Fluorescent reverse transcription recombinase-aided amplification assay for sensitive and rapid detection of six respiratory viruses

Xudong Guo[1], Dongli Gao[1], Minghui Song[2], Yi Yang[1], Dan Sun[2], Wanying Liu[1], Hua Shi[1], Rongtao Zhao[1], and Hongbin Song[1]

[1]Chinese PLA Center for Disease Control and Prevention
[2]Hainan Hospital of Chinese PLA General Hospital

May 17, 2023

Abstract

Background: Rapid detection of pathogens is important for the timely control of outbreaks, especially for respiratory infectious diseases that are prone to spread and outbreaks. Methods: In this work, we developed a sensitive reverse transcription recombinase-aided amplification (RT-RAA) assay for the rapid detection of six common respiratory viruses including respiratory syncytial virus type A (RSVA), influenza A virus (Flu A), influenza B virus (Flu B), human parainfluenza virus (HPIV), SARS-CoV-2 and adenovirus (ADV). The nucleic acid standards and pharyngeal swab samples were used to test the sensitivity, specificity, reliability of the established RAA assay. Results: The assay could be completed within 20 minutes at 39 using a portable built-in power device. The detection limits for the six viruses were all less than 1000 copies/mL and reached 10 copies/mL for ADV. Excellent specificity was demonstrated by cross-testing with 21 different pathogen nucleic acids. The results of RT-RAA and RT-PCR were consistent in 85 laboratory-conserved pharyngeal swab samples, but RT-RAA was more time-saving and portable. Meanwhile, the RT-RAA assay using the same test procedure for six viruses could allow operators the flexibility to select the number of samples and pathogens to be detected in one test. Conclusions: This portable, sensitive and reliable RT-RAA assay for rapid detection of multiple respiratory viruses could be applied to health resource-poor areas and outbreak sites.

Fluorescentreverse transcription recombinase-aided amplification assay for sensitive andrapid detection of six respiratory viruses

Xudong Guo[1+], Dongli Gao[1,2+], Minghui Song[3+], Yi Yang[1], Dan Sun[3], Wanying Liu[1], Hua Shi[1], Rongtao Zhao[1,*], Hongbin Song[1,2,*]

[1]Chinese PLA Center for Disease Control and Prevention, Beijing 100071, China

[2]College of Public Health, Zhengzhou University, Henan 450001, China

[3] Hainan Hospital of Chinese PLA General Hospital, Hainan 572013, China

[+]These authors contributed equally to this work

*Corresponding author:

Rongtao Zhao, Email: zhaorongtao1984@163.com;

Hongbin Song, Email: hongbinsong@263.net

Abstract

Background: Rapid detection of pathogens is important for the timely control of outbreaks, especially for respiratory infectious diseases that are prone to spread and outbreaks.

Methods: In this work, we developed a sensitive reverse transcription recombinase-aided amplification (RT-RAA) assay for the rapid detection of six common respiratory viruses including respiratory syncytial virus type A (RSVA), influenza A virus (Flu A), influenza B virus (Flu B), human parainfluenza virus (HPIV), SARS-CoV-2 and adenovirus (ADV). The nucleic acid standards and pharyngeal swab samples were used to test the sensitivity, specificity, reliability of the established RAA assay.

Results: The assay could be completed within 20 minutes at 39 using a portable built-in power device. The detection limits for the six viruses were all less than 10^3 copies/mL and reached 10^1copies/mL for ADV. Excellent specificity was demonstrated by cross-testing with 21 different pathogen nucleic acids. The results of RT-RAA and RT-PCR were consistent in 85 laboratory-conserved pharyngeal swab samples, but RT-RAA was more time-saving and portable. Meanwhile, the RT-RAA assay using the same test procedure for six viruses could allow operators the flexibility to select the number of samples and pathogens to be detected in one test.

Conclusions: This portable, sensitive and reliable RT-RAA assay for rapid detection of multiple respiratory viruses could be applied to health resource-poor areas and outbreak sites.

Key words

Isothermal amplification; RAA; Respiratory virus; Rapid detection;

1.Introduction

Respiratory viruses can be transmitted through airborne as well as close contact, usually proliferate in the mucosal epithelium of the respiratory tract, causing localized infection of the respiratory tract or lesions of tissues and organs outside the respiratory tract[1-3]. Infected individuals always present with non-specific symptoms such as fever, cough, malaise and loss of smell, which in severe cases can lead to pneumonia, respiratory failure, multiple organ damage, and even shock or death[4, 5]. Acute respiratory diseases caused by respiratory viruses, such as COVID-19, have posed a serious threat to human health and social stability in the past decades[6, 7]. The respiratory syncytial virus, influenza A virus, influenza B virus, parainfluenza virus, and adenovirus are the most common respiratory viruses and can be easily and fast transmitted from person to person[8, 9]. Especially the COVID-19 outbreak in 2020, it is estimated that as of April 8, 2023, the global cumulative number of infections has exceeded 684 million and the cumulative number of deaths exceeded six million[10]. The epidemic is still spreading worldwide[11]. Early and accurate diagnosis are essential to control pandemics caused by respiratory viruses because of the non-specific symptoms and insidious transmission of asymptomatic infections[12, 13]. Therefore, rapid detection of respiratory viruses is of great importance.

