Journal of Food and Drug Analysis

Volume 20 | Issue 4 Article 19

2012

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Journal of Food and Drug Analysis, Vol. 20, No. 4, 2012, Pages 938-946 doi:10.6227/jfda.2012200424

Establishment of a Serological Panel for *In Vitro* Diagnostics of Severe Acute Respiratory Syndrome

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(Received: May 23, 2012; Accepted: November 2, 2012)

ABSTRACT

Severe acute respiratory syndrome (SARS) is an infectious disease with a high mortality rate. Because SARS suddenly disappeared after an approximately 1-year-long pandemic period (November 2002 to July 2003), insufficient data was available for the development of *in vitro* diagnostics for SARS (SARS IVD). To rapidly identify cases in the event of future epidemics, it is necessary to establish a SARS serological panel. In this study, 20 SARS convalescent sera and 20 normal sera from the Taiwan CDC were used to establish a SARS serological panel. This can be used as a standard for the Taiwan FDA to evaluate the effectiveness of SARS IVDs during the premarket approval process. To characterize the immunological activity, protein extracts containing SARS coronavirus (SARS-CoV) proteins, synthetic viral peptide fragments and recombinant viral proteins, were used to detect antibody reactivity. Results demonstrated that synthetic S, M, N peptide fragments and whole SARS-CoV protein extracts had stronger antigenicity than individual recombinant viral proteins. Moreover, results of the ELISA and the immunofluorescence assay indicated that our SARS panel had no cross-reactivity with the human coronavirus 229E, and displayed weak cross-reactivity with human coronavirus OC43. These findings suggested that our SARS serological panel is suitable for evaluating SARS IVDs.

Key words: severe acute respiratory syndrome, serological panel, SARS coronavirus protein, human coronavirus 229E, human coronavirus OC43

INTRODUCTION

A worldwide outbreak of severe acute respiratory syndrome was first reported in China in late 2002. The disease was eventually termed "SARS" by the World Health Organization (WHO) in 2003. The SARS outbreak resulted in over 8,000 cases of infection with 10% mortality rate[(1)]. The etiological agent of SARS is a novel coronavirus named SARS-CoV[(2)]. The common human coronavirus strains 229E (HCoV-229E) and OC43 (HCoV-OC43) cause upper respiratory tract infections, but SARS-CoV infects the lower respiratory tract, causing fever, cough without phlegm, headache, hypoxemia, dyspnea, and eventually death from respiratory failure[(3)]. The SARS-CoV virion, similar to other coronaviruses, is composed of a spike (S) glycoprotein, a small envelope (E) glycoprotein, a membrane (M) glycoprotein, a nucleocapsid (N) phosphoprotein, and a hemagglutinin-acetylesterase (HA) glycoprotein. All of these proteins play a critical role in the viral infection process. S protein is responsible for receptor binding and can fuse the viral E protein to host cells. E protein is a viroporin involved in virus morphogenesis and budding. M protein, a typical transmembrane glycoprotein, is the most abundant structural protein in the SARS virion. Evidence indicates that N protein can block IFN-β production during the very early stage of infection[(4-11)]. These proteins not only serve as markers of virus infection but also act as potential targets for antiviral drug discovery[(12)].

The SARS diagnostic methods, announced by WHO, posed a number of challenges. For example, all procedures for SARS-CoV culture had to be performed in biosafety level 3 containment because of the high infectivity during SARS-CoV amplification. In addition, a study by Hsueh *et al.* showed that the viral load rapidly declines at 9 or 10 days after SARS onset[(13)]. Thus, molecular diagnosis is only suitable for early detection. It is difficult to estimate the initial onset of SARS infection based on clinical symptoms[(13)]. Hence, clinics need more effective diagnostic tools for SARS detection. A study by Drosten *et al.* showed that patients with acute SARS-CoV infection had virus-specific IgG

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seroconversion[14]. The specificity and sensitivity of SARS detection can reach > 90% by using SARS-CoV structural proteins, derived from mammalian cells or *Escherichia coli*, as antigens for the detection of anti-SARS-CoV IgG and IgM in enzyme-linked immunosorbent assays (ELISAs)[15-17]. In addition, reports indicate that the specificity and sensitivity of serological diagnostic methods can be improved to nearly 100% by using a combination of viral S, M, and N proteins for detection[13]. As demonstrated in these studies, peptide fragment recognition is commonly used in the development of serological diagnosis of SARS. However, a reliable reference panel is needed to evaluate the laboratory and clinical performance of these immunological detection methods. The SARS reference panel could also be used as a standard for national authorities to evaluate the effectiveness of SARS IVDs during the premarket approval process.

