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An improved non-crosslinking gold nanoprobe-NASBA based on 16S rRNA for rapid discriminative bio-sensing of major salmonellosis pathogens

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ABSTRACT

In the absence of an appropriate detection method for simple and rapid differentiation of major salmonellosis-causing agents, nano-bio-sensing could provide ideal molecular detection approaches for food-borne pathogens. In addition, the 16S ribosomal RNA-based detection techniques would enhance specificity and sensitivity due to the presence of multiple copies of 16S rRNA per cells and also the presence of hypervariable regions in the genes. In this study, we developed a novel detection technique based on 16S rRNA gold nanoprobe-nucleic acid sequence-based amplification (NASBA) in an improved non-crosslinking mode for nanodiagnosis of the most important serovars of the *Salmonella* genus: *Salmonella enteritidis* and *Salmonella typhimurium*. In that regard, we designed a specific set of primers along with a thiolated oligonucleotide gold nanoprobe for one of the hypervariable regions of 16S rRNA gene. The NASBA-gold nanoprobe method was improved to lower the overall assay time to about 80 min and enhance the specificity and reproducibility of the detection process using various closely related non-*Salmonella* species as well as some *Salmonella* serotypes. Finally, the sensitivity of the developed method was determined to be around 5 CFUs *Salmonella* per amplification tube. The developed nano-bio-sensing method could provide a cost-effective and promising assay especially suited for quick identification of food-borne pathogens in the event of outbreaks.

1. Introduction

Elegant techniques for rapid and specific detection of *Salmonella enteritidis* and *Salmonella typhimurium*, the main causes of salmonellosis, are presently at high global demand particularly in medicine and food industries (Greenwood et al., 2002; Swaminathan et al., 2001; Torrence and Isaacson, 2008). It is believed that these species are the second main cause of bacterial food-borne infections in the northern US (Lynch et al., 2006) and the main cause of gastrointestinal diseases in Iran (Zali et al., 2003). Detection of *Salmonella* serovars is routinely achieved on the basis of phenotypic and/or genotypic criteria (Mandal et al., 2011). However, conventional phenotype-based detection methods suffer from prolonged analysis time, low sensitivity and specificity, labor intensity and the high cost. In addition, further experimental data are required for final confirmation of the results obtained by the aforementioned methods (Gracias and McKillip, 2004). Similarly, most genotype-based detection methods such as nucleic acid-based approaches including (in)direct probe hybridization and DNA/RNA-based polymerase chain reaction (PCR) as well as serological assays e.g. enzyme-linked immunosorbent assay (ELISA) are almost associated with the same technical problems besides high dependency on advanced instrumentation (Rijpens and Herman, 2002).

In our previous work, we reported on the development of a new non-crosslinking gold nanoprobe-nucleic acid sequence-based amplification (NASBA) method to solve some of the aforementioned problems (Mollasalehi and Yazdanparast, 2012). The developed nano-bio-sensing technique is in fact an isothermal transcription-based molecular method for RNA amplification along with gold nanoprobes for specific detection of the amplified amplicons (Mollasalehi and Yazdanparast, 2012). In the new technique, RNA content of cells is targeted by two sense and antisense (carrying T7 RNA polymerase promoter) primers, so that the single-stranded RNA products are produced using the combination of three enzymes: avian myeloblastosis virus reverse transcriptase (AMV-RT), ribonuclease H and T7 RNA polymerase. AMV-RT is responsible for cDNA reverse transcription and production of RNA–cDNA hybrid. Subsequently, RNase H removes the RNA component of the hybrid which is replaced by a complementary DNA strand using DNA polymerization activity of AMV-RT. This new dsDNA with the incorporated promoter makes the substrate for the third enzyme, T7 RNA polymerase resulting in the production of the antisense

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RNA amplicon. Finally, each generated RNA amplicon is also targeted by the NASBA primer set and transcription-based amplification system in a cyclic mode and exponential accumulation of target RNA is accomplished (Compton, 1991). The NASBA products are then exposed to the fabricated gold nanoprobes which are expected to hybridize specifically to their complementary target sequences in the amplified amplicons. Through hybridization, a change in the surface plasmon resonance (SPR) from 520 nm to 600–650 nm (associated with a change in color from red to purple/gray) would provide the discriminative tool of detection. In non-crosslinking mode, the elevation of the ionic strength of the reaction environment which affects the stability of gold nanoprobes, depending upon their hybridization status, provides the main strategy of differentiating the positive samples from the negative ones (Baptista et al., 2006; Sato et al., 2005). In the absence of amplicon-gold nanoprobe hybridization, gold nanoprobes' aggregation occurs after salt addition (Sato et al., 2003, 2005). Otherwise, after complete hybridization, the concentration of negatively charged nucleic acid phosphate groups around polarizable gold nanoprobes would increase. Therefore, the nanoprobes are protected against aggregation as a result of charge repulsion between them (Doria et al., 2007; Sandström et al., 2003). Regardless of simplicity, low-cost and the speed of analysis, the new approach benefits from high sensitivity and specificity (Doria et al., 2007; Mollasalehi and Yazdanparast, 2012). Based upon these criteria, the developed technique would certainly constitute the basis for a highly acute diagnostic approach.

