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Relative rates of non-pneumonic SARS coronavirus infection and SARS coronavirus pneumonia

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Summary

Background Although the genome of severe acute respiratory syndrome coronavirus (SARS-CoV) has been sequenced and a possible animal reservoir identified, seroprevalence studies and mass screening for detection of subclinical and non-pneumonic infections are still lacking.

Methods We cloned and purified the nucleocapsid protein and spike polypeptide of SARS-CoV and examined their immunogenicity with serum from patients with SARS-CoV pneumonia. An ELISA based on recombinant nucleocapsid protein for IgG detection was tested with serum from 149 healthy blood donors who donated 3 years previously and with serum positive for antibodies against SARS-CoV (by indirect immunofluorescence assay) from 106 patients with SARS-CoV pneumonia. The seroprevalence of SARS-CoV was studied with the ELISA in healthy blood donors who donated during the SARS outbreak in Hong Kong, non-pneumonic hospital inpatients, and symptom-free health-care workers. All positive samples were confirmed by two separate western-blot assays (with recombinant nucleocapsid protein and recombinant spike polypeptide).

Findings Western-blot analysis showed that the nucleocapsid protein and spike polypeptide of SARS-CoV are highly immunogenic. The specificity of the IgG antibody test (ELISA with positive samples confirmed by the two western-blot assays) was 100%, and the sensitivity was 94.3%. Three of 400 healthy blood donors who donated during the SARS outbreak and one of 131 non-pneumonic paediatric inpatients were positive for IgG antibodies, confirmed by the two western-blot assays (total, 0.48% of our study population).

Interpretation Our findings support the existence of subclinical or non-pneumonic SARS-CoV infections. Such infections are more common than SARS-CoV pneumonia in our locality.

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Introduction

Severe acute respiratory syndrome (SARS) has now affected 30 countries in five continents, with more than 8400 cases and more than 910 deaths. A novel virus, the SARS coronavirus (SARS-CoV), is known to be the aetiological agent.^{1–9} The viral genome has been completely sequenced.^{10,11} We have also reported the isolation of viruses resembling SARS-CoV from Himalayan palm civets found in a live animal market in the Guangdong Province of China; this finding implied that animals could be a reservoir of the virus.¹²

Detection of SARS-CoV from a non-pneumonic case in Singapore (http://www.who.int/csr/don/2003_09_16/en/) suggested that non-pneumonic and subclinical SARS-CoV infections are possible. However, extensive seroprevalence studies and mass screening for detection of subclinical and non-pneumonic infections are still lacking. At present, the most widely used methods for detection of antibodies against SARS-CoV are indirect immunofluorescence assay and ELISA with cell-culture extract.^{1,5} However, antibody detection by these methods is difficult to standardise and has not been compared with recombinant-antigen-based antibody-detection tests. A recent seroprevalence study, which used the indirect immunofluorescence assay for antibody detection, did not detect SARS-CoV antibodies in any of 674 health-care workers from a hospital in which a SARS outbreak had occurred.¹³

An approach of ELISA-based antibody-detection tests using recombinant antigens with positive results confirmed by western-blot assays that use two different antigenic proteins offers higher sensitivity, specificity, and reproducibility than indirect immunofluorescence assay and ELISA with cell-culture extract and is easier to standardise.^{14–19} In this study, we used a sensitive and specific ELISA based on the highly immunogenic nucleocapsid protein of SARS-CoV and confirmed positive results by western-blot assays with recombinant nucleocapsid protein and recombinant spike polypeptide (another highly immunogenic protein) of SARS-CoV to examine the seroprevalence of non-pneumonic SARS-CoV in the general population, non-pneumonic patients in hospital, and health-care workers during the SARS epidemic.

