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Evaluation of a Recombinant Nucleocapsid Protein-Based Assay for Anti-SARS-CoV IgG Detection

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A high throughput accurate assay for anti-SARS-CoV IgG detection is needed for large-scale epidemiological studies. The evaluation of a commercial recombinant nucleocapsid protein-based microtitre plate enzyme immunoassay, ELISARS™ is described. The results on 150 sera from SARS patients and 450 sera from non-SARS controls showed that this assay had a high level of sensitivity (96.2% for late serum samples) and specificity (97.8%). The performance and setup of this assay fulfills the requirement as a screening test for large-scale studies. A vast majority of SARS patients developed antibodies against the nucleocapsid protein. In some patients (10/45), a high level of anti-nucleocapsid antibody appeared very early in the course of the illness. In contrast, a minority (4 of 105 patients) never developed these antibodies. The implication of differences in antibody response to the nucleocapsid protein deserves further investigation. ***J. Med. Virol. 75:181–184, 2005.***

KEY WORDS: coronavirus; diagnosis; enzyme immunoassay; SARS; serology

INTRODUCTION

In March 2003, a global alert was raised by a newly recognized infectious disease, Severe Acute Respiratory Syndrome (SARS), which was later identified to be caused by the SARS-associated coronavirus (SARS-CoV) not observed previously in humans [Drosten et al., 2003; Fouchier et al., 2003; Ksiazek et al., 2003; Kuiken et al., 2003; Peiris et al., 2003a]. This novel coronavirus has clinical and epidemiological characteristics distinct from the known human coronaviruses, HCoV-229E and HCoV-OC43 [Lee et al., 2003; Peiris et al., 2003b; Rainer, 2004]. Although research on SARS-CoV has been progressing very rapidly, a substantial body of knowledge is still missing. To this end, high throughput assays for large-scale sero-epidemiological studies are needed. Recent studies have shown that the nucleocapsid protein reacted to a large proportion of sera collected from SARS patients, and with a sensitivity of upto 100% has been reported for enzyme immunoassays based on the nucleocapsid protein [Wang et al., 2003; Timani et al., 2004]. However, the specificity of nucleocapsid protein-based assays is still unknown. Here, we report on the evaluation of a commercially available assay, ELISARS™ (IgGENE, Hong Kong) for the detection of anti-SARS CoV-immunoglobulin (Ig) G from human serum samples.

MATERIALS AND METHODS

Anti-SARS-CoV IgG

The enzyme immunoassay (EIA), ELISARS™ (IgGENE) is based on *Escherichia coli* BL21 expressed, bacterial glutathione S-transferase (GST)-fused recombinant nucleocapsid proteins of SARS-CoV. This recombinant protein was selected based on the observation of a previous study [Leung et al., 2004]. All assay procedures

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were performed as instructed by the manufacturer's protocol. Briefly, serum samples were added to the antigen-coated wells of the microtitre plate, after an incubation step, the wells were washed, followed by the addition of anti-human IgG antibodies conjugated with horseradish peroxidase. After a second wash, 3,3′, 5,5′-tetramethylbenzidine (TMB) was added as a substrate for color development. The optical density (OD) was measured at 450 nm. Results were interpreted according to the manufacturer's criteria. Specimens with an OD reading = 0.5 were considered negative, whereas specimens with an OD reading >0.5 were considered positive. The cut-off value of 0.5 was according to the manufacturer's recommendation.

Serum samples were also tested by an "in-house" indirect immunofluorescence assay (IFA) as described previously [Chan et al., 2004a]. This assay is based on acetone-fixed Vero cells infected with SARS-CoV, strain CUHK-W1 (GenBank accession no. AY278554). This method was used as a reference for this study as it has been shown to be highly sensitive and specific [Chan et al., 2004a].

SARS Cases

A total of 112 SARS patients aged 4–36 (mean: 19.3; SD: 7.5) years, with 66 females and 46 males were recruited in this study. All these patients fulfilled the modified criteria defined by the World Health Organization for a probable case of SARS [World Health Organization, 2003]. All these patients had either seroconversion or fourfold rise in anti-SARS-CoV IgG antibody levels as detected by our in-house IFA. Three of these patients died, 12 required intensive care but eventually recovered, all the others developed a milder degree of pneumonia that did not required intubation or intensive care. A total of 105 serum samples were collected between day 21—36 (median: 24; interquartile range: 21–26) after the onset of fever. For the purpose of study analysis, these samples were referred as late samples. Another 45 samples were collected between day 4–15 (median: 10; interquartile range: 7–12) after the onset of fever. These samples were referred as early samples.

