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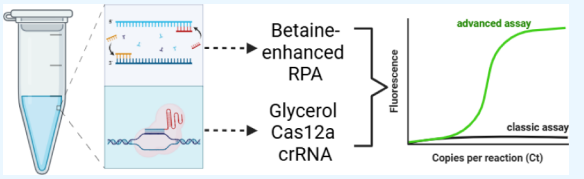
Advanced One-Pot RPA-CRISPR/Cas12a Reaction with Glycerol and Betaine for High-Sensitivity Diagnosis of *mecA*-Carrying Strains in Clinical Samples

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ABSTRACT: The *mecA* gene confers methicillin resistance in both MRSA and MR-CoNS by encoding the PBP2a protein and poses a significant public health threat due to its resistance to beta-lactam antibiotics. Rapid and accurate detection of *mecA* is critical for timely treatment, reducing morbidity, and preventing its spread in healthcare settings. In this study, we developed an advanced one-pot recombinase polymerase amplification (RPA)-CRISPR/Cas12a system, enhanced with glycerol and betaine, for ultrasensitive detection of the *mecA* gene. Glycerol's viscosity effect prevents premature interaction between Cas12a and early amplification products, while betaine enhances nucleic acid amplification. The assay demonstrated superior sensitivity, detecting as low as 5 copies/μL of *mecA* DNA within 60 min. Specificity testing against a panel of bacterial species confirmed the high selectivity of the assay for *mecA*-carrying strains with negligible cross-reactivity. Furthermore, this method exhibited excellent performance across various clinical samples, including blood, urine, and bronchoalveolar lavage fluid. Our findings underscore the potential of this advanced RPA-CRISPR/Cas12a assay as a powerful diagnostic tool for rapid, cost-effective, and highly sensitive *mecA* detection, offering a promising solution for clinical diagnostics and infection control.



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INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MR-CoNS) are types of bacteria that have developed resistance to methicillin and other beta-lactam antibiotics, including penicillins and cephalosporins.[1,2] This resistance makes infections difficult to treat with standard antibiotic therapies, leading to more severe health outcomes. Among them, MRSA can cause severe infections such as bloodstream infections (bacteremia), pneumonia, and surgical site infections. These conditions often result in longer hospital stays, more intensive treatment, and higher rates of complications compared to infections caused by methicillin-sensitive *Staphylococcus aureus* (MSSA). According to the WHO bacterial priority pathogens list 2024, MRSA maintains its position in the high-priority pathogen category, in line with its high estimated burden.[3] *S. aureus* was identified as one of the six leading pathogens for deaths associated with resistance in 2019, and 100,000 deaths caused by MRSA were attributable to antimicrobial resistance.[4]

The rapid, sensitive, and accurate diagnosis of MRSA and MR-CoNS is crucial because it allows for quick and effective treatment, reduces the risk of severe illness and death, prevents the misuse of antibiotics, and helps control the spread of the infection in healthcare settings.[5,6] It also lowers healthcare costs by reducing hospital stays and supports public health efforts to manage outbreaks. Current diagnostic methods offer distinct advantages and disadvantages that affect their utility in managing infections. Culture-based techniques, the gold standard method, provide high specificity and detailed antibiotic susceptibility profiles, which are cost-effective and widely accessible. However, they are labor-intensive and time-consuming, requiring 24−48 h to yield results, which delays the initiation of appropriate therapy.[7,8] Molecular methods, targeting *mecA* gene which is a biomarker for methicillin-resistant strains, such as polymerase chain reaction (PCR) offer rapid and highly sensitive detection.[9,10] Nevertheless, PCR is expensive and requires specialized equipment and expertise, which may not be available in all healthcare settings. Advanced techniques like MALDI-TOF mass spectrometry provide rapid and accurate identification, but they also come with high initial costs and technical challenges, making their widespread adoption a hurdle in resource-limited environments.[11]

The emerging use of recombinase polymerase amplification (RPA) coupled with CRISPR/Cas12a technology in bacterial diagnostics marks a significant advancement in the field, providing rapid, highly sensitive, and specific detection capabilities.[12−14] The RPA amplifies target DNA at a constant temperature, eliminating the need for thermal cycling and enabling quick, efficient amplification. The amplified product is