Methods

RNA extraction

Coronavirus isolated from a patient with SARS-CoV pneumonia in Hong Kong was inoculated into Vero cells at confluence in Dulbecco's modified Eagle's medium (Gibco BRL, Carlsbad, CA, USA) with 10% fetal calf serum in a tissue-culture flask. After 48 h, the supernatant was transferred to a new tube and centrifuged at 12 000 *g* for 15 min at 4°C. Viral RNA was extracted from 100 μ L of the supernatant with TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. The RNA

Primer sequences**Nucleocapsid protein**

LPW723 5'-CGCGGATCCGATGTCTGATAATGGACC-3'

LPW726 5'-CGGAATTCTTATGCCTGCCTGAGTTGAATC-3'

Spike protein

LPW742 5'-CGCGGATCCGAGTGACCTTGACCGGTGC-3'

LPW931 5'-CGGGGTACCTTAACGTAATAAAGAAACTGTATG-3'

pellet was resuspended in 10 μ L DNase-free, RNase-free double-distilled water and was used as the template for RT-PCR.

Cloning and purification of (His)₆-tagged recombinant proteins

To produce a fusion plasmid of the nucleocapsid protein of the SARS-CoV for protein purification, primers LPW723 and LPW726 (panel) were used to amplify the gene encoding this protein by RT-PCR. The sequence coding for aminoacid residues 1–422 of the nucleocapsid protein was amplified and cloned into the *Bam*HI and *Eco*RI sites of expression vector pET-28b(+) (Novagen, Madison, WI, USA) in frame and downstream of the series of six histidine residues. The (His)₆-tagged recombinant nucleocapsid protein was expressed and purified by means of the nickel-loaded HiTrap Chelating System (Amersham Pharmacia, Piscataway, NJ, USA) according to the manufacturer's instructions. Roughly 3 mg purified protein was routinely obtained from 1 L *Escherichia coli* carrying the fusion plasmid.

For the spike protein of the SARS-CoV, primers LPW742 and LPW931 (panel) were used to amplify the gene encoding aminoacid residues 14–667 by RT-PCR. This portion of the spike protein was used because the complete protein could not be expressed in *E. coli*. The PCR product was cloned into the *Bam*HI and *Kpn*I sites of vector pQE-31 (Qiagen, Hilden, Germany). The resulting clone was digested by *Pst*I, and the fragment that contained the gene encoding aminoacid residues 250–667 of the spike protein was cloned into expression vector pQE-30 (Qiagen, Hilden, Germany) in frame and downstream of the series of six histidine residues. Expression and purification of the (His)₆-tagged recombinant spike polypeptide were done as described for the nucleocapsid protein.

Western-blot analysis

Western-blot analysis was done according to our published protocols, with slight modifications.^{20–22} Briefly, 200 ng purified (His)₆-tagged recombinant nucleocapsid protein or (His)₆-tagged recombinant spike polypeptide was loaded into each well of a sodium dodecyl sulphate 10% polyacrylamide gel, then electroblotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blot was cut into strips, and the strips were incubated separately with 1 in 1000 dilution of three serum samples, obtained from three patients with SARS-CoV pneumonia, positive for antibodies against SARS-CoV detected by our indirect immunofluorescence assay.¹ Antigen–antibody interaction was detected with an ECL fluorescence system (Amersham Life Science, Buckinghamshire, UK). Serum samples from three healthy blood donors were used as controls.

Assessment of recombinant nucleocapsid protein ELISA

Serum samples from 149 healthy blood donors who donated blood 3 years previously (aged 18 years or older)

