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ARTICLE

Genomic nano-biosensor for rapid detection of carbapenem-resistant gene *bla*$_{NDM-1}$ in carbapenemase-producing bacteria

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Abstract: Antimicrobial resistance (AMR) has become one of the major public health concerns causing serious obstacles to the successful prevention and treatment of infectious diseases. To curb the spread of AMR, well-equipped laboratories for early detection of disease-causing pathogens and resistant genes are crucial, something that remains unmet in developing countries due to resource constraints and inadequate infrastructure. This paper presents an affordable and simple nanoparticle-based biosensor for rapidly detecting the *bla*$_{NDM-1}$ gene in carbapenemase-producing (CP) bacteria. The biosensor employed thiol-ligand surface functionalized gold nanoparticles (GNPs) with conjugated oligonucleotide probe specific to detect the bla$_{NDM-1}$ gene. The biosensor was evaluated using DNA extracted from CP bacteria having the target *bla*$_{NDM-1}$ gene, two non-NDM-1 CP bacteria, and five susceptible bacterial strains. Tuning of the localized surface plasmon resonance (LSPR) of the GNPs was achieved by reducing the surrounding pH of the GNPs, hence inducing aggregation. With the binding of GNPs-probe-target DNA, the stability of GNPs was enhanced and was confirmed by the red colour being maintained when an optimized amount of 0.1 M HCl was added to induce aggregation. The absence of target DNA was indicated by the aggregation of GNPs after the addition of the acid, which corresponded with a colour change from red to blue/purple and shifting the LSPR band to a longer wavelength on average at 620 nm. The biosensor visual detection results were quantified with absorbance spectra measurements and results were achieved within 30 minutes. The biosensor successfully detected the target DNA from *bla*$_{NDM-1}$ positive bacteria and distinguished the non-targets. The analytical sensitivity achieved was 2.5 ng/µL which corresponds to approximately 10^3 colony-forming units per milliliter. These findings were confirmed through PCR amplification. This nano-biosensor advances an inexpensive, simple, rapid, and sensitive method for detecting *bla*$_{NDM-1}$ gene in carbapenemase producers, and readily implementable in resource-limited settings.

1.0 Background

Early detection of pathogenic bacteria and their resistance genes is a crucial factor in healthcare and overall public health. Antimicrobial resistance (AMR) is one of the most worrying public health concerns challenging the world today **[1]**. Indiscriminate use of antibiotics in medical care, agriculture, and veterinary care has resulted in selection pressure favouring the survival and spread of such resistant microorganisms, leading to an increased hospital stay, high morbidity, and mortality **[2,3]**. An estimated 700,000 people are currently losing lives to AMR infections annually and this number is projected to reach 10 million by 2050, according to the US Centers for Disease Control and Prevention (CDC) **[4]**. But, even with the advent of effective antibiotic therapy, bacteria are continuously mutating to evade the treatment programs in question, giving rise to the global threat **[5,6]**.

Carbapenems play a significant role in the antibiotic landscape, as they offer the broadest spectrum of activity and highest potency among the β-lactam antibiotics. They are the most widely used drugs to treat bacterial infections due to their safety and efficacy **[7,8]**. However, the expression of β-lactamase genes is one of the mechanisms by which pathogens become antibiotic resistant. These pathogens produce carbapenemase type of β-lactamase enzymes, which subvert the lethal action of the antibiotics, thus, they have been identified as critical priority pathogens by the World Health Organization (WHO) **[9,10]**. They include a serine-β-lactamase type *Klebsiella pneumoniae* carbapenemases (KPC) and metalloid-β-lactamase types, such as Imipenemase (IMP), Verona integrin encoded metalloid β-lactamase (VIM), and New Delhi metallo-lactamase (NDM) carbapenemases **[8]**. The New Delhi metallo-lactamase (NDM) was first identified in 2009 in *Klebsiella pneumoniae* and *Escherichia coli* isolated from a patient in Sweden who had previous hospitalization in New Delhi, India **[11,12]**. To date, there are twenty-eight NDM variants classified as NDM-1 through NDM-28, with NDM-1 and NDM-5 being commonly detected in *Enterobacterales* **[13,14]**.

NDM-1 is the most common variant in cases of clinical infections, and the gene that encodes β-lactamase, *bla*$_{NDM-1}$, has rapidly spread to different Gram-negative pathogenic species worldwide since its detection, through transfer of the *bla*$_{NDM-1}$ gene among mobile plasmids and clonal outbreaks **[15,16]**. Even though the *bla*$_{NDM-1}$-containing bacteria display severity like other enterobacteria, they are resistant to a broad spectrum of β-lactam antibiotics, including carbapenems, penicillins, and cephalosporins **[17]**. A threatening scenario to public health is the existence of NDM-1 genes, which

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have been detected in the food chain **[18,19]** and several compartments of the environment: hospital sewage **[20]**, drinking tap waters **[21]**, and municipal waste waters **[22]**.

