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Multiplex detection and genotyping of pathogenic bacteria on paper-based biosensor with a novel universal primer mediated asymmetric PCR

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ABSTRACT

Traditionary multiplex asymmetric polymerase chain reaction (PCR) can be applied to detect multiplex target organisms simultaneously, but complex optimizations of primer concentrations and staggered additions of primers are required to achieve equal amplification of multiplex genes. To overcome this shortcoming, we propose a novel method based on multiplex asymmetric PCR and paper-based nucleic acid diagnostics (PBNAD). In the asymmetric PCR, a universal primer was introduced to break the bottlenecks of low sensitivity and self-inhibition among different sets of primers. Amplification using the novel multiplex asymmetric PCR boosted the quantity of single-stranded amplicons, and the amplified products contained the same sequence at the 5' end. Therefore, only one gold nanoparticle-based signal probe was needed for the simultaneous detection of three genes using the PBNAD platform, and the detection signals could be observed with the naked eye. With this highly efficient, novel multiplex asymmetric PCR, as little as 1 pg/μL genomic DNA can be detected. This method can also be applied to genotyping for reliable epidemiological investigations. This proof-of-concept study highlights the potential of the PBNAD platform for cost- and labor-effective applications in the detection of pathogenic bacteria.

1. Introduction

The microbiological safety of food is a serious concern of consumers and the food industry owing to the significant public health and global economic impact of foodborne diseases (Akhtar et al., 2014). In particular, headache, vomiting, bleeding, meningitis and even abortion can be caused by bacterial pathogens, particularly in developing countries (Torgerson et al., 2014). *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* are three of the most important pathogens that spread through food and water, afflicting people worldwide. Recently, *E. coli* is listed as the most serious foodborne infection because of its high risk (Elaine et al., 2011). *Salmonella* strains cause salmonellosis, a zoonotic disease of considerable importance (Feasey et al., 2012). *L. monocytogenes* can cause listeriosis and serious illness in humans and animals, particularly strains 4b and 1/2a (Cossart et al., 2008; Lee et al., 2013). Therefore, a method is urgent needed that can quickly and easily detect multiplex pathogens simultaneously, thereby reducing cost.

Traditionally, the detection and enumeration of bacterial pathogens have been based largely on the use of selective culture and standard biochemical methods (Cadnum et al., 2014; Gracias and McKillip, 2004). Such methods suffer from a number of drawbacks. First, pathogenic bacteria that normally occur in low numbers tend to incur large errors in sampling and enumeration. Second, culture-based methods are time-consuming, invariably monospecific, and low-throughput. Third, many pathogenic organisms in the environment, although viable and capable of causing illness, are difficult to culture or even non-culturable (Kong et al., 2002).

Paper-based nucleic acid diagnostics (PBNAD) refers to a rapid detection strategy with wide application in clinical diagnosis, food quality control and environmental monitoring (Yang et al., 2013; Martinez et al., 2010; Parolo and Merkoci, 2013). PBNAD can be used quantitatively or semi-quantitatively to detect amplification products of nucleic acids or many antibody reactivities in 10–15 min. Compared to other technical methods, such as capillary electrophoresis (Frost et al., 2010), electrochemiluminescence detection (Jie et al., 2012) and enzyme-linked immunosorbent assays (Rissin et al., 2010; Lequin, 2005), this method is more simple, more efficient and easier to carry out with inexpensive, biodegradable and renewable materials. PBNAD is usually combined

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with PCR to achieve high detection efficiency. However, conventional symmetric PCR generates double-stranded amplicons, with a subsequent pre-hybridization for further analysis (Elsholz et al., 2006; Wei et al., 2014). Asymmetric PCR uses conventional PCR primers at unequal concentrations to generate single-stranded DNA (ssDNA) amplicons. However, this method requires extensive optimization of the proper primer ratios, the amount of starting material, and the number of amplification cycles to generate a reasonable amount of products for individual template–target combinations (Ang et al., 2012).

In this proof-of-concept study, we developed a paper-based multiplex detection system using an advanced form of multiplex asymmetric PCR. In this asymmetric PCR, a universal primer was introduced to simultaneously detect multiplex genes without the need to optimize the amplification parameters. By using this novel multiplex asymmetric PCR technique, single-stranded products resulting from the amplification of three genes can be detected simultaneously using one gold nanoparticle (AuNP) signal probe without the need for manual addition of probes or a pre-hybridization step. The whole detection procedure on the paper platform can be performed at ambient temperature. The detection limit was 1 pg/μL genomic DNA. This PBNAD method eliminates the need for preheated buffers or additional equipment and greatly simplifies the protocol for sequence-specific PCR amplicon analysis. The method not only can be applied to multi-detection but also can be used for bacterial genotyping widely.

