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Visual and spectrophotometric detection of Pseudomonas aeruginosa based on gold nanoparticles probe biosensor and endonuclease enzyme

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ABSTRACT: Colorimetric DNA detection is preferable in comparison with the other methods used in clinical molecular diagnosis, because of no need to expensive equipment's. In present study, the colorimetric method based on gold nanoparticles was used for the detection of *P. aeruginosa*. Firstly the primers and probe for *P. aeruginosa* exotoxin A gene were designed. Then, GNPs were synthesized using the citrate reduction method and conjugated with the prepared probe to develop the new nanobiosensor. Next, the extracted DNA of the bacteria was added to GNPs-probe complex to check its efficacy for *P. aeruginosa* diagnosis. When, GNP-probe-target DNA was cleaved into the small fragments by Bam HI endonuclease, the right shift of absorbance peak from 530 to 562 nm. It was measured using a UV-Vis spectroscopy that indicates the existence of *P. aeruginosa*. The sensitivity was determined in the presence of target DNA. The results obtained from the optimized conditions showed that the absorbance value has linear correlation with concentration of target DNA (R: 0.9850) in the range of 10-50 ng mL^{-1} with the limit detection of 9.899 ng mL^{-1}. The specificity of the new method for detection of *P. aeruginosa* was shown in comparison with other bacteria.

Keywords: *Colorimetric assay, Endonuclease, Exotoxin A; Gold nanoparticles; Probe; Pseudomonas aeruginosa*

Abbreviations: *GNPs (Gold Nanoparticles); DTT (Dithiothreitol); ETA (Exotoxin A); LB-broth (Luria-Bertani broth); TEM (Transmission Electron Microscopy); DLS (Dynamic Light Scattering)*

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic microorganism that can cause fatal infections in a wide range of immunocompromised diseases including cancer, cystic fibrosis (CF), burns and etc (Wolf, *et al.*, 2009). This bacterium has a variety of virulence factors such as different types of proteases, exotoxins, polysaccharides, flagella and etc. (Wolf, *et al.*, 2009, Driscoll, *et al.*, 2007). *P. aeruginosa* is the second most common pathogen bacteria in surgery and the third common cause of nosocomial infections after E. coli and *Staphylococcus aureus*, which make up about 10 percent of hospital infections (Mendiratta, *et al.*, 2005). The natural and acquired resistance exhibited by this

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microorganism to a variety of antibiotics and consequent enhanced mortality has led researchers to seek new methods for rapid detection of bacteria in order to prevent infections caused by the *P. aeruginosa* (Wolf, *et al.*, 2009, Mendiratta, *et al.*, 2005, Adawi, *et al.*, 2012).

Many methods, including radioactive labeling, gel electrophoresis, high-performance liquid chromatography, electrochemical assay, immune-based assay, nanomaterial-based assay, enzymatic end point analysis, fluorescence sensors, DNA-based biosensors and colorimetric sensing method have been developed for the specific detection of *P. aeruginosa*. While some of these approaches made great contributions to the sensing of *P. aeruginosa*, most of them require expensive instrumentation techniques and complicated analysis procedures (Krishna, *et al.*, 2017). Of these methods, colorimetric sensing method has an advantage over the methods mentioned above. This method opens the scope for an easy operation, inexpensive, flexibility in design rapid and sensitive for detection of *P. aeruginosa* based on gold nanoparticles (Rui, *et al.*, 2016, Heidari, *et al.*, 2015).

GNPs have useful optical property that derives from their surface Plasmon resonance. The optical property is dependent on the size of particles or the distance between each particle. So the aggregation of GNPs could cause the change of their optical property (Wai, *et al.*, 2014). These unique optical properties have been widely applied in the field of chemical and biochemical sensing including metal ions, DNA detection, amino acids, antibodies, cancer cell imaging and photo thermal therapy (Heidari, *et al.*, 2015). Recently, Wai, *et al.*, managed to detect *Staphylococcus aureus* through this way (Jing, *et al.*, 2012, Venkatesan, *et al.*, 2011). In another study by Rui, *et al.*, E. coli species were detected in a short time and with high sensitivity (Rui, *et al.*, 2016). The key of the sensor is how to trigger the aggregation of the particles by the target DNA. The typical method is to design a GNP-probe to capture a target DNA, causing the aggregation of GNPs. However, the sensitivity of this assay is just around 20-30 nM (Bingjie, *et al.*, 2015). In addition, the detection sensitivity can be improved by the introduction of appropriate nucleic acid amplification strategies such as strand displacement amplification (SDA), nicking enzyme signal amplification (NESA), rolling circle amplification (RCA) and Endonucleases enzyme (Krishna, *et al.*, 2017).

