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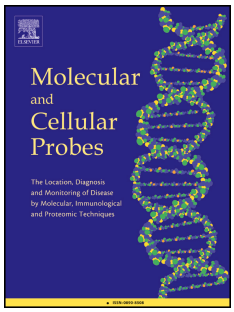

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Reza Shahbazi, Mojtaba Salouti, Bahram Amini, Ahmad Jalilvand, Ebrahim Naderlou, Ali Amini, Arash Shams

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Highly Selective and Sensitive Detection of *Staphylococcus aureus* with Gold Nanoparticle-based Core-shell Nano Biosensor

Reza Shahbazi [a], Mojtaba Salouti *[a], Bahram Amini **[b], Ahmad Jalilvand [c], Ebrahim Naderlou [a], Ali Amini [b], Arash Shams [a]

a) Department of Microbiology, Faculty of Sciences, Zanjan Branch, Islamic Azad University, Zanjan, Iran.

b) Department of Biology, Faculty of Science, University of Zanjan, Zanjan, Iran

c) University of Medical Sciences and Health Services, Mousavi Teaching Hospital, Gavazang road, Zanjan, Iran.

***1st Corresponding author:** saloutim@yahoo.com, Tel/fax: +98 24 33460056.

****2nd Corresponding author:** bamini50@yahoo.com, Tel/fax: +98 21 82482562.

Abstract

Staphylococcus aureus is a gram-positive and opportunistic pathogen that is one of the most common causes of nosocomial infections; therefore, its rapid diagnosis is important and valuable. Today, the use of nanoparticles is expanding due to their unique properties. The purpose of the present study is the determination of *S. aureus* by a colorimetric method based on gold nanoparticles (AuNPs). Firstly, *S. aureus* was cultured on both LB media (broth and agar) and their chromosomal DNA was extracted. Afterwards, primers and biosensors were designed based on Protein A sequence data in the gene bank. PCR assay was performed under optimal conditions and the PCR product was electrophoresed on 2-percent agarose gel. The synthesized biosensors were conjugated with AuNPs and, eventually, a single-stranded genome was added to the conjugated AuNPs and hybridization was performed. The results were evaluated based on color change detected by the eye, optical spectrophotometry, and transient electron microscopy. Finally, the sensitivity and specificity of the biosensor were determined. The results of the present study showed a 390 bp band on the agarose electrophoresis gel, which confirmed the presence of Protein A genes on the chromosome of the bacteria. The PCR and colorimetric methods were compared with each other. The sensitivity of the PCR and colorimetric methods were 10 ng μL^{-1} and 30 ng μL^{-1}, respectively. The limit of detection (LOD) equaling 8.73 ng μL^{-1} was determined and the specificity of the method was confirmed by the DNA of other bacteria. According to the results, the present method is rapid and sensitive in detecting *S. aureus*.

Keywords: *S. aureus*; Colorimetry; AuNPs; Protein A gene.

Abbreviation: *Staphylococcus aureus*; *S. aureus*, gold nanoparticles; AuNPs, limit of detection; LOD, Transient Electron Microscopy; TEM, Dynamic Light Scattering; DLS, Dithiothreitol; DTT.

