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FEN1-assisted LAMP for specific and multiplex detection of pathogens associated with community-acquired pneumonia†

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Lower respiratory tract infections (LRITs), including community-acquired pneumonia (CAP), are the fifth leading cause of death worldwide over the last ten years, posing a serious threat to global healthcare. Conventional laboratory assays for detecting pathogens are hindered by complicated procedures, a long turnaround time and a lack of multiplex detection capabilities. In this study, a flap-endonuclease 1 (FEN1)-assisted loop-mediated isothermal amplification (LAMP) method was designed, and an assay based on this method was developed to identify three leading pathogens for CAP, namely, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae* and *Haemophilus influenzae*. FEN1-assisted LAMP utilized a sequence-specific probe with a flap structure to generate an amplified signal, demonstrating high specificity and sensitivity with a low limit of detection (100 copies per μL). Based on the cleavage of flap probes by FEN1, our assay was able to detect three pathogens in a single reaction. This method is highly consistent with the polymerase chain reaction (PCR) in clinical sample testing. This simple, specific and multiple detection method has the potential to identify CAP and could be applied to detect other pathogen infections.

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Introduction

Over the past decades, lower respiratory tract infections (LRTIs), which have high incidence and mortality rates, have remained among the most common infectious diseases worldwide, particularly affecting individuals over the age of 70 and children under the age of 5.[1–3] LRTIs are the fifth leading cause of death globally, which resulted in approximately 2.49 million deaths (35.72 deaths per 100 000 people) in 2019,[1–3] according to the World health statistics 2024: monitoring health for the SDGs, sustainable development goals (WHO) (**https://www.who.int/publications/i/item/9789240094703**). Community-acquired pneumonia (CAP) is one of the most common types of LRTIs and has brought heavy healthcare and economic burden all around the world.[4–7] CAP is defined as the lung parenchyma infection acquired outside the healthcare setting or before hospitalization.[8,9] CAP is typically caused by bacterial or viral infections. *Streptococcus pneumoniae* (SP), *Mycoplasma pneumoniae* (MP) and *Haemophilus influenzae* (HI) are the main pathogens responsible for CAP,[10,11] despite the increasing viral morbidity in recent years.[12] Pneumonia caused by different pathogens shows no significant distinction in clinical presentations, especially in the early stages of the disease, which significantly increases the difficulty of diagnosis and hinders the rapid and accurate selection of antibiotics.[3,9,13–15] Therefore, there is a need for developing an accurate, timely and multiplex method to identify these pathogens to ensure effective clinical management and treatment, as well as to monitor the epidemic.

Conventional laboratory methods for pathogen detection of CAP are predominantly microbiology-based and immunology-based tests. While the bacterial culture from sputum or blood samples is the gold standard,[16,17] it is hindered by a long turnaround time (commonly more than 48 hours) and limited sensitivity.[18,19] Immunology-based tests, such as urinary antigen detection of SP and serological tests for antibodies of MP, present insufficient specificity.[20–23] Therefore, immunology-based tests typically need to be combined with other methods to accurately confirm pneumonia.[22–24] The polymerase chain reaction (PCR), a classic molecular amplification technique, is widely used for rapid pathogen detection owing to its high specificity and sensitivity.[22,25] Furthermore, the

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TaqMan probe-based PCR enables multi-pathogen detection in one reaction, which is achieved through the hydrolysis of sequence-specific probes by the 5′ to 3′ exonuclease activity of Taq polymerase during amplification.[26] However, PCR requires specialized equipment to control the reaction temperature precisely and a strict laboratory setting, which hinders their accessibility, especially in resource-limited regions.[27,28]

