Infection and Drug Resistance

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ORIGINAL RESEARCH

A Method for Detecting Five Carbapenemases in Bacteria Based on CRISPR-Cas12a Multiple RPA Rapid Detection Technology

Huaming Xu[1], Chunhui Lin[2,3], Hao Tang[4], Rongrong Li[2,3], Zhaoxin Xia[2,3], Yi Zhu[3], Zhen Liu[2,3], Jilu Shen[2,3]

[1]The First Affiliated Hospital of Anhui University of Chinese Medicine, Hefei, People's Republic of China; [2]The First Affiliated Hospital of Anhui Medical University, Hefei, People's Republic of China; [3]Anhui Public Health Clinical Center, Hefei, People's Republic of China; [4]The Second Affiliated Hospital of Anhui Medical University, Hefei, People's Republic of China

Correspondence: Jilu Shen, Tel +86 151 5515 2963, Email shenjilu@ahmu.edu.cn; 1464675852@qq.com

Introduction: As the last line of defense for clinical treatment, Carbapenem antibiotics are increasingly challenged by multi-drug resistant bacteria containing carbapenemases. The rapid spread of these multidrug-resistant bacteria is the greatest threat to severe global health problems.

Methods: To solve the problem of rapid transmission of this multidrug-resistant bacteria, we have developed a rapid detection technology using CRPSPR-Cas12a gene editing based on multiple Recombinase polymerase amplification. This technical method can directly isolate the genes of carbapenemase-containing bacteria from samples, with a relatively short detection time of 30 minutes. The instrument used for the detection is relatively inexpensive. Only a water bath can complete the entire experiment of Recombinase polymerase amplification and trans cleavage. This reaction requires no lid during the entire process while reducing a large amount of aerosol pollution.

Results: The detection sensitivity of this method is 1.5 CFU/mL, and the specificity is 100%.

Discussion: This multi-scene detection method is suitable for screening populations in wild low-resource environments and large-scale indoor crowds. It can be widely used in hospital infection control and prevention and to provide theoretical insights for clinical diagnosis and treatment.

Introduction

In recent years, multidrug-resistant bacteria containing carbapenemase have spread rapidly worldwide. The high incidence and mortality rates of these bacteria, the limited drugs used to treat such bacteria, the prolonged hospitalization, and the high cost of hospitalization pose a severe threat to patients' health.[1] Carbapenems antibiotics are potent in fighting *Pseudomonas aeruginosa* and multidrug-resistant Gram-negative bacilli infections and play an essential role in clinical anti-infection treatment. Due to its broad anti-bacterial spectrum and strong and stable anti-bacterial effect,[2] β–lactamases are commonly used in treating severe infections caused by multidrug-resistant strains. Therefore, these antibiotics are widely used in clinical anti-infection treatments. Under antibiotic selection pressure, clinical isolates with carbapenemase-coding genes, but no phenotypic expression, can quickly transform into carbapenemase-expressing bacteria through gene replication, resulting in drug-resistant strains, limited clinical treatment options, and poor therapeutic effectiveness.[3]

Currently, the laboratory detection methods for Enterobacteriaceae containing carbapenemase are mainly divided into phenotypic, genotypic, and other detection methods. Phenotypic detection methods mainly include the Hodge experiment, Carba NP experiment, mCIM and eCIM experiment, micro broth dilution experiment, and macro broth dilution experiment. Genotype detection methods include real-time molecular Xpert Carba-R detection, PCR, multiplex PCR,

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nested PCR, RT-PCR combined with microfluidic technology, and isothermal amplification technology.[4] Molecular detection has the characteristics of fast, accurate, high sensitivity, strong stability, and high specificity. It can accurately identify five different carbapenemases, which help clinical workers choose anti-bacterial drugs while avoiding adverse reactions caused by drug abuse.[5] Clinicians can scientifically formulate treatment plans and guide clinical medication based on genetic testing results. In addition, other new carbapenemases detection technologies include MALDI-TOF MS, selective and color development culture, enzyme immunochromatography, and identification of carbapenemases from volatile metabolites.[6]

With the rapid development of molecular detection technology, the diagnosis method based on nucleic acid detection has been widely used in the laboratory detection of human diseases. Recombinant enzyme polymerase amplification (RPA) is a new isothermal amplification technique. Similar to the traditional PCR[7] method, the principle of this method is to design positive and negative primers for specific gene sequences. The protein-DNA complex formed by the combination of recombinant enzyme and primer can find homologous sequences in double-stranded DNA. Once the primer locates the homologous sequence, a chain exchange reaction occurs and DNA synthesis is initiated, and the target region on the template is amplified exponentially. The replaced DNA strand is combined with SSB to prevent further replacement. Different from the conventional polymerase chain reaction, the primer length of this method is generally above 30bp, and the variation at 37°C does not require initial heating and temperature change. Therefore, the RPA reaction can amplify a large amount of nucleic acid[8] in a water bath within 10 minutes. At the same time, RPA primers allowed several base mismatches during amplification, which significantly increased the tolerance rate of the whole reaction.[9]

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), an adaptive immune system for prokaryotes, is an emerging technology for molecular diagnosis and biological analysis.[10] CRISPR-Cas12a has been applied to clinically test a new type of coronary pneumonia virus. The principle is that the trans-cracking activity of CRISPR-Cas can be specifically activated by the amplified sequence, cutting the target with the single-stranded DNA probe in the reaction system.[11] Consequently, the probes cleaved by the Cas12a protein can be detected by immunochromatographic strips and fluorescence detection instruments. The detection results display the detection line and fluorescence value.[12]

CRISPR-Cas12a gene-editing technology is a potential, fast, accurate, and portable diagnostic tool that is worth promoting and implementing in large-scale clinical applications. The CRISPR-Cas12a platform (Figure 1) can compensate for the impact of non-specific amplification of RPA reactions and improve the sensitivity and efficiency of molecular diagnosis.[13] This study successfully established a CRISPR-Cas12a-RPA rapid detection method, which can accurately identify and detect five carbapenemases within 30 minutes without opening the lid of EP pipe.

Materials and Methods

Primers and crRNA Design Sequences

Other Reagents and Equipments

PCR premix and plasmid extraction kits were purchased from Shenggong Biotechnology Co., Ltd. (Shanghai, China). Mineral oil was purchased from Shenggong Biology Co., Ltd. (Shanghai, China). Primers (listed in Table 1) was designed by Shenggong Biology Co., Ltd (Shanghai, China). CrRNA (Figure 2) was designed by GeneBiogist (Shanghai, China). Ezup column saliva and urine genomic DNA extraction kit were purchased from Biotechnology and Biotechnology (Shanghai, China). The RPA sequencing result was performed by Shanghai Biotechnology Co., Ltd. TwistAmp ® DNA amplification kit was purchased from TwistDx Company (Cambridge, UK). Nucleic acid detergent was purchased from General Biology (Anhui, China). Blood plates were purchased from Kemaga Biotechnology Co., Ltd. (Shanghai, China). CRISPR-Cas12a immunochromatographic test strip was purchased from Wobo Biotechnology Co., Ltd. (Jiangsu, Nanjing). Cas12a protein was purchased from New England Biological Laboratory Co., Ltd. (UK). The instruments used for isothermal amplification in this study were water or metal baths. Electrophoresis results of PCR and RPA were observed by the gel imaging system (Bio-RAD, USA). Fluorescence curve monitoring was completed on a qPCR (ABI7500) instrument. Fluorescence visual detection results were observed using U/V blue light imager (Bio-RAD, USA).

