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Original Article

CRISPR/Cas13-assisted carbapenem-resistant *Klebsiella pneumoniae* detection

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Abstract *Background/Purpose:* Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is capable of causing serious community and hospital-acquired infections. However, currently, the identification of CRKP is complex and inefficient. Hence, this study aimed to develop methods for the early and effective identification of CRKP to allow reasonable antimicrobial therapy in a timely manner.
Methods: K. pneumoniae (KP)-, *K. pneumoniae* carbapenemase (KPC)- and New Delhi metallo-β-lactamase (NDM)- specific CRISPR RNAs (crRNAs), polymerase chain reaction (PCR) primers and recombinase-aided amplification (RAA) primers were designed and screened in conserved sequence regions. We established fluorescence and lateral flow strip assays based on CRISPR/Cas13a combined with PCR and RAA, respectively, to assist in the detection of CRKP. Sixty-one clinical strains (including 51 CRKP strains and 10 carbapenem-sensitive strains) were collected for clinical validation.
Results: Using the PCR-CRISPR assay, the limit of detection (LOD) for KP and the blaKPC and blaNDM genes reached 1 copy/μL with the fluorescence signal readout. Using the RAA-

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CRISPR assay, the LOD could reach 10^1 copies/μL with both the fluorescence signal readout and the lateral flow strip readout. Additionally, the positivity rates of CRKP-positive samples detected by the PCR/RAA-CRISPR fluorescence and RAA-CRISPR lateral flow strip methods was 92.16% (47/51). The sensitivity and specificity reached 100% for KP and blaKPC and blaNDM gene detection. For detection in a simulated environmental sample, 1 CFU/cm^2 KP could be detected.

Conclusion: We established PCR/RAA-CRISPR assays for the detection of blaKPC and blaNDM carbapenemase genes, as well as KP, to facilitate the detection of CRKP.

Introduction

The emergence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains has undoubtedly become a serious public health problem. In the last few decades, the number of multidrug-resistant Gram-negative bacteria has increased dramatically, and it has become one of the most dangerous threats to public health security worldwide.[1] *K. pneumoniae* (KP), a significant Gram-negative opportunistic bacterial pathogen, is capable of causing several types of clinical infections, including pneumonia, urinary tract infection, soft tissue infection and sepsis.[2] Carbapenem antibiotics are common β-lactam drugs, and these drugs are considered the "last line agents" in the clinical treatment of severe Gram-negative bacterial infections because of their potential antibacterial activity.[3] Nevertheless, with the indiscriminate use of carbapenem antibiotics in clinical treatments, CRKP strains have gradually emerged worldwide and have become a pressing global public health issue.[4]

The primary mechanism for drug resistance in these isolates is the production of acquired carbapenemases, which are able to hydrolyze carbapenem antibiotics to cause drug resistance.[5,6] Carbapenemases are commonly classified into classes A, B, and D β-lactamases according to the Ambler classification system.[7] In addition to the more popular *K. pneumoniae* carbapenemase (KPC)-type enzymes, *Serratia marcescens* enzyme (SME), Guiana extended-spectrum (GES), nonmetallo-enzyme carbapenemase (NMC) and imipenem-hydrolyzingβ-lactamases (IMI) variants also belong to class A carbapenemases, although they are less common. The class B carbapenemases consist of the New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM), and imipenemase (IMP) enzymes.[8,9] The class D carbapenemases mainly include oxacillinase (OXA)-type enzymes. Of these carbapenemases, the most predominant type is KPC, followed by NDM and a few OXA-48-like enzymes.[10] Carbapenemase genes can be transmittde horizontally among strains via mobile genetic elements carrying multiple resistance genes, leading to the epidemic spread of carbapenemases.[11] Hence, the rapid detection and identification of CRKP is vital as it would allow patients to obtain effective treatment while providing opportunities for hospitals and communities to implement infection control measures in a timely manner to prevent its dissemination. However, CRKP detection in clinical diagnosis usually requires routine bacterial culture and isolation from specimens to identify *K. pneumoniae* by VITEK MS, followed by a drug sensitivity test to obtain a definitive result, such as an eCIM/mCIM or VITEK2 Compact assay. This process mostly requires an additional overnight culture for a result. It is currently difficult to identify CRKP infection and initiate effective antibiotic treatment for critically ill patients within the first 24 h.

Furthermore, depending on the main resistance mechanism of CRKP, the detection of carbapenemase, the main factor that breaks down antibiotics, can also assist in the detection of CRKP. Clinical diagnostic methods generally include antigen testing and gene detection.[12] Molecularly detection techniques such as qPCR are the gold standard, and the testing process and reading results depend on expensive machinery.[13] Although antigen-based carbapenemase detection methods have the advantage of convenience, the product is expensive because of the high cost of the raw materials. Hence, new molecular detection methods need to be established to conveniently detect CRKP.

Over the years, CRISPR technology has been gradually discovered and used for gene editing and molecular diagnosis.[14,15] Cas13a, as an RNA-guided RNase, is a important member of the CRISPR-Cas effector family. This effector protein has an accessory cleavage activity, which triggers the enzymatic cleavage of the targeted sequence and the untargeted subsidiary cleavage of all single stranded RNA (ssRNA) in its neighborhood as soon as the target sequence is combined with CRISPR RNA (crRNA), enabling signal amplification.[16] In 2017, a platform called SHERLOCK was established by Feng Zhang et al., who combined the Cas13a system with isothermal amplification technology to achieve attomolar sensitivity without relying on expensive equipment.[17]

In this study, we established two novel detection assays combining the CRISPR—Cas13a system with PCR amplification or recombinase-aided amplification (RAA) techniques to assist in the detection of CRKP. Furthermore, using a FAM-biotin-labeled reporter in the RAA-CRISPR method, in which the results can be visualized via a lateral flow test strip readout, will aid in rapid CRKP screening and prevent widespread transmission in underdeveloped countries.