1. Introduction

S. aureus is one of the most common human pathogens which, due to its high pathogenicity and resistance to most antibiotics, cause many acquired infections; this has made it one of the major challenges for the WHO [1-2]. The virulence factors of *S. aureus* include hemolysis, oxytocin, enterotoxin, and toxic shock syndrome (TSST-1) [3–4]. In addition, the cell wall of the bacterium has Protein A; a large portion of this is located on the cell surface and is connected to peptidoglycan with covalent bonds. This protein has a molecular weight of 42,000 Da, with 5 similar Ig extracellular strands, which consist of A, B, C, D, and E, respectively. The presence of two active and distinct Igs makes Protein A secrete and membranous. Each of the chains can be connected to Fc and Fab. Protein A has multiple biological effects that increase pathogenic activity by stimulating lymphocyte production, severe allergic reactions, preventing phagocytosis as well as activating complement and cytotoxicity [5]. There are several methods for the identification and diagnosis of *S. aureus*, including the characteristics of the bacterial colony on blood agar, Mannitol salt agar, Muller Hinton agar. However, these methods usually require several days to identify the bacteria which are a long-term process [6]. The latex agglutination (LA) method is not directly capable of diagnosis and requires the isolation of a suspected colony by culture [7-8]. In recent years, genetic-identification methods, especially using the technique of polymerase chain reaction (PCR) allows significant expansion, quicker identification ability, and more accuracy than the old method [9−10]. The analysis of PCR results by electrophoresis are needed for equipment, materials, and a long duration of time because of the presence of PCR inhibitors; in this method, sometimes, the result can be falsely negative. In advanced PCR models, such as real-time PCR, resonant fluorescence energy transmissions are utilized that accelerate the detection process providing probes, however, is still expensive [11- 12].

Nanoparticles have properties such as very small sizes, surface-to-volume ratios, and unique physical and chemical characteristics, which large-scale materials lack. In fact, the size of particle makes it a possibility to overcome the quantum force. This also affects the particle's properties during its reaction to other particles [13–17]. Gold nanoparticles (AuNPs) exhibit different physical and chemical properties when they reach the nanoscale; there are significant changes in the color of the nanoparticles that depending on the size of the nanoscale [18–23]. The principles of the AuNPs colorimetric method are that if the genomic DNA is present in the environment, the probes are complemented by them. Then, the addition of salt causes the accumulation of AuNPs and, as a result, changes the color of environment. In the absence of genomic DNA, the probes will remain with the AuNPs in the solution without any reaction; thus, no color change occurs. The purpose of present study was to set up a new method based on nanobiotechnology to detect *S. aureus* by using the AuNPs colorimetric method.

2. Materials and Methods

2.1. Materials

The standard strains of *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. dysentery* PTCC 1188, *K. pneumoniae* ATCC7881 and *S. epidermidis* ATCC 12228 were prepared from Pasteur Institute, Tehran, Iran. Hydrogen tetrochloroaurate (III) trihydrate (HAuCl4· 3H2O) and sodium citrate dehydrate were purchased from Sigma (St. Louis, MO, USA). Taq DNA polymerase, dNTP, PCR Buffer, 100 bp DNA size marker, $MgCl_2$, ethidium bromide, and TBE buffer were purchased from the Sina Clone Company, Iran. All the oligonucleotides (probe and primers) were synthesized by Bioneer (Korea).

2.2. Culturing Bacteria

At first, the bacteria were cultured in LB-Agar for 18–24 h at 37°C. Then, one of the colonies was cultured in LB-broth for 18 h at 37°C. The obtained colonies were examined by the biochemistry test as well as macroscopic and microscopic methods. In order to ensure that no contamination arose from the culture medium, gram-staining was prepared.

2.3. DNA Extraction

A bacterial colony was cultured in the LB-Broth for 18 h at 37°C. The genomic DNA was extracted by the genome extraction kit (Pouya Gen Azema Company, Iran). The DNA extracted was electrophoresed on 0.8-percent agarose gel, and then, the concentration of the DNA extracted was quantitatively investigated by using the UV-VIS Array Spectrophotometer Teif Sanj Pishro Pajohesh (Laboratory Industrial & Research System, Iran) and was stored at –20°C.

2.4. Designing the Primers and the Probe

The conservative regain sequence of Protein A gene (accession numbers X61307.1 and BX571856.1) was obtained from the gene bank database (NCBI). The probes as well as the forward and reverse primers were designed by using the DNasis and Oligo (Cinna Gen, version 3, Iran) Get Primers NCBI software (www.ncbi.nlm.nih.gov). The oligonucleotide probe and the primers were synthesized by Bioneer (Korea; it is described in Table 1). The specificity of the nucleotide sequence of the probe and primers for the detection of the Protein A gene was verified and confirmed by the Blast Online Software (www.Pubmed.Com/blast).

