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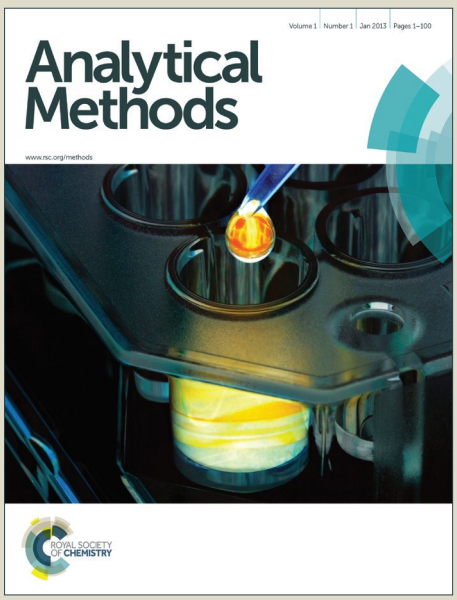

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Visual detection of *Listeria monocytogenes* using unmodified gold nanoparticles based on a novel marker

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Abstract:

Listeria monocytogenes (*L. monocytogenes*) make people and animals have Listeria disease. After infection, the main symptoms of Listeria disease are sepsis, meningitis and mononucleosis. For this purpose, a visual detection of *L. monocytogenes* was developed with unmodified gold nanoparticles. The species-specific probes, in the presence of PCR target products of *L. monocytogenes*, cause the gold nanoparticles to aggregate irreversibly, producing a red to purple colorimetric change. As little as 1.304 fg/μl of DNA of *L. monocytogenes* were thus detected visually, by the naked eye, without the need for any sophisticated, expensive instrumentation and biochemical reagents, which was approximately seventy-fold higher sensitivity over conventional PCR gel electrophoresis method. The results indicate that this assay is highly species-specific, simple, low-cost, and visual for easy detection of *L. monocytogenes*.

Keywords: visual detection; *Listeria monocytogenes*; gold nanoparticles

Introduction

L. monocytogenes has been recognized as a cause of disease in humans and animals and has been responsible for listeriosis. it can be found in dairy products, meat, poultry, vegetables, as well as in frozen food because of growing at refrigeration temperatures. The consumption of food products contaminated with this bacterium can cause listeriosis, a serious disease with a 30% mortality rate[1]. At home and abroad the damage of *L. monocytogenes* gets more and more valued, and WHO listed it as one of the four major food-borne pathogens in 1990s. Recent consumption of frozen food in China has increased, the potential risk of *L. monocytogenes* is becoming more and more outstanding, simultaneously, risk assessment data from the United States ranks several categories of dairy products as high-to moderate risk in terms of predicted listeriosis cases on a per serving basis[2]. The ubiquitous distribution of this pathogen in nature, its ability to grow at refrigeration temperatures and its tolerance to certain preservative agents make its elimination from food very difficult. A fast and sensitive detection of this pathogen would be extremely important for early prevention of disease spreading.

Nowadays, many different kinds of methods have been evaluated for detecting *L. monocytogenes* in food samples, including conventional culture-, biochemical-, and immunological-based assays[3], polymerase chain reaction (PCR) and RT-PCR based methods[4-6], loop-mediated isothermal amplification assays[7-9], biosensors [10, 11], matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy[12], Fourier transform infrared (FT-IR) spectroscopy [13], Among these methods, calculating the number, biochemical characteristics and identification of colony are time-consuming, immunological assays can suffer from low specificity, resulting in false-positive results[14], because most commercially developed antibodies are not

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species-specific. RT-PCR, biosensors, MALDI-TOF, and FT-IR assays are not routinely used because of the expensive requirements. LAMP can easily produce false-positive results even though a very small amount of contaminant is in reaction systems[7].