The first case of SARS was reported in China in mid-November 2002, and a global outbreak started in late February 2003. However, the disease suddenly disappeared after the last confirmed infection on July 2003, apart from the subsequent laboratory-acquired infections. At present, little is known about SARS pathogenesis, and there is yet no effective treatment. An insufficient number of SARS specimens are available for the development of serological diagnostic devices. In this study, SARS specimens collected in Taiwan in 2003 by the Centers for Disease Control of Taiwan (Taiwan CDC) were used to establish a human anti-SARS serum panel as a reference standard for SARS diagnosis. The relative titers of our SARS reference panel were characterized against inactivated whole SARS-CoV, various virus structural proteins, as well as their peptide fragments. Moreover, the cross-reactivity of this panel with HCoV-229E and HCoV-OC43 was evaluated by immunofluorescence assay (IFA).

MATERIALS AND METHODS

I. *Materials*

Minimum essential medium (MEM), *N*-acetyl-L-alanyl-L-glutamine, antibiotics (100×, lyophilized), fetal bovine serum (FBS), and trypsin/ethylenediaminetetraacetic acid (trypsin/EDTA) solution were purchased from Gibco BRL (Grand Island, NY, USA). Phosphate-buffered saline (PBS) was from BioChromAG (Berlin, Germany). Coomassie brilliant blue R-250 was from Sigma-Aldrich (St. Louis, MO, USA). Specific SARS-CoV ELISA kit and recombinant viral envelope and nucleocapsid protein were from United Biomedical Inc. (Taipei, Taiwan) and Serotech Laboratories Limited (Toronto, Canada), respectively. Goat anti-human IgG-HRP antibody was from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). Mouse anti-coronavirus 229E monoclonal antibody (clone 401-4A), mouse anti-coronavirus OC43 monoclonal antibody (clone 541-8F), and goat anti-mouse IgG- Fluorescein isothiocyanate (FITC) antibody were from Chemicon (MA, USA).

Table 1. List of human SARS sera specimens

Serum no.	Serum type	Serum titer	Days after onset at collection	Serum no.	Serum type
1	Positive	1:1,600	119	2	Negative
3	Positive	1:1,600	111	4	Negative
5	Positive	1:1,600	132	6	Negative
7	Positive	1:1,600	119	8	Negative
9	Positive	1:6,400	132	10	Negative
11	Positive	1:1,600	134	12	Negative
13	Positive	1:1,600	121	14	Negative
15	Positive	1:1,600	121	16	Negative
17	Positive	1:1,600	132	18	Negative
19	Positive	1:1,600	135	20	Negative
21	Positive	1:1,600	150	22	Negative
23	Positive	1:6,400	180	24	Negative
25	Positive	1:400	149	26	Negative
27	Positive	1:1,600	133	28	Negative
29	Positive	1:6,400	149	30	Negative
31	Positive	1:6,400	152	32	Negative
33	Positive	1:1,600	139	34	Negative
35	Positive	1:1,600	150	36	Negative
37	Positive	1:1,600	155	38	Negative
39	Positive	1:6,400	125	40	Negative

II. *Specimens Collection*

Twenty independent human anti-SARS convalescent sera [SARS (+)], collected from confirmed SARS patients during the SARS outbreak in Taiwan by the Taiwan CDC, were used in this study. Moreover, 20 independent normal human sera [SARS (-)], obtained from healthy individuals in Taipei Blood Center before the SARS outbreak, were also used (Table 1). The average age of 20 healthy donors ranged from 20 to 40 years old. According to regulations regarding patient privacy in Taiwan, the SARS patient information, including age, gender and geography, from the Taiwan CDC were not disclosed. All SARS (+) sera were inactivated with gamma irradiation to reduce the potential risk of infection. The panel was filled with SARS (+)/(-) sera at 0.1 mL per vial, and samples were stored at -20℃.

III. *Relative Titers of IgG/IgA/IgM Antibodies in SARS Sera against Whole SARS-CoV Viral Protein Extracts*

The indirect ELISA protocol using inactivated SARS-CoV was modified from a previous study[18]. Briefly, the SARS-CoV-infected Vero cells were sonicated in a chilled cup horn for 10 min and then centrifuged at 10,000 ×g for 5 min at 4°C. The supernatant was inactivated with gamma-irradiation (20,000 Gy) and used as viral antigen. ELISA plates (Immulon II HB) were coated with SARS-CoV viral antigen. Plates were washed 3 times with PBS Tween-20 (PBST), followed by the addition of different dilutions of sera (1:100-1:6,400) in solutions containing PBST with 5% skim milk, and incubated at 37°C for 1 h. Plates were washed, and subsequently, diluted goat anti-human IgG/IgA/IgM horseradish peroxidase conjugate antibody (1:4,000) was added, and the plates were incubated at 37°C for 1 h. Wells were washed with PBST 3 times, the peroxidase color reaction was performed using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as substrate (KPL, Gaithersburg, MD, USA) at 37°C for 30 min, and the reaction was terminated using ABTS stop solution. Absorbance was read at 405 nm with a 490-nm reference filter. Antibody titers were determined at the highest dilution where the positive adjusted optical density (O.D.) value was ≥ 0.21. For a sample to be considered positive, the sum-adjusted O.D. (the sum of the differences between the positive and negative wells) for the 1:100 through 1:6,400 dilutions must exceed 1.25, and the antibody titer of the sample must be 1:400 or greater.

