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Expression and purification of recombinant SARS-CoV-2 nucleocapsid protein in inclusion bodies and its application in serological detection

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ABSTRACT

The current standard for the diagnosis of COVID-19 is the nucleic acid test of SARS-CoV-2 RNA, however, virus antibody detection has the advantages of convenient sample collection, high throughout, and low cost. When combining detection with nucleic acid detection, antibody detection can effectively compensate for nucleic acid detection. Virus infection always induce high antibody titer against SARS-CoV-2 nucleocapsid protein (N protein), which can be used to detect COVID-19 at both infected and convalescent patients. In this study we reported the expression and purification of N protein in *E.coli* from inclusion bodies by a combination of two cation exchange chromatography, and the yield of N protein was around 50 mg/L fermentation broth with more than 90% purity. A corresponding colloidal gold detection kit prepared with our purified N protein was used to verify the efficiency and accuracy our N protein in antibody detection method. Of the 58 COVID-19 PCR positive patients' inactivated serum samples, 40 samples were IgM positive (69.0%), and 42 samples were IgG positive (72.4%), and all 95 COVID-19 negative patients' inactivated serum samples were both IgM and IgG negative. Our results indicates that the refolded soluble N protein could be used for the preliminary detection of IgG and IgM antibodies against SARS-CoV- 2.

1. Introduction

The epidemic of COVID-19 is spreading worldwide. It is foreseeable that the epidemic will have a significant and far-reaching impact on human health management, social behavior and habits, and even international economic and trade relations. COVID-19 is a pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS CoV-2), which belongs to the β - coronavirus of coronaviridae, a spherical, enveloped positive-strand RNA virus. The genome mainly encodes four structural proteins: spike protein, envelope protein, membrane protein, nucleocapsid protein (N protein). N protein is composed of 413 amino acid residues and binds to the viral genome RNA to form a nucleocapsid. N protein has strong immunogenicity and can induce a high antibody response in the serum of convalescent patients [1–3].

Since the outbreak of the epidemic, the research on N protein has been expanding. Studies have revealed that N protein is a compact, interwoven dimer, similar to SARS coronavirus and other related coronaviruses [4]. Bioinformatics analysis combined with the existing experimental spiritual evidence showed that SARS-CoV-2 N protein could form or regulate biomolecular aggregates in vivo by interacting with RNA and essential host cell proteins. The N protein of SARS-CoV-2 may use this activity to control the virus life cycle and host cell response to virus infections [5]. Static light scattering, size exclusion chromatography, and small-angle X-ray scattering (SAXS) showed that the purified N protein was mainly dimer in solution. Fluorescence polarization analysis showed that the purified N protein had a nonspecific nucleic acid binding ability. Western blotting confirmed IgA, IgM and IgG antibodies against N protein in the blood serum of COVID-19 patients, which proved the importance of this antigen in host immunity and diagnosis [6].

The luciferase immunoprecipitation analysis system was used to detect nucleocapsid protein and spinin antibodies with 100 COVID-19 patients. Researchers tested and compared samples with and without thermal inactivation. The results showed that anti SARS-CoV-2 N

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protein antibody was more sensitive than spike protein antibodies at early infection [7]. Rapid developments of immunoassays have been made to detect SARS-CoV-2 antibodies, including the neutralizing antibody assay, enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA) and lateral flow immunoassay (LFA). These detection methods have the potential to improve the diagnosis and control of different types of infection. These methods are based on detecting IgG and IgM antibodies against N protein and S protein in serum [8–10]. ELISA based on recombinant N protein was used to detect antibodies (IgM and/or IgG), the detection rates of positive samples were 68.2% and 70.1%, respectively. The positive rate of IgM and IgG increased as days exceeded but decreased after 35 days [11]. The Lanthanide-Doped Nanoparticles-Based Lateral Flow Immunoassay using N protein shown that N protein could be used for rapid and sensitive anti SARS-CoV-2 IgG detection [12]. With the continuous improvement of methodology, the serological method is used as an auxiliary tool for RT-PCR detection protocol [13].

The vaccine formulations and diagnostic reagents needs a large number of high purity, high activity and low cost N protein, but some studies only expressed N protein fragment, or used methods with complex operation such as gel filtration chromatography and other [4,6,14]. In this study, we developed an efficient and ingenious method to express and purify N protein, the process is simple and suitable for mass production.