Methods

CRISPR—Cas13a-based assay for the detection of CRKP

Sputum, urine, blood and other clinical specimens were treated by conventional bacterial culture to obtain single colonies. After DNA extraction, the bacterial DNA was

analyzed by PCR/RAA-CRISPR–Cas13a to detect KP DNA to identify *K. pneumoniae*; blaKPC and blaNDM were detected to determine if the isolates carried the two carbapenemase genes, and the results are displayed by two models, fluorescence signal readout and lateral flow strip readout (Fig. 1a).

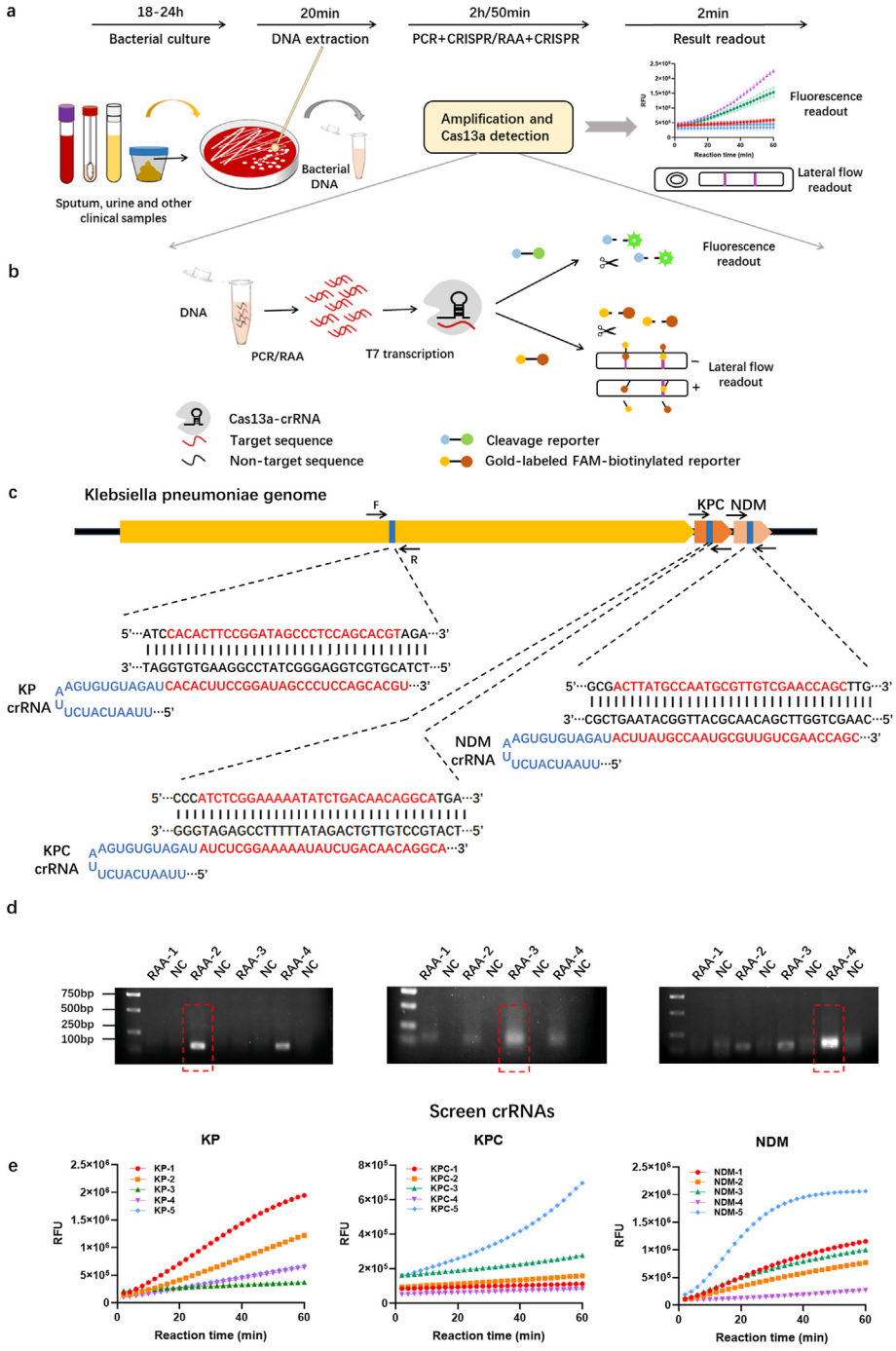

Figure 1. CRISPR–Cas13a-based assay for the detection of CRKP. (a) Schematic of the workflow for CRKP detection via the CRISPR–Cas13a-based assay. **(b)** Schematic description of PCR/RAA-CRISPR detection. Target sequences were amplified by PCR/RAA and then by CRISPR-based detection. When the amplicon is mixed with the Cas13a-crRNA complex, the cleavage reporter or the FAM-biotin reporter is trans-cleaved, and the results are visualized with a fluorescent readout or a lateral flow strip. **(c)** Visualization of KP, KPC, and NDM crRNA in the CRKP genome. The genome of CRKP was used as a reference, and blaKPC and blaNDM genes were mostly carried as plasmids in the KP genomic sequence. CrRNAs are indicated by blue rectangles. **(d, e)** Screening of RAA primers and crRNAs for KP, KPC, and NDM DNA detection by agarose gel electrophoresis. RAA1-4 refers to RAA primers 1–4 that were designed for the three target genes. RFU, relative fluorescence units.

