

Detection of the nucleocapsid protein of severe acute respiratory syndrome coronavirus in serum: Comparison with results of other viral markers

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Abstract

A capture enzyme-enhanced chemiluminescence immunoassay (ECLIA) based on three specific monoclonal antibodies to detect the nucleocapsid (N) protein of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in the serial serum samples from SARS patients was developed. The anti-SARS-CoV IgG and the viral RNA were also detected in the sera by ELISA and RT-PCR, respectively. During the first 10 days after onset, anti-SARS-CoV IgG, SARS-CoV RNA and the N protein were detected in 21.4, 42.9, and 90% of the patients' sera, respectively. The detection rate of the N protein during days 11–15 of the disease was still significantly higher than those of anti-SARS-CoV IgG and SARS-CoV RNA. The data demonstrated that detection of the N protein with the capture ECLIA appears to be more useful than detection of other viral markers for rapid diagnosis of SARS in patients.

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1. Introduction

Severe acute respiratory syndrome (SARS), an infectious disease which occurred firstly in the Guangdong province, southern part of China, in November 2002, caused outbreaks in 29 countries and areas in spring 2003 with 8098 clinical cases and 774 deaths, resulting in an overall mortality rate of 9.6% (WHO, 2003b). After the outbreaks were contained in mid-July 2003, three events of laboratory-associated infections caused a total of 11 clinical SARS cases (Normile and Vogel, 2003; Normile, 2004a, 2004b). Again in Guangdong, four clinical SARS cases occurred sporadically without contact histories from December 16, 2003 to January 8, 2004 (Liang et al., 2004). On the other hand, the origin of the etiological agent of SARS, SARS-CoV (Peiris et al., 2003), remains elusive although SARS-CoV-like virus was isolated from civet cats (Guan et al., 2003). The fact that 11 initial clinical SARS cases occurred independently without any contact history in different areas of Guangdong and the find-

ings that the viral sequences isolated from these patients were grouped in different subtype clusters (Chinese SARS Molecular Epidemiology Consortium, 2004) suggest that the patients in China were infected by SARS-CoV from multiple infectious sources, rather than from a single source. Therefore, SARS may be recurrent in human beings.

SARS-CoV is an enveloped, single-stranded positive-sense RNA virus classified in a new group of the *Coronaviridae* family. The viral genome is approximately 30 kb in length with 11 open reading frames. SARS-CoV contains four major structural proteins, including spike (S), membrane (M), small envelope (E), and nucleocapsid (N) (Marra et al., 2003; Rota et al., 2003). The S protein mediates attachment of the virus to cellular receptors (Li et al., 2003b) and virus entry by fusion with cell membranes (Hofmann et al., 2004; Yang et al., 2004). Both M and E proteins are integral membrane proteins and form the minimum protein units for virus assembly. The N protein is an extensively phosphorylated, highly basic protein, which interacts with viral RNA and makes up the viral core and nucleocapsid (Lai, 2003).

SARS is indistinguishable clinically at the early stage from pneumonia caused by other infectious agents. Currently,

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the diagnosis of SARS depends basically upon detecting SARS-CoV RNA by RT-PCR and/or testing specific antibodies directed against SARS-CoV by assays based on cultured virus or recombinant viral antigens. Although RT-PCR is a reliable technique for the diagnosis of SARS in the early phase, the detection rate for probable SARS was only 37.5–50% (Tang et al., 2004; Wu et al., 2004). Antibodies directed against SARS-CoV, examined by immunofluorescent assay, immunochromatographic test, enzyme-linked immunosorbent assays (ELISA), or Western blot, can detect over 90% of the cases (Wu et al., 2004; Guan et al., 2004; He et al., 2004a; Li et al., 2003a), however, it takes 2–3 weeks after the onset for the measurable specific antibodies to develop. Therefore, detecting anti-SARS-CoV IgG is not a practical strategy for rapid diagnosis.

