Methods and Applications

Establishment of a Lateral Flow Dipstick Detection Method for Influenza A Virus Based on CRISPR/Cas12a System

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ABSTRACT

Objective: This study aimed to develop a rapid, visual PCR-CRISPR/Cas12-LFD method for detecting influenza A by utilizing the conserved region of the matrix protein gene.

Method: We crafted universal degradation primers and clustered regularly interspaced short palindromic repeats RNA (CRISPR RNA, crRNA) targeting the conserved matrix protein gene of the influenza virus (IFV), integrated with lateral flow dipstick (LFD) technology. This new PCR-CRISPR/Cas12-LFD approach was designed to determine its sensitivity and specificity through the analysis of various clinical samples collected in 2023.

Results: The developed nucleic acid assay for influenza A viruses (IAV) demonstrated a sensitivity of 10 copies/μL without cross-reactivity with other respiratory pathogens. Evaluation of 82 clinical samples showed high concordance with results from fluorescent Polymerase Chain Reaction (PCR), achieving a kappa value of 0.95.

Conclusion: A highly sensitive and specific PCR-CRISPR/Cas12-LFD method has been successfully established for the detection of influenza A, offering a robust tool for its diagnosis and aiding in the prevention and control of this virus.

Influenza (flu) is an acute respiratory illness caused by the influenza virus (IFV), characterized by symptoms such as fever, cough, headache, and muscle pain, posing a significant threat to public health (*1*). The virus falls within the Orthomyxoviridae family, known for single-stranded, negative-sense RNA viruses divided into four subtypes — A, B, C, and D (*2*). The influenza A virus (IAV) comprises eight distinct RNA segments and is further subclassified into 16 HA and 9 NA subtypes. The high mutation rates of HA and NA facilitate recombination and reassortment events, significantly contributing to IAV's potential to cause recurring seasonal outbreaks and pandemics (*3*–*4*). Recent surveillance data from the China CDC indicates that IAV was responsible for 86.1% of influenza cases in China in 2023 (https://ivdc.chinacdc.cn/cnic/). The ongoing COVID-19 pandemic has further exacerbated the incidence of clustered influenza A outbreaks since 2023, underscoring the urgent need for enhanced influenza prevention and control, notably through early diagnosis and continuous monitoring (*5*).

Molecular diagnosis is crucial for detecting influenza A (*6*). Isothermal methods, characterized by longer primer lengths, often suffer from non-specific amplification, leading to false-negative outcomes. Conversely, gene chips and sequencing require highly skilled personnel, limiting their utility in standard laboratory settings (*7*). Polymerase Chain Reaction (PCR) remains the preferred method due to its high sensitivity, specificity, accuracy, and ease of use, establishing it as the "gold standard" in molecular diagnostics. The CRISPR/Cas system, a defense mechanism in bacteria and archaea against phages, includes CRISPR and associated proteins (Cas). This system is categorized into two types and six subtypes based on its components and functions (*8*). Cas12, a member of the second type, subtype five, utilizes crRNA (CRISPER RNA) to locate specific 5'-TTTN-3' sites in the PAM sequence. It achieves precise binding via the complementarity between crRNA and the target sequence, activating Cas12's cleavage activity to indiscriminately cut trans-acting single-stranded DNA (ssDNA). Exploiting this mechanism, ssDNA probes, labeled with fluorescent signals and biotin, can be tailored for nucleic acid detection (*9*).

In this study, we developed a PCR-CRISPR/Cas12-LFD method for detecting IAV by designing amplification primers and crRNAs targeting the M gene, as characterized in recent GeneBank entries. This approach utilized dual-labeled ssDNAs — modified with fluorescent FAM and biotin motifs — as probes, integrated with lateral flow dipstick (LFD) technology

(Figure 1). We further analyzed the sensitivity and specificity of this method and validated it using clinical samples.

