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Development of a Method for Concentrating and Purifying SARS Coronavirus RNA by a Magnetic Bead Capture System

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ABSTRACT

Severe acute respiratory syndrome (SARS) is a recently emerged infectious disease caused by a novel coronavirus, which has been designated the SARS coronavirus (SARS-CoV). To date, molecular assays for the detection of SARS-CoV has focused mainly on reverse transcriptase-PCR (RT-PCR) analysis of specimens. However, RT-PCR assays currently available have low sensitivity during the early stage of the disease in which the viral load in specimens is very low. A method for concentrating and purifying SARS-CoV RNA by a magnetic bead capture system was developed and followed by an RT-PCR assay in this study with the goal of improving the sensitivity of the RT-PCR method. This approach takes advantage of the cooperative interaction between adjacently hybridized oligonucleotides. A capture probe was covalently coupled to magnetic beads and a second probe, which anneals adjacent to the capture probe site, was prehybridized in solution to the target. It was shown that, when applied to SARS RNA samples, the sensitivity of nucleic acid capture RT-PCR was about 10-fold greater than routine RT-PCR. This nucleic acid capture system was effective in improving the sensitivity of the RT-PCR, due to enriching and purifying SARS-CoV RNA. The method will be helpful for the early detection of the SARS-associated coronavirus.

INTRODUCTION

THE SEVERE ACUTE RESPIRATORY SYNDROME (SARS) is a recently emerging disease caused by a novel coronavirus, designated the SARS coronavirus (SARS-CoV). The disease is unusual due to its highly infectious nature and severity (Drosten *et al*., 2003; Ksiazek *et al*., 2003). To date, molecular testing for SARS has focused mainly on reverse transcriptase-polymerase chain reaction (RT-PCR) assays, and several assays based on RT-PCR for SARS-CoV have been devised using genetic information provided by several groups (Marra *et al*., 2003; Peiris *et al*., 2003a; Poon *et al*., 2003). However, the World Health Organization pointed out that existing PCR tests are very specific but lack sensitivity. This means that negative tests cannot rule out the presence of the SARS virus in patients [Severe Acute Respiratory Syndrome (SARS): Laboratory diagnostic tests; http://www.who.int/sars/diagnostictests/en/]. Therefore, it is important to devise a method that is sufficiently sensitive to effectively screen for SARS-CoV.

In PCR, as in any other diagnostic test, the risk of creating a false-negative result exists. In such a case, the most probable source besides human error, low target, or technical problems is an inhibition caused by interfering substances in the patient's sample (Burkardt, 2000). Human error can be minimized by careful and regular education, training, and supervision of personnel; technical problems can be tackled by the use of a positive run control. A false-negative result, because of low target concentration and PCR inhibition by interfering substances in patients' samples, could be a serious problem. For these cases, the correct method is to remove the inhibition and enrich the target.

Nucleic acid capture using magnetic bead technology is a novel method to isolate, concentrate, and purify target nucleic acid from samples. The technique incorporates streptavidin-coated magnetic beads. A biotinylated capture probe was coupled to the magnetic beads. The complex can capture specific target RNA. This system has been applied to the molecular detection of HCV, HAV, enterovirus, and poliovirus in clinical specimens and environmental samples (Muir *et al*., 1993; van Doorn *et al*., 1994a; Regan and Margolin, 1997; Jothikumar *et al*., 1998).

In this study, we develop a nested RT-PCR method to detect SARS-CoV RNA based on the concentration and purifica-

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tion of SARS-CoV RNA by the magnetic beads capture system, with the ultimate aim of improving the sensitivity of the RT-PCR method.

MATERIALS AND METHODS

SARS-CoV RNA

SARS-CoV RNA isolated from SARS coronavirus-type GDH culture using TRIzol LS (Invitrogen, Carlsbad, CA) was generously provided by the First Military Medical University, Guangzhou, China. SARS-CoV RNA samples were generally provided by Sun Yat-sen University of Medical Sciences.

