Short communication

Development of a point-of-care test to detect SARS-CoV-2 from saliva which combines a simple RNA extraction method with colorimetric reverse transcription loop-mediated isothermal amplification detection

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ABSTRACT

The new coronavirus infection (COVID-19) is a major public health concern, with a high burden and risk for infection among patients and healthcare workers. Saliva droplets containing SARS-COV-2 are a major vector for COVID-19 infection, making saliva a promising alternative for COVID-19 testing using nasopharyngeal swab samples. To diagnose COVID-19 patients in the field, a point-of-care test (POCT) using saliva was conceptualized. We have developed a simple method for extracting RNA from saliva samples using semi-alkaline proteinase, a sputum homogenizer typically used for preparing samples for tuberculosis testing, and a subsequent simple heating step with no need for centrifugation or RNA extraction. Further, we newly developed a triplex reverse transcription loop-mediated isothermal amplification approach (RT-LAMP) which utilizes colorimetric readout using a heat block, with results evaluated with the unaided eye. In 44 clinical patients suspected of having COVID-19 infection, the test took 45 min, and resulted in a diagnostic sensitivity of 82.6% (19/23) and diagnostic specificity of 100% (21/21), compared to the reference standard. The limit of detection was 250 copies/reaction (25,000 copies/mL). Our newly developed POCT approach achieved simple RNA extraction and constant RT-LAMP detection. This POCT has the potential to be used for simple inspection stations in a field setting, helping reduce the risk of infection by simplifying and accelerating testing for COVID-19.

1. Introduction

With progressing globalization, the worldwide spread of infectious diseases has accelerated. The new coronavirus infection (COVID-19) is a major public health concern [1], with a high burden and risk for infection among patients and healthcare workers. A reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) testing for the diagnosis of COVID-19 has increased the use of saliva as an alternative to nasopharyngeal swab sampling [2–6].

Angiotensin-converting enzyme II (ACE2), the main receptor for SARS-CoV-2 entry into human cells, is highly expressed on oral cavity mucosa, especially tongue epithelial cells [3,7]. In Japan, many

COVID-19 cases have been found in metropolitan entertainment districts, where food, drink, and conversation are frequent. Saliva droplets containing SARS-CoV-2 are a major vector for COVID-19 infection, making saliva a promising candidate for COVID-19 testing [2,4,8]. While the viral load of SARS-CoV-2 in saliva samples is lower than in nasopharyngeal swab samples [3,9], several researchers have reported comparable or better diagnostic sensitivity with saliva testing [2,4–6,8].

The combined use of the direct-to-test addition by heating and RT-qPCR using a nasopharyngeal swab sample has proven successful due to reduced inhibitory effects [10]. However, the saliva sample must undergo high-speed centrifugation at 20,000 *g* for 5−30 min following purification with a commercial RNA extraction kit to remove potential

inhibitors [2–5,8,9]. This tedious RNA extraction process requires a well-equipped laboratory and is a major hurdle for performing sensitive point-of-care testing (POCT) for COVID-19 using saliva.

Saliva is easier to sample and carries less risk of contamination to the collector than nasopharyngeal swabs [6]. For immunochromatographic assays, the sensitivity is relatively high for nasopharyngeal swabs with low carryover of sample-derived inhibitors, but decreases for saliva with high inhibitors [4]. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a more attractive option for POCT with robustness to inhibitors in the sample over immunochromatographic assays. Also, it has broader utility when a portable real-time detector is used with an in-house battery or combined use with a cost-effective heat block and colorimetric reagent [11–14].

We have developed a simple method for extracting RNA from saliva samples using semi-alkaline proteinase (SAP), a sputum homogenizer typically used for preparing samples for tuberculosis testing, and a subsequent simple heating step with no need for centrifugation or RNA extraction. Further, we newly developed a triplex RT-LAMP approach which utilizes colorimetric readout usable of a heat block, with results evaluated with the unaided eye. Of note, colorimetric readout is more unambiguous than turbidimetric readout with the unaided eye, and more practical in the field than fluorescent LAMP analysis without color change dye. Here, we preliminarily evaluated the POCT performance of this approach with 44 clinical saliva samples.

