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Lateral flow biosensor for DNA extraction-free detection of salmonella based on aptamer mediated strand displacement amplification

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ABSTRACT

Convenient and sensitive point-of-care rapid diagnostic tests for food-borne pathogens have been a long-felt need of clinicians. Traditional approaches such as culture-based methods have good sensitivity and specificity, but they tend to be tedious and time-consuming. Herein we present a simple and sensitive aptamer based biosensor for rapid detection of *Salmonella enteritidis* (*S. enteritidis*). One of the aptamers specific for the outmembrane of *S. enteritidis* was used for magnetic bead enrichments. Another aptamer against *S. enteritidis* was used as a reporter for this pathogen, which was amplified by isothermal strand displacement amplification (SDA) and further detected by a lateral flow biosensor. As low as 10^1 colony forming unit (CFU) of *S. enteritidis* was detected in this study. Without DNA extraction, the reduced handling and simpler equipment requirement render this assay a simple and rapid alternative to conventional methods.

1. Introduction

Food-borne disease is one of the major public health problems, especially in developing countries. Failure to detect food-borne pathogens may lead to a dreadful effect. The World Health Organization (WHO) reported that in 2005 alone 1.8 million people died from diarrheal diseases, a great proportion of these cases can be attributed to contaminated food and drinking water ("Food safety and foodborne illness" Fact sheet, https://apps.who.int/inf-fs/en/fact237.html). *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli*, commonly found in food and drinking water, pose a threat to public health (Hedberg, 1999; Sockett, 1991; Crump and Mintz, 2010). They were responsible for majority of food-borne illness and outbreaks (Alocilja and Radke, 2003; Chemburu et al., 2005). Patients with these pathogen infections can cause gastrointestinal disease, fever, diarrhea, abdominal cramps, vomiting, and nausea (Helms et al., 2005; Raguenaud et al., 2010; Allerberger and Wagner, 2010; Pichler et al., 2009; Aureli et al., 2000; Oh and Lau, 2012; Colgan et al., 2011; Peterson, 1996).

Significant improvements of disinfection in food processes have been achieved, such as the rigorous adherence to good manufacturing practices (Mucchetti et al., 2008) and good agricultural practices (Kay et al., 2008), the outcome of food-borne pathogenic microorganism control is still not optimistic. Therefore, routine monitoring of the quality and safety of food is important for public health.

Conventional culture-based method for pathogen diagnosis is labor intensive, time consuming, and costly. It usually takes up to 72 h to obtain confirmed results (Fang et al., 2010). They are unable to meet the needs of real-time pathogen detection. Therefore, rapid methods such as antibody-based immunoassay (Kang et al., 2009; Pappert et al., 2010), polymerase chain reaction (PCR) based methods (Donhauser et al., 2011; Kumar et al., 2010; Lee et al., 2009; McCarthy et al., 2009; Park et al., 2011; Pui et al., 2011; Wolter et al., 2008), and loop-mediated isothermal amplification methods (Maruyama et al., 2003; Ravan and Yazdanparast, 2012a, 2012b; Wang et al., 2012) have been developed for the detection of food-borne pathogens. These methods have good sensitivity and specificity, but expensive, specialized equipments and highly trained personnel are still required. Besides, DNA extraction also adds complexity to the detection. In addition, DNA-based methods only tell the presence of target nucleic acid in the sample, false-positive results may be encountered when cells have broken down (Josephson et al., 1993). Thus, antibody-based methods such as enzyme-linked immunosorbent assay (ELISA) that depend on the integrity of pathogens have better performance in viable pathogen detection (Kumar et al., 2008). But the low sensitivity is still a major limitation for ELISA. Therefore, magnetic bead based enrichment emerges as an attractive pre-analytical

