Development and evaluation of an efficient 3′-noncoding region based SARS coronavirus (SARS-CoV) RT-PCR assay for detection of SARS-CoV infections

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Abstract

The severe acute respiratory syndrome (SARS) epidemic originating from China in 2002 was caused by a previously uncharacterized coronavirus that could be identified by specific RT-PCR amplification. Efforts to control future SARS outbreaks depend on the accurate and early identification of SARS-CoV infected patients. A real-time fluorogenic RT-PCR assay based on the 3′-noncoding region (3′-NCR) of SARS-CoV genome was developed as a quantitative SARS diagnostic tool. The ideal amplification efficiency of a sensitive SARS-CoV RT-PCR assay should yield an E value (PCR product concentration increase per amplification cycle) equal to 2.0. It was demonstrated that the 3′-NCR SARS-CoV based RT-PCR reactions could be formulated to reach excellent E values of 1.81, or 91% amplification efficacy. The SARS-CoV cDNA preparations derived from viral RNA extract and the cloned recombinant plasmid both exhibit the identical amplification characteristics, i.e. amplification efficacy using the same PCR formulation developed in this study. The viral genomic copy (or genomic equivalences, GE) per infectious unit (GE/pfu) of SARS-CoV used in this study was also established to be approximate 1200–1600:1. The assay's detection sensitivity could reach 0.005 pfu or 6–8 GE per assay. It was preliminarily demonstrated that the assay could efficiently detect SARS-CoV from clinical specimens of SARS probable and suspected patients identified in Taiwan. The 3′-NCR based SARS-CoV assay demonstrated 100% diagnostic specificity testing samples of patients with acute respiratory disease from a non-SARS epidemic region.
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Keywords: Severe acute respiratory syndrome; SARS coronavirus; Real-time RT-PCR; Quantitative RT-PCR; 3′-Noncoding region.

1. Introduction

Severe acute respiratory syndrome (SARS) is a newly recognized infectious disease with significant health and economic impacts worldwide. SARS was initially identified in Guangdong province, China in November 2002 and subsequently in Hong Kong, Singapore, Vietnam, Taiwan, countries in Europe and in North America by March and April of 2003 (CDC, MMWR 2003a; Lee et al., 2003; CDC, MMWR 2003b; WHO, 2003; Riley et al., 2003). The 2003 SARS epidemic was caused by a previously uncharacterized coronavirus, SARS-CoV (Peiris et al., 2003a; Drosten et al., 2003; Ksiazek et al., 2003). Typical coronavirus particles were readily visible under electron microscope and the specific coronavirus sequences were detected by reverse transcription polymerase chain reaction (RT-PCR) from most SARS-CoV infected patients (Peiris et al., 2003a; Drosten et al., 2003; Lawrence, 2003). SARS-CoV could be isolated and cultivated from sputum specimens and respiratory secretions of SARS patients using *Vero* cell cultures before the appearance of SARS-specific antibodies in serum, average 21 days following exposure (Peiris et al., 2003b; Booth et al., 2003). The incubation period for SARS ranges

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from 2 to 10 days, though viremia can be documented approximately 2–5 days following exposure. The challenge of early detection of SARS-CoV infection is the low viremia of infected patients. Low viremia yields low to modest specific signal/noise ratio making distinction of the early-infected victims from non-SARS febrile patients difficult.

Initial efforts to control SARS were directed toward identification of travel-associated cases that originated from China and other SARS epidemic areas, such as Hong Kong (Riley et al., 2003; Lipsitch et al., 2003; Twu et al., 2003). Despite enormous efforts to implement extensive control measures, there were still unrecognized cases of SARS that led to nosocomial clusters and subsequent spread to other health-care facilities and community settings in different regions of the world (Twu et al., 2003; Seto et al., 2003). Several factors might contribute to difficulties to recognize cases of SARS. Early symptoms of SARS are nonspecific and more commonly associated with other respiratory illnesses (updated interim US case definition for severe acute respiratory syndrome (SARS), CDC, 12 December 2003). Patients with SARS who are immuno-compromised or who have chronic conditions, e.g. diabetes mellitus or chronic renal insufficiency may remain afebrile when acutely ill or have symptoms attributable to underlying disease, delaying SARS diagnosis. The reappearance of SARS in Toronto, Canada illustrates the possibility that future outbreaks of SARS might occur during the flu season in different parts of the world. Thus, it would not be adequate to rely solely on the preliminary clinical symptoms, such as fever and cough to detect and differentiate SARS-CoV infections from other common upper respiratory infections.