Design and synthesis of nucleic acids and crRNA

All KPC and NDM genomic sequences were downloaded from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) Genbank, and the sequences were aligned and analyzed using MEGA7.0 and Jalview to obtain conserved sequences. We used the *K. pneumoniae* hemolysin (khe) gene as a target gene to identify *K. pneumoniae* species. Two hundred to 600 base pairs were selected from the khe gene and KPC/NDM respective conserved sequences to be cloned and inserted into PUC cloning vectors to construct recombinant plasmids as synthesized DNA targets by Sangon Biotech Co. (Shanghai, China). CrRNA sequences were also designed from the synthesized plasmid sequences, but the complete crRNA was obtained by synthesizing single-stranded DNA (Supplement Table 1) and then using a primer with a T7 promoter sequence for PCR amplification. The upstream and downstream primers for the crRNA DNA were mixed with T7 polymerase and incubated overnight at 37°C using the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs). The final step was to purify the crRNA with an RNA Rapid Concentration Purification Kit (Sangon Biotech).

Design and screening of PCR/RAA primers

Using the Primer 3 program, three pairs of PCR primers and four pairs of specific RAA primers were designed for the khe gene and conserved zone of the KPC and NDM genomic sequences (Supplement Table 2) and synthesized by Sangon Biotech Co. (Shanghai, China). All primers were screened for amplification efficiency in optimization experiments.

PCR and RAA reactions

The PCR mixture contained 14.5 μL of PCR mix (Biomed), 2 μL (1 mM) of sense and antisense primers and 2 μL of template DNA, and nuclease-free water was added to 25 μL. The reaction was performed as follows: a pre-denaturation step at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 50 s; a final extension step of 72°C for 5 min; ending at 4°C. Nuclease-free water was used as the negative control.

The RAA amplification kit used was provided by Hangzhou ZC Biotechnology Co., Ltd. (Hangzhou, China). The final total volume was 50 μL, including 2 μL of F (10 mM) and 2 μLof R (10 mM) and $MgCl_2$. The reaction was performed at 37°C for 30 min, and the negative control was nuclease-free water.

Target detection

Fluorescence signal detection

According to a previous study, the following procedure was used for detection.[18] The reaction system contained 2.5 mM NTP MIX (Sangon Biotech Co.), 2 μL of murine RNase inhibitor (New England Biolabs), 20 mM HEPES solution, 0.5 μL of T7 RNA polymerase (New England Biolabs), 0.25 μL of 10 mM $MgCl_2$, 22.5 nM crRNA, 45 nM Cas13a (Hangzhou ZC Bio-Sci &Tech Co., Ltd. In Hangzhou, China), 125 nM quenched fluorescent reporter RNA (RNAse Alert, Thermo Scientific, Waltham, MA, USA) 5 μL of target nucleic acid. The fluorescence signal was recorded every 2 min at 37°C for 1 h.

Lateral flow readout

The test line (T) was coated with streptavidin (Thermo Fisher Scientific, Waltham, MA, USA), and the control line (C) was coated with goat anti-rabbit immunoglobulin G (IgG) (Solarbio, Beijing, China). The products were purchased from Hangzhou ZC Biotechnology Co., Ltd. (Hangzhou, China). According to a previous study, after the amplification step was completed, 5 μL of amplicon was combined with 45 μL of CRISPR reaction mixture and incubated for 30 min at 37°C. The mixture contained 2.5 mM NTP MIX (Sangon Biotech Co.), 2 μL of murine RNase inhibitor (New England Biolabs), 20 mM HEPES solution, 1 μL of T7 RNA polymerase (New England Biolabs), 0.5 μL of 10 mM $MgCl_2$, 22.5 nM crRNA, 25 nM Cas13a (ZC Biotechnology Co.), 2 nM reporter RNA (5′6-FAM/UUUUUUUUUUUUUUUUU UUU-Bio/3′) and RNase. After completion of the CRISPR reaction (37 °C for 30 min), the product was transferred to the sample pad of the lateral flow strip, and the result was visualized with the naked eye after approximately 2 min.[18]

Evaluation of the CRISPR–Cas13a-based detection assay

The synthetic target DNA and positive samples were serially diluted 10-fold in water to concentrations of 10^4, 10^3, 10^2, 10^1, and 10^0 copies/μL and amplified by PCR-CRISPR and RAA-CRISPR detection to evaluate sensitivity. Several common gram-negative strains (*Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) and other carbapenemase genomes were amplified by PCR or RAA for CRISPR–Cas13a detection to evaluate specificity.

Quantitative PCR (qPCR) analysis

To compare the Cas13a assay to qPCR methods, we serially diluted the synthesized target DNA and implemented qPCR using an ABI ViiA7 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). qPCR was implemented using specific primers and a probe that we designed by Primer 3 (Supplement Table 3). The primers and probes were designed by Primer 3. The reaction conditions were as follows: denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s and annealing at 58°C for 45 s.