2. Materials and methods

2.1. Design of LAMP primers

We used Primer Explorer V5 (primerexplorer.jp/lampv5e/index.html) to design three primer sets targeting the ORF 1ab, S, and ORF 7a regions to simultaneously detect SARS-CoV-2 with high sensitivity and robustness. To identify highly conserved nucleotide sequences with low similarity to SARS-CoV-1 sequences, we performed multiple sequence alignment using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo) of 490 whole-genome SARS-Cov-2 sequences from the DDBJ/EMBL/GenBank databases (www.ncbi.nlm.nih.gov/nucleotide/) submitted by researchers from five continents. The details of each primer and in-house reagent are shown in Appendix Tables A1–A3. Artificially synthesized SARS-CoV-2 sequences (Eurofins, Tokyo, Japan) corresponding to the three target regions and RNA extracted from healthy human saliva were used as positive and negative controls, respectively, for the triplex RT-LAMP format.

2.2. Reference method for SARS-CoV-2 detection from saliva

Saliva samples were collected from a total of 165 patients suspected of COVID19 infection at Kyoto University Hospital and Kyoto City Hospital between May and July 2020. Saliva samples were collected into a disposable sterilized plastic container through the drooling technique. Forty-five and 120 samples were determined to be positive and negative, respectively, according to the reference method shown below [15]. By random sampling, 44 of these samples were used for this study. The saliva samples were mixed thoroughly with 3 strength-volume of SAP (semi-alkaline protease, Suputazyme; Kyokuto Pharmaceutical Industrial, Tokyo, Japan) manually or using a vortex mixer for 15 s. After incubation for 15 min at room temperature to dissolve the saliva components, each of the saliva-SAP mixtures (SS mixtures) was added to a 1.5-mL microcentrifuge tube. Subsequent procedures were completed without interruption, or else the SS mixture was stored at −80 °C until used. For RNA extraction, 140 µL of each SS mixture was eluted into 60

Table 1
LOD determination of the triplex RT-LAMP with the heat-inactivated SARS-CoV-2, ATCC VR-1986HK in phosphate-buffered saline.

Copies/mL	50,000	25,000	12,500	10,000
Copies/reaction	500	250	125	100
Positive/tested	20/20	20/20	14/20	9/20
(%)	(100%)	(100%)	(70%)	(45%)

ATCC, American Type Culture Collection.
LOD, Limit of detection.
RT-LAMP, Reverse transcription loop-mediated isothermal amplification detection.
SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2.

µL using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). RT-qPCR using the N2 primers and probe was performed with 5 µL of the extracted RNA in 20 µL of reaction mixture in the LightCycler 480 System II (Roche, Basel, Switzerland) according to the protocol recommended by the National Institute of Infectious Diseases, Japan [15]. Ten-fold serial dilutions (5 to 5×10^6 copies/reaction) of the synthesized RNA containing the target sequence [15] were used to calculate the viral loads in clinical samples.

2.3. POCT for SARS-CoV-2 detection from saliva

In parallel, for POCT, the remaining SS mixture was heated at 95 °C for 5 min, and then, if visible large amount of the droplet had adhered on the 1.5-mL microcentrifuge tube lid, the tube was swung manually 1–2 times to quickly remove the aqueous droplet, as the prevention measure of contamination. Using 50 µL of RT-LAMP reaction mixtures (including 10 µL of template), the RT-LAMP reaction was monitored in real-time using a LightCycler 480 System II (Roche) at 63 °C for 30 min. The threshold time of positivity was set at 25 min, and time of positivity was automatically calculated. Amplification was also judged by endpoint readout with the unaided eye, which was interpreted as positive when the color change was observed from violet to sky blue.

2.4. Determination of limit of detection (LOD)

The LOD of triplex RT-LAMP was defined as the lowest concentration at which 95% of positive samples were detected using a 2-fold series of phosphate-buffered saline (PBS) dilutions with 20 replicates of 50,000, 25,000, 12,500, and 10,000 copies/mL of heat-inactivated SARS-CoV-2 (ATCC VR-1986HK; American Type Culture Collection, Manassas, VA, USA) (Table 1). The SARS-CoV-2 dilutions by PBS were heated at 95 °C for 5 min. After removal of the aqueous droplet on the tube lid by swinging the tube manually 1–2 times, 10 µL of the template was applied to 50 µL of RT-LAMP reaction mixture, as described above.

