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ORIGINAL ARTICLE

POCT Detection of 14 Respiratory Viruses Using Multiplex RT-PCR

Ji Su Lee[1] · Jeong Jin Ahn[2] · Seung Jun Kim[2] · So Yeon Yu[1] · Eun Jung Koh[3] · Seung Hwan Kim[3] · Heung Sup Sung[4] · Jin Won Huh[5] · Seung Yong Hwang[1]

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Abstract

Over the past 6 years, acute respiratory infections have constituted an average of more than 70,000 cases in South Korea. It results in a high mortality rate in infants and the elderly with weak immunity. There are several types of respiratory viruses that invade the human respiratory tract and cause infectious disease. Reverse transcription PCR (RT-PCR) is mainly used for respiratory virus detection owing to its high sensitivity and reproducibility. In response, a multiplex real-time RT-PCR (rRT-PCR) assay was developed for the detection of influenza A and B viruses, parainfluenza viruses 1–4 (PIV1-4), human metapneumovirus, adenovirus, human rhinovirus, respiratory syncytial virus (RSV), and SARS-CoV-2. Detection ability of RT-PCR assay was confirmed by applying it to a portable device capable of point-of-care testing (POCT). Amplicons were synthesized using primer pairs and probe sets designed for each target virus, and a standard curve was constructed to confirm the limit of detection. An experiment using nasopharyngeal swab samples was conducted to understand the field applicability of the rRT-PCR assay. Detection was confirmed in most samples. This study demonstrated that rapid and accurate detection results can be obtained using the multiplex rRT-PCR based POC test, and that it is possible to detect 14 types of respiratory viruses that are generally difficult to distinguish at the same time, enabling timely treatment. Furthermore, we expect that the portable PCR device can significantly reduce the processing procedure of clinical samples before testing, which is the main disadvantage of common RT-PCR tests and can help reduce costs.

Keywords Respiratory virus · RT-PCR · Point-of-care · Molecular diagnosis · SARS-CoV-2

1 Introduction

Respiratory tract infections are disorders that develop in the lungs or other parts of the respiratory system and can cause various symptoms ranging from a common cold to pneumonia. Respiratory viruses are the most frequent cause of disease in humans and have a significant impact on hospitalization and mortality worldwide [1, 2]. They have become a crucial factor in respiratory diseases affecting all ages, especially in children ≤ 5 years of age and adults with weakened immune systems [3, 4]. The seasonality of infection varies from virus to virus, and infectious diseases can occur throughout the year [5]. Viruses that cause these infections include influenza viruses (types A and B), respiratory syncytial viruses (RSV), parainfluenza viruses (PIV), human metapneumovirus (hMPV), human rhinovirus (HRV), coronavirus, adenovirus (ADV), and bocavirus. Some of these viruses cause serious disease in immunocompromised hosts, and other respiratory viruses are also recognized as causes

✉ Seung Yong Hwang
syhwang@hanyang.ac.kr

1 Department of Molecular and Life Science, Hanyang University, 55, Hanyangdeahak-ro, Sangnok-gu, Ansan, Gyeonggi-do 15588, Republic of Korea

2 GeneTrigger, 20, Magokjungang 5-ro 1-gil, Gangseo-gu, Seoul, Republic of Korea

3 Department of Bio-Nanotechnology, Hanyang University, 55, Hanyangdeahak-ro, Sangnok-gu, Ansan, Gyeonggi-do 15588, Republic of Korea

4 Department of Laboratory Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

5 Department of Pulmonary and Critical Care Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

of upper and lower respiratory tract diseases [6–8]. In particular, Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which appeared in 2019, has been an urgent problem worldwide with a high mortality and infectivity since its appearance. For this purpose, rapid and accurate diagnostic tests are continuously being developed [9, 10].

In general, respiratory viruses commonly infect the respiratory system and cause similar and atypical clinical symptoms; therefore, it is very difficult to distinguish the causative pathogen using only general practice [11]. Several diagnostic methods have been used to detect respiratory viruses. Molecular diagnostics are widely used because they can achieve more precise results in a shorter time [12]. Diagnosing viruses using reverse transcription PCR (RT-PCR) is rapid and sensitive. Therefore, it has become the most important technique for detecting and monitoring Multiplex RT-PCR, which can detect multiple respiratory viruses simultaneously in a single reaction and has the advantage of facilitating timely treatment decisions, thereby helping to prevent infection [4, 13, 14]. Indeed, it has been demonstrated that rapid diagnosis of respiratory viral infections significantly reduces patient hospitalization, antibiotic use, and laboratory utilization compared to diagnosis through viral culture [12, 15].