There are various RT-PCR tests to detect SARS-CoV available using different SARS-CoV genomic sequences (Drosten et al., 2003; Peiris et al., 2003b). The formats of SARS-CoV RT-PCR assays range from conventional agarose gel based assays to fluorogenic real-time RT-PCR assays. Real-time RT-PCR may be preferred format due to its simplicity and ability to generate quantitative data. However, most of the current real-time SARS-CoV RT-PCR assays have not been validated to document consistent detection sensitivity and specificity to detect low SARS-CoV titers during early stages of illness. An expected characteristic of SARS-CoV similar to other RNA viruses, such as flaviviruses is the high rate of genetic mutations, which leads to evolution of new viral variants (Leitmeyer et al., 1999; Mangada and Igarashi, 1998; Sanchez and Ruiz, 1996). It is possible that the high mutation rate of SARS-CoV would render any given RT-PCR assay useless if the assay's target sequence is subject to mutational changes. Thus, a negative test result may not rule out SARS-CoV infection especially for the patients showing early symptoms of SARS. In this study, we report the development of a real-time RT-PCR assay based on the highly conserved 3′-NCR of the genome as a quantitative SARS diagnostic system (Marra et al., 2003; Ruan et al., 2003). It was retrospectively demonstrated

that the assay could be used as an efficient diagnostic to identify SARS-CoV positive samples derived from SARS probable, or suspected patients during Taiwan's 2003 SARS outbreak.

2. Materials and methods

SARS-CoV RNA and cDNA preparation. The SARS-CoV strain used in this study originated from a deceased WHO physician in Vietnam (US CDC isolate known as strain Urbani; Rota et al., 2003) and was obtained from the US Army Medical Research Institute of Infectious Diseases at Fort Detrick, Maryland. Twenty throat swabs of acute respiratory disease (ARD) patients from Fort Jackson, South Carolina were obtained from collections of the Walter Reed Army Institute of Research, Silver Spring, Maryland as negative controls for the SARS-CoV assay (McNeill et al., 2000). The viral RNA was extracted and transcribed into SARS-CoV cDNA through reverse transcription (RT) reaction using a commercial RNA extraction kit (Qiagen Inc.) and a RT kit (PE Applied Biosystems Inc., Foster City, California). RNA was mixed with RT reaction mixture containing anti-sense SARS-CoV RT primer (5′-CATTATTCACTGTACCCTCGATCG-3′, 20 pmol/reaction) according to the manufacture's instruction. The RT mixture was incubated as followings, 25 °C, 10 min; 48 °C, 30 min; 95 °C, 5 min. The resultant cDNA was stored at −20 °C until tested.

