



Loop-mediated isothermal amplification for visual detection of *Vibrio parahaemolyticus* using gold nanoparticles

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Abstract

Loop-mediated isothermal amplification (LAMP) eradicates the need of thermocycler in DNA amplification. Signals are usually obtained via fluorometry or turbidimetry, but such methods need improvement in order to become more effortless and reliable. The authors describe a set of six specific primers targeting the species-specific *tlh* gene of *Vibrio parahaemolyticus* which were used in accelerated LAMP reaction. Gold nanoparticles (AuNPs) were functionalized with streptavidin (Avidin-AuNPs), and engineered to signal the LAMP reaction. Two of the loop primers for LAMP were biotinylated and then can produce a DNA that can cause clusterization of Avidin-AuNPs based on the formation of avidin-biotin complex. This leads to a color change of the solution from red to blue. Amplification is completed within 30 min and can be visually detected within 5 min. The detection limit of the method is found to be 8.6 cfu per reaction. This visual detection scheme does not require any fluorescent reagents and detection instruments. Conceivably, the method has a wide scope because such Avidin-AuNPs can be used as nanoprobes for a variety of other LAMP products. This rapid and universal strategy holds promise in point of care testing and food testing, particularly in resource-limited regions.

Keywords Electrophoresis · Gene detection · Point of care detection · Field detection · Streptavidin · Biotin · Nucleic acid amplification · Nanosensor

Introduction

Vibrio parahaemolyticus (*V. parahaemolyticus*), a Gram-negative, halophilic marine bacterium, is one of the most prevalent seafood-borne pathogens worldwide, causing acute

gastroenteritis associated with exposure of raw or undercooked seafood in the diet [1]. Early diagnosis and timely treatments are critical to avoid subsequent complications. Therefore, the accurate detection of *V. parahaemolyticus* in food samples is of the utmost importance and urgent necessity regarding the public health [2]. The most commonly used methods for the identification and quantification of *V. parahaemolyticus* can be sorted as biochemical and nucleic acid amplification based methods. API 20E system has been considered as ‘gold standard’ for biochemical identification of bacteria in clinical and food laboratories, including *V. parahaemolyticus* [3]. However, the operation procedures are quite time-consuming and laborious. Moreover, it shows non-specificity between similar species and inability to the non-cultivable bacteria [4]. Besides, conventional PCR methods require well-trained skills, sophisticated procedures, well-prepared laboratory environment, and expensive reagents [5]. As a result, there is a demand of rapid, sensitive, specific, effortless and low-cost methods for the detection of *V. parahaemolyticus* [6, 7].

Loop-mediated isothermal amplification (LAMP) can directly amplify sequences of DNA under isothermal conditions

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(60–65 °C) [8] due to the intrinsic strand displacement activity of *Bacillus stearothermophilus* (Bst) DNA polymerase. It does not require the complicated and expensive thermocycler. [9]. In particular, these reaction can be initiated with few DNA targets and are less sensitive to inhibitor substances, which usually eradicates the need for high-quality extracted DNA [10]. The final product can be identified qualitatively or quantitatively through its turbidity derived from magnesium pyrophosphate precipitation or fluorescent signal by indicators [11, 12]. It holds high promise for use in field-friendly detection, at the point of care tests or in resource-limited regions. Therefore, the LAMP is a promising candidate in numerous fields related to molecular amplification detection [13]. However, the visible white precipitation-induced turbidity is usually too weak to be observed by unaided eye, and can lead to inter-observer bias [14]. Also, Calcein fluorescent indicator is less noticeable compared to conventional nucleic acid dye [15]. For this purpose, there are several reports aiming to improve these methods or explore novel strategies for its product detection [16–18]. As LAMP usually needs four primers to initiate and continue reactions, Notomi et al. further developed an accelerated LAMP reaction by using two more loop primers. Less than half of the time was achieved compared to original LAMP method [19].

