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Rapid detection of human influenza A viruses by HFman probe-based loop-mediated isothermal amplification assays

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ABSTRACT

Since China abandoned the zero-COVID policy at the end of 2022, a wave of severe Flu pandemic emerged in China. Rapid and accurate diagnosis of Influenza A virus (IAV) is critical for clinical management and therapeutic decision-making of patients with fever. Here, we reported a novel IAV HF-LAMP assay, which can be performed with purified RNA or directly using clinical samples. The assays with purified RNA and clinical samples have high sensitivity with limit of detection (LOD) of 9.6 copies/reaction, 9900 copies/mL, and short sample-to-answer times of 36 and 50 min, respectively. Both assays showed high specificity and significantly higher IAV detection rate than the rapid antigen detection (RAD) assays. Furthermore, we found the vast majority (91.2 %) of children with fever during the pandemic were infected by IAV, and current IAV infection has a very narrow detectable window. The novel IVA HF-LAMP assays will provide robust tools to facilitate early diagnosis of IAV infection in current and future seasonal influenza epidemics.

1. Introduction

Influenza (Flu) affects nearly 10 % of the world's population, with about half a million deaths every year [1]. Influenza A viruses (IAV) belong to the family of *Orthomyxoviridae* of RNA viruses, and are the major cause of Flu epidemics worldwide. During the past three years of the COVID-19 pandemic, non-pharmaceutical interventions (NPIs) well reduced the spread of not only SARS-CoV-2, but also other respiratory viruses such as IAV [2]. Lack of immune stimulation to influenza viruses during the COVID-19 pandemic may render the population more vulnerable to IAV. IAV activity started to revive since the summer of 2022 in China, and the prevalence of IAV seemed to exceed a model-predicted level with NPIs [3]. In particular, a wave of severe influenza pandemic emerged since China abandoned the zero-COVID policy at the end of 2022, and the positive rate of IAV rapidly increased to over 50 % in patients with fever by March 2023 [4].

Rapid and accurate diagnosis of IAV is critical for clinical management and therapeutic decision-making of patients with fever, and rapid sample-to-answer multiplex RT-PCR assays were demonstrated to reduce time to result and improve clinical care (e.g. shorter

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hospitalization) [5]. Various multiplex RT-PCR assays have been developed for detection, typing and sub-typing of influenza viruses [6,7]. However, the expensive costs of the mulitplex RT-PCR assays and relatively longer detection time (1.5 h) limit their wide application in routine clinical laboratory tests for patients with fever. Instead, rapid antigen detection (RAD) assays are widely used in fever clinics in China due to their cheap cost and simplicity of use. However, the poor sensitivity of RAD than nucleic acid amplification (NAA) methods and shorter duration of viral shedding of IAV (about 6 days) than SARS-CoV-2 (about 10 days) often lead to false-negative results [8,9], which explains why most patients with fever were negative for IAV. The false-negative results might affect the clinical management of patients with fever. Therefore, the development of simple, fast, sensitive, and specific NAA tests is urgently needed to facilitate detection of IAV.

Loop-mediated isothermal amplification (LAMP) is a promising point-of-care testing (POCT) method and has comparable sensitivity to PCR method [10,11]. A number of RT-LAMP assays had been developed for the detection of IAV [12,13]. As a POCT method, few studies compared the performance of RT-LAMP-based IAV assays with RAD method. Recently, we developed an HFman probe-based loop-mediated isothermal amplification (HF-LAMP) method to avoid the effect of non-specific amplification and demonstrated that it has comparable specificity and sensitivity to qPCR, but shorter reaction time and superior tolerance to viral variation [14–16].

In this study, we used the HFman probe-based RT-LAMP assay to rapidly detect IAV and compared its performance with RAD using clinical samples. Further, we developed a duplex assay for simultaneous detection of both IAV and SARS-CoV-2 in a single tube. The development of the HF-LAMP assay has great significance for timely detection of IAV and/or SARS-CoV-2 infection.

