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Research paper

Evaluation of a safe and sensitive Spike protein-based immunofluorescence assay for the detection of antibody responses to SARS-CoV

Ivanus Manopo, Liqun Lu, Qigai He, Li Lian Chee, Shzu-Wei Chan, Jimmy Kwang*

Animal Health Biotechnology, Temasek Life Sciences Laboratory, 1 Research link, National University of Singapore, 117604 Singapore

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Abstract

Previously, we have identified a truncated antigenic fragment named protein C [441 to 700 amino acids (a.a.)] as the immunodominant fragment of Spike (S) protein of severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV). We have now successfully expressed protein C using the baculovirus system in *S. frugiperda* (Sf-9) cells. This recombinant baculovirus expressing protein C was first characterized using five SARS convalescent human sera and five normal human sera. The results showed that protein C is an authentic antigen against SARS-CoV antibody. Our Spike protein-based immunoflourescence assay (IFA) based on this recombinant baculovirus-Sf-9 system was further assessed with a panel of 163 clinical samples collected during the SARS epidemic in Singapore, which include samples from 21 clinically confirmed SARS, 42 non-SARS patient sera, and 100 normal sera. The results were compared to a commercial SARS IFA kit (EUROIMMUN, Germany) and a conventional IFA test performed in Singapore General Hospital. All of the 21 SARS-positive serum samples could be recognized by our IFA, giving a specificity and sensitivity of 100%, which was compatible with both whole virus-based IFA assays. No cross-reactivity with serum samples against infectious bronchitis virus (IBV) and transmissible gastroenteritis virus (TGEV) were detected in our assays. Thus, our Spike protein-based IFA could offer a safer procedure which can be performed in a BSL-2 laboratory as it could mimic the whole virus based-IFA without any loss of sensitivity and specificity. It is also more user-friendly and cost-effective than the whole virus-based IFA.

Keywords: SARS-CoV; Spike protein; IFA; Antibody detection

1. Introduction

Severe acute respiratory syndrome (SARS), an atypical pneumonia of unknown aetiology, was first identified in Guangdong Province, China, in Novem-

* Corresponding author. Tel.: +65 68727473; fax: +65 68727007.
E-mail address: kwang@tll.org.sg (J. Kwang).

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ber 2002. By May 2003, the disease outbreak spread worldwide affecting thousands of individuals, resulting in 764 deaths. The causative agent, SARS-associated coronavirus (SARS-CoV), a novel CoV (order Nidovirales, family Coronaviridae, genus *Coronavirus*), was recognized at the end of February 2003 and declared a global threat to health by WHO. This disease is highly contagious, and if SARS-CoV maintains its pathogenicity and transmissibility, it could become the first severe new disease of the 21st century with global epidemic potential.

Phylogenetic analyses indicate that the SARS coronavirus is not closely related to any of the previously characterized coronaviruses and forms a distinct group within the genus (Ruan et al., 2003). The Coronaviridae family contains enveloped positive-stranded RNA viruses that cause respiratory and enteric diseases in human and animals. Their genome of around 30 kb is the largest found in RNA viruses, encoding 23 putative proteins, which include 4 major structural proteins: Spike (S), Nucleocapsid (N), membrane (M), and small envelope (E). The Spike protein is a large membrane glycoprotein that forms 180- to 190-kDa peplomers that bind to receptors on CoV-susceptible cells and induce cell fusion. S protein is also a well-known neutralizing antigen of many coronavirus (Homberger, 1994; Jackwood and Hilt, 1995; Callebaut et al., 1996; Gomez et al., 1998). SARS Spike protein has a low level of similarity [20% to 27% pairwise amino acid (a.a.) identity] when compared to Spike proteins of other CoV (Lu et al., 2004).

Various diagnostic methods for SARS-CoV detection are available, such as sequence detection, serological assay, etc. Sequence detection of SARS-CoV infection using real-time RT-PCR has been used to detect viral RNA in clinical samples. Although it is more sensitive and specific than a serological assay, the drawback of using real-time RT-PCR is that the equipment is very expensive. Differences in optimization of RNA extraction, delays in processing specimens, and deterioration of samples may further lower the efficiency of the assay. These drawbacks make real-time RT-PCR a difficult procedure to use in the majority of clinical laboratories, especially those in developing countries.