Gold Nanoparticles (AuNPs), an ideal loader for nanoprobe after binding with recognition unit [20], can offer amplified signal readout for various detection strategies [21, 22]. The aggregation of AuNPs, introduced through the recognition process of nanoprobe, normally results in an apparent color change from red to blue, which can be attributed to the size and distance dependent optical properties of these particles [23, 24]. The process is favorable to be visually read out with unaided eye to identify the positive or negative sensing process without any instrument. Therefore, AuNPs based colorimetric strategies were preferable for the development of effortless, fast, field-directed detection methods.

Based on the advantages of LAMP and AuNPs sensing which combines rapid, convenient and on-site techniques of nucleic acid detection, we aimed to establish a simplified and generalized method that specifically amplify the target gene by LAMP and detect through amplification induced AuNPs aggregation. Considering the importance of *V. parahaemolyticus* detection, it was chosen as a potential target. The *tlh* gene, encoding thermolabile hemolysin, has been regarded as a reliable specific marker for *V. parahaemolyticus* for various detection methods [25]. In this research, an accelerated LAMP method was tested by a set of six specific primers for *tlh* gene. As is shown in Fig. 1, the two loop primers (TLH-LF, TLH-LB) were modified with biotin, which may bind with streptavidin functionalized AuNPs (Avidin-AuNPs) after the LAMP process and may result in a clustered Avidin-AuNPs in the solution mediated by the amplified DNA product. The aggregation of Avidin-

AuNPs offers an obvious color change from red to blue, indicating the presence of *tlh* gene. As a result, the positive detection of *V. parahaemolyticus* can be achieved. Apparently, the color of Avidin-AuNPs solution should keep red for negative detection of the target as no aggregation occurred. The strategy was developed and the performance was examined in seafood matrix.

Experimental

Information about the reagents, bacterial strains, culture media, instruments and primers are included in the electronic supplementary material.

AuNPs preparation

AuNPs were prepared according to previously reported research [26]. Briefly, to 50 mL of deionized water, 0.5 mL of 1% HAuCl₄·3H₂O was added, and the mixture was stirred for 1 min and heated to boil. Then, 500 μL of 1% sodium citrate was added under vigorous stirring. The color of the mixture changed from yellow to red within 2 mins. The solution was kept stirring and boiling for 15 mins and then left to restore to room temperature with stirring. After filtered with 0.8 μm Gelman membrane, the solution showed deep red color with a substantial absorbance peak at 527 nm. The final AuNPs was transferred in a brown bottle and kept at 4 °C, which is ready to be used within one month.

