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The design and application of DNA chips for early detection of SARS-CoV from clinical samples

Yi-ming Zhou[a,1], Ren-quan Yang[b,1], Sheng-ce Tao[a,1], Ze Li[c,d], Qiong Zhang[c,d], Hua-fang Gao[a], Zhi-wei Zhang[a], Jian-yu Du[c,d], Pei-xuan Zhu[c,d], Li-li Ren[b], Liang Zhang[c,d], Dong Wang[a], Lan Guo[b], Yan-bin Wang[b], Yong Guo[a], Yan Zhang[a], Chuan-zan Zhao[c,d], Can Wang[c,d], Di Jiang[c,d], Yan-hua Liu[c,d], Hua-wei Yang[c,d], Li Rong[c,d], Yan-jun Zhao[c,d], Shuang An[c,d], Zhe Li[c,d], Xiao-dong Fan[c,d], Jian-wei Wang[b,**], Yun Cheng[e], Ou Liu[f], Zhong Zheng[g], Huan-cong Zuo[g], Quan-zhong Shan[f,***], Li Ruan[b], Zhan-xiu Lü[e], Tao Hung[b], Jing Cheng[a,c,d,*]

[a] *State Key Laboratory of Biomembrane and Membrane Biotechnology, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China*

[b] *Institute of Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100052, China*

[c] *National Engineering Research Center for Beijing Biochip Technology, 18 Life Science Parkway, Changping District, Beijing 102206, China*

[d] *CapitalBio Corporation, 18 Life Science Parkway, Changping District, Beijing 102206, China*

[e] *No. 302 Hospital of the People's Liberation Army, Beijing 100039, China*

[f] *Tsinghua University Jiuxianqiao Hospital, Beijing 100016, China*

[g] *Tsinghua University Yuquanlu Hospital, Beijing 100039, China*

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Abstract

Background: SARS coronavirus has been identified as the cause of severe acute respiratory syndrome (SARS). Few tests allow confirmation or exclusion of SARS within the first few days of infection. A gene chip is a useful tool for the study of microbial infections mainly for its capability of performing multi-target analysis in a single test.

Objectives: Investigate the possibility of early detection of SARS virus from clinical samples using the gene chip-based method.

Study design: We purified RNA from SARS-CoV obtained from routinely collected peripheral blood and sputum samples of 34 patients who had been identified as probable SARS patients by following the interim U.S. case definition. Four segments of the SARS-CoV were amplified using reverse transcription-nested PCR and the products examined using the 70-mer gene chips for SARS-CoV detection.

Results: A blind-test of both peripheral blood and sputum specimens lead to the positive detection of SARS-CoV in 31 out of 34 patients. SARS-CoV was not found in peripheral blood or sputum specimens from three patients. Two of the 34 patients were only 3 days post-onset of symptoms and were subsequently confirmed to be SARS positive. Our results indicate that the gene chip-based molecular test is specific for SARS-CoV and allows early detection of patients with SARS with detection rate about 8% higher than the single PCR test when the sputum sample is available.

Keywords: SARS; Coronavirus; SARS-CoV; Early detection; Gene chip

Abbreviations: SARS-CoV, severe acute respiratory syndrome-coronavirus; HEX, hexa-chloro-6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine

* Corresponding author. Tel.: +86 10 62772239; fax: +86 10 62773059.

** Co-corresponding author. Tel.: +86 10 63581580; fax: +86 10 63529809.

*** Co-corresponding author. Tel.: +86 10 64361322; fax: +86 10 64361322.

E-mail addresses: wangjw@vip.sina.com (J.-w. Wang), brma@public.bta.net.cn (Q.-z. Shan), jcheng@tsinghua.edu.cn (J. Cheng).

[1] These authors equally contributed to the article.

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1. Introduction

Severe acute respiratory syndrome (SARS) is a newly emerged disease associated with pneumonia in infected patients (World Health and Organization, 2003). From November 2002 to June 3, 2003, SARS virus infected 8398 people and caused 772 deaths worldwide (www.who.int/csr/sars/country/2003-06-03/en/, accessed July 2003). The genome of the SARS-CoV has been sequenced (Marra et al., 2003; Ruan et al., 2003). The earlier that a case of SARS virus is detected, the less chance there is that a patient will spread the disease to others. Various serological and molecular tests have been developed for the detection of this virus or to confirm infection. These methods include enzyme-linked immunosorbent assay (ELISA) (Ksiazek et al., 2003), indirect immunofluorescent assay (IFA) and reverse transcription (RT) followed by a real-time PCR using either the TaqMan technique or the intercalating fluorescent dye approach (Drosten et al., 2003; Peiris et al., 2003). As yet, few tests allow confirmation or exclusion of SARS within the first few days of infection. Serological methods cannot close the window period, and are not useful for early detection. Real-time PCR can only detect one target sequence a time, which means that false negative results are possible. The genome of SARS-CoV is a positive ssRNA, which means that, the mRNA sequence of SARS-CoV is same as the genome sequence, hence the copy number of SARS-CoV can not be calculated directly from the quantitative result of real-time PCR. Gene chip provide a useful tool to study microbial infections (Cheng and Kricka, 2001; Wang et al., 2002; Tao et al., 2002) mainly for its capability of performing multi-targets analysis in only one test. Recently, we have reported the use of gene chips for the detection of pathogenic bacteria and viruses in clinically relevant samples or changes in gene expression among drug-treated and untreated microbes (Cheng et al., 1998; Tao et al., 2003; Zhang et al., 2002). In this paper, we report a method for early detection of the SARS virus in clinical specimens from SARS patients. The method combines a convenient sample preparation method, nucleic acid amplification using a modified nested PCR, and a gene chip array with probes selected to cover the representative regions of the SARS-CoV genome.