Fluorogenic probe/primers and PCR conditions. The fluorogenic probe (FAM-TTTCATCGAGGCCACGCGGAG-TAMRA) and its flanking primer pair (sense primer, 5′-GGACTTGAAAGAGCCACCACA-3′ & and anti-sense primer, 5′-CATTATTCACTGTACCCTCGATCG-3′) were designed based on the conserved SARS-CoV 3′-NCR of Canadian Tor2 isolate (Marra et al., 2003; GenBank accession #278554) and were purchased from PE Applied Biosystems Inc. (Foster City, California). PCR master mixture contained the following ingredients: 1X ABI PCR buffer II, 2 mM $MgCl_2$, 0.4 mM dNTPs', 200 nM HCV-U4 upper primer, 200 nM HCV-L4 lower primer, 20 nM HCV-P4 fluorogenic Taqman probe labeled with 3′-FAM reporter dye and 5′-TAMRA quencher dye, 1.25 unit Taq DNA polymerase (AmpliTaq Gold, ABI, Foster City, California). Two microliter of viral cDNA derived from the RT reaction was mixed with 48 μl PCR master mixture. The PCR-cDNA mixture was subjected to the following amplification conditions: 95 °C, 10 min heat activation of AmpliTaq Gold enzyme; 40 cycles of 95 °C, 15 s; 58 °C, 40 s. Two commercially available fluorogenic thermocyclers were used to perform the real-time RT-PCR assay developed in this report, the Rotor-Gene Real-Time PCR thermocycler (Westburg BV Inc., Leusden, The Netherlands), and the MJ Opticon I DNA Engine (MJ Inc., Massachusetts).

Construction and isolation of the recombinant plasmid containing the SARS-CoV 3′-NCR. The sense and

anti-sense primers of SARS-CoV were used to generate SARS-CoV cDNA through 35 cycles of PCR amplification. The SARS-CoV 3′-NCR PCR amplicon was then cloned into the *Sma*I site of pUC19 vector using TA-cloning protocol (Mezei and Storts, 1994). The ligated vector-cDNA mixture was transformed into *E. coli* JM109 host. One of the recombinant plasmid clones, pHCV1 was identified as X-gal negative after incubating overnight at 37 °C on selective LB agar plate containing ampicillin and adequate X-gal, IPTG substrates. The pHCV1 plasmid was extracted and purified by modified alkaline lysis and CsCl-gradient centrifugation from 50 ml of overnight LB broth culture containing 50 mg/ml ampicillin.

3. Results

3.1. Quantitative real-time detection of SARS-CoV viral RNA using the 3′-NCR based fluorogenic RT-PCR assay

The assay was optimized by exploring combinations of assay parameters, such as Mg^{2+} concentration, flanking primers/probe ratio and concentration, annealing temperature to achieve the highest possible detection efficiency of the SARS-CoV template. The optimal RT and PCR assay conditions obtained were described above, and were used throughout this study. Various concentrations of viral RNA were used to demonstrate the quantitative nature of the assay. Fig. 1a shows typical sigmoid plots representing formation of the SARS-CoV specific PCR products from samples derived from Vero cell culture containing five-fold

serial dilutions of viral RNA by showing accumulation in fluorescence (ΔR) as amplification cycle number increases. Threshold cycle (C_T) for each concentration of cDNA is defined as the cycle number where the application software detects the increase of product fluorescence above the calculated background fluorescence (Holland et al., 1991). The C_T values for the described viral cDNA concentrations were automatically calculated by application software as the intercepts of the fluorescence signals and the base line of background fluorescence (set as the average of all tested samples from cycle 3 to 15). Parallel sigmoid plots representing serial dilutions of SARS-CoV cDNA template are shown and displayed in sequential order. Higher concentrations of template cDNA resulted in lower C_T values, i.e. greater initial viral cDNA template copy numbers required fewer amplification cycles or a smaller C_T value to reach the detectable fluorescence level. These preliminary results demonstrated that the fluorogenic RT-PCR assay was capable of detecting SARS-CoV viral cDNA in a dose-dependent fashion over at least seven serial five-fold dilutions (4.9 logs dilution). In addition, the assay was also capable of discriminating specific SARS-CoV viral stock cDNA from background levels (non-template controls showed no fluorescence signal).

