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# Diagnosis of Severe Acute Respiratory Syndrome (SARS) by Detection of SARS Coronavirus Nucleocapsid Antibodies in an Antigen-Capturing Enzyme-Linked Immunosorbent Assay

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**Recombinant severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein was employed to establish an antigen-capturing enzyme-linked immunosorbent assay (ELISA). Antinucleocapsid protein antibodies could be detected in 68.4% of probable SARS patients 6 to 10 days after illness and in 89.6% of the patients 11 to 61 days after illness. No false-positive results were observed in 20 non-SARS fever patients, 24 non-SARS respiratory illness patients, and 20 health care workers. Among 940 other non-SARS clinical serum samples, only 1 was found to be weakly positive. This method provides a new, sensitive, and specific approach for SARS diagnosis.**

An accurate, rapid, and cost-effective laboratory etiologic method is of great importance for the diagnosis of severe acute respiratory syndrome (SARS). The isolation of the virus (4) led to the development of some specific diagnostic techniques, including indirect fluorescent-antibody detection, indirect enzyme-linked immunosorbent assay (ELISA) using virus lysates as antigen, and reverse transcription PCR for the detection of the SARS coronavirus (SARS CoV) genome (5). However, as observed in the clinical practices of China and shown in this paper, indirect ELISA gave about 2% false-positive results among healthy people; SARS-CoV infection could be confirmed only if seroconversion from negative to positive status was observed.

Antigen-capturing ELISA is a superior method to indirect immunoassay because of its high specificity and sensitivity. The basis of the assay is that antibodies are at least bivalent, i.e., one valence is used in attaching the antibody to the immobilized antigen, leaving the other(s) free to bind to the labeled antigen. Both capture and detection of the target antibody depend on its specificity toward the antigen, so if the antigen is correctly chosen and purified, the assay can be made very specific. And principally, all types of antibodies (immunoglobulin G [IgG], IgM, IgA, etc.) could be detected (1). It has been demonstrated previously that, at least in early responses, the antibodies to the nucleocapsid protein (N protein) predominate as assayed by Western blotting (3). Therefore, the N protein was chosen to be produced as a recombinant protein for establishing an antigen-capturing ELISA for SARS diagnosis.

The SARS CoV N gene was obtained by reverse transcription PCR amplification from blood samples of a SARS patient in Beijing by using the following primer pair: 5′-CGCATATG TCTGATAATGGACCCCA-3′ and 5′-CGGATCCTTATGC CTGAGTTGAATCAGCA-3′. The DNA fragment was then cloned into a T7 promoter-based prokaryotic expression vector, pET22b (Novagen). The resulting recombinant plasmid (pMG-N) was subjected to DNA sequencing and showed 100% identity with the N gene reported in the SARS CoV Toronto strain (GenBank accession number NC_004718). pMG-N was then transformed into *Escherichia coli* BL21a (DE3) and induced with 0.5 mmol of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, Mo.) per liter for overexpression. The recombinant N protein was purified by S-Sepharose fast-flow ion-exchange chromatography followed by gel filtration with Superdex 200 (Amersham Pharmacia, Uppsala, Sweden) to a purity of more than 97% as determined by laser densitometry of silver-stained sodium dodecyl sulfate-polyacrylamide gel.

The purified N protein was diluted to a concentration of 1 μg/ml with 50 mM carbonate buffer (pH 9.6) and used to coat the wells of 96-well microplates at 4°C overnight, followed by blocking with 5% fatal bovine serum for 4 h at room temperature. In addition, N protein was conjugated to horseradish peroxidase (Sigma). An antigen-capturing ELISA was established for the detection of antinucleocapsid antibody present in sera. One hundred microliters of serum was added to the well coated with recombinant N protein; the plate was incubated at 37°C for 30 min and then washed five times with phosphate-buffered saline containing 0.05% Tween 20. One hundred microliters of labeled antigen was added, and the plate was incubated for another 30 min followed by washing as just described. Then, 100 μl of TMB substrate solution (0.1 mg of tetramethylbenzidine hydrochloride–0.01% $H_2O_2$ per ml in 0.1 M acetate buffer [pH 5.8]) was added and incubated at 37°C

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for 20 min, the reaction was terminated by adding 50 μl of 2 N sulfuric acid, and the absorbance at 450 nm ($A_{450}$) was determined.

