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Quantitative and sensitive detection of the SARS-CoV spike protein using bispecific monoclonal antibody-based enzyme-linked immunoassay

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The severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein is known to mediate receptor interaction and immune recognition and thus it is considered as a major target for vaccine design. The spike protein plays an important role in virus entry, virus receptor interactions, and virus tropism. Sensitive diagnosis of SARS is essential for the control of the disease in humans. Recombinant SARS-CoV S1 antigen was produced and purified for the development of monoclonal and bi-specific monoclonal antibodies. The hybridomas secreting anti-S1 antibodies, F26G18 and P136.8D12, were fused respectively with the YP4 hybridoma to generate quadromas. The sandwich ELISA was formed by using F26G18 as a coating antibody and biotinylated F26G18 as a detection antibody with a detection limit of 0.037 μg/ml ($p < 0.02$). The same detection limit was found with P136.8D12 as a coating antibody and biotinylated F26G18 as a detection antibody. The sensitivity was improved (detection limit of 0.019 μg/ml), however, when using bi-specific monoclonal antibody (F157) as the detection antibody. In conclusion, the method described in this study allows sensitive detection of a recombinant SARS spike protein by sandwich ELISA with bi-specific monoclonal antibody and could be used for the diagnosis of patients suspected with SARS.

1. Introduction

The severe acute respiratory syndrome-coronavirus (SARS-CoV) is the RNA virus containing positive sense single stranded RNA (genome of 27–32 kb). The virus contains several structural proteins such as the spike (S) protein, nucleocapsid protein, membrane protein, as well as the envelope protein. All these proteins replicate in the host cell (Marra, 2003). The outbreak of SARS-CoV occurred in the Guangdong province of Southern China in 2002 and spread subsequently to 32 countries in Asia, Europe and North America. It was brought under control due to concerted world-wide efforts led by the World Health Organization (WHO). The SARS-CoV is the causative agent of SARS in humans. According to the WHO, the outbreak of SARS epidemic in 2002–2003 infected over 8400 persons and led to the death of over 900 people with a fatality rate of 9.6%. SARS-CoV-like viruses almost identical to an isolate from a patient were found in palm civet cats during the same period. Recently, the Chinese horseshoe bat has been identified as an important natural reservoir of the virus (Lau et al., 2005). In the past 10 years, the highly pathogenic SARS-CoV has been identified in humans and animals (Yang et al., 2007; Li et al., 2008; Yip et al., 2009; Yuan et al., 2010; Peiris and Poon, 2011; Balboni et al., 2012).

The vital step in preventing and controlling future epidemics is to block the transmission of infection by an effective quarantine policy which in turn depends upon early diagnosis and confirmation of the disease by laboratory tests (Poon et al., 2004). Presently, serological detection of viruses and their components by monoclonal antibodies (mAbs) is known to be a powerful method for investigating the structure and function of viral components. The production of mAbs specific for SARS-CoV aids in the study of viral pathogenesis and the development of diagnostic and therapeutic strategies. Since the development of serum antibody can take one to three weeks after SARS-CoV infection (Li et al., 2003a,b), a sensitive assay that can detect the viral nucleic acid or protein may be preferred for rapid detection of SARS-CoV infection. The profiles of antibody responses to SARS-CoV have been well established (Li et al., 2005). Reports indicate that specific viral proteins might be better markers for serological detection of infection by SARS-CoV (Tan et al., 2004; Lu et al., 2004; Berry et al., 2010).

The spike proteins of CoV are large transmembrane-glycoproteins that mediate receptor association, membrane

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fusion, and viral entry (Gallagher and Buchmeier, 2001; Bosch et al., 2003; Colman and Lawrence, 2003). They play a key role in the infection of targeted cells that possess the specific receptor (Gallagher and Buchmeier, 2001; Holmes, 2003; Hofmann et al., 2004). The cellular receptor of SARS-CoV and the receptor binding domain on S-protein have been identified (Babcock et al., 2004). Two subunits of SARS-CoV S-protein, S1 and S2, have been described (Xiao et al., 2003; Huang et al., 2006). The S1 subunit is responsible for virus binding to cellular receptor(s) and contains neutralizing epitopes, whereas the S2 subunit contains a hydrophobic fusion peptide that mediates membrane fusion following receptor association (Bosch et al., 2003). Earlier studies have shown that the receptor binding domain in the S1 region of S-protein induces neutralizing antibody, β-cell production, angiotensin-converting enzyme 2 (ACE-2) binding and virus entry (He et al., 2006). Depletion of receptor binding domain reduced significantly a humoral immune response, indicating that this domain is dominant in inducing neutralizing antibody (Lu et al., 2007). SARS-CoV S2 subunit has a highly conserved ten-residue sequence Y(V/1)KWPW(W/Y)VWL, which is rich in aromatic amino acids with 3–4 tryptophan residues. The last five residues of this region, probably from the beginning of the membrane-spanning domain, are also called the transmembrane domain (Sainz et al., 2005; Siu et al., 2008).

