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A rapid point of care immunoswab assay for SARS-CoV detection

Sriram Kammila^a, Dipankar Das^a, Pravin K. Bhatnagar^a, Hoon H. Sunwoo^a,
Gustavo Zayas-Zamora^b, Malcolm King^b, Mavanur R. Suresh^{a,*}

^a Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, 11304-89 Avenue, Edmonton, Alberta, Canada T6G 2N8

^b Mucophysiology Laboratory, 173 Heritage Medical Research Centre, Department of Medicine, Faculty of Medicine & Dentistry, University of Alberta, Alberta, Canada T6G 2S2

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ABSTRACT

The emergence of severe acute respiratory syndrome (SARS) resulted in several outbreaks worldwide. Early tests for diagnosis were not always conclusive in identifying a SARS suspected patient. Nucleocapsid protein (NP) is the most predominant virus derived structural protein which is shed in high amounts in serum and nasopharyngeal aspirate during the first week of infection. As part of such efforts, a simple, easy to use immunoswab method was developed by generating a panel of monoclonal antibodies (MAbs), Bispecific MAbs and chicken polyclonal IgY antibody against the SARS-CoV nucleocapsid protein (NP). Employing the MAb-based immunoswab, an NP concentration of 200 pg/mL in saline and pig nasopharyngeal aspirate, and 500 pg/mL in rabbit serum were detected. BsMAB-based immunoswabs detected an NP concentration of 20 pg/mL in saline, 500 pg/mL in rabbit serum and 20–200 pg/mL in pig nasopharyngeal aspirate. Polyclonal IgY-based immunoswabs detected an NP concentration of 10 pg/mL in pig nasopharyngeal aspirate providing the most sensitive SARS point of care assay. Results show that the robust immunoswab method of detecting SARS-CoV NP antigen can be developed into an easy and effective way of identifying SARS suspected individuals during a future SARS epidemic, thereby reducing and containing the transmission. The key feature of this simple immunoswab diagnostic assay is its ability to detect the presence of the SARS-CoV antigen within 45–60 min with the availability of the body fluid samples.

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1. Introduction

In 2003, a potential lethal variant of human SARS-CoV emerged in Guangdong province of China resulting in close to 800 deaths worldwide (Drosten et al., 2003; Peiris et al., 2003; Poon et al., 2004; WHO). Sporadic instances of laboratory (SARS) cases were also reported after the containment of the SARS outbreak (Lim et al., 2004; Normile, 2004a,b; Normile and Vogel, 2003) including the four clinical cases in the Guangdong province (Liang et al., 2004). The impact of the SARS outbreak affected the social and economic activity regionally and globally (Tan et al., 2005). This led to a world wide collaborative effort to analyze the virus structure, pathology, routes of infection and possible diagnostic and therapeutic interventions.

The common mode of transmission of SARS-CoV was shown to be the infectious respiratory airborne droplets which contributed to a global outbreak (WHO, 2003a,b). The development of rapid diagnostic procedures that can detect suspected cases of SARS infection

could limit clinical and nosocomial infection. The viral nucleocapsid protein (NP) is a 48 kDa highly phosphorylated basic antigen (422 aa) that interacts with the membrane (M) protein (Chang et al., 2006; Fang et al., 2006; Luo et al., 2005a,b; Rota et al., 2003) to make up viral RNA and nucleocapsid (Chen et al., 2007; Hsieh et al., 2005; Huang et al., 2004b; Lai, 2003). NP is also the most predominant virus derived protein throughout the infection, probably because its template mRNA is the most abundant subgenomic RNA (Di et al., 2005; Hiscox et al., 1995; Lau et al., 2005; Rota et al., 2003) making it a viable target for diagnostics.

The current diagnosis of SARS-CoV depends basically on laboratory-based tests since the clinical symptoms are nonspecific when compared with other respiratory illness caused by non-SARS pathogens (Ksiazek et al., 2003). Hence development of specific methods for detection of SARS-CoV virus is crucial in identification and prevention of future SARS outbreaks. To date, three methods are reported for laboratory detection of SARS-CoV virus: (a) Virus isolation from patient samples by inoculating cell cultures was used to determine the presence of infection. This is often time consuming, tedious and prone to false results (Keyaerts et al., 2005; Yamashita et al., 2005). (b) Conventional and reverse transcriptase polymerase chain reactions (RT-PCR) used in the direct detection of viral RNA

* Corresponding author. Tel.: +1 780 492 9233; fax: +1 780 492 1217.