METHOD

Primer and crRNA Design and Synthesis

The M gene sequences from 126 IAVs, encompassing various subtypes such as H3N2, H1N1, H1N2, H5N1, H5N6, H5N8, H7N7, H7N9, H9N2, H10N3, among others, were retrieved from the NCBI GenBank database and analyzed individually. Sequence alignment was conducted using VectorNTI software, focusing on identifying highly homologous conserved sequences while avoiding the formation of secondary and hairpin structures. To target these conserved regions, multiple sets of primers and probes were designed using Primer 5 software and assessed with Oligo software, incorporating degenerate bases to accommodate variations among sequences. Specifically, the M gene of the H3N2 subtype (GenBank: OR865615.1) was utilized to design three sets of primers for both forward and reverse orientations, as detailed in Table 1. Additionally, for crRNA design, the reverse 20 nucleotide segment of the PAM sequence (TTTN or AAAN) was selected from the amplified targets, with a backbone sequence of UAAUUUCUACUAAGUGUAGAU, also listed in Table 1. The synthesis of all primers and crRNA sequences was performed by Shanghai Sangong Biotechnology.

PCR Reaction and Primer Selection

The M gene was cloned into the pUC57 plasmid

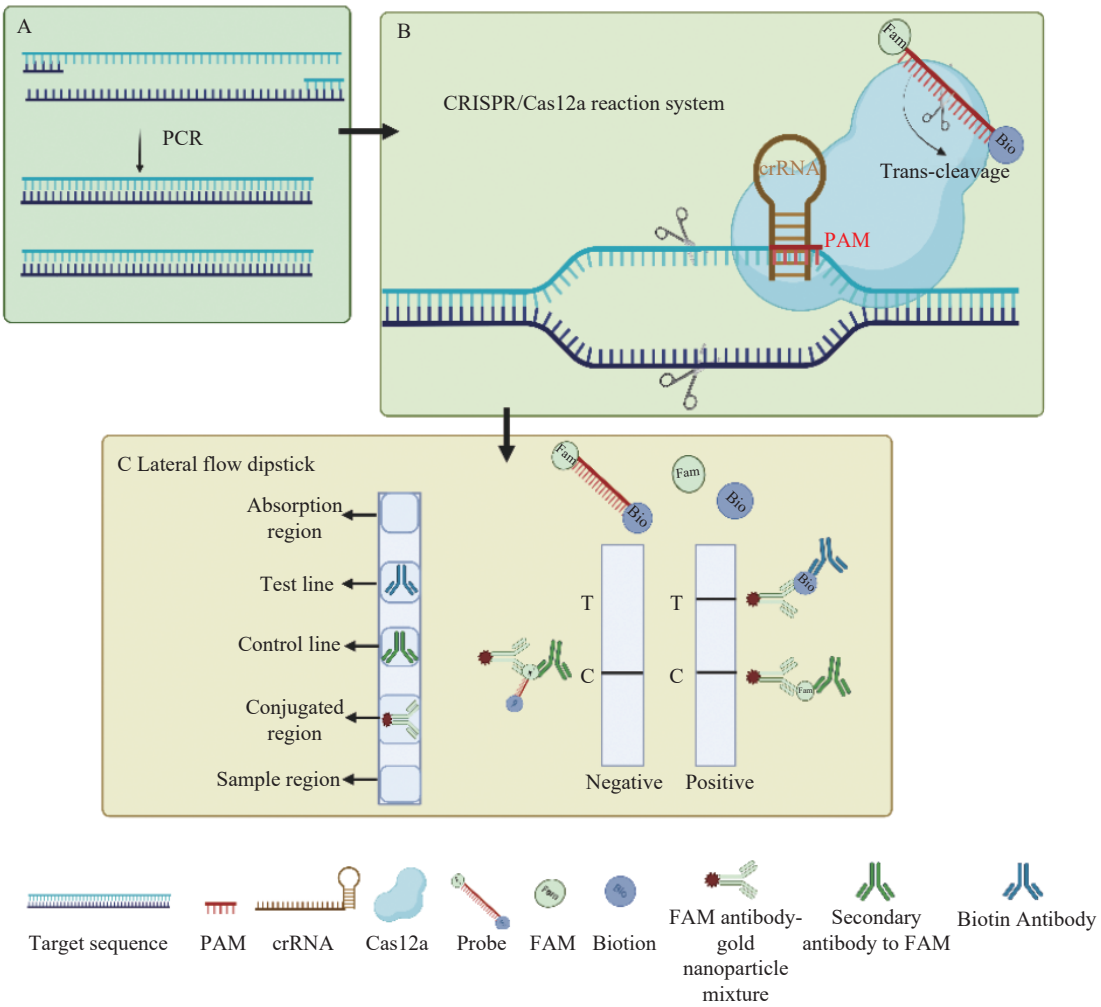

FIGURE 1. Schematic diagram of the PCR-CRISPR/Cas12a-LFD technology. (A) PCR amplification process; (B) the CRISPR/Cas12a and crRNA reaction system; (C) detection of reaction products using LFD.
Abbreviation: LFD=lateral flow dipstick; crRNA= CRISPER RNA.

TABLE 1. List of the primers and crRNA sequences.

Primer/crRNA	Sequence (5'–3')	Position
F1	GAGGTCGAAACGTATGTTCT	41–60
R1	TCACTGGGCACGGTGAGCGT	218–237
F2	ACCGAGGTCGAAACGTATGT	38–57
R2	CGTCTACGCTGCAGTCCCCG	239–258
F3	CTTCTTACCGAGGTCGAAAC	32–51
R3	CGTAGACGCTTTGTCCAAAA	251–270
crRNA-1	UAAUUUCUACUAAGUGUAGAUCUGGGAAAAACACAGAUCUU	98–117
crRNA-2	UAAUUUCUACUAAGUGUAGAUCCAGCAAAGACAUCUUCAAG	82–101

Abbreviation: crRNA=CRISPER RNA.