Non-SARS Controls

A total of 450 control serum samples were included in this study, of which 200 were from medical students aged 19–31 (mean: 22.51; SD: 2.19) years, comprising of 110 females and 90 males. These samples had been collected in 2000 for a pre-varicella vaccination examination. Two hundred and fifty serum samples that had been collected from patients admitted to the Prince of Wales Hospital in 2000 for fever or pneumonia served as the non-SARS pneumonia controls. These comprised of 150 serum samples from adults aged 17–65 (mean: 42.81; SD: 2.75) years of which 69 were females and 81 were males; 50 from elderly aged 65–104 (mean: 77.69; SD: 10.77) years of which 20 were females and 30 were males; and 50 from pediatrics aged 1–16 (mean: 6.28; SD: 4.5) years of which 22 were females and 28 were males.

RESULTS

SARS Patients

Of the 150 serum samples obtained from the SARS group, 111 (74.0%) were tested positive for anti-SARS-CoV IgG by the ELISARS^{TM} kit (IgGENE) (Table I). All except one of these EIA-positive specimens were also tested positive by IFA. This IFA-negative specimen was collected on day 14 after the onset of fever. A later serum collected from this patient was positive by both EIA and IFA. Of the 39 EIA-negative specimens, 11 (28.2%) were tested positive by IFA. A majority (8/11) of these EIA-negative, IFA-positive samples had high IFA titres detected, ranging from 1:160 to 1:1,280. The correlation between OD readings and IFA titres of the 150 samples collected from SARS patients is shown in Figure 1. Overall, a positive association between EIA OD reading and IFA titre was observed ($P < 0.001$ by Kruskal–Wallis test). However, one IFA-negative (<1:40) sample had a high EIA OD reading (>2.5), and 4.9% (3/61) of samples with high IFA titre (=1:640) showed a low level of OD reading (<0.5).

When correlated with the time of specimen collection, the ELISARS^{TM} kit (IgGENE) showed a sensitivity of 96.2% (101/105) for late specimens (≥day 21), and 22.2% (10/45) for early specimens (≤day 15), respectively (Table I). The corresponding sensitivity for IFA was 100% and 35.5%, respectively. The OD readings of SARS and non-SARS samples that were positive for anti-nucleocapsid protein IgG by EIA are shown in Figure 2. Majority (91.9%, 102/111) of the true-positive samples (those taken from SARS patients) had an OD

TABLE I. Results of Anti-SARS-CoV IgG Antibody Detected by ELISARS^{TM} Kit

Study group	No. tested	Anti-SARS-CoV IgG positive	Sensitivity (%)	Specificity (%)
Non-SARS controls	450	10	—	97.8
Health adult	200	5	—	97.5
Pneumonia pediatrics	50	0	—	100
Pneumonia adult	150	3	—	98.0
Pneumonia elderly	50	2	—	96.0
SARS patients	150	111	74.0	—
Convalescent (≥21 days)	105	101	96.2	—
Early serum (≤15 days)	45	10	22.2	—

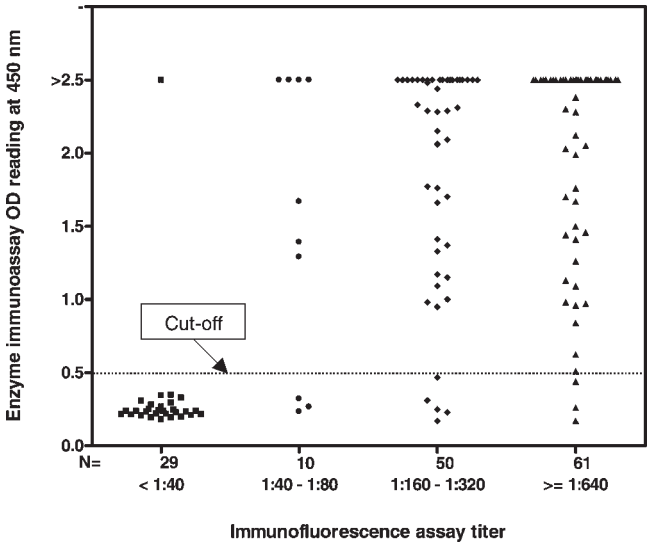

Fig. 1. Correlation between enzyme immunoassay optical density (OD) reading and immunofluorescence assay titre for anti-SARS-CoV IgG antibody. The 150 sera collected from SARS patients are grouped according the antibody titre as detected by immunofluorescence assay (IFA). The horizontal broken line represents the cut-off OD for the nucleocapsid protein-based enzyme immunoassay (EIA) ELISARS^{TM}. A positive correlation between EIA OD readings and IFA titres was observed ($P < 0.001$ by Kruskal–Wallis test).

reading twice above the cut-off (>1.0), whereas all the false-positive samples (those taken from non-SARS groups) had low levels of OD reading between cut-off (0.5) and 1.0.

Non-SARS Controls

Altogether 10 of the 450 samples from non-SARS control groups were positive for anti-SARS-CoV IgG by the ELISARS^{TM} kit (Table I). All these samples were subsequently tested negative by the IFA, suggesting nonspecificity of the EIA. The OD readings of these 10 EIA false-positive specimens are shown in Figure 2.