E-mail address: msuresh@pharmacy.ualberta.ca (M.R. Suresh).

are laborious and expensive, require highly skilled personnel and with 37.5–50% success rates in identification of early infection (Wu et al., 2004). (c) The serological detection of viral antibodies (IgG, IgM) by enzyme linked immunosorbent assay (ELISA), immunofluorescence (IFA), or Western blot was found to be sensitive (90%), but the time lag in detecting the antibodies (2–3 weeks) makes this method less viable for early diagnosis (Chan et al., 2005; Guan et al., 2004; Saijo et al., 2005; Yu et al., 2007).

SARS, with high rates of transmission and mortality rates, needs a rapid, sensitive and inexpensive detection method that can be used to effectively quarantine an infected person for further clinical and therapeutic monitoring to mitigate the rapid spread of the infection. Recent studies have shown that SARS-CoV NP can be detected in the acute phase of SARS infection by specific monoclonal antibodies (MAbs) (Che et al., 2004a; Huang et al., 2004a; Liu et al., 2003; Shang et al., 2005; Tan et al., 2004; Woo et al., 2004). The comparison of viral markers by enzyme-enhanced chemiluminescence immunoassay (ECLIA) showed that NP could be detected in 90% of the patients sera within 1–10 days after infection with a gradual decrease over time (Li et al., 2005). Studies also showed that circulating shed antigen detection is also used for a variety of viral diseases such as HIV, Hepatitis B, Hepatitis C, viral hemorrhagic fever and cytomegalovirus (Cano et al., 2003).

The objective of this study was to develop a sensitive immunoassay utilizing a panel of MAbs, bispecific antibodies (BsMAbs) and chicken IgY polyclonal antibody against SARS-CoV NP for early screening or point of care applications. IgY polyclonal antibodies are also an attractive source for development of sensitive immunoassays compared to mammalian antibodies (Cova, 2005; Miyamoto et al., 2007a,b; Sunwoo et al., 2006). The generation of BsMAbs as bifunctional immunoconjugates bearing two different binding sites (paratopes) for SARS-CoV NP avoids the need for random chemical coupling strategies. The development of BsMAbs, the first of its kind against SARS-CoV NP, was subsequently used as part of immunoswab assays. The presence of intrinsic enzyme binding activity within the BsMAb makes it a useful tool in the development of specific diagnostics against SARS-CoV NP with clean backgrounds.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) was purchased from PAA cell culture company (Ontario, Canada) and Streptomycin–penicillin–glutamine was obtained from Gibco (NY, USA). Polyethylene glycol (PEG) 1300–1600, HAT and HT supplement, goat anti-mouse IgG conjugated with horseradish peroxidase (GAM–HRPO), bovine serum albumin (BSA), fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), horseradish peroxidase (Type VI), Protein G-agarose, Long chain–sulfosuccinimidyl NHS biotin, low molecular weight dextran sulphate and rabbit anti-chicken IgY–HRPO were sourced from Sigma (St. Louis, MO, USA). Cell strainers for collection of spleen and Streptavidin–HRPO (St–HRPO) were obtained from BD Biosciences Pharmingen (MA, USA). IMABTM bags were obtained from BioVectra (Canada). Tetramethylbenzidine (TMB) and Hydrogen peroxide (H₂O₂) detecting reagent was obtained from KPL laboratories (Gaithersburg, MD, USA). Slide-A-Lyzer^R for dialysis was obtained from Pierce (Rockford, IL, USA). Pig nasopharyngeal aspirates were obtained from Surgical Medical Research Institute (SMRI), University of Alberta with the help of Dr. Gustavo Zayas-Zamora and Dr. Malcolm King. Pooled naïve rabbit serum was obtained from Health Sciences Laboratory Animal Services (HSLAS), University of Alberta. Calcium alginate fiber tipped

ultra fine aluminum applicator swabs were obtained from Fisher scientific (USA). Tetramethylbenzidine (TMB) stabilized substrate for HRP was obtained from Promega Corporation (Madison, USA). Non-sterile flat bottom 96-well ELISA plates were obtained from Nunc International Maxisorp (Rochester, NY, USA). Sterile flat bottom 96-well cell culture plates for production of hybridoma clones, cell culture flat bottom plates (6, 12, 24, and 48 well) and cell culture flasks (25, 75 and 175 cm²) were, respectively, obtained from Corning Incorporated (NY, USA).

2.2. Preparation of SARS-CoV nucleocapsid protein (NP)

A full-length codon optimized NP gene was cloned in a bacterial expression vector and the expressed protein was purified from *E. coli* cultures (Das and Suresh, 2006). The non-glycosylated NP was used to generate anti-NP MAbs, anti-NP IgY, for screening BsMAbs, and in the development of immunoswab assays.