Table 1

2.5. PCR Amplification

The presence of the Protein A gene on the chromosomal DNA was determined by using PCR assay. To achieve this purpose, PCR assay was performed in a volume of 50 μL, which contains PCR Buffer (10 X), $MgCl_2$ (1.5 mM), dNTP (0.2 mM), forward and reverse primers (each one 0.4 mM, 10 pm μL^{-1}), a DNA template (50 ng μL^{-1}), and Taq DNA polymerase (2.5 U). PCR assay was initiated with the initial denaturation program at 95°C for 5 min in 30 cycles; secondary denaturation was carried out at 94°C for 30 seconds; annealing the primers to the target DNA was carried out at 54°C for 30 seconds; the extension temperature was set at 72°C for 30 seconds; and the final incubation temperature was 72°C for 5 min. The PCR product was electrophoresed on 2-percent agarose gel.

2.6. Synthesis of AuNPs

Colloidal AuNPs, 6–10 nm in diameter, were synthesized by using the citrate-reduction method [12, 19]. Typically, 100 mL of a $HAuCl_4$ (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 the mixture. The solution color changed from pale yellow to wine red within 1–2 min. The solution was then heated under reflux for another 20 min. The heat source was thereafter removed and the solution was continuously stirred until it cooled to room temperature (25 ± 2°C). The resultant AuNPs colloids were stored at 4°C.

2.7. Characterization of AuNPs

After the sonication of nanoparticles by using an ultrasonic homogenizer (Development of Ultrasonic Technology, Iran), the morphology and size of the synthesized AuNPs 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. The TEM experiment was performed on a Philips CM208 microscope that was operated at 80 kV. The size distribution of the AuNPs was studied by dynamic light scattering measurement by using a Malvern DLS. The absorption spectra of the AuNPs were measured by the UV-VIS Array Spectrophotometer Teif Sanj Pishro Pajohesh (Laboratory Industrial & Research System, Iran).

2.8. Conjugation of Probes

For the conjugation of the probe with AuNPs, at first, the thiol probe should be activated. Thus, 15 μL (1 μM) of the probe was dissolved in 100 μL DTT (Dithiothreitol solution) for 1 h. To remove DTT, 200 μL of ethyl acetate was added; after mixing for 5 min and centrifuging at 12000 g for 10 min, the organic phase of the aqueous phase was separated by a sampler. This process was repeated three times to

completely remove DTT. Then, purification probes were added to the AuNPs solution and it was incubated at 45°C for 2 h. The thiol groups in the probes were bound to the surface of the AuNPs [19, 24].

2.9. Colorimetric Assay

For colorimetric assay, standard samples were prepared from Pasteur Institute, Tehran, Iran and clinical samples were isolated of stool and urine samples of Mousavi Hospital, Zanjan, Iran. Then, the target DNA was extracted by Kit. For the hybridization of the bacterial genome with probe-conjugated AuNPs, first, the bacterial genome (100 ng μL^{-1}) was incubated into the micro-incubator at 95°C for 5 min to convert the double strand genome (dsDNA) into a single-stranded genome (ssDNA). Eventually, the ssDNA was added to 50 µL of the AuNP-probe. The hybridization was performed at 45°C for 10-15 min. On completion of this step, 10 µL (0.1 M) of NaCl solution was added to the mixture reaction and the color change of the environment was investigated through the naked eye and UV-VIS spectrophotometry [Fig.1].

Fig.1

2.10. Sensitivity

To determine the sensitivity of the colorimetry method, different concentrations of the genomes of *S. aureus* (1, 5, 10, 15, 20, 40, 60, 80, and 100 ng μL^{-1}) isolated of standard sample and clinical samples were used. The results were measured and compared with the naked eye, UV-VIS spectrophotometry, and PCR methods.