Nucleic acid detection is still the gold standard for pathogen diagnosis. Polymerase chain reaction (PCR) is one of the most common methods for nucleic acid detection[14]. However, some disadvantages limit its widespread use, especially grassroots and field applications, such as the need for complex and time-consuming thermal cycling processes, expensive detection equipment, and specialized laboratory conditions[15]. Immunological assays such as enzyme-linked immunosorbent assay (ELISA) and colloidal gold assay are prone to false negatives due to their relatively low sensitivity[14]. Therefore, the development of sensitive, rapid and simple methods for detecting respiratory viruses is of great practical significance in early screening of cases and reducing the risk of transmission[16].

In recent years, the continuous development of various isothermal amplification techniques has brought new opportunities for field detection[17, 18]. They could perform nucleic acid amplification at a constant temperature, and do not depend on thermal cycling instruments[19]. Several promising isothermal amplification methods are already available, but some of them require relatively high temperatures, long reaction times, or complex primer designs. For example, loop-mediated isothermal amplification (LAMP) requires 4-6 primers, leading to the failure to design appropriate primers for some pathogen genes, and a high working temperature of 65°C[20, 21]. Nucleic acid sequence-based amplification (NASBA) requires a longer amplification time

(typically 90 min)[22]. In contrast, the recombinase aided amplification (RAA) method works successfully over a wide temperature range of 37 ˜ 42°C using specific primer pairs, thus enabling amplification even at room temperature or human body temperature[23, 24]. RAA is considered to be a promising alternative to PCR for rapid nucleic acid detection[25]. The schematic diagram of the RAA amplification is shown in Figure 1. The recombinant enzyme in RAA reaction system, obtained from bacteria or fungi, can tightly bind to the primer DNA at room temperature to form an enzyme-primer polymer. When the primer searches for a complementary sequence on the template DNA, the recombinant enzyme opens the double-stranded structure of the template DNA with the aid of single-stranded DNA binding protein. Under the action of DNA polymerase, a new DNA complementary strand is formed, and the amplification product grows exponentially[26]. The detection results can be obtained in 20 minutes using fluorescent probes. RAA has the characteristics of simple operation, fast amplification speed, high sensitivity and specificity, and is very promising for application, which can meet the needs of not only the front-line field detection, but also the lack of conditions in the community, grassroots and other scenarios.

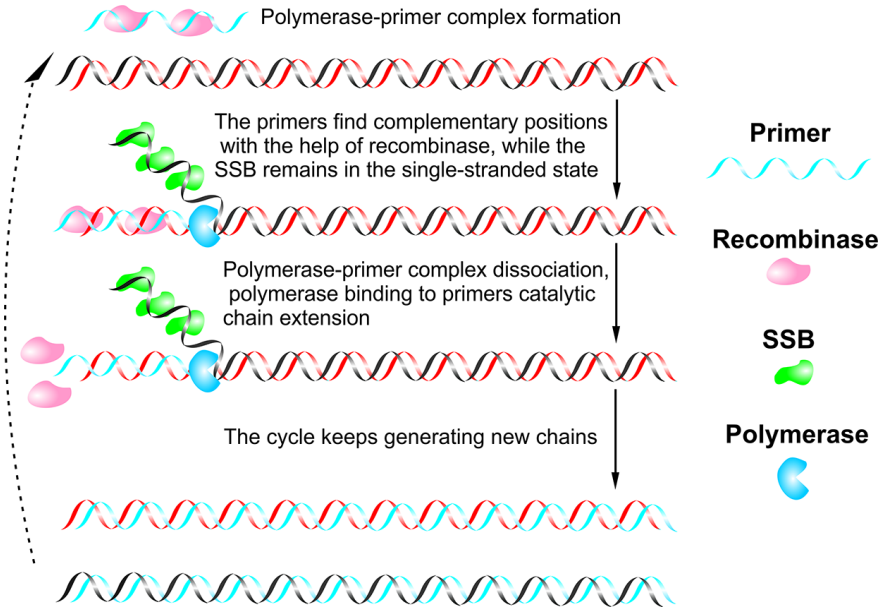

Figure 1. Principle diagram of amplification of primers and recombinant enzyme reaction by RAA technique.

Zhang et al. established a method for the fluorescence detection of SARS-CoV-2 clinical samples by RT-RAA, and the results showed that the clinical detection performance of RT-RAA kits was comparable to that of RT-PCR kits, and RT-RAA has the advantages of simple operation and portability, and is considered an important alternative to RT-PCR to meet the needs of community-level medical institutions[27]. Nie et al. constructed a method for detecting JEV using RT-RAA. It can detect multiple genotypes of JEV, which is simpler and more convenient than the traditional PCR assay. The detection limit of JEV plasmid is 5.5 copies/μL, which is similar to JEV RT-LAMP and TaqMan RT-qPCR, slightly higher than SYBR Green I RT-qPCR, and 100 times higher than RT-PCR. The established JEV RT-RAA fluorescence assay has been used in clinical diagnosis with rapidity, high sensitivity and specificity[28].

