Infection and Drug Resistance

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METHOD

Cas12a/Guide RNA-Based Platform for Rapidly and Accurately Detecting *bla*$_{KPC}$ Gene in Carbapenem-Resistant *Enterobacterales*

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Purpose: Accurate detection and identification of pathogens and their associated resistance mechanisms are essential prerequisites for implementing precision medicine in the management of Carbapenem-resistant *Enterobacterales* (CRE). Among the various resistance mechanisms, the production of KPC carbapenemase is the most prevalent worldwide. Consequently, this study aims to develop a convenient and precise nucleic acid detection platform specifically for the *bla*$_{KPC}$ gene.

Methods: The initial phase of our research methodology involved developing a CRISPR/Cas12a detection framework, which was achieved by designing highly specific single-guide RNAs (sgRNAs) targeting the *bla*$_{KPC}$ gene. To enhance the sensitivity of this system, we incorporated three distinct amplification techniques—polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA)—into the CRISPR/Cas12a framework. Subsequently, we conducted a comparative analysis of the sensitivity and specificity of these three amplification methods when used in combination with the CRISPR/Cas12a system. Additionally, we assessed the clinical applicability of the methodologies by evaluating fluorescence readouts from 80 different clinical isolates. Furthermore, we employed lateral flow assay technology to provide a visual representation of the results, facilitating point-of-care testing.

Results: Following a comparative analysis of the sensitivity and specificity of the three methods, we identified the RPA-Cas12a approach as the optimal detection technique. Our findings demonstrated that the limit of detection (LoD) of the RPA-Cas12a platform was 1 aM (~1 copy/μL) for plasmid DNA and 5 × 10^3 fg/μL for genomic DNA. Furthermore, both the sensitivity and specificity of the platform achieved 100% upon validation with 80 clinical isolates.

Conclusion: These findings suggest that the developed RPA-Cas12a platform represents a promising tool for the cost-effective, convenient, and accurate detection of the *bla*$_{KPC}$ gene.

Keywords: Klebsiella pneumoniae carbapenemase, CRISPR-Cas12a, isothermal amplification, polymerase chain reaction, loop-mediated isothermal amplification, recombinase polymerase amplification, lateral flow strips

Introduction

Carbapenem-resistant *Enterobacterales* (CRE), defined as in vitro resistance to at least one carbapenem, was once considered the last-resort antibiotic against multidrug-resistant gram-negative pathogens.[1] In February 2017, as per the mandate from the World Health Organization (WHO), the position held by CRE is of utmost precedence (critical priority) in the sphere of novel and effective antibiotics development.[2] In the past decade, the incidence and prevalence of CRE were constantly high in the United States, Europe, the Middle East, and China.[3–6] Data from the China Antimicrobial Surveillance Network (CHINET, www.chinets.com) revealed a significant increase in the resistance rates of Klebsiella pneumoniae to imipenem and meropenem, rising from 3.0% and 2.9% in 2005 to 26% and 27.5% in 2022, respectively. In Europe, the prevalence of carbapenem-resistant Klebsiella pneumoniae was approximately 60% in Greece and 40% in

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Italy.[4,5] According to the Centers for Disease Control and Prevention (CDC), approximately 13,100 cases of CRE occurred in the United States in 2019.[3]

The carbapenem resistance of CRE is conferred by an increase in efflux pumps or a decrease in membrane permeability, alterations to the penicillin-binding protein, and the production of carbapenemase.[7,8] Reportedly, approximately 85% of CRE resistance mediates via the production of carbapenemases worldwide.[9] Between 2016 and 2018, pooled analyses from 36 hospitals across 24 provinces and cities in China demonstrated that the most prevalent carbapenemase among Klebsiella pneumoniae in adult patients was Klebsiella pneumoniae carbapenemase-2 (KPC-2), accounting for 64.6% (457/709) of cases.[6] Likewise, the production of KPC remains the most common resistance mechanism of CRE in the United States, Latin America, Italy, Greece, and the Middle East.[5,10–14]

The widespread dissemination of carbapenem-resistant Enterobacteriaceae (CRE) poses a significant public health threat. Numerous studies have indicated that CRE bloodstream infections are associated with adverse clinical outcomes, with mortality rates among immunocompromised patients ranging from 40% to nearly 70%.[15–19] A multinational prospective cohort study found that CRE was associated with prolonged hospital stay and increased mortality in patients with bloodstream infections in low-income and middle-income countries.[20] An economic prediction model revealed that the societal cost of each CRE infection is expected to reach between $59,692 and $86,940 in the United States.[21] In summary, CRE represent a severe threat to human health due to prolonged hospital stays, increased mortality rates, significant economic burden, and the limited availability of effective antibiotics.

Therefore, novel and effective antibacterial agents are urgently required to treat patients with CRE infections. In recent years, several new pharmaceutical compounds have successfully reached the market or entered the drug development pipeline. These include ceftazidime-avibactam (effective against both KPC and oxacillinase-48-like (OXA-48-like) Enterobacterales), meropenem-vaborbactam (effective against KPC Enterobacterales), imipenem-relebactam (effective against KPC Enterobacterales), plazomicin (effective against KPC and OXA-48-like Enterobacterales), eravacycline (effective against KPC and OXA Enterobacterales), cefiderocol (potent against isolates with all classes of carbapenemases, ie, class A, B, C, and D), and aztreonam-avibactam (effective against KPC, New Delhi metallo-β-lactamase (NDM), and OXA Enterobacterales), which offer opportunities for employing precision medicine to target antibiotic therapy against specific CRE resistance mechanisms.[22–24] Hence, accurate detection and identification of pathogens and their resistance mechanisms is considered a prerequisite to ensure precision medicine and the appropriate antibiotic.[25]

The standard approaches used for carbapenemase detection in Enterobacterales isolates can be divided into phenotypic methods and molecular techniques. The phenotypic detection methods from CLSI recommendations include CarbaNP, mCIM, and eCIM, which detect the hydrolysis of carbapenems in vitro. However, despite meeting the criteria for carbapenemase detection standards, the culture-based method has several drawbacks, including being time-consuming, generating excessive medical waste, and having limited sensitivity due to the absence of typical bacterial colonies. Several carbapenemase gene tests have gotten to the market, including Unyvero P55 Pneumonia Cartridge (Curetis AG), GeneXpert Carba-R assay, FilmArray® Blood Culture Identification Panels (BioFire Diagnostics LLC, Salt Lake City, UT) and Verigene® Gram-negative blood culture test (Nanosphere, North-brook, IL). Despite these molecular detection methods being sensitive and specific, most require highly costly reagents, professional personnel, and sophisticated machinery that make them unsuitable for deployment in resource-limited settings.