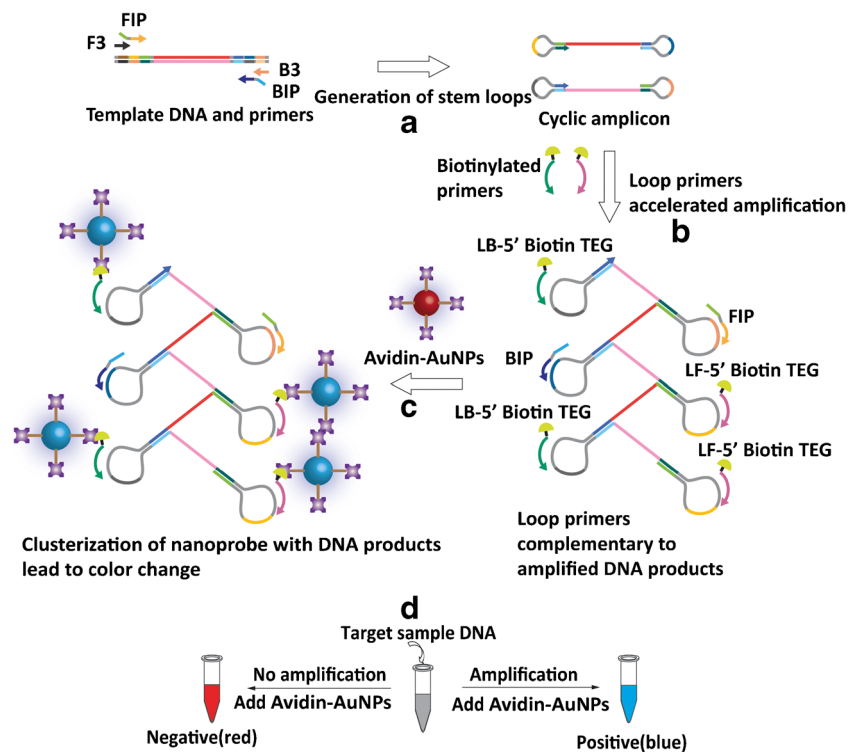
Streptavidin functionalized AuNPs

The streptavidin functionalization on AuNPs was carried out according to previous literature with modification [27]. Firstly, the pH of high concentrated AuNPs was adjusted to 7.4 using 0.1 mol·L⁻¹ K₂CO₃. After that, it was diluted until its absorbance dropped to c.a. 1.5 at 450 nm. Then, 1 mL of 33 μg·mL⁻¹ streptavidin (in water) was added into 10 mL of the prepared AuNPs solution. The mixture was stirred for 5 mins, and 0.1 mL of 10% PEG20000 was added afterwards. The mixed solution was remained still for 1 h, and centrifuged for 30 mins at 15000×g, followed by removal of the supernatant. The bottom Avidin-AuNPs was resuspended in 3 mL of 10 mM Tris-HCl buffer (pH 8.0) containing 0.5 mg·mL⁻¹ PEG20000 solution. After the addition of 300 μL of 1 mg·mL⁻¹ NaN₃, the solution was stored in the refrigerator for further use.

Bacterial isolation and DNA extract

V. parahaemolyticus were isolated and cultured in 3% NaCl alkaline peptone water under 37 °C for 16 h according to the national standard of China (GB 4789.7–2013). After

Fig. 1 Schematic illustration of the nanosensing of Loop-mediated isothermal amplification. The target gene is amplified through initially four primers to get cyclic amplicon for abundant DNA production(a), and the additional two biotinylated loop primers will also take part in the following amplification, resulting in cauliflower-like DNA structure (b), the Avidin-AuNPs, as a nanoprobe, bind to biotinylated site on the DNA clusters, forming the color change from red to blue(c). In the whole process, the presence of target gene results in a blue color, while the negative result would keep its original red in the reaction tube (d)



overnight grown, the well mixed bacterial suspension was kept still for 5 mins. And then 5 mL of the suspension was transferred in a 10 mL sterile tube, centrifuged at $6500\times g$ for 2 mins. After decanting the supernatant, 1 mL of cell lysate containing 2.0% Triton X-100, $2.5 \text{ mg}\cdot\text{mL}^{-1}$ NaN_3 and $0.1 \text{ mol}\cdot\text{L}^{-1}$ Tris-HCl buffer (pH 8.0) was added and bathed with boiled water for 10 mins, quickly chilled in ice bath for 10 mins. At last, the solution was centrifuged at $11000\times g$ for 5 mins, and the supernatant was kept frozen and was served as DNA template in the next step.

DNA extract for other non-*V. parahaemolyticus* strains was performed according to the similar procedures, except that the different culture mediums were employed, in which *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Vibrio vulnificus* were inoculated into LB Broth, 7.5% NaCl, LB1 Broth and 3% NaCl alkaline peptone water respectively. Finally, these strains were incubated overnight at 37°C before DNA extract.

LAMP

LAMP was performed with a final $40 \mu\text{L}$ of reaction solution containing $2.0 \mu\text{mol}\cdot\text{L}^{-1}$ each of TLH-FIP and TLH-BIP, $0.3 \mu\text{mol}\cdot\text{L}^{-1}$ each of TLH-F3 and TLH-B3, $1 \mu\text{mol}\cdot\text{L}^{-1}$ each of TLH-LF and TLH-LB, $20 \text{ mmol}\cdot\text{L}^{-1}$ of Tris-HCl (pH 8.8), $1.2 \text{ mol}\cdot\text{L}^{-1}$ Betaine, $7 \text{ mmol}\cdot\text{L}^{-1}$ of MgSO_4 , $10 \text{ mmol}\cdot\text{L}^{-1}$ each of KCl and $(\text{NH}_4)_2\text{SO}_4$, 0.1% Tween 20, $2.0 \text{ mmol}\cdot\text{L}^{-1}$ each of dNTP, $5 \text{ U}\cdot\mu\text{L}^{-1}$ of Bst polymerase large fragment and $2 \mu\text{L}$ DNA template sample. The mixture was vortexed and

centrifuged before incubation at 62°C in water bath for 30 mins, and then at 80°C for 5 mins to terminate the reaction. All assays were conducted in 3 replicates with certified *tlh* gene extract or pure sterile water as a positive or negative control.