LAMP, an isothermal amplification technology established by Notomi *et al.* in 2000,[29] has emerged as a promising alternative for identifying various pathogenic microorganisms. LAMP is driven by Bst DNA polymerase and a set of target-specific primers, which offers advantages in both speed and sensitivity.[29] Unlike the DNA polymerases used in PCR, the Bst DNA polymerase possesses strong strand displacement activity, which allows the LAMP reaction to be performed under isothermal conditions.[29] Therefore, the LAMP assay could be carried out on simpler and cheaper equipment with lower requirements of a laboratory setting. However, isothermal amplification, including LAMP, is susceptible to non-specific amplification due to the formation of primer dimers or mis-hybridization.[30] In addition, the Bst DNA polymerase lacks the 5′ to 3′ exonuclease activity;[31] thus, it is unable to hydrolyse the sequence-specific probe akin to the TaqMan probe-based PCR method. As a result, conventional LAMP is unsuitable for developing multiplexed assays based on sequence-specific probes. To address these issues, the Combined Flap Probe Amplification (CFPA) method has been proposed, which integrates Flap Endonuclease 1 (FEN1) with Bst DNA polymerase-mediated isothermal amplification to enable the isothermal probe-based nucleic acid detection.[27] FEN1 is a structure-specific 5′ to 3′ endonuclease essential for DNA replication and repair.[32] It recognizes and binds the flap base (the junction of single- and double-stranded DNA), threads the 5′ flap into its active site through a helical arch that selectively accommodates the single-stranded DNA, and hydrolyses the phosphodiester bond *via* a phosphate steering mechanism, ensuring precise cleavage specificity.[32–34] Therefore, FNE1 specifically cleaves the 5′ DNA and RNA flaps of branched double-stranded DNA substrates. Owing to its specificity, the developed FEN1-assisted CFPA can diminish the risk of false positives and provide a robust strategy for nucleic acid detection. To date, several FEN1-mediated isothermal amplification methods have been developed for the detection of infectious diseases or food allergens encoding genes, suggesting its superior analytical performance and broad application potential.[27,28,35–38]

In this study, we propose an SP, MP and HI triplex assay based on the FEN1-assisted LAMP method for multiplex detection of three primary bacterial causes of CAP: SP, MP and HI. By combining the LAMP with FEN1, this method achieved high specificity and sensitivity and detected multiple pathogens in a single reaction. The clinical sample testing results demonstrated that the FEN1-assisted LAMP method enables the accurate diagnosis of pathogens. Our method would provide significant enhancement to the diagnosis and management processes of CAP in clinical laboratories and public health agencies and holds promise for the detection of multiplex targets with specificity and simplicity.

Results and discussion

Principle of the proposed FEN1-assisted LAMP method

The reaction principle of the FEN1-assisted LAMP method is presented in Fig. 1A. First, the flap probe was designed to contain two regions: the binding region, shown in purple, is specifically complementary with the target sequence, while another region (flap region, red) is unpaired with the target DNA. The binding region of the flap probe is located between the F1c and F2c, or B1c and B2c on the target sequence. Each flap probe was modified with different fluorescent groups to identify the three pathogens of CAP. When the target is present in the reaction system, the target sequence would be amplified by the action of primers, subsequently forming a dumbbell structure through the extension of the Bst DNA polymerase.[29] Then, the flap probe could recognize and bind the binding region of the amplified target at the location of its dumbbell structure, specifically through complementary base pairing. Therefore, a flap structure was formed between the amplified target and the flap probe. Ultimately, the FEN1 enzyme can specifically recognize and cleave the flap region of the probe owing to its distinctive cleavage characteristics,[27,35,36] which results in the fluorescent group releasing from the quenching group on the flap probe, thus generating a fluorescent detection signal. Three flap probes labelled with different fluorescent groups (FAM, ROX, and HEX) were designed to differentiate MP, SP, and HI in this triplex assay, thus enabling the multiplex detection of pathogens for CAP in a single reaction system (Fig. 1B).

Screening of the LAMP primer sets for SP, MP and HI

4–5 sets of primers of SP, MP and HI were designed based on the website: **https://primerexplorer.jp/e/index.html**. To determine the optimal primer set of each pathogen in this method, a real-time fluorescence LAMP reaction was carried out, which used SYTO-9 (a fluorescent dye) for monitoring the fluorescence change. As the amplification proceeds, the fluorescence signal of the target gradually intensifies until it reaches a plateau phase. The time at which the amplification signal reaches a certain intensity is referred to as the threshold time (T_t value). T_t value is defined as the assessment criterion. Fig. 2A–C show the amplification results of the three pathogens using different primer sets, respectively. The fluorescent signals indicate the effectiveness of the primer design despite variations in the T_t value (ranging from minutes to tens of minutes). Based on the minimum T_t value, the P1 of MP (Fig. 2A), P3 of SP (Fig. 2B) and P4 of HI (Fig. 2C) are identified as the most suitable primer sets in this study. The selected primer sets and target sequence are listed in Table S2.†

