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Development of the CRISPR-Cas12a-Based Biosensing System for Rapid, Ultrasensitive, and Highly Specific Detection of *Streptococcus pyogenes*

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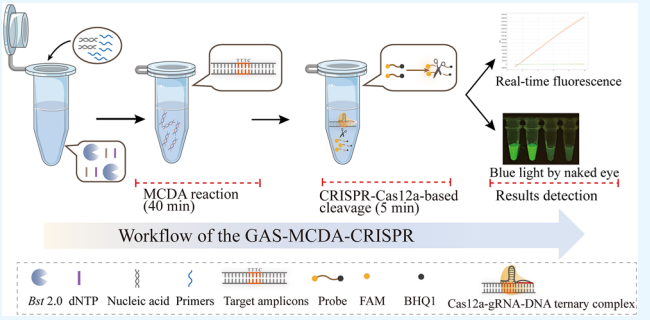
ABSTRACT: *Streptococcus pyogenes* (group A streptococcus, GAS) is the leading bacterial cause of acute pharyngitis in children and adolescents. Rapid and reliable diagnosis of GAS pharyngitis is essential for guiding a timely antibiotic treatment. Here, we developed a rapid, highly sensitive, and specific test platform for the detection of GAS, designated GAS-MCDA-CRISPR. In this diagnostic platform, the multiple cross displacement amplification (MCDA) technique was utilized to preamplify the specific *speB* gene of GAS. Subsequently, the CRISPR-Cas12a-based biosensing system was employed to decode the MCDA products. MCDA primers, a guide RNA (gRNA), and a quenched fluorescent single-stranded DNA (ssDNA) reporter were designed to target the *speB* gene of GAS. The GAS-MCDA-CRISPR assay demonstrated the ability to detect GAS genomic DNA at a concentration as low as 45 fg per microliter while exhibiting no cross-reactivity with other non-GAS pathogens. Moreover, 56 clinical samples were correctly detected by the GAS-MCDA-CRISPR assay. These data highlighted that the GAS-MCDA-CRISPR assay is a reliable diagnostic tool for the reliable and quick diagnosis of GAS infection.



INTRODUCTION

Streptococcus pyogenes (*S. pyogenes*), also known as group A streptococcus (GAS), is a Gram-positive, host-adapted bacterial pathogen.[1] GAS is the most common bacterial cause of acute pharyngitis in children worldwide, causing 15−30% of cases in children aged 5−15 years.[2−4] A meta-analysis showed that the pooled prevalence of GAS infection among children seeking medical care for sore throat was 37% with a prevalence of 24% among children younger than five years.[5] Moreover, pharyngitis caused by GAS can lead to various complications, including otitis media, tonsillar abscess, sinusitis, impetigo, sepsis, scarlet fever, and severe invasive infections such as septicemia, necrotizing fasciitis, and streptococcal toxic shock-like syndrome (STSS).[3,6−11] In addition, recurrent GAS infections can cause autoimmune diseases such as acute rheumatic fever, rheumatic heart disease (RHD), and acute poststreptococcal glomerulonephritis.[12,13] In the absence of a commercial GAS vaccine, early and effective antibiotic therapy could shorten the duration of symptoms by 1−2 days, reduce the incidence of complications, improve clinical symptoms, and prevent the spread of the disease.[14,15] It is therefore imperative to develop a diagnostic tool that can rapidly and accurately diagnose acute GAS infections, enabling patients to receive prompt antibiotic treatment and preventing the misuse of antimicrobial agents in patients with nonstreptococcal pharyngitis.[3]

Oropharyngeal swab culture and rapid antigen detection tests (RADTs) are the primary laboratory diagnostic tools for confirming suspected GAS pharyngitis in children older than 3 years.[3] However, the oropharyngeal swab culture method is a time-consuming technique that typically requires 18−48 h for culture and identification.[4] In addition, culture results are highly susceptible to conditions of specimen collection, storage, transport, and prior antibiotic use. In real-world studies, the data showed that diagnostic culture had unsatisfactory sensitivities ranging from 72 to 87%.[16] Although RADTs can diagnose GAS infection in a short time, they have low sensitivity with false negative results.[16,17] Therefore, the Infectious Diseases Society of America recommended that negative RADTs be confirmed by throat culture in children and adolescents.[3] PCR-based diagnostic methods provide more accurate results for patients with suspected GAS pharyngitis and eliminate the need for subsequent validation of negative results.[16] However, this technique requires more expensive thermocycling equipment to amplify sequences and

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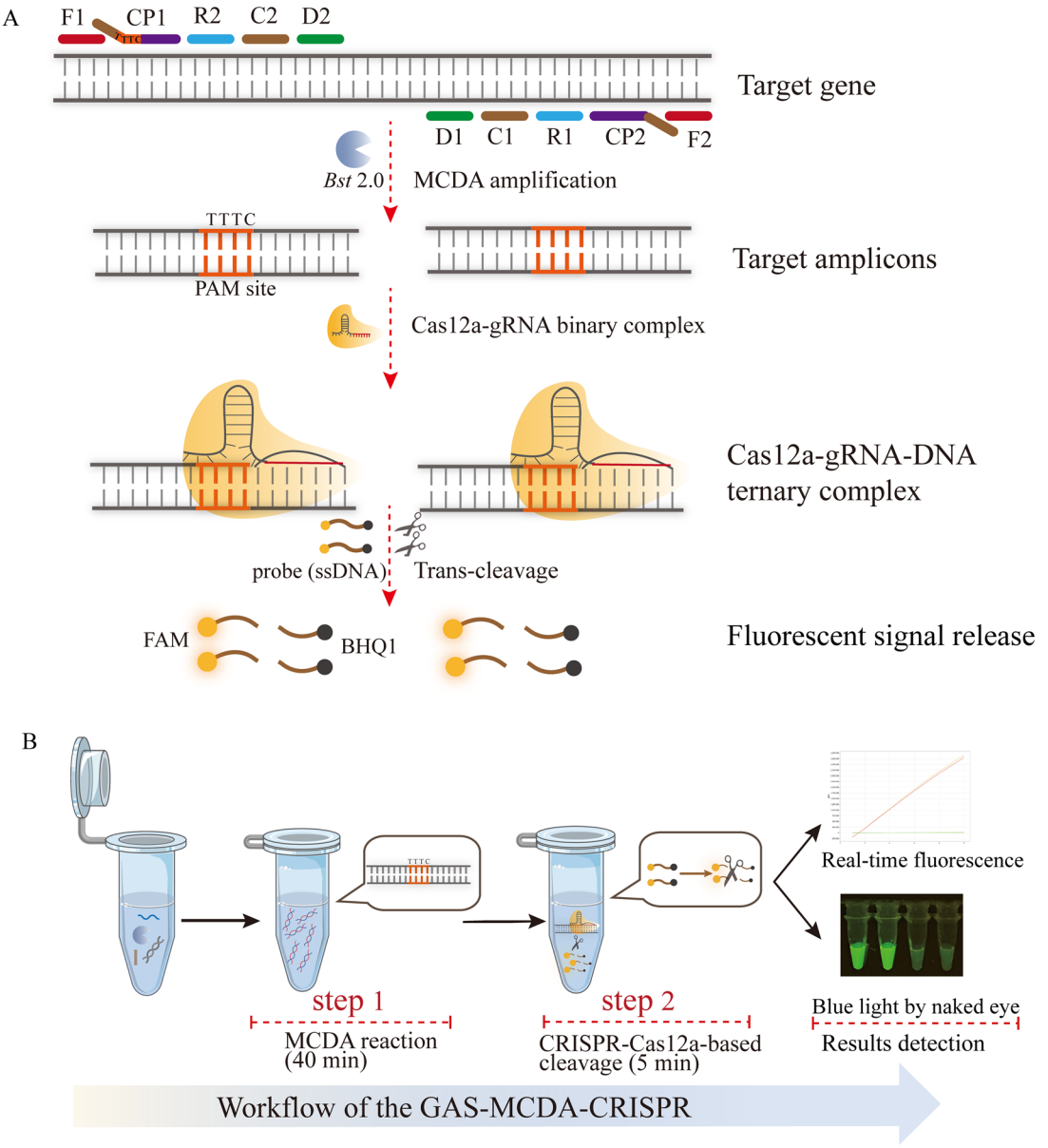

