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Solid-Phase PCR Based on Thermostable, Encoded Magnetic Microspheres for Simple, Highly sensitive and Multiplexed Nucleic Acid Detection

**Zhejia Gu^a, Simin Zhao^a, Gaolian Xu^a, Cang Chen^a, Yao Wang^a, Hongchen Gu^a, Yi Sun^{b*},
Hong Xu^{a*}**

^a School of Biomedical Engineering / Med-X Research Institute, Shanghai Jiao Tong University, Shanghai 200030, P. R. China.

^b Department of Health Technology, Technical University of Denmark, Kgs. Lyngby, 2800, Denmark.

Abstract: Multiplexed detection of nucleic acids is important for pathogen identification and disease diagnosis. Though real-time quantitative PCR allows for simultaneous amplification of different genes in a single test, the interferences between various primers inevitably cause difficulty in assay design, and pose limitation on multiplexing degree. Conventional solid-phase PCR (SP-PCR) on plenary microarrays can assess more targets than real-time quantitative PCR. However, they are suffering from low reaction efficiency, high background noise, and requiring special equipment and expertise for reliable analysis. Here, we addressed the challenges by performing multiplex PCR on thermostable, tailor-made host-guest encoded magnetic microspheres (Beads based PCR, BB-PCR), which were constructed by coupling magnetic host microspheres with fluorescent guest nanoparticles. As proof of concept, a 3-plex barcoded microbeads with high thermostability and low non-specific fluorescence absorption were selected as model BB-PCR carriers to amplify and detect three gene targets in *Salmonella Enteritidis*. After BB-PCR reaction, the barcoding and reporting signals were easily identified through flow cytometry. Attributed to the effective interactions between reagents and microspheres during PCR reaction, this method showed high sensitivity of 10 copies/reaction, which was comparable to that of liquid PCR. Thus, BB-PCR developed here allowed for simple, rapid, highly sensitive and high-throughput detection of nucleic acid targets, which paves the way for routine multiplexed molecular diagnostics in clinical laboratories.

Keywords: Beads-based multiplex PCR, encoded magnetic microsphere, thermostable, *Salmonella Enteritidis*, high sensitivity

1. Introduction

With the advances in genomics, multiplexed detection of genomic targets has attracted increased attention [1-4]. Based on conventional ~~PCR~~ nucleic acids amplification technology, multiplex PCR and isothermal amplification technology combined with electrophoresis analysis [5] or fluorescence detection have been developed. Attributed to the the ability of quantification and extremely low detection limit, multiplex real-time quantitative PCR (qPCR) method that detects various fluorescent signals from multiple targets has been widely used [6-8]. However, due to the interferences between different sets of primers in one reaction and the limited number of fluorescence detector channels on real-time PCR instrument, it is difficult to simultaneously analyze more than 6 targets in a single tube. To meet the demand of high-throughput nucleic acid detection, Next Generation Sequencing (NGS) has been applied, which is specific, with ultra-high throughput and able to detect unknown nucleic acid molecules [9]. Whereas, it is time-consuming and relatively expensive, so it is not suitable for use in the routine clinical laboratory test. Recently, amplification-free methods based on nucleic acid hybridization and novel ultra-sensitive detection technologies, , such as Surface-enhanced Raman spectroscopy (SERS) [2,10] and electrochemistry [11] have been demonstrated. Unfortunately, these methods also face challenges in clinical application due to e. g. , the poor reproducibility and stability of SERS-active substrates [12], cross-reaction between the affinity ligands and target [13] or poor reliability and robustness of conventional electrochemical sensors [14]. Alternatively, solid phase PCR (SP-PCR) that was conducted with at least one primer fixed on a solid surface, has emerged [15-18]. This technology allows for physical isolation of primers and specific amplicons, which could effectively reduce the interference between primers as well as providing multiplex detection by position coding. To date, chips or DNA microarrays as solid phase carriers have shown significant advantages in realizing high throughput, multiplexed detection and fulfilling requirements for integration and miniaturization [19-21]. However, in multiplex detection on chip, non-specific binding of fluorescent-labeled primers often causes high background noise; and the inaccessible immobilized primers or probes and the inefficient diffusion of target templates also result in a low reaction efficiency. Moreover, it requires special equipment and expertise for reliable analysis. Consequently, SP-PCR has rarely been exploited in real applications.

