

Coupling multiplex RT-PCR to a gene chip assay for sensitive and semiquantitative detection of severe acute respiratory syndrome-coronavirus

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An early and accurate diagnostic assay for severe acute respiratory syndrome (SARS) is crucial for infection control. However, most of the diagnostic methods available today, such as real-time reverse transcriptase-polymerase chain reaction (RT-PCR), require a second detection method for confirmation because they detect a single sequence region of the SARS-coronavirus (SARS-CoV). For sensitive and accurate early diagnosis, we report a novel assay system combining multiplex RT-PCR and a diagnostic gene chip to detect multiple virus-specific genomic sequences of SARS-CoV. With 53 clinical specimens, we successfully demonstrate that this technique offers not only a high-accuracy diagnosis for early SARS infection but also a semiquantitative assay. *Laboratory Investigation* (2004) 84, 1085–1091, advance online publication, 14 June 2004; doi:10.1038/labinvest.3700136

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Severe acute respiratory syndrome (SARS) is a novel worldwide infectious disease, caused by a newly identified coronavirus, SARS-CoV.^{1–3} As this disease is rapidly spreading with a relatively high mortality rate (close to 10%), and tends to spread within hospitals or communities, accurate diagnosis in the early course of illness and isolation of suspect cases for appropriate clinical care becomes the best strategy for the control of possible recurrent SARS outbreaks.⁴

However, the viral dynamics and pathogenesis of SARS-CoV *in vivo* has mostly remained unknown. It is believed that the incubation period of SARS can range from 2 to 16 days before the onset of symptoms, and the viral load during the first week of the illness is low.⁵ Therefore, the disease stage of

the patient may greatly affect the diagnostic accuracy of a chosen assay. Serological testing has been shown to be very reliable for confirmation of the diagnosis of SARS-CoV. To date, there are two prevailing serological diagnostic methods, enzyme-linked immunosorbent assays (ELISA)^{6,7} and indirect immunofluorescence assays (IFA),^{6,8} which are very suitable for the SARS diagnosis while the virus titer is relatively high in convalescent phase.³ For early diagnosis, which is critical for the control of disease spread, real-time reverse transcriptase-polymerase chain reaction (RT-PCR) becomes the prevailing option because of its higher sensitivity for the detection of a lower virus titer in the early infection.⁹ Nonetheless, this PCR-based assay only identifies one region of the SARS-CoV sequence per reaction. As suggested by the WHO (<http://www.who.int/csr/sars/diagnostictests/en/>) and the Centers for Disease Control and Prevention, USA (<http://www.cdc.gov/ncidod/sars/labdiagnosis.htm>), the detection of more than one genome region with different primer sets (or by another diagnostic method) is required to confirm the SARS infection. To overcome this limitation, the multiplex RT-PCR

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(MRT-PCR) seems to be a good alternative because it can simultaneously detect multiple target sequence regions of a pathogen genome in one assay. For several respiratory infectious diseases, this method has been reported useful in detecting viruses,¹⁰ such as influenza,^{11–13} parainfluenza,^{14,15} and respiratory syncytial viruses (RSV).^{12,15} However, some practical problems, including poor sensitivity and specificity, limit this method as a general diagnostic tool.

As gene chip technology requires hybridization with its sequence-specific probes to allow for specific and sensitive detection of targets, it is emerging as a powerful tool for the diagnosis of infectious pathogens.¹⁶ Gene chip protocols can also be coupled with PCR amplification technique to increase the sensitivity of diagnosis. Here, we show a complementary method, which combines the advantages of MRT-PCR and gene chip systems for the SARS early diagnosis.

Materials and methods

Extraction of Viral RNA

Viral RNA was extracted from the supernatant fraction of viral culture medium and its concentration was determined using the RealArt™ HPA-Coronavirus LC RT-PCR Reagents (Artus, USA) according to the manufacturer's instructions. The titer of calibrated viral RNA was adjusted to 1.0×10^6 copies/ μ l as RNA stock. To create a set of

RNA standards, the viral RNA stock was serially diluted in a 10-fold manner to give various concentrations ranging from 10^6 to 10^0 copies/ μ l. To isolate viral RNA from clinical specimens, throat swabs of clinical cases were collected by contracted hospitals of the Center for Disease Control, Taiwan during May 2003. Viral RNA was extracted from 200 μ l of viral transport medium with the QIAamp Viral RNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions and eluted in 50 μ l of RNase-free water, and frozen at -70°C until use.

