

## Development of immunoglobulin G enzyme-linked immunosorbent assay for the serodiagnosis of severe acute respiratory syndrome

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### Summary

Severe acute respiratory syndrome (SARS) is an emerging infectious disease caused by a novel SARS-associated coronavirus (SARS-CoV). The clinical characteristics are high fever, rapidly progressive diffuse pneumonitis and respiratory distress. It is highly infectious through intimate contact or direct contact with infectious body fluids. Outbreaks within communities and hospitals have been reported. Development of rapid and reliable diagnostic tools is urgently needed. We developed an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA), using whole virus antigen of SARS-CoV. Eighty-six serum samples collected from patients who were hospitalized for other causes were examined to determine the cut-off O.D. value. The cut-off O.D. value was defined as 0.175 by calculating the mean O.D. value of the 86 sera plus 3 standard deviations. To determine the sensitivity and specificity of the ELISA, 56 positive sera and 204 negative sera were tested. The sensitivity was 96.4% and the specificity was 100%. The results suggest that the IgG ELISA using whole virus antigen of SARS-CoV has a high sensitivity and specificity in detecting SARS IgG antibodies. This IgG ELISA is a powerful tool for serodiagnosis of SARS.

### Introduction

Severe acute respiratory syndrome (SARS) is an emerging infectious disease. The clinical characteristics are fever, dry cough, myalgia, dyspnea, lymphopenia, and abnormal chest radiograph

results [1,2]. A significant proportion of patients with SARS have a fulminant course characterized by high fever, rapidly progressive diffuse pneumonitis and respiratory failure [3]. Cases of SARS were first reported in Guangdong Province of southern China in November 2002, followed by reports from Vietnam, Hong Kong, Singapore, Canada, and Taiwan [1,2,4,5]. As of 29 September 2003, there have been 8098 persons reported as infected and 774 patients have died because of

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SARS. The case fatality ratio has reached 9.6% [5].

The responsible pathogen for SARS is a newly discovered SARS-associated coronavirus (SARS-CoV) [6,7]. This virus is transmitted through contact with respiratory droplets, fomites, or respiratory secretions. The virus is highly contagious and able to cause outbreaks within communities and hospitals [1,2,4]. Delayed diagnosis and failure to isolate SARS patients in hospitals can lead to the transmission of this virus to health-care workers and other patients [1,4,8]. However, it is very difficult to distinguish SARS-CoV infection from other acute viral respiratory diseases, such as influenza, based on the clinical presentations [8,9]. Current laboratory diagnosis of SARS-CoV infection is achieved by isolating the virus by cell culture or detecting the viral nucleic acids by reverse-transcriptase polymerase chain reaction (RT-PCR). These methods are either time-consuming or of inadequate sensitivities. For that reason, developing a rapid and reliable diagnostic tool is of urgent need. In this study, we describe a sensitive and specific ELISA in detecting SARS-CoV IgG for use in routine diagnosis and for large-scale population surveillance.

## Materials and methods

### *Preparation and identification of whole-virus antigen for ELISA*

SARS-CoV isolate (TW1) [4] was propagated in Vero E6 cells, in MEM medium (JRH, Lenexa, USA) supplemented with 2% fetal calf serum (FCS). Viruses were harvested from culture supernatant at 48 h after inoculation when more than 90% of cells showed cytopathogenic effects. After centrifugation ( $600 \times g$  for 10 min) and discarding the pellets containing exfoliated cells, the supernatants were heated at  $56^\circ\text{C}$  for 1 h to inactivate SARS-CoV and were filtered through  $0.2 \mu\text{M}$  pores (Sartorius, Goettingen, Germany) to dispose of bacteria. The supernatants were suspended in 20% polyethylene glycol (Sigma, St. Louis, MO, USA) and centrifuged again at  $8000 \times g$  for 4 h at  $4^\circ\text{C}$ . The pellets containing viruses were resuspended in PBS buffer and purified by 35–80% sucrose gradients centrifugation ( $8000 \times g$  for 16 h at  $4^\circ\text{C}$ ). The

viral fraction was washed with PBS and then centrifuged at  $8000 \times g$  for 4 h at  $4^\circ\text{C}$ . The pellets contained the purified whole cell viral antigens. The concentration of purified viral proteins was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

The purified whole virus antigen was subjected to polyacrylamide electrophoresis and stained with Coomassie blue and Western blotting analysis using SARS patients' sera to understand the proteins contained in this preparation.