2.3. Preparation of anti-SARS-CoV NP mouse monoclonal hybridomas

The 6–8 week old female BALB/c mice were immunized intraperitoneally 3 times with 25 µg of NP antigen on day 0, and 14 using complete and incomplete Freund's adjuvant, and once with 10 µg of antigen on day 28 using PBS pH 7.3. The immune response to the antigen was assessed by measuring the titer of polyclonal antibody in mouse serum using indirect ELISA. The mice with highest titer were splenectomized on day 3 after the last antigen injection. The spleen cells were fused with SP2/0 myeloma cells at a ratio of 5:1 using 50% (w/v) polyethylene glycol (PEG) according to the technique described previously by Kohler and Milstein (1975) and Shahhosseini et al. (2007). Five SARS-CoV anti-NP MAbs were developed and characterized (unpublished data). These MAbs were used for generation of quadromas and subsequent immunoswab assay development. The isotypes of the MAbs were determined using specific HRPO-antibodies from SIGMA, USA.

2.4. Immunization and purification of anti-NP IgY antibody

Chickens were immunized with recombinant NP antigen to obtain NP-specific IgY loaded eggs according to published methods (Sunwoo et al., 1996). Immunization of hens was carried out (50 µg of NP) with an equal volume of Freund's incomplete adjuvant to immunize 23-week-old Single Comb White Leghorn chickens intramuscularly. A booster immunization was given at 2 weeks after the initial immunization. Eggs were collected daily and IgY was purified from egg yolk for antibody titer by ELISA (Sunwoo et al., 2002) and for development of immunoswab assay.

2.5. Cell lines for quadroma fusion

The anti-HRPO YP4 is a well-characterized rat hybridoma that was previously selected for drug resistance to 8-azaguanine, making it sensitive to aminopterin in HAT medium. This cell line (YP4) along with anti-NP SARS-CoV MAbs were chosen for developing quadromas (hybridoma × hybridoma) (Suresh et al., 1986a,b). YP4 secretes (IgG2a) monospecific anti-horseradish peroxidase (HRPO) antibodies and was obtained from the late Dr. C. Milstein, Medical Research Council for Molecular Biology, Cambridge, United Kingdom.

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

The development of anti-NP/anti-HRPO quadromas involved maintaining the two hybridoma cell lines (anti-NP and anti-HRPO)

in logarithmic growth phase containing RPMI medium with 10% FBS at 37 °C supplemented with 5% CO₂. Trypan blue staining of over 90% was observed 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 the working solution. The following steps as reported earlier were then followed for successful completion of a quadroma fusion (Das and Suresh, 2005; Tang et al., 2004). Briefly, 2×10^7 cells/mL of anti-NP hybridomas (P140.20B7, P140.19B6, P140.19C7) and YP4 hybridomas were separately resuspended in RPMI pH 7.4 and 6.8, respectively. Anti-NP hybridomas were then labelled with TRITC (red fluorescence) and YP4 cells were labelled with FITC (green fluorescence). Following 30 min incubation at 37 °C in a CO₂ incubator the hybridoma cell suspensions were washed and mixed in a 50 mL tube and centrifuged at $459 \times g$ for 7 min. To the cell pellet, 2 mL of PEG was added drop by drop over a period of 2 min, with gentle mixing. Upon the addition of PEG, the cell suspension was then placed at 37 °C in a CO₂ incubator for 3 min, followed by addition of 20 mL of FBS free RPMI medium to dilute the toxic effects of PEG. Flow cytometry (Epics Elite cell sorter, Coulter Corporation, 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% CO₂. The clones were then screened twice using a bridge ELISA (Suresh et al., 1986a,b). The cloning of quadromas was performed based on the limiting dilution culture method. The four best clones of each fusion P140.20B7/YP4, P140.19C7/YP4, P140.19B6/YP4 were then chosen to undergo recloning (3–4 times) to select positive and highly specific quadromas secreting BsMab against SARS-CoV NP.

