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ARTICLE

Diagnosing Antibiotic Resistance Using Nucleic Acid Enzymes and Gold Nanoparticles

Mohamed A. Abdou Mohamed,[∞] Hannah N. Kozlowski,[∞] Jisung Kim,[∞] Kyryl Zagorovsky, Melinda Kantor, Jordan J. Feld, Samira Mubareka, Tony Mazzulli, and Warren C. W. Chan*

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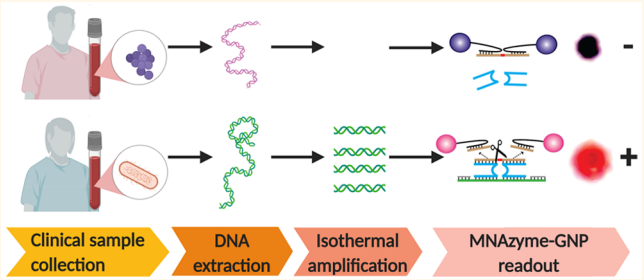
ABSTRACT: **The rapid and accurate detection of antimicrobial resistance is critical to limiting the spread of infections and delivering effective treatments. Here, we developed a rapid, sensitive, and simple colorimetric nanodiagnostic platform to identify disease-causing pathogens and their associated antibiotic resistance genes within 2 h. The platform can detect bacteria from different biological samples (*i.e.*, blood, wound swabs) with or without culturing. We validated the multicomponent nucleic acid enzyme–gold nanoparticle (MNAzyme-GNP) platform by screening patients with central line associated bloodstream infections and achieved a clinical sensitivity and specificity of 86% and 100%, respectively. We detected antibiotic resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) in patient swabs with 90% clinical sensitivity and 95% clinical specificity. Finally, we identified *mecA* resistance genes in uncultured nasal, groin, axilla, and wound swabs from patients with 90% clinical sensitivity and 95% clinical specificity. The simplicity and versatility for detecting bacteria and antibiotic resistance markers make our platform attractive for the broad screening of microbial pathogens.**



KEYWORDS: *antibiotic resistance, MRSA, gold nanoparticles, DNAzyme, RPA, central line infection, sepsis*

Resistance to antimicrobial drugs in bacteria, viruses, and parasites is a public health concern. It leads to more than 700 000 deaths annually, and this number could grow to 10 million annually.[1] A major factor contributing to antibiotic resistance is extensive and improper use of antimicrobial drugs.[2−5] Bacteria that are exposed to inadequate doses or incorrect classes of antibiotics can become resistant. Minimizing the impacts of antibiotic resistance is a global priority. To achieve this, a sensitive and robust diagnostic approach is required to rapidly identify multiple disease-causing bacteria and their associated antimicrobial resistance profiles. Healthcare workers can then provide appropriate therapy in a timely manner, thereby avoiding empiric treatment and reducing the development of new resistance mechanisms.[5−9] These detection methods should be simple and adaptable for use in countries with limited resources where screening for antibiotic resistance is inadequate.[9,10]

Current gold standard techniques for detecting antibiotic resistance are culture based. These methods may take 20−72 h for an accurate diagnosis, thereby delaying treatment.[6,7,11−13] Pathogens carrying antibiotic resistance genes can also appear sensitive to antibiotics due to a lack of gene expression or poor growth producing false negative results.[14−17] Inconclusive culture results cannot inform treatment decisions until they are confirmed using nucleic acid tests (*i.e.*, polymerase chain reaction, PCR) that directly detect the presence of antibiotic resistance genes.[18] PCR has high analytical sensitivity, but this technique requires specialized equipment and skilled technicians, precluding its use in resource limited areas. Researchers have started to address the above limitations by identifying antibiotic resistance using microfluidics and nanomaterial assays.[19−26] Keays et al. determined the resistance phenotype in >2 h by manipulating small volumes of bacteria and antibiotic using droplet microfluidics.[26] Veigas et al. used PCR and GNP probe aggregation to identify resistance mutations in *Mycobacterium tuberculosis*.[19] However, these methods have the same limitations as standard phenotypic tests, have insufficient sensitivity, or detect only a single antibiotic resistance determinant.

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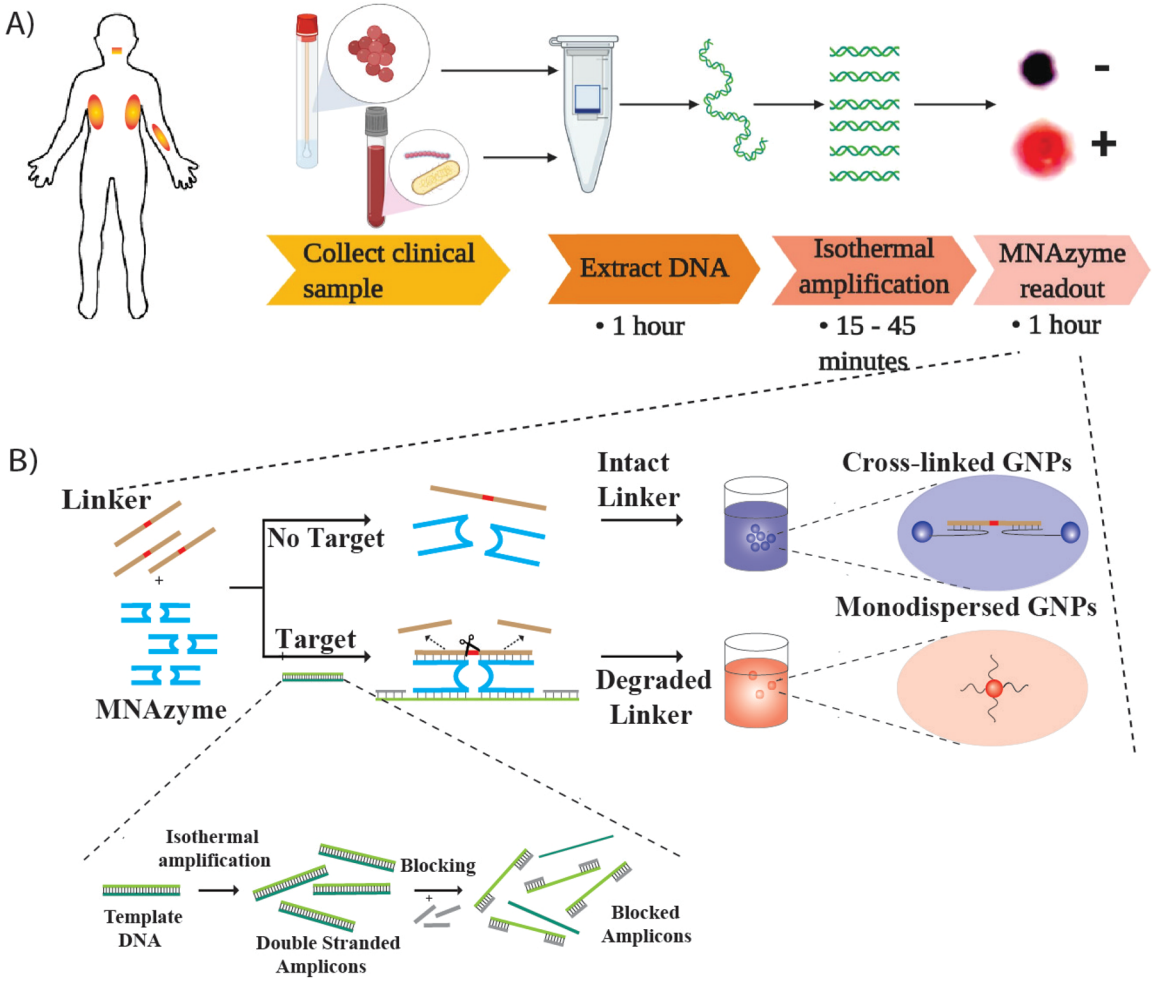

