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Rapid Differential Diagnosis of Seven Human Respiratory Coronaviruses Based on Centrifugal Microfluidic Nucleic Acid Assay

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ABSTRACT: With the global outbreak of the coronavirus disease 2019 (COVID-19), the highly infective, highly pathogenic, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has attracted great attention. Currently, a method to simultaneously diagnose the seven known types human coronaviruses remains lacking and is urgently needed. In this work, we successfully developed a portable microfluidic system for the rapid, accurate, and simultaneous detection of SARS-CoV, middle east respiratory syndrome coronavirus (MERS-CoV), SARS-CoV-2, and four other human coronaviruses (HCoVs) including HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1. The disk-like microfluidic platform integrated with loop-mediated isothermal amplification provides highly accurate, sensitive, and specific results with a wide linear range within 40 min. The diagnostic tool achieved 100% consistency with the "gold standard" polymerase chain reaction in detecting 54 real clinical samples. The integrated system, with its simplicity, is urgently needed for the diagnosis of SARS-CoV-2 during the COVID-19 pandemic.

Infectious diseases caused by emerging viruses pose a substantial threat to social public health. Millions of people around the world were affected, and this has resulted in a huge number of deaths worldwide.[1,2] At the end of December in 2019, a novel type of human infectious coronavirus of unknown origin emerged. It was later identified by the World Health Organization (WHO) as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to the Coronaviridae family. Seven classic types of coronavirus are known to infect humans including the most recently discovered SARS-CoV-2. According to the clinical symptoms, human coronaviruses (HCoVs) including HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 cause mild respiratory tract infections. In contrast, SARS-CoV, middle east respiratory syndrome coronavirus (MERS-CoV), along with SARS-CoV-2, which belong to the genus *Betacoronavirus*,[3,4] can cause severe pneumonia and the need for significant in-hospital treatment. The SARS-CoV, MERS-CoV, or SARS-CoV-2 pandemics have imposed huge economic burdens on global healthcare systems. Therefore, discovering the main antiviral strategies and establishing various diagnostic platforms are key for the COVID-19 pandemic.[3,5] Because there is no vaccine or drugs available, many companies and laboratories are now racing to develop highly effective point-of-care diagnostic methods for the purpose of initiating timely treatment.[6]

Chest-computed tomography (CT) plays an important role in the diagnosis of COVID-19.[7,8] However, the detection method is very expensive, and patients are only tested in hospitals. CT, therefore, cannot satisfy the demand for rapid and real-time analysis. With the advance of nucleic acid diagnostics, polymerase chain reaction (PCR) such as reverse transcriptional PCR (RT-PCR) has been considered and developed as the primary considered diagnostic strategy.[9,10] Noh et al. developed a duplex RT-PCR system for the detection of SARS-CoV and MERS-CoV, which also monitored bat-related CoVs.[11,12] For the detection of the recent COVID-19, also known as SARS-CoV-2, Corman et al. developed a RT-PCR workflow that effectively distinguished SARS-CoV-2 from SARS-CoV in 297 original clinical specimens, thus determining the feasibility of the RT-PCR diagnostic in epidemic coronavirus research.[13] Nevertheless, RT-PCR lacks convenience in the heating−cooling reactions and also lacks sensitivity.[14,15] RT-PCR also cannot easily and simultaneously detect the seven types of human respiratory coronaviruses, which means it is time-consuming for differential diagnosis. To address these difficulties, a non-PCR-based diagnostic method utilizing loop-mediated isothermal amplifi-

