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Research Article

Research Article

Rapid and accurate detection of carbapenem-resistance gene by isothermal amplification in *Acinetobacter baumannii*

Shuang Liu[1,2], Guangtao Huang[3], Yali Gong[3], Xiaojun Jin[4], Yudan Meng[1], Yizhi Peng[3], Junning Zhao[6], Xiaolu Li[5,6,*] and Qin Li[7,*]

[1]Department of Plastic & Burns Surgery, The Affiliated Hospital of Southwest Medical University, Tai Ping Street, Luzhou, 646000, China, [2]Department of Dermatology, Dazhou Central Hospital, Nanyue Temple Street, Dazhou, 635000, China, [3]Institute of Burn Research, Southwest Hospital, The Army Medical University, Gao Tan Yan Street, Chongqing, 400038, China, [4]Department of Emergency, The First Affiliated Hospital, Zhejiang University School of Medicine, Qingchun Road, Hangzhou, 310003, China, [5]School of Medicine, Chengdu University, Xindu Avenue, Chengdu 610106, China, [6]Sichuan Translational Medicine Center of Chinese Medicine, Sichuan Academy of Chinese Medical Sciences, Ren Min Nan Lu Road, Chengdu, 610041, China and [7]Department of Infectious Diseases, The Affiliated Hospital of Southwest Medical University, Tai Ping Street, Luzhou, 646000, China

*Correspondence. Xiaolu Li, Email: ls809434281@163.com; Qin Li, Email: 279825702@qq.com

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Abstract

Background: *Acinetobacter baumannii* (A. *baumannii*) is one of the pivotal pathogens responsible for nosocomial infections, especially in patients with low immune response, and infection with carbapenem-resistant *A. baumannii* has been increasing in recent years. Rapid and accurate detection of carbapenem-resistance genes in *A. baumannii* could be of immense help to clinical staff.

Methods: In this study, a 15-μL reaction system for recombinase polymerase amplification (RPA) was developed and tested. We collected 30 clinical isolates of *A. baumannii* from the Burn Institute of Southwest Hospital of Third Military Medical University (Army Medical University) for 6 months and tested antibiotic susceptibility using the VITEK 2 system. *A. baumannii* was detected based on the $bla_{\text{OXA-51}}$ gene by PCR, qPCR and 15 μL-RPA, respectively. Sensitivity and specificity were evaluated. In addition, PCR and 15 μL-RPA data for detecting the carbapenem-resistance gene $bla_{\text{OXA-23}}$ were comparatively assessed.

Results: The detection limit of the $bla_{\text{OXA-51}}$ gene by 15 μL RPA was 2.86 CFU/ml, with sensitivity comparable to PCR and qPCR. No positive amplification signals were detected in non-*Acinetobacter* isolates, indicating high specificity. However, only 18 minutes were needed for the 15 μL RPA assay. Furthermore, an antibiotic susceptibility test showed that up to 90% of *A. baumannii* strains were resistant to meropenem and imipenem; 15 μL RPA data for detecting $bla_{\text{OXA-23}}$ showed that only 10% ($n = 3$) of *A. baumannii* isolates did not show positive amplification signals, and the other 90% of ($n = 27$) isolates were positive, corroborating PCR results.

Conclusion: We demonstrated that the new 15 μL RPA assay for detecting $bla_{\text{OXA-23}}$ in *A. baumannii* is faster and simpler than qPCR and PCR. It is a promising alternative molecular diagnostic tool for

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rapid and effective detection of *A. baumannii* and drug-resistance genes in the field and point-of-care testing.

Key words: *Acinetobacter baumannii*, Recombinase polymerase amplification, Rapid detection, Carbapenem-resistance gene, bla_{OXA-51}, bla_{OXA-23}

Background

Acinetobacter baumannii (A. *baumannii*), a ubiquitous and opportunistic gram-negative pathogen, is one of the pivotal pathogens responsible for hospital-acquired infections. It has been reported that >36% cases of hospital-acquired pneumonia are likely due to infection by *A. baumannii* [1]. Nearly 12% of cases of hospital-acquired bloodstream infections caused by *A. baumannii* in intensive care units have been reported [2]. A prospective study showed that *A. baumannii* is the most frequently cultured organism among gram-negative bacteria in cases with inhalation injury [3]. In addition, *A. baumannii* is also involved in community-acquired infections. *A. baumannii* often leads to outbreaks, because of the extent of its antimicrobial resistance, and *Acinetobacter* spp. are renowned for their ability to survive in the environment under dry conditions for a prolonged period of time; environmental contamination may also play a vital role in such outbreaks [4]. *A. baumannii* can rapidly acquire antimicrobial resistance and shows insusceptibility to a wide range of antimicrobial agents, including β-lactamases, which are frequently used in clinic [5]. However, infection with acquired multidrug-resistant *A. baumannii* (MDR-AB) is associated with increased risk of patient mortality [6]. The World Health Organization published a priority list of antibiotic-resistant bacteria including 12 bacterial families/genera most dangerous for human health and for which new antibiotics are urgently needed: *A. baumannii* is considered 'priority 1' (critical) [7].

Carbapenem antibiotics are the mainstay of therapy for infections caused by *A. baumannii*. Meanwhile, an increased number of carbapenem-resistant *A. baumannii* (CRAB) isolates have been reported since the 1980s [8,9]. This evolution may be due to misuse and overuse of carbapenems for the management of *Acinetobacter* infections, and the accumulation of various resistance mechanisms, including carbapenemase production, loss of membrane pore channel proteins and overexpression of efflux pump and penicillin-binding protein mutations [10]. Carbapenemase production is considered a key factor in CRAB occurrence [11–13]. Among these mechanisms, the carbapenem-resistance gene bla_{OXA-23} remains the most crucial drug-resistance determinant [14,15]. Sun *et al.* reported that 114/117 *A. baumannii* isolates in Southwest Hospital (Chongqing, China) had the bla_{OXA-23} gene, indicating the widespread presence of *A. baumannii* harboring bla_{OXA-23} [16].

Bacterial culture is considered the 'gold standard' for detecting bacterial pathogens in clinical samples. However, the disadvantages of this method are low detection speed and sensitivity [17]. Currently, PCR-based methods are used to detect pathogens and drug-resistance genes in most molecular diagnostic techniques. However, a major drawback of these approaches is the need for precise thermocycling devices. Meanwhile, limitations of conventional PCR also include a high rate of false-positives, prolonged detection time and the requirement of highly skilled laboratory technicians. Isothermal amplification is a promising tool in the field of molecular diagnostic technology. Recombinase polymerase amplification (RPA) is an isothermal nucleic acid amplification technology that amplifies nucleic acids at low constant temperatures (25–42°C) for only 10–20 minutes [18]. Recombinase, single-stranded binding protein and strand-replacement DNA polymerase are the crucial components of RPA, making RPA independent of precise thermal cycling equipment to achieve isothermal exponential amplification [19]. Furthermore, the amplification product in RPA can be detected by a fluorescent probe, lateral flow chromatography, gel electrophoresis, biochip, etc. RPA has many advantages, such as simple instrument, simple operation, rapid detection, high specificity and high sensitivity, which are very suitable for point-of-care testing (POCT). This work focused on utilizing real-time RPA to achieve rapid and sensitive detection of a carbapenem-resistance gene in *A. baumannii* by exonuclease enzyme and exo probe. Furthermore, we analysed the relationship between carbapenem resistance and bla_{OXA-23} presence.