IV. *IgG Antibody in SARS Sera against Different SARS-CoV Proteins*

(I) *Synthetic S, M, and N Peptide Fragments*

The Tor2 SARS-CoV genome sequence was used to obtain the coding sequences for S, M, and N peptide synthesis according to the previous study[19]. SARS (+) and SARS (-) sera were diluted 1:200, transferred to the S, M, and N peptide-coated microplate (containing 2 μg/mL of a mixture of the S, M, and N protein-derived peptides), and incubated at 37°C for 60 min. The experimental ELISA protocol followed the operation manual of the specific SARS-CoV EIA kit. Distilled water was used as a negative antigen control. Finally, the O.D. was read at 450 nm. In determining the variant reactivity of SARS sera against synthetic peptide fragments, the lower cutoff value for the assays was set by calculating the mean absorbance value of SARS (-) sera plus 2 standard deviations (SD)[20-21].

(II) *Recombinant E and N Proteins*

Recombinant full-length E and N proteins (1 μg/μL) were individually coated on different 96-well plates at 4°C for 16 h. The experimental ELISA was performed according to the manufacturer's protocol.

V. *Relative Titers of IgG Antibody in SARS Sera against Different SARS-CoV Proteins*

Different SARS sera were serially diluted from 1:200 to 1:6,400 and individually added to the microplates precoated with synthetic peptides (S, M, and N peptides) or recombinant E and N proteins. The ELISA protocol was as described above.

VI. *Cross-Reactivity between Different SARS Sera and HCoV-229E/HCoV-OC43*

(I) *Cell Culture*

The MRC-5 human diploid fibroblast cell line, obtained from the American Tissue Culture Collection (Manassas, VA, USA; CCL-171), was maintained in MEM supplemented with 10% FBS, 100 mg/L streptomycin, and 100 units/mL penicillin. The culture was incubated at 37°C in a humidified atmosphere containing 5% CO_2. Upon reaching confluence, cells were washed with PBS, trypsinized with 0.25% trypsin-EDTA, washed with fresh culture medium, and transferred to 6-well plates (1×10^5 cells/mL) for virus infection assays.

(II) *Immunofluorescence Assay*

Before the assay, MRC-5 cells were trypsinized and seeded on a 6-well plate (1×10^5 cells/mL) with MEM containing 2% FBS. Upon reaching confluence, cells were infected with HCoV-229E (ATCC VR-740) or HCoV-OC43 (ATCC VR-759) at 37°C for 2 h and maintained in MEM with 2% FBS at 37°C in a 95% humidified atmosphere containing 5% CO_2 for 3 days. After virus infection, the culture medium was aspirated and cells were treated with Carnoy's fixation solution for 15 min. Fixed MRC-5 cells were treated with 5% normal goat serum and individually reacted with mouse anti-HCoV-229E, anti-HCoV-OC43 monoclonal antibody, SARS (+) sera, or SARS (-) sera at 37°C for 2 h. Mouse anti-HCoV-229E and anti-HCoV-OC43 monoclonal antibodies were used as positive controls. MRC-5 cells without

HCoV-229E or HCoV-OC43 infection acted as the negative control. FITC-conjugated goat anti-mouse IgG or anti-human IgG antibody reacted with MRC-5 cells at a 1:10,000 dilution. Immunofluorescence images were observed under an Olympus inverted fluorescence microscope (Tokyo, Japan).

(III) *Enzyme-Linked Immunosorbent Assay*

Virus peptides were extracted from MRC-5 cells infected with HCoV-229E and used as the antigen for microplate coating. In brief, the HCoV-229E-infected MRC5- cells were lysed with radio immunoprecipitation assay (RIPA) solution on ice. Cell lysate solution was then centrifuged at 13,000 ×g for 10 min at 4°C, while the supernatant was collected as virus peptides. The microplate coating procedure and ELISA protocol were carried as described above.

VII. *Statistical Analysis*

Data are presented as the mean and SD. Statistical significance was determined using Student's *t*-test.

RESULTS

Our SARS panel is composed of 20 SARS-positive sera from convalescent individuals and 20 normal sera from healthy individuals collected before the SARS outbreak. The panel was designated as the reference panel for the clinical evaluation of SARS IVDs by the Taiwan FDA. The analytical results of the tests conducted with this panel would be used to evaluate SARS IVDs by the Taiwan FDA during the premarket approval process.

I. *Relative Titers of SARS (+) Sera against Whole SARS-CoV Protein Extracts*

To assess the immunological responses of our SARS (+) sera to SARS-CoV, SARS-CoV protein extracts from SARS-CoV-infected Vero cells were used to determine the relative human IgG/IgA/IgM antibody titers. The results demonstrated that all SARS (+) sera have immunological titers ranging from 1:1,600 to 1:6,400, except for serum #25 (Table 1). Sera #9, #23, #29, and #31 had the highest immunological reactivity against the whole SARS-CoV protein extracts, and serum #25 had the lowest reactivity. These results confirmed that the 20 SARS convalescent sera in our panel have immunological activities against SARS-CoV.