Primers and probes

The oligonucleotide primer and probe sequences were selected from a highly conserved region of the SARS-associated coronavirus. The capture probe P1 consisted of the complement to nucleotides (5′-AGC CAC TAC ATC GCC ATT CAA GTC TG-3′). The capture probe was biotinylated at the 5′ end during synthesis. The prehybridized probe PR1 was adjacent to the capture probe, and consisted of the complement to nucleotides (5′GGA AGA ATG TGA GAG ATA GCT C-3′). Primers used for nested PCR are the following outer primer pairs: CN1S (sense: 5′-GAA AGT CAA CAA CCC ACC T-3′) and CN1As (antisence: 5′-TTC CAC ATA AGC AGC CAT-3′); inner primer pair CN2S (sense: 5′-ACT ACC GAA GTT GTA GGC AAT G-3′) and CN2As (antisense: 5′-CCA CAT AAG CAG CCA TAA GA- 3′).

Preparation of recombinant SARS-CoV RNA (rRNA) standard

The vector PCR2.1 (Invitrogen) was used to generate *in vitro* transcripts from the T7 promoter containing the target region. The SARS-RNA product appears as a single band. The concentration (molecules/μl) of the SARS-RNA product was determined by its absorption at 260 nm.Then, the SARS-RNA product was used as a standard to evaluate the sensitivity of the RT-PCR method.

Preparation of mock SARS RNA samples

Total RNA was extracted from normal serum samples using TRIzol LS (Invitrogen) and dissolved in DEPC H_2O, according to the manufacturer's instructions. SARS rRNA were diluted serially by the normal total RNA with a ratio of 1:10 (rRNA: total RNA).

Reverse transcription and PCR

Sensitivity was determined by preparing 10-fold dilutions of target RNA from 10^3 to 10^{-1} molecules/μl. Each RNA dilution was assayed in a 20-μl final volume, with a final concentration of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 10 mM DTT, 0.5 mM each of the deoxynucleoside triphosphates (Invitrogen), and 0.125 μM downstream primer, 40 U of RNase inhibitor (Invitrogen), and 20 U of M-MLV reverse transcriptase (Invitrogen). Each 10 μl of RNA dilution was added per reaction tube. The RT temperature was 50°C for 10 min. Following RT, 30 μl of PCR mix was added to each RT tube. The final PCR concentrations were 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM each of the deoxynucleoside triphosphates (Takara, Japan), 0.5 μM each of primer CN1S and CN1As, and 2.5 U of ExTaq polymerase (Takara). Amplification was performed for 30 cycles, 5 cycles at 62°C, 5 cycles at 60°C, 5 cycles at 58°C, and 15 cycles at 56°C, followed by a final extension at 72°C for 10 min in a Takara PCR Thermal Cycler. Two microliters of the first PCR product was added to 48 μl of PCR mix. The final PCR concentrations were identical with the first PCR. Amplification was conducted for 30 cycles at 60°C, followed by a final extension at 72°C for 7 min. Negative controls were included for every test to monitor for contamination as a source of false-positive results. Ten microliters of the second PCR product was analyzed by 2.5% agarose gel electrophoresis and ethidium bromide staining.

Streptavidin-coated beads

Magnetic beads (2.8 $\pm$ 0.2 μm diameter), with streptavidin covalently attached, were purchased from Dynal (Norway). Beads were washed with the aid of a magnetic particle concentrator (MPC) according to the instructions of the manufacturer. Ten microliters of bead suspension (containing 100 μg of beads) was added to a 300 pool of biotinylated oligonucleotide. The suspension was mixed for 45 min at room temperature and then washed three times with 50 μl of freshly prepared 6 $\times$ SSPE(1 $\times$ SSPE is 0.18 M NaCl, 10 mM Na_3PO_4, and 1 mM EDTA[pH 7.7]) by using a MPC to facilitate the removal of wash fluid. The prepared beads were then finally suspended in 100 μl of 6 $\times$ SSPE and stored at 4°C.