vector, and the synthesis was carried out by Shanghai Sangong Biotechnology. To amplify the cloned plasmid containing the target gene, three pairs of primers were utilized. The optimal primer pairs were determined through PCR. The composition of the PCR mixture was as follows: 25 μL of 2×*EasyTaq*® PCR SuperMix, 1 μL of 10 μmol/L forward primer, 1 μL of 10 μmol/L reverse primer, 2 μL of template DNA, and 21 μL of nuclease-free water (Supplementary Material). The PCR conditions included an initial denaturation at 94 °C for 30 seconds; followed by 40 cycles of 94 °C for 30 seconds, 58 °C for 15 seconds, and 72 °C for 10 seconds. The PCR products were analyzed using agarose gel electrophoresis, employing 2 μL of the PCR products for analysis; the electrophoresis was conducted at 120 V for 40 minutes.

CRISPR/Cas12a-LFD Detection and CrRNA Selection

The CRISPR/Cas12a reaction setup was as follows: the reaction mixture contained 2 μL of 10x NEB buffer, 2 μL of 500 nmol/L crRNA, 2 μL of 500 nmol/L Cas12a, and 11 μL of nuclease-free water. This mixture was incubated at 25 °C for 10 min. Subsequently, 1 μL of ssDNA-LFD probe (10 μmol/L, 5'-FAM-AAAAAAAA-Bio-3') and 2 μL of PCR products were added. The mixture was thoroughly mixed by shaking, followed by centrifugation, and then incubated in a 37 °C water bath for an additional 10 minutes. After the incubation, an LFD was inserted into the reaction tube, and results were typically observed within 5–10 minutes. The reactions were conducted separately using crRNA-1 and crRNA-2 to determine the more effective crRNA, as indicated by the intensity of the test strip's color.

PCR-CRISPR/Cas12-LFD Sensitivity Analysis

The plasmid encoding the M gene template was synthesized following the previously mentioned procedures. Nucleic acid concentrations were measured using a Qubit 2.0 fluorometer, and the copy number was calculated using the formula: Concentration (copies/μL) = [(6.02 × 10^{23}) × Concentration (ng/μL) × 10^{-9}] / [DNA length (bp) × 660]. The plasmid was serially diluted in tenfold increments from 10^7 to 10^0 copies/μL. PCR reactions utilized these varying concentrations of plasmids as templates and were analyzed through qPCR and CRISPR/Cas12a diagnostic strips. For each dilution level, 2 μL of the sample was added to the reaction mix, with eight replicates conducted for each dilution.