Figure 1. Workflow for the MNAzyme-GNP Platform. (A) Workflow with timeline for MNAzyme-GNP platform using clinical samples. Samples are collected and extracted following institutional protocols. DNA is amplified at one temperature, removing the need for a thermocycler. The final DNA product is identified using the colorimetric readout of the MNAzyme-GNP platform. (B) Design of MNAzyme-GNP platform. Amplified target gene is chemically denatured and blocked to prevent rehybridization. When activated by blocked amplicons, MNAzyme cleaves the linker DNA, rendering GNPs monodispersed. In the absence of the target gene, the linker DNA remains intact owing to inactive MNAzyme and causes GNPs to aggregate.

We first proposed the use of a multicomponent nucleic acid enzyme–gold nanoparticle (MNAzyme-GNP) for diagnostics in 2013.[27] While a number of researchers have advanced similar technologies for measuring miRNA in culture and lysed cells, there is a need to advance this technology for clinical use.[28−32] Clinical translation is complex due to patient variability, matrix effects, and clinical requirements for analytical sensitivity. Here, we engineered a diagnostic platform and workflow for the diagnosis of antimicrobial resistance. Our approach uses a simple, rapid, and highly sensitive platform for detecting multiple genetic markers in parallel, bringing MNAzyme-GNP assays one step closer to use in surveilling antimicrobial resistance in patients.

RESULTS AND DISCUSSION

Overall Workflow for Diagnosing Pathogens Using the MNAzyme-Gold Nanoparticle Platform. Figure 1A depicts the workflow for our platform from sample collection to final colorimetric readout. In the first step a clinical specimen is collected from an ill patient. The nucleic acid is extracted and amplified using isothermal amplification. The amplified product is detected using the MNAzyme-gold nanoparticle (MNAzyme-GNP) platform. A MNAzyme is an enzyme composed of two nucleic acid strands instead of amino acids. It has the ability to selectively cleave DNA. The DNA cleaving domain has a specific DNA sequence that does not bind target and becomes active when both MNAzyme strands are in close proximity.[33] Figure 1A describes the MNAzyme-GNP workflow using blood and swabs, but it can be applied to other sample types. DNA was extracted from bacterial cells, followed by isothermal amplification of target genes by recombinase polymerase amplification (RPA) at 37−39 °C. We added an isothermal amplification step because the MNAzyme-GNP assay does not have the required analytical sensitivity to detect clinically relevant levels of antimicrobial resistant pathogens. The amplified product was identified using the MNAzyme-GNP assay.

Following isothermal amplification, purified amplicons were chemically denatured using sodium hydroxide to obtain single-stranded sequences (Figure 1B).[34] Chemical DNA denaturation is more suitable for use in low resource settings compared to thermal denaturation, which requires a high-temperature heating device. The denatured amplicons were mixed with short blocking oligonucleotide strands, and the solution was

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Table 1. Clinical Sensitivity and Specificity of Antibiotic Resistance Genes in Clinical Isolates

antibiotic class	antibiotic	antibiotic resistance genes	% sensitivity	% specificity
β-lactam	oxacillin	*mecA*	100 (80/80)	100 (20/20)
	penicillin	*blaZ* and *mecA*	94 (94/100)	NA[a]
glycopeptides	vancomycin	*vanA* and *vanB*	NA[b]	100 (100/100)
tetracyclines	tetracycline	*tetK* and *tetM*	100 (10/10)	100 (90/90)
macrolides	erythromycin	*ermA* and *ermC*	41.7 (25/60)	100 (40/40)
macrolides	erythromycin	*ermA*, *ermC*, and *msrA*	86.7 (52/60)	87.5 (35/40)

[a]All isolates were tested to be resistant to penicillin using culture method. [b]All isolates were tested to be sensitive to vancomycin using culture method.

neutralized with hydrochloric acid to allow hybridization of blocking strands with our target gene. Such a blocking strategy has been proven to be effective in preventing rehybridization of the sense-strand of target DNA with its antisense strand, allowing unbound single-stranded DNA to hybridize to DNA probes.[35] The blocked amplicons were mixed with the MNAzyme solution to allow hybridization of blocked amplicons with the MNAzyme sensor arms. In the absence of target, the inactive MNAzyme was present as two DNA strands in solution and GNPs were cross-linked by linker DNA. The cross-linked nanoparticles had a dark purple color in solution. The target (amplified DNA) acted as a scaffold for MNAzyme assembly. Only when the target was bound to both MNAzyme strands was the catalytic activity of the MNAzyme triggered. The activated MNAzyme then bound the linker DNA and cleaved it. A single activated MNAzyme could sequentially cleave multiple linker strands, resulting in additional signal amplification. This signal amplification step is an advantage as it is not present in GNP aggregation or other colorimetric detection methods.[19,21] Cleavage of the linker DNA released the GNPs, rendering them monodispersed. Monodispersed GNPs had a distinct red color in solution. The resulting GNP solution was spotted on a thin-layer chromatography (TLC) plate to visualize the results. A dark purple color indicated a negative test, while a red color indicated a positive test result. DNA sequences of RPA primers, sense-strand of target DNA, MNAzyme components, linker DNA, GNP probes, and blocking strands are presented in Tables S1−S4.

Characterizing the MNAzyme-GNP Platform. Our previous study showed that a standalone MNAzyme-GNP assay can detect 10^7−10^9 synthetic DNA copies/μL.[27] This limit of detection is insufficient for clinical detection of bacteria that cause central line-associated bloodstream infections (CLABSI) and their antibiotic resistance markers. We used antibiotic resistance genes from *Staphylococcus aureus* (*S. aureus*), the leading cause of CLABSIs, to characterize the assay limit of detection. The clinical detection of antibiotic resistance in bacteria requires analytical sensitivity of 10^5 CFU/mL, corresponding to 10^2 DNA copies/μL.[11,36] We incorporated an RPA amplification step into the MNAzyme-GNP platform to significantly improve the limit of detection.