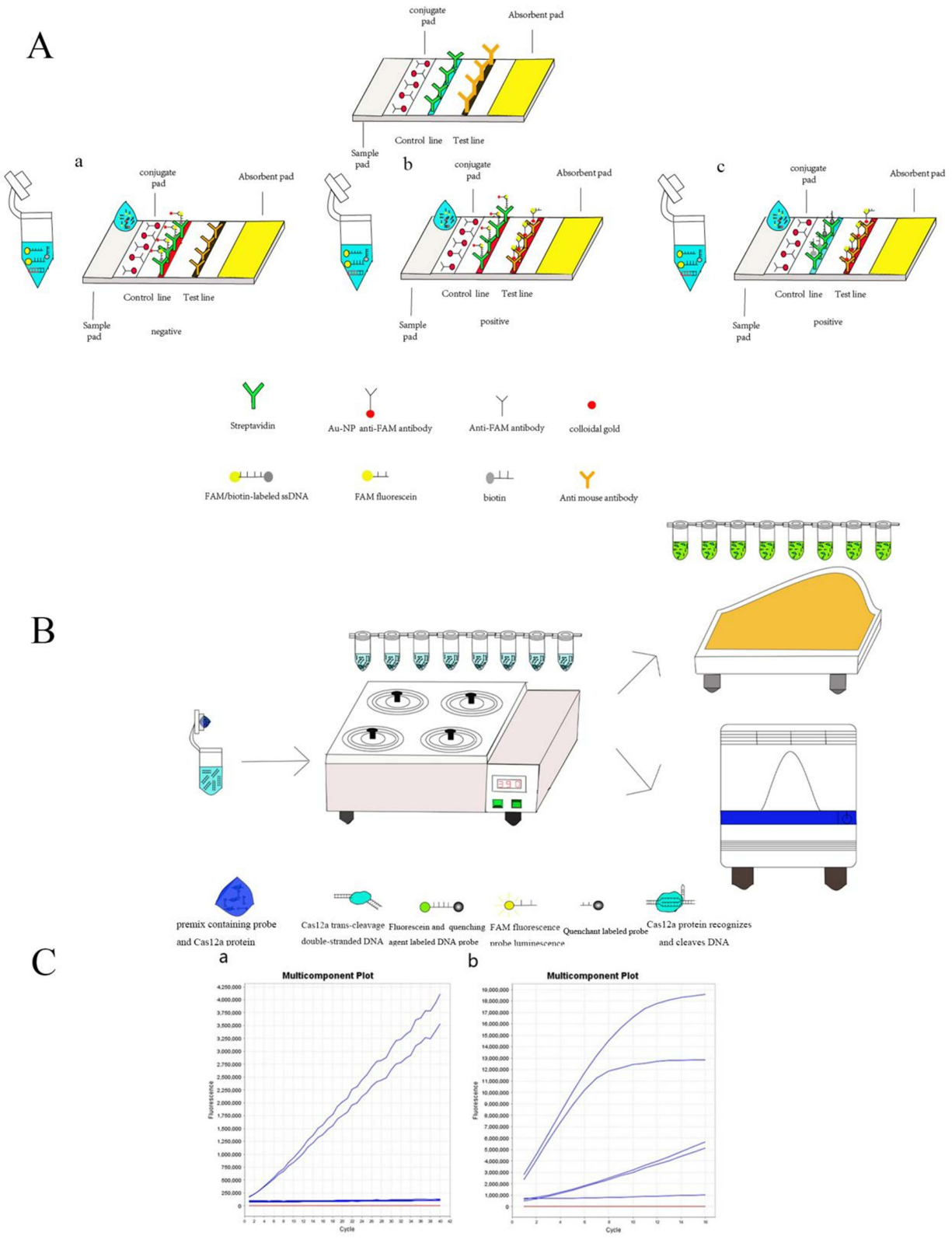

Figure 1 CRISPR-Cas12a platform.

Notes: (**A**) **a**, the cut probe was captured by streptomycin on the control line, while the anti-mouse antibody on the test line did not capture the probe, and the test result was negative; **b**, the cut probe was captured by streptomycin on the control line, and the anti-mouse antibody on the test line also captured the corresponding probe, and the test result was positive; **c**, the cut probe was not captured by streptomycin on the control line, but the anti-mouse antibody on the test line captured the corresponding probe at the same time, and the test result was positive. (**B**) After being incubated in water bath, the cut product was detected by Umurv UV and FQ-PCR. (**C**) Fluorescence quantitative PCR shows a fluorescence curve, where **a** represents that the probe in the reaction has not been cut, and **b** indicates that the probe has been cut.

Table 1 Oligonucleotide Sequences Used in This Study

Oligonucleotides	Sequence (5'-3')	
Single stranded DNA reporter molecule	FAM-TTATTTTATT-biotin/FAM-TTAAAATATT-BHQ	This study
RPA primer		
KPC Forward primer	ATCTCGGAAAAATATCTGACAACAGGCATGACGGTG	This study
KPC Reverse primer	CGGTCGTGTTTCCCTTTAGCCAATCAACAAAACTGCT	This study
NDM Forward primer	TCGCACCGAATGTCTGGCAGCACACTTCCTAT	This study
NDM Reverse primer	GTTCGACAACGCATTGGCATAAGTCGCAATCC	This study
OXA Forward primer	ATTATCGGAATGCCAGCGGTAGCAAAGGA	This study
OXA Reverse primer	TCGAGGGCGATCAAGCTATTGGGAATTTT	This study
IMP Forward primer	AGGCAGTATTTCCTCTCATTTTCATAGTGACAGC	This study
IMP Reverse primer	ATTTTCCTTTCAGGCAGCCAAACTACTAGGTTAT	This study
VIM Forward primer	GGGAGCCGAGTGGTGAGTATCCGACAGT	This study
VIM Reverse primer	TTTTCGCACCCCACGCTGTATCAATCAA	This study
CrRNA sequence		
CrRNA KPC	UAAUUUCUACUAAGUGUAGAUUUGCUGAAGGAGUUGGGCGG	This study
CrRNA NDM	UAAUUUCUACUAAGUGUAGAUGGGGCAGUCGCUUCCAACGG	This study
CrRNA OXA	UAAUUUCUACUAAGUGUAGAUCCAAUAAUCUUAAACGGGCG	This study
CrRNA IMP	UAAUUUCUACUAAGUGUAGAUGCGGGGUUAACUAUUGGCUA	This study
CrRNA VIM	UAAUUUCUACUAAGUGUAGAUAUGGCGCAGUCUACCCGUCC	This study
PCR primer		
$KPCF_I$	ATGTCACTGTATCGCCGTCT	This study
$KPCR_I$	TTACTGCCCGTTGACGC	This study
$NDMF_I$	CATTAGCCGCTGCATTGATG	This study
$NDMR_I$	GGCTGCGAGCCAGCACAGC	This study
$OXAF_I$	CCCCGAGTCAGATTGTTC	This study
$OXAR_I$	GCTTCATGGCTTCTCCTAG	This study
$VIMF_I$	GCTGTCGCAAGTCCGTTA	This study
$VIMR_I$	CCGCTCGATGAGAGTCCT	This study
$IMPF_I$	GGCAGTATTTCCTCTCATTT	This study
$IMPR_I$	GCAGCTCATTAGTTAATTCAG	This study

Strain Collection and DNA Extraction

A total of 75 clinical strains (listed in Table 2) were isolated from the First Affiliated Hospital of Anhui Medical University. The concentrations of all strains were adjusted to 0.5 McFarland units, and bacterial DNA was extracted according to the instructions of the bacterial genomic DNA extraction kit. GenBank login numbers: KPC gene (MT415074.1), NDM gene(NZ_QETJ01000331.1), OXA gene (AM263454.1), IMP gene (HQ263342.1), VIM gene (MT185944.1)

The patient's sputum samples were collected in a 1.5mL centrifuge tube, and 250 μL of saliva was added. Then, 350 μL of protease K Buffer were added to the centrifuge tube and mixed thoroughly. After 20 minutes of incubation in a 65 °C water bath, the tubes were shaken and mixed repeatedly. After mixing, 300μL of anhydrous ethanol was added to the centrifuge tube and shaken thoroughly. The solution was removed, and sediment was transferred to a silica gel membrane adsorption column. The adsorption column was placed in a collection tube and centrifuged for 1 min at 10,000 rpm. The solution was then poured into the collection tube. The adsorption column was removed, and placed in a new 1.5 mL centrifuge tube, followed by adding 50μL TE buffer to the center of the adsorption membrane. The adsorption column was left standing for 3 min and centrifuged at 12,000 rpm for 2 min to obtain a DNA solution, which was stored at - 20 °C or directly used for subsequent experiments.