2. Materials and methods

2.1. Selection of clinical specimens and control samples

Peripheral blood and sputum samples were collected from 34 patients suffering from SARS in Tsinghua University Jiuxianqiao Hospital. These patients were identified as probable SARS patients by following the interim U.S. case definition. The probable SARS case definition is based on clinical criteria and epidemiologic linkage to other SARS cases with radiographic evidence. The negative controls include 100 peripheral blood samples and 100 sputum samples that were collected from university students at Tsinghua who were determined to be free of infection based on radiological and temperature measurements. In the following experiment one negative blood control sample or sputum sample was included with 17 clinical blood samples or sputum samples during the sample pretreatment and extraction stages as a control to monitor any occurrence of contamination that may appear during the sample treatment process. In addition, one blank control using water as template was included with the 17 RT-PCR reactions on clinical samples to control the PCR procedure.

2.2. Design and construction of the gene chip

The sequence data of SARS-CoV were obtained from the curated database in GenBank. The unique and conserved regions of SARS-CoV were selected by align the released SARS-CoV sequences with each other and the latest non-redundant nucleic acid sequence database of NCBI (ftp://ftp.ncbi.nih.gov). To enable the early detection of SARS-CoV, multiple regions from open reading frame (orf) replicase 1a, spike glycoprotein and nucleocapsid protein (Rota et al., 2003) were selected as the target for hybridization. To minimize cross-hybridization, oligonucleotides of 70mer were designed following the rule from http://www.westburg.nl/download/arrayposter.pdf. To increase the immobilization efficiency of the probes, 10 thymidines were added to the 5′ end of each probe.

After an initial screening test, a set of four oligonucleotides (Sangon, Shanghai, China) were chose as the probes for identifying SARS-CoV, see Fig. 1 (a). Additionally, a set of probes for control purposes was also included. The QC probe was used to confirm the efficiency of the attachment chemistry on the surface of the substrate. For all tests this probe should always generate a strong and consistent fluorescence signal. The IC probe was designed to guarantee the sample processing procedure and the entire nested RT-PCR process operates as expected. The EC probe was used to monitor the efficiency of the hybridization process and also as a reference for quantifying the amplicons' hybridization signal. The BC was DMSO spotted on the substrate to ensure no signal detected on these spots indicating to guarantee no carry-over of the previously spotted samples.

The probes were suspended in 50% dimethyl sulfoxide (DMSO) at a concentration of 10 μM and printed on glass slides modified with amino groups (AminoSlide^{TM}, CapitalBio Corp., Beijing, China). The 6×6 arrays was printed according to the spotting pattern (Fig. 1 (b)) in duplicate on each slide. The sequence information of the probes is listed in Table 1. Concerning the current gene chip designing there are fifteen possible combinations for the occurrence of SARS-CoV positive signals. As long as one of the fifteen combinations shown in Fig. 1 (c) is detected the presence of SARS-CoV can be then ensured.