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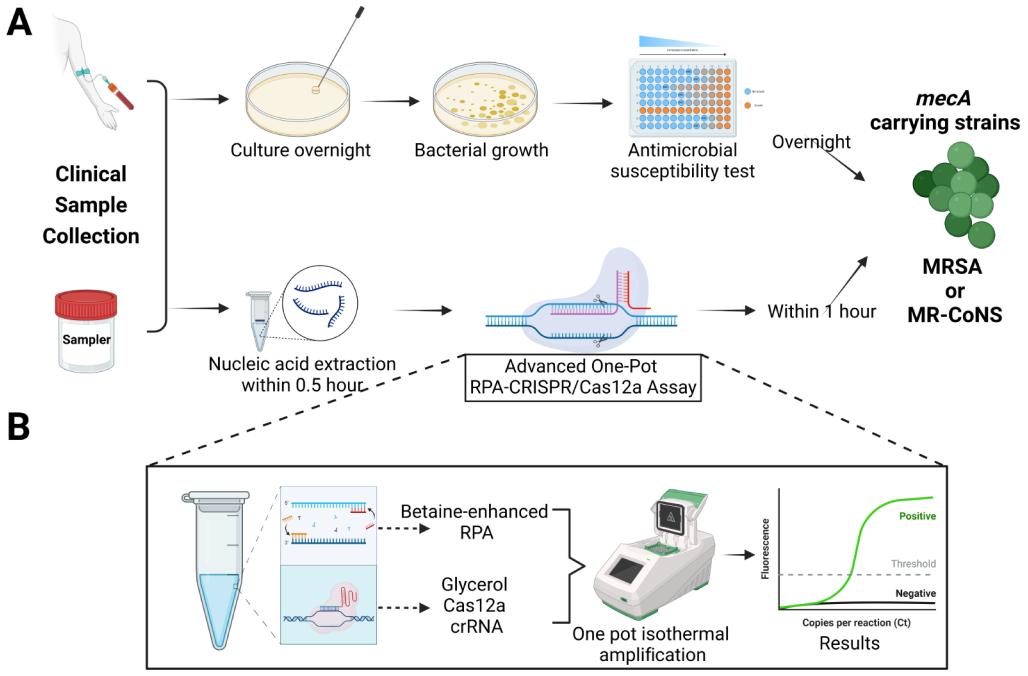


Figure 1. Comparison of traditional and advanced one-pot RPA-CRISPR/Cas12a assay workflows for *mecA*-carrying strains detection. (A) The traditional diagnostic method results in a diagnosis within 24 h, whereas the advanced method delivers results within 1 h. (B) The advanced one-pot RPA-CRISPR/Cas12a assay utilizes betaine-enhanced RPA for efficient amplification and glycerol-stabilized Cas12a−crRNA complexes to prevent premature interaction with amplification products, ensuring high sensitivity and specificity in *mecA* detection.

then recognized by the CRISPR/Cas12a system, where guide RNA (crRNA) directs Cas12a to the target sequence. Upon binding, Cas12a is activated, exhibiting trans-cleavage activity that indiscriminately cleaves single-stranded DNA reporters, producing a detectable signal. This innovative approach leverages the isothermal amplification of RPA, which can amplify target DNA sequences within 20−30 min, combined with the precise targeting and cleavage activity of CRISPR/Cas12a, to detect bacterial pathogens directly from clinical samples without the need for extensive laboratory infrastructure. Additionally, the simplicity and cost-effectiveness of RPA-CRISPR/Cas12a assays facilitate their use in resource-limited settings and point-of-care applications, providing a powerful solution for timely and accurate bacterial diagnostics in diverse clinical environments.[15,16]

Although the emergence of the RPA-CRISPR/Cas12a technology has revolutionized traditional molecular diagnostics, providing a rapid, sensitive, and specific method for pathogen detection, some studies have revealed a critical challenge. Early amplification products generated during the initial phase of the RPA reaction can be recognized by crRNA, which subsequently activates the Cas12a-mediated cleavage effect. This premature activation can ultimately diminish the sensitivity of the detection assay. To address this issue, various approaches such as microfluidics and light-activated crRNA probes have been developed, aiming to physically separate the RPA and Cas12a systems.[17−19] These methods are designed to prevent the activation of Cas12a until a sufficient amount of amplification product has been generated by RPA. While these designs have demonstrated significant efficacy, they also increase the complexity and cost of the assay, thereby limiting their applicability in resource-constrained settings. Recent studies found that additives like glycerol and agarose hydrogel delayed the fusion of the Cas12a detection system and the RPA amplification system in a tube on account of their viscosity, which boosts the detection sensitivity.[20,21] This is a promising approach that can enhance detection sensitivity while maintaining the method's simplicity.