2. Materials and methods

2.1. Materials

Beijing BioMed Gene Technology Co., Ltd. provided plasmid vector PET28a, *E. coli* BL21 (DE3). BCA protein quantitative kits and plasmid small extraction kits were purchased from Sangon Bioengineering (Shanghai) Co., Ltd.. Peptone, yeast powder and agar powder were purchased from Oxid Ltd., and casein was purchased from Beijing Solarbio Biotechnology Co., Ltd. Our laboratory synthesized colloidal gold particle. Sino Biological Inc. kindly donated rabbit monoclonal antibody against SARS -CoV-2 N protein. Isopropyl-β-D-thiogalactopyranoside(IPTG) and G25 dextran gel were purchased from GE Healthcare, and Ni NTA agarose resin, SP strong cation agarose resin, and CM weak cation agarose resin were purchased from Tosoh Bioscience.

2.2. Construction of expression vector in E. coli

The codon of the SARS-CoV-2 N gene(Gene ID: 43740575) was optimized to make it suitable for *E. coli* expression system. The optimized gene was synthesized and constructed into expression vector pET28a(+), and then transformed into T1 *E.coli* bacteria for plasmid DNA verification. Briefly, the transformation was plated at LB plate containing 50 μg/ml kanamycin and incubated at 37 °C incubator for 12–16 h. Single colonies were selected and inoculated into 5 ml LB medium with kanamycin (50 μg/ml) for plasmids extraction. The plasmids were digested by XhoI and ApaI, and the fragments were visualized and identified by DNA agarose gel electrophoresis.

2.3. Expression of N protein in the E. coli

A single recombinant expression colony of transformation of recombinant plasmid with *E.coli* BL21 (DE3) was selected from the plate and inoculated into 3 ml LB medium supplemented with kanamycin (50 μg/ml) and cultured overnight at 37 °C and 200 rpm. The next day, the bacterial culture was inoculated into 1L LB medium containing kanamycin (50 μg/mL). The bacterial solution was cultured at 37 °C and 250 rpm until the OD600 value reaches 0.8–1.0 (about 5hrs.). IPTG was added to the final concentration of 1 mmol/L, and then bacteria was induced overnight at 37 °C. Auto-induction based on Studier medium was also used for expression of N protein [15]. After induction, the bacteria were pelleted by centrifugation at 4500*g* for 30min at 4 °C. The bacterial pellet was resuspended in 50 ml of Phosphate buffer solution (pH 9.0) with 1% Triton X100, and then was broken by sonication on ice (setting: power 600 W, ultrasonic time 3s, pause time 7s, total time 30 min). After high speed centrifugation, the target protein was identified in the inclusion body by SDS-PAGE electrophoresis with the whole lysate, supernatant, and pellet of bacteria. The inclusion body was extracted and purified by several time washing pellet after sonication, and the washed inclusion body was dissolved at 8 M urea solution.

2.4. Purification of SARS-CoV-2 N protein

2.4.1. Preliminary purification of recombinant N protein by SP cation exchange chromatography

Recombinant N protein was purified and refolded by SP cation exchange chromatography. Briefly, SP column were pre-equilibrated with 50 mM phosphate buffer solution (pH 9.0) containing 6 M urea, the inclusion body dissolved in 8 M urea was loaded at a flow rate of 5 ml/min. After sample loading, 6 M urea buffer washed till the 280 UV absorption line returned to the baseline, and then urea concentration was gradually reduced to 0 at the flow rate of 1 ml/min and 800min length. Finally, the recombinant N protein was eluted with 50 mM phosphate buffer containing 0.45 M NaCI.

2.4.2. Fine purification of N protein by CM cation exchange chromatography

Recombinant N protein primary refolded and purified by SP cation exchange chromatography still have host protein contamination, and further polish purification by CM cation exchange chromatography needed to remove contaminants. Briefly, CM cation exchange chromatography was pre-equilibrated with 50 mM phosphate buffer solution (pH 9.0), and then elutes from SP column 5 times dilution with 50 mM phosphate buffer (pH9.0) was loaded to column at a flow rate of 5 ml/min. After sample loading, column was washed with 3 times of column volume with 50 mM phosphate buffer (pH9.0) containing 0.25 M NaCI, and recombinant N protein was eluted by 50 mM phosphate buffer (pH9.0) containing 0.30 M NaCI.