Gene Chip Design

Three virus-specific probes (SCP1, SCP2, and SCP3) for SARS-CoV were designed (Table 1) by alignment of genomic sequences of several documented strains of SARS-CoV to decide on three distinct 60-nucleotide sequence regions so that each of them is conserved through SARS-CoV strains, while unique among genomic sequences of all species available in GenBank database at NCBI. In addition to the three SARS-CoV-specific probes, we also designed three control probes (RTCP, HCP, and NCP) for RT-PCR, hybridization, and negative control, respectively (Table 1). Each of the six synthetic 60-mer oligonucleotides was dissolved in spotting solution and spotted onto CMT-GAPS™ slide (Corning, USA) in triplicate.

Table 1 Sequence-specific probes and primers used for the gene chip-based detection of SARS-CoV

Fragment	Polarity ^a	Sequence (5'–3')	Length (mer)	Product (bp)
<i>Probe^b</i>				
SCP1	S	TGGCTGTAGTTGTGACCAACTCCGCGAACCCTTGATGCAGTCTGCGGATGCATCAACGTT	60	
SCP2	S	ACTCTGTGCTCTACAACCTCAACATTTTTTCAACCTTTAAGTGCTATGGCGTTTCTGCCA	60	
SCP3	S	GGTGGTGAAACTGCCCTCGCGCTATTGCTGCTAGACAGATTGAACCACTTGAGAGCAAAA	60	
RTCP	S	GGTCCGAAGGTTTACCACATCACTGGCTCGCCTCTCAACGATTTCCGTAACCTTTGGCCCC	60	
HCP	S	AGCATTCGGAGTAACTCCTCAACCTGGAGTCCACCTGAAGAAGCAGGGGCCGCGGTAGC	60	
NCP	S	TGTGCTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCCTTTTA	60	
<i>Primer^c</i>				
SC1	S	AGAAACACAGTCTGTACCGT	20	183
	A	AAGCCCTGTAGACGACATCA	20	
SC2	S	AAATTCCCTTCTGTCTATGC	20	478
	A	GGTAGCCAATGCCAGTAGTG	20	
SC3	S	GCAGTCAAGCCTCTTCTCGC	20	228
	A	ATGCCTCAGCAGCAGATTTC	20	
RTC	S	TGAAAACATCCCTCTCATCG	20	616
	A	GACCCAACCAAGATCATCGC	20	
HC	S	GTAAGTCCATCGGTCCATAC	20	141
	A	GAGTACCAAACCAAGGATAC	20	
NC	S	CCTCCGGCCCCCTGAATGCGG	20	154
	A	ATTGTCACCATAAGCAGCCA	20	

^aS, sense strand; A, antisense strand.

^bSCP, SARS-CoV probe; RTCP, RT-PCR control probe of the Arabidopsis *GA4* gene; HCP, hybridization control probe of the plant chloroplast *rbcl* gene; NCP, negative control probe of the Picornavirus 5'-UTR region.

^cSC, SARS-CoV; RTC, RT-PCR control; HC, hybridization control; NC, negative control.

Multiplex Reverse Transcriptase-Polymerase Chain Reaction (MRT-PCR)

To produce cDNA from viral RNA, a mixture of 1 μ l extracted viral RNA and 10 pmol of each primer (Table 1) in final 10 μ l of solution was heated to 70°C for 5 min and then cooled down on ice for 3 min and then added with 10 μ l of reaction mixture (4 μ l M-MLV reverse transcriptase reaction buffer (5 \times ; Promega Inc., USA), 1 μ l dNTP mix (10 mM each), 0.5 μ l M-MLV reverse transcriptase (100 U; Promega Inc., USA), 0.5 μ l RNasin ribonuclease inhibitor (20 U; Promega Inc., USA), and 4 μ l RNase-free water) for reverse transcription at 42°C for 30 min.

The PCR reactions were performed by mixing 20 μ l of cDNA from previous reverse transcription with 80 μ l of reaction mixture (10 μ l 10 \times PCR reaction buffer, 1 μ l *Taq* DNA polymerase, 1 μ l dNTP mix (10 mM), and 68 μ l distilled water) and amplified under the following conditions: an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s, and final extension of 72°C for 10 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., USA) and eluted with 50 μ l of distilled water.

