

## NOTES

# Retrospective Serological Investigation of Severe Acute Respiratory Syndrome Coronavirus Antibodies in Recruits from Mainland China

Sumeng Yu,<sup>1†</sup> Maofeng Qiu,<sup>2†</sup> Zeliang Chen,<sup>2†</sup> Xiaobo Ye,<sup>1</sup> Yaling Gao,<sup>1</sup> Aimin Wei,<sup>1</sup> Xiaoyi Wang,<sup>2</sup> Ling Yang,<sup>3</sup> Jin Wang,<sup>2</sup> Jie Wen,<sup>3</sup> Yajun Song,<sup>2</sup> Decui Pei,<sup>2</sup> Erhei Dai,<sup>2</sup> Zhaobiao Guo,<sup>2</sup> Cheng Cao,<sup>4</sup> Jian Wang,<sup>3</sup> and Ruifu Yang<sup>2\*</sup>

*Infectious Disease Control Unit, Logistic Department for Air Forces, Beijing Military Area,<sup>1</sup> Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences,<sup>2</sup> Beijing Genomics Institute, Chinese Academy of Sciences,<sup>3</sup> and Beijing Institute of Bioengineering,<sup>4</sup> Beijing, People's Republic of China*

Received 19 October 2004/Returned for modification 3 January 2005/Accepted 19 January 2005

**Different assays were used to analyze 1,621 serum specimens collected from military recruits from the People's Republic of China in 2002 for severe acute respiratory syndrome (SARS) coronavirus antibodies. The results demonstrated that the subjects either had rarely been exposed to the virus before the 2003 SARS outbreak or had not been exposed but the nucleocapsid protein cross-reacted with other antibodies in humans.**

In mid-November 2002, the first case of a unique atypical infectious pneumonia was found in Foshan City, Guangdong Province, People's Republic of China. This disease then spread throughout many areas in China and other regions and countries around the world. The World Health Organization (WHO) recognized it as severe acute respiratory syndrome (SARS) (9). In May 2003, a WHO-coordinated scientific team identified a novel coronavirus, the SARS coronavirus (SARS-CoV), as the causative agent of SARS (4).

The natural reservoir of SARS-CoV has not been discovered, although SARS-CoV-like viruses were isolated from Himalayan palm civets, and evidence of virus infection was also documented in other animals (including a raccoon dog [*Nyctereutes procyonoides*]) and in humans in a retail market in Guangdong Province of China (2). Using immunofluorescence and neutralization assays, Zheng et al. (10) detected antibodies to human SARS-CoV and/or an animal SARS-CoV-like virus in 17 of 938 (1.8%) healthy adults from Hong Kong in 2001, suggesting that a small proportion of healthy people in Hong Kong had been exposed to SARS-related viruses at least 2 years before the first SARS outbreak.

In this study, to investigate whether the people from mainland China had been exposed to the SARS-CoV before the first SARS outbreak in 2003, 1,621 serum samples were screened by enzyme-linked immunosorbent assay (ELISA), and the positive samples were further analyzed by various serological assays. Serum samples were taken from healthy military recruits for routine examination in March 2002, about 1

year before the first SARS outbreak; and all serum samples were stored at  $-86^{\circ}\text{C}$  before SARS-CoV-related analysis. These soldiers came from 17 provinces, including Guangdong Province, of China; they were all 18-year-old men whose health status met the standards for army recruitment. In addition, a positive control serum sample was taken from a patient with clinically confirmed SARS in Beijing, and a negative control serum sample was collected from a healthy volunteer.

All serum samples were heat inactivated at  $56^{\circ}\text{C}$  for 30 min before analysis. They were first screened by using an ELISA kit (Beijing GBI Biotechnology Co. Ltd.) that detects immunoglobulin G (IgG) antibodies against the SARS-CoV. This whole-virus lysate-based kit was the only ELISA kit approved by the China State Food and Drug Administration for SARS-CoV antibody detection during the 2003 SARS outbreak, and it was the most frequently used assay in mainland China. Eleven of 1,621 (0.68%) serum samples were positive for IgG antibodies against SARS-CoV, and these ELISA-positive samples were further tested by immunofluorescence assay (IFA) and neutralization assay, which are more specific than ELISA. Four hundred ELISA-negative serum samples were also randomly selected for confirmation of the results by IFA, and the results showed that they were all negative by IFA.

IFA was performed by using an IFA kit that was approved for use by the China State Food and Drug Administration during the 2003 SARS outbreak and that was manufactured by the Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing.