### *IgG ELISA using SARS-CoV whole virus antigen*

Purified viral protein ( $5 \mu\text{g}$ ) was diluted in  $200 \mu\text{l}$   $50 \text{ mM Na}_2\text{CO}_3$  (pH 9.5) and was used to coat the wells of microtiter plates at  $4^\circ\text{C}$  overnight. The plates were then blocked with  $380 \mu\text{l/well}$  blocking buffer [5% FCS in Superblock (PIERCE, Rockford, IL, USA)] at room temperature for 1 h. The sera were pre-treated at  $56^\circ\text{C}$  for 30 min and then diluted at 1:50 in blocking buffer. Two hundred microliters of diluted sera were added to each well and incubated at room temperature for 30 min. The plates were then washed five times in washing buffer containing PBS and 0.5% Tween 20. Thereafter,  $200 \mu\text{l}$  of goat anti-human IgG antibody (Chemicon, Temecula, CA, USA) diluted at 1:80,000 in blocking buffer was added to each well and incubated at room temperature for 30 min. The plates were washed five times in washing buffer again. The specific anti-SARS-CoV IgG was detected by adding  $100 \mu\text{l}$  of TMB (KPL, Gaithersburg, MD, USA). Absorbance was measured at 450 nm after adding  $100 \mu\text{l}$  of  $\text{H}_2\text{SO}_4$  solution (1N).

### *Cut-off value determination*

We tested sera collected from patients visiting National Taiwan University Hospital for diseases other than SARS to determine the cut-off O.D. value for the IgG ELISA. None of the patients whose sera were used for cut-off value determination had a prior contact history with SARS patients. All the sera had been confirmed to be seronegative for SARS-CoV by immunofluorescence assay (IFA) at the dilution of 1:25. The mean O.D. value of the sera plus 3 standard deviations was calculated as the cut-off O.D. value of the IgG ELISA.

### ELISA diagnostic sensitivity and specificity

To determine the sensitivity and specificity of the ELISA, true positive and true negative control sera were defined and tested. True positive control sera were those collected from patients of SARS and tested positive by IFA. Clinical SARS patients were diagnosed according to WHO criteria [10]. True negative control sera were those collected in 2000 and 2001 before SARS had been first reported and tested negative by IFA.

### Statistical analysis

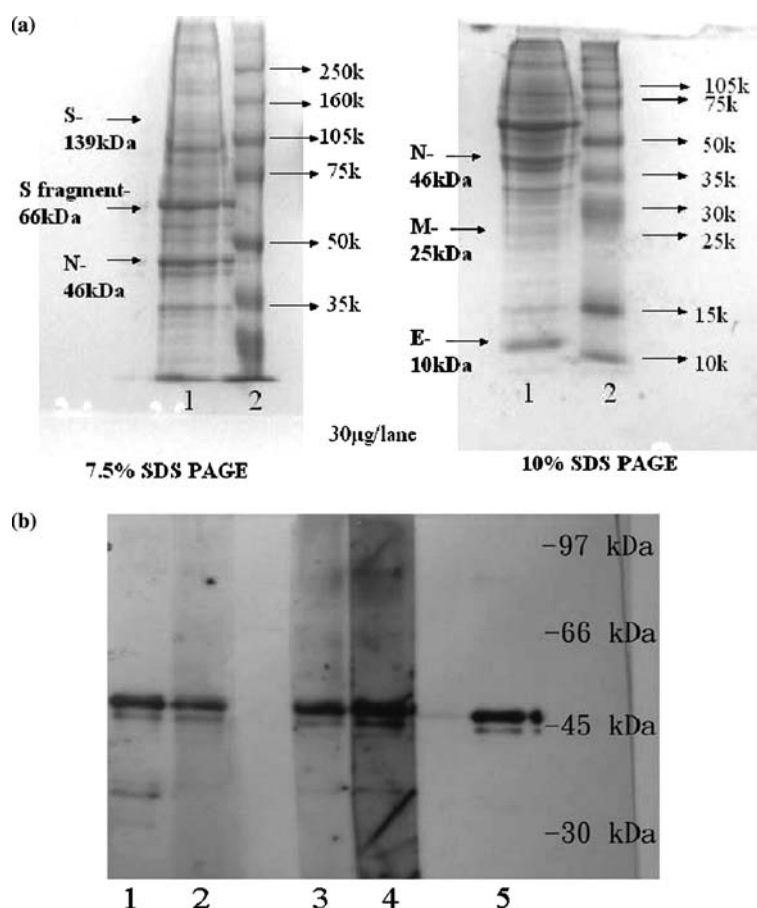
Data were analyzed with the SPSS Statistical Package (Version 11.5, Leadtools, Chicago, IL, USA). Spearman rank order correlation was used

to examine the correlation between O.D. values and time of patients' sera collection.