2. Materials and methods

2.1. Reagents and apparatus

Bovine serum albumin (BSA), formamide, sodium phosphate, sodium dodecyl sulfate (SDS), Tween-20, phosphate-buffered saline (PBS, pH 7.4, 0.01 M), and sodium chloride-sodium citrate (SSC) buffer (20× concentrate, pH=7) were purchased from Sangon Biotech (Shanghai). Streptavidin and Tris (2-carboxyethyl)-phosphine (TCEP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). $HAuCl_4 \cdot 4H_2O$ was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Sodium chloride (NaCl) and sodium citrate dihydrate were purchased from the Guangzhou Chemical Reagent Factory (Guangzhou, China). An HM3020 X-Z two-dimensional dispenser and a GD300 Goldbio cutting module were purchased from Shanghai Goldbio Tech. Co., Ltd. (Shanghai, China). Cellulose fiber sample pads, conjugate pads, plastic adhesive backing pads, absorption pads and nitrocellulose membranes were purchased from Millipore (Billerica, MA). All reagents used in this study were of analytical reagent grade, and all solutions were prepared using ultrapure water from an ELGA water purification system. All oligonucleotides purified by ULTRAPAGE were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China), and their sequences are listed in Supplemental Table S1.

2.2. Preparation of AuNPs

AuNPs were prepared according to a published protocol (Fu et al., 2013). First, 15-nm-diameter AuNPs were prepared by the citrate reduction of $HAuCl_4 \cdot 4H_2O$. All glassware was cleaned in aqua regia (HCl:HNO_3=3:1) and rinsed with pure H_2O. An aqueous solution of $HAuCl_4$ (1 mM, 100 mL) was brought to reflux while stirring, and then 10 mL of a 38.8 mM trisodium citrate solution was added quickly, which resulted in a change in the solution color from pale yellow to deep red. After the color changed, the solution was refluxed for an additional 15 min to cool to room temperature. The resulting solution of AuNPs was characterized by an absorption maximum at 520 nm.

2.3. Functionalization of AuNPs

The AuNP–probe conjugate was prepared according to previously reported methods, with slight modifications (Liu et al., 2014). Prior to the conjugation, 10 μL of 100 μM thiolated DNA probe was activated with 3.3 μL of 50 mM acetate buffer (pH 5.2) and 5 μL of 1 mM TCEP. After 1 h of incubation, 1 mL of the AuNPs was transferred into a microcentrifuge tube and then stirred for at least 16 h. Then 10 μL of 500 mM Tris acetate (pH 8.2) buffer was added drop-wise to the tube with gentle shaking, and 100 μL of 1 M NaCl was quickly added to the mixed solution with rapid stirring. The tube was stored in a dark room for at least one day. Subsequently, the conjugate was resuspended in 1 mL of buffer containing 20 mM sodium phosphate, 0.25% Tween 20, 10% sucrose, and 5% BSA. The conjugate then dispensed onto the polyester cellulose membrane with the HM3020 X-Z dispenser, then dried at 37 °C for 2 h and stored at 4 °C before further use.

2.4. Immobilization of capture probes

In this study, four biotinylated capture probes were used in one PBNAD. The capture probes were first linked to streptavidin to maintain their entrapment within a designated area of the detection region and then dispensed at specific concentrations onto a NC membrane (25×4 mm^2) that was pre-affixed to the center of a PVC sheet (60×4 mm^2). That is, the capture probes were separately immobilized on the NC membrane and served as test lines and a control line (CL), respectively.

The capture probes were separately immobilized on the nitrocellulose (NC) membrane with the HM3020 X-Z dispenser at a rate of 5 μL/cm. The concentration of probes was 50 μM, and the paper-based biosensor was 4 mm wide. So the calculations show that the quantity of the capture probes immobilized on the NC membrane was 100 pmol. The membrane was then dried at 37 °C for 1 h and stored at 4 °C in a dry state.

2.5. Construction of PBNAD

The dry-reagent PBNAD (60 mm × 4 mm) was composed of a sample pad, a conjugate pad, a nitrocellulose membrane and an absorbent pad. Capture reagents for the test lines and the control line (CL) were applied separately onto the nitrocellulose membrane using a HM3020 X-Z two-dimensional dispenser at a rate of 5 μL/cm. The nitrocellulose membrane was dried overnight in a desiccator. The buffer application pad, conjugate pad, nitrocellulose membrane and absorbent pad were assembled with a 2 mm overlap between each component, and the assembled master card was cut into 4 mm wide strips with a programmable shear. The strips were stored in a desiccator or sealed in aluminum pouches containing silica gel.

2.6. PCR conditions

The PCR reactions were prepared in a 25 μL reaction mixture containing 1× PCR buffer, 200 μM dNTPs, 0.25 U of *Taq* DNA polymerase, forward and reverse primers for each template, universal primer, different concentrations of the target DNA template, and deionized water. The detail bacteria culture conditions and the agarose gel electrophoresis (AGE) analysis were showed in Supplementary materials Fig. S1. Genomic DNAs were extracted using a Nucleospin Tissue Kit. The concentrations of the primers were optimized in the single, duplex, and triplex PCR system, ranging from 200 to 2 nM with a series of decimal dilutions; the concentration of the universal primer was 300 nM for all reactions.