**Neutralization assay.** The serum samples were serially diluted from 1:10 to 1:640 and then mixed with 100 times the 50% tissue culture infective dose of SARS-CoV. After incubation for 1 h at  $37^{\circ}\text{C}$ , the mixture was inoculated onto Vero E6 cell monolayers in a 96-well plate, with 4 wells used for each dilution. The results were determined after a 4-day incubation at  $37^{\circ}\text{C}$ .

\* Corresponding author. Mailing address: Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, No. 20, Dongdajie, Fengtai District, Beijing 100071, People's Republic of China. Phone: 86-10-66948595. Fax: 86-10-83820748. E-mail: yangrf@nic.bmi.ac.cn.

† S.Y., M.Q., and Z.C. contributed equally to this work.

TABLE 1. Results for ELISA-positive samples further analyzed by IFA, neutralization assay, protein microarray analysis, and nucleocapsid protein-based antigen-capturing ELISA

Sample no.	Result by:				
	ELISA	IFA	Neutralization assay	Protein microarray analysis <sup>a</sup>	Antigen-capturing ELISA <sup>b</sup>
1	+	+	-	+	+
2	+	+	-	+	+
3	+	+	-	+	+
4	+	+	-	-	-
5	+	-	-	-	-
6	+	-	-	-	-
7	+	-	-	-	-
8	+	-	-	-	-
9	+	+	-	-	-
10	+	+	-	-	-
11	+	-	-	-	-
Positive control	+	+	+	+	+
Negative control	-	-	-	-	-

<sup>a</sup> The results obtained by protein microarray analysis are only those obtained for the N protein.

<sup>b</sup> N-protein-based antigen-capturing ELISA.

As Table 1 shows, 6 of the 11 ELISA-positive samples were positive for IgG antibodies by IFA. However, all of those were negative by the neutralization assay; these findings differed from those described in previous reports by Zheng et al. (10) for samples from individuals in Hong Kong. To explore the reason for the differences in the findings, we further used a protein microarray to screen the specific antibodies to individual proteins of the SARS-CoV and a nucleocapsid (N) protein-based antigen-capturing ELISA to confirm the results for anti-N antibodies.

In our previous report (5), the protein microarray assay was performed to detect IgG antibodies against the individual proteins of the SARS-CoV. Briefly, 13 recombinant proteins associated with 4 structural proteins (S, E, M, and N) and 5 putative uncharacterized proteins (3a, 3b, 6, 7a, and 9b) of SARS-CoV were prepared and used to screen 58 serum samples from convalescent-phase SARS patients for IgG antibodies specific for these proteins by use of the protein microarray. The cutoff value of the fluorescence intensity for each protein was calculated by using the fluorescence intensity values for this protein with normal human serum. Antibodies to proteins S, 3a, N, and 9b were detected in the sera from convalescent-phase SARS patients, whereas antibodies to proteins E, M, 3b, 6, and 7a were not detected. In the present study, when the protein microarray described above was applied to the ELISA-positive serum specimens, among the 13 recombinant proteins, only the N protein reacted with 3 of 11 samples, whereas all 11 samples were negative for antibodies to the other main antigenic proteins of SARS-CoV (Table 1). The results revealed by protein microarray analysis were further confirmed by an N protein-based antigen-capturing ELISA (Table 1). This recombinant full-length N protein-based ELISA kit (Sino-American Bioengineering Co.) could detect the total antibodies (IgG, IgM, IgA, etc.) to the N protein of SARS-CoV (7).

Anti-N and anti-S antibodies have been proved to be the first and second most frequent antibodies, respectively, in the sera from SARS patients (3). However, among the structural proteins, only the S protein was identified as a significant neutral-

ization antigen and protective antigen, and anti-N antibody lacks neutralization activity (1).

In the present study, all ELISA-positive samples were negative by the neutralization assay, although at least three samples were positive by other tests. These findings suggest two possibilities.

First, 3 of the 1,621 (0.18%) recruits might have exposed to SARS-CoV or the SARS-CoV-like virus before the first SARS outbreak in 2003. In contrast to the report of Zheng et al. (10), the sera from these people were negative by the neutralization assay. The reason is likely that the concentrations of neutralizing antibodies, such as anti-S antibodies, were too low to neutralize the virus, while the detectable anti-N antibody lacked neutralization activity. Although the exposure history and symptoms of the participants studied were unavailable for assessment, the existence of subclinical or nonpneumonic SARS-CoV infections is possible (8).