Nucleic acid amplification additives such as betaine, polyethylene glycol (PEG), and dimethyl sulfoxide (DMSO) play crucial roles by reducing DNA secondary structure formation, facilitating DNA strand separation, and promoting amplification efficiency.[22−24] Herein, we optimized the nucleic acid amplification efficiency for the RPA-CRISPR/Cas12a system by screening additives and leveraging the viscosity effects of glycerol to prevent premature diffusion of Cas12a into the RPA system at the reaction initiation stage, thereby protecting early-stage amplification products. This advanced one-pot RPA-CRISPR/Cas12a reaction with glycerol and betaine was successfully applied for ultrasensitive detection of the *mecA* gene across various clinical samples, providing a novel, user-friendly, sensitive, and accurate diagnostic tool for the prevention and control of *mecA*-carrying strains, especially MRSA.

MATERIALS AND METHODS

Sample Collection and Preparation. Clinical samples, including blood, urine, and bronchoalveolar lavage fluid, were collected from The First Affiliated Hospital of Xi'an Jiaotong University. We synthesized a plasmid carrying the *mecA* gene and used it as a standard sample of a known concentration. The sequence of the *mecA* gene used in this study is provided in Table S1. The samples were spiked with known concentrations of *mecA* DNA to assess the assay's detection limits. Each sample type was handled under sterile conditions to prevent contamination, and aliquots were prepared for downstream analysis. This study was approved by the hospital's ethics committee.

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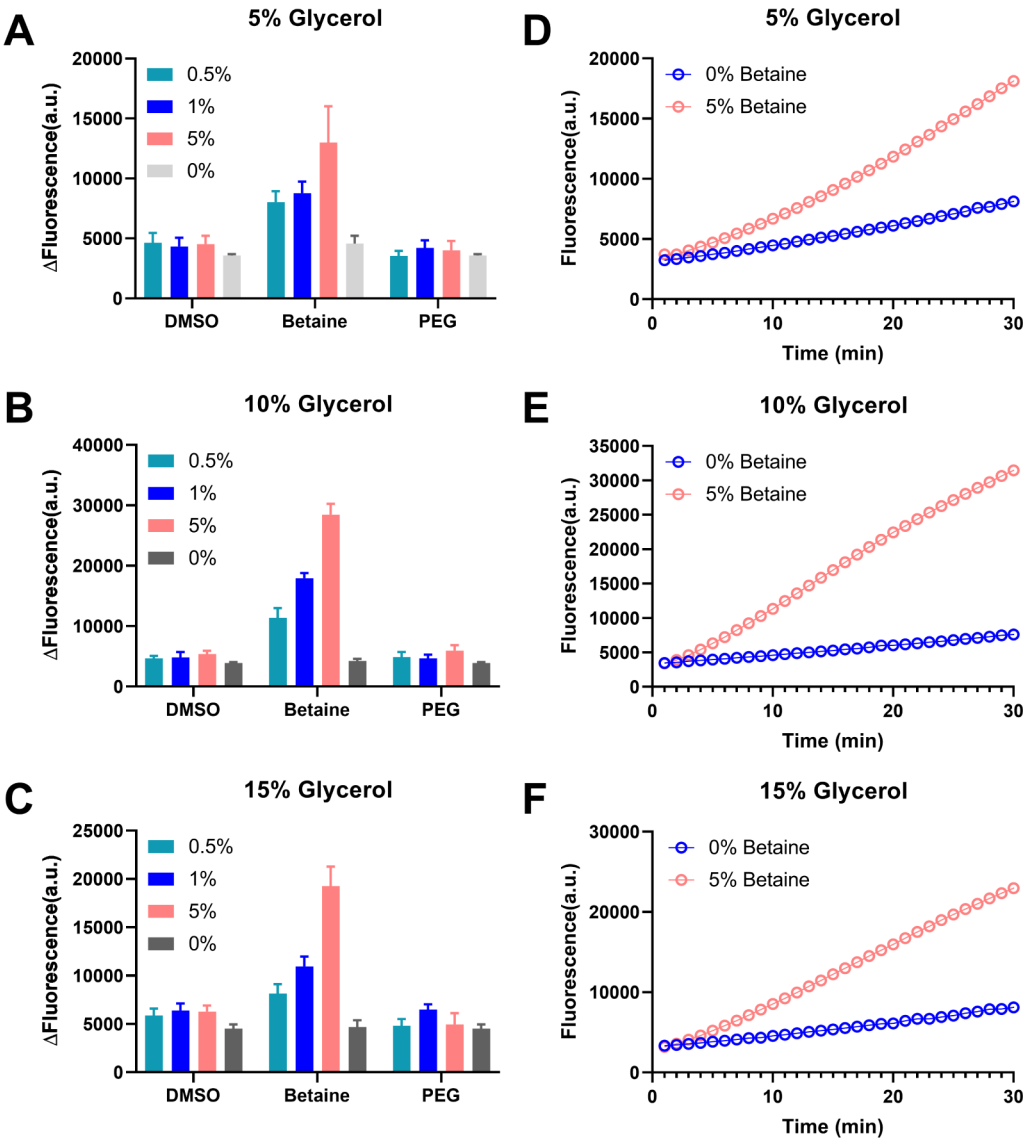