Regarding the choice of nucleic acid target structure(s), 16S ribosomal DNA (16S rDNA) which is commonly used in phylogenetic evaluations for species and/or subspecies discrimination purposes (Weisburg et al., 1991), appears a good candidate. This choice is mainly due to the presence of hypervariable regions in 16S rRNA gene (Chakravorty et al., 2007), the existence of multiple operons in the genome of most bacteria including *Salmonella* serovars (Klappenbach et al., 2000), higher copy number and deposition of much more16S rDNA sequence information in the relevant database relative to other kinds of ribosomal RNA genes such as 23S rRNA (Clarridge, 2004). Regarding these advantages, we targeted 16S rRNA gene of *Salmonella* serotypes Enteritidis and Typhimurium, and designed against the hypervariable region a degenerate sense primer along with a specific antisense primer, carrying on its 5′ site terminus the RNA polymerase promoter sequence. Additionally, a thiolated oligonucleotide probe, for specific recognition of a unique region within the amplified amplicon, was designed which was further fabricated into gold nanoprobe particles for nanodetection of the NASBA products. The combined NASBA-gold nanoprobe method was upgraded to decrease the overall assay time and enhance the specificity and reproducibility of the detection process using closely related non-*Salmonella* species as well as several *Salmonella* serotypes. Finally, the sensitivity and the overall assay time of the developed method were determined to be around 5 CFUs of *Salmonella* per amplification tube and 80 min, respectively, including amplification and nanodetection.

This study is both an improvement of the previous work (Mollasalehi and Yazdanparast, 2012) in cases of specificity, sensitivity, simplicity and speed and also an application of the method to rapid bacterial pathogen detection which is a step forward for designing applied biosensors. The method is especially suited for situations where target DNA is not available (e.g. RNA viruses), increasing the sensitivity of detection, decreasing cross-contamination and false positive results as well as viability approaches in laboratories. However, because of RNA-based nature of the method and critical condition of handling RNA in comparison to DNA, it is hardly possible to make the method applicable to the field for layman. Furthermore, quantification, calibration study and limit of detection (LOD) were demonstrated besides specificity and speed improvement studies. In that regard, quantification was performed using spectroscopic analysis at different wavelengths, calibration studies were done to adjust the ionic strength and hybridization period to decrease the overall assay time from 150 min to 80 min and finally the LOD was determined to be 5 CFUs per amplification tube.

2. Experimental

2.1. Materials

The enzymes and NTP mixture used in this study were acquired from Roche (Germany) and the dNTP mixture was from Takara (Japan). The fetal bovine serum (FBS) was purchased from Gibco (USA) and the culture media including Nutrient Broth (NB) and Brain–Heart Infusion (BHI) broth were obtained from Merck (Germany) and Tryptic Soy Broth/Agar (TSB/TSA) was from Liofilchem (Italy). The oligonucleotides were synthesized by Bioneer (South Korea) and the molecular biology grade chemicals were from Sigma-Aldrich (USA) except ethanol and acetone which were from Merck (Germany). Bacterial strains were purchased from Razi Vaccine and Serum Research Institute (RVSRI) and Iranian Research Organization for Science and Technology (IROST) (Iran).

2.2. Apparatus

Nucleic acid horizontal electrophoretic instruments and UV trans-illuminator were from Biorad (USA). UV–visible spectroscopy evaluation was performed using NanoDrop 2000c (Thermo Scientific, USA). The sequences were aligned using the standard nucleotide Basic Local Alignment Search Tool (BLAST).

2.3. Bacterial culture

The bacteria were cultured overnight at 37 °C either in Nutrient Broth (NB) or Tryptic Soy Broth (TSB) supplemented with 10% FBS and Brain–Heart Infusion (BHI) broth and harvested at their exponential phase.

2.4. Isolation of genomic nucleic acids

Silica-based guanidine thiocyanate nucleic acid extraction method presented by Boom et al. (1990) was employed. Briefly, the pellet of the centrifuged (12,000*g* for 30 s) bacterial culture (1 mL) was dissolved in 50 μL of TE buffer (pH 8.0) containing 10 mM Tris–hydrochloride (Tris–HCl) and 1 mM ethylenediaminetetraacetic acid (EDTA). Subsequently, 940 μL reaction buffer containing 40 μL activated-silica and 900 μL of lysis buffer was added. The lysis buffer contained 1.2*g* guanidine thiocyanate in 1 mL of 0.1 M Tris–HCl (pH, 6.4), 220 μL of 0.2 M EDTA and 26 mg Triton X-100. The mixture was then vortexed, incubated at room temperature for 10 min, vortexed again and centrifuged at 12,000*g* for 15 s. Finally, the silica particles were washed twice with washing buffer (containing 1.2*g* guanidine thiocyanate in 1 mL Tris–HCl (pH, 6.4)), twice with ethanol 70% (v/v) and once with acetone. Following complete acetone evaporation, the nucleic acids were desorbed from the silica particles using 150 μL of TE buffer.

2.5. Primer design and RNA amplification

One set of NASBA primers was designed using BLAST and GenBank database at the National Center for Biotechnology Information (NCBI) for 16S rDNA sequences of various available *Salmonella* serotypes as well as non-*Salmonella* species for simultaneous