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Evaluation of Reverse Transcription-PCR Assays for Rapid Diagnosis of Severe Acute Respiratory Syndrome Associated with a Novel Coronavirus

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The reverse transcription (RT)-PCR protocols of two World Health Organization (WHO) severe acute respiratory syndrome (SARS) network laboratories (WHO SARS network laboratories at The University of Hong Kong [WHO-HKU] and at the Bernhard-Nocht Institute in Hamburg, Germany [WHO-Hamburg]) were evaluated for rapid diagnosis of a novel coronavirus (CoV) associated with SARS in Hong Kong. A total of 303 clinical specimens were collected from 163 patients suspected to have SARS. The end point of both WHO-HKU and WHO-Hamburg RT-PCR assays was determined to be 0.1 50% tissue culture infective dose. Using seroconversion to CoV as the "gold standard" for SARS CoV diagnosis, WHO-HKU and WHO-Hamburg RT-PCR assays exhibited diagnostic sensitivities of 61 and 68% (nasopharyngeal aspirate specimens), 65 and 72% (throat swab specimens), 50 and 54% (urine specimens), and 58 and 63% (stool specimens), respectively, with an overall specificity of 100%. For patients confirmed to have SARS CoV and from whom two or more respiratory specimens were collected, testing the second specimen increased the sensitivity from 64 and 71% to 75 and 79% for the WHO-HKU and WHO-Hamburg RT-PCR assays, respectively. Testing more than one respiratory specimen will maximize the sensitivity of PCR assays for SARS CoV.

A global outbreak of a new emerging illness, severe acute respiratory syndrome (SARS), was associated with a novel coronavirus, SARS CoV (5, 8, 11). By the end of April 2003, more than 1,500 patients were diagnosed with SARS in Hong Kong. Transmission within hospitals was a major contributor to disease amplification. Rapid laboratory confirmation of SARS CoV infection was important for managing patient care and for preventing nosocomial transmission. While serological testing was reliable as a retrospective diagnostic method, diagnosis of the infection in the early phase of the illness was important for patient care. The identification of the etiological agent and its partial gene sequence data made it possible to develop molecular diagnostic methods for SARS CoV (4, 9). The protocols were made available through the World Health Organization (WHO) website (http://www.who.int/csr/sars/primers/en). This study evaluates two of the first-generation reverse transcription (RT)-PCR assays that were used during this outbreak.

MATERIALS AND METHODS

Patients and specimen collection. Specimens were available for 163 patients who presented with clinically suspected SARS according to the WHO definition (13) and who were admitted to three acute regional hospitals in Hong Kong between 26 February and 17 April 2003. For each patient, paired acute- and convalescent-phase serum samples and at least one respiratory specimen were collected for study. A total of 303 specimens (124 nasopharyngeal aspirate specimens, 65 throat swab specimens, 95 urine specimens, and 19 stool specimens) were available for study. Respiratory specimens were collected between days 1 and 5 after admission, whereas urine and stool specimens were collected between days 5 and 10. The acute-phase sera were collected in the first week of illness, and the convalescent-phase sera were collected ≥21 days after the onset of clinical symptoms. Nasopharyngeal aspirate specimens were assessed by rapid direct immunofluorescent antigen detection for influenza virus A and B, parainfluenza virus types 1, 2, and 3, respiratory syncytial virus (RSV), and adenovirus as described previously (2). Paired serum samples were assayed for increasing titer against CoV. Nasopharyngeal aspirate and stool specimens from patients suffering from unrelated diseases were collected as controls.

Extraction of CoV RNA. Nasopharyngeal aspirate and throat swab specimens were suspended in viral transport medium. Urine specimens were transported in sterile containers. Stool specimens were mixed in viral transport medium (diluted 1:10) and microcentrifuged at 10,000 $\times$ g for 1 min, and supernatant was collected. Viral RNA was extracted from 140-μl samples using a Qiagen viral RNA mini kit (Qiagen, Hilden, Germany). The initial processing of specimens was performed under biohazard level 2 containment conditions. After lysis of the sample by the lysing buffer, the mixture was applied to a spin column as described by the manufacturer. The extracted RNA was eluted in a total volume of 50 μl of RNase-free water before RT-PCR amplification.