Feasibility of the FEN1-assisted LAMP method for assaying SP, MP and HI

To verify the feasibility of the FEN1-assisted LAMP method using flap probes, three flap probes were designed based on the sequence of three pathogens, and different fluorescent and quenching groups were labelled into the 5′ and 3′ end of flap

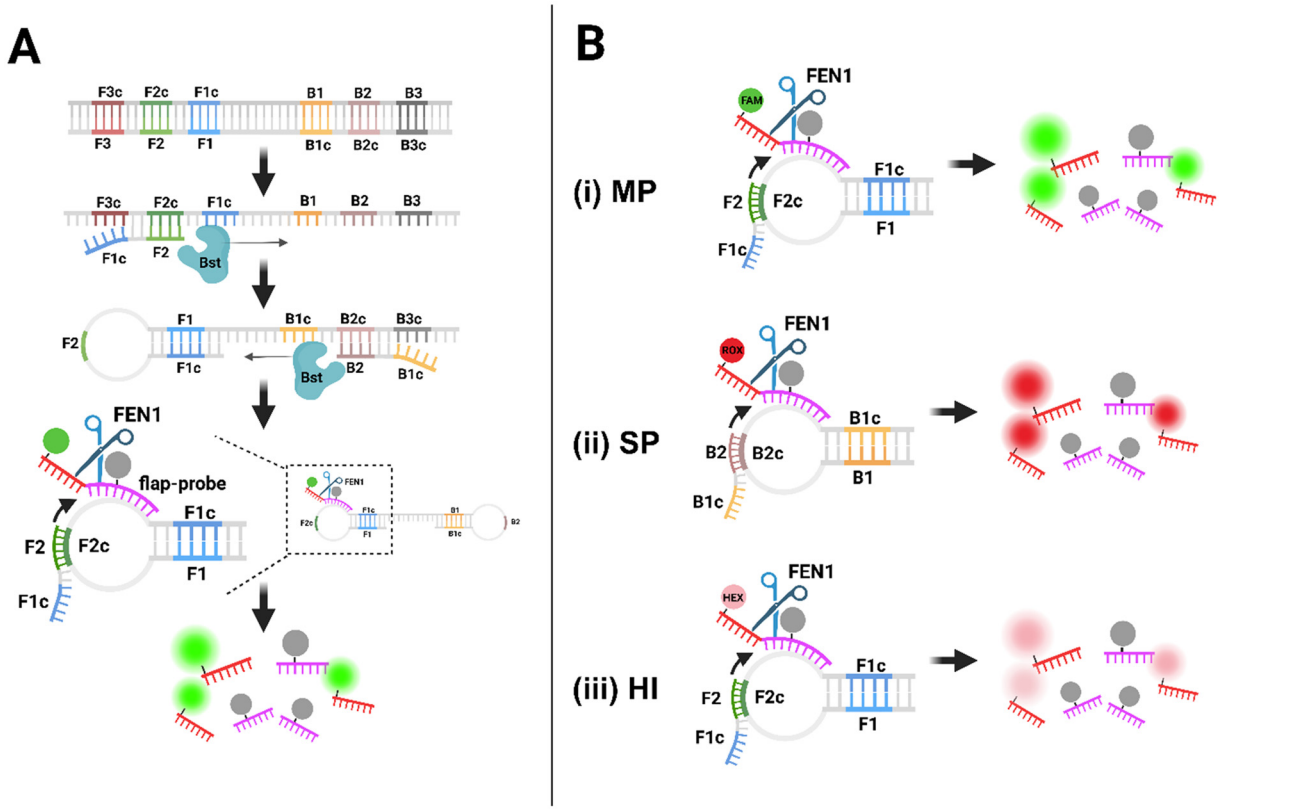

Fig. 1 Reaction mechanism of the FEN1-assisted LAMP method. (A) Schematic of this method. (B) The strategy of identifying three pathogens for CAP. Bst DNA polymerase was used for amplification of the target sequence and strand displacement. FEN1 was used for cleaving flap probes to generate signals. Each flap probe was labelled with FAM, ROX, and HEX for identifying MP, SP and HI, respectively. The Scheme was created on https://BioRender.com.