The polymerase chain reaction (PCR) assay has the advantages in that it can be highly specific, sensitive, rapid and may permit direct detection of the pathogen in foods and other samples without the need for isolation of pure cultures. Various genes have been targeted for the detection of *L. monocytogenes*, including *hlyA*[15], *iap*[16], *inlA*[17], *inlAB*[15], *actA*[18] and *lmo*0733[19]. Although lmo0202hly gene fragment is a potential molecular detection marker for *L. monocytogenes* in bioinformatics analysis in our previous research[20], it still needs to be identified by biological experiments in this study.

Gold nanoparticles (AuNPs) have been used as sensing material for colorimetric detection due to their unique optical properties[21]. It presents a color change of AuNPs colloids based on electrostatic interaction between AuNPs with nucleotide sequences. Therefore, AuNPs can selectively aggregate due to the different characteristics of the single and double strand DNA, and provide a simple, inexpensive and colorimetric detection of various DNA sequences. However, many color assays are time consuming due to about a two-day's preparation for modified AuNPs[22]. In order to overcome limitations, a reliable, simple, cost-effective, and visual detection of *L. monocytogenes* based on unmodified gold nanoparticles and PCR has been developed in the study.

Experimental

Materials and reagents

L.monocytogenes, *L.ivanuii*, *L.innocua*, *L.seeligeri*, *L.grayi*, *Listeria welshimeri*, *Salmonella choleraesuis*, *Salmonella typhimurium* , *Serratia marcescens* were supplied by Shanghai Entry-Exit Inspection and Quarantine Bureau, Shanghai, China. *Staphylococcus aureus* and *Carnation ringspot virus* (CRSV) were kept in our laboratory. HAuCl4 (99.999%) and sodium citrate dehydrate were purchased from Sinopharm Group Chemical Regent Co., Ltd., Shanghai, China. All glass was cleaned in aqua regia (3 parts HCl, 1 part HNO_3) rinsed with pure H_2O. The species-specific fragment from *Staphylococcus aureus* (289bp) and the species-specific fragment from *Carnation ringspot virus* (476bp) were supplied by our laboratory and used as controls. All oligonucleotides used in the study were synthesized and purified by Sangon Biotech.

Extraction of Genomic DNA or RNA

For DNA isolation, 1 mL of overnight pure culture BHI broth was centrifuged and washed once with physiologic saline. Genomic DNA was isolated from the pellet using the TIANamp Bacteria DNA Kit (TianGenBiotech, Beijing, China) according to the manufacturer's instructions and dissolved in ddH_2O. Viral genomic RNA was extracted using TIANamp Virus RNA Kit (TianGenBiotech, Beijing, China) according to the manufacturer's instructions. The RNA was eluted with 60 μL of elution buffer and stored at -80°C pending analysis.

Preparation of negative control

289-bp fragment from *Staphylococcus aureus* and 475-pb fragment from *Maize chlorotic mottle*

virus (MCMV) were used to be controls and prepared as followed, respectively. The 289 bp of PCR products were gained according to our previous research[23]. 475-pb fragments from CMMCV were amplified with the primers (Forward primer: 5'-TCAGGTTTCATGCCCTCT-3', reverse primer: 5'- ATGCTTGCTCCATCCACT-3'). RT-PCR assays were performed on Eppendorf gradient thermocycler using One-Step PrimeScriptTM RT-PCR Kit (Takara, China). All sets of reactions were carried out in a final volume of 25μl each containing: 0.5 μl of RNA (MCMV), 12.5 μl of 2×OneStep RT-PCR Buffer, 1μl of PrimeScript enzyme (5U/μl), 0.5μl of MCMV PF(20 μM),0.5 μl of MCMV PR(20 μM), and 10 μl of RNase-free ddH_2O. Amplification reactions were consisted of a reverse transcript at 42 ℃for 25 min, an initial denaturing step at 94 ℃for 2 min followed by 35 cycles at 94 ◦C for 30 s, 55 ◦C for 30 s and 72 ◦C for 30s followed by a final extension at 72 ◦C for 10 min.