2.7. Bridge ELISA screening of anti-NP/anti-HRPO quadromas secreting BsMAbs

Bridge 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 μ L of 5 μ g/mL SARS-CoV NP antigen as solid phase. The microtiter plate was then incubated at 4 °C overnight, washed with PBS-T (phosphate buffer saline, pH 7.4 with 0.05% Tween-20) 3 times and blocked with 2% dialyzed bovine serum albumin (DBSA in (PBS-T) for 2 h at 37 °C. After washing, 100 μ L of the serially diluted (neat, 1:10, 1:100, 1:1000, 1:10,000) quadroma supernatant was added to the plate and then incubated for 1 h at 37 °C. A 100 μ L aliquot of 10 μ g/mL (diluted in 2% DBSA) of HRPO was then added to the microtiter plate followed by a wash step. Finally, 100 μ L of TMB substrate was added to the wells. The positive quadromas were then selected after 15 min of color development and the plate was read at 650 nm on V_{max} ELISA plate reader. RPMI media and PBS were used as blanks. Negative controls were wells without NP antigen and HRPO.

2.8. Purification of anti-NP/anti-HRPO BsMab

The purification of P143.19C7 was done by a new novel method using *m*-aminophenylboronic acid agarose column (APBA) as described (Bhatnagar et al., 2008). Briefly the APBA column was saturated with HRPO to capture the BsMab along with the monospecific anti-HRPO MAB eliminating the monospecific anti-NP species. Following a short wash procedure, the bound antibodies were eluted with potassium phosphate buffer containing 0.1 M sorbitol.

2.9. Biotinylation of anti-NP MAbs

Anti-NP MAbs were biotinylated by using long arm biotinimido hexanoic acid-3-sulfo-*N*-hydroxysuccinimide ester. 1 mg each of protein-G purified (five anti-NP MAbs) in PBS pH 7.4 were added to 20 μ L of long chain biotin (3 mg/mL) and incubated at room temperature (RT) for 1 h. A 10 μ L of glycine (100 μ g/ μ L) was added and the solution kept on a shaker for 10 min (Delos et al., 2000). The solution was then dialyzed in a slide-A-lyzer against PBS pH 7.4 overnight at 4 °C.

2.10. Design of immunoswab assays

The development of immunoswab assays involved use of calcium alginate tipped swabs with aluminum shafts based on the previous published method (Tang et al., 2004). The pointed tip of the swab was used to coat the capture antibody (P140.19B6) and later used in identification of NP in different matrices as discussed in the next section (Fig. 1).

2.11. Preparation of different matrices for spiking NP antigen

Three different matrices were used for spiking the NP antigen for heterosandwich-based detection. Normal saline at pH 7.3–7.4 and naïve neat rabbit serum was used to spike the NP antigen. Pig nasopharyngeal aspirate was used as the third matrix to mimic human nasopharyngeal aspirate-based detection. An endotracheal tube was inserted in the nasopharynx of a pig and the sticky mucous was then further processed with a modification of the published method (Liu et al., 2005; Sudo et al., 2000). The aspirate was suspended in Low Molecular weight dextran sulphate (5000 MW, 200 mg/mL) in the ratio of 1:5 for 45 min at RT. The suspended mucous was then centrifuged at $459 \times g$ for 5 min. The supernatant was collected for spiking of SARS-CoV NP antigen to develop the immunoswab assay.

2.12. Development of prototype MAb, BsMab and IgY-based immunoswabs for NP diagnostics

Different concentrations of NP antigen were spiked in saline, neat rabbit serum, pig nasopharyngeal aspirate and aliquoted in eppendorf tubes (50 μ L). The tagged nasopharyngeal calgiswabs were incubated with a constant volume (50 μ L) of P140.19B6 capture antibody (100 μ g/mL) in PBS pH 7.4 at RT for 20 min and dried at RT for 5 min. They were then fixed with 50 μ L of 95% ethanol (50 μ L) for 1 min and dried for 5 min at RT. The swabs were then blocked with 5% DBSA (50 μ L) for 10 min at RT and washed with PBS (0.05% BSA) at pH 7.4 for 5 times by simple fill and aspiration steps in a test tube. The washed swabs were incubated for 30 min with different concentrations of NP antigen spiked in saline, neat rabbit serum or pig nasopharyngeal aspirate. Next the swabs were washed with PBS and incubated with biotinylated P140.19C7 MAB (100 μ g/mL) or IgY (10 μ g/mL) for 30 min in a separate eppendorf tube (50 μ L) at RT. In the BsMab immunoswab assay the P143.19C7 BsMab at a concentration of 100 μ g/mL aliquoted at 50 μ L diluted in PBS at pH 7.4 was used. The swabs were again washed with PBS (0.05% BSA) for 5 times by simple fill and aspiration method in a test tube. The detection of the biotinylated murine antibody or chicken IgY bound to antigen was done by incubating the swabs in 50 μ L of St-HRPO (1:1000 diluted in 1% DBSA) or rabbit anti-chicken IgY-HRPO (1:5000 diluted in 1% DBSA) for 15 min at RT. However, for the BsMab assay, the addition of HRPO was not needed since the BsMab is already complexed to HRPO. The swabs were then carefully washed and incubated with 50 μ L of TMB substrate for color development. Control swabs were also incubated at the same time in saline, rabbit serum and pig nasopharyngeal aspirate without NP