In present study, we used endonucleases enzyme to increase sensitivity. Endonucleases are a branch of nucleases which recognize and cleave DNA sequences with high specificity. DNA cleavage by the nuclease is of significant importance in many biological processes, such as genotyping, mapping and colorimetric method. We employed a novel and sensitive method based on GNP-bound probe and endonuclease enzyme for detection of target DNA extracted from *P. aeruginosa*. In this method, GNPs are conjugated with probes. Then, the prepared biosensor is added to the target DNA. In the presence of target DNA, the coordinating groups amino (GC, AT) present in the target DNA interacts with probes on the surface of GNPs. After adding endonuclease which induces the cross linking of neighboring nanoparticles, the endonucleases leading to cleave of target DNA into the small fragment (Krishna, *et al.*, 2017). As a result of aggregation of GNPs the ensemble takes place that changes the color of the solution from red to violet or blue (Initially the color of synthesized GNPs are red), because one target DNA can yield around 1000 cleaved linker probes, the sensitivity increases in comparison with the conventional GNPs-based sensors. This sensing process was demonstrated using UV-Vis spectroscopy, transmission electron microscopy (TEM) and dynamic light scattering (DLS) analyses. The principle involved in detection of target DNA by the present assay has been illustrated in the schematic shown in Fig. 1.

EXPERIMENTAL

Materials

Hydrogen tetrochloroaurate trihydrate ($HAuCl_4.3H_2O$) and sodium citrate dehydrate were purchased from Sigma (St. Louis, MO, USA). Dithiothreitol (DTT), Ethyl acetate, NaCl and LB-broth were purchased from Merck, Germany. All oligonucleotides were synthesized by Bioneer, Korea. Marker 100 bp, dNTP, PCR buffer, $MgCl_2$ and Taq poly were purchased from SinaClon BioScience Co, Tehran, Iran. *P. aeruginosa* ATCC27853 (positive control) was purchased from

Institute Pasteur, Tehran, Iran. Other strains including *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysentery* and *Vibrio cholera bacteria* (negative controls) were purchased from patient hospital in Zanjan, Iran

Bacterial DNA Preparation

P. aeruginosa was grown on LB-broth for 12 h. Then, 5 ml of the bacteria culture was centrifuged at 3000 g for 5 min. Next, the chromosomal DNA was extracted using DNA purification kit (Roche Diagnostics GmbH, Mannheim, Germany High Pure PCR Template Preparation Kit). Genomic DNA was electrophoresed on % 0.8 agarose gel. The genomic DNA concentration was quantified at 260 nm using UV-Vis spectroscopy (Cary 4000, U.S) and was stored at -20°C.

Primers and probe designing

ETA gene was selected on bacteria chromosome as a target DNA for the detection of *P. aeruginosa* (GenBank, NCBI). ETA is a 1917 bp gene whose nucleotide sequence was extracted from gene bank database and one part of it was selected and designed using Oligo (Cinna Gen, version 3, Iran) Get Primers NCBI software (www.ncbi.nlm.nih.gov). Subsequently, the forward primer, reverse primer and the biosensor were synthesized by Bioneer Company, Korea. The sequences of upstream primer, downstream primer and probe were shown in Table 1.

PCR amplification

Primers designed for ETA gene were used for PCR amplification. PCR was carried out with a final volume of 50 µl, a mixture containing 5 µl 10X buffer, $Mgcl_2$ 1.5 mM, dNTP 0.2 mM, 1.0 U Taq polymerase enzyme, 2 µl of each primer (10 pm/µl) and 1 µl extracted bacterial DNA samples. This mixture was placed in a thermal cycler and was cycled with primary denaturation at 94°C for 5 min. Then 30 cycles with denaturation program at 94°C for 30s. Annealing primers to target DNA was performed at 60°C for 30s and the extension was conducted at 72°C for 30s and final incubation at 72°C for 5 min. In order to test the PCR product, 5 µl of it was transferred to %2 agarose gel for electrophoresis, and then it was stained with ethidium bromide. The PCR product was analyzed.

Synthesis of GNPs

Colloidal GNPs of 6-10 nm in diameter were synthesized using the citrate reduction method (Heidari, *et al.*, 2015, Amini, *et al.*, 2017). Typically, 100 ml of $HAuCl_4$ (1 mM) solution was heated to reflux with vigorous stirring, the plug was removed and then, 10 ml of sodium citrate 38.8 mM was added to mixture. The solution color changed from pale yellow to wine red within 1-2 min. The solution was heated under reflux for another 20 min. Then, the heating source was removed and the solution was continuously stirred until it cooled to room temperature (25±2°C). The resultant GNPs colloids were stored at 4°C. The morphology and the size of the synthesized GNPs were studied by transmission electron microscopy (TEM) and dynamic light scattering (DLS). The sample was diluted in absolute ethanol (1:10) and one drop of the diluted suspension was slowly evaporated on a 300 mesh carbon membrane-coated copper grid. TEM experiment was performed on a Philips CM208 Biotwin microscope operated at 80 kV. The size distribution of the GNPs was studied by dynamic light scattering measurement using Malvern Zeta sizer.