NG-test CARBA 5

NG-Test® CARBA-5 (NG Biotech, Guipry, France) is a lateral flow immunoassay for the detection of the five most common carbapenemase families (KPC, NDM, IMP, VIM and OXA-48).

Detection of simulated environmental sample

After serial dilution of a single colony, bacterial liquids of different concentrations were obtained, sprayed onto a clean table, dried on the surface for 2 h and sampled with cotton swabs. Individual colonies of bacterial cultures were diluted with 5 ml of normal saline followed by serial 10-fold dilutions to obtain 1∼6 tubes with different concentrations of bacterial liquid samples, sprayed onto a clean area (the area was 20 $cm^{2)}$, and sampled with cotton swabs. After sampling with a cotton swab, 5 ml of normal saline was added and mixed well, and 500 μL of bacterial liquid was used for plate coating. At the same time, 1 ml of bacterial solution was used to extract DNA for CRISPR detection.

Cultivation, DNA extraction, and clinical sample pretreatment method

CRKP strains were obtained from the clinical laboratory of Beijing Youan Hospital Capital Medical University and Beijing Chui Yang Liu Hospital, and purified bacterial colonies were obtained after incubation on solid medium at 37°C for 18 h—24 h. A single colony was selected and placed into an Eppendorf (EP) Micro Test Tube containing Luria—Bertani (LB) liquid medium. The bacterial DNA was extracted using a bacterial genome extraction kit (method establishment section) or the boiling method (clinical validation section), and the bacterial DNA was stored in a −80°C freezer for use. The CFU in the culture solutions were counted by a hemocytometer and by the flat colony counting method, and the culture solutions were diluted to the desired concentration and stored at −80°C.

Statistical analysis

GraphPad Prism 8 was used for the calculation of means and standard deviations. All of the statistical tests were two-sided, and $p < 0.05$ was considered indicative of statistical significance. The fluorescence signal of the CRISPR reaction was expressed as the mean of ≥3 technical replicates ± SD, and the bars represent the mean ± s.e.m.

Other methods are detailed in the supplementary materials.

Results

Confirmation of the efficient primers and crRNA for KP, KPC and NDM gene detection

To obtain efficient primers and crRNAs applicable to detect target DNAs, we designed and compared the amplification effects of PCR primers (F1—F3), RAA primers (F1—F4) and the detection efficiency of five crRNAs for the detection of KP gene, the KPC gene and the NDM gene (Fig. 1d, e, Fig. S1). We determined the amplification or detection efficiency by observing the agarose gel electrophoresis results after RAA amplification of synthesized DNA targets, and the fluorescence signal allowed CRISPR—Cas13a-based detection. For KP detection, KP-PCR-3 and KP-RAA-2 showed higher amplification efficiency (Fig. 1d, Fig. S1). Similarly, KP-1crRNA yielded higher fluorescence signals than the other four crRNAs (Fig. 1e). Hence, KP-PCR-3, KP-RAA-2 and KP-1CrRNA were finally selected for subsequent detection. Similarly, we selected KPC-PCR-3, KPC-RAA-3 and KPC-5crRNA to detect the blaKPC gene. NDM-PCR-2, NDM-RAA-2 and NDM-5crRNA were chosen for the detection of the blaNDM gene.

Optimization of the RAA-CRISPR—Cas13a assay for accurate detection of CRKP

To further improve the detection sensitivity, we optimized the RAA- CRISPR—Cas13a detection system to determine some reaction conditions of the detection process based on the final fluorescence signal intensity of the assay. Some factors that may affect the reaction were accounted for, such as the temperature of RAA, reaction time, quantities of the templates and steps of bacterial DNA extraction. To summarize the optimization results, we chose to add 6 μL of template DNA in all further studies and incubated the samples at 37°C for 20 min (Figs. S2a, b, c). For optimization of the extraction steps for bacterial DNA, we finally chose to wash the bacterial solution a second time for extraction (Fig. S2d).

KP, blaKPC and blaNDM gene detection by PCR/RAA-CRISPR

To evaluate the limit of detection (LOD) of these methods, 10-fold serial dilutions of the synthetic target DNA were tested by PCR-CRISPR and RAA-CRISPR. The results suggested that in the PCR-CRISPR detection system, 1 copy per test of target DNA could be detected for each of the three target DNA samples within 30 min (Fig. 2a). In the RAA-CRISPR detection system, the LOD was shown to reach 1 copy/μL for the KP and blaKPC genes, and 10^1 copies per test of blaNDM gene could be detected (Fig. 2b). Moreover, we observed the results of qPCR and found that the LOD was 10^2 copies/μL for all three target DNAs (Fig. 2e). In contrast, our method could achieve high sensitivity for the three target DNAs.

To further simulate clinical applications, we used bacterial genomic DNA (10-fold serial dilutions of extracted bacterial DNA) instead of synthetic DNA to complete PCR-CRISPR and RAA-CRISPR detection. The results showed a consistent LOD compared with synthetic DNA. In the PCR-CRISPR detection system, for the three target DNAs, the LOD reached 1 copy/μL. In the RAA-CRISPR detection system, the LOD of this method was 10^1 copies/μL. Moreover, bacterial DNA was extracted from 1 ml of culture medium of KP at different concentrations (0, 1, 10^1, 10^2, 10^3 and 10^4 CFU/ml) for RAA-CRISPR detection, and we found that culture solutions with 1 or more CFU/mL KP exhibited meaningful fluorescence signals with fluorescence signal readouts (Fig. S4a).