Figure 2. Effect of betaine, DMSO, and PEG on the fluorescence intensity of the RPA-CRISPR/Cas12a assay at varying glycerol concentrations. Bar graphs depicting the change in fluorescence (ΔFluorescence) at 30 min for different concentrations of betaine (0.5%, 1%, 5%, 0%) in comparison with DMSO and PEG under 5%, 10%, and 15% glycerol conditions, respectively (A−C). The amplification curves showing the fluorescence intensity over time (up to 30 min) for 0% and 5% betaine at 5%, 10%, and 15% glycerol, respectively (D−F).

Nucleic Acid Extraction. Total DNA was extracted from the clinical samples using the QIAamp DNA Mini Kit following the manufacturer's protocol. The extracted DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and stored at −20 °C until use.

The Composition of the Advanced and Classic RPA-CRISPR/Cas12a Assay. The advanced RPA-CRISPR/Cas12a assay was performed by using a one-pot isothermal amplification approach. For RPA, a commercially available kit (TwistAmp Basic Kit, TwistDx, UK) was used. The reaction was enhanced with 5% betaine (Sigma-Aldrich, USA) to stabilize GC-rich regions and reduce secondary structure formation, thus improving the amplification efficiency. The RPA reaction reagent contained 10 ng of template DNA, 29.5 μL of rehydration buffer, 1.2 μL of each primer (10 μM), 1 μL of betaine (5%), 5 μL of nuclease-free water, and 2 μL of the RPA enzyme mix. The Cas12a−crRNA complex was preassembled by mixing 1 μL of crRNA (100 nM) with 1 μL of LbCas12a enzyme (New England Biolabs, USA) in a NEB 2.1 buffer containing 5% glycerol (final concentration), which was incubated at room temperature for 10 min to ensure complex formation. Subsequently, the above Cas12a−crRNA mix was added with 1 μL of a quenched fluorescent reporter at the bottom of the tube; then, an equal volume of the RPA reaction reagent was added into the tube well with 2.4 μL of magnesium acetate (280 mM), and the reaction was incubated at 37 °C. Fluorescence was monitored in real-time using a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, USA) over 60 min.

The formulation of the classic reaction is the same as that of the advanced reaction, except that betaine and glycerol are not added.

The forward primer sequence used was 5′-GATTATGGCT-CAGGTACTGCTATCCACCCTC-3′, and the reverse primer sequence was 5′-GCCAACCTTTACCATCGATTTTA-TAACTTG-3′. The crRNA sequence was 5′-UAAUUUCUA-CUAAGUGUAGAUUCUUUUUGCCAACCUUUACC-3′.

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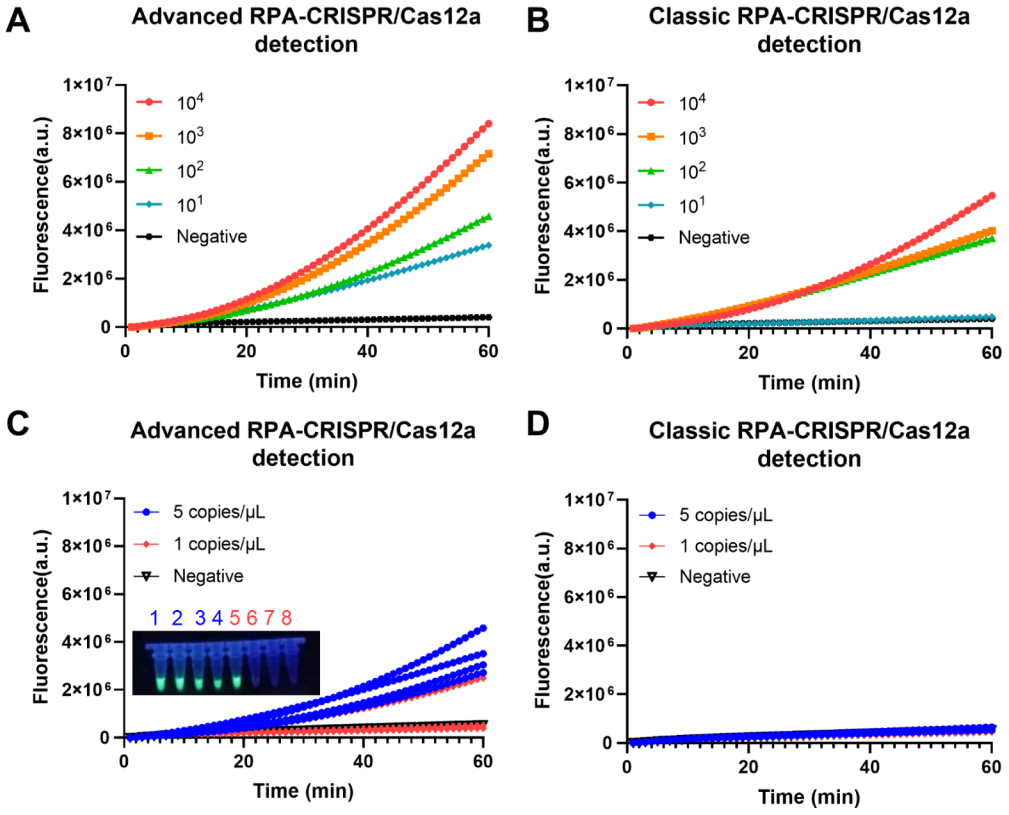