Fluorescent Dye Labeling of RT-PCR Products

The eluted cDNAs yield from RT-PCR were added to 10 μ l of the reverse primer mixture (10 pmol) (Table 1), and denatured at 95°C for 3 min. After cooling down on ice for 3 min, a 40 μ l of labeling reaction mixture (10 μ l Klenow fragment buffer (10 \times ; Takara Bio Inc., Japan), 0.5 μ l Klenow fragment (2 U; Takara Bio Inc., Japan), 1 μ l dUTP mix (1 mM dATP, 1 mM dCTP, 1 mM dGTP, and 0.5 mM dTTP), 0.5 μ l Cy5-dUTP (1 mM; Amersham Biosciences Inc., USA), and 28 μ l distilled water) was added to the denatured cDNAs and incubated at 37°C for 30 min for the Cy5 labeling. The labeled probes were purified using the QIAquick PCR Purification Kit (Qiagen Inc., USA), eluted with 40 μ l of distilled water, and then dried by Eppendorf® Vacufuge™ Concentrator.

Gene Chip Hybridization and Image Acquisition

The gene chip was first blocked with prehybridization solution (25% formamide, 5 \times SSC, 0.1% SDS, and 1% BSA) at 42°C for 45 min. The Cy5-labeled cDNA was resuspended in 6 μ l of hybridization solution (25% formamide, 5 \times SSC, 0.1% SDS, 0.5 mg/ml of oligonucleotide, 0.5 mg/ml of yeast tRNA, and 0.5 mg/ml of salmon sperm DNA) and denatured in boiling water for 3 min. After cooling down to room temperature, labeled cDNA was hybridized to a gene chip. The gene chip was hybridized at 42°C for 1 h, and then washed

sequentially with a primary solution (2 \times SSC and 0.1% SDS) at 42°C for 10 min, a second solution (0.1 \times SSC and 0.1% SDS) at room temperature for 10 min, and a final solution (0.1 \times SSC) at room temperature for 10 s. After a 100% ethanol alcohol rinse, the gene chip was dried with compressed air. An image of the gene chip was obtained by using a laser confocal slide scanner (GenePix® 4000B) and was analyzed by using GenePix® Pro software (Axon Instruments Inc., USA).

Results

Designs of Primers and Probes

To optimize the diagnostic sensitivity of early infection while the viral load in clinical specimens is still low, we amplified the copy number of virus-specific sequences by RT-PCR with specific primer pairs before hybridization detection by gene chip analysis. By careful selection of primer pairs corresponding to different regions of the viral genome (as listed in Table 1), specific and efficient amplicons with expected sizes were detected while no nonspecific band was found by gel electrophoresis (data not shown). The RT-PCR products were labeled by fluorescent dye as described in 'Materials and methods' and hybridized to the SARS-CoV gene chip. After hybridization, the fluorescent intensities of probes were determined using a laser confocal scanner. The amplified cDNA targets, including SARS-CoV and control sequences, were specifically hybridized to their corresponding probes without noticeable cross-hybridization background (Figure 1a).

To further evaluate the dynamic range of detection by the gene chip assay, serial 10-fold dilutions of calibrated SARS-CoV RNA (10⁶ copies/ μ l) were used as templates for RT-PCR amplification. The signal intensity of each probe was extracted from the gene chip for analysis. Figure 1b shows the signal intensity of individual probe plotted against viral RNA input. Notably, the SCP1 and SCP3 probes are capable of detecting the PCR products with as few as a theoretically single copy of starting SARS-CoV RNA. In contrast, the SCP2 appears to be less efficient in detecting low titer virus (<10² copies), suggesting that the design of the SC2 primer pair for RT-PCR amplification and/or the SCP2 probe for hybridization was not optimized for detection of trace virus. On the other hand, we also noted that the fluorescent signals of SCP1 and SCP3 plateaued early at low virus titer, whereas that of SCP2 seems to exert a wider linear dynamic range; a result that suggests that SCP2 is valuable for quantitative assay. In the same chip, we also noted that the RTCP control probe showed invariant signal magnitude to the constant input of control target (Figure 1c), thus making it a useful normalization standard for semiquantification assay.

