JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2005, p. 4262–4265 Vol. 43, No. 8
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.8.4262–4265.2005

Performance of Single-Step Gel-Based Reverse Transcription-PCR (RT-PCR) Assays Equivalent to That of Real-Time RT-PCR Assays for Detection of the Severe Acute Respiratory Syndrome-Associated Coronavirus

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Received 11 March 2005/Returned for modification 18 April 2005/Accepted 2 May 2005

Simple gel-based one-step reverse transcription-PCR (RT-PCR) assays, used to investigate patients during the 2003 severe acute respiratory syndrome (SARS) outbreak in Singapore, were found to be as sensitive as commercial and in-house real-time RT-PCR assays. The detection limit was approximately 1 genome equivalent (GE) per 5 μl PCR mixture. One PFU of SARS coronavirus was estimated to be 258 ± 46 GE.

Severe acute respiratory syndrome (SARS) emerged in 2002–2003 as a global outbreak caused by a newly recognized coronavirus (SARS-associated coronavirus [SARS-CoV]). In the absence of confirmatory tests, the diagnosis was based on World Health Organization (WHO)-defined clinical criteria (17). WHO stressed the need for simple tests (2, 10, 18) that meet the capabilities of institutions in the developing world. Many PCR assays (7, 9) targeting the RNA-dependent RNA polymerase region (3, 13), the nucleocapsid (4, 8), and the 3′ noncoding region (6) have been reported. Here we demonstrate the sensitivity of two simple gel-based systems and one real-time system targeting the proteinase gene region. We compare these assays with the Artus kit, using RNA standards, and present some results from clinical samples collected during the outbreak.

WHO criteria (17) were used to define probable or suspect cases of SARS. Serial $10^{0.5}$-fold dilutions of RNA were prepared from a culture of SARS CoV isolate 2003VA2774 (16). RNA was extracted from patient samples, from cultured SARS-CoV, and directly from the stock vial of other viruses (ATCC) with a QIAGEN viral RNA kit and from the MRC-5 fibroblast cell line (ATCC CCL171) with the QIAGEN RNA extraction kit. The Artus RealArt HPA-Coronavirus reverse transcription-PCR (RT-PCR) kit, which targets the RNA polymerase region NSP9, was performed on a LightCycler (Roche). In-house Institute of Molecular and Cell Biology (IMCB) primers and probe were selected by using the Urbani strain (GenBank accession no. AY278741) sequence. The proteinase gene (position 6652 to 7003, NSP1 region) was chosen, as it is well conserved in SARS-CoV (15, 16), mutations are rare (16), and there are no matches with other coronaviruses, unlike the NSP9 (RNA polymerase) region, which shows homologous sequences (16). Three sets of primers, designated IMCB1 (upper, 5′-ACATCAAATTGCGCTAAGA-3′; lower, 5′-ACAATTCTCTAACGCCATTAC-3′), IMCB2 (upper, 5′-GCCGTAGTGTCAGTATCAT-3′; lower, 5′-CACCTAACTCTGTACGCTGTC-3′), and IMCB3 (upper, 5′-GCACTTTGTAGAAACAGTTTCTTTGG-3′; lower, 5′-CACCTAACTCTGTACGCTGTCCTG-3′), and one TaqMan probe for the IMCB3 set (6FAM 5′-TGGCTCTTACAGAGATT-3′MGBNFQ [where 6FAM is 6-carboxyfluorescein]) were used. In the gel-based IMCB assay, one-step RT-PCR was performed with the QIAGEN one-step RT-PCR kit (catalog no. 210210), 5 μl of RNA, 10 units of RNase inhibitor, and each primer at a final concentration of 0.6 μM on a Stratagene Robocycler 40 (La Jolla, California) by using the following steps: reverse transcription at 50°C for 30 min and initial denaturation at 95°C for 15 min, followed by 42 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 80 s, and extension at 72°C for 50 s, and a final extension at 72°C for 10 min. Conventional electrophoresis was used. The expected amplicon sizes for the IMCB1 and IMCB2 primer sets are 352 bp and 157 bp. The real-time IMCB assay was performed using TaqMan one-step RT-PCR master mix reagents (catalog no. 4309169; Applied Biosystems) with 5 μl of RNA. The upper and lower primers were at a final concentration of 0.3 μM and 0.9 μM, the TaqMan probe at 0.2 μM. Thermal cycling was performed by Applied Biosystems Prism 7500 or Stratagene Mx3000P using the following steps: reverse transcription at 48°C for 30 min and initial denaturation at 95°C for 10 min, followed by denaturation at 95°C for 15 s and annealing and extension at 60°C for 60 s. The cycle was repeated 50 times. This assay was evaluated only on cultured SARS-CoV material.

