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Sensitive colorimetric detection of *Listeria monocytogenes* based on isothermal gene amplification and unmodified gold nanoparticles

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ABSTRACT

Listeria monocytogenes (*L. monocytogenes*), one of most problematic food-borne bacteria, is mainly transmitted through the food chain and may cause listeriosis. Therefore, the development of rapid and sensitive *L. monocytogenes* detection technique has become an urgent task. In this study, we proposed a method using hyperbranching rolling circle amplification (HRCA) combined with gold nanoparticle (GNP) based colorimetric strategy to offer an isothermal, highly sensitive and specific assay for the detection of *L. monocytogenes*. First, a linear padlock probe targeting a specific sequence in the *hly* gene was designed and followed with a ligation by Taq DNA ligase. After ligation, further amplification by HRCA with a thiolated primer and an unlabeled primer is performed. The resulting thiolated HRCA products were then captured onto GNP surface and made GNP more salt-tolerant. Detection of the bacteria can be achieved by a facilitated GNP based colorimetric testing using naked eyes. Through this approach, as low as 100 aM synthetic *hly* gene targets and about 75 copies of *L. monocytogenes* can be detected. The specificity is evaluated by distinguishing target *L. monocytogenes* from other bacteria. The artificial contaminated food samples were also detected for its potential applications in real food detection. This method described here is ideal for bacteria detection due to its simplicity and high sensitivity.

1. Introduction

Listeria L. monocytogenes is a human pathogen widely distributed in environment [1]. Taking *L. monocytogenes* contaminated foods can result in listeriosis, resulting symptoms such as, meningitis, encephalitis, septicaemia, central nervous system damage and miscarriage. The fatality rate of *L. monocytogenes* has approached 30%, which far exceeds other foodborne pathogens [2–4]. The International Commission on Microbiological Specification on Foods has declared that food sample containing more than 100 CFU *L. monocytogenes* per gram at the point of consumption is considered unacceptable for individuals [2]. Thus, it is crucial to develop a rapid, specific and highly sensitive method for *L. monocytogenes* detection.

Conventional methods for the pathogenic bacteria detection are culture-based and require individual biochemical confirmation of the species in a number of isolated colonies [3]. These methods are labor-intensive, time consuming and not always reliable. Therefore, much effort has been devoted to the designing of new methods for efficient pathogen detection. Although other methods, such as the immunological based biosensors including immunoblotting, enzyme-linked immunosorbent assay (ELISA) [4,5] and electrochemical assays [6,7], have overcome the aforementioned drawbacks, they are not always the perfect choice because of their lack of sensitivity and specificity [8]. It has been revolutionized in the field of bacteria detection with the advent of PCR-based methods [9,10]. However, PCR is considered to be complicated for requiring thermal cycling steps and thus need sophisticated and expensive equipment, which have limited its use in many laboratories. Instead of PCR, recent developments in isothermal amplification techniques provide a wide variety of signal-amplification methods; in which rolling circle amplification (RCA) is an admirable technology due to its simplicity and high efficiency [14–17]. However, to be adopted in solution-based diagnostic and find a wider application, the sensitivity of RCA must be evidently enhanced [11]. Thus, the development of a highly sensitive, specific and low-cost method for food hygiene inspection remains a challenge.

Recently, hyperbranched rolling circle amplification (HRCA) has acquired significant attention as a powerful amplification method [12]. It initiates from hybridizing a linear padlock probe to a sample DNA and then ligated by DNA ligase to form a circular probe. The resulting circular probe can be used as the template for amplification, continue as a turn-by-turn cascade of multiple hybridization, primer extension and strand displacement involving two primers [13,14]. This simply procedure of DNA strand displacement ensures a 10^9-fold signal amplification of each circle within 90 min [12]. Moreover, as it can avoid complicated thermocycling and ensure high sensitivity, this technique can be applied in variety

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fields, such as DNA [15,16], RNA [17], and protein detection [18]. Currently, electrophoresis and fluorescence based methods are dominantly available for HRCA product detection, however, electrophoresis is generally considered as inefficiency due to its labor-intensive characteristic. For fluorescence based methods, the high background resulting from free fluorescent probes or dyes as well as the reflection and scattering of light source is a limitation [19].

Colorimetric assay is a simple, speedy and a direct visual detection without the need for any complicated equipment [27–29]. Gold nanoparticle (GNP) has been used as sensing material for colorimetric detection due to their unique optical properties and ease of surface modifications [30]. It presents a color change of GNP solution by modifying the surface of GNPs with thiolated oligonucleotide probes and followed by hybridising with the target DNA, due to the cross-linking reaction between nanoparticles. Furthermore, as it could selectively aggregate GNPs due to the different characteristics of the single and double stranded DNA, unmodified GNPs have been used in DNA analysis [20]. Thus, colorimetric method provides a possibility for a quick and inexpensive detection of various DNA sequences [21]. However, conventional GNP colorimetric assay is inapplicability in foodborne bacteria detection due to its lack of high sensitivity. Therefore, it is feasible by combining HRCA with GNP colorimetric strategy and applying them in food pathogenic bacteria detection.