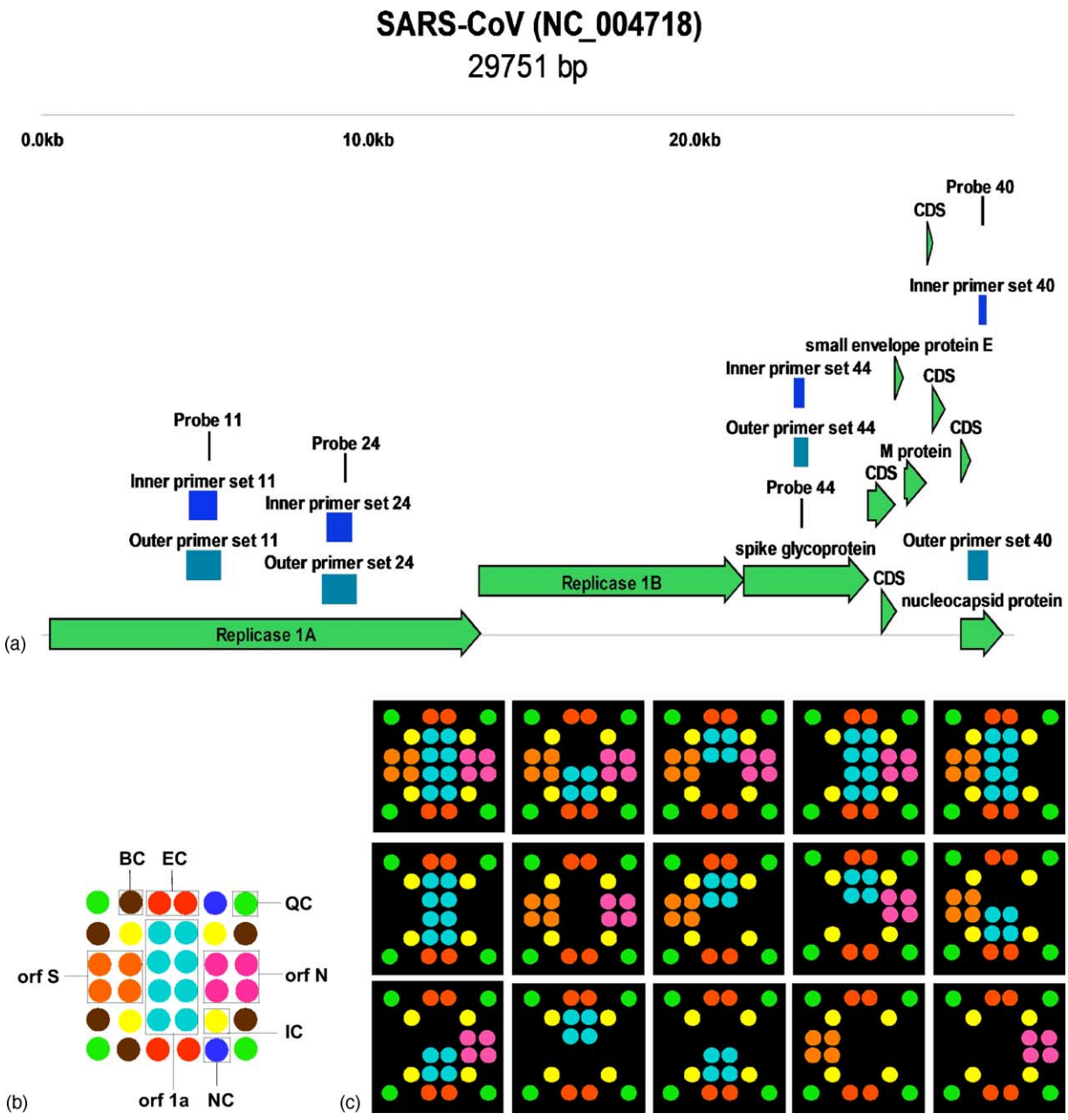

Fig. 1. The selection and design of the probes for SARS-Cov gene chips. (a) The four sets of primers and probes in the open reading frames of SARS-CoV genome. (b) Gene chip design for the detection of SARS-CoV. (c) Fifteen different gene chip signal patterns for detecting the presence of SARS-CoV. BC–DMSO spotted as blank control. QC–Hex-labeled oligonucleotide used for quality control of surface chemistry. IC–internal control probe for nested RT-PCR and hybridization. EC–external control probe for hybridization-based quantitation. NC–negative control probe. 1a–probes selected from SARS-CoV's orf 1a (011, 024). N–the probe selected from the SARS-CoV's orf for nucleocapsid protein (040). S–the probe selected from the SARS-CoV's orf for spike glycoprotein (044).

2.3. Collection and preparation of samples

Fresh EDTA-anticoagulated peripheral blood (1.8 mL) was spun at 1200 G for 10 min. The upper-layered plasma was replaced by a volume of physiological saline solution equivalent to the amount of plasma to resuspend the blood cells. To a 10 mL test tube was added 3.6 mL Ficoll solution followed by the resuspended blood cells. The Ficoll gradient was centrifuged at 220 G for 20 min. Subsequently the lymphocytes at the interface was harvested into a 1.5 mL eppendorf tube and spun at 8000 × *g* for 5 min. When the supernatant was removed, 560 μL of the buffer AVL from the QIAamp Viral RNA Mini Kit (Qiagen, Chatsworth, CA) was added to lyse the pelleted lymphocytes.

The sputum specimen was collected into a 50 mL test tube and kept at −80 °C before processing. The sputum sample was shaken at room temperature with an equal volume of 1% acetylcysteine (Sigma, St. Louis, MO) and 0.9% NaCl at

Table 1
The sequence information of the probes printed on the gene chip

Name	Probe sequences
QC	5′-TTTTTTTTTTTGTCTTCCACCAGGAGTCAGCAGAGTGCTTGG TGCCATAAC-HEX
IC	5′-TTTTTTTTTTCGTCAAGGCTGAGAACGGGAAGCTTGTCATCAATGGAAATCCCATCACCATCTTCCAGGAGCGAGATCCC
EC	5′-TTTTTTTTTTAAAGTTAAAGCAGACCGAAGTGGATTGCGAGTATTTGAAAAGATGTGTTGAGAAATTAACGGAAGAGAA
NC	5′-TTTTTTTTTTAGTGGTGGACCTGACCTGCCGTCTAGAAAAACCTGCCAAATATGATGACATCAAGAAGGTGGTGAAGCAG
011	5′-TTTTTTTTTTCTACGTAGTGAAGCTTTCGAGTACTACCATACTCTTGATGAGAGTTTTCTTGGTAGGTACATGTCTGCTT
024	5′-TTTTTTTTTTTCATAGCTAACATCTTTACTCCTCTTGTGCAACCTGTGGGTGCTTTAGATGTGTCTGCTTCAGTAGTGGC
040	5′-TTTTTTTTTTGAGGTGGTGAAACTGCCCTCGCGCTATTGCTGCTAGACAGATTGAACCAGCTTGAGAGCAAAGTTTCTGG
044	5′-TTTTTTTTTTCACCTGGAACAAATGCTTCATCTGAAGTTGCTGTTCTATATCAAGATGTTAACTGCACTGATGTTTCTAC