PCR procedure

In order to amplify the species-specific fragment for *L.monocytogenes*, the primes and probe were designed to target *lmo0202hly* gene fragment and were synthesized as follows: F: 5'-ATCAACCAgATgTTCTCCCTg TA-3', R: 5'-GATTCACTgTAAgCCATTTCgTC-3'. PCR was carried out in a 50 μl reaction mixture composed of 5.0 μl of 10×Taq DNA polymerase buffer, 3.0 μl of 2.5 mmol/L dNTPs, 1.0 μl of Taq DNA polymerase, 1.0 μl of prime and reverse primer, 38 μl of ddH_2O and 1.0 μl of DNA. PCR reaction was performed in an Eppendorf gradient thermocycler. The cycling procedures for amplifying target sequence were set as follows: an initial step of denaturation at 95□ for 2 min; 30 cycles of denaturation at 95 □ for 45 s, anneal at 53 □ for 30 s and elongation at 72 □ for 55 s; final extension at 72 □ for 10 min. 5.0 μl of PCR product was analyzed by gel electrophoresis using a 1.5% agarose gel containing 0.5 pg of EB dye. The running conditions were stable voltage at 120 V for 30 min. After electrophoresis, the relative amount of PCR products was analyzed by image analysis software (Quantity OneTM, Bio-Rad, CA, USA). 600-bp DNA markers, which contain 600, 500, 400, 300, 200, and 100 bp DNA fragments, were used as standards for the evaluation of the gel electrophoresis results. The expected PCR product is the length of 226 bp.

Preparation of gold nanoparticles (AuNPs)

AuNPs with the average diameter of 13nm were synthesized by a citrate reduction method. Briefly, AuNPs were prepared by boiling an aqueous solution of 0.01% $HAuCl_4$ (100 ml) under rapid stirring and adding 3.5 ml of 1% sodium citrate. After boiling for15 min and further rapid stirring for 30 min, the solution was allowed to be cooled to room temperature and filtered through a 0.8 μm membrane. Concentration of the as-prepared AuNPs was estimated to be 3.5 nM, which was calculated from the quantity of starting material ($HAuCl_4$) and the size of the nanoparticles.

Effect of NaCl concentration on the complex of AuNPs with DNA

In order to achieve the optimal NaCl concentration to the complex of AuNPs with DNA, six different concentration of NaCl were induced in to the complex of AuNPs with double strand DNA, single strand DNA (probe) respectively.

Specificity of visual detection of *L. monocytogenes*

1 μl of 10-fold diluted PCR product and 1 μl of 10 mM probe (5'-

AAAAAATGCCACTAAATCAAA -3') were mixed completely in a sterile PCR tube. The mixture was denatured at 95°C for 2min, annealed at 53°C for 6min, and then cooled to room temperature for 10 min. After addition of 30 μl of colloidal AuNPs to the reaction mixture, 2.89 μl of 0.11M PBS buffer and ddH_2O, the total volume reached 35μl and kept for 10 min at room temperature , the color changes in the solutions were observed visually and recorded by cameras. There existed 65 mM NaCl in the final mixture. To validate the specificity of this method, 39 strains were tested using the above procedures.

Sensitivity of visual detection of *L. monocytogenes*

The sensitivity of the assay was evaluated by performing the above procedures at a series of diluted DNA of *L.monocytogenes*.

Results

The design of visual detection of *L.monocytogenes* based on AuNPs colorimetric assay

