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Research article

Rapid and sensitive detection of *Haemophilus influenzae* using multiple cross displacement amplification combined with CRISPR-Cas12a-based biosensing system

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ABSTRACT

Haemophilus influenzae (*H. influenzae*, Hi) is an opportunistic bacterium that colonizes the upper respiratory tract of humans and frequently causes meningitis, pneumonia, sepsis, and other severe infections in children. Early and accurate detection of *H. influenzae* is essential for effective diagnosis and treatment. In this study, we established a novel diagnostic method by integrating the CRISPR-Cas12a detection platform with multiple cross-displacement amplification (MCDA), termed the Hi-MCDA-CRISPR assay. This method offers an efficient and highly precise diagnostic tool for the identification of *H. influenzae*. In the Hi-MCDA-CRISPR system, the outer membrane protein (OMP) *P6* of *H. influenzae* was pre-amplified using the MCDA assay. The CRISPR-Cas12a-gRNA complex specifically recognized and bound to the amplified gene, forming a ternary complex that triggered the nonspecific trans-cleavage of the Cas12a effector, which subsequently degraded the fluorescent-quenched single-stranded DNA (ssDNA) probes, resulting in the emission of detectable fluorescent signals. After optimizing the reaction conditions, the Hi-MCDA-CRISPR assay proved capable of completing *H. influenzae* detection within 45 min, including a 40 min MCDA pre-amplification at 62 °C and a 5 min CRISPR-Cas12a cleavage at 37 °C. The assay was able to detect *H. influenzae* genomic DNA at concentrations as low as 50 fg and showed no cross-reactivity with non-*H. influenzae* pathogens. Furthermore, the Hi-MCDA-CRISPR assay successfully analyzed 65 clinical sputum samples. These findings suggest that the Hi-MCDA-CRISPR assay is a promising new detection tool for the rapid and reliable diagnosis of *H. influenzae* infection.

1. Introduction

Haemophilus influenzae (*H. influenzae*, Hi) is a small, facultative anaerobic, and polymorphic gram-negative coccobacillus, generally

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regarded as an opportunistic pathogen that colonizes the upper respiratory tract in humans [1,2]. The bacterium is classified into encapsulated and non-encapsulated strains, the latter referred to as non-typeable *H. influenzae* (NTHi). The encapsulated strains are further categorized into six serotypes (a-f) based on their agglutination reaction with specific sera [3,4]. Historically, prior to the widespread adoption of the *H. influenzae* type b (Hib) conjugate vaccine, Hib was the dominant cause of invasive diseases, including epiglottitis, meningitis, and sepsis, in children under five years of age [1,3]. In contrast, NTHi is closely associated with non-invasive mucosal infections such as conjunctivitis, otitis media, and sinusitis [3]. Despite a significant reduction in invasive Hib disease following vaccine introduction, there has been a consistent global rise in invasive NTHi infections, particularly among perinatal neonates, young children, and older individuals [1,3,5–8]. Furthermore, studies have shown that NTHi strains are linked to longer hospitalizations and higher mortality rates compared to those caused by encapsulated *H. influenzae* strains [5].

The traditional methods for detecting *H. influenzae*, including morphological analysis, culture, and serological identification, have limitations. Culture-based methods are time-consuming, complex, and highly susceptible to interference from factors such as antibiotic pretreatment and sample handling issues including acquisition, transportation, and storage conditions [9,10]. The standard slide agglutination serotyping has been shown to exhibit low sensitivity, poor specificity and subjective interpretation, often leading to inconsistent results [10,11]. Currently, molecular diagnostic techniques based on real-time PCR technologies have become indispensable tools for the identification of *H. influenzae* because of their high stability, sensitivity, specificity, and superior detection rates compared to traditional diagnostic methods. However, real-time PCR assays require sophisticated thermal cycling equipment, labor-intensive protocols, and highly trained personnel, limiting their applicability for on-site diagnosis, especially in resource-constrained environments [12–15]. Consequently, there is an immediate necessity for an accurate and economical molecular diagnostic technique for identifying *H. influenzae* to provide improved surveillance.

In recent years, the evolution and refinement of CRISPR/Cas (clustered regularly interspaced short palindromic repeats and their associated protein) gene-editing tools have greatly advanced the development of rapid molecular diagnostic technologies. The CRISPR/Cas system, an adaptive immune defense mechanism present in many archaea and bacteria, protects against invasive foreign elements (DNA and RNA sequences) to maintain genomic stability. Over time, the CRISPR/Cas9 system has gradually replaced earlier genome tools, emerging as a faster and more convenient gene-editing tool [16]. CRISPR/Cas gene-editing tools rely on RNA-guided Cas nucleases to precisely recognize and cleave specific DNA sequences [17]. Further exploration of the Cas protein family revealed the substantial potential of Cas12 and Cas13 nucleases in biosensing applications, primarily because of their collateral cleavage activity. This property allows them to rapidly release detectable signals upon binding to specific nucleic acid sequences, enabling efficient signal transduction and amplification, and enhancing biosensor sensitivity [18–20]. In the development of CRISPR/Cas biosensing systems, various strategies for optimizing the gRNA design can significantly reduce off-target effects and ensure high specificity for target sequence recognition [19]. Leveraging the inherent collateral cleavage activity and high specificity afforded by gRNA, various CRISPR-based biosensors have recently been developed to detect various pathogens, including *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, SARS-CoV-2, and Hepatitis B virus [13,21–23]. Importantly, these diagnostic tests offer analytical sensitivity and specificity that are equivalent to or above those of PCR-based technologies while eliminating the need for sophisticated equipment. This advancement presents a simplified and cost-effective option for on-site monitoring of infectious diseases [13,24].