Therefore, this study aimed to develop an affordable and convenient *KPC* gene detection method suitable for deployment in underdeveloped areas. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR) and Cas (CRISPR-associated proteins) system has been developed for nucleic acid detection. The non-specific RNA-guided DNase activity of Cas12a, which is one of the Cas proteins, can be precisely activated after a specific cis target hybridizes with a sgRNA-programmed Cas12a. This enables Cas12a to cleave single-stranded DNA probes for nucleic acid detection.[26] Furthermore, when combined with nucleic acid amplification techniques, the CRISPR-Cas12a system can achieve high sensitivity in detecting target DNA, with single-base resolution.[27]

Recombinase Polymerase Amplification (RPA), Loop-mediated Isothermal Amplification (LAMP), and Polymerase Chain Reaction (PCR) represent distinct DNA amplification techniques, each possessing unique applications and advantages. RPA operates at room temperature, utilizing specialized enzymes such as DNA polymerase and Single Strand Binding proteins (SSB).[28] This method allows for rapid DNA amplification typically within 30 minutes,

simplifying the process by eliminating the need for thermal cyclers. LAMP proceeds at a constant temperature, employing Bst DNA polymerase and highly specific primer sets. Compared to PCR, LAMP can amplify DNA more quickly, offering high specificity and sensitivity.[29] The reaction's end product, characterized by a visible white precipitate, can be directly observed. PCR, on the other hand, replicates specific DNA fragments through precise temperature cycling including denaturation, annealing, and extension phases. This technique is crucial in genetic research and disease diagnostics.[30] Together, these technologies provide robust tools for contemporary biotechnology and medical research.

In this study, we developed an affordable and precise nucleic acid detection method for bla_{KPC} gene detection using the CRISPR/Cas12a system in combination with PCR, LAMP, and RPA amplification techniques. In addition, real-time and endpoint fluorescence and lateral flow test strip technology were employed to visualize the results.

Materials and Methods

Design and Synthesis of Nucleic Acid Targets and sgRNAs

The bla_{KPC} gene sequences were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). Multiple bla_{KPC} gene sequence alignments were performed using MEGA 7 software (www.megasoftware.net). After the sequence alignment, corresponding specific sgRNAs were designed based on the conserved sequences of the bla_{KPC} gene that match the Cas12a protospacer adjacent motif (5′-TTTN). Subsequently, the sgRNA was synthesized by Takara Bio (Beijing, China). The specificity of the target sequence for the bla_{KPC} gene and the sequence alignment was examined using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition, a partial sequence of the bla_{KPC} gene was synthesized by Sangon Biotech (Shanghai, China), cloned into *pET-28a* vectors, and transformed into DH5α Competent Cells. The recombinant pET-28a vector containing the bla_{KPC} gene was extracted using a mini-prep plasmid extraction kit (Qiagen, Hilden, Germany).

Sequence Analysis

To design primers for detecting the five major families of carbapenemase genes (bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, and $bla_{OXA\text{-}48\text{-}like}$), reference sequences were obtained from the GeneBank database (https://www.ncbi.nlm.nih.gov/genbank/). Primer design was conducted using PrimerV5 (http://primerexplorer.jp/lampv5e/index.html), adhering to criteria such as no mutation sites at the 3'-end of the forward primer, optimal Tm values, terminal stability, and the absence of secondary structures. These principles ensure that the primers can theoretically detect a broader range of reported variants within each carbapenemase gene family compared to previous studies. According to the Beta-Lactamase DataBase (BLDB) (http://www.bldb.eu/Enzymes.php), bla_{NDM} has 31 variants, bla_{KPC} has 79, bla_{IMP} has 89, and bla_{VIM} has 73. The sequences of $bla_{OXA\text{-}48\text{-}like}$ mutants were aligned with $bla_{OXA\text{-}48}$. Our primer sets can detect all known variants of bla_{NDM} and bla_{KPC}, the bla_{IMP} group including $bla_{IMP\text{-}1}$ to $bla_{IMP\text{-}14}$, $bla_{IMP\text{-}22}$, $bla_{IMP\text{-}32}$, $bla_{IMP\text{-}33}$, $bla_{IMP\text{-}48}$, and $bla_{IMP\text{-}68}$, the bla_{VIM} group except for $bla_{VIM\text{-}7}$, $bla_{VIM\text{-}10}$, $bla_{VIM\text{-}51}$, and $bla_{VIM\text{-}65}$, as well as the $bla_{OXA\text{-}48\text{-}like}$ group including $bla_{OXA\text{-}48}$, $bla_{OXA\text{-}181}$, $bla_{OXA\text{-}232}$, $bla_{OXA\text{-}204}$, $bla_{OXA\text{-}162}$, and $bla_{OXA\text{-}244}$.

Cas12a Detection Reactions

The Cas12a trans-cleavage assay was carried out using the EnGen® LbaCas12a (NEB, Ipswich, MA, USA). First, 1 μM LbaCas12a was preassembled with 1.25 μM sgRNA (Takara Bio) and 10 U RNase inhibitor (Takara Bio, Beijing, China) at room temperature for 10 min. Subsequently, the DNA target was dissolved in 10× NEBuffer 2.1 (NEB) solution, then mixed with LbCas12a-sgRNA complexes and 500 nM custom ssDNA reporter (Sangon Biotech, Chengdu, China) in a 20μL reaction volume. The terminal concentrations of LbaCas12a and sgRNA within this solution were determined to be 50 and 62.5 nM, respectively. The 20-μL combined reaction solutions were then distributed to a 384-well microplate (Corning Life Sciences, Corning, NY, USA). Real-time and endpoint fluorescence signals were subsequently acquired using a fluorescence plate reader (Tecan Infinite 200 PRO, Tecan, Grödig, Austria), with measurements taken every 60 seconds over 60 minutes at 37°C (ssDNA FQ reporter = λex: 492 nm; λem: 522 nm). In addition, the Cas12a trans-cleavage signals were detected using lateral flow strips (LFS; Milenia Hybridetect 1; TwistDx, Cambridge, UK),

following the manufacturer's guidelines. This procedure involved the use of an ssDNA FB reporter, which was specifically modified with 6-FAM and biotin at both ends.