This paper outlines a colorimetric assay for the HRCA amplified *L. monocytogenes hly* gene, which can be directly distinguished by naked eyes. The *hly* gene which encodes a cholesterol-dependent cytolysin responsible for clinical symptoms and is highly conserved in *L. monocytogenes* was chosen as a target for specific detection [22]. It is described here when thiolated HRCA primer is applied in *hly* gene amplification, HRCA products which thiolated on DNA double strands were obtained. Mixing unmodified GNP solution with the thiolated HRCA products could result in the formation of GNP-HRCA products, which are more salt-tolerant than HRCA primers linked to GNPs. The results show that the target pathogenic bacteria sample could be specifically recognised and detected. The whole strategy relies on the use of thiolated primers based HRCA and unmodified GNPs. Sensitive detection of foodborne pathogenic bacteria in artificial contaminated food samples was also permitted.

2. Materials and methods

2.1. Materials

All oligonucleotides were synthesised and then purified by Invitrogen. The padlock probes are chemically 5′-phosphorylated, the HRCA primer 1 are thiolated (Table 1). *L. monocytogenes* (CMCC54007), *Salmonellaenterica* (CMCC50040) and *Escherichiacoli O157:H7* (GW1.2020) are obtained from Guangzhou Institute of Microbiology China. $HAuCl_4$ (99.999%) and sodium citrate dehydrate were purchased from Sinopharm Group Chemical Regent Co., Ltd., Shanghai, China.

The Taq DNA ligase, Bst DNA polymerase large fragment and their corresponding buffer are purchased from New England Biolabs(NEB), the deoxynucleotide solution mixture (dNTPs), Exonuclease I, Exonuclease III and their corresponding buffer are purchased from Takara Bio (Shiga, Japan).

13 nm diameter GNPs were prepared by the citrate reduction of $HAuCl_4$. All glass ware was cleaned in aqua regia (3 parts HCl, 1 part HNO_3) and rinsed with pure H_2O. An aqueous solution of $HAuCl_4$ (1 mM, 500 mL) was brought to a reflux while stirring, and then 50 mL of a 38.8 mM trisodium citrate solution was added quickly which resulted in a change in solution color from pale yellow to deep red. After the color change, the solution was refluxed for additional 15 min to cool it to room temperature. The typical solution of these 13 nm diameter gold particles exhibited a characteristic surface plasmon band centred at 520 nm [23]. As 13 nm GNPs has a strong absorption band at 520 nm in the visible light spectrum, the UV–vis absorption spectroscopic picture for our GNPs was shown in Fig. 1A. To further illustrate the characteristic feature of GNPs in our colorimetric experiment, GNPs were characterizeed by transmission electron microscopy (TEM) (Fig. 1B).

2.2. Genomic DNA extraction

L. monocytogenes was grown in brain heart infusion broth at 37 °C with shaking overnight. The bacteria DNA isolated from 1 mL cultures was extracted according to the manufacturer's protocol from the TIANamp Bacteria DNA Kit which was purchased from Tiangen biotech (Beijing) Co., LTD (Beijing, China) and quantified by measuring the optical density at 260 nm with a spectrophotometer (Eppendorf BioPhotometer, AG 22331 Hamburg, Germany).

2.3. Ligation and exonuclease treatment

Genomic DNA was fragmented by digestion using Bsp1286 I and EcoT14 I for 60 min and then used as template. Hybridization of target sequences with the padlock probes was carried out in 20 μL ligation system containing 20 mM Tris-HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol, 1 mM nicotinamide adenosine dinucleotides, 0.1% Triton X-100 and 12 U of Taq DNA ligase, 100 nM linear padlock probes, different concentration of *hly* gene or genomic DNA template. The mixture was incubated at 60 °C for 60 min, 95 °C for 10 min to inactivate the enzyme. After that, 10 μL ligation products were added to 10 μL exonuclease mixture containing 67 mM Glycine-KOH (PH 9.5), 1 mM DTT, 6.7 mM $MgCl_2$, 10 U Exonuclease I and 20 U Exonuclease III. The mixture was incubated at 37 °C for 2 h, followed by inactivation at 95 °C for 10 min, then purified according to the manufacturer's protocol from the Dr.GenTLE® Precipitation Carrier Kit which were purchased from Takara Bio (Shiga, Japan).