2.4.3. SDS-PAGE and western blotting

The purified protein was desalted by G25 desalting column with 20 mM Tris and 50 mM glycine (pH8.0). After G25 column pre-equilibrated, buffer exchange was done with same buffer at 1 ml/min. SDS was added to the final concentration of 0.1%, then the recombinant protein stored at −20 °C for further purpose. The purified recombinant N protein was subjected to 12% SDS-PAGE electrophoresis, and protein was stained by Coomassie blue dye. Another samples were separated by SDS-PAGE and transferred to nitrocellulose membranes using a Bio-Rad Mini Trans-Blot Cell (Bio-Rad). The membranes were probed with the rabbit monoclonal antibody (1:2000) against SARS-CoV-2 N followed by goat anti-rabbit serum (1:10000) conjugated to horseradish peroxidase, and the target protein was visualized by TMB color development solution.

2.5. Preparation of colloidal gold immunoassay kit in-house

The colloidal gold conjugation was prepared in-house. Briefly, 10 mL colloidal gold solution (40 nm diameter, prepared in our laboratory) mixed with 65 mL of potassium carbonate (0.1 mol/L) in a clean beaker, then the purified N protein was added and incubated for 30 min at room temperature. In order to block unconjugated sites, 100 mL of 20% bovine serum albumin solution was added to the mixture and incubated at room temperature for an additional 30 min. The mixture was centrifuged at 12,000 r/min for 20 min and washed with 5 mL phosphate-buffered saline (PBS; 50 mmol/L, pH 7.4). The colloidal gold particles were resuspended in PBS buffer, and the final concentration of N protein is 15 μg/mL.

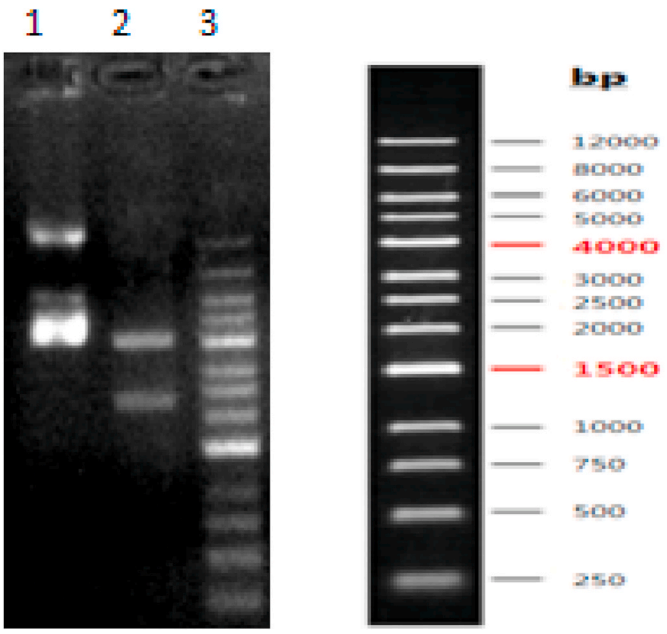

Fig. 1. Double restriction enzymes digestion of expression vectors. Lane 1, Undigested plasmid; Lane 2: Plasmid digested with *Xho* I + *Apa* I; Lane 3: DNA Marker.

The lateral flow rapid colloidal gold immunoassay antibody test kit was also developed in-house. Briefly, N protein labeled with colloidal gold was mixed with chicken IgY labeled with colloidal gold at the ratio of 5:1, and sprayed to membrane with the speed of 5 μl/cm with the gold spraying film plotter, and remembrance dried overnight at 37 °C. The monoclonal anti-human IgM (2 mg/ml), monoclonal anti-human IgG (2 mg/ml) and goat anti-chicken IgY (1 mg/ml) were sprayed on the nitrocellulose membrane with the speed of 1 μl/cm with the gold spraying film maker and marked as M line, G line and C line respectively, then remembrance dried at 45 °C overnight.

