 0             | 0                |
| E          | 0                              | 2    | 0              | 0             | 0                |
| GST-N      | 100                            | 0    | 0              | 0             | 0                |

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<sup>a</sup> Counted as reactive to the respective proteins if any one of the three bands in the region appeared.

<sup>b</sup> Sample sizes for the respective cohorts were as follows: patients with SARS, 40; healthy donors, 50; non-SARS patients with tuberculosis (TB), 50; non-SARS patients with fever, 50. A total of 18 samples [column “ELISA (FP)” previously identified as falsely positive for SARS were also screened. Sample sizes and categories are identical in Tables 2 and 3.

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**TABLE 2. Sensitivity and specificity of Western immunoblots based on N protein combined with respective proteins**

| Combination | SARS | Healthy donors | Non-SARS (TB) | Non-SARS (fever) | ELISA (FP) | Sensitivity | Specificity | Specificity | Specificity | Specificity |
|-------------|------|----------------|---------------|------------------|------------|-------------|-------------|-------------|-------------|-------------|
| N + S       | 78   | 100            | 100           | 100              | 100        |
| N + 97-kDa  | 78   | 100            | 100           | 100              | 100        |
| N + 60-kDa  | 23   | 96             | 92            | 100              | 94         |
| N + 28-kDa  | 75   | 100            | 100           | 100              | 100        |
| N + M       | 75   | 100            | 100           | 100              | 100        |
| N + E       | 0    | 100            | 100           | 100              | 100        |
| N + GST-N   | 100  | 100            | 100           | 100              | 100        |

<sup>a</sup> A test result was considered positive only when the nucleocapsid (both N<sub>high</sub> and N<sub>low</sub>) and the respective protein were detected simultaneously. Values are in percentages.
the Western immunoblot at around a molecular mass of 45 kDa were confirmed to be associated with the N protein by the specific mouse anti-N antiserum. The appearance of a shorter form of the N protein (N\textsubscript{low}) in addition to the N\textsubscript{high} form is consistent with previous observations with other CoVs such as transmissible gastroenteritis CoV (3). It has been demonstrated that host cell caspases, a family of cysteine proteases, is responsible for the cleavage of the N protein of CoVs during an infection (3). With our immunoblot, the two forms of the N protein of the SARS-CoV were found to be equally immunoreactive. Both N\textsubscript{high} and N\textsubscript{low} reacted to all the 40 SARS-related samples as well as a fair portion of the samples from the healthy donor and disease control groups (34 to 68%) (Table 1). In fact, these proteins reacted to 15 of 18 samples identified as false positive from a population of 1,066 healthy donors from the United States by a SARS-specific ELISA in a previous study (6). In view of this finding, it is perhaps noteworthy that the genomic sequence of the N protein in its natural form does predict a highly conserved motif found in all CoVs (15). Thus, it is not surprising that the viral lysate-derived N protein on the Western immunoblot presented cross-reactivities with those non-SARS-related samples. This result is consistent with the finding of a recent report of antibodies cross-reactive to human and animal SARS-CoV in samples of healthy adults collected 2 years prior to the SARS outbreak (22). Although the report suggested the existence of a SARS-related virus predating the SARS outbreak, the possibility of the observed cross-reactivity due to a more distant but CoV-related agent cannot be completely ruled out in view of our present findings. In this respect, a further study of those samples with the new Western blot analysis may provide useful information and thus warrant further investigation. Interestingly, our findings here also echoed a very recent report of the cross-reactivity of the N protein of SARS-CoV to antigenic group I animal CoVs (17).

It is perhaps also noteworthy that the cross-reactivity could in theory be eliminated by reducing the amount of the N protein used for the production of the immunoblot, since there was a consistent difference in the protein band intensities between the SARS samples and the controls (Fig. 2). However, it is impracticable for the production of an immunoblot to manipulate only the amount of the N protein without affecting the other proteins in the same lysate mixture that already produced weaker band intensities on the same immunoblot (Fig. 2A). Furthermore, the results from the study with mouse or rabbit antisera seemed to suggest that the high intensity of the N protein might be due to the highly immunogenic nature of the protein itself rather than the amount used in the production of the immunoblot. Among the three specific antisera raised to the S, N, and M proteins in mice, the anti-N serum had the highest titer. This antiserum was used at a 1:100,000 dilution for the assay but produced the same band intensity as that of the anti-S serum at a 1:1,000 dilution (Fig. 1B).

Nevertheless, the N proteins on the Western immunoblot are a useful indicator provided they are used in combination with other immunoreactive proteins. In this aspect, although the S, 97-kDa, 28-kDa, and M proteins were found to be less sensitive individually, they were critical indicators and if combined, produced a sensitivity of 95% without compromising specificity. The further introduction of the GST-N recombinant protein to the above criterion proteins appeared synergistic and necessary for performance and result interpretation of the immunoblot. This recombinant protein contains a deletion of a conserved motif found in other CoVs (19), which may explain the lower cross-reactivity than its full-length counterpart expressed in infected cells, as discussed above. When the GST-N protein was used as an interpretation criterion in combination with the above-mentioned critical proteins, it yielded a kit capable of differentiating and confirming samples from the SARS patients or the healthy or disease controls with excellent sensitivity and specificity of 100% for the tested population (Table 3).

Consequently, the selected criterion markers included not only the lysate N protein but also the S, 97-kDa, 28-kDa, M, and GST-N proteins. As a result, a test with the new Western immunoblot will be considered positive only if the lysate N protein and at least one of the other criterion proteins were detected simultaneously. This prerequisite of multiple markers in combinations limited the possible impact of the cross-reactivity of the N proteins observed here and elsewhere (17) without compromising the sensitivity of the immunoblot and thus provided an advantage over some currently available tests that relied on only a single N protein.

**ACKNOWLEDGMENTS**

We thank Hoe Nam Leong, Yee Sin Leo, and Ai Ee Ling for providing the SARS patient sera.

This project was partially supported by an EDB (Economic Development Board of Singapore) grant under its Innovation Development Scheme.

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