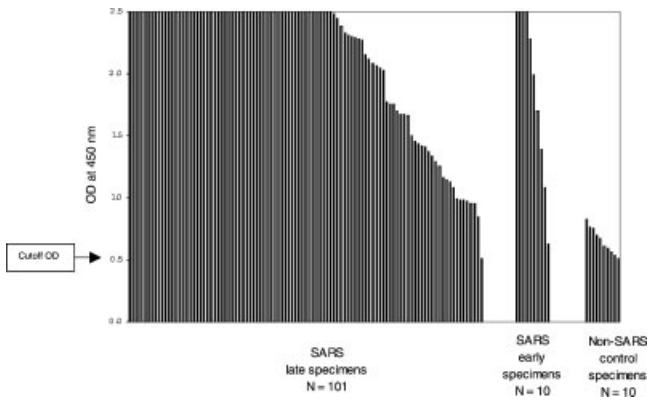

Fig. 2. OD readings of anti-SARS IgG-positive specimens. The 121 specimens tested positive by the nucleocapsid protein-based enzyme immunoassay are grouped according to the SARS status and the time of collection. All false-positive specimens had OD below 1.0, whereas 102/111 true-positive specimens had OD readings above 1.0.

The overall specificity of the ELISARS^{TM} kit (IgGENE) was 97.8% (range: 96.0%–100%), and without significant variation among different groups of controls ($P < 0.01$ by Exact test).

DISCUSSION

Seroprevalence studies play an important role in revealing the epidemiology of an infectious disease. A highly sensitive and specific assay with a high throughput capacity is required for this purpose. In this study, it was found that the recombinant nucleocapsid protein-based microtitre plate EIA, ELISARS^{TM} (IgGENE), provided a high degree of sensitivity (96.2% for late serum samples) and specificity (97.8%) for the detection of anti-SARS-CoV IgG antibody from human serum samples. Thus, ELISARS^{TM} (IgGENE) fulfills the criteria to be a screening assay for large-scale studies. The good performance of ELISARS^{TM} is in line with those reported for other nucleocapsid protein-based assays [Shi et al., 2003; Chang et al., 2004; Guan et al., 2004; Huang et al., 2004; Liu et al., 2004; Woo et al., 2004a,b], indicating the nucleocapsid protein is a promising target for the development of serological assays for SARS-CoV infection.

It was found that a small fraction (0%–4%) of the non-SARS samples had false-positive results. Whether these were due to cross-reactivity with recent infections with other human coronaviruses such as 229E and OC43 requires further studies. All samples positive by ELISARS^{TM} should be followed by a confirmatory assay.

When compared to IFA, ELISARS^{TM} (IgGENE) was less sensitive in early samples as 28.2% of the EIA-negative specimens were tested positive by IFA. However, it is worth noting that one of the IFA-negative specimens showed a high OD reading when tested by ELISARS^{TM} (IgGENE), suggesting that the recombinant nucleocapsid proteins used in the ELISARS^{TM} (IgGENE) were able to detect a fraction of anti-SARS-CoV antibodies that were not revealed by the IFA. These antibodies might target epitopes that were not exposed in the acetone-fixed SARS-CoV-infected Vero cells that were used for IFA. Of the 61 samples with high IFA titres (=1:640), 3 were tested negative by the ELISARS^{TM} (IgGENE). This observation is in line with the hypothesis that the recombinant nucleocapsid protein-based assay is detecting a different spectrum of antibodies. Thus, the sensitivity may be increased by using a combination of assays in particular when applied to the early stage of infection. The majority (101/105, 96.2%) of SARS patients developed antibodies against the nucleocapsid protein after 3 weeks of onset of fever, and a small portion (10/45, 22.2%) had these antibodies developed within the first 2 weeks. On the other hand, a small fraction of patients (4/105, 3.8%) never developed these antibodies. Understanding the implication of differences in antibody response to the nucleocapsid protein is important for the development of nucleocapsid protein- or sequences-based vaccines which is in progress [Kim et al., 2004; Zhu et al., 2004].

An attempt was made to investigate whether the ELISARS^{TM} (IgGENE) assay can be applied for IgM antibody detection. Only 10.8% of early samples, and 61.5% of late samples were found positive for anti-SARS-CoV IgM, indicating that the recombinant nucleocapsid proteins used were suboptimal for anti-SARS-CoV IgM detection.

Another clinical application of serological assay is to confirm the infection status of suspected cases. This is important, as the currently available viral RNA and virus isolation system are not perfect in terms of sensitivity [Chan et al., 2004b; Chen et al., 2004]. In this regard, the IFA is still more superior as it provides an overall higher sensitivity and can detect anti-SARS-CoV antibody at an earlier stage. However, the preparation of IFA requires handling of a high concentration of infectious viruses where high containment facilities are required. Further studies to develop a reliable IgM assay is urgently needed to improve the diagnostic accuracy for SARS-CoV infection.

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