RAA-CRISPR combined with a lateral flow visualization system

To realize the portability and visualization of detection, the lateral flow strip was introduced as an ideal signal readout

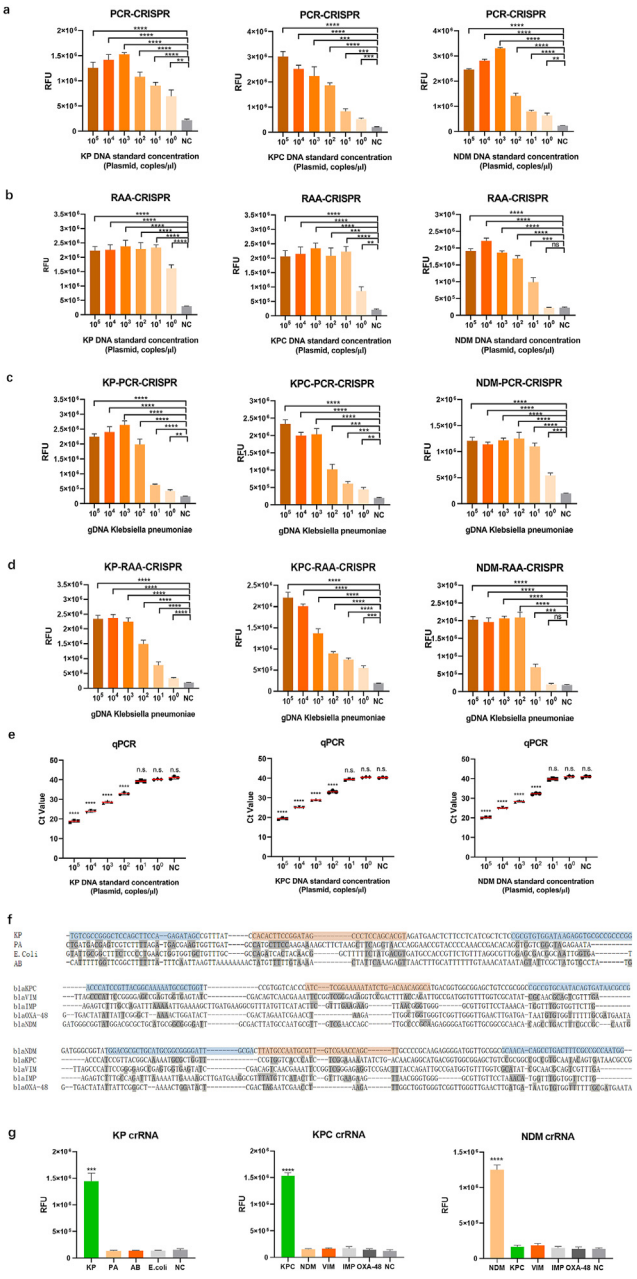

Figure 2. Determination of the LOD of the PCR/RAA-CRISPR assay and the specificity of the CRISPR–Cas13a assay. (a, b) LOD of the PCR/RAA-CRISPR assay for the different synthetic DNA targets. **(c, d)** LOD of the PCR/RAA-CRISPR assay for bacterial gDNA containing the target sequence. The fluorescence signal was collected after PCR or 20 min of RPA and 30 min of CRISPR detection. **(e)** LOD of the qPCR assay for the different synthetic DNA targets (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, ****P < 0.0001; the negative control is nuclease-free water. **(f)** Specificity analysis of primers and crRNA. For the detection of the KP gene, alignment of the region associated with the primer and crRNA with other common gram-negative bacterial genomes, including those of *E. coli*, *P. aeruginosa* and *A. baumannii*, was performed. For the detection of the blaKPC gene and blaNDM gene. Alignment of the region associated with the primer and crRNA with other common carbapenemase sequences, including the OXA-48, VIM, the IMP gene sequences, was performed. RAA primers and crRNAs are indicated by blue and orange rectangles, respectively. **(g)** The specificity of the CRISPR–Cas13a assay. Shown from left to right are the cross-reaction detection results for KP crRNA, KPC crRNA and NDM crRNA for nontarget sequences. Fluorescence signals were collected after 30 min of CRISPR reaction in the cross -reaction tests. DNase/RNase-free water was used as the negative control. *p < 0.05, **p < 0.01, ***p < 0.001, ****P < 0.0001; the negative control is nuclease-free water.

method. The test line was coated with avidin, and the control line was coated with anti-FAM antibody. Upon recognition of the target DNA, the cleavage function of the cas effector protein is activated and the reporter molecule is cleaved. The biotin is captured in the test line, the reporter molecule continues to flow to the control line, and the anti-FAM antibody binds to the reporter molecule, showing a colorimetric signal. When the target DNA is not recognized, avidin can capture the reporter molecule on the test line, and the anti-FAM antibody can also capture the colorimetric signal on the control line (Fig. 3b). Tenfold serial dilutions of the synthetic dsDNA targets were tested, and the LOD reached 10^1copy/μL in RAA-CRISPR detection with lateral flow (Fig. 3c). The signal intensities of the C- and T-bands were generated using ImageJ software (Fig. S3). For the detection of different concentrations of bacteria in culture solutions, 10^1 or more CFU/mL KP do not cause the appearance of a visible stripe in the lateral flow strip. Hence, the fluorescence readout may be more sensitive than the lateral flow strip readout, but the latter is more portable than the former in terms of testing equipment and operations (Figs. S4a, b).