## Results

### Constituents of purified virus antigen

The purified SARS CoV antigen underwent an SDS PAGE and was stained with Coomassie blue as well as patients' sera (Figure 1). Several antigens were identified by the Coomassie blue stain in our whole virus preparation, including the four structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N) [11]. From the results of Western blotting, the major antigen recognized by the patients' sera was the 46 kDa N protein.



**Figure 1.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Western blot analysis of SARS-CoV whole virus antigen preparations. (a) Purified whole virus antigens were subjected to electrophoresis on 7.5 and 10% SDS-PAGE gels (lane 1). Proteins were visualized by staining with Coomassie Blue. Bands corresponding to S, S fragment, N, M, and E proteins are identified. The molecular masses of protein standard (lane 2) are shown at the right. (b) Western blot analysis of SARS-CoV whole virus antigen preparations using four different patients' sera is shown in lane 1 to lane 4. There is a major antigen recognized by patients' sera. The size of the major antigen is compatible with the mouse monoclonal anti-N antibody (lane 5).

### Cut-off value determination

Eighty-six sera collected from patients visiting National Taiwan University Hospital for other causes were tested for cut-off value determination, including 33 with respiratory tract infections, 30 with non-respiratory infections, and 23 with non-infectious diseases. All the sera were seronegative when tested by IFA. The mean O.D. value and the standard deviation were calculated to be 0.064 and 0.037, respectively. Thus, the cut-off value was defined to be 0.175 from the 86 sera.

### ELISA diagnostic sensitivity and specificity

#### Sensitivity

Fifty-six serum samples collected serially from 18 SARS patients were tested by both IFA and ELISA. There were three males and 15 females. Their ages ranged from 11 to 70 years old. All 18 patients had fever and pneumonia during April and May of 2003. Among them, eight were health-care workers of two hospitals that had nosocomial SARS outbreaks [12]. Six had visited or had been admitted to those two hospitals during the outbreak period. Three had household contact with a SARS patient. Detection of SARS-CoV nucleic acid by RT-PCR in plasma, throat swab, or stool samples was done in 11 patients, and 5 patients had positive results.

All the sera were positive for IgG anti-SARS CoV using IFA. They were collected from 3 to 110 days (median, 17.5 days) after fever onset. Fifty-four of the 56 sera were reactive in the IgG ELISA (O.D. value higher than the defined cut-off of 0.175). The O.D. value of the 56 positive sera ranged from 0.115 to 0.686, and the mean O.D. value was 0.286 (Figure 2). Forty-nine (87.5%) sera had an O.D. value higher than 0.2. The two false negative sera were collected 10 and 12 days after fever onset, respectively. The five sera that had O.D. values between 0.175 and 0.2 were collected 4, 10, 13, 14 and 20 days after onset of fever, respectively. The O.D. values tended to be higher, while the sera were collected later in the disease course ( $p < 0.001$ ) [Figure 3]. The sensitivity of the SARS-CoV IgG ELISA was 96.4%.

#### Specificity

Two hundred and four sera collected in 2000 and 2001 before SARS had been first reported were

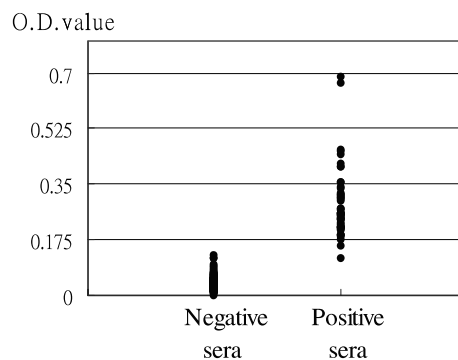


Figure 2. Scatter chart of 204 negative control sera and 56 positive control sera tested by the SARS-CoV IgG ELISA. The cut-off O.D. value was defined as 0.175.