2. Materials and methods

2.1. Construction of the RNA standard

IAV M gene was the major target for LAMP assays. A PUC-57 plasmid carryingIAV M gene (GenBank accession no: MT540704.1, 1–635 nt) of IAV was synthesized by Sangon Biotech (Shanghai) Co., Ltd. Using primers containing a T7 promoter (Table S1), the M gene fragment was amplified by PCR from the plasmid. For standard RNA synthesis, an *in vitro* transcription reaction was carried out using a HiScribe T7 high-yield RNA synthesis kit (New England Biolabs, Ipswich, USA) according to the instruction. The *in vitro*-synthesized RNA was treated with DNase I at 37 °C for 15 min to remove DNA,and then the RNA was purified using LiCl precipitation, and quantified with a Qubit® 4.0 Fluorometer (Thermo Fisher Scientific, USA). The copy number of RNA standard was calculated using the following formula: RNA copies/mL = [RNA concentration (g/mL)/(nt transcript length × 340)] × 6.022 × 10^{23}.

2.2. Primers and probe of the IAV HF-LAMP assay

Five sets of IAV RT-LAMP primers have been described previously (Table S1) [17–21]. The primer set-2 was used to develop the IAV HF-LAMP assay. The LF of the Set-2 was designed as an HFman probe by labeling a Cy5 fluorophore and a BHQ2 quencher group at its 3′- and 5′-ends, respectively. The primers and HFman probe were synthesized by Sangon Biotech (Shanghai) Co., Ltd,.

2.3. Reaction system of the IAV HF-LAMP assay

A 25 μL HF-LAMP reaction was established, including 8 U Bst 4.0 DNA polymerase (Haigene, Harbin, China), 0.15 U High-fidelity DNA polymerase (New England Biolabs, Ipswich, USA), 10 × isothermal amplification buffer, 8 mmol/L $MgSO_4$, 1.8 mmol/L dNTPs., 0.1 μM each of F3 and B3, 1.0 μM each of FIP and BIP, 0.6 μM LB, 0.3 μM LF and 0.3 μM HFman probe. 5 μL RNA standard, purified RNA from nasopharyngeal swab (NP) specimen or direct NP specimen were used in the HF-LAMP reaction. The reaction was run at 64 °C for 50 min with real-time monitoring by CFX 96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) or a portable fluorescent visual POC device (Yijian Technology, Hangzhou, China).

2.4. Specificity and sensitivity of the IAV HF-LAMP assay

The specificity of the HF-LAMP assay was validated by 11 other human respiratory viruses, including influenza virus (IBV and ICV), respiratory syncytial viruses (groups A and B), human rhinovirus, Human bocavirus, human coronaviruses (SARS-CoV-2, HCoV-NL63, HCoV-229E, and HCoV-OC43) and Human metapneumovirus. The sensitivity of the LAMP system was evaluated using ten-fold serially diluted IAV standard (from 10^4 to 10° copies/μL), and 3 μL each dilution was added in each reaction.

2.5. Limit of detection (LOD) of the IAV HF-LAMP assay

LOD of the IAV HF-LAMP assay was determined by 10 replicates of five-fold serial dilution of RNA standard (from 3000 to 5 copies per reaction). For the nucleic acid extraction-free HF-LAMP assay, simulated clinical samples were prepared by adding various dilutions of RNA standard into NP specimen collected from healthy individuals. The LOD was defined as the lowest amount of target RNA that has a 95 % probability of being true positive, using the probit regression analysis (SPSS 17.0 software). 2.6 Nucleic acid extraction and nucleic acid extraction-free processing.