In this work, we established a rapid, highly sensitive and specific fluorescent RAA assay for six respiratory viruses. The flow chart of the fluorescent RT-RAA experiment is shown in Figure 2. With a portable built-in power supply device, nucleic acid amplification of various pathogens could be completed in one test within 20 minutes at 39, and the test results can be observed in real time. This study could provide technical support for the portable, reliable and sensitive detection of multi-pathogen on the infectious disease site.

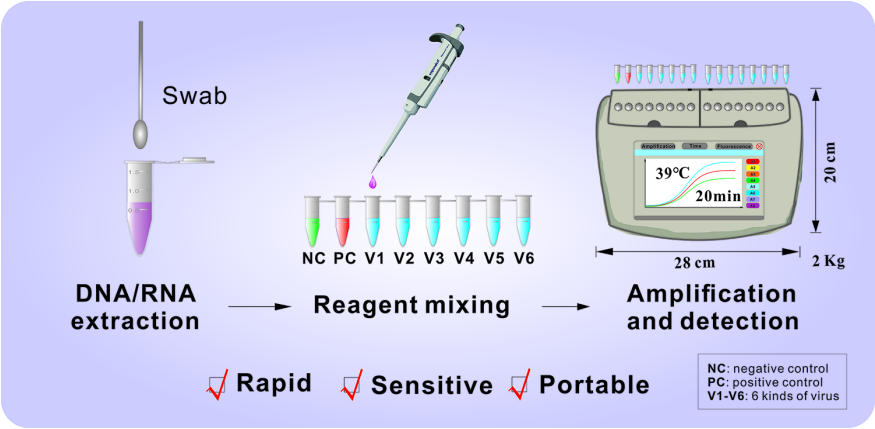

Figure 2. Flow chart of fluorescent RT-RAA experiment operation.

2. Material and methods

2.1 Material

The RNA standards of RSVA, HPIV, Flu A, Flu B, and SARS-CoV-2 were purchased from Shanghai Institute of Measurement and Testing Technology. The ADV was obtained from the chicken embryo culture in our laboratory. Genomic DNA/RNA extraction kits were purchased from Tiangen Biochemical Technology (Beijing) Co. The primers and probes were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The information of the respiratory virus nucleic acid is shown in Table S1. The PCR kits for nucleic acid detection of six viruses were purchased from Shenzhen Aodong Inspection and Testing Technology Co. 71 positive and 14 negative pharyngeal swab samples conserved in our laboratory were used for validation of the method.

2.2 Instruments and equipment

The Qitian RAA-B6100 thermostatic oscillation mixer was purchased from Jiangsu Qitian Genetic Biotechnology Co., Ltd., and the Genchek real-time RT-RAA fluorescence detector was purchased from Hangzhou Zhongce Bio-Sci&Tech Co. Ltd. The fluorescent quantitative PCR instrument was purchased from Bio-Rad Laboratories, Inc.

2.3 Design of primers and probes

Primers and probes were designed based on the sequence fragments in Table S2, and the corresponding sequences were downloaded from NCBI (at least 20 sequences for each virus were downloaded). Sequences were compared by DNA MAN software to obtain conserved sequences, and primers and probes were designed by custom parameters using the Primer-BLAST website and Primer 5 software, setting amplification products between 120 bp and 350 bp, primer length between 28 and 35 nucleotides, primer GC content between 20% and 70%, primer max self-complementary group was set to 4 bp and the TM value was set to 57-63°C. Several primer pairs were designed for each virus.

The probe is positioned between the forward primer (F), reverse primer (R), and is roughly 46-52 nucleotides long. The 3' end of the probe is modified with C3 Spacer and the tetrahydrofuran (THF) in the middle of the probe serves as the cleavage site for the nucleic acid exonuclease (exo). The two T bases adjacent to the THF site are modified with a FAM fluorescent motif and a BHQ1 quenching motif, respectively. 5' end is at least 30 nucleotides away from THF and 3' end is at least 15 nucleotides away from THF.

2.4 Primer and probe screening

After the candidate primers and probes were identified, their relative performance had to be evaluated, compared and screened. Candidate primers and probes were screened using 10^5 copies/mL of template

sample, and primers and probes with high fluorescence value of amplification curve, early amplification time and no non-specific amplification in negative control were selected according to the fluorescence curve detected by RAA.

2.5RT-RAA fluorescence assay.

According to the instruction of RT-RAA fluorescence amplification kit, the required amount of reaction system was prepared with the mixture solution containing enzyme-free water, A Buffer, pre-primer, forward primer, reverse primer, in Table S3. Mix all of them and add them into the assay unit tube preloaded with reaction dry powder, then add RNA/DNA template to the unit tube, and finally add 2.5 μL of B Buffer on the cap of the assay unit tube and cap the tube. Put into Qitian RAA-B6100 thermostatic oscillatory mixer to pretreat for 4 min, and put the assay unit tube into Genchek fluorescence detector and set the reaction at 39°C for 20 min.