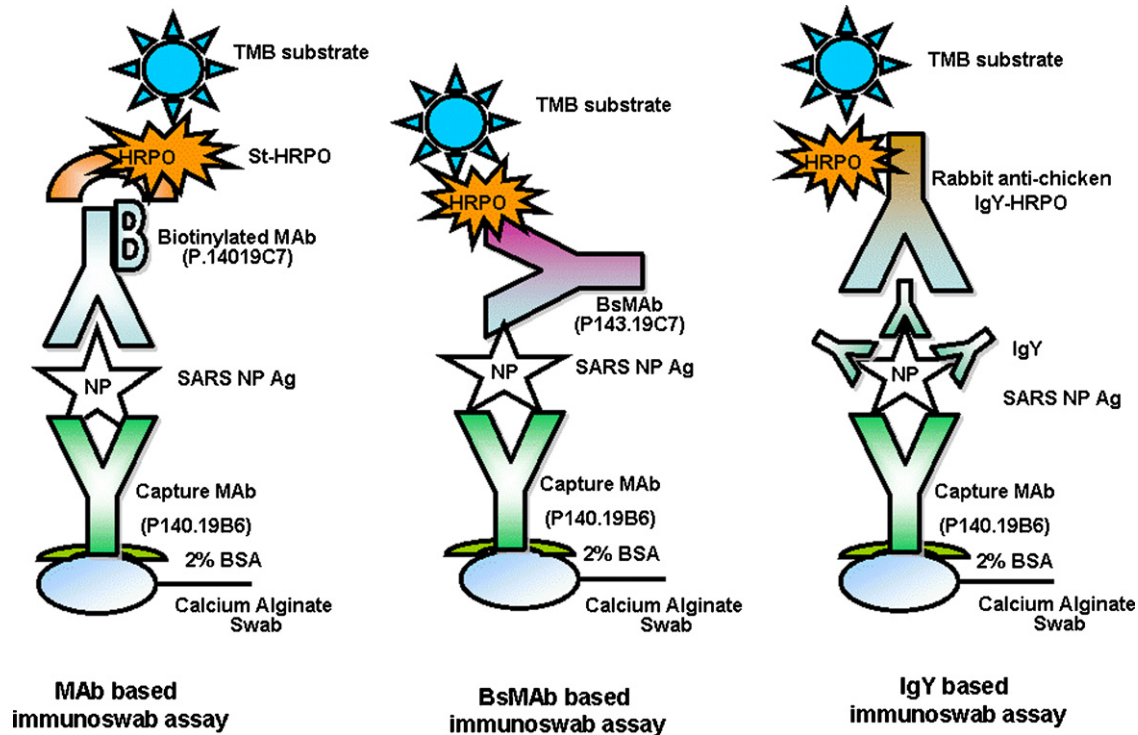


Fig. 1. Schematic illustration of anti-NP MAb, anti-NP/HRPO BsMAb and anti-NP IgY-based immunoswab assays.

antigen. A digital camera with high pixel size and optical zoom was used to capture images of the blue color. A shelf-life study on the robustness of MAb and BsMAb immunoswabs was also performed at three different temperatures (RT, 4, and -20°C).

3. Results

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

The development of anti-SARS-CoV NP hybridomas was accomplished by classical hybridoma technology. The recombinant NP antigen expressed in *E. coli* was injected into BALB/c mice for the development of anti-SARS-CoV NP MAbs. Five anti-SARS-CoV NP hybridomas were developed namely P140.20B7, P140.19B6, P140.19C7, P140.19D3 and P140.14D6. The primary hybridomas secreting anti-NP antibodies namely P140.20B7 (IgG₁), P140.19B6 (IgG_{2a}) and P140.19C7 (IgG₁), were fused with the YP4 anti-HRPO hybridoma to generate quadromas. The proportion of double positive cells in the bispecific fusions was in the range of 3.8% (data omitted). The recloning process involved cloning the quadromas by limiting dilution such that one cell per well was plated. Quadromas

exhibit polyploidy and hence were shown to have a high level of instability. The process of repeated recloning helps in obtaining and identifying a strong BsMAb secreting quadromas. Three Quadroma clones (P141.20B7, P142.19B6, and P143.19C7) were selected based on their high optical density (O.D.) values (Table 1) in the bridge ELISA (Suresh et al., 1986a,b). In Table 1 the O.D. value of all the BsMAbs increase when the neat culture supernatant was initially diluted at 1:10 and decrease at higher dilutions. The purified yield of BsMAb was 3.8 mg/L of culture supernatant and was later utilized in the development of immunoswab assays.