2.11. Specificity

In order to determine the specificity, the target DNA of *S. aureus* ATCC 25923, S. aureus ATCC 29213, *E.coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. dysentery* PTCC 1188, *K. pneumoniae* ATCC7881 and *S. epidermidis* ATCC 12228 were extracted by using a genome DNA extraction kit (Pouya Gen Azema Company, Iran). Then, 60 ng μL^{-1} of each one was added to the AuNPs and the result was examined by the naked eye and UV-VIS spectrophotometry.

3. Result and Discussion

3.1. PCR Amplification

In the present study, in the *S. aureus* standard strain, small and clear colonies were observed on the LB agar medium. Furthermore, gram-staining of the colonies' cluster-like cocci in violet color, confirmed the characteristics of *S. aureus*. Amplified products (5 µL) were analyzed based on their sizes by using 2-percent agarose gel electrophoresis in the TBE buffer. A 100 bp size marker (Fermentas, Germany) was

used as a molecular size standard, which was stained with ethidium bromide, and visualized under a Bio-Rad UV Trans illuminator (Hercules, CA, USA). The results showed a fragment of 390 bp (lane 1, 2) of the *S. aureus* Protein A gene (Fig. 2).

Fig. 2

3.2. Characterization of AuNPs

3.2.1. UV-VIS Spectrophotometry

The absorbance ratio of the AuNPs was measured by UV-VIS spectrophotometry. The results of these studies are shown in Fig. 3. The synthesized AuNPs had a maximum absorbance wavelength of 520 nm and were deep-red in color. The conjugating and binding of the AuNPs with the probe, the target DNA and NaCl of the bacteria were investigated. The results showed that by connecting the probe and the target DNA of the bacterium to the AuNPs, the absorbance wavelength changed from 520 to 523 nm and 560 nm, respectively. Furthermore, the color of the AuNPs changed from red to blue. Finally, the optical absorption rate of AuNPs-probe DNA-target DNA complex at 520 nm was reduced. However, the optical absorption was increased at 560 nm wavelength that as shown in Fig. 3.

Fig. 3

3.2.2. TEM and DLS

The structure of the AuNPs was measured by using TEM and DLS. The size and potential of the synthesized AuNPs was 15 nm and −28 mV as shown in Figs. 4A and 4B. Using TEM, the positive control reaction specimens (containing the target DNA of the bacterial and AuNPs probe) and a negative control reaction (no target DNA of bacterial and AuNPs-probe) were investigated. The accumulation, change of size, and potential of AuNPs indicates the bond between the AuNPs of the probes and the target DNA of the bacteria. In the present study, the size and potential of AuNPs, the AuNPs-probe, and the AuNPs-probe-target DNA were changed from 15 nm and −28 mV to 18 nm and −20 mV 23 nm and −12 mV, respectively, as shown in Figs. 4C, D and 4E, F. The lack of accumulation of AuNPs in the negative control specimens (lacking the target DNA bacterial) indicates the absence of target DNA bacterial.

Fig. 4

3.3. Sensitivity

In order to determine the sensitivity of the colorimetric method, different concentrations of *S. aureus* target DNA (1, 5, 10, 15, 20, 40, 60, 80, and 100 ng μL^{-1}) were added to the 47.5 µl of AuNPs,

respectively. After adding 0.1 M NaCl, the color of the AuNPs were changed from red to blue for 5 min at a concentration above 10 ng mL of the bacterial target DNA. In lower concentrations of the bacterial target DNA, no change in the color appears, as shown in Fig. 5A. The results demonstrated the good linearity relationship between the absorbance and concentration of the bacteria target DNA from 5 to 40 ng μL^{-1}. This is shown in Fig. 5B and can be expressed as Y = 0. 647X + 0.6197, where R^2 = 0.9959. The established method for bacteria detection had a broad linear range of 5– 40 ng μL^{-1}, with a detection limit of 8.73 ng μL^{-1} for colorimetric assay, as estimated from the derived calibration curve (Fig. 5B). Finally, in different concentrations of the target DNA, the sensitivity of the PCR method was investigated, and the results of both methods were compared with each other. The sensitivity of colorimetric assay with eye naked, UV-VIS spectrophotometry and the PCR method were determined as 8.73 ng μL^{-1}, 4.3 ng μL^{-1} and 30 ng μL^{-1}, respectively. Therefore, the colorimetric assay was about 3.45 times more sensitive than the PCR method (Fig. 5C).