We selected 10 different antibiotic resistance biomarker genes that conferred resistance to five different classes of antibiotics in *S. aureus* strains: *blaZ* gene for penicillin resistance, *mecA* gene for oxacillin resistance, *vanA* and *vanB* genes for vancomycin resistance, *tetK* and *tetM* genes for tetracycline resistance, *tetM* gene for minocycline resistance, *acc6* and *aph3iiia* genes for gentamicin and kanamycin resistance, and *ermA* and *ermC* for erythromycin resistance (Table 1).[37−42] While there were a number of other genes that could potentially cause antibiotic resistance in *S. aureus*, analysis of 24 research papers covering 2599 *S. aureus* isolates from different sources and geographical regions indicated that choosing the two most common genes responsible for resistance to each antibiotic should be sufficient to detect the majority of resistant strains. As shown in Table S5, the selected antibiotic resistance genes had high combined prevalence rates between 83.0% and 99.5%. By selecting the most prevalent antibiotic resistance genes, we could achieve high diagnostic sensitivity without the need to screen a larger set of rarer antibiotic resistance genes.[16,38,39,41−61] To detect the chosen antibiotic resistance biomarkers with high specificity and minimal cross reactivity, we selected the most conserved regions of their genetic sequences using the basic local alignment search tool (BLAST) and designed the primers to amplify these regions. The amplified biomarkers were detected with sequence-specific probes using our MNAzyme-GNP readout platform.

To determine the limit of detection using RPA, synthetic DNA was serially diluted down to 10^0 DNA copies/μL and amplified. The amplified products were qualitatively assessed using gel electrophoresis (Figure S1). Both the amplified and nonamplified (*i.e.*, serially diluted genes without RPA) targets were chemically denatured, blocked, and added to the MNAzyme-GNP mixture for the signal readout. The samples (3 μL) were deposited onto a TLC plate and observed for a color shift from purple to red indicating positive signal. Figure S2 illustrates that the RPA step significantly increased the analytical sensitivity for all genes by 8−9 orders of magnitude yielding detection limits of 10^2−10^3 DNA copies/reaction (2−20 DNA copies/μL). The ability to detect 10^2 DNA copies/μL was indicated by the color shift of GNPs from purple to red and was confirmed quantitatively by measuring the peak absorbance wavelength using a UV−vis spectrophotometer (Figure S3). These data showed that there are statistically significant differences in the peak absorbance wavelength for 10^2 DNA copies/reaction or greater for *tetM*, *acc6*, *aph3iiia*, and *ermA* genes (P value of <0.0001 or <0.00001) and for 10^3 DNA copies/μL or greater for all other genes (P value of <0.0001 or <0.00001) when RPA is used compared to the negative control (*i.e.*, 0 DNA copies/reaction). This confirmed the need of a RPA preamplification step to achieve a clinically relevant limit of detection.

Next, we used DNA extracted from clinical isolates to confirm that we can achieve a clinically relevant limit of detection of at least 10^5−10^7 CFU/mL, which corresponds to clinical positivity for bloodstream infections.[10,62,64] We screened three isolates of *S. aureus*: *S. aureus* ATCC BAA-44 (MRSA-44), *S. aureus* ATCC BAA-41 (MRSA-41), and *S. aureus* ATCC 29213 (methicillin-susceptible *S. aureus*, MSSA) for the presence of antibiotic resistance genes. Bacteria were

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grown at a high concentration of 10^7 CFU/mL to ensure that negative detection of antibiotic resistance genes was not due to insufficient amount of target DNA. DNA was extracted from the three isolates and screened for the presence of antibiotic resistance genes. Two types of negative controls were used throughout this study to ensure proper interpretation of our test results. No template controls (NTCs) used water instead of antibiotic resistance genes in the RPA reaction to rule out contamination of RPA reagents or nonspecific RPA products. To examine any false-positive signals that could arise from improper aggregation of GNPs, we used "EBS" controls, which are composed of DNA elution buffer and blocking strands. This control was mixed directly with the MNAzyme assay components and transferred to GNP solutions to confirm proper aggregation of GNPs with inactive MNAzyme and intact linker DNA.

The results show that MRSA-44 had five antibiotic resistance genes *blaZ*, *mecA*, *tetM*, *acc6*, and *ermA* genes, MRSA-41 contained three antibiotic resistance genes *blaZ*, *mecA*, and *ermA*, while MSSA contained only *blaZ* gene (Figure S4). The presence of these antibiotic resistance genes was confirmed by PCR (Figure S5), which detected the same genes in the three isolates. Next, we measured the analytical sensitivity of our assay in detecting the five antibiotic resistance genes from MRSA-44 using serial dilutions of this bacteria (10^7–10^0 CFU/mL). Media without bacteria served as a negative control (*i.e.*, 0 CFU/mL). We achieved limits of detection that are between 10^2 and 10^3 CFU/mL, which corresponded to 2–20 DNA copies/μL (Figure 2). This limit

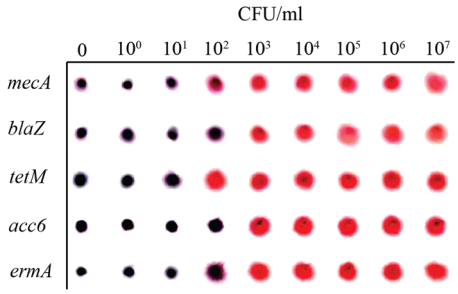

Figure 2. Determining sensitivity for antibiotic resistance genes from MRSA-44. MRSA-44 was serially diluted, and the extracted DNA was amplified via RPA and then identified using the MNAzyme-GNP platform. Spot images capture TLC plate results. ImageJ was used to correct for lightness and contrast. Red indicates a positive result.

of detection was similar to the analytical sensitivity measured with synthetic antibiotic resistance genes. This result was confirmed quantitatively by measuring the peak absorbance wavelength using a UV–vis spectrophotometer (Figure S6). We observed statistically significant differences in the peak absorbance wavelength at or greater than 10^2 CFU/mL for *mecA* and *tetM* genes and 10^3 CFU/ml for *blaZ*, *acc6*, and *ermA* genes (P value of <0.0001 or <0.00001) compared to the negative control (*i.e.*, 0 CFU/mL). Our assay can detect antibiotic resistance genes in MRSA isolates at concentrations required for clinical applications.

Detecting Bacteria Causing Central Line Associated Bloodstream Infection. Following analytical characterization of our platform, we designed a bacterial identification panel to detect seven different bacteria known to cause CLABSI, each of which occurs in ≥4% of CLABSIs.[63] The CDC defines CLABSI as the recovery of a nonskin pathogen from one blood culture or the recovery of skin pathogens from two or more blood cultures in a patient who had a central line within 48 h of developing symptoms of infection. To identify each bacteria, we targeted the *chuA* gene in *Escherichia coli*, *ddl* gene in *Enterococcus faecalis* and *Enterococcus faecium*, *16S rRNA* gene in *Klebsiella* species (spp.), *16S rRNA* gene in *Pseudomonas aeruginosa*, *fib* gene in *Staphylococcus aureus* (*S. aureus*), and *nrdE* gene in *Staphylococcus epidermis* (Table S4).[64,65] The amplified regions were detected using our MNAzyme-GNP platform for simple, sequence-specific detection.