The integration of CRISPR/Cas biosensing systems with amplification techniques can further enhance target gene recognition and expand the detection capabilities. Methods based on isothermal amplification, including multiple cross-displacement amplification (MCDA), loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA), facilitate the amplification of nucleic acids at constant temperatures using accessible heating devices [25–27]. Owing to their reduced need for advanced equipment, quicker turnaround times, and lower operating expenses, isothermal amplification-based detection technologies are more suitable than PCR for point-of-care (POC) testing [28]. Recently, many studies have combined CRISPR-based biosensing systems with isothermal amplification technology to establish novel molecular detection platforms, such as DETECTR [29], SHERLOCK [30], and MCCD [13], which enable rapid, ultra-sensitive, and specific detection of DNA molecules. However, few CRISPR-Cas-based diagnostic methods for *H. influenzae* are currently available, highlighting the need for further development in this area.

This study presents the establishment of an innovative Hi-MCDA-CRISPR assay that combines CRISPR-Cas12a detection with MCDA, an efficient isothermal nucleic acid amplification technique. The assay provides a highly accurate, rapid, ultrasensitive, and specific tool for detecting *H. influenzae* infection. Furthermore, we designed an amplification primer that incorporates a protospacer adjacent motif (PAM) site, enabling PAM-independent detection of target sequences. The principles underlying the Hi-MCDA-CRISPR assay have been thoroughly outlined and its clinical applicability has been robustly validated using clinical samples.

2. Materials and methods

2.1. Primer and gRNA design

The MCDA primers, which target the outer membrane protein (OMP) *P6* gene (L42023.1:402127-402588), were designed using PRIMER PREMIER 5.0 software (*http://*www.premierbiosoft.com/primerdesign). The specificity of the primers was validated through the NCBI Primer-BLAST analysis tool. The MCDA primer set included two displacement primers (F1 and F2), two cross-primers (CP1 and CP2), and six amplification primers (C1, D1, R1, C2, D2, and R2). For CRISPR-Cas12a-based detection, the primer CP1 was further modified by adding a PAM sequence (TTTC) to the junction region. In addition, a gRNA based on the OMP *P6* gene was designed in accordance with the CRISPR-Cas12a detection principle. All oligomers were synthesized by TianYi-Huiyuan Bioscience and Technology Co., Ltd. (Beijing, China). Principle of MCDA reaction and mechanism of CRISPR-Cas12a fluorescent detection are illustrated in Fig. 1, while the sequences and locations of the primers and gRNA, as well as the probe details, are shown in Fig. 2 and Table 1.

2.2. Preparation of clinical samples and nucleic acid templates

In this study, the clinical applicability of the Hi-MCDA-CRISPR assay was evaluated using DNA templates extracted from 65 sputum samples. In addition, nucleic acid templates were prepared from five *H. influenzae* strains and 27 non-*H. influenzae* strains to verify the analytical specificity of the assay. After cultivation, *H. influenzae* pure cultures were subjected to DNA extraction utilizing the EasyPure