However, while using specialized equipment and technical expertise, PCR tests are conducted in centralized laboratories. This may delay the turnaround time due to sample transportation, and the use of batch testing is common, leading to unnecessary infection [16]. In fact, most multiplex real-time RT-PCR (rRT-PCR) kits for detecting respiratory viruses use large-scale equipment due to multiple fluorescence signals. The drawback, however, is the length of the testing time (more than 2 h), excluding the nucleic acid extraction. The point-of-care testing (POCT) enables rapid diagnosis by conducting diagnostic tests near the location where the patient is treated and shows outstanding economic efficiency [17, 18]. As the importance of the POCT increases due to SARS-CoV-2, the sensitivity and accuracy of the assay will continue to improve, and new methods and technologies for detecting nucleic acids are being integrated into the POCT, highlighting the continuous progress in RT-PCR testing and detection [19, 20].

Therefore, we developed a multiplex rRT-PCR detection method targeting 14 viruses known to have high disease severity and infectivity among respiratory viruses, and the target list is as follows: influenza A virus, influenza A virus H3N2 (seasonal influenza virus), H1N1pdm09 virus, influenza B virus, PIV type 1–4, hMPV, ADV, HRV, RSV, and SARS-CoV-2. By applying this to PCR equipment capable of POCT, it was confirmed that it has high detection sensitivity and specificity, as well as rapid diagnosis.

2 Results and Discussion

2.1 Primer and Probe for Target Virus

Primers and probes for target viruses were synthesized based on the consensus sequences of each virus gene. To distinguish between influenza A and B viruses, the genetically conserved matrix (M) gene in influenza A virus, and the nonstructural protein (nuclear export protein, NS & NEP) genes, which are abundantly present in cells infected with influenza B virus, were targeted [21, 22]. In addition, for subtyping influenza A virus (H3N2, pdm09), primers and probes targeting the subtype-specific hemagglutinin (HA) gene of each subtype were prepared. PIV targets the hemagglutinin-neuraminidase (HN) gene and the phosphoprotein (P) gene, which have the largest antigenic and genetic differences between types, to target subtypes PIV1, PIV2, PIV3, and PIV4, respectively [23, 24]. Considering the existence of several subtypes of the virus, two probes were prepared for hMPV and ADV. The hMPV targeted the fusion protein (F) gene, while the ADV targeted the hexon (H) gene [25–27]. The HRV targets a polyprotein (5'UTR) gene, and two probes were designed based on mutations in the sequence [28]. For RSV, primers and probes were designed to target the consensus region of RSVA and RSVB matrix protein genes. To target SARS-CoV-2, primers and probes were designed with the most conservative reverse-dependent reverse-polymerase (RdRp) gene and nucleocapsid (N) gene with high virus content [29]. The designed primer and probe sequences and Tm values are listed in Table 1.

2.2 Sensitivity of Multiplex real-time PCR

To confirm that the designed primers and probes worked correctly, multiplex rRT-PCR of the entire set of synthetic plasmid samples was performed. Each plasmid template was diluted from 1.0×10^6 to 1.0×10^0 copies to detect the limit of detection. The tests were conducted using Bio-Rad CFX96 (Bio-Rad, USA), which is mainly used in general RT-PCR to confirm whether the designed primer and probe sets reacted well in common detection tests. To check the possibility of POCT, GENECHECKER UF-300 (Genesystem, Korea), a portable device with dual detection channels (FAM/ROX), was used. For analysis using portable equipment, diluted samples were applied to a ten-well PCR chip, and a set of primer pairs and probes were injected into each well to check whether simultaneous detection in a single reaction was possible. The experiment confirmed that all primer and probe sets were detected up to 10^1 or 10^0 copies/rxn of the plasmid in