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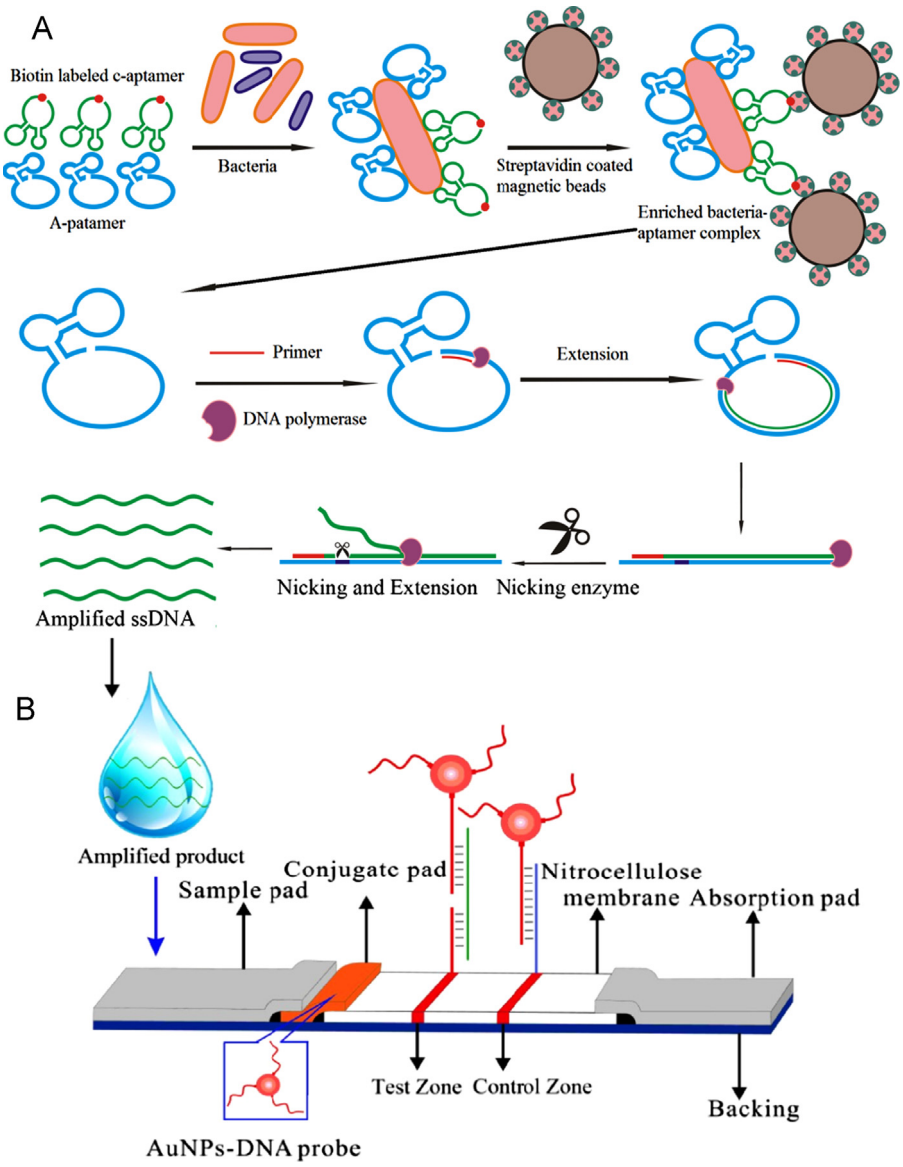

Fig. 1. Schema of the aptamer based detection. (A) A schematic description of the magnetic enrichment of bacteria and SDA amplification of aptamer. Bacteria are first mixed with excess amount of aptamers. After incubation, streptavidin coated magnetic beads are added, and the MNB–Ap–bacteria complexes are collected by a magnet separator. The collected MNB–Ab–bacteria complexes are directly amplified by isothermal strand displacement amplification. (B) The amplified product is loaded onto the sample pad for visual detection.

sample processing method for sensitive detection (Liandris et al., 2011; Vasanth et al., 2011; Wang et al., 2011; Yang et al., 2013).

Aptamers are single stranded oligonucleotides that have high affinity to certain targets as antibodies do (Pestourie et al., 2005; Savran et al., 2004; Stoltenburg et al., 2007; Tuerk and Gold, 1990). They are widely used for analyte detection. Moreover, aptamers are inexpensive, stable, and can be chemically synthesized in high purity and modified with relative ease. In this study, we developed an aptamer based biosensor for simple and rapid detection of viable *Salmonella*. Two aptamers against different outermembrane proteins were used in the detection. Biotin modified capture-aptamer (c-aptamer) was used for the bacteria enrichment. Another unmodified amplification-aptamer (a-aptamer) against another outermembrane protein was used as template for strand displacement amplification (SDA). The amplified single strand DNA was further detected with a lateral flow biosensor (Fig. 1). Thus, a simple and reliable biosensor for pathogen diagnosis was established.