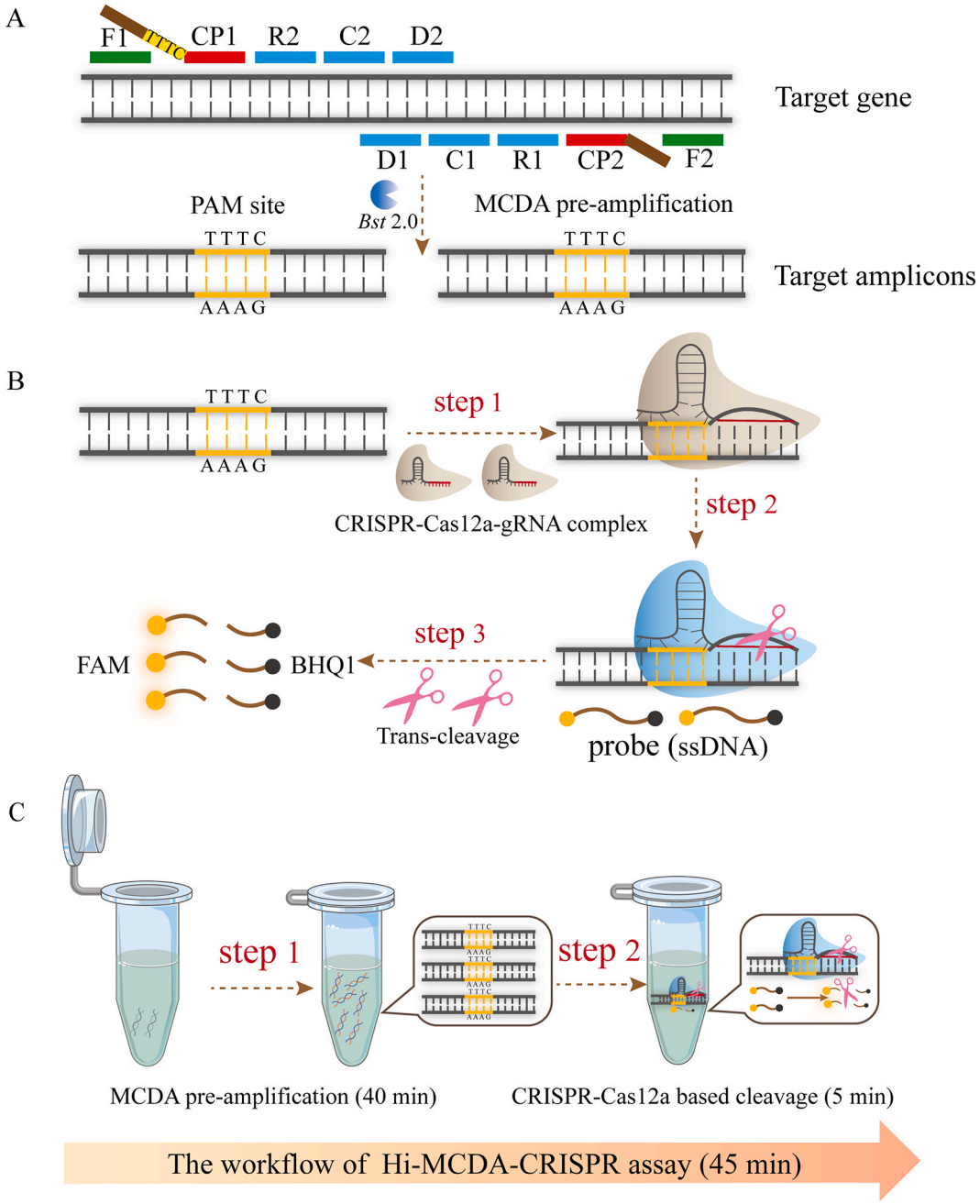

Fig. 1. Outlines of the principle of the Hi-MCDA-CRISPR assay. (A) Schematic illustration of the principle of the MCDA with a modified primer CP1. A PAM site (TTTC) was added to the linker region of the primer CP1. During the MCDA reaction, numerous target amplicons containing PAM sites are generated, enabling detection by the CRISPR-Cas12a effector. (B) Schematic representation of the CRISPR-Cas12a detection system. Upon recognizing the target amplicons, the tans-cleavage ability of the CRISPR-Cas12a effector is activated, resulting in the cleavage of ssDNA reporters labeled with FAM and BHQ1 and the subsequent release of fluorescent signals. (C) Workflow of the Hi-MCDA-CRISPR assay. Detection of *H. influenzae* involves two main steps: MCDA amplification (step 1) and CRISPR-Cas12a-based cleavage (step 2). The entire process is completed within 45 min.

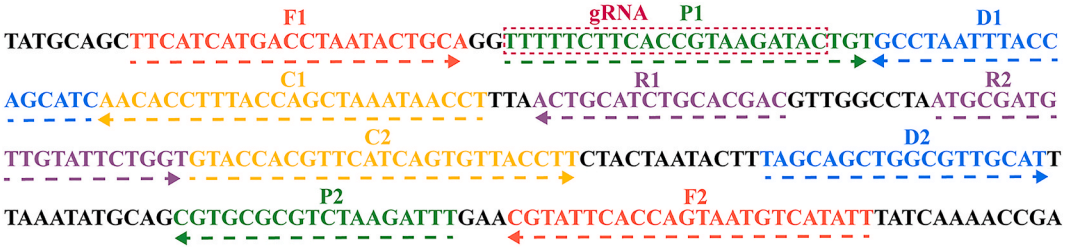

Fig. 2. Design of primers and gRNA for the Hi-MCDA-CRISPR assay in this study. Right arrows and left arrows represent the sense and complementary sequences, respectively. The locations of primers are underlined, and the gRNA position is highlighted with a dashed box.

Table 1
The primers, probe, and gRNA used in this study.

Primers/probe/gRNA	Sequences (5′-3′)	Length[b]
F1	TTCATCATGACCTAATACTGCA	22 nt
F2	AATATGACATTACTGGTGAATACG	24 nt
CP1[a]	AGGTTATTTAGCTGGTAAAGGTGTT-TTTC-TTTTTCTTCACCGTAAGATACTGT	54 nt
CP2	GTACCACGTTCATCAGTGTTACCTT-AAATCTTAGACGCGCACG	44 nt
C1	AGGTTATTTAGCTGGTAAAGGTGTT	25 nt
D1	GATGCTGGTAAATTAGGC	18 nt
R1	GTCGTGCAGATGCAGT	16 nt
C2	GTACCACGTTCATCAGTGTTACCTT	25 nt
D2	TAGCAGCTGGCGTTGCAT	18 nt
R2	ATGCGATGTTGTATTCTGGT	20 nt
gRNA	UAAUUUCUACUAAGUGUAGAUUUUUUCUUCACCGUAAGAUAC	42 mer
Probe	FAM-TATTATTATTATTATTT-BHQ1	17 mer

[a] Nucleotide sequence 'TTTC' in CP1 primer is the PAM site for Hi-MCDA-CRISPR detection.
[b] nt, nucleotide; mer, monomeric unit.