Methods

Preparation of the 5% Chelex-100 lysate

The 5% Chelex-100 lysate was prepared with 2.5 g Chelex-100 (Bio-Rad, USA) and 500 µL TritonX-100 (Sigma-Aldrich, USA) mixed with 50 mL TE buffer (Tris 10 mM, EDTA 1 mM). It was stored at room temperature.

DNA extraction

A. baumannii (ATCC19606) was obtained from North Carolina Souren Biotechnology Research Institute (Beijing, China). *Pseudomonas aeruginosa*(P. *aeruginosa*), *Candida albicans* (C. *albicans*), *Staphylococcus aureus* (S. *aureus*) and *Escherichia coli* (E. *coli*) were supplied by the Institute of Burn Research, Southwest Hospital of Third Military Medical University (Army Medical University). A total of 30 non-repetitive *A. baumannii* isolates were collected from patients at the Institute of Burn Research, Southwest Hospital of Third

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Table 1. Primers and probe for the RPA assay

Name	Type	Sequence (5′–3′)
bla$_{OXA-51}$-F1	Forward primer	AGCTCGTCGTATTGGACTTGAGCTCATGTC
bla$_{OXA-51}$-F2	Forward primer	ACATGACCCTAGGCGATGCCATGAAAGCTTCCGC
bla$_{OXA-51}$-F3	Forward primer	ATGACCCTAGGCGATGCCATGAAAGCTTCCGC
bla$_{OXA-51}$-R1	Reverse primer	GCTAGCTTGTAAGCAAACTGTGCCTCTTGCTGAGG
bla$_{OXA-51}$-R2	Reverse primer	CTTGTAAGCAAACTGTGCCTCTTGCTGAGG
bla$_{OXA-51}$-R3	Reverse primer	TAGCTTGTAAGCAAACTGTGCCTCTTGCTGAGGAG
bla$_{OXA-51}$-P	Exo probe	AAGCGTGTTGGTTATGGCAATGCAGATA[FAM-dT]C[THF]G[BHQ-dT]ACCCAAGTCGATA[C3-spacer]
bla$_{OXA-23}$-F	Forward primer	GCAGTCCCAGTCTATCAGGAACTTGCGCGACG
bla$_{OXA-23}$-R	Reverse primer	GGCGTAACCTTTAATGGTCCTACCAACCAG
bla$_{OXA-23}$-P	Exo probe	ATGCAAAAAGAAGTAAAACGTATTGGTT[FAM-dT]C[THF]G[BHQ-dT]AATGCTGAAATTG[C3-spacer]

FAM-dT fluorescein coupled to a thymine, *THF* tetrahydrofuran, *BHQ-dT* Black Hole Quencher-1 coupled to a thymine, *RPA* recombinase polymerase amplification

Military Medical University (Army Medical University), for 6 months from May to October 2013. DNA extraction was performed by the Chelex-100 rapid DNA extraction method developed by our research group. The entire extraction process only takes 15 minutes, with the following steps. After culturing pure colonies of various strains on Luria-Bertani Liquid Medium (*C. albicans* was cultured in Sabouraud dextrose broth) at 37°C in a 10% CO_2 shake box for 12–16 hours. A total of 1 mL broth was centrifuged at 13,000 rpm for 2 minutes on a Legend Micro17 centrifuge (Thermo Scientific, USA). The pelleted bacterial cells were suspended in 5% Chelex-100 lysate and incubated at 97–100°C with shaking at 300 rpm on a Bioer Mixing Block MB-102 (Bioer Technology, China). After 10 minutes, the suspension was centrifuged at 13,000 rpm for 2 minutes. DNA concentrations were quantified by spectrophotometry on a NanoDrop ND-1000 spectrometer (Thermo Scientific, USA). In addition, genomic DNA standard containing 50 ng/μl and 25 ng/μl of DNA in molecular biology-grade H_2O extracted with TIANamp Bacteria DNA Kit (Tiangen Biotech, China) was prepared for screening candidate RPA primers of the *bla*$_{OXA-51}$ gene.

Real-time RPA primers and the exo probe

The *bla*$_{OXA-51}$ gene, an innate gene of *A. baumannii*, is encoded on the chromosome. Amplification of the *bla*$_{OXA-51}$ gene is a new method for the detection *A. baumannii*, which is attributed to the highly conserved nature and species specificity of *bla*$_{OXA-51}$ [20–22]. To develop an effective RPA assay, three forward (*bla*$_{OXA-51}$-F1 to *bla*$_{OXA-51}$-F3) primers, three reverse (*bla*$_{OXA-51}$-R1 to *bla*$_{OXA-51}$-R3) primers and an exo probe (*bla*$_{OXA-51}$-P) were designed according to the sequence of the *bla*$_{OXA-51}$ gene (NCBI reference sequence: KJ584925.1) and the manufacturer's guidelines (TwistDx Inc., UK) (Table 1). The three forward and three reverse primers were screened for reactivity to purified *A. baumannii* DNA using a 50 μL RPA assay and a probe was designed that would accommodate all primer sets. For screening reverse primers, one forward primer was selected randomly to respectively pair with the three reverse primers (*bla*$_{OXA-51}$-R1–R3) to test *A. baumannii* with the 50 μL-RPA assay, with a DNA concentration of 50 ng/μl. The primer sets were then tested and compared for time to fluorescence threshold. The reverse primer with the shortest fluorescence threshold time was selected as the optimal one. If similar fluorescence threshold times were encountered in certain primer sets, these were further tested by reducing the DNA concentration to 25 ng/μl. After determining the optimal reverse primer, the same method was applied to screen forward primers, but DNA was used directly at 25 ng/μl. After the initial screening, one optimal primer set was selected for experimental evaluation. The primers and probes for *bla*$_{OXA-23}$ gene detection in *A. baumannii* were designed and screened strictly according to the manufacturer guidelines (Table 1). The sequence of the *bla*$_{OXA-23}$ gene is NC_025109.1 (GenBank). All primers and probes were provided by Sangon Biotech (China).