The final thermal cycling program included an initial 5 min denaturation at 94 °C; 45 cycles of 30 s at 94 °C, 30 s at 54 °C, and 30 s at 72 °C; and a final extension for 10 min at 72 °C. Conventional multiplex PCR systems do not include universal primers, and the concentration of each specific primer is 300 nM. The other reagents were used in this novel asymmetric PCR at the same concentrations used in conventional multiplex PCR. Five microliters of each PCR product was separated by electrophoresis in 20 g/L agarose in 1 × TAE containing 1 × SYBR GREEN II. Images were recorded with a GelDoc UV gel documentation system. Another 20 μL of each amplification product was snap-cooled on ice before proceeding with the PBNAD assay.

2.7. PBNAD assay protocal

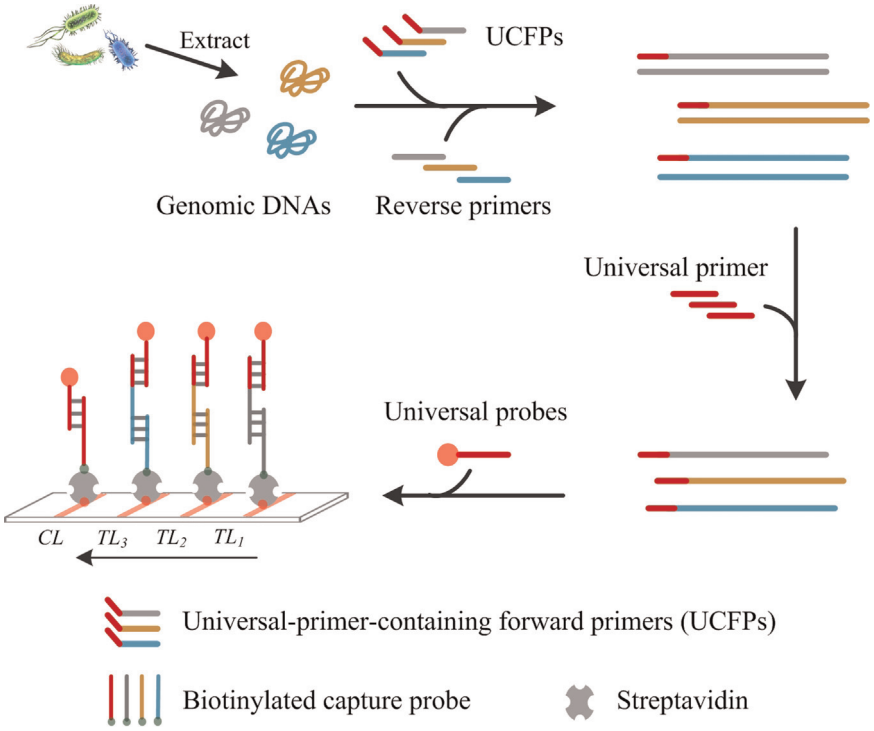
The test sample for the PBNAD was prepared by adding an equal volume of hybridization buffer (4 × SSC) to the PCR products and mixing by pipetting the sample. A 40 μL sample of the mixture was applied to the sample application region. After waiting for 10 min, the biosensor was washed twice by adding 20 μL of running buffer to the sample pad at 3 min intervals. Red bands were observed on the PBNAD due to the accumulation of conjugates of amplified products and the AuNP–probe. The results were visualized as a red signal by the naked eye. The results were further analyzed using ImageJ software.

3. Results and discussion

3.1. Development of multiplex asymmetric PCR

Single-stranded products have been shown to be very useful for DNA hybridization detection with high efficiency and do not need to be denatured before hybridization (Citartan et al., 2012). Asymmetric PCR aims to maximize the yield of ssDNA targets in a single reaction for hybridization and thereby increase the sensitivity of the PBNAD platform (Hao et al., 2011). However, conventional asymmetric PCR is usually inefficient and difficult to optimize because limiting the concentration of one primer lowers its melting temperature below the reaction annealing temperature. Furthermore, the process requires extensive optimization of the primer ratio, the amount of starting material, and the number of PCR cycles (Sanchez et al., 2004). Multiplex asymmetric PCR could be applied to detect multiple target organisms simultaneously to save time and labor, but there is always disproportionate amplification resulting from the disparity of different amounts of primers. For the purpose of detecting different products specifically, at least two AuNP–probes are needed for the multiplex detection (Shin et al., 2009).