Bacteria Genomic DNA Kit (TransGen Biotic Co., Ltd, Beijing, China). DNA concentrations were quantified using a NanoDrop One spectrophotometer (Thermo, USA), and the extracted DNA samples were stored at −20 °C for subsequent analyses.

2.3. The standard MCDA assay

Target sequences were amplified by the MCDA method using an isothermal amplification kit (Huidexin Biotech Co. Ltd., Tianjin, China). The MCDA reaction system was of 25 μl, containing 12.5 μl 2 × reaction buffer, 1.0 μl of *Bst* 2.0 DNA polymerase, 0.4 μM each of F1 and F2, 0.8 μM each of C1, C2, D1, D2, R1 and R2, 1.6 μM each of CP1 and CP2, and either 1 μl of DNA template from pure culture or 5 μl from clinical sample. The reaction mixture was incubated at 63 °C for 40 min using a real-time turbidimeter (Eiken Chemical Co., Ltd., Tokyo, Japan). The result was interpreted according to the turbidity value, with a value > 0.1 considered as positive. Furthermore, performance of the MCDA process was optimized by conducting standard MCDA reactions at temperatures between 59 and 66 °C, with intervals of 1 °C.

2.4. CRISPR-Cas12a-based fluorescent detection assay

The CRISPR-Cas12a detection was performed according to a previous report [22], utilizing a 50 μl reaction system that included 25 μl of 2 × NEBuffer (New England Biolabs Inc., MA, USA), 1 μl of MCDA products, 9 μl of CRISPR-Cas12a-gRNA complex, 1.25 μl of single-stranded DNA (ssDNA) reporter molecule, and 13.75 μl of distilled water. Reactions were incubated for 10 min at 37 °C, and results were recorded using a real-time PCR instrument (Applied Biosystems, USA). Particularly, the CRISPR-Cas12a-gRNA complex was prepared by incubating 5 μl of Cas12a (10 μM, New England Biolabs Inc., MA, USA) with 5 μl of gRNA (10 μM) in 2 × NEBuffer at 37 °C for 8–10 min. The complexes must be utilized promptly or kept at 0–4 °C for no more than 12 h.

2.5. Sensitivity and specificity evaluation of the Hi-MCDA-CRISPR assay

To assess the sensitivity and determine the limit of detection (LOD) of the Hi-MCDA-CRISPR assay, the genomic DNA template of *H. influenzae* was serially diluted tenfold increments, ranging from 5 ng to 5 fg per microliter. Specifically, 1 μl of each templates was added to the Hi-MCDA-CRISPR assay reaction system with three replicates. The lowest concentration at which a positive result was reliably detected was defined as the LOD. The assay's specificity was evaluated using five *H. influenzae* strains and 27 non-*H. influenzae* strains (Table S1). All strains were tested in triplicate.

2.6. Clinical application of the Hi-MCDA-CRISPR assay

To further assess the performance of the Hi-MCDA-CRISPR assay in clinical practice, it was useed to identify *H. influenzae* in 65 sputum samples from individuals at the Capital Institute of Pediatrics, who were suspected of having *H. influenzae* infections. Concurrently, these specimens were analyzed using real-time PCR for comparative validation. The real-time PCR assay was performed as previously reported [31], with a procedure of holding at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The results obtained from both methods were compared for detection accuracy and efficiency.

3. Result

3.1. Schematic mechanism of the Hi-MCDA-CRISPR assay

The Hi-MCDA-CRISPR assay is comprised of two main stages: the initial pre-amplification of the target sequence through the MCDA reaction, followed by the subsequent fluorescent detection of the target amplicons via the CRISPR-Cas12a collateral cleavage activity. The MCDA reaction was initially conducted to pre-amplify the target sequence for 40 min based on previously described principles [28]. To enable CRISPR-Cas12a detection, the CP1 primer was modified with a PAM sequence (TTTC) specific to the Cas12a effector, which resulted in the production of amplicons containing PAM sequences (Fig. 1A). In the second stage, the binary CRISPR-Cas12a-gRNA complex identified and bound to the target sequence guided by the gRNA and PAM sequence, resulting in the formation of a ternary complex that initiated the trans-cleavage activity of the Cas12a effector (Fig. 1B, steps 1 and 2). This activated Cas12a effector non-specifically cleaved the ssDNA probe, resulting in the emission of fluorescent signal (Fig. 1B, step 3). The entire diagnostic workflow of the Hi-MCDA-CRISPR assay, including 40 min of MCDA amplification and 5 min of CRISPR-Cas12a detection, was completed within 45 min (Fig. 1C).

3.2. Verification of the Hi-MCDA-CRISPR assay

The feasibility and specificity of the Hi-MCDA primer set was verified through MCDA pre-amplification procedures conducted at 63 °C for 40 min, both in the presence and absence of *H. influenzae* genomic DNA. The amplification products were verified using real-time turbidity measurement and 2 % agarose gel electrophoresis. The results showed increased turbidity and the presence of ladder-like DNA bands on the agarose gel exclusively in MCDA reactions containing *H. influenzae* genomic DNA template (Fig. 3A and B). During the CRISPR-Cas12a detection stage, significant fluorescent signals were observed only in reactions containing pre-amplified products of *H. influenzae* genomic DNA (Fig. 3C). These results suggest the viability of using the Hi-MCDA-CRISPR system developed here for the identification of. *influenzae*.