Conjugation of GNPs with the probe

The thiol group of probes was unable to spontaneously form a layer on the surface of the nanoparticles. Therefore, in order to solve the problem, the oligonucleotides (probes, 20 µl, 100 pm/µl) were dissolved in 100 µl dithiothreitol solution (DTT 0.1 M, PBS 170 mM, pH 7.5) for 2 h at room temperature until the di-

Table1. Sequence of synthesized probe and primers

Primers and probe	Sequence
Primer forward	5′TGCTGCACTACTCCATGGTC3′
Primer reverse	5′ATCGGTACCAGCCAGTTCAG3′
Probe	5′SHCAACGACGCACTCAAGCTG3′

sulfide bond was broken. Then, to remove DTT solution and extract recovered probes, 200 µl ethyl acetate was added to the probes. After stirring for 2 min, the upper phase liquid (organic) was separated from the lower phase liquid (water) by a sampler. This was repeated 3 times until the DTT was dissolved in organic phase and it was removed. The probe concentration was determined by Nano drop UV-Vis spectrophotometer after being extracted. The GNPs (2000 µl) and the purified thiolate DNA probe (2 µM) was then mixed together and incubated at 40°C for 2 h. Thiolate probes would form a self-assembled monolayer on the surface of GNPs (Amini, *et al.*, 2017). Fig. 1 shows the schematic of GNPs functionalization.

Detection of target DNA

Hybridization of GNPs-probe with the target DNA was conducted on the basis of method described by Amini, *et al.*, (Amini, *et al.*, 2017). In summary, after the target DNA denaturation (50 ng mL^{-1}, 50 µl) at the temperature of 95◦C for 5 min and putting it on ice for 5 min, the prepared single strand DNA was added to GNPs-probe (200 µl, 10 mM). The mixture was incubated at the temperature of 45°C for 30 min and well mixed followed by the addition of 2.5 µl of Bam HI enzyme at 37°C for 10 min. After adding NaCl (0.1 M), the absorption change of solution was measured by UV-Vis spectroscopy from 500 to 600 nm wave length. It should be noted that all experiments were performed at room temperature and all measurements were done three times with triplicate samples. The overall strategy of the new procedure method was presented in Fig. 1 (Jung, *et al.*, 2010).

Sensitivity

The sensitivity of hybridization of GNPs-bound-probe with the target DNA was monitored by different concentrations of *P. aeruginosa* DNA. *P. aeruginosa* target DNA was serially diluted of 1, 10, 12.5, 15, 20, 25, 35, 50, 65, 80 and 100 ng mL^{-1} on the basis of method described at section 2.7. After adding Bam HI enzyme and NaCl to each dilution, the absorption rate of change was measured using a UV-Vis spectroscopy.

Specificity

To determine the specificity of designed probe to detect the target DNA (ETA gene), the bacteria *Staphylococcus aureus*, Escherichia coli, Shigella dysentery and Vibrio cholera were used as the negative controls. After extraction of DNA molecules from all the men-