II. *Reactivity of SARS Sera against Synthetic Peptide Fragments and Recombinant Viral Proteins*

Since the SARS outbreak in 2003, several types of SARS ELISA IVDs have been developed using a single SARS viral protein or viral protein compositions. Various designs were developed to obtain optimal reaction sensitivity and specificity. To clarify the immunological response of different SARS viral proteins against our panel, an ELISA kit containing commercial synthetic SARS S, M, and N peptide fragments and an ELISA kit containing recombinant SARS E or N proteins were used. In the former kit, the assay plates were precoated with S, M and N peptide fragments for analysis. In the latter, the assay plates needed to be manually coated with recombinant SARS E or N proteins before testing.

(I) *Synthetic S, M and N Peptide Fragments*

The cutoff value for the analysis with synthetic S, M and N peptide fragments was determined to be 0.332. An absorbance value higher than the cutoff value was considered a positive response. The mean signal/cutoff (S/C) ratios for SARS (+) and SARS (-) sera were 7.477 and 0.731, respectively. The scatter charts of absorbance values with SARS (+) and SARS (-) sera showed that a total of 18 SARS (+) sera had positive IgG reactivity against synthetic peptide fragments (Figure 1A). Only two sera samples, #25 and #27, had no IgG reactivity with peptide fragments among 20 SARS (+) sera, so that the sensitivity was 90% (18/20). In addition, only the negative serum #36 displayed the positive response among 20 SARS (-) sera, while the specificity was 95% (19/20). The relative titers of sera #25 and #27 against the whole SARS-CoV protein extracts were 1:400 and 1:1,600, respectively. Therefore, a quantitative analysis was performed to calculate the relative IgG titers against synthetic S, M, and N peptide fragments. Results showed that sera #9, #29, and #39 had higher titers (1:3,200), but sera #25 and #27 still had no titer or had a very low titer (1:50). The other SARS (+) sera had titers ranging from 1:200 to 1:800.

(II) *Recombinant N and E Proteins*

The SARS N protein is widely used as a detection target for serological diagnosis of SARS. Recombinant SARS N protein was coated on a 96-well plate and reacted with SARS (+) sera. Figure 1B showed the scatter charts for the absorbance value of the SARS panel against the recombinant N protein, while the cutoff value was calculated to be 1.183. Only 12 SARS (+) sera showed positive IgG responses. Eight of the 20 sera had no IgG reactivity against the recombinant N protein. In addition, in the quantitative analysis, the relative IgG titers of the 20 SARS (+) sera against the recombinant N protein ranged from 1:100 to 1:25,600 (Figure 2). Eight SARS (+) sera had no IgG reactivity, but their relative titers were still within the the range of 1:100 to 1:200.

The recombinant SARS E protein was also used to evaluate IgG titers, with a cutoff value of 0.384. All SARS (+) sera had positive IgG reactivity (Figure 1C). The mean S/C ratios for the SARS (+) and SARS (-) sera were 2.284 and 0.861, respectively. Quantitative titers against the recombinant E protein ranged from 1:800 to 1:6,400 (Figure 2). The immunological responses of the SARS (+) sera against the recombinant E protein were more consistent than those against the recombinant N protein.

III. *Cross-Reactivity with HCoV-229E and HCoV-OC43*

SARS-CoV is a member of the *Coronaviridae* family. HCoV-229E and HCoV-OC43 are human coronaviruses that commonly cause upper respiratory tract infections. Therefore, the cross-reactivity between SARS-CoV and these two other CoVs should be elucidated. In this study, HCoV-229E and HCoV-OC43, inoculated into MRC-5 cells, were used as the reaction targets for the immunofluorescence assay (IFA). In images obtained under the fluorescence microscope, 5 SARS (+) sera (#9, #23, #29, #31, #39) and 5 SARS (-) sera, reacted with HCoV-229E-infected cells, showed the same fluorescence intensity (Figure 3A). Comparison of the fluorescence signal obtained using the mouse anti-HCoV-229E monoclonal antibody with the fluorescence signal obtained with the SARS (+) sera revealed that the latter was due to background (non-specific) labeling. In addition, all of these sera, 5 SARS (+) and 5 SARS (-) sera, reacted with HCoV-OC43-infected cells and displayed weak fluorescence signals, which was weaker than our positive control, the mouse anti-HCoV-OC43 monoclonal antibody (Figure 3B). Furthermore, ELISA results deduced the absorbance values of the anti-HCoV-229E antibody, SARS (+) sera, and SARS (-) sera to be 1.708, 0.616, and 0.616, respectively (Figure 4). These results indicated that our SARS serological panel exhibited weak cross-reactivity with HCoV-OC43, but has weak or no cross-reactivity with HCoV-229E.