We first validated this assay using 37 clinical isolates from Mount Sinai Hospital to assess the clinical sensitivity and specificity of our panel for detecting six organisms (*S. aureus*, *Klebsiella* spp., *E. faecalis*, *E. faecium*, *Pseudomonas* spp., and *S. epidermis*). Using clinical isolates, we achieved an overall sensitivity of 100% and specificity of >98% when compared to gold standard culture results (Table S7). The specificity of *S. aureus* and *E. faecalis* was 97% and 95%, respectively, while all other targets had a specificity of 100%.

Following validation with clinical isolates, we conducted a single blind study to detect CLABSI-associated pathogens in febrile patients with catheters.[66] In these suspected cases, blood was drawn from two sites and then cultured before the organisms were identified using the MNAzyme-GNP platform. We obtained blood culture samples from Mount Sinai Hospital and used the MNAzyme-GNP platform to identify pathogenic bacteria. Researchers performing the amplification and MNAzyme assay did not know which bacteria were present in the samples. The overall sensitivity and specificity of this assay was 86% and 100%, respectively. The sensitivity of detecting *E. faecium*, *Klebsiella* spp., and *Pseudomonas* spp. was 100%, while the sensitivity of detecting *S. epidermis*, *S. aureus*, and *E. coli* was 75%, 80%, and 60%, respectively, when compared to phenotypic culture results (Figure 3).

Clinical Sensitivity and Specificity in Clinical Isolates. We screened clinical isolates for the presence of antibiotic resistance genes. 100 *S. aureus* isolates were collected from Toronto General Hospital and Toronto Western Hospital to assess the clinical sensitivity and specificity levels of our system. For this investigation, we initially examined the samples for the presence of eight genes (*mecA*, *blaZ*, *vanA*, *vanB*, *tetK*, *tetM*, *ermA*, and *ermC*) and tested for resistance to oxacillin, penicillin, vancomycin, doxycycline, and erythromycin in the clinical isolates. We did not test for resistance to gentamicin (*i.e.*, presence for *acc6* and *aph3iiia* genes) because it was not included in the panel of the automated culture VITEK 2 system used in the hospital. The isolates were first cultured to adjust the final concentration to 10^5 CFU/mL, and DNA was extracted in three replicates for each isolate using Nulisens easyMag instrument (bioMerieux Canada, Inc. Quebec, Canada). Extracted DNA was amplified and purified, chemically denatured, blocked, incubated with MNAzyme-GNP reagents, and spotted on a TLC plate for the final readout. As shown in Table 1 and Figure S7, our assay was able to profile antibiotic resistance for 100 clinical isolates with high sensitivity and specificity when compared to culture results obtained by the hospital diagnostic facilities. Our assay achieved 100% sensitivity and 100% specificity for determining *S. aureus* resistance and susceptibility to oxacillin, vancomycin, and tetracycline and 94% sensitivity for determining resistance to penicillin. We initially obtained 100% specificity but only 40% sensitivity when detecting erythromycin resistance. To

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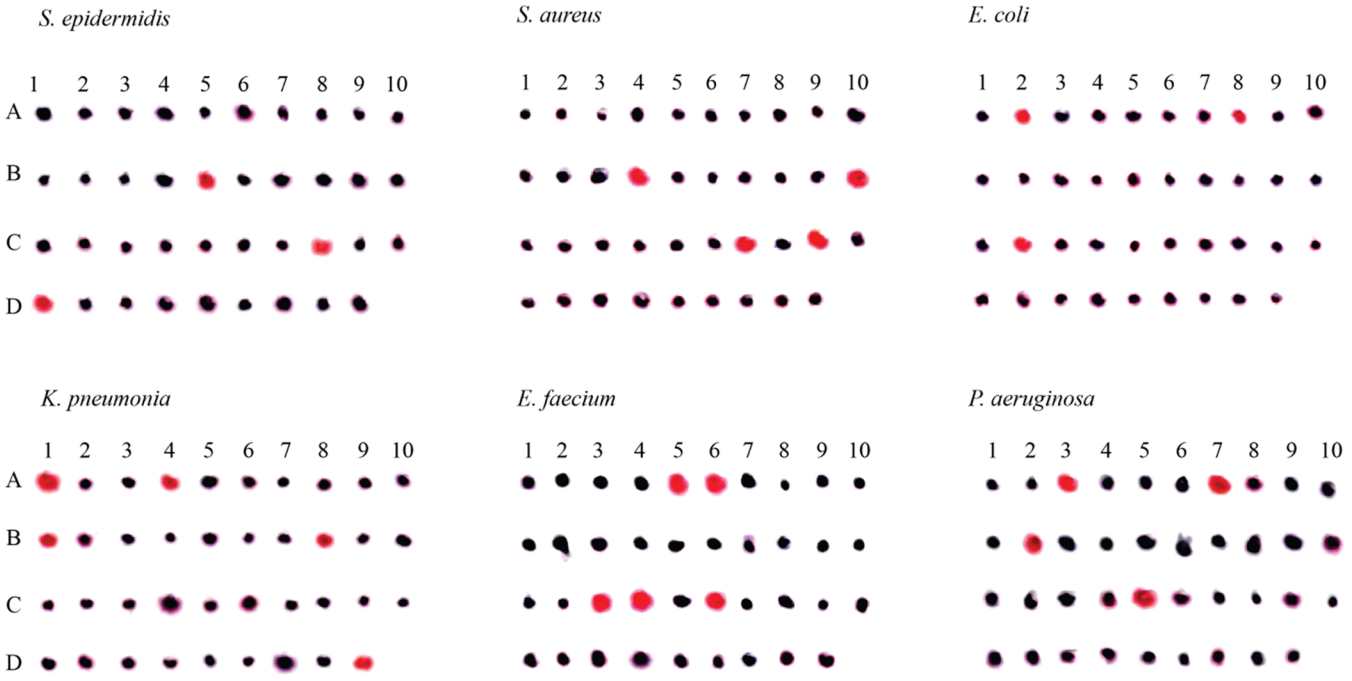

Figure 3. Identifying CLABSI-associated bacteria in clinical specimens. Extracted DNA was amplified via RPA and identified using the MNAzyme-GNP platform. The bacteria listed above each group is the target bacteria being detected. Spot images capture TLC plate results. ImageJ was used to correct for lightness and contrast. Red indicates a positive result.