LAMP product detection

The LAMP product was detected by adding $20 \mu\text{L}$ Avidin-AuNPs into the terminated reaction solution, and vigorously mixed for 2 mins. For the positive assay, the solution turned from red to blue, while the negative reaction remained unchanged. To further confirm the amplification process, $2 \mu\text{L}$ of the reaction solutions from negative and positive tubes were electrophoresed on a 2% agarose gel, and stained with Cyber Green, visualized and photographed under UV exposure.

Specificity, detection limit and practicability

Standard strains of *V. parahaemolyticus*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Vibrio vulnificus* were inoculated and cultured in different mediums at 37°C for 12 h respectively. Their bacterial suspension was used to collect DNA template following above mentioned procedure. These extracted DNA templates were tested by LAMP amplification. The specificity of the method was evaluated by the detection profile of these bacteria.

The detection limit of the method was examined by the detection of a series of 10-fold diluted *V. parahaemolyticus* collected from the overnight culture suspensions of standard strains. 100 μL of diluted bacteria were plated onto the nutrient agar containing 3% NaCl respectively and cultured at 30 $^{\circ}\text{C}$ for 48 h. And then, the bacterial population for each dilution was counted by the average colony forming unit on 3 replicas of the agars. 1 mL of each dilution was used for DNA extract, LAMP reaction and further DNA product detection. The lowest detected population was correlated with the plate counting method.

Three batches of clams were sampled from the local market and verified to be negative for *V. parahaemolyticus* through both the National standard of China (GB 4789.7–2013) (LOD, 3 cfu·g $^{-1}$) [28] and the LAMP method. These samples homogenate were used as blank and spiked with 100 μL overnight cultured *V. parahaemolyticus*. And then, the samples were detected following the National standards and the LAMP method. Furthermore, to check the consistency of our method compared with the national standard, 30 samples of different batches of scallops, oysters, shrimps and clams were collected from different suppliers, and tested by both methods.

Results and discussion

Preparation of the AuNPs nanoprobe

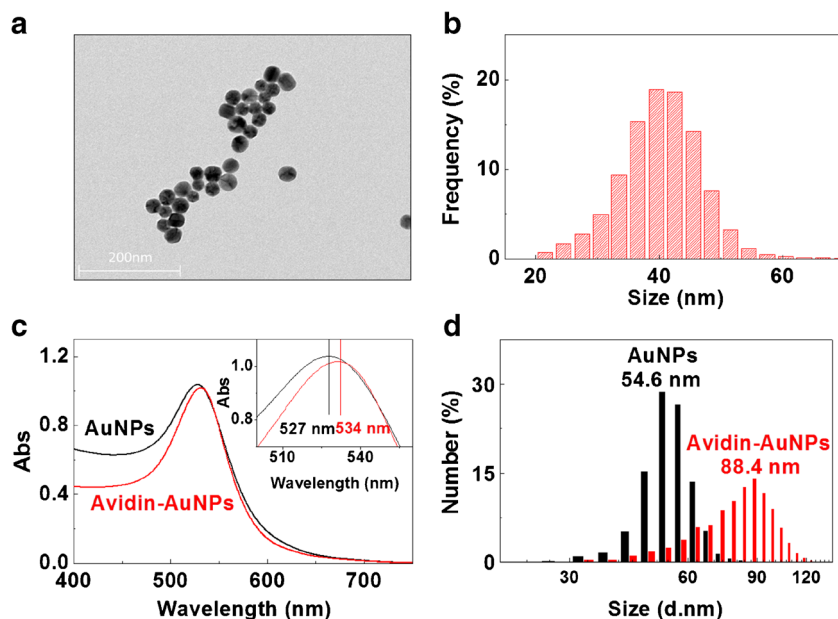
The quality of the nanoparticles and the surface modification are critical for the performance of analytical system. As is shown in Fig. 2a, AuNPs have been successfully prepared