300 revolution/min for 30 min. Subsequently 560 μL of the buffer AVL was added to a 1.5 mL eppendorf tube followed by the addition of 140 μL homogenate. Isolation of the total RNA (60 μL elution volume) was accomplished with the QIAamp Viral RNA Mini Kit.

2.4. Nested RT-PCR

To improve the detection limit, a modified nested RT-PCR method was applied. To amplify the four segments from three orfs in the genome of SARS coronavirus, four sets of outer primers and inner primers were selected (Table 2). These four outer primer pairs were designed with Primer3 by setting the optimal T_m to 68 °C and PCR product size between 400 and 1200 bp (Rozen and Skaletsky, 2000). Two universal primers were allowed to bind to the end of the PCR product amplified by the inner primers for efficient labeling of the PCR product. The primers were aligned using BLASTN with the latest non-redundant nucleic acid sequence database to avoid mispriming and aligned with all the released SARS-CoV sequences to ensure the efficient prime. The primers for amplifying the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession: NM_002046) gene from human RNA as the internal control for the entire process namely sample preparation, PCR amplification and chip hybridization, the primers for amplifying the gene (GenBank accession: BT006112) from *Arbidopsis thaliana* as the control for hybridization and the universal primers for incorporating fluorescent dye are listed in Table 2. The above-mentioned primers were synthesized by Sangon.

The reaction conditions for RT-PCR were as follows: the first round reaction of the nested RT-PCR is one step RT-PCR and the second round reaction is the PCR using the product from the first round as the template. The one step RT-PCR kit from TaKaRa (Dalian, China) was used for the first round reaction and the conditions were as follows: The total volume for each reaction was 20 μL including 5 μL total RNA (including human total RNA and viral RNA) from either patient's sample or normal person's sample, or 5 μL diethyl pyrocarbonate treated deionized water as blank control, 1× One Step RNA PCR Buffer, 5 mmol/l $MgCl_2$, 0.8 U/μL RNase Inhibitor, 0.1 U/μL AMV RTase XL, 0.1 U/μL AMV-optimized Taq, 0.5 μmol/L outer primer or IC primers, and 1 mmol/L each deoxynucleoside triphosphate. The reactions were performed on a PTC-225 thermal cycler (MJ Research Inc., Miami, FL). The thermal conditions were as follows: one cycle at 50 °C for 30 min; one cycle at 94 °C for 3 min; 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; one cycle at 72 °C for 10 min. 2× Master mixture (TW-times, Beijing, China) was used for the second round reaction and the conditions was as follows: The total volume for each reaction was 25 μL including 5 μL RT-PCR product from the first round as template, or 21 ng of plasmid p127 as template for EC, or 5 μL deionized water as blank control, 1× master mixture, 0.4 mmol/L dUTP (Sangon), 0.01 U/μL Uracil-DNA Glycosylase (Invitrogen, Carlsbad, CA), 20 ng/mL RNase A (Sigma), 0.2 μmol/l inner primer or EC primers, 1.0 μmol/l universal primers. The reactions were performed on a PTC-225 thermal cycler. The thermal conditions were as follows: one cycle at 37 °C for 10 min; one cycle at 68 °C for 10 min; one cycle at 94 °C for 10 min; 32 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; one cycle at 72 °C for 10 min.

The PCR products were loaded onto 1.2% agarose gel along with 5 μL DL2000 DNA molecular marker (TaKaRa, Dalian, China) and run at 8 V/cm for 30 min, then photographed using a UVP system (Ultraviolet Products, Cambridge, UK).

2.5. DNA hybridization assay

To prepare the hybridization sample, an equal volume of the amplicon (1.7 μL) was removed from each nested RT-PCR reaction and the positive control reaction, respectively, and mixed. Then to the above hybridization sample was added 6 μL of the hybridization solution (7.5× SSC, 12.5× Denhart's solution and 0.5%SDS). The mixture was briefly centrifuged. To denature the double-stranded DNAs, the hybridization sample was heated at 95 °C for 5 min followed by snap chilling on ice for 5 min. The slide with four reaction wells was placed into the hybridization chamber preloaded with 200 μL of double-distilled water. The plastic cover with 4 molded sample-loading holes, SmartCover™ from CapitalBio (Beijing, China), was placed on the top of the slide. The denatured DNA samples were applied to the individual reaction well on the glass slide through the loading