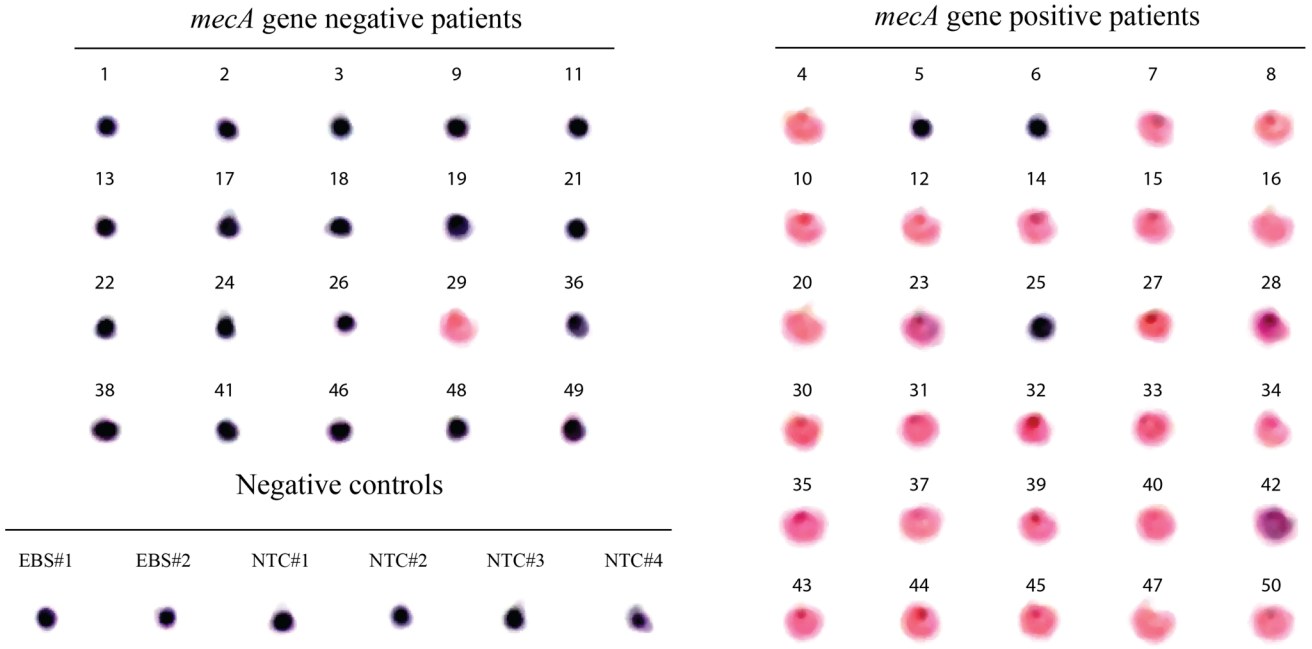

Figure 4. Identifying the *mecA* gene in clinical specimens. Extracted DNA was amplified via RPA and identified using the MNAzyme-GNP platform. "EBS" control is composed of elution buffer from the DNA purification kit and blocking strands. Spot images capture TLC plate results. ImageJ was used to correct for lightness and contrast. Red indicates a positive result. NTC: nontemplate control.

increase the assay sensitivity for erythromycin resistance, we added an additional *msrA* gene to the erythromycin panel. This successfully increased the sensitivity of erythromycin resistance detection from 41.7% to 86.7% but decreased the specificity from 100% to 87.5% (Table 1, Figure S7). This suggests that adding an extra antibiotic resistance gene to our panel can significantly improve the sensitivity with a mild reduction in specificity. Our assay successfully profiled the resistance of *S. aureus* with a high level of sensitivity and specificity compared to culture method.

In a similar manner to the *S. aureus* antibiotic resistance genes, we selected the predominant four antibiotic resistance genes that confer carbapenem resistance in CLABSI-associated gram-negative bacteria: bla_{KPC} for KPC carbapenemase, bla_{NDM} and bla_{VIM} for metallo-β lactamases, and $bla_{OXA\text{-}48}$ for OXA-48-like carbapenemases (Table S3). The most predominant are KPC and OXA-48-like carbapenemases. KPC is endemic in the

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USA, Israel, and South America, while OXA-48-like carbapenemases are dominant in North Africa and Europe.[67−69] We screened for these antibiotic resistance genes in 12 clinical isolates from *E. coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., and *Citrobacter* spp. collected from Toronto General Hospital. Our assay achieved 100% sensitivity and 100% specificity (Table S6, Figure S7).

Detecting Antibiotic Resistance Genes in Patient Swabs. We examined the ability of our system to detect the presence of antibiotic resistance genes directly in clinical samples without culture. We tested 50 clinical admission screening swabs collected at Toronto General Hospital, Toronto Western Hospital, and Mount Sinai Hospital for the presence of the *mecA* gene. The performance of our assay was compared to real-time PCR, which is the gold standard for genetic molecular detection. The collected admission screening swabs (nasal, groin, axilla, and wound swabs) were frozen and stored up to 1 day at −20 °C. DNA was extracted from frozen swabs, followed by detection of the *mecA* gene using our assay. When compared to real-time PCR, our assay achieved 90% sensitivity (95% CI, 72−97%) for identifying *mecA* gene positive patients and 95% specificity (95% CI, 73−100%) for identifying *mecA* gene negative patients in this clinical screen (Figure 4).

The work presented herein describes the development of a rapid diagnostic platform that identified bacteria and their associated resistance genes within 2 h. The MNAzyme-GNP concept was first presented in 2013.[27] It took us seven years to optimized the chemistry and amplification procedures for tthe clinical evaluation of the MNAzyme-GNP platform. The translation of technology from proof-of-concept to a tool for diagnosing patients is challenging. There are no detailed studies of MNAzyme-GNPs for clinical use published in the literature as most researchers stopped at the proof-of-concept stage where synthetic DNA is detected. The translation of a technology should be a focus for many researchers as the impact of a study depends on the technology's use for patients For clinical use, we had to carefully design the probes and choose targets for the MNAzyme-GNP diagnostic system. This step was not easy, but it is required move beyond an idea and to real-world use.

We see significant advantages of the MNAzyme-GNP platform for infectious disease diagnostics. Unlike many versatile detection systems, the MNAzyme-GNP platform does not require specialized equipment, making it practical for use in low resource areas without pre-existing laboratory infrastructure. Our platform accurately detected target from clinical samples with concentrations as low as 10^2−10^3 CFU/mL and had high clinical sensitivity and specificity for profiling pathogenic bacteria and antibiotic resistance genes when compared to culture and PCR. The detection range achieved by our platform is in alignment with 2009 guidelines from the Infectious Disease Society of America, which states that growth of >100 CFU from a catheter using quantitative culture should guide clinical management.[66] The simple diagnostic platform we developed can detect antibiotic resistance genes from gram-negative and gram-positive bacteria. These bacteria were found in different clinical specimens, demonstrating the versatility of this platform. The workflow can be adapted to other emerging diagnostics that are in the "academic" proof-of-concept stage.