and characterized. The SEM image of the particles displays well monodispersity in size and shape. The statistical size distribution graph (Fig. 2b) shows that the sizes of most of the particles are around 35–45 nm (statistics on 557 particles). Furthermore, the absorbance spectrum of the red AuNPs solution exhibits a major peak at 527 nm (Fig. 2c), which precisely correlates surface plasmonic resonance of these particles with the determined size range. After modification with streptavidin, the absorbance spectrum is slightly shifted to 534 nm. To further examine the streptavidin loading, these particles were characterized through dynamic light scattering measurement before and after streptavidin modification. From the number size distribution obtained for Avidin-AuNPs (Fig. 2d), it is evident that the average size of the original particles grows from 54.6 nm to 88.4 nm after modification, indicating the success of this nanoprobe assembly. The stable Avidin-AuNPs are stored in refrigerator, and are ready to be used in further studies.

LAMP reaction and detection

There are several genes related to the *V. parahaemolyticus* strains, such as *tlh*, *trh*, *tdh*. However, *trh* and *tdh* are not species-specific genes. Rather, they are characteristic genes for the virulence of *V. parahaemolyticus*. Primers based on these two genes may not be in good agreement with current biochemical method for total *V. parahaemolyticus* counting. The *tlh* gene has been proved to be a species-specific gene in various literature sources, and correlated well with other methods for total *V. parahaemolyticus* identification [29]. As a result, this gene was selected as the target to analyze and compare with the commonly used biochemical method such

Fig. 2 The representative TEM image of AuNPs (a), and the statistical size distribution of AuNPs (b). The absorbance spectrum of AuNPs and Avidin-AuNPs (c). The number size distribution of AuNPs and Avidin-AuNPs by dynamic light scattering measurement (d)



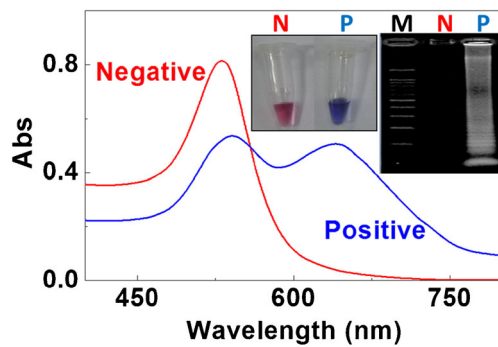


Fig. 3 The absorbance spectra of the LAMP reaction solution after addition of Avidin-AuNPs for the negative (red line) and positive (blue line) sensing process; the photograph showing the different color for negative and positive result (inset, left), and the agarose electrophoresis result for the LAMP reaction solution without addition of particles (inset, right), N, Negative, P, Positive, M, 100 bp molecular marker

as the national standard of China (GB 4789.7–2013) [28]. The design and preparation of optimal primers are critical for the beginning and elongation of the amplification reaction. It requires certain key points in the AT-rich region, T_m values, length of the primers and amplified region, primers purity [11]. Our procedures for LAMP reaction followed most of the previous research for LAMP based detection methods [4], except the biotinylation of the two loop primers. The biotinylated loop primers were integrated into the amplified DNA complexes and were ready for Avidin-AuNPs binding, in order to form clusters of DNA-AuNPs complexes. The absorbance spectra of negative and positive detections are shown in Fig. 3, which display different peaks of Avidin-AuNPs for the negative result and a new broad peak between 580 and 700 nm for positive detection. Furthermore, as presented in the left inset of Fig. 3, after LAMP amplification and DNA-AuNPs complexes formation, a rapid color change from red to blue is recorded, while the solution of negative control keeps its original red. The newly appeared broad peak accounts for the blue color caused by the clusterization of Avidin-AuNPs after the addition of the amplified DNA solution. Moreover, from the result of electrophoresis (the right inset of the Fig. 3), the negative control shows no bands visible due to the lack of DNA amplification, while the positive tube

displays ladder bands, indicating the amplified DNA products by LAMP reaction.

