Evaluation and Validation of an Enzyme-Linked Immunosorbent Assay and an Immunochromatographic Test for Serological Diagnosis of Severe Acute Respiratory Syndrome

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A newly developed severe acute respiratory syndrome (SARS)-specific enzyme-linked immunosorbent assay (ELISA) was further validated to confirm cutoff values and evaluate its diagnostic performance with clinical samples. In parallel, an immunochromatographic test was also evaluated. A total of 227 clinical serum specimens collected from SARS patients were used in the study, together with 385 samples from healthy donors. By use of an immunofluorescent (IF) test as the “gold standard,” both the ELISA and the immunochromatographic test were able to detect immunoglobulin G antibodies to SARS not only from late-convalescent-stage samples (>21 days from the onset of clinical symptoms), as previously established, but also from early-acute-phase samples (1 to 10 days from onset). The ELISA, using an optical density (OD) of 0.25 as its cutoff value, produced the best sensitivity while maintaining high specificity. It detected SARS-specific antibodies in 58, 70, 75, and 95%, respectively, of the four groups of samples collected from patients 1 to 10 days, 11 to 20 days, 21 to 30 days, and more than 30 days after the onset of clinical symptoms. Similarly, the immunochromatographic test detected SARS-specific antibodies in 55, 68, 81, and 79% of the four groups, respectively. The overall specificities for the ELISA and the rapid test were 99.5 and 97.7%, respectively. Although the positive correlation observed between the ELISA OD values and the IF titers was moderate (r = 0.6915; P < 0.001), the detection rates of both the ELISA and the rapid test were found well in agreement with the IF titers.

The newly emerged severe acute respiratory syndrome (SARS) is a serious respiratory illness of global significance. Its highly contagious nature, combined with its high mortality rate, has proven disruptive and costly in an increasingly globalized world. As new cases appear to be reemerging in January 2004, the urgency for prompt identification and isolation of infected patients remains. Although the identification of the novel coronavirus SARS coronavirus (SARS-CoV) as the etiological agent for SARS (3, 4, 7, 9) made it possible for various tests to be developed, providing tools for laboratory diagnosis remains a priority, as suggested by the World Health Organization (WHO). To date, there is still no standardized test for diagnosing SARS regardless of whether tests are antigen based or antibody based (http://www.who.int/csr/sars/conference/june_2003/materials/presentations/en/laboratorydiagnosis.pdf). In this regard, various immunofluorescent (IF) tests were essentially used as the “gold standards” for serodiagnosis of SARS during the 2003 outbreak. Although these IF protocols were found to be well correlated with PCR and clinical outcomes (9), they differed from laboratory to laboratory and were technically very demanding. In addition, these protocols utilized virus-infected materials and thus required laboratories with biosafety-level-3 facilities under current regulations. Hence, alternative tests with standardized approaches addressing these shortcomings are very much needed. However, only a thorough evaluation with standardized panels or by a trial using large numbers of clinical specimens will help to firmly establish the usefulness of new tests.

In this study, two newly developed serological tests were evaluated and validated with large numbers of samples. The two tests, an enzyme-linked immunosorbent assay (ELISA) and an immunochromatographic test, are both based on recombinant proteins, and both demonstrated excellent performance when tested with convalescent-phase samples from SARS patients in a previous study (5). The present expansion to include earlier specimens from acute-phase patients as well as large numbers of relevant controls provided a more realistic assessment of the performances of the two tests for serological diagnosis of SARS in clinical settings.

MATERIALS AND METHODS

Serum specimens. Serum specimens were collected from patients who presented with suspected clinical SARS according to the WHO definition (13) and who were admitted to one of three acute-care regional hospitals in Hong Kong between 18 March and 24 May 2003. A total of 227 serum samples from these patients were tested by using an IF test (14) and were confirmed to have IF titers of >1:10 to 1:2,560. In the meantime, 385 serum samples collected locally from healthy donors in Hong Kong were used as controls. These samples were also tested by the same IF test and confirmed to have IF titers of <1:10. In addition, 1,066 serum samples from healthy donors purchased from BioClinical Partners Inc. (Franklin, Mass.) were included in the study as additional healthy controls. For the disease controls, serum samples from various previous studies in the
TABLE 1. Different cutoff values selected for the ELISA with their corresponding sensitivities, specificities, PPVs, NPVs, and delta values

| Cutoff (OD) | Sensitivity | Specificity | PPV | NPV | Delta |
|-------------|-------------|-------------|-----|-----|-------|
| 1.5          | 0.25        | 100% (385/385) | 100% (385/385) | 100% (385/385) | 0.75 |
| 2.0          | 0.50        | 99.7% (382/383) | 99.7% (382/383) | 99.7% (382/383) | 0.50 |
| 2.5          | 0.75        | 99.4% (379/380) | 99.4% (379/380) | 99.4% (379/380) | 0.25 |

Statistical analysis. Delta values, defined as the distance of the mean OD/cutoff ratio of the sample population from the cutoff, measured in standard deviations (SD) (2), were calculated for validation of the cutoff values. The kappa statistic was used to measure the strength of an agreement between the results by the new rapid test and the new ELISA. A kappa statistic of >0.75, 0.40 to 0.75, or <0.40 represents excellent, good to fair, or poor agreement, respectively (10).