Our diagnostic development addresses a persistent challenge of antimicrobial infections which is a lack of diagnostic tests that can rapidly identify infectious pathogens and antibiotic resistance markers. This challenge burdens clinicians who struggle to manage their patients appropriately (*i.e.*, prescribe highly specific antibiotics).[70] While progress has been made to quickly identify bacterial species, long wait times between bacterial identification and antimicrobial susceptibility results have persisted. In clinical practice, physicians usually prescribe empiric broad-spectrum antibiotics as the initial therapy based on clinical diagnosis.[4] Once antibiotic susceptibility test results are available, an appropriate or narrow spectrum antibiotic is prescribed.[13] This time window between initial and definitive therapy is critical to the final clinical outcome of a patient. A delay in the administration of appropriate antibiotics due to turnaround times from current clinical methods (24−72 h) can lead to increased risk of a patient's mortality, higher costs due to extended hospitalization times, and increased risk of antibiotic resistance development.[13,71,72] Therefore, the ability of our platform to identify both the pathogen and its antibiotic susceptibility much sooner than the current laboratory techniques will help reduce unnecessary prescriptions and has the potential to conserve the currently available drugs, reduce health-care costs, and mitigate the risk of further antibiotic resistance development.

Current testing for antibiotic resistance is either phenotypic or genotypic. Phenotypic testing is currently the gold standard. It indicates the antibiotics to which bacteria are resistant or susceptible. Although phenotypic methods such as disk agar diffusion, microbroth dilution, or selective chromogenic media are cost-effective, they may take 20−72 h for an accurate diagnosis, thereby delaying treatment.[6,7,11−13] Phenotypic methods are known to have interlab variability resulting in questionable reliability.[73,74] Pathogens that carry antibiotic resistance genes can appear as antibiotic sensitive using phenotypic tests due to the lack of gene expression, poor bacterial growth, or decreased antibiotic potency.[11,16,55,75−78] These issues produce false-negative results. Unlike phenotypic tests, genotypic tests can directly detect the presence of antibiotic resistance genes and are often used to confirm inconclusive phenotypic test results.[42,79−81] The Clinical and Laboratory Standards Institute has acknowledged that molecular assays may be more sensitive than typical phenotypic methods.[82] Phenotypic tests can misclassify bacteria that are transiently susceptible to antibiotics. Prior studies have shown that *E. faecium* with wild-type *vanA* tested as susceptible using phenotypic tests but became resistant after treatment.[17,83] Genotypic tests have a unique clinical role as they provide information that cannot be captured using traditional phenotypic tests.

The detection of multiple genes for diagnosis of a pathogen can increase the diagnostic sensitivity of an assay and allow for epidemiological tracking.[83] Our panel includes two antibiotic resistance genes for each antibiotic class. These panels can be expanded to include multiple genes for each antibiotic class or microorganism, which can further increase the diagnostic sensitivity of the assay. For instance, when we initially selected *ermA* and *ermC* genes based on previous reports to test the resistance of clinical isolates to erythromycin, our assay achieved only 41.7% detection sensitivity. With the addition of *msrA* gene to our panel, the sensitivity of our assay increased to 86.7%. This might suggest that the prevalence of antibiotic resistance genes is geographically dependent. As indicated in Table S5, previous reports showed a high prevalence of *ermA* and *ermC* genes for erythromycin resistance in the U.S. and Europe; however, we showed that *msrA* gene seems to be more

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prevalent in our local hospitals in Toronto, Canada. This demonstration is important from an epidemiological point of view as our assay could help epidemiologists and microbiologists track select antibiotic resistance genes or viral strains to identify community or hospital spread. These epidemiological studies require the study of single point mutations. Our platform currently detects conserved regions. The MNAzyme can be redesigned to detect single point mutations by using truncated sensor and stabilizer arms.[84] In future work we plan to adapt our MNAzyme-GNPs assay to detect antibiotic resistance associated with single point mutations.

We used the MNAzyme-GNP platform to detect the cause of CLABSI in patients with suspected infections. *Klebsiella* spp., *E. faecalis*, *E. faecium*, and *Pseudomonas* spp. were detected with 100% sensitivity and specificity. When detecting *E. coli* from blood culture samples, two samples were incorrectly identified as negative. The *chuA* gene used for *E. coli* detection is present in the majority of virulent extra-intestinal strains.[85,86] To detect additional strains of *E. coli*, another primer set could be added to increase sensitivity as demonstrated with the *msrA* gene. Additionally, three of four *S. epidermis* blood culture samples tested positive by the MNAzyme-GNP platform. The one sample that tested negative was positive for both *S. hominis* and *S. epidermidis* using the Vitek 2 system. The Vitek system identifies <63% of *S. hominis* samples correctly, 3−43% of which are incorrectly identified as *S. epidermis*.[87−89] The Vitek classified one sample as positive for *S. epidermis* and *S. hominis*. This sample appeared negative for *S. epidermis* using the MNAzyme-GNP platform. Due to the Vitek's poor accuracy for detecting coagulase negative staphylococci, it is likely that this sample was negative for *S. epidermis* and that the MNAzyme-GNP result was correct. Overall, the high sensitivity and specificity of the MNAzyme-GNP bacterial identification panel demonstrated that the MNAzyme-GNP platform can guide health professionals to prescribe targeted therapy and make appropriate infection control decisions.

In this study we focused on single-plexed detection of genomic markers. We can further advance our diagnostic test to run multiplexed detection. For instance, our preliminary experiment demonstrates that we can successfully amplify five antibiotic resistance genes simultaneously using multiplexed RPA (Figure S8), followed by multiplexed MNAzyme reaction and parallel GNP readout. Such a system provides high detection selectivity through both RPA and MNAzyme steps and was confirmed to have no cross-reactivity in detection of the five genes (Figure S9). Multiplex detection allows us to expand the number of genetic targets that can be detected in parallel while minimizing the number of necessary reactions.

CONCLUSION

Our diagnostic platform provides colorimetric results that can be interpreted by the naked eye. It can be easily adapted to both centralized and limited resource testing locations and modified to detect different classes of pathogens by simply altering primer and MNAzyme sequences.

MATERIALS AND METHODS

Design of Target Regions, Primers, and MNAzyme Constructs. Fourteen different antibiotic resistance genes representing eight different families of antibiotics were selected for this study. Ten genes were selected based on their common presence in *S. aureus* strains, and four genes were selected based on their presence in CRE. The antibiotic resistance gene sequences were obtained from the Antibiotic Resistance Genes Database (https://ardb.cbcb.umd.edu) and the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov). All whole genome sequences for bacteria were also obtained from NCBI. The gene sequences were screened using BLAST (https://www.ncbi.nlm.nih.gov) to select the most conserved regions. RPA primers have been then designed for each conserved region using primerQuest Tool from Integrated DNA Technologies, Inc. (https://www.idtdna.com/Primerquest/Home/Index) and Geneious (Geneious 11.1.5). The cross-reactivity of all primers was examined using OligoAnalyzer 3.1 from Integrated DNA Technologies, Inc. (https://www.idtdna.com/calc/analyzer). DNA oligonucleotides were purchased from Biobasic Canada Inc. or Integrated DNA Technologies Inc.