3.2. Comparison of the cloned recombinant plasmid, pHCV1 containing the 3′-noncoding SARS-CoV cDNA and viral cDNA preparation as SARS-CoV copy number standards

Thus far, the SARS-CoV cDNA copy standards (presented in plaque-forming units, pfu) used in this study
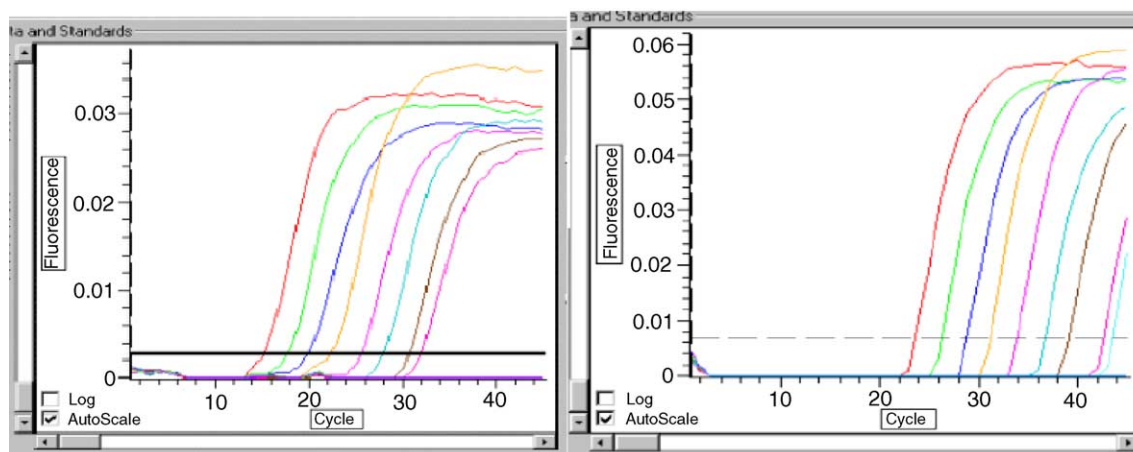

Fig. 1. (a) Real-time amplification and detection of SARS-CoV using the fluorogenic SARS-CoV 3′-NCR based RT-PCR system. SARS-CoV viral stock was serially diluted (dynamic range from 18,000 to 0.24 pfu/reaction) and used to generate viral cDNA to demonstrate that the assay is a copy number dependent reaction. Wells A1–A8 contained serially diluted viral copy numbers, i.e. pfu/reaction, to yield sequential sigmoid curves arranged from high through low copy number (left to right). B6–B8 wells were non-template controls that showed no noise, or background for this assay. (b) Real-time amplification and detection of the cloned SARS-CoV 3′-NCR, pHCV1 using the fluorogenic SARS-CoV 3′-NCR based RT-PCR system. Five-fold serial dilutions of the cloned SARS-CoV plasmid, pHCV1 (from 700,000 to 2 GE/reaction) were used to demonstrate that the assay is a copy number dependent reaction. Wells A1–A8 containing serially diluted pHCV1 yielded sequential sigmoid curves arranged from high through low copy number (left to right of the figure). B6–B8 wells were non-template controls that showed no noise, or background for this assay.

were obtained from laboratory derived SARS-CoV stock. Live SARS-CoV cannot be safely used in most clinical laboratories and it is also difficult to maintain consistent viral RNA standards for routine usage due to the labile nature of RNA molecules. Therefore, a recombinant plasmid containing the 3′-noncoding SARS-CoV cDNA was constructed to serve as a SARS-CoV genomic equivalence standard. The recombinant plasmid, pHCV1 containing the SARS-CoV 3′-noncoding region was constructed and isolated as a possible SARS-CoV genomic reference standard (see Section 2). Fig. 1b demonstrated that the pHCV1 plasmid can be used as a SARS-CoV genomic copy number standard for the assay developed in this study. The C_T values of serially diluted plasmid concentration reflect a copy number dependent reaction. The slope of the pHCV1 standard curve (0.28) is identical to the slope of SARS-CoV viral standard curve (see Fig. 2). This illustrates that the pHCV1 DNA and viral cDNA can be amplified by the same method with the same amplification efficacy.