3.2. Development of anti-SARS-CoV NP IgY

Chicken IgY polyclonal antibodies were developed against SARS-CoV NP and characterized. Heterosandwich assay was developed using different MAbs as capture antibody and IgY as detector antibody. It was demonstrated that all the five MAbs made good heterosandwiches and the best heterosandwich pair was when P140.19B6 was used as capture antibody and IgY as detector antibody (unpublished data). The best pair was chosen for subsequent immunoswab assays.

Table 1
Screening of quadroma cell lines secreting BsMAb (anti-NP/anti-HRPO) at different dilutions

	Dilution of BsMAb culture supernatant for screening of positive clones					Blank
	Neat	1:10	1:10 ²	1:10 ³	1:10 ⁴	
Primary quadroma clones						
2B7	0.326	0.523	0.203	0.102	0.042	0.067
4D2	0.296	0.525	0.190	0.090	0.060	
3G6	0.301	0.493	0.210	0.102	0.057	
Quadroma cell lines after 3rd recloning						
P141.20B7	0.843	1.109	0.423	0.107	0.087	
P142.19B6	0.787	1.196	0.321	0.114	0.091	
P143.19C7	0.887	1.090	0.434	0.106	0.070	

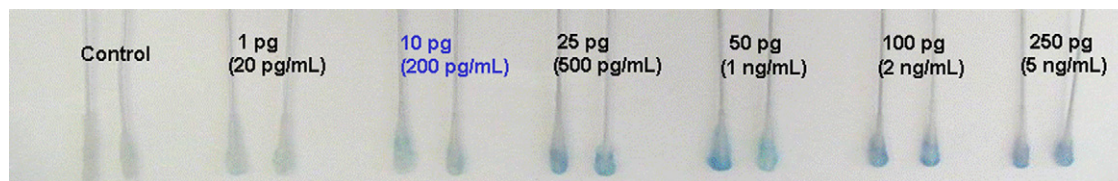


Fig. 2. Anti-NP/anti-HRPO BsMAB immunoswab assay for detection of SARS-CoV. The MAb P140.19B6 captured NP was detected by a heterobifunctional BsMAB (P143.19C7) which has two binding sites. One binding arm (paratope) of the BsMAB binds to the NP while the other arm was pre-complexed to HRPO. The colored swabs from pig nasopharyngeal aspirate medium (after detection of NP) were taken out of the eppendorf tubes to capture the developed blue color on the swab as an image for better visualization. This reveals the increased intensity of the color with increasing NP concentrations (from left to right). The control swabs tested in the absence of antigen showed no color development upon addition of substrate.

3.3. Diagnostic evaluation of MAb, BsMAB and IgY-based immunoswab assays

The comparative testing of immunoswab assays involved detection of the antigen spiked in different matrices such as saline, serum and pig nasopharyngeal aspirate. This simulated detection of NP antigen was done to understand its potential use in screening applications where minimally trained persons can perform the assay during a future SARS outbreak. Preparation of pig nasopharyngeal aspirate involved the use of dextran sulphate (Liu et al., 2005; Sudo et al., 2000) which was used as a mucolytic agent to break the covalent, ionic and hydrogen bonds that exist in the thick mucous gel.

The time required to perform the assay with a set of 14 swabs (each concentration in duplicate) with the monoclonal or polyclonal antibody was approximately 60 min. In the BsMAB immunoswab assay design (Fig. 2) the swabs were coated with MAb P140.19B6 to capture NP followed by detection with BsMAB–HRPO complex (P143.19C7). The time required to perform the assay was approximately 45 min. The assay time was reduced with BsMAB immunoswab assay when compared with MAb or IgY-based detection due to the absence of an additional washing step. In the MAb-based assay the biotinylated antibody needs the addition of St–HRPO followed by a wash. In IgY-based assay the addition of rabbit anti-chicken IgY–HRPO is followed by a washing step.