Developing the RPA Recombinant Enzyme Polymerase and PCR Amplification Methods

The extracted DNA was used as a template for RPA and PCR amplification reactions. The composition of a single RPA reaction system included: 2.4 μL of forward primer (10 μM), 2.4 μL of reverse primer (10 μM), 29.5 μL of primer-free

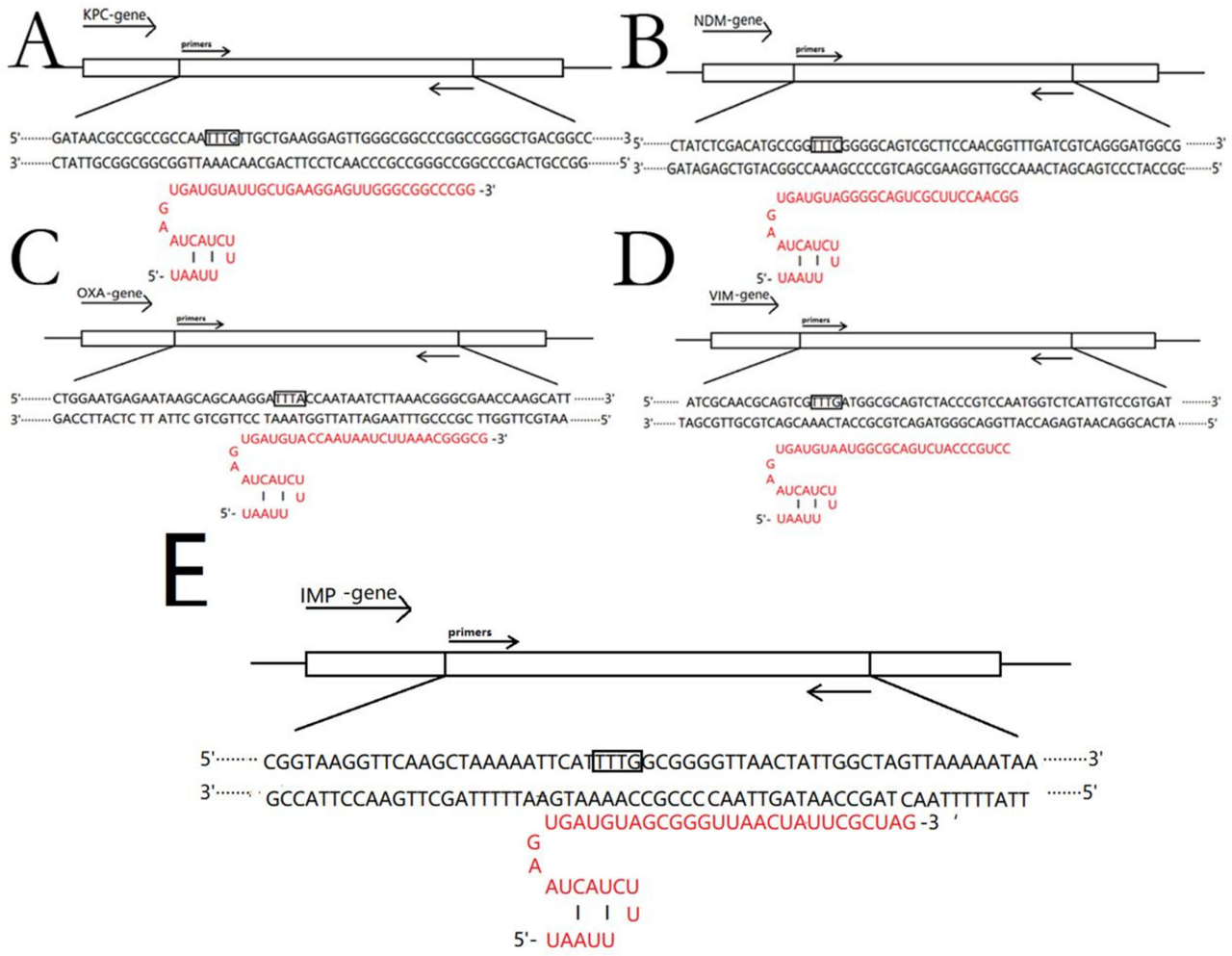

Figure 2 Target sequences of five carbapenemases and their corresponding red CrRNA sequences.
Notes: (**A**) KPC-gene, (**B**) NDM-gene, (**C**) OXA-gene, (**D**) VIM-gene, (**E**) IMP-gene.

rehydration buffer, 13.2 μL of template, and water was added to a total volume of 47.5 μL. The 47.5 μ L rehydrated mixture was transferred to a reaction microsphere and mixed repeatedly until the entire reaction system was resuspended. After that, 2.5 μL of 280 mM magnesium acetate (MgOAc) was added to each sample and mixed thoroughly. The volume of the mixture increases five times for five-fold RPA reaction systems. The single-tube reaction was carried out in a PCR octuple tube. The tube cap was carefully closed and centrifuged to allow MgOAc to enter the rehydrated solution

Table 2 Basic Information of 75 Bacterial Strains

Species	Number of Strains	Source	Carbapenemase Gene
Klebsiella pneumoniae	11	Clinical isolates	KPC
Klebsiella pneumoniae	7	Clinical isolates	NDM
Escherichia coli	9	Clinical isolates	KPC
Escherichia coli	8	Clinical isolates	NDM
Klebsiella pneumoniae	3	Clinical isolates	OXA
Klebsiella pneumoniae	4	Clinical isolates	IMP
Klebsiella pneumoniae	5	Clinical isolates	VIM
Klebsiella pneumoniae	15	Clinical isolates	NONE
Escherichia coli	12	Clinical isolates	NONE

and activate the reaction, followed by rapid oscillation and centrifugation. Finally, 80 μL of mineral oil was added to the amplification system to prevent aerosol spillage when opening the lid. Incubation was performed in a suitable thermostat (39 °C) for 22 minutes, followed by observing the RPA amplification band using agarose gel electrophoresis.

The PCR reaction system consisted of 2.0 μL of forward primer (10 μM), 2.0 μL of reverse primer (10 μM), 25 μL of 1×Taq Premix, 1− 2μL of extracted genomic DNA (200ng), and ddH_2O to a final reaction volume of 50 μL. The reaction was carried out in a PCR octuple tube with a firmly closed cap. After the reaction, the amplified PCR bands were observed using agarose gel electrophoresis.

Establishment of Cas12a Trans Cleavage Experiment Combined with Immunochromatographic Strip Detection Experimental Method

The Cas12a cracking reaction system (25μL) included: 5μL NEB 3.1 buffer, 3μL crRNA (500nM), 1μL LbCas12a (1μM), 1 μL fluorescein biotin double labeled reporter molecule (60 nM), 3 μL RPA amplification product, and ddH_2O 12μL up to a final volume of 25μL. The reaction was incubated at 39 °C for 10 minutes. The test strip was inserted into the reaction tube according to the arrow direction. All areas to be analyzed were then soaked in the liquid, followed by adding a diluent based on the concentration of the target substance. A total of 80 μL supernatant was removed and slowly dripped onto the sample hole of the test strip. The test strips were interpreted by their color development after 7–10 minutes.

Cas12a Trans Cleavage Experiment and Fluorescence Detection

Cas12a cracking reaction system (25μL) contained: 5 μL NEB 3.1 buffer, 3μL crRNA (500nM), 1 μL LbCas12a (1μM), 1 μL fluorescein quenching agent double labeled reporter molecule (60 nM), 3 μL RPA amplification product, and 12μL ddH_2O. The mixture was incubated at 39°C for 10 minutes. After the reaction, the reaction tube was placed in a U/V fluorescent flat panel reader or fluorescence monitoring instrument and observed for fluorescence.