Synthesis and Surface Modification of Gold Nanoparticles. A solution containing 1 mL of 25 nM $HAuCl_4$ and 98 mL of water was heated to boiling in an aqua regia (30 mL of HCl, 10 mL of nitric acid) prewashed 250 mL flask. We rapidly added 1 mL of 33 mg/mL of sodium citrate tribasic solution. The solution was continuously stirred. After 10 min the solution was put on ice. Nanoparticle size and monodispersity were measured by dynamic light scattering. We added Tween-20 to a final concentration of 0.01% (v/v), and nanoparticles were concentrated by centrifugation at 12 000*g* for 35 min. Nanoparticle concentration was measured using UV−vis spectrophotometry at λ = 520 nm (extinction coefficient 2.33×10^8 M^{-1} cm^{-1}), then adjusted to 100 nM using 0.01% (v/v) Tween-20 solution. To functionalize the nanoparticle surface with thiol-terminated DNA, 100 μL of 100 nM GNPs was mixed with 100 μL of DNA strand (1:4 ratio of GNP probe 1:GNP probe 2), 40 μL Tween-20 0.1% (v/v), and 60 μL water.[90] We incubated the solution at room temperature for 5 min, added 100 μL of 100 mM trisodium citrate buffer with a pH of 3, further incubated at room temperature for 30 min. We backfilled the nanoparticle surface with polyethylene glycol (PEG) by adding 50 μL of 2 mM methoxy and thiol terminated PEG (1 kDa) and incubating at 60 °C for 30 min. Conjugated nanoparticles were centrifuged at 16 000*g* for 45 min three times. Particles were resuspended in Tween-20 solution (0.01% v/v) and diluted to a concentration of 11 nM.

DNA Extraction from Bacterial Cells. DNA was extracted from bacterial cells using a manual extraction kit (GeneJET genomic DNA purification kit, ThermoFisher Scientific Inc.) or automated NucliSENS easyMAG instrument (BioMerieux Canada, Inc.). The manual extraction kit was used for the development of sensitivity curves, where the number of freshly grown cells was adjusted to 10^7 CFU/mL and serially diluted to 10^0 CFU/mL in LB media. Cells were centrifuged at 5000*g* for 10 min. The supernatant was removed, and the cells were resuspended in 160 μL of lysis buffer (2× TE, 1.2% Triton X-100) followed by 10 μL of lysostaphine (Sigma-Aldrich) and 10 μL of 20 mg/mL lysozyme (Bioshop Canada). The cells were incubated at 37 °C for 30 min. After incubation, 200 μL of lysis solution (kit component) was added and the mixture was incubated at 56 °C for 30 min. The mixture was treated with 20 μL of proteinase K and 20 μL of RNase A. 400 μL of 50% ethanol was added, and the mixture was transferred to the spin column for purification. The purified DNA was eluted in 50 μL and stored at 4 °C for further use. For multiplex reaction, the DNA was manually extracted from bacterial cells at a concentration of 10^5 CFU/mL and purified following the same procedure.

Automatic extraction method was used to extract DNA from clinical isolates and specimens and to measure clinical sensitivity and specificity levels. For extraction of DNA from clinical isolates, a number of freshly grown cells was adjusted to 10^5 CFU/mL, which were first mechanically lysed using glass beads (Sigma-Aldrich, G4649). Mechanically lysed bacterial suspension was transferred to easyMAG vessel and subjected to on-board lysis using the generic protocol. Extracted DNA was eluted in 50 μL volume and stored at −20 °C for later use. For extracting DNA from clinical specimens, admission screening swabs (nasal, groin, axilla, and wound swabs) were first collected and stored using ESwab Liquid Amies Collection and Transport system (Copan Diagnostics Inc., Brescia, Italy). Bacteria resuspended in this media were mechanically lysed using

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ACS Nano 2021, 15, 9379−9390

glass beads (Sigma-Aldrich, G4649), transferred to easyMAG vessel, and subjected to on board lysis using the generic protocol. Extracted DNA was eluted in 50 μL volume and stored at −20 °C for later use.

Recombinase Polymerase Amplification. RPA was performed using TwistAmp Basic kit (TwistDx). Primers and target DNA genes were purchased from Bio Basic Inc. HPLC-purified primers were prepared at a concentration of 100 pmol/μL in TE buffer, diluted to 10 pmol/μL aliquots, and stored at 4 °C until later use. For RPA reaction using synthetic DNA targets, we combined 2.4 μL of each forward and reverse primers (10 pmol/μL), 12.2 μL of sterile water, 29.5 μL of rehydration buffer, 2.5 μL of magnesium acetate (280 mM), and 1 μL of serially diluted DNA samples (10^0–10^{11} copies/μL) to make a master mix. The no template controls used 1 μL of TE buffer in place of target DNA. The master mix was combined with the lyophilized enzyme pellet. This solution was vortexed and incubated at 37 °C for 30 min.

For RPA reaction using clinical isolates and specimens, a similar master mix was prepared using 3.2 μL of sterile water and 10 μL of extracted DNA from bacteria. The no template controls used 10 μL of solution extracted from the media without bacteria in place of extracted DNA. The master mix and nontemplate control solution were combined with the lyophilized enzyme pellet. The solution was vortexed and incubated at 37–39 °C for 30 min.

The EZ-10 spin column DNA gel extraction kit (Bio Basic Inc.) or GeneJET PCR purification kit (Thermo Fisher Scientific) was used to purify RPA products using the manufacturer's protocol. Gel electrophoresis using a 3% agarose gel was used for visualization. Gels include a low molecular weight ladder from New England Biolabs. Purified products were stored at 4 °C.

Denaturation and Blocking of Recombinase Polymerase Amplification Products. For clinical sensitivity, cross reactivity, and analytical performance tests, 3 μL of RPA product or elution buffer (EBS control), 2 μL of blocking strand mix (pmol/μL final concentration for each), and 1 μL of NaOH (0.1 M) were mixed, incubated at room temperature for 5 min, and neutralized by adding 1 μL of HCl (0.1 M) to the solution mix.