Currently, the more commonly used diagnostic assay for the detection of SARS-CoV is the detection of antibodies by immunoflourescence assay (IFA). IFA is recognized as the "gold standard" (Tang et al., 2004). It uses inactivated SARS-CoV-infected cells fixed on a slide as antigen to detect SARS antibody in serum samples. It is known that conventional whole virus IFA can detect positive SARS-CoV infection using sera collected as early as 8 days. However, conventional whole virus IFA is not so convenient since it initially utilizes infectious SARS-CoV to infect cell culture before fixating on the slides. Although the inactivated IFA slides can be handled in biosafety level 2 (BSL-2) laboratories, the initial infection procedure needs to be carried out in a BSL-3 research facility. This makes the whole virus IFA difficult to perform in most laboratories, as there is a risk of contamination when dealing with live virus. Due to the current problems and safety issues with the whole virus IFA, a safer serological assay with little risk of contamination is needed for the diagnosis of SARS-CoV infection. This has led to the development of Nucleocapsid protein-based ELISA and immunoblot assays. However, until recently, little information has been available to evaluate Spike protein-based immunoassays for the detection of specific antibodies in SARS patients.

There are three serotypes of coronaviruses, class 1 and 2 containing mammalian viruses and class 3 containing only avian viruses (Enjuanes et al., 2000). Analysis of the SARS-CoV genome shows that this virus does not belong to any of the three classes described above, although it has the same genome organization as other coronaviruses. Further interpretation suggests that SARS-CoV proteins share low homology with other known coronaviruses (Rota et al., 2003). The SARS-CoV Spike gene encodes a glycoprotein of approximately 150 kDa containing 1255 amino acids, which is normally cleaved into an S1 domain and an S2 domain (Jackwood et al., 2001). Recently, an angiotensin converting enzyme 2 (ACE2)-recognizing site has been identified in the Spike protein S1 domain as the functional receptor for SARS-CoV in humans (Li et al., 2003). The Spike protein may mediate membrane fusion and induce neutralization antibodies in the host, raising the possibility that

antibodies against the SARS-CoV Spike protein may be a good marker for early detection and neutralization of SARS-CoV infections.

In our previous study, we identified a major immunodominant domain of SARS-CoV Spike protein from 441 a.a. to 700 a.a. Named protein C, this represents a segment of the S1 domain (Lu et al., 2004). To study the diagnostic potential of our Spike protein-based immunoassay based on protein C expressed by recombinant baculovirus in Sf-9 cell, a panel of sera containing 21 SARS-positive samples collected 7–76 days after the onset of SARS symptom, 42 non-SARS serum samples, and 100 normal serum samples were subjected to our Spike protein-based IFA test. The specificity and sensitivity of our test were compared with a commercially available IFA (EUROIMMUN, Germany) and a conventional IFA, performed in Singapore General Hospital (SGH).

2. Material and methods

2.1. Viruses and cell lines

The SARS-CoV used in this experiment was isolated and provided by the SGH (SIN2774, GenBank accession no. AY283798). *S. frugiperda* (Sf-9) cells were maintained and propagated in SF-900 II serum-free (Gibco) medium. Vero cells were propagated in DMEM medium with a supplement of 10% FBS. Viral titration and infection were performed as described previously (Lu et al., 2003).

2.2. Sera

Human sera used in this study were collected from SGH, Singapore and Tan Tock Seng Hospital, Singapore. Included in this study were 163 serum samples with available clinical records. Twenty-one sera from confirmed SARS patients, 10 sera from autoimmune disease patients, 10 sera from dengue fever patients, 12 sera from patients with community acquired pneumonia, 10 sera from patients with renal failure, and 100 sera from healthy donors. All sera were kept on ice after collection and were heat-inactivated at 60 °C for 1 h before testing. Four infectious bronchitis virus (IBV)-infected chicken sera and seven transmissible gastroenteritis virus (TGEV)-infected swine sera obtained from the Temasek Life Science laboratory, were used to check for cross-reactions.