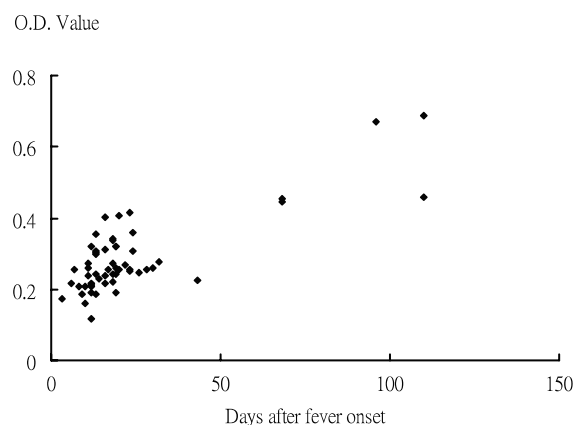


Figure 3. Correlation between the O.D. values and the time of patients' sera collection. The Spearman coefficient was 0.586 and  $p$ -value  $< 0.001$  ( $N = 56$ ).

considered as true negative controls. These sera were collected from healthy children and teenagers of different age groups: 57 from children aged less than 2 years old, 47 from 2 to 5 years old, 50 from 6 to 10 years old, and 50 from 11 to 15 years old. None of these sera had IgG antibody against SARS-CoV when tested by IFA. All 204 sera were seronegative by the ELISA with a mean O.D. of 0.036 (Figure 2). Two hundred and one sera (98.5%) had O.D. values less than 0.1. The specificity of the SARS-CoV IgG ELISA was 100%.

### Discussion

In this study, we developed a SARS-CoV IgG ELISA using whole SARS-CoV antigen. This IgG

ELISA is able to differentiate between positive and negative sera. The mean O.D. values for the positive sera and negative sera were 0.286 and 0.036, respectively. With the cut-off O.D. value set at 0.175, all 204 negative control sera collected in 2000 and 2001 had O.D. values lower than the cut-off, while 54 of the 56 positive controls collected from SARS patients had O.D. values higher than the cut-off. The sensitivity of the SARS-CoV IgG ELISA using whole SARS-CoV antigen is 96.4%, and the specificity is 100%. The positive predictive value is 100% and the negative predictive value is 99%. The results also correlate well with the IFA results.

There are two key steps in developing a good ELISA to detect antibodies against viral antigens. The first step is defining an appropriate cut-off value for positive readings, while the second step involves reducing the background noise. We set the cut-off value at the mean O.D. value plus 3 standard deviations after testing 86 negative serum samples. With the derived cut-off value (0.175), we were able to accurately differentiate seropositive from seronegative serum samples with high sensitivity (96.4%) and specificity (100%).

Initially we used skim milk-based blocking buffer as suggested by several protocols [13,14]. However, the background O.D. values were unacceptably high (data not shown). Therefore, we tried a new blocking buffer containing fetal calf serum and Superblock. We also increased the concentration of Tween 20 to 0.5% in the washing buffer. These modifications successfully reduced the non-specific reaction in the SARS-CoV IgG ELISA. Fetal calf serum has been used in the blocking buffer in some protocols [15,16]. One previous study also showed that blocking buffers containing fetal calf serum are able to reduce the background noise that is present in protocols using skim milk-based blocking buffers [16].

In this study, the whole SARS-CoV (TW1) was used as the antigen for the specific ELISA. Compared with recombinant viral protein as antigen, the advantages in using whole virus antigen are speed and simplicity of preparation without the need for recombinant-DNA techniques and expression, and purification of viral proteins. Recombinant protein antigen-based ELISA was found to be of low specificity in one ELISA for enterovirus 71 antibody detection [14]. Furthermore, infected patients usually mount variable

antibody responses to several antigens of a virus [17]. Using multiple virus antigens theoretically could be more sensitive in determining antibody response compared with assays using single viral antigen.

Safety issues are always a concern when developing a whole virus-based ELISA. In addition to operating in a level 3 biosafety laboratory, this protocol used prolonged heat of 60 min to inactivate the virus. This is necessary because there is evidence suggesting that heating less than 60 min is ineffective in sterilizing SARS CoV (S.R. Shih, personal communication).

Most positive sera in this study had O.D. values higher than 0.2. In the 7 sera with O.D. values lower than 0.2 (including two false negative sera), six were collected no later than 14 days after fever onset. Thus, we believe that a low O.D. value for a serum sample obtained within 2 weeks of fever onset should be interpreted with caution. Serial sampling is recommended to establish the diagnosis.

The 204 negative control sera were collected from children and teenagers under 16 years old during 2000 and 2001. These subjects were young and were supposedly experiencing frequent virus infections. None of these sera were reactive. Thus, it is suggested that cross-reactions to other prevalent viruses among children and teenagers is not a major concern in the SARS-CoV IgG ELISA.

In conclusion, we successfully developed an IgG ELISA using whole SARS-CoV antigen. The protocol is quick and simple, with high sensitivity and specificity in detecting SARS IgG antibodies. The IgG ELISA is a powerful tool for rapid serodiagnosis of SARS and for seroepidemiology survey.

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