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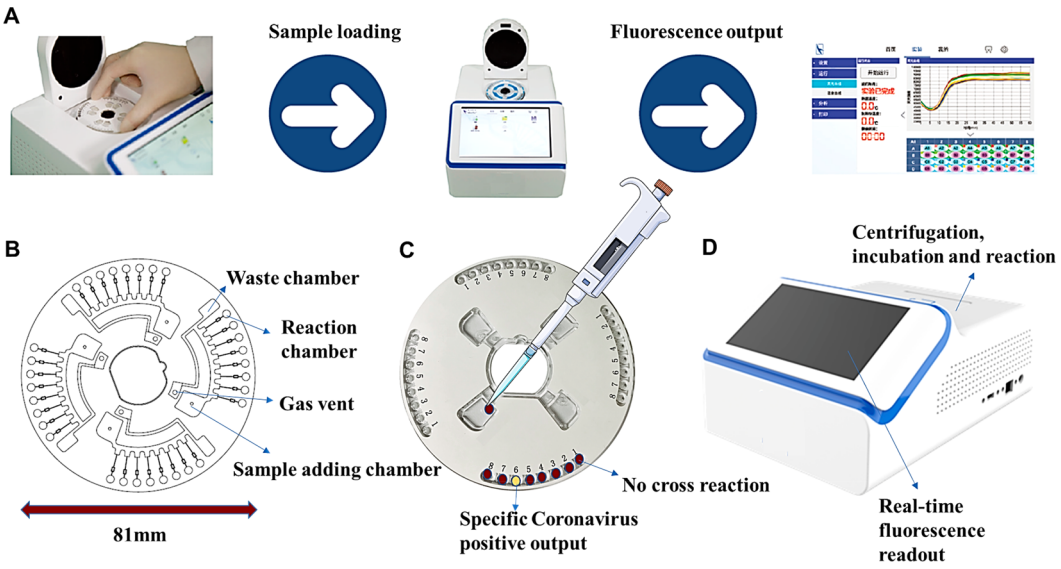

Figure 1. (A) Flow diagram of the LAMP-microfluidic assay. (B) Details of the LAMP-microfluidic chip with its sample adding chamber, reaction chamber, gas vent, and waste chamber. (C) Specific reaction of the LAMP-microfluidic chip. Corresponding primers preloaded in the reaction chamber: 1. HCoV-OC43; 2. HCoV-HKU1; 3. HCoV-229E; 4. MERS-CoV; 5. SARS-CoV; 6. HCoV-NL63; 7. SARS-CoV-2 targeting N; 8. SARS-CoV-2 targeting ORF1ab. (D) Photograph of the real-time fluorescence detection apparatus.

cation (LAMP) has been proposed as a promising tool.[16,17] RT-LAMP provides high accuracy and efficiency when amplifying target sequences.[18−21] Huang et al. combined RT-LAMP with a vertical flow visualization strip for MERS-CoV detection and achieved a low detection limit of 10 copies/mL.[22] RT-LAMP also served as a rapid and reliable diagnostic tool for the newly emerged SARS-CoV-2 and could, therefore, make great contributions to the control of coronavirus-related disease.[23,24]

In our present work, an integrated microfluidic system was originally constructed for the rapid detection of African swine fever.[25] It also displayed the potential of an integrated LAMP-microfluidic system for the accurate, highly sensitive, and specific detection of coronaviruses. However, coronaviruses from the same family origin have similar pathogenesis and symptomatology. Therefore, a tool for the accurate differentiation and diagnosis within the Coronaviridae family is urgently needed. In this work, we presented a small disk-like microfluidic chip (size of 81 mm) based on the LAMP method. The instrument is portable (length 280 mm, width 200 mm, height 135 mm) and allows for the rapid genotyping of all seven known human coronaviruses in small sample volumes and at reduced cost. The microfluidic chip with excellent performance was capable of discriminating HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV, and SARS-CoV-2 simultaneously within 40 min, thus providing a promising tool for the fight against coronaviruses.

RESULTS AND DISCUSSION

Construction of LAMP Microfluidic Chip. The proposed centrifugal disk-like microfluidic chip was developed based on the classic LAMP reaction (This microfluidic chip was provided by Shanghai IgeneTec Diagnostics Co. Ltd.). The photographs of specific responses on the LAMP-microfluidic chip under 480 nm excitation light and the real-time product of the LAMP-microfluidic chip under sunlight are shown in Figure S1. As shown in Figure 1A, the prepared microfluidic chip was sealed and then loaded on the corresponding instrument. After centrifugation, incubation, and reaction, the fluorescence output was displayed on the instrument. The LAMP-based microfluidic chip reduced sample waste and increased automation and standard reliability. Most importantly, the integrated microfluidic chip realized a fast response and accomplished seven coronavirus detections in 40 min. Four independent experiments for coronaviruses genotyping on a single microfluidic chip were processed simultaneously. The integrated microfluidic chip consisted of four main chambers including sample adding chamber, reaction chamber, gas vent, and waste chamber (Figure 1B). The sample was added to the inner chamber, while the LAMP reaction was conducted in the outer chamber. With the blocking effect of the capillary microvalve, the reaction mixture was prevented from reflux. In the presence of the middle circle, the reaction chamber and the sample addition chamber were separated to reduce contamination. Thirty-two reactions with four independent experiments operated simultaneously. Primers for each of the seven coronaviruses were preloaded into eight outer chambers (SARS-CoV-2 primer sets targeting N and ORF1ab sequences). The samples were distributed evenly to eight parallel outer chambers by centrifugal forces to ensure data reliability. Each coronavirus sample reacted with primers and produced a positive signal in the corresponding reaction chamber with specific primers; negative signals were obtained in the other reaction chambers (Figure 1C). The supporting apparatus was well-matched to accommodate our disk-like microfluidic chip (Figure 1D). In the small-volume device, the temperature could quickly reach 65 °C to enable nucleic acid amplification and maintain 65 °C during the centrifugation, incubation, and automatic fluorescence output process without manual intervention, thus decreasing interferences. Specific coronavirus positive samples displayed "S"-shaped real-time fluorescence curves from the corresponding primer chamber. The chip produced rapid results with high sensitivity and specificity for coronavirus genotyping.