Table 2
The primer sequences

Name	Primer sequence	Amplicon location
Outer primers 11	F: 5′-GCATCGTTGACTATGGTGTCCGATTCT B: 5′-ACATCACAGCTTCTACACCCGTTAAGGT	SARS-CoV orf 1a
Inner primers 11	F: 5′-TCACTTGCTTCCGTTGAGGAGCCGCTTGT CACAATGCCAATT B: 5′-GGTTTCGGATGTTACAGCGTCATCACCAA GCTCGCCAACAGTT	SARS-CoV orf 1a
Outer primers 24	F: 5′-GCTGCATTGGTTTGTTATATCGTTATGC B: 5′-ATACAGAATACATAGATTGCTGTTATCC	SARS-CoV orf 1a
Inner primers 24	F: 5′-TCACTTGCTTCCGTTGAGGTAGCCAGCGT GGTGGTTCATACAA B: 5′-GGTTTCGGATGTTACAGCGTCTCCCGGCA GAAAGCTGTAAGCT	SARS-CoV orf 1a
Outer primers 40	F: 5′-CCTCGAGGCCAGGGCGTTCC B: 5′-CACGTCTCCCAAATGCTTGAGTGACG	SARS-CoV orf N
Inner primers 40	F: 5′-TCACTTGCTTCCGTTGAGGTCCTCATCACG TAGTCGCGGTAATTC B: 5′-GGTTTCGGATGTTACAGCGTGGCTTTTTAG ATGCCTCAGCAGCA	SARS-CoV orf N
Outer primers 44	F: 5′-TTAAATGCACCGGCCACGGTTTG B: 5′-CCAGCTCCAATAGGAATGTCGCACTC	SARS-CoV orf S
Inner primers 44	F: 5′-TCACTTGCTTCCGTTGAGGATGCACCGGC CACGGTTTGTG B: 5′-GGTTTCGGATGTTACAGCGTATGCGCCAA GCTGGTGTGAGTTGA	SARS-CoV orf S
Outer primers IC	F: 5′-ATGGGGAAGGTGAAGGTCGG B: 5′- TGGTGAAGACGCCAGTGGAC	Human GAPDH gene
Inner primers IC	F: 5′-TCACTTGCTTCCGTTGAGGCGTATTGGGC GCCTGGTCAC B: 5′-GGTTTCGGATGTTACAGCGTCCAGCATCG CCCCACTTGAT	
EC primers	F: 5′-TCACTTGCTTCCGTTGAGGATCCAAAGAT CAATCCGCAG B: 5′-GGTTTCGGATGTTACAGCGTCACATCAAAA GTGTGGTCGG	*Arbidopsis thaliana*
Universal primers	F: 5′-TAMRA-TCACTTGCTTCCGTTGAGG B: 5′-TAMRA-GGTTTCGGATGTTACAGCGT	

holes on the plastic cover. Finally, the sealed cartridge was placed in a 55 °C water bath for 3 h. When the hybridization is completed, the slide was removed from the cartridge and then the cover slip removed. The slide was washed sequentially in the pre-warmed (45 °C) washing solution A (2× SSC and 0.2% SDS) for 3 min followed by washing solution B (0.2× SSC) for 3 min. Afterwards, the slide was rinsed in the double-distilled water three times and dried by centrifugation at 110 G for 2 min.

2.6. Laser-induced fluorescence scanning

The slide was scanned using the GenePix 4000B from Axon Instrument (Union City, CA). The scanning conditions were as follows: wavelength: 532 nm, laser power: 33%, pixel size: 10 μm, PMT voltage: 550, brightness: 90 and contrast: 90.

To minimize the effects of nonspecific hybridization and false positive, a threshold ratio of signal-to-noise > 2.5 was implemented, based on the statistical analysis of the hybridization data obtained from 200 negative control samples.

3. Results

There are four different SARS-CoV specific probes immobilized on the gene chip. When any one of the following 15 signal patterns is detected (Fig. 1 (c)) it signifies that the partial sequence of SARS-CoV is present and the sample is considered positive for SARS-CoV.

Fig. 2 shows the DNA electrophoresis results and hybridization images from different controls including blank control using double distilled water as template, negative control using blood from a healthy control and sputum samples, and positive control using the Vero cells inoculated with SARS-CoV. All blank controls and negative controls tested were of negative results whereas the positive control produced the expected positive hybridization results.

Fig. 3 (a–d) show the DNA electrophoresis results and gene-chip hybridization images from a patient's blood (patient 1) and sputum specimens respectively. The blood sample was taken 2 days post-onset of symptoms and the sputum sample taken 3 days post-onset of symptoms. The results show that the sputum specimen is positive for the presence