Figure 1. Schematic illustration of the GAS-MCDA-CRISPR assay. (A) Schematic diagram of the CRISPR-Cas12a-based trans-cleavage detection. The MCDA reaction generated a large number of target amplicons containing a PAM site (−TTTC−). The Cas12a-gRNA binary complex then correctly recognized these amplicons to form a ternary complex, which was then activated and nonspecifically cleaved the FAM/BHQ1 ssDNA reporter, resulting in the release of multiple fluorescent signals. (B) Workflow of the GAS-MCDA-CRISPR assay. The entire assay includes MCDA amplification (step 1), CRISPR-Cas12a trans-cleavage (step 2), and result detection. The results of the GAS-MCDA-CRISPR assay could be recorded using a real-time fluorescence detector or visually interpreted by the naked eyes under blue light.

well-trained technicians to analyze results, limiting its popularity and application in the field of on-site diagnosis.[18,19]

More recently, the development of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) (CRISPR-Cas) systems has demonstrated significant advantages and potential as a practical nucleic acid detection tool in the field of point-of-care testing (POCT).[20,21] The principle of molecular diagnostics using the CRISPR-Cas system is based on the capacity to accurately identify the specific nucleic acid sequences, as well as collateral cleavage activities of Cas effectors (such as Cas12a, Cas12b, Cas13a, and Cas14a).[22] To illustrate, the Cas12a protein (Cpf1), after navigation by guide RNA (gRNA) to recognize the target sequence and the adjacent suitable protospacer adjacent motif (PAM) site, can cleave the surrounding nontarget fluorophore-labeled single-stranded DNA (ssDNA) in a nonspecific and indiscriminate manner, resulting in the formation of the detectable fluorescent signals.[23,24] In particular, the CRISPR-Cas biosensing system is commonly employed in conjunction with isothermal amplification techniques, such as multiple cross displacement amplification (MCDA) and recombinase polymerase amplification (RPA), to improve the sensitivity and accuracy of detection.[25,26] Currently, a variety of CRISPR-Cas-based platforms, such as DETECTR (DNA endonuclease-targeted CRISPR trans reporter),[27] SHERLOCK (specific high-sensitivity enzymatic reporter unlocking),[28] and HOLMES (a one-hour low-cost multipurpose highly efficient system),[29] have been developed and applied in molecular diagnostics, with the advantages of rapidity, efficiency, high sensitivity, and specificity. Never-

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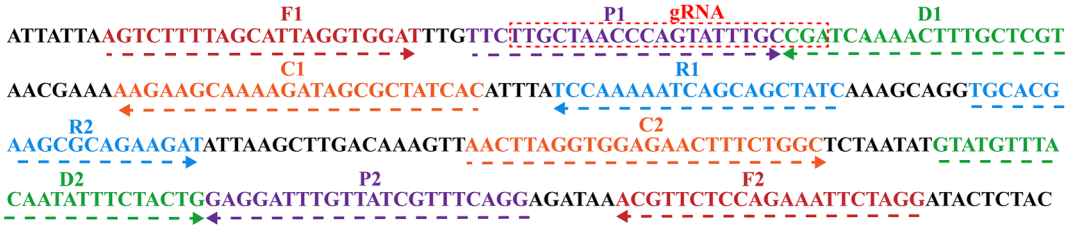

Figure 2. Positions and sequences of the *speB* gene of GAS used to design the MCDA primers and gRNA. Right and left arrows represent the sense and complementary sequences used in this study, respectively. Primer positions are highlighted with colored text, and gRNA is marked with a dashed frame.

Table 1. Primers, Probe, and gRNA Used in This Study

primers/probe/gRNA	sequences (5′−3′)	length[c]
F1	AGTCTTTTAGCATTAGGTGGAT	22 nt
F2	CCTAGAATTTCTGGAGAACGT	21 nt
CP1[a]	GTGATAGCGCTATCTTTTGCTTCTT-TTCTTGCTAACCCAGTATTTGC	47 nt
CP2	AACTTAGGTGGAGAACTTTCTGGC−CCTGAAACGATAACAAATCCTC	46 nt
C1	GTGATAGCGCTATCTTTTGCTTCTT	25 nt
D1	ACGAGCAAAGTTTTGATCG	19 nt
R1	GATAGCTGCTGATTTTTGGA	20 nt
C2	AACTTAGGTGGAGAACTTTCTGGC	24 nt
D2	GTATGTTTACAATATTTCTACTG	23 nt
R2	TGCACGAAGCGCAGAAGAT	19 nt
gRNA	UAAUUUCUACUAAGUGUAGAUUUGCUAACCCAGUAUUUGCCGA	43 mer
probe[b]	FAM-TATTATTATTATTATTT-BHQ1	17 mer

[a]The bolded nucleotide sequence "TTTC" in the CP1 primer is the PAM site for GAS-MCDA-CRISPR detection. [b]The 5′ end labeled with FAM and the 3′ end labeled with BHQ1. [c]nt, nucleotide; mer, monomeric unit.

theless, few CRISPR-Cas-based diagnostic methods have been developed for GAS infections, underscoring the imperative for further research.

In this study, we developed a novel diagnostic tool targeting the *speB* gene of GAS, designated GAS-MCDA-CRISPR, for the simple, rapid, and sensitive diagnosis of GAS infections. The detection platform, which was designed by employing MCDA for rapid amplification of the *speB* gene at 63 °C for 40 min and the CRISPR-Cas12a technique for detecting the target amplicons and results reporting at 37 °C for 5 min, was expected to accurately detect GAS in POCT settings. The performance of the GAS-CRISPR-Cas12a method was evaluated by using DNA templates of GAS strains and other non-GAS strains. Additionally, its applicability in clinical settings was validated with oropharyngeal swabs.

RESULTS

Schematic of the GAS-MCDA-CRISPR Assay. The schematic illustration of the GAS-MCDA-CRISPR assay is shown in Figure 1. The GAS-MCDA-CRISPR assay, which combines the MCDA with the CRISPR-Cas12a biosensing system, is employed for the detection of GAS. First, the *speB* gene sequence was preamplified by the MCDA assay for 40 min. This step resulted in the generation of a substantial number of amplicons containing the PAM site, which can be identified with the prepared Cas12a-gRNA binary complex under the guidance of the complementary gRNA. Once the PAM site and the target sequence were recognized, the Cas12a effector was activated to cleave the ssDNA reporter non-specifically, resulting in the production of a large number of fluorescence signals. The intensity of the fluorescence signals can be measured by the real-time fluorescence platform or observed visually under blue light.

Confirmation of the GAS-MCDA-CRISPR Assay for GAS Detection. A set of 10 primers targeting the *speB* gene was designed for the detection of GAS based on the principle of the MCDA assay (Figure 2 and Table 1). To test the validity and specificity of the primers for the GAS-MCDA-CRISPR assay, the MCDA preamplification stage was conducted at a constant temperature of 63 °C. The genomic DNA of GAS was used as the positive control, while *Streptococcus pneumoniae* served as the negative control, and DW was utilized as the blank control. The initial analysis of the MCDA products was conducted by using a real-time turbidity system and 2.5% agarose gel electrophoresis. As illustrated in Figure 3A,B, the tubes containing the GAS products demonstrated a notable increase in turbidity and exhibited bright ladder-like bands on the gel, whereas the negative control and blank control did not show any turbidity or band. Next, the preamplified MCDA products were subjected to detection using the CRISPR-Cas12a-based biosensing system. The reaction mixtures were incubated at 37 °C for 10 min using a real-time fluorescence PCR instrument, and the fluorescence intensities were measured in real time. As shown in Figure 3C,D, the positive control reaction mixtures showed remarkable fluorescence signals on the real-time fluorescence detector system and were readily discernible with the naked eyes under blue light. Thus, the MCDA primers and the novel GAS-MCDA-CRISPR assay were capable of amplifying the *speB* gene and detecting it based on the CRISPR-Cas12a biosensing system, either using the real-time fluorescence system or visual inspection under blue light.