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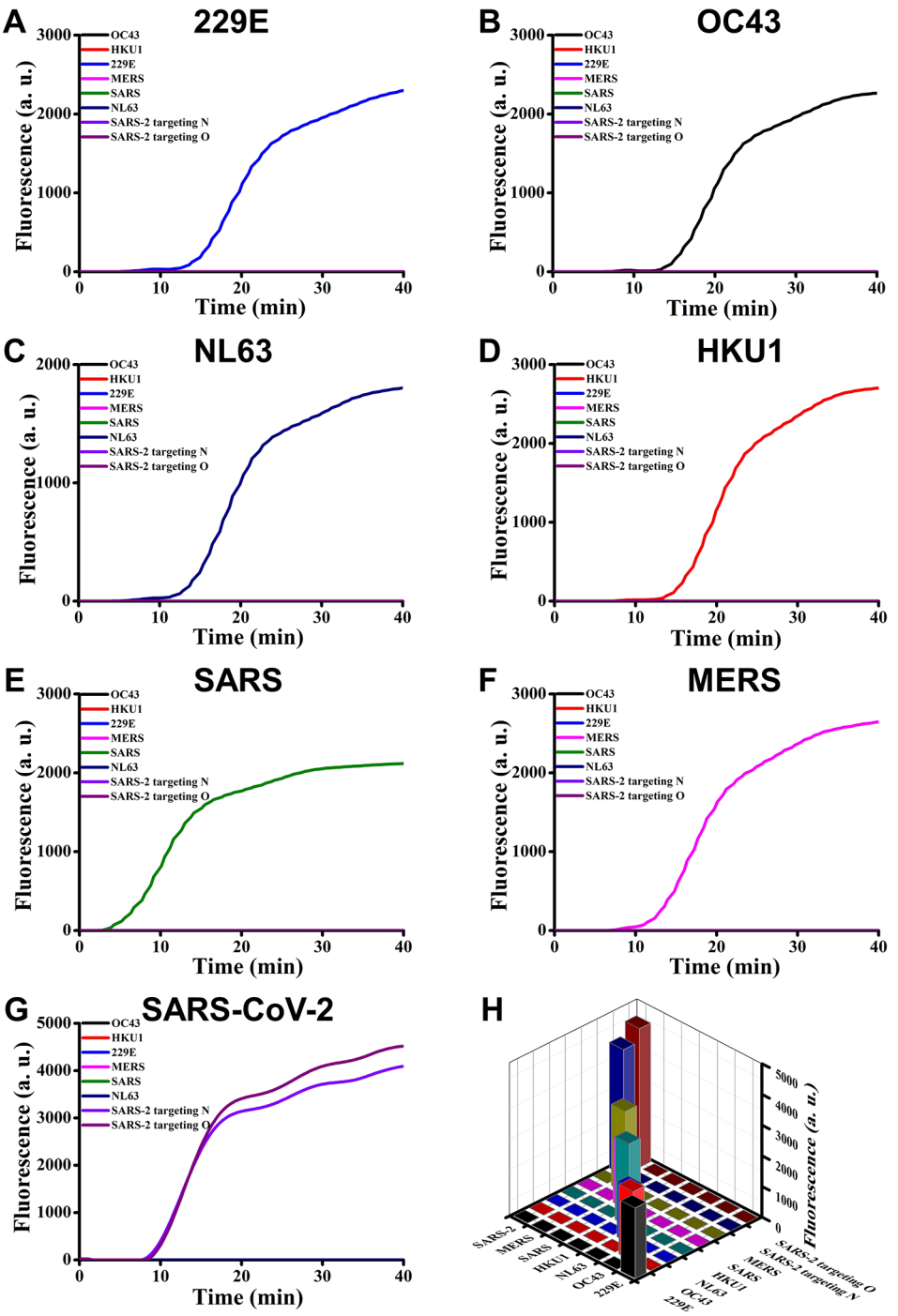