RNA-capture RT-PCR assay

For hybridization of SARS-CoV RNA to the magnetic beads, 100 μl of pretreated sample was mixed with 10 μl of probe-linked bead suspension for 90 min at room temperature with continuous rotating. Magnetic beads were then washed three

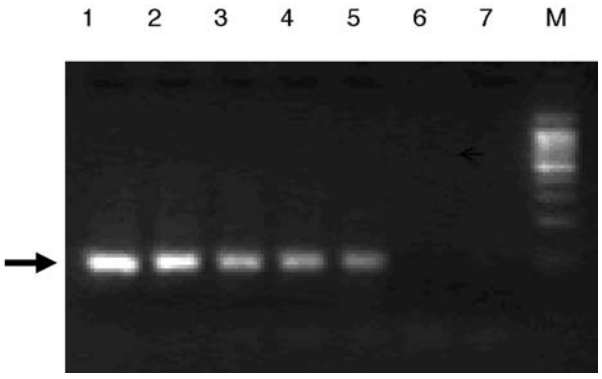

FIG. 1. Reverse transcription and PCR amplification of SARS rRNA. Dilutions (10-fold) of SARS rRNA ranging from 10^4 to 10^{-1} copies/μl (lanes 1–6, respectively); lane 7 is the negative control (no target) and M is the molecular weight marker pBR322 *Hae* III. Ten microliters of each dilution were assayed. Amplification product were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide. The arrow indicates the position of the 100-bp band.

times with 100 μl of B/W buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl], twice in 100 μl of 1 $\times$ PCR buffer (and changed to a new centrifuge tube prior to the final washing step), and resuspended in 10 μl of DEPC H_2O. Each 10 μl RNA-binding beads suspension was analyzed by an RT-PCR assay (according to method 2.5).

Analysis of mock SARS RNA samples by RNA-capture RT-PCR assay

A prehybridization procedure was performed by incubation of 50 μl of mock SARS RNA sample or control sample at 54°C for 15 min in 100 μl of 6 $\times$ SSPE containing 1.0 μM probe PR1.

Analysis of clinical SARS RNA samples by RNA-capture RT-PCR assay

SARS RNA was extracted from a probable SARS patient's serum specimen. The RNA sample was diluted serially and 50 μl of the dilution was prehybridized in 100 μl of 6 $\times$ SSPE containing 1.0 μM probe PR1 at 54°C for 15 min. The 100-μl pretreated sample was assayed according to the method 2.7.

RESULTS

RT-PCR

Initial experiments evaluated and optimized the primers to ensure RT-PCR would result in the generation of the predicted 100-bp product. A nested PCR cycling procedure was used, and consisted of a gradient anneal-extension (from 62 to 56°C) step in the first-round of PCR and a 60°C anneal-extension step in the second-round of PCR. Results demonstrated a single 100-bp product. Other nonspecific bands were not detectable by agarose gel electrophoresis (Fig. 1).

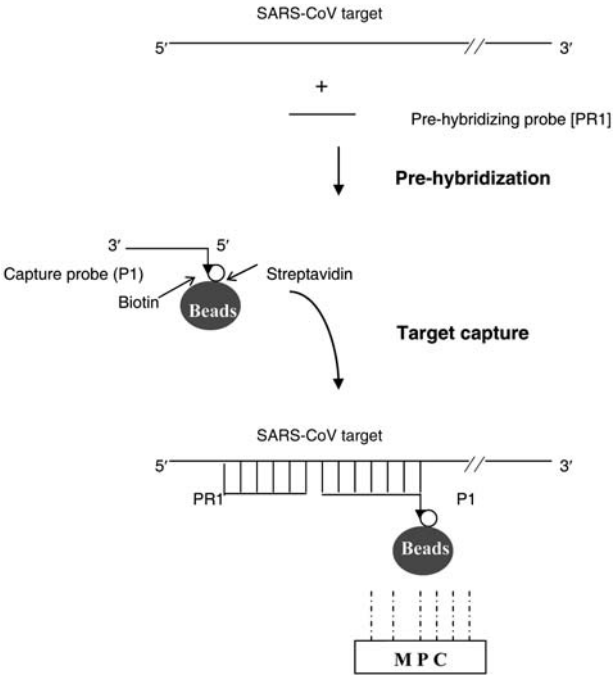

FIG. 2. The target SARS-CoV RNA is initially prehybridized with PR1, and then hybridized in solution with a biotinylated capture probe covalently coupled to magnetic beads. The product is then concentrated and washed by using a magnetic particle concentrator.