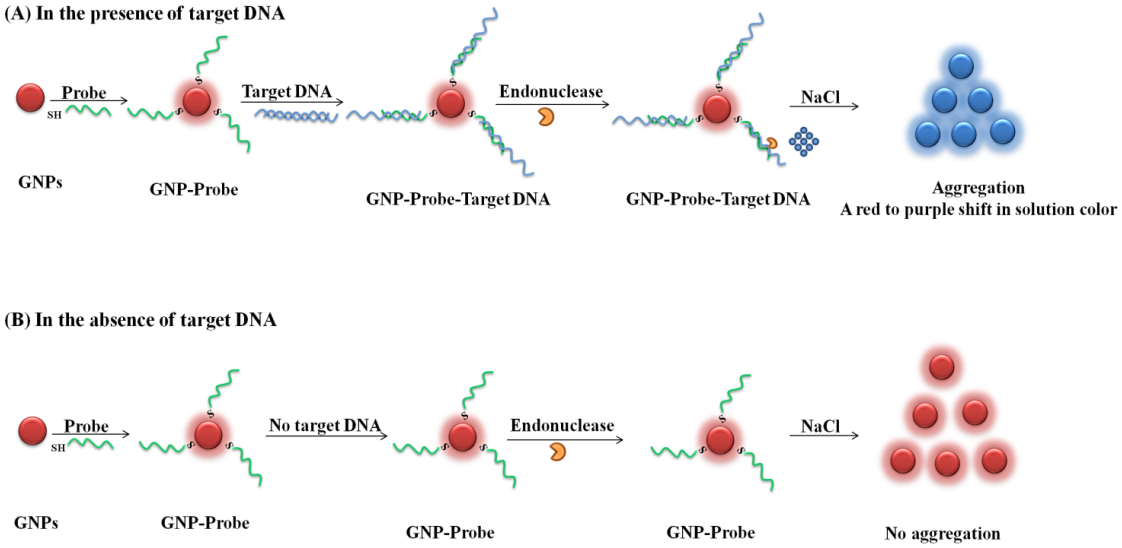

Fig. 1. Schematic representation of the colorimetric detection of target DNA based on GNP-probe hybridization and enzyme controlled cleavage and aggregation of GNPs upon the addition of NaCl. (A) In the presence of target DNA, GNP-DNA probe-target DNA hybridization occurs which initiates Bam HI enzyme cleavage of target DNA structure. The target DNA recycles until the entire DNA is cleaved, allowing for subsequent GNPs aggregation in the presence of NaCl and observable color change from red to violet. (B) In the absence of target DNA, no hybridization occurs, thus there is no cleavage site DNA-probe structure to act on and the Bam HI enzyme is inactive. Therefore the particles remain stable upon the addition of NaCl.

tioned bacteria, their DNA bacterial (100 ng mL^{-1}) were hybridized with designed GNPs-probe on the basis of method described at section 2.7 and analyzed using a UV-Vis spectroscopy.

RESULTS AND DISCUSSION

Primers and probe designing

In this study, we designed primers from the N-terminal gene because this area was protected compared to other areas (between nucleotides 200-389 of the ETA gene). The PCR result showed that that the designed primers have 100% specificity for the detection of *P. aeruginosa*. Nucleic acid amplification assay for detecting the ETA gene of *P. aeruginosa* was first described by Deschaght, *et al.*, (Deschaght, *et al.*, 2011). In our study, DNA was directly detected in the standard sample obtained from Institute Pasteur, Tehran, Iran. For this purpose, a homology search with the BLAST software with the sequences currently available in the NCBI database (http://blast.ncbi.nlm.nih) showed that the ETA gene sequence between the nucleotides 200-389 (190 bp) was similar to that found in the pseudomonads (> 99%). In the primers region, the sequence was 100% identical in all of the Pseudomonas species in the gene bank. Homology analysis indicated that the PCR assay targeting the ETA gene sequence between the forward and reverse primers may not be suitable for detecting *P. aeruginosa* DNA. Unlike the ETA gene sequence present between the forward and revers primers, the ETA gene sequence from nucleotides 200 to 389 is not similar to that found in other pseudomonads. These results strongly suggested that the region from nucleotides 200 to 389 is the best target for designing the PCR primers for detection of *P. aeruginosa*.

PCR amplification

In general, PCR amplification performed for standard sample by upstream and downstream primers. The results showed that the ETA gene was present in the bacteria (a standard sample) by PCR method. Amplified products (5 µl) were analyzed based on appoint of their sizes using 2% agarose gel electrophoresis in TBE buffer. A 100 bp size marker (Fermentas, Germany) was used as a molecular size standard, stained with ethidium bromide, and visualized under a Bio-Rad UV Trans illuminator (Hercules, CA, U.S). The results showed a fragment of 190 bp (lane 1, 3) of ETA gene *P. aeruginosa* in the standard sample (Fig. 2A).

Characterization of the GNPs

The TEM and DLS experiment was performed to study the morphology and size distribution of the produced GNPs and understand how the aggregation process occurs with increasing concentration of target DNA. Fig. 2B-E shows the TEM and DLS images of the GNPs before and after probe-conjugated GNPs. It is clear from the TEM and DLS images that there are no aggregated. GNPs in the absence of target DNA (Fig. 2B), have a well-defined spherical shape and an average dimension of 6-12 nm (Fig. 2B and D). It was also obvious from Fig. 2 that there is a significant increase in the amount and size of aggregated GNPs with the increase of target DNA. Upon probe-conjugation, the average diameter of probe-conjugated GNPs of 15-20 nm was found (Fig. 2C and E). The increase of 7-10 nm is consistent with the theoretical full layer coating with probe as the hydrodynamic diameter of a probe is approximately 6-8 nm.