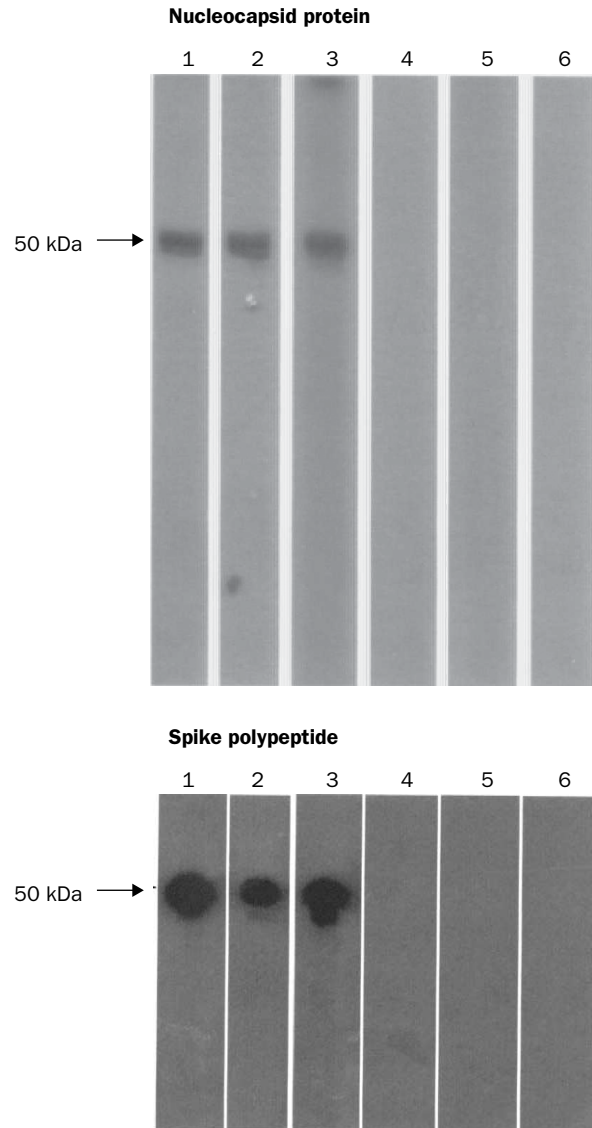


Figure 1: Western-blot analysis of purified recombinant SARS-CoV nucleocapsid protein and purified recombinant SARS-CoV spike polypeptide

Prominent immunoreactive protein bands of about 50 kDa were visible for both antigens with serum from three patients with SARS-CoV pneumonia, indicating antigen–antibody interactions between the recombinant SARS-CoV nucleocapsid protein and the patients' antibodies (lanes 1–3). No immunoreactive band was detected for serum from three healthy blood donors (lanes 4–6).

and 106 patients with pneumonia positive for antibodies against SARS-CoV detected by our indirect immunofluorescence assay¹ were used for the assessment of the ELISA-based IgG antibody test. The test was modified from our previous publications.^{15,17} Briefly, each well of a Nunc immunoplate (Roskilde, Denmark) was coated with 20 ng purified (His)₆-tagged recombinant nucleocapsid protein for 12 h, then blocked in phosphate-buffered saline with 2% bovine serum albumin. 100 μ L diluted human serum (1 in 40) was added to each well of the protein-coated plates, and they were incubated at 37°C for 2 h. After five washes with washing buffer, 100 μ L horseradish-peroxidase-conjugated goat antibody to human IgG (1 in 4000 dilution; Zymed Laboratories Inc, South San Francisco, CA, USA) was added to each well, and the plates were incubated at 37°C for 1 h. After a further five washes with washing buffer, 100 μ L diluted