Fig. 4

3.4. Limit of Detection

Limit of quantitation (LOQ) of the colorimetric assay were estimated by using (standard deviation of the blank solution, n = 6 and the background signals of the absorbance spectrophotometer). The limit of detection (LOD) is the minimum concentration of the target DNA in producing the absorbance ratio by UV-VIS absorption spectroscopy [25-27]. In our method, we took advantage of the definitions and equations provided in the guideline EP17, which was published by the Clinical and Laboratory Standards Institute (CLSI) to calculate the values of LOD and LOQ [19]. Thereafter, the LOD and LOQ were determined by using the equation LOD = 3.3 SD/b and LOQ = 3.3 × LOD, where SD is the standard deviation of the blank measurements (n = 6) and b is the slope of the calibration curve. Subsequently, the LOD and LOQ were calculated as 8.73 and 28.81 ng μL^{-1}, respectively.

3.5. Specificity

Firstly, the target DNA of *E.coli*, *P. aeruginosa*, *S. dysentery*, *K. pneumoniae*, *S. epidermidis*, and *S. aureus* were extracted. Then, 60 ng μL^{-1} from the target DNA of each bacterium was added to the mixture reaction containing the AuNP-probe conjugated with functionalized AuNPs. The results indicate that in the percentage of the target DNA of *S. aureus*, the color of mixture reaction was changed from red to blue; other bacteria, however, did not observed color change. Furthermore, absorbance wavelength was shifted from 523 nm to 560 nm; in the other strains, however, it was not changed (Fig. 6).

Fig. 5

4. Conclusion

In present study, we report an AuNP-based probe for colorimetric assay and the selective detection of *S. aureus* target DNA. This method is rapid and convenient, and can be accomplished within 10–15 min based on the eye naked and UV–VIS spectra, and a significant color change that is easily recognized by the naked eye. The results of the present study indicated that the sensitivity of colorimetry and PCR methods were 8.73 ng μL^{-1} and 30 ng μL^{-1}, respectively. Therefore, colorimetric assay was about three times more sensitive than the PCR method. The purpose of the present assay presents a satisfactorily linear range, low detection limit, good accuracy and specificity in the detection of the *S. aureus* target DNA. Finally, the results indicate that AuNPs, with a relative higher aspect ratio (longer ones), demonstrate advantages. Furthermore, we have demonstrated that our method is suitable to efficiently monitor the amount of target DNA in food and clinical samples, which can be applied as a promising candidate for the on-site detection of *S. aureus* [28].