Optimization Conditions for the GAS-MCDA-CRISPR Assay. The isothermal amplification efficiency of the MCDA assay was evaluated at eight temperatures, spanning a range from 60 to 67 °C for a duration of 40 min, using the DNA

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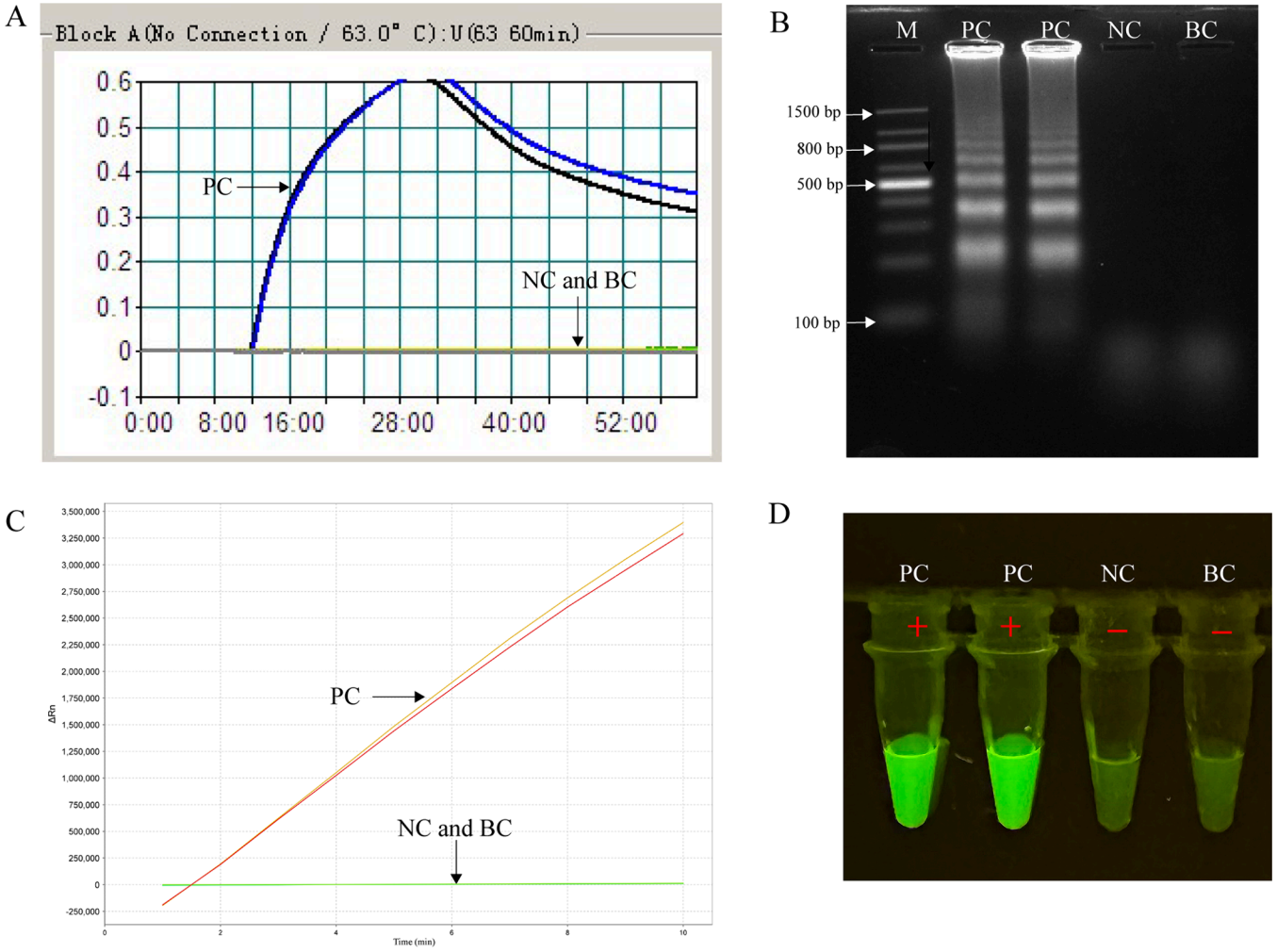

Figure 3. Confirmation of the GAS-MCDA-CRISPR assay for GAS detection. MCDA products amplified with the designed primer set were analyzed by the real-time turbidity method (A) and interpreted by 2.5% agarose gel electrophoresis (B). The CRISPR-Cas12a biosensing system was used for the detection of MCDA products. The generated fluorescence signals were recorded by a real-time fluorescence instrument (C) and visualized under blue light (D). PC, positive control; NC, negative control; BC, blank control.

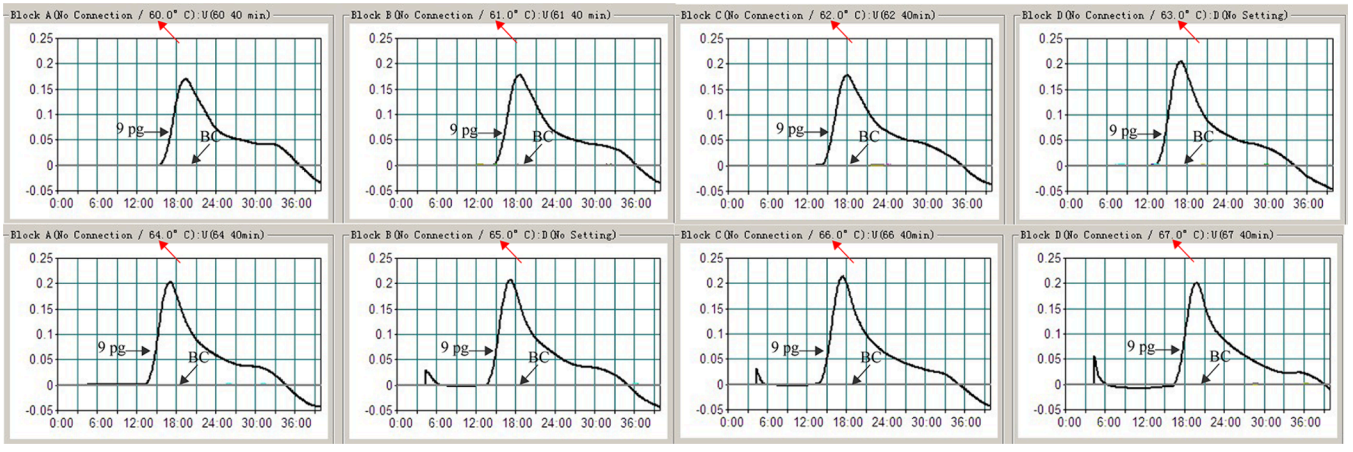

Figure 4. Temperature optimization for the GAS-MCDA assay. The optimal reaction temperature of the GAS-MCDA assay was analyzed by a real-time turbidimeter, and turbidity greater than 0.1 was considered positive. Eight kinetic curves were recorded at different temperatures ranging from 60 to 67 °C (1 °C intervals) with DNA templates of GAS at the 9 pg level, which showed 63 °C as the optimal temperature.

template of GAS (9 pg per microliter). The results indicated that 63 °C was the optimal temperature for the MCDA preamplification stage in this study, as the fastest turbidity threshold value (0.1) was reached at this condition (Figure 4). Then, the optimal reaction time for CRISPR-Cas12a detection was determined. According to the results identified by the real-