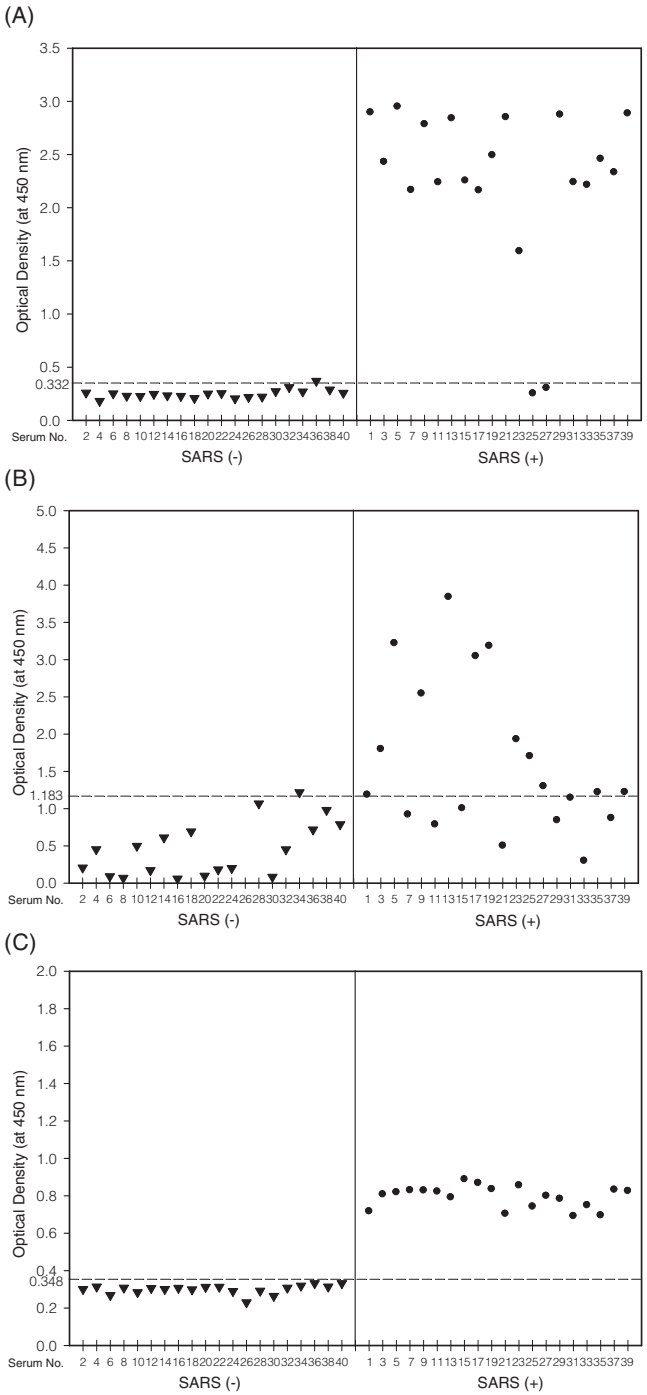

Figure 1. IgG antibody against SARS-CoV synthetic peptide in SARS sera. Synthetic S, M, and N peptide fragments (A); recombinant N protein (B); and recombinant E protein (C). ▼: SARS (-) sera; ●: SARS (+) sera. The dotted line indicates the cutoff value for each group. The cutoff value was calculated as the mean absorbance value of SARS (-) sera plus 2 SD.

DISCUSSION

One of the most important issues in developing IVDs is obtaining standard reference materials. Analytical sensitivity and specificity, which are determined using reference standards or panels, are required for the evaluation of clinical specimens. A global SARS outbreak suddenly appeared between late 2002 and mid 2003. This highly contagious and deadly disease then suddenly disappeared after July 5, 2003 (with the exception of several laboratory-associated infections)[22]. The brevity of the pandemic made specimen collection extremely challenging, and thus, it was very difficult to develop SARS diagnostic methods and devices for academic or industrial use. The Taiwan FDA is a government agency responsible for establishing national standards

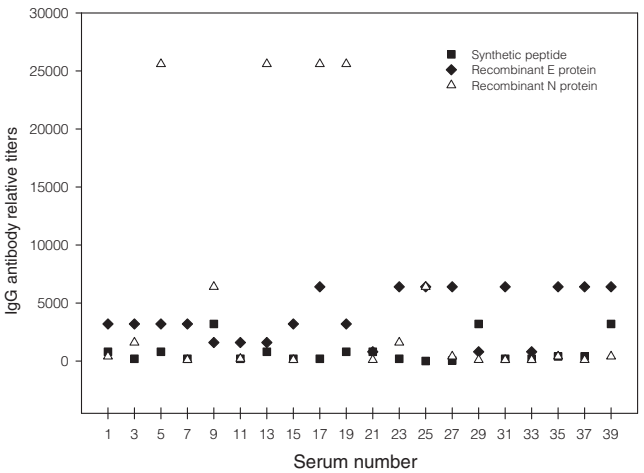

Figure 2. Relative titers of IgG antibody in our reference panel against different SARS-CoV antigens.