Effective prevention of the spread of NDM-1 producers requires a rapid screening assay that can detect the NDM-1 gene in food, water and the environment early enough **[23,24]**. In addition, to reduce the misuse of antibiotics, a fast, robust, and affordable antimicrobial susceptibility test (AST) is imperative, as about 50% of antibiotic treatments are initiated with wrong antibiotics and without a proper detection of the pathogen **[25]**.

AST assays presently used in medical practice for carbapenemase detection include growth-based assays, that measure carbapenem resistance based upon the growth of bacteria in the presence of a carbapenem antibiotic, namely the modified Hodge test and the modified carbapenem inactivation method **[26,27]**. Secondly, hydrolytic methods detect carbapenem degradation products, examples being Carba NP test and matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF) **[28,29]**. And thirdly, lateral flow immunoassays usually detect carbapenemase enzymes using specific antibodies **[30,31]**. The Carba NP test is preferred by the Clinical and Laboratory Standards Institute (CLSI) as a reliable phenotypic method for carbapenemase detection **[32]**. However, these phenotypic methods are time-consuming and sometimes, costly in equipment and supplies **[33,34]**.

Genotypic AST identifies given resistance genes or genetic mutations using molecular or genomic DNA amplification **[35]**. Polymerase Chain Reaction (PCR)-based methods, whole genome sequencing (WGS), loop-mediated isothermal amplification (LAMP), DNA microarray and chips, and fluorescence in situ hybridization (FISH) are some of the genotypic techniques for the detection of these resistant genes **[34]**. Real-time PCR methods capable of detecting bla_{NDM}-positive isolates directly from clinical samples have been reported **[36]**. The PCR method allows the exponential amplification of specific sequences of DNA and RNA. Additionally, a high specificity, fast and dependable multiplex PCR technique for rapid screening of carbapenemase genes has been developed **[37,38]**.

In general, genotypic methods are attributed to be rapid, sensitive, explicit, and specific in detection of resistance genes. Nevertheless, they also suffer from drawbacks including expensive equipment and reagents, and a need for skilled personnel, decreasing their clinical utility **[39–41]**, especially in resource constrained regions. Thus, a timely and accurate detection method for the resistant genes is particularly important for the control of the spread and treatment of infections **[42]**. Biosensors have emerged as specific, sensitive, and cost-effective techniques for early-stage diagnosis, a fundamental value for health care management **[43]**, and they come in various platforms **[44]**. Among them, a novel electrochemical biosensor was developed to detect the drug-resistant gene bla_{NDM-1} **[17]**. In another study, a thermometric NDM-1 biosensor enabled the detection of β-lactamases (metallo- and serine- carbapenemases) in clinical bacterial isolates with 100% accuracy **[45]**. Similarly, a disposable lateral flow biosensor detected *A. baumannii* strains harboring bla_{OXA-23}-like gene with a specificity of 100% **[46]**.

The unique and highly tunable optical properties, high surface area to volume ratio and chemical stability of gold nanoparticles (GNPs) have shown enormous potential in the development of state-of-the art biosensing techniques. In addition, the ease of modification with a wide variety of thiol-terminated organic biomolecules with formation of strong Au–S bonds, and ligand exchange reactions, leads to stable colloidal suspensions in aqueous solutions **[47]**.

A GNP-based biosensing is based on localized surface plasmon resonance (LSPR) giving rise to a strong absorption band in the visible region. A bathochromic resonance/wavelength shift occurs due to changes in interparticle distance **[48,49]**. The pH change leads to modification in the dielectric properties triggering the aggregation of GNPs resulting in a shift of the LSPR band from 520 to 620 nm and a change in the colour of the colloidal solution from wine red (dispersed) to purple/blue (aggregated) due to interparticle surface plasmon coupling **[47]**. GNP-based optical biosensors have enormous potential to be used as point-of care devices owing to their cost-effectiveness, high sensitivity, and reliable analytical results. A plasmonic nano-biosensor able to detect CP pathogens directly from urine within 2.5 hours has been reported previously **[50]**. In a most recent study, dextrin-coated GNPs, genomic-based plasmonic biosensor has been developed for the detection of the KPC-producing carbapenem-resistant bacteria in 30 min **[51]**.

In this research paper, we describe a rapid, cost effective, simple, visual and label free GNP-based nano-biosensor for the detection of the NDM-1 gene expressed by CP bacteria. The biosensor is applicable in environmental surveillance and in clinical diagnostics.