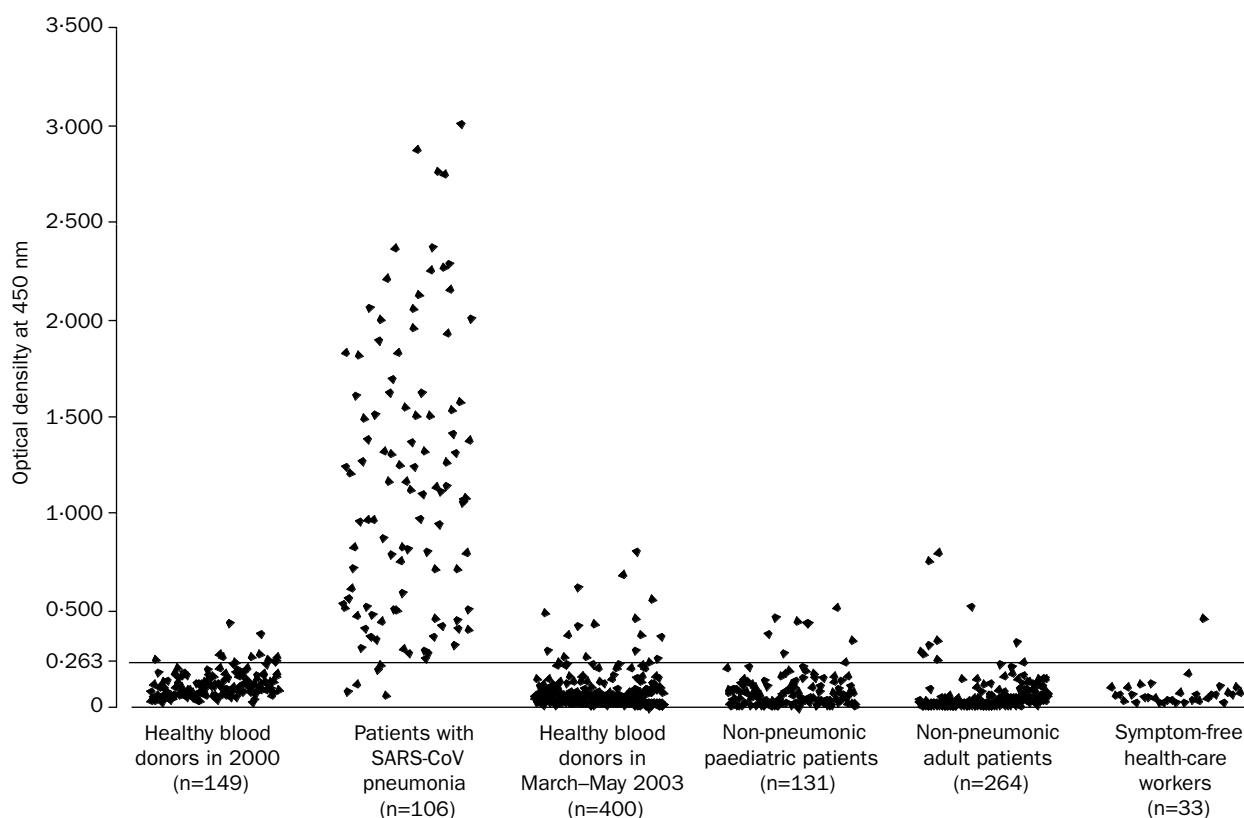


Figure 2: **ELISA based on recombinant nucleocapsid protein IgG antibody for SARS-CoV infection**

3,3',5,5'-tetramethylbenzidine (Zymed Laboratories) was added to each well and incubated at room temperature for 15 min. 100 μ L 0.3 mol/L sulphuric acid was added, and the absorbance at 450 nm of each well was measured. Each sample was tested in duplicate, and the mean absorbance for each sample was calculated. The presence of specific antibodies in positive samples was confirmed by retesting of the sample by the ELISA based on recombinant nucleocapsid protein and western-blot assays with recombinant nucleocapsid protein and recombinant spike polypeptide separately.

Seroprevalence of SARS-CoV

Using the protocol described above, we tested for the presence of IgG antibodies against the nucleocapsid protein in serum samples from 400 healthy blood donors (aged 18 years or older) who donated blood in March–May, 2003; 131 non-pneumonic paediatric inpatients (aged less than 18 years) and 264 non-pneumonic adult inpatients (aged 18 years or older) admitted to Queen Mary Hospital in May, 2003; and 33 symptom-free health-care workers. The presence of specific antibodies in all positive samples was confirmed by two separate western-blot assays, one with recombinant nucleocapsid protein and the other with recombinant spike polypeptide as the antigen.

Role of the funding source

The study sponsors had no role in the study design; collection, analysis, or interpretation of data; or the writing of the report.

Results

The purified (His)₆-tagged recombinant nucleocapsid protein and spike polypeptide were separated on denaturing polyacrylamide gels followed by western-blot

analysis with serum from three patients with pneumonia positive for antibodies against the SARS-CoV. Prominent immunoreactive protein bands of about 50 kDa were visible on the western blots that used either antigen (figure 1). These sizes were consistent with the expected size of 49.6 kDa for the full-length (His)₆-tagged nucleocapsid protein and 48.6 kDa for the (His)₆-tagged spike polypeptide.