PCR and Cas12a Detection Reaction

Leveraging the sgRNA target sequences of the bla_{KPC} gene, appropriated PCR primers were conceived utilizing the Primer3Plus software (http://primer3plus.com/). Analysis of PCR primer specificity, accompanied by sequence alignment, ensured through the application of BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Thereafter, the bla_{KPC} target gene was subject to amplification using PCR with the application of the earlier established primer pairs. In the subsequent stage, the generated PCR product was meticulously examined through 1% agarose gel electrophoresis, followed by direct sequencing to ensure thorough analysis. In the ensuing steps, 1 μL of the unpurified PCR product, serving as the DNA target, was deployed in executing the Cas12a detection process.

LAMP and Cas12a Detection Reaction

By the sgRNA target sequence specific to the bla_{KPC} gene, the primers necessary for LAMP were engineered utilizing the PrimerExplorer v5 software (http://primerexplorer.jp/lampv5e/index.html). The distinctiveness of LAMP primers and sequence alignment were executed through the use of BLAST. The execution of the LAMP process was facilitated via a WarmStart® Colorimetric *LAMP* 2X Master Mix Kit (DNA & RNA; New England Biolabs, Hitchin, United Kingdom), abiding by the manufacturer's guidelines. Subsequently, 1 μL of the untreated LAMP amplified product was employed as the target DNA in the execution of the Cas12a detection assay.

RPA and Cas12a Detection Reaction

Utilizing the sgRNA target sequence of the bla_{KPC} gene as a reference, the primers for RPA were formulated through the use of Primer3Plus software, adhering to the procedural guidelines provided by the manufacturer (TwistDx). The specificity of the constructed RPA primers and sequence alignment were worked through using the BLAST. Furthermore, the experimental protocol for RPA was effectuated with the help of TwistAmp® Basic Kit (TwistDx, Cambridge, United Kingdom), strictly in compliance with the manufacturer's directives. In the subsequent step, 1 μL of the unpurified RPA amplified product was incorporated into the Cas12a detection assay.

Bacterial Strain Collection and Identification

In this investigation, a total of thirteen clinically verified whole-genome sequencing strains were utilized, encompassing 1 bla_{OXA-48}, 1 bla_{NDM-5}, 1 bla_{NMD-6}, 1 bla_{IMP-13}, 1 bla_{IMP-17}, 1 $bla_{VIM-232}$, 1 $bla_{VIM-212}$, 1 bla_{KPC-2}, 1 bla_{KPC-3}, 1 bla_{KPC-4}, 1 bla_{KPC-5}, 1 bla_{KPC-6}, and 1 bla_{KPC-7} (Table S1). These strains were acquired from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). Moreover, we collected 40 clinical isolates harboring the *blaKPC* gene and an additional 40 clinical isolates possessing various other β-lactamase genes, as detailed in Table S2. All these bacterial isolates were obtained from clinical samples provided by the microbiology laboratory of the specified institution. The extraction of genomic DNA from the bacterial strains was achieved by employing a Bacterial Genomic DNA Extraction Kit (TIANGEN, Beijing, China), following the manufacturer's guidelines. The identification of all clinical isolates was performed with a VITEK®2 COMPACT system (bioMérieux, Inc., Durham, NC, USA), while the bla_{KPC} gene was ascertained via PCR amplification and Sanger sequencing methods.

Evaluation of Sensitivity and Specificity

The sensitivity of the *CRISPR/Cas12*a system was evaluated using serially diluted plasmids (1–1000 nM) carrying the bla_{KPC} gene without any nucleic acid amplification steps. We incorporated PCR, LAMP, and RPA amplification methods into the *CRISPR/Cas12a* system to strengthen the sensitivity of the system. To choose the optimal bla_{KPC} gene identification method, we evaluated the sensitivity and specificity of the three different amplification methods combined with the *CRISPR/Cas12a* platform. The clinical performance of our method for bla_{KPC} gene identification was validated by analyzing 40 clinical isolates containing bla_{KPC} gene, and 40 clinical isolates containing other β-lactamase genes (Table S2). Furthermore, the sensitivity of the three platforms was evaluated using serially diluted plasmids (1–10^6 aM) carrying the bla_{KPC} gene. In addition, the specificity of

the three platforms for the bla_{KPC} gene was evaluated by testing DNA from the sequencing-verified strains carrying the bla_{KPC} gene, and non-bla_{KPC} bacterial strains (Table S3). Finally, the sensitivity of our method to bacterial strain was evaluated using serially diluted *Klebsiella pneumoniae* (carrying *blaKPC* gene) bacterial genomic DNA (50–5 × 10^{11} fg/μL).

Statistics

Cas12a detection assays were conducted in triplicate across three independent experimental repetitions. In every graphical representation, the mean values are displayed accompanied by the standard error of the mean (SEM). The construction of graphs and the execution of statistical evaluations were facilitated by employing GraphPad Prism v.8.1.2 software. (GraphPad Software, San Diego, USA).

Ethical Statement

Bacterial strain collection was according to the relevant clinical practice guidelines. Ethical approval was waived for this study, as all individual patient identifiers were removed during the experiments.

