Rapid Visual Detection of Anti-Microbial Resistant Genes for Far-Forward Wound Assessment

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Background

Forward-deployed warfighters suffering from wound infections caused by multi-drug resistant organisms (MDROs) require extensive treatment that reduces operational readiness. Modern conflict has introduced challenges for medical evacuation and transport in deployed settings. Antibiotic resistant bacteria have been isolated from over half of extremity wound infections (EWIs). A recent study of injuries in soldiers in the Ukraine War conflict, bacterial infections were found in 56% of wound cases, of which over 85% of the isolated colonizing strains were multi-drug resistant.[1] Current methods of MDRO detection rely on specific organism identification, requiring extensive time and significant resources unavailable forward of Role 3 settings. Thus, there is a need for reliable and rapid MDRO detection from environmental and wound samples, especially in austere and low-resource environments.

The solution is targeted detection of anti-microbial resistance (AMR) genes. AMR genes are often shared across MDROs, so specific strain identification is not necessary. In particular, the mecA gene confers mild resistance to all beta lactam antibiotics while CTX-M-15 codes for group 2 ESBLs. Thus, focusing on low-resource methods for rapid and reliable detection of these causative AMR genes will allow for earlier diagnosis and informed treatment. Furthermore, reducing equipment requirements could expand the reach of AMR detection into resource-poor environments.

General Methodology

Isothermal approaches to genomic amplification are known for their potential to function with little-to-no equipment, and the sensitivity of amplification with low gDNA template amounts. Primer sets were previously evaluated and optimized in vitro using traditional thermocycling PCR with extracted nucleic acids as template. Recombinase polymerase amplification (RPA) was utilized as an isothermal amplification methodology, which utilizes unique proteins to initiate the replication fork needed for amplification. Two methods of incubation were tested, both steady-state incubation utilizing a heating mantle set at a steady temperature of 37-40 °C for 20-30 minutes, as well as a body-heat method wherein the RPA reaction tubes were taped to the skin and held against the armpit for 30 minutes. Generated amplicons were purified utilizing the QIAGEN QIAQuick PCR purification quick kit before being utilized in the detection assay regime.

The developed a gold nanoparticle (AuNP) reporting system utilizes gene baits that target the AMR amplicon. A change in the dimensionality of the gold nanoparticle results a color change corresponding to the aggregation of the nanoparticles. The purified RPA product is combined with gene baits that complement sections of the target AMR gene. This mixture is then heated to 95 °C for 3 minutes and annealed for a minute at 55 °C to allow bait-target hybridization if target is present. AuNPs are then added to the bait-RPA product solution. Thiolated ends on the baits form chemical bonds to the gold nanoparticle surface. A solution of sodium chloride salt is added to the mixture. If no target is present, the salt induces rapid AuNP aggregation, forcing a color shift of the sample from red to purple. With target, hybridization between the baits and target prevents aggregation of the AuNP, halting the color shift and allowing for clear visual confirmation of AMR presence without specialized detection equipment. This process is illustrated in Figure E.

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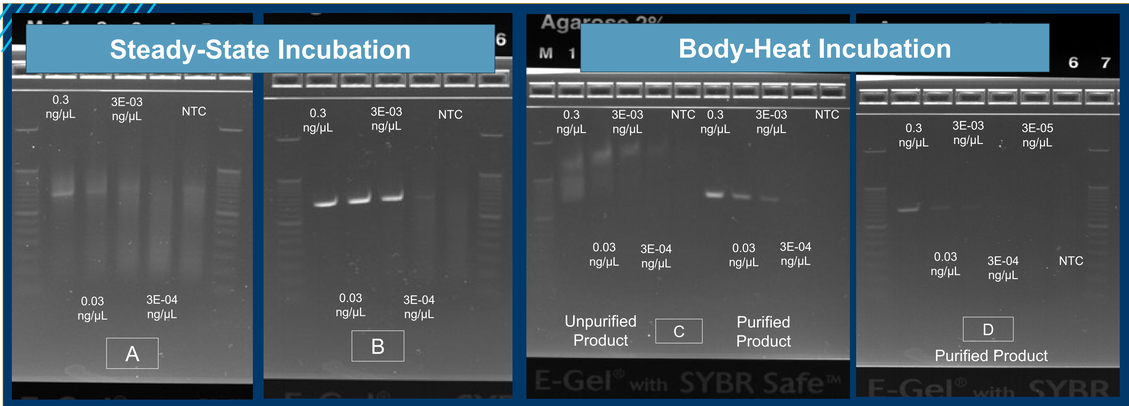