3. Results and discussion

In 44 clinical patients suspected of having COVID-19 infection, 23 and 21 saliva samples resulted positive and negative by the reference method. The diagnostic sensitivity and specificity were 82.6% (19/23; 95% confidence interval [CI], 61.2%–95.1%) and 100% (21/21, 95% CI, 83.8%–100%) for the new POCT, which took 45 min from starting crude RNA extraction to final judgement (Fig. 1, Table 2). Colorimetric endpoint readout with the unaided eye achieved robust judgement from violet to sky blue (Fig. 1). The results of the fluorescent real-time RT-LAMP detection by using the qPCR equipment, LightCycler 480 System II and endpoint judgement by the colorimetric readout with the unaided eye were constantly matched each other.
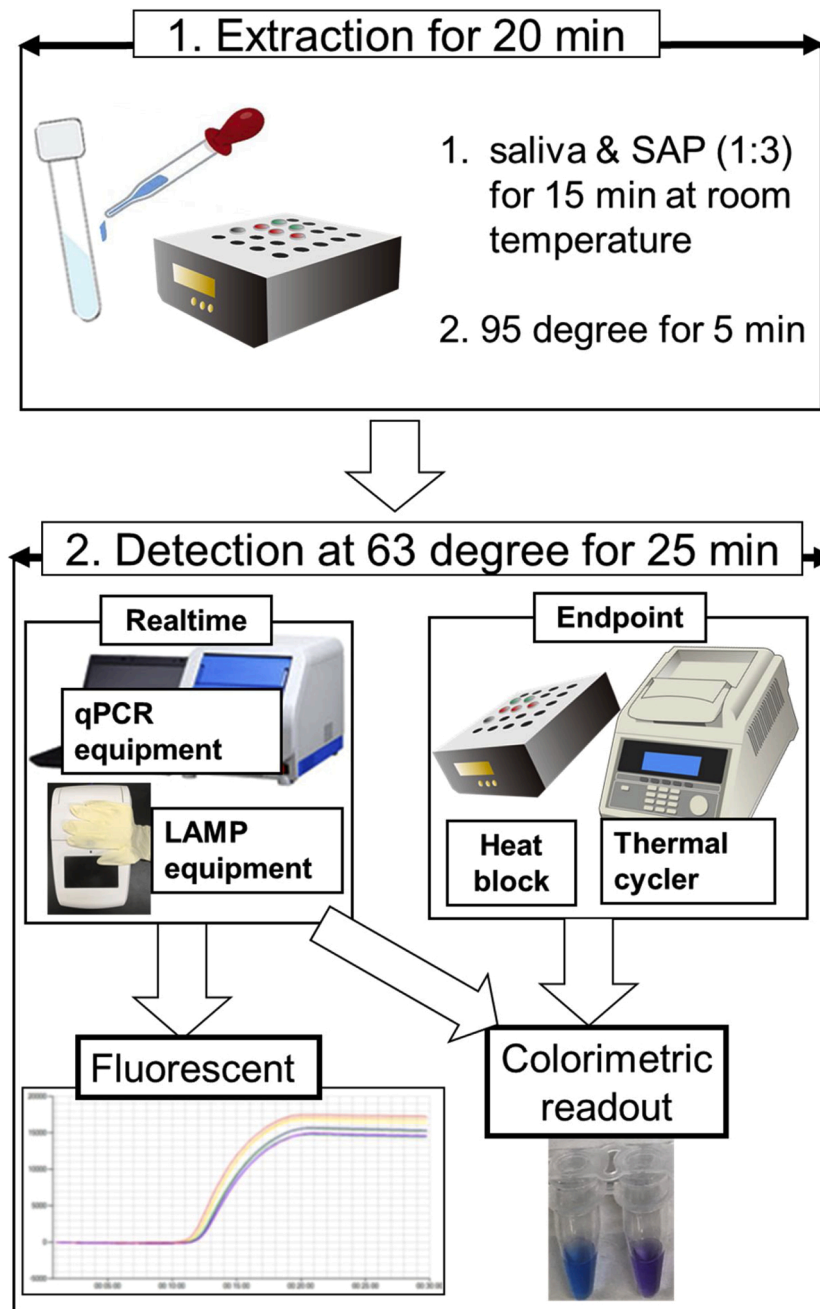
Fig. 1. Concept of POCT using a combination of simple RNA extraction and LAMP equipment.

Vortexing or manual mixing was used to mix the saliva sample with the SAP solution. If a large amount of visible droplets adhered to the 1.5-mL microcentrifuge tube lid after heating at 95 °C for 5 min, the droplets were removed by swinging the 1.5-ml microcentrifuge tube 1–2 times. Neither of these manual operations affected the detection by POCT. Our newly developed POCT is capable of both simple endpoint judgement using a heat block and more rapid real-time judgement using a qPCR equipment or real-time LAMP equipment, as shown in Fig. 1.