Specificity of the PCR/RAA-CRISPR assay

All of the primers and crRNA sequences involved were specific to the target DNAs to minimize the off-target affinity to other easily confused and common bacterial or carbapenemase genomes. To evaluate the specificity of this method, several common Gram-negative strains were collected as the sample to experiment with our methods, including *E. coli*, *P. aeruginosa* and *A. baumannii*. Similarly, the synthetic KPC, NDM, VIM, IMP and OXA-48 DNA were also applied to conduct the cross reactions. All test results showed no cross reaction with any related genes (Fig. 2f and g).

Comparison of PCR-CRISPR and RAA-CRISPR with a fluorescence signal readout, the RAA-CRISPR assay with a lateral flow strip readout and the NG-test CARBA 5 method using CRKP strains

Given the excellent performance of this method, the test was further used to detect clinical strains. Sixty-one clinical strains were collected and 51 of these strains were definitively identified as CRKP by mCIM/eCIM, the remaining 10 strains were carbapenem-sensitive strains (including *E. coli*, *A. baumannii*, and *P. aeruginosa* strains). The 51 strains include 41 strains in which the gene carriage status of carbapenemase was not clear and 10 CRKP strains

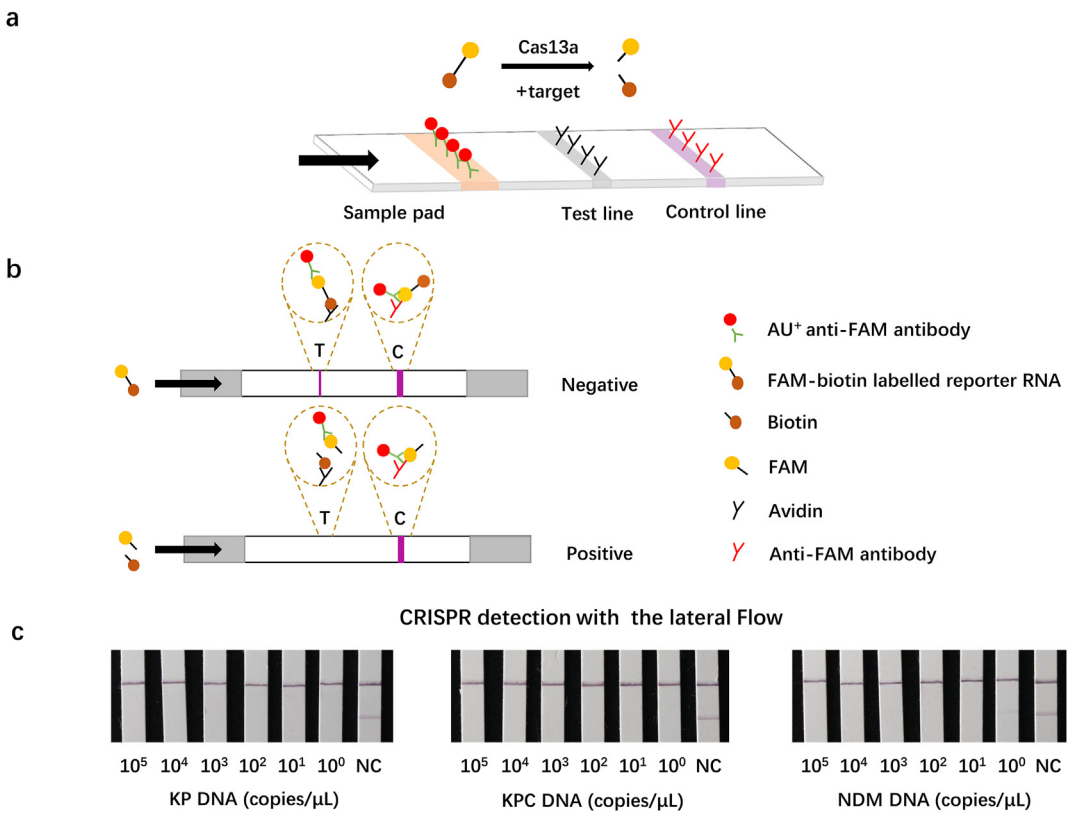

Figure 3. Lateral flow strip based on CRISPR–Cas13a detection. (a) The structure of the lateral flow strip. Samples flow from the sample pad to the colloidal gold pad, followed by the test line and control line. The test line was coated with avidin, and the control line was coated with anti-FAM antibody. **(b)** Schematic of CRISPR–Cas13a detection with the lateral flow strip and the colorimetric outcomes for positive and negative samples. When the target DNA was not identified, the gold-labeled FAM-biotin-labeled reporter conjugate flowed to the test line, and the superfluous gold nanoparticles continued to flow to the control line. Once recognition of the target DNA occurred, the reporter conjugate was cleaved, was not captured in the test line and flowed to the control line. **(c)** LOD of the RAA-CRISPR assay with the lateral flow strip.