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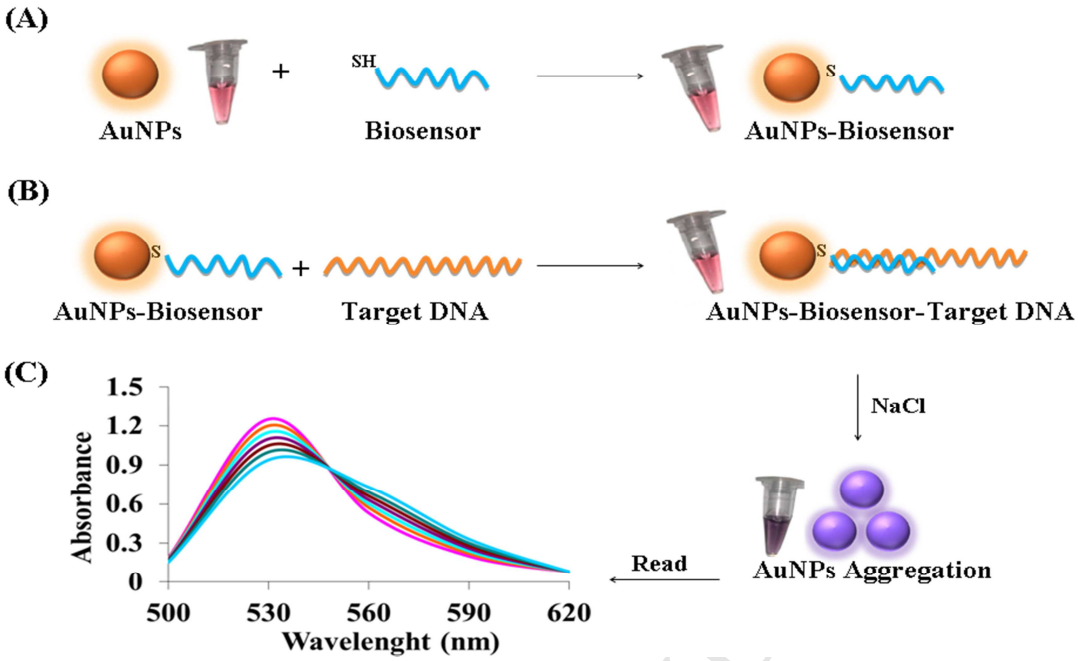

Fig.1. Schematic illustration of the AuNPs-probe for detection of target DNA

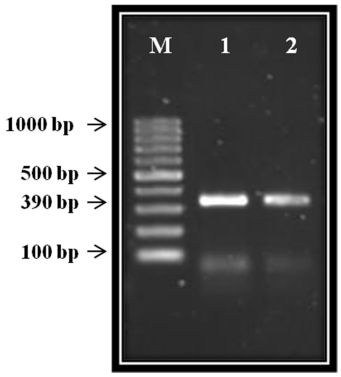

Fig. 2. PCR analyses of the *S. aureus* Protein A gene; (M): marker 100 bp; (1, 2): Standard strain of *S. aureus*.

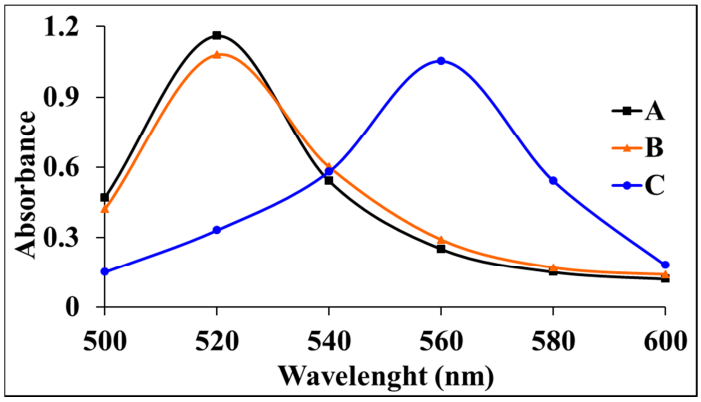

Fig. 3. UV-VIS spectrophotometry of synthesized AuNPs; (A) Wavelength and absorbance ratio of AuNPs prior to conjugation with a probe, (B) wavelength and absorbance ratio of AuNPs after conjugation with a probe, and (C) wavelength and absorbance ratio of AuNPs after hybridization with the bacterial target DNA.