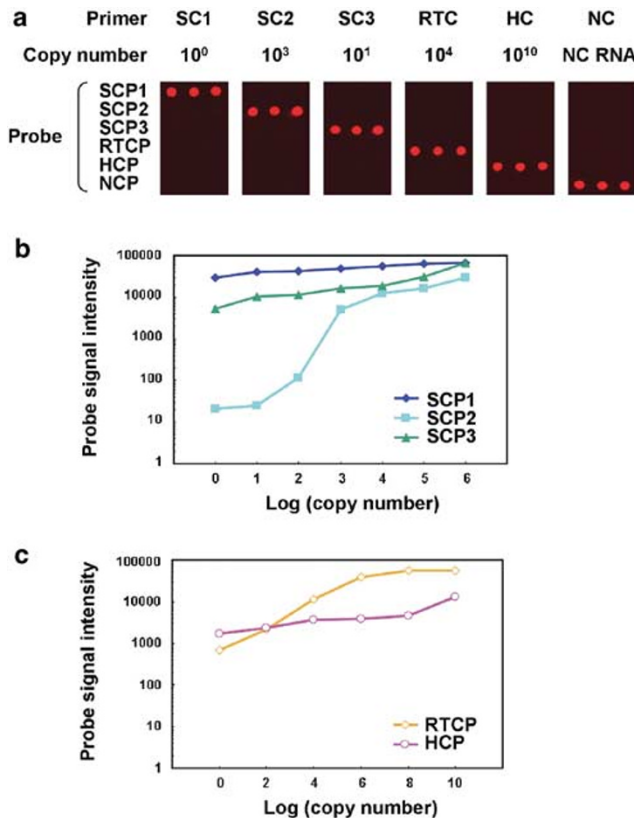


Figure 1 (a) Specific RT-PCR products were labeled with Cy5 and hybridized to the probes on the SARS-CoV gene chip. No background cross-hybridization was detected. SCP, SARS-CoV probe; RTCP, RT-PCR control probe of the Arabidopsis *GA4* gene; HCP, hybridization control probe of the plant chloroplast *rbcl* gene; NCP, negative control probe of the Picornavirus 5'-UTR region; SC, SARS-CoV primers; RTC, RT-PCR control primer; HC, hybridization control primer; NC, negative control primer. The dynamic range of sensitivities in detecting specific RT-PCR products by three SARS-CoV-specific probes (b) and two control probes (c).

Optimization of MRT-PCR for Gene Chip Assay

Given the fact that the specific RT-PCR amplification is compatible with the gene chip detection of SARS-CoV, we tested the feasibility of coupling MRT-PCR to the same gene chip assay. We first optimized the concentration of each primer set and RT-PCR conditions to provide highly efficient RT-PCR amplification in multiplex reactions with four primer sets. Consistent with the single RT-PCR result, the agarose gel electrophoresis analysis revealed that all but SC2 were markedly amplified (Figure 2a). The products of MRT-PCR were then labeled with fluorescent dye for the chip-based detection. The result showed that both viral and control probes were specifically hybridized to their corresponding probes (Figure 2b). In particular, the SC2 PCR amplicon, which was undetectable by the agarose gel electrophoresis (Figure 2a), became detectable by the SCP2 probe in the gene chip (Figure 2b). Clearly, the gene chip assay is more