The detection strategy is shown in Scheme 1. Single strand DNA (ssDNA) adsorbs on citrate-coated AuNPs, and this adsorption increases the negative charge on the AuNPs, which give rise to increasing repulsion between the particles, thus preventing aggregation. The adsorption of ssDNA on AuNPs occurs due to the fact that ssDNA can uncoil and expose its nitrogenous bases, and the attractive electrostatic forces between the bases and the AuNPs allow adsorption of the ssDNA. Inversely, double strand DNA (dsDNA) does not adsorb on AuNPs due to the repulsion between its negatively-charged phosphate backbone and the negatively-charged coating of citrate ions on the surfaces of the AuNPs[24, 25]. Therefore, when AuNPs are added to a saline solution containing the target DNA and its complementary, specific probes, AuNPs aggregate (since the probes are not free to stabilize the AuNPs) and the solution color changes to grey blue. However, in the absence of the target or the presence of a non-complementary target, the probes remain free to stabilize the AuNPs thus preventing their aggregation, and the solution color remains red (Scheme.1). The denaturation and annealing help species-specific probes sufficiently absorb the targets. In appearance, the color difference of AuNPs can indicate whether the RCR product can combine complementally with species-specific probe or not, and identified the samples.

Optimal concentration of NaCl to color change of the complex of AuNPs colloid

Single strand DNA probes can absorb into the surface of AuNPs through electric interaction to increase the stability of colloidal of AuNPs, and the electric double layers of colloidal of AuNPs can also retain good stability. However, there exist a critical salt concentration, which can induce the complex of AuNPs with double strand DNA to deposit, but the complex of AuNPs with probes is still steadily stable. As shown in Fig.1, the color of four samples was clearly red at the concentration of NaCl below 60 mM (Fig.1A, B,C), however, the color of three samples turned to grey blue at the concentration of NaCl over 65 mM (Fig.1F), the color of one sample begin turning to grey blue when the concentration of NaCl was 62 mM (Fig.1D), only one sample was grey blue and others appeared red color (Fig.1E), this results demonstrated that the critical concentration of NaCl was 65 mM, which was sufficient for aggregation of AuNPs and guaranteed the color change of visual detection.

Specificity of visual detection of *L. monocytogenes*

Based on our previous result[20], lmo0202*hly* gene fragment was used to be a novel molecular marker for detection of *L. monocytogenes*, and PCR product was expected as the 226 bp fragment. As we supposed that all of *L. monocytogenes* (39 strains) could gain about 226 bp fragments in lane 1-39 in Fig.2A according to our designing specific primers and simultaneously, other non- *L. monocytogenes* (8 strains) cannot be successfully amplified in lane 40-47 in Fig. 2A with the same primers, the results showed that the novel molecular marker and the PCR assay are very high species-specific to *L. monocytogenes*.

The color change is the prominent indicator which determines the accuracy of the visual detection of *L. monocytogenes* assay. In the experiment, there were many non-specific amplification based on negative controls. Simultaneously there existed some specific amplification because of *L. monocytogenes*. The PCR of negative controls could not combine with the specific probe because that there were lack of target temples in lane 3-10 in Fig. 2B, which lead to free probes to absorb on the surface of AuNPs, increased NaCl resistance, and prevented the aggregation of AuNPs induced by NaCl, and the color in mixtures is always red in lane 1 and 3-10 in Fig. 2B. In the other hand, the specific probes and the specific amplifications hybridized polymeric substance in lane 2 in Fig.2B, which lead to absence of probes, thus AuNPs could not be protected from salt induction to cause aggregation, and the color changed grey blue in lane 2in Fig.2B. Compared with PCR assay, visual detection assay was highly species-specific to *L. monocytogenes*; In addition, only 0.1μl of PCR product in visual detection assay could make the same detection conclusion as the 7 μl of PCR assay, which indicated that visual detection assay was seventy-fold (7/0.1) higher sensitivity than conventional PCR assay.