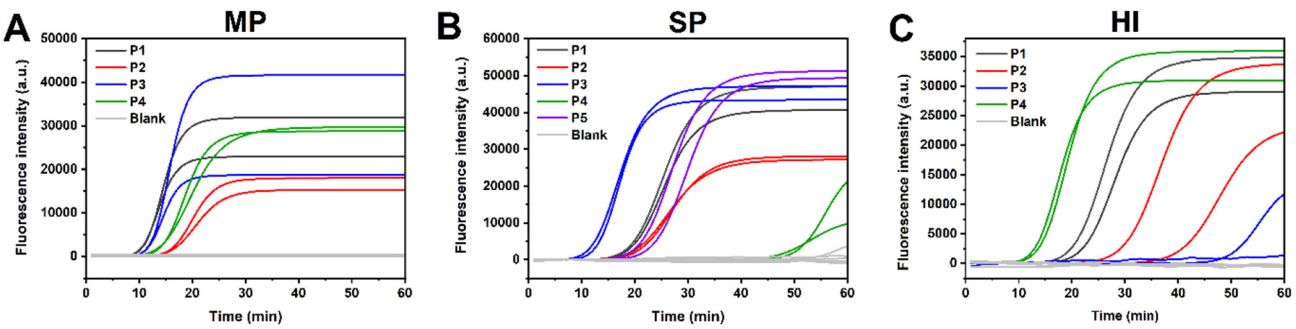

Fig. 2 Results of the screening of LAMP primers for MP, SP and HI. Real-time fluorescence monitoring of amplification curves of (A) MP, (B) SP and (C) HI using different sets of primers. The concentration of each gene plasmid is 2.5 × 10^6 copies per μL. Ultrapure water was used as the blank control. Primer sets are displayed as P1–P5 for each target. The LAMP reaction was carried out using different sets of primers. The blank results from all primers (P1–P4/P5) are overlapped. Data represents duplicates (n = 2).

probes, respectively (Table S3†). The FEN1-assisted LAMP assay was established, which is comprised of a primer set, Bst DNA polymerase, FEN1, and flap probe. As shown in Fig. 3, each target plasmid being detected generated a strong fluorescence signal, while blank controls showed no signal. Although the FEN1-assisted LAMP method exhibits a slightly reduced amplification efficiency compared to traditional LAMP (Fig. 2), the above results illustrated that flap probes were successfully recognized and cleaved by FEN1 during amplification, thereby confirming the feasibility of the FEN1-assisted LMAP method.

Key analytical performance parameters for the SP, MP and HI assay

The appropriate reaction conditions for this method were established based on prior studies (detailed parameters shown

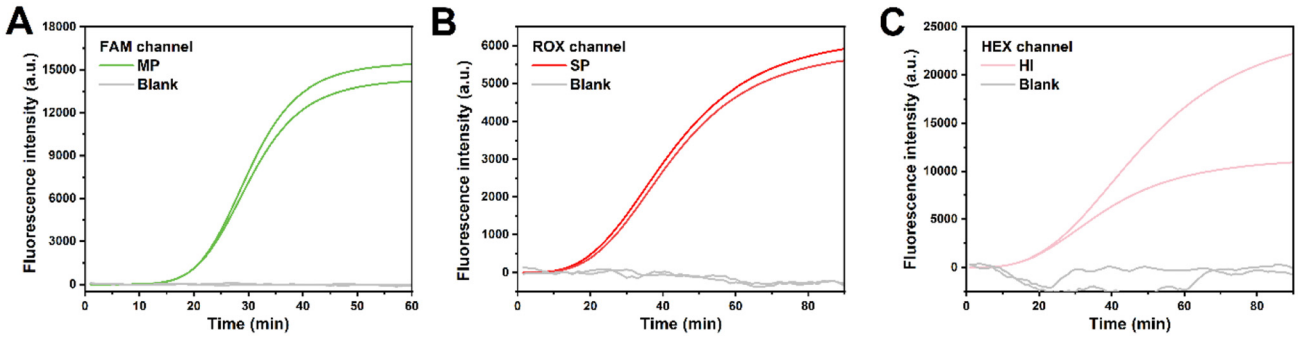

Fig. 3 Results of the feasibility of the FEN1-assisted LAMP method. Real-time fluorescence monitoring of amplification reactions of (A) MP, (B) SP and (C) HI using flap probes. The concentration of each plasmid is 2.5×10^6 copies per μL. Ultrapure water was used as the blank control. Data represents duplicates (n = 2).