To overcome the above limitations, we developed a novel universal primer mediated multiplex asymmetric PCR by introducing a universal primer to allow the simultaneous detection of multiplex genes without optimization of the primer ratio (Yuan et al., 2009). The selection of universal primers depends on the purpose of PCR amplification and on the type of samples. Universal primers, as described here, may be any arbitrary sequence which is compatible with the PCR conditions employed and not being present within the template genome's DNA sequence. Three symmetrical primer pairs were designed for use at the same concentration with an extra specific sequence at the 5' end of each forward primer, which was the same as the sequence of the universal primer in this method. We called these primers universal primer-containing forward primers (UCFPs) (Scheme 1). First, the amplification of different targets was initiated by symmetrical UCFP pairs with an exponential phase. Next, the obtained double-stranded DNA products were used as templates for the universal primers to initiate linear amplification to yield the final ssDNA products. All of the ssDNA products contained the universal primer at their 5' end. Then, we added AuNP–probes that were designed to hybridize only with the universal primer and fix the



Scheme 1. Schematic illustration of bacteria detection based on novel universal primer mediated multiplex asymmetric PCR and paper-based nucleic acid diagnostics.

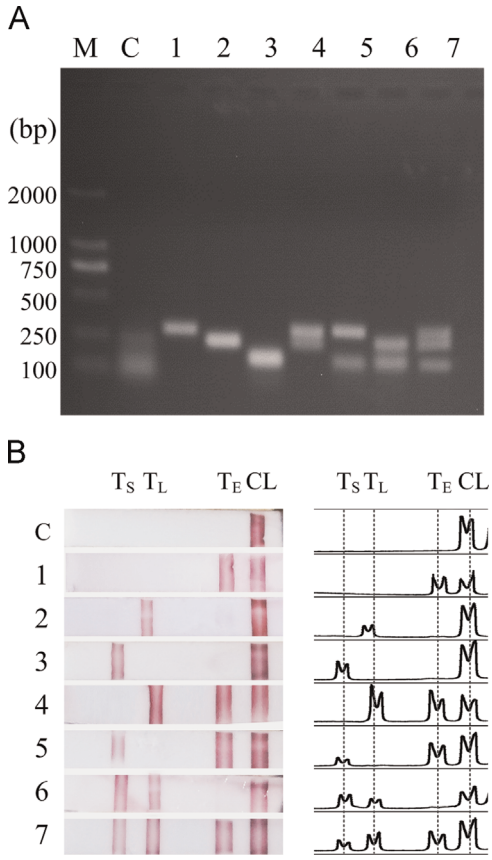


Fig. 1. Performance of the paper-based nucleic acid diagnostics (PBNAD) with the novel asymmetric PCR. (A) 1% AGE analysis of the amplification products. (B) Photographs (left) and corresponding optical responses (right). Each lane represents the amplification result of complex PCR with a different DNA target; M: 2000 bp DNA Marker; C: negative control without target; lanes 1–7, amplicons of different DNA targets: 1: *Escherichia coli*; 2: *Listeria monocytogenes*; 3: *Staphylococcus aureus*; 4: *Escherichia coli* and *Listeria monocytogenes;* 5: *Escherichia coli* and *Staphylococcus aureus;* 6: *Listeria monocytogenes* and *Staphylococcus aureus;* 7: *Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus* (For interpretation of the references to color in this figure the reader is referred to the web version of this article.).

ssDNA products to the current PBNAD platform. In the later cycles of the multiplex asymmetric PCR, the universal primer was the only one primer for amplification; therefore, all the single-stranded products were amplified with almost the same efficiency by universal primer. By contrast, traditional multiplex PCR cannot avoid disproportionate amplification between different primers during the whole reaction because different primer pairs have different amplifying efficiencies.

3.2. Verification of the specificity and the feasibility of the primer

A comparative study of the specificity was performed between the specific primer pairs and the UCFP pairs. Each PCR system was prepared with the same reagents but using different primer pairs to target the corresponding organisms. The results showed that the DNA templates from 3 target organisms were efficiently amplified by specific primer pairs with a high specificity (See Supplemental Fig. S2, lanes 1–3). Because the sets of UCFP pairs had a longer universal sequence (24 bp) at the 5' end of each forward primer than the common specific primer pairs, the amplified fragments were larger than 24 bp. The UCFP pairs produced nearly the same concentration of amplicon with a high specificity, which showed that these primers also had equivalent amplifying specificity to the corresponding common specific primer pairs (See Supplemental Fig. S2, lanes 4–6). *E. coli*, *S. aureus* and *L. monocytogenes* were chosen to perform the experiments. The universal primer mediated reaction was investigated by comparing the amplification with and without the universal primer in single PCRs. Two sets of PCR with the same primer concentration gradient (without and with universal primers) were performed. With the concentration of UCFP pairs (Sta-UCFP in this experiment) decreasing from 200 to 2 nM, the concentration of amplified fragments fell markedly from 200 to 20 nM (See Supplemental Fig. S3, lanes 1–3) in the absence of the universal primer, but the amplified fragments were detected at a concentration of 20 nM with universal primers. These results indicate that the universal primer was well designed and could be used for efficient PCR amplification. Similar results were obtained using the primer pairs Esc-UCFP and LM-UCFP. The concentrations of the primers strongly influence the efficiency and ratio of the ssDNA products in PCR and are among the most important factors for PCR, particularly in multiplex PCR. Finally, 20 nM for the UCFP pairs and 300 nM for the universal primer were chosen as the optimal concentrations for universal primer single PCR.