RT-PCR amplification. The RT-PCR protocols of two WHO SARS network laboratories (Table 1) were evaluated in this study. The WHO SARS network laboratory at The University of Hong Kong (WHO-HKU) used a single RT step to synthesize cDNA, followed by subsequent PCR amplification with specific primers in another reaction tube (8). The WHO SARS network laboratory at the Bernhard-Nocht Institute in Hamburg, Germany (WHO-Hamburg) used a single RT-PCR step, followed by transfer of the initial PCR products to the nested PCR amplification mixture (4). Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed. For nested PCR, RT-PCR amplicon tubes were spun (in pulses) before the tubes were opened using separate Eppendorf tube openers for transferring RT-PCR products to the nested PCR mix. Negative control was incorporated for every five nested PCRs to monitor cross-contamination. Amplified products were electrophoresed through a 2% agarose gel in Tris-borate buffer. Target bands were visualized by staining with ethidium bromide.

CoV immunoglobulin G serology. Smears of CoV-infected Vero cells were prepared, fixed in acetone for 10 min, and stored at −80°C before use (8). Each batch of SARS CoV-infected cell smears with 60 to 70% infected cells was prepared and tested with a high-titer, positive-control serum sample from a confirmed SARS patient as a standard to assess sensitivity and batch-to-batch variations. Serial twofold dilutions starting with a 1:10 dilution of each patient serum sample were added to the smears and incubated for 30 min at 37°C. After two 5-min washes in phosphate-buffered saline, fluorescein isothiocyanate-con-

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TABLE 1. RT-PCR protocols for rapid diagnosis of CoV associated with SARS[a]

Characteristic or component of protocol	WHO-HKU		WHO-Hamburg	
	RT	PCR	RT-PCR	Second PCR
Primer sequences				
Sense		TACACACCTCAGCGTTG	ATGAATTACCAAGTCAATGGTTAC	GAAGCTATTCGTCACGTTCG
Antisense		CACGAACGTGACGAAT	CATAACCAGTCGGTACAGCTAC	CTGTAGAAAATCCTAGCTGGAG
Reagent formulation	Superscript II RTA (Invitrogen) (i) 4 μl of 5× first-strand buffer (ii) 10 mM DTT (iii) 500 μM dNTP (iv) 0.15 μg of random primer (v) 200 U of Superscript II (vi) 12 μl of RNA extract (vii) Make up to total volume of 20 μl	AmpliTaq Gold (Roche) (i) 5 μl of 10× reaction buffer (ii) 200 μM dNTP (iii) 2.5 mM $MgSO_4$ (iv) 250 nM (each) primer (v) 2 U of AmpliTaq Gold (vi) 2 μl of RT product (vii) Make up to total volume of 50 μl	Superscript II RT-PCR (Invitrogen) (i) 10 μl of 2× reaction buffer (ii) 2.45 mM $MgSO_4$ (iii) 500 nM (each) primer (iv) 0.4 μl of RTA-*Taq* mixture (v) 2 μl of RNA extract (vi) Make up to total volume of 20 μl	AmpliTaq Gold (Roche) (i) 5 μl of 10× reaction buffer (ii) 200 μM dNTP (iii) 2.5 mM $MgSO_4$ (iv) 200 nM (each) primer (v) 2 U of AmpliTaq Gold (vi) 1 μl of RT-PCR product (vii) Make up to total volume of 50 μl
Thermal cycling profile	(i) 25°C, 10 min (ii) 42°C, 50 min (iii) 94°C, 3 min	(i) 94°C, 10 min (ii) 40 cycles (a) 94°C, 30 s (b) 50°C, 40 s (c) 72°C, 15 s (iii) 72°C, 10 min	(i) 45°C, 30 min (ii) 95°C, 3 min (iii) 10 cycles (a) 95°C, 10 s (b) 60°C, 10 s (decrease by 1°C/cycle) (c) 72°C, 30 s (iv) 40 cycles (a) 95°C, 10 s (b) 56°C, 10 s (c) 72°C, 30 s	(i) 95°C, 5 min (ii) 10 cycles (a) 95°C, 10 s (b) 60°C, 10 s (decrease by 1°C/cycle) (c) 72°C, 20 s (iii) 20 cycles (a) 95°C, 10 s (b) 56°C, 10 s (c) 72°C, 20 s
Expected PCR product size (bp)		182	189	108