Table 1
DNA sequences of probes for HRCA.

DNA	Sequence(5′-3′)
Padlock probe	Phosphate-GCGTCTTAGGACTTGCAGGCGG**ATTAGGTTACTGCGATTAGCACAAG***CACCAAGAGCAACTACACGAATTC*CTGTTTCTTTTCGATTG
Hly target	TCTCCGCCTGCAAGTCCTAAGACGA|CAATCGAAAAGAAACACGC
HRCA primer 1	SH-CTTGTGCTAATCGCAGTAACCTAAT
HRCA primer 2	CTTGTGCTAATCGCAGTAACCTAAT
HRCA primer 3	ACCAAGAGCAACTACACGAATTC

Single underlining indicates sequences complimentary to *hly* genes of pathogenic *L. monocytes*; underlining in bold face type indicates the binding region for thiolated primer 1; italics indicates the binding region for primer 2. As to *hly* target, single underlining indicates the padlock region, the vertical bar indicates ligation site of the 5′ and 3′ ends of the padlock.

2.4. HRCA reaction

After ligation and circularization of the probe, a followed step was performed to amplify the circular probe by using Bst DNA polymerase with strand displacing activity. HRCA reactions were performed in a 60 μl volume containing 8 U Bst DNA polymerase, 400 μM deoxynucleoside triphosphate mix, 1 μM of each HRCA primer and 20 μl of the post-digestion mixture. Circularized probe signals were amplified by incubation at 63 °C.

2.5. GNPs-based colorimetric assay

The GNPs extinct peak is at 520 nm. Its concentration was calculated to be about 10 nM, assuming all the $HAuCl_4$ was reacted. For the colorimetric assay, 6 μL of HRCA product was added to 50 μL of the gold colloid and after mixing for some minutes, additional 4 μL 2 M NaCl was added. Then the resulted solutions can be observed by naked eye and/or quantified by UV–vis absorption spectra (Perkin Elmer Instruments, USA). Due to the difference between the aggregation degree of GNP between absorbance at wavelength 520 and 650 nm, the ratio of Absorbance 520/Absorbance 650 (Abs 650/Abs 520) were chosen to examine the effect of proposed method.

3. Result and discussion

3.1. The principle of HRCA based GNP colorimetric assay for detecting target DNA

The basic principle of the proposed method is illustrated in Scheme 1. In colorimetric assay, the key is the control of the colloidal GNPs dispersion and aggregation [24]. Mainly, the stabilization or aggregation of GNP was caused by the net potential between interparticle attractive and pulsive forces [25]. The crucial factor for GNP electrostatic stabilization was the surface charges form a repulsive electric double layer that stabilizes colloids against Vander Waals attractive forces [26]. High sensitivity to the bulk ionic strength is a characteristic feature of electrostatic repulsion. When the electric double layer is highly suppressed, the force of electrostatic repulsion diminishes significantly at high salt concentrations [27]. This is the origin that citrate-capped GNPs are stabilized in water, but aggregated at elevated salt concentrations [24]. Therefore, taking advantage of double strand DNA has plenty of negative charges on its surface, we designed our strategy that bind amplified DNA to GNP surface in *L. monocytogenes* detection.

In the HRCA, the padlock probe includes four distinct regions: a special region hybridized with thiolated primer, a region that is the same as the unlabeled primer, and two target complementary sequences located at the 5′ and 3′ termini, and is 89 nucleotides (nt) in length. The linear padlock probe can be specifically ligated and circularized by the Taq DNA ligase when the two termini of padlock probe are perfectly hybridized with the target sequences. Then un-reacted padlock and excess oligonucleotides in the ligation products were digested by Exonuclease I and III [28] in order to reduce subsequent ligation-independent amplification. Following with digestion, the thiolated primers which hybridized with a special region of resulting circular padlock probes are extended isothermally at its 3′ end by Bst DNA polymerase and generates multimeric single-stranded DNA (ssDNA). This ssDNA can be further amplified by HRCA through unlabeled primer extension and downstream DNA displacement; this displaced strand will in turn contain multiple binding sites for thiolated primer. Thus, strand placement processes generate a continuously expanding pattern of DNA branches connected to the original circle and a discrete