Results

Schematic Overview of the Study

Recently, two revolutionary DNA detection methods based on the Cas12a trans-cleavage activity, DETECTR and HOLMES, have been developed by two independent groups.[26,31] Both employed technological methodologies that displayed efficacy and accuracy in accurately discerning the intended DNA through the systematic development of target-oriented sgRNA. As a result, we postulate the establishment of a Cas12a-derived nucleic acid detection apparatus for the detection of the bla_{KPC} gene. In the present research, we devised tailor-made sgRNA focusing on the bla_{KPC} gene. As depicted in Figure 1, the initiation stage encompassed the employment of three prominent techniques, namely PCR, RPA, and LAMP, to amplify the selected DNA templates. In the subsequent step, the obtained amplicons underwent conjugation with the Cas12a/sgRNA molecular complex. In the formation of the ternary complex involving Cas12a/sgRNA/target DNA, the dormant fluorescent ssDNA reporter underwent trans-cleavage, initiating a fluorescent signal that can be observed with a plate reader. An alternative visualization method involved the employment of lateral flow strips, employing a FAM-Biotin-tagged ssDNA reporter (Sangon Biotech, Chengdu, China) for the interpretative result.

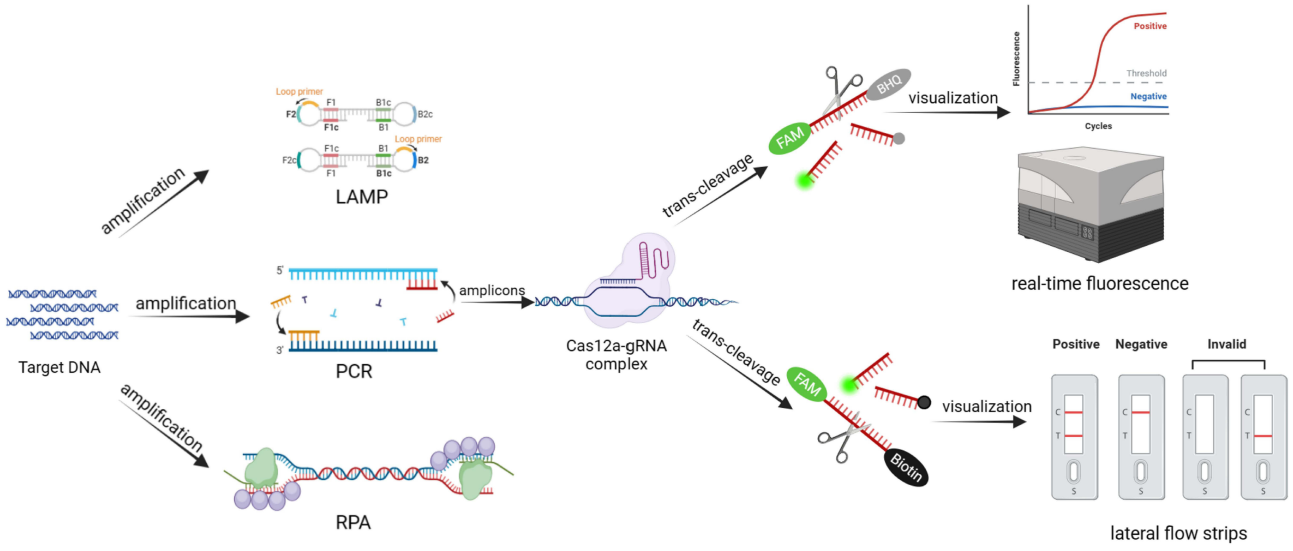

Figure 1 Schematic diagram overview of the study.

Limit of Detection of CRISPR/Cas12a Detection Platform and Activity Validation of Synthetic sgRNA

To develop the *CRISPR/Cas12a*-based detection platform and validate the activity of sgRNA, we used recombinant *pET-28a* plasmid containing partial bla_{KPC} gene as DNA target to perform Cas12a detection reaction. A comprehensive assessment of the sensitivity of the CRISPR/Cas12a system was conducted with the implementation of progressively diluted plasmids carrying the bla_{KPC} gene, with DNA concentrations ranging from 1 to 1000 nM, importantly, this was performed without any steps involving nucleic acid amplification. The investigation detailed in Figure 2 demonstrates a gradual intensification in fluorescence intensity of the Cas12a detection reaction as both reaction time and DNA concentration escalate, with the culmination almost reached at the 30-minute mark. Upon further analysis, it was uncovered that the detection threshold of the CRISPR/Cas12a platform attains levels at the 10-nM scale. Thus, we demonstrated that synthetic sgRNA has an ability to detect various concentrations of recombinant plasmids.

Sensitivity and Specificity of PCR, LAMP, and RPA Amplification Combined with the CRISPR/Cas12 Platform

To strengthen the sensitivity of the proposed assay, we incorporated PCR, LAMP, and RPA amplification methods into the *CRISPR/Cas12a* system. Furthermore, we compared the sensitivity of the three different amplification methods combined with the *CRISPR/Cas12a* system for the bla_{KPC} gene plasmid. The LoDs for the PCR-Cas12a-based, LAMP-Cas12a-based, and RPA-Cas12a-based methods were 100, 1, and 1 aM, respectively (Figure 3). Taken together, these Results indicate that both the RPA-Cas12a-based and LAMP-Cas12a-based methods demonstrated a sensitivity that was 200% greater than that of the PCR-Cas12a-based method for detecting the bla_{KPC} gene plasmid.

Specificity of RPA-Cas12a-based and LAMP-Cas12a-based detection methods for bla_{KPC} gene.To select the optimal detection method for the bla_{KPC} gene, we also evaluated the cross-reactivity of the different methods. For the cross-reactivity of RPA-Cas12a-based and LAMP-Cas12a-based detection methods for the bla_{KPC} gene, the specificity of the methods to the sequencing-verified strains carrying the bla_{KPC} gene, and non-bla_{KPC} bacterial strains (Table S3) were evaluated. As shown in Figure 4, the LAMP-Cas12a-based method showed cross-reactivity with the tested bacteria for the bla_{KPC} gene, whereas cross-reactivity was not observed in the RPA-Cas12a-based detection method.

Therefore, based on careful consideration of both sensitivity and specificity, the RPA-Cas12a-based method was selected as the optimal detection method for identifying the bla_{KPC} gene. The sensitivity of our method to the *Klebsiella pneumoniae* strain carrying the bla_{KPC} gene was subsequently analyzed, and the results showed that the LoD of the RPA-Cas12a-based detection method is 5×10^3 fg/uL bacterial genomic DNA.