Since SARS-CoV is highly contagious and the disease is severe with a high mortality rate, rapid diagnosis of SARS has significant importance both for treating patients and for preventing the spread of the disease. Recently, assays based on monoclonal antibodies (Che et al., 2004a, 2004b) or polyclonal antibodies (Lau et al., 2004) directed against the N protein were developed to detect the N protein of SARS-CoV in serum samples and in other clinical specimens. In the present study, a capture ECLIA was developed based on three monoclonal antibodies directed against the N protein of SARS-CoV, and the N protein in the longitudinal serum samples from the SARS patients were detected with this method. Anti-SARS-CoV IgG and SARS-CoV RNA were also detected in the serial sera from probable SARS patients. The usefulness of the N protein, SARS-CoV RNA, and anti-SARS-CoV IgG in identifying the infection at the early phase was evaluated.

2. Materials and methods

2.1. Serum samples

Three hundred and thirty-six serial serum samples, collected from 117 probable SARS patients in Xiongke Hospital of Beijing, were used in this study. Of the 117 probable patients, 73 were female, and 44 were male. The patients were aged between 19–61 years. All patients had clinical features of probable cases with SARS defined by WHO (WHO, 2003a). In addition, 410 serum samples from the close contacts that did not develop any symptom and 813 samples from healthy blood donors were also used in this study. The study to review the clinical, radiological and laboratory features of the patients was approved by the Ethics Committee of the Beijing Xiongke Hospital.

2.2. ELISA for anti-SARS-CoV IgG

Anti-SARS-CoV IgG was detected using a commercial indirect ELISA kit (BDI-GBI Biotech, Beijing), which was developed based on SARS-CoV lysates as coating

antigens. Briefly, 100 μ l/well of each serum diluted 10-fold in 0.05% Tween 20 in PBS (PBS-T) was added to the viral protein-coated plates and incubated at 37 °C for 30 min. Following five washes with PBS-T, 100 μ l/well of peroxidase-conjugated mouse anti-human IgG (Fc-gamma specific) antibody diluted 2000-fold in PBS-T was added. Following incubation at 37 °C for 30 min, the plates were washed as above and 100 μ l/well of the substrate tetramethylbenzidine solution was added. After incubation at room temperature for 15 min, the color development was stopped by adding 2 M H₂SO₄. Optical density (OD) at 450 nm was measured with an ELISA reader. Blank, negative, and positive controls were always included in each plate. The cut-off value was 0.18, which was calculated as the mean plus 2S.D. of normal serum controls.

2.3. RT-PCR for SARS-CoV detection

Total RNA from 140 μ l of each serum from the SARS patients was extracted using the QIAamp Virus RNA Mini Kit (Qiagen). The extracted RNA was subjected to a single step RT-PCR, followed by a second round of PCR to amplify the target viral sequences essentially the same way as described elsewhere (Drosten et al., 2003; Yam et al., 2003). The resultant products were electrophoresed through a 2% agarose gel in Tris–borate buffer. Target bands were visualized by staining with ethidium bromide. Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed.

2.4. Capture ECLIA for detecting the N antigen

A capture ECLIA was developed in collaboration with Beijing Weixiao Biotech to detect the N antigen of SARS-CoV in the sera from the SARS patients based on the assay developed by Che et al. (2004a) with modifications. Microtiter plates were coated with 100 μ l/well of a mixture of three anti-N protein monoclonal antibodies, N10E4, N1E8, and N8E1 (kindly provided by Dr. Che, Zhujiang Hospital, South Medical University, China) (Che et al., 2003) each at a concentration of 10 μ g/ml in carbonate–bicarbonate buffer (0.05 M, pH 9.6) and incubated at 4 °C overnight. The plates were washed five times with PBS-T and then blocked with 150 μ l of 1% casein in PBS-T for 1 h at 37 °C. Following five washes with PBS-T, 100 μ l of each serum diluted two-fold in PBS-T was added to each well and then incubated at 37 °C for 1 h. After five washes, 100 μ l/well of peroxidase-conjugated rabbit anti-SARS-CoV polyclonal antibodies (Beijing Weixiao Biotech) diluted 400-fold in PBS-T was added and again incubated at 37 °C for 1 h. After five washes, 100 μ l/well of chemiluminescence substrate solution was added and incubated for 10 min at room temperature. Finally, the relative luminescence value (RLV) was read by the microplates single photon counter (Beijing Weixiao Biotech). The cut-off value was 2.1 times of the mean RLV of negative controls.

