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Molecular identification of agrobacterium tumefaciens containing pCAMBIA 1305.2 plasmid using multiplex PCR and Gold nanoparticles multiplex probe

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ABSTRACT: Conventional microbiology methods used to detect bacteria include multiple cultures and identification processes, so the results of lab work are painstaking and time-consuming. In recent years, more and more tend to use the diagnostic tests which are based on DNA; hence, DNA diagnostic biosensors have been created to perform DNA identification better. In this study, GUS and *hpt* genes were used to identify the species of Agrobacterium tumefaciens containing pCAMBIA1305.2 plasmid and to design primers and probes. PCR results for both GUS and *hpt* genes indicated amplification of two expected fragments. The specificity of the primers designed in Agrobacterium tumefaciens bacteria containing pCAMBIA1305.2 plasmid and negative control samples was evaluated. Synthesis of the gold nanoparticles was performed with a diameter of about 20 nm. Genomic DNA of the bacteria was used to detect the gold nanoparticles and the markers. Finally, using probes designed for GUS and *hpt* genes, simultaneous detection of the bacteria was done using multiplex probes attached to gold nanoparticles. The results showed a change of color in gold nanoparticles in the presence of the target molecule. In addition, the hybridization probes were examined with target molecules at wavelengths between 400 and 700 nm. The most significant changes occurred in the wavelength ranges of 550 to 650 nm. The results showed that using detectors attached to gold nanoparticles had higher speed and specialty than biochemical and molecular methods, and of course, it had lower costs.

Keywords: *Agrobacterium tumefaciens; Gold nanoparticles; GUS gene; HPT gene; pCAMBIA1305.2 plasmid*

INTRODUCTION

Conventional microbiology methods, which are commonly used to detect bacteria, include multiple cultures and identification processes, which lead to painstaking and time-consuming lab work. Emergence of specialized investigative techniques using genes paved the way to development of powerful test by which certain bacteria can be identified quickly and without the need to isolate pure cultures. Polymerase chain reaction (PCR) is a technique used for amplifying specific fragments of DNA using pairs of primers. With proliferation of more than a million times from a particular area among plenty of other genes, it often allows sensitive detection of specific genes. This reaction is also useful for replication of specific genes related to gene classification and the genes creating bacterial

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diseases (Prasad, and Vidyarthi, 2011). On the other hand, there are DNA Diagnostic Biosensors in which the basis of identifying the existing DNA systems is hybridization of a target DNA with its complementary probe. By performing it in solution or on a solid surface (Vo-Dinh & Vo-Dinh, 2000); DNA identification operation can be performed. The use of this type of biosensor, especially DNA detection nano-biosensors identifying genetic mutations at the molecular level provides the opportunity to identify the bacteria even before its appearance (Sassolas, *et al.*, 2008).The use of gold nanoparticles (GNPs) is widely common in biological studies in recent decades (Wang, *et al.*, 2008). As a new generation of diagnostic biosensors, DNA probes of gold nanoparticles offer innovations in biological sciences, which are of value in rapid and sensitive processes such as detection of pathogenic microorganisms. In fact, their optical properties can be useful for connection diagnosis of available analytics (Vaseghi, *et al.*, 2013).

Gene transfer by Agrobacterium tumefaciens, which is present naturally in many plant cases in soil, leads to Crown gall disease in which Agrobacterium enters the plant via wounded sites. In 1907, this bacterium was initially introduced as the main factor causing the scabies disease (Zaenen, *et al.*, 1974).

Gal disease is caused by transmission of a specific piece of T-DNA from macro Agrobacterium Ti plasmid into the plant genome (Chilton, *et al.*, 1977). Three factors contribute to transfer of T-DNA into plants: vir genes on main Agrobacterium chromosome (Gelvin, 2000), a fragment of 25 bp with direct iterative in T-DNA (Zambryski, *et al.*, 1983), and virulence genes (vir) in Ti plasmid in an area out of the T-DNA which is responsible for production of proteins needed for transfer of T-DNA into the plant (Godelieve & Angenon, 1998).