Sensitivity of RPA-Cas12a Platform for Klebsiella Pneumoniae Strain Carrying bla_{KPC} Gene

Based on the above analysis, the RPA-Cas12a-based method was selected as the optimal detection method for the bla_{KPC} *gene*. To optimize our detection platforms, it is necessary to go one step further to observe the performance of bacterial

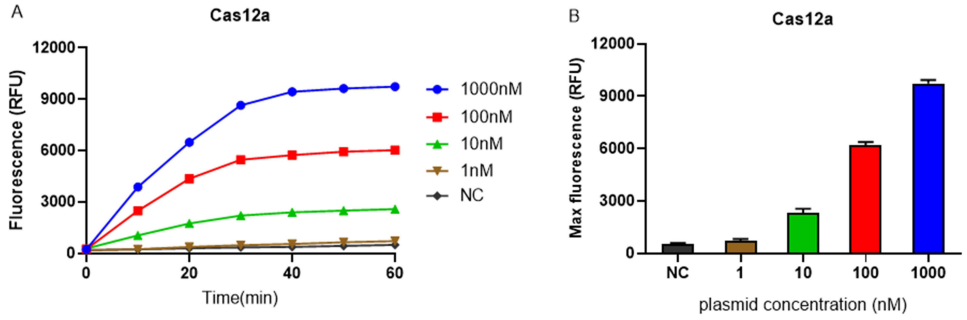

Figure 2 Limit of detection of CRISPR/Cas12a detection platform and activity validation of synthetic sgRNA. (**A**) The real-time fluorescence signals from 0 min to 60 min with different concentrations of target plasmids are shown. (**B**) The endpoint fluorescence signals with different concentrations of target plasmids are shown. Negative control (NC) utilized RNase-free water as input instead of target plasmid.

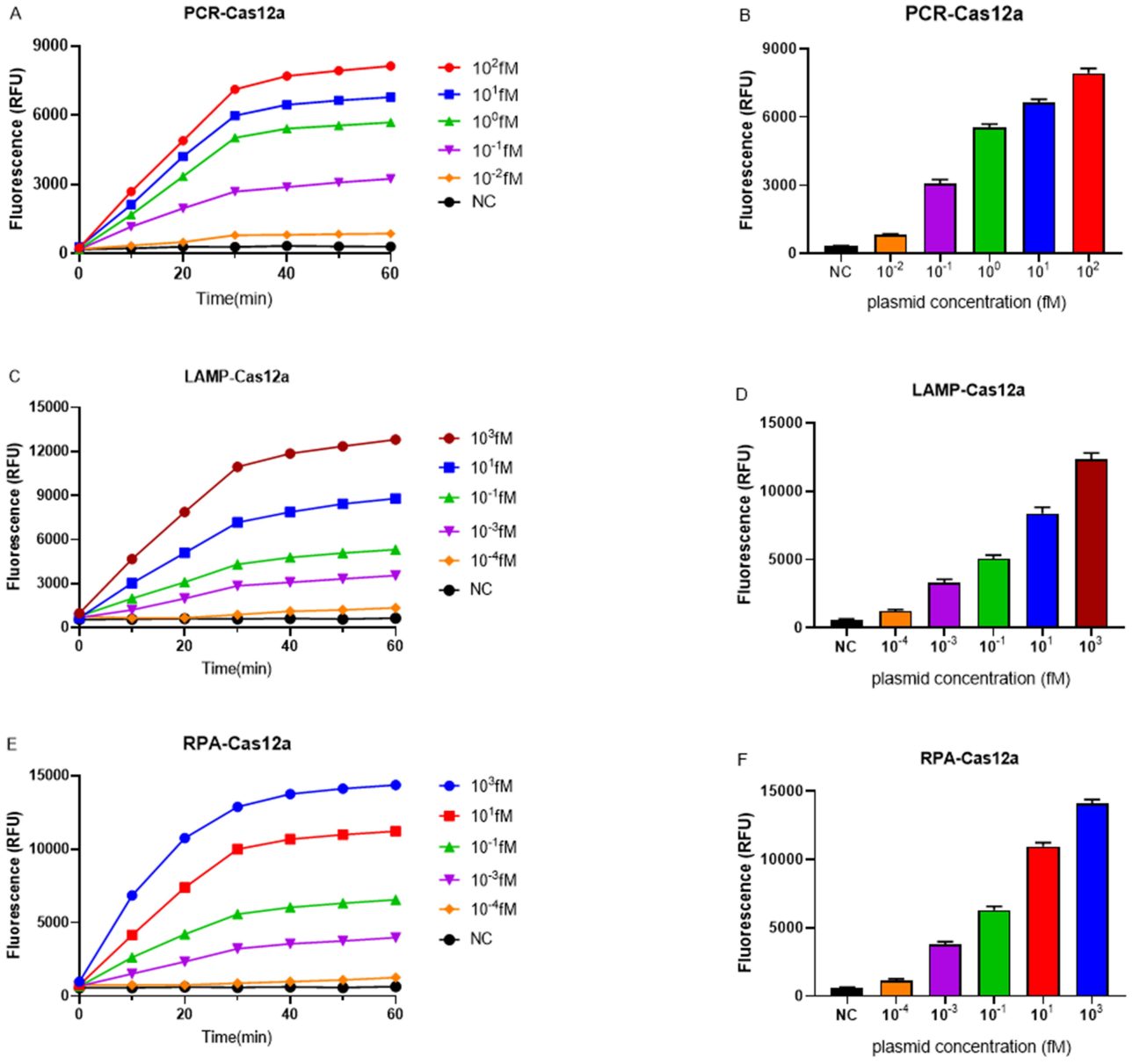

Figure 3 Sensitivity of PCR, LAMP and RPA combined with the CRISPR/Cas12 platform. (**A,C** and **E**) The real-time fluorescence signals of PCR-Cas12a, LAMP-Cas12a and RPA-Cas12a from 0 min to 60 min with different concentrations of the target plasmid are shown. (**B,D** and **F**) The endpoint fluorescence signals of PCR-Cas12a, LAMP-Cas12a and RPA-Cas12a with different concentrations of the target plasmid are shown. Negative control (NC) utilized RNase-free water as input instead of target plasmid.

strains. We first extracted the genomic DNA of the *Klebsiella pneumoniae* strain using a Bacterial Genomic DNA Extraction Kit. Subsequently, we applied the RPA-Cas12a platform to detect serially diluted *Klebsiella pneumoniae* bacterial genomic DNA. As shown in Figures 5, the LoDs of the RPA-Cas12a method was 5×10^{3} fg/uL bacterial genomic DNA.