Information is available on mAbs for the detection of SARS-CoV (Kammila et al., 2008), however there is limited information on a sensitive detection method for SARS-CoV S-protein. Hybridomas for monoclonal antibody (mAb) and quadromas were proudced for a construction of bi-specific mAb (bsmAb) against SARS-CoV S1 antigen. The aim of this study was to evaluate a sensitive ELISA using a panel of mAbs and/or bsmAbs for the detection of SARS-CoV S1 antigen in vitro.

2. Materials and methods

2.1. Materials

Fetal bovine serum was purchased from the PAA laboratories (Etobicoke, ON, Canada) and Streptomycin–penicillin–glutamine was obtained from Gibco (Burlington, ON, Canada). Polyethylene glycol 1300–1600, HAT and Ht supplement, goat anti-mouse IgG conjugated with horseradish peroxidase (HRPO), bovine serum albumin, fluorescein isothicyanate, tetramethylrhodamine isothicyanate, horseradish peroxidase (Type IV), m-aminopheylboronic acid agarose (binding capacity to HRPO: 8–14 mg/ml of the gel), Protein G-sepharose, long chain-sulfosuccinimidyl NHS biotin, low molecular dextran sulphate, RPMI media and rabid anti-chicken IgY–HRPO were obtained from Sigma (St. Louis, MO, USA). Cell strainers for collection of spleen and streptavidin–HRPO were obtained from BD Biosciences Pharmingen (Mississauga, ON, Canada). IMAB^{TM} bags were obtained from BioVectra (Charlottetown, PE, Canada). Tetramethylbenzidine and hydrogen peroxide (H_2O_2) detecting reagent were obtained from KPL laboratories (Gaithersburg, MD, USA). Slide-A-Lyzer^{R} for dialysis was obtained form Pierce (Rockford, IL, USA). Dialysis tubing (12,000 MW cut off) was obtained from BioDesign Inc. (Carmel, NY, USA). IMAC protein purification resin was purchased form Thermo Scientific (Rockford, IL, USA). F26G18 hybridoma was kindly provided by National Microbiology Laboratory, Health Canada (Winnipeg, MB, Canada).

2.2. Preparation of SARS-CoV S1

The SARS-CoV S1 nucleotide sequence was codon optimized for prokaryotic expression and synthesized from GENEART (Burlington, ON, Canada). The optimized S1 gene was amplified by PCR and cloned in the proper reading frame in the pBM802 vector along with the His6 tag at the C-terminal for higher expression of proteins in inclusion bodies of *Escherichia coli*. The recombinant clones were analyzed by restriction digestion fragment mapping and the correct clones were selected for protein expression. Protein purification was done by IMAC chromatography from inclusion bodies. The non-glycosylated S1 protein was used to generate anti-S1 mAb and bsmAb for the development of this sensitive immunoassay.

2.3. SDS-PAGE and Western blot analyses

Purified S1 was electrophoresed on SDS-PAGE using 10% polyacrylamide gel to check the purity according to a published method (Laemmli, 1970). The protein band was electroblotted onto Hybond ECL nitrocellulose membranes (Towbin et al., 1979). The membrane was blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T) for 1 h. The membrane was washed with PBS-T and incubated for 1 h with anti-SARS-CoV S1 (1 μg/ml). After washing with PBS-T, the nitrocellulose membrane was incubated with rabbit anti-S1-HRPO for 1 h. All incubations were carried out at room temperature. Finally, the membrane was washed with PBS and electrochemiluminescence detection was performed to visualize specific binding.