The number of SARS-CoV genome equivalents (GE) was estimated to be 258 ± 46 GE per PFU by the Artus system in repetitive tests with dilutions at $10^{-5.5}$ and 10^{-6} PFU per ml. The sensitivity was estimated to be 3×10^{-3} to 9×10^{-3} PFU per 5 μl reaction mixture or 0.7 GE to 2 GE per 5 μl reaction mixture (Table 1).

Of 505 saliva and 70 sputa tested, 6 saliva and 30 sputa were positive by IMCB gel-based assays. Eight of these positive specimens were not from clinically diagnosed SARS cases. Serological correlation was not possible, as our data were

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TABLE 1. Sensitivities of different SARS-CoV RT-PCR assays[a]

SARS RNA standards (PFU/5 μl, copies/5 μl)[b]	No. of specimens positive by indicated assay/total				Result(s) for Artus (real-time) assay[f]		
	IMCB1[c]	IMCB2[d]	IMCB3 (real-time)[e]		No. of positive specimens/total	Avg no. of copies/μl	SD, CV (%)
			Run 1	Run 2			
$10^{-5.5}$ (0.277, 71)	12/12	8/8	ND	ND	7/7	12.164	0.923, 7.6
$10^{-6.0}$ (0.088, 23)	12/12	8/8	12/12	6/6	7/7	5.215	0.457, 8.8
$10^{-6.5}$ (0.028, 7)	12/12	8/8	12/12	6/6	7/7	2.520	0.479, 19.0
$10^{-7.0}$ (0.0088, 2)	12/12	8/8	12/12	6/6	8/8	0.393	0.232, 59.0
$10^{-7.5}$ (0.0028, 0.7)	12/12	8/8	10/12	6/6	6/7	0.413	0.318, 77.0
$10^{-8.0}$ (0.0009, 0.2)	4/10	1/8	2/12	1/6	1/7	0.014	0.034, 244.9
$10^{-8.5}$ (0.0003, 0.1)	2/10	2/6	1/12	1/6	1/6	0.061	0.137, 223.6

[a] Sample volume per reaction is 5 μl for all RT-PCR assays.
[b] Copies/5 μl estimated from the results of $10^{-5.5}$ and $10^{-6.0}$ dilutions.
[c] Results of one-step RT-PCR with primer set 1; products were analyzed by agarose gel/ethidium bromide.
[d] Results of one-step RT-PCR with primer set 2; products were analyzed by agarose gel/ethidium bromide.
[e] Results of one-step real-time RT-PCR with TaqMan probe determined by AB ABI 7500 real-time PCR system. ND, not done.
[f] Results of RealArt HPA-Coronavirus LC RT-PCR assay. CV, coefficient of variation.

TABLE 2. Results from clinical samples obtained with IMCB and Artus assays

Sample	Sample description[a]	Artus results (copies/ml)	Assay results[b]		SARS status[c]	No. of days of illness	Note[d]
			IMCB1	IMCB2			
6	Saliva	1,637	+	ND	N	11	
8	Saliva	2,658	++	ND	P	5	
10	Saliva	123,379	+++	ND	P	9	
29	Saliva	Negative	Negative	ND	N	5	
M43	Saliva	602	++	ND	P	7	
N61	Saliva	677	+	+	P	7	
119	Stool	1,045	+	ND	P	57	
120	Stool	105,700	+++	ND	P	14	
602	Stool	440,000	++	ND	P	54	
604	ETT, S	39,127	+++	ND	P	14	
606	ETT, C	258,900	+++	ND	P	14	
607	ETT, C	23,385	++	ND	P	15	
B68	Plasma	1,090	++	ND	P	10	
B71	Plasma	2,975	++	ND	P	11	
C77	Plasma	185,825	+++	ND	P	2	
C79	Plasma	875	++	ND	P	9	
M30	Plasma	Negative	+	ND	P	3	
G03	Serum	1,249	+	ND	P	10	
G04	Serum	8,795	+	ND	P	4	
G12	Serum	740	+	ND	P	7	
H75	Serum	9,837	+	ND	S	8	
M17	Serum	Negative	+	ND	P	7	
D55	Sputum, S	227,925	+++	ND	P	14	
	Sputum, C	65,670	+++	ND			
D41	Sputum, S	2,655	+	ND	P	21	
	Sputum, C	15,097	++	ND			
E14	Sputum, S	22,163	+++	ND	N	3	
	Sputum, C	47,572	+++	ND			
C78	Sputum, S	666	ND	+	P	35	
	Sputum, C	2,876	ND	+			
J7	Sputum, S	4,509	+	+	P	13	
	Sputum, C	73,222	++	++			
L65	Sputum, S	185	W+	+	P	23	
N83	Sputum, S	462	+	ND	P	25	
	Sputum, C	14,228	++	ND			
P38	Sputum, S	23,625	+++	ND	P	62	
	Sputum, C	161,850	+++	+++			
D23	Sputum, S	Negative	Negative	ND	S	1	Flu B positive
	Sputum, C	Negative	Negative	ND			
D32	Sputum, S	Negative	Negative	ND	S	11	RSV positive
	Sputum, C	Negative	Negative	ND			
R95	Sputum, S	Negative	Negative	Negative	NA	NA	Flu A positive
	Sputum, C	Negative	Negative	Negative			
P83	Sputum, S	Negative	ND	Negative	NA	NA	Flu A positive
	Sputum, C	Negative	ND	Negative			