Recently, encoded microbeads technologies have attracted significant attention due to their enhanced reaction kinetics and the remarkable capability for multiplexing. In general, different oligonucleotide probes were immobilized on microbeads with unique barcodes, and then different target molecules in the sample could be identified, captured and manipulated specifically by their corresponding barcoded microbeads, thus realizing highly multiplexed detection in one reaction tube. For instance, Hsu et al. (2013) coupled DNA probes onto 15 Luminex microbead sets, and demonstrated the use of the suspension array to simultaneously identify and quantify 11 pathogens [22]. Our group has also developed a 3D encoding library of 100 dual-color barcoded microspheres with ultrahigh stability and extraordinary coding ability, and has utilized them for six-plex detection of influenza viruses and five-plex tumor marker [23,24]. However, up to now, most of work required two discrete steps for nucleic acid detection: DNA PCR amplification and labeling reaction in solution, and a subsequent sequence-specific hybridization to the probe-loaded capture microbeads. This two-step operation is very likely to cause aerosol contamination when the cap of PCR reaction tube is opened between the two steps, leading to false positivity. Nevertheless, performing one-step SP-PCR directly on encoded microbeads has seldom been reported, while the main challenges probably lie in: I) the difficulties in developing temperature-tolerant microbeads which can withstand repeated heating-cooling cycles with rapid and high temperature gradients, and II) lack of proper surface chemistry that can minimize non-specific binding of fluorescence primers.

In this paper, we developed a new type of thermostable, tailor-designed encoded magnetic microbeads for multiplexed SP-PCR applications (Beads based PCR, BB-PCR). As shown in **scheme 1a**, the microbeads consisted of a host-guest structure: a six-micrometer magnetic sphere as host and 200-nm silica nanoparticles as guest. The guest nanoparticles contain two types: one with FITC doping, and the other without FITC. The microbeads were encoded by mixing two types of guest particles at different ratios, and a unique barcode library with ultrahigh encoding capacity was easily achieved [23, 24]. Next, the surface of barcode microbeads was further carboxyl functionalized with polyacrylic acid (PAA). Here, to reduce the non-specific adsorption of microspheres during PCR, we redesigned the surface modification molecules of host beads by decorating them with only one layer of polyethylenimine (PEI), instead of depositing 8 layers of polyelectrolytes as reported before [23].

The concept of multiplex BB-PCR was illustrated in **scheme 1b**. Here, we chose three typical gene targets existing in *Salmonella Enteritidis*, which were called *hlyA*, *sefA*, *sdf* genes. Three 5' terminal amino modified DNA probes specific to the three genes were immobilized on three barcoded microbeads. The microbeads were directly added to the PCR reaction mixture, which consists of forward and reverse primers of the three genes, PCR mix buffer, DNA polymerase and BSA. After first few cycles of asymmetric amplification in solution, DNA amplicons were captured by the immobilized nested probes, and then the probes were extended by polymerase taking the captured amplicons as template. Importantly, each unique barcoded probe could specifically recognize one target among large number of genomic DNA, and be extended due to its free 3' -end. Besides, the nested structure of probes could efficiently reduce non-specific binding in amplicons to improve the specificity of amplification. Subsequently, Cy5-labelled primers annealed to the extended probes and generated the new products which were covalently coupled to the microbeads. Repeatedly, the encoded microspheres became the microreactor, where amplicons were captured and extended constantly by the immobilized probes. Finally, a large number of amplified products were obtained on the surface of the encoded microspheres. After BB-PCR reaction, fluorescence signals from microbeads were detected by flow cytometry, where different DNA targets were identified based on intensity of the two fluorescent dyes: FITC for decoding and Cy5 for quantification.

Furthermore, we investigated the physiochemical properties of the encoded microbeads, and studied the effects of probe length, immobilized probe density and number of thermal cycles on amplification efficiency of BB-PCR. The microbeads showed excellent thermal stability and much reduced background noise. At optimal conditions, a high sensitivity of 10 copies/reaction was achieved, which was comparable to that of liquid PCR. Moreover, the method was successfully validated using 60 clinical diarrhea patients' samples. In contrast to real-time PCR and SP-PCR on planar chips, encoded magnetic microsphere-based BB-PCR provides apparent advantages, including high-multiplexing level, enhanced reaction kinetics, high sensitivity and specificity, and ease of operation. It paves the way to establish an innovative multiplex BB-PCR detection platform for routine clinical diagnosis.