2.6 Performance verification

2.6.1 Sensitivity evaluation

The sensitivity of the assay was characterized by serial tenfold dilutions of nucleic acid standards (from 10^5 to 1 RNA/DNA copies/mL), with ddH_2O as a negative control and the same system configuration is shown in Table S3.

2.6.2 Specificity evaluation

The specificity of the detection systems of the six pathogen primer probes was evaluated using RSVA, RSVB, HPIV, H1N1, influenza B virus, SARS-CoV-2, adenovirus, rhinovirus type A, rhinovirus type B, rhinovirus type C, H7N9, OC43, 229E, NL63, and S. aureus, Chlamydia pneumoniae, Mycoplasma pneumoniae, HMPV, SARS-CoV-2, MERS, and Klebsiella pneumoniae mock sample nucleic acids, respectively, at a template concentration of 10^5copies/mL.

2.6.3 Comparison of the consistency of pharyngeal swab results using real-time polymerase chain reaction and RT-RAA

71 positive pharyngeal swab samples were tested by the RT-RAA and PCR method, including 11 syncytial viruses, 7 influenza A viruses, 30 influenza B viruses, 4 parainfluenza virus, 11 novel coronaviruses, and 8 adenoviruses.

Real-time PCR was performed on a Bio-rad instrument, in which a commercial virus detection kit (Shenzhen Aodong Inspection and Testing Technology Co.) was used. Reactions were conducted in a 25 μL volume following kit instructions. RNA was extracted from 71 positive pharyngeal swab samples for method comparison experiments. All samples were tested by two methods, one-tube RT-RAA and real-time PCR assays. The concordance between these two methods was compared using SPSS 24.0 software.

3. Results

3.1 Primers and fluorescent probes screening

We downloaded 20 sequences of each virus from NCBI for alignment and obtained the relatively conserved sequences of each virus, and the conserved sequences are shown in Table S2. According to the RT-RAA design principles, the appropriate probes were first designed in the selected conserved sequences of each virus, as shown in Figure 3, and the probe positions of each virus were P (132-179) in RSV gene sequence, P (108-156) in HPIV gene sequence, P (714-763) in Flu A gene sequence, P (557-603) in Flu B gene sequence probe sequence, P (330-377) in SARS-CoV-2 gene sequence, and P (412-474) in ADV gene sequence. Then, several forward and reverse primers were designed surrounding to the probe, while some primers that might form secondary structures by themselves were excluded, and the relative positions of the candidate primers are shown in Figure 3. The ideal primers were screened by RT-RAA experiments based on the fluorescence intensity and peak onset time of each group of designed primers (Figure S1). The sequence information of the ideal primer pairs is shown in Table 1.

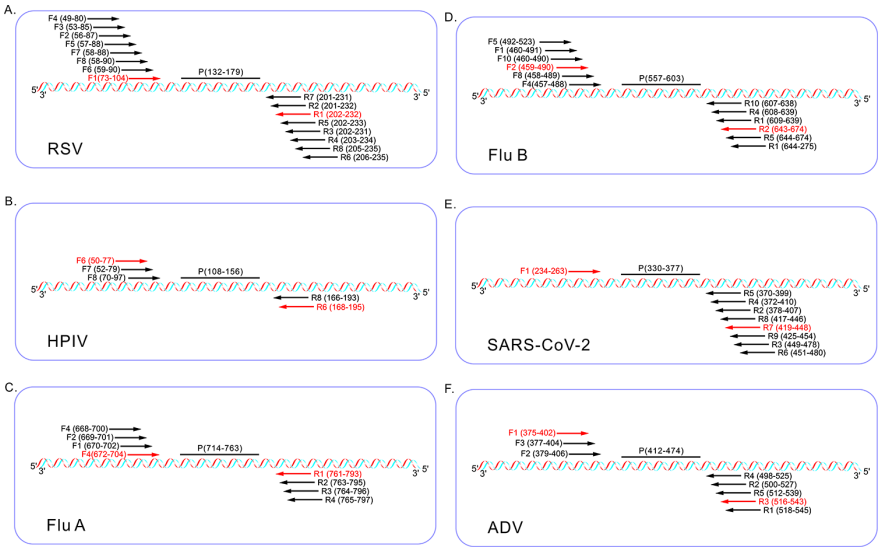

Figure 3. The relative position map of fluorescent probe and candidate primers in the conserved sequence screened for each virus. The optimal primers for each virus were shown in red. The numbers in parentheses represent relative positions within conserved sequence of (A) RSVA (GenBank accession no. KX655697.1: 4610-5503), (B) HPIV (GenBank accession no. MF554715.1: 82-1656), (C) Flu A (GenBank accession no. NC_002018.1: 1-1014), (D) Flu B, (GenBank accession no. CY018656.1:45-1727), (E)SARS-CoV-2, (GenBank accession no. OQ253304.1: 28214-29464), (F) ADV (GenBank accession no. MW816018.1:18385-21189).