2. Materials and methods

2.1. Reagents and chemicals

Oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology (Shanghai, China). Vent (exo-) DNA polymerase, Nb.BbvCI, *Taq* DNApolymerase, deoxynucleoside triphosphates (dNTPs) were purchased from New England Biolabs (New England, USA). $HAuCl_4$ was from Sigma-Aldrich (Steinheim, Germany). Bovine serum was obtained from Dingguo Biotech. Co. (Beijing, China). Nitrocellulose membrane was purchased from Sartorius (Goettingen, Germany). Fiberglass and absorbent paper were purchased from Shanghai Kinbio (Shanghai, China). All buffer solutions used in this study were prepared in our lab. Other chemicals were purchased from standard commercial sources and were of analytical grade purity.

2.2. Preparation of gold-nanoparticle (AuNP)–DNA conjugates

AuNPs with an average diameter of 15 nm were prepared according to our previously reported method (Fang et al., 2010).

Briefly, 4 mL of 1% trisodium citrate was added into 100 mL of a rapidly stirred and boiling $HAuCl_4$ solution (0.01%) in a 500-mL round bottom flask. After turning red, the solution was boiled for additional 10 min. It was cooled to room temperature with gentle stirring. The AuNPs were collected and concentrated 4 times by centrifugation (12×10^3 rpm, 20 min). The resulting AuNPs solution was stored at 4 °C until use.

To prepare AuNP–DNA conjugate, 100 μL of ultra-pure water (18.2 MΩ/cm, Millipore, Billerica, MA, USA) containing 1 OD of thiolated DNA was added to 1 mL of the AuNPs solution (4 times concentrated) and shaken gently overnight at 4 °C. The DNA coated AuNPs (1.1 mL) were subjected to "aging" by adding 110 μL 100 mM phosphate buffer (pH 7.0) with 1% sodium dodecyl sulfate and 1.5 M NaCl. The solution was kept at 4 °C for 12 h. Particles were centrifuged (12×10^3 rpm, 20 min) and rinsed three times with rinsing buffer (20 mM Na_3PO_4, 5% BSA, 0.25% Tween-20 and 10% sucrose) to remove any unbound DNA. The red pellet was re-suspended in 150 μL of rinsing buffer and then stored in a refrigerator at 4 °C until use. The AuNP–DNA conjugate solution was dispensed onto fiberglass as conjugate pad.

2.3. Construction of lateral flow biosensor

Thirty microliters of 100 μM DNA probes (test line probe and control line probe) were dispensed onto the nitrocellulose membrane simultaneously with a lateral flow dispenser (Shanghai Kinbio, Shanghai, China). The membrane was then dried at room temperature for 12 h. Fiberglass was used as sample pads after being soaked in sample pad buffer (0.5% Triton, 1% BSA, 2% sucrose, 50 mM boric acid, pH 8.0). It was dried and stored in low-humidity at room temperature. Sample pad, conjugate pad, nitrocellulose membrane and absorbent pad were attached along the long axis of an adhesive plate with an overlap of 1–2 mm, and cut into 4-mm-wide strips using a paper cutter (Fig. 1B).

2.4. Bacteria culture

Strains of *Salmonella typhimurium*, *S. enterica*, *E. coli* O157:H7, *Pseudomonas aeruginosa*, and *Citrobacterfreundio* were obtained from Guangzhou Microbiology Institute. Strains were cultured aerobically for 16 h at 37 °C in 50 mL of Luria-Bertani (LB) medium (final OD > 1 for each sample).

For colony formation test, bacteria were incubated at 37 °C for 30 min. Fifty microliters of the bacteria suspension were diluted and plated on dishes with LB agar in triplicate. The dishes were incubated at 37 °C for 24 h to allow bacterial growth. The colonies were then counted.