2.3. Immunoblot

Total cell lysate from Sf-9 cells infected with recombinant baculovirus expressing protein C was immunoblotted on nitrocellulose membranes (0.45μm, Bio-Rad). All sera, including five SARS convalescent human sera and five normal human sera were screened at a dilution of 1:100, followed by peroxidase-conjugated secondary antibody (Dako, Denmark) according to the manufacturer's instructions. DAB (3, 3′-diaminobenzidine tetrahydrochloride; Pierce, IL, USA) was used as the horseradish peroxidase (HRP) substrate when developing reactions on the membrane. Negative controls were included in the test.

2.4. IFA and microscopy analysis

Sf-9 cells grown in 96-well microplates (NUNC, Nunclon Delta Surface, Cat no. 167008) were infected with recombinant baculovirus expressing protein C at MOI of 5. The Sf-9 cells were incubated with recombinant baculovirus for 1 h at 28 °C before changing the inoculum. After incubation for another 36 h at 28 °C, the infected Sf-9 cells were fixed to the plate with 100% ethanol for 30 min at room temperature. The cells in each well were reacted with human serum samples at 1:100 dilution in phosphate-buffered saline (PBS) for 1 h at 37 °C in a moist chamber. Following this, the cells were washed in PBST twice at 10-min intervals. FITC-conjugated rabbit antihuman immunoglobulin G, IgG (DAKO, Cat no. F0202) at 1:50 dilution was used for fluorescent studies. The optimal serum dilution was previously determined to be 1:100 for Ig-total detection (data not shown). As a positive control, antibody-specific protein C at 1:100 dilution and FITC-conjugated rabbit anti-guinea pig immunoglobulins (DAKO, Cat no. F0233) at 1:50 were used. To test for cross-reactivity, the Sf-9 cells were incubated with TGEV and IBV serum samples at 1:100 dilution and FITC-conjugate rabbit antiswine immunoglobulins (DAKO, Cat No. F0235) and FITC-conjugate rabbit antichicken immunoglobulins

(DAKO, Cat No. F888), respectively. The staining patterns of Sf-9 cells were examined with an inverted fluorescence microscope (Olympus) using a 20× objective and a 10× eye piece with appropriate barrier and excitation filters for optimized FITC visualization. Signals were captured by a digital camera imaging system (Nikon). The optimal conditions were defined to be the reaction in which maximum fluorescent signals were observed with no background.

For confocal microscopy, Sf-9 cells were cultured on four-well chamber slides (IWAKI). Infection, fixation, and IFA were performed as described above. Following IFA, the slides were observed under an inverted confocal inverted laser microscope (Zeiss) using an argon laser and a FITC filter.

2.5. Conventional IFA

This was performed in a BSL-3 laboratory in SGH as described previously (He et al., 2004). SARS-CoV was propagated in Vero E6 cells at 37 °C until cytopathogenic effects were seen in 75% of the cell monolayer, following which the cells were harvested, spotted onto 12-well Teflon-printed microscope slides (Precision Lab Products, LLC. Cat no. PL2021) and fixed with 80% cold acetone. Uninfected Vero E6 cells were used as controls in this experiment. Human serum samples were tested at 1:10 dilution and FITC-labeled antihuman Ig-total at 1:5 dilution.

2.6. Commercial SARS IFA kit (EUROIMMUN)

A commercial SARS IFA kit was purchased from Medizinische Labordiagnostika, Germany. The test was conducted according to the manufacturer's instruction. The recommended serum samples dilutions used were 1:10 for the primary antibody and 1:5 for the FITC-labeled secondary antibody. The results were interpreted in accordance with the instructions provided.