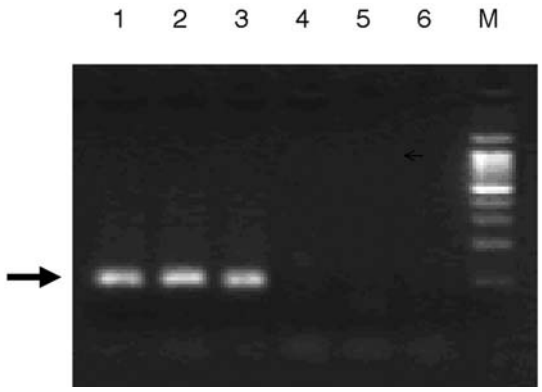

FIG. 3. RNA-capture followed by reverse transcription and PCR amplification of SARS rRNA. Lanes 1–5 contain SARS rRNA at concentrations of 2×10^1, 2×10^0, 5×10^{-1}, 2×10^{-1}, 2×10^{-2} copies/μl, respectively; lane 6 is the negative control (no target); and M is the molecular weight marker pBR322 *Hae* III. Fifty microliters of each dilution were concentrated and assayed. Amplification product was resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide. The arrow indicates the position of the 100-bp band.

The sensitivity of the RT-PCR procedure was determined. Dilutions (10-fold) of SARS rRNA were prepared and assayed by RT-PCR. A control sample (no rRNA) was included to monitor any crosscontamination. Amplification was detected out to 10^1 copies per reaction as demonstrated by agarose gel electrophoresis and ethidium bromide staining (Fig. 1).

RNA-capture RT-PCR

As a result of the successful nested RT-PCR analyses the target rRNA was 10-fold serially diluted and further evaluated for the magnetic beads RNA-capture RT-PCR method with a covalently bound capture probe (P1) and a prehybridized probe (PR1).

As illustrated in Figure 2, an oligonucleotide module (PR1), designed to anneal adjacent to the capture probe (P1), was prehybridized to the serially diluted templates at 54°C for 15 min. The hybridization mixtures were subsequently incubated with magnetic beads at room temperature for 90 min to facilitate specific RNA capture. All samples were tested in duplicate, and a control bead sample (no rRNA) was included to monitor any crosscontamination during the washing steps, etc. After incubation, the beads were washed and transferred to PCR tubes containing reagents and primers for reverse transcription. A nested PCR was carried out following RT.

The results are depicted in Figure 3, and the sensitivity of the RNA-capture RT-PCR procedure was determined. Amplification was detected out to 25 copies per reaction, as demonstrated by agarose gel electrophoresis and ethidium bromide staining.