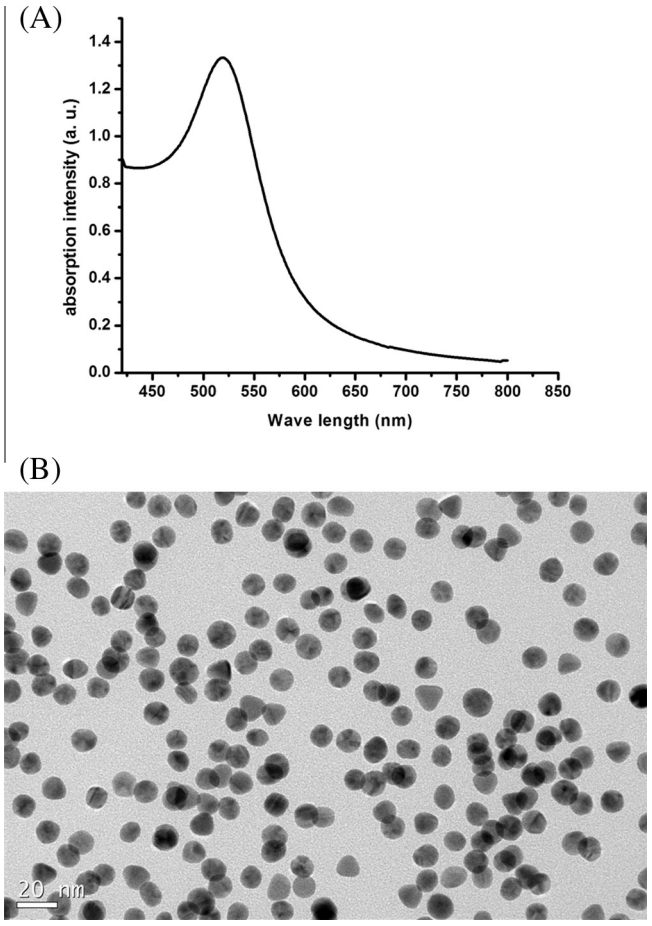
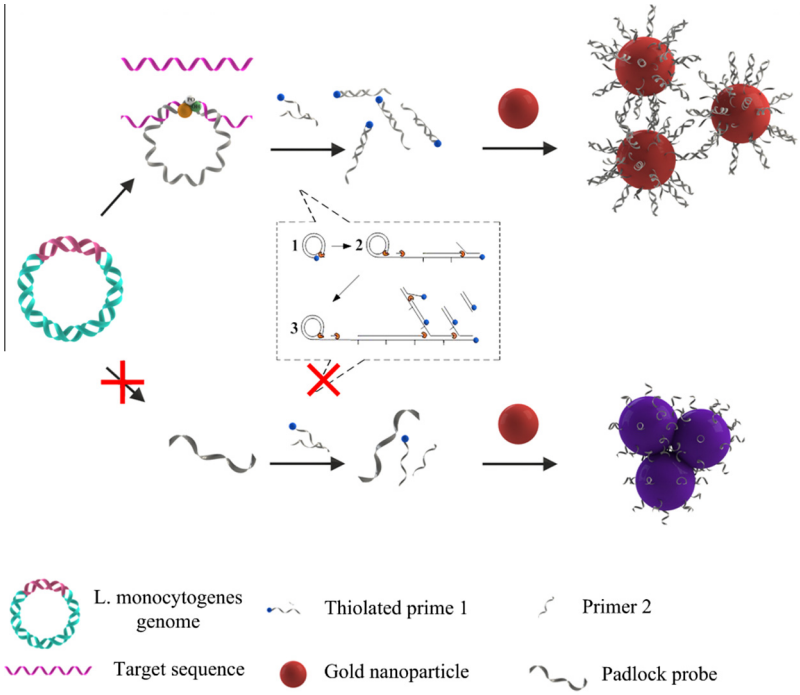


Fig. 1. Characterization of GNPs with UV–vis absorption spectroscopy (A) and transmission electron microscopy (B).

set of free thiolated DNA fragments comprising double-stranded pieces of the unit length of a circle and mutiples thereof.

The resulted DNA strands would bind to unmodified GNPs surface and creates SH–Au bond when adding the HRCA products into GNP solution [29]. A thick barrier containing negative charges has built up on the surface of GNPs by these DNA strands. This barrier results in repulsion between DNA-bound GNPs, which indicates negative tendency to a color change from red to blue as the result of the salt-induced aggregation. Meanwhile, thiolated HRCA primer may not be extended and, instead, it would bind to GNPs surface itself after the HRCA reaction when there is no target DNA presents in HRCA mixture. As the primers contain considerably less bases than the amplified HRCA products, primer-GNPs would be less salt-tolerant than those of HRCA product-bound GNPs, which undergoes an obvious color change after salt concentration adjusting and can be observed by naked eyes. Moreover, GNPs in the unlabelled HRCA product would aggregate easily after the addition of salt, with a rapid color change from red to blue [30].