Probe-conjugated GNPs

GNPs were prepared using the citrate reduction method which produces negatively charged nanoparticles due to the citrate coating on their surfaces. This negative charge prevents their aggregation and a red color is obtained. We have conjugated the GNPs surface with ETA probe, which has high specificity to ETA gene. The basis of detection is in the presence of ETA. ETA conjugated with GNPs-probes undergoes aggregation due to the specific recognition between N-terminal gene of ETA and probe. This binding will result in a network of aggregated nanoparticles, causing a significant change in color that can be observed visually or can be demonstrated by UV-Vis spectroscopy. As shown in Fig. 3, curve A, the GNPs solution exhibited a band around 520 nm. The conjugation of GNPs with probes does not change the color of GNPs (Fig. 3, curve B) and does not cause aggregation, which is confirmed by absorption spectra. Although ssDNA probes adsorb on GNPs and prevent their aggregation,

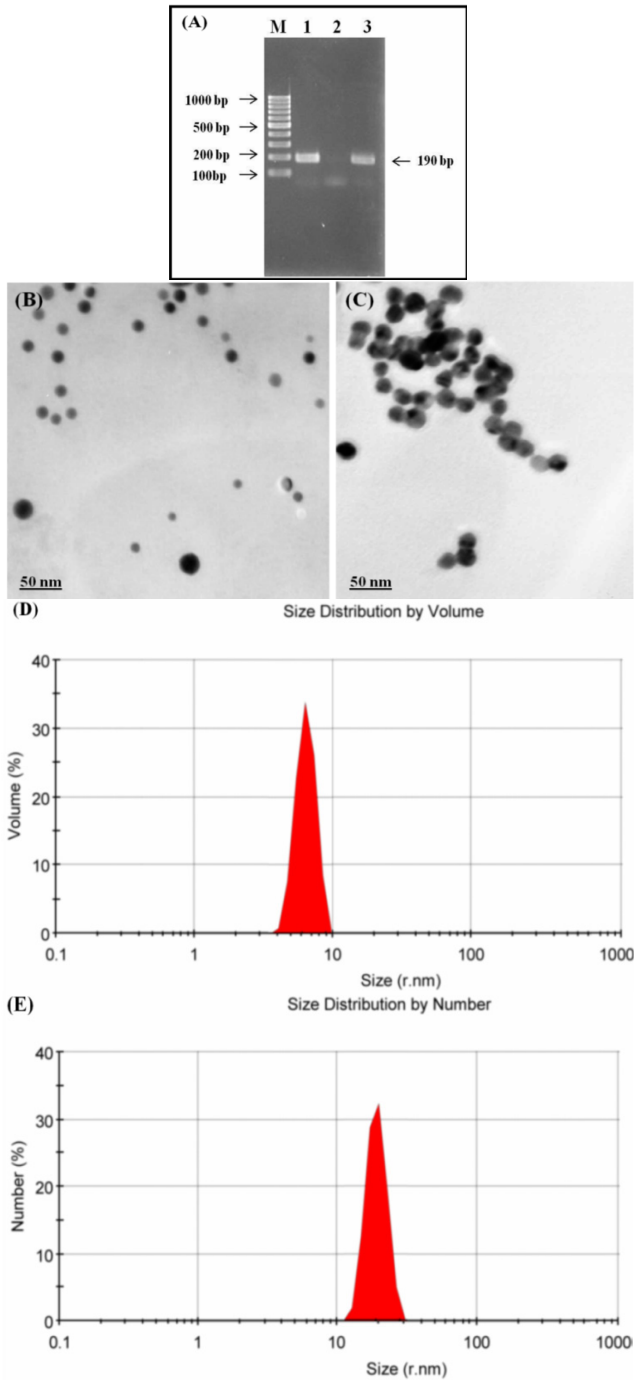

Fig. 2. Agarose gel electrophoresis photos (A), 100 bp size marker (M), PCR products of 190 bp strain of P. aeruginosa ATCC27853 standard (1), negative control (H_2O) (2), PCR products of 190 bp strain of P. aeruginosa ATCC27853 standard (3), TEM micrograph of synthesized GNPs and GNP-probe. Mon dispersed GNPs (B), aggregated GNP-probe in the presence of P. aeruginosa target DNA (B). Analysis of the size distribution of the prepared GNPs measured by dynamic light scattering (DLS), analysis of GNPs size distribution before conjugation with probe (D), analysis of GNPs size distribution after conjugation with probe and in the presence of P. aeruginosa target DNA (E).

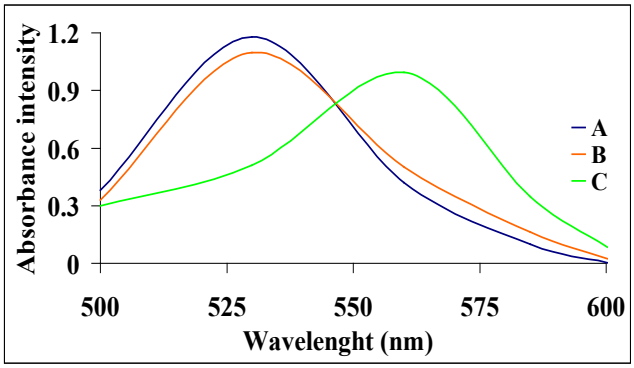

Fig. 3. UV-Vis spectra show the absorption peak of GNPs (None aggregated GNPs: blue) at 520 nm (Curve A), probe-conjugated GNPs (None aggregated GNPs: red) at 525 nm (Curve B), the GNPs-probe-target DNA complex (aggregated GNPs in present of target DNA: green) at 562 nm (Curve C).