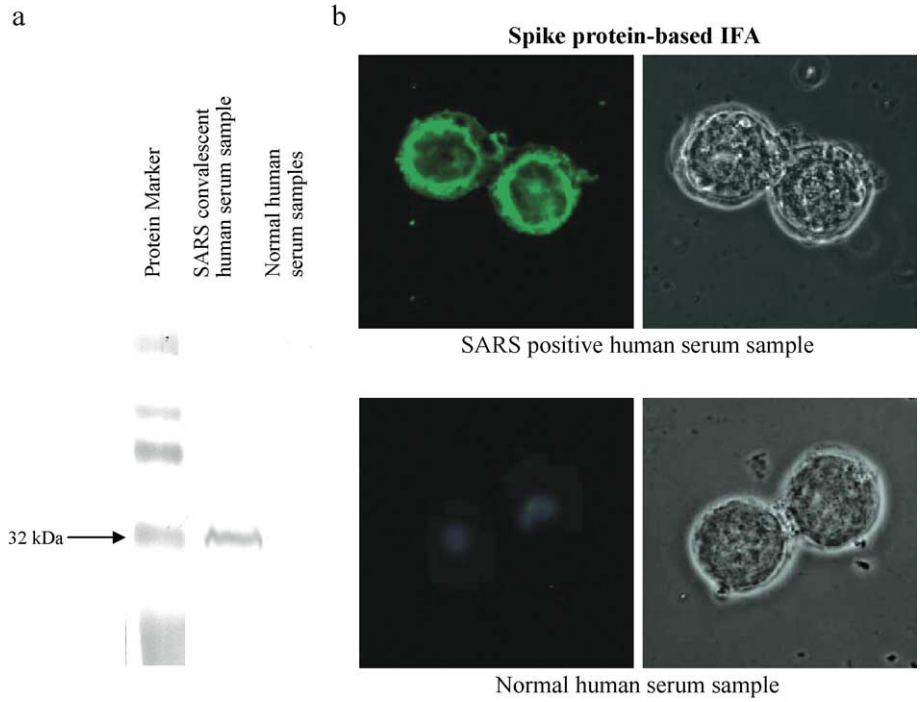

Fig. 1. Representative immunoblot (a) and IFA (b) tests. (a) Western blot assay of Sf-9 cells with recombinant baculovirus expressing protein C using one representative SARS convalescent human serum sample and one normal human serum sample. (b) IFA test of protein C with one representative SARS-positive human serum viewed using a confocal microscope (Zeiss, 100×). The two panels shown from left to right are cell images viewed with and without an FITC filter, respectively.

3. Results

3.1. Antigenicity confirmation of recombinant Spike protein C expressed in a baculovirus system

To confirm the authenticity of protein C expressed in a baculovirus system as a specific antigen for SARS-CoV infection, immunoblot and IFA were carried out using five SARS convalescent human sera and five normal human sera (Fig. 1). The immunoblot showed a 32-kDa band, indicating that protein C expressed in a baculovirus system is antigenic for antibody detection of SARS-CoV infection (represented by Fig. 1a). In our IFA test using a confocal microscopy, all five SARS convalescent human sera showed a fluorescence ring around Sf-9 cells, while all the normal human sera showed no specific staining (represented by Fig. 1b). Fig. 1b also indicates that fluorescence was confined to the cytoplasm. These findings showed that protein C maintains the same antigenicity as the SARS-CoV whole Spike protein. Thus, our Spike protein-based IFA has the potential to replace the whole virus IFA in detecting SARS-CoV infection.

4. Analysis of protein C of SARS-CoV

We have investigated the specificity of protein C by reacting IBV-infected chicken serum samples and TGEV-infected swine serum samples with our Spike protein-based IFA. Negative results were observed indicating no cross-reactivity of protein C with these animal coronaviruses (Table 1).

We have also conducted pairwise nucleotide identity analysis of protein C against spike protein of human coronavirus HCoV-OC43, HcoV-229E, and HCoV-NL63. The analysis reveals a very low level of similarity between protein C and other human coronavirus, as well as animal coronaviruses (Table 1), indicating that protein C is unique to SARS-CoV and is unlikely to cause cross reaction.