Scheme1 a) Schematic illustration of synthesis of the—dual fluorescent encoded magnetic

microspheres with a host–guest structure. The steps included: 1) modifying magnetic microspheres with branched poly (ether imide) (PEI), 2) mixing FITC doped nanoparticles and non-fluorescent nanoparticles at various ratios as guest part, 3) coupling host and guest part forming encoded magnetic microspheres, and 4) immobilizing 5-NH₂ modified DNA probes to the surface of encoded magnetic microspheres. b) Proof of concept for multiplex BB-PCR. PCR mixture contained encoded microbeads, small amount of forward primers and Cy5-labelled reverse primers. During the first few cycles, the template was preferentially amplified in solution. Then accumulated amplicons tended to hybridize with probes on microbeads, and probes were further extended by polymerase. Finally, Cy5-labelled amplicons were immobilized on encoded magnetic microspheres, and the fluorescence signals from these complexes were measured by flow cytometry.

2. Experimental section

2.1 Materials

N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), 2-(N-Morpholino) ethanesulfonic acid (MES) were purchased from Sigma-Aldrich, China. Components of buffer were from Sinopharm Chemical Reagent Co., Ltd. All buffers were prepared with deionized water. DNA sequences were synthesized from Sangon Biotech, Shanghai. The DNA sequences are shown in Table S1.

2.2 Fabrication of encoded micro magnetic beads

PEI modification. 75 mg of carboxyl modified magnetic microspheres (purchased from Micromod Partikeltechnologie GmbH) were washed by MEST (100 mM, pH 4.0) containing 0.02 wt% Tween-20 for four times. Then the microspheres were re-suspended in 9.99 mL of MEST containing 26.17 mg of EDC and sonicated for 2 min. Afterwards, 9.99 mL of PEI buffer (39 mg/mL) was added and this mixture was rotated for 3h to obtain PEI-modified magnetic microspheres (MBs@PEI).

Preparing for guest nanoparticles. Two types of silica nanoparticles were used, and the ratios of them decided the final intensity of encoded microbeads. For high fluorescent encoding microspheres, guest part contained 100% of the nanoparticles doped with FITC, while the low fluorescent barcode microspheres were loaded with 100% of the non-fluorescent nanoparticles. For medium fluorescent encoding microspheres, guest mixture contained the 92% of non-fluorescent nanoparticles and 8% of FITC-doped nanoparticles.

Assembling process of Host-Guest structure barcode microspheres. MBs@PEI were washed by 100 mM of MEST (pH 4.0) twice and sonicated to be well re-suspended in MEST. Guest nanoparticles with different fluorescent ratios were added dropwise to MB@PEI in an ultrasound

bath. Then the mixture was rotated for 30 min. After that, 1.668 mL of MEST containing EDC (50 mg/mL) and NHS (50 mg/mL) were added and rotated for 3 h. Finally, the supernatant was removed. Encoded microspheres were washed by 1mM of NaOH solution for three times and deionized water for three times.

2.3 Template DNA extraction

Salmonella Enteritidis used was from Chinese Center for Disease Control and Prevention of Guangzhou. The template DNA used in optimization and sensitivity experiment was extracted through nucleic acid extraction kit purchased from Beijing Bohui Innovation Biotechnology Co., Ltd. Clinical samples were extracted by a simple method as following. 100 μ L of inactivated bacterial solution was incubated with 200 μ g/mL of proteinase K in 50 mM of Tris-HCl(pH 8.5) buffer containing 0.5% SDS and 1mM of EDTA at 55°C for 45 min. Then, the mixture was heated to 95 °C to inactive proteinase K, and centrifuged at 12000 g for 15 min then cooled to 4 °C. The supernatant was used as template.