1.1 Novelty of the research study. The nano-biosensor was designed to detect the NDM-1 resistant gene in CP bacteria. The novelty of the study includes the use of a 50-mer oligonucleotide probe specific for detecting the NDM-1 resistant gene in CP bacteria without the need for PCR amplification. Additionally, the biosensor demonstrates the capability to monitor resistant genes in water samples.

2.0 Materials and Methods

2.1. Materials. This study utilized eight bacterial stock cultures, including the NDM-1 resistant target strain *Escherichia c*oli (BAA-2471) and non-target resistant strains*, Klebsiella pneumoniae* subsp. *pneumoniae* (BAA-13883) and *E. coli* (BAA-2340). Additionally, two susceptible strains were procured from the American Type Culture Collection (ATCC). Three other susceptible strains were obtained as frozen cultures from the Nano-Biosensors Laboratory at Michigan State University. The DNA extraction kits were purchased from Qiagen (Germantown, MD, USA). NanodropOne from ThermoFisher Scientific (Waltham, MA, USA) was used to assess the quality and quantity of the DNA samples and measure the absorption spectra. Oligonucleotide probes were designed and ordered from Integrated DNA Technologies (IDT; Coralville, Iowa). Phosphate Buffer Saline (PBS), Nutrient Agar (NA) and Nutrient Broth (NB), Hydrochloric acid (HCl), gold (III) chloride ($HAuCl_4$), sodium carbonate (Na_2CO_3), 11-mercaptoundecanoic acid (MUDA: $HS(CH2)_{10}CO_2H$), sodium dodecyl sulfate (SDS, $CH_3(CH_2)_{11}OSO_3Na$) and dextrin from potato starch were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2 Bacterial strains. The bacterial strains utilized in this study are detailed in Table 1.

2.3. DNA Extraction. Overnight bacterial inoculum from various strains was used to extract DNA with the Qiagen kit. The extracted DNA was then suspended in elution buffer (pH 8). The concentration and quality of the extracted DNA were measured using a Nanodrop spectrophotometer, with acceptable quality ratios for A260/A280 being approximately 1.8 and for A260/A230 being around 2.0. These DNA samples were then used for the biosensor assay. Additionally, overnight fresh bacterial cultures grown in nutrient broth (NB) were used to inoculate the water samples. The strains included the target NDM-1 positive *E. coli* strain and the non-targets *E. coli* C3000 and the enterotoxigenic *E. coli*, ETEC. Samples not contaminated were used as negative control representing the natural microflora. To artificially contaminate the water samples, 1 ml of 10^3 CFU/mL from serially diluted overnight bacterial culture was added to the 25 ml of

tap water samples. They were left to acclimatize for 1 h at room temperature before 225 mL of PBS was added to each sample. A volume of 100 ml was transferred into 100 ml Whirl Pak bags, followed by addition of 1 mL of MNP to the bag. They were mixed, and allowed to incubate at room temperature for 5 min. The Whirl Pak bag was then attached to a magnetic rack for another 5 min before supernatant removal. The remaining sample was resuspended in 1 mL PBS. For each concentrated sample, 500 µL was then transferred to 4.5 mL of NB and incubated for 12 hrs. DNA extraction was then performed using the Qiagen kit and quantified using the Nanodrop spectrophotometer. DNA extracted from target-inoculated water, two non-target-inoculated samples, and DNA from uncontaminated water were used for biosensor tests.

2.4. Synthesis of dextrin capped GNPs and Surface Modification. Dextrin-capped gold nanoparticles (GNPs) were synthesized under alkaline conditions using a greener method as previously described by Anderson *et al* [**52**]. In brief, gold (III) chloride trihydrate was dissolved in distilled sterile water and reduced in alkaline conditions by adding sodium carbonate. Dextrin was then added, and the hot plate was heated to 150 °C. The solution temperature was maintained between 90-95 °C for 30-60 minutes under continuous stirring conditions until it turned wine red. The absorption maxima at approximately 520 nm (wine red colour) of the synthesized GNPs was then confirmed using a Nanodrop UV-VIS spectrophotometer. The GNPs were then surface functionalized with 25 µM 11-mercaptoundecanoic acid (MUDA) and suspended in 0.1 M borate buffer. For biosensor applications, MUDA functionalization allows for amine-thiol ligand interaction, and the oligo probe then hybridizes with the target DNA, to form a stable GNP-probe-DNA complex. The ready-to-use surface-modified GNPs were stored at 4 °C until further use.

2.5. Probe Design. An NDM-1-specific oligonucleotide probe with a single-stranded complementary sequence was designed to target the NDM-1 gene in carbapenemase-producing (CP) bacteria. The probe was designed using the bla_{NDM-1} gene sequence of carbapenem-resistant *E. coli* (ATCC BAA-2471), utilizing the design tools from the National Center for Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST). The E-values were then checked to ensure that no cross-reactivity with non-target sequences and confirm that the oligonucleotide gene sequence was specific to the NDM-1 gene. The single-stranded 50-mer aminated oligonucleotide probe, specific to the genomic DNA of the NDM-1 gene, was employed for this study: 5′-CAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGC-3′.