To investigate how sensitive and selective the assay is, a panel set up by the National Institute for the Control of Pharmaceutical and Biological Products was employed; it included 18 positive sera, 20 negative sera, and 1 serially diluted serum as a sensitivity control (which showed positive at a maximum dilution of 1:4 as assayed by immunofluorescent and indirect ELISA that used virus lysates as antigen). Of the 18 positive sera, 2 showed an $A_{450}$ that ranged from 0.397 to 0.500, 4 had $A_{450}$s that ranged from 0.500 to 1.000, 2 had $A_{450}$s that ranged from 1.0 to 2.0, and the remaining 10 serum samples had $A_{450}$s that were greater than 2.0. All 20 negative samples showed an absorbance lower than 0.075 (0.038 ± 0.021 [mean ± standard deviation]), so we set 0.15 (2 × 0.075) as the cutoff value. Moreover, when the sensitivity control serum was diluted 1:64, it gave an $A_{450}$ of 0.211. All the results above demonstrated that the new method was a very sensitive and specific assay for SARS virus antibodies.

In our primary trials with clinical samples, the antigen-capturing ELISA showed promising sensitivity and specificity too. Of 940 serum samples from clinically confirmed non-SARS patients in the Affiliate Hospital of the Medical School of Zhengzhou University, only 1 (0.106%) showed a weak reaction ($A_{450}$, 0.23 compared with the cutoff value of 0.15); the patient did not have any symptoms related to SARS and should thus be considered as false positive. No positive results were observed in 24 non-SARS respiratory illness patients (excluded from SARS by World Health Organization-recommended diagnostic criteria) from the General Hospital of Guangzhou Command of the People's Liberation Army (PLA). Similar background values were obtained in 20 non-SARS fever patients and 20 health care workers from the 309 Army Hospital of the PLA. Moreover, the antigen-capturing ELISA showed a very low absorbance in the above-mentioned negative serum samples (0.023 ± 0.014 [mean ± standard deviation]).

Serum samples from 200 probable SARS patients from the General Hospital of Guangzhou Command of the PLA and the 309 Army Hospital of the PLA were subjected to the N protein antigen-capturing ELISA. As shown in Table 1, 4 out of 27 (14.8%) patients developed antinucleocapsid antibody within 5 days after illness. In the 38 patients who had been ill for 6 to 10 days, 26 (68.4%) were shown to be positive ($A_{450} \geq 0.15$), Further, 121 out of 135 (89.6%) samples from the patients who had been ill for more than 10 days scored positive for anti-N protein antibodies.

As a control for the effectiveness of the N protein antigen-capturing ELISA, all the samples were also analyzed by an indirect ELISA, the most widely used method in Chinese hospitals, which uses SARS CoV lysates as antigen. This kit showed a substantially higher level of false-positive results in non-SARS patients and healthy people (7%) than did the antigen-capturing ELISA, and it demonstrated lower sensitivity in serum samples from patients 5 to 10 days after illness (44 versus 68.4% [$P < 0.01$ as confirmed by $\chi^2$ test]).

TABLE 1. Development of antinucleocapsid antibody in patient types

| Patient type (days after illness) | No. of samples | No. of positive samples | % Positive |
|---|---|---|---|
| Probable SARS (0–5) | 27 | 4 | 14.8 |
| Probable SARS (6–10) | 38 | 26 | 68.4 |
| Probable SARS (10–61) | 135 | 121 | 89.6 |
| Non-SARS respiratory illness | 24 | 0 | 0 |
| Non-SARS fever | 20 | 0 | 0 |
| Health care workers | 20 | 0 | 0 |
| Other non-SARS | 940 | 1 | 0.106 |

Due to the relatively low percentage of false-positive determinations in non-SARS samples, and considering that overdiagnosis does exist in present SARS clinical diagnostic criteria (2), the N protein antigen-capturing ELISA might potentially be used in the confirmation of SARS infection. Because the assay employs recombinant N protein rather than virus lysates, it provides a safer, cost-effective, and more sensitive approach for SARS diagnosis.

Y.S., Y.Y., and P.L. contributed equally to this work.

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