the concentration of the probes should be optimized. This is because, in the absence of the target, a very low probe concentration will not be sufficient to prevent aggregation that is leading to a false positive result. On the other hand, in the presence of the target, a very high probe concentration will prevent aggregation that is leading to a false negative result. In this study, at a final Bam HI concentration of 2.5 units, a probe concentration less than 0.1 µM was unable to prevent aggregation of GNPs in the absence of the target DNA. On the other hand, a final probe concentration more than 5 µM was too high for any aggregation to occur in the presence of the target DNA. Consequently, the optimal probe concentration was found to be 1 µM in the total assay volume.

Sensitivity

To perform a concentration-dependent of target DNA detection, the different concentrations of target DNA between 1-100 ng mL^{-1} was added to the probe-conjugated GNP. As the concentration of target DNA increased, the color of the probe-conjugated GNP gradually changed from red to violet (Fig. 4A). The color changing was due to the aggregation of probe-conjugated GNP upon binding with target DNA attributed to strong affinity between probe and target DNA. As illustrated in Fig. 4, the colorimetric assay was sensitive enough to visually detect as low as 12.5 ng mL^{-1} of target DNA, caused by a decrease in interparticle distance, in the present of endonuclease enzyme. The

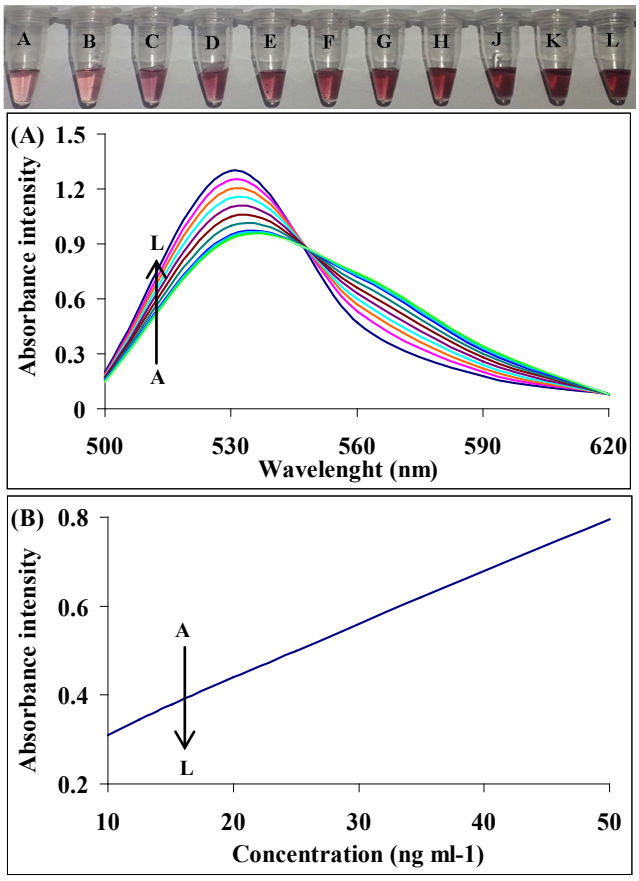

Fig. 4. (A) UV-Vis spectra and calibration curve of P. aeruginosa. A600/A520 ratio and hybridization solution at different concentration of target DNA (from A to L: 1, 10, 12.5, 15, 20, 25, 35, 50, 65, 80 and 100 ng mL^{-1}) in the presence of the target DNA and endonuclease enzyme. Insert image: visual detection and determining sensitivity of hybridization of GNP-probe with target DNA from P. aeruginosa by testing different concentrations of target DNA. (B) The calibration curve showing the relationships between different concentrations of target DNA, absorbance intensity.