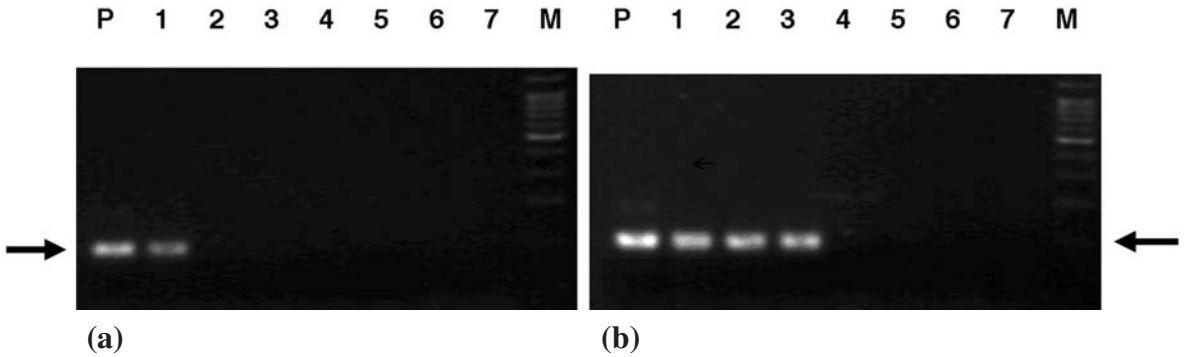

FIG. 4. Routine RT-PCR (**a**) and RNA-capture RT-PCR (**b**) amplification of mock SARS RNA samples. Lanes 1–6 contain SARS rRNA at concentrations of 2×10^1, 2×10^0, 1×10^0, 2×10^{-1}, 2×10^{-2}, and 2×10^{-3} copies/μl, respectively; lane 7 is the negative control (no target); lane P is the positive control (SARS rRNA, 2×10^3 copies/μl); and M is the molecular weight marker pBR322 *Hae* III. The arrow indicates the position of the 100-bp band.

Sequence of amplified product

The result of the nested PCR product identity was compared against GenBank published sequence data. The sequenced cDNA product was found to have a 100% match when compared with the published SARS-CoV sequence. This confirmed the specificity of the primers to amplify SARS-CoV.

Detection of mock SARS RNA samples by RNA-capture RT-PCR assay

Mock SARS RNA samples were prepared and 10-fold diluted serially by total RNA sample extracted from normal serum. Mock SARS RNA samples, due to its lack of infectivity, convenience, and similarity with real SARS RNA samples, were tested to evaluate the two assays. In RNA-capture RT-PCR reactions, 50 μl of the mock RNA sample was analyzed; in routine RT-PCR reactions, 10 μl of the mock RNA sample was analyzed. A positive control (10^4 copies/μl SARS rRNA) was used with each sample to monitor the amplification. The results (Fig. 4) show all of the positive controls exhibited amplification; the minimum sample concentration detected by routine RT-PCR was 2×10^1 copies/μl; however, the minimum sample concentration detected by RNA-capture RT-PCR was 10^0 copies/μl.

Detection of clinical SARS RNA samples by RNA-capture RT-PCR assay

A SARS RNA sample from a probable clinical patient specimen was used for evaluating the RNA-capture RT-PCR assay. Initially, the SARS RNA sample was fourfold diluted and further diluted serially by 10-fold. Fifty microliters of each dilution was tested by an RNA-capture RT-PCR method, and 10 μl of each dilution was tested by the routine RT-PCR method. All samples were tested in duplicate, and a positive control sample (10^4 copies/μl SARS rRNA) and a negative control sample were included in all reactions. The results (Fig. 5) showed that the 4×10^{-1} dilution was amplified in RNA-capture RT-PCR tests; the 4 dilution was amplified in routine RT-PCR tests.

DISCUSSION

Molecular testing for SARS has focused mainly on reverse transcriptase-polymerase chain reaction (RT-PCR) assays. Previous studies demonstrated that viral loads in nasopharyngeal aspirate (NPA) are low in the first few days of illness and peak

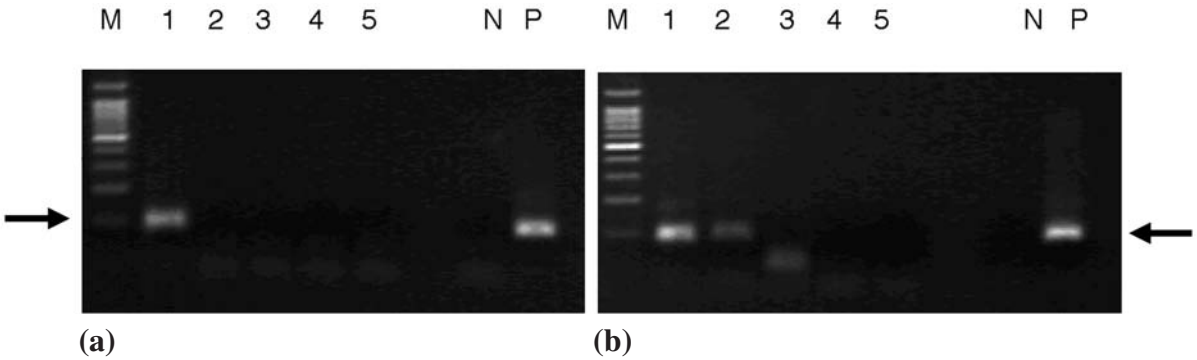

FIG. 5. Routine RT-PCR (**a**) and RNA-capture RT-PCR (**b**) amplification of clinical SARS RNA samples. Lanes 1 to 5 correspond to dilutions of 4 to 4×10^{-4}, respectively; lane N is the negative control (no target); lane P is the positive control (SARS rRNA, 2×10^3 copies/μl), and M is the molecular weight marker pBR322 *Hae*III. The arrow indicates the position of the 100-bp band.