Table 1
Pairwise identity and cross-reactivity of protein C with other groups of coronaviruses

Group	Virus	Pairwise amino acid identity (%)	Cross-reactivity
G1	HCoV-229E	5.3	No (Huang et al., 2004)
	HCoV-NL63	4.1	NA
	TGEV	3.1	No (Lu et al., 2004)
G2	HCoV-OC43	4.2	No (Huang et al., 2004)
G3	IBV	2.6	No (Lu et al., 2004)
G4	SARS-CoV	100	

Table 2
Comparison of detection rate between our Spike protein-based IFA, a conventional IFA, and a commercial SARS IFA kit (EUROIMUN), excluding the 42 non-SARS serum samples and 100 normal control serum samples

IFA Detection rate				
Days postinfected	Total number of sera	Spike-based IFA	SGH IFA	EUROIMMUN SARS detection kit
7–14	6	6	6	6
14–76	15	15	15	15
Detection rate		100%	100%	100%

4.1. Comparison of Spike protein-based IFA with whole virus-based IFA in the detection of antibodies against SARS-CoV

The results of our Spike protein-based IFA were found to be comparable with the conventional IFA in term of sensitivity and specificity (Table 2). Our IFA could identify all of the 21 positive sera, giving a sensitivity of 100%. There was no false positive result from the 42 serum samples collected from non-SARS patients sera or from the 100 serum samples collected from normal donors, indicating 100% specificity. We also tested for specificity of all of the abovementioned sera by reacting them with noninfected Sf-9 cells. No positive reactions were obtained, indicating that our Spike protein-based IFA is very specific.

Fig. 2 shows representative IFA results in the detection of positive serum samples using our Spike protein-based IFA, compared to the conventional IFA performed by SGH and the commercial SARS IFA kit (EUROIMMUN). In the conventional IFA and the commercial IFA (EUROIMMUN) using SARS-pos-

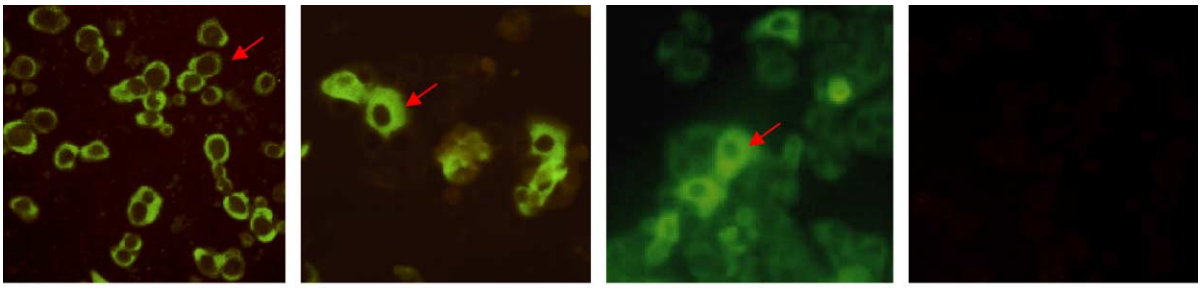

Fig. 2. Representative IFA tests SARS-positive human serum samples in the Spike-based IFA, conventional IFA performed by SGH, and commercial IFA (EUROIMMUN). Images were captured under a normal inverted fluorescence Microscope (Olympus, 40×). "Ring" fluorescence was observed in three IFAs using SARS-positive human sera. Fluorescence can be seen in the cytoplasm of the Sf-9 cells in the Spike protein-based IFA and the Vero cells of the conventional IFA and commercial IFA (EUROIMMUN; red arrow).

itive serum samples, fluorescence signals were also observed in the cytoplasm of the Vero cells.

5. Discussion

Spike protein of SARS-CoV is made up of two subunits, the S1 fragment near the N-terminus and the S2 fragment near the C-terminus. The S1 and S2 subunits form the globular head and the stalk of the Spike protein (Bosch et al., 2003) and play an important role in specific receptor recognition and cell fusion. Spike protein contains important virus neutralization epitopes, and amino acid changes in the Spike protein are known to have a profound effect on the virulence and tropism of known coronavirus (Leparc-Goffart et al., 1998; Sanchez et al., 1999).