Figure 3. Comparison of advanced vs classic RPA-CRISPR/Cas12a assays for *mecA* detection. Fluorescence intensity over time for the detection of serially diluted *mecA* DNA (10^4−10^1 copies/μL) using the advanced RPA-CRISPR/Cas12a assay (A) and the classic RPA-CRISPR/Cas12a assay (B). Sensitivity analysis comparing the advanced (C) and classic (D) assays for detecting low concentrations of *mecA* DNA (1 and 5 copies/μL). The inset in (C) shows a fluorescence image confirming the presence of amplified products at different DNA concentrations.

The reporter sequence was 5′-FAM-NNNNNNNNNNNN-BHQ1-3′.

Specificity and Sensitivity Analysis. To evaluate specificity, DNA from a panel of bacterial species, including MRSA, MSSA, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Enterococcus faecium*, and *Klebsiella pneumoniae*, was tested using the advanced RPA-CRISPR/Cas12a assay. Sensitivity was assessed by testing serial dilutions of MRSA DNA in the clinical matrices mentioned above, ranging from 10^4 to 1 copy/μL.

Data Analysis. Fluorescence data were analyzed using QuantStudio Design and Analysis Software (Thermo Fisher Scientific, USA). The threshold for positive detection was set based on the mean fluorescence of negative controls plus three standard deviations. The performance of the assay was evaluated by receiver operating characteristic (ROC) curves using GraphPad Prism (ver. 8.0, GraphPad Software, USA).

RESULTS AND DISCUSSION

Figure 1 presents a comparison between the traditional diagnostic approach and an advanced one-pot RPA-CRISPR/Cas12a assay for detecting *mecA*-carrying strains, highlighting the significant benefits of the latter. Figure 1A describes the traditional method, where clinical samples (e.g., blood, urine, bronchoalveolar lavage) undergo overnight bacterial culture followed by antimicrobial susceptibility testing, leading to a diagnosis after more than 48 h.[25] In contrast, the advanced method incorporates rapid nucleic acid extraction followed by the one-pot RPA-CRISPR/Cas12a assay, delivering diagnostic results within 1 h. This approach streamlines the diagnostic process by eliminating the time-consuming culture step, thereby drastically reducing the time to diagnosis.

Figure 1B provides a detailed examination of the advanced one-pot RPA-CRISPR/Cas12a assay, focusing on the use of betaine-enhanced recombinase polymerase amplification (RPA) for target DNA amplification and glycerol-stabilized Cas12a with crRNA for specific detection. Betaine, an osmotic protectant, improves the RPA process by stabilizing DNA structures, particularly in regions with a high GC content or complex secondary structures that could otherwise impede amplification. Specifically, betaine interacts with negatively charged groups on the DNA strands, decreasing electrostatic repulsion between them, thereby minimizing secondary structure formation. By lowering the DNA melting temperature, betaine facilitates denaturation and promotes more uniform primer binding, resulting in more efficient and reliable amplification, which enhances the yield and sensitivity of the RPA process.[26,27] Glycerol, leveraging its viscosity, prevents premature interaction between the Cas12a−crRNA complex and early amplification products, ensuring that these initial products are not degraded.[20,21] This preservation of early amplification events is crucial for maintaining the assay's sensitivity and accuracy. The overall simplicity of this assay, performed in a single isothermal amplification step with results visualized via a fluorescence-based readout, enables clear differentiation between positive and negative samples based on a predefined threshold. This method not only offers rapid and precise *mecA* detection but also supports timely and informed clinical decision-making, facilitating the prompt initiation of targeted antimicrobial therapy.