The formation of CAMBIA vectors originates from pzp plasmid made by Hajdukiewicz. General structure of pCAMBIA vectors is shown in Figure 1.Pzp vectors have limitations that are removed in pCAMBIA vectors. pCAMBIA vectors have numerous copies in E. coli bacteria (Hajdukiewicz, *et al.*, 1994). Pvs1 of these vectors increases their stability in presence of Agrobacterium. Moreover, the size of these vectors is small and about 7-12 kb and have enzyme cutting sites for adding DNA fragments. Selection among bacteria is performed by genes resistant to Kanamycin, Chloramphenicol and Streptomycin of these vectors. Likewise, the genes resistant to Hygromycin, kanamycin and phosphinotricin, do selection among plants. T-DNA region is placed in these sectors. Moreover, they own GUS and GFP reporter genes and PUC18, PUC8, PUC9 poly- linkers are used in the vectors (Hellens, *et al.*, 2000).

PCAMBIA 1305.2 plasmid is a binary vector with 11921 base pairs. It has both a gene resistant to previous have groves (*hpt*) to select between plants and a gene resistant to kanamycin for selection in bacteria (Hoekema, *et al.*, 1983). This vector owns a GUS reporter gene with intron that is only expressed in eukaryotes. It is not expressed in prokaryotes (Komori, *et al.*, 2007).

MATERIALS AND METHODS

DNA extracted from bacteria by boiling method

1 mL of medium containing bacterial solution was poured in a 1.5 mL tube, centrifuged in 14000 rpm, at 4° C, for 2 minutes; then, the supernatant was discarded. The resulted sediment was dissolved in 1000 µL of sterile water or PBS solution. Then, it was again centrifuged and the supernatant was discarded (This

Table 1: primer sequences.

Gene	Primers	Primer sequences(5'-3')	Length of primers	Tm	GC%	Size (bp)
GUS	F	GTCGTGATCGACCAGACTCC	20	62.5	60	601
	R	GCTCACCCACGAAGTTCTCA	20	60.5	55	
hpt	F	GGGCAGTCCTCGGCCCAAAG	20	60.5	55	500
	R	GGGCGTGGATATGTCCTGCG	20	62.5	60	

Fig. 1. Shows a part of the *GUS* gene at the junction of Primers and probe

step was repeated two or three times). The resulted sediment was dissolved in 100 µL of distilled water in the rmoheater at 94 °C for 18 minutes. The microtube content was centrifuged in 14000 rpm, at 4°C for 5 minutes. Upper phase of the tube, containing DNA, was transferred to a new tube and stored for subsequent steps at -20°C. To see the extracted DNA on agarose gel, 1% was loaded and electro fused (Queipo-Ortuno, *et al.*, 2008). After extracting DNA, it is necessary to analyze the quality and quantity of the extracted sample. For this purpose, spectrophotometry and agarose gel methods were used.

Designing specific primer

In order to confirm the transfer of pCAMBIA1305.2 plasmid to bacterium, specific primer was designed.

GUS reporter gene sequence

GUS reporter gene sequence was obtained from the NCBI database with the access no. primer both *GUS* and *hpt* genes were designed using Oligo7 software. Their sequence is shown in Table 1.

AF354046.1. the area used in designing primers and their sequences are shown in Fig. 1.

Hpt gene sequence

Hpt gene sequence was obtained from the NCBI database with the access no. AF354046.3. The area used in designing primers and primer sequences are shown in Fig. 2.

Synthesis of gold nanoparticles

Gold nanoparticles were synthesized using sodium citrate (Frens, 1973, Turkevich, *et al.*, 1951). Then, the generated gold nanoparticles were kept in the dark and at room temperature.

In order to evaluate the morphology of the synthesized gold nanoparticles, three methods were used: electron microscopy of transmission, analysis of absorption spectrum of gold nanoparticles using a spec-

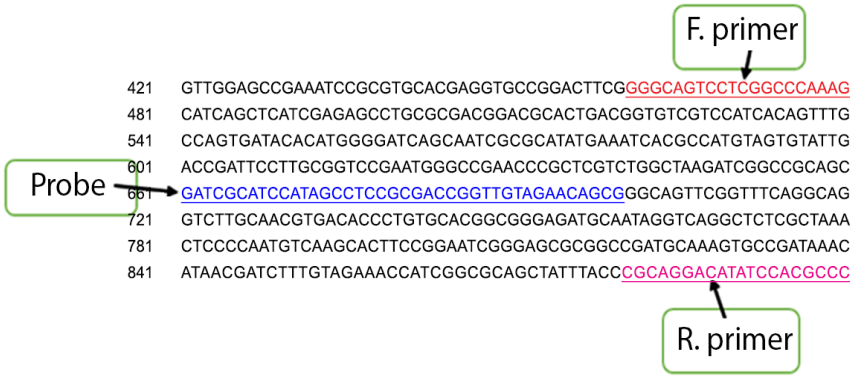

Fig. 2.Part of the *hpt* gene, and position of designed Primers and probe

Table 2: Sequence of designed markers used to bind to DNA

Sequence of Probes	Gene
GCC TCG TTG GCG ATG CTC CA	GUS
CGC CCT CTT CCT CAG TCG GC	
CAA TGA CCC CTG TTA TGC GG	hpt
CTC CCA GGG CGA AGA ATC TC	

trophotometer and using of FT-IR analysis.