3.3. Optimal conditions for Hi-MCDA-CRISPR assay

The optimal temperature for MCDA pre-amplification was determined by comparing the performance over eight temperatures, ranging from 59 to 66 °C in 1 °C increments. As shown in Fig. 4, 62 °C was identified as the optimal temperature, providing the highest amplification efficiency for the Hi-MCDA assay. To optimize the reaction time for CRISPR-Cas12a trans-cleavage, intervals of 5, 10, and 15 min were tested at 37 °C, with 5 min found to be sufficient for effective cleavage of the ssDNA reporter. The resulting optimal Hi-MCDA-CRISPR assay conditions were used in subsequent experiments.

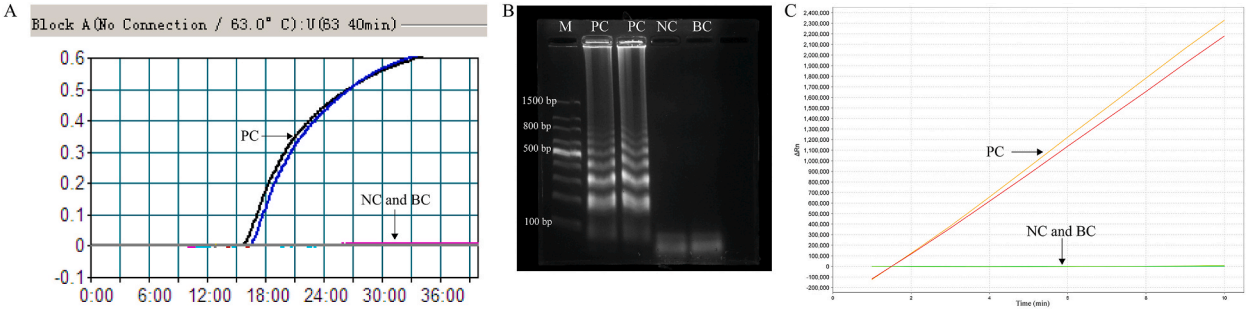

Fig. 3. Confirmation of the Hi-MCDA-CRISPR assay for *H. influenzae* detection. (A) The performance of the primer set for the Hi-MCDA reaction was determined using the real-time turbidity method. (B) The MCDA products were confirmed by agarose gel electrophoresis. (C) The CRISPR-Cas12a biosensing system was employed to detect the target amplicons, with fluorescence signals analyzed using a real-time fluorescence (RTF) instrument. Genomic DNA extracted from pure *H. influenzae* cultures was used as the positive control, DNA from *Staphylococcus aureus* as the negative control and DW as the blank control.

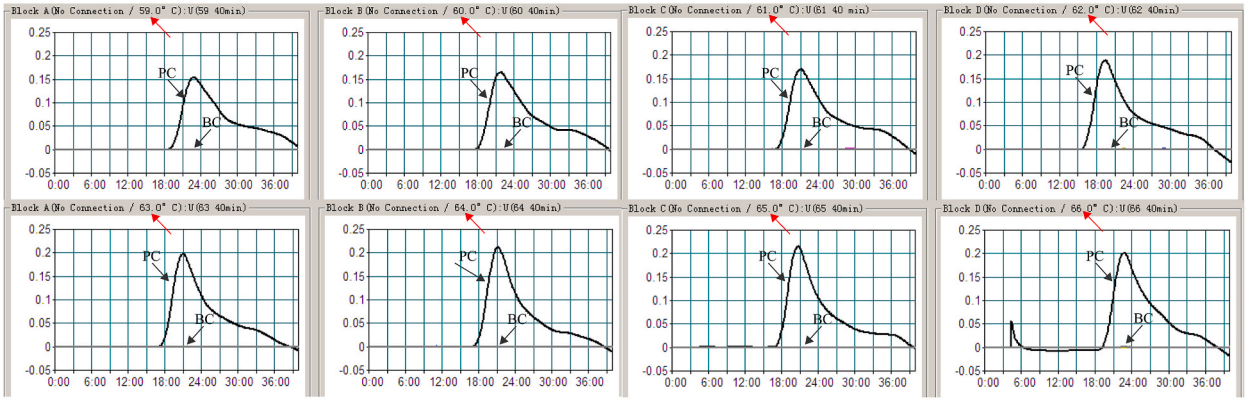

Fig. 4. Temperature optimization for the Hi-MCDA assay. The optimal temperature for *H. influenzae* pre-amplification was determined using the real-time turbidity method. Eight kinetic curves were generated under constant temperatures ranging from 59 to 66 °C. The results indicated 62 °C as the optimum temperature for the Hi-MCDA assay. BC, blank control.