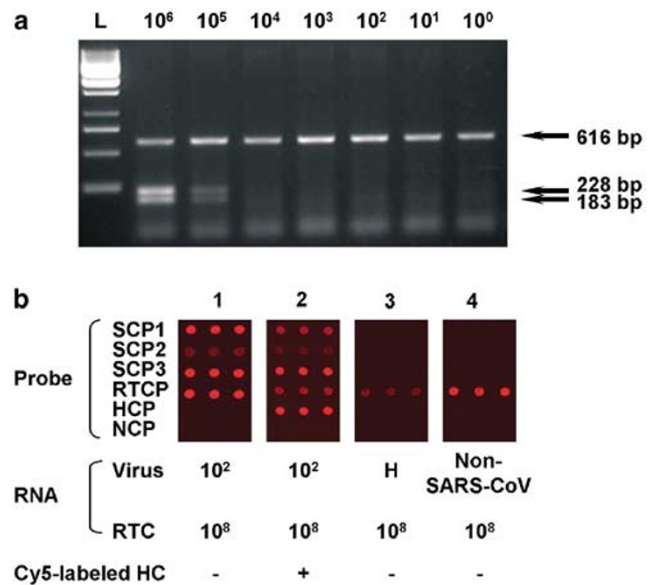


Figure 2 Coupling MRT-PCR and gene chips for detection of SARS-CoV. (a) Quadruplex RT-PCR was carried out with three virus-specific plus one RT-PCR control primer sets, serial dilutions of SARS-CoV were used as templates and amplicons were analyzed by agarose gel electrophoresis. The 616, 228, and 183 bp amplicons represent RTC, SC3, and SC1 PCR products, respectively. (b) Specific fluorescent signals on a gene chip hybridized with the Cy5-labeled MRT-PCR products; chip #1 without hybridization control (HC); chip #2 with HC; chip #3 is a negative control with only human RD cell RNA (H); chip #4 is a negative control with non-SARS-CoV RNA (non-SARS-CoV).

sensitive than conventional agarose gel electrophoresis in detecting MRT-PCR products of trace SARS-CoV.

Diagnostic Accuracy of Clinical Samples

The potential application of this chip-based assay for clinical diagnosis of SARS-CoV infection was evaluated with clinical specimens. In this study, throat swabs were randomly selected from 53 different patients who were either suspect or probable SARS cases in the Center for Disease Control, Taiwan. The definition of suspect or probable cases relies upon WHO criteria (<http://www.who.int/csr/sars/casedefinition/en/>). For example, a suspect case of SARS has high fever (> 38°C), symptoms of cough or breathing difficulty, and has history of exposure or contact with person who is a suspect or probable case of SARS. In our study, the probable cases, which may represent people who had similar symptoms with those of suspect cases except the history of contact is not clear, were also included in the sampling pool. All cases were tested in single-blinded manner. In addition to chip-based assay, all specimens were analyzed in parallel by single-round RT-PCR (Artus), real-time nested PCR,¹⁷ and subsequently confirmed

by seroconversion unless convalescent sera were not available due to the fatal cases or sera were only collected at acute phase.

Table 2 summarizes the accuracy of different diagnostic methods. Both the chip-based assay and real-time nested PCR detected 18 positive cases in 53 clinical specimens. In contrast, only 15 cases were identified as SARS-CoV-positive and three were missed by the single-round RT-PCR. Interestingly, we determined that these three missed samples contained <10 copies of viral genome in the test of real-time nested PCR, suggesting that the gene chip is a sensitive method to detect SARS-CoV in early infection. For those three cases missed by single-round RT-PCR (Artus) and another eight positive cases (total 11 out of 18 positive cases), infection was confirmed by seroconversion (Table 2).

A Semiquantitative Method by Gene Chip

Quantitation of viral load in clinical samples has important implications for treatment strategies. To establish a semiquantitative model for the gene chip assay of SARS-CoV, a serial 10-fold dilution of SARS-CoV RNAs was amplified by MRT-PCR and hybridized to gene chips (Figure 3a). The dynamic range of signal magnitude of each probe to different

viral RNA input was shown by plotting the signal intensity extracted from each probe on the gene chip against the copy number of viral RNA (Figure 3b). The results reveal that three viral probes exhibited distinct linear ranges of sensitivity for detection of viral RNA concentration.

Based on the observed signals of those viral and control probes, statistical analysis was undertaken to establish a quantitative model. The average signal intensity of RTCP control in each gene chip was first adjusted with respect to the average of the RTCP controls of all gene chips, and the average intensities of SCP_{*i*} (*i*=1,2,3) on each gene chip were then normalized according to the adjusted average intensity of RTCP control. Using the logarithm of base 10, we denote the copy number of virus as *V*, and the normalized average intensity of SCP_{*i*} as *F*_{SCP_{*i*}} (*i*=1,2,3). We proposed the following quantitative model for evaluation of viral load:

$$V = a_0 + a_1 \times F_{SCP1} + a_2 \times F_{SCP2} + a_3 \times F_{SCP3} \quad (1)$$

which transforms the normalized average signal intensities of SCP_{*i*} (*i*=1,2,3) into copy number of virus. Using least-squares method, the coefficients of *a*₀, *a*₁, *a*₂ and *a*₃ were estimated based on the data generated from the controlled hybridization results of titration SARS-CoV. The quantitative model can be represented as the following equation:

$$V = -4.14 + 0.69F_{SCP1} - 0.05F_{SCP2} + 1.03F_{SCP3} \quad (2)$$

The value of *R*² is 0.91, which indicates that the normalized average intensities of SCP_{*i*} (*i*=1,2,3), could explain, for the most part, the variation of