Identification of bacteria using gold nanoparticle probe

GUS and *hpt* genes sequence is identified using the NCBI database with the accession number and the probes are designed using its exon regions (Table 2). The designed probes were put beside each other head-to-tail. These two detectors will create an open oligonucleotide fragment with a length of 40.

In order to identify bacteria using gold nanoparticles probe is taking advantage of the method used for detection of telomeric DNA introduced in 2009 by Qi (Qi, *et al.*, 2008). However, it coincided with changes in the amount and concentration of the used materials and buffers. Moreover, simple probes (Unmodified) were also used in this research.

Using gold nanoparticle probe to Identification of Agrobacterium tumefaciens

In order to identify Agrobacterium tumefaciens, the following protocol was used.

2 µl DNA was mixed and vertexed with 10 µl phosphate buffer 0/02 M. 5 ml of each probe and some NaCl 0.25 M were first added to the tubes resulted from the additional reaction mixture, then they were vortexed and spinned. The tube was placed for 5 minutes at a temperature of 94°C to strain DNA. Then, be hybridized between the probe and RNA and after cooling at room temperature, 120 ml of gold nanoparticles was added to it. Finally, after observing the desired color variation, its ansorption rate was read by spectrophotometer. After cooling at the room temperature, 120 micro liters of gold particles is added to create stronger connections between the DNA and the probe. As soon as the change in the color of its absorption is observed, it is read by a spectrophotometer (Fig. 3).

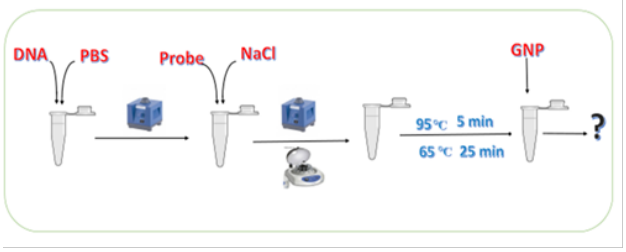

Fig. 3. Identification of bacteria using gold nanoparticle probe

pCAMBIA1305.2 - free Agrobacterium was used as a negative control to determine specificity of the designed markers in all experiments.

RESULTS AND DISSCUTION

Results

PCR results for *GUS* reporter gene, 600 bp, were confirmed approving presence of plasmids in bacterium. In addition, no band was detected in plasmid- free bacteria (Fig. 4).

PCR results of *hpt* gene are shown in Fig. 5 indi-

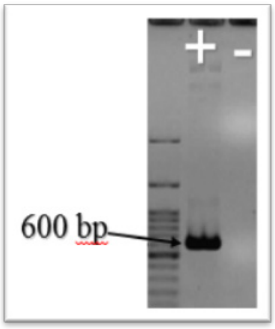

Fig. 4. PCR results for *GUS* reporter gene. PCR product for Agrobacterium containing pCAMBIA1305.2 plasmid (+), PCR product for Agrobacterium without pCAMBIA1305.2 plasmid (-)

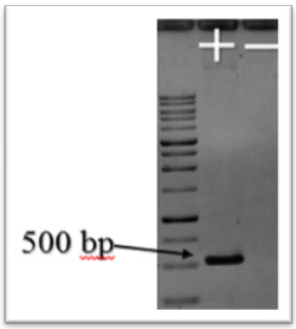

Fig. 5. PCR results for *hpt* gene. PCR product for Agrobacterium containing pCAMBIA1305.2 plasmid (+), PCR product for Agrobacterium without pCAMBIA1305.2 plasmid (-)

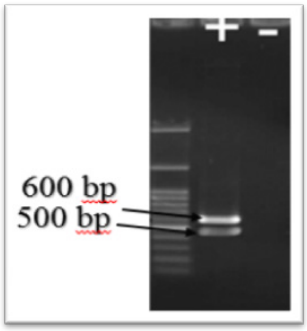

Fig. 6. Multiplex PCR results for both *GUS* and *hpt* genes in bacteria containing plasmid (+) and bacteria without plasmid (-).

cating a 500 bp band for the bacterium containing plasmid, whereas no band was observed in plasmid –free bacterium.