in ESI†).[27,37,39] In the clinical scenario, the capability of identifying low-abundance pathogens with high accuracy is fundamental and essential for an assay. To explore the detection sensitivity of the SP, MP and HI assay in detecting each pathogen, the serial dilution ranging from 10^7 to 10^1 copies per μL of each target plasmid was prepared. This concentration range generally covers the common concentration range of clinical infections.[40] The amplification curves in Fig. 4A–C illustrated that this assay achieved a limit of detection (LOD) down to 10^2 copies per μL in detecting MP and SP, and 10^3 copies per μL in detecting HI. Besides, the results in Fig. 4D–F show a good linear relationship between the T_t value and logarithmically transformed concentration. This assay displays excellent sensitivity for detecting these pathogens for the precise diagnosis of CAP.

Multiplex detection strategies are important because they can rapidly identify pathogen infections with high testing efficiency. Here, we evaluated the multiplex detection capability of the SP, MP and HI assay by observing the amplification signal in different fluorescence channels (FAM, ROX and HEX). We found that the fluorescent signal could only be detected when the corresponding target was present (Fig. 5). This experiment demonstrates that each flap probe accurately identified its target without cross-interference. This result confirmed that the FEN1-assisted LAMP method has multiplex detection capability as well as excellent specificity in a single