An ELISA-based SARS-CoV antibody test was developed for the detection of specific antibodies against the (His)₆-tagged recombinant nucleocapsid protein. Box titration was carried out with different dilutions of (His)₆-tagged recombinant nucleocapsid protein coating antigen and pooled serum from three patients with pneumonia positive for antibody against the SARS-CoV. The results identified 20 ng purified (His)₆-tagged recombinant nucleocapsid protein per ELISA well as the ideal amount for plate coating for IgG detection (data not shown).

To establish the baseline for the tests, serum samples from 149 healthy blood donors who donated blood 3 years previously were tested in the ELISA. For these samples, the mean optical density at 450 nm for IgG detection was 0.127 (SD 0.068). An absorbance value of 0.263 was selected as the cut-off value (the mean value for the healthy controls plus 2 SD; figure 2). Seven of the samples from 149 healthy blood donors had values of more than 0.263 in the IgG ELISA (figure 2), but none of them had the specific antibody when tested by the nucleocapsid protein or the spike polypeptide western-blot assay. The specificity of the IgG antibody test (ELISA confirmed by western-blot assays) was 100%.

The mean value for the samples obtained from the 106 patients with SARS-CoV pneumonia, positive for IgG antibodies against the SARS-CoV by our indirect immunofluorescence assay, was 1.153 (SD 0.702). 100 samples had optical density of more than 0.263 in the

IgG ELISA (figure 2). All 100 were confirmed to have the specific antibody by both the nucleocapsid protein and the spike polypeptide western-blot assays. The sensitivity of the IgG antibody test, with the immunofluorescence assay as the gold standard, was therefore 94.3%.

16 (4.0%) of the 400 healthy blood donors who donated blood in March–May, 2003, eight (6.1%) of the 131 non-pneumonic paediatric patients, eight (3.0%) of the 264 non-pneumonic adult patients, and one (3%) of the 33 symptom-free health-care workers who had cared for the patients with SARS-CoV pneumonia were positive for IgG antibodies by ELISA (figure 2). However, only three (0.8%) healthy blood donors who donated blood in March–May, 2003, and one (0.8%) non-pneumonic paediatric patient were confirmed to have specific SARS-CoV antibodies by both the nucleocapsid protein and spike polypeptide western-blot assays.

Up to the end of May, 1728 patients from a population of about seven million in Hong Kong (0.025%) had developed SARS-CoV pneumonia, compared with a rate of non-pneumonic SARS-CoV infections in our study population of about 0.48% ($p < 0.001$ by Poisson exact test of equality).

Discussion

Previous studies in animal coronaviruses, such as infectious bronchitis virus, have shown that the nucleocapsid protein and spike protein are highly immunogenic, are abundantly expressed during infection, and can be used for serodiagnosis of animal coronavirus infections.^{23–25} In this study, detection of IgG antibodies to both proteins was highly sensitive and specific for SARS-CoV infections. Six (5.7%) serum samples that were seropositive by the immunofluorescence assay were negative by the ELISA. The reason for this discrepancy may be that the nucleocapsid protein did not elicit antibody response in this minority group of patients. Conversely, of five patients with SARS-CoV pneumonia who were seronegative by the immunofluorescence assay but RT-PCR positive for SARS-CoV, two were seropositive by our ELISA, with clearly positive optical-density values of 0.874 and 1.228 and confirmation by western-blot assay (unpublished). Furthermore, in four of 20 patients with SARS coronavirus pneumonia who had serial serum samples, IgG was detected earlier by the ELISA than by the immunofluorescence test (unpublished). In another study that used ELISA based on cell-culture extract, five of 15 patients with SARS-CoV pneumonia were positive according to that ELISA but negative by indirect immunofluorescence during the time when the ELISA titres were low.⁵ This finding accords with the results of a previous study on human coronavirus 229E, which showed that three of 51 serum samples were positive by recombinant-nucleocapsid-protein-based western blot but negative by immunofluorescence, and six of 51 serum samples were positive by immunofluorescence but negative by recombinant-nucleocapsid-protein-based western blot.²⁶ All these findings show that our ELISA may be able to detect additional cases that the immunofluorescence assay has missed.