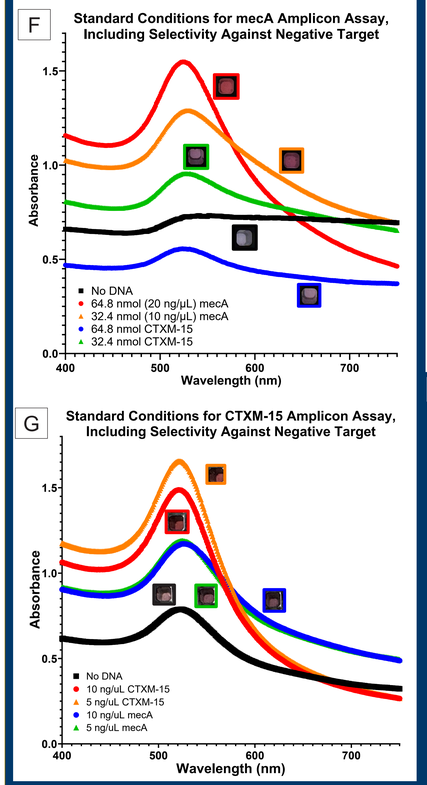
Figures A-B: Gels of mecA amplicon production via steady-state incubation RPA amplification LOD at 37 °C pre-optimization (A) and 40 °C post-optimization (B) of primer concentrations. Lanes are labeled with the concentration of gDNA template used from 0.3 ng/µL to 0.0003 ng/µL.

Figures C-D: Gels of mecA amplicon production via body-heat RPA amplification run for 30 minutes LOD showing post amplification products both unpurified and purified. Lanes are labeled with the concentration of gDNA template used from 0.3 ng/µL to 0.0003 ng/µL. Both figures have the same optimized conditions, illustrating the variance in body-heat amplification as a method.

The pre-optimized RPA reaction with steady-state incubation utilized a 0.48 µM concentration of primers, per RPA kit manufacturer recommendation, which led to undesired byproducts and less amplification of the target gene (Figure A). Primer concentration was lowered to 0.12 µM which significantly reduced byproducts and increased target gene production (Figure B). Based on gel images and quantification of produced amplicons, the LOD of RPA amplification for the mecA target gene is 0.003 ng/µL of template, or just under 1000 genomic equivalents.

Transitioning to body-heat incubation, multiple experiments show that RPA amplification of target gene is highly viable. The LOD of the RPA reaction with mecA utilizing body-heat incubation is also 0.003 ng/µL, the same as the optimized steady-state incubation with thermocycler at 40 °C (Figures C and D). The quantity of amplification product is both lower and more variable at this lower template (3.7 ng/µL or lower) with steady-state incubation.

General Detection Assay Performance



Figures F-G: Standard conditions for detection of mecA (F) and CTXM-15 (G) target amplified genes as first established with PCR-amplified target gene. Both cases show selective stabilization of AuNPs in the presence of target over off-target DNA.

1. **Hybridization buffer with thiolated baits (9 µL) is combined with 1 µL of amplification product in tube**
2. **Tube is heated to 95 °C for 3 minutes, then 55 °C for one minute to incubate & anneal baits with amplicon target**
3. **13 nm diameter gold nanoparticles (30 µL) are added into tube to interact with thiolated baits**
4. **1 µL of NaCl added to force aggregation and tube is monitored visually for 10 minutes**

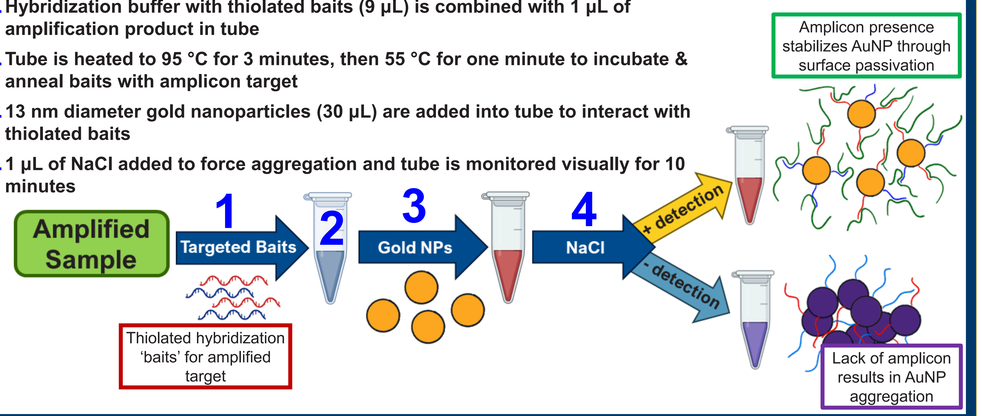


Figure E: Schematic of gold nanoparticle-based detection assay, illustrating the assay steps and the stabilization caused by bait hybridization to amplified target.