The probe was aminated at the 5' end. A C6 hexyl linker was used to attach the amine group to the probe, providing flexibility and preventing steric hindrance during hybridization.This 50-mer probe was chosen to balance specificity and sensitivity. Preliminary studies in our lab demonstrated that shorter probes, while offering higher specificity, suffered from lower sensitivity. Conversely, longer probes exhibited greater sensitivity but at the expense of specificity. Thus, a 50-mer length was selected to optimize both aspects. Additionally, the probe was meticulously designed to avoid cross-reactivity with non-target sequences, ensuring accurate and reliable detection of the NDM-1 gene. The PCR primer sequence used for validation of results was F-5′ GGTTTGGCGATCTGGTTTTC, R-5′ CGGAATGGCTCATCACGATC using the protocol adapted from a previous study [53]. The amplified DNA of the different samples were analysed on a 2% agarose gel in Tris Acetate EDTA (TAE) buffer at an applied voltage of 120 V for 1 hr.

2.6. Biosensor Design and Optimization. The biosensor design was adapted from an earlier study by Dester *et al*.,[54]. First, the DNA of bacterial inoculum was extracted using a commercial kit. For each repeated biosensor trial, 5 µL of 25 µM DNA probe, 5 µL of GNPs, and 10 µL of DNA sample were mixed in a single tube. The tubes were then placed in a thermocycler for one cycle for denaturation at 95 °C for 5 min and annealing at 55 °C for 10 min then cooling for 5 min at 25 °C. Upon hybridization, an optimized amount of 0.1 M HCl was used for the aggregation of GNPs after a specified optimized response time. In the presence of the target DNA the red colour of the GNP-probe-DNA complex was maintained while in absence, the GNPs aggregated turning blue/purple. The change in colour of GNPs was observed visually and by measuring their light absorption using a spectrophotometer in a wavelength range of 400 nm to 800 nm. The observed visual results were quantified using the absorbance ratio at 520 nm and 620 nm.

2.7. Analytical sensitivity test. The biosensor sensitivity was evaluated at different DNA concentrations ranging from 20 to 1.25 ng/µL to evaluate the minimum DNA concentration detected. For each test, both the target and nontarget DNA samples were serially diluted to the desired concentration. And then, a series of nine trials of the target NDM-1 positive were compared with a susceptible non-target sample (*E. coli* C-3000). The observable colour changes and absorption measurements were used to verify the difference in GNP aggregations between the two samples. The $A_{520/620}$ values were statistically analysed at a 95% confidence interval. The sensitivity of the biosensor was determined by the lowest DNA concentration it could detect.

2.8. The Specificity Tests. The biosensor was validated by evaluating DNA samples of seven non-target bacterial strains, among them 5 susceptible and 2 resistant KPC producing strains. A DNA concentration of 20 ng/µL was used for all the DNA samples for each set of nine trials. Each specificity trial included a negative control (DNA-free), targets (NDM-1 positive), and non-target (NDM-1 negative) samples. Their absorbance spectral measurements and images were collected during the experiment. The differences in $A_{520/620}$ values between target and non-target samples were analysed at a 95% confidence interval. Table 2 represents a list of the strains of bacteria used for specificity tests.

2.9. Statistical Analysis. Statistical analysis was conducted at 95% confidence interval level (α=0.05) and the ratio of absorbance 520 to 620 corresponding to a peak absorbance of the target and non-target DNA samples was compared. All the experiments in this study were ran in 9 trials and averages and standard deviations were used to report the data. The various group mean differences for the specificities and sensitivities were accomplished by using the One-way Analysis of Variance (ANOVA) and Tukey's HSD (Honestly significant difference) test.

3.0 Results and discussion

3.1 The working principle of the NDM-1 nano-biosensor. The biosensor concept in this study relies on the shift of Localized Surface Plasmon Resonance (LSPR) of gold nanoparticles (GNPs), causing their aggregation and resulting in a visual colour change. This phenomenon was confirmed through spectrophotometric measurements. GNP aggregation is a consequence of the distribution of electrostatic repulsion, leading to a shift in their absorption maxima due to the distance-dependent nature of the LSPR. The biosensor employed the alkaline synthesized dextrin capped and 11-mercaptoundecanoic (11-sulfanylundecanoic) acid surface functionalized GNPs, which have an absorption peak at around 520 nm as displayed in Fig. 1a and a particle size ranging from 10-50 nm,

previously determined using transmission electron microscopy (TEM). Furthermore, the hybridized GNP-probe-DNA sample complex showed no shift in the absorbance maxima for the gold nanoparticles, indicating that the GNPs remained stable after undergoing the hybridization process in the thermocycler [55,58]. This stability suggests that the biological probe molecule retains its biorecognition properties.