Viral RNA was extracted from 200 μL NP specimen using Magnetic Viral DNA/RNA Kit (Tiangen Biotech, Beijing, China) and re-dissolved with 50 μL nuclease-free water for storage at −80 °C until use. Nucleic acid extraction-free HF-LAMP was performed directly

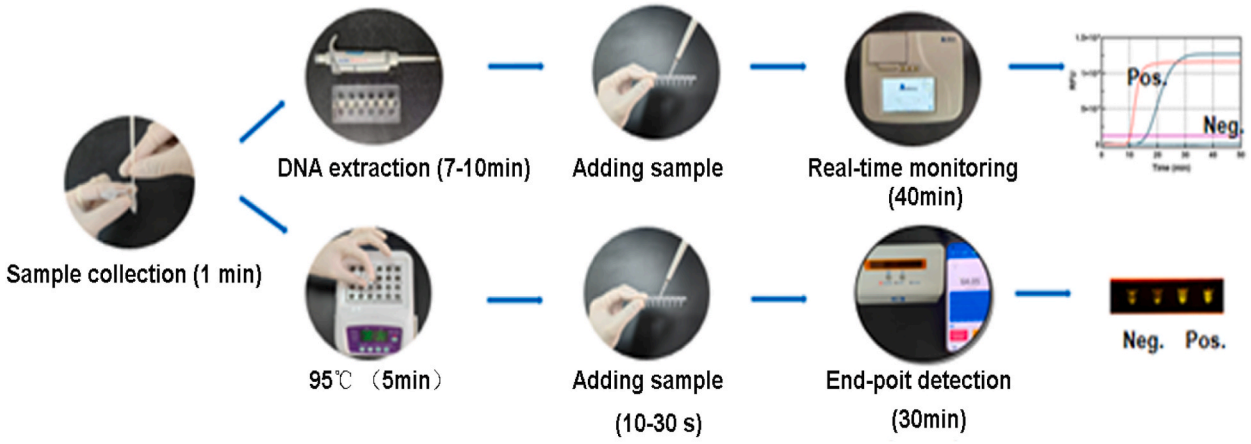

Fig. 1. Workflow of the HF-LAMP assay. Pos.: positive result; Neg.: negative result.

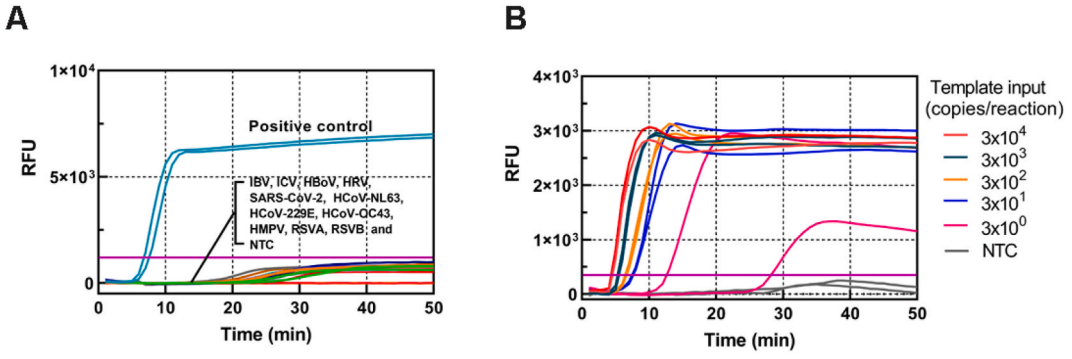

Fig. 2. Sensitivity and specificity of the HF-LAMP assay. (A) Specificity. 11 other human viruses were used. (B) Sensitivity. Purple horizontal line: time threshold (Tt). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

using 5 μL of NP specimen after heat-inactivated at 95 °C for 5 min.

2.6. Evaluation of IAV HF-LAMP using clinical samples

To assess the performance of the IAV HF-LAMP assay, 68 NP specimens were collected from children with fever who visited the fever clinic of Shanghai Public Health Clinical Center during March 19, 2023 to April 12, 2023. Ethical approval for this study was obtained from the Shanghai Public Health Clinical Center ethics committee (2021Y082-01) and all patients signed written informed consent. Standard HF-LAMP (with purified RNA), nucleic acid extraction-free HF-LAMP, and RAD (Bioline Influenza Antigen kit, Abbott, Korea) were applied to analyze the NP specimens in parallel. Positive rate was calculated with the following formula: (number of positive results/total number) × 100 %.