RESULTS

Evaluation and validation of the ELISA. As presented in Table 1, three cutoff values at OD of 0.45, 0.30, and 0.25 were used to evaluate the performance of the ELISA. With the cutoff value set at an OD of 0.45, the ELISA produced the best specificity, 100% (385 of 385), but a low overall sensitivity of 60% (136 of 227) (Table 1). However, improved performance was obtained when the cutoff value was adjusted lower, to an OD of 0.3 or 0.25. The ELISA de-
tected almost 12% more SARS-associated samples overall with an OD of 0.25 as the cutoff value than with the OD 0.45 setting (Table 1). An improved delta value of 0.53 for positive results was also obtained. In a supplementary test, a further 1,066 samples from healthy controls were tested, and a mean OD of 0.0432 was obtained. An OD of 0.25 was found to be equivalent to the mean OD + 3 SD, whereas an OD of 0.45 was found to be equivalent to the mean OD + 5 SD. The two cutoff values of 0.25 and 0.45 produced similarly high specificities of 98 versus 99.2%, 100 versus 100%, and 92 versus 98% for the supplemental healthy-control group, the non-SARS controls with respiratory illness, and the non-SARS controls with fever, respectively (Table 1).

With the cutoff set at an OD of 0.25, the ELISA detected IgG antibodies to SARS-CoV not only in late-convalescent-stage samples (with a high sensitivity of 95%) but also in acute-phase specimens. It detected IgG antibodies to SARS-CoV in 58, 70, and 75% of samples collected from SARS patients 1 to 10 days, 11 to 20 days, and 21 to 30 days after the onset of clinical symptoms, respectively. The specificity obtained with the cutoff OD of 0.25 remained high at 99.5% (383 of 385) for the healthy-control group tested at the same testing site (Table 1). The test thus provided an overall positive predictive value (PPV) of 94.7 and 84.9%, respectively, with the populations tested (Table 2). Again, in a supplementary test, the rapid test was further evaluated with disease control groups consisting of non-SARS patients who suffered from respiratory illness or fever and was found to cross-react to only 3 or 4 of the 50 samples tested in the respective groups (Table 2). When the results were compared, the rapid test and the ELISA gave an excellent overall agreement of 92.5%, with a kappa statistic of 0.81 (Table 3). In addition, the rapid test was also found to perform well in concordance with the IF titers, detecting progressively more patient samples as the titers increased (Fig. 2).
DISCUSSION

SARS is new to humans, and thus many aspects of the disease and its etiological agent, SARS-CoV, have yet to be fully understood. For example, the apparently contradictory phenomenon of a high infection rate for health care workers but a low infection rate for family members has yet to be fully explained. It appears that viral loads are low both during early infection and after about 3 weeks of infection. This situation renders PCR techniques relatively insensitive during the early stage of infection. Serological approaches such as IF tests, as demonstrated, have been welcome complementary tools for combating SARS. In fact, IF tests were practically used as the gold standard for serodiagnosis of SARS in hospitals during the outbreak despite their limitations. Nevertheless, new approaches are needed, and the IF test should be a useful reference for our evaluations.

In a previous study, the newly developed ELISA and the rapid immunochromatographic test demonstrated excellent performances, with PPVs and NPVs higher than 95%, suggesting great potential at least for exclusion of SARS or as study tools for epidemiologists (5). However, the study was limited to convalescent-phase samples from SARS patients, and the usefulness of the new tests for clinical diagnosis of SARS thus remained to be fully verified. In the present study, the two new tests were further evaluated for their performances with 227 patient samples and 385 samples from healthy controls. Furthermore, the study included a validation of the cutoff values using an additional 1,066 samples from healthy donors and 100 samples from disease controls. The study thus allowed us to fine-tune the cutoff value for the ELISA. The data showed that the adjusted cutoff OD of 0.25 produced the best sensitivity for the ELISA without compromising its specificity much (Table 1).