Table 1. Primer and probe sequences of the screened respiratory pathogens.

Name [1]	Sequences (5'–3')
RSVA-F	TTCATATCATCGTGCTTATACAAGTTAAATCT
RSVA-R	CTTGTATGATTGCAGTTGTTAGTGTGACTTT
RSVA-P	TATTTTGGCAATGATAATCTCAACCTCACT[FAM-dT][THF][BHQ1-dT]AATTGCAGCCATCAT[C3-s
HPIV- F	AGAGCATCAATAAGTCTGGCGGAGGAGC
HPIV- R	ATCTGTATCCAGTGAGTGGGCTAAGAAA
HPIV-P	TGTCTTCACATTAGGCCCGAGTGTGACAGA[FAM-dT][THF]A[BHQ1-dT]GCAGATAAATTATTA[C
Flu A-F	TGTGTAAATGGTTCATGTTTTACTATAATGACT
Flu A-R	TTAGGTGCATTCAACTCTATTGATTTAGTAACC
Flu A-P	AGTGATGGGCTGGCCTCGTACAAAATTT[FAM-dT]CA[THF]GA[BHQ1-dT]CGAAAAGGGGAAGGT
Flu B-F	CTTTTACAAGATGGTAAGAGATGATAAAAC
Flu B-R	ATTAATGAAGGATCAAGTCCAACTCTTTTTAG
Flu B-P	TGGGGAGTGATGGCTTCAGTGGATTAAA[FAM-dT]C[THF]CA[BHQ1-dT]AATGATTGGGCA[C3-sp
SARS-Cov-2-F	TACGCAGAAGGGAGCAGAGGCGGCAGTCAA
SARS-Cov-2-R	CCTTGTTGTTGTTGGCCTTTACCAGACATT
SARS-Cov-2-P	AAGAGCAGCATCACCGCCATTGCCAGCCAT[FAM-dT][THF][BHQ1-dT]AGCAGGAGAAGTTCC[C3
ADV-F	CCTATGAGCAGGCAGGTGGTTGATGAGG
ADV-R	CGGCAGTAGTTCCGATGAGCGGGTATGG
ADV-P	CGTCACCTTACCATATCAACACAACAACTC[FAM-dT]G[THF]C[BHQ1-dT]TTGTAGGATACCTTG[C

[1]F, Forward primer; R, Reverse primer; P, Probe.

3.2Sensitivity evaluation

To evaluate the sensitivity of RT-RPA assay for six respiratory viruses, serial 10-fold dilutions of RNA/DNA standards were prepared, and fluorescence-time curves were obtained with a real-time RT-RAA fluorometric detector, The results were shown in Figure 4. The detection limits for these viruses were: 10^2 copies/mL for RSVA (4A), 10^2 copies/mL for HPIV (4B), 10^2 copies/mL for Flu A (4C), 10^3copies/mL for Flu B (4D), 10^2 copies/mL for SARS-Cov-2 (4E), and 10^2 copies/mL for ADV (4F), respectively.

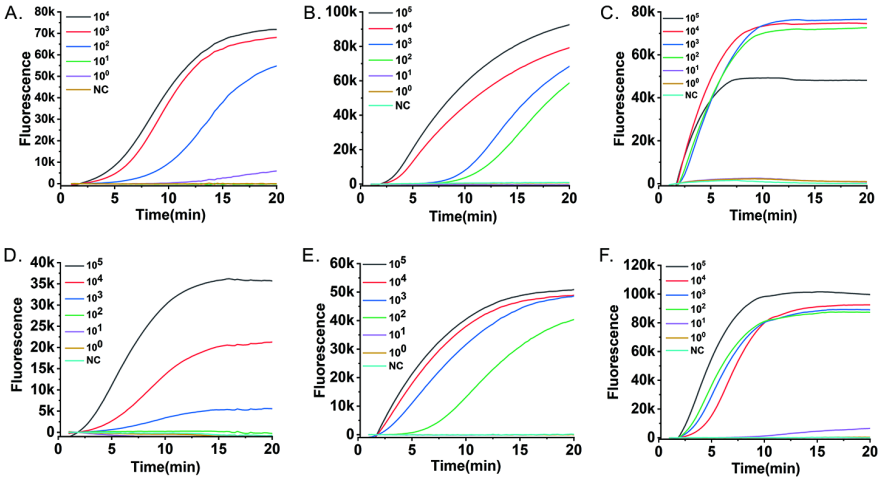

Figure 4. Sensitivity of the RT-RAA detection system. (A) Fluorescence-time curve for detection of serial 10-fold dilutions of RSVA RNA standards (10^4-10^0 copies/ml). (B) HPIV RNA standards (10^5-10^0 copies/ml). (C) Flu A RNA standards (10^5-10^0 copies /mL). (D) Flu B RNA standards (10^5-10^0 copies/mL). (E) SARS-Cov-2 RNA standards (10^5-10^0 copies/ml). (F) ADV DNA (10^5-10^0 copies/mL). The detection limit of each pathogen can be detected by repeating 3 times.