2.7. Single-tube duplex HF-LAMP reaction for IAV and SARS-CoV-2

The single-tube duplex HF-LAMP was performed in a 25 μL reaction system. The reaction mix and condition are consistent with HF-LAMP assay except primer concentrations. The concentrations of the IAV and SARS-CoV-2 primers were halved compared with the IAV HF-LAMP assay. The primers used for the SARS-CoV-2 E gene have been described previously [14]. All primer information are listed in Table S1. The HFman probe of SARS-CoV-2 E gene was designed by labeling LB primer with a fluorophore FAM at its 3′ end and a quencher BHQ1 at 5’ end.

3. Results and discussion

3.1. Establishment of the HF-LAMP assay for IAV

Five RT-LAMP assays were previously developed for detection of IAV [5–9]. All these assays yielded non-specific amplification (false-positive) in absence of IAV RNA or have very low amplification efficiency (Fig. S1). Primer set 2 was demonstrated to have the

Table 1
Limit of Detection (LOD) of the HF-LAMP assays for IAV detection.

Standard HF-LAMP assay	Template input (copies/25 μL reaction)	3000	600	120	24	5	LOD (copies/25 μL reaction)
	IAV (positive/total)	10/10	10/10	10/10	10/10	5/10	9.6
Extraction-free HF-LAMP assay	Simulated NP specimen (copies/mL)	6.0×10^5	1.20×10^5	2.4×10^4	4.8×10^3	9.6×10^2	LOD (copies/mL)
	IAV (positive/total)	10/10	10/10	10/10	5/10	1/10	9.9×10^3

Note: The simulated samples were prepared by mixing IAV RNA standard into negative NP specimens. 5 μL Simulated NP specimen was directly used in each extraction-free HF-LAMP reaction.

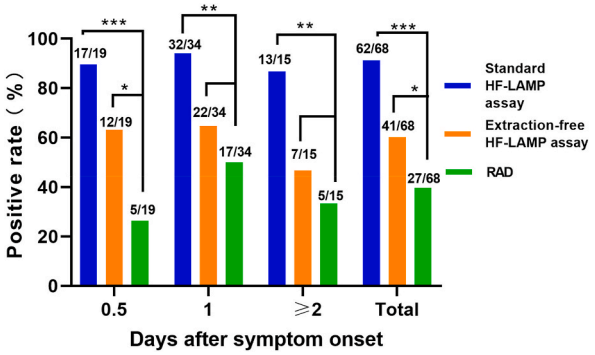

Fig. 3. Clinical validation. NTC: non-template control, Pos.: positive result; Neg.: negative result; min: minute; s: second.

highest amplification efficiency (Fig. S1 and Table S1), and selected for the development of a novel HF-LAMP assay. The selected primers target the most conserved M gene of IAV, and at least covers H1 to H3 IVA subtypes [18]. Set 2 LF primer was designed as an HFman probe. Using the previous LAMP primer set 2, we developed a novel HF-LAMP assay for POC diagnosis of IAV (Fig. 1).

3.2. Specificity, sensitivity of the HF-LAMP assay

The specificity of the HF-LAMP assay was validated by 11 other human respiratory viruses, including influenza virus (IBV and ICV), respiratory syncytial viruses (groups A and B), human rhinovirus, Human bocavirus, human coronaviruses (SARS-CoV-2, HCoV-NL63, HCoV-229E, and HCoV-OC43) and Human metapneumovirus (Fig. 2A). The sensitivity of the assay was determined as low as 3 copies per reaction within 30 min using 10-fold serially diluted RNA standards from 10^4 to 1 copies/μL (Fig. 2B).

3.3. LOD of the HF-LAMP assay

The LOD was determined as 9.6 copies per reaction (equal to 480 copies/mL specimen) using 10 replicates of five-fold serial dilutions of RNA standard (from 3000 to 5 copies per reaction) (Table 1). The extraction-free HF-LAMP assay has a LOD of 9.9×10^3 copies/mL NP specimen (Table 1), and can detect the presence of transmissible IAV in upper respiratory tract [9].