2.4. Production of mouse monoclonal hybridomas against SARS-CoV S1 antigen

Immunizations were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Briefly, 6–8-week-old female BALB/c mice (Charles River) were immunized intraperitoneally 3 times with 25 μg of S1 antigen on days 0 and 14 using complete and incomplete Freund's adjuvant, respectively, and once with 10 μg of antigen on day 28 using PBS (pH 7.3). The polyclonal antibody to the antigen was measured in mouse serum using indirect ELISA. Mice with the highest titer were spleenectomized on day 3 after the last antigen injection. Spleenocytes were fused with SP2/0 myeloma cells in a ratio of 5:1 using 50% (w/v) polyethylene glycol (PEG) (Kohler and Milstein, 1975). Five SARS-CoV anti-S1 mAbs (P135.3F3, P136.8D12, P147.4R4, P147.2R8, and P147.2R16) were developed and used subsequently for production of quadromas. The isotypes of the mAbs were determined by using isotype specific HRPO conjugated antibodies.

2.5. Cell lines for quadroma fusion

A cell line of YP4 known to secrete (IgG2a) monospecific anti-HRPO hybridoma was obtained from the late Dr. C. Milstein (Medical Research Council for Molecular Biology, Cambridge, United Kingdom). A cell line of F26G18 known to secrete monospecific anti-S1 hybridoma was kindly provided by NML (Winnipeg, MB, Canada). Anti-HRPO YP4 is a well-characterized rat hybridoma that was selected previously for drug resistance to 8-azaguanine, making it sensitive to aminopterine in HAT medium. Anti-SARS-CoV S1 F26G18 is a mouse hybridoma that can bind the receptor binding domain in S1 region of SARS CoV (Berry et al., 2010). Two cell lines of YP4 and F26G18 were chosen for developing quadromas (hybridoma × hybridoma) (Kammila et al., 2008).

2.6. Development of anti-S1/anti-HRPO quadromas

The development of anti-S1/anti-HRPO quadromas involved maintaining the two hybridoma cell lines (YP4 and F26G18, anti-HRPO and anti-S1, respectively) in the logarithmic growth phase containing RPMI medium with 10% fetal bovine serum (FBS) at 37 °C supplemented with 5% CO_2. Trypan blue staining was observed over

90% before the cells were used for fusion. A stock solution of tetramethyl rhodamine isothiocyanate (TRITC, 0.5 mg/ml) and fluorescein isothiocyanate (FITC, 0.5 mg/ml) was diluted in 1:5 ratios to be used as a working solution. The following steps as published earlier were then followed for successful completion of a quadroma fusion (Tang et al., 2004). Briefly, 2×10^7 cells/ml of anti-S1 hybridomas (F26G18, P1353F3, P136.8D12, P1474R4, P1472R8 and P1472R16) and YP4 hybridomas were resuspended separately in RPMI at pH 7.4 and 6.8, respectively. Anti-S1 hybridomas were then labeled with TRITC (red fluorescence) and YP4 hybridomas were labeled with FITC (green fluorescence). Following 30 min incubation at 37 °C in a CO_2 incubator the hybridomas cell suspensions were washed and mixed in a 50 ml tube and centrifuged at $459 \times g$ for 7 min. Two ml of PEG was added to the cell pellet, drop-wise over a period of 2 min, with gentle mixing. After adding PEG, the cell suspension was placed at 37 °C in a CO_2 incubator for 3 min, followed by addition of 20 ml of FBS free RPMI medium to dilute the toxic effects of PEG. Flow cytometer (Epics Elite cell sorter, Coulter, Hialeah, USA) with an argon ion 488 nm air cooled laser (Cross Cancer Institute, University of Alberta) was then used to sort cells with dual fluorescence and were seeded at 1 cell/well in 96 well sterile tissue culture plates with RPMI containing 20% FBS. The plates were incubated at 37 °C with 5% CO_2. The clones were screened twice using a direct ELISA (see Section 2.7.2). The cloning of quadromas was carried out using the limiting dilution method. The three clones, P135.3F3/YP4 (P144), P136.8D12/YP4 (P145) and F26G18/YP4 (F157), were then selected for recloning (3–4 times) to select positive and highly specific quadromas secreting bsmAb against the SARS-CoV S1 antigen.