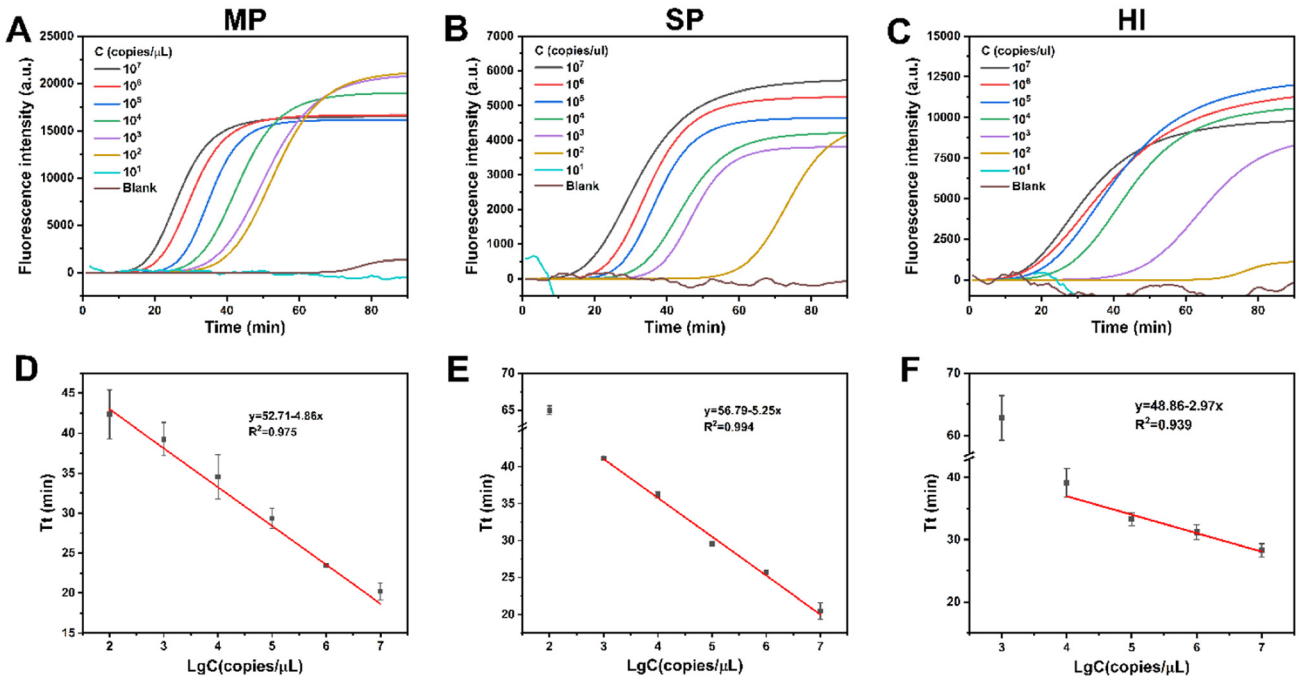

Fig. 4 Evaluation of the detection sensitivity of the FEN1-assisted LAMP method. Real-time fluorescence monitoring of amplification reactions of (A) MP, (B) SP and (C) HI with concentration ranging from 1×10^7 to 0 (blank) copies per μL at a constant temperature of 65 °C. Ultrapure water was used as the blank control. Standard linear fitting curve of T_t (threshold time) *vs.* lg C (concentration) generated by the FEN1-assisted LAMP assay of (D) MP, (E) SP and (F) HI. Data represents the mean (n = 3). Error bars are from triplicate measurements.

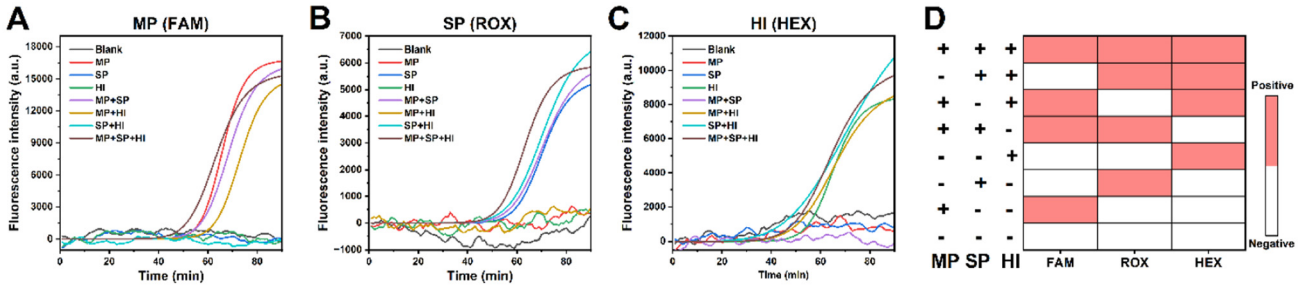

Fig. 5 Outcome of triplex FEN1-assisted LAMP consisting of universal primers and flap probes of MP, SP and HI. Real-time fluorescence monitoring of amplification reactions of (A) MP, (B) SP and (C) HI with a concentration of 1×10^4 copies per μL. Ultrapure water was used as the blank control. Flap probes with the flap structure were labelled with FAM, ROX, and HEX for identifying MP, SP and HI, respectively. (D) Heatmap of the triplex FEN1-assisted LAMP assay. Data represents the mean (n = 3).