Sensitivity of RPA-Cas12a Platform for Direct Detection of *KPC* Gene in Blood Specimens

To verify that the CRISPR-Cas12a platform can detect directly the *KPC* gene in complicated blood specimens, we spiked serially diluted bla_{KPC} plasmids ranging from 1 aM to 10^{6} aM in serum samples of healthy people and performed the CRISPR-Cas12a detection. Even though the high background of the serum sample affects the sensitivity of this assay, the LoD of the RPA-Cas12a platform still reached 100 aM (~100 copies µL-1) of plasmid (Figure 6). Thus, this demonstrates the potential of our method to be applied in clinical routine and molecular epidemiology investigation.

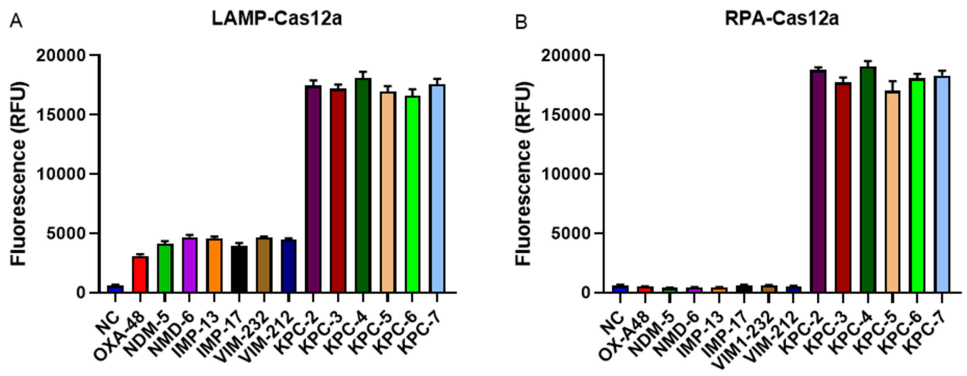

Figure 4 Specificity of RPA-Cas12a-based and LAMP-Cas12a-based detection methods for bla_{KPC} gene. (**A**) The specificity of LAMP-Cas12a-based method for bla_{KPC} gene. (**B**) The specificity of RPA-Cas12a-based method for bla_{KPC} gene. Negative control (NC) utilized RNase-free water as input instead of target plasmid.

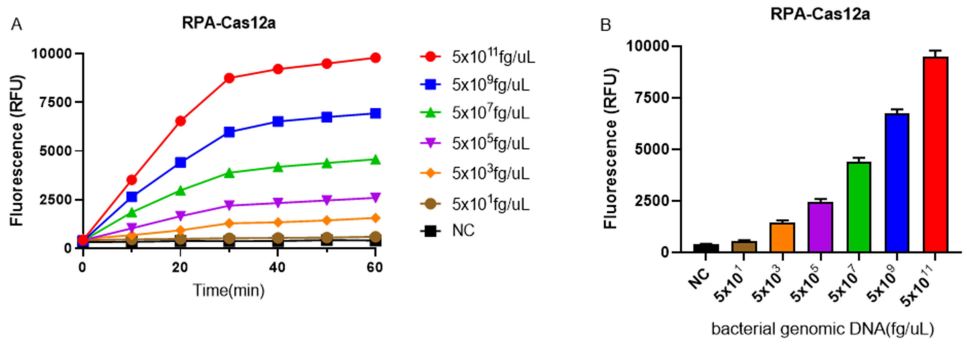

Figure 5 Sensitivity of RPA-Cas12a platform for Klebsiella pneumoniae strain carrying bla_{KPC} gene. (**A**) The real-time fluorescence signals of RPA-Cas12a from 0 min to 60 min with different concentrations of the target plasmid are shown. (**B**) The endpoint fluorescence signals of RPA-Cas12a with different concentrations of the target plasmid are shown.Negative control (NC) utilized RNase-free water as input instead of target plasmid.

Application of RPA-Cas12a Platform for Clinical Isolates

To further investigate the clinical performance of our platform, we applied the RPA-Cas12a platform to detect the 40 clinical isolates containing bla_{KPC} gene, and 40 clinical isolates containing other β-lactamase genes (Table S2). As shown in Figure 7, 40 clinical isolates containing the bla_{KPC} gene were correctly identified (40/80), and 40 clinical isolates without the bla_{KPC} gene tested negative using the RPA-Cas12a detection platform (40/80). The sensitivity and specificity of this method are both 100%.

Application of the Lateral Flow Strip Visualization System

We randomly selected seven strains out of the 40 clinical isolates containing the bla_{KPC} gene and two strains out of the 40 clinical isolates without the bla_{KPC} gene for the visual LFS detection of the RPA-Cas12a platform. As shown in Figure 8, seven KPC-positive clinical strains were correctly identified (7/9), and two negative clinical isolates tested negative (2/9) both for fluorescence and LFS readout. The sensitivity and specificity of this method are both 100%.