3.4. Clinical evaluation of the HF-LAMP assay

Clinical information of involved patients are shown in Supplementary Table S2. Apart from a standard HF-LAMP assay using purified RNA, an extraction-free HF-LAMP assay was also performed directly using heat-inactivated NP specimens (Fig. 1A). Of 68 NP specimens, the standard HF-LAMP assay detected 62 (91.2 %) positive for IAV (Fig. 3), indicating that the vast majority of children with fever were infected by IAV during this Flu pandemic. Only 27 (39.7 %) NP specimens were detected positive for IAV by the RAD assay, significantly lower than both HF-LAMP assays, implying a poor sensitivity. The extraction-free HF-LAMP assay detected 41 (60.3 %) positive for IAV, lower than the standard HF-LAMP assay but significantly higher than the RAD assay (Fig. 3). Compared to the standard HF-LAMP assay, relatively low detection rate by the extraction-free assay can be explained by two reasons. First, only 5 μL NP specimen was used in the extraction-free assay, about one quarter of the amount (5 μL purified RNA corresponding to 20 μL NP specimen) used in the standard assay. Second, the presence of some inhibitors in NP specimen might slightly reduce the sensitivity of the assay. Furthermore, all positive specimens by both HF-LAMP assays showed a fast amplification with a Time threshold (Tt) of less than 20 min (Table S2), implying a short sample-to-answer time.

In this study, we found that the vast majority (91.2 %) of children with fever were infected by IAV, substantially higher than that in a recent observation in another hospital in Shanghai [3]. This wave of IAV prevalence in spring, 2023 significantly exceeded its prevalence before the COVID-19 pandemic [3,6], which might be ascribed to the lack of immune stimulation caused by reduced

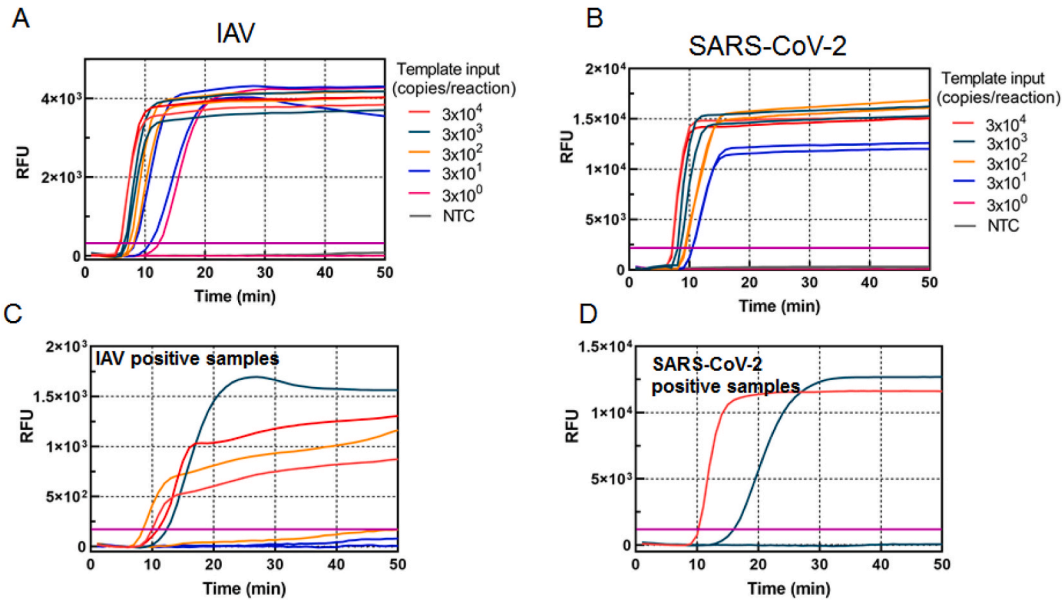

Fig. 4. A multiplex LAMP for IAV and SARS-CoV-2 (A, B) Sensitivity. Purple horizontal line: time threshold (Tt). (C, D) Clinical validation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