3.4. Sensitivity and specificity evaluation of the Hi-MCDA-CRISPR assay

The analytical sensitivity of the Hi-MCDA-CRISPR method was assessed by testing 10-fold serial dilutions (5 ng–5 fg) of *H. influenzae* genomic DNA. Fluorescence signals were observed in reactions with concentrations between 5 ng and 50 fg, as shown in Fig. 5. No fluorescence was observed in the blank control or in the reaction with a DNA concentration of 5 fg. These results indicate that the LOD for the method was 50 fg of *H. influenzae* genomic DNA per reaction. To assess the specificity of the assay, nucleic acid templates from five *H. influenzae* strains and 27 non-*H. influenzae* pathogens were analyzed. Positive results were obtained exclusively from tubes containing *H. influenzae* genomic DNA, whereas all reactions with non-*H. influenzae* pathogens yielded negative results (Fig. 6), demonstrating the high specificity (100 %) of this assay for detecting *H. influenzae*.

3.5. Clinical application of the Hi-MCDA-CRISPR assay

In a clinical evaluation of 65 samples, 28 tested positive and 37 tested negative for *H. influenzae* in the Hi-MCDA-CRISPR assay. These results are in agreement with those obtained from the concurrent real-time PCR analysis (Fig. 7, Table 2, Table S2). Therefore, the Hi-MCDA-CRISPR assay developed in this work serves as a rapid, accurate, and specific diagnostic tool for identifying *H. influenzae,*

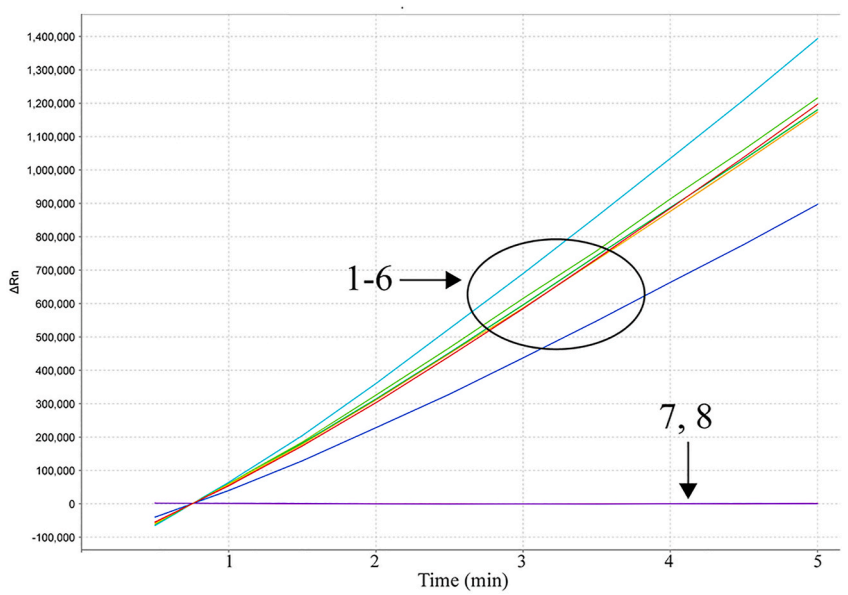

Fig. 5. Sensitivity evaluation of the Hi-MCDA-CRISPR detection system. The analytical sensitivity of the system was assessed using RTF analysis. Curves 1–7 correspond to DNA template concentrations of 5 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, and 5 fg, respectively, while curve 8 represents the blank control. The RTF results indicated that the detection limit of the Hi-MCDA-CRISPR assay was 50 fg per reaction.