3.3. Amplification efficiency and detection sensitivity of the SARS-CoV 3′-NCR based PCR cycling reaction

The ideal amplification efficiency of any given double helical cDNA substrate should yield a two-fold increase in PCR product during each amplification cycle. The *E* value, PCR product increment per amplification cycle of any quantitative real-time PCR assay can be estimated from the slope of standard curves consisting of C_T values of serially diluted target cDNA; either viral cDNA preparations or the cloned plasmid, pHCV1 may be used for this purpose (shown in Fig. 2). The SARS-CoV 3′-NCR PCR assay consistently yielded standard curves with the slope of 0.28 producing an *E* value of 1.81 ($-1/\log(\text{slope})$) or amplification efficiency of $E_{\text{observed}}/E_{\text{ideal}} \times 100 = 91\%$. It was demonstrated that the RT-PCR assay with 91% amplification efficiency could be used for consistent detect ion of the SARS-CoV viral RNA extracted from samples containing as little as 0.005 pfu per reaction with an anticipated C_T value of 40 cycles (data not shown). Fig. 2 illustrates that the assay could be used
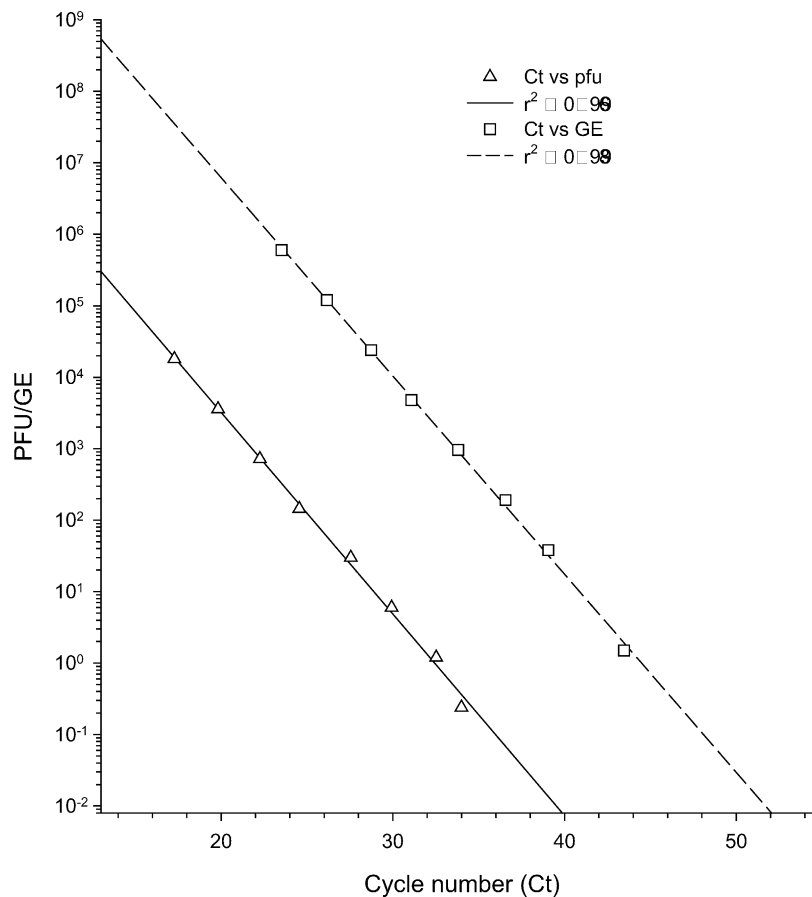

Fig. 2. Comparison of quantitative SARS-CoV standard curves derived from direct viral cDNA preparation and the cloned 3′-NCR SARS-CoV plasmid DNA, pHCV1. It was shown that both standard curves yield the same slope (two parallel lines taken from Fig. 1a and b). The GE/pfu ratio of 1:1600 could be derived from the direct readout of intercepts difference at Y-(GE/pfu). The GE/pfu ratio can also be calculated from the intercepts difference at X-(C_t cycle) of 12.0 cycles between viral cDNA (pfu) and cloned plasmid (GE) standard curves as followings: GE/pfu = E^{12} or $1.81^{12.0}$ = 1239.