2.6. Test of the performance of the kit

In order to verify the sensitivity and specificity of the kit, we collected 58 inactivated serum samples from COVID-19 patients confirmed by nucleic acid detection and 95 inactivated serum samples from patients with nonrelated diseases for clinical study. All the samples were from the First Hospital of Yueyang City. All procedures involving human participants in this study were in accordance with the Helsinki Declaration and approved by the hospital ethics committee. The lateral flow rapid antibody test was carried out in the biosafety cabinet. After adding 10 μl inactivated serum sample into the kit's sample pad, and 100 μl PBS was quickly added to the kit's sample pad. 15 min later, the test results were determined qualitatively by the staining of IgM and/or IgG lines and control lines.

3. Results and discussion

3.1. Construction of vectors

Restriction enzymes *Apa* I and *Xho* I were used for enzyme digestion of the expression plasmid DNA. The products of digestion were identified by agarose gel electrophoresis, a 4200 bp vector band and a 2300 bp insert gene fragment could be obtained after enzyme digestion, which was consistent with the theoretical value of 4201bp and 2349bp. With additional of the DNA sequencing result (data not shown), this restriction digestion results showed that the recombinant plasmid was successfully correctly constructed (Fig. 1).

3.2. Expression of N protein in E. coli BL21 (DE3)

The recombinant SARS-CoV-2 N protein contained 447 amino acids and the expected molecular weight was 48.85 kDa. Different induction conditions (20 °C and 37 °C) and medium (IPTG induction and auto-induction) were tried to express soluble protein, however few soluble proteins were expressed, most of target protein was in the inclusion body. As the prediction, without induction N protein did not express at either supernatant or pellet (Fig. 2, lane 1,5). After IPTG induction, there was an obvious target protein band at 49 kDa (Fig. 2, lane 6,7). However N protein was expressed at the inclusion body either at 20 °C or 37 °C (Fig. 2, lane 2,3,6,7). Surprisingly, N protein was not induced with auto-induction medium either at supernatant or inclusion body (Fig. 2, lane 4,8). Recombinant N protein was expressed at BL21 (DE3) in the inclusion body with remarkably high expression level.

3.3. Purification of N protein

In this study, it was found that N protein could bind strongly with SP strong cation resin and eluted at a relatively high concentration of NaCl (0.45 M) (Fig. 3). However, the elution peak also contained a small amount of host protein and nucleic acid of *E.coli*, and it was difficult to

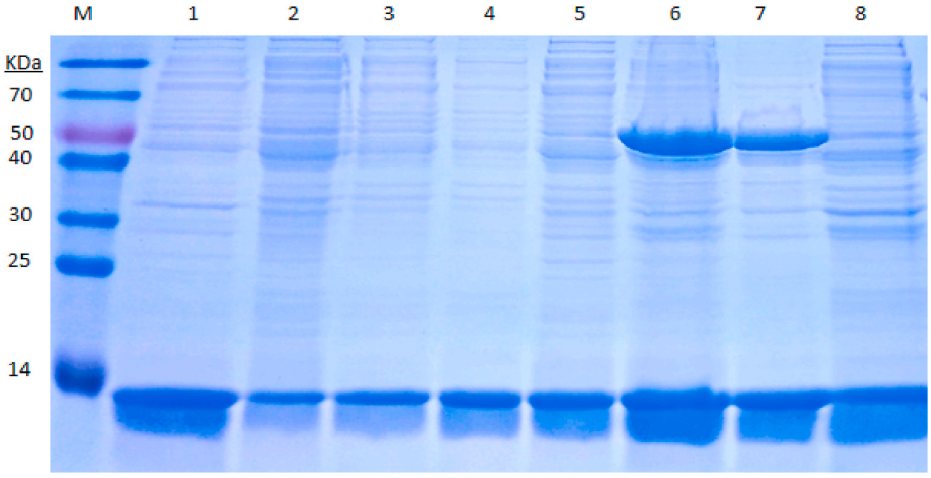

Fig. 2. SDS–PAGE analysis of N protein expression. Lane M: marker (100, 70, 50, 40, 30, 25 and 14 kDa); Lane 1, uninduced supernatant; lane 2, IPTG induced supernatant (37 °C); lane 3, IPTG induced supernatant (20 °C); lane 4, Auto-induction supernatant; lane 5, uninduced cell lysate; lane 6, The whole cell lysate by IPTG induction (37 °C); lane 7, The whole cell lysate by IPTG induction (20 °C); lane 8, the whole cell lysate by auto-induction.