[a] ETT, aspirate from an endotracheal tube; S, supernatant; C, cell pellet.
[b] +, positive; ++, strong positive; +++, very strong positive; W+, weak positive; ND, not done.
[c] P, probable; S, suspect; N, not clinically SARS; NA, not available.
[d] Flu, influenza virus; RSV, respiratory syncytial virus.

made anonymous. The WHO criteria for laboratory-defined SARS (19) were fulfilled, and the specificity of these eight results was corroborated by positive Artus results obtained in another laboratory. This further confirms the specificity shown by negative results on related and unrelated viruses: human coronaviruses 229E and OC43, avian infectious bronchitis virus, dengue virus type 1, yellow fever virus (11), human enteric coronavirus, various animal coronaviruses (bovine, rabbit, canine, rat, and feline), mouse hepatitis virus, respiratory syncytial virus, influenza A virus, influenza B virus, and total human cell RNA (2 μg/reaction). Representative results from clinical samples obtained with IMCB and Artus assays are shown in Table 2. Confirmation that an amplicon is indeed from a SARS-CoV either by restriction enzyme digestion or sequencing improves specificity (1). However, this requires further manipulation of the amplified products outside PCR tubes and may increase the chance of contamination in the laboratory. We believe this would be unnecessary and unduly hazardous if positive results were confirmed by another assay targeting a different site on the genome.

Two groups reported loop-mediated isothermal amplification for SARS-CoV detection in clinical samples (5, 14). The loop-mediated isothermal amplification method uses isothermal conditions, and the results can be obtained after 1 h of incubation (12). This assay will become a valuable test only if the system is demonstrated to have a high sensitivity.

These gel-based assays are simple to use and are particularly suited to laboratories without expensive real-time equipment. Many protocols which require a nested assay to reach a similar level of sensitivity have been published, but nested assays carry a much higher risk of contamination and are longer, more expensive, technically demanding, and require "double time" use of manpower and equipment. These IMCB gel-based protocols can be used as primary or confirmatory tests to complement other assays, including real-time PCR, as they target a different site on the genome and are of equivalent sensitivity. The high costs and short shelf life of commercial kits increase the attraction of these simpler assays. We would advise spiking a duplicate sample to provide an internal control.

A real-time version of these assays, IMCB3, was developed for better-equipped laboratories. Its performance was equivalent to the gel-based assays (Table 1), but this assay brings the additional specificity of a probe. Real-time amplification plots for IMCB3 (data not shown) showed the fluorescence signals obtained from the $10^{-7.5}$ dilution to be significantly higher than that of the no-template control.

The apparent detection of less than 1 GE per reaction is statistically unlikely and suggests the Artus quantification standards undercount. Hourfar et al. reported the Artus assay detection limit to be 0.189 molecule per 5 μl reaction mixture (7). The detection of one molecule at this level depends on freak chance and thus, in theory, should be difficult to reproduce. Houng et al. reported that the number of viral genomic copies per PFU in their preparation was estimated to be 1,200 to 1,600 by using cloned plasmid DNA (6). Our Artus results estimate that the SARS-CoV culture lot used in this report contained 258 ± 46 copies/PFU.

These two single-step gel-based RT-PCR assays have performances equivalent to that of the Artus assay on both clinical material and RNA standards. This fact, combined with their simplicity and low cost, especially as testing for SARS becomes less frequent and commercial kits expire before being used up, has made them our primary tests. As these IMCB assays target the proteinase region, they offer an excellent method for confirming results obtained with assays that detect other parts of the genome.

The work at the IMCB was in part supported and funded by the Biomedical Research Council, which is part of Singapore's Agency for Science, Technology, and Research (A*STAR). The work at Tan Tock Seng Hospital (TTSH) was in part supported and funded by the Biomedical Research Council. Ethical approval was granted by the TTSH Ethics Committee.

We thank Ooi Eng Eong from National Environment Agency, Singapore, for SARS-CoV RNA extracts. We thank Vijaya Kumari Rajagopal, Samuel Tiang Shee Tiung, and Seet Swee Kai from TTSH, Khng Jiaying from IMCB, and Tan Hwee Cheng from Environmental Health Institute, National Environment Agency for their expert technical assistance. We also acknowledge Ling Ai Ee from Singapore General Hospital for the virus isolate.

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