Table 1
Kinetic anti-SARS-CoV IgG response in probable SARS patients

Days after onset	Serum number	Positive number (%)
1–10	14	3 (21.4)
11–15	36	15 (41.7)
16–20	51	41 (80.4)
21–25	53	50 (94.3)
26–62	182	182 (100)

3. Results

3.1. Anti-SARS-CoV IgG in probable SARS patients

All 336 serum samples from 117 probable SARS patients were tested for anti-SARS-CoV IgG by an indirect ELISA which was developed based on cultured SARS-CoV lysates as coating antigens. As shown in Table 1, during the first 10 days of the disease, anti-SARS-CoV was detected only in three of 14 patients. Thereafter, more patients seroconverted and the positive rate of anti-SARS-CoV IgG increased from 41.7% during 11–15 days after onset to 94.3% during 21–25 days. All 117 patients had detectable specific antibodies at 26 days after onset. To observe at least how long the antibodies lasted, anti-SARS-CoV in the subsequent sera collected afterwards from 65 patients was also measured. All these 65 sera were still positive for anti-SARS-CoV, indicating that anti-SARS-CoV IgG lasted at least 62 days after the onset. In addition, the result also showed that the concentration of anti-SARS-CoV IgG increased during the convalescent phase since the OD values of the convalescent sera were higher than those of the acute samples (data not shown). On the other hand, all 410 serum samples from the subjects who had close contacts with probable SARS patients but did not develop any symptom were negative for anti-SARS-CoV IgG. Similarly, none of the 813 serum samples from healthy blood donors collected during the period of SARS outbreak in 2003 was positive for these antibodies.

3.2. SARS-CoV RNA in sera of SARS patients

A total of 205 serum samples collected during different phases of the disease from 63 SARS patients were tested for viral RNA of SARS-CoV by RT-PCR. Table 2 shows the positive rates of the viral RNA in the circulation at various stages of the disease. During the first 10 days, six (42.9%) of the 14 patients were positive for SARS-CoV RNA. In the subsequent phases, the positive rates of viral RNA in the

Table 2
SARS-CoV RNA in sera of SARS patients at different phases

Days after onset	Serum number	Positive number (%)
1–10	14	6 (42.9)
11–15	36	6 (16.7)
16–20	46	4 (8.7)
21–25	51	2 (3.9)
26–62	58	0

Table 3
The N protein of SARS-CoV in sera of SARS patients

Days after onset	Serum number	Positive number (%)	
		N protein	SARS-CoV RNA
1–10	10	9 (90.0)	4 (40.0)
11–15	12	9 (75.0)	3 (25.0)
16–20	14	8 (57.1)	0
21–25	15	1 (6.7)	0
26–62	39	0	0

serum samples decreased from 16.7% during days 11–15 to 3.9% during days 21–25. At 25 days after onset, the SARS-CoV RNA could not be detected in all 63 SARS patients.

3.3. N antigen in sera of SARS patients

The N protein of SARS-CoV was tested in a total of 90 sera from 34 SARS patients with an antigen-capture ECLIA based on monoclonal antibodies directed against the N protein of SARS-CoV. As shown in Table 3, the N protein was detected in nine of the ten patients in the first 10 days after onset. The positive rate remained to be 75% during days 11–15. On the other hand, during the first 10 days, SARS-CoV RNA was detected only in four of the ten patients, and the positive rate was only 25% during days 11–15 (Table 3). The N antigen still remained to be positive in eight of the 14 and one of the 15 samples during days 16–20 and days 21–25 of the disease, respectively. However, SARS-CoV RNA became negative during these periods. The N protein was not detected in 39 serum samples collected during days 26–62 after onset while anti-SARS-CoV IgG was positive in all these serum samples. The N protein was not detected in the sera from 200 normal blood donors (data not shown).

