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

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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.

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

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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). IMAB^{TM} bags were obtained from BioVectra (Canada). Tetramethylbenzidine (TMB) and Hydrogen peroxide (H_2O_2) 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 cm^2) 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 aminopterine 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)