to detect viral cDNA preparations as well as the cloned SARS-CoV 3′-NCR at the same amplification efficacy. A linear regression model was used to determine the number of infectious units (pfu) present in a given SARS-CoV preparation and the results from this calculation indicates that the ratio of defective to infectious particles could be deduced from the difference of standard curves of assays using viral cDNA preparations as well as the cloned SARS-CoV 3′-NCR. The GE/pfu ratio of 1:1600 was derived from the direct readout of intercepts difference at Y-(GE/pfu). The GE/pfu ratio was calculated from the intercepts difference at X-(C_t cycle) of 12.0 cycles between viral cDNA (pfu) and cloned plasmid (GE) standard curves as followings: GE/pfu $= E^{12}$ or $1.81^{12.0} = 1239$.

3.4. Laboratory diagnosis of SARS-CoV infection from clinical specimens of probable and suspected SARS patients in Taiwan

To demonstrate the utility of the SARS-specific quantitative RT-PCR assay for diagnostic purposes, the assay was used for quantitative detection of SARS-CoV from nasopharyngeal aspirates obtained from patients that were diagnosed as probable or suspected SARS cases using clinical criteria.

Table 1
Quantitative detection of SARS-CoV from nasopharyngeal aspirates of probable and suspected SARS patients on 14 May 2003, Taichung, Taiwan

Sample ID	GE/R_x	GE/ml	Pfu/ml*	C_t
STD7	1.8	360	0.31	45.0
STD6	18.0	3600	3.06	40.9
STD5	180.0	36000	30.60	34.5
STD4	1800.0	360000	306.00	29.7
STD3	18000.0	3600000	3060.00	25.9
STD2	180000.0	36000000	30600.00	22.5
BLANK	0.0	0	0.00	44.8
D8	0.0	0	0.00	45.0
E6	0.0	0	0.00	45.0
E7	0.0	0	0.00	45.0
F2	0.0	0	0.00	45.0
F3	0.0	0	0.00	45.0
G2	21.1	4220	3.59	39.6
G3	0.0	0	0.00	45.0
G5	0.0	0	0.00	45.0
G6	239.0	47800	40.63	34.7
H2	2.3**	460	0.39	44.1
H3	0.0	0	0.00	45.0
D6	9460.5	1892100	1608.29	27.4
D8	0.0	0	0.00	45.0
D9	62.3	12460	10.59	37.4
CDC-PC	6870.60	1374120	1168.00	28.0
A2	4403.63	880726	748.62	28.9
D9	0.0	0	0.00	45.0

The assay used 5 µl of extracted aspirate wash per reaction and the assay was set to run for 45 cycles of amplification. CDC-PC was the SARS positive control RNA provided by the Taiwanese CDC, and BLANK served as the negative control. The cloned pHCV1 template (STD2-10) was used as the SARS-CoV GE copy control.
* Based on GE/pfu ration of 1200:1.
** Below the cutoff threshold (SARS negative).

Table 1 indicates that the assay developed from this study could efficiently detect SARS-CoV from infected patients in Taiwan. SARS-CoV RNA provided by the Center for Diseases Control (CDC) of the Ministry of Health, Taiwan and the cloned pHCV1 were used as positive controls. Five out of 16 samples tested were identified as SARS-CoV positive (31.2% positive rate for SARS identification). SARS-CoV RT-PCR positive samples indicated the presence of low concentrations of viral nucleic acid. Unfortunately, no other laboratory testing results, such as the rise of SARS-specific antibodies and SARS-CoV viral isolation were available to corroborate the RT-PCR data in this study. We further investigated and confirmed the specificity of SARS-CoV RT-PCR in this study. Twenty clinical samples derived from acute respiratory disease (ARD) patients caused by adenovirus in US were employed as negative controls for SARS-CoV assay (McNeill et al., 2000) None of these ARD samples yield any positive fluorescence signal for SARS-CoV (specificity = 100%).

