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Detection of severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein in human serum using a localized surface plasmon coupled fluorescence fiber-optic biosensor

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ABSTRACT

In order to enhance the sensitivity of conventional immunoassay technology for the detection of SARS coronavirus (SARS-CoV) nucleocapsid protein (N protein), we developed a localized surface plasmon coupled fluorescence (LSPCF) fiber-optic biosensor that combines sandwich immunoassay with the LSP technique. Experimentally, a linear relationship between the fluorescence signal and the concentration of recombinant SARS-CoV N (GST-N) protein in buffer solution could be observed from 0.1 pg/mL to 1 ng/mL. In addition, the concentration of GST-N protein in diluted serum across a similar range could also be measured. The correlation coefficients (linear scale) for these two measurements were 0.9469 and 0.9624, respectively. In comparison with conventional enzyme linked immunosorbent assay (ELISA), the detection limit of the LSPCF fiber-optic biosensor for the GST-N protein was improved at least 10^4-fold using the same monoclonal antibodies. Therefore, the LSPCF fiber-optic biosensor shows an ability to detect very low concentration (~1 pg/mL) of SARS-CoV N protein in serum. The biosensor should help with the early diagnosis of SARS infection.

1. Introduction

Severe acute respiratory syndrome (SARS) is a highly infectious disease that results in death in a great portion of patients (Drosten et al., 2004). SARS is caused by the SARS coronavirus (SARS-CoV) which is detectable in respiratory secretions of patients after infection (Fouchier et al., 2003). This disease is highly contagious and still has the potential to cause a very large-scale epidemic in the future in the absence of a vaccine or effective therapeutic drugs (Wang et al., 2004). The key to preventing and controlling a future outbreak of SARS is to block transmissions of infection through a strict quarantine policy. Therefore, a rapid, sensitive, specific, and accurate diagnostic method is essential so that suspected patients can be correctly assessed (Jiang et al., 2004; Che et al., 2004). Currently, there are several diagnostic methods used for the detection of SARS. The first option is based on nucleic acid detection. Since the genome of SARS-CoV has been sequenced completely (Marra et al., 2003; Rota et al., 2003), reverse transcription-polymerase chain reaction (RT-PCR) is the most common method that is used to detect the SARS-CoV during the early phase immediately after the onset of clinical symptoms (Hourfar et al., 2004; Louie et al., 2006; Petrich et al., 2006). However, the RT-PCR assay is probably not sensitive enough to detect SARS-CoV in secretions or serum until 3 days after the onset of symptoms (Yam et al., 2003). Statistically, about half of SARS patients cannot be identified at an early stage based on viral RNA detection (Li et al., 2005). Besides, the RT-PCR tests require not only a thermal cycler for conventional PCR or a more complicated machine for real-time PCR (Liu et al., 2005), but also a specific laboratory with expertise in molecular diagnostics to confirm SARS in the acute phase (Drosten et al., 2004; Che et al., 2004; Fujimoto et al., 2008). Unfortunately, other sequence based methods such

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as a real-time loop-mediated amplification assay (Poon et al., 2004, 2005; Hong et al., 2004), a gold film with enzymatic electrochemical genosensor (Abad-Valle et al., 2005) and a rolling circle amplification PCR-based assay (Wang et al., 2005) exhibit lower sensitivity than the RT-PCR method. Serological testing is another diagnostic option. However, serological tests have shown a relatively low sensitivity of only 65.4% using sera obtained 6–10 days after the onset of symptoms (Shi et al., 2004). The third option is based on detecting antibodies against SARS-CoV after infection (Peiris et al., 2003a). However, the antibody response in more than 93% of SARS patients takes at least 10–28 days to develop after symptoms onset, so this approach is not a good method for early detection (Peiris et al., 2003b). The final possible approach is to detect specific SARS-CoV antigens such as the spike protein (Zhao et al., 2005; Haynes et al., 2007; Manopo et al., 2005) and particularly the nucleocapsid (N) protein. The N protein is one of the early expressed proteins and should have diagnostic value. In the serum of SARS patients, it has been shown that N protein can be detected as early as 1 day after infection (Che et al., 2004). Thus, detection of SARS-CoV N proteins is a valuable approach to diagnose and monitor disease activity and makes it possible to develop a rapid and accurate diagnostic method at an early stage. In addition, one significant advantage of detecting SARS-CoV N protein in serum is to eliminate the risk of infection while collecting nasopharyngeal aspirates (Che et al., 2004). Among several possible methods are available for the detection of SARS-CoV N protein, conventional antigen capture enzyme-linked immunosorbent assay (ELISA) is the most widely used method (Chen et al., 2005; Chan et al., 2005; Qiu et al., 2005). Yet another method of detecting SARS-CoV N protein is based on an immunofluorescence assay, which was able to detect SARS-CoV from SARS patients as early as 2 days after the onset of symptoms (Liu et al., 2005). Recently, Professor Okamoto and his co-workers proposed a highly sensitive immunoassay based on an enzyme-linked immunosorbent assay using chemiluminescence (CLEIA) for the detection of SARS-CoV N protein and this method has pushed the detection limit to 1.56 pg/mL (Fujimoto et al., 2008).