One-Tube Multiplex RPA-CRISPR-Cas12a Detection Method Without Opening the Cover

A: Negative result: there are stripes on the control line but no stripes on the test line; b: Positive result: there are strips on the test line and also on the control line; c: Positive result: there are no stripes on the control line, and there are stripes on the test line.

B: The process of gene fluorescence detection by constant temperature amplification in water bath at 39 °C.

C: The RPA amplification product containing the target gene will show an increase in fluorescence curve after cleavage, while the fluorescence value of the RPA amplification product without the target gene remains unchanged.

The RPA octuple tube was placed in a water bath and amplified at a constant temperature of 37 °C. After the RPA reaction, the CRISPR-Cas product in the tube cap was centrifuged into the reactant and heated in a water bath. The product of CRISPR-Cas cleavage containing the target gene would emit fluorescence, while the product without the target gene would not.

Sensitivity Evaluation of RPA-CRISPR-Cas12a Rapid Detection Platform

The concentration of bacteria containing carbapenemases was adjusted to 0.5 McFarland units and diluted sequentially eight times from 1.5×10^8 CFU/mL to 1.5CFU/mL. The RPA-CRISPR-Cas12a rapid detection platform was used to detect the different dilution templates and determine the minimum detection limit for the different genes.

Evaluation of the Specificity of RPA-CRISPR-Cas12a Rapid Detection Platform

We used the RPA-CRISPR-Cas rapid detection platform (Figure 3) to detect the genome of 75 clinical specimens. We retained 250 μL of sputum for detection using the RPA-CRISPR-Cas rapid detection platform. The corresponding bands were observed on the test line to detect carbapenemase-positive samples, while the fluorescence value was detected on the fluorescence detection instrument. The positive test line and fluorescence signjal peak would not be detected for carbapenemase-free samples.

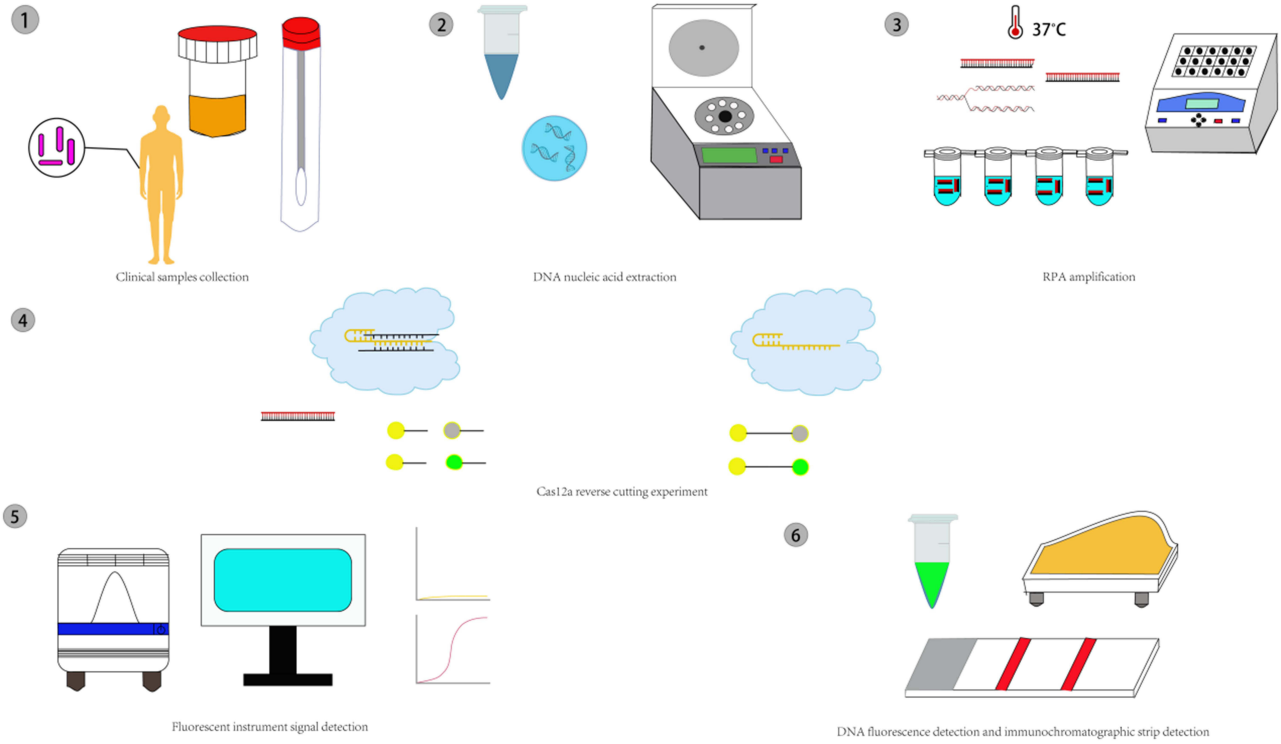

Figure 3 The CRISPR-Cas12a Rapid Detection Platform.

RPA-CRISPR-Cas12a Rapid Detection Platform System Optimization

The RPA-CRISPR-Cas12a rapid detection platform mainly consists of two parts. The first part is the Cas12a protein trans-cleavage reaction. To shorten the reaction time, we sequentially set the reaction time for Cas12a cutting to 5 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, and 40 minutes. The second part was the reaction time of RPA. The reaction time of RPA was set to 5 minutes, 8 minutes, 10 minutes, 12 minutes, 15 minutes, 18 minutes, 20 minutes, and 22 minutes respectively. The results were obtained through fluorescence signals. The results were displayed through fluorescence signals.

We optimized the detection performance of the RPA-CRISPR-Cas12a rapid detection platform. The first optimization targeted the RPA reaction. We first optimized. The primer concentration was optimized to keep the positive and negative primer concentrations of RPA amplification consistent. We set the primer concentration gradient in the following order: 10.0 μM, 5.0 μM, 2.0 μM, 1.5 μM, 1.0 μM, 0.5 μM, 0.25 μM. After setting the concentration gradient, RPA amplification was performed. The second optimization was applied to the Cas12a trans cleavage reaction. In order to achieve the best detection performance, we successively optimized the Cas12a protein and CrRNA concentrations.

First, we set the crRNA concentration to 50 nM, 100 nM, 200 nM, 250 nM, 300 nM, 400 nM, and 500 nM series concentrations. We optimized the Cas12a protein concentration and set the Cas12a concentration to 0.1 μM, 0.2 μM, 0.4 μM, 0.5 μM, 0.6 μM, 0.7 μM, 0.8 μM, and 1.0 μM. At the same time, the reaction system was increased by a factor of five to detect five carbapenemases in one tube and improve the detection efficiency. Finally, 12.5 μL magnesium sulphate was added to the tube cap. After mixing, the reaction was activated by quick centrifugation and immediately incubated at 37°C for 15 minutes.

Results

Results of Five Carbapenemase crRNA Design

The total length of crRNA is 41 bp, including a stem ring structure of 21 bp leading sequence and a matching sequence of 20 bp. The sequence of 20 bp was determined based on the complementary base pairing of the 20 bp sequence after the PAM site.

Establishment of Multiple RPA-CRISPR-Cas12a Rapid Detection Platform

As shown in Figure 3, sputum and urine samples were collected from clinical specimens, and genomic DNA was quickly extracted. After RPA amplification, the products were transferred to the Cas12a detection system containing KPC, NDM, OXA, IMP, and VIM gene-specific crRNAs. The positive sample containing carbapenemase exhibits fluorescence after Cas cutting, and the detection line would display on the detection plate. Fluorescence was detected using a fluorescent plate reader or fluorescence detection instrument. Negative samples showed no fluorescence or detection line on the test line.