4. Discussions

The SARS-CoV genomic sequence differs significantly from other known coronaviruses, particularly in the 5′- and 3′-NCR (Marra et al., 2003; Ruan et al., 2003). There are more than forty 3′-NCR SARS-CoV sequences deposited in the GenBank database, and a Blast search demonstrated 100% homology among various SARS-CoV isolates obtained from different parts of the world. It was demonstrated that the 3′-NCR based fluorogoenic RT-PCR developed in this study could be used as an efficient diagnostic assay for laboratory grown SARS-CoV detection and quantification. It was preliminarily illustrated that the assay has a wide dynamic range of detection (more than 4.9 logs) with excellent linearity (linear coefficient greater than 0.99) using cDNA templates derived from serial dilutions of viral RNA through reverse transcription (RT) reaction. The data presented in Fig. 1 showed the detection range of SARS-CoV from 18,000 through 0.25 pfu per assay. The C_t value of 34 cycles obtained for the 0.25 pfu/assay did not represent the ultimate end point of the assay. It was later demonstrated that the end point, or lower detection limit of the assay could reach as low as 0.005 pfu per assay with the C_t value approximate at 40 cycles (data not shown). The cloned 3′-NCR SARS-CoV cDNA pHCV1 was also established in this study as a molecular copy standard, or genomic equivalent standard for the SARS-CoV assay. It was demonstrated in this study that the cloned cDNA pHCV1 could mimic the viral cDNA derived from SARS-CoV RNA preparations as a copy number standard for the assay. Fig. 2 showed the minimal detectable pHCV1 copy number of two GE per assay with C_t value greater than 40 cycles. However, the population size of cloned SARS-CoV standard at two copies per reaction was too small to be represented as a "normal distribution" (Gaussian distribution). Thus, multiple repeats

of the same assay using such a low substrate copy number, two copies per assay would yield the result of "hit or miss" phenomenon. In order to obtain more reproducible results of the assay, the cutoff, or end point of pHCV1 for the assay was raised to 6–8 GE per assay with the C_t value at 40 cycles. Based on the calculated SARS-CoV GE/pfu ratio from Fig. 3, and the actual experimental data using serial dilutions of viral cDNA preparations and cloned pHCV1, the lower detection limit of SARS-CoV assay could be conservatively defined as 0.005 pfu or 6–8 GE per assay.

The traditional expression for viral titer, plaque-forming unit (pfu) per ml must be determined through a conventional viral plaquing assay. This culturing method identifies viable viruses that can generate cytopathic effects, or plaques, on the confluent lawn of susceptible host cells, such as *Vero* cells (Drosten et al., 2003). The viral plaquing assay can only be used to score the total viable viruses that can infect the host cells. Thus, it cannot be used to distinguish, or detect any viral contaminants that are capable of infecting the host cells used for the plaquing assay (Porterfield, 1959; De Madrid and Porterfield, 1969). In contrast, the SRAR-CoV RT-PCR assay developed in this study is specific for the SARS-CoV detection. However, the SARS-CoV PCR assay developed from this study cannot be used to differentiate among living versus dead viral particles (Freeman et al., 1999; Bae et al., 2003; MacKay et al., 2002). Any viral particle or genomic segment containing intact PCR target sequence, such as the 3'-NCR SARS-CoV would be detected as one genomic equivalence (GE). The difference between pfu (viable viruses) and GE (total viral counts including defective and functional viruses) of SARS-CoV can be mathematically expressed as GE/pfu ratio (Freeman et al., 1999; Bae et al., 2003). The GE/pfu ratio of approximate 1200–1600:1 obtained in this study only represents the GE/pfu ratio of the *Vero* cell derived SARS-CoV stock used in this study. The finding of high GE/pfu ratio from this study supported the hypothesis of SARS-CoV cultures and SARS-CoV infected clinical samples containing both infectious and defective (or subgenomic) viral RNA, i.e. each SARS-CoV infectious unit is represented by multiple viral RNA species. It is likely that the 3'-noncoding based SARS-CoV RT-PCR assay could detect both infectious and defective viral RNA from clinical samples (Poon et al., 2004). It is possible that viral stocks of different preparations might contain the same pfu per ml, but the total viral particles, or RNA copy numbers would be different (Bae et al., 2003; Peccoud and Jacob, 1996). The pfu, infectious activity of any given viral stock is greatly affected by the percentage of viable virus. There are numerous factors that will determine, or affect the viability of each individual viral stock, such as culturing conditions (nutrients, ionic strength, incubation time and temperature), storage conditions (duration and temperature), and viral harvesting conditions. Thus, it is difficult to obtain a universal reference viral stock containing a precisely standardized viral copy number with persistent viability data. It was demonstrated in this study that the cloned pHCV1 plasmid could be used to replace viral cDNA as a stable and rational SARS-CoV copy number standard for the SARS-CoV RT-PCR assay.