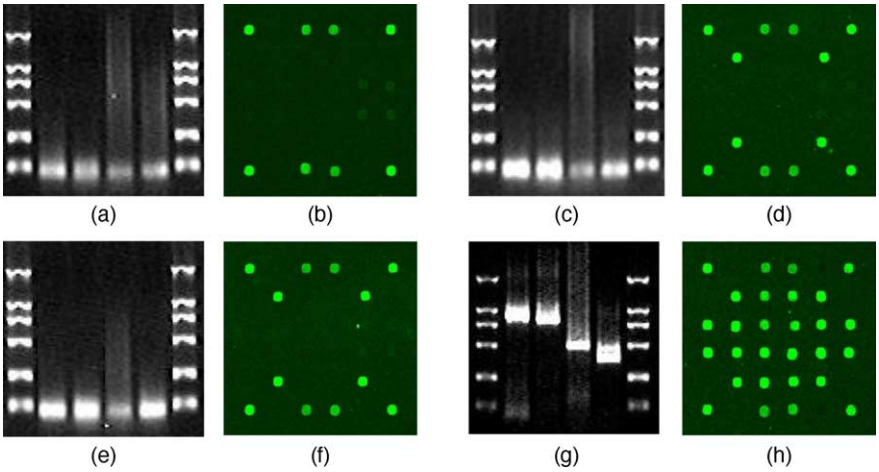

Fig. 2. The DNA electrophoretic and DNA hybridization results obtained from different controls. (a) The electrophoretic result of amplicons generated from double distilled water. Lane 1 and 6 was the molecular weight marker. Lanes 2–5 were amplicons generated using primer sets 11, 24, 40 and 44, respectively (same arrangement for the rest). (b) The hybridization image of amplicons in (a). (c) The electrophoretic result of amplicons produced from the healthy control's blood specimen. (d) The hybridization image of amplicons in (c). (e) The electrophoretic result of amplicons generated from the healthy control's sputum specimen. (f) The hybridization image of amplicons in (e). (g) The electrophoretic result of amplicons produced from the SARS-CoV-infected Vero cells. (h) The hybridization image of amplicons in (g).

Table 3
Comparison of the detection rate with a single probe and the four-combined probes for blood specimens only, sputum specimens only and both

	Probe 11 (%)	Probe 24 (%)	Probe 40 (%)	Probe 44 (%)	Four reactions combined (%)
Blood	0/34 (0)	6/34 17.6	13/34 (38.2)	13/34 (38.2)	19/34 (55.8)
Sputum	8/34 (23.5)	16/34 (47.1)	25/34 (73.5)	22/34 (64.7)	27/34 (79.4)
Blood + sputum	8/34 (23.5)	22/34 (64.7)	28/34 (82.4)	28/34 (82.4)	31/34 (91.2)

of SARS-CoV whereas the blood sample was negative (no signal). Fig. 3 (e–h) show the electrophoretic results and hybridization images from the blood and sputum specimens, respectively, of a second patient (patient 2). The blood sample was taken 2 days post-onset of symptoms and the sputum sample taken 3 days post-onset of symptoms. It is clear that only the sputum specimen is positive for the presence of SARS-CoV (no signal was detected from the blood sample). Fig. 3 (i–l) show the electrophoretic results and hybridization images from the blood and sputum specimens respectively of a third patient (patient 3). Both samples were taken 6 days post-onset of symptoms. The results indicate that both blood and sputum specimens are positive for the presence of SARS-CoV. Fig. 3 (m–r) shows the electrophoretic results and hybridization image from the blood and sputum specimens respectively, of a fourth patient (patient 4). The blood sample was taken 7 days (Fig. 3 m and n) and 9 days (Fig. 3 o and p) post-onset of symptoms and the sputum sample taken 8 days (Fig. 3 q and r) post-onset of symptoms. The results indicate that both blood and sputum specimens are positive for the presence of SARS-CoV.

The comparative study of the positive detection rate on gene chip with single probe and the four-combined probes for blood or sputum or blood and sputum are summarized in Table 3. From all 34 patients, each run of nested RT-PCR may or may not produce amplicons depending on the type of the specimen and time interval of sampling. However, when the four amplification reactions from both blood and sputum were combined in, the positive detection rate on chip is maximized, increasing from 55.8% for blood and 79.4% for sputum to 91.2% when blood and sputum results were combined. When a single probe was used on the chip, the highest detection rate was 83.3%. This rate is approximately 8% lower than the rate (91.2%) when all four probes were used with both blood and sputum.

4. Discussion

The early detection of SARS-CoV present in the samples collected from SARS patients represents a rare event detection that requires the efficient isolation of the targeted viral RNA, the highly efficient amplification and specific detection of the isolated RNA. The efficiency of clinical sample preparation was ensured by the application of the QIAamp Viral RNA Mini Kit, the amplification efficiency of the target RNA amplification was guaranteed by nested PCR, and the high specificity in detection was provided by chip hybridization. Furthermore, the false negative rate has been greatly reduced by the combined use of four probes (Table 3)—a "quadruple security" strategy, which is quite different from the existing methods such as serological method or real-time PCR. Only one target was detected in one test using the ex-