PCR

The optimal forward and reverse primer set was used to identify *A. baumannii* by PCR, as well as for the determination of the specificity and sensitivity of the optimal primer set by the following PCR protocol. PCR was performed with a reaction mix containing 10 μL 2 × TaqPCR Master Mix (Sangon Biotech), 0.4 μL each of forward and reverse primers (10 μM), 7.2 μL molecular biology-grade H_2O and 2 μL template DNA. The PCR program consisted of an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of DNA denaturation at 94°C for 30 seconds, primer annealing (bla$_{OXA-51}$, 60°C for 30 seconds; *bla*$_{OXA-23}$, 56°C for 30 seconds), primer extension at 72°C for 40 seconds and final extension at 72°C for 5 minutes. PCR products were assessed by agarose-gel electrophoresis, and gel images were captured on a Gel Doc^{TM} XR^{+} Gel Imaging System (Bio-Rad).

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qPCR

qPCR was performed on a CFX96^{TM} Thermal Cycler (Bio-Rad). The reaction system contained 5 μL Eva Green nucleic acid dye (Applied Biological Materials, Canada), 0.2 μL of each 10 μM optimal forward and reverse primers of the *bla*$_{OXA-51}$ gene, 3.6 μL molecular-grade H_2O and 1 μL of template DNA. The CFX96^{TM} Thermal Cycler was programmed as follows: pre-incubation at 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

The 50 μL RPA assay

In this study, the 50 μL RPA assay was only used to screen the optimal primer set, with the TwsitAMP exo kit (TwistDx Inc.). The reaction system of 50 μL RPA contained 2.1 μL of 10 μM forward primer and reverse primers, 0.6 μL exo probe (10 μM), 29.5 μL rehydration buffer and 12.2 μL molecular biology-grade H_2O. After preparation, the mix was distributed into reaction tubes supplied with the TwsitAMP exo freeze-dried enzyme powder. A total of 1 μL of DNA at the corresponding concentration and one magnetic bead were then added to each tube. To start the reaction, 2.5 μL of 280 mM magnesium acetate was pipetted into the lid of each tube. The tube was immediately centrifuged using a mini-centrifuge before placement into the T8-ISO fluorescence detector (TwistDx Inc.) set at 42°C for 15 minutes.

The 15 μL RPA assay

In this study, the 15 μL RPA assay was performed in a 15 μL with the TwsitAMP exo kit containing 2.1 μL of each forward and reverse primers (10 μM), 0.6 μL exo probe (10 μM), 29.5 μL rehydration buffer and 9.2 μL molecular biology-grade H_2O, which were mixed in a 1 mL EP tube. Then, exo freeze-dried enzyme powder was added into the tube and fully mixed. A total of 10.5 μL of the master mix was distributed into 0.2 mL reaction tubes, each supplemented with 4 μL of template DNA and one magnetic bead. Finally, 0.5 μL of 280 mM magnesium acetate was pipetted into the lid of each tube. The tubes were closed, centrifuged using a mini-centrifuge and quickly placed into the CFX96^{TM} Thermal Cycler, which was programmed to run for 18 minutes comprising 60 cycles at 42°C and to collect fluorescence signals at 10-second intervals.

Specificity and sensitivity assessment

The specificity of the optimal primer set was determined by amplifying DNA from *A. baumannii* and four negative control strains, *P. aeruginosa, C. albicans, S. aureus* and *E. coli*, by PCR, qPCR and 15 μL RPA. In addition, the feasibility of 15 μL RPA in detecting *A. baumannii* was evaluated. To determine the analytical sensitivity of the 15 μL RPA assay, a bacterial suspension of *A. baumannii* ATCC19606 at 1 OD (2.86×10^9 CFU/ml) was serially diluted 10-fold with physiological saline (10^9–10^0 CFU/ml). Then, the target DNA of the diluted bacterial suspension was extracted by the Chelex-100 rapid DNA extraction method. Next, the target DNA was amplified with the optimal primer set of the *bla*$_{OXA-51}$ gene by PCR, qPCR and 15 μL RPA assay, comparatively assessing detection limit and sensitivity among the three methods. Furthermore, the relationship between bacterial suspension concentration and fluorescence threshold time (cycle number) was assessed for qPCR and 15 μL RPA with GraphPad Prism 6.02. Finally, the detection rate of DNA at each bacterial suspension concentration was detected.

Detection of clinical isolates

The genomic DNAs of 30 clinical isolates of *A. baumannii* were tested, respectively, by 15 μL RPA and PCR, analysing the detection rates of 15 μL RPA and PCR.

Antibiotic susceptibility test

Susceptibility testing of bacterial isolates was carried out on the VITEK 2 system (Biomerieux, France) using antimicrobials (OXOID. UK) including amikacin, ampicillin-sulbactam, polymyxin B, sulfamethoxazole-trimethoprim, ciprofloxacin, meropenem, minocycline, netilmicin, gentamicin, tetracycline, ceftazidime, cefepime, cefoperazone-sulbactam 1:1, cefotaxime, tobramycin, imipenem, levofloxacin, piperacillin and piperacillin-tazobactam. *P. aeruginosa* (ATCC25923) and *E. coli* (ATCC25922) were used for quality assurance. Antimicrobial susceptibility was interpreted according to Clinical and Laboratory Standards Institute guidelines [23].

Drug resistance gene detection

The carbapenem-resistant gene *bla*$_{OXA-23}$ in 30 clinical isolates, as well as one *bla*$_{OXA-23}$ negative isolate, was amplified by the 15 μL RPA and PCR to analyse the feasibility of 15 μL RPA for detecting resistance genes. The detection rates of *bla*$_{OXA-23}$ were compared between the above amplification methods.

Results

Screening of candidate RPA primers for *bla*$_{OXA-51}$ gene detection

The results of reverse primer screening are shown in Figure 1a. Amplification signal threshold times were significantly reduced for *bla*$_{OXA-51}$-F3-R2 (261 seconds) and *bla*$_{OXA-51}$-F3-R3 (271 seconds) primer sets compared with the *bla*$_{OXA-51}$-F3-R1 primer set (311 seconds). The fluorescence threshold time differed only by 10 seconds between *bla*$_{OXA-51}$-R2 and *bla*$_{OXA-51}$-R3. To further determine the optimal reverse primer, we reduced the DNA concentration to 25 ng/μl and both primer sets, including *bla*$_{OXA-51}$-F3-R2 and *bla*$_{OXA-51}$-F3-R3, were tested again. The results showed that the amplification signal threshold time for the *bla*$_{OXA-51}$-F3-R2 primer set (261 seconds) was obviously shorter than that of the *bla*$_{OXA-51}$-F3-R3 primer