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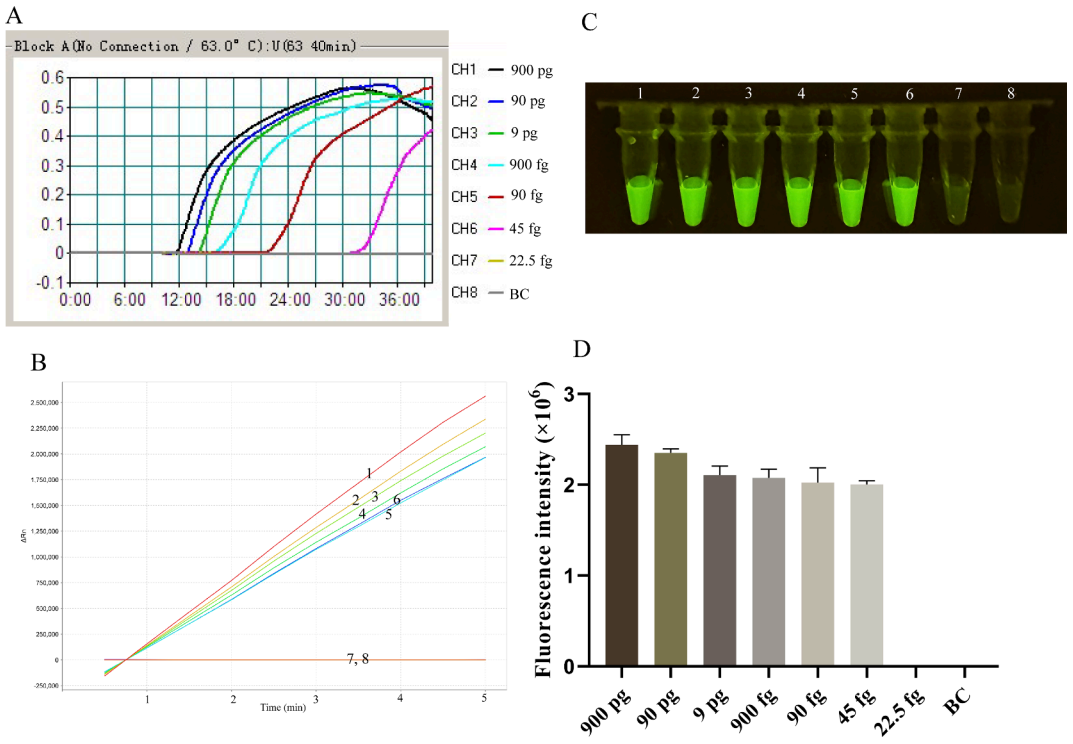

Figure 5. Sensitivity assessment of the GAS-MCDA-CRISPR assay. Analytic sensitivity was evaluated using serially diluted pure GAS genomic DNA templates. The results of the MCDA preamplification step were measured by a real-time turbidimeter (A). The fluorescence signals from CRISPR-Cas12a detection step were recorded by the real-time fluorescence instrument (B, D) and visually interpreted by the naked eyes under blue light (C). Signals/tubes 1−7 refer to the concentration level of the GAS DNA template as 900 pg, 90 pg, 9 pg, 900 fg, 90 fg, 45 fg, and 22.5 fg, and signal/tube 8 refers to the blank control. Bars and error bars represent means ± SD, where n = 3 replicates.

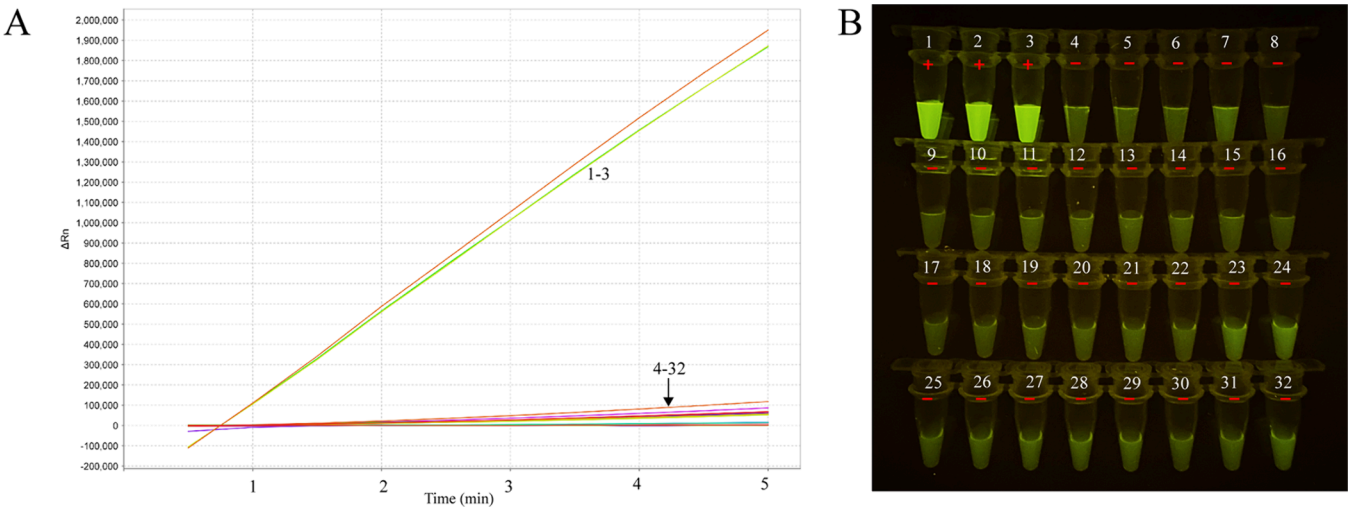

Figure 6. Specificity evaluation of the GAS-MCDA-CRISPR assay. Analytic specificity was tested using nucleic acid templates from three GAS strains and 28 non-GAS pathogens. All detection results were analyzed by means of measuring the fluorescence intensity (A) and observed by the naked eyes under blue light (B). Signals/tubes 1−3, three GAS strains; signals/tubes 4−31, 28 non-GAS strains; signal/tube 32, blank control.

time fluorescence system and naked eyes under blue light, a reaction time of 5 min was sufficient for the CRISPR-Cas12a-based detection (Figure S1). Hence, the optimal reaction conditions identified in the above tests were recommended for the GAS-MCDA-CRISPR assay.

Specificity and Sensitivity of the GAS-MCDA-CRISPR Assay. The sensitivity of the GAS-MCDA-CRISPR assay was determined by monitoring the reaction products from serially diluted pure GAS genomic DNA templates (900 pg, 90 pg, 9 pg, 900 fg, 90 fg, 45 fg, and 22.5 fg per microliter). As shown in Figure 5, reaction tubes containing DNA template concentrations ranging from 900 pg to 45 fg exhibited an increase in turbidity and intense fluorescence signals. In contrast, no turbidity or fluorescence signal was detected in the blank control or in the tube with a concentration of 22.5 fg. These results were in accordance with those observed visually under blue light. In addition, the sensitivity was further assessed in simulation samples containing both GAS and non-GAS cells. These samples were prepared with the concentration of the GAS genomic DNA templates ranging from 1.5×10^6 to $1.5 \times$