Results of Multiplex PCR for *GUS* and *hpt* genes in bacteria containing plasmid (sample positive control) represent two 600 and 500 bands simultaneously. On the other hand, the results of Multiplex PCR for bacteria without plasmid (negative control samples) showed no band (Fig. 6).

Analysis of synthesized gold nanoparticles using an electron microscope

Synthesized gold nanoparticles are the result of regeneration of gold salt with trisodium citrate with regenerative properties. The taken Images indicate spheric and uniform state of the synthesized gold nanoparticles (Fig. 7). Fig. 7 shows the image of gold nanoparticles taken by TEM.

FT-IR analysis results

FTIR spectrum of the synthesized gold nanoparticles is shown in Fig. 8. Despite the relatively broad absorption, within the range of 3443 cm^{-1}, the presence of hydroxyl functional groups is confirmed. Aliphatic C-H bonds create severe peaks in the range of 2850-3000 cm^{-1}. Existence of these peaks is evident in the range of 2925 cm^{-1} in the structure of synthesized gold nanoparticles. The average peak, 1624 cm^{-1} is related to stretching vibration C=O. Respectively, peak observed in 1398 cm^{-1} represent stretching vibration of the peak and 620 cm^{-1} represents C-H of the bending alkaline (Logaranjan, *et al.*, 2012). These results highlight the presence of hydroxyl and carbonyl functional groups of trisodium citrate molecules present on the surface of the nanoparticles which play an important role in reduction of Au^{+} ions and result in stability of nanoparticles.

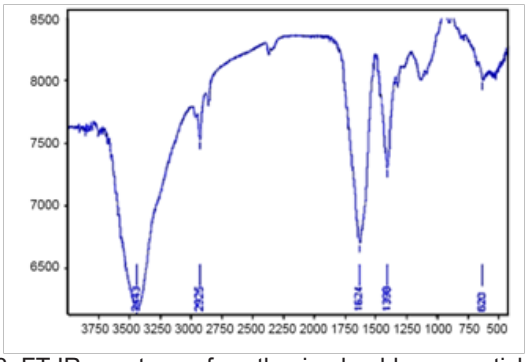

Fig. 8. FT-IR spectrum of synthesized gold nanoparticles

Absorption spectrum of gold nanoparticle synthesis

The maximum absorption (OD) of nanoparticles synthesized after viewing the cherry-red color was achieved with a spectrophotometer at a wavelength of 525 nm (Fig. 9).

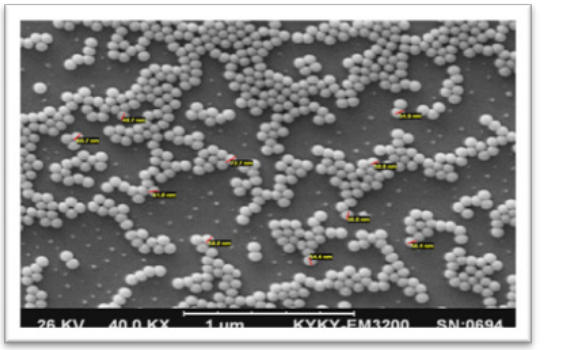

Fig. 7. Image of gold nanoparticles synthesized by trisodium citrate taken by TEM

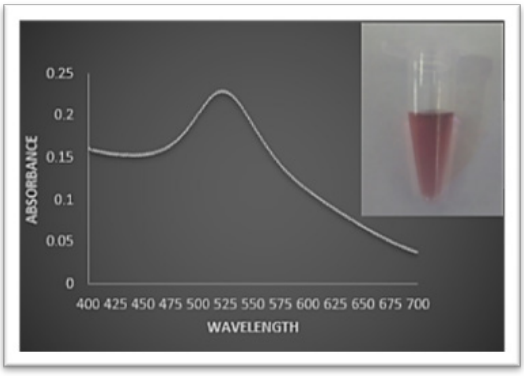

Fig. 9. Absorption spectrum of gold nanoparticles, as shown in the picture, the maximum absorption of gold nanoparticles occurred at the wavelength of 525 nm