With this potentially more sensitive ELISA for IgG antibody detection, we assessed the seroprevalence of non-pneumonic SARS-CoV infections in both the general population and our hospital population; all positive serum samples detected by ELISA were confirmed by two separate western-blot assays, with two immunologically unrelated antigens. The spike polypeptide was used in addition to the nucleocapsid protein for western-blot confirmation to eliminate the possibility of cross-reactivity

between antibodies against the nucleocapsid proteins of other human coronaviruses and that of SARS-CoV. However, the aminoacid identities between the nucleocapsid protein of SARS-CoV and those of the human coronaviruses OC43 and 229E are only 32.7% and 21.3%, respectively, and there were no cross-reactions between 13 pairs of OC43 and 14 pairs of 229E human coronavirus serum samples and the SARS-CoV.⁵ In fact, of the 33 individuals who were IgG positive by ELISA, 26 (79%) were positive by the nucleocapsid-protein-based western-blot assay, but only four were positive by both the nucleocapsid protein and the spike polypeptide western-blot assays. This apparent high rate of false-positive non-pneumonic cases, as detected by the recombinant nucleocapsid protein ELISA, is due to the use of a single antigen for screening asymptomatic or non-pneumonic infections. It is well known that the positive predictive values of serological tests are much affected when the prevalence of the infection is low, especially in clinically incompatible cases. This is the reason why an immunologically unrelated antigen, the spike protein, has to be used for confirmation of the cases detected as “positive” by the nucleocapsid-protein-based assays. Cross-reactivity with human coronavirus OC43 or other SARS-CoV-like viruses remains an important issue for future studies on SARS-CoV serology. Three of the four individuals with non-pneumonic SARS-CoV infections were healthy blood donors, and one was a paediatric inpatient. This patient was a 19-month-old girl who was admitted to hospital in May, 2003, because of fever for 2 days. She was also noted to have had a cough for the previous 2 weeks and repeated vomiting before admission. There had been no breathing difficulty or diarrhoea. The child had no history of direct contact with patients with SARS-CoV pneumonia. However, a colleague in her mother’s workplace had recently been diagnosed as having SARS-CoV pneumonia. Physical examination of the girl revealed only shotty (palpable but too small to be measured) cervical lymph nodes and congested throat. Her chest radiograph was normal. Apart from mild lymphopenia (lymphocyte count $0.63 \times 10^9/L$), her blood results were normal. Nasopharyngeal aspirate was negative for influenza viruses, parainfluenza viruses, adenovirus, and respiratory syncytial virus antigens.²⁷ She was given antipyretic treatment and was discharged the next day. Subsequent antibody testing showed that her serum was positive for both IgG and IgM antibodies against the nucleocapsid protein as well as IgG antibodies against the spike polypeptide of SARS-CoV.

The difference between the rate of non-pneumonic SARS-CoV infections in our study population and the rate of SARS in Hong Kong suggests that non-pneumonic infections are more common than SARS-CoV pneumonia and may explain cases of SARS-CoV pneumonia in patients who had no obvious contact with other patients with SARS.

Contributors

P C Y Woo and K Y Yuen are coprincipal investigators, jointly wrote the report, and coordinated and supervised the study. S K P Lau supervised the analysis of all serological data and corrected the report. H W Tsoi cloned and purified the nucleocapsid protein and did the serological assays. K H Chan supervised the immunofluorescence assay. B H L Wong and V K P Tam did the serological assays and analysed the data. X Y Che cloned and purified the spike polypeptide. S C F Tam coordinated the collection of clinical specimens. V C C Cheng and I F N Hung managed the patients’ database. S S Y Wong, B J Zheng, and Y Guan corrected the report.

Conflict of interest statement

None declared.

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