[a] The RT-PCR protocols of two WHO SARS network laboratories, WHO-HKU (8) and WHO-Hamburg (4), are also available online (http://www.who.int/esr/sars/primers/en). Abbreviations: RTA, reverse transcriptase; DTT, dithiothreitol; dNTP, deoxynucleoside triphasphate.

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TABLE 2. Performance of RT-PCR assays for rapid detection of CoV associated with SARS

Specimens (no.)	No. of specimens tested	Seroconversion[a]	No. of specimens positive by RT-PCR assay		
			WHO-HKU	WHO-Hamburg	Both WHO-HKU and WHO-Hamburg
Clinically suspected SARS					
Nasopharygneal aspirate specimens (124)	72	+	44	49	43
	52	−	0	0	0
Throat swab specimens (65)	54	+	35	39	33
	11	−	0	0	0
Urine specimens (95)	78	+	39	42	39
	17	−	0	0	0
Stool specimens (19)	19	+	11	12	11
Controls					
Nasopharygneal aspirate specimens	22[b]	ND	0	0	0
Stool specimens	21[c]	ND	0	0	0

[a] A fourfold rise or more in antibody titer against CoV was considered seroconversion (+). ND, not done.
[b] Samples positive for other viral pathogens included nine samples positive for influenza virus A, one sample positive for influenza virus B, six samples positive for adenovirus, and six samples positive for RSV by immunofluorescence (2).
[c] No intestinal pathogens detected.

jugated goat anti-human immunoglobulin G (INOVA Diagnostics, Inc., San Diego, Calif.) was added to the smears, and the smears were incubated for 30 min at 37°C. Acute- and convalescent-phase serum samples from each patient were assayed for SARS CoV antibodies in the same experiment to minimize experimental variations. The titer was determined as the highest dilution of serum exhibiting fluorescence of the infected cells. A weakly positive patient serum sample was included as a control in each run. A sample was scored as a positive result if the fluorescent intensity was equal to or higher than that of the positive control.

Determination of the end points of the RT-PCR assays. A 96-well microtiter plate containing 0.1 ml of confluent Vero cells was used to determine the 50% tissue culture infective dose ($TCID_{50}$) of SARS CoV under biohazard level 3 containment conditions. Tenfold serial dilutions of a cell-adapted SARS CoV strain from 10^{-1} to 10^{-8} were prepared. One hundred microliters of each dilution was added to each well of four replicate wells and incubated at 37°C for 2 to 3 days to observe cytopathic effect. $TCID_{50}$s were determined by the Kärber method (1). For the same serial dilutions of virus, 100-μl samples were subjected to RNA extraction, and the end points of the two RT-PCR assays were determined.