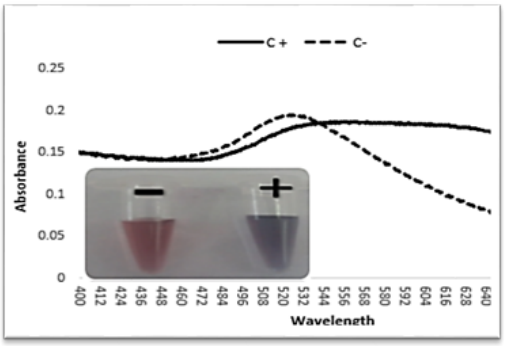

Fig. 10. Changes in wavelength and colorimetric resulting from single-bind probe for GUS gene to target sequence in positive control sample (+) compared to the negative control (-)

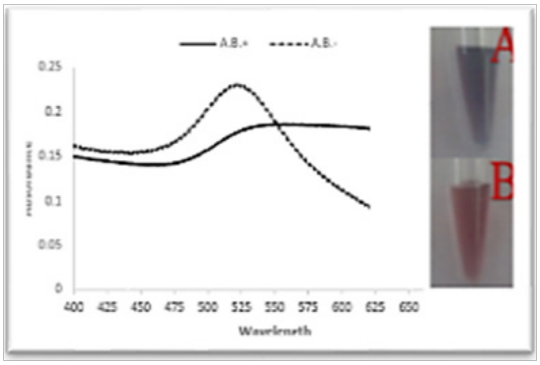

Fig. 12. Changes of wavelength and colorimetric resulting from connection of multiplex probes to target sequences in a positive control sample (A) compared to the negative control (B)

Results of probes bound to genomic DNA

The results of aggregation of gold nanoparticles and changes of their color or wavelength in presence of a probe for GUS gene and genomic DNA are in accordance with Fig. 10 indicating DNA probe's connection with genomic DNA.

The results obtained from the aggregation of gold nanoparticles and changes of color in presence of a single wavelength probe for *hpt* gene and genomic DNA, (Fig. 11), represent the connection between the probe and the genomic DNA.

The results of the aggregation of gold nanoparticles and changes in their color or wavelength in presence of multiplex probes (GUS and *hpt*) and genomic DNA (Fig. 12) represent probe's connection with genomic DNA.

Discussion

After amplification of target genes for identifying Agrobacterium tumefaciens, detection was performed with the use of gold nanoparticles. In comparison with other procedures such as biochemical and molecular detection, or PCR methods, modern methods of nano-biosensors have greater sensitivity and specificity. Designing primers in molecular methods like multiplex PCR sign can suffer from some faults such as proliferation of non-specific deficiencies as well as unwanted bands, low efficiency ofDNA replication in the selective pattern or absence of PCR product, and nucleotide mutations that results from errors in connection of neucloids to each other. However, due to the optical properties, using biosensors and nano-biosensors can remove this limitation. For detectors attached to gold nanoparticles, it is possible to design two oligonucleotides simultaneously as a unique detector. After identifying the position, it increases more than 40 specifies to nearly 100 percent. It is also probable that identical points in the other genome, even with a different base pair, get closer to zero, because it uses gold nanoparticles to illustrate even a single nucleotide along with its color variation and absorption spectrum. Taking advantage of detectors attached to gold nanoparticles is a more specific and rapid way to detect than the biochemical and molecular techniques. It can also be achieved spending lower costs.

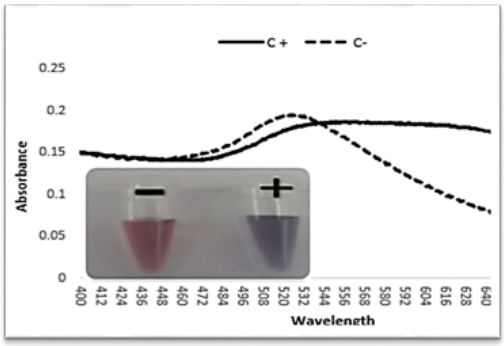

Fig. 11. colorimetric and wavelength changes resulting from single probes for hpt gene binding to target sequences in a positive control sample (+) in comparison with the negative control (-)