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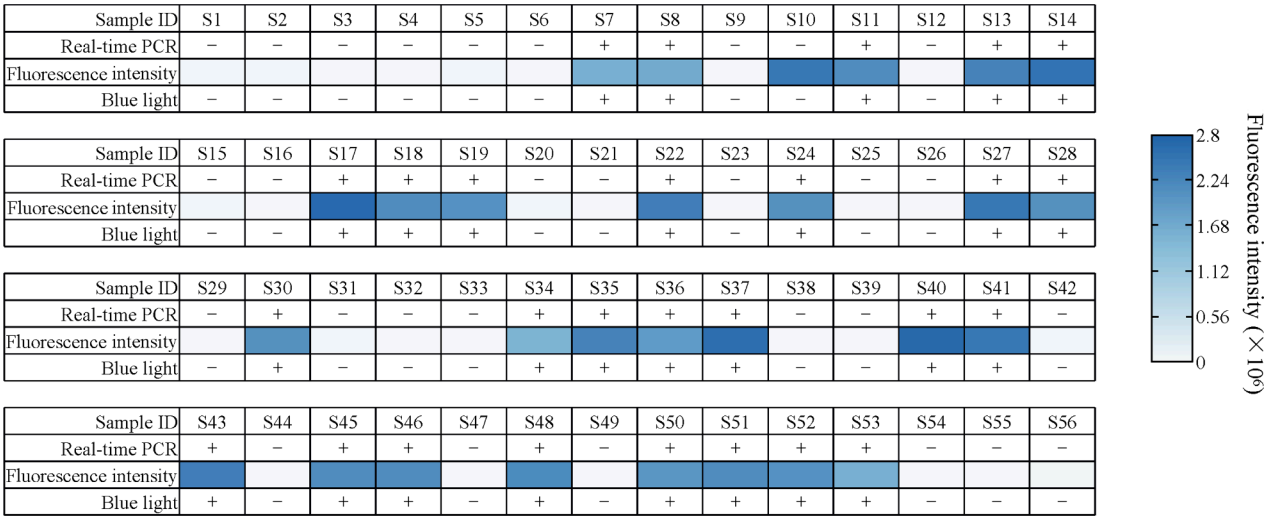

Figure 7. Performance of the GAS-MCDA-CRISPR assay in clinical samples. A total of 56 swab samples were tested by the GAS-MCDA-CRISPR assay to confirm the clinical feasibility. All of the positive samples tested by the real-time PCR were positive when detected by the GAS-MCDA-CRISPR assay, and the negative samples were also negative.

10 °CFU per microliter. As shown in Figure S2, the LOD of the GAS-MCDA-CRISPR assay in simulation samples was lower, to 1.5×10^1 CFU per microliter. Then, the specificity of the GAS-MCDA-CRISPR assay was tested with three GAS strains and 28 non-GAS pathogens (Table S1). Significant fluorescence signals were observed in the tubes containing genomic DNA of GAS strains, whereas no signals were detected in the tubes containing non-GAS pathogens or the blank control (Figure 6 and Figure S3). These findings demonstrated that the developed GAS-MCDA-CRISPR assay did not cross-react with non-GAS pathogens, exhibiting a high specificity of 100%.

Clinical Feasibility of the GAS-MCDA-CRISPR Assay. Methods of the GAS-MCDA-CRISPR assay and the real-time PCR simultaneously detected 56 randomly mixed genomic DNA templates extracted from oropharyngeal swabs. Of the 56 oropharyngeal swabs, 28 were identified as GAS-positive and 28 as GAS-negative by the GAS-MCDA-CRISPR assay, yielding identical results to those obtained by the real-time PCR (Figure 7). Consequently, the novel GAS-MCDA-CRISPR assay has been demonstrated to possess 100% analytic sensitivity and specificity, thereby indicating its potential for extensive application in clinical settings.

DISCUSSION

Group A streptococcus is the most prevalent bacterium responsible for infectious pharyngitis.[30] Previous studies have estimated that more than 616 million new cases of GAS pharyngitis occur annually, and up to 30% of acute pharyngitis cases in the pediatric population are caused by GAS.[31,32] GAS pharyngitis can result in a number of inflammatory complications, which may, in turn, lead to the development of chronic diseases. Such complications include chronic tonsillitis, scarlet fever, and postinfection sequelae such as acute rheumatic fever (ARF) and poststreptococcal glomerulonephritis.[33] Severe GAS disease presents a significant global public health concern, with an estimated 50,000 deaths annually.[31] Hence, early and rapid detection of GAS infection is essential for the subsequent clinical treatment and management of GAS pharyngitis. However, the symptoms of GAS pharyngitis are nonspecific and overlap broadly with those of viral causes of acute pharyngitis, rendering an accurate differential diagnosis based on symptoms alone a significant challenge.[34,35] In this context, we have developed an advanced detection technology that combines MCDA isothermal amplification technology with the CRISPR-Cas12a biosensing platform with the objective of rapidly and accurately identifying GAS infection.

Currently, the traditional methods for diagnosing GAS infection rely primarily on throat culture, RADTs, and PCR analysis.[35] However, the culture method is complex and time-consuming, rendering it unsuitable for rapid clinical diagnosis. Although the RADTs can provide immediate results in a POCT setting, their negative results were recommended to be confirmed by throat culture, which greatly increased the complexity of their widespread use.[3,17,36] PCR-based techniques are highly sensitive; however, they are not applicable in low- and middle-income areas due to the high cost and the necessity for specialized equipment and well-trained personnel.[35] Recently, several CRISPR-Cas12a-based technologies have emerged as promising next-generation molecular diagnostic tools for pathogen detection, particularly when integrated with nucleic acid isothermal amplification (NIA) techniques.[24,37,38] For GAS detection, Cheng et al. reported a rapid CRISPR/crRNA-based assay that uses RPA for nucleic acid preamplification and lateral flow biosensor (LFB) technology for CRISPR product visualization, achieving exceptional sensitivity and specificity.[38] In this study, we introduce the MCDA assay as a superior alternative to traditional NIA methods for GAS-CRISPR diagnostics, leveraging its rapid reaction kinetics, cost-effectiveness, and compatibility with portable detection devices.[39]

A key advantage of the MCDA assay over RPA is its cost-efficiency, as it requires only a single-strand-displacing DNA polymerase for amplification. Furthermore, the MCDA assay employs 10 primers targeting 10 distinct binding sites, enabling highly specific hybridization to the target sequence and potentially offering greater specificity compared to other NIA techniques.[39] Additionally, the newly developed GAS-MCDA-CRISPR assay eliminates the need for complex thermocycling equipment, relying instead on a simple isothermal heater (e.g., a heating block or a thermocup) to facilitate the entire

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detection process. Beyond its simplified instrumentation, the assay also demonstrates exceptional detection efficiency, with only 40 min for MCDA amplification and 5 min for CRISPR-Cas12a cleavage, significantly reducing turnaround time (Figure 1). These features make the assay particularly well-suited for POCT in resource-limited settings, outperforming traditional PCR-based methods in terms of speed and accessibility.

The GAS-MCDA-CRISPR assay targeting the *speB* gene was developed. At the stage of MCDA amplification, a set of 10 specific MCDA primers was designed for preamplifying the *speB* gene of GAS, enabling the generation of a substantial quantity of amplified products within 40 min.[39] In addition, to optimize the GAS-MCDA-CRISPR conditions, different experimental temperatures were initially tested and analyzed in this reaction. The result showed that 63 °C was recommended as the optimal temperature for the MCDA preamplification step (Figure 4). Then, the CRISPR-Cas12a-based detection platform was implemented for the detection of MCDA preamplified products in this assay. The specific gRNA for the *speB* gene was successfully designed to navigate the Cas12a effector protein to correctly recognize the target sequence. As a result, the GAS-MCDA-CRISPR assay achieved a high specificity of 100%, which could clearly identify the GAS and did not cross-react with other non-GAS pathogens (Figure 6). In addition to its strong specificity, the novel GAS-MCDA-CRISPR assay also demonstrated superior detection sensitivity, capable of detecting as little as 45 fg of GAS genomic DNA templates from pure GAS cultures and as little as 1.5×10^1 CFU of GAS cells in simulated swab samples. This limit of detection (LOD) is significantly superior to that of previously developed assays, including the LAMP-based assay (1.49 pg), the RPA-linked lateral flow strip (LFS) platform (0.908 ng), and the MCDA-LFS platform (200 fg).[40−42] This high sensitivity facilitates the effective detection of GAS infections at the early stages of disease, enabling a timely diagnosis, treatment, and prevention of disease progression. These findings suggest that the GAS-MCDA-CRISPR assay established here is a highly sensitive and specific diagnostic tool for the detection of GAS.