carrying the NDM carbapenemase gene, as shown by qPCR (6 $bla_{NDM\text{-}1\text{-}positive}$ isolates and 4 $bla_{NDM\text{-}5\text{-}positive}$ isolates). These strains were tested blindly by PCR-CRISPR and RAA-CRISPR fluorescence detection, RAA-CRISPR strip detection, NG-test CARBA 5 detection and qPCR. At the same time, we analyzed the advantages and disadvantages of different methods according to the detection results (Table S6). Of the 51 CRKP strains, the results showed that 47 CRKP-positive strains were detected using the above five methods, and the positive coincidence rate of CRISPR detection in the detection of CRKP was approximately 92.16% (47/51) (Table 1). Four negative strains were identified as the strains that did not carry blaKPC and blaNDM genes through qPCR, and the remaining ten strains were considered non-CRKP strains without KP, blaKPC and blaNDM genes detected (Fig. 4b, c, d, Figs. S5a and S6a). Among the 61 clinical strains, 51 strains showed positive results for the KP gene by the PCR-CRISPR assay and RAA-CRISPR assay, in accordance with expectations. For the detection of the blaKPC gene, 36 positive samples were detected by the above five methods, and the sensitivity and specificity of the KPC gene assay was 100% (Figs. S5b and S6b and Table S4). Interestingly, the NG-test CARBA 5 method showed a weakly positive sample (sample No.6), but our results still showed significant differences in fluorescence signal or band depth compared with the negative control (Fig. 4c, e, Figs. S5b and S6b). For the detection of the blaNDM gene, five methods indicated that 11 samples carried the blaNDM gene, and the sensitivity and specificity of the blaNDM gene assay was 100% using qPCR as the gold standard (Fig. 4e, Figs. S5c and S6c and Table S5).

CRISPR–Cas13a-based assay for the detection of KP, blaNDM and blaKPC carbapenemase genes in simulated environmental samples

To determine suitability for clinical application, we simulated the detection of bacterial samples in the environment using a single colony obtained by culture (Fig. S7a). Bacterial culture and CRISPR detection were simultaneously performed on the collected samples. The results showed that a 1 CFU/cm^2 environmental sample could be used for detection of the KP, and an 8 CFU/cm^2 environmental sample could be used for detection of the blaKPC gene and blaNDM gene (Fig. S7b, Fig. S8). Interestingly, with very low concentrations of samples such as that in the "5" tube (less than 1 CFU/cm^2), the KP was detected by the CRISPR assay

Table 1 The detection of CRKP in 61 clinical strains.

		mCIM/eCIM		Sensitivity	Specificity	PPA	NPA
		Positive	Negative				
PCR-CRISPR- Cas13a assay with fluorescence readout	Positive	47	0	92.16%	100%	92.16%	100%
	Negative	4	10				
	Total	51	10				
RAA-CRISPR- Cas13a assay with fluorescence readout	Positive	47	0	92.16%	100%	92.16%	100%
	Negative	4	10				
	Total	51	10				
RAA-CRISPR-Cas13a assay with lateral readout	Positive	47	0	92.16%	100%	92.16%	100%
	Negative	4	10				
	Total	51	10				
NG-test CARBA 5	Positive	47	0	92.16%	100%	92.16%	100%
	Negative	4	10				
	Total	51	10				
qPCR	Positive	47	0	92.16%	100%	92.16%	100%
	Negative	4	10				
	Total	51	10				

The 61 clinical strains (including 51 CRKP strains and 10 carbapenem-sensitive strains) were collected for clinical validation. PPA, positive predictive agreement; NPA, negative predictive agreement; The gold standard for KP, KPC and NDM DNA is qPCR.

even when no single colony was obtained after 24 h, and after 48 h of bacterial culture, few colonies grew on solid medium (Fig. S8).

Discussion

In this study, we developed a PCR/RAA-CRISPR−Cas13a-based detection platform to assist in CRKP detection. We tested the assay for the detection of KP. KPC and NDM, as the two major types of carbapenemases produced in CRKP worldwide were tested to identify the production of carbapenemase. Therefore, we constructed a multi-detection system to assist in the detection of CRKP. In our method, the sensitivity of the PCR-CRISPR detection system was able to reach an LOD of 1 copy/μL with the fluorescence readout for the different target DNAs. The sensitivity of the RAA-CRISPR detection system reached an LOD of 10 copies/μL with the fluorescence readout and the lateral flow strip readout for the different target DNAs.

Currently, the emergence of CRKP strains is a global public health problem, especially in endemic areas.[19] Different studies have reported that the mortality rate associated with CRKP infection could reach 30—60% (except for urinary tract infection).[20—23] Appropriate treatment time is an independent predictor of the 30-day mortality in patients with bloodstream infection caused by carbapenemase-producing KP.[24] However, it is currently difficult to initially identify CRKP infection and initiate effective antibiotic treatment for critically ill patients within the first 24 h due to the need for additional overnight susceptibility testing. Our method described here has a short turnaround time (within 24 h) and incorporates a convenient result readout format, such as the lateral flow strips. It is of great significance for critically ill patients who cannot wait for the results of drug sensitivity testing. Compared to other methods for the detection of carbapenemases at the genetic level, such as RT−PCR or antigen detection, our method shows the advantage of ultra-sensitivity and specificity and no requirement for complicated equipment or expensive raw materials (the test strip costs less than $1). This will facilitate rapid screening and diagnosis of CRKP in communities or hospitals in developing or underdeveloped countries. Moreover, the method established in this study can improve the antimicrobial resistance monitoring system and provide a new tool for the detection of carbapenemase resistance in communities or underdeveloped areas, which can help in the rapid identification of resistant strains in the clinic, allowing improvement of the therapeutic regimen and control of nosocomial infections in a timely manner. Notably, a specific crRNA was designed to detect the KP due to the complexity of bacterial species after the initial bacterial culture of the sample, which is an obvious advantage of our approach and will facilitate confirmation of bacterial type through genetic testing.