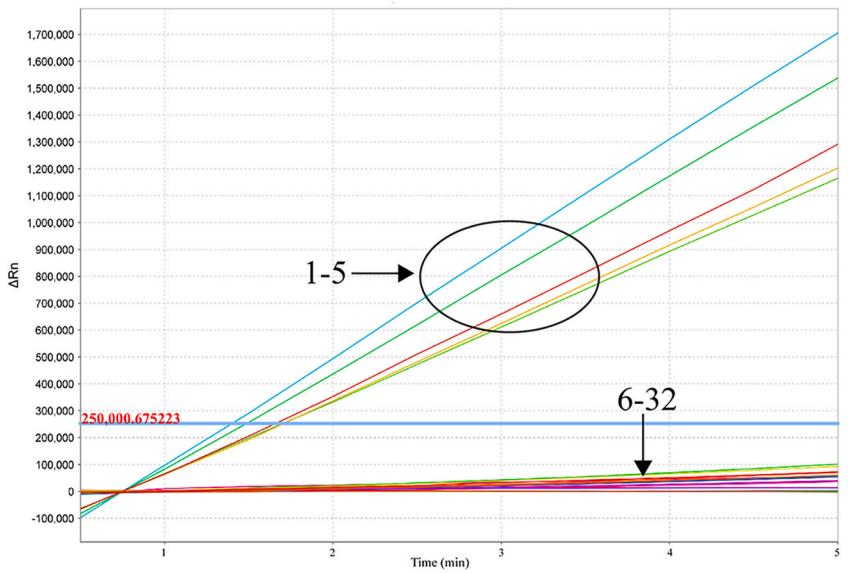

Fig. 6. Specificity assessment of the Hi-MCDA-CRISPR detection system. The specificity of the Hi-MCDA-CRISPR assay was evaluated using five *H. influenzae* strains (curves 1–5) and 27 non-*H. influenzae* strains (curves 6–32). Fluorescence signal intensities were measured using the real-time fluorescence (RTF) method. Signals 1–5, *H. influenzae*; signal 6, *Streptococcus pyogenes*; signal 7, *Pseudomonas aeruginosa*; signal 8, *Corynebacterium striatum*; signal 9, *Staphylococcus Haemolyticus*; signal 10, *Mycoplasma pneumoniae*; signal 11, *Shigella boydii*; signal 12, *Shigella sonnei*; signal 13, *Enteropathogenic E. coli*; signal 14, *Enteroaggregative E. coli*; signal 15, *Enterotoxigenic E. coli*; signal 16, *Neisseria lactate*; signal 17, *Staphylococcus succinis*; signal 18, *Enterococcus faecium*; signal 19, *Mycobacterium tuberculosis*; signal 20, *Staphylococcus epidermidis*; signal 21, *Neisseria meningitidis*; signal 22, *Streptococcus salivarius*; signal 23, *Enterinvasive E. coli*; signal 24, *Listeria monocytogenes*; signal 25, *Moraxella catarrhalis*; signal 26, *Staphylococcus aureus*; signal 27, *Klebsiella pneumoniae*; signal 28, *Entercoccus faecalis*; signal 29, Influenza virus A; signal 30, Influenza virus B; signal 31, Parainfluenza virus; signal 32, Respiratory syncytial virus. A reaction was considered positive when the fluorescence intensity exceeded 250,000.

demonstrating considerable promise for use in clinical settings.

4. Discussion

H. influenzae is a common pathogen that is responsible for community-acquired pneumonia (CAP) in children [32]. It predominantly colonizes the human nasopharynx, with unvaccinated children being particularly susceptible to Hib colonization by the age of five [33]. The incidence of Hib-associated infections, including meningitis, pneumonia, sepsis, and other severe conditions, has markedly decreased due to widespread vaccination with the Hib conjugate vaccine [34]. However, increasing cases of both invasive and non-invasive infections caused by NTHI are raising global health concerns [33,35]. Hence, the development of advanced detection technologies for *H. influenzae* is essential for improving diagnostic accuracy and epidemiological surveillance.

The discovery of the CRISPR technology, particularly its associated proteins (such as Cas9, Cas12, and Cas13), has provided more advanced options for pathogen diagnostics owing to its accuracy, portability, and sensitivity, paving the way for revolutionary POC testing [23,36]. The Cas12a protein (Cpf1, type V) is an RNA-guided DNA-targeting enzyme utilized in various pathogen diagnostics that induces collateral cleavage of ssDNA reporter molecules tagged with a fluorophore/quencher upon binding to target sequences adjacent to PAM sites [29]. This collateral cleavage separates the fluorophore from the quencher, producing detectable fluorescent signals [37], which can be monitored and analyzed using real-time fluorescence detectors. By adjusting the gRNA sequence, these systems offer flexibility to specifically detect various nucleic acids, making them invaluable for pathogen detection [37]. The CRISPR-Cas12a biosensing system has proven effective in detecting pathogens such as *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and SARS-CoV-2, with detection limits as low as a few copies [13,22,23,38]. Here, we introduce the first integration of a CRISPR-Cas12a detection platform with MCDA amplification technique, termed the Hi-MCDA-CRISPR assay, to enhance the efficiency of *H. influenzae* detection.

We aimed to develop an optimal onsite diagnostic method for the rapid detection of *H. influenzae* infection with excellent specificity and sensitivity. Preamplification of the target gene (OMP *P6*) is crucial for improving the sensitivity of *H. influenzae* detection. To achieve this, we employed the MCDA assay to pre-amplify the OMP *P6* gene of *H. influenzae* because of its rapidity and sensitivity. MCDA is a novel isothermal amplification method that solely relies on strand displacement enzyme (*Bst* DNA polymerase) to efficiently amplify the target gene. In particular, a set of 10 target-specific primers ensured high specificity by binding to 10 distinct sites on the target gene [28]. In addition, the CP1 primer includes a unique PAM sequence (TTTC), which is essential for guiding the CRISPR-Cas12a-gRNA complex to the target amplicons, enabling the detection platform to identify pathogens even in the absence of a native PAM site [23]. The MCDA amplification process can be accomplished in 60 min by employing basic isothermal heating devices,