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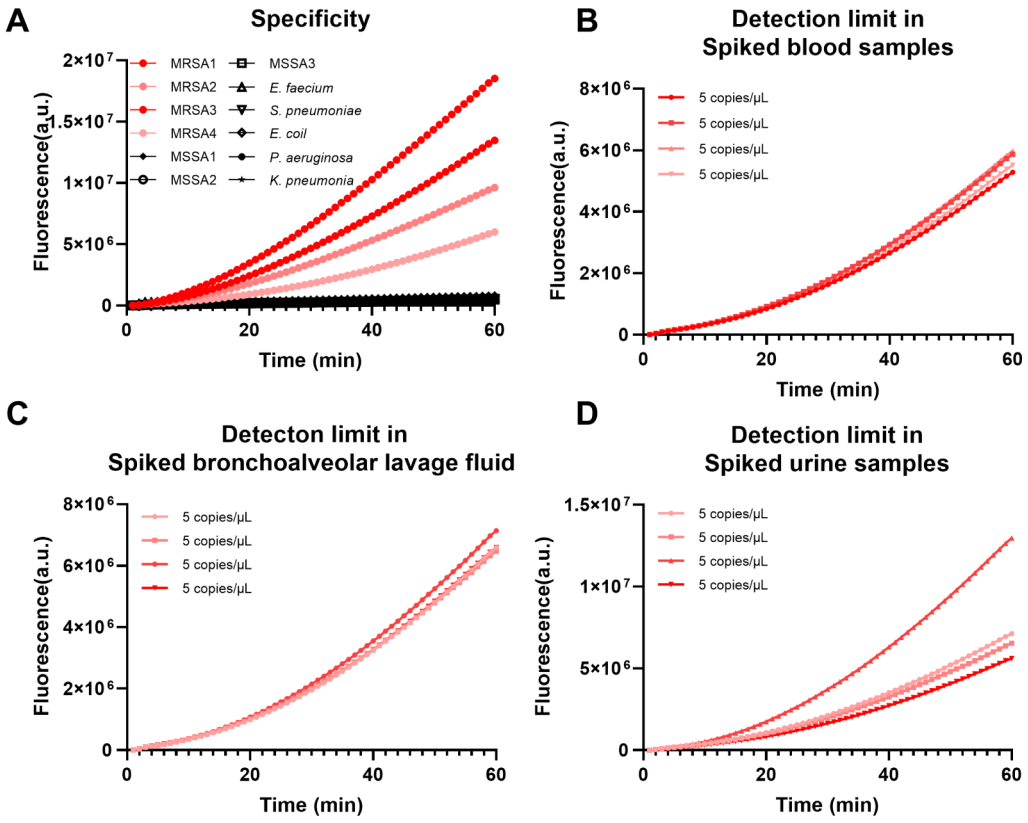


Figure 4. Specificity and detection limit of the advanced RPA-CRISPR/Cas12a assay in various spiked biological samples. (A) Specificity analysis showing fluorescence intensity over time for *mecA* detection in MRSA compared to various other bacterial strains, including MSSA (methicillin-sensitive *Staphylococcus aureus*), *Enterococcus faecium*, *Streptococcus pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Detection limit of the advanced assay in spiked biological samples, including (B) blood, (C) bronchoalveolar lavage fluid, and (D) urine, was 5 copies/μL of *mecA* DNA.

The data presented in Figure 2 demonstrate the impact of different additives, particularly betaine, DMSO, and PEG, on the fluorescence intensity of the RPA-CRISPR/Cas12a assay under varying glycerol conditions. In all tested glycerol concentrations (5%, 10%, and 15%), betaine, especially at a 5% concentration, significantly enhanced fluorescence signals compared to DMSO and PEG. The bar graphs in Figure 2A−C reveal that 5% betaine consistently produces the highest ΔFluorescence values, indicating superior target DNA amplification and increased Cas12a activity. This enhancement is crucial as it suggests that betaine not only facilitates the RPA process by stabilizing DNA structures but also effectively improves the sensitivity of the assay across different glycerol concentrations.

The amplification curves in Figure 2D−F further support these findings, showing that the presence of 5% betaine leads to a more rapid increase in fluorescence intensity over time compared to reactions without betaine. The fold change in fluorescence value for reactions with or without 5% betaine was 5.36, 9.98, 7.73 and 2.70, 2.34, 2.62, respectively, which have a significant difference (p = 0.0185). This trend is consistent across all glycerol levels, indicating that betaine's beneficial effects are robust and reliable. The combination of betaine and glycerol appears to synergize, maintaining the integrity of the Cas12a−crRNA complex during amplification, thereby preventing premature degradation of early amplification products. These results underscore the potential for optimizing the reaction conditions to enhance the efficiency and accuracy of CRISPR-based diagnostics, particularly in clinical applications where rapid and precise detection is critical.