High sensitivity of the visual detection assay

In order to know the lowest detection limit of this new colorimetric assay system, PCR was performed on decreasing amounts of genome DNA. Mixtures of extensively amplified target, probes and AuNPs (lanes 1-7 in Fig.3B), changed gray blue after salt addition. Simultaneously, in cases where low DNA amplification yields could not appear in electrophoresis (lane 8-9) in Fig.3A, the solutions retained the red color after salt addition. the color difference among the various concentrations of DNA temples were clearly distinguishable owing to the strong aggregation of AuNPs occurring in the solution not containing enough templates. The observations described that as few as 1.304 fg/μl of the DNA of *L. monocytogenes* could be detected by observing the color change arising during the extended incubation time of 10 min following salt addition, it suggested that over the 1.304 fg/μl DNA temple could amplify enough specific PCR product to combine with the specific probes, so that there were not adequate free probes to prevent from strong aggregation of AuNPs after salt induction. The result implied the extent of amplification dominated the sensitivity of this colorimetric DNA detection methodology. In addition, 0.1μl of DNA used in visual detection method had consistent results with 7μl of DNA used in PCR, which indicated the visual detection of *L. monocytogenes* was more seventy fold sensitive than gel electrophoresis.

Discussion

L. monocytogenes spreads widely in fresh meat, aquatic product, vegetables, and ubiquitous and capable of growth at refrigeration temperature, which may contaminate foods at any point of production, distribution or storage. Nowadays, several methods have been developed to identify *L.*

monocytogenes infection. PCR has been a simple and rapid detection method for detection *L. monocytogenes*. In fact, the signal transduction gene, vicK has been identified as a molecular marker for detection of *Staphylococcus aureus* in our previous research based on comparative genome and sequences alignment analysis [26].With the similar strategy, 226-bp sequence (lmo0202 hly) has been identified as a novel molecular marker for detection of *L. monocytogenes* by means of bioinformatics in our previous research[20], and tested in the biological experiment in this study.

NaCl concentration is an important contributor to affect the color of AuNPs colloidal solution. Although single strand DNA probes adsorb on AuNPs to help them more stable and prevent their aggregation, the increase in NaCl concentration will destroy gradually the double electrostatic layer of AuNPs, and once NaCl concentration is over its critical value, AuNPs will aggregate unavoidably, and the solution turns blue. Certainly, the critical concentration of NaCl can vary according to various target, probes and AuNPs.

Except for NaCl concentration, the concentration of the probes also affects the AuNPs state. In the presence of the target, a very high probe concentration will not only hybrid with target, and the rest of probes can absorb the surface of AuNPs to prevent aggregation leading to a false negative result. On the other hand, in the absence of the target the low concentration probes absorb AuNPs, and excess AuNPs will aggregate after salt induction leading to a false positive result. Consequently, the optimal probe concentration was found to be 10 μM in the total assay volume.

The denaturation and annealing steps are deemed necessary before the addition of the AuNPs to increase the specificity of the assay, this because that the extent of PCR targets combination with the species-specific probe is the key of the successful assay, and increasing the time of the denaturation and annealing steps also increases the percentage of positive results. In our opinion, increasing the time of denaturation and annealing might increase the probability of the probes annealing specifically to species-specific PCR products in *L. monocytogenes*, hence, after adding the species-specific probes to PCR products, the mixture was subjected to denaturation at 95 °C for 2 min, annealing at 53 °C for 6min, and then cooling to room temperature for 10 min in our experiment.

Excellent sensitivity, specificity, and speed have made molecular assays an attractive alternative to culture or enzyme immunoassay methods. Wang et al reported the sensitivity of detection of *L. monocytogenes* by cross-priming amplification of lmo0733 gene is 2.5 pg DNA per reaction [19]; Liu et al described the sensitivity of a novel paper-based microfluidic enhanced chemiluminescence biosensor for detection of *L. monocytogenes* is 6.3×10^{-2} pmol/l of DNA[27]. In our experiment, the sensitivity of detection *L. monocytogenes* is 1.304 fg/μl of DNA temples, it means the sensitivity of the novel detection developed in our study is 1.304 fg DNA per reaction, and 1.98×10^{-2} pmol/l, all of these suggest visual detection of *Listeria monocytogenes* using unmodified gold nanoparticles based on *lmo0202hly* gene in our study has more sensitivity than others.