PCR-CRISPR/Cas12-LFD Specificity Analysis

Nucleic acids from positive samples of common respiratory viruses, including IBV, RSV, HRV, MP, HAdV, and 2019-nCoV, were detected using the established PCR-CRISPR/Cas12-LFD method. Additionally, the specificity of this method was analyzed.

Clinical Detection

Fifty-six IAV-positive clinical samples, along with 26 negative controls, underwent nucleic acid extraction followed by reverse transcription using the specified reagents: 4 μL of 5×ES RT Buffer, 1 μL of Total RNA, 1 μL of 10 mmol/L dNTPs, 1 μL of Anchored Oligo(dT)$_{18}$ Primer, 1 μL of *EasyScript*®, and 12 μL of nuclease-free water. The reverse transcription conditions were set at 42 ℃ for 30 minutes. Subsequently, the samples were analyzed using PCR-

CRISPR/Cas12-LFD and fluorescent PCR methods. The concordance between these two methods was then assessed using kappa statistics.

RESULTS

Establishment of PCR-CRISPR/Cas12-LFD detection.

The plasmid was identified utilizing three primer pairs and two crRNAs targeting the M gene, with results in Figure 2. All primer pairs successfully amplified the target gene. The two crRNAs detected the amplification products. Notably, the F2R2 primer exhibited higher amplification efficiency compared to the other pairs (Figure 2A), and crRNA-2 produced the most robust signal (Figure 2B). Consequently, F2R2 and crRNA-2 were chosen to develop the PCR-CRISPR/Cas12-LFD method.

PCR-CRISPR/Cas12-LFD Sensitivity Analysis

The M gene of IAV underwent further sensitivity analysis and reproducibility evaluation. Eight dilution gradients from 10^7 to 10^0 were established, and detection utilized both the qPCR and the PCR-CRISPR/Cas12-LFD method. The detection limit for the PCR-CRISPR/Cas12-LFD method was identified as 10 copies/μL. Repeated experiments (eight repetitions per dilution) demonstrated consistent results, affirming the method's superior stability (Figure 3).

PCR-CRISPR/Cas12-LFD Specificity Analysis

This study established a PCR-CRISPR/Cas12-LFD method that successfully detected twenty-six common

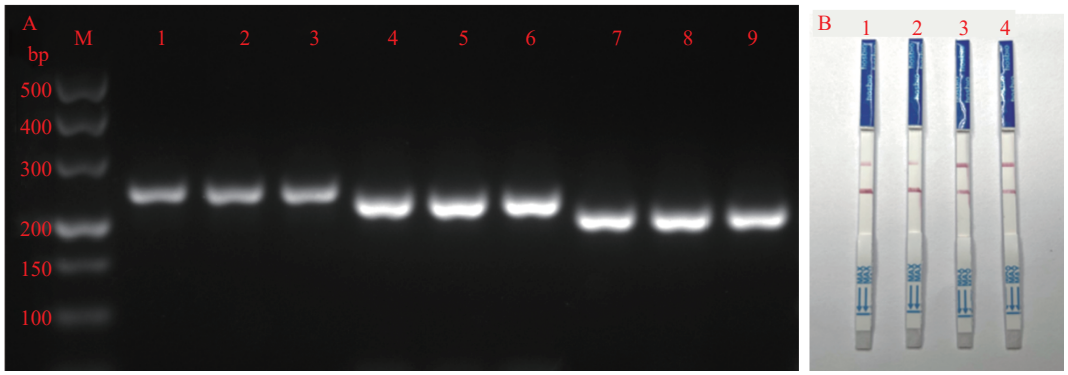

FIGURE 2. Establishment of the PCR-CRISPR/Cas12-LFD Detection Method. (A) Electrophoretic analysis of PCR products with three primer pairs; (B) CRISPR RNA (crRNA) selection using the LFD method.
Note: Panel A, 1–3: three replicated amplicons obtained with F1R1 primers; 4–6: three replicated amplicons using F2R2 primers; 7–9: three replicated amplicons generated by F3R3 primers. Panel B, 1–2: two replicates tested with crRNA-1; 3–4: two replicates tested with crRNA-2.
Abbreviation: LFD=lateral flow dipstick; crRNA= CRISPER RNA.