3.3 Specificity evaluation

To determine the specificity of the assay, 21 different pathogen nucleic acids were investigated in the experiment, including RSVA, RSVB, HPIV, Flu A, Flu B, SARS-CoV-2, ADV, rhinovirus type A, rhinovirus type B, rhinovirus type C, H7N9, HCoV-OC43, HCoV-229E, HCoV-NL63,*S. aureus* , Chlamydia pneumoniae, Mycoplasma pneumoniae, HMPV, SARS-CoV-2, MERS, and Klebsiella pneumoniae mock sample nucleic acids were used as templates to validate the 6 sets of primers and probes. As shown in Figure 5, except for the detection curves of target pathogen, other curves of non-target pathogens were all negative. It indicated there was no cross-reactivity between different pathogens, reflecting good specificity.

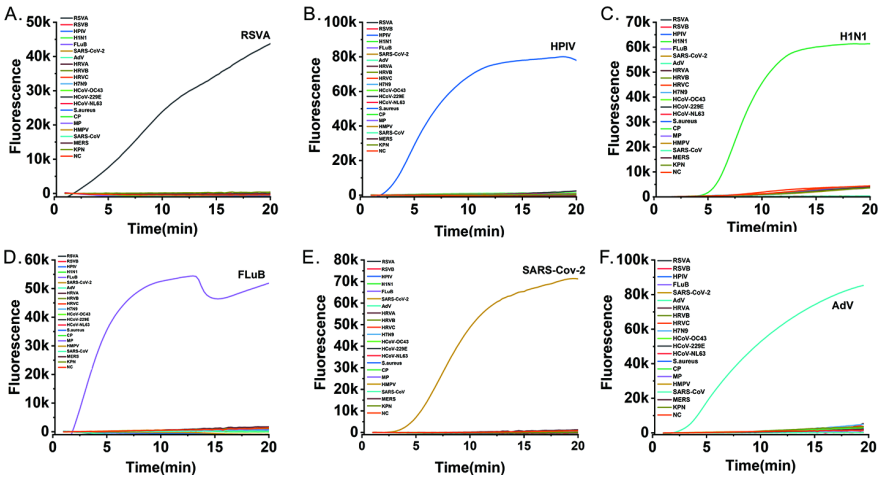

Figure 5. Specificity of the RT-RAA detection system. Fluorescence-time curves for the detection of nucleic acids from 21 different pathogens. The curves of (A) RSVA, (B) HIPV, (C) Flu A, (D) Flu B, (E) SARS-CoV-2 and(F) ADV detection systems, respectively.

3.4Pharyngeal swab samples testing

To evaluate the performance, 71 positive pharyngeal swabs (11 samples of RSVA, 5 samples of HPIV, 6 samples of Flu A, 30 samples of Flu B, 11 samples of SARS-CoV-2 and 8 samples of ADV, respectively) and 14 negative samples (not containing the six viruses) were extracted for nucleic acid and parallelly detected by both RT-RAA and RT-PCR. The established RT-RAA assay could accurately detect all positive samples as positive within 20 minutes (Figure 6), while all negative samples as negative (data not given). The parallel detection results of RT-RAA and RT-PCR were shown in Table S4, the positive and negative coincidence rates of the two methods were both 100%, but RT-RAA was more time-saving than RT-PCR while used a more portable instrument.

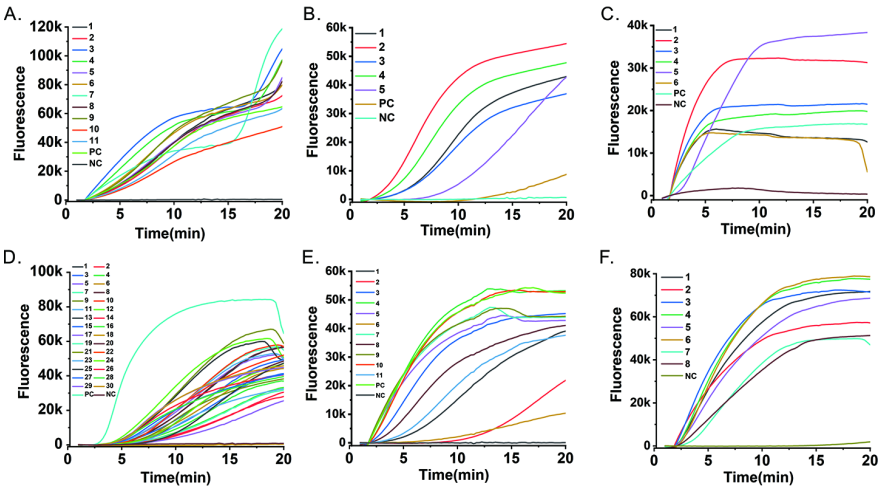

Figure 6. RT-RAA test results of pharyngeal swab samples of A-F virus. Fluorescence-time curves of (A) 11 samples of RSVA; (B) 5 samples of HPIV; (C) 6 samples of Flu A; (D) 30 samples of Flu B; (E) 11 samples of SARS-CoV-2 and (F) 8 samples of ADV.