In order to validate the HRCA efficiency, HRCA and gel electrophoresis experiment were performed for analyzing the amplified products. Fig. 2 shows both HRCA results amplified by thiolated primers and unlabelled primers. It is found that the amplification efficiency of HRCA by thiolated primer and unlabelled primer were almost identical. It would be rational to assume that when applying thiolated HRCA primer, the *hly* gene in HRCA method could be amplified as same as HRCA using unlabelled primers. Based on this assumption, the colorimetric assay was introduced to reinforce designated strategy. The average time that takes to run the entire measurement for *L. monocytogenes* includes the preparation of



Scheme 1. Strategy for the HRCA based gold nanoparticle colorimetric assay for detection of *L. Monocytogenes*.

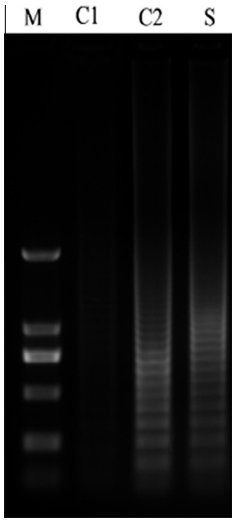


Fig. 2. Agarose gel electrophoresis result of HRCA method. Lane M represents marker, the C1 lane represents HRCA reaction without target sequence using thiol-labeled primer, the C2 lane represents HRCA reaction using unlabeled primer with target sequence in the system, and the S lane was the HRCA reaction using thiol-labeled primer with target sequence in the system.

hly segments, whole HRCA running time and the time cost in colorimetric assay. The preparation of *hly* segments takes about 2 h (either from food sample or from bacteria culture). The HRCA amplification takes about 2 h. The whole colorimetric assay takes about 10–30 min, depends on using a naked eye qualitative observation or semi-quantitative spectral detection. Therefore, the entire detection process is expected to complete within 4–5 h.

3.2. The sensitivity of HRCA based GNP colorimetric assay

To define *L. monocytogenes hly* gene is existed in the detect sample, a cut-off value was calculated base on the average $V_{control}$ standard deviation V_{dev} of the Absorption spectroscopic analysis reading from the control sample, shown as in the following fomula:

$$V_{LOD} = V_{control} + 3V_{dev}$$

To evaluate the sensitivity of HRCA-GNP colorimetric method, *hly* target was serially diluted from 100 nM to 100 aM, and the 12 different concentrations of *hly* target were amplified and then colorimetric assays were carried out. As shown in Fig. 3A, after added 4 μL 2 M NaCl respectively in every colorimetric sample to adjust the salt level, a dramatic red shift can be observed with the decreasing of target concentration from the absorbance spectra of the detection samples. Compared with the case above, abroad SPR band (530–650 nm) had appeared in the solution containing the unlabeled product. Meanwhile, abroad SPR band (530–650 nm) could be observed in the solution containing the thiolated primer product with the absence of *hly* gene. The color change of GNPs was shown in the inset photo in Fig. 3A. Due to the differences between the aggregation degree of GNP between absorbance at wavelength 520 and 650 nm, the absorbance intensity ratio of Absorbance 520/Absorbance 650 (Abs 650/Abs 520) were chosen to examine the effect of proposed method. In Fig. 3B, the result of absorption spectroscopic analysis shows that as little as 100 aM *hly* sequence DNA can be detected. At this concentration, the Abs 520/Abs 650 of *hly* gene was 1.2 ± 0.12. The control protocol suggests that the Abs 520/Abs 650 of GNPs mixed with unlabelled HRCA products was 0.86 ± 0.06 and of the GNPs mixed with thiol-labeled HRCA primer was 0.92 ± 0.06, respectively.

Fig. 3B indicates that the absorbance intensity ratio Abs 520/Abs 650 increased linearly with the increasing of target concentration from 100 aM to 10 pM, the *X* axle in Fig. 3B presents the *hly* gene concentration(10^{x-8} nM) and the best fit line is:

$$Y = 0.25x + 0.95$$

To investigate the precision of the proposed assay, Relative standard deviation (RSD) was determined by measuring absorption spectroscopic at fixed concentration with six replicates. RSD of about 10% for detecting for the concentration of 1 fM target is achieved, which suggests an acceptable reproducibility.