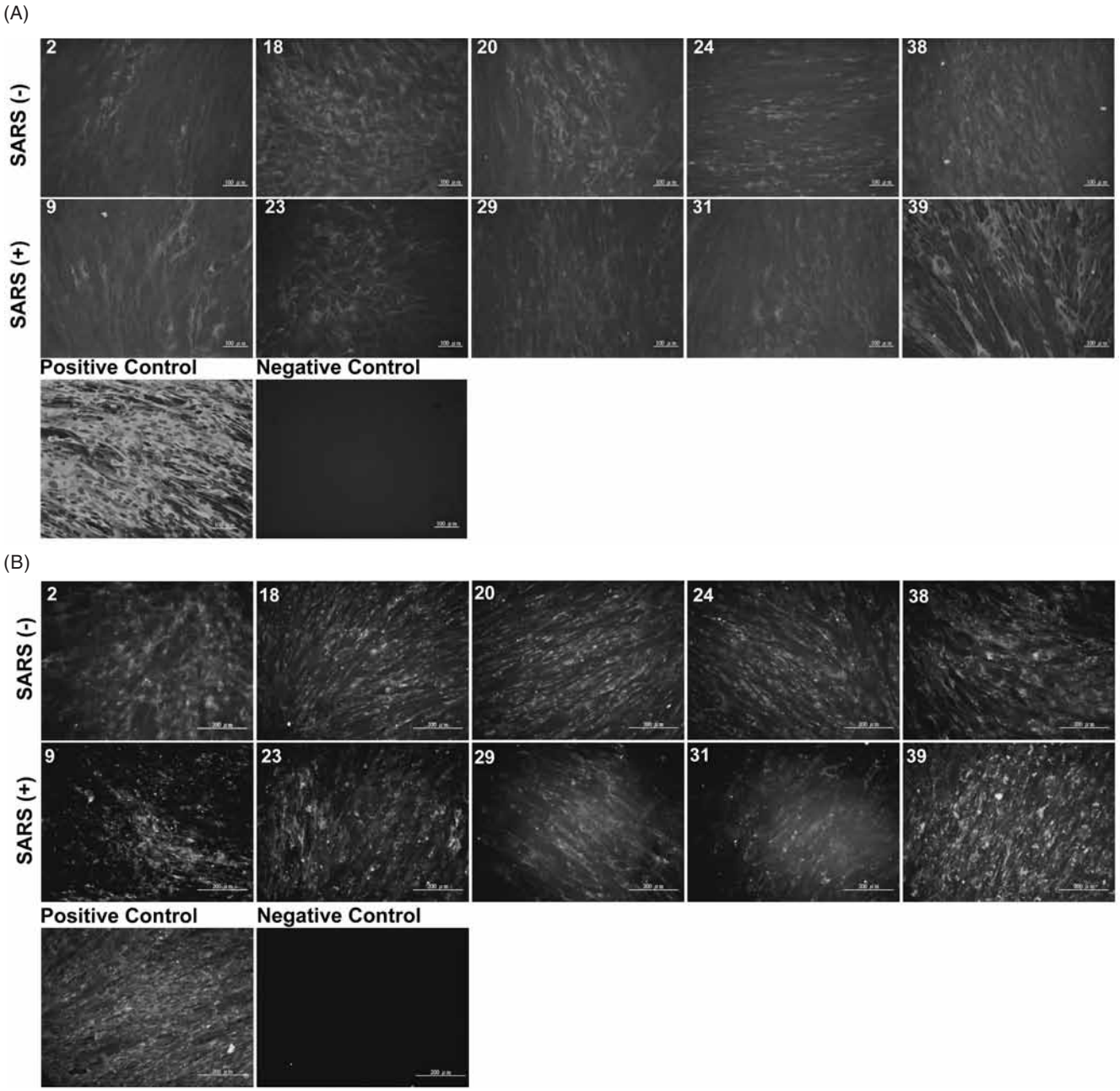

Figure 3. Immunofluorescence staining of SARS sera against HCoV-229E-infected (A) and HCoV-OC43-infected MRC-5 cells (B). SARS (-) sera: #2, #18, #20, #24, and #38; SARS (+) sera: #9, #23, #29, #31, and #39; Positive controls: mouse anti-HCoV-229E monoclonal antibody reacted with HCoV-229E-infected cells in panel A and mouse anti-HCoV-OC43 reacted with HCoV-OC43-infected cells in panel B, respectively. Negative controls: MRC-5 cells without HCoV-229E or HCoV-OC43 infection.

and serological reference panels for the control of biological products. To prepare for a potential future SARS outbreak, the Taiwan CDC and FDA collaborated to establish a serological SARS reference panel for the clinical evaluation of newly developed SARS IVDs. The Taiwan FDA has 30 sets of the SARS reference serological panel. This panel is provided free of charge to biological industries if research program proposals are submitted for approval by the agency.