With regard to its clinical applicability, the GAS-MCDA-CRISPR assay demonstrated a satisfactory diagnostic performance for identifying GAS infection. The novel GAS-MCDA-CRISPR assay accurately identified the swab specimens with suspected bacterial pharyngitis, exhibiting performance comparable to that of the real-time PCR method (Figure 7). Notably, the assay allows for direct observation of results under blue light, eliminating the need for fluorescence detectors or LFB-based visualized analysis.[38] This feature greatly simplifies the detection process, making the assay particularly suitable for rapid bedside diagnosis and field monitoring, streamlining detection, and reducing operational complexity. Nevertheless, several limitations of the GAS-MCDA-CRISPR assay remain for further discussion. This method cannot avoid DNA extraction and purification steps, which increase the complexity of the procedure. Future efforts will focus on simplifying sample preparation procedures and developing a one-step CRISPR operation for GAS detection. Moreover, in this study, the assay was validated using oropharyngeal swabs of children, which are the most clinically relevant samples for GAS pharyngitis. However, the fundamental mechanism of the MCDA-CRISPR-Cas12a system suggests that the assay could potentially be applied to other clinical sample types, such as blood and conjunctival secretions, provided that target DNA can be efficiently extracted. Future studies will aim to evaluate the assay's performance across a broader range of clinical samples to extend its utility in diverse diagnostic settings.

In conclusion, we successfully developed a CRISPR-Cas12a-based detection platform following the MCDA assay for the rapid and accurate detection of GAS and named it the GAS-MCDA-CRISPR assay. The assay results can be visualized on a real-time fluorescence detector or observed by the naked eyes under blue light. The entire detection process was completed within 45 min, obviating the need for sophisticated thermal cycling equipment. The results of analytical sensitivity, specificity, and clinical applicability tests demonstrated that the GAS-MCDA-CRISPR assay described here was able to detect GAS simply, rapidly, portably, and accurately with high sensitivity and specificity. In light of these findings, the GAS-MCDA-CRISPR assay shows promise as a potentially powerful tool for laboratory diagnosis of GAS infections, particularly in resource-limited settings.

MATERIALS AND METHODS

Reagents and Apparatuses. An EasyPure Bacteria Genomic DNA Kit for nucleic acid extraction and purification was purchased from Transgen Biotech Co., Ltd. (Beijing, China). A DNA isothermal amplification kit was obtained from Huidexin Biotech Co., Ltd. (Tianjin, China). EnGen Lba Cas12a (Cpf1) and NEBuffer r2.1 (10×) were supplied by New England Biolabs, Inc. (MA, USA). A Premix Ex Taq (Probe qPCR) kit was obtained from Takara Biotechnology Co., Ltd. (Tokyo, Japan). The specific primers, gRNA, and fluorescent probe used in this study were synthesized by Dia-up Biotech Co., Ltd. (Beijing, China). The real-time turbidimeter (LA-320C) and the ABI7500 real-time PCR instrument were manufactured by Eiken Chemical Co., Ltd. (Japan) and Applied Biosystems (USA), respectively. The BluSight Pro Blue LED transilluminator was supplied by Monad Biotech (Suzhou, China).

Preparation of Pathogens and Clinical Samples. In this study, genomic DNA from a pure culture GAS strain was used for optimizing the reaction conditions and performing sensitivity analysis. In addition, 28 isolated non-GAS strains obtained from the Chinese Center for Disease Control and Prevention (CDC) were employed for specificity analysis (Table S1). Furthermore, to further verify the feasibility of the GAS-MCDA-CRISPR assay in clinical settings, a total of 56 oropharyngeal swabs were collected from patients with suspected GAS infection at the Capital Institute of Pediatrics (CIP). The nucleic acids of all strains and oropharyngeal swab samples were extracted and purified with a Genomic DNA Kit according to the manufacturer's instruction. The initial concentration of extracted DNA from the pure culture GAS strain was quantified by a spectrophotometer (NanoDrop One, Thermo, USA). All extracted nucleic acids were stored at −20 °C prior to use.

Primers and gRNA Design. Five paired primers for GAS detection targeting the *speB* gene were designed by using PREMIER 5.0 software based on the MCDA reaction mechanism. The specificity of each primer pair was determined using the BLAST tool. The MCDA primers include a pair of displacement primers (F1 and F2), a pair of cross primers (CP1 and CP2), and three pairs of amplification primers (C1, D1, R1, C2, D2, and R2). Notably, the designed CP1 primer contains a PAM site (−TTTC−) specific for the Cas12a

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effector. After primer screening, gRNA complementary to the P1 primer was designed according to the principle of the Cas12a biosensing system. The ssDNA reporter molecule labeled with FAM (5′-end) and black hole quencher (BHQ1, 3′-end) was also prepared. Positions and sequences of the primers and gRNA, and the details of the probe, are shown in Figure 2 and Table 1.

The Standard MCDA Assay. The target gene was preamplified by the MCDA method in a 25 μL mixture using an isothermal amplification kit. The reaction system consisted of 12.5 μL of a 2× reaction buffer, 1 μL of a *Bst* 2.0 DNA polymerase, 0.4 μM each of F1 and F2, 1.6 μM each of CP1 and CP2, 0.8 μM each of C1, C2, D1, D2, R1, and R2, and 1 μL (pure culture bacteria) or 5 μL (clinical samples) of a nucleic acid template and was made up to 25 μL with double distilled water (DW). To evaluate the efficacy and specificity of the GAS-MCDA-CRISPR primers, the DNA template of the GAS strain was used as a positive control, while that of *Streptococcus pneumoniae* served as a negative control and DW was used as a blank control. The MCDA mixtures were incubated with a real-time turbidimeter at 63 °C for 40 min. Subsequently, the products were subjected to analysis via real-time turbidimetry and agarose gel electrophoresis. In addition, to determine the optimal temperature for the MCDA stage, MCDA reactions were conducted and compared at temperatures ranging from 60 to 67 °C (1 °C intervals) for 40 min, with the amplification curves monitored and plotted by the real-time turbidimeter.

CRISPR-Cas12a-Based Assay. The CRISPR-Cas12a-based biosensing system was used to detect the preamplified products on the basis of its nonspecific trans-cleavage activity. The CRISPR-Cas12a detection protocol was based on a previous study.[24] First, the CRISPR-Cas12a-gRNA binary complex was prepared by mixing 100 nM Cas12a with 100 nM gRNA in 2× NEBuffer. The complex was incubated at 37 °C for 10 min and then used immediately or stored at 4 °C for not more than 12 h. Subsequently, the CRISPR-Cas12a-based detection reaction was conducted in a 50 μL mixture comprising 25 μL of NEBuffer (2×), 9 μL of the CRISPR-Cas12a-gRNA complex, 1.25 μL of the ssDNA reporter molecule, and 1 μL of the MCDA product. The real-time fluorescence detector (ABI7500 real-time PCR system) was employed to quantify the fluorescence intensity. Moreover, the fluorescence signals can be readily observed with the naked eyes under blue light (BluSight Pro Blue LED trans-illuminator). Furthermore, the optimal reaction time for trans-cleavage was determined by performing the CRISPR-Cas12a-based reaction with serially diluted DNA templates of the GAS strain at 37 °C for 5−15 min (5 min intervals).

Sensitivity and Specificity of the GAS-MCDA-CRISPR Assay. For specificity analysis, a total of three GAS strains (one from pure culture and two from clinical swabs) and 28 non-GAS strains were employed in this study (Table S1). Moreover, to evaluate sensitivity and ascertain the limit of detection (LOD) of the GAS-MCDA-CRISPR assay, reactions were performed with serially diluted pure GAS genomic DNA (900 pg, 90 pg, 9 pg, 900 fg, 90 fg, 45 fg, and 22.5 fg). A 1 μL portion of each diluted GAS genomic DNA was subjected to the GAS-MCDA-CRISPR assay, with DW serving as the blank control. The sensitivity was also assessed with simulated swab samples containing both GAS and non-GAS cells. After liquid cultivation, a pure GAS bacterial solution with a concentration of 1.5×10^8 CFU/μL was obtained; then, it was 10-fold serially diluted from 1.5×10^7 to 1.5×10^1 CFU/μL. Then, 10 μL of each bacterial dilution was mixed with the 90 μL of swab lotions from the GAS negative samples. Following extraction and purification of total genomic DNA using the Bacterial Genomic DNA Kit, the resulting nucleic acid templates were subjected to the GAS-MCDA-CRISPR assay, with the sample containing non-GAS genomic DNA serving as the negative control. The experiments with the GAS-MCDA-CRISPR assay were conducted in accordance with the aforementioned conditions. To ensure the reliability of the results, each experiment was conducted in triplicate.