3.2 Biosensor Optimization. The biosensor operates on the principle of LSPR absorption peak shift, which occurs due to the aggregation of gold nanoparticles (GNPs). This aggregation is visually indicated by a colour change from red to blue or violet. This was quantified spectrophotometrically on a Nanodrop spectrophotometer. In this study, we utilized the GNPs' absorption spectra peak at around 520 nm and a shift in the peak of maxima to approximately 620 nm. The optimization parameters involved the amount of HCl added and the time between HCl addition and response reading of visual results, which were then quantified by spectroscopic measurements. The optimized parameters resulted in the optimal and most consistent bathochromic peak shift difference between target and non-target analytes along with a clearly visible red target sample when compared to the purple/blue non-target and control. The optimization procedure led to a 7 µL of 0.1 M HCl and 10 min response time. As previously reported [51,54,55], the biosensor assay, from sample preparation to colour change assessment, could be completed in approximately 30 min.

3.3 Analytical sensitivity of the nano-biosensor. The lowest DNA concentration detected by the nano biosensor was evaluated using target DNA (resistant *E. coli)* and the non-target DNA (susceptible *E. coli* C3000). The dilutions of a factor of two of the DNA samples for both the target and non-target ranged from 20 to 1.25 ng/µL.

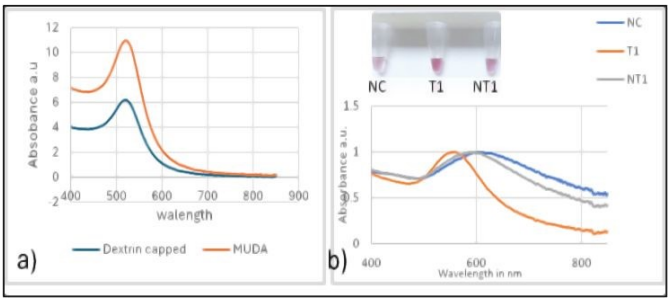

Fig. 1a. Absorbance spectra for dextrin coated and MUDA surface functionalized GNPs, **1b**. Normalized absorbance spectra for the NDM-1 biosensor at 20 ng/µL: Inset a visual image NC (Nuclease free water), T1-Target *E. coli* and NT1 Non-target *E. coli* C3000.

The colour of the target and non-target DNA samples at similar concentrations were visually assessed and compared with absorbance spectra measurements.

Fig. 1b depicts the absorbance spectra of the biosensor at 20 ng/µL with an inset image of the colour differences for the negative control NC (water free of DNA), the target T and the non-target NT (*E. coli*). For the nine trials, there was an average clear shift ≈ 100 nm to a longer wavelength for the negative control NC and the non-target. The target had a minimum shift therefore maintained the red colour as observed visually in the inset photo and evidenced by a narrower band on the absorbance spectrum Fig. 1b.

The absorbance measurements for each comparable concentration were quantified and compared with the colour change. The absorbance ratio $A_{520/620}$ of GNP-probe-DNA complex aggregation were also used to show the differences between the target and non-targets. The tube images together with the absorbance ratios ($A_{520/620}$) of the target and non-targets are displayed in Fig. 2.

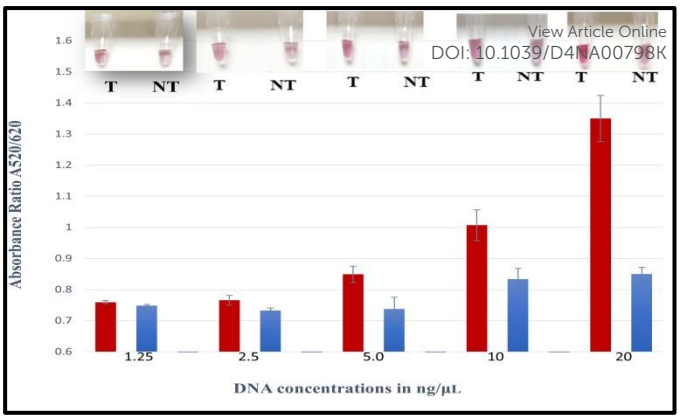

Figure 2. Analytical sensitivity assessment: $A_{520/620}$ absorbance ratios target *E. coli* 2471 (Red bars-T) and non-target *E. coli* C3000 **(**blue bars-NT) at 1.25–20 ng/µL, each concentration tested in 9 trials. Inset are visual results.