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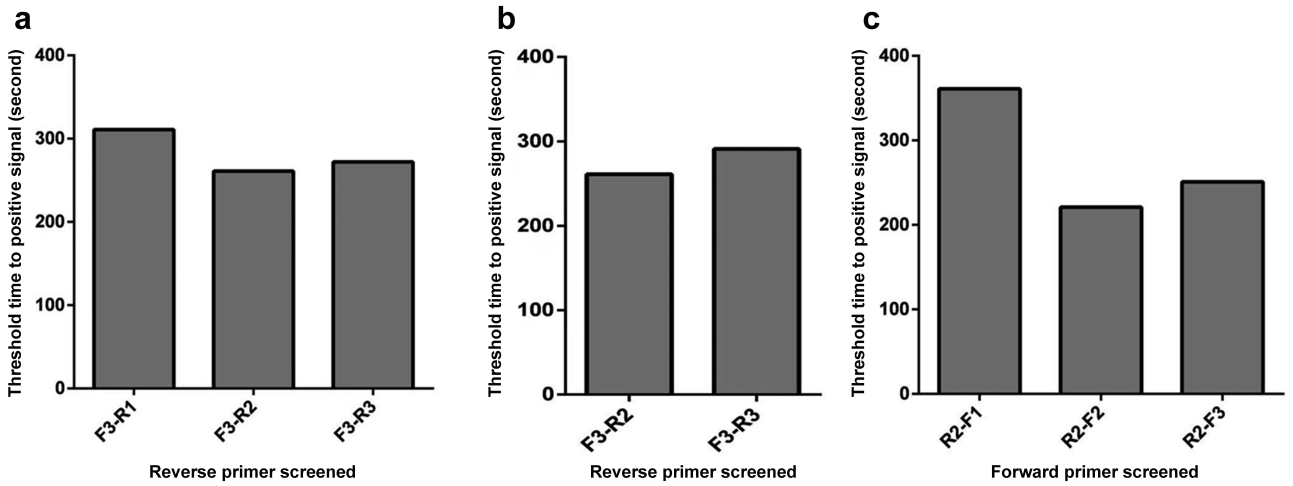

Figure 1. Threshold times to positive signal detection of different recombinase polymerase amplification (RPA) primer sets. Reverse primer screening. *bla*$_{OXA-51}$-F3 paired with reverse primers to detect *Acinetobacter baumannii* (A. *baumannii*) at DNA concentrations of (**a**) 50 ng/μl and (**b**) 25 ng/μl, respectively. Forward primer screening. (**c**) *bla*$_{OXA-51}$-R2 paired with forward primers to detect *A. baumannii* at a DNA concentration of 25 ng/μl

set (291 seconds), with the fluorescence value of the former increasing faster (Figure 1b). Obviously, after reducing target DNA concentration, the amplification signal threshold time for *bla*$_{OXA-51}$-F3-R2 did not increase significantly; therefore, the *bla*$_{OXA-51}$-R2 primer was selected as the optimal reverse primer. Next, the optimal forward primer was screened with *bla*$_{OXA-51}$-R2 paired, respectively, with all three forward primers. Figure 1c shows that the amplification signal threshold time was shorter for *bla*$_{OXA-51}$-F2-R2 (221 seconds) compared with *bla*$_{OXA-51}$-F1-R2 (361 seconds) and *bla*$_{OXA-51}$-F3-R2 (251s). Therefore, *bla*$_{OXA-51}$-F2 was selected as the optimal forward primer. In summary, *bla*$_{OXA-51}$-F2-R2 of the *bla*$_{OXA-51}$ gene as the optimal primer set was used for evaluating the specificity and sensitivity of the method.

Analytical specificity

When the optimal primer set was tested for specificity only *A. baumannii* showed a typical amplification curve in the 15 μL RPA assay. No positive signals were detected for *P. aeruginosa, C. albicans, S. aureus and E. coli* (Figure 2). The specificity data for 15 μL RPA were consistent with those of PCR and qPCR. These findings showed that the *bla*$_{OXA-51}$-F2-R2 primer set was highly specific for the detection of *A. baumannii*, also indicating the feasibility of 15 μL RPA in amplifying *A. baumannii*.

Analytical sensitivity

To test the analytical sensitivity (detection limit) of the 15 μL RPA assay, serial dilutions of *A. baumannii* suspension were tested by PCR, qPCR and 15 μL RPA using *bla*$_{OXA-51}$-F2-R2. Except for PCR, both qPCR and 15 μL RPA only detected bacteria at concentrations of 10^7–10^0 CFU/ml. The data sets of triplicate bacterial fluid standards for both 15 μL RPA and qPCR were used in semi-log and probit analyses. Surprisingly, the detection limit of 15 μL RPA was only 2.86 CFU/ml (Figure 3), which was consistent with PCR and qPCR data. The threshold time required for the detection limit of 2.86 CFU/ml was below 8 minutes in the 15 μL RPA assay, while more than 60 minutes were required for qPCR (Figure 4). In the probit analysis, the limits of detection with 95% probability were 2.86 CFU/ml for both 15 μL RPA and qPCR (Figure 5).

Detection of clinical isolates

The clinical applicability of 15 μL RPA was evaluated by amplifying 30 clinical isolates of *A. baumannii* by PCR and 15 μL RPA. All 30 clinical strains showed positive signals in 15 μL RPA, as shown in Figure S1, for a positive rate of 100%, consistent with PCR data (Figure S2). Figure S3 depicts the sequencing results of PCR amplification products shows that the clinical isolates were 98% homologous compared to the NCBI sequence.

Antibiotic susceptibility

As shown in Figure 6 and Table S1, >95% of the clinical isolates were multi-drug resistant. All clinical isolates were resistant to sulfamethoxazole-trimethoprim, while no isolate was resistant to polymyxin B. Except for cefoperazone-sulbactam and minocycline, more than 90% of the assessed isolates were resistant to the remaining 15 antibiotics. It is worth noting that 90.0% of isolates were resistant to meropenem and imipenem. The resistance rates of *A. baumannii* isolates towards cefoperazone-sulbactam and minocycline were 56.7% and 26.7%, respectively.