Results of Gel Electrophoresis of Five Carbapenemases

Figure 4A: RPA primer amplification of target fragment representing KPC in lanes 1 and 2; lane 3 being a negative control. Lanes 4 and 5 represent the amplification target fragment of the RPA primer for NDM. Lane 6 is a negative control. Lanes 7 and 8 represent the target fragment amplified by RPA primers for OXA, and lane 9 is a negative control. Lanes 10 and 11 represent the target fragment amplified by the RPA primer of IMP, and lane 12 represents its negative control. Lanes 13 and 14 represent the target fragment amplified by VIM primers, and lane 15 lanes its negative controls. Lane 16 includes the 3000 bp markers.

Figure 4B: 1 and 2 represent positive results of KPC, 3 represents negative result; 4 and 5 represent positive results of NDM, 6 represents negative result; 7 and 8 represent positive results of OXA, 9 represents negative result; 10 and 11 represent positive results of IMP, 12 represents negative result; 13 and 14 represent positive results of VIM, 15 represents negative result.

Sensitivity Results of RPA-CRISPR-Cas12a Rapid Detection Platform

Figure 5A (a) Represents KPC carbapenemase: lanes 1–8 are the RPA agarose gel electrophoresis results of the diluted templates; (b) Represents the results of CRISPR-Cas immunochromatographic strips diluted by the template for KPC type carbapenemase. Figure 5B (c) Represent the agarose gel electrophoresis result of the RPA of NDM carbapenemase after diluting the template; 2–9 represents the RPA positive result of the diluted template. Figure 5B (d) The results of CRISPR-Cas immunochromatographic bands representing NDM carbapenemase after dilution of the template: 1–8 are positive, 9 and 10 are negative. Figure 5C (e) It represents the agarose gel electrophoresis result after diluting the template with OXA type carbapenemase: 1–8 is the RPA positive result of the diluted template. Figure 5C (f) The result of CRISPR-Cas immunochromatography representing OXA-type carbapenemase after diluting the template: 1–8 is positive, 9 and 10 are negative. Figure 5D (h) It represents the RPA result of IMP carbapenemase after diluting the template: 2–9 is the RPA positive result of diluted template agarose gel electrophoresis. (i) The result of CRISPR-Cas immunochromatography representing IMP-type carbapenemase after diluting the template: 1–8 is positive, 9 and 10 are negative. Figure 5E (j) It represents the RPA positive result of agar gel electrophoresis 1–8 swimlanes of VIM carbapenemase after diluting the template, and (k) represents the detection results of immunochromatographic strips of VIM carbapenemases 1–8 after diluting the template, 9, 10 are negative.

Evaluation of the RPA-CRISPR-Cas12a Rapid Detection Platform Specificity

From August 2021 to October 2022, 75 sputum samples were collected from hospital. Use Ezup column saliva and urine genomic DNA extraction kit to extract DNA according to the instructions. The significant increase in fluorescence value after CRISPR-Cas cleavage demonstrates the existence of target genes. We reserved 250 μ l of sputum for the RPA-CRISPR-Cas rapid detection platform (Figure 6).

RPA-CRISPR-Cas12a Rapid Detection Platform Optimization

In order to obtain the best detection performance, we optimized the Cas12a reaction system through fluorescence kinetics. First, we optimized the concentrations of Cas12a, crRNA, and RPA primers (Figure 7A–C). Based on the amplification curve and endpoint fluorescence values, we found that the optimal crRNA concentration was 500 nM. The results of fluorescence detection by the fluorescent flat panel reader were consistent with the detection results of the instrument. The amplification curve and endpoint fluorescence values indicated that the optimal Cas12a concentration was 1.0μM. The optimal concentration of RPA primers was 10.0 μM (Figure 7A and D). In order to shorten the reaction

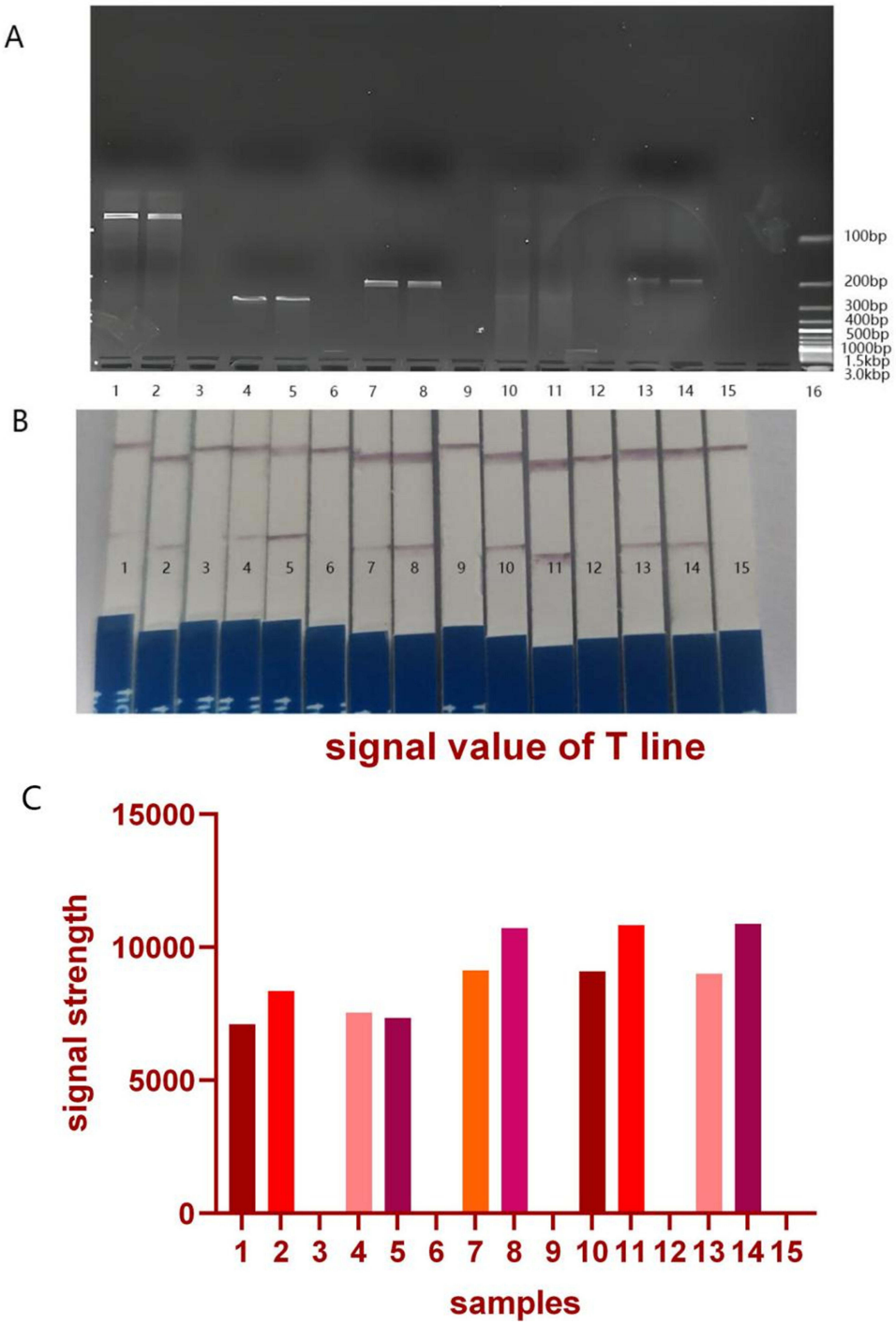

Figure 4 Electrophoresis and immunochromatographic strip detection results of five carbapenemases.