4. Discussion

In this study, the anti-SARS-CoV IgG, SARS-CoV RNA, and the N protein were examined in consecutive serum samples from the probable SARS patients. The detection of the N protein of SARS-CoV in serum samples by ECLIA appears to be superior to the detection of the viral RNA by RT-PCR in rapid diagnosis of SARS patients.

All 117 probable cases in this study were confirmed retrospectively to be infected with SARS-CoV by detecting anti-SARS-CoV IgG in their convalescent serum samples. The ELISA for anti-SARS-CoV IgG used in this study was shown to be highly specific (Li et al., 2003a; Chen et al., 2004). The findings that none of the 813 sera from healthy blood donors was positive for anti-SARS-CoV also indicated that the antibodies detected in the probable cases were directed specifically against SARS-CoV. In addition, the anti-SARS-CoV IgG was not detected in any serum from the 410 people with close contact histories to SARS patients, suggesting that occult infection of SARS-CoV occurs rarely.

Usually, human beings develop specific IgM antibody earlier than IgG after being infected with microorganisms. However, based on an ELISA which was also used in this study, anti-SARS-CoV IgG and IgM antibodies almost simultaneously appeared in the patients' sera (Li et al., 2003a), therefore, only anti-SARS-CoV IgG antibody was detected, and anti-SARS-CoV IgM was not measured in the patients' sera in this study.

Since the genome of SARS-CoV was sequenced completely (Marra et al., 2003; Rota et al., 2003), RT-PCR has been used widely for the detection of the viral genome in clinical specimens as the most sensitive method for rapid diagnosis of SARS. However, the positive rate in the early phase of the disease was generally about 50% or less. In the present study, SARS-CoV RNA was only detected in 42.9% of the patients during the first 10 days after the onset (Table 2). This is in agreement with the results reported by others (Tang et al., 2004; Wu et al., 2004). Apparently, about half of SARS patients cannot be diagnosed at the early phase based on detection of the viral genome.

The N protein of coronavirus forms the viral core and nucleocapsid and probably is the most abundant component during replication since all transcripts of the virus may carry the nucleotide from the N gene. Based on a specific monoclonal antibody, the N protein of coronavirus infectious bronchitis virus was detected in tracheal smears and sliced tracheas from chickens infected experimentally (Yagyu and Ohta, 1990). It is likely that the N protein of SARS-CoV is also abundant during replication since the N protein is detected easily in the SARS-CoV cell culture lysates (Lau et al., 2004). This feature makes it possible to detect the N protein of SARS-CoV as a rapid diagnostic strategy. Recently, it is reported that most of SARS patients had detectable N protein in their sera at the early stage of the disease (Che et al., 2004a, 2004b). The other clinical samples including nasopharyngeal aspirate, urine, and stool samples from SARS patients also had detectable N protein (Lau et al., 2004).

In the present study, the N protein was tested in 90 consecutive sera from 34 SARS patient with an assay based on specific monoclonal antibodies. In the meantime, the SARS-CoV RNA was also amplified in the same samples. Nine sera from patients collected during days 1–10 after onset were positive for the N protein but only four of the patients were positive for the viral RNA. Of those sera collected during days 11–15, 75% were positive for the N protein, however, the positive rate of SARS-CoV RNA was only 25% (Table 3). Fig. 1 shows that the detection rates of the N protein, the viral RNA, and the anti-SARS-CoV IgG at the different times after the onset. The data demonstrated that detection of the N protein appeared to be more sensitive than detection of the viral RNA and the specific antibodies during the early phase, although the sample number in this study was relatively small.