MNAzyme-Gold Nanoparticle Assay. Clinical sensitivity, cross reactivity, and analytical performance tests were completed using similar mixtures. We mixed 5 μL of blocked RPA amplicons or elution buffer with 1 μL of 10× MNAzyme buffer (0.5 M KCl (pH 8.3), 0.1 M Tris-HCl), 1 μL of 300 mM $MgCl_2$, 1 μL of MNAzyme (4 μM), 1 μL of 1 μM of linker DNA, and 1 μL of water. We incubated the solution at 50 °C for 1 h. We mixed the samples or negative control with 10 μL of the GNPs probe mixture. This solution was incubated at 50 °C for 20 min to facilitate GNP aggregation. TLC plates were used for visualization. 3 μL of sample was pipetted onto the plate to form a spot. A UV–vis spectrophotometer was used to measure the peak absorbance wavelength of each sample. For the measurement of clinical sensitivity and specificity of the clinical isolates and clinical specimens, 6 μL of blocked RPA amplicons was used and no water was added.

For testing cross-reactivity and screening clinical isolates of the first multiplexed group, 18 μL of multiplexed blocked amplicons (five genes from each group) or elution buffer was mixed with 3 μL of 10× MNAzyme buffer, 3 μL of 300 mM $MgCl_2$, 3 μL of 5 MNAzyme mixtures of the five genes (4 μM), and 3 μL of the five DNA linkers mixture. We incubated the solution at 50 °C for 1 h. After incubation, both the positive and negative control mixtures were pipetted into five different tubes (5 μL in each tube), and 5 μL of five different GNPs probes was added to the samples and negative control. These were incubated at 50 °C for 20 min. 3 μL of sample was pipetted on the TLC plate.

Antimicrobial Susceptibility Test Using Agar Diffusion Method. Eight different antibiotic disks were purchased from Bacterius LTC, U.S., and used in this experiment: penicillin (10 units), oxacillin (1 μg), vancomycin (30 μg), tetracycline (30 μg), minocycline (30 μg), gentamicin (10 μg), kanamycin (30 μg), and erythromycin (15 μg). The procedure was done following the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing guideline.[11] Briefly, fresh cultures of *S. aureus* strains were prepared from single colony on fresh Luria agar (LA) plates. Plates were incubated at 37 °C for 24 h. Three to five colonies of each culture were transferred to 1 mL of 0.85% NaCl solution, and the solution turbidity was adjusted to 0.5 McFarland standard. A sterile cotton swab was immersed into the cell suspension and rotated firmly several times against the tube's upper inside wall to remove excess fluid. The swab was used to inoculate the surface of Mueller–Hinton agar plates (MHA) plates by spreading the swab over the agar surface. The desired antibiotic disks were applied to the surface of the MHA plates using sterile forceps. The plates were incubated at 35 °C for 16 h. After incubation, the diameter of inhibition zone was measured and compared to the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing guidelines to determine the antibiotic susceptibility of the bacterial strains.

Antimicrobial Susceptibility Test Using Vitek 2. Susceptibility testing using the Vitek 2 system (bioMerieux Canada, Inc., Quebec, Canada) was performed according to the manufacturer's instructions. Briefly, a suspension of each *S. aureus* isolate was prepared in Vitek saline tubes and adjusted to a 0.5 McFarland turbidity standard. This was used to inoculate a Vitek ASTp580 card for each isolate which was then placed on the Vitek 2 instrument. Results were read automatically by the instrument and reported for each isolate.

Statistical Analysis. Statistical analysis was completed with GraphPad Prism 6 and Microsoft Excel 2013. The Student *t* test (two tails) was used for hypothesis testing between data pairs.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.0c09902.

Methods for multiplex detection of antibiotic resistance genes, gel electrophoresis data showing antibiotic resistance gene products after RPA (single target and multiplex), analytical sensitivity of MNAzyme-GNP assay with and without RPA, detection of antibiotic resistance genes in bacterial strains, MNAzyme-GNP assay results for clinical isolates, cross reactivity of MNAzyme-GNP assays for antibiotic resistance genes, tables of DNA sequences used in detection, table of antibiotic resistance gene prevalence for *S. aureus*, and tables summarizing clinical sensitivity and specificity of MNAzyme-GNP panels (PDF)

AUTHOR INFORMATION

Corresponding Author

Warren C. W. Chan − *Institute of Biomedical Engineering, University of Toronto, Toronto, Ontario M5S 3G9, Canada; Terrence Donnelly Centre for Cellular and Bimolecular Research, University of Toronto, Toronto, Ontario M5S 3E1, Canada; Centre for Global Engineering, University of Toronto, Toronto, Ontario M5S 1A4, Canada; Department of Chemistry, University of Toronto, Toronto, Ontario M5S 3H6, Canada; Department of Chemical Engineering, University of Toronto, Toronto, Ontario M5S 3E5, Canada; Department of Materials Science and Engineering, University of Toronto, Toronto, Ontario M5S 3E4, Canada;* orcid.org/0000-0001-5435-4785; Phone: 416-946-8416; Email: warren.chan@utoronto.ca

Authors

Mohamed A. Abdou Mohamed − *Institute of Biomedical Engineering, University of Toronto, Toronto, Ontario M5S 3G9, Canada; Terrence Donnelly Centre for Cellular and Bimolecular Research, University of Toronto, Toronto, Ontario M5S 3E1, Canada; Botany and Microbiology*

Department, Faculty of Science, Zagazig University, Zagazig 44519, Egypt

Hannah N. Kozlowski − *Institute of Biomedical Engineering, University of Toronto, Toronto, Ontario M5S 3G9, Canada; Terrence Donnelly Centre for Cellular and Bimolecular Research, University of Toronto, Toronto, Ontario M5S 3E1, Canada; Centre for Global Engineering, University of Toronto, Toronto, Ontario M5S 1A4, Canada*

Jisung Kim − *Institute of Biomedical Engineering, University of Toronto, Toronto, Ontario M5S 3G9, Canada; Terrence Donnelly Centre for Cellular and Bimolecular Research, University of Toronto, Toronto, Ontario M5S 3E1, Canada; Centre for Global Engineering, University of Toronto, Toronto, Ontario M5S 1A4, Canada*

Kyryl Zagorovsky − *Institute of Biomedical Engineering, University of Toronto, Toronto, Ontario M5S 3G9, Canada; Terrence Donnelly Centre for Cellular and Bimolecular Research, University of Toronto, Toronto, Ontario M5S 3E1, Canada*

Melinda Kantor − *Department of Microbiology, Mount Sinai Hospital and University Health Network, Toronto, Ontario M5G 1X5, Canada*

Jordan J. Feld − *Sandra Rotman Centre for Global Health, University Health Network, Toronto, Ontario M5G 1L7, Canada*

Samira Mubareka − *Divisions of Microbiology and Infectious Diseases, Sunnybrook Health Sciences Centre, Toronto, Ontario M4N 3M5, Canada; Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5S 3H6, Canada*

Tony Mazzulli − *Department of Microbiology, Mount Sinai Hospital and University Health Network, Toronto, Ontario M5G 1X5, Canada; Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5S 3H6, Canada*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsnano.0c09902

Author Contributions

[∞]M.A.A.M., H.N.K., and J.K. contributed equally. The manuscript was written with contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): W.C.W.C, M.A.A.M., and K.Z. started a company based on this technology.

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