4. Discussion

In the past decades, frequent outbreaks of infectious diseases caused by respiratory viruses have posed a great threat to human health. In order to prevent and control infectious disease epidemics as soon as possible, rapid and accurate diagnostic methods are essential to identify the pathogens. The use of PCR is limited by the need for complex and time-consuming thermal cycling processes, expensive testing equipment and specialized laboratory conditions, so it is not suitable for grassroots and resource-poor areas. In this work, we established a simple, rapid, highly sensitive and specific assay based on fluorescent RT-RAA for the detection of six respiratory viruses (RSVA, Flu A, Flu B, HPIV, SARS-CoV-2 and ADV), each of which could be completed within 20 min at a mild reaction temperature of 39°C. The sensitivity of the RAA assay was tested at 10^2 copies/mL for RSVA, 10^2 copies/mL for HPIV, 10^2copies/mL for Flu A, 10^3 copies/mL for Flu B, 10^2 copies/mL for SARS-CoV-2, and 10^1 copies/mL for ADV, and was confirmed by cross-testing with 21 different pathogen nucleic acids with good specificity. Although the results of parallel testing of pharyngeal swab samples by the RAA method were consistent with those of RT-qPCR, the results obtained by the RAA method within 20 min were more time-saving compared with the 1.5 h required by PCR, because the reaction conditions were mild. Moreover, the required equipment by RT-RAA in this work was more portable (only 2 kg) than that usually required by RT-PCR, and the RT-RAA detector had a built-in battery, which did not require additional power supply and could be carried to the field for testing.

In addition, different from PCR, which requires different heating and cooling processes for the amplification of different pathogenic nucleic acids, RT-assay established in this work can achieve the amplification and detection of six respiratory virus nucleic acids using the same thermostatic procedure. Therefore, in field detection, 16 separate channels of RT-RAA portable detector can be used to flexibly choose single pathogen detection or multiple pathogen detection for test samples. For example, 14 samples (in addition to negative and positive controls) can be tested for single pathogen, or the same sample can be added to different channels for multiple detection of 1 to 6 respiratory viruses at the same time.

5.Conclusions

The fluorescence RT-RAA based respiratory virus detection assay established in this work was of advantages of rapidity, portability, high sensitivity and specificity. It could provide technical support for the development of portable and accurate pathogen detection equipment suitable for application in health resource-poor areas and outbreak filed.

Author Contributions

Author Contributions: Conceptualization, R.Z. and H.S.; writing—original draft preparation, X.G. and D.G.; primer and probe design, X.G.; PCR and RAA assays, D.G.; sample collection, M.S. and D.S.; data curation, Y.Y.; writing—review and editing, H.S. and W.L.; All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors have no competing interests to declare.

Funding

This work was supported by the Hainan Provincial Key Research and Development Program, China (grant number ZDYF2021SHFZ262); the National Key Research and Development Program of China (grant number 2021YFC2301100); and the National Natural Science Foundation of China (grant number 32141003).

Ethical approval

Not applicable.

Acknowledgments

Not applicable.