Table 2 Comparison of 53 diagnoses of suspected or probable cases of SARS by different assays

Test	Positive results ^a		Negative results	Total
	> 10 copies per test	< 10 copies per test		
SARS-CoV gene chip				
No. of results	9	9	35	53
Seroconversion ^b	6	5	0	11
Real-time nested PCR^c				
No. of results	10	8	35	53
Seroconversion ^b	7	4	0	11
Single-round RT-PCR^d				
No. of results	10	5	38	53
Seroconversion ^b	7	1	3	11
Shared results				
No. of results	9 ^e	5	35	49
Seroconversion ^b	6	1	0	7

^aResults of four independent tests.

^bData are number of samples obtained from patients for whom seroconversion was also noted, by ELISA and neutralizing antibody responses for available serum samples obtained during the convalescent phase of illness (ie, > 28 days after onset of illness).

^cResults obtained by real-time nested PCR.¹⁷

^dResults obtained by hybridization probe-based detection using the RealArt HPA-coronavirus RT-PCR kit (Artus).

^eA total of nine samples were detected in the '>10 copies per test' with the gene chip method, and 10 samples with both nested and single-round PCR methods, because the virus copy number in case 18 was underestimated (see Table 3).

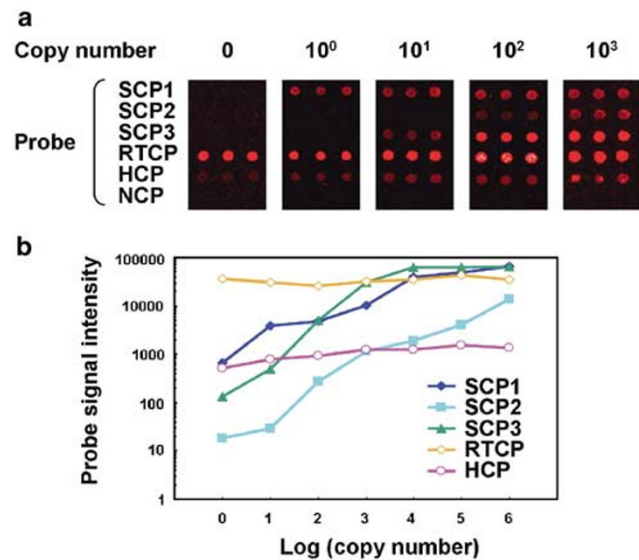


Figure 3 Range of detection sensitivities of three virus-specific probes on the gene chip. (a) Differential hybridization signal intensities of three independent SARS-CoV probes were detected using a serial dilution of viral RNA input. (b) Hybridization signal of each individual probe was plotted against the log concentration of viral RNA; order of detection sensitivity: SCP1 > SCP3 > SCP2.

viral titers. In practice, we can predict the copy number of SARS-CoV in clinical samples by this model.

Accordingly, we tested the model with gene chips hybridized with 18 SARS-CoV-positive clinical samples. Table 3 shows that the gene chip estimates of viral RNA concentration correlates highly with the result determined by real-time nested PCR. Plots of chip-based semiquantitation and real-time nested PCR quantitation also showed good correlation (Figure 4, correlation coefficient = 0.92). Thus, the

Table 3 Quantitative analysis of viral copy number in clinical specimens by gene chip and real-time nested PCR

Case no.	Chip image	Estimation of viral copy number	
		By gene chip ^a	By real-time nested PCR ^b
1		1241	609
2		273	750
3		2	1
4		32	18
5		42	52
6		123	869
7		981	1360
8		2	3
9		5653	2889
10		1	7
11		1	4
12		6	1
13		10	7
14		6	1
15		1407	1268
16		50	41
17		2	2
18		5	18

^aThe viral copy number estimated by gene chip assay equals to 10^V ; $V = -4.14 + 0.69F_{SCP1} - 0.05F_{SCP2} + 1.03F_{SCP3}$.