Detection limit, specificity and practicability

A series of 10-fold diluted *V. parahaemolyticus* were prepared to examine the detection limit of the method. The results were correlated with the population counted through plating on nutrient agar containing 3% NaCl. As summarized in **Table S1** (see electronic supplementary material), the lowest concentration detected in all 3 replicas is correlated to be $430 \text{ cfu}\cdot\text{mL}^{-1}$ by plating count method, which represents for 8.6 bacteria in each reaction tube. This detection limit is comparable to other LAMP methods, but the rapid reaction (30 min) and detection time (5 min) are realized by our method. The comprehensive comparison between the method and previous methods is shown in Table 1. Furthermore, comparing to the fluorescent or turbidity monitoring of the LAMP reaction, our method offers facilitated and fast analysis of the readout, with apparent color change and no need of UV light source. Moreover, five of the common bacteria presented in seafood were collected and detected by our method to test its specificity. As all these strains were inoculated and cultured overnight in a suitable medium, their populations before DNA extraction were all higher than $10^5 \text{ cfu}\cdot\text{mL}^{-1}$. As a result, only the *V. parahaemolyticus* showed positive outcome with the method, demonstrating the high specificity of the designed primers and sensing system. The specificity and detection limit promise the feasibility of this method for detection of *V. parahaemolyticus*.

To investigate the performance of this method in practical samples, the verified blank samples were spiked with *V. parahaemolyticus* and detected according to the national standard of China and our method [28]. All the spiked samples were tested as positive by the nanosensing LAMP method, implying the high tolerance of the LAMP method to the biological matrix. Furthermore, 30 real samples from the markets were detected through our method and national standard. The results showed 7 consistent positive for *V. parahaemolyticus*

Table 1 Comparison of the method with previous methods

Target gene	Detection method	Detection time	Detection limit	References
tlh	Colorimetric	~1 h (DNA extract, amplification, detection)	8.6 cfu per reaction	This work
ldh	Resistance measurement	1–2 h (amplification and detection)	$10 \text{ cfu}\cdot\text{mL}^{-1}$	[2]
tlh	Fluorescent	6–8 h for enrichment, ~1.5 h for DNA extract, amplification and detection	1.9 to $0.19 \text{ cfu}\cdot\text{g}^{-1}$ (with enrichment)	[4]
tlh/ldh	Fluorescent	20 min reaction, 60 min (whole record)	7 cfu per reaction	[5]
tlh	Colorimetric	~1 h (sample preparation to detection)	$3 \times 10^3 \text{ cfu}\cdot\text{g}^{-1}$	[30]
tlh	Turbidity	~60 min (DNA extract, amplification, detection)	$5.3 \times 10^2 \text{ cfu}\cdot\text{g}^{-1}$	[31]

by both methods, and two false positive samples by LAMP method which were examined to be negative by the national standards. The national standard (biochemical method) is suitable to detect viable strains, while LAMP method is designed to detect strains regardless of viability, therefore, the discrepancy between the results from these two methods is reasonable [32, 33]. In our practical research, two false samples were detected in thirty samples, due to the existence of dead strains in the samples [34, 35]. These results demonstrate the applicability of the nanosensing LAMP method in practical applications.

Conclusion

In summary, these results have proved that the rapid amplification of target DNA can be achieved within 30 mins using optimized LAMP method, and can be readily identified by unaided eye. The nanosensing LAMP method has excellent specificity to *V. parahaemolyticus* compared with other similar coexist microorganisms. It can attain detection limit at 8.6 cfu per reaction and shows good consistency with the current biochemical method. Furthermore, the method eradicates the need of complicated thermocycler during the process, and no utilization of fluorescent reagents or detection instruments are involved. The Avidin-AuNPs can therefore be used as simplified and generalized nanoprobe, which are feasible for the sensing of many similar LAMP products, as long as the primers are elaborately designed with the two biotinylated loop primers. It should be noted that this method can also give false positive results, due to the existence of nucleic acid in dead cells after sterilization or heat treatment, which may induce inaccurate evaluation. However, these merits derived from LAMP and AuNPs nanosensing promise its suitability in practical, rapid and on-site detection of bacteria.

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Compliance with ethical standards There is no conflict of interest about this article.

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