Generally, the N protein and viral RNA circulate together in the peripheral blood as SARS-CoV particles, thus both components should have been detected at a comparable rate; however, the detection rate of the N protein was higher than

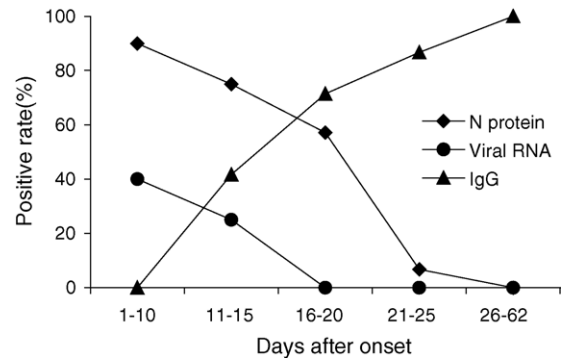


Fig. 1. Detection rates of SARS-CoV N protein, viral RNA and anti-SARS-CoV IgG in sera of SARS patients during the different stages of disease.

that of the viral RNA. It is likely that the current RT-PCR is not sensitive enough to detect the low concentration of SARS-CoV RNA. Indeed, only half of the SARS patients are positive for the viral RNA (Tang et al., 2004; Wu et al., 2004), which was also observed in the present study. Instability of viral RNA, inefficient extraction of RNA, unoptimized primers, and potential existence of inhibitory substances may contribute to the less sensitivity of the RT-PCR. Indeed, the sensitivity of RT-PCR may be improved by optimizing the reaction conditions (Poon et al., 2003; Yam et al., 2005).

Since the N protein of SARS-CoV shares low homology with nucleocapsid proteins of other members of the coronavirus family (Marra et al., 2003; Rota et al., 2003), the monoclonal antibodies directed against the N protein of SARS-CoV should not capture the N proteins of other coronaviruses. It is indeed the case that the antibodies directed against the N protein of SARS-CoV did not recognize the nucleocapsid proteins of two human coronaviruses OC43 and 229E (Che et al., 2004b). In this study, all 200 healthy blood donors were negative for the N protein of SARS-CoV, indicating that the assay was highly specific. Therefore, the N protein, which was detected in the sera of the SARS patients, should be derived from SARS-CoV.

Notably, of fourteen sera collected during days 16–20 after the onset, eight were positive for the N protein but none was positive for viral RNA (Table 3), indicating that the N protein of SARS-CoV tends to persist in the circulation longer than the viral RNA. In chickens infected experimentally with coronavirus infectious bronchitis virus, the N protein also lasts longer than the virus in tracheal smears and sliced tracheas (Yagyu and Ohta, 1990). This is likely because the N protein is not as degraded easily as viral RNA in the circulation since the N protein, when expressed in mammalian cells, can self-interact to form dimers or oligomers (He et al., 2004b), which may stabilize its helical structure. Therefore, the ECLIA for the N protein can detect the viral marker in SARS patients in whom RT-PCR shows negative.

Recently, a competition ELISA based on two monoclonal antibodies against SARS-CoV developed by Berry et al. (2004) appears to be more sensitive than the current indirect ELISA in detecting anti-SARS-CoV. That method may

be an alternative to rapidly diagnose SARS-CoV infection. The comparison of the ECLIA used in this study and Berry et al.'s ELISA in identifying SARS-CoV infection remains to be further studied.

In conclusion, although the number of the patients included in the present study was limited, the data demonstrated that the ECLIA for the N protein of SARS-CoV is more sensitive than RT-PCR for the viral RNA in identifying SARS-CoV infection at early stage of the disease. In addition, ECLIA has the advantages of avoiding special expertise, time-consuming procedures, and cross-contamination over RT-PCR and can be used easily in clinical laboratories. Therefore, the N antigen-capture ECLIA is a promising method for early diagnosis of SARS-CoV infection.

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