RESULTS

Of 303 specimens from clinically suspected SARS cases (Table 2), 145 were positive by one or both PCR assays and more than 87% of PCR-positive samples were identified by both PCR assays. Common respiratory viral pathogens, including influenza virus A and B, parainfluenza virus types 1, 2, and 3, RSV, and adenovirus, were not detected in the 124 nasopharyngeal aspirate specimens. The end point for both WHO-HKU and WHO-Hamburg RT-PCR methods was determined to be 0.1 $TCID_{50}$. The acute-phase serum samples from all patients were seronegative for SARS CoV. Eighty-six patients were confirmed to have SARS CoV infections on the basis of seroconversion. Using seroconversion as the gold standard for SARS diagnosis, the sensitivities of the WHO-HKU and WHO-Hamburg RT-PCR assays were found to be 61 and 68% (nasopharyngeal aspirate specimens), 65 and 72% (throat swab specimens), 50 and 54% (urine specimens), and 58 and 63% (stool specimens). A specificity of 100% was exhibited by both RT-PCR assays, as none of the seronegative patient samples and control samples gave a positive PCR result. Among the 163 patients, two or more respiratory specimens (nasopharyngeal aspirate or throat swab specimens) were available from 41 patients. Of the 41 patients, 28 were subsequently confirmed to have SARS CoV on the basis of seroconversion. In these 28 patients, the numbers of first specimens positive for WHO-HKU and WHO-Hamburg RT-PCR were 18 and 20, respectively, but testing a second specimen increased the overall sensitivity from 64 and 71% to 75 and 79%, respectively.

DISCUSSION

In Hong Kong, SARS is a serious respiratory illness that led to significant morbidity and mortality (3). The diagnosis depends mainly on the clinical findings of an atypical pneumonia not attributed to another cause and a history of exposure to a suspect or probable case of SARS or to the respiratory secretions and other bodily fluids of individuals with SARS. Definitive diagnosis of this novel CoV relies on classic tissue culture isolation, followed by electron microscopy studies to identify the virus on cell culture, which is technically very demanding. Serological testing for increasing titer against SARS-associated CoV was shown to be highly sensitive and specific (8) but was not suitable for rapid laboratory diagnosis.

The rapid isolation and characterization of the novel CoV associated with SARS allowed for the timely development of diagnostic tests (6, 10). RT-PCR protocols of two WHO SARS network laboratories were evaluated for rapid diagnosis of SARS-associated CoV in Hong Kong. The end point for the novel CoV by both RT-PCR assays was similar to the previous finding for human CoV (12), yet sufficient diagnostic sensitivity was not achieved, despite attaining a specificity of 100%. A recent study using real-time RT-PCR revealed that the viral load in nasopharyngeal aspirate specimens peaked in the second week of the illness (9). Results indicated a more sensitive RT-PCR assay is essential for rapid diagnosis of SARS CoV during the early stage of disease. Due to the nature of respiratory specimens with inconsistent pathogen loads at various sample times, testing of multiple specimens has been shown to increase the sensitivity of laboratory diagnosis for *Mycobacterium tuberculosis* (7). Testing a second respiratory specimen by RT-PCR increased the sensitivity of diagnosis for SARS CoV.

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The examination of more than one respiratory specimen is necessary to maximize the sensitivity of RT-PCR assays for SARS CoV. As molecular characterization of this novel CoV is ongoing, targeting genomic segments of the virus for diagnostic application is still unclear. Amplification of a second genome region may further increase test specificity. In this study, the high specificity and concordance of both RT-PCR assays verified that the amplified genomic segments for both protocols are suitable for diagnostic application. Incorporation of internal probe hybridization will probably increase the sensitivity of the WHO-HKU RT-PCR assay.

In this global outbreak of SARS, prompt communication and exchange of information among the WHO collaborating laboratories facilitate development of rapid diagnostic assays with shortened turnaround time. The availability of the protocols on the WHO website was helpful to diagnostic laboratories. The collaborative approach can be invaluable in our efforts to understand and control emerging pathogens in the future.