concurrent change in the plasmon band of nanoparticle was also monitored by UV-Vis spectroscopy (Fig. 4A). As shown in Fig. 4A, with increasing concentration of target DNA, the absorbance at plasmon band 525 nm decreased and the absorbance at plasmon band 562 nm increased. In addition, under optimal conditions, the corresponding absorbance ratio (A600/A525) versus the concentration of target DNA was evaluated for quantitative analysis. A linear relationship was found between the absorbance ratio and target DNA of over the range of 10-50 ng mL^{-1} (Fig. 4B). Further increasing of the concentration above 50 ng mL^{-1} kept the absorption ratio almost unchanged. This could be explained as the ratio of plasmon band changing become insignificant with the formation of large amounts of aggregates for higher concentrations of target DNA. Fig. 4B illustrates the linear regression curve which shows the relationship between absorbance and different concentrations of target DNA. DNA concentrations from 10 to 50 ng mL^{-1} were within the linear range with R^2= 0.9850. When we performed these experiments, in target DNA concentrations below 10 ng mL^{-1} there was not detected any visible color changing (Fig. 4A), but Nano drop analysis indicated a change in the absorbance spectrum. We detected the target DNA as low as 10 ng mL^{-1} (9.899 ng mL^{-1}) and 12.5 ng mL^{-1} with visual detection of the color changing and UV-Vis spectroscopy, respectively which was consistent with the results obtained by other researchers (Chiang, *et al.*, 2012, Lavenir, *et al.*, 2007, Junillon, *et al.*, 2014, Veigas, *et al.*, 2010, Adikaram, *et al.*, 2014).

Detection range and limit of detection

Limit of Blank (LOB), Limit of Detection (LOD), and Limit of Quantitation (LOQ) are terms used to describe the smallest concentration of a measuring that can be reliably measured by an analytical procedure (David, *et al.*, 2008). In our method, we took advantage of definitions and equations provided in the guideline EP17 published by Clinical and Laboratory Standards Institute (CLSI) to calculate the values of LOB, LOD and LOQ. To determine these parameters two major methods are used: statistical and experimental. To perform experimental method a series of samples containing increasingly lower concentrations of target DNA were analyzed (Table 2).

LOB was estimated by measuring replicates of a

Table 2. Different concentrations of target DNA for determining LOB.

Number of samples	Target concentration (ng mL^{-1})
1	7.5
2	5
3	1
4	0.1
5	0
Total	13.6
Mean	2.72
SD (standard deviation)	3.364

blank sample and calculating the mean result and the standard deviation (SD). LOD was determined by utilizing both the measured LOB and test replicates of a sample known to contain a low concentration of target DNA. The mean and SD of the low concentration sample was then calculated according to:

LOB= mean blank + 1.645 (SD blank) → LOB = 2.72 + 1.645 (3.364) → LOB = 8.254 ng mL^{-1}.

LOD= LOB + 1.645 (SD low concentration sample) → LOD= 8.254 + 1.645 → LOD= 9.899 ng mL^{-1}.

To compare the values of LOD and LOQ, we also used statistical method to calculate LOD and LOQ. In this approach, LOD is the mean blank value plus 3SDs and LOQ is the mean blank value plus 10 SDs.

LOD = 3 (SD blank) → LOD = 3 (3.364) → LOD = 10.092 ng mL^{-1}.

LOQ = 10 (SD blank) → LOQ = 10 (3.364) → LOQ = 33.64 ng mL^{-1}.

Specificity

To determine the specificity of our detection approach, we evaluated the response of the assay against other bacteria including *Staphylococcus aureus*, Escherichia coli, Shigella dysentery and Vibrio cholera. As shown in Fig. 5, there was no significant change in the color of probe-conjugated GNPs in presence of other bacteria (Fig. 5, columns B, C, D and E). To study the specificity further, the ratio of extinction intensity between aggregated nanoparticle (562 nm) and dispersed nanoparticle (520 nm) was plotted in Fig. 5. As expected the extinction intensity ratio was significantly higher for ETA gene (Fig. 5, column A) than the other competing DNA. These showed excellent specificity of the assay for ETA gene over other bacteria.

Our assay proved to be rapid, highly specific, cost-effective and time-saving with potential for early detection of *P. aeruginosa* via the ETA gene and other pathogens. The GNPs-based colorimetric method has the capacity to be automated as chips. This widely extends the applications of GNPs in the detection of a great variety of pathogenic microorganisms including

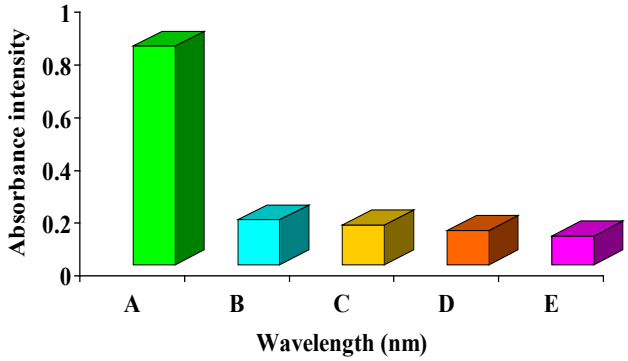

Fig. 5. The absorbance spectrum obtained after adding GNPs-probe to DNA isolated from bacterial strains selected as positive and negative control including P. aeruginosa (column A), Staphylococcus aureus (column B), Escherichia coli (column C), Shigella dysentery (column D) and Vibrio cholera (column E). Adding GNPs-bound probe to DNA of negative control bacteria did not lead to any alteration in absorbance spectrum for bacterial DNA (100 ng mL^{-1}).

bacteria, viruses and fungi (Ma, *et al.*, 2014, Wang, *et al.*, 2015). Moreover, the procedure can be promising due to the properties such as high sensitivity and selectivity for target DNA, fast response time, the elimination or simplification of sample preparation steps and the ability to obtain measurements with minimal perturbation of the sample (Fu, *et al.*, 2013).