As shown in Table 2, 18 clinical samples exceeding 380 copies/reaction (>33,000 copies/mL of saliva) according to reference standard measurement were positive on POCT between 9 min 02 s and 16 min 12 s from the start of amplification. Regarding the 3 samples showing low

copy numbers of 170–230 copies/reaction (15,000–20,000 copies/mL of saliva) on RT-qPCR, 1 was ultimately positive at 23 min 32 s of amplification, while the other 2 were false negatives on POCT. This discrepant result is not surprising. This is because, as shown in Table 1, the LOD determined by 20 repetitions was 250 copies/reaction (25,000 copies/mL), but the frequency was 70% (14/20) positive at 125 copies/reaction (12,500 copies/mL). Samples with copy numbers in this range are on the LOD borderline between positive and negative, so we speculate that the results were unstable. Therefore, these findings were interpreted to indicate borderline samples near the LOD of POCT. Two other samples showing low copy numbers of 13–86 copies/reaction (1,100−7,400 copies/mL of saliva) were also false negatives, likely due to their low

Table 2
Results of POCT and reference standard in 44 clinical patients.

Sample ID	Time of positivity by POCT (min:s)	Copies/reaction tube by reference standard	Copies/mL of saliva by reference standard
N = 23		RT-qPCR positive	
AR+26	Negative	13	1100
AR+23	Negative	86	7400
AR+24	23:32	170	15,000
AR+03	Negative	180	15,000
AR+11	Negative	230	20,000
AR+22	12:05	380	33,000
AR+20	14:35	590	51,000
AR+04	14:34	1300	110,000
AR+16	13:37	2500	210,000
AR+21	12:11	3800	330,000
AR+17	16:12	8000	690,000
AR+06	13:11	9300	800,000
AR+25	12:11	16,000	1,400,000
AR+18	14:43	24,000	2,100,000
AR+07	12:56	34,000	2,900,000
AR+08	11:59	45,000	3,900,000
AR+14	11:36	120,000	10,000,000
AR+09	10:58	130,000	11,000,000
AR+10	10:17	190,000	16,000,000
AR+15	09:49	530,000	45,000,000
AR+27	09:02	1,600,000	140,000,000
AR+13	09:06	1,700,000	150,000,000
AR+12	13:51	3,600,000	310,000,000
N = 21			
	Negative	RT-qPCR negative	

POCT, Point-of-care test.
RT-qPCR, Reverse transcription quantitative real-time polymerase chain reaction.

copy numbers being below the detection power of POCT.

4. Conclusions

Our newly developed POCT approach achieved simple RNA extraction and constant RT-LAMP without the need for centrifugation, or nucleic acid extraction kits. While this POCT does not require expensive equipment and performs comparably to an existing reference standard, of course, requiring further improvement for higher sensitivity and larger scale evaluation with clinical samples. There is a high need for POCT using saliva, due to a low risk of contamination and easiness of sample collection, but the immunochromatography is not a realistic option because of the high inhibitory effects in saliva [4]. This POCT using saliva thus has the potential to be used for simple inspection stations in a field setting, helping reduce the risk of infection by simplifying and accelerating testing for COVID-19.

Declaration of Competing Interest

The authors report no declarations of interest.

Ethics approval

The Ethics Committee of Kyoto University Graduate School and the Faculty of Medicine approved this study (R2379) and waived the need for obtaining informed consent from each patient.

CRediT authorship contribution statement

Wataru Yamazaki: Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing - original draft, Writing - review & editing. **Yasufumi Matsumura:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Visualization, Writing - original draft, Writing - review & editing. **Uraiwan Thongchankaew-Seo:** Data curation, Investigation. **Yasuko Yamazaki:** Data curation, Investigation. **Miki Nagao:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.

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Appendix A

Table A1
Primers used for RT-LAMP assay.

Primer	Sequences (5′-3′)	Location*	Target
F3−1ab	TGCTAGATTTTACTTTTACACCAG	4522−4545	
B3−1ab	CAGGTGTTTTAGAAGAAGAAGT	4757−4736	
FIP-1ab	CCAAGTGGCATTGTAACAAGAGTTT-TAAAACAACTGTAGCGTCAC	4617−4593, 4546−4565	
BIP-1ab	TGCTCGGTATATGAGATCTCTCAA-CGCTGTAACAGCATCAGG	4651−4674, 4720−4674	ORF1ab
FLP-1ab	CATTTAGATCGTTAAGTGTGTTGA	4592−4569	
BLP-1ab	AGTGCCAGCTACAGTTTCT	4675−4693	
F3-S	TCTTTCACACGTGGTGTT	21653−21670	
B3-S	GTACCAAAAATCCAGCCTC	21885−21867	
FIP-S	CATGGAACCAAGTAACATTGGAAAA-CCTGACAAAGTTTTCAGATCC	21761−21737, 21677−21697	Spike glycoprotein
BIP-S	CTCTGGGACCAATGGTACTAAGAG-GACTTCTCAGTGGAAGCA	21772−21795, 21855−21838	
FLP-S	GGTAAGAACAAGTCCTGAGTTGAAT	21732−21708	
BLP-S	GTTTGATAACCCTGTCCTACCATT	21796−21819	
F3-7ab	GCACTGACTTGCTTTAGC	27556−27573	
B3-7ab	TGAAAGTTCAATCATTCTGTCTT	27770−27748	
FIP-7ab	AGGTGAAACTGATCTGGCACG-AATTTGCTTTTGCTTGTCCTG	27645−27625, 27578−27598	
BIP-7ab	AGACAAGAGGAAGTTCAAGAACTTT-TGAGTGTGAAGCAAAGTGT	27658−27682, 27742−27724	ORF7ab
FLP-7ab	GATAGACGTGTTTTACGCCGT	27619−27599	
BLP-7ab	CTCCAATTTTTCTTATTGTTGCGGC	27686−27710	