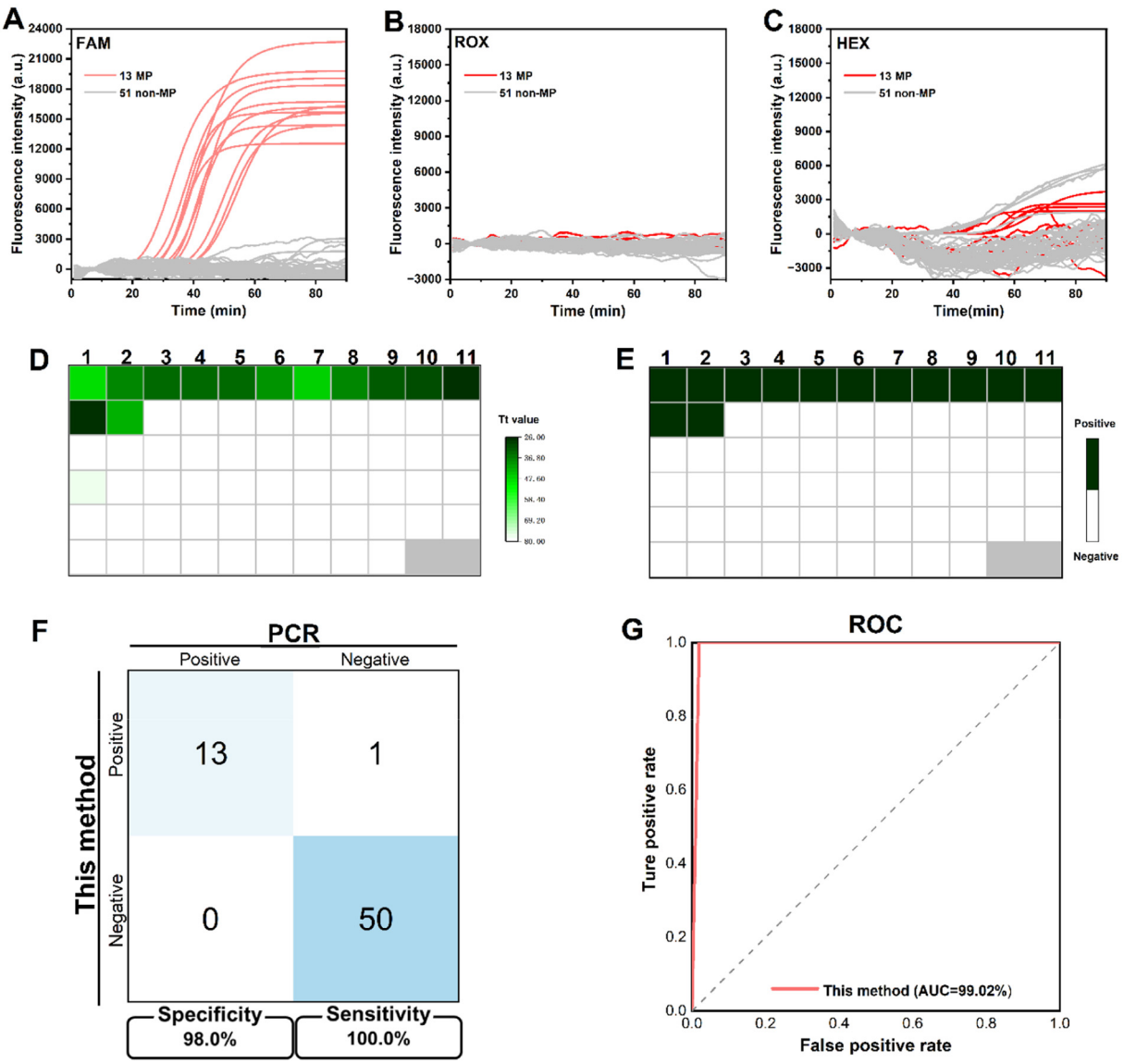

Fig. 6 Evaluation of the detection performance of the triplex FEN1-assisted LAMP method in clinical samples. Real-time fluorescence monitoring of the amplification reactions of MP (A), SP (B), and HI (C) in clinical samples. Red and grey lines represent the positive (13 cases) and non-positive MP (including 23 other pathogen cases and 28 negative samples), respectively. Heatmap of the triplex FEN1-assisted LAMP assay (D) and PCR (E) for detecting MP in clinical samples. Confusion matrix (F) and ROC-AUC plot (G) of the FEN1-assisted LAMP method. Positive results: CT value ≤40; negative results >40. CT represents cycle threshold of PCR.

Table 1 The comparison of the FEN1-assisted LAMP and other FEN1-mediated methods

Method	LOD (copies per μL)	Specificity	Turnaround time	Multiplex capacity	Operation procedure
FRAME[41]	4.76×10^5	No cross-interference with other targets	>150 min	Single-plex	Complex
FEN1-mediated CHA[42]	2.17×10^2	No cross-interference with other targets	>3 h	Single-plex	Complex
CFPA for allergen genes[36]	100	No cross-interference with other targets	60 min	Single-plex	Easy
CFPA for SARS-CoV-2[43]	100	No cross-interference with other targets	60 min	Single-plex	Easy
CFPA for ASF virus[35]	10	No cross-interference with other targets	30 min	Single-plex	Easy
This method	100	No cross-interference with other targets	90 min	Triplex	Easy

amplification reaction. It was also observed that the reaction rate of the same target in a multi-reaction system is slightly faster than that in a single-reaction system (Fig. 5A and B). We hypothesize that this phenomenon is attributed to the increased intermolecular interactions in the multi-reaction system, which subsequently alters the reaction kinetics.