Then, Figure 3 compares the performance of the advanced RPA-CRISPR/Cas12a assay with that of the classic version for detecting *mecA* DNA across different concentrations. Figure 3A,B illustrates that the advanced assay achieves significantly higher fluorescence intensity across all tested DNA concentrations (10^4−10^1 copies/μL) compared to that of the classic assay. In Figure 3A, the advanced RPA-CRISPR/Cas12a detection system displays a clear, time-dependent increase in fluorescence across all target concentrations, achieving a robust signal even at 10^1 copies/μL. By contrast, in Figure 3B, the classic RPA-CRISPR/Cas12a detection system shows a lower sensitivity, particularly at lower concentrations (10^1 copies/μL), where the signal is barely distinguishable from the negative control.

At ultralow concentrations (5 and 1 copies/μL tested in four replicates), as shown in Figure 3C, the advanced RPA-CRISPR/Cas12a system is highly sensitive, successfully detecting as low as 1 copy/μL within 60 min. In contrast, Figure 3D demonstrates that the classic RPA-CRISPR/Cas12a system is unable to generate a detectable signal at these low concentrations, with fluorescence levels remaining indistinguishable from the negative control. The amplification products shown in Figure 3C were placed under UV light. Tubes 1−4 show positive results, which correspond to four blue amplification curves (5 copies/μL tested in four replicates), while tubes 5−8 correspond to four red amplification curves (1 copy/μL tested in four replicates).

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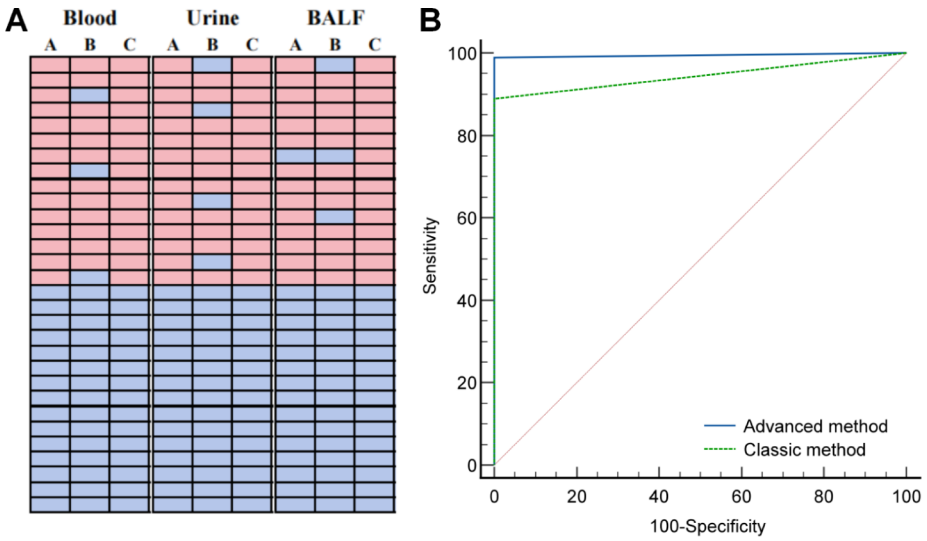


Figure 5. Comparison of detection consistency and diagnostic accuracy between advanced and classic RPA-CRISPR/Cas12a methods. (A) Heatmap analysis showing the detection results of the advanced RPA-CRISPR/Cas12a method across different biological samples: blood, urine, and bronchoalveolar lavage fluid (BALF). A: advanced method; B: classic method; C: culture method (gold standard method). Pink indicates positive detection, while blue indicates negative detection. (B) Receiver operating characteristic (ROC) curve comparing the diagnostic accuracy of the advanced RPA-CRISPR/Cas12a method and the classic method.

Only tube 5 shows a positive product, which is consistent with the results of the fluorescence amplification curves. The results clearly highlight the superior sensitivity and efficiency of the advanced RPA-CRISPR/Cas12a detection system compared to those of the classic version. The advanced system demonstrated a significantly faster and stronger fluorescence signal across all tested concentrations, including the ultralow range (1−5 copies/μL), which is crucial for early and accurate pathogen detection in clinical samples. The classic system, while functional at higher concentrations (10^2 and 10^3 copies/μL), exhibited substantial limitations in detecting lower concentrations, failing to produce reliable results at 1−5 copies/μL, thus limiting its clinical utility in scenarios requiring highly sensitive detection.