3.3. Performance of the PBNAD with this novel asymmetric PCR

We employed specific primer pairs for the *rbfE* gene (*E. coli O157:H7*), the *hlyA* gene (*L. monocytogenes*), and the *nuc* gene (*S. aureus*) to detect their presence in the sample according to the corresponding test line. Therefore, in this study, multiplex asymmetric PCR and PBNAD are described for the detection of pathogenic bacteria.

PBNAD consists of five distinct components, each of which played an important role in this study. The first part consisted of a sample pad where the sample to be analyzed was applied. The sample then moved along the strip due to capillary action and was collected at the fourth part, referred to as the absorption pad. As the solution passed the second part, referred to as the conjugate pad, hybridization occurred between the products and AuNP–probes, and the formed complexes continued to migrate along the strip. At the third part, referred to as the test zone, the formed complexes were captured by a second hybridization between the products and the CP_test (capture probes for test, apart complementary to the products). The accumulation of AuNPs produced three characteristic red bands on the test lines, which are visible to the naked eye. This color change enabled the determination of the concentration of products in the sample because the color intensity was proportional to target concentration. Then, the excess AuNP–probes continued to migrate through the control zone and were captured by CP_control (capture probes for control, complementary to the AuNP–probe). Finally, four red bands appeared. Comparison between current biosensor and existing sensing mechanisms for foodborne pathogen detection was showed in the Supplementary material (Table S2)

A positive result necessarily is accompanied by the appearance of test lines (Fig. 1, lines 1–7). The appearance of these lines indicated that the sample contained the corresponding bacteria. If only one red band was observed in the control zone (Fig. 1, line c), the sample contained no bacterium. The control line gives the visible indication to excluding insufficient specimen, improper assay execution, and/or deterioration of test reagents. A visible red line at the control line of the biosensor should be present each time. If no red control line is seen, adequate specimen flow has not occurred or reagents on the biosensor were deteriorated, and the test is considered invalid.

The sensitivity of the fabricated strip biosensor was assessed using genomic DNA targets in triplex PCR. We tested the sensitivity of each bacterium separately and compared it with the

sensitivity of the multiplex detection to judge whether the sensitivity decreased as the number of genes increased.

A set of universal primer single PCRs were performed with template concentrations of 1 ng/μL, 100 pg/μL, 10 pg/μL, and 1 pg/μL. Then the proportionate target DNA were produced and detected by electrophoresis assay and PBNAD (Fig. 2). As the concentration of the genes decreased, the amplified products decreased in abundance, as did the intensity of the bands in electrophoresis and PBNAD. The red band on the test zone was quite visible even at a target concentration of 1 pg/μL; however, when the target template was lower than 1 pg/μL, there was no corresponding amplicon, so the threshold for the visual determination of target genes without instrumentation was 1 pg/μL target DNA per reaction in single samples. Then we tested the sensitivity of the multiplex detection of this method. The bright band observed after gel electrophoresis and the red bands on the test zone were also visible at 1 pg/μL target, as shown in Fig. 3, indicating that this method has no definite limitation for the simultaneous detection of multiple genes. No distinct red band was observed on the test zone in the absence of target DNA (Fig. 3, line c), indicating