Moreover, we tested 61 clinical strains isolated from clinical samples, and the results indicated that the positive coincidence rate of the CRISPR−Casa assay was 92.16%. According to the detection results for the carbapenemase gene in the CRKP strains that we collected, we found that the blaKPC carbapenemase gene was still the most frequently carried carbapenemase gene in CRKP strains, and this conclusion was consistent with previous research.[25] Notably, blaKPC and blaNDM genes also exist in bacteria other than KP, such as other Enterobacteriaceae bacteria, *A. baumannii* and *P. aeruginosa*.[25,26] The method developed in this study can be used to detect blaKPC and blaNDM genes in different bacteria.

This assay had several limitations. First, although the blaKPC and blaNDM genes are the two carbapenemase genes most commonly carried by CRKP strains, missed diagnoses will still occur in clinical applications. However, this did not lead to a striking difference because these strains that produce other carbapenemase are so rarely

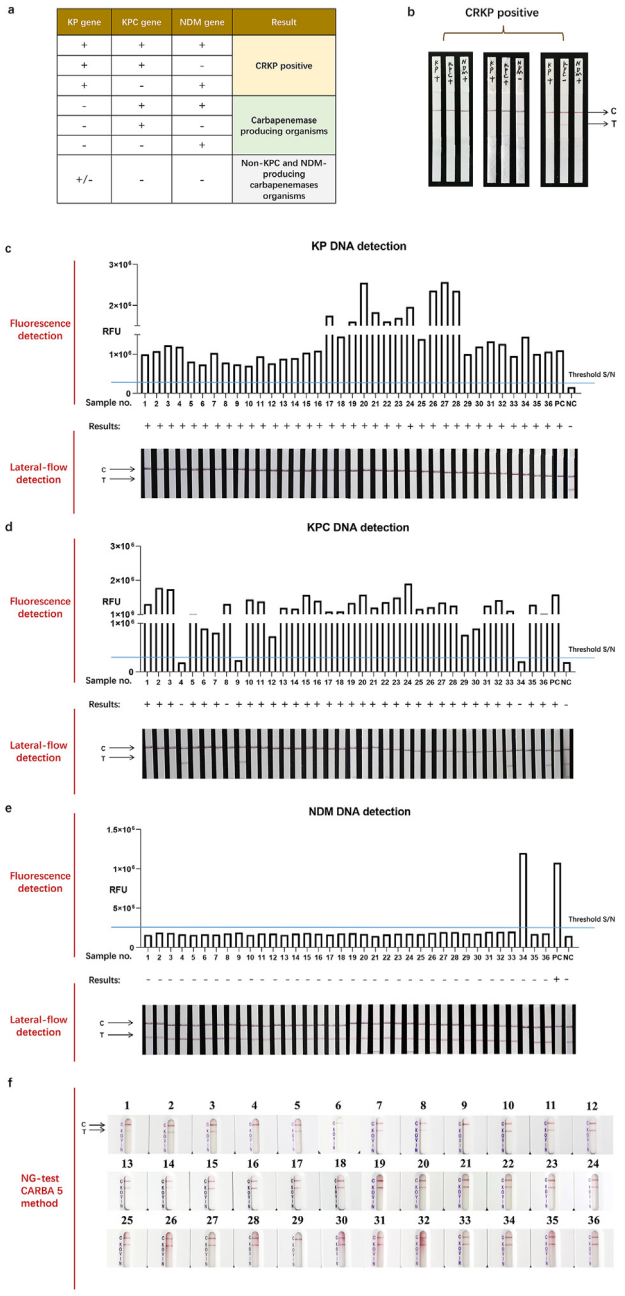

Figure 4. Detection of clinical samples with the CRISPR–Cas13a-assay and NG-test CARBA 5 method. (a) Display and interpretation of lateral flow strip assay readout results. A positive result requires detection of KP target and at least one of the two carbapenemase gene targets (blaKPC gene or blaNDM gene, as indicated in the color table) for DNA extracted from a single colony. **(b)** Lateral flow strip assay readout. A positive result demands detection of the KP gene and at least one of the two carbapenemase gene targets (blaKPC gene or blaNDM gene, as indicated in the illustration matrix). **(c, d, e)** Detection of CRKP strains by the CRISPR–Cas13a-assay with the fluorescence signal readout and lateral flow strip readout. For the fluorescence signal readout, we set the threshold of fluorescence signal intensities (the blue line; with noise being the fluorescence signal intensity from a negative control with nuclease-free water as input tested in parallel); if the fluorescence signal exceeded that, we considered it positive. **(f)** The detection of CRKP strains by the NG-test CARBA 5 method.

detected. Second, a few CRKP strains may be missed because they have other resistance mechanisms for drug resistance, such as the production of β-lactamase combined with membrane impermeability, without carrying carbapenemase genes.[27] Further studies will focus on combining microfluidic technology to achieve multichannel detection and include different types of carbapenemases into the detection processes for convenience. Additionally, a recent study found that the process of RAA and CRISPR can be accomplished in the same system to realize "one-step" detection. RAA isothermal amplification technology is now commercially available and can be used for large-scale applications.[28]

Conclusion

In summary, we developed two CRISPR-cas13a-based assays for KP and blaKPC and blaNDM carbapenemases gene detection to assist in the detection of CRKP. These assays provide a portable and visual tool for CRKP detection without specialized equipment, which can aid patients in acquiring appropriate prescriptions and provide opportunities for hospitals and communities to implement infection control measures in a timely manner to prevent CRKP dissemination.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.10.010.