Second, none of the recruits have been exposed to the SARS-CoV; and the positive results of ELISA, IFA, protein microarray analysis, and antigen-capturing ELISA might have been false positive. Since the ELISA and IFA kits used in this study were the first and second most frequently used during the SARS outbreak in Mainland China in 2003, respectively, the presence of possible false-positive results indicates that the results obtained with these kits should be combined with the results obtained by other diagnostic tests and with clinical signs and symptoms for the diagnosis of SARS. Use of the recombinant N-protein-based protein microarray assay and the antigen-capturing ELISA could reduce the probability of false-positive results, but the possible cross-reactivity of the SARS-CoV N protein to other antibodies in humans should also be taken into account. More specific recombinant truncated N protein (6) or other potential candidate antigens, such as S protein, could be further screened and developed for use in reliable serological assays.

In conclusion, our results suggest that the people from mainland China either had only rarely been exposed to SARS-CoV before the 2003 SARS outbreak or had not been exposed to SARS-CoV at all but the SARS-CoV N protein cross-reacted with other antibodies in humans, which needs to be further verified.

This work was supported by grant 2003AA208211 from the National High Technology Research and Development Program of China (863 Program).

We are grateful to David Bastin, Tianjin Biochip Co., Tianjin, People's Republic of China, for careful reading of the manuscript and valuable suggestions.

#### REFERENCES

- Buchholz, U. J., A. Bukreyev, L. Yang, E. W. Lamirande, B. R. Murphy, K. Subbarao, and P. L. Collins. 2004. Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity. *Proc. Natl. Acad. Sci. USA* **101**:9804-9809.
- Guan, Y., B. J. Zheng, Y. Q. He, X. L. Liu, Z. X. Zhuang, C. L. Cheung, S. W. Luo, P. H. Li, L. J. Zhang, Y. J. Guan, K. M. Butt, K. L. Wong, K. W. Chan, W. Lim, K. F. Shorridge, K. Y. Yuen, J. S. Peiris, and L. L. Poon. 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science* **302**:276-278.
- Krokhin, O., Y. Li, A. Andonov, H. Feldmann, R. Flick, S. Jones, U. Stroehner, N. Bastien, K. V. Dasuri, K. Cheng, J. N. Simonsen, H. Perreault, J. Wilkins, W. Ens, F. Plummer, and K. G. Standing. 2003. Mass spectrometric characterization of proteins from the SARS virus: a preliminary report. *Mol. Cell Proteomics* **2**:346-356.
- Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery,

- S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, and L. J. Anderson. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* **348**:1953–1966.
5. Qui, M., Y. Shi, Z. Guo, Z. Chen, R. He, R. Chen, D. Zhou, E. Dai, X. Wang, B. Si, Y. Song, J. Li, L. Yang, J. Wang, H. Wang, X. Pang, J. Zhai, Z. Du, Y. Liu, Y. Zhang, L. Li, J. Wang, B. Sun, and R. Yang. Antibody responses to individual proteins of SARS coronavirus and their neutralization activities. *Microbes Infect.*, in press.
  6. Qiu, M., J. Wang, H. Wang, Z. Chen, E. Dai, Z. Guo, X. Wang, X. Pang, B. Fan, J. Wen, J. Wang, and R. Yang. 2005. Use of the COOH portion of the nucleocapsid protein in an antigen-capturing enzyme-linked immunosorbent assay for specific and sensitive detection of severe respiratory syndrome coronavirus. *Clin. Diagn. Lab. Immunol.* **12**:474–476.
  7. Shi, Y., Y. Yi, P. Li, T. Kuang, L. Li, M. Dong, Q. Ma, and C. Cao. 2003. Diagnosis of severe acute respiratory syndrome (SARS) by detection of SARS coronavirus nucleocapsid antibodies in an antigen-capturing enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **41**:5781–5782.
  8. Woo, P. C., S. K. Lau, H. W. Tsoi, K. H. Chan, B. H. Wong, X. Y. Che, V. K. Tam, S. C. Tam, V. C. Cheng, I. F. Hung, S. S. Wong, B. J. Zheng, Y. Guan, and K. Y. Yuen. 2004. Relative rates of non-pneumonic SARS coronavirus infection and SARS coronavirus pneumonia. *Lancet* **363**:841–845.
  9. World Health Organization. 2003. Severe acute respiratory syndrome (SARS). *Wkly. Epidemiol. Rec.* **78**:181–183.
  10. Zheng, B. J., K. H. Wong, J. Zhou, K. L. Wong, B. W. Young, L. W. Lu, and S. S. Lee. 2004. SARS-related virus predating SARS outbreak, Hong Kong. *Emerg Infect. Dis.* **10**:176–178.