2.7. ELISA techniques

2.7.1. Indirect ELISA

Hybridoma culture supernatants were assayed for binding to the SARS-CoV S1 coated 96 well plates. Plates were coated with 100 μl of purified S1 (5 μg/ml) in 50 mM carbonate buffer (pH 9.6) and incubated at 4 °C overnight. After thorough washing with PBS-T buffer 5 times, the remaining sites on the well surface were blocked with 200 μl of blocking buffer (3% (w/v) BSA in PBS-T) at 37 °C for 1 h. After washing, 100 μl of hybridoma supernatants (1:2000 dilution in 1% (w/v) BSA in PBS-T) were added to the wells and incubated at 37 °C for 1 h, which was followed by additional washing steps. A volume of 100 μl of goat anti-mouse IgG HRPO (1:2000 dilution in 1% (w/v) BSA in PBS-T) was added to the wells and incubated at 37 °C for 1 h. The plate was washed again with PBS-T. Positive binding was detected with commercial TMB substrate and the plate was read at 650 nm after 5 min using a V_{max} ELISA plate reader. Mouse immune and preimmune sera were diluted 1:1000 with 1% (w/v) BSA in PBS-T for use as positive and negative controls, respectively.

2.7.2. ELISA format for detection of bsmAb in quadroma culture supernatants

A direct ELISA was used for all the screening procedures after 10 days of quadroma fusion. This method involved coating the 96-well ELISA plate with 100 μl of 5 μg/ml SARS-CoV S1 antigen in 50 mM carbonate buffer (pH 9.6). The plate was then incubated at 4 °C overnight, washed with PBS-T and blocked with 2% dialyzed BSA (w/v) in PBS-T at 37 °C for 2 h. After washing, 100 μl of serially diluted (neat, 1:10; 1:100; 1:1000) quadroma supernatants were added to the plate and incubated at 37 °C for 1 h. Following a washing step, 100 μl of 10 μg/ml (diluted in 2% BSA (w/v) in PBS-T) HRPO were added to the wells followed by a washing step. Finally, 100 μl of TMB substrate was added. The positive quadromas were then selected after 5 min of color development and the plate was read at 650 nm using an ELISA plate reader. RPMI media and PBS were used as blanks. Negative control was wells without S1 antigen and HRPO. This bridge ELISA measures only the activities of bsmAbs that bind to the solid phase with one paratope and to HRPO in solution with the other paratope of bsmAb. Monospecific antibodies are not detected by this assay since they do not cross-link the two different antigens.

2.7.3. Sandwich ELISA with biotinylated mAb

Except as otherwise indicated, all incubation steps were performed at 37 °C for 1 h. Washing five times was conducted by PBS-T between each step. Plates were coated with 100 μl of purified anti-S1 mAb (F26G18 or P136.8D12, 5 μg/ml) in 50 mM carbonate buffer (pH 9.6). The remaining sites on the well surface were blocked with 200 μl of blocking buffer (3% (w/v) BSA in PBS-T). A volume of 100 μl of SARS-CoV S1 (serial dilution in 1% (w/v) BSA in PBS-T) was added to the wells, which was followed by additional 100 μl of biotinylated probing mAb (F26G18, 1 μg/ml in 1% (w/v) BSA in PBS-T). Plates were reacted with streptavidin–HRPO for 10 min and then the TMB substrate was added. The color developed was read at 650 nm after 5 min using an ELISA reader.

2.7.4. Sandwich ELISA with bsmAb

Except as otherwise indicated, all incubation steps were performed at 37 °C for 1 h. Washing five times was conducted by PBS-T between each step. Plates were coated with 100 μl of purified anti-S1 mAb (F26G18 or P136.8D12, 5 μg/ml) in 50 mM carbonate buffer (pH 9.6). The remaining sites on the well surface were blocked with 200 μl of blocking buffer (3% (w/v) BSA in PBS-T) at 37 °C for 1 h. A volume of 100 μl of SARS-CoV S1 (serial dilution in 1% (w/v) BSA in PBS-T) was added to the wells, which was followed by additional 100 μl of bsmAb-HRPO complex (F157, 1 μg/ml in 1% (w/v) BSA in PBS-T). Plates were washed and TMB substrate was added. The color developed was read at 650 nm after 5 min using an ELISA reader.

2.8. Purification of anti-S1/anti-HRPO bsmAb

The purification of the bsmAb (F157 quadroma) fused with F26G18 × YP4 was used by m-aminophenylboronic acid agarose column (APBA). Briefly the APBA column was saturated with HRPO to capture the bsmAb along with the monospecific anti-HRPO mAb eliminating the monospecific anti-S1 species. Following a short wash with PBS, the bound antibodies were eluted with potassium phosphate buffer containing 0.1 M sorbitol.