To our knowledge, this is the first approach employing probes for visual detection of *L. monocytogenes* using unmodified AuNPs. The new method requires only addition of AuNPs with the solution after PCR. Surface modification of AuNPs is not required. Although it still requires a PCR step, post-PCR analysis such as gel electrophoresis is eliminated in this method. As a consequence of its simplicity, the methodology should find wide application in assays performed

in small-scale corporations or in developing countries, where only inexpensive equipment and nonhighly trained personnel need to be available.

Conclusion

In this work, we took full advantage of interaction AuNPs with nucleic acids and developed a low-cost, facile, sensitive method for visual detection of *L. monocytogenes* with the unmodified AuNPs. The assay described is easily read with the naked eye. In comparison with other methods for detection of *L. monocytogenes*, the method is more attractive because of its high sensitivity, low cost, ready availability and simple manipulation. This is the first application of the unmodified AuNPs-based biosensing platform for detection of *L. monocytogenes*.

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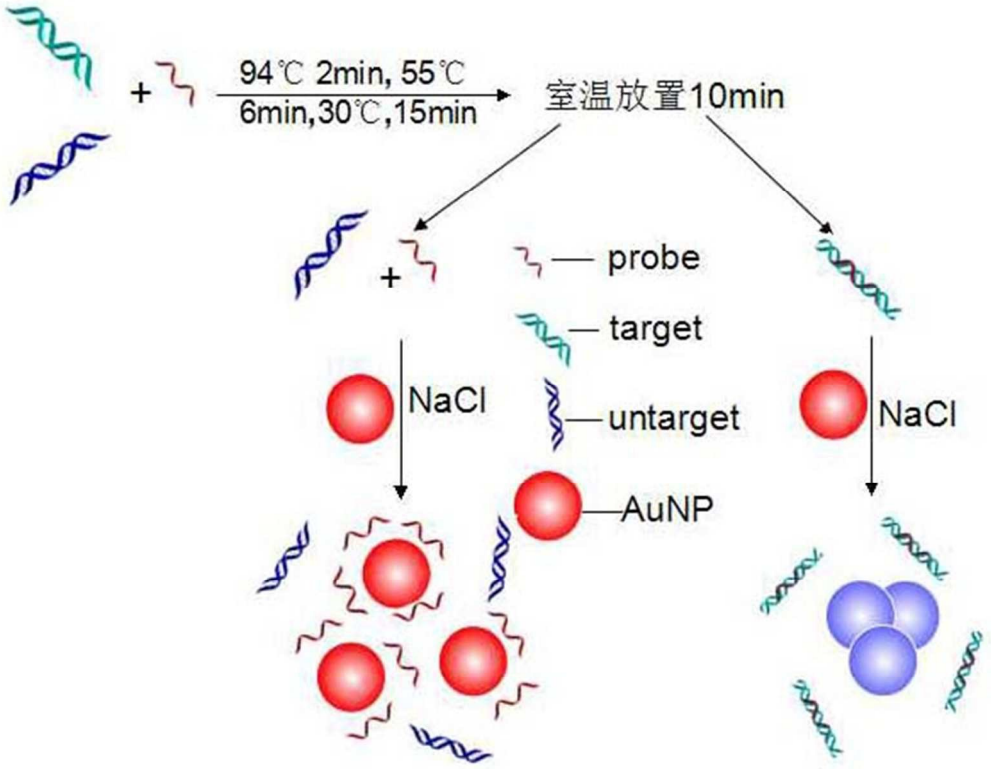