Applicability of the GAS-MCDA-CRISPR Assay for Clinical Samples. A total of 56 clinical samples were used to evaluate the applicability of the GAS-MCDA-CRISPR assay for the detection of GAS in clinical settings. These clinical samples were obtained from oropharyngeal swabs of patients exhibiting symptoms suggestive of bacterial pharyngitis. In addition, the real-time PCR method was performed for comparative purposes and to corroborate the outcomes of the GAS-MCDA-CRISPR assay. The sequences of the PCR primers and TaqMan probe for *speB* were referenced from a previous study.[43] The amplification procedure was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s, in accordance with the manufacturer's instructions of the Premix Ex Taq (probe qPCR) kit. All samples used in this study had informed consent signed by the guardians of the participants and approved by the Ethics Committee of the Capital Institute of Pediatrics (Ethical Approval No. SHERLL2024033).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c00479.

Pathogen information used in this study, optimal CRISPR-Cas12a time, sensitivity results of the GAS-MCDA-CRISPR assay in simulated samples, and specificity results of the GAS-MCDA-CRISPR assay (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GAS, group A streptococcus; MCDA, multiple cross displacement amplification; ssDNA, single-stranded DNA; gRNA, guide RNA; ssDNA, single-stranded DNA; RADTs, rapid antigen detection tests; CRISPR, clustered regularly interspaced short palindromic repeats; CRISPR-Cas, CRISPR-associated protein; POCT, point-of-care testing; RPA, recombinase polymerase amplification; ARF, acute rheumatic fever; NIA, nucleic acid isothermal amplification; CDC, Chinese Center for Disease Control and Prevention; CIP, Capital Institute of Pediatrics; LoD, limit of detection; PCR, polymerase chain reaction; LAMP, loop-mediated isothermal amplification; FAM, 6-carboxyfluorescein; BHQ1, black hole quencher; BC, blank control; NC, negative control; DW, distilled water; FAM, carboxyfluorescein; Bst, *Bacillus stearothermophilus*

■ REFERENCES

(1) Brouwer, S.; Rivera-Hernandez, T.; Curren, B. F.; Harbison-Price, N.; De Oliveira, D. M. P.; Jespersen, M. G.; Davies, M. R.; Walker, M. J. Pathogenesis, Epidemiology and Control of Group a Streptococcus Infection. *Nat. Rev. Microbiol.* **2023**, *21*, 431−447.

(2) Pichichero, M. E. Group a Streptococcal Tonsillopharyngitis: Cost-Effective Diagnosis and Treatment. *Ann. Emerg. Med.* **1995**, *25*, 390−403.

(3) Shulman, S. T.; Bisno, A. L.; Clegg, H. W.; Gerber, M. A.; Kaplan, E. L.; Lee, G.; Martin, J. M.; Van Beneden, C. Clinical Practice Guideline for the Diagnosis and Management of Group a Streptococcal Pharyngitis: 2012 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2012**, *55*, e86−102.

(4) Thompson, T.Z.; McMullen, A.R. Group a Streptococcus Testing in Pediatrics: The Move to Point-of-Care Molecular Testing. *J. Clin. Microbiol.* **2020**, *58*, No. e01494-19.

(5) Shaikh, N.; Leonard, E.; Martin, J. M. Prevalence of Streptococcal Pharyngitis and Streptococcal Carriage in Children: A Meta-Analysis. *Pediatrics* **2010**, *126*, e557−564.

(6) Anthony, B. F.; Kaplan, E. L.; Wannamaker, L. W.; Briese, F. W.; Chapman, S. S. Attack Rates of Acute Nephritis after Type 49 Streptococcal Infection of the Skin and of the Respiratory Tract. *J. Clin Invest.* **1969**, *48*, 1697−1704.

(7) Begovac, J.; Kuzmanović, N.; Bejuk, D. Comparison of Clinical Characteristics of Group a Streptococcal Bacteremia in Children and Adults. *Clin. Infect. Dis.* **1996**, *23*, 97−100.

(8) Bisno, A. L.; Stevens, D. L. Streptococcal Infections of Skin and Soft Tissues. *N. Engl. J. Med.* **1996**, *334*, 240−245.

(9) Schwartz, B.; Elliott, J. A.; Butler, J. C.; Simon, P. A.; Jameson, B. L.; Welch, G. E.; Facklam, R. R. Clusters of Invasive Group a Streptococcal Infections in Family, Hospital, and Nursing Home Settings. *Clin. Infect. Dis.* **1992**, *15*, 277−284.

(10) Stevens, D. L. Invasive Group a Streptococcus Infections. *Clin. Infect. Dis.* **1992**, *14*, 2−11.

(11) Tejani, A.; Ingulli, E. Poststreptococcal Glomerulonephritis. Current Clinical and Pathologic Concepts. *Nephron* **2004**, *55*, 1−5.

(12) Walker, M. J.; Barnett, T. C.; McArthur, J. D.; Cole, J. N.; Gillen, C. M.; Henningham, A.; Sriprakash, K. S.; Sanderson-Smith, M. L.; Nizet, V. Disease Manifestations and Pathogenic Mechanisms of Group a Streptococcus. *Clin. Microbiol. Rev.* **2014**, *27*, 264−301.

(13) Karthikeyan, G.; Guilherme, L. Acute Rheumatic Fever. *Lancet (London, England)* **2018**, *392*, 161−174.

(14) Spinks, A.; Glasziou, P.P.; Mar, C.B.D. Antibiotics for Sore Throat in children and adults. *Cochrane Database Syst. Rev.* **2013**, No. CD000023.

(15) Little, P.; Hobbs, F.D.R.; Moore, M.; Mant, D.; Williamson, I.; McNulty, C.; Cheng, Y.E.; Leydon, G.; McManus, R.; Kelly, J.; Barnett, J.; Glasziou, P.; Mullee, M.; investigators, P. Clinical Score and Rapid Antigen Detection Test to Guide Antibiotic Use for Sore Throats: Randomised Controlled Trial of Prism (Primary Care Streptococcal Management). *BMJ* **2013**, 347.

(16) Rao, A.; Berg, B.; Quezada, T.; Fader, R.; Walker, K.; Tang, S.; Cowen, U.; Duncan, D.; Sickler, J. Diagnosis and Antibiotic Treatment of Group a Streptococcal Pharyngitis in Children in a Primary Care Setting: Impact of Point-of-Care Polymerase Chain Reaction. *BMC Pediatr.* **2019**, *19*, 24.

(17) McIsaac, W. J.; Kellner, J. D.; Aufricht, P.; Vanjaka, A.; Low, D. E. Empirical Validation of Guidelines for the Management of Pharyngitis in Children and Adults. *JAMA* **2004**, *291*, 1587−1595.

(18) Navarro, E.; Serrano-Heras, G.; Castaño, M. J.; Solera, J. Real-Time Pcr Detection Chemistry. *Clin. Chim. Acta* **2015**, *439*, 231−250.

(19) Bae, N.H.; Lim, S.Y.; Song, Y.; Jeong, S.W.; Shin, S.Y.; Kim, Y.T.; Lee, T.J.; Lee, K.G.; Lee, S.J.; Oh, Y.-J.; Park, Y.M. A Disposable and Multi-Chamber Film-Based Pcr Chip for Detection of Foodborne Pathogen. *Sensors (Basel, Switzerland)* **2018**, *18*, 3158.