Notes: (**A**) Numbers 1, 2 represent the positive result of KPC gene test, 3 represents negative result; 4, 5 represent the positive results of NDM gene test, 6 represents negative result; 7, 8 represent the positive results of OXA gene test, 9 represents negative result; 10, 11 represent positive results of VIM gene test, 12 represents negative result; 13,14 represent positive results of IMP gene test, 15 represents negative result. (**B**) Numbers 1 and 2 showed positive detection of KPC gene in immunochromatographic strips and negative in 3; 4 and 5 were positive for NDM gene in immunochromatographic strips and negative in 6 cases; 7 and 8 were positive results of OXA gene detection in immunochromatographic strips, while 9 were negative results; 10 and 11 were positive for VIM gene in immunochromatographic strips and 12 cases were negative; 13 and 14 were positive for IMP gene in immunochromatographic strips, 15 were negative. (**C**) Numbers 1, 2 represent the positive result of KPC gene test, 3 represents negative result; 4, 5 represent the positive results of NDM gene test, 6 represents negative result; 7, 8 represent the positive results of OXA gene test, 9 represents negative result; 10, 11 represent positive results of VIM gene test, 12 represents negative result; 13,14 represent positive results of IMP gene test, 15 represents negative result.

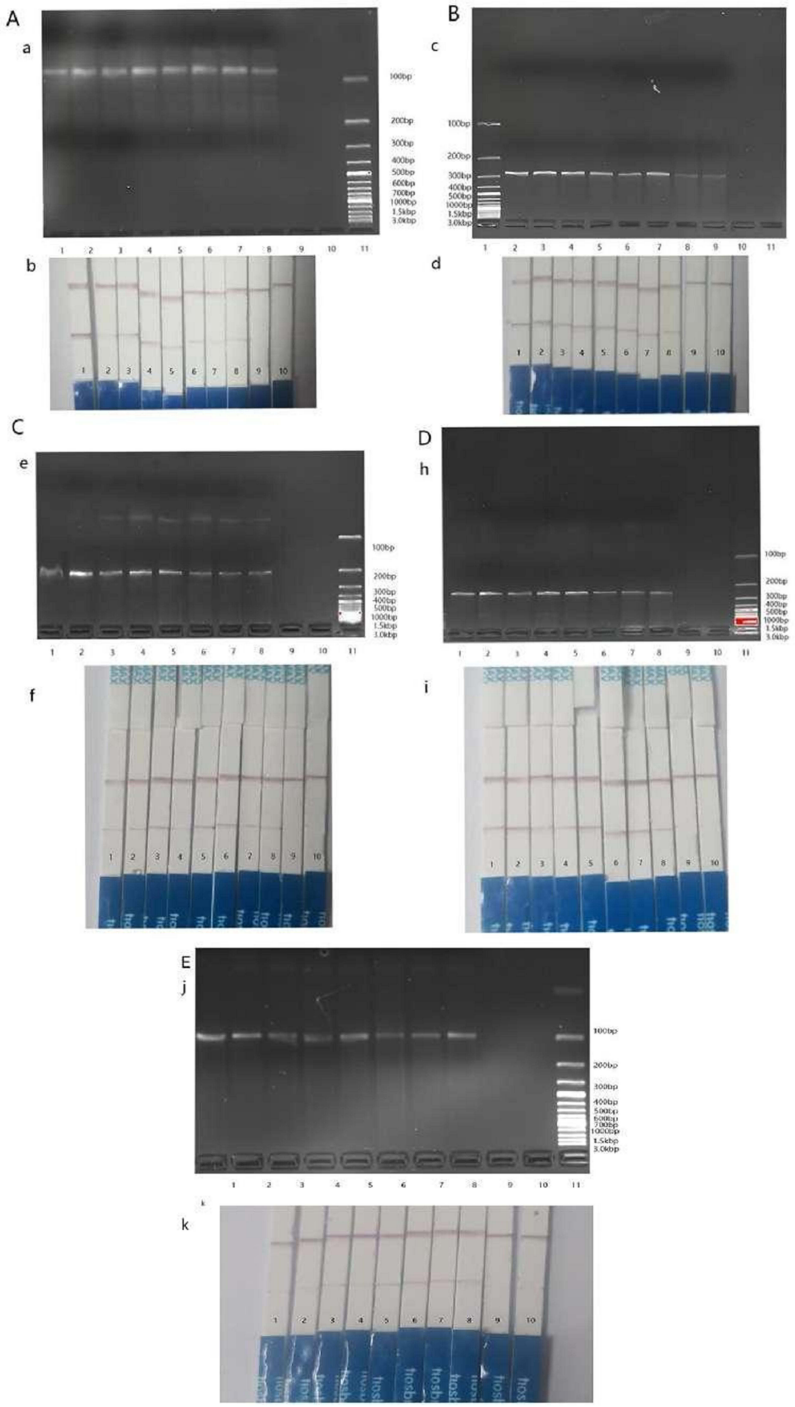

Figure 5 (**A**–**E**) RPA results of five carbapenems enzymes KPC, NDM, OXA, IMP, and VIM after diluting the template (from 1.5X10^{8}CFU/mL to 1.5CFU/mL), as well as the corresponding CRISPR-Cas12a immunochromatographic strip detection results.

Notes: (**A**) **a**, KPC carbapenemase: lanes 1–8 are the RPA agarose gel electrophoresis results of the diluted templates; (**b**) results of CRISPR-Cas immunochromatographic strips diluted by the template for KPC type carbapenemase. (**B**) **c**, agarose gel electrophoresis result of the RPA of NDM carbapenemase after diluting the template: lames 2–9 represents the RPA positive result of the diluted template; **d**, results of CRISPR-Cas immunochromatographic bands representing NDM carbapenemase after dilution of the template: numbers 1–8 are positive, 9 and 10 are negative. (**C**) **e**, agarose gel electrophoresis result after diluting the template with OXA type carbapenemase: lanes 1–8 is the RPA positive result of the diluted template; **f**, result of CRISPR-Cas immunochromatography representing OXA-type carbapenemase after diluting the template: numbers 1–8 is positive, 9 and 10 are negative. (**D**) **h**, represents the RPA result of IMP carbapenemase after diluting the template: lanes 2–9 is the RPA positive result of diluted template agarose gel electrophoresis; **i**, result of CRISPR-Cas immunochromatography representing IMP-type carbapenemase after diluting the template: numbers 1–8 is positive, 9 and 10 are negative. (**E**) **j**, RPA positive result of agar gel electrophoresis 1–8 swimlanes of VIM carbapenemase after diluting the template; **k**, detection results of immunochromatographic strips of VIM carbapenemases 1–8 after diluting the template, 9, 10 are negative.