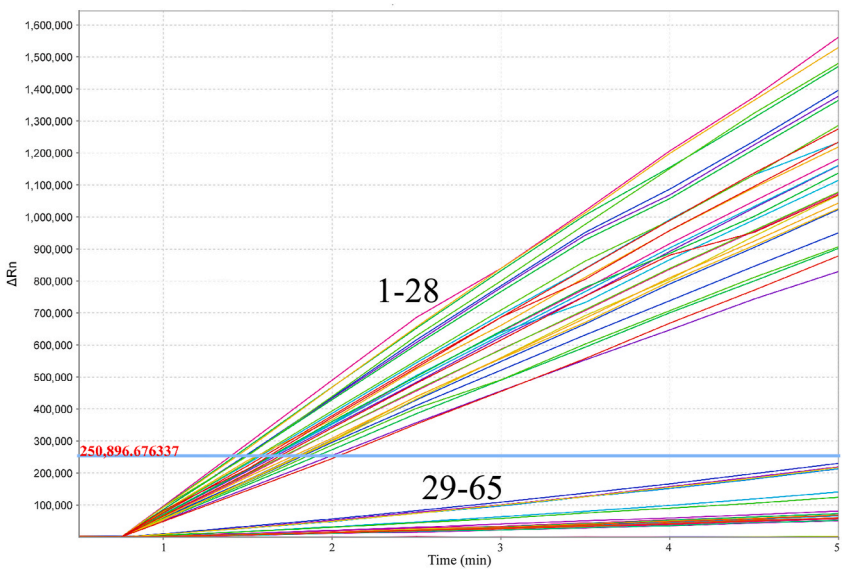

Fig. 7. Clinical application of the Hi-MCDA-CRISPR assay. A total of 65 sputum samples were analyzed using the Hi-MCDA-CRISPR assay. Fluorescence signal intensities were measured using the RTF method. The results identified 28 positive and 37 negative samples. Signals 1-28 correspond to the 28 samples positive by real-time qPCR; while signals 29-65 correspond to the 37 negative samples. A reaction was considered positive when the fluorescence intensity exceeded 250,000.

Table 2
Comparison of Hi-MCDA-CRISPR and PCR method for *H. influenzae* detection in sputum samples.

Detection methods		Hi-MCDA-CRISPR		Comparison of two methods		
		Positive	Negative	Sensitivity (%)	Specificity (%)	kappa
Real-time PCR	Positive	28	0	100	100	1
	Negative	0	37			

such as a thermostatically regulated heating block [28]. In this study, it is recommended to perform the pre-amplification at 62 °C for 40 min. Compared to PCR-based amplification methods, the MCDA assay eliminates the need for time-consuming amplification protocols, expensive thermocyclers, and laborious product analysis procedures, thus providing a simple nucleic acid amplification platform for POC applications [25]. The Hi-MCDA-CRISPR assay, in conjunction with the CRISPR-Cas12a detection platform, achieved a LOD of 50 fg of *H. influenzae* genomic DNA, matching the sensitivity of real-time PCR and exceeding that of the MCDA-LFB platform [39,40]. Alternative detection methods, including LAMP-LFB and ERT-LAMP (endonuclease restriction real-time LAMP), exhibit comparable turnaround times and analytical specificity. However, their detection sensitivity remains inferior to that of the Hi-MCDA-CRISPR method [41,42]. Thus, the Hi-MCDA-CRISPR assay may be conceptualized as a suitable approach for the on-site identification of *H. influenzae* compared to other molecular diagnostics.

The Hi-MCDA-CRISPR assay demonstrated its specificity through testing 27 non-*H. influenzae* pathogens, none of which produced a positive fluorescence signal. This result confirms 100 % specificity and highlights the assay's effectiveness in accurately detecting *H. influenzae*. The assay was further evaluated in a clinical setting using 65 sputum samples suspected of being infected with *H. influenzae*. Our assay correctly identified 28 positive and 37 negative samples, showing complete agreement with PCR-based results. These findings indicate that the Hi-MCDA-CRISPR technique is a valuable method for the accurate identification of *H. influenzae* diseases in clinical settings. Overall, the Hi-MCDA-CRISPR assay exhibited notable sensitivity by pre-amplifying the OMP *P6* gene using the MCDA technique, while also ensuring exceptional specificity by utilizing custom-designed MCDA primers and gRNA. Moreover, the non-specific cleavage of the fluorescence-labeled ssDNA probes contributes to the easy interpretation of outcomes. However, unlike PCR methods, the Hi-MCDA-CRISPR assay has some limitations, including the inability to provide quantitative results. Additionally, the extensive production of amplified products during the MCDA process can lead to aerosol contamination, potentially increasing background fluorescence and false-positive results [38]. To address this, strict protocols, such as separating the sample preparation area from the amplification area, frequent glove changes, and cleaning the workspace with 70 % ethanol and sodium hypochlorite solution are recommended [25]. Further optimization could include conducting the test in a single step without opening the reaction tube lid to minimize aerosol contamination.

In conclusion, we developed a novel *H. influenzae* Hi-testing platform called Hi-MCDA-CRISPR. This two-step detection process, including MCDA pre-amplification and CRISPR-Cas12a detection, was completed within 45 min using simple instruments. The Hi-MCDA-CRISPR assay successfully identified 50 fg of *H. influenzae* genomic DNA and showed no cross-reactivity with non-

H. influenzae pathogens. This newly established Hi-MCDA-CRISPR method offers a simple, rapid, and accurate alternative for the clinical diagnosis of *H. influenzae* infections.

CRediT authorship contribution statement

Xinbei Jia: Writing – original draft, Software, Investigation, Formal analysis. **Wenjian Xu:** Writing – original draft, Validation, Methodology, Investigation. **Fei Xiao:** Writing – original draft, Methodology, Formal analysis. **Nan Jia:** Writing – original draft, Visualization, Supervision. **Xiaolan Huang:** Writing – original draft, Software, Methodology. **Yiqin Zhang:** Writing – original draft, Formal analysis. **Juan Zhou:** Writing – review & editing, Supervision, Resources, Data curation, Conceptualization. **Yi Wang:** Writing – review & editing, Visualization, Project administration, Methodology, Data curation, Conceptualization. **Jun Tai:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Ethics statement

This study was reviewed and approved by the Human Ethics Committee of the Institute of Pediatrics with the approval number: SHERLL2024033. All participants and their legal guardians provided written informed consent to participate in the study and for their data to be published.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e41535.

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