Discussion

Infections caused by CRE pose a severe threat to global health. Reportedly, the production of KPC carbapenemase was the most common resistance mechanism of CRE worldwide. In recent years, some new pharmaceutical compounds successfully got to the market and the drug development pipeline, which offers the opportunity for employing precision medicine to target antibiotic therapy against specific CRE resistance mechanisms. In other words, the ultimate goal of precision medicine is the prescription of individualized antibiotic therapy based on the CRE resistance mechanism. A previous study demonstrated that mortality increases by 7.6% for every hour delay in the effective antibiotic prescription to sepsis.[32] Therefore, in this context, accurate and rapid identification of the CRE resistance mechanism

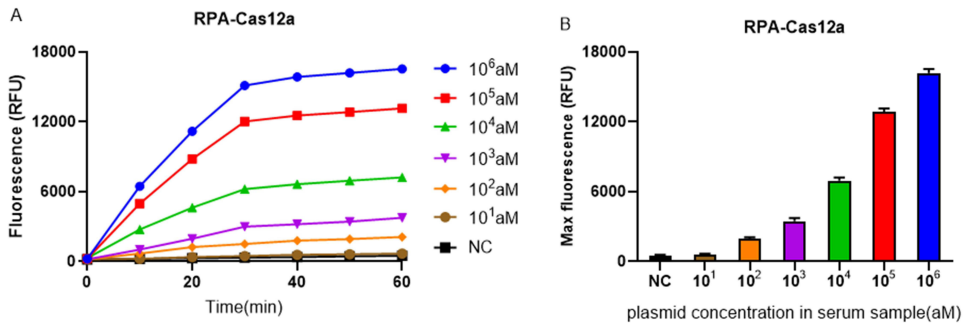

Figure 6 Sensitivity of RPA-Cas12a platform for Klebsiella pneumoniae strain carrying *bla*$_{KPC}$ gene. (**A**) The real-time fluorescence signals of RPA-Cas12a from 0 min to 60 min with different concentrations of the target plasmid are shown. (**B**) The endpoint fluorescence signals of RPA-Cas12a with different concentrations of the target plasmid are shown. Negative control (NC) utilized RNase-free water as input instead of target plasmid.

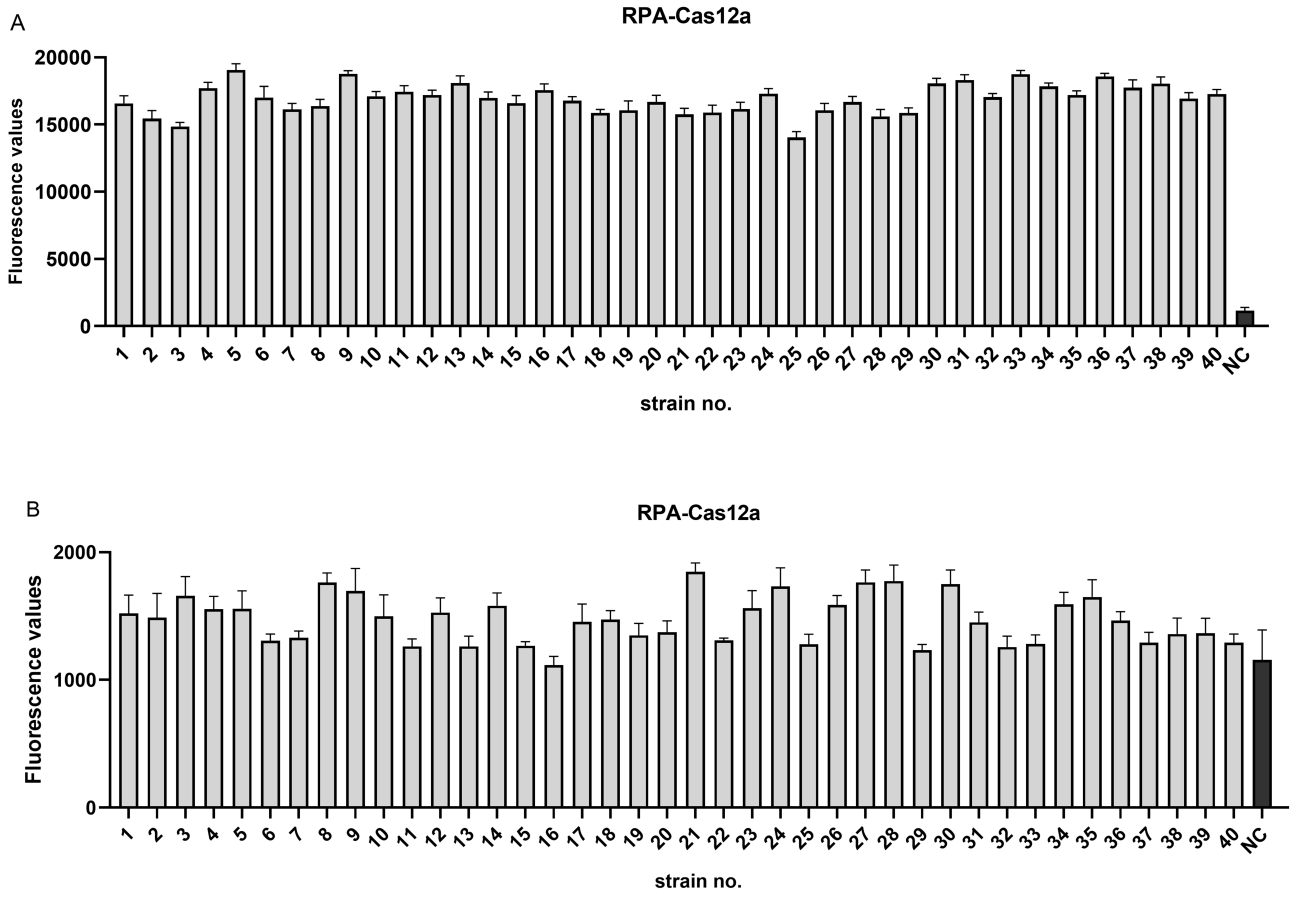

Figure 7 Application of RPA-Cas12a platform in clinical isolates. (**A**) 40 clinical isolates containing the *bla*$_{KPC}$ gene. (**B**) 40 clinical isolates containing other β-lactamase genes. Negative control (NC) utilized RNase-free water as input instead of target plasmid.

has important clinical implications. Additionally, the accurate and rapid identification of carbapenemase-producing CRE allows prompt implementation of infection control interventions, rather than days delay based on traditional methods. The positive influence is well demonstrated by the example of active surveillance of CRE colonization using point-of-care detection methods in the Israeli national KPC-producing Klebsiella pneumoniae outbreak.[33] To summarize, the accurate and rapid CRISPR/Cas12a detection platform could allow timely initiation of effective antimicrobial therapy and infection control interventions, which result in better patient outcomes, less transmission of carbapenemase-producing CRE, and lower economic burden. Therefore, we developed a convenient and low-cost nucleic acid detection