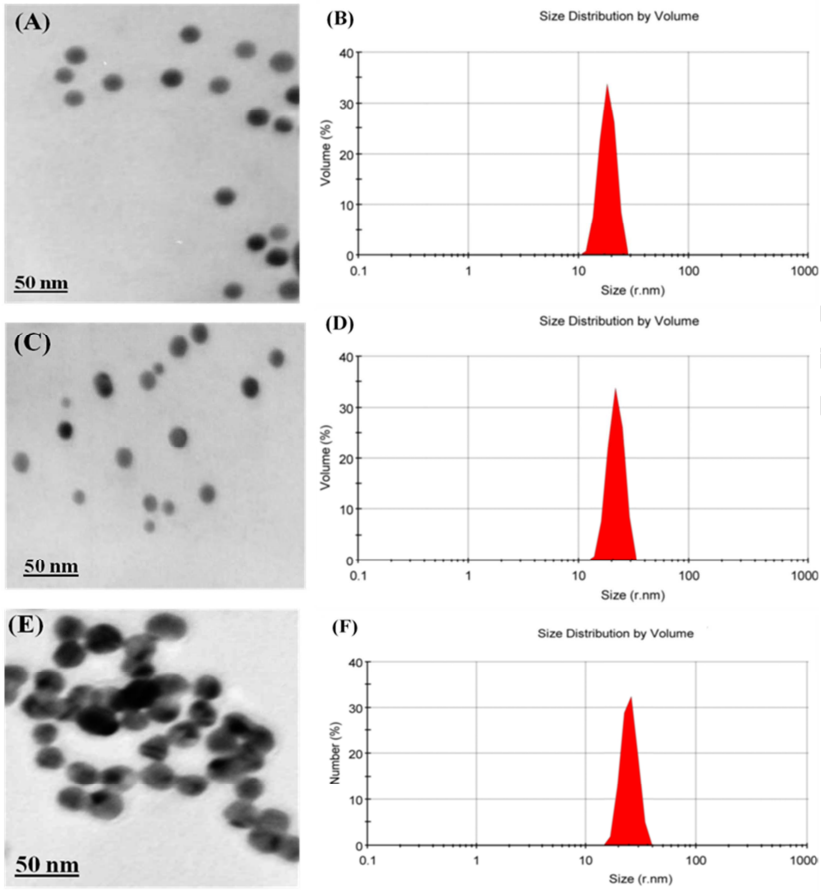

Fig. 4. Size and morphology analysis of AuNPs and a system of nanomaterials by TEM and DLS: pure AuNPs (A, B), AuNPs-probe DNA (C, D) and AuNPs-probe DNA-target DNA accumulation in the presence of NaCl (E, F).