2.4 Primer design and synthesis

Three pairs of forward and reverse primers used in the experiment were designed for hliA gene, sefA gene and sdf gene amplification [18]. These three genes could determine serotype Salmonella Enteritidis. Each reverse primer was labelled with Cy5 and the amplicons extended from the reverse primers could be finally detected by flow cytometry. The nested probes were modified with amino group at the 5' end for immobilization on encoded micro magnetic beads by NHS/EDC method. The sequences of the probes are shown in **table S1**.

2.5 Probe immobilization

Microbeads were washed with 100 mM of MES buffer (pH 4.0) for three times. After discarding the supernatant, microbeads were incubated with probes in 100 mM of MES buffer containing 37.5 mg/mL of EDC in a total volume of 200 μ L on a rotate mixer for 2 h at room temperature. Then, probe-microbead complexes were washed with 10 mM of borax buffer (pH 9.5) for three times to remove excess probes. To reduce non-specific adsorption, microbeads were treated with 10 mM of PBS (pH 7.4) containing 5 mg/mL of bovine serum albumin (BSA) at 4 °C overnight and re-suspended in 100 μ L of Milli-Q water after washing two times with Milli-Q water.

The probe concentration was quantified by qubitTM 2.0 fluorometer. The probe contents in the

original probe solution and supernatant after coupling were measured to calculate probe immobilized density onto the microbeads.

2.6 Beads based PCR (BB-PCR)

One μL of DNA samples were mixed with microbeads, 0.2 μM of sdf forward and 0.8 μM of sdf reverse primers, 0.2 μM of sefA forward and 0.8 μM of sefA reverse primers, 0.4 μM of hilA forward and 1.6 μM of hilA reverse primers, 1 $\times$ Phusion Human Specimen PCR buffer, 16 $\mu\text{g/mL}$ of BSA, and 0.05 U/ μL of Phusion Human Specimen DNA Polymerase in a total volume of 25 μL . BB-PCR was conducted in the following temperature sequence: preheating at 94 $^{\circ}\text{C}$ for 5 min; 31 cycles consisting of 94 $^{\circ}\text{C}$ for 10 s, 60 $^{\circ}\text{C}$ for 20 s, 72 $^{\circ}\text{C}$ for 20 s; 24 cycles consisting of 94 $^{\circ}\text{C}$ for 10 s, 65 $^{\circ}\text{C}$ for 20 s, 72 $^{\circ}\text{C}$ for 20 s. After amplification, microbeads were washed with 0.1 $\times$ SSC containing 0.1% SDS for three times and re-suspended in 100 μL Milli-Q water for detection by flow cytometry.

2.7 Data analysis

Encoded microbeads with Cy5-labelled amplicons were analyzed by Accuri C6 (BD, USA). The fluorescence detection channels were 515 ± 10 nm and 675 ± 12.5 nm for FL1 and FL4, which were excited by 488 nm or 640 nm laser, respectively. The type of encoded microbeads was determined by the mean fluorescence intensity (MFI) from FL1 channel and the Cy5-labelled amplicon on microbeads was detected by MFI from FL4.

3. Results and Discussion

3.1 Characterization of encoded magnetic microbeads

Firstly, we evaluated the thermal stability of encoded magnetic microspheres during the PCR reaction. **Fig. 1a** shows the Scanning Electron Microscope (SEM) image of a typical host-guest encoded magnetic microbead before and after cyclic heating in PCR buffer. The diameter of host magnetic microbead and guest silica nanoparticle were about 6.2 μm and 190 nm, respectively. There was no significant difference in morphology of encoded microbeads before and after the PCR reaction, which indicated both the materials and the coupling chemistries could tolerate repeated heating-cooling cycles. **Fig. 1b** depicts three encoded magnetic beads with different fluorescent intensity (denoted as weak, medium and high fluorescent intensity) by laser-scanning confocal

microscopy, respectively. No leakage of fluorescent dye molecules was observed during PCR and three different types of beads could be identified easily via fluorescence image after thermal cycling. As shown in **Fig. 1c**, the fluorescence intensity was quantified by flow cytometry, and three distinguished peaks with no overlap were identified, which confirmed the potential of encoded microbeads for multiplex BB-PCR. To further evaluate the influence of encoded microbeads on PCR efficiency, the microbeads were added to conventional liquid PCR reaction. The sensitivity of the reaction was with the same as that of liquid PCR without microbeads, showing that the microbeads had no inhibitory effect on PCR, even when presenting at high concentration (40,000 beads/25 μ l reaction) (**Fig. S1**).