RT-LAMP, reverse transcription loop-mediated isothermal amplification.
* Corresponding to the GenBank accession No. NC_045512.2.

Table A2
Composition of the RT-LAMP reaction mixture including the homemade master mix.

RT-LAMP reaction mix	Triplex	
4x LAMP Master Mix	12.5	μL
Distilled water	11.5	μL
1x Primer mix for 1ab	2.6	μL
1x Primer mix for S	2.6	μL
1x Primer mix for 7a	2.6	μL
CFI	2	μL
Bst 2.0 DNA polymerase (8 units/μL)	6	μL
AMV Reverse Transcriptase for Genie III (20 units/μL)	0.2	μL
RNA template	*10*	*μL*
Total	50	μL

Homemade 4x LAMP master mix	For 500		For 1000	
5 M Betaine	4	mL	8	mL
1 M Tris-HCl (pH8.8)	0.5	mL	1	mL
3 M Potassium chloride (KCl)	0.08	mL	0.16	mL
5.6 M $(NH_4)_2SO_4$	0.04	mL	0.08	mL
1 M $MgSO_4$	0.2	mL	0.4	mL
100 mM dATP	0.35	mL	0.7	mL
100 mM dCTP	0.35	mL	0.7	mL
100 mM dGTP	0.35	mL	0.7	mL
100 mM dTTP	0.35	mL	0.7	mL
Tween20	0.05	mL	0.1	mL
Total (for 500/1000 samples)	6.27	mL	12.54	mL

CFI	For 500	
Distilled water	0.6965	mL
10 mM HNB*	0.3	mL
Gel green (×10,000)	0.0035	mL
Total (for 500 samples)	1.0	mL
* Distilled water:HNB powder =1 mL:6 mg		

Primer mix	For 500	
FIP (Inner F) (100 μM)	0.4	mL
BIP (Inner B) (100 μM)	0.4	mL
LF (Loop F) (100 μM)	0.2	mL
LB (Loop B) (100 μM)	0.2	mL
F3 (Outer F) (100 μM)	0.05	mL
B3 (Outer B) (100 μM)	0.05	mL
Total (for 500 samples)	1.3	mL

HNB, hydroxynaphthol blue; CFI, colori fluorescent indicator; RT-LAMP, reverse transcription loop-mediated isothermal amplification.

Table A3
Manufacturers' list for RT-LAMP reaction mix.

LAMP reagents	Manufacturers	Locations
4x LAMP Master Mix		
Tris-HCl	Wako Pure Chemical Industries	Osaka, Japan
Potassium chloride (KCl)	Sigma–Aldrich	St. Louis, MO, USA
$(NH_4)_2SO_4$	Sigma–Aldrich	St. Louis, MO, USA
Tween20	Sigma-Aldrich	St. Louis, MO, USA
$MgSO_4$	New England Biolabs	Ipswich, MA, USA
Illustra dNTP set 100 mM, solutions 4 × 25 μmol	GE Healthcare	Little Chalfont, UK
5M Betain Solution 5 mL	Sigma–Aldrich	St. Louis, MO, USA
CFI		
HNB	MP Biomedicals	Aurora, OH, USA
Gel green (x10,000)	Biotium	Hayward, CA, USA
Bst 2.0 DNA polymerase (8 units/ μL)	New England Biolabs	Ipswich, MA, USA
AMV Reverse Transcriptase, for Genie III (20 units/μL)	Nippon Gene	Tokyo, Japan
Primer (Recommended cartridge grade purification)	Hokkaido System Science	Sapporo, Japan

HNB, hydroxynaphthol blue; CFI, colori fluorescent indicator; RT-LAMP, reverse transcription loop-mediated isothermal amplification.

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