Scheme 1. Schematic illustration for visual detection of MCMV based on unmodified AuNPs.
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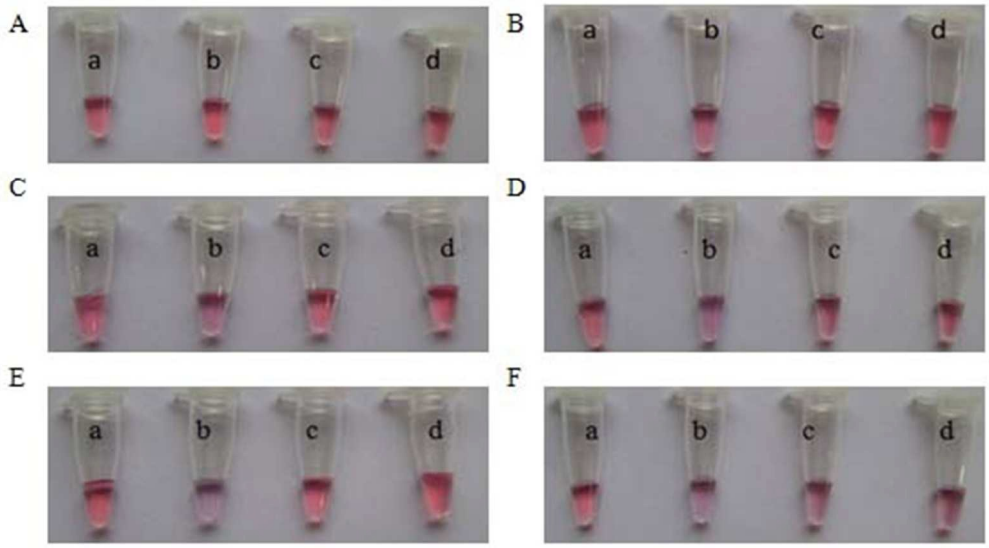

Fig. 1 Critical NaCl concentration for the color change of existing status of AuNPs collides. 35 μl of mixtures (containing 0.1 μl DNA or distilled water, 1μl of 10 μM probes, 30μl of AuNPs, and some distilled water) was subjected to denaturation at 95 °C for 2 min, annealing at 53 °C for 6min, and then cooling to room temperature for 10 min before adding different concentration of NaCl induction for 90 min. (a) distilled water as control; (b) 226-bp PCR of L. monocytogenes; (c) 476-bp RT-PCR of MCMV RNA; (d) 289-bp PCR of Staphylococcus aureus; (A) 40 mM NaCl; (B) 50 mM NaCl; (C) 60 mM NaCl; (D) 62 mM NaCl;(E) 65 mM NaCl; (F)70 mM NaCl.
192x106mm (72 x 72 DPI)