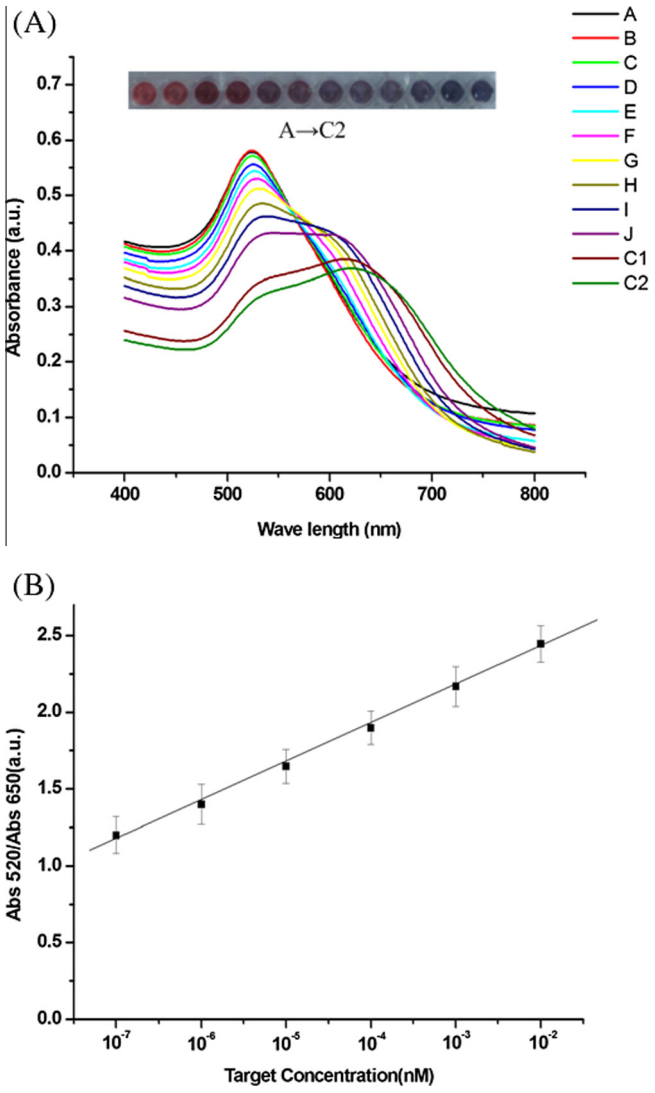


Fig. 3. Sensitivity of target sequence in colorimetric assay. (A) A to J represent colorimetric results while the HRCA reaction with target concentration from 100 nM to 100 aM (A was sample with 100 nM target DNA, B was 10 nM and J was 100 aM), C1 represents HRCA reaction without target sequence using tihol-labeled primer, and C2 represents HRCA reaction using unlabeled primer with target sequence in the system. The inner photo shows the color change of GNPs. (B) Linear analysis of Abs 520/Abs 650 from different target concentration of the colorimetric assay.

The high sensitivity of GNP colorimetric-HRCA assay in detecting *hly* gene was well demonstrated by the results of sensitivity testing. The factors that affecting the limit of detection mainly includes two points: HRCA amplification efficiency and the optimal salt concentration added in the colorimetric system. In current assay, the Bst DNA polymerase has shown a strong and reliable ability to displace newly synthesized DNA strands under isothermal conditions, so a large number of thiolated DNA fragments will bind with the GNPs. In this colorimetric system regulated by salt, the salt concentration not only affects the dynamic range of the method, but also affects the detection sensitivity. In this assay, the best sensitivity result was obtained by employing 4 μL 2 M NaCl.

3.3. Detection of bacterial genomic DNA

To evaluate the sensitivity of HRCA-GNP colorimetric method, *L. monocytogenes* genome was calculated from mass fraction to copy number, and diluted from 7.5×10^4 copies to 75 copies, and the 4 different concentrations of *L. monocytogenes* genome were amplified and then colorimetric assays were carried out, as shown in Fig. 4A. It is shown that the detection limit down to 75 copies/reaction was achieved, in which absorbance intensity ratio (Abs 520/Abs 650) was 1.41 ± 0.10. The control protocol suggests that the Abs 520/Abs 650 of GNPs mixed with unlabelled HRCA products was 1.00 ± 0.10 and of the GNPs mixed with thiolated non-target HRCA products was 1.01 ± 0.10, respectively. The color change of GNPs was shown in the inset photo in Fig. 4A.

Fig. 4B indicates that the absorbance intensity ratio Abs 520/Abs 650 increased linearly with the increasing of target, the X axle in Fig. 3B presents the quantity of *L. Monocytogenes* genome (7.5×10^x copies), and the best fit line is:

$$Y = 0.27x + 1.14$$

The factors that affecting the limit of detection mainly includes the aforementioned two points: HRCA amplification efficiency and the optimal salt concentration added in the colorimetric system in