2.5. Experimental design

Two aptamers, capture-aptamer and amplification-aptamer, were used in this assay. The capture-aptamer was pre-labeled with biotin moiety, which was captured by streptavidin coated magnetic beads for enrichment. The two aptamers were first incubated with pathogens. Then streptavidin coated magnetic beads were added into the mixture. After three times of magnetic washing, the enriched amplification-aptamer that anchored on the outermembrane was used as template for single direction strand displacement amplification. The amplified single strand DNA (ssDNA) was further detected with lateral flow biosensor (Fig. 1). Thus, a simple, reliable, point-of-care rapid diagnostic tests (RDTs) for pathogen diagnosis was established.

2.6. Enrichment of bacteria

Bacteria were first mixed with excess amount of aptamers in a 500 μL of phosphate buffered saline (PBS) supplied with 0.1 mg/mL of salmon sperm DNA. The ultimate concentration of each aptamer was 1 μM. The mixture was incubated at room temperature for 30 min. Ten microliters of streptavidin coated magnetic beads (Invitrogen, M-270 Streptavidin) were then added to the mixture. The mixture was incubated at room temperature for another 10 min with intermittent shaking. Magnetic bead–aptamer–bacteria complexes (MNB–Ap–bacteria) were collected by a magnet separator. The precipitant was transferred to another tube and washed three times with PBST (PBS supplied with 0.05% Tween-20). The collected MNB–Ap–bacteria complexes were suspended with 10 μL of ultrapure water. Half of the bacteria suspension was detected by the present method, and the other half was used for PCR confirmation.

2.7. Detection of bacteria

For the aptamer based detection, 5 μL of previously prepared bacteria suspension was added to 15 μL of reaction solution (1 μM primer, 1 U Nb. BbvCI, 5 U polymerase Klenow fragment exo-, 50 μM dNTPs, and 1 × polymerase Klenow fragment exo-reaction buffer). It was incubated at 50 °C for 30 min. After amplification, 10 μL of the product was diluted in 50 μL of 4 × saline-sodium citrate (SSC) and loaded onto the sample pad in the biosensor. Test line and control line in biosensor were visually determined in 5 min.

The performance of this method was also tested using bacteria spiked food products. Milk powder obtained from local supermarket was analyzed. Ten grams of milk powder was mixed with 100 mL of PBS, and analyzed for the absence of endogenous *S. enteritidis* using PCR assay. The confirmed *S. enteritidis* negative milk was spiked with target bacteria and analyzed immediately. Briefly, 50 μL of target bacteria cell suspension was added into 950 μL of milk, mixed thoroughly, and centrifuged at 500*g* for 5 min to remove insoluble particles. Target bacteria in the supernatant were detected as described above.

To make sure the milk powder was free of *S. enteritidis*, PCR based *S. enteritidis* detection was carried out according to previously reported method with some modification (Kumar et al., 2010). Total DNA was isolated from milk sample by DNA extraction kit (Tiagen biotech, Beijing). Aliquots (3 μL) of DNA solution were directly added to the PCR reaction buffer, and the reaction volume was adjusted to 20 μL. The PCR system consisted of 125 mM dNTP mixture, 200 nM primer, 2 U of Taq polymerase. The PCR was carried out as follows: 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 10 min. The PCR products were run on 2% agarose gel and stained with SYBR green.

For the PCR detection of amplification-aptamer on the enriched bacteria, 5 μL of the magnetic collected bacteria suspension was directly added into 15 μL of PCR reaction buffer. The PCR system consisted of 125 mM dNTP mixture, 200 nM primer, 2 U of Taq polymerase. The PCR was carried out as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 72 °C for 10 min. The PCR products were run on 2% agarose gel and stained with SYBR green.

3. Results and discussion

3.1. Design of probes for S. enteritidis detection

Two aptamers were synthesized in this study (c-aptamer and a-aptamer) according to a previously reported research (Kolovskaya

et al., 2013). The c-aptamer was modified with biotin for magnetic separation. A primer sequence and a Nb.BbvCI recognition site were added at 3′ of the a-aptamer for SDA amplification. AuNP-probe was modified with a thiol group at the 5′ for AuNPs coating. Test-probe and AuNP-probe are complementary to two fragments of the amplified target, hence forming a sandwich link between test zone and AuNPs. Control-probe is a DNA sequence complementary to the AuNP-probe (Table 1).