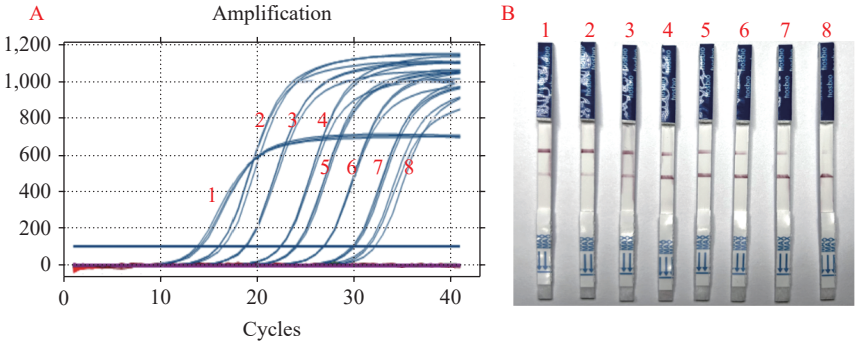

FIGURE 3. Sensitivity analysis of PCR-CRISPR/Cas12-LFD for the detection of IAV. (A) Sensitivity of qPCR in detecting IAV, with three replicates for each of eight dilutions ranging from 10^7 to 10^0; (B) Sensitivity of PCR-CRISPR/Cas12-LFD in detecting IAV, with eight dilutions from 10^7 to 10^0.
Abbreviation: LFD=lateral flow dipstick; IAV=influenza A virus.

respiratory viruses, including IBV, RSV, HRV, MP, HAdV, and 2019-nCoV, without any false positive results, demonstrating the method's high specificity.

Evaluation with Clinical Samples

The practicality of the PCR-CRISPR/Cas12-LFD method developed in this study was assessed using 82 clinical samples, including 56 IAV-positive and 26 IAV-negative samples identified by qPCR. For comparative purposes, the Universal Nucleic Acid Detection Kit for Influenza A Virus from SunSure Biotechnology was used as a control. Results indicated a kappa coefficient of 0.95 between the two methods, indicating identical performance (Table 2).

DISCUSSION

Data show that between November 15, 2023, and December 30, 2023, the incidence of IFV infection among outpatients exhibiting respiratory symptoms in North China, particularly in the Beijing area, ranged from 20% to 50%. Influenza is expected to continue its prevalence across different regions, potentially leading to localized outbreaks. It is anticipated that the IAV, specifically strains H3N2 and H1N1, will remain predominant in these pandemic occurrences (https://www.nmdc.cn/). Concurrently, other respiratory pathogens such as RSV and mycoplasma infections are also imposing significant public health challenges (*10*–*11*). Accurate and fast identification of these pathogens is crucial for effective disease prevention and control.

The CRISPR/Cas system, renowned for its high precision and ease of use, has evolved swiftly into an effective tool for pathogen detection (*12*). Initial research primarily concentrated on the CRISPR/Cas9 system, yet the inherent limitations associated with the Cas9 protein, such as its size, have restricted broader applications of this technology (*13*). Recently, the CRISPR/Cas12 system, also known as CRISPR/Cpf1, has emerged as a focal area of interest. This system falls under the V type category and includes Cas proteins ranging from Cas12a to Cas12e, with Cas12a being more extensively utilized in nucleic acid detection. This interaction activates the RuvC structural domain of the protein for DNA cleavage and initiates trans-cleavage activity, a distinct feature of the CRISPR/Cas12 system (*14*–*15*). The CRISPR/Cas12 system offers advantages such as increased reaction speed, lower off-target rates, and higher editing efficiency, making it particularly suitable for the precise identification of pathogens (*16*). Moreover, leveraging the trans-cleavage activity of Cas proteins, CRISPR-Cas has been integrated with photoelectrochemical biosensors for high-throughput detection (*17*). Additionally, the high sensitivity of the CRISPR-Cas system allows for the direct detection of pathogens using electrochemical methods without the need for nucleic acid amplification (*18*).