It was very important to minimize the non-specific fluorescence adsorption on the encoded microbeads since the reverse primers for PCR were Cy5-labelled, and there were still a large number of unreacted fluorescent-labeled short-chain primers in the reaction system in the process of the amplification reaction. Previously, our group developed a surface chemistry to fabricate encoded magnetic microbeads with host-guest structures, where 8 layers of polyelectrolytes were assembled layer by layer in situ on the host beads to afford highly stable guest silica nanoparticle landing and covalently conjugating [22]. However, the multi-layer polymer coating caused extremely high non-specific adsorption during PCR. As shown in **Fig. S2**, for the PCR reaction with no DNA template added (negative control), the mean fluorescence intensity (MFI) of encoded magnetic microbeads could reach 10^7 whereas the original ones before PCR was only 10^2 . It could be because the soft polymer chains repeatedly became stretched and shrunk with the periodical change of temperature during PCR, which directly caused the polymer coating to become sparse and fluffy, resulting in significant adsorption of Cy5-labelled primers. To reduce the background noise, we redesigned the surface chemistry by depositing one layer of hyperbranched PEI consisting of amine group, which could covalently form amide bond with carboxyl group on the silica guest nanoparticles, instead of the 8-layer modification. **Fig. 1d** indicated that with the new surface chemistry, the non-specific adsorption was dramatically reduced from 10^7 to less than 2000, which has negligible effect on the final BB-PCR results.

Fig.1 a) SEM image of encoded magnetic microspheres (high fluorescent encoded particles were

chosen as example) before and after PCR reaction. b) Confocal fluorescence microscope image of mixture of three encoded beads (top left), high magnification image of encoded beads with weak fluorescent intensity (top right), encoded beads with medium fluorescent intensity (bottom left), and encoded beads with high fluorescent (bottom right), before and after PCR. For weak and high fluorescent encoding microbeads, guest mixture contained 100% non-fluorescent nanoparticles and 100% of FITC-doped nanoparticles, respectively. For medium fluorescent encoded microspheres, guest mixture contained 92% of non-fluorescent nanoparticles and 8% of FITC doped nanoparticles. c) Histogram of flow cytometry of three encoded microbeads populations identified by fluorescence intensity in the FL1 channel (510/20 nm). The wavelength of the laser was 488 nm. d) Histogram of flow cytometry representing non-specific adsorption state of encoded microbeads.

3.2 Effects of probe length, immobilized probe density and cycle numbers on BB-PCR

The length of oligonucleotide probes and their density on microbeads could significantly influence the accessibility of immobilized probe molecules, which would further affect the efficiency of hybridization between probes and targets as well as binding capacity of PCR polymerase [18]. To determine the optimal parameters, we explored the effects of probe length and immobilized probe density on amplification efficiency of BB-PCR, as illustrated in **Fig. 2a**. Here, we chose *hlaA* gene as the model template. At the same probe immobilization density, MFI was significantly increased with the length of probes from 45 bp to 75 bp, probably due to the exposure of more complementary hybridization sites for annealing and extension [26-28]. To obtain a series of immobilized densities, various initial amounts of probes were utilized, achieving the immobilization probe densities from 2.77×10^{11} , 2.28×10^{11} , 1.90×10^{11} , 1.76×10^{11} , 1.27×10^{11} , 1.20×10^{11} , 5.00×10^{10} , 4.69×10^{10} , to 3.48×10^{10} molecules/mm² and the corresponded amount of probes ranged from 3.34×10^7 to 4.05×10^6 molecules /bead respectively. When the density increased, MFI generated from the 45 bp probe was greatly improved and finally reached the maximum value at probe density of 2.77×10^{11} molecules/mm². However, when much longer probes were used, MFI was slightly increased with probe immobilized density and then reached a plateau. The optimal densities for probes of 60 bp and 75 bp were 1.90×10^{11} molecules/mm² and 1.76×10^{11} molecules/mm², respectively. The different optimal immobilization density for probes at various length might be explained by the collective effects of both amplification efficiency and the interactions of adjacent probe molecules. As the density increased, the concentration of probes in a single test was improved, which may lead to higher amplification efficiency. Meanwhile, the interactions between probes also became stronger, making them less accessible to DNA target or primers. The interference between longer probes was