^bThe vial quantitation was performed as described previously.¹⁷

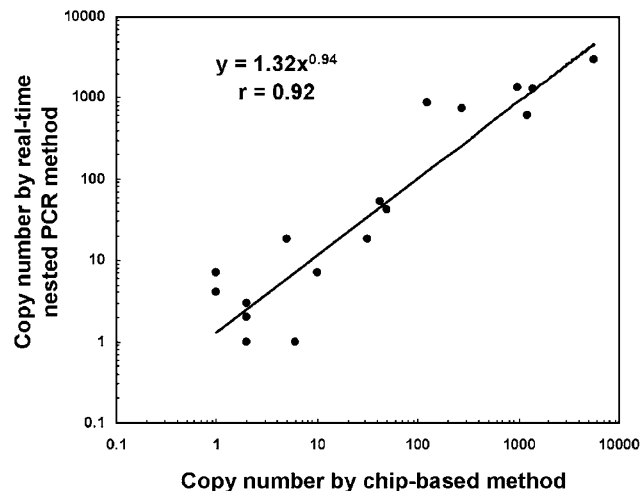


Figure 4 Correlation between virus titers determined by the chip-based assay and by a real-time nested PCR method. Viral RNA concentration was determined by gene chip and plotted against those by real-time nested PCR. The correlation coefficient = 0.92.

chip-based assay not only provides a qualitative but also a semiquantitative diagnosis of SARS-CoV in clinical specimens.

Discussion

In the present study, we report a chip-based assay for early SARS-CoV infection. We tested 53 clinical specimens and demonstrate that the gene chip can accurately detect SARS-CoV. This assay is highly specific and sensitive and is applicable to clinical samples. Our findings suggest several advantages over conventional single-round real-time RT-PCR. First, the chip-based assay is highly sensitive for detection of trace virus in early infection. We have shown that the detection sensitivity of chip-based assay (<10 copies of SARS-CoV) is better than, or at least comparable to, the single-round real-time RT-PCR method for diagnosis of clinical specimens. Second, the gene chip-based diagnosis is highly specific. It requires hybridization with multiple sequence-specific probes (three virus-specific and three control probes on our gene chip) to allow for specific detection of virus targets of more than one genome region. Therefore, the gene chip assay not only specifically identifies the SARS-CoV but also simultaneously confirms the diagnosis in one assay, thus reducing the false-positive and false-negative results. In contrast, conventional single-round real-time RT-PCR can only detect one virus-specific sequence per reaction without stringent hybridization with probes. Third, this assay is simple and rapid. The entire assay, including MRT-PCR and chip-based assays, can be easily completed within 6 h, including a 3-h MRT-PCR followed by a 3-h gene chip analysis, thereby making it a clinically applicable alternative for screening of SARS-CoV in laboratories that are not equipped with a real-time PCR instrument. Although this diagnosis method requires a 3-h post-PCR process for gene chip detection, it is a more time-efficient and cost-effective assay, because this gene chip can concurrently identify the virus target and confirm the diagnosis result. Last and perhaps most importantly, one may expand the present gene chip to simultaneously detect other related coronaviruses or multiple respiratory pathogens in one assay. SARS is an acute pneumonic illness and is clinically difficult to distinguish from other types of atypical pneumonia in the absence of a clear epidemiological link to other patients with the disease. The major advantage of gene chip diagnosis is that one can add to this system a large number of alternative pathogens responsible for community-acquired pneumonia with little modification.

On the other hand, it seems clear that, if MRT-PCR primers of sufficiently broad utility cannot be designed, the extension of this technology to simultaneously detect other related pathogens will be seriously hampered because only high efficiency

of PCR amplification can provide maximum sensitivity for detection of low titer RNA in clinical specimens. We are currently expanding the content of this gene chip to permit detection of a greater selection of viral and other pathogens.

For the semiquantitative assay by gene chip, we designed three virus-specific probes and the corresponding primer sets for a diverse range of sensitivity in detecting SARS-CoV (Figure 3). A semiquantitation model was successfully established and validated using clinical specimens to resolve the relative virus copy numbers. Taken together, our data suggest that this gene chip assay system can be ready for clinical application to detect, confirm, and semiquantify the virus in early SARS infection.

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