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Cloning, sequencing, expression, and purification of SARS-associated coronavirus nucleocapsid protein for serodiagnosis of SARS

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Abstract

A novel coronavirus has been associated with a worldwide outbreak of atypical pneumonia referred to as Severe Acute Respiratory Syndrome (SARS-CoV). SARS-CoV nucleocapsid (N) protein has been cloned sequenced and expressed in Escherichia coli strain. Purified N protein was used to measure the SARS-CoV specific IgG antibodies from 16 SARS-CoV infected patients' sera and from 131 control subjects using ELISA assay. Specific antibody responses to the purified recombinant N protein after 10, 20, and 30 days of disease onset were observed in 13 of 16 (81.3%), 16 of 16 (100%) and 16 of 16 (100%) SARS patients sera, respectively. Comparison of detection results with a commercially available diagnostic kit coated with a mixture of SARS-CoV viral proteins showed 9 of 16 (56.3%), 13 of 16 (81.3%), and 15 of 16 (93.7%) positive responses, respectively. None of 131 control sera gave positive reaction in either assay. This data suggests that the N protein of SARS-CoV is immunodominant and this ELISA based test assay for detecting the SARS-CoV N antigen may hold a significant value for SARS diagnosis.

Keywords: SARS-CoV; Nucleocapsid protein; Expression and purification; ELISA

1. Introduction

Severe Acute Respiratory Syndrome (SARS) is a recently recognized febrile respiratory illness that first appeared in southern China in November 2002, and has since spread to several other countries. The disease was caused by a novel coronavirus, now provisionally named “SARS-associated coronavirus” (SARS-CoV) (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). The severity of this disease is such that the mortality rate appears to be 9.6% and diagnostic methods for SARS are urgently needed (Lipsitch et al., 2003; Riley et al., 2003). The SARS-CoV is an enveloped virus of a positive single-stranded RNA genome ~29.7 kb in length and has 11 open reading frames. The genome organization is similar to that other coronavirus, where phylogenetic analyses and sequence comparisons showed that the SARS-CoV is not closely related to any of previously characterized coronavirus (Marra et al., 2003; Rota et al., 2003). The structural protein genes of SARS-CoV contain four major open reading frames; including the surface spike (S) glycoprotein, the small membrane (M) protein, the envelope (E) glycoprotein, the nucleocapsid (N) protein, and a set of accessory proteins whose number and sequence vary among different coronaviruses (Siddell, 1995). The N proteins have been shown to be strong immunogens in several coronaviruses, such as murine coronavirus (Wage et al., 1993), Turkey coronavirus (Akin et al., 2001), porcine reproductive and respiratory syndrome coronavirus (Casal et al., 1998). It has been reported that N protein accumulates intra-cellularly even before it is packed in the mature virus (Collisson et al., 2000) and is the most abundant virus derived-protein throughout the infection, probably because its template mRNA is the most abundant subgenomic RNA (Hiscox et al., 1995; Keck et al., 1988). These features make it a suitable candidate for raising antibodies for diagnostic applications.

In this study, SARS-CoV N protein gene was cloned, sequenced, expressed in Escherichia coli strain and purified to homogeneity. Using the purified N protein as an antigen for SARS diagnosis and comparing it with a commercially available detection kit coated with a mixture of SARS viral proteins, we demonstrated that the antibody to N protein has high sensitivity and specificity for SARS diagnosis.
2. Materials and methods

2.1. Clinical samples

Serum samples from 16 SARS-CoV infected patients (the clinical diagnostic for SARS followed the clinical description of SARS released by WHO), 3 from Hubei, 1 from Beijing, and 12 from Henan were collected from 10, 20, and 30 days of disease onset. One hundred and thirty-one serum samples of symptom free direct contacts to the above patients (physician nurses, and their family members) or non-SARS patients who showed symptoms of fever >38 °C, cough and sore throat, were collected as controls by Dr. Zuojiong Gong from Renmin Hospital of Wuhan University, Wuhan, China.

2.2. Cells and virus infection

VeroE6 cells were maintained in minimal essential medium (GIBCO) with 10% fetal bovine serum (Hyclone), 1 mM of each dNTP, 20 pmol of each primer, and 2.5 U of LATaq™ DNA polymerase (TAKARA Biomedical, Japan). The viral cDNA was made by adding 5 μl of extracted viral material RNA was extracted by Trizol reagent (Invitrogen) according to the manufacturer’s instructions.

2.3. RT-PCR

The cDNA was made by adding 5 μl of extracted viral RNA to 20 μl of total reaction mixture using AMV-reverse transcriptase kit (TAKARA Biomedical, Japan), containing 50 μM random primer. The Viral cDNA was used as a template for PCR by adding 3 μl to 50 μl of total reaction mixture containing 10× transcription buffer, 20 mM MgCl₂, 1 mM of each dNTP, 20 pmol of each primer, and 2.5 U of LTaq™ DNA polymerase (TAKARA Biomedical, Japan). The primers were synthesized according to HKU39849 accession number (AGCTGAATTCCATCATGAGTGTTTATGCCTGAGT). The results of the N protein ELISA were compared with results from a commercially available ELISA kit based on viral S, N, and E proteins prepared by the Beijing Genomics Institute, Chinese Academy of Sciences, Beijing, China.