Time required to identify the target genome expressed in this study is less than 1 hour, which is less than time needed in the traditional methods and even PCR. There is no need for toxic substances such as ethidium bromide. Due to using RNA, total step of the fragment reproduction is removed by PCR. These

tests can be performed with a little attention and initial optimization as well as minimal facilities in research centers. Moreover, they do not require special equipment. Using gold nanoparticles can optimize factors such as temperature, size, shape, and salt concentration. Controlling the size and shape of gold nanoparticles by sodium citrate concentration can act as an inhibitor. The common principle is based on the ability of mono- strand primers and fixing them by van der Waals links and electrostatic Nforces at the level of gold nanoparticles and their resistance to deposition in presence of some electrolytes such as NaCl. Negative loads of citrate ions on the surface of gold nanoparticles in the solution inhibit gold nanoparticles' deposition. Nevertheless, in the presence of salt solution, they undergo changes and precipitation. Likewise, in presence of target DNA and after hybridization reaction, the primers connect to their complements in case of salt solution and phosphate buffer leading to sediment and its color changes from red to blue. Due to their optical properties, whenever gold nanoparticles are placed side by side and become dense, they change color. Using the wavelength measurement tools such as nano-drop and spectrophotometer, they become visible. Color changes can also be seen with eyes, but there is no clear- cut definition of color change, it cannot be certainly used for identification. High temperature itself can cause sedimentation and change the color of gold nanoparticles. Among the advantages of the diagnostic method used in this study, the absence of gold nanoparticles at high temperatures as well as hybridization detection with RNA target is spatially unimpeded.

The benefits of the diagnostic methods used in this study are: removal of the PCR process and using total RNA, removal of toxic and expensive materials such as Ethidium bromide and agarose, lack of need for cost-based laboratory equipment and fluorescent markers, reduced diagnostic costs in every reaction compared with other diagnostic methods, early detection (in less than 60 minutes), and application of testing in any research laboratory. In fact, there is a similarity between the results of using gold nanoparticle-based detection method and the results in most published papers and the other results observed in the field (Logaranjan, 2012, Khalil, *et al.*, 2014).

Using this method to detect pig's DNA (Ali, *et al.*, 2011), reported the specificity and sensitivity of this technique respectively as 90- 95% and 6 ng / µL in the sample. Using this method, they also stated that, it is possible to express and identify changes in samples at least with one open pair. In addition (Deng, *et al.*, 2013) used non-modified gold nanoparticles to detect Bacillus anthracic bacterium with the help of the PCR product. Likewise (Khalil, *et al.*, 2014) identified Acinetobacterbaumannii bacterium using genome replication and probe's connection to the target molecule followed by addition of gold nanoparticles. Using either protocol needs to optimize factors such as temperature, size and shape of the gold nanoparticles, the amount of probe, and salt concentration. Here, after locating at the binding temperature, the gold nanoparticles are added to a product in order to prevent their instability at high temperatures. Moreover, using different ratios of oligonucleotides in the presence of proper concentration of salt should be considered (Khalil, *et al.*, 2014). Like PCR method, gold nanoparticles have extremely high specificity. Due to presence of unwanted bonds, resulted from some failure and unwanted amplification of PCR, lack of differentiation in amplification of fragments with single nucleotide differences, it is expected to observe specificity of the gold nanoparticles which reflects detection of differences, up to one nucleotide. However, in this study, both available samples have represented full specificity.

Simultaneous use of designed probes for GUS and *hpt* genes contributes to saving performance time. In addition, cost reduction is another benefit of multiplex probe method. Probe multiplex has all benefits of PCR multiplex, but probe multiplex does not have disadvantages of PCR multiplex. For example, while designing primers for PCR multiplex reaction, it should be noted that the size of the piece, which is set by the primers to be amplified, should differ so that it would be possible to distinguish the components on Agars gel (Prasad & Vidyarthi, 2011), while there is not such problem in multiplex probe. In PCR multiplex, due to proliferation of all sequences in a single tube, sequences for similar lab materials such as DNA polymerase and dNTP compete and each sequence is able to stop sequence of other sequences. Hence, it is nec-

essary to define the same laboratory materials for PCR precisely to minimize the competition (Henegariu, *et al.*, 1997). Yet, in case of using multiplex probe, this problem does not exist since there is no practical need of amplification process.

CONCLUSIONS

Using detectors attached to gold nanoparticles for detecting biochemical and molecular techniques has unique specificity and high speed and lower costs. The time required to identify the target genome, as expressed in the protocol discussed in this study, is less than 1 hour which is less than that of the conventional methods and even PCR.There is no need for toxic substances such as ethidium bromide. Moreover, due to the use of genome, the amplification process of the desired fragment is removed by PCR. These tests can be performed with little care and initial optimization in research centers with the least possible facilities, since they do not need any special equipment.

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