prevalence of IAV and low uptake of flu vaccination during the COVID-19 pandemic [22]. Interestingly, we found that the detection rate of IAV was highest at one day after symptom onset and reduced since the second day after symptom onset regardless of any methods to be used (Fig. 3), implying a very narrow detectable window for IAV infection. This result might be ascribed to a very short duration of IAV shedding at the upper respiratory tract [9]. Only 26.3 % of IAV infection at 0.5 days after symptom onset can be detected by RAD assay, implying that the RAD assays are insufficient for early diagnosis of IAV infection [8,9]. Importantly 89.5 % and 63.2 % of them can be found by the standard and extraction-free HF-LAMP assays, respectively, suggesting that the HF-LAMP assays are robust POCT tools to facilitate early diagnosis of IAV infection [8,9].

3.5. Single-tube duplex HF-LAMP assay for IAV and SARS-CoV-2

SARS-CoV-2 and IAV spread through similar mechanisms and present with overlapping clinical symptoms. Co-infection with SARS-CoV-2 and IAV were highly prevalent and coinfection with influenza A virus enhances SARS-CoV-2 infectivity [23]. The mortality rate of co-infected individuals was 1.53 times higher than SARS-CoV-2 alone [24]. Surveillance of co-infection with IAV and SARS-CoV-2 should be strengthened globally. In view of multiplex detection capacity, the current IAV HF-LAMP assay was further developed into a duplex assay for simultaneous detection of both IAV and SARS-CoV-2 by combining our previous SARS-CoV-2 assay [14] (Fig. 4). A ten-fold serial diluted IAV and SARS-CoV-2 RNA standards from 10^4 to 1 copies/μL were used to determine the sensitivity of duplex HF-LAMP assay. The results show that the duplex RT-LAMP can detect as few as 3 copies of IAV and 10 copies of SARS-CoV-2 RNA per reaction within 15 min (Fig. 4A and B). The cost of the duplex HF-LAMP assay was about 0.61 US dollar per reaction) (Table S3).

To verify the clinical validation of the duplex HF-LAMP assay, four IAV positive samples confirmed by RAD, two SARS-CoV-2 positive samples confirmed by qRT-PCR and 6 healthy individuals' samples were tested. The results showed 100 % concordance with clinical diagnosis (Fig. 4C and D).

Our study has two limitations. First, the HF-LAMP assay was not designed for typing and sub-typing IAV. Therefore, we were unable to determine the IAV genotypes. Second, the sample size was relatively small. Further evaluation of the new assay is still needed.

4. Conclusion

In summary, we developed a rapid, cheap, sensitive and specific HF-LAMP assay for the detection of IAV. The standard and extraction-free versions of the assay have LOD of 480 copies/mL and 9.9 × 10^3 copies/mL of NP specimen and short sample-to-answer times of less than 50 and 36 min, respectively. The reaction can be conducted in a portable isothermal fluorimeters for real-time monitoring or low-cost simple heating block for end-point fluorescence observation by naked eye. The novel HF-LAMP assays will be especially useful for IAV surveillance in current and future seasonal influenza epidemics.

Ethical approval

The study was approved by the Shanghai Public Health Clinical Center ethics committee (2021Y082-01).

Funding information

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Authors' contributions

Chiyu Zhang: conceived and designed the experiments, analyzed and interpreted the data, contributed reagents, materials, analysis tools or data, wrote the paper. Yongjuan Zhao: performed the experiments, analyzed and interpreted the data, wrote the paper. Weimin Tian: conceived and designed the experiments, contributed reagents, materials, analysis tools or data, analyzed and interpreted the data. Bing Li: contributed reagents, materials, analysis tools or data, analyzed and interpreted the data. Zhenzhou Wan, Yi Zeng and Xiaoling Zhang: analyzed and interpreted the data.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21591.

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