High absorbance ratios were realized for target samples for each sample concentration assessed indicating minimum aggregation. Samples that exhibited lower absorbance ratios were non-targets. The mean differences of $A_{520/620}$ of the target and non-target DNA samples were also plotted as a function of the different DNA concentrations as demonstrated in Fig. 3. It was observed that at a concentration of 20 ng/µL, the difference of the absorbance ratio between the target and non-target sample, was high followed by 10, 5 and 2.5 ng/µL, respectively.

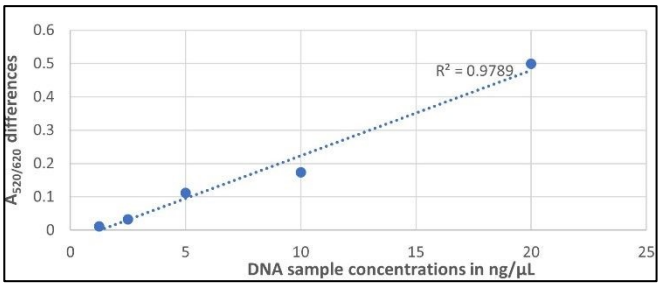

Fig. 3. A linear graph showing the linearity of $A_{520/620}$ differences between the target and non-target samples against concentration ng/µL.

The statistical mean differences between target and non-target samples for each concentration were evaluated using ANOVA followed by Tukey's test at 95% confidence level. The differences between the target and non-target samples at 20, 10, 5, 2.5 ng/µL were found to be significantly different ($\rho<0.05$) at 95% confidence interval. The observed $A_{520/620}$ difference at 2.5 ng/µL was minimal but nevertheless, there was a significant difference between the target and non-target sample ($\rho< 0.05$). Whereas for 1.25 ng/µL there was no significant difference ($\rho>0.05$), nor a clear visual colour distinction between the target and non-target sample as in Fig 2. The analytical sensitivity for the biosensor was therefore identified as 2.5 ng/µL corresponding to ~10^3 CFU/ml substantiating recent findings for a nanoparticle-based plasmonic biosensor for detecting *Klebsiella pneumoniae* (*bla*$_{KPC}$) gene in CP strains [51]. Other colorimetric assays reported lower sensitivities. For instance, a genomic biosensor for detecting *E. coli* C3000 had a detection limit of 10 ng/µL [55]. Likewise, a colorimetric biosensor using SEB-binding aptamer (SEB2) and unmodified GNPs for the detection of *Staphylococcal enterotoxin* B (SEB) had a sensitivity of 50 ng/µL for visual detection [56]. Other assays from literature included an LSPR-based label free aptasensor designed to detect whole-cell multidrug resistant *Pseudomonas aeruginosa* strain PAO1 that was found to have a limit

of detection of 10 CFU/mL with ~3h detection time [57]. An earlier study of a plasmonic nano sensor for a bedside detection of CP producing pathogens demonstrated higher detection limits of >10^5 CFU/mL[50].

3.4 Specificity of the nano-biosensor. Seven non-target bacterial strains, including two CP producing bacteria, were evaluated for specificity studies for the bla_{NDM-1} resistant gene. The results were validated for the presence or absence of bla_{NDM-1} gene by the PCR amplification as shown in Figs 4 and 5 below. Fig. 4a and 4b shows the biosensor, successfully distinguishing all the susceptible strains as negative non-targets (NT1-NT5). Nuclease-free water was used as a negative control (NC). The colour change for the non-targets was visually differentiated as shown in the inset image of Fig. 4a. The mean absorbance ratios $A_{520/620}$ for the target (red bars) and the non-target samples (blue bars) at 20 ng/µL were significantly different (ρ< 0.05) at 95% confidence interval. The absorbance readings from the spectrum for the same tested strains confirmed the shift of the plasmon resonance peak on average to around 620 nm for the non-targets as observed in Fig. 4b. The validated results for the presence or absence of bla_{NDM-1} gene by the PCR amplification are shown in the inset photo of Fig. 4b. The amplification confirmed the presence of *E. coli* BAA 2471 target DNA band, and no bands were observed for the non-targets.

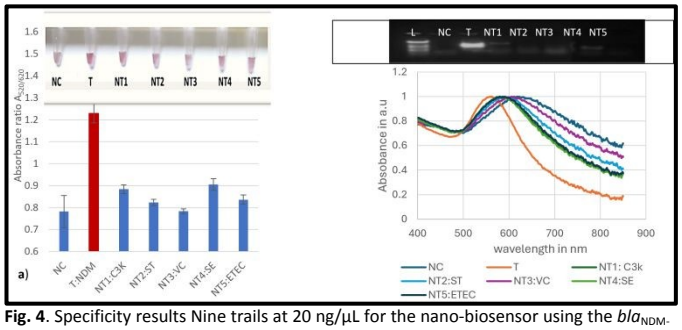

Fig. 4. Specificity results Nine trails at 20 ng/µL for the nano-biosensor using the bla_{NDM-1} probe for tested non-target susceptible strains : a) Mean absorbance ratios $A_{520/620}$ of the negative control NC, target (T) E. *coli* -BAA 2471 in and non-targets (NT1; *E. coli* C3000, NT2; *S. typhimurium*, NT3*; Vibrio cholerae,* NT4; *S. enteritidis,* NT5*; Enterotoxigenic E. coli*) inset: Visual detection (b) Normalized absorbance spectra and inset: PCR amplification results of the strains along with the 1000 bp ladder