Table 1 List of primer pairs and probe sets for target virus detection

Set	Virus	Target	Name	Sequence (5´–3´)	Tm (℃)
1	Influenza A	M	Forward	ACAAGACCAATCCTGTCACCT	63
			Reverse	TGGACAAAGCGTCTACGCT	64
			Probe	FAM-CTCACCGTGCCCAGTGAGC-BHQ1	67
	Influenza B	NS & NEP	Forward	GGATCCTCAACTCACTCTTCGA	63
			Reverse	CGGTGCTCTTGACCAAATTGG	64
			Probe	CAL Fluor Red 610-CAATTCGAGCAGCTGAAACTGCG-BHQ2	66
2	Influenza A (H3N2)	HA	Forward	CCATGCAGTACCAAACGGAAC	64
			Reverse	CACACTGAGGGTCTCCCAATAG	64
			Probe	FAM-TGACCGAATTGAAGTTACTAATGCTACTGA-BHQ1	66
	Influenza A (pandemic 09, H1N1)	HA	Forward	GGGGTAGCCCCATTGCA	64
			Reverse	AGTGATTCACACTCTGGATTTCCC	64
			Probe	CAL Fluor Red 610-TGGGTAAATGYAACATTGCTGGCTG-BHQ2	66–68
3	PIV1	HN	Forward	CAGGAAACCCAGACTGCAAC	63
			Reverse	GCGTACAGTGTGGTTGTAGC	63
			Probe	FAM-TGTCCGAGAGAATGCATATCAGGTGT-BHQ1	67
	PIV3	HN	Forward	AGATGTATATCAACTGTGTTCAACTCC	63
			Reverse	GCCTTTGTAGTATATCCCTGGTCC	64
			Probe	CAL Fluor Red 610-TGAAAGATCAGATTATGCATCATCAGGC-BHQ2	65
4	PIV2	HN	Forward	CAGGACTATGAAAACCATTTACCTAAGT	63
			Reverse	GAAAGCAAGTCTCAGTTCAGCTAG	63
			Probe	FAM-AATCAATCGCAAAAGCTGTTCAGTCA-BHQ1	66
	PIV4	P	Forward	CCTGGAGTCCCATCAAAAGTAAG	63
			Reverse	GCATCTATACGAACACCTGCTC	63
			Probe	CAL Fluor Red 610-TGTCTCAAAATTTGTTGATCAAGACAATACAAT-BHQ2	66
5	hMPV	F	Forward	GTTGTGVGGCARTTTTCAGA	60–64
			Reverse	CCTGCWGATGTYGGCATGT	63–65
			Probe1	FAM-AAYGCTGGAATAACACCAGCAAT-BHQ1	64–66
			Probe2	FAM-AATGCAGGGATAACACCRGCAAT-BHQ1	66–68
	ADV	H	Forward	CCCATGGAYGAGYCCAC	60–65
			Reverse	TCRATGACGCCGCGGT	65–67
			Probe1	FAM- TTCGACGTSGTCMGAGTGCAC-BHQ1	67–69
			Probe2	FAM- TTTGACGTGGTCCGTGTGCAC-BHQ1	67
6	HRV	Poly	Forward	TGAGTCCTCCGGCCC	62.2
			Reverse	GAAACACGGACACCCAAAGT	62.8
			Probe1	FAM-CTGAATGCGGCTAATCYWACCCC-BHQ1	65–68
			Probe2	FAM-CTGAATGYGGCTAACCTTAACCCYG-BHQ1	65–69
	RSV	M	Forward	GGGCAAATATGGAAACATACGTGAA	64
			Reverse	GGCACCCATATTGTWAGTGATGC	64
			Probe	CAL Fluor Red 610-CTTCACGAGGGCTCCACATACAC-BHQ2	66
7	SARS-CoV-2 (RdRp)	RdRp	Forward	GATGATACTCTCTGACGATGCTGT	64
			Reverse	TCTCAGTCCAACATTTTGCTTCAG	64
			Probe	FAM-ATGCATCTCAAGGTCTAGTGGCTAG-BHQ1	65
	SARS-CoV-2 (N)	N	Forward	GCTTCAGCGTTCTTCGGAATG	64
			Reverse	RTCATCCAATTTGATGGCACCT	63
			Probe	CAL FLUOR RED 610-TTGGCATGGAAGTCACACCTTCG-BHQ2	67