more obvious when compared to shorter probes, which could be the reason for the lower optimal density with longer probes. In theory, when the surface footprint of the probe is larger than the average surface area for each of them, interactions between adjacent probes occur and tend to be enhanced as the probe immobilization density increases [27,28].—For the 45 bp, 60 bp and 75 bp probes, the theoretical critical immobilized probe densities for the interactions to happen are 6.58×10^{10} , 4.93×10^{10} , 3.92×10^{10} molecules/mm² [27,28]–^{20–21}, respectively. Thus, when the probe density was above these values, MFI increased slowly or even decreased.

To evaluate the specificity, multiplex PCR mix containing three encoded microbeads coupled with probes for *hlaA*, *sefA*, *sdf* genes was prepared, whereas only two plasmids containing two specific genes mentioned above were added (**Fig. 2b**). The specificity of the three probes at 60 bp and 75 bp were shown in **Fig. 2c** and **2d**, respectively. Although 75 bp probe gave higher MFI, the specificity of 75 bp probe for the *sefA* gene detection was poor, as shown in **Fig. 2d**, where the microbeads with *sefA* gene-specific probes gave positive signals when there was no *sefA* gene in the test. The nonspecific signal was likely due to complementary hetero-dimer sequences of 10 consecutive base pairs and 5 A-T pairs exist between 75 bp *sefA* probe and the *sdf* amplicon based on integrated DNA Technologies (IDT) analysis. It indicated that longer probe was more likely to be complementary to nonspecific target. Thus, 60 bp probe was used in the following experiments **due to the** high specificity.

Fig.2. a) Effect of length and density of probes on BB-PCR for *hlaA* gene detection. b) Four experimental groups were designed for evaluating the specificity of each type of probes. The minus sign means this gene was not added to the reaction and the plus sign means the opposite. Specificity of three probes for *hlaA*, *sefA*, and *sdf* gene detection at c) 60 bp and d) 75 bp, respectively.

In order to further improve amplification efficiency of multiplex BB-PCR, we increased number of thermal cycles from 47 to 60. As expected, **Fig. 3a** indicated *hlaA* gene could be detected at a much lower concentration (below 10copies/reaction), when the cycle number was increased to 55. **Fig. 3b, 3c** showed that the fluorescent intensity from *sdf* and *sefA* genes were significantly enhanced when more cycles were added, whereas the limit of detection for these two genes were not improved. As such, 55 cycle was adopted.

Fig.3 Effect of number of thermal cycles on multiplex BB-PCR for a) *hilA*, b) *sdf* and c) *sefA* gene detection. The column graph corresponds to the mean fluorescence intensity of BB-PCR.

After optimization of the probe length, immobilization density, number of thermal cycle and addition of encoded microbeads (**Fig. S3**), BB-PCR reactions were performed to detect the *hilA*, *sefA*, *sdf* gene, respectively. Three 60 bp probes were immobilized on encoded microbeads with a density of $1.90 \times 10^{11}/\text{mm}^2$, and 10,000 microbeads were added to each reaction. Although BB-PCR is usually intuitively considered to have lower sensitivity due to relatively inefficient diffusion of immobilized probes compared with primers in solution, it was worth noting that the limit of detection of BB-PCR for *hilA*, *sefA*, *sdf* genes were all around 10 copies/reaction shown in **Fig. 4**. Besides, electrophoresis results demonstrated the detection sensitivity of *hilA* and *sdf* genes in liquid PCR was below 10 copies/reaction and *sefA* genes was around 10 copies/reaction (**Fig. S4**), which was much closer to sensitivity of BB-PCR. For the three genes, MFI values were increased linearly with the concentration of target DNA. Besides, MFI value of microbeads corresponding to each gene differed greatly when the same concentration of template was added, which might be caused by the different secondary structures of three probes and different amplification efficiency of these primers.