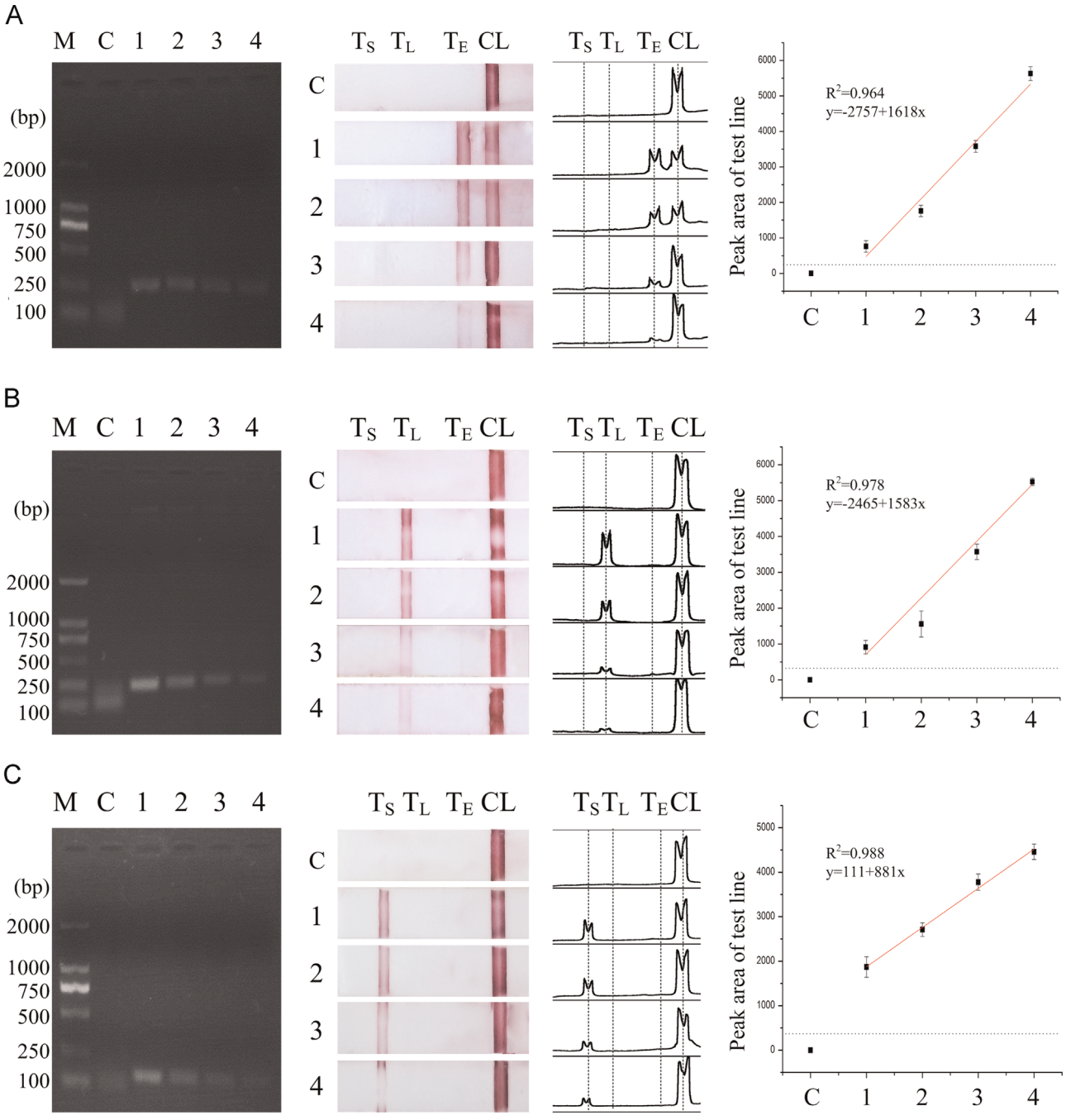


Fig. 2. Sensitivity analysis of the paper-based biosensor for *Escherichia coli* (A), *Listeria monocytogenes* (B), and *Staphylococcus aureus* (C), respectively. Each lane of 1% AGE analysis (left) represents the amplification result of different concentration of target. M: 2000 bp DNA Marker; C: negative control without target; lane 1: 1 ng/μL; lane 2: 100 pg/μL; lane 3: 10 pg/μL; lane 4: 1 pg/μL. Photographs and corresponding optical responses were shown in the middle graphs. The calibration plots were shown in the right graphs.