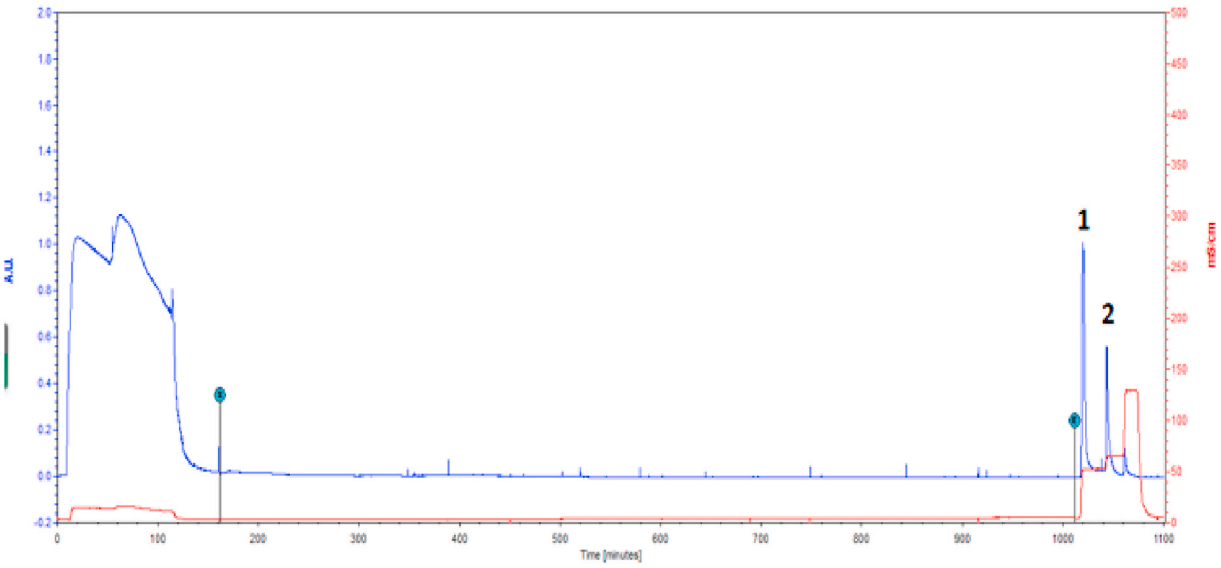

Fig. 3. SP strong exchange chromatography.