**TABLE 1. Performance of the rapid immunochromatographic test in detecting IgG antibodies to SARS in samples from patient groups and healthy controls**

| Recombinant Protein | Sensitivity | Specificity | PPV | NPV |
|---------------------|-------------|-------------|------|-----|
| SARS-CoV (respiratory) | 97 (96)     | 99 (98)     | 100  | 97 (96) |
| SARS-CoV (gastrointestinal) | 98 (97)    | 99 (98)    | 99 (98) | 99 (98) |
| Total               | 98 (97)     | 99 (98)     | 99 (98) | 99 (98) |

**TABLE 2. Performance of the rapid immunochromatographic test in detecting IgG antibodies to SARS in samples from patient groups and healthy controls**

| Recombinant Protein | Sensitivity | Specificity | PPV | NPV  |
|---------------------|-------------|-------------|------|------|
| SARS-CoV (respiratory) | 97 (96)    | 99 (98)    | 100  | 97 (96) |
| SARS-CoV (gastrointestinal) | 98 (97) | 99 (98) | 99 (98) | 99 (98) |
| Total               | 98 (97)    | 99 (98)     | 99 (98) | 99 (98) |

**TABLE 3. Agreement between the new rapid test and the new ELISA with sera from SARS patients and healthy controls**

| Result with rapid test | No. of ELISA results that were: | Agreement (%) |
|------------------------|----------------------------------|---------------|
| Positive               | 144                              | 25            | 85.2          |
| Negative               | 21                               | 422           | 95.3          |

* The kappa statistic obtained was 0.81. A kappa statistic of ≥0.75 represents excellent agreement, 0.40 to 0.75 represents good to fair agreement, and <0.40 represents poor agreement (11).
the case of borderline positives, will be helpful in resolving false-positives. Thus, further studies are warranted.

With the detection rate of 95% for samples obtained more than 30 days after the onset of clinical symptoms (Table 1), the present data confirmed previous findings that the ELISA was excellent at detecting antibodies to SARS in convalescent-phase samples (5). Although the ELISA yielded an overall sensitivity of about 72%, most of its shortcomings are attributable to its performance with samples obtained earlier, 1 to 10 days after the onset of clinical symptoms (Table 1). This new test detected only 58% of this group (Table 1). However, it is noteworthy that this detection rate is a significant improvement over that of a capture ELISA, reported very recently, that detected IgG antibodies in only 46% (30 of 65) of an equivalent group (12). It is perhaps also noteworthy that IgG antibody to SARS CoV is believed to reach its peak value around 60 days after the onset of obvious symptoms (8). The Centers for Disease Control and Prevention has recommended including, as part of its case definition for exclusion of SARS, the absence of antibodies to the virus in convalescent-phase serum samples obtained more than 21 days after onset of the illness (1).

Similarly, the rapid test was found to have an overall detection rate comparable to that of the ELISA, at about 70%, with most of its shortcomings, again, due to results for the early group (Table 2). Although the rapid test utilized two recombinant proteins, Gst-N and Gst-U274, the latter appeared to contribute little to the sensitivity of the kit but more to the nonspecific reactivity with healthy controls. Gst-U274 detected only 18 of the 227 patient samples but cross-reacted with 8 of the 385 healthy-control samples (Table 2). As discussed previously, the biological function of Gst-U274 has yet to be unraveled, and the utility of this protein as a diagnostic marker thus has yet to be fully understood (5). The present study appears to suggest a limitation of this protein. However, it is interesting that the only three samples that were detected by Gst-U274 but not Gst-N were those collected from patients in the earlier group, 1 to 20 days from the onset of clinical symptoms.

The positive correlation observed between the ELISA ODs and the IF titers was moderate, with a correlation coefficient \( r = 0.6915 \) \((P < 0.0001 [Fig. 1])\). This finding suggested that the ELISA OD and the IF titers were only moderately associated; the difference between the two approaches could be due to the fact that the new test utilized recombinant proteins rather than the virus-infected cells used in the IF test. However, it is noteworthy that the overall performances of both the ELISA and the rapid test were well in concordance with the IF titers. Both new tests detected progressively more patient samples as IF titers increased (Fig. 2). This finding, thus, suggested that the shortcomings of the new tests were mostly limited to samples with IF titers lower than 1:40 (Fig. 2). Nevertheless, the two new tests had similar overall sensitivities of 70.5 to 71.8% and specificities of 97.7 to 99.5% relative to the IF test, which was used as the gold standard (Tables 1 and 2). When the two tests were compared with each other, they gave an overall agreement of 92.4% with a kappa statistic of 0.81 (Table 3). This confirmed our previous findings and suggested that although the immunochromatographic test is a simple and rapid test that needs no special training to use, its performance was fully compatible with that of the ELISA. The immunochromatographic test should be a valuable addition to current options for combating SARS, especially in areas where laboratory facilities are not available.

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