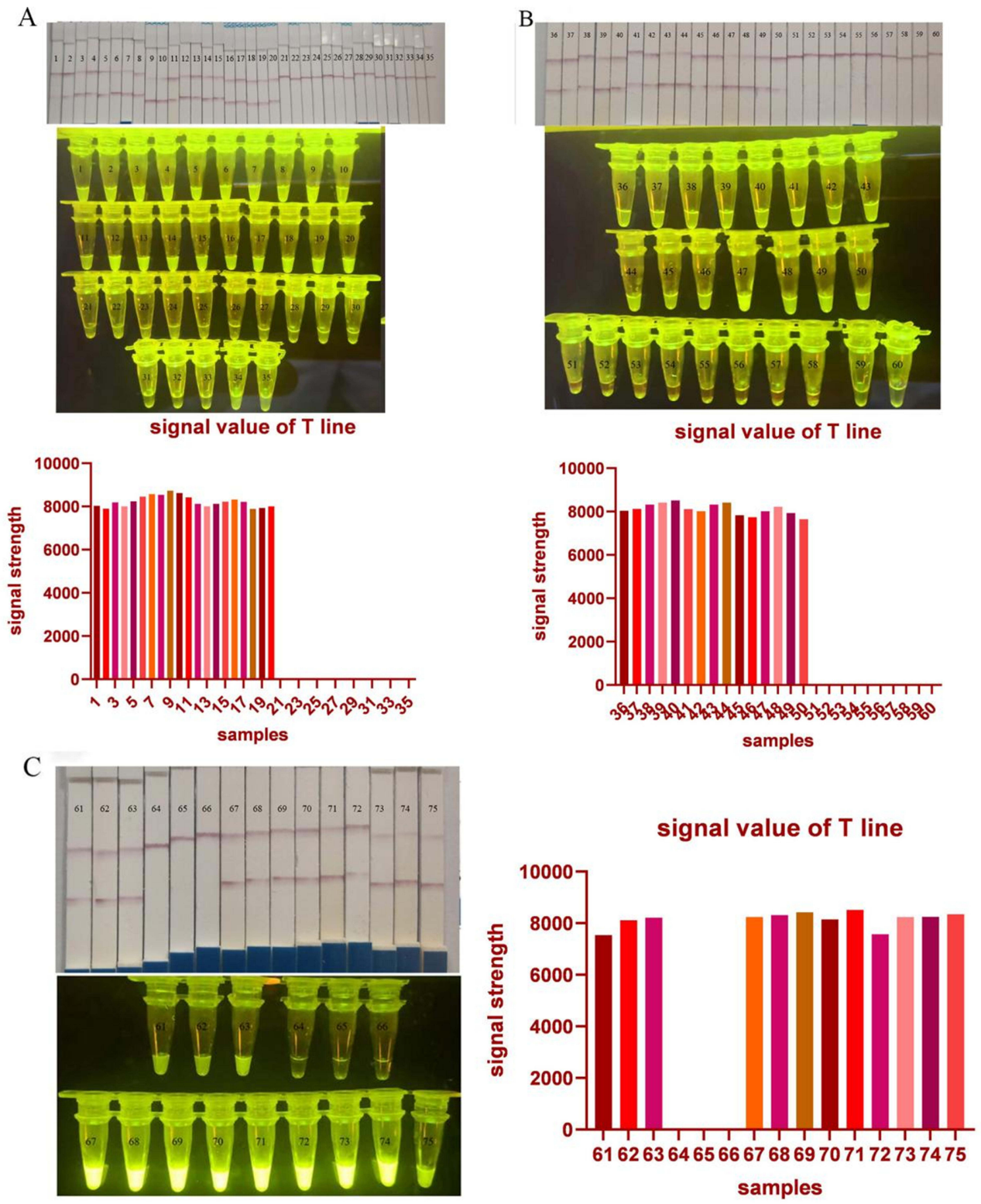

Figure 6 Test results of 75 clinical specimens.

Notes: (**A**) Test results of samples containing KPC enzyme: numbers 1–20 represent positive samples, and 21–35 represent negative samples. (**B**) Test results of samples containing NDM enzyme: numbers 36–50 represent positive, and 51–60 represent negative samples. (**C**) Test results of OXA samples: numbers 61–63 represent positive samples, while 64–66 represent negative; test result of the representative IMP sample: 67–70 represent positive samples ; test results of representative VIM samples: 71–75 are positive samples.

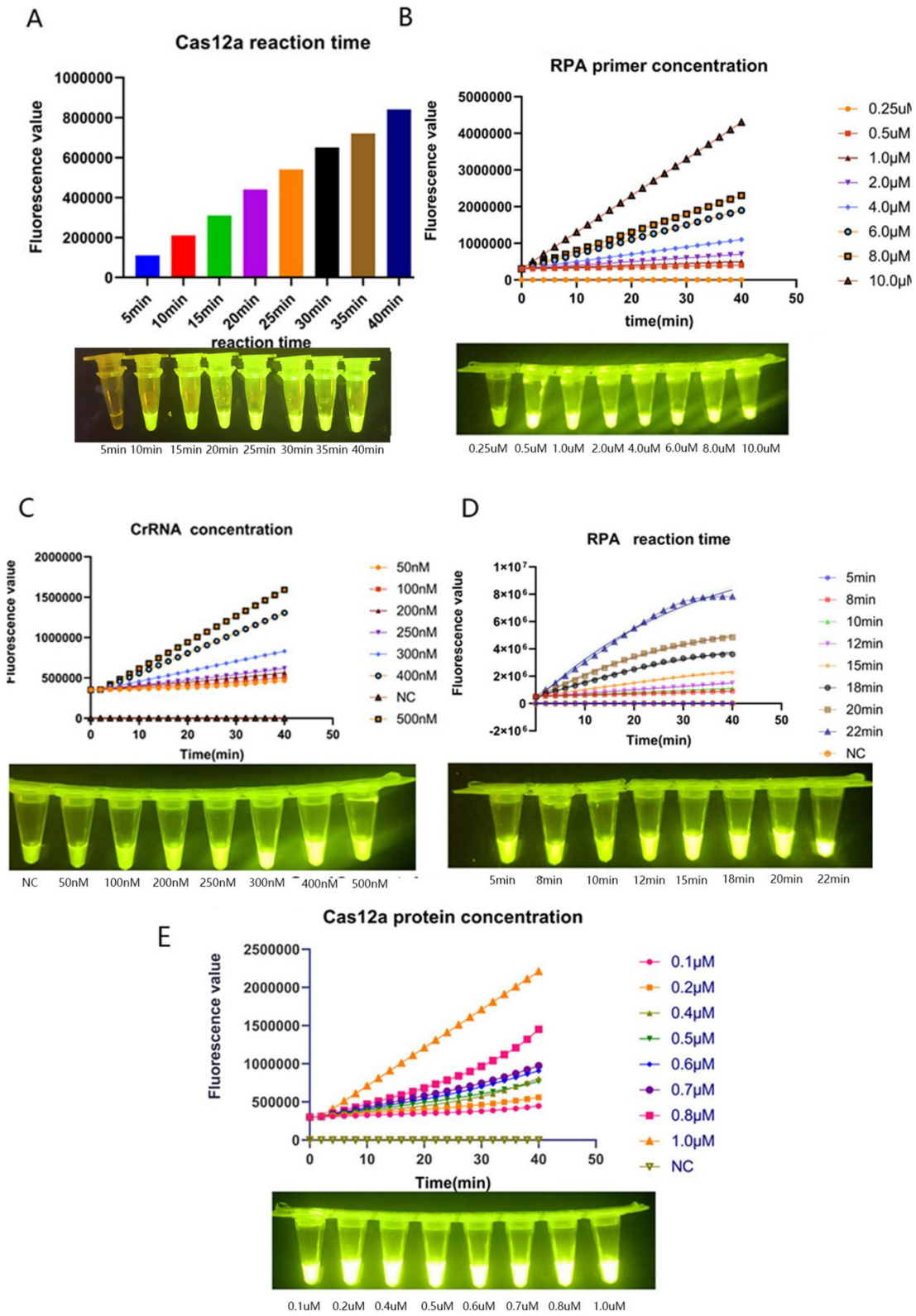

Figure 7 Optimization of the CRISPR-Cas12a reaction system.
Notes: (**A**) Optimization of different Cas12a reaction times. (**B**) Optimization of RPA primer concentrations. (**C**) Optimization of crRNA concentrations. (**D**) Optimization of RPA reaction times. (**E**) Optimization of Cas12a protein concentrations. All experiments were repeated at least three times.
Abbreviation: NC, negative control.

time, we optimized the detection time of the RPA-CRISPR-Cas12a rapid detection platform: the fluorescence signal was generated when the Cas12a reaction time was 5 minutes, and the fluorescence signal was strongest after 40 minutes. The fluorescence intensity of the RPA reaction curve was highest at 22 minutes.

Comparison Results Between Ordinary PCR and RPA-CRISPR-Cas12a Rapid Detection Platform

It is well known that PCR is the gold standard for nucleic acid detection. We validated the clinical application of the detection method based on RPA-CRISPR-Cas12a by comparing it (listed in Table 3) with conventional PCR (Figure 8).