The assay sensitivity of NP detection in different matrices also varied based on the detection by MAb, BsMAB and IgY (Table 2). The sensitivity of BsMAB was high in saline (1 pg/swab or 20 pg/mL) compared to 10 pg/swab or 200 pg/mL seen in the case of MAb-based immunoswab assay. In rabbit serum, MAb and BsMAB-based immunoswab assay showed a sensitivity of 25 pg/swab or 500 pg/mL of NP. MAb and BsMAB immunoswab assay sensitivity of NP in pig nasopharyngeal aspirate were 200 pg/mL (10 pg/swab) and 20–200 pg/mL (1–10 pg/swab), respectively. The

IgY immunoswab assay sensitivity was 10 pg/mL or 0.5 pg/swab (Table 2). The higher sensitivity of IgY compared to MAb and BsMAB was likely due to polyvalent binding to NP antigen. The detection of NP by IgY immunoswab was performed only in pig nasopharyngeal aspirate matrix. The control tubes without NP antigen showed no color in all the assays. The visual quality of MAb-based immunoswabs was relatively low, compared to BsMAB-based immunoswabs which had clear backgrounds (Fig. 2). This higher sensitivity can be due to high specific activity and molecular uniformity of BsMABs unlike the heterogenous chemical conjugates of St–HRPO complexed with the MAb. The immunoswab assays were repeated independently 2–3 times with each concentration in duplicate. The performance of the assays under similar conditions showed good concordance in visual sensitivity.

The MAb and BsMAB swabs were tested after storage for 2, 6, 10 and 14 week time periods to understand the shelf-life under different conditions. The pig nasopharyngeal aspirate was used as surrogate to human samples to test visual end points at low concentrations of NP antigen. The lowest concentration chosen for the study was 200 pg/mL (10 pg/swab) and was used as a reference point for NP sensitivity. This was also the lowest level of NP detected when assays were initially performed. MAb and BsMAB-based detection of NP in terms of sensitivity was constant for 2 and 6 weeks for all the three storage conditions. The detection of NP by MAb and BsMAB for swabs stored at RT at 10 weeks showed poorer sensitivity to NP (500 pg/mL). MAb-based detection of NP with swabs stored at -20°C on week 10 was more sensitive (200 pg/mL) compared to BsMAB-based detection (500 pg/mL). However, the detection of NP with swabs stored at 4°C on week 10 with MAb and BsMAB immunoswab assay was found to be sensitive and capable of detecting 200 pg/mL. On the 14th week, 500–1000 pg/mL of NP could be detected for RT, 4 and -20°C by MAb-based detection exhibiting some deterioration of the assay. In comparison BsMAB-based immunoswabs showed better sensitivity in detecting NP on week 14 with 200 pg/mL at 4°C and 500 pg/mL for RT and -20°C .

Table 2

Detection limits of NP in various matrices using anti-NP MAb immunoswab assay (A), BsMAB assay (B) and IgY assay (C)

Medium	Detection limit concentration (pg/mL)	Amount/swab (pg)
A		
Saline, n = 2	200	10
Rabbit serum, n = 2	500	25
Pig nasopharyngeal aspirate, n = 3	200	10
B		
Saline, n = 3	20	1
Rabbit serum, n = 2	500	25
Pig nasopharyngeal aspirate, n = 3	20–200	1–10
C		
Pig nasopharyngeal aspirate, n = 2	10	0.5

'n' refers to separate complete independent immunoswab assays and batch preparations.

4. Discussion

Human coronaviruses (229E, HCoV-NL, OC43 and HKU1) were shown to cause mild upper respiratory tract infections and were rarely associated with lower respiratory tract infections (El-Sahly et al., 2000; Falsey et al., 2002; Makela et al., 1998). In the case of SARS-CoV, a severe lower respiratory tract infection is seen resulting in acute respiratory distress syndrome (Lo et al., 2006) with a fatality rate of 10–15% with >60% in elderly patients (Drosten et al., 2003; Holmes, 2003; Ksiazek et al., 2003). The detection of NP was demonstrated in various body fluids of suspected SARS patients which include serum, urine, fecal matter, nasopharyngeal aspirate, throat wash samples, and saliva, during the early days of infection indicating that NP is rapidly shed in high amounts (Che et al., 2004b; Di et al., 2005; Lau et al., 2004; Liu et al., 2005; Wang et al., 2004). Serum and pig nasopharyngeal aspirate were selected in

this study as high amounts of shed NP antigen were found in SARS infected patients. MAb, BsMAb and IgY were developed to compare the sensitivity of detection of NP for point of care applications. The heterobifunctional BsMAbs (P141.20B7, P142.19B6, and P143.19C7) showed high specific activity towards NP by bridge ELISA. The anti-HRPO binding arm conjugated with the HRPO enzyme of the BsMAb also reduced the time course of the assay and showed excellent signal to noise ratio in the qualitative immunoswab assay.