References

1. Subbarao K, Mahanty S. Respiratory Virus Infections: Understanding COVID-19. Immunity 2020;52(6):905-909.
2. Kesson AM. Respiratory virus infections. Paediatr Respir Rev 2007;8(3):240-248.
3. Zhao Z, Huang C, Huang Z, Lin F, He Q, Tao D, Jaffrezic-Renault N, Guo Z. Advancements in electrochemical biosensing for respiratory virus detection: A review. Trends Analyt Chem 2021;139:116253.
4. Zhang N, Wang L, Deng X, Liang R, Su M, He C, Hu L, Su Y, Ren J, Yu F, Du L, Jiang S. Recent advances in the detection of respiratory virus infection in humans. J Med Virol 2020;92(4):408-417.
5. Albuquerque MC, Pena GP, Varella RB, Gallucci G, Erdman D, Santos N. Novel respiratory virus infections in children, Brazil. Emerg Infect Dis 2009;15(5):806-808.
6. Joshee S, Vatti N, Chang C. Long-Term Effects of COVID-19. Mayo Clin Proc 2022;97(3):579-599.
7. Bartoszko JJ, Farooqi MAM, Alhazzani W, Loeb M. Medical masks vs N95 respirators for preventing COVID-19 in healthcare workers: A systematic review and meta-analysis of randomized trials. Influenza Other Respir Viruses 2020;14(4):365-373.
8. Mahony JB, Petrich A, Smieja M. Molecular diagnosis of respiratory virus infections. Crit Rev Clin Lab Sci 2011;48(5-6):217-249.
9. Wrenn JO, Pakala SB, Vestal G, Shilts MH, Brown HM, Bowen SM, Strickland BA, Williams T, Mallal SA, Jones ID, Schmitz JE, Self WH, Das SR. COVID-19 severity from Omicron and Delta SARS-CoV-2 variants. Influenza Other Respir Viruses 2022;16(5):832-836.
10. COVID-19 CORONAVIRUS PANDEMIC.*https://www.worldometers.info/coronavirus/*; 2023 [Accessed 20 February 2023].
11. Alafeef M, Pan D. Diagnostic Approaches For COVID-19: Lessons Learned and the Path Forward. ACS Nano 2022;16(8):11545-11576.
12. Kang T, Lu J, Yu T, Long Y, Liu G. Advances in nucleic acid amplification techniques (NAATs): COVID-19 point-of-care diagnostics as an example. Biosens Bioelectron 2022;206:114109.
13. Valera E, Jankelow A, Lim J, Kindratenko V, Ganguli A, White K, Kumar J, Bashir R. COVID-19 Point-of-Care Diagnostics: Present and Future. ACS Nano 2021;15(5):7899-7906.
14. Lazcka O, Del Campo FJ, Munoz FX. Pathogen detection: a perspective of traditional methods and biosensors. Biosens Bioelectron 2007;22(7):1205-1217.
15. Xiao M, Tian F, Liu X, Zhou Q, Pan J, Luo Z, Yang M, Yi C. Virus Detection: From State-of-the-Art Laboratories to Smartphone-Based Point-of-Care Testing. Adv Sci (Weinh) 2022;9(17):e2105904.
16. Hocking L, George J, Broberg EK, Struelens MJ, Leitmeyer KC, Deshpande A, Parkinson S, Francombe J, Morley KI, de Carvalho Gomes H. Point of Care Testing for Infectious Disease in Europe: A Scoping Review and Survey Study. Front Public Health 2021;9:722943.
17. Boonbanjong P, Treerattrakoon K, Waiwinya W, Pitikultham P, Japrung D. Isothermal Amplification Technology for Disease Diagnosis. Biosensors (Basel) 2022;12(9).
18. Reid MS, Le XC, Zhang H. Exponential Isothermal Amplification of Nucleic Acids and Assays for Proteins, Cells, Small Molecules, and Enzyme Activities: An EXPAR Example. Angew Chem Int Ed Engl 2018;57(37):11856-11866.
19. Gao D, Guo X, Yang Y, Shi H, Hao R, Wang S, Li ZJ, Zhao R, Song H. Microfluidic chip and isothermal amplification technologies for the detection of pathogenic nucleic acid. J Biol Eng 2022;16(1):33.
20. Zhang H, Xu Y, Fohlerova Z, Chang H, Iliescu C, Neuzil P. LAMP-on-a-chip: Revising microfluidic platforms for loop-mediated DNA amplification. Trends Analyt Chem 2019;113:44-53.
21. Sherrill-Mix S, Hwang Y, Roche AM, Glascock A, Weiss SR, Li Y, Haddad L, Deraska P, Monahan C, Kromer A, Graham-Wooten J, Taylor LJ, Abella BS, Ganguly A, Collman RG, Van Duyne GD, Bushman FD. Detection of SARS-CoV-2 RNA using RT-LAMP and molecular beacons. Genome Biol 2021;22(1):169.
22. Heo S, Kim HR, Lee HJ. Development of a Quantitative Real-time Nucleic Acid Sequence based Amplification (NASBA) Assay for Early Detection of Apple scar skin viroid. Plant Pathol J 2019;35(2):164-171.
23. Fan X, Li L, Zhao Y, Liu Y, Liu C, Wang Q, Dong Y, Wang S, Chi T, Song F, Sun C, Wang Y, Ha D, Zhao Y, Bao J, Wu X, Wang Z. Clinical Validation of Two Recombinase-Based Isothermal Ampli-

fication Assays (RPA/RAA) for the Rapid Detection of African Swine Fever Virus. Front Microbiol 2020;11:1696.
24. Chen Y, Zong N, Ye F, Mei Y, Qu J, Jiang X. Dual-CRISPR/Cas12a-Assisted RT-RAA for Ultrasensitive SARS-CoV-2 Detection on Automated Centrifugal Microfluidics. Anal Chem 2022;94(27):9603-9609.
25. Wang ZH, Li P, Lin X, Jia H, Jiang YT, Wang XJ, Hou SH. Application of portable real-time recombinase-aided amplification (rt-RAA) assay in the clinical diagnosis of ASFV and prospective DIVA diagnosis. Appl Microbiol Biotechnol 2021;105(8):3249-3264.
26. RAA technology.*http://www.hz-zc.cn/raajs*; 2023 [Accessed 25 February 2023].
27. Zhang R, Qi S, Li J, Liu S, Han G, Shen X, Li Q, Xu W, Ma X. Field Evaluation of a Duplex RT-RAA Assay for Rapid Detection of SARS-CoV-2 - Hebei Province, China, January 2021. China CDC Wkly 2022;4(23):504-508.
28. Nie M, Zhou Y, Li F, Deng H, Zhao M, Huang Y, Jiang C, Sun X, Xu Z, Zhu L. Epidemiological investigation of swine Japanese encephalitis virus based on RT-RAA detection method. Sci Rep 2022;12(1):9392.