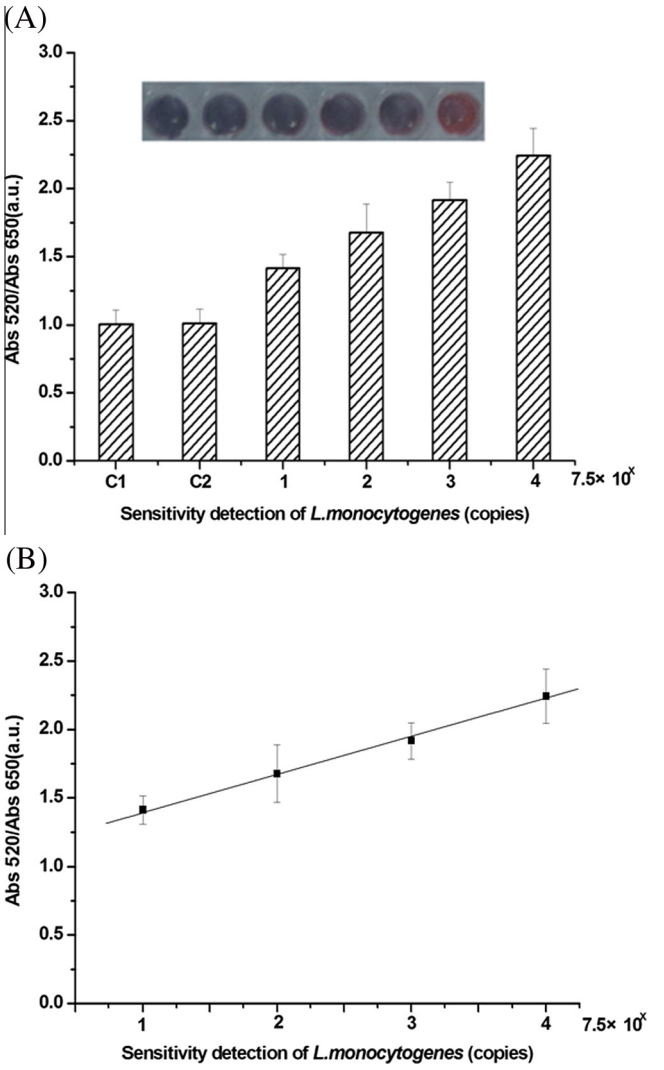


Fig. 4. Sensitivity testing of *L. monocytogenes* genomic DNA in colorimetric assay (A) The absorbance intensity ratio (Abs 520/Abs 650) from different concentration of *L. monocytogenes*. C1 represents HRCA reaction without target sequence using tihol-labeled primer and C2 represents HRCA reaction using unlabeled primer with target sequence in the system. The inner photo shows the color change of GNPs. (B) Linear analysis of Abs 520/Abs 650 from different target concentration of the colorimetric assay.

current assay. Moreover, the exonuclease has shown a strong and reliable ability to digest *L. monocytogenes* genome while short *hly* fragments were achieved, which brings this method a high specificity.

3.4. The specificity of HRCA based GNP colorimetric assay

To validate the specificity of this method, GNP colorimetric HRCA was applied to detect genomic DNA from other foodborne pathogens, *L. monocytogenes, S. enterica* and *E. coli*. Padlock probe was designed to detect a complementary strand of its virulence gene, *hly* (GeneBank GeneID 223702383), encoding a cholesterol-dependent cytolysin, listeriolysin O (LLO) [22]. When the linear padlock probe hybridizes to the target gene, the circular padlock probe can be formed to start HRCA. BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov) search of the 38-base target in *hly* gene showed highly specific for *L. monocytogenes*, and no exact match against *S. enterica*, *E. coli* genome.

For the *L. monocytogenes* detection, we applied thiolated HRCA primer of *hly* gene in *L. monocytogenes*, *S. enterica* and *E. coli* genome amplification. In the colorimetric assay, the resulted HRCA products were added to GNP solution. After adding 4 μL 2 M NaCl respectively in every colorimetric sample to adjust the salt level, an obvious color change was shown in the GNPs solution containing non-amplified HRCA product, while the GNPs solution containing thiolated HRCA products remained red. This result could be identified with naked eyes. The result of absorption spectroscopic analysis shows that the Abs 520/Abs 650 of 7.5×10^4 copies of *L. monocytogenes* genes was 2.30 ± 0.20. This result could be easily specified with the non-amplified control samples (Fig. 5).

It was shown that the proposed method was highly selective to *L. monocytogenes* over other bacteria, which attributes to the specific padlock probe, in which the *Tm* value of 5′ arm was close to or above the ligation temperature and the *Tm* value of 3′ arm is 10–15 °C below ligation temperature. Thus, the 5′ end arm served as an anchor sequence and the 3′ end arm was an equilibrium process. According to BLAST result, the 5′ arm was selected highly specific for *L. monocytogenes*. Therefore, the presented assay could detect *L. monocytogenes* with high specificity.