2.9. Biotinylation of anti-S1 mAbs (F26G18)

Anti-S1 mAbs were biotinylated by using long arm biotinaimdo hexanoic acid-3-sulfo-N-hydroxysuccinimide ester. A volume of 1 μg each of protein-G purified mAb (F26G18) in PBS, pH 7.4 was added to 20 μl of long chain biotin (30 μg/ml) and incubated at room temperature for 1 h. Ten microliter of glycine (100 μg/μl) was added and the solution kept on a shaker for 10 min. The solution was then dialyzed in a slide-A-lyzer against PBS, pH 7.4 at 4 °C overnight.

2.10. Statistical analysis

Values represent the mean of experiments done in triplicate. Comparison of the data was performed using Student's "*t*" test. Significance was defined as p value <0.05.

3. Results

3.1. Expression and purification of the S1 fragment of spike protein

The purified S1 was analyzed by SDS-PAGE (Fig. 1A). The specificity was determined by Western blotting, which demonstrated

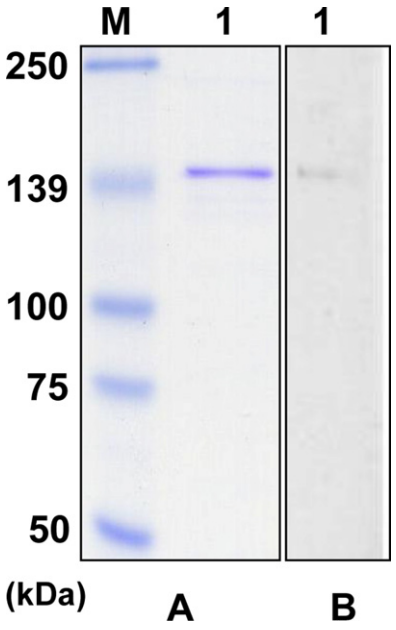

Fig. 1. Reactivity of anti-SARS-CoV S-protein mAb (P136.812) to SARS-CoV S1. (A) SDS-PAGE analysis. Lane M: standard protein molecular weight markers. Lane 1: SARS-CoV S1. (B) Western blot analysis of S-protein probed with anti-SARS-Cov S1 mAb (P136.812) in lane 1.

Table 1
Anti-SARS-CoV spike protein mAbs.

SARS spike hybridoma	Concentration (mg/ml)	Total purified antibody
F26G18	0.8	13 ml (10 mg)
P135.3F3	0.7	13 ml (9.1 + 3 = 12 mg)
P136.8D12	0.68	10 ml (6.8 mg)

that the anti-SARS-CoV S1 mAb (P136.8D12) binds strongly to the S1 antigen (Fig. 1B). A similar result was also found with anti-SARS-CoV S1 mAb F26G18 (data not shown). There was no cross-reaction with other viral recombinant proteins including nonstructural proteins derived from Dengue virus or Ebola virus (data not shown).

3.2. Development of anti-SARS-CoV hybridomas and quadromas

Hybridomas producing antibodies for SARS-CoV S1 were established successfully from spleenocytes following immunization with S1 antigen. Among six hybridomas against SARS-CoV S1 antigen, three hybridomas of P135.3F3, P1368D12 and F26G18 showed approximately 5-fold higher titer than those of P147.4R4, P147.2R8 and P147.2R16. Tables 1 and 2 show the isotypes of SARS-CoV S1 mAbs and the yield of SARS-CoV S1 purified mAbs, respectively.

The hybridomas secreting anti-S1 mAb namely F26G18 was fused respectively with YP4 hybridoma to produce quadromas according to a method described previously (Tang et al., 2004) and selection was done by a direct ELISA. The proportion of double positive cells in the bispecific fusions was in the range of 3.8% (data not shown). The recloning process involved cloning the quadromas by limiting dilution such that one cell per well was plated. Quadromas

Table 2
Isotyping of anti-SARS-CoV spike protein mAbs by ELISA.