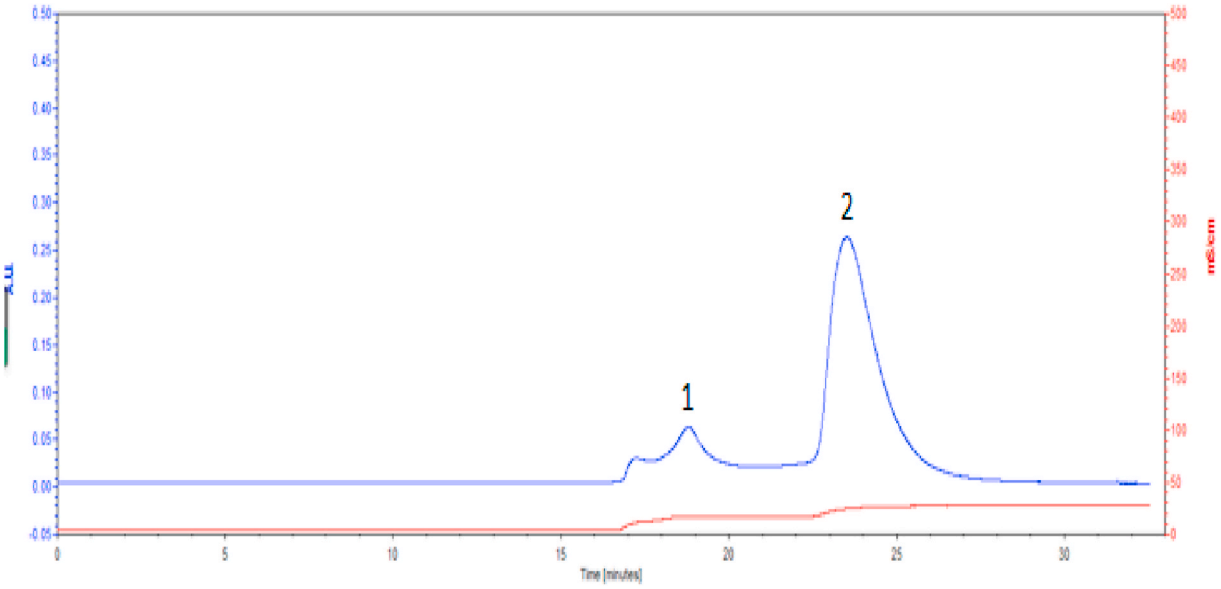

Fig. 4. CM cation exchange chromatography.

further improve the purity after trying different several conditions. We then used CM weak cation resin to adsorb SP cation elute after five times dilution with PB buffer, N protein could be eluted at a relatively low concentration of NaCl (0.35 M), and more importance is that the impurity protein could be efficiently separated from N protein (Fig. 4). With two ion-exchange chromatography steps, we could obtain 50 mg refolded soluble N protein per liter of fermentation broth. This purification method could acquire high purity N protein with high efficiency and simplicity. Compared with the commonly used affinity chromatography, such as Ni-NTA, CM weak cationic resin had higher binding selectivity for N protein, however, the target N protein could not be eluted by high imidazole concentration with Ni NTA resin, resulting lower N protein yield (data not shown).

SDS-PAGE electrophoresis showed that the molecular weight of the purified N protein was about 50 kDa, which was consistent with the expectation, and the purity of the protein was more than 90%. The recombinant N protein was identified by western-blotting using rabbit monoclonal antibody against SARS-CoV-2 N protein as the primary antibody, and there was a specific reaction band at 49 kDa (Fig. 5).

3.4. Kit performance test

Our in-house prepared colloidal gold immunoassay kit was tested the specificity and sensitivity with 58 COVID-19 and 95 unrelated patients' inactivated serum samples, which were verified with a nucleic acid detection kit (Fig. 6). Serum samples were obtained from COVID-19 patients' symptom started from 7 days to 40 days, and random labeled with number. Experiment conductor did not know patient information and disclosed only after all tests were completed. The results showed that the IgM and IgG antibodies of 95 samples with unrelated diseases were negative. Among 58 COVID-19 patients' samples, 40 samples showed IgM positive, and 42 samples showed IgG positive, and the specificity was 69.0% and 72.4%, respectively (Table 1). This result is consistent with the reported enzyme-linked immunosorbent assays using soluble N protein, and results indicates that our in-house prepared kit with refolded soluble N protein could be used to detect SARS-CoV-2

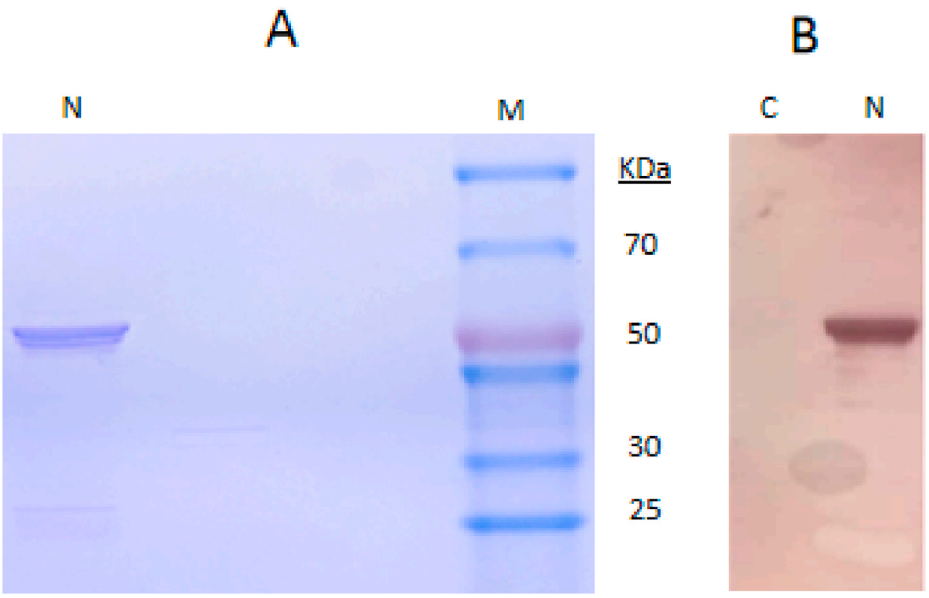

Fig. 5. Analysis of purified SARS-CoV-2 N protein. A: SDS–PAGE analysis of the purified N protein; B: Western blotting by anti- SARS-CoV-2 N protein rabbit monoclonal antibodies. BSA was used as negative control(lane C).