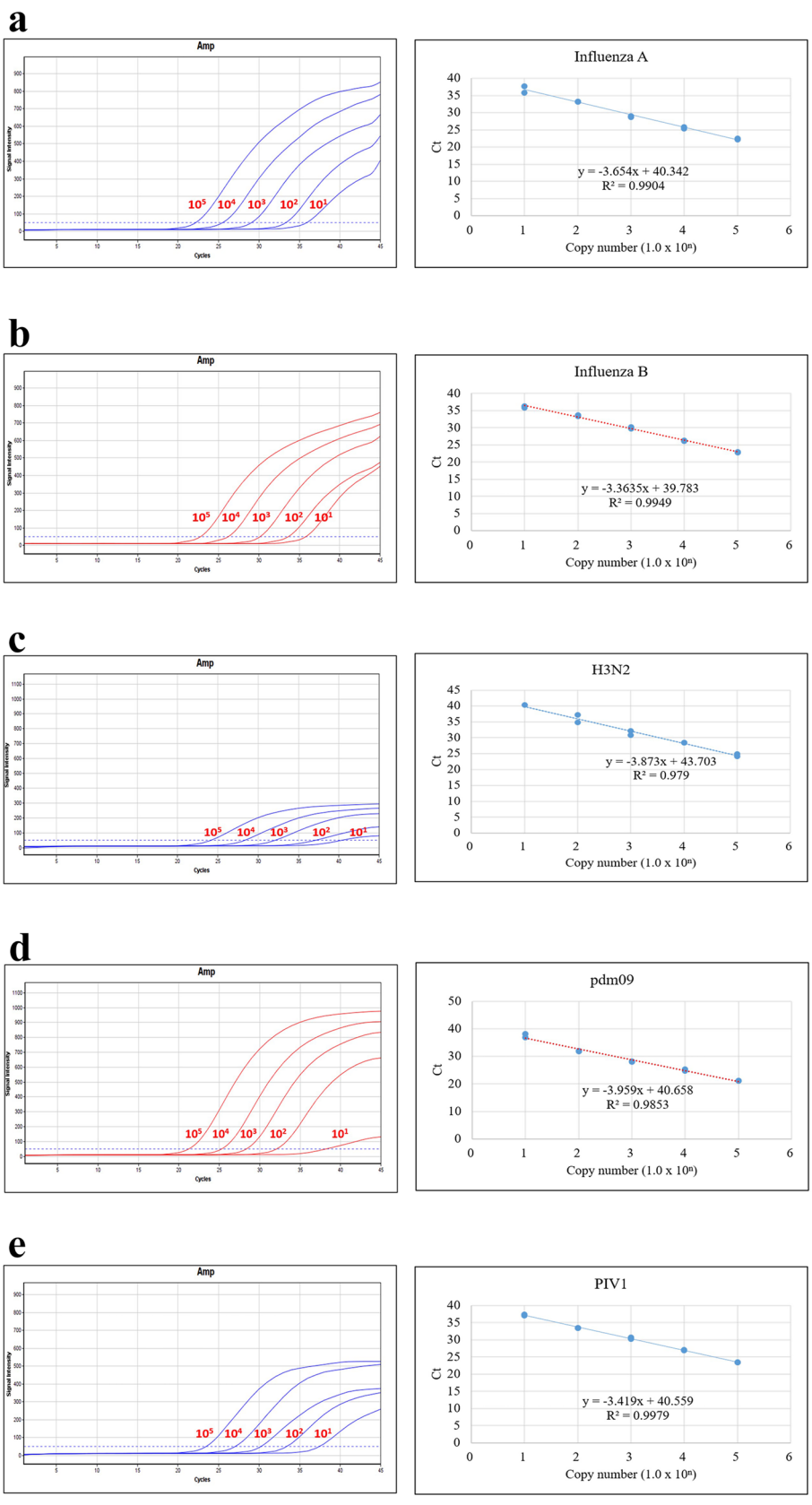


Fig. 1 Standard curves produced by a portable PCR device. **a** Influenza A virus, **b** influenza B virus, **c** Flu A H3N2, **d** Flu A pdm09, **e** PIV1, **f** PIV3, **g** PIV2, **h** PIV4, **i** hMPV, **j** ADV, **k** HRV, **l** RSV, **m** SARS-CoV-2 RdRp gene, **n** SARS-CoV-2 N gene

Fig. 1 (continued)