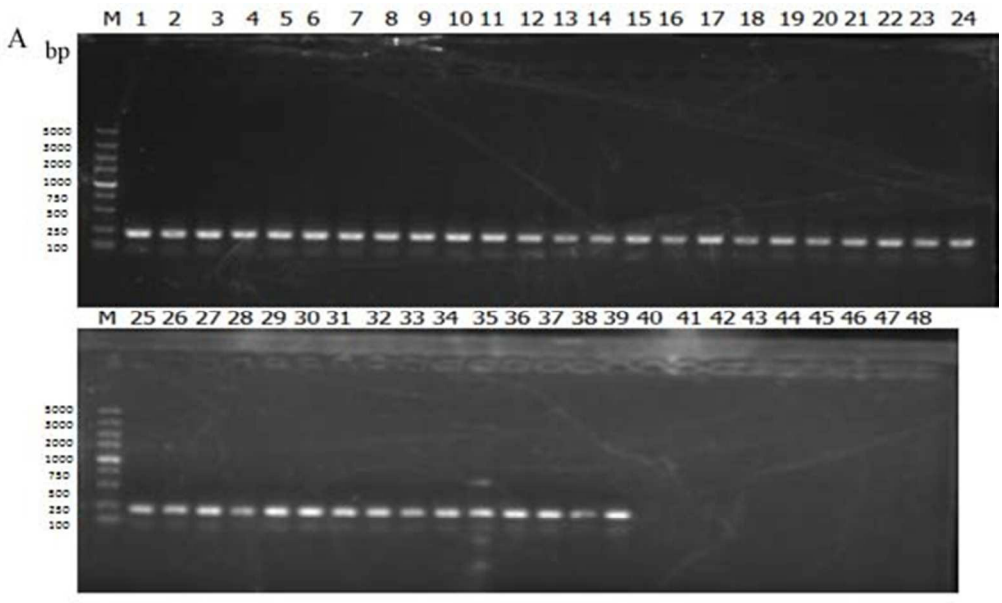

Fig. 2 Specificity of visual detection of L. monocytogenes. (A) Electrophoresis of PCR products from different microorganisms and control. M was the DNA ladder; Lane 1-39 represented L. monocytogenes; 40-47 were L.ivanuii, L.innocua, L.seeligeri, L.grayi, Listeria welshimeri, Salmonella choleraesuis, Salmonella typhimurium , Serratia marcescens, respectively; lane 48 was distilled water ; (B) Different results of PCR combination with a species-specific probe after salt induction. Lane 1 was distilled water and used as control; lane 2 was L. monocytogenes; lane 3-10 represented L. ivanuii, L. innocua, L. seeligeri, L. grayi, L. welshimeri, Salmonella choleraesuis, Salmonella typhimurium, Serratia marcescens, respectively.
196x121mm (72 x 72 DPI)

(B) Different results of PCR combination with a species-specific probe after salt induction. Lane 1 was distilled water and used as control; lane 2 was L. monocytogenes; lane 3-10 represented L.ivanuii, L.innocua, L.seeligeri, L.grayi, Listeria welshimeri, Salmonella choleraesuis, Salmonella typhimurium , Serratia marcescens, respectively.
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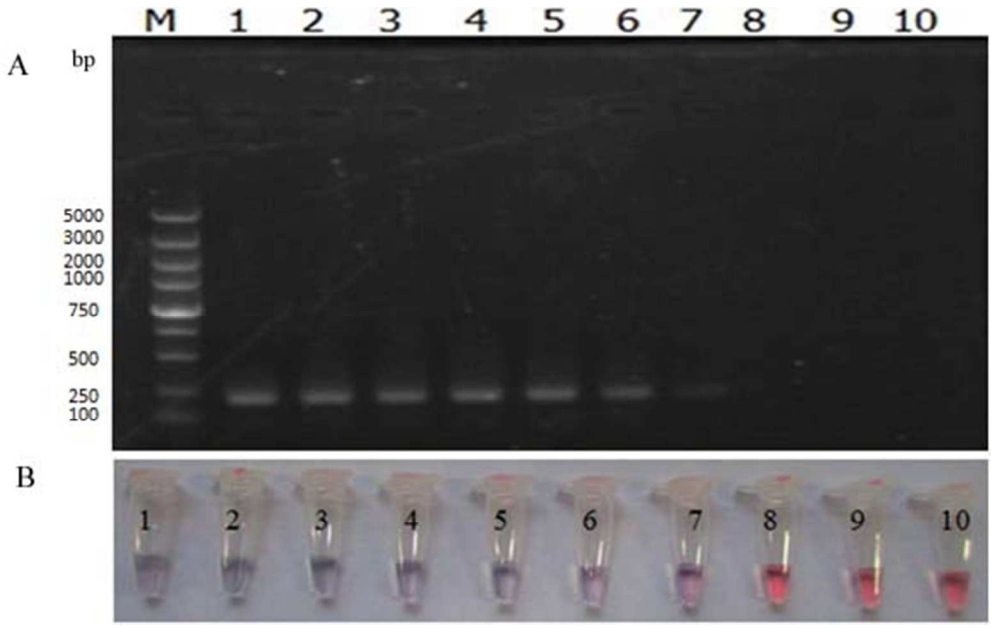

Fig.3 Sensitivity of visual detection of DNA of L. monocytogenes. (A) M: 5000 bp DNA ladder; lane 1: 1.304 ng/μl; lane 2 : 130.4 pg/μl; lane 3: 13.4 pg/μl; lane 4: 1.304 pg/μl; lane 5:130.4 fg/μl; lane 6: 13.4 fg/μl; lane 7: 1.304 fg/μl; lane 8: 0.1304 fg/μl; lane 9:0.01304 fg/μl; Lane 10: distilled water.
207x128mm (72 x 72 DPI)