Fig. 5 shows the evaluation of two non-targets bacterial strains of KPC producing *K. pneumoniae* (BAA-13883 NT6) and *E. coli* (BAA-2340 NT7) by the biosensor. The visual detection results in Fig. 5a showed that the biosensor positively detected the target NDM-1 and differentiated it from the non NDM-1 CP producers NT6 and NT7. The mean absorbance ratios $A_{520/620}$ for the target DNA samples and the negative control and the non-target samples (blue bars) at 20 ng/µL were significantly different (ρ< 0.05). This was confirmed with the absorbance peaks of Fig 5b. The non-targets and negative control samples showed broader peaks.

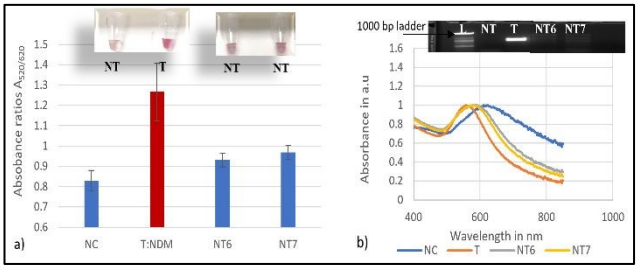

Fig. 5: Specificity results for the nano-biosensor using the bla_{NDM-1} probe. (a) Mean absorbance ratio $A_{520/620}$ of Negative control NC, target (T) and non-targets NT6 (KPC) and NT7 (KPC), in-set are visual images (b) Absorbance spectra of the same strains and inset PCR amplification results along the 1000 ladder.

In summary, the designed genomic nano-biosensor assay was specific and successfully detected the NDM-1 gene target sample and distinguished the non-targets within a turnaround time of 30 min. The biosensor accurately detected the target NDM-1 CP producer and discriminated against all seven non-targets. The absorbance ratios for the target sample were in the range of 1.12-1.6, indicating that the peak shift was smaller with minimum aggregation, hence positive detection and 0.80-0.99 for a larger shift for the non-targets implying aggregation and therefore successful differentiation. Statistical analysis using ANOVA followed by Tukey's method (p < 0.05) confirmed significant differences for the absorbance ratios $A_{520/620}$ between target and all not-target samples and negative control. The biosensor results agreed with the PCR amplification results as shown in-set in Fig 5b.

3.5 Detection of target bla_{NDM-1} gene from inoculated water samples using the nano-biosensor. To establish proof of concept for this biosensor's applicability in water, magnetic extraction of bacterial cells from artificially contaminated water with target *E. coli* (BAA 2471) and the non-targets *E. coli* C3000 and ETEC was conducted. The glycan coated magnetic nanoparticle (gMNP) enrichment step enabled the capture and concentration of the bacteria from larger volumes to smaller volumes. Successful binding between gMNP and *E. coli (*BAA 2471) in PBS was confirmed with images from Transmission Electron Microscopy (TEM). Fig. 6 displays the TEM images of *E. coli* BAA-2471 NDM-1 producer without MNPs (Fig. 6a) and the binding of the magnetic nanoparticles with the *E. coli* resistant bacteria and therefore illustrating the capture in Fig. 6b.

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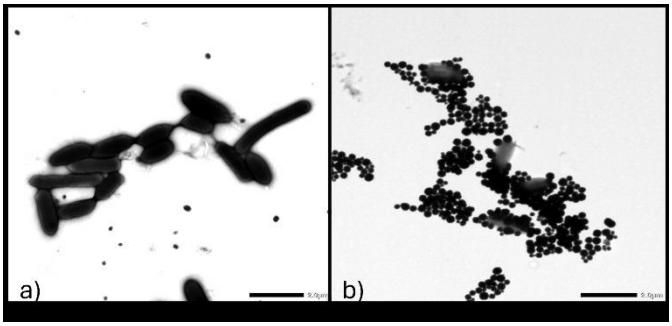

Fig. 6: TEM images of E. *coli* BAA-2471 NDM-1 producer a) without MNPs and b) with MNPs

The capture and concentration were followed by growth in NA and extraction of DNA of the inoculated samples. A control (NF) with no inoculation was also used to represent the natural microflora. The developed nano-biosensor assay was used to assess the DNA of the samples from water for the bla_{NDM-1} resistant gene. The results of the mean absorbance ratio $A_{520/620}$ for the target and the non-targets were found to be significantly different at 95% confidence interval as shown in Fig. 7a. These differences were validated using the absorbance readings by a shift of a plasmon resonance peak on average to around 620 nm for the non-targets in Fig. 7b.