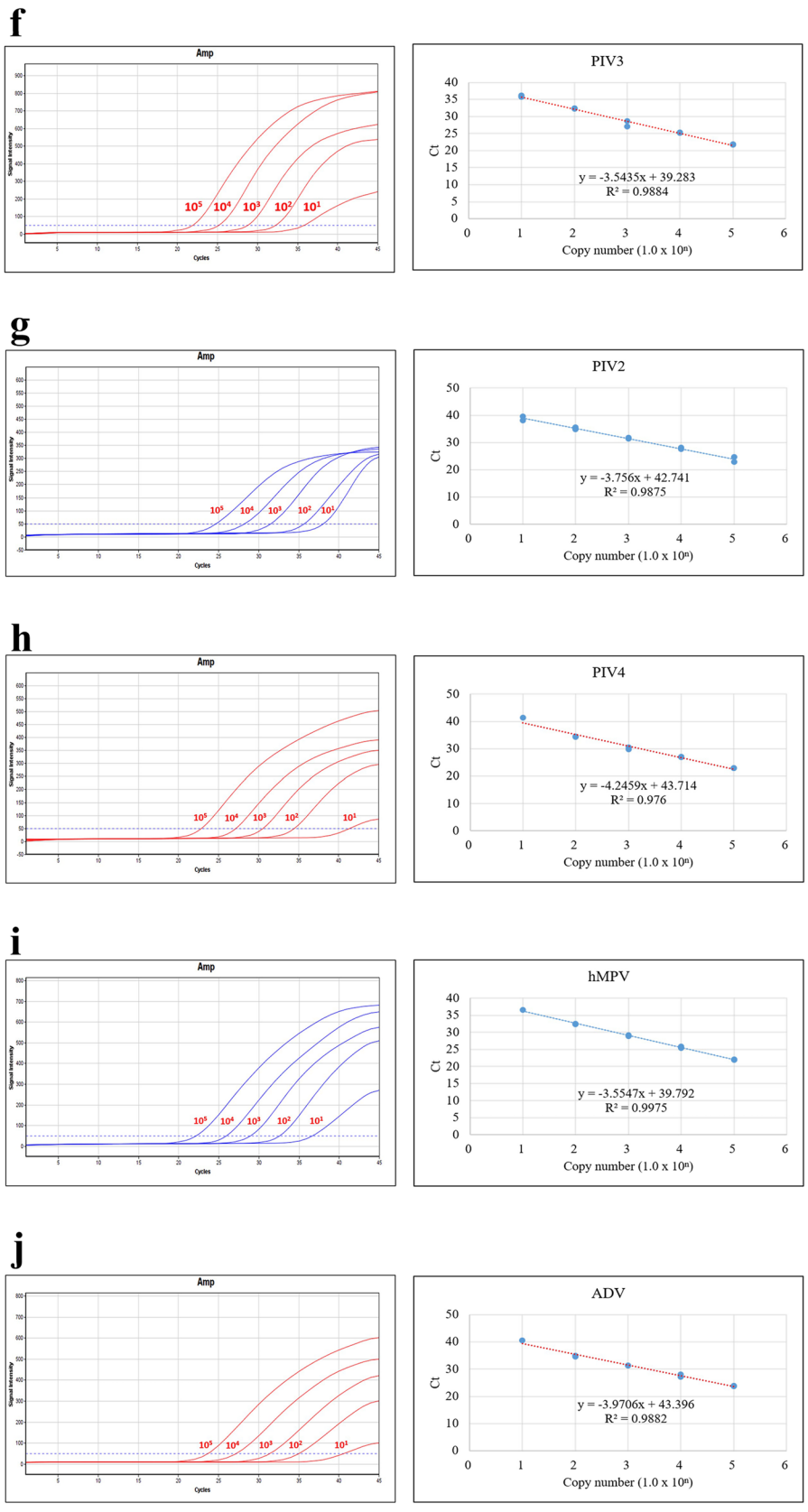