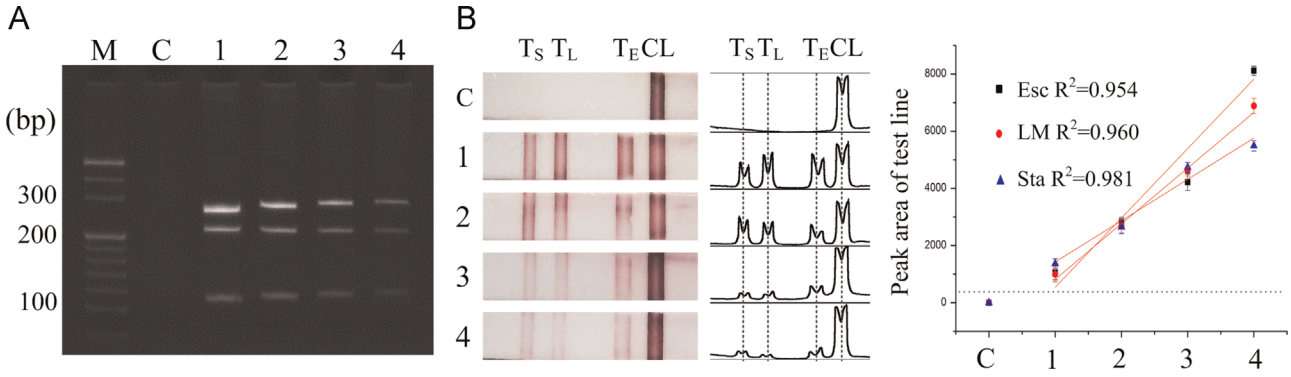


Fig. 3. The sensitivity of multiplex detection. (A) 1% AGE analysis of the amplification products. (B) Photographs and corresponding optical responses were shown in the middle graphs. The calibration plots were shown in the right graphs. Each lane represents the amplification result of complex PCR with a different concentration of three DNA targets: M: 2000 bp DNA Marker; C: negative control without target; lane 1: 1 ng/μL; lane 2: 100 pg/μL; lane 3: 10 pg/μL; lane 4: 1 pg/μL (For interpretation of the references to color in this figure the reader is referred to the web version of this article.).

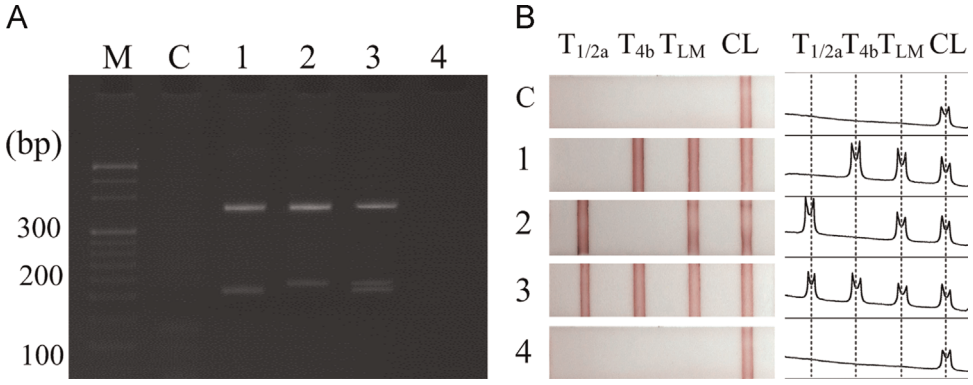


Fig. 4. Paper-based genotyping of *L. monocytogenes*. (A) 10% PAGE analysis of the amplification products for serovar 4b (line 1), serovar 4b (line 2) and both of them (line 3). Sample 4 is *Escherichia coli*. The C is the negative control without target. (B) Photographs (left) and corresponding optical responses (right).

negligible nonspecific adsorption during the strip assay in multiplex detection.

3.4. Application to genotyping

Genotyping of pathogenic bacteria, a commonly used method in epidemiological surveillance, is widely accepted in food safety analysis and clinical diagnosis (Koopmans et al., 2013). The method presented here not only can be used for multiplex detection, it also can be used for genotyping pathogenic bacteria. Compared with traditional discriminatory subtyping methods, such as pulsed-field gel electrophoresis (PFGE) (Chenal-Francisque et al., 2013), amplified fragment length polymorphism (AFLP) (Feng et al., 2013), and multilocus variable-number tandem repeat analysis (MLVA) (Saleh-Lakha et al., 2013), this method is simpler, more efficient and easier to carry out using inexpensive, biodegradable and renewable materials.

Listeriosis, one of the most important zoonotic bacterial diseases with a worldwide distribution, is caused by *L. monocytogenes*. Isolates of serotypes 4b, 1/2a and 1/2b are key contributors to food listeriosis (Ferreira et al., 2014). Serotype 1/2a accounts for $>50\%$ of the *L. monocytogenes* isolates recovered from food and the environment. Most major outbreaks of human listeriosis have been caused by serotype 4b strains (Doumith et al., 2004; Pan et al., 2009). Identifying the infection source of *L. monocytogenes* is required for reliable epidemiological investigations (Birtles et al., 2002). We employed three primer pairs for the *hlyA* gene, the *orf0799* gene and the *lmo0737* gene, which are specific to the *Listeria* genus, the 4b serovar, and the 1/2a serovar, respectively. Through the advanced multiplex PCR technique described here, three fragments with different lengths (222 bp, 107 bp, and 102 bp, respectively) were obtained. We determined whether the sample contained *Listeria* according to the presence of the *hlyA* gene. If *Listeria* was present, we used the *orf0799* gene and *lmo0737* gene to determine whether the sample contained serovar 4b or serovar 1/2a.

The identification of a positive result is confirmed by the appearance of three lines (Fig. 4). Fig. 4-1 shows the detection of *L. monocytogenes* serovar 4b, which contained *hlyA* and *orf0799*. However, a negative result on the T_{4b} line but a positive result on the $T_{1/2a}$ line indicated that the samples was serovar 1/2a, containing *hlyA* and *lmo073* (Fig. 4-2). The appearance of four lines indicated that the sample contained serovar 4b and 1/2a (Fig. 4-3). If only one red band was observed in the control zone, the sample included no *hlyA*, *orf0799*, or *lmo0737*, meaning that it was not *Listeria* (Fig. 4-4). Any test result obtained when the chromatography control line was negative was considered an invalid result. The threshold for the visual determination of a target gene without instrumentation was 1 pg/μL target DNA per reaction for genotype detection (Fig. 5).