(20) Fapohunda, F. O.; Qiao, S.; Pan, Y.; Wang, H.; Liu, Y.; Chen, Q.; Lü, P. Crispr Cas System: A Strategic Approach in Detection of Nucleic Acids. *Microbiol. Res.* **2022**, *259*, No. 127000.

(21) Pickar-Oliver, A.; Gersbach, C. A. The Next Generation of Crispr-Cas Technologies and Applications. *Nat. Rev. Mol. Cell Biol.* **2019**, *20*, 490−507.

(22) Aman, R.; Mahas, A.; Mahfouz, M. Nucleic Acid Detection Using Crispr/Cas Biosensing Technologies. *ACS Synth Biol.* **2020**, *9*, 1226−1233.

(23) Gootenberg, J. S.; Abudayyeh, O. O.; Kellner, M. J.; Joung, J.; Collins, J. J.; Zhang, F. Multiplexed and Portable Nucleic Acid Detection Platform with Cas13, Cas12a, and Csm6. *Science.* **2018**, *360*, 439−444.

(24) Jia, N.; Wang, C.; Liu, X.; Huang, X.; Xiao, F.; Fu, J.; Sun, C.; Xu, Z.; Wang, G.; Zhou, J.; Wang, Y. A Crispr-Cas12a-Based Platform for Ultrasensitive Rapid Highly Specific Detection of Mycobacterium Tuberculosis in Clinical Application. *Front. Cell. Infect. Microbiol.* **2023**, *13*, No. 1192134.

(25) Liu, S.; Huang, S.; Li, F.; Sun, Y.; Fu, J.; Xiao, F.; Jia, N.; Huang, X.; Sun, C.; Zhou, J.; Wang, Y.; Qu, D. Rapid Detection of Pseudomonas Aeruginosa by Recombinase Polymerase Amplification Combined with Crispr-Cas12a Biosensing System. *Front. Cell. Infect. Microbiol.* **2023**, *13*, No. 1239269.

(26) Zhu, X.; Wang, X.; Li, S.; Luo, W.; Zhang, X.; Wang, C.; Chen, Q.; Yu, S.; Tai, J.; Wang, Y. Rapid, Ultrasensitive, and Highly Specific Diagnosis of Covid-19 by Crispr-Based Detection. *ACS Sens.* **2021**, *6*, 881−888.
(27) Chen, J. S.; Ma, E.; Harrington, L. B.; Da Costa, M.; Tian, X.; Palefsky, J. M.; Doudna, J. A. Crispr-Cas12a Target Binding Unleashes Indiscriminate Single-Stranded Dnase Activity. *Science.* **2018**, *360*, 436−439.
(28) Gootenberg, J. S.; Abudayyeh, O. O.; Lee, J. W.; Essletzbichler, P.; Dy, A. J.; Joung, J.; Verdine, V.; Donghia, N.; Daringer, N. M.; Freije, C. A.; Myhrvold, C.; Bhattacharyya, R. P.; Livny, J.; Regev, A.; Koonin, E. V.; Hung, D. T.; Sabeti, P. C.; Collins, J. J.; Zhang, F. Nucleic Acid Detection with Crispr-Cas13a/C2c2. *Science.* **2017**, *356*, 438−442.
(29) Li, S.-Y.; Cheng, Q.-X.; Wang, J.-M.; Li, X.-Y.; Zhang, Z.-L.; Gao, S.; Cao, R.-B.; Zhao, G.-P.; Wang, J. Crispr-Cas12a-Assisted Nucleic Acid Detection. *Cell Discovery* **2018**, *4*, 20.
(30) Murray, R. C.; Chennupati, S. K. Chronic Streptococcal and Non-Streptococcal Pharyngitis. *Infect. Disord. Drug Targets.* **2012**, *12*, 281−285.
(31) Carapetis, J. R.; Steer, A. C.; Mulholland, E. K.; Weber, M. The Global Burden of Group a Streptococcal Diseases. *Lancet Infect. Dis.* **2005**, *5*, 685−694.
(32) Weber, R. Pharyngitis. *Prim Care.* **2014**, *41*, 91−98.
(33) Soderholm, A. T.; Barnett, T. C.; Sweet, M. J.; Walker, M. J. Group a Streptococcal Pharyngitis: Immune Responses Involved in Bacterial Clearance and Gas-Associated Immunopathologies. *J. Leukoc. Biol.* **2018**, *103*, 193−213.
(34) Mustafa, Z.; Ghaffari, M. Diagnostic Methods, Clinical Guidelines, and Antibiotic Treatment for Group a Streptococcal Pharyngitis: A Narrative Review. *Front. Cell. Infect. Microbiol.* **2020**, *10*, No. 563627.
(35) Cohen, J.F.; Bertille, N.; Cohen, R.; Chalumeau, M. Rapid Antigen Detection Test for Group a Streptococcus in Children with Pharyngitis. *Cochrane Database Syst. Rev. 7* **2016**, No. CD010502.
(36) Tanz, R. R.; Gerber, M. A.; Kabat, W.; Rippe, J.; Seshadri, R.; Shulman, S. T. Performance of a Rapid Antigen-Detection Test and Throat Culture in Community Pediatric Offices: Implications for Management of Pharyngitis. *Pediatrics* **2009**, *123*, 437−444.
(37) Jia, X.; Xu, W.; Xiao, F.; Jia, N.; Huang, X.; Zhang, Y.; Zhou, J.; Wang, Y.; Tai, J. Rapid and Sensitive Detection of Haemophilus Influenzae Using Multiple Cross Displacement Amplification Combined with Crispr-Cas12a-Based Biosensing System. *Heliyon* **2025**, *11*, No. e41535.
(38) Cheng, Y.; Lyu, J.; Han, J.; Feng, L.; Li, X.; Li, P.; Zhang, S.; Zang, W. A Specific and Ultrasensitive Cas12a/Crrna Assay with Recombinase Polymerase Amplification and Lateral Flow Biosensor Technology for the Rapid Detection of Streptococcus Pyogenes. *Microbiology spectrum* **2024**, *12*, No. e0034524.
(39) Wang, Y.; Wang, Y.; Ma, A.-J.; Li, D.-X.; Luo, L.-J.; Liu, D.-X.; Jin, D.; Liu, K.; Ye, C.-Y. Rapid and Sensitive Isothermal Detection of Nucleic-Acid Sequence by Multiple Cross Displacement Amplification. *Sci. Rep.* **2015**, *5*, 11902.
(40) Dou, Z.; Xie, L.; Gao, M.; Liu, D. Development of a Multiple Cross Displacement Amplification Combined with Nanoparticles-Based Biosensor Assay for Rapid and Sensitive Detection of Streptococcus Pyogenes. *BMC Microbiol.* **2024**, *24*, 51.
(41) Zhao, X.; He, X.; Li, H.; Zhao, J.; Huang, S.; Liu, W.; Wei, X.; Ding, Y.; Wang, Z.; Zou, D.; Wang, X.; Dong, D.; Yang, Z.; Yan, X.; Huang, L.; Du, S.; Yuan, J. Detection of Streptococcus Pyogenes Using Rapid Visual Molecular Assay. *FEMS Microbiol. Lett.* **2015**, *362*, fnv148.
(42) Gao, X.-Z.; Cao, Y.-D.; Gao, Y.-Z.; Hu, J.; Ji, T. Efficient Detection of Streptococcus Pyogenes Based on Recombinase Polymerase Amplification and Lateral Flow Strip. *Eur. J. Clin. Microbiol. Infect. Dis.* **2024**, *43*, 735−745.
(43) Virtaneva, K.; Graham, M. R.; Porcella, S. F.; Hoe, N. P.; Su, H.; Graviss, E. A.; Gardner, T. J.; Allison, J. E.; Lemon, W. J.; Bailey, J. R.; Parnell, M. J.; Musser, J. M. Group a Streptococcus Gene Expression in Humans and Cynomolgus Macaques with Acute Pharyngitis. *Infect. Immun.* **2003**, *71*, 2199−2207.

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