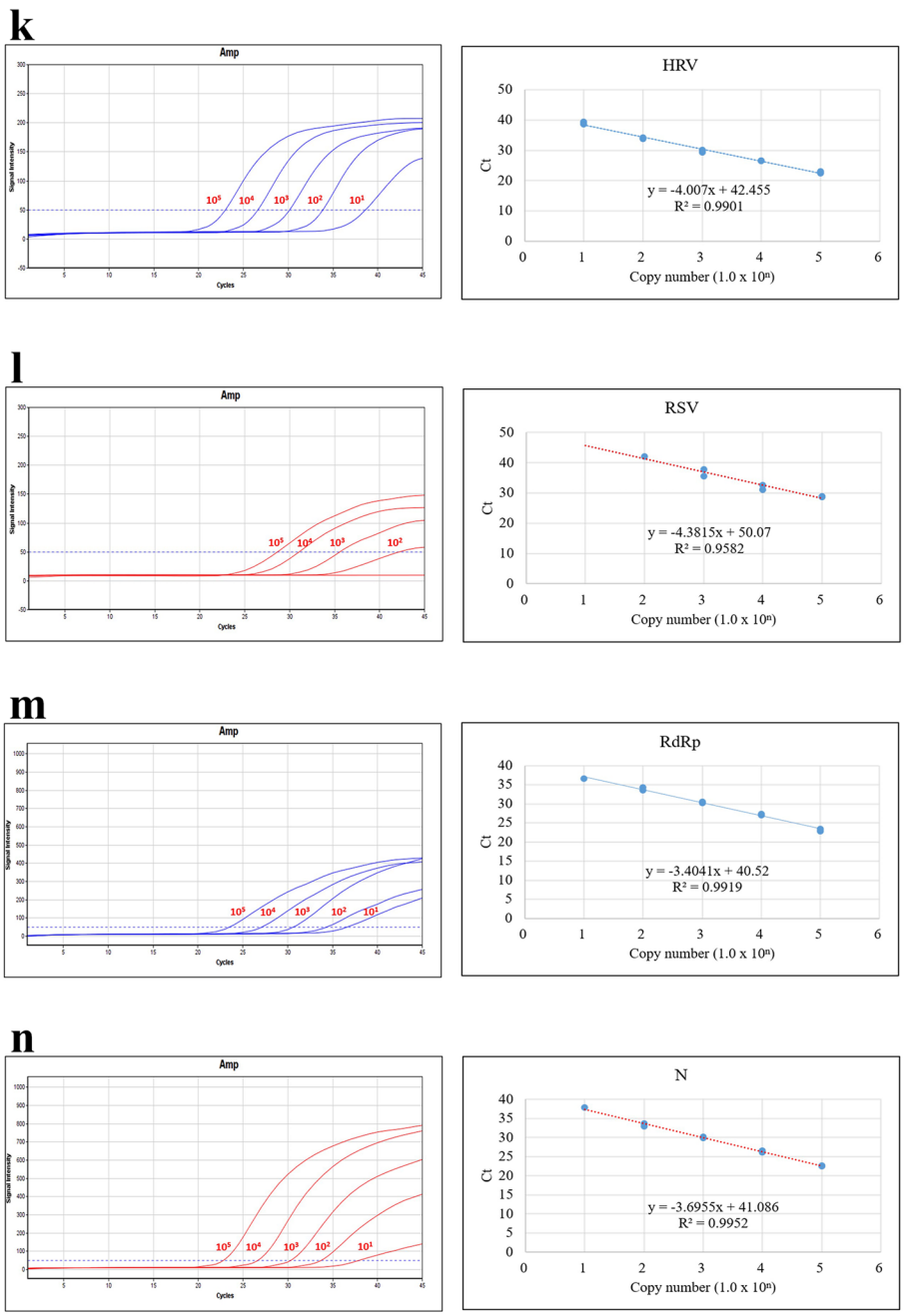