Drug resistance gene detection

The carbapenem-resistance *bla*$_{OXA-23}$ gene was tested in 30 non-repetitive *A. baumannii* isolates by PCR and 15 μL RPA,

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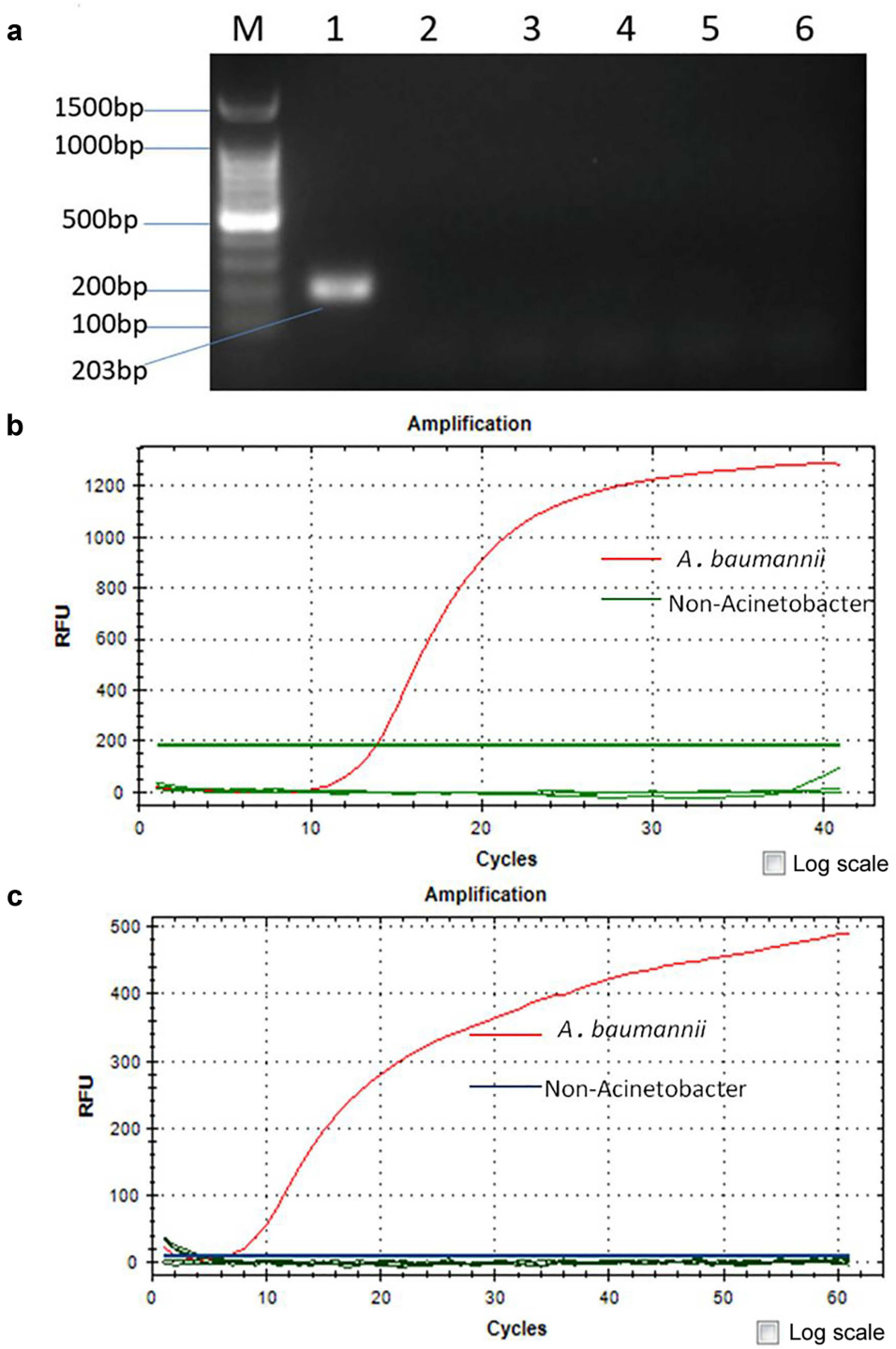

Figure 2. Analytical specificities of PCR, qPCR and 15 μL recombinase polymerase amplification (RPA) for the detection of the bla_{OXA-51} gene. **(a)** Analytical specificity of PCR. M is the DNA marker; lines 1–5 show *Acinetobacter baumannii* (A. *baumannii*), *Pseudomonas aeruginosa* (P. *aeruginosa*), *Candida albicans* (C. *albicans*), *Staphylococcus aureus* (S. *aureus*), *Escherichia coli* (E. *coli*) and line 6 shows blank control (purified water), respectively. **(b)** Analytical specificity of qPCR. **(c)** Analytical specificity of the 15 μL RPA assay. Non-*Acinetobacter* were *P. aeruginosa, C. albicans, S. aureus* and *E. coli*. *RFU* relative fluorescence units

respectively. In 15 μL RPA, only 3/30 (10%) of *A. baumannii* isolates showed no positive amplification signals, and the remaining 27/30 (90%) isolates were positive (Figures 7 and S4), consistent with PCR data (Figure S5). Furthermore, based on the antibiotic susceptibility test, the three isolates with no amplification signals were sensitive to meropenem and imipenem, while the remaining 27 with amplification were resistant to these antibiotics (Table S1). These findings demonstrated that the bla_{OXA-23} resistance gene was not present in carbapenem-sensitive *A. baumannii* (CSAB).

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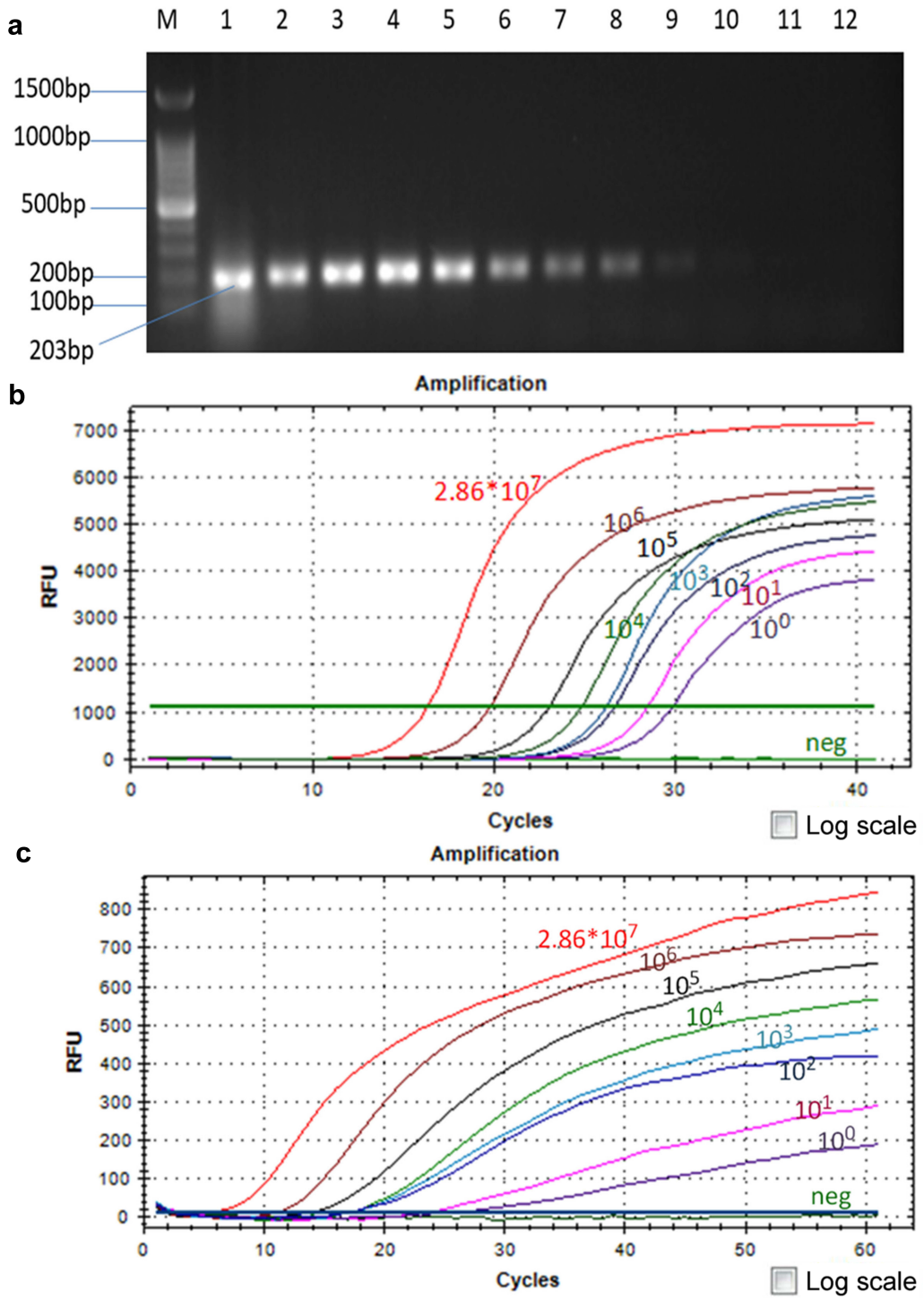