Evaluation of the SP, MP and HI assay by clinical sample testing

To test the capability of the FEN1-assisted LAMP method for detecting MP in clinical samples, we applied the SP, MP and HI triplex assays. A total of 36 infected samples (13 MP and 23 other pathogens), which had previously been verified by the PCR assay (Sansure Biotech Inc) and 28 negative samples were collected (detailed information is shown in Table S4†). The triplex assay includes three detection channels: FAM for MP, ROX for SP, and HEX for HI. In the FAM channel, the fluorescent signals were detected in the sample infected by MP, while no amplification was identified in other infected and negative samples (Fig. 6A). In the ROX and HEX channels, no positive fluorescent signal was detected in any samples (Fig. 6B and C). These results demonstrated that the FEN1-assisted LAMP method is capable of detecting clinical samples and exhibits a potential for multiplex detection. The sensitivity of each target in multiplex detection would need to be further evaluated in future practical application development.

Then, we compared the SP, MP and HI triplex assay with the clinical diagnosis results obtained by PCR. A total of 14 positive samples (13 strong and 1 weak) were identified through T_t values by the FEN1-assisted LAMP method (Fig. 6D). Among these positive results, the 13 strong positive samples were consistent with the PCR (Fig. 6E). One very weak positive result tested by our method was identified as negative by the PCR; we hypothesize that the observed differences in performance could be attributed to the slight variations between our method and the PCR method. Additionally, it is possible that contamination might have occurred during the testing process utilizing our method or due to improper experimental procedures. However, our assay reached 98% sensitivity and 100% specificity compared with the PCR results (Fig. 6F), demonstrating the promising potential of our method in clinical applications. Moreover, the area under the receiver operating characteristic (ROC-AUC) curve from our results achieved 99.02% (Fig. 6G), illustrating its excellent detection performance. Nevertheless, the sensitivity of this method is currently not as good as that of the PCR methods[44,45] and needs to be further improved. However, this method is an isothermal reaction that does not rely on precise temperature control instruments or the expensive ddPCR technology. It holds significant advantages in on-site detection and screening applications, such as those in community settings. This FEN1-assisted LAMP was further compared with other FEN1-mediated isothermal amplification detection methods, focusing on their LOD, specificity, turnaround times, multiplex capabilities, and operational complexity (Table 1). The proposed method exhibits superior multiplexing capabilities and comparable sensitivity performance to existing methods. There is still potential for enhancing detection sensitivity and reducing the time to obtain the results. This method has the potential to be a candidate for molecular diagnostics, with room for further optimization to improve its utility in clinical and research settings.

Conclusions

In summary, we successfully established an SP, MP and HI triplex assay based on the FEN1-assisted LAMP method for identifying pathogens associated with CAP. The sequence-specific flap probes were designed to identify the three targets. Owing to the high efficiency of LAMP and the specific cleavage of FEN1, this method exhibits excellent sensitivity and specificity. Additionally, the FEN1-assisted LAMP method was developed for multiplex detection in a single reaction tube, which overcomes the limitations of conventional LAMP. The FEN1-assisted LAMP method can generate comparable results with PCR in clinical sample testing. These results validate that the FEN1-assisted LAMP method holds great promise for clinical applications.

Author contributions

Guopeng Teng: methodology, data curation, and writing the original draft. Gongde Lin: validation and data curation.

Pengfan Wei: methodology. Lizhi Li: sample collection, resources, modified the language, and investigation. Hongyuan Chen: methodology, reviewing and editing the manuscript. Qingquan Chen: project supervision as well as reviewing and editing the manuscript. Qiuyuan Lin: conceptualization, supervision, writing – review & editing, manuscript checking and proofreading.

Data availability

The Experiment section and data supporting this article have been included in the ESI.†

Conflicts of interest

There are no conflicts to declare.

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