Fig. 1 (continued)



CFX96. In the GENECHECKER test results, all sets were detected up to 10^2 or 10^1 copies/rxn (Fig. 1). These results demonstrate that the newly designed primer pair and probe sets can detect the viral genome at a low load with high accuracy. Moreover, it is possible to evaluate the viral load at a given time point by quantification using a standard curve and monitoring the response to treatment.

2.3 Specificity of Multiplex rRT-PCR

One-step multiplex rRT-PCR was performed with clinical sample genomes to confirm the potential application of the primer and probe sets in the field. The test was conducted with the Bio-Rad CFX96 and GENECHECKER UF-300 using the DNA or RNA extracts of a positive clinical sample

Table 2 Specificity test

Sample	AMC		Bio-Rad		GENECHECKER	
	Positive	Negative	Positive	Negative	Positive	Negative
Influenza A virus	29	–	29	–	28	1
Influenza B virus	20	–	20	–	20	–
Influenza A virus (H3N2)	19	–	18	1	18	1
Influenza A virus (pandemic09)	10	–	10	–	10	–
PIV1	10	–	10	–	10	–
PIV2	10	–	10	–	10	–
PIV3	10	–	10	–	10	–
PIV4	10	–	10	–	10	–
hMPV	29	–	29	–	29	–
ADV	30	–	27	1	26	2
HRV	9	1	9	–	10	–
RSV	10	–	10	–	10	–
SARS-CoV-2 (RdRp gene)	30	–	29	1	28	2
SARS-CoV-2 (N gene)	30	–	30	–	28	2

of the target respiratory virus ($n = 196$) provided by the Asan Medical Center (AMC), Seoul, Korea. Overall, we confirmed that most samples were identified by multiplex RT-PCR (Table 2). Then, to check cross-reactivity for each target set, the detection was confirmed through 13 types of respiratory virus clinical samples excluding the target, and no nonspecific reaction or cross-reactivity was observed. As a result of verifying our multiplex rRT-PCR data in AMC and sequencing, the false-negative samples were estimated to be degraded during sample transportation and storage before the test. Through this result, we demonstrated that respiratory virus targets can be detected in infected samples using the developed platform.

3 Conclusion

This study describes a multiplex rRT-PCR diagnostic method for 14 respiratory viruses using newly designed primer pairs and probe sets. The target viruses are known to cause frequent infectious diseases worldwide and severe or even fatal consequences in children and immunocompromised patients. With the radical increase in resistance to antiviral agents, personalized medicine for the treatment of infectious diseases is gaining importance, suggesting that the identification of pathogen-specific factors may trigger further research on this topic. Therefore, we confirmed that test results with a high sensitivity and accuracy could be obtained using this diagnostic method. Moreover, by applying this method to a portable POCT PCR equipment with a ramp rate four times faster than common PCR devices, it has been demonstrated that rapid and accurate test results can be obtained in less than an hour, with half the amount of common PCR reaction volume. This explains that this is a new study that has have never been tried before. Point-of-care testing with portable equipment can be used in places where laboratory acceptance is not possible, and the immediate identification of causative agents can facilitate appropriate treatment and infection control measures [30]. With this newly developed platform, we can consider the composition of the kit through freeze-drying of the PCR mixture and with this kit, we are investigating a way to purify viral RNA in situ.

4 Materials and Methods

4.1 Ethics Statement

This study was approved by the Institutional Review Board of Asan Medical Center (IRB No.: 2020-0312).

4.2 Platform Conformation and Oligo Design

For the simultaneous detection of 14 virus targets, the detection platform consisted of two targets constituting one set with a total of eight sets (with internal control). The fluorophores of the probes in one set were attached to each FAM and ROX (CAL FLUOR RED 610), allowing target discrimination through fluorescence (Fig. 2). SARS-CoV-2, which is currently considered to have an important detection accuracy, and influenza virus, which is the most commonly used for simultaneous detection with SARS-CoV-2, can be double-checked to improve the reliability of test results. The gene sequences of each target virus for primer pair and probe design were obtained from the National Center for Biotechnology Information (NCBI) database. The obtained sequences were aligned using MAFFT (Multiple Alignment

Set	Target	Fluorescence
1	Influenza A	FAM
	Influenza B	ROX
2	Influenza A H3N2	FAM
	Influenza A pandemic 09	ROX
3	Parainfluenza virus 1	FAM
	Parainfluenza virus 3	ROX
4	Parainfluenza virus 2	FAM
	Parainfluenza virus 4	ROX
5	Human Metapneumovirus	FAM
	Adenovirus	ROX
6	Human Rhinovirus	FAM
	Respiratory syncytial virus	ROX
7	SARS-CoV-2 (RdRp gene)	FAM
	SARS-CoV-2 (N gene)	ROX
8	Internal Control (IC)	FAM