2.4. Plasmid construction

The N protein gene cDNA was cloned into pGEM®-T Easy vector (Promega, UK) by standard techniques then transformed into E. coli strain DH5α. The constructed pGEMT-NP was digested with BamHI and EcoRI (MBI Fermentas, Germany), sub-cloned into pETHis expression vector thereby introducing a small hexahistidine-T7 Tag at the N-terminus of the protein to facilitate subsequent purification. The recombinant plasmid was transformed into DH5a strain. Seven positive colonies were analyzed, confirmed by PCR and sequenced.

2.5. Expression and purification of SARS-CoV nucleocapsid protein

Fifty nanogram purified N protein, dissolved in coating buffer (0.016 M Na₂CO₃, 0.034 M NaHCO₃ pH 9.6), were added to a 96-well microplate and incubated at 4 °C overnight. Wells were then blocked with 4% non-fat milk, 0.5% BSA in phosphate buffer saline (PBS), incubated for 1 h at RT, washed with PBS and dried at 37 °C for 1 h. For detection by chemiluminescent method, sera were diluted in sample buffer (1:25) then incubated at 37 °C for 30 min. Each well was washed then incubated with peroxidase-conjugated rabbit anti-human antibody at 37 °C for 30 min.

3. Result

The nucleocapsid protein gene sequence of SARS-CoVHB was performed and the sequence (1269 nt) was deposited at the National Centre for Biotechnology Information (NCBI). The full length of N protein (422 amino acids) with an amino terminal hexahistidine-tag was expressed in Bacteroides thetaiotaomicron (BTH101) and purified by Ni-NTA affinity column. After 2 h induction
Table 1: Results of the SARS-CoV recombinant N protein ELISA in comparison to the commercially available ELISA for the sero-diagnosis of SARS.

| Coated antigen          | Number positive/number tested (% positive) | SARS infected patients sera | Control sera* |
|-------------------------|-------------------------------------------|-----------------------------|---------------|
|                         | 10 days                                   | 20 days                     | 30 days       |               |
| Nucleocapsid protein    | 13/16 (81.3%)                             | 16/16 (100%)                | 16/16 (100%)  | 0/131 (0%)    |
| Mixture of antigens     | 9/16 (56.3%)                              | 13/16 (81.3%)               | 15/16 (93.7%) | 0/131 (0%)    |

* Serum samples of both the symptom free direct contacts to the above patients (physicians, nurses, and their family members) and non-SARS patients who showed symptoms of fever >38°C, cough and sore throat.

with IPTG, high level expression of N protein was observed (Fig. 1, lane 1) compared to uninduced cells lysate (Fig. 1, lane 2). The His-tagged protein was purified from the soluble fraction resulting in a single band of the expected mass of 47 kDa (Fig. 1, lane 3). This data demonstrates that the SARS-CoV N protein was successfully expressed in E. coli and purified to homogeneity. The N protein was identified by western blot using human SARS-CoV infected serum (data not shown).

To investigate the immunoreactivity of the nucleocapsid protein with the 16 SARS-CoV infected patient’s sera collected at 10, 20, and 30 days after disease onset, an ELISA assay was established using the purified recombinant N protein. Interestingly, we found that 13 of 16 (81.3%), 16 of 16 (100%), and 16 of 16 (100%) SARS patients had a significant positive response to the N protein after 10, 20, and 30 days of disease onset, respectively. When comparing this result with a commercially available detection ELISA kit coated with a mixture of viral proteins (S, N, and E), prepared by Beijing Genomics Institute, Chinese Academy of Science, Beijing, China, we observed that the IgG antibodies to N protein are commonly present in all 16 SARS patients sera and could be detected as early as 10 days of disease onset. The mean OD ± S.D. obtained using 131 control sera were 0.079 ± 0.052 and 0.0688 ± 0.034 for N protein coated ELISA and commercially available detection kit, respectively.

4. Discussion

Despite the initial rapid progress in the discovery of the causative agent and the early development of diagnostic tests, there is a critical clinical need for further laboratory tests that can confirm a diagnosis of SARS-CoV infection in its early stages. A real time RT-PCR with optimized RNA extraction methods could detect 40 of 50 (80%) nasopharyngeal aspirate samples from confirmed SARS patients at the first 3 days of disease onset (Poon et al., 2003). However, comparable improvements in serological methods are needed.

We used purified N protein as an antigen in an ELISA assay and studied its sensitivity and specificity for sero-diagnosis of SARS. The results indicate that the ELISA based on the SARS-CoV N protein has high sensitivity and high specificity. Eighty-one percent of the SARS patients had a positive result 10 days after disease onset while 100% had positive results at 20 and 30 days post-infection. When comparing this result with the commercially available diagnosis kit using a mixture of viral proteins, only 56.3% of the sera were positive 10 days after disease onset while 81.3% and 93.7% were positive after 20 and 30 days, respectively. No reactivity was observed in the control samples in either assay. The difference in the positive response between these two assays may be due to the quantity of coated N protein or to the expression and purification procedures that has been used for the recombinant viral proteins. Our work suggests that the high specificity and sensitivity of nucleocapsid protein ELISA indicates that it has potential to be an useful option for the sero-diagnosis of SARS, and will be particularly in laboratories that lack BSL 3 facilities. However, the cross reactivity of this assay in patients...
with recent infection with human coronaviruses 229E and OC43 require to be studied more closely. Therefore, until further experience is accumulated with this assay, it is suggested that the nucleocapsid protein ELISA can be useful as a screening test, with positive results being confirmed by immunofluorescence or virus neutralization tests.

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