There is also a low level of similarity (20% to 27% pairwise amino acid identity) between the predicted amino acid sequence of the S protein of SARS-CoV and other coronaviruses (Lu et al., 2004). Pairwise analysis of the C domain of spike protein with other human coronaviruses showed even lower similarity (4% to 5%) when compared to the existing human coronaviruses 229E, OC43, and Ho-NL63 (Table 1). Moreover, it has been found that there is no serological reactivity against SARS-CoV among patients with respiratory or other diseases in Hong Kong (Peiris et al., 2003B), suggesting that there is no cross-reactivity between the antibodies against previously known coronaviruses and antibodies against SARS-CoV (Huang et al., 2004).

Recently, Li et al. (2003) has reported that the angiotensin converting enzyme 2 (ACE2) recognizing site in the Spike protein S1 domain is the functional receptor for SARS-CoV. We have compared the sequences of our protein C, 441 to 700 a.a. of Spike protein with the ACE2 recognizing site and found that the ACE2 recognition domain is overlapping in our protein C. This sequence is found in the S1 domain of SARS-CoV spike protein and has a high affinity to ACE2. This ACE2 recognition domain is important as it grants SARS-CoV the ability to bind and mediate virus entry in cells, causing infection and hence inducing an early immune response. Because protein C overlaps with the ACE2 recognition site, we believe that antibody against protein C may be a good candidate for the development of effective and sensitive detection assays for SARS-CoV. In this study, we are the first to show that the immunogenic protein C-based IFA is a sensitive and specific method for detecting SARS-CoV infection.

IFA is considered as the "gold standard" for the detection of SARS-CoV infection. The technique is simple and inexpensive to perform with acceptable sensitivity and specificity in the detection of SARS-CoV infection. IFA is also known for its ability to detect antibody responses, as early as 8 days. Therefore, we have developed an IFA technique using recombinant baculovirus expressing the C domain of Spike protein in Sf-9 cells. Our Spike protein-based IFA using the antigenic protein C had a sensitivity and specificity of 100%, which was comparable to the conventional and commercial IFAs (Table 2).

We observed a "ring" fluorescence pattern in our Spike protein-based IFA, as well as in the conventional and commercial SARS IFA when analyzing positive human sera. Further localization studies using an inverted confocal microscope showed that protein C was localized in the cytoplasm of Sf-9 cells, thus forming a "ring" fluorescence pattern (Fig. 1b). However, in terms of sensitivity in the detection of SARS-CoV antibody in serum samples, our Spike protein-based IFA utilized one-tenth of the serum used in the commercial IFA. We have conducted experiments to optimize the conditions used for the IFA and we have identified the best dilution of serum (data not shown). We found that the Spike protein-based IFA can detect SARS-CoV specific antibody at a lower dilution of 1:100, compared to the 1:10 dilution for commercial and conventional IFAs. This might be due to the overexpression of protein C in our system. Thus, our Spike protein-based IFA is more sensitive compared to the existing IFAs. Moreover, protein C is able to mimic the antigenicity of the whole SARS-CoV although it was developed on the basis of a truncated immunogenic fragment, from 441 to 700 a.a. of Spike protein. Future development of an assay with protein C of the Spike protein will now be possible.

Manipulation of live SARS-CoV is biohazardous work, which needs a BSL-3 laboratory. Recent SARS outbreaks due to laboratory escapes of live virus have occurred in Singapore, Taiwan, and China. Safety has therefore become a paramount issue. Initial work on a whole virus IFA requires a high stringency facility, as it is dealing with infectious virus. A diagnostic technique is needed which eliminates the possibility of contamination or the escape of pathogenic virus. Our Spike protein-based IFA which has a comparable detection rate to the two available existing IFAs, could be conducted in a biosafety level 2 (BSL-2) laboratory and is a safer diagnostic procedure than the existing IFAs.

In summary, our Spike protein-based IFA is (1) a cost-effective method to detect antibody against SARS-CoV with little risk of infection; (2) as reliable, sensitive, and accurate as the whole virus-based IFA; and (3) easy to start up, user-friendly, and can be utilized easily in the majority of laboratories without fear of infection. We believe that our Spike protein-based IFA is an appropriate method for daily, routine diagnosis of SARS-CoV infection.

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