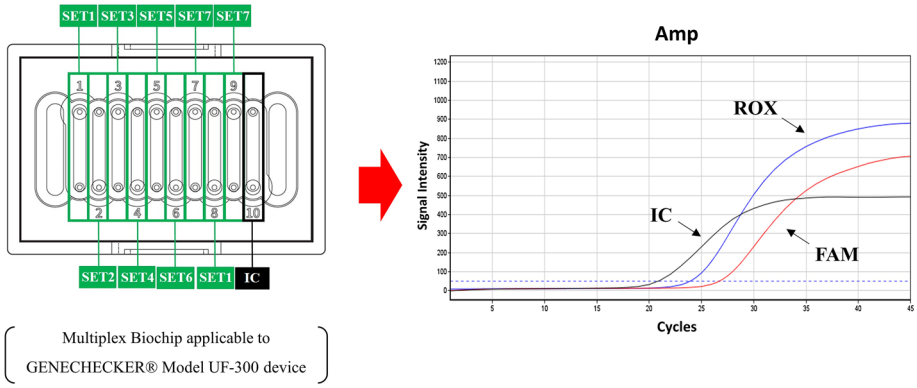


Fig. 2 Overall plan of the development platform

using Fast Fourier Transform), and primer and probe designs were performed using BioEdit. The designed sequences were checked using the Integrated DNA Technologies (IDT) OligoAnalyzer Tool and NCBI BLAST (Basic Local Alignment Search Tool).

4.3 Confirmation of Primer and Probe Set

The detection limit of the multiplex rRT-PCR was determined by testing tenfold dilutions of the synthetic template for each detection set. Serial dilutions were performed from 1×10^6 to 1×10^0 (copy number/rxn), and detection of the target primer and probe set was performed for each copy number. The test was conducted using GENECHECKER and Bio-Rad. The composition of Bio-Rad reaction mixture is: 10 µL of master mix (Takara Bio, Japan), 0.5 µM, of primer, 0.25 µM of probe, 1 µL of template, and the remaining volume was supplemented with H_2O to 20 µL. The composition of the GENECHECKER reaction mixture is 10 µL in total, consisting of 5 ul of genechecker mix (Genesystem, Korea), 1 µM of primer, 0.5 µM of probe, and 4 µL of template. The PCR conditions consisted of an initial predenaturation at 95 °C for 30 s, followed by denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 30 s with 45 cycles. The detection results were analyzed using Bio-Rad CFX Manager 3.1 (Bio-Rad) and UF300Viewer (Genesystem).

4.4 Viral Nucleic Acid Samples

Extracted nucleic acids of respiratory viruses and bacteria commonly found in the respiratory tract were used to determine the specificity of the multiplex PCR. Specimens were collected from sputum or nasopharyngeal swabs using FLOQSwab (Copán, Brescia, Italy) in Universal Transport Medium (Copán). Viral nucleic acids were extracted using the eMAG (bioMérieux, Marcy-l'Etoile, France) system using NUCLISENS easyMAG (bioMérieux, Marcy-l'Etoile, France). Before providing the samples, all samples were confirmed to be positive for each virus through multiplex rRT-PCR with Allplex Respiratory Panel assays (Seegene, Seoul, Korea). Samples were stored at − 70 °C immediately upon shipment.

4.5 One-Step rRT-PCR

rRT-PCR experiments were performed on the viral DNA or RNA extraction samples to confirm the actual detection ability of the prepared primer and probe sets. The reaction mixture of Bio-Rad is a total of 20 µL consisting of 10 µL, master mix (Bioline, Meridian Bioscience, USA), 0.2 µL reverse-transcriptase, 0.4 µL RNase inhibitor, 0.5 µM primer, 0.25 µM probe, 4 µL template, and the remaining volume was supplemented with H_2O. The components of the rRT-PCR reaction for GENECHECKER were 10 µL in total, with 5 µL of genechecker mix (Genesystem, Korea), 0.5 µM of primer, 0.25 µM of probe, and template (4 µL). Cycling conditions were reverse transcription for 5 min at 50 °C, pre-denaturation for 30 s at 95 °C, followed by 45 cycles of denaturation for 5 s at 95 °C, and annealing and extension for 30 s at 60 °C. The data analysis was performed using Bio-Rad CFX Manager 3.1 (Bio-Rad) and UF300_Viewer (Genesystem) (Table 2).

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Author contributions Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals.

Availability of data and material Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest Not applicable.

Ethics approval This study was approved by the Institutional Review Board of Asan Medical Center (IRB No.: 2020–0312).

Consent to participate Not applicable.

Consent for publication Not applicable.

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