Recently, gold nanoparticles (GNPs) have been introduced into biosensing and proved to be one of the most efficient ways to increase the detection limit of biosensors (Manso et al., 2008; Cui et al., 2008). It is well known that GNPs possess special optical properties such as localized surface plasmons (LSPs) which result in wavelength selective absorption with extremely large molar extinction coefficients and significant enhancement of the localized electromagnetic field close to the GNP surface within 50–60 nm (Mock et al., 2003; Sonnichsen et al., 2002; McFarland and Van Duyne, 2003). Based on the property of localized surface plasmon coupled fluorescence (LSPCF) combined with the sandwich immunoassay, a novel fiber-optic biosensor has been proposed by our group to study protein–protein interactions. Experimentally, the detection limit of LSPCF fiber-optic biosensor has been demonstrated to be as low as 1 pg/mL when detecting mouse immunoglobulin G (IgG) interacting with anti-mouse IgG (Hsieh et al., 2007). In addition, we have also demonstrated that the LSPCF fiber-optic biosensor is able to measure alpha-fetoprotein in human serum as low as 0.1 ng/mL (Chang et al., 2009). In this report, the LSPCF fiber-optic biosensor is applied to enhance the detection sensitivity of SARS-CoV N protein in diluted serum to a limit of 0.1 pg/mL. Clearly, the biosensor has significant potential for the early detection of clinical SARS-CoV infection.

2. Materials and methods

2.1. Materials

Human serum was prepared from a healthy donor. Bovine serum albumin (BSA); GNP conjugate protein A (Au-PA, GNP diameter = 20 nm); phosphate buffer saline (PBS) tablet; ethyl acetate and 2-propanol were purchased from Sigma Inc. (St. Louis, MO, USA). The fluorescent labeling kit (DyLight™ 649) was purchased from Pierce Co. (Rockford, IL, USA). The plastic optical fiber was purchased from Mitsubishi Rayon Co., LTD. (Tokyo, Japan).

2.2. Plasmid construction

The RNA genome sequence of SARS-CoV was utilized as a template to synthesize the cDNA sequence with the proper codon usage for *E. coli*. The cDNA sequence encoding the N protein was synthesized by RT-PCR reaction using the specific primers, F5′-GCCGAATTCATGTCTGATAA TGGACCCCA-3′ and R5′-GCGCGTCGACGTTATGCCTGAGTTGAATCA-3′. The cDNA fragment (1.3 kD) of N protein so obtained was digested with *Eco*RI and *Sal*I restriction enzymes and then ligated into the vector, pGEX-5X-1, which contains a glutathione S-transferase (GST) tag sequence. The expression vector encoding recombinant SARS-CoV N protein was named GST-N protein.

2.3. Expression and purification of recombinant SARS-CoV N protein

The GST-N plasmid was transformed into *E. coli* BL21 for expression of recombinant SARS-CoV N protein. After 3 h of induction with isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM), the bacteria were harvested and pellet resuspended in NETN buffer (20 mM Tris base in pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40) for protein purification. The protein mixture was next incubated with glutathione-agarose beads (Sulphur linkage, Sigma St. Louis, MO, USA,) for 12 h. After the elution, the GST-tagged N protein was concentrated for immunization.

2.4. Preparation of anti-SARS-CoV N protein antibodies

Six-week-old BALB/c mice were immunized and twice-boosted with 25 μg of GST-N protein in 0.1 mL of PBS emulsified with an equal volume mixture of complete/incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA). After the antibody titers against GST-N protein had been confirmed by ELISA and immunoblotting, splenocytes were harvested and fused with NS-1 myeloma cells. The hybridoma culture supernatants were then screened by ELISA and immunoblotting against the GST-N protein. Single clones producing a specific antibody were selected by the limiting dilution method. Polyclonal antibodies against GST-N protein were also prepared using New Zealand white rabbits, which were immunized and boosted with 100 μg of GST-N proteins in 0.5 mL PBS emulsified with an equal volume mixture of complete/incomplete Freund's adjuvant twice or three times. After the antibody titers against GST-N fusion protein were confirmed, then blood was taken from the rabbits for further purification. The monoclonal and polyclonal antibodies were purified from mouse ascites and rabbit serum, respectively, using protein A affinity columns (GE Healthcare, Buckinghamshire, UK). Two monoclonal antibodies against GST-N protein used in this study and were named anti-N-1 antibody and anti-N-2 antibody.

2.5. Immunoblotting analysis

In total, 1.0 μg of purified GST-N protein was loaded into each well of a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel for electrophoresis. After the transfer of the separated proteins to polyvinylidene difluoride membrane (PVDF) (Millipore, Billerica, MA, USA), the blot was cut into strips and incubated separately with 2.5 μg/mL of anti-N-1 antibody or 5 μg/mL of anti-N-2 antibody.