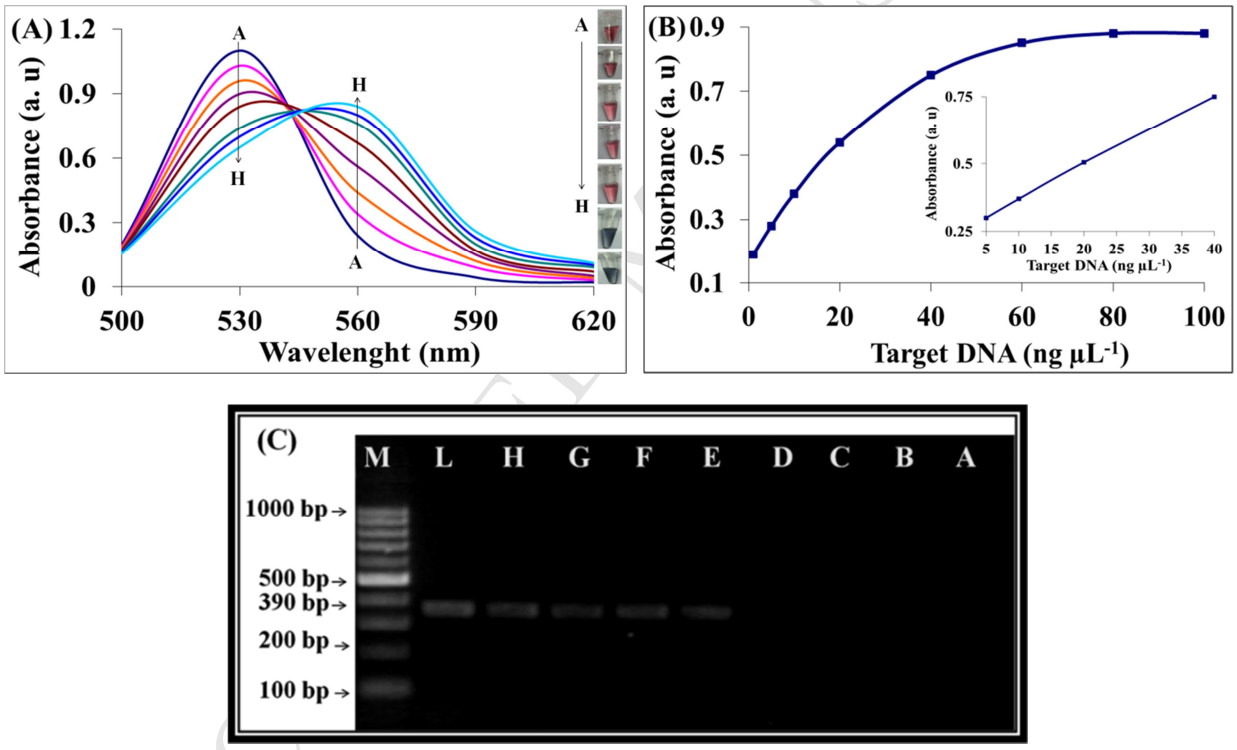

Fig. 5. Comparison of the sensitivities of the PCR and colorimetry methods; (A): determination of sensitivity of *S. aureus* at different concentrations of the target DNA (1, 5, 10, 20, 40, 60, 80, and 100 ng μL^{-1}) from A to H, respectively; (B): linear relationship between the different concentrations of the target DNA and absorbance changes from 1–100 ng μL^{-1}, and the calibration curve showing the relationships between the different concentrations of the target DNA and absorbance from 5–40 ng μL^{-1} (insert); (C): PCR analysis at different concentrations of the target DNA (1, 5, 10, 20, 30, 40, 60, 80, and 100 ng μL^{-1} from A to H, respectively) and M: markers 100 bp.

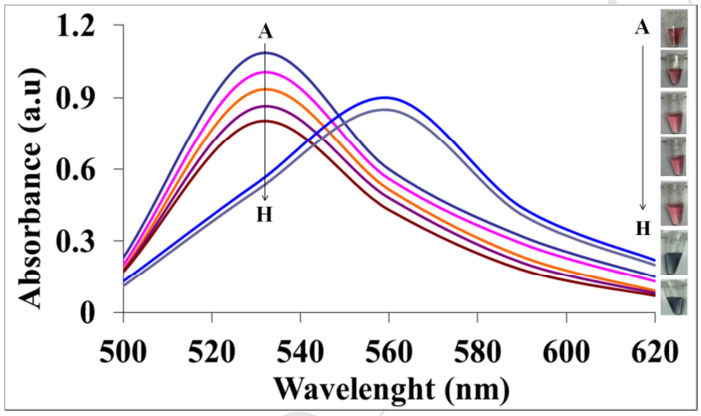

Fig. 6. Specificity of the colorimetric method A to F containing the target DNA (60 ng μL^{-1}) of *E.coli*, *P. aeruginosa*, *S. dysentery*, *K. pneumoniae*, *S. epidermidis*, and *S. aureus*, respectively.

Table 1. Probe and Primers sequence

Primers and Probe	Sequence	Tm (°C)	CG (%)	Length (bp)
Primer forward	5′CGAATCTCAAGCACCGAAAG3′	57.2	50	20
Primer reverse	5′CAGGCTTGTTGTTGTCTTCC3′	57.22	50	20
Probe	5' (SH)GCCTAACTTGAACGAAGAAC3′	54.67	45	20

. The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined 8.73 ng μL^{-1} and 28.81 ng μL^{-1}, respectively.

. The sensitivity of nanomaterials system was determined at different concentration of the target DNA of *S. aureus*.

. The colorimetric assay based on AuNPs was used for detection of *S. aureus* gene protein A for first time.