Figure 3. Analytical sensitivities of **(a)** PCR, **(b)** qPCR and **(c)** 15 μL-recombinase polymerase amplification (RPA) for the detection of the blaOXA-51 gene. Target DNA was extracted after 10-fold dilution of bacterial suspension, **(a)** Detection sensitivity for the blaOXA-51 gene of Acinetobacter baumannii by PCR. M shows the DNA marker; lines 1–10 show DNA concentrations of 2.86×109 CFU/ml, 2.86×108 CFU/ml, 2.86×107 CFU/ml, 2.86×106 CFU/ml, 2.86×105 CFU/ml, 2.86×104 CFU/ml, 2.86×103 CFU/ml, 2.86×102 CFU/ml, 2.86×101 CFU/ml and 2.86×100 CFU/ml, respectively; lines 11–12 show negative and blank controls, respectively. **(b)** qPCR and **(c)** 15 μL RPA assay only detected 2.86×107–2.86×100 CFU/ml. *RFU* relative fluorescence units

Discussion

Multiple isothermal amplification techniques have been proposed for the detection of pathogens in recent years, including nucleic acid sequence based amplification, transcription mediated amplification, helicase-dependent amplification, RPA, loop-mediated isothermal amplification (LAMP),and single primer isothermal amplification. Li et al. performed LAMP to detect the target DNA of *A. baumannii* within

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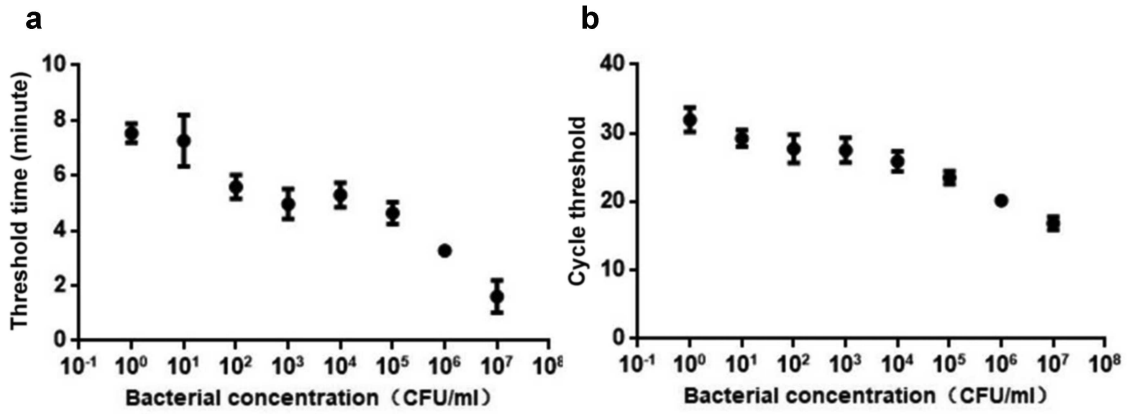

Figure 4. Semi-log regression assay for the **(a)** 15 μL-recombinase polymerase amplification (RPA) and **(b)** qPCR assays using PRISM; data sets of three 15 μL RPA and qPCR assays. The threshold time required for the detection limit of 2.86 CFU/ml was below 8 minutes in the 15 μL RPA assay, and >60 minutes in qPCR

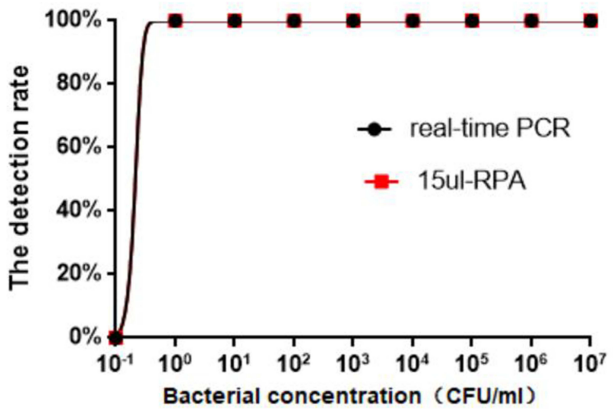

Figure 5. Probit analysis of the 15 μL recombinase polymerase amplification (RPA) (red) and qPCR (black) assays using PRISM. Data sets of three 15 μL RPA and qPCR assays as shown in Figure 3 were used. Limits of detection with 95% probabilities were 2.86 CFU/ml in the RPA and qPCR assays, respectively

60 minutes at 65°C, with a detection limit of 50 pg/μl (i.e. ~10-fold greater than that of PCR) [21]. Lars D. Renner combined vacuum degassed microfluidic cartridge preloaded and lyophilized RPA assays to detect the targeted ESKAPE pathogens, including *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, A. baumannii, P. aeruginosa and Enterobacter spp.*, with a detection limit of 1 pg/μl for *A. baumannii* on the bacterial detection chip (B-chip) [24].

In this study, we evaluated the clinical feasibility of RPA compared to the reference PCR and qPCR assays. The isothermal RPA method was selected for the following reasons: (1) low amplification temperature, as low as 25°C [25]; (2) exponential amplification within 15 minutes using a fluorescent probe; (3) the lyophilized enzyme powder in RPA contains the single-strand binding protein GP32, which enhances nucleic acid detection [26]; (4) reagents can be stored for several days even at room temperature [27, 28]; and (5) the operation and equipment are simple and cheap, making the assay amenable to use in the field and POCT.