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REFERENCES

1. **Ballew, H. C.** 1992. Neutralization, p. 229–241. *In* S. Specter and G. Lancz (ed.), Clinical virology manual. Elsevier, New York, N.Y.
2. **Chan, K. H., N. Maldeis, W. Pope, A. Yup, A. Ozinskas, J. Gill, W. H. Seto, K. F. Shortridge, and J. S. M. Peiris.** 2002. Evaluation of the Directigen FluA+B test for rapid diagnosis of influenza virus type A and B infections. J. Clin. Microbiol. **40:**1675–1680.
3. **Donnelly, C. A., A. C. Ghani, G. M. Leung, A. J. Hedley, C. Fraser, S. Riley, L. J. Abu-Raddad, L. M. Ho, T. Q. Thach, P. Chau, K. Chan, T. H. Lam, L. Y. Tse, T. Tsang, S. H. Liu, J. H. B. Kong, E. M. C. Lau, N. M. Ferguson, and R. M. Anderson.** 2003. Epidemiological determinants of spread of causal agent of severe acute respiratory syndrome in Hong Kong. Lancet **361:**1761–1766.
4. **Drosten, C., S. Günther, W. Preiser, S. van der Werf, H.-R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. M. Fouchier, A. Berger, A.-M. Burguière, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J.-C. Manuguerra, S. Müller, V. Rickerts, M. Stürmer, S. Vieth, H.-D. Klenk, A. D. M. E. Osterhaus, H. Schmitz, and H. W. Doerr.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1967–1976.
5. **Lee, N., D. Hui, A. Wu, P. Chan, P. Cameron, G. M. Joynt, A. Ahuja, M. Y. Yung, C. B. Leung, K. F. To, S. F. Lui, C. C. Szeto, S. Chung, and J. J. Sung.** 2003. A major outbreak of severe acute respiratory syndrome in Hong Kong. N. Engl. J. Med. **348:**1986–1994.
6. **Marra, M. A., S. J. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, G. S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T. F. Booth, D. Bowness, M. Drebot, L. Fernando, R. Flick, M. Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G. A. Tipples, S. Tyler, R. Vogrig, D. Ward, B. Watson, R. C. Brunham, M. Krajden, M. Petric, D. M. Skowronski, C. Upton, and R. L. Roper.** 2003. The genome sequence of the SARS-associated coronavirus. Science **300:**1399–1404.
7. **Nelson, S. M., M. A. Deike, and C. P. Cartwright.** 1998. Value of examining multiple sputum specimens in the diagnosis of pulmonary tuberculosis. J. Clin. Microbiol. **36:**467–469.
8. **Peiris, J. S. M., S. Lai, L. L. M. Poon, Y. Guan, L. Yam, W. Lim, J. Nicholls, W. Yee, W. Yan, M. Cheung, V. Chen, K. H. Chan, D. Tsang, R. Yung, T. Ng, and K. Y. Yuen.** 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet **361:**1319–1325.
9. **Peiris, J. S. M., C. M. Chu, V. C. Cheng, K. S. Chan, I. F. Hung, L. L. Poon, K. I. Law, B. S. Tang, T. Y. Hon, C. S. Chan, K. H. Chan, J. S. Ng, B. J. Zheng, W. L. Ng, R. W. Lai, Y. Guan, K. Y. Yuen, and HKU/UCH SARS Study Group.** 2003. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. Lancet **361:**1767–1772.
10. **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rassmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, and W. J. Bellini.** 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300:**1394–1399.
11. **Tsang, K. W., P. L. Ho, G. C. Ooi, W. K. Yee, T. Wang, M. Chan-Yeung, W. K. Lam, W. H. Seto, L. Y. Yam, T. M. Cheung, P. C. Wong, B. Lam, M. S. Ip, J. Chan, K. Y. Yuen, and K. N. Lai.** 2003. A cluster of cases of severe acute respiratory syndrome in Hong Kong. N. Engl. J. Med. **348:**1977–1985.
12. **Vabret, A., F. Mouthon, T. Mourez, S. Gouarin, J. Petitjean, and F. Freymuth.** 2001. Direct diagnosis of human respiratory coronaviruses 229E and OC43 by the polymerase chain reaction. J. Virol. Methods **97:**59–66.
13. **World Health Organization.** 2003. Severe acute respiratory syndrome (SARS). Wkly. Epidemiol. Rec. **78:**86–87.

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