SARS Mab	ELISA value @ 650 nm			
	IgG1	IgG2a	IgG2b	IgG3
P135.3F3	0.094	0.098	0.098	0.717
P136.8D12	0.166	0.177	0.809	0.206
F26G18	0.217	0.220	0.757	0.161

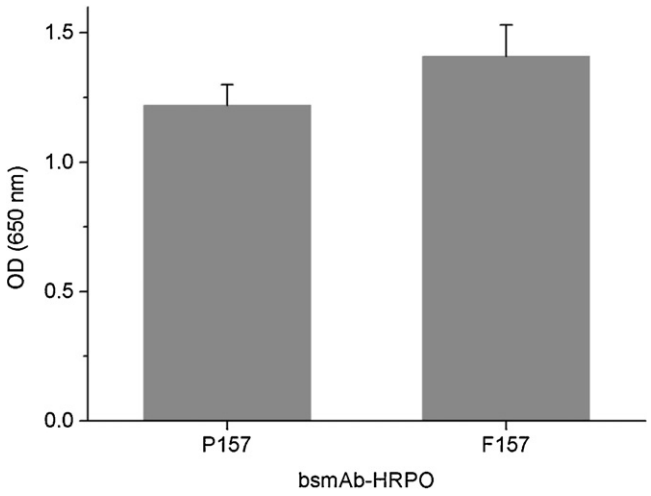

Fig. 2. Titer of purified bsmAb-HRPO complex (P157 and F157 at a concentration of 20 ng/ml) by ELISA.

exhibit polyploidy and hence were shown to have a high level of instability. The process of repeated recloning helped in the development and identification of a strong bsmAb secreting quadromas. The sensitivity of the purified bsmAb–HRPO complex was examined by ELISA, demonstrating that OD (absorbance units) values of two quadromas, P157 (P136.8D12 × YP4, 20 ng/ml in PBS) and F157 (F26G18 × YP4, 20 ng/ml in PBS), were at 1.2 and 1.4, respectively (Fig. 2). Quadroma clones (F157) obtained by the fusion of F26G18 and YP4 were selected based on their higher OD values. The purified yield of bsmAb was approximately 2 mg/ml of culture supernatant and was subsequently used in the development of immunoassays.

3.3. Diagnostic evaluation of sandwich ELISA with biotinylated mAb

Fig. 3 illustrates the sandwich ELISA for detection of the SARS-CoV S1 antigen using mAb F26G18 as a coating antibody and biotinylated mAb F26G18 as a detection antibody. The limit of detection was 0.037 μg/ml ($p < 0.02$) (Fig. 3A). A similar result was found when mAb P136.8D12 was used as a coating antibody and biotinylated F26G18 used as a detection antibody at 0.037 μg/ml ($p < 0.02$) (Fig. 3B).

3.4. Diagnostic evaluation of sandwich ELISA with bsmAb

Fig. 4 illustrates the sandwich ELISA for detection of the SARS-CoV S1 antigen using mAb F26G18 as a coating antibody and bsmAb-HRPO complex (F157) as a detection antibody. The limit of detection was 0.019 μg/ml (Fig. 4A). A similar result was found when P136.8D12 mAb was used as a coating antibody and bsmAb-HRPO complex (F157) was used as a detection antibody at 0.019 μg/ml ($p < 0.02$) (Fig. 4B).

4. Discussion

SARS-CoV S-protein is an essential structural protein of the virus. It has an important functional relationship to the pathogenesis of SARS (Ho et al., 2004). It not only plays a significant role in viral infection, but may also be used as a potential target for a vaccine. The S-protein of SARS-CoV is heavily glycosylated and contains many disulfide bonds (Spiga et al., 2003; Krokhin et al., 2003; Tripet et al., 2004; Ying et al., 2004). However it is difficult to obtain S-protein expression in eukaryotic systems, which would otherwise be ideal for detection of anti-spike antibodies in vitro. It