We first described a tool for detecting *A. baumannii* and the carbapenem-resistance gene bla_{OXA-23} using 15 μL RPA, which can be performed rapidly without precision thermal cycling instruments and may be used in rural health clinics or battlefields. In this study, a 15 μL RPA assay was designed to specifically and efficiently amplify the bla_{OXA-51} gene of *A. baumannii*. In specificity analysis, amplification signals were only observed in *A. baumannii* without cross-reactivity with non-*Acinetobacter* isolates. The detection limit of the 15 μL RPA assay was as little as 2.86 CFU/ml of DNA template; that is, the same sensitivity as qPCR and PCR. The high specificity of the 15 μL RPA assay was partly due to the high specificity of the designed primers and probes; on the other hand, it may be due to inherent proofreading capabilities of the recombinant enzyme [29, 30]. Meanwhile, the fluorescence threshold time gradually decreased with increasing bacterial concentration in 15 μL RPA. Surprisingly, only 18 minutes were needed to detect *A. baumannii* by 15 μL RPA assay, with an amplification threshold time as short as 2 minutes; the threshold time was 8 minutes for amplification at the detection limit of 2.86 CFU/ml, which is still significantly shorter than that of qPCR. However, the detection rate obtained at the detection limit of 2.86 CFU/ml was consistent with qPCR data. Next, 30 non-repetitive *A. baumannii* isolates were collected to evaluate the clinical applicability of 15 μL RPA. As expected, the positive rate for the clinical isolates of *A. baumannii* was 100% by the 15 μL RPA assay, which was consistent with PCR data. The high analytical specificity and sensitivity of 15 μL RPA makes this assay amenable to clinical applications. Overall, these results indicated that 15 μL RPA is specific, sensitive and fast in the detection of *A. baumannii* with our designed primer set bla_{OXA-51}-F2-R2 and bla_{OXA-51}-P. Therefore, this common pathogen can be rapidly identified by the 15 μL RPA assay in poorly equipped areas, such as battlefields, farms and communities.

Treatment with carbapenems is considered the optimal therapeutic strategy for *A. baumannii* infection. However, CRAB has rapidly increased worldwide in recent years. Huang et al. reported a resistance rate of *A. baumannii*

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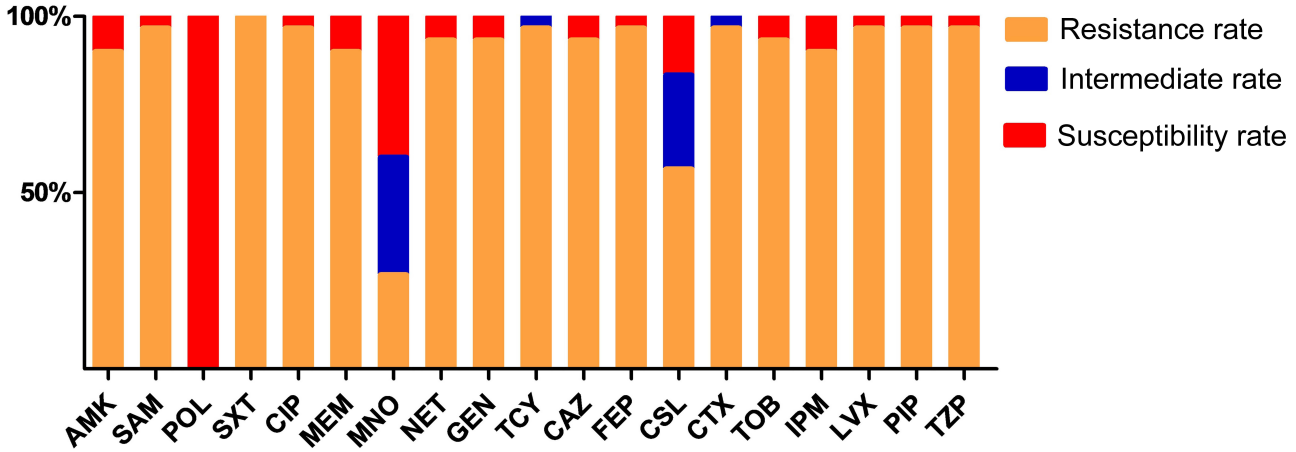

Figure 6. Antibiotic susceptibility data. *AMK* amikacin, *SAM* ampicillin/sulbactam, *POL* polymyxin B, *SXT* sulfamethoxazole-trimethoprim, *CIP* ciprofloxacin, *MEM* meropenem, *MNO* minocycline, *NET* netilmicin, *GEN* gentamicin, *TCY* tetracycline, *CAZ* ceftazidime, *FEP* cefepime, *CSL* cefoperazone/sulbactam, *CTX* cefotaxime, *TOB* tobramycin, *IPM* imipenem, *LVX* levofloxacin, *PIP* piperacillin, *TZP* piperacillin/tazobactam