Next, we demonstrated the high specificity and sensitivity of the advanced RPA-CRISPR/Cas12a assay in detecting *mecA* in various biological samples. We used 4 MRSA strains and 13 non-MRSA strains to evaluate the specificity of the method. Figure 4A highlights the assay's specificity, where fluorescence signals are significantly higher for MRSA strains (MRSA1 through MRSA4) compared to non-MRSA strains, including MSSA and other common pathogens. This lack of cross-reactivity with nontarget species underscores the assay's precision in identifying *mecA* without false positives, a crucial feature for accurate clinical diagnostics. The strong discrimination between *mecA*-carrying and non-*mecA*-carrying strains is particularly important in mixed bacterial infections, ensuring that MRSA and MR-CoNS are reliably detected, even in the presence of other pathogens.

The detection limit of the advanced assay is further confirmed in Figure 4B−D, which depicts its performance in blood, bronchoalveolar lavage fluid, and urine samples spiked with low concentrations of *mecA* DNA (5 copies/μL). Across all repeated tested matrices, the assay consistently generates robust fluorescence signals, reflecting its ability to detect even small amounts of *mecA* DNA in different sample types. The consistent detection limits observed across these clinically relevant samples highlight the versatility and reliability of the assay in various diagnostic contexts. This capability to maintain high sensitivity in complex biological matrices positions the advanced RPA-CRISPR/Cas12a assay as a powerful tool for early *mecA*-carrying strain detection, potentially enabling more timely and targeted therapeutic interventions.

Finally, the detection consistency and diagnostic accuracy between the advanced RPA-CRISPR/Cas12a method, the classic RPA-CRISPR/Cas12a method, and the culture method (gold standard method) in clinical samples were compared. In Figure 5A, the heatmap reveals consistent and reliable *mecA*-carrying MRSA (culture confirmed) detection across various biological samples (30 blood samples, 30 urine samples, and 30 BALF samples) using the advanced method. The uniformity of positive detections (pink cells) across different concentrations and sample types underscores the assay's robustness. This consistency is crucial in clinical diagnostics, where variable sample types and conditions can often lead to inconsistent results. The advanced method's ability to maintain high detection consistency across diverse matrices further demonstrates its potential for widespread clinical application.

Figure 5B presents a receiver operating characteristic (ROC) curve, which quantitatively compares the diagnostic accuracy of the advanced method against that of the classic method. The advanced method achieves near-perfect sensitivity and specificity, as indicated by the ROC curve's proximity to the top-left corner of the plot. In contrast, the classic method shows lower diagnostic accuracy, with a less steep curve. The AUC values for the advanced method and classic method were 0.994 and 0.944, respectively. The Z statistic was 3.145, with a p-value of 0.0017, indicating that the detection performance of the advanced method is significantly superior to that of the classic method. The superior performance of the advanced RPA-CRISPR/Cas12a method, as evidenced by this analysis, highlights its efficacy in accurately identifying the *mecA* gene. This high level of diagnostic precision is critical for ensuring timely and appropriate therapeutic interventions, ultimately improving patient outcomes.

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CONCLUSION

The advanced one-pot RPA-CRISPR/Cas12a assay demonstrates significant improvements in both specificity and sensitivity over traditional detection methods, as evidenced by its robust performance across various clinically relevant biological samples. The assay's high specificity ensures accurate *mecA* detection without cross-reactivity to non-*mecA*-carrying strains, reducing the likelihood of false-positive results. Moreover, its consistent and reliable detection of low concentrations of *mecA* DNA in complex matrices, such as blood, urine, and bronchoalveolar lavage fluid, underscores its potential as a powerful diagnostic tool in diverse clinical settings.

These findings highlight the advanced one-pot RPA-CRISPR/Cas12a method's capability to provide rapid, accurate, and reliable diagnostics, making it a useful tool for the early detection and management of *mecA*-carrying strain infections. The method's enhanced diagnostic accuracy, as reflected in the superior ROC curve, further emphasizes its suitability for widespread clinical implementation, where timely and precise detection is critical for effective patient care. However, we acknowledge that this remains a preclinical study focusing on the development of the method. Before it can be applied in clinical practice, further validation with a larger number of samples is required. Overall, this assay represents a significant advancement in *mecA*-carrying strain diagnosis, with the potential to improve patient outcomes through more targeted and timely therapeutic interventions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c09078.

The sequence of *mecA* gene used in this study (PDF)

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Notes

The authors declare no competing financial interest.

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