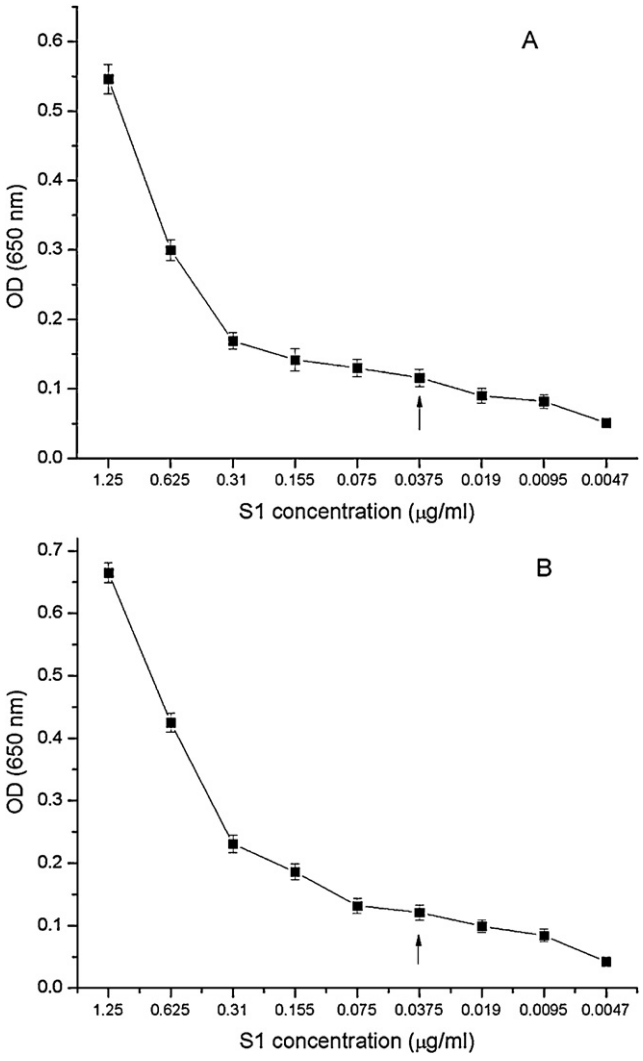

Fig. 3. ELISA for detection of SARS-CoV S1 antigen using biotinylated mAb (F26G18). (A) Sandwich ELISA with anti-SARS-CoV S1 mAb (F26G18) as a coating antibody and same mAb labeled with biotin as a detection antibody. The biotinylated mAb (F26G18) was detected using Streptavidin-HRPO. (B) Sandwich ELISA with anti-SARS-CoV S1 mAb (P136.8D12) as a coating antibody and another mAb labeled with biotin (F26G18). The biotinylated mAb was detected using Streptavidin–HRPO. The results are expressed as absorbance reading at 650 nm wavelength. Arrows indicate the limit of detection.

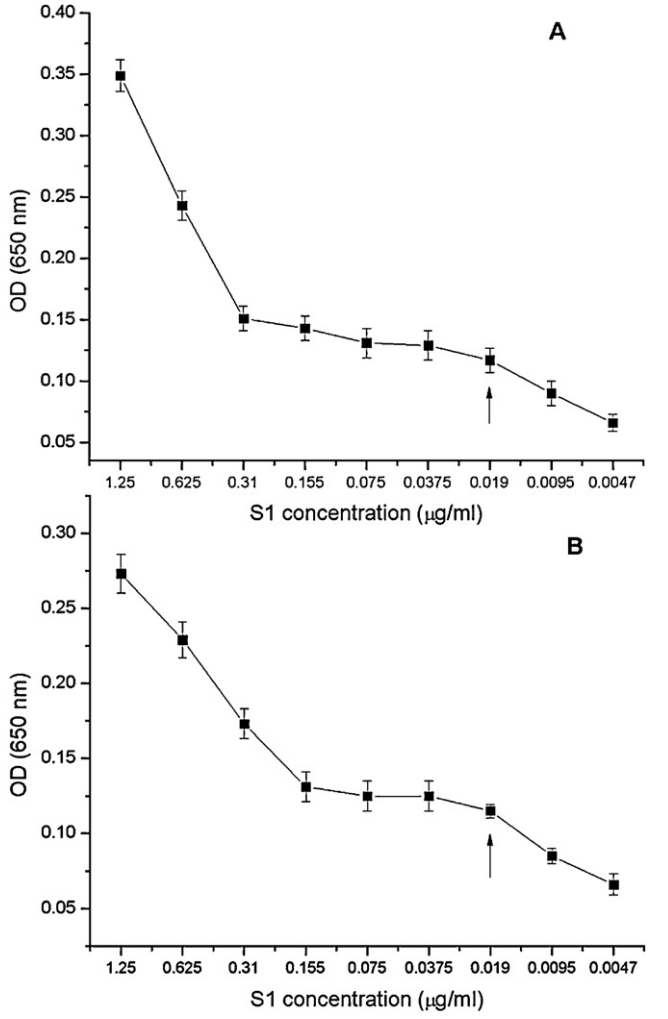

Fig. 4. ELISA for detection of SARS-CoV S1 antigen using bsmAb (F157). (A) Sandwich ELISA with anti-SARS-CoV S1 mAb (F26G18) as a coating antibody and bsmAb-HRPO complex (F157) as a detection antibody. (B) Sandwich ELISA with anti-SARS-CoV S1 mAb (P136.8D12) as a coating antibody and bsmAb-HRPO complex (F157) as a detection antibody. The color was developed using TMB substrate. The results are expressed as absorbance reading at 650 nm wavelength. Arrows indicate the limit of detection.