The practical application of the SARS-CoV RT-PCR developed in this study is to accurately identify SARS-CoV in suspected SARS patients. The key to the successful control of SARS outbreaks is to identify SARS-CoV infected individuals in the early stages of infections. This will allow public health officers to apply adequate physical quarantine measures before the SARS infected individuals become highly contagious. This requires the deployment of highly sensitive SARS-CoV diagnostics to detect low SARS-CoV titers. In this study, the assay was used to test a limited numbers of total RNA extracts derived from SARS suspected, or probable patients in Taiwan. The tested results indicated that it is feasible to use the developed assay to identify the SARS-CoV infected patients with viremia loads ranging from 3.59 pfu through 1608.29 pfu/ml. Even though only a limited numbers of samples were tested in this study, the assay was confirmed to be specific, i.e. none of 20 non-SARS ARD patients' samples tested positive using the SARS-CoV assay. Even though we did not have specific information on the onset dates of disease for those clinical samples tested in this study. We demonstrated that the 3'-noncoding based RT-PCR developed in this study could be used to detect both infectious and defective viral RNA. Thus, the assay should have fairly high diagnostic sensitivity of detecting SARS-CoV during the early stage of infections (Poon et al., 2003). It was previously reported that the mean time between onset of symptoms and sample collection ranged from 3.2 to 4.3 days for SARS-CoV detection directly from nasopharyngeal aspirate using real-time RT-PCR (Peiris et al., 2003a,b; Tsang et al., 2003). It was proposed to further verify and validate the assay developed in this study by using careful selection panel of reference samples consisting of wide dynamic range of naturally infected SARS samples, i.e. from 10^6 to 1 pfu/ml as well as SARS negative control samples. However, it is very difficult to obtain credible SARS reference samples with known infectious titers since there is no known major outbreak of SARS after the last multi-country outbreak in 2003. The conserved 3'-NCR feature of other RNA viruses, such as dengue viruses had been reported and utilized to develop type-specific RT-PCR assays for dengue virus identification and quantification throughout the world (Sudiro et al., 1997, 1998; Houng et al., 2000, 2001). Thus, it would be reasonable to predict that the 3'-NCR based SARS-CoV specific RT-PCR assay can be used to detect different SRAS-CoV originated from outbreaks of various geographic origins. It was demonstrated that the SARS-CoV 3'-NCR based assay using Canadian SARS-CoV Tor2 sequence could be used to detect laboratory grown SARS-CoV Urbani strain as well as various SARS-CoV infected samples in Taiwan. Based on the assay's excellent performance capacity (high detection sensitivity at 0.005 pfu/assay and robustness in detecting SARS-CoV of different origins and preparations including

direct viral cDNA as well as cloned recombinant plasmid) and superior specifications (100% specificity and 91% amplification efficacy) shown in this report, the 3′-NCR based SARS-CoV RT-PCR should be able to serve as an efficient SARS-CoV diagnostics in the event of future outbreak.

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