The standard conditions established via PCR-generated amplicons (Figures F & G) were utilized as a starting point for further optimization of assay performance utilizing target mecA amplicons generated using body-heat incubated RPA and purified via the QIAGEN QIAQuick kit. The difference in reagents between PCR product and the RPA product gave rise to the need for fine-tuning of hybridization buffer composition and bait buffer concentrations to prevent false-negatives from reagent-induced aggregation of the AuNPs.

Optimized conditions for detection of the body-heat mecA amplicon were determined, with clearly visible color changes beginning immediately and resolving within 10 minutes of final salt addition. The assay has a reliable limit of detection down to 5 ng/µL of amplified gene product (Figure H). Visual detection of target amplicon was possible but unreliable with amplicon amounts as low as 2.5 ng/µL.

Rapid Visual Detection of Body-Heat Amplified AMR Genes

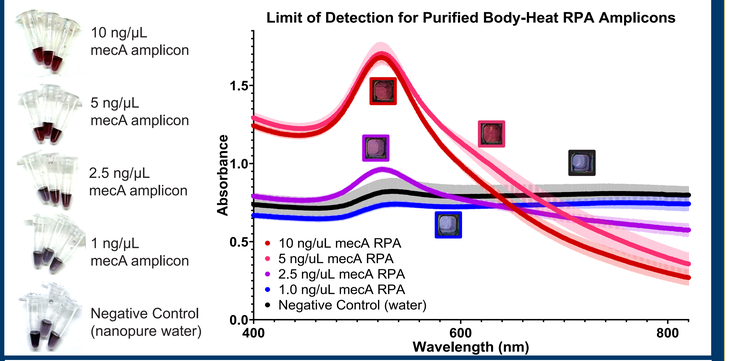


Figure H: LOD of detection assay with purified RPA amplicons. (*Left*) Photographs of the assay tubes after 10 minutes. (*Right*) UV-Vis of assay samples plated from 400 nm – 820 nm with adjoining pictures of wells.

Results & Discussion

Ruggedization of target amplification post-initial optimization was pursued via elimination external heating devices for body-heat incubation. The RPA reaction was taped under the armpit to incubate for 30 minutes, with the arms kept down and additional movement minimized. After 30 minutes, amplification was evaluating using gel electrophoresis both with and without additional purification of amplicons. Simple kit purification was determined to be ideal for detection assay purposes. The limit of detection for body-heat amplification matched that of the steady-state isothermal amplification; however greater variance was observed in amount of copies produced between body-heat amplification runs (Figures C&D).

The gold nanoparticle-based colorimetric detection assay was developed utilizing a modification of the bait-buffer regime outlined by Khalil et. al,[3] outlined in Figure E. Uniquely to many such AuNP-based assays, the product of the amplification reaction are combined with the baits prior to bait passivation of the nanoparticle. It is after the hybridization between target and bait occurs that the AuNPs are added, at which point the thiolated tails bind to the AuNP surface. Performing bait-target hybridization before surface passivation was found to prevent interference/steric blocking by the nanoparticle.

The development of this assay was first completed utilizing target amplicons generated via PCR and purified, during which standard conditions for assay performance were determined for both mecA and CTXM-15 target amplicons (Figures F&G). After the optimization of the isothermal RPA amplification of the target amplicons, the assay conditions were re-optimized for use with RPA-generated amplicons to account for additional RPA buffer reagent interference with the nanoparticle aggregation process. Utilizing body-heat generated mecA amplicons, the ideal conditions were found to be 0.80 µM each of hybridization baits comprised of adjacent sequences of complementary nucleotides to target, with thiolated tails on the 5' and 3' end for the first and second bait sequences, respectively. Ideal hybridization buffer conditions were determined to be 11 mM sodium phosphate buffer at pH 7.4 with an additional 210 mM sodium chloride (Figure H).

Future Outlook

In the interest of further ruggedization, several steps of future optimization are being pursued. First, the validation of the body-heat amplification and assay performance with the CTXM-15 target is desired. Second, further detection assay optimization to eliminate the need for external heating during the incubation and anneal process is desired, ideally also utilizing body-heat, though an increase in incubation and annealing time would likely be required with reduced temperature. An alternative vector under consideration would be the utilization of currently-deployed heating devices, such as MRE heaters or commercial hand-warmers. Finally, a complete elimination for the need to purify the amplification product post body-heat amplification is being explored. Several components of the current RPA buffer kit are being investigated as primary initiators for false-negative AuNP aggregation, and alternative reagents for use in the RPA process are under investigation.

Acknowledgements

Citations

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