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

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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.

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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.
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 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. 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 Juxianqiao 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.
2.3. 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 2200 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
optimal outer primer pairs were designed with Primer3 by setting the primers and inner primers were selected (Table 2). These four orfs in the genome of SARS coronavirus, four sets of outer primers were synthesized by Sangon.

To improve the detection limit, a modified nested RT-PCR method was applied. To amplify the four segments from three 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 Arbidiposis 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 of the nested RT-PCR is one step RT-PCR. 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 1 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), 0.2 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 amiploc (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 glass slide 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'-GCACGTTGACTATGGTGTCCGATTCT | SARS-CoV orf 1a |
|               | B: 5'-ACATCAGCTTCTACACCCGTTAAAGGT | |
| Inner primers 11 | F: 5'-TCACACTCCCGCCTGAGGAGCCGTITT | SARS-CoV orf 1a |
|               | C: 5'-CACATGCCAATT | |
|               | B: 5'-GCTTGCGAATCCTACACCCGTTAAAGGT | SARS-CoV orf 1a |
| Outer primers 24 | F: 5'-GCTGCATTGGTTTGTTATATCGTTATG | SARS-CoV orf 1a |
|               | B: 5'-ATACAGAAGACAGAAATTCTGTGCTT | |
| Outer primers 40 | F: 5'-GCTGCATTGGTTTGTTATATCGTTATG | SARS-CoV orf 1a |
|               | B: 5'-ATACAGAAGACAGAAATTCTGTGCTT | |
| Outer primers IC | F: 5'-ATGGGGAAGGTGAAGTGCGGGTTTGG | Human GAPDH |
|               | B: 5'-TGGTGAAGACGCCAGTGGAC | |
| Inner primers IC | F: 5'-TCACACTCCCGCCTGAGGAGCCGTITT | Arabidopsis thaliana |
|               | B: 5'-GCTTGCGAATCCTACACCCGTTAAAGGT | |

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

|                  | Probe 11 (%) | Probe 24 (%) | Probe 40 (%) | Probe 44 (%) | Four reactions combined (%) |
|------------------|--------------|--------------|--------------|--------------|----------------------------|
| Blood            | 0/34 (0)     | 0/34 (0)     | 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)               |

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 nested RT-PCR amplicons derived 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 produced from the patient 4’s sputum specimen 8 days post-onset of symptoms. (p) The hybridization image of amplicons in (o).

Listed 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 amplification products, 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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