3.5. Detection of L. monocytogenes genomic DNA in food samples

In order to evaluate its potential capability in food pathogenic bacteria detection, GNP colorimetric-HRCA assays were applied

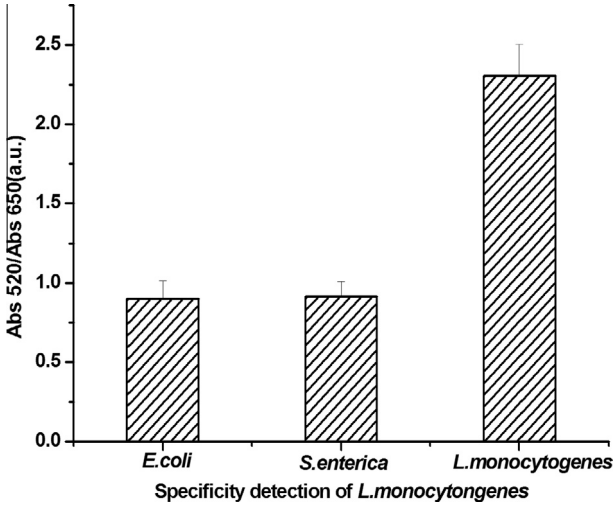


Fig. 5. Specificity testing of colorimetric assay of *L. monocytogenes*.

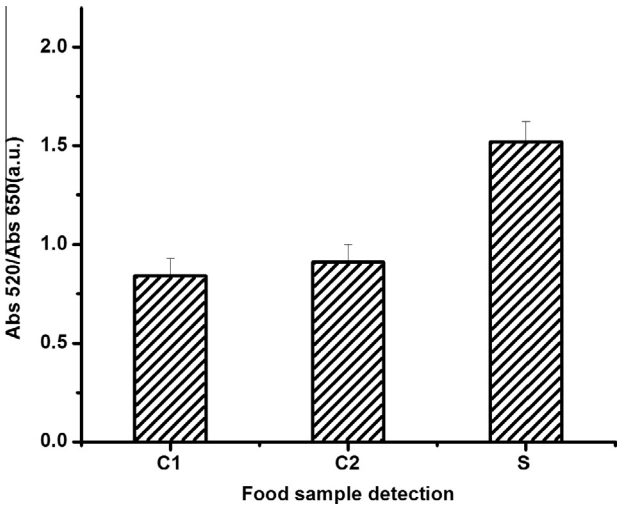


Fig. 6. Detection of *L. monocytogenes* contaminated milk, analysis of Abs 520/Abs 650 for GNPs mixed with HRCA products: (S) HRCA products from *L. monocytogenes* contaminated milk. (C2) Unlabelled HRCA products from *L. monocytogenes* contaminated milk, (C2) HRCA products from clean milk.

in food sample detection. A group of milk samples were artificially contaminated with an overnight culture of *L. monocytogenes*. The DNA were isolated from 1 mL culture after an overnight incubation at 37 °C and subjected to HRCA. The genome concentration of bacteria isolated from the *L. monocytogenes* contaminated milk was 2.8 μg mL^{-1}, includes genomes of bacteria that exist in milk and *L. monocytogenes*. The assays were carried out in either the presence or absence of the target template using the predetermined primer set; thiolated primers were used in clean milk as controls. The results of these assays are showed in Fig. 6. In Fig 6, when thiolated primers were used in clean milk, after adding 4 μL 2 M NaCl the colour of GNP solution changed from red to blue, and the Abs 520/Abs 650 value was 0.91 ± 0.09. This result suggests that salt induced an aggregation of the GNPs. On the other hand, if the target template was present, when thiolated primer was used in contaminated milk, the GNP solution remained red color after adding 4 μL 2 M NaCl to adjust the salt level. This result suggests that the GNP were still remaining dispersed, while the Abs 520/Abs 650 of *hly* gene was 1.52 ± 0.10. When it comes to the HRCA product using unlabeled primer, the GNP solution turned blue after salt addition. The differences among the samples and the controls could be clearly distinguished by analyzing the absorption spectroscopic result.

4. Conclusions

In summary, HRCA based GNP colorimetric method is novel and useful to detect *L. monocytogenes*. Taking the advantages of the high amplification efficiency of HRCA, the speediness and convenience of colorimetric assay, this method leads to a limit of detection of 100 aM *hly* gene with high specificity. The HRCA colorimetric method presented here is sensitive, specific, and robust, which has advantages over the PCR method. Moreover, since HRCA could avoid complicated thermal cycling steps, heating in a short of time using a simple incubator such as a water bath or a block heater is sufficient to amplify DNA to detectable levels. Furthermore, the proposed method could detect food pathogenic bacteria even by naked eyes, so that this technique entails no specialised devices. This can also significantly reduce the cost. To sum up, it is anticipated that the proposed method has enormous potential for a variety of biorelated detection.

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