Detection of P. aeruginosa by real samples

To evaluate the feasibility of established colorimetric sensor for detection of actual *P. aeruginosa*, the absorbance values of different concentrations *P. aeruginosa* were measured. The cultivate bacteria was processed as described steps by extracting genomic DNA with DNA purification kit (Roche Diagnostics GmbH, Mannheim, Germany High Pure PCR Template Preparation Kit) and the PCR was performed using the extracted target DNA. Then the PCR analysis was performed by both colorimetric biosensor and agarose gel electrophoresis. The amplification of a 190 bp fragment of ETA gene was successfully achieved, which could be verified by 2% agarose gel electrophoresis (Fig. 6A). The colorimetric-PCR assay can be applied to analyze the denatured PCR products for *P. aeruginosa* from 2.8×10^3 to 2.8×10^8 CFU mL^{-1}. The absorbance values to different PCR samples were shown in Fig. 6B. However, no target band could be observed

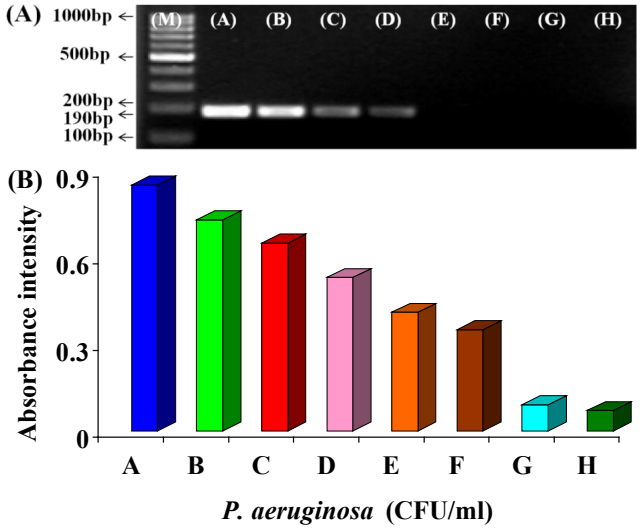

Fig. 6. Comparison of the sensitivities of the PCR and colorimetry assays detection of *P. aeruginosa* by real samples. (A) Gel electrophoresis photos of 100 bp size maker (M) and PCR products of 2.8×10^{8} (A), 2.8×10^{7} (B), 2.8×10^{6} (C), 2.8×10^{5} (D), 2.8×10^{4} (E), 2.8×10^{3} (F), 2.8×10^{2} (G) and 2.8×10^{1} (H) CFU mL^{-1} *P. aeruginosa*. (B) Typical OD curves of colorimetric assay with target DNA obtained from serial dilutions of P. aeruginosa in the range of 2.8×10^{8} (A), 2.8×10^{7} (B), 2.8×10^{6} (C), 2.8×10^{5} (D), 2.8×10^{4} (E), 2.8×10^{3} (F), 2.8×10^{2} (G) and 2.8×10^{1} CFU mL^{-1} (H).

in PCR products when the concentration was below 2.8×10^{5} CFU mL^{-1}. This is attributed to the low EtBr straining efficiency for ssDNA. On the contrary, the established colorimetric sensor could effectively detect *P. aeruginosa* as low as 2.8×10^{3} CFU mL^{-1}, which was much lower than other methods reported previously for the detection of *P. aeruginosa* (Veigas, *et al.*, 2010, Adikaram, *et al.*, 2014). Thus, the designed colorimetric assay is simple, fast and sensitive, which can be regarded as a valid alternative to conventional assays for the detection of *P. aeruginosa*.

CONCLUSIONS

In this study, it has been demonstrated that the GNPs/probe/target DNA on the basis of endonuclease enzyme, with their high capture capacity, can be employed as a highly sensitive and specificity tool for the sensing of *P. aeruginosa*. We detected *P. aeruginosa* target DNA with the limit detection of 9.899 and 12.5 ng mL^{-1} visually and UV-Vis spectroscopy, respectively. This new diagnostic method may be useful in molecular diagnosis of infectious diseases and offer great potentials for the development of hand-held DNA diagnostic devices that can be used to detect pathogenic microorganisms at point-of-care.

Diagn Microbiol Infect Dis

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