The CRISPR-Cas12a-RPA test showed that 75 strains were positive, 48 strains were positive and 27 strains were negative, which was consistent with the results of ordinary PCR, with a sensitivity of 100%, a specificity of 100% and a coincidence rate of 100% with conventional polymerase chain reaction.

Discussion

At the same time, the resistance of clinically isolated bacteria to carbapenems is crucial for hospital patient management, nosocomial infection control, and clinical public health work.[14] The diversity of these enzymes and changes in susceptibility phenotypes make testing them a challenging task. It is risky for clinicians to start empirical treatment before obtaining results, which increases the likelihood of unreasonable drug use. The emergence of multidrug-resistant strains increases the failure rate of empirical treatment.[15] Therefore, it is crucial to provide a rapid diagnosis of bacterial resistance and accurately classify the five carbapenemases.[16]

The selection of an appropriate carbapenem enzyme test (CDT) depends on several factors; the epidemiological factor, the diagnostic performance of the disease, the labor intensity of testing personnel, the complexity and cost of testing, and the testing duration. These factors determine carbapenems' usage in future infection control or surveillance studies.[17] Most phenotypic experiments require bacterial culture, which results in long detection cycles, low detection rates, complex operations, and high detection costs. Similarly, conventional molecular detection instruments have disadvantages like complexity and limited testing space. Most importantly, the inability to distinguish the types of carbapenemases leads to the inability to guide clinical medication accurately. These shortcomings lead to limitations in the detection ability of carbapenemases.[18] This raises the urgent need for a new technology that is rapid, simple, and accurate. Our method would solve the "bottleneck" problem of rapid detection and treatment of bacterial resistance. The characteristic of this technology is its suitability for both detection of large-scale indoor populations and the screening of small-scale external populations.[19]

Based on the above diagnostic conditions, we have established an RPA-CRISPR-Cas12a rapid detection platform. The importance of this detection platform is its ability to detect infections caused by carbapenemase-positive bacteria, as well as detect other pathogens that cause significant public health problems. It is a flexible and convenient detection method suitable for multiple scenarios.[20] First, the entire RPA amplification reaction can be achieved by constant temperature amplification at 37 °C. Unlike other nucleic acid amplification techniques, isothermal amplification has the advantages of being fast, efficient, and specific. Multiple amplification can be achieved with an amplification efficiency consistent with conventional PCR. Second, RPA enzymes stored in a freeze-dried form are convenient for storage, transportation. Finally, RPA reaction can amplify target nucleotides in the presence of PCR inhibitors and conduct RPA experiments relatively quickly with limited resources.

Table 3 Comparative Coincidence Test Between CRISPR-Cas12a-RPA and Conventional PCR

	CRISPR-Cas12a-RPA	PCR
Positive	48	48
Negative	27	27
Total	75	75

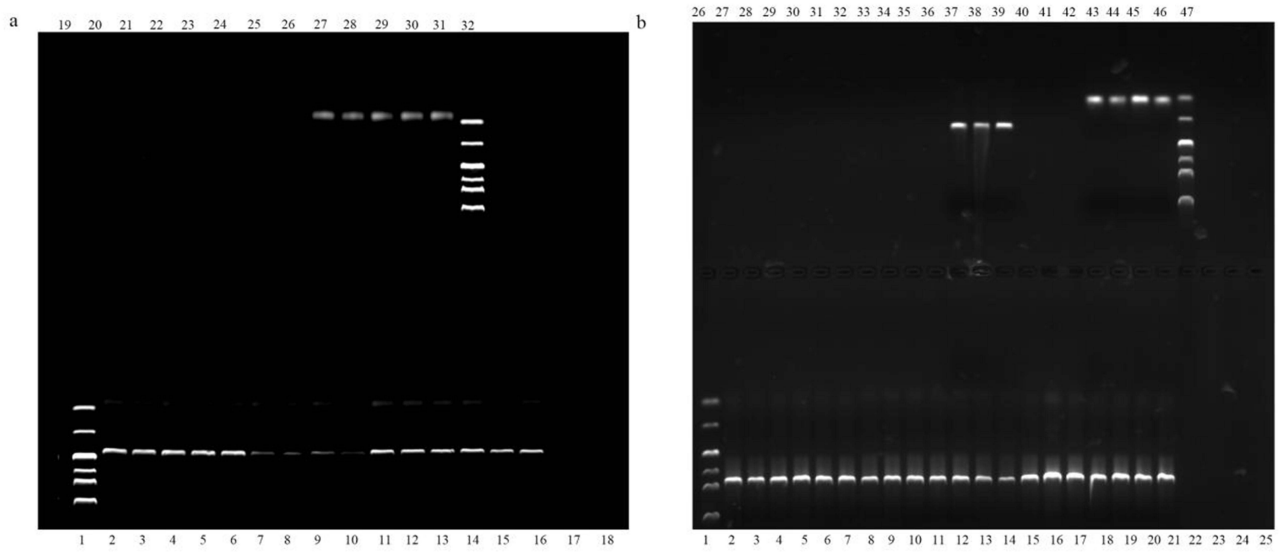

Figure 8 PCR results of 75 samples containing carbapenems.
Notes: (**a**) Test results of samples containing NDM enzyme: numbers 2-16 represent positive samples, and 17-26 represent negative samples; test results of samples containing VIM enzyme: numbers 27-31 represent positive samples. (**b**) Test results of samples containing KPC enzyme: numbers 2-21 represent positive samples, and numbers 22-36 represent negative samples; test results of samples containing OXA enzyme: numbers 37-39 represent positive samples,40-42 represent negative samples; test results of samples containing IMP enzyme: numbers 43-46 represent positive samples.

Furthermore, isothermal amplification can include enzymes with different activities and primers to amplify different target genes rapidly. The CRISPR-Cas genotyping system can also run at 37 °C, allowing the visualization of results by naked eyes, mobile phones, immunochromatographic strips, or UV blue light stimulators. The detection methods can share reagents, facilities, and equipment.[21] In the future, microfluidic chips, digital PCR, and other auxiliary technologies can also be used for dual diagnosis and genotyping, improving the sensitivity and diagnostic efficiency of molecular diagnosis. The rapid detection platform requires less than 1 hour, and the entire reaction is conducted under constant temperature. The testing platform can significantly reduce testing costs without sophisticated instrumentation and professionally trained personnel. At present, it has been reported that this method has been used to detect bacteria or pathogens such as Staphylococcus aureus, novel coronavirus, Leptospira and mycoplasma. At the same time, the technology can also be connected to electronic devices such as mobile phones or chips for portable reading tests.[22–26] The single-tube detection method we established placed the RPA reaction reagent at the bottom of the test tube. After the amplification reaction, the product was mixed with the CRISPR mixture previously placed on the tube cap. This one-step approach reduces liquid transfer operations, shortens detection time, reduces waste of consumables, avoids capping, and reduces aerosol pollution.[27] The sensitivity of this method can reach as low as 1.5 cfu/mL. The specificity is 100%, and the gene can be directly detected in the sample. It is well known that conventional PCR is the gold standard for nucleic acid detection. The consistency rate of our method compared to conditional PCR reached 100%. Based on these results, the detection method of CRISPR/Cas12a we developed is reliable and can be developed into a portable detection tool, which will significantly promote the development of clinical diagnosis in the future.

Conclusion

In conclusion, the detection platform based on CRISPR-Cas-RPA carbapenemase gene is fast, convenient and accurate, and is a new multi scene detection method.

Ethics Approval and Consent to Participate

The studies involving human participants were reviewed and approved by The First Affiliated Hospital of Anhui Medical University. This study involved the use of the patient's saliva, which had been given with the informed consent of the

subjects, and all specimens were numerically numbered and did not affect the patient's privacy. All procedures performed in studies involving human participants were in accordance with the ethical standards of the medical ethics committee of the First Affiliated Hospital of Anhui Medical University (Anhui Public Health Clinical Center) (reference number: LISC20210802) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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 in 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.

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Disclosure

The authors declare that they have no competing interests for this work.

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