4. Conclusions

We have successfully developed a PBNAD system for the visual detection of pathogenic bacteria with a novel universal primer

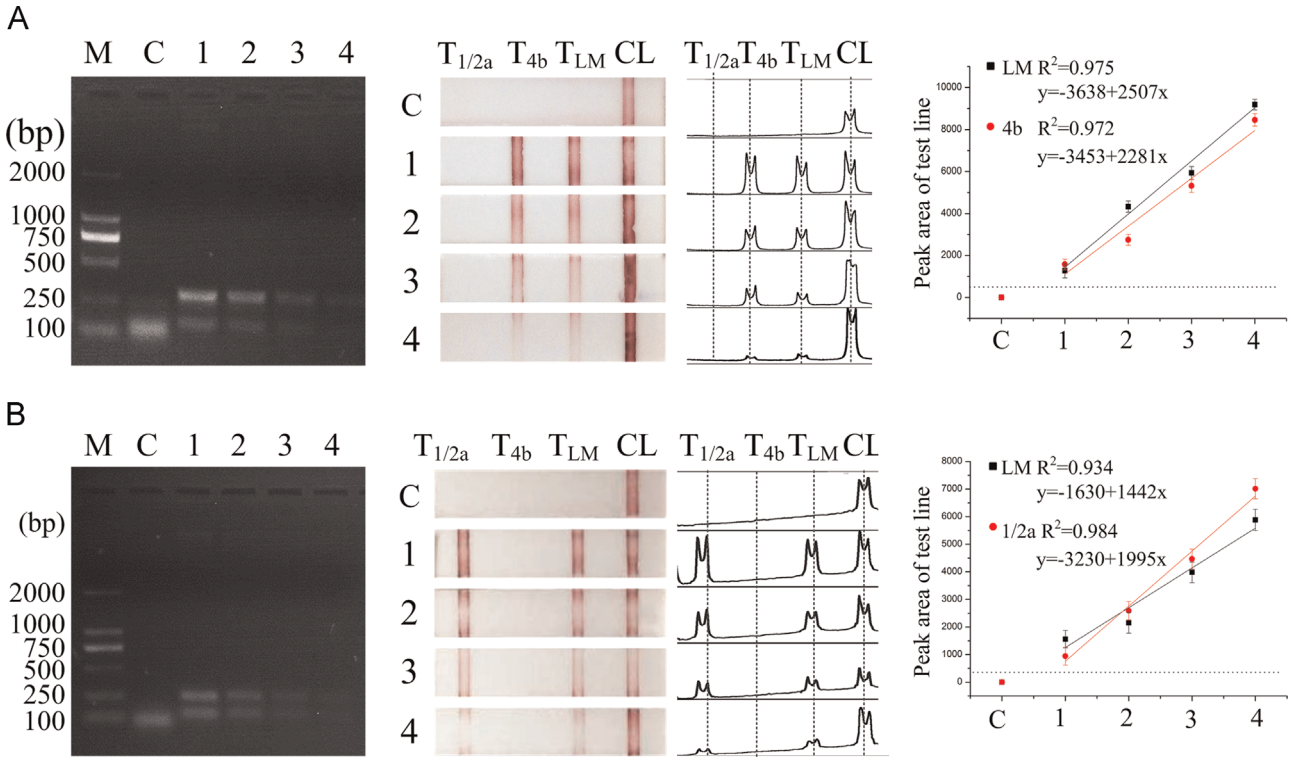


Fig. 5. Sensitivity analysis of the paper-based biosensor for serovar 4b (A) and serovar 1/2a (B). Each lane of 1% AGE analysis (left) represents the amplification result of different concentration of target. M: 2000 bp DNA Marker; C: negative control without target; lane 1: 1 ng/μL; lane 2: 100 pg/μL; lane 3: 10 pg/μL; lane 4: 1 pg/μL. Photographs and corresponding optical responses were shown in the middle graphs. The calibration plots were shown in the right graphs.

mediated multiplex asymmetric PCR. With the high efficiency of this amplification method, single or multiplex target genes can be simultaneously detected, and the detection limit was 1 pg/μL genomic DNA. This cost-effective, disposable and robust biosensor poses no health risk to the end user, and the generated signal can be unambiguously read by the naked eye. More importantly, our method may be extended from the detection of pathogenic bacteria and genotyping to other applications, such as screening and/or identification of other infectious disease agents. These characteristics make the strip assay an ideal candidate for the development of a rapid detection kit, which will facilitate the translation of diagnostics from the laboratory to point-of-care or field settings.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2014.05.063.

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