Patients and methods

SARS patients: Forty four patients (admitted to 302 Hospital, Beijing, P.R.China from March to June, 2003) with acute SARS symptoms (high fever, cough, lung x-ray positive) were clinically diagnosed according to the SARS criteria established by the Health Ministry of China and WHO. All the patients were positive in either CoV or anti-CoV IgG test. Among 44 SARS patients tested, 28 were male and 16 female, aged from 10 to 74 with a mean age of 28. Among them, 17 patients were in 1 to 7 days after fever onset, 18 patients in 13 to 36 days after fever onset and 9 patients in 79 to 91 days after fever onset. Another 5 patients were discharged from the hospital and lived among the healthy population for 2 months. The patients were aware of and willing to participate in this study.

Collection of samples: Blood of patients was collected in the presence of EDTA followed by centrifugation (2,000g for 5 min) to separate the plasma. The lymphocytes were isolated by a standard procedure using Ficoll.

RNA extraction and reverse transcription: Two hundred µl of plasma or one million lymphocytes were mixed with 1 ml of Trizol (Gibco BRL, Grand Island, NY), followed by 200 µl of chloroform. The mixture was centrifuged at 15,000 g for 15 min at 4°C. The supernatant was then mixed with 0.6 ml of isopropanol and centrifuged (15,000 g, 15 min, 4°C). The pellet was washed twice with 75% ethanol, dried and then directly re-suspended in 20 µl of RT reaction mixture containing 200 U of Superscript reverse transcriptase (Invitrogen, Carlsbad, CA), 0.5 mM dNTP, 10 mM dithiothreitol and 0.5 µg of CoV specific primer that is 5 nucleotides longer than the PCR primer as indicated

below. The RT reaction was carried out at 42°C for 1 hour and stopped at 75 ^{o}C for 15 min.

Real-time quantitative PCR: A real-time quantitative PCR was performed with 10 µl of RT sample (half of the reaction product) and 10 µl of reaction mixture containing 0.5 µg of primers: sense 5'- TTATC ACCCG CGAAG AAGCT-3'; antisense 5'- CTGTA GAAAA TCCTA GCTGG AG-3' (fragment length 123 bp) and TaqMan probe: FAM TCGTGCGTGGAT TGGCTTTGATGT-TAMRA with 1 U of Platinum Taq DNA polymerase. Following the treatment at 95°C for 2 min to further inactivate reverse transcriptase and denature the cDNA, two rounds of PCR were performed. The first round consisted of 5 cycles at 94°C for 10 sec, 50°C for 15 sec, and 72°C for 12 sec. The second round consisted of a total of 45 cycles at 92°C for 10 sec and 58°C for 42 sec. Data acquisition and analysis were performed with the *LightCycler*® Instrument (Roche Diagnostics). The reference standards contain 10^5, 10^4, 10^3or 10^2 RNA copies of CoV with a correlation coefficient greater than 0.95. Viral copies in each sample were converted from cycle time (Ct = X) using a function of the form Y= log10 (-0.3417*X+11.688).

Data analysis:

The mean and standard deviation were calculated from the raw data, and then the data were analyzed by student *t* test. The p value < 0.05 was regarded as statistically significant.