Figure 2. Specificity analysis of optimized primer sets with loop primers for each virus. (A) HCoV-229E; (B) HCoV-OC43; (C) HCoV-NL63; (D) HCoV-HKU1; (E) SARS-CoV; (F) MERS-CoV; (G) SARS-CoV-2. (H) Histogram overview of specificity analysis. Other viruses as negative controls.

Currently, the total number of SARS-CoV-2 infected individuals is underestimated due to the presence of asymptomatic cases. Additionally, doctors have been unable to quickly identify the coronavirus type in patients with cough and pneumonia. Our microfluidic chip distinguished seven coronaviruses and diagnosed clinical samples with high accuracy regardless of the presence or absence of symptoms in the patients. Therefore, this system has prospects for broad clinical applications.

Performance Verification of a LAMP-Based Microfluidic Platform. To evaluate the specificity of the fabricated system, cross-reactivity tests using RNA were carried out at Qingdao Administration of Entry & Exit Inspection and Quarantine Bureau. Eight reaction chambers were preloaded with seven specific coronavirus-targeted primers, and reactions were performed to verify specificity. A positive signal was displayed according to the real-time fluorescence, while negative results produced a flat line. All seven samples representing single-coronavirus infection exhibited positive responses in the corresponding designed primer chambers (Figure 2A−G). Other reaction chambers exhibited negative responses, which showed that the specificity of our integrated

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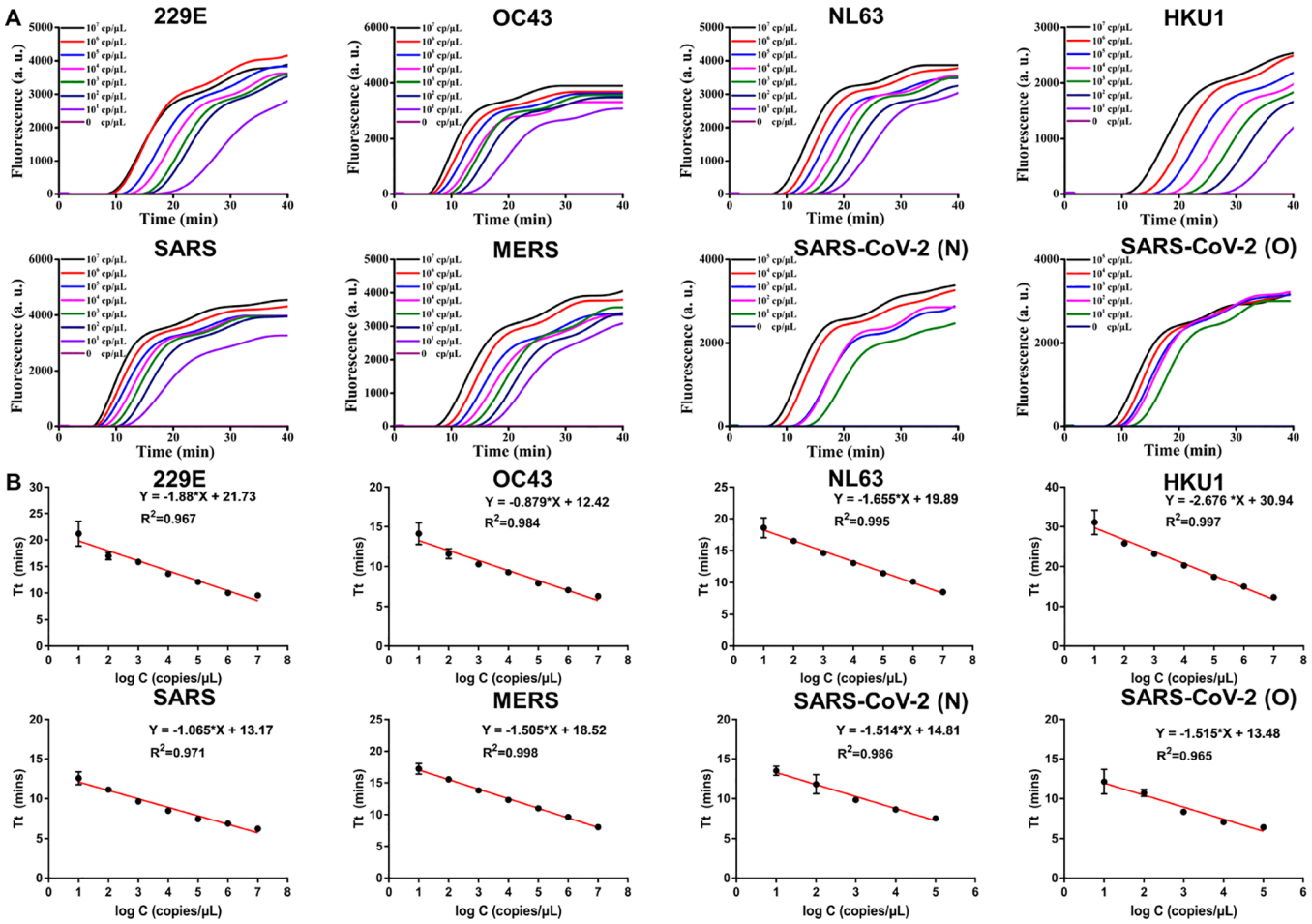