around day 10 of the disease (Peiris *et al*., 2003a). Using first-generation conventional RT-PCR assay (Peiris *et al*., 2003a, 2003b), only 22% of NPA samples collected from days 1–3 of disease onset from SARS patients were shown to have SARS-CoV RNA; by increasing the initial volume for RNA extraction from 140 to 540 μl, the proportion of positive cases was increased to 44% (Poon *et al*., 2003). That means it is effective for improving sensitivity of RT-PCR methods by increasing the target concentration.

This study was undertaken to develop a method for the concentration and purification of SARS-CoV RNA samples. The principal objective was to incorporate a procedure to enrich specific RNA, remove RT-PCR inhibitors and permit the analysis of a larger RNA sample, and finally, to improve the sensitivity of the RT-PCR method. Nucleic acid capture using streptavidin-coated magnetic beads was shown to accomplish both objectives.

Magnetic capture systems were originally developed for use in immunoassays (Heerman *et al*., 1994). Recently, magnetic beads have been used in efforts to develop closed diagnostic system (Jungkind, 2001). However, these capture assays often show losses in sensitivity in comparison to the standard protocols (van Doorn *et al*., 1994b). A novel method to design a prehybridized probe, which anneals adjacent to the capture probe site, were used to recover directly HCV RNA from clinical samples, and the capture efficiency was increased up to 25-fold in comparison to capture with a single probe (O'Meara *et al*., 1998). In this study, a second probe was designed to prehybridize initially with the SARS RNA target; the capture efficiency was increased up to about 10-fold in comparison to capture without the prehybridized probe (data not shown).

The efficacy of the magnetic capture system in our study was initially determined with SARS rRNA. In routine RT-PCR tests, amplification was detected out to 10 copies per reaction. In RNA-capture RT-PCR tests, amplification was detected out to 25 copies per reaction. This was somewhat less sensitive than what was found in routine RT-PCR. The loss in sensitivity is likely due to the additional steps used in nucleic acid capture, such as in the bead-washing procedure. Another area to consider is the efficiency of biotinylation during the oligonucleotide synthetic process. There is a chance that a nonbiotinylated probe could hybridize and prevent RNA recovery.

Mock SARS RNA samples were prepared to evaluate the effect of the magnetic beads system, compared with the routine RT-PCR system. The results demonstrated that using 10-μl samples to be tested by routine RT-PCR, the minimum concentration detected was 20 copies/μl. This was less sensitive than what was determined in the routine RT-PCR assays for SARS rRNA samples. It was indicated that PCR inhibitors might exist in the total RNA samples extracted from normal human serum. Furthermore, using 50 μl samples tested by the RNA-capture RT-PCR assay the minimum concentration detected by was one copy/μl. This was more sensitive than what was found in routine RT-PCR. The increase in sensitivity may have been the result of RNA enrichment and inhibitors elimination through the magnetic bead capture system.

Clinical SARS RNA samples were used to evaluate the system further. The results had shown that a 4×10^{-1} dilution was detected in RNA-capture RT-PCR tests, and a 4-dilution was detected in routine RT-PCR tests. The result was consistent with that found in mock SARS RNA samples.

In summary, target nucleic acid can be effectively concentrated and purified by the magnetic bead system, so we could therefore analyze 50-μl RNA samples. This is useful for improving the sensitivity of the RT-PCR assay. Based on the success with the 50-μl sample size, future efforts will focus on increasing the sample volume and developing the capture system as a rapid and practical method for extracting specific RNA from SARS clinical specimens. Moreover, the system can be adapted easily to other viruses simply by generating new probes and a primer set.

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