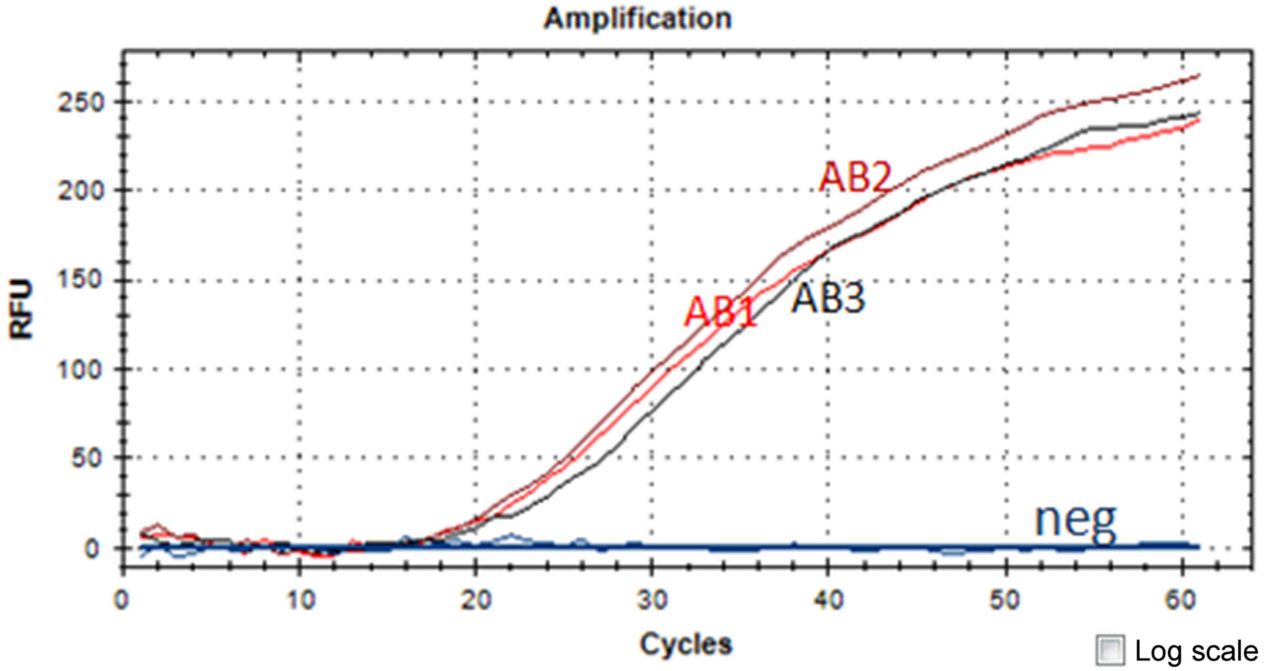

Figure 7. Detection of the bla_{OXA-23} drug-resistance gene by 15 μL recombinase polymerase amplification. All three clinical isolates showed exponential amplification curves, while the bla_{OXA-23} negative strains were not amplified. *RFU* relative fluorescence units

towards imipenem and meropenem of about 85% in Southwest Hospital, while the resistance rate of bloodstream isolates towards imipenem and meropenem is >90% [31, 32]. In this study, 30 clinical isolates of *A. baumannii* were collected from the burn ward, most of which were MDR-AB. The resistance rate of *A. baumannii* isolates towards meropenem and imipenem reached 90%; in contrast, polymyxin B was the most effective agent, with a susceptibility rate of 100%, indicating that polymyxin B could be used to treat infections caused by CRAB. However, the side effects of polymyxin B must be considered. The resistance gene bla_{OXA-23} plays an important role in resistance to carbapenems in *A. baumannii*, and strains carrying bla_{OXA-23} are steadily increasing worldwide. In a burn ward in Iran, 96.92% of clinical isolates of *A. baumannii* were shown to be resistant to meropenem and imipenem, with CRAB isolates carrying the oxacillinase determinants of bla_{OXA-23} accounting for 74.6%, suggesting that high-level CRAB with bla_{OXA-23} in burn patients is a reason for *A. baumannii* resistance to carbapenems [33]. Abdulzahra revealed that 80% of *A. baumannii* isolates carry bla_{OXA-23}, which implies that it is a crucial healthcare concern in Egypt that necessitates strict interventions to eliminate such infections [34]. Lowo et al. collected 141 *A. baumannii* strains from two tertiary academic hospitals (A

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and B) in South Africa; the results showed that these clinical isolates carried the bla_{OXA-23} gene at a rate of 96% (69/72) in hospital A versus 91% (63/69) in hospital B, implying that greater infection control and prevention strategies need to be developed [35]. In this study, we focused on testing the resistance gene bla_{OXA-23} by 15 μL RPA to develop the RPA assay for detecting carbapenem-resistance genes. The results of 15 μL RPA showed that 90% of isolates showed positive amplification signals and only 10% were negative, consistent with PCR data (Figure S5). To further analyse the relationship between CRAB and the bla_{OXA-23} gene, we analysed the antibiotic susceptibility of 30 isolates. Interestingly, the 27 isolates with positive amplification signals were resistant to imipenem and meropenem, while the three isolates with negative amplification signals were CSAB. CRAB carrying the bla_{OXA-23} resistance gene were 100% in this study, implying that bla_{OXA-23} has inescapable responsibility for *A. baumannii* resistance to carbapenem. We inferred that the initial screening of antibiotics could be carried out by detecting the bla_{OXA-23} gene by the 15 μL RPA assay. This also implied that it is necessary and important to monitor and control the spread of bla_{OXA-23}-producing *A. baumannii*.

Finally, regarding cost, the standard 50 μL RPA assay is expensive and costs approximately $8 per reaction, which hinders large-scale promotion and use. In this study, we developed a reliable real-time 15 μL RPA assay for efficient detection of *A. baumannii* and the carbapenem-resistance gene, with shorter time-to-result than qPCR and PCR. In addition, the method is simple to operate with low equipment requirements and does not need a thermal cycler. In addition, the 15 μL RPA assay only costs approximately $2.50 per test.

The limitations of this study should be mentioned. First, there were too few strains in the specificity analysis, and it especially lacked other *Acinetobacter* species. In addition, the CFX96™ Thermal Cycler was used as a fluorescence reader in this study, which is impossible in the field and POCT. Furthermore, only the carbapenem-resistance bla_{OXA-23} gene was assessed, with no validation against other drug-resistance genes. Notwithstanding its limitations, this study clearly demonstrated that 15 μL RPA for the detection of *A. baumannii* and the carbapenem-resistance gene bla_{OXA-23} is rapid, accurate and effective. Moreover, these issues could be solved in future investigation, including the development of portable equipment for fluorescence detection, detection of diverse drug-resistance genes and further verification of the specificity of bla_{OXA-51} gene primers as well as the clinical applicability of 15 μL RPA.

Conclusions

This study demonstrated that the new real-time 15 μL RPA assay is a clinically useful molecular diagnostic tool with good specificity and sensitivity for the detection of carbapenem-resistance genes in *A. baumannii*. The 15 μL RPA assay is faster and much easier to handle than PCR and qPCR, and cheaper than the 50 μL RPA assay. We consider that 15 μL RPA has great potential as a promising alternative molecular diagnostic method for rapid and effective detection of *A. baumannii* and drug-resistance genes in the field and POCT, which has a vital use for early diagnosis, detection and treatment.

Abbreviations

MDR-AB: multidrug-resistant *A. baumannii*; CRAB: carbapenem-resistant *A. baumannii*; RPA: recombinase polymerase amplification; POCT: point-of-care testing; CSAB: carbapenem-sensitive *A. baumannii*; LAMP: loop-mediated isothermal amplification

Supplementary data

Supplementary data is available at *Burns & Trauma Journal* online.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Authors' contributions

Shuang Liu performed the main experiments, analysed the data, prepared the figures and wrote the manuscript. Guangtao Huang, Yali Gong and Yudan Meng performed the experiments and prepared Figures 5–7. Yizhi Peng, Junning Zhao, Li Yang and Xiaolu Li helped conceived the project and designed the experiments. Xiaolu Li revised the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

This study was carried out in accordance with the approved guidelines of the Ethics Committee of Southwest Hospital, Army Medical University.

Conflicts of interest

The authors declare that they have no competing interests.

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negative control strains. We sincerely thank Xiaojun Jin, Peng He, Peiting Zhou, Zhilin Tu and Yan He for help during the experiments.

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