In this study, we used different ELISA methods to evaluate the immunological reactivity of the SARS panel against recombinant SARS proteins and synthetic peptide fragments. In addition, Dr. Lia Haynes in the Centers for Disease Control and Prevention, United States (US CDC), assisted us in evaluating the immunoactivity of this panel against the whole SARS-CoV protein extracts. Previous reports have shown that seroconversion of the IgG/IgA/IgM antibody occurs at an average of 10 days after infection, and serum levels of this antibody can be detected 210 days after onset[13,23]. Hence, we chose the IgG/IgA/IgM antibody to analyze the immunological response against

Table 2. Summary of our SARS reference panel against different antigens by IgG relative titers

Antigen for ELISA	Relative immunological titers of SARS (+) sera			
	> 1:3,200 (High)	1:1,600-1:800 (Moderate)	1:400-1:200 (Low)	<1:100 (None)
SARS-CoV extract	9, 23, 29, 31, 39	1, 3, 5, 7, 11, 13, 15, 17, 19, 21, 27, 33, 35, 37	25	-
Synthetic S/M/N peptide	9, 11, 29, 39	1, 5, 13, 19, 21	3, 7, 15, 17, 23, 31, 33, 35, 37	25, 27
Recombinant E protein	1, 3, 5, 7, 15, 17, 19, 23, 25, 27, 31, 35, 37, 39	9, 11, 13, 21, 29, 33	-	-
Recombinant N protein	5, 9, 13, 17, 19, 25	3, 23	1, 11, 27, 35, 39	7, 15, 21, 29, 31, 33, 37

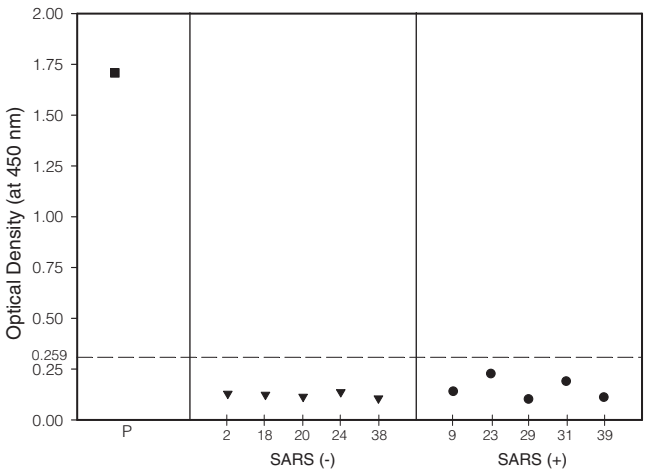

Figure 4. IgG antibody against HCoV-229E peptide in SARS sera. Positive control (P, ■): mouse anti-HCoV-229E monoclonal antibody, ▼: SARS (-) sera, ●: SARS (+) sera. The dotted line indicates the cutoff value, which was calculated as the mean absorbance value of SARS (-) sera plus 2 SD.

the whole protein extracts of SARS-CoV. The analytical results obtained using the whole protein extracts may mimic the immunological responses against SARS-CoV by the relevant antibodies in the human body. As shown in Table 1, all SARS (+) sera collected between 111 and 180 days after disease onset were confirmed to be seroconverted. Because of the diversity of human serum proteins, the relative titers of 20 SARS (+) sera ranged from 1:400 to 1:6,400. A study by Liu *et al.* showed that the antigenicity percentage of SARS-CoV polypeptides in SARS convalescent plasma ranged from 3.5 to 96.5%[24]. We compared antibody titers against the whole protein extracts, synthetic peptide fragments, and individual recombinant proteins to characterize the immunological responses of the SARS panel (Table 2). We found that SARS (+) sera that reacted with whole SARS-CoV extracts had an immunological response similar to those reacted with synthetic S, M and N tripeptide fragments. In this study, Tor2 sequence was used to synthesize the antigen fragments because Tor2 sequence was firstly sequenced from the clinical specimen and mostly used for diagnosis. In 2003, a study by Marco *et al.* sequenced the 29,751-base genome of SARS-associated coronavirus, which was isolated from a fatal SARS patient belonging to the original case cluster from Toronto, Canada. After that, many reports discussed about the Tor2 sequences[25,26]. Our result indicates that the synthetic S, M and N peptide fragments, derived from Tor2 SARS-CoV genomic coding sequences, are adequate ELISA targets for serological diagnosis of SARS[19]. However, further investigation is required to identify the optimal composition of viral peptide fragments for the detection of SARS antibodies.