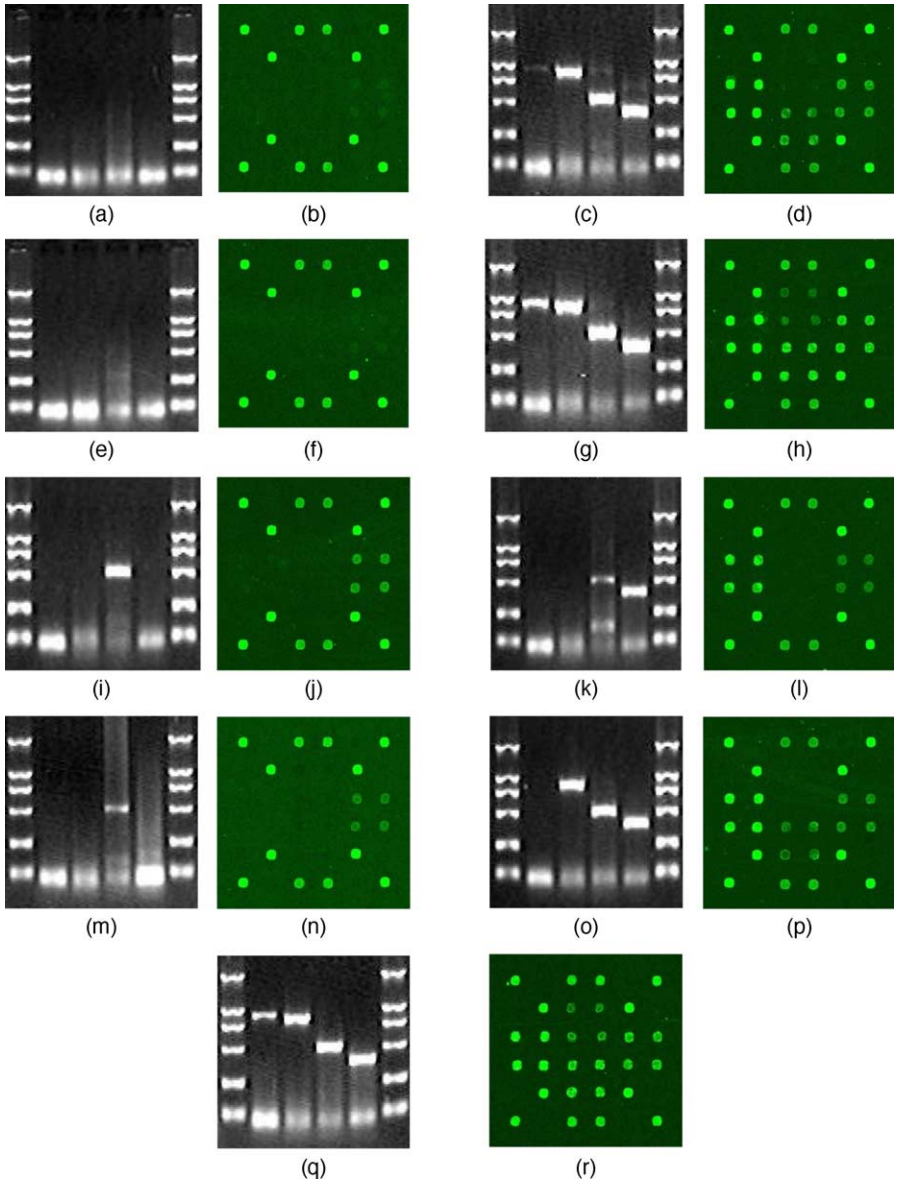

Fig. 3. The DNA electrophoretic and gene chip hybridization results obtained from Patients 1–4. (a) The electrophoretic result of the nested RT-PCR amplicons derived from the patient 1's blood specimen 2 days post-onset of symptoms. From left to right, lane 1 and lane 6 were the molecular weight marker DL2000, lane 2, 3, 4, 5 were the amplicons from primers 11, 24, 40 and 44. (b) The hybridization image of amplicons in (a). (c) The electrophoretic result of amplicons produced from the patient 1's sputum specimen 3 days post-onset of symptoms. (d) The hybridization image of amplicons in (c). (e) The electrophoretic result of nested RT-PCR amplicons derived from the patient 2's blood specimen 2 days post-onset of symptoms. (f) The hybridization image of amplicons in (e). (g) The electrophoretic result of amplicons produced from the patient 2's sputum specimen 3 days post-onset of symptoms. (h) The hybridization image of amplicons in (g). (i) The electrophoretic result of nested RT-PCR amplicons derived from the patient 3's blood specimen 6 days post-onset of symptoms. (j) The hybridization image of amplicons in (i). (k) The electrophoretic result of amplicons produced from the patient 3's sputum specimen 6 days post-onset of symptoms. (l) The hybridization image of amplicons in (k). (m) The electrophoretic result of nested RT-PCR amplicons derived from the patient 4's blood specimen 7 days post-onset of symptoms. (n) The hybridization image of amplicons in (m). (o) The electrophoretic result of amplicons derived from the patient 4's blood specimen 9 days post-onset of symptoms. (p) The hybridization image of amplicons in (o). (q) The electrophoretic result of amplicons produced from the patient's sputum specimen 8 days post-onset of symptoms. (r) The hybridization image of amplicons in (q).

isted methods ("miss one, miss all") and a higher risk of false negative was inevitably encountered. During a SARS crisis a false negative diagnostic could be disastrous to the public. We found that the combination of four individually nested RT-PCR reactions and chip hybridization produced maximal sensitivity in gene chip-based early detection of SARS-CoV.