Fig.4 Sensitivity of BB-PCR for a) *hilA* gene, b) *sefA* gene, c) *sdf* gene and d) triple genes detection. The concentration of template varied from 1 to 10^6 copies/reaction with 10-fold dilution. The insets show the MFI of negative control and 10^0 copies/reaction.

To further examine the sensitivity of multiplex BB-PCR, three types of encoded microbeads with specific probes were added to a single test. As shown in **Fig. 4d**, for these three genes, MFI signals were firstly enhanced with the concentration of template then came to a plateau, the detection limit of multiplex BB-PCR for three genes were also around 10 copies/reaction, which was similar to that of PCR for each gene, indicating that the amplification efficiency of multiplex PCR could be comparable with that of single gene PCR when performed on microbeads. Moreover, most noteworthy was that the detection limit of multiplex PCR in solution for three genes was worse than that for each genes and even multiplex BB-PCR. The detection sensitivity of multiplex PCR in solution for *sdf* gene was decreased to 100 copies / reaction (**Fig. S4 and S5**), which demonstrated

the usefulness of physical isolation of immobilized primes for reducing the interactions and achieving the efficient and specific amplifications.

3.3 Comparative evaluation of BB-PCR using clinical real samples

As we all know, Salmonella is a common bacterium that not only causes various poultry and livestock disease, but also leads to food poisoning in humans. Among various subtypes, S. Enteritidis is one of the major pathogenic bacteria, which could do harm to human health. To confirm the potential clinical application of multiplex BB-PCR on encoded microbeads for S. Enteritidis identification, we tested 60 diarrhea patients' cultured samples from Chinese Center for Disease Control and Prevention (CDC) in Guangzhou in a double-blind experiment. **Fig. 5** shows the two distinct groupings in MFI values corresponding *hilA*, *sefA*, and *sdf* gene between positive and negative samples ($P < 0.0001$), indicating this method could be used to identify triple targets in a real sample. The data of the 60 samples is shown in **Table S2**. The traditional bacterial culture method is time consuming and may be affected by subjective experience of inspectors or antibiotic contamination. In comparison, BB-PCR method could attain quicker and more accurate identification for multiple bacterium by nucleic acid detection, though MFI could not reflect the initial amount of the targets.

Despite of the different mechanisms between BB-PCR and traditional bacterial culture method, S. Enteritidis detection with 100% specificity and 100% sensitivity compared with culture methods was obtained (**Fig. 5**), showing the effectiveness of BB-PCR strategy.

Fig.5 Results of identification of S. Enteritidis in 60 real samples by simultaneous detection of *hilA*, *sefA* and *sdf* genes.

4. Conclusions

In summary, we reported a multiplex BB-PCR detection platform based on thermostable, encoded microbeads. To obtain the high sensitivity comparable to that of liquid PCR, parameters such as probe length, immobilization density, number of thermal cycle and number of microbeads have been optimized. We found longer immobilized probes caused higher efficiency but might result in poor

specificity. Besides, the relative optimal density of immobilization depended on the length of probe, where the longer probes could correspond to lower optimal density. Increasing the number of the thermal cycle was less effective to improve the sensitivity when it was above a certain value. Under the optimal conditions, the detection limit of multiplexed BB-PCR was about 10 copies/reaction, which was comparable to that of liquid PCR. Moreover, in double blind experiment, the result of *S. Enteritidis* detection using multiplex BB-PCR was highly consistent with that of the traditional culture method, but the detection time was greatly shortened. As encoded microbeads with host-guest structure are thermostable, easy to prepare and show ultrahigh encoding capacity, this method has great potential to realize sensitive detection for tens of DNA targets in a single tube, thus providing an innovative multiplexed BB-PCR platform for personalized, easily manipulated and automatic nucleic acid detection.

Conflict of interests

The authors declare no competing financial interests.

Author statement

Hong Xu, Yi Sun and Hongchen Gu conceived the research, acquired funding and supervised the whole project; Zhejia Gu and Gaolian Xu designed the experimental plan; Yao wang gave some important advice for decreasing non-specific adsorption of microbeads. Zhejia Gu, Simin Zhao and Cang Chen performed the experiments; Zhejia Gu and Hong Xu analyzed data and wrote the paper. This paper was revised and approved by all the co-authors.

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