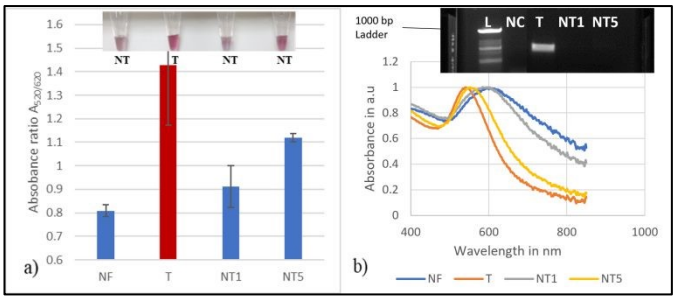

Fig. 7. a) Mean absorbance ratios $A_{520/620}$ of the Natural microflora NF, target strain (T), differentiated non-targets (NT1 E. coli C3000 and NT5 ETEC) from inoculated tap water, b) Absorbance spectra of same strains and inset PCR amplification results along the 1000 bp ladder.

However, the non-target NT5 had a weakly correlated colour change as in set image Fig. 7a nevertheless there was a significant difference in the absorbance ratio $A_{520/620}$ which was validated by PCR amplification Fig. 7b. The biosensor can be extended to monitor food products for the presence of NDM-1, ensuring that contaminated supplies are identified and removed from the supply chain before reaching consumers. Early detection of NDM-1 in food products can prevent public health crises by allowing for rapid response and mitigation efforts. The assay offers an alternative assay for detection of bla_{NDM-1} in a short experimental time and limited analytical equipment compared to other rapid methods that have proven to be costly and less portable [58,59].

Conclusions and Future

A genomic-based nano-biosensor designed using GNPs stabilized by dextrin and surface functionalized with 11-mecarptoundecanoic acid notably identified NDM-1-gene in carbapenem-resistant bacteria within 30 min. The nano-biosensor differentiated the target having NDM-1 gene, from the non-target samples, through visual colour assessment at a low concentration of 2.5 ng/µL and a bacteria load of ~10^3 CFU/mL for the unamplified DNA samples with a turnaround time of approximately 7 hrs. The biosensor successfully detected the target and differentiated non-targets in water samples. For future work, this biosensor could be adapted to detect a wider range of bacterial strains in clinical, food, and environmental samples. This includes strains that carry similar resistance genes, allowing for a comprehensive assessment of its cross-reactivity profile. Moreover, integrating smartphone imaging to distinguish between aggregated and non-aggregated GNPs by identifying colour changes would eliminate the need for a spectrophotometer. This advancement would enhance the technology's applicability in low-resource settings, especially in Africa and Asia, where surveillance data is limited, and contamination of food and water is a significant concern.

Author Contributions:

Conceptualization, E.C.A and R.K.M.; methodology, R.K.M.; Validation, E.C.A, investigation, R.K.M.; formal analysis, R.K.M.; writing—original draft preparation, R.K.M.; writing—review and editing, R.K.M and E.C.A.; supervision, E.C.A.; project administration, E.C.A.; All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study can be obtained upon request from the corresponding author at Michigan State University, USA.

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Table 1: Bacterial strains and description

Strain Name	Description	Category
Escherichia coli BAA-2471	NDM-1 producing resistant strain	resistant
Klebsiella pneumoniae subsp. *pneumoniae* (BAA-13883)	KPC-producing resistant strain	resistant
Escherichia coli (BAA-2340)	KPC-producing resistant strain	resistant
Escherichia coli C-3000 (BAA-15597)	Susceptible strain	susceptible
Enterotoxigenic *Escherichia coli* (BAA-35401)	Susceptible strain	susceptible
Vibrio cholerae (BAA-2163)	Susceptible strain	susceptible
Salmonella enterica serotype *Typhimurium*	Susceptible strain	susceptible
Salmonella enterica serovar *Enteritidis*	Susceptible strain	susceptible

Table 2: Bacterial strains examined for specificity test

Positive target	Negative non- target (non-NDM-1) producing bacteria	Negative non-target Susceptible bacteria
E. coli (BAA-2471)	*K. pneumoniae* (BAA-13883)	*E. coli* C3000
	E. coli (BAA-2340)	*E. coli* ETEC
		Salmonella (2)
		V. cholerae