The nucleocapsid protein is the predominant viral structural protein and is shed in large amount into serum, nasopharyngeal aspirate, throat wash samples, fecal matter and urine during the early days of infection. A study by Che *et al.* demonstrated successful detection of SARS-CoV N protein in various body fluids using specific monoclonal antibodies[27]. The immunodominant epitopes of N protein are already used in ELISA, IFA, enhanced chemiluminescence immunoassay, and Western blotting[28]. A study by Suresh *et al.* showed that SARS-CoV N protein can be detected during the acute phase of SARS infection[29]. However, we found that the IgG titers of SARS (+) sera against individual N proteins were different from those against the whole viral protein extracts. At least 7 SARS (+) sera had no IgG reactivity against N protein. This may be due to the fact that our convalescent SARS (+) sera were collected > 110 days after disease onset. A study by Nicholls *et al.* showed that viral replication and the amount of SARS-CoV nucleocapsid rapidly decreased after the first 2 weeks of infection[30]. This may explain why the recombinant N protein exhibited the poorest immunoactivity in their study.

Viral envelope proteins are also used for SARS detection with monoclonal antibodies[31]. In our study, SARS (+) sera showed stronger immunoactivity against individual viral E proteins than against N proteins. On the basis of our findings, we suggest that our SARS panel may be suitable for evaluating the performance of SARS confirmatory IVDs. SARS diagnosis can be divided into 2 parts: early detection and disease confirmation. Patients suspected to have SARS are first screened using early detection kits, and then those with a positive SARS response are managed with suitable medical treatments. SARS is a highly contagious disease. All patients suspected to have SARS have to be quarantined. SARS confirmatory IVDs could be used to confirm SARS-CoV infection in these patients. Through a combination of

early detection and disease confirmation, nosocomial SARS infection can be prevented. Our SARS serological panel is adequate for assessing SARS confirmatory IVDs and is a valuable tool for the rapid identification of SARS patients.

There were 4 major strains of human coronaviruses worldwide. Among them, HCoV-229E and HCoV-OC43 were found in the 1960s, while HCoV-NL63 and HCoV-HKU1 were found after the SARS epidemic. Based on the phylogeny of CoVs RNA-dependent RNA polymerase gene, HCoV-229E and HCoV-NL63 are classified as group 1b coronaviruses, while HCoV-OC43 and HCoV-HKU1 are classified as group 2a coronaviruses. SARS-CoV is classified into group 2b[32]. Research has shown that antigenic cross-reactivity is found between SARS-CoV and other CoVs. Furthermore, a study by Chan *et al.* revealed that most SARS patients or healthy blood donors have preexisting antibodies to CoVs[17,23]. Hence, we chose HCoV-229E from group 1 and HCoV-OC43 from group 2 for the cross-reactivity analysis of our SARS panel. Our results demonstrated that HCoV-229E had negligible or very weak cross-reactivity with SARS (+) sera, as determined by IFA and ELISA. However, HCoV-OC43 displayed weak cross-reactivity with SARS (+) and SARS (-) sera (Figure 3B). A study by Chan *et al.* showed that the 12 of 20 SARS patients (60%) had 4-fold elevated antibody titers against HCoV-OC43 or HCoV-229E, or both. In addition, SARS-CoV antibody responses were positively correlated with the increasing titers of preexisting HCoV-229E, HCoV-OC43, and HCoV-NL63 antibodies[22]. Furthermore, 3 HCoV-229E positive patients and 11 HCoV-OC43 positive patients with no prior exposure to SARS-CoV displayed no cross-reacting antibody responses to SARS-CoV. Therefore, the likely reason for the cross-reactivity of our SARS panel is that our SARS sera had very weak or no immunological memory toward HCoV-229E, but contain preexisting antibodies against HCoV-OC43[17]. Taken together, the results suggested that our SARS panel, using SARS antibody detection, may provide a relatively specific assay for the development of SARS IVDs, even if the sera from SARS patients possess weak cross-reactivity with HCoV-OC43.

Although there has been no native SARS infection since 2004, preparedness for a potential future SARS pandemic might have the same relevance as pandemic flu preparedness. We established a SARS serological panel and examined its immunological characteristics against different types of SARS viral proteins. One set of the SARS panel was provided to a diagnostic biotechnology company in Boston in the United States for SARS diagnostic evaluation in 2006. The preliminary values obtained for analytical sensitivity and specificity ranged from 94 to 100% and 84 to 100%, respectively (data not shown). Our SARS serological panel was also provided to the US CDC for SARS coronavirus diagnostic and antibody kinetic studies[33]. Because only few SARS serological panels have been established, our SARS panel is important and useful for clinical, epidemiological, and public health research.

ACKNOWLEDGMENTS

We thank Dr. Lia M. Haynes from the US CDC for performing the test on IgG/IgA/IgM antibody relative titers in the SARS sera experiment, Drs. Eric T.T. Suen and Chia-Po Lin for technical support, and Dr. Mei-Ying W. Yu for facilitating communication with the Center for Biologics Evaluation and Research/US FDA and US CDC. We would like to thank the Department of Health, Executive Yuan, Taiwan (Grant no. DOH94-FD-2010) for financially supporting this study.

DISCLAIMER

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the US CDC.

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