TABLE 2. Comparison of PCR-CRISPR/Cas12-LFD and qPCR assay results.

PCR-CRISPR/Cas12-LFD	qPCR		
	Positive	Negative	Total
Positive	54	0	54
Negative	2	26	28
Total	56	26	82

LFD has become a commonly employed technique for nucleic acid detection due to its rapidity, convenience, and cost-effectiveness (*19*). In our study, we utilized double-antibody sandwich immunochromatography for the swift identification of nucleic acid amplification products. The front end of the test strip was pre-coated with a mixture of FAM antibody and gold nanoparticles, the control line with a secondary antibody to the FAM antibody, and the test line with a biotin antibody. We labeled the ends of the probe with FAM and biotin, respectively. Upon activation of Casase's cutting activity, colorful precipitates were observed at both the detection and quality control lines. In the absence of probe cleavage, a precipitate formed only at the quality control line. This reaction with the amplified product allows for visual results within 2–10 minutes (*20*). Due to the lower cost of traditional PCR combined with LFD compared to fluorescence quantitative PCR, it is especially favored in primary-level laboratories and field detection.

The CRISPR/Cas12 system holds promise as a diagnostic tool for pathogen detection (*21*–*23*). Recent advancements have incorporated isothermal detection combined with CRISPR and test strips to identify IFA and other prevalent pathogens. However, these methods face challenges, such as the need for 30–35 bp primers in recombinase polymerase amplification (RPA) and 4–6 pairs of primers in Loop-mediated isothermal amplification (LAMP), complicating primer design. This complexity can lead to mismatches and false-positive results, thereby reducing specificity (*24*). In contrast, PCR remains a traditional method with advantages including lower costs for instruments and

reagents, along with enhanced sensitivity and specificity. The integration of PCR, CRISPR-Cas12a, and LFD offers superior sensitivity and specificity, making it particularly suitable for local primary laboratories.

Challenges to this combined detection include less sensitivity than qPCR, and requirement for integrated microfluidics for portability, in which we will develop further.

In this study, we developed a rapid diagnostic method for detecting IAV that integrates the benefits of PCR, CRISPR/Cas12, and LFD techniques. This method exhibits high sensitivity and specificity while eliminating the need for fluorescent PCR instruments and allowing for visual interpretation of results. When testing a positive plasmid containing the M gene, the method achieved a sensitivity of 10 copies/μL. We evaluated the method on 82 clinical samples, and it demonstrated a high degree of consistency with real-time fluorescence PCR, except for two samples not detected with a qPCR CT value exceeding 35. Tests on various common respiratory samples indicated no cross-reactivity, confirming the method's high specificity. Furthermore, this study innovatively detects different subtypes of IAV by including degenerate bases in the primer design and conducts optimized screening of crRNA to enhance both sensitivity and specificity of the detection process.

Conflicts of interest: No conflicts of interest

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SUPPLEMENTARY MATERIAL

2x EasyTaq® PCR SuperMix and EasyScript® Reverse Transcriptase were acquired from Beijing TransGen Biotech Company. The CFX96TM Real-Time System was purchased from Bio-Rad. Rapid Nucleic Acid Extraction Kit (Magnetic Bead Method) was sourced from BioPerfectus Company, while Tiosbio Cas12/13 Test Strips were obtained from Nanjing WarBio Technology Company. EnGen® Lba Cas12a was procured from New England Biolabs and AceQ qPCR SYBR Green Master Mix from Nanjing Vazyme Company. The Influenza A Virus Universal Nucleic Acid Detection Kit (PCR-fluorescent probe method) was purchased from SunSure Company. Zhumadian Central Hospital provided samples of several respiratory pathogens, including IAV, influenza B virus (IBV), human adenovirus (HAdV), respiratory syncytial virus (RSV), human rhinovirus (HRV), Mycoplasma pneumonia (MP), COVID virus (2019-nCoV), and 26 other common respiratory pathogens. Additionally, 56 IAV clinical samples were supplied by the Inspection Department of Zhumadian Central Hospital.