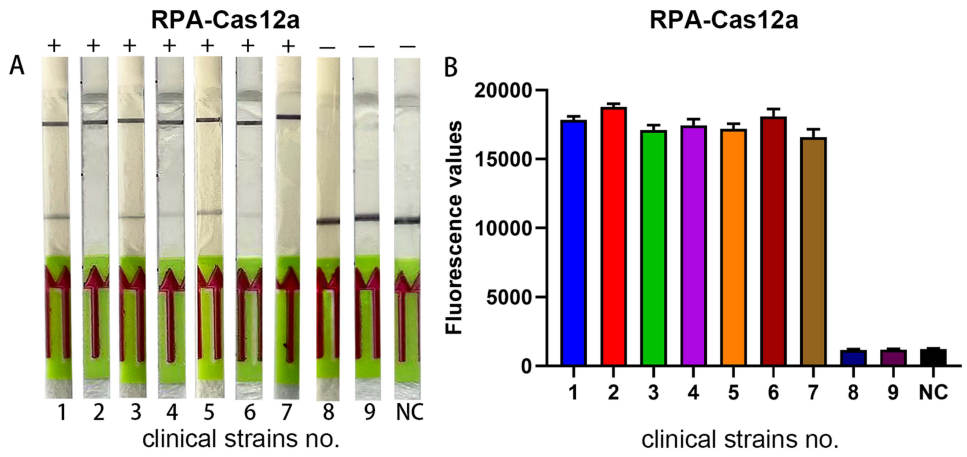

Figure 8 Application of the lateral flow strip visualization system. (**A**) Lateral flow strip results of clinical specimens. (**B**) endpoint fluorescence signal of clinical specimens. Clinical strains No. 1–7 are KPC-positive clinical isolates, and Clinical strains No. 8–9 are KPC-negative clinical isolates. Negative control (NC) utilized RNase-free water as input instead of target plasmid.

platform to detect the bla_{KPC} gene in Enterobacterales isolates using a CRISPR/Cas12a system in combination with PCR, LAMP, and RPA amplification techniques, as well as real-time and endpoint fluorescence and lateral flow test strip technology to visualize the results.

In this study, we first applied a Cas12a/sgRNA-based nucleic acid detection platform to detect the bla_{KPC} gene. By designing specific sgRNA targeting the bla_{KPC} gene, we developed a *CRISPR/Cas12a* detection system using real-time and endpoint fluorescence and lateral flow strip-based tests for signal detection. Using this approach, we were able to detect bla_{KPC} gene plasmids up to 10 nM without a nucleic acid amplification step. These results agree with the findings of other studies in which the range of LoD for the *CRISPR/Cas12a* system was between 10 and 100 nM for different targets.[34,35]

To strengthen the sensitivity of this assay, we incorporated PCR, LAMP, and RPA amplification methods into the *CRISPR/Cas12a* system. Unlike PCR which requires expensive thermal cycling instruments and time-consuming amplification, RPA and LAMP reactions only require a constant-temperature environment, which is more suitable for point-of-care testing in resource-limited areas. Furthermore, we compared the sensitivity of the three different amplification methods combined with the *CRISPR/Cas12a* system for the bla_{KPC} gene plasmid. The LoDs for the LAMP-Cas12a-based and RPA-Cas12a-based methods both were 1 aM (~1 copy μL^{-1}). Two methods appear to be as sensitive as those of other studies in which the LoD was lowered to attomolar levels for different targets or using different endonucleases, which is adequate to achieve clinically relevant detection levels.[27]

Concerning sensitivity performance, both the RPA-Cas12a-driven and LAMP-Cas12a-driven approaches exhibited enhanced sensitivity in comparison to the PCR-Cas12a-driven method when detecting the bla_{KPC} gene plasmid. A thorough evaluation of cross-reactivity across these varied methodologies was conducted to identify the most suitable detection approach for the bla_{KPC} gene. In the context of cross-reactivity, the LAMP-Cas12a-driven method displayed cross-reactivity with the bacterial samples tested for the bla_{KPC} gene, while the RPA-Cas12a-driven method showed no such cross-reactivity. Based on the acquired outcomes, the RPA-Cas12a-driven method was determined to be the optimal detection approach for the bla_{KPC} gene.

The clinical performance of the RPA-Cas12a-based detection method was validated by analyzing 80 clinical isolates. As shown in Figure 6, 40 clinical isolates containing the bla_{KPC} gene were correctly identified, and 40 clinical isolates without the bla_{KPC} gene tested negative using the RPA-Cas12a detection platform. Considering the clinical performance of our method, the RPA-Cas12a detection platform could sensitively and specifically detect the bla_{KPC} gene.

Also, our proposed method was adapted to a convenient readout system, similar to a pregnancy test, for which the result could be read by the naked eye on a paper strip. Thus, this study provides an affordable, convenient, and precise nucleic acid platform for detecting bla_{KPC} genes that can be applied almost anywhere. This is important in resource-constrained areas because sophisticated molecular experimental equipment might not be affordable in low- and middle-income countries.

While the established platforms yielded both positive and negative predictive values of 100%, cautious interpretation of these findings is advised in light of the confined number of strains utilized in the clinical analysis. Consequently, a broader and multicenter cohort study incorporating additional clinical isolates is imperative to corroborate the clinical efficacy of our established assays. A secondary constraint of the current investigation is the absence of direct clinical specimen evaluation using the RPA-Cas12a-driven detection platform. As a result, supplementary research employing clinical specimens is warranted.

Conclusion

In summary, we have successfully developed an RPA-Cas12a detection platform for identifying the bla_{KPC} gene. This study integrates isothermal amplification technology with lateral flow assay devices, circumventing the need for complex molecular diagnostic equipment and enabling real-time diagnostics. Looking ahead, this platform is anticipated to be extensively adopted in clinical microbiology laboratories, significantly enhancing the diagnosis, treatment, and monitoring of CRE-producing KPC enzymes.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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