For the nested RT-PCR amplification systems we adopted here, competitive amplification among the four sets of primers for SARS-CoV RNA exists. When the amount of the templates is limited, we found that the orf N, which is the closest to the 3′ proximal region of the SARS-CoV genome, is the most easily amplifiable template (463 bp, 25 positives for sputum specimens and 13 for blood samples) and the

next most easily amplified is the orf S (349 bp, 22 positives for sputum specimens and 13 for blood samples). This may be explained by the previous report that the nidovirus structural proteins, which are encoded in the 3′-proximal region of the genome, are individually expressed from a nested set of subgenomic mRNAs generated by a unique discontinuous transcription mechanism (Ziebuhr et al., 2000). At least five subgenomic mRNAs were detected by Northern hybridization of RNA from SARS-CoV-infected cells, using a probe derived from the 3′-untranslated region. The calculated sizes of the five predominant bands correspond to the sizes of five of the predicted subgenomic mRNAs of SARS-CoV (Rota et al., 2003). However, when the quantity of the templates is increased to a certain level one or even two of the remaining two products representing orf 1a were also amplified (797 bp, 16 positives for sputum specimens and 6 for blood samples and 858 bp, 8 positives for sputum specimens and 0 for blood samples). Additionally, extreme precautions have been taken to prevent cross contamination during the experiment, which include the use of dUTP and Uracil-DNA Glycosylase, the remote physical isolation of laboratories for sample preparation, PCR preparation, electrophoretic examination and hybridization of amplicons. The results in Fig. 2 (a–f) showed no nonspecific amplifications occurred in the blank control and no nonspecific hybridization signals for any of the probes. In Fig. 2 (b), it appears as if the probe for orf N generated a weak positive signal, yet in fact the hybridization result was categorized as negative because the signal-to-noise ratio was 1.34 (74/55.25) and much less than the threshold value of 2.5. Both blood and sputum samples from the healthy control produced negative hybridization signals for all four SARS-CoV specific probes. Yet, the results from Fig. 2 (g) and (h) demonstrate that all four SARS-CoV specific PCR products were amplified and detected as expected when sufficient amount of SARS-CoV RNA was used as the template. From the results shown in Fig. 3 (a–h) it can be clearly seen that in patients only 2 days post-onset of symptoms, no RNA from SARS-CoV was detected in their blood samples, whereas abundant copies of RNA from SARS-CoV were detected from these two patients' sputum specimens. These results imply that sputum is a good specimen for early detection of the presence of SARS-CoV. The results in Fig. 3 (i, j, m, n) showed that the earliest detection of RNA from SARS-CoV in blood specimen was 6 to 7 days post-onset of symptoms. From Fig. 3 (m–p) we can see that the presence of SARS-CoV in the blood increased dramatically 9 days after the onset of symptoms compared to the results obtained 7 days after the onset of symptoms.

Among the 34 patients tested, four early patients were positively identified for the presence of SARS-CoV, and specimens from the other 27 patients were also positive for the presence of SARS-CoV. One of the patients who was 24 days post-onset of symptoms, still tested positive for the presence of SARS-CoV. Both blood and sputum samples from the remaining three patients were negative for SARS-CoV. ELISA tests were conducted on blood samples collected from these three patients, and the patients 9 days and 30 days post-onset of symptoms was found positive. It was incomprehensible that the gene chip test result did not match the ELISA result for the former patient 9 days post-onset of symptoms. However, both gene chip and ELISA results could be interpreted easily for the patient 30 days post-onset of symptoms, as the viral load may be reduced to zero 30 days after medical treatment according the time course study of the viral load (data not shown). The patient 15 days post-onset of symptoms tested negative with both ELISA test and gene chip could well be the misidentified non-SARS patient.

We have shown that the nucleic acid analysis was highly effective for the early detection of SARS-CoV. One of the major advantages for adopting a gene chip based strategy is the multi-target analysis possible in a single test, which means higher throughput, sensitivity and higher specificity. When a single probe is used on the gene chip, the highest detection rate was 83.3%, which was approximately 8% lower than the rate (91.2%) when all four probes were used. So if only one target was chosen, such as the case for real-time PCR, a higher risk of false negative is inevitable. Although the agarose gel electrophoresis could be used to examine the RT-PCR amplicons, the results were inconclusive for clinical purposes because the sequence information of the amplicons is not available and hence false positive results may be produced.

Further improvements are desirable in the current gene chip approach to SARS diagnosis. The four-separate nested RT-PCR reactions were slow, labor-intensive, and expensive. The results shown here, however, were from a primary study, which was designed to meet the requirements imposed by the SARS-crisis. In our modified PCR, only the universal primers, not the gene-specific primers, were labeled, which were designed initially for the multiplex amplification to avoid the preferential amplification in the ordinary multiplex PCR. Based on the success of the current study, an effort is underway to develop a multiplex nested RT-PCR. This will allow the separate nested RT-PCR reactions to be combined in a single reaction tube. Once this method is developed, the operation will be more convenient and faster and this gene chip based method could be transferred from research to a service setting and applied to the identification of other microorganisms.

In summary, early detection of SARS-CoV has been made possible by combining four nested RT-PCR reactions and a gene chip assay. Sputum, not blood, is the ideal sample for detection of SARS-CoV for early diagnosis of SARS. Furthermore, the gene chip-based early detection strategy can be applied to other newly emerging infectious pathogens.

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