Figure 3. (A) Sensitive analysis using serially diluted plasmid samples targeting HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV, SARS-CoV-2 targeting N, and SARS-CoV-2 targeting ORF1ab, respectively. (B) Relationship between fluorescence time to threshold values and logarithmic values of plasmid concentration for HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV, SARS-CoV-2 targeting N, and SARS-CoV-2 targeting ORF1ab, respectively.

microfluidic chip was 100%. The newly designed microfluidic chip exhibited excellent specificity, and the corresponding reaction chambers produced obvious amplification fluorescence signals (Figure 2H).

All plasmid samples, SARS-CoV, MERS-CoV, SARS-CoV-2, HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1, were serially diluted 10-fold. A dilution level of 10^1 copies/μL for HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, MERS-CoV, SARS-CoV, and SARS-CoV-2 each remained detectable by the integrated microfluidic platform (Figure 3A). The relationship between the plasmid concentration and the Tt value for the detection of seven human coronaviruses is shown in Figure 3B. The Tt value was logarithmically related to the concentrations of the plasmid samples in the range from 10^7 to 10^1 copies/μL (R^2 = 0.967) for HCoV-229E, from 10^7 to 10^1 copies/μL (R^2 = 0.984) for HCoV-OC43, from 10^7 to 10^1 copies/μL (R^2 = 0.995) for HCoV-NL63, from 10^7 to 10^1 copies/μL (R^2 = 0.997) for HCoV-HKU1, from 10^7 to 10^1 copies/μL (R^2 = 0.971) for SARS-CoV, from 10^7 to 10^1 copies/μL (R^2 = 0.998) for MERS-CoV, from 10^5 to 10^1 copies/μL (R^2 = 0.986) for SARS-CoV-2 targeting N sequence, and from 10^5 to 10^1 copies/μL (R^2 = 0.965) for SARS-CoV-2 targeting ORF1ab sequence. The novel LAMP microfluidic platform demonstrated the potential for quantitative detection capability. These results show that the LAMP-microfluidic chip is suitable for the rapid and accurate differential diagnosis of human respiratory coronavirus.

Genotype Detection in Clinical Specimens of Microfluidic LAMP Assays. To further estimate the practical potential of the integrated platform, a total of 27 specimens collected from single-virus-specific patients representing seven genotypes were detected successfully. The specimens were confirmed positive for HCoV-229E (n = 3), HCoV-OC43 (n = 3), HCoV-NL63 (n = 3), HCoV-HKU1 (n = 3), MERS-CoV (n = 3), and SARS-CoV-2 (n = 12). A total of 27 healthy controls were confirmed negative for the seven mentioned coronaviruses using a 1 h RT-PCR system. For SARS-CoV-2, reaction chambers containing primer sets targeting N and ORF1ab sequences both displayed positive results (Figure 4). The SARS-CoV case occurrence rate at the time of the study was very low, and fresh specimens of SARS-CoV were not available. Our LAMP-microfluidic chip produced the RT-PCR results with 100% consistency in each case, and the time consumption was surprisingly far less than that of the commercial RT-PCR. The sensitivity and specificity both reached 100%. The LAMP-microfluidic system improved the diagnosis efficiency when compared with RT-PCR, which indicated that our microfluidic chip has potential as a commercial product for clinical diagnostics.