Recent ELISA studies have reported that the sensitivity of recombinant NP was 50 pg/mL using polyclonal antibody (pAb) when spiked in human sera (Che et al., 2004b). Another study by rapid immunochromatographic test (RICT) method showed detection of NP at 31 pg/mL in nasopharyngeal aspirate samples of SARS patients (Kogaki et al., 2005). In comparison the MAb capture of NP followed by detection with BsMAb using immunoswab assay showed NP detection limits of 10 pg/mL (1 pg/swab) in saline, 20–200 pg/mL (1–10 pg/swab) in pig nasopharyngeal aspirate and 500 pg/mL (25 pg/swab) in rabbit serum (Table 2). It is likely that the NP could be partially degraded in the serum matrix. The MAb capture of NP and detection with a biotinylated MAb shows a sensitivity limit of 200 pg/mL (10 pg/swab) in saline, pig nasopharyngeal aspirate, and 500 pg/mL (25 pg/swab) in rabbit serum. In IgY immunoswab assay format the sensitivity was 10 pg/mL (0.5 pg/swab) in pig nasopharyngeal aspirate which is 3 times more sensitive than the literature value (Kogaki et al., 2005).

The immunoswab detection of SARS-CoV NP antigen spiked in saline, rabbit serum, and pig nasopharyngeal aspirate is a simple point of care test when compared to ELISA's and IFA's (Di et al., 2005). A set of 14 swabs were completed in 60 min for MAb and IgY formats and 45 min for BsMAb-based assays. RICT SARS assay is also a rapid test but requires trained personnel to operate the test, and higher cost can be an issue in underdeveloped parts of the world. In contrast, an immunoswab assay can be done by a minimally trained person as a low cost home testing device in the future. The study on robustness of immunoswab assays has shown that swabs precoated with capture antibody can be utilized for up to 6 weeks with no decrease in sensitivity of NP detection and can be further optimized for longer stability. We propose to extend this study with human mucus or pig nasopharyngeal aspirate intended for simulating aspirates of SARS infected patients. The relative ease of accessing nasopharyngeal aspirate from patients gives it an added advantage for testing within a short period of time during a future SARS outbreak with no invasive procedures. In terms of sensitivity, MAb and BsMAb swabs appear to show good binding and intensity of color at 4 °C followed by storage at –20 °C and at RT; however, we have not yet tested IgY immunoswabs for shelf-life stability. The optimized and most effective conditions of this stability assay showed better results for storage at 4 °C for both MAb and BsMAb immunoswabs.

The SARS-CoV NP sequence shares low amino acid homology with other human coronaviruses (229E and OC43). It was also reported that MAbs and polyclonal antibodies raised against SARS-CoV NP showed little or no cross-reactivity with NP of human coronavirus (Che et al., 2005; Lau et al., 2004). Nevertheless we propose to test the antibodies developed in this study for potential cross-reactivities.

In conclusion, the panel of MAbs, BsMAb and IgY could be effectively used in a sandwich format on a simple easy-to-use swab to detect NP from patient body fluids. There are clear advantages of adopting an immunoswab assay in a clinical and primary healthcare setting. First, the ease of use and detection of highly infective strains of virus could help in rapid detection and potentially isolating patients, mitigating the spread of infection. An immunoswab assay could be utilized as a simple, rapid point of care diagnostic test at all ports of mass human entry (airports, seaports, bus,

train and border stations). Since the testing time is rapid the use of immunoswab-based SARS-CoV detection during an outbreak will not unnecessarily cause inconvenience to the passengers or delay their travel plans. If successful, this type of assay can reduce the unprecedented paranoia associated with such outbreaks involving people traveling across the globe. This type of test can also be used in populated centers as well as in areas where adequate health care is not available. The other main advantage of this test is its low cost/low tech approach. The use of immunoswab also does not require complicated technology or highly qualified staff to perform the test. Any social worker with some minimal training can use this test in a primary healthcare or rural setting. It will not be a heavy burden to people in underdeveloped countries or developing countries who have to invest in costly procedures such as RT-PCR in case of large outbreaks. This assay is particularly useful in parts of the world where lack of technology can be an impediment in detecting SARS-CoV infection before further diagnostic and therapeutic procedures can be procured. The immunoswab detection of NP can also set a precedent for detection of other viral and bacterial diseases such as avian influenza and tuberculosis. These diseases have a similar route of air borne infection as SARS-CoV and also shed antigens into body fluids.

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