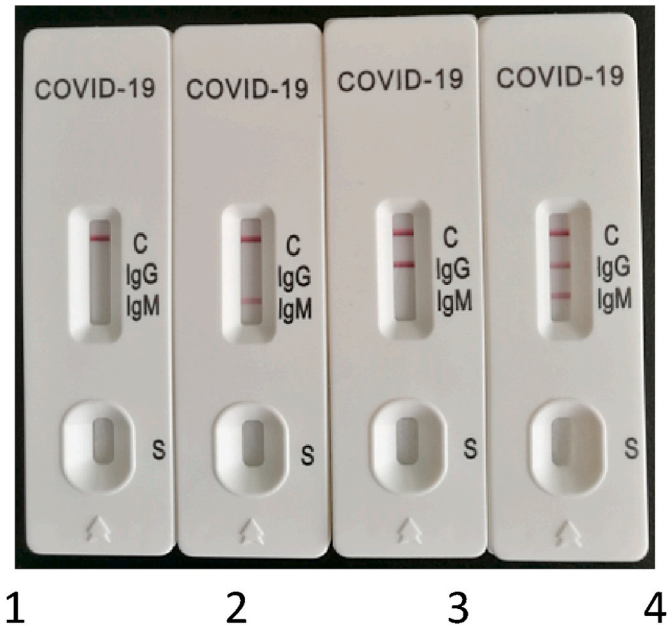

Fig. 6. Detection of serum samples using colloidal gold test kit.1, IgG and IgM negative; 2, IgM positive; 3, IgG positive; 4, Both IgG and IgM positive.

Table 1
Diagnostic result of IgM and IgG Antibodies for serum samples.

	IgG against SAR CoV-2		IgM against SAR CoV-2	
	Positive	Negative	Positive	Negative
COVID-19 patient	42	16	40	18
Unrelated disease patient	0	95	0	95

IgG and IgM antibodies [11].

There were 13 confirmed COVID-19 positive samples shown negative results with our IgG and IgM test kit. Antibody response against SARS-CoV-2 nucleocapsid protein after virus infections is later than virus PCR detection window. Negative antibody detection results with PCR positive patient may be due to these patients are in the early infection "window period". The antibody aggregation or degradation occurs after serum long-term storage may also cause false negative results with antibody detection method. Due to the limitation of the patient sample in China, the sample size used in this study is small, and more samples are needed for further verification.

4. Conclusion

Compared with the eukaryotic expression system, the prokaryotic expression system has the advantages of a higher efficiency rate and a lower cost, *E.coli* is a good choice to express N protein. We found that soluble N protein could be expressed under mild induction conditions, but the expression level was very low, this make a very big challenge for large scale production. Inclusion body expression harvested a large amount of N protein and was very conducive to further purification and application. Like most of nucleocapsid protein, SAR CoV-2 N protein has a high pI value (10.07), could bind with RNA and some host cell proteins which will copurify with N protein in the final products. These contaminations may eventually lead to false positive result when it was used for the immunoassay detection. The conventional affinity chromatography for N protein purification by nickel resin did not achieve the desired effect in our lab, so we designed a purification process based on the subtle differences between strong ion exchange chromatography and weak ion exchange chromatography. This combination purification procedures could get highly purified N protein and could get rid of most nucleic acid and host protein contaminations, and the process is simple, convenient and low cost, which is very conducive to the large-scale production and application of N protein. The colloidal gold immunoassay kit prepared with refolded soluble N protein could meet the needs of rapid and preliminary detection. This method may be helpful in the purification of other inclusion body proteins with a suitable chromatographic matrix that is to be carefully selected for the specific proteins.

Declaration of competing interest

The author(s) declare no competing interests.

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