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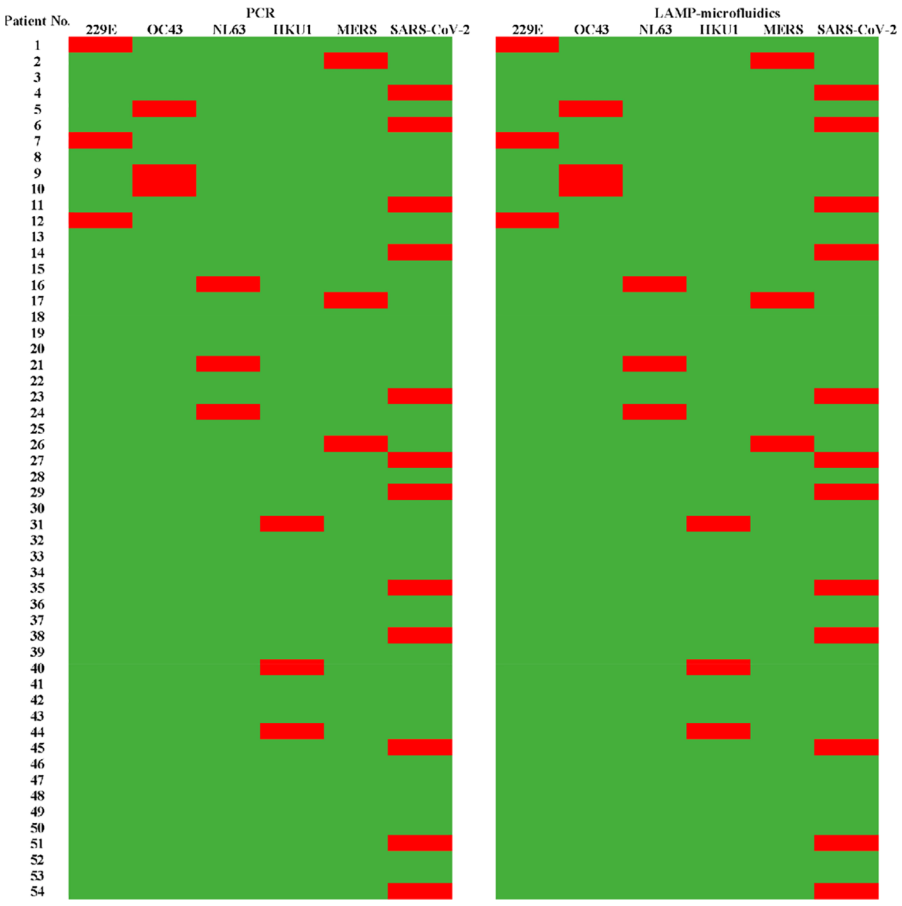

Figure 4. Comparison of results from LAMP-microfluidic assays and PCR assays in the detection of coronavirus genotype in clinical specimens (red zones: positive; green zones: negative).

CONCLUSIONS

Under the background of the SARS-CoV-2 outbreak, the establishment of rapid and accurate point-of-care devices has become crucial. In this work, a LAMP-microfluidic chip for rapid genotype detection and diagnosis of seven coronaviruses is presented. Our LAMP-based centrifugal microfluidic chip fully demonstrated the rapid, portable, accurate, and effective discrimination of the known human coronaviruses. The results were similar to those obtained by PCR regarding sensitivity and specificity, and the testing time was substantially reduced. In terms of current issues, our proposed system is relatively low-cost and could serve as a reliable diagnostic tool in the fight to control the COVID-19 pandemic, thus providing a huge commercial and practical potential.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c03364.

Experimental section; photograph of LAMP-microfluidic chip under 480 nm emission light and sunlight; the primer set selection results for each virus; primer sets for each coronavirus microfluidic LAMP assay (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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