has been suggested that the N-glycans of the S-protein could have a significant effect on its antigenicity, as their presence contributes to the proper folding and biological function of glycoproteins. The fragments of the S-protein (non-glycosylated) expressed in bacteria could also maintain its antigenicity (Zhou et al., 2004; Lu et al., 2004). Identification of neutralizing epitopes in the S-protein is of great importance for vaccine design and also to our understanding of the anti-SARS-CoV humoral immune response. It has been demonstrated that the receptor binding domain (residue 318–510) contains immunodominant B cell epitopes, some of which are recognized by neutralizing antibodies (Sui et al., 2004; He et al., 2005). The neutralizing antibodies were raised successfully in mice after immunization using prokaryotically expressed S486-625 of SARS-CoV (Zhou et al., 2004). Currently there are many diagnostic methods for detection of SARS, such as real-time RT-PCR and ELISA with virus lysates as an antigen. However, RT-PCR is expensive for small rural hospitals. Also, its use was found to be very limited in clinical settings due to difficulties in sample collection. Furthermore, inconsistent readings at lower level of concentrations were observed (Poon et al., 2003). The peptide based SARS ELISA, however, demonstrates distinct advantages (Huanga et al., 2008). The peptide is easy and safe to prepare as compared to whole virus lysates. This new assay should improve test sensitivity and reliability.

The S-protein, a projection on the viral surface, is the major neutralizing antigen of the known CoVs. It plays an important role in the initial stages of infection, forming the characteristic corona of large, distinctive spikes in the viral envelope (Giménez et al., 2009). It also mediates the initial stages of viral entry and serves as the major targets recognized by both humoral and cellular immune responses (Li et al., 2003a,b). The development of a murine mAb which recognizes the SARS-CoV S1 antigen that can then be measured by ELISA is described in the current study. A sandwich ELISA using a panel of mAbs and bsmAbs was developed using recombinant SARS-CoV S1 antigen. The production of bsmAb as a bifunctional immunoconjugate bearing two different binding sites (paratopes) for SARS-CoV S-protein avoids the need of random chemical coupling strategies. The development of bsmAb, the first of its kind against SARS-CoV S1 was used subsequently as part of this assay. The presence of intrinsic enzyme binding activity within the bsmAb results in a clear

background, making it a useful tool in the development of specific diagnosis against SARS-CoV S1. The assay is able to detect the S1 antigen first, suggesting that this assay could be useful for detecting the whole virus as soon as the infection occurs, rather than when the antigen is secreted in the body fluids.

An earlier study also showed that different combinations of mAb, bsmAb, and IgY polyclonal antibodies can detect the SARS-CoV nucleocapsid protein by immunoswab assay (Kammila et al., 2008) and sandwich ELISA (Palaniyappan et al., 2012) with a sensitivity of 18.5 pg/ml of recombinant NP antigen in vitro. Antibodies against the nucleocapsid protein have longer shelf life and occur in greater abundance in SARS patients than antibodies against other viral components such as the S-protein, membrane and envelope protein (Rota et al., 2003). This may be due to the presence of higher levels of nucleocapsid protein, compared with other viral proteins, after SARS-CoV infection (Wang et al., 2010). A recombinant nucleocapsid protein-based IgG ELISA was more sensitive than a recombinant S1-based IgG ELISA for serodiagnosis of SARS-CoV (Woo et al., 2005; Yu et al., 2007), duet to the highly immunogenic region of N2. It may help in explaining the present results which show less sensitivity of S-protein detection, compared to a previous nucleocapsid protein detection study (Palaniyappan et al., 2012).

The present results suggest that mAbs and bsmAb could be useful reagents for the diagnosis of SARS-CoV, as well as for functional analysis of S-protein during infection. Further immunological studies with two different mAbs against two targeted SASR-CoV specific antigens such as nucleocapsid protein and S-protein remain to be evaluated.

5. Conclusions

In conclusion, both mAb and bsmAb against SARS-CoV S1 antigen were generated by fusion technology and evaluated by ELISA for use in serological diagnosis. The present study shows the development of a novel sandwich ELISA test with a potential use for the diagnosis of SARS-CoV infections based on bsmAb that recognize simultaneously the S-protein of SARS-CoV and the enzyme peroxidase.

Conflict of interest

The authors declare that they have no competing interests.

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