Table 1
Oligonucleotide sequences [a,b,c,d,e].

Oligonucleotide	Sequence 5′-3′
Amplify-aptamer	TCGGCAACAAGGTCACCCGGAGAAGATCGGTGGTCA-AACTGCATAGGTAGTCCAGAAGCCGAACAAGCTGAGG-**ATGAAGAACAACGGCT**
Capture-aptamer	B-n-TCGGCAACAAGGTCACCCGGAGAAGATCGGT-GGTCAAACTGCATAGGTAGTCCAGAAGCCGAACAAGC-TGAGGATGAAGAACAACGGCT
Primer	AGCCGTTGTTCTTCAT
AuNPs-probe	S-n-TAGTCCAGAAGCCGAACAA
Control-probe	TTGTTCGGCTTCTGGACTA
Forward primer	TCGGCAACAAGGTCACCC
Reverse primer	AGCCGTTGTTCTTCAT

[a] Solid underlines indicate the recognition sequence of Nt.BstNBI.
[b] Bold characters indicate the additional primer sequence.
[c] B indicates a 5′ biotin modification.
[d] n Indicates a C_6 linker.
[e] S indicates a 5′ thiol modification.

3.2. Establishment of the biosensor for S. enteritidis detection

Primary experiments were carried out using 10^4 CFU of *S. entertidis.* A-aptamer and c-aptamer in excess with a molar ratio of 1/1 was used for the enrichment. Besides, *S. entertidis* without a-aptamer or c-aptamer was also tested. After 30 min of amplification, the mixture was loaded onto the sample pad. Fig. 2 shows the typical photo-images of the biosensor. No test line was observed in the absence of *S. entertidis*, c-aptamer or a-aptamer. The distinctive red test line could only be observed in the presence of two aptamers and *S. entertidis*. The results showed that this biosensor works in *S. enteritidis* detection.

3.3. Sensitivity of the biosensor for S. enteritidis detection

After optimization the proportion of these two aptamers (Fig. S1 Supporting information), the sensitivity of the biosensor was evaluated using serially diluted *S. enteritidis*. After magnetic separation, the enriched *S. enteritidis* was divided in two parts, which were detected by the present biosensor and PCR. Fig. 3A presented the biosensors for the detection of different concentrations of *S. enteritidis*. From left to right, the concentration of *S. enteritidis* are 10^{12} CFU/mL, 10^8 CFU/mL, 10^6 CFU/mL, 10^4 CFU/mL, 10^2 CFU/mL, 10^1 CFU/mL, 0 CFU/mL. There was no red test line in the absence of *S. enteritidis* (0 CFU/mL). Visible test lines were observed as different concentrations of *S. enteritidis* were tested (Fig. 3A). Semi-quantitative detection can be further performed by recording the intensities of the test line with a strip reader. The other part of *S. enteritidis* was also verified by PCR. Specific bands were observed in the presence of *S. enteritidis* (Fig. 3B). The minimum number of bacteria that can be detected by

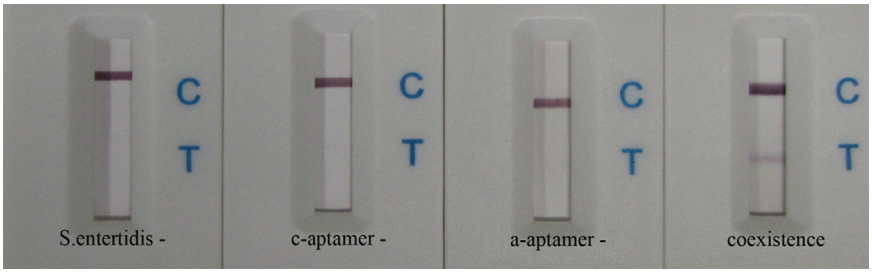

Fig. 2. Typical images of the biosensor for *S. enteritidis* detection with different conditions. From left to right are detection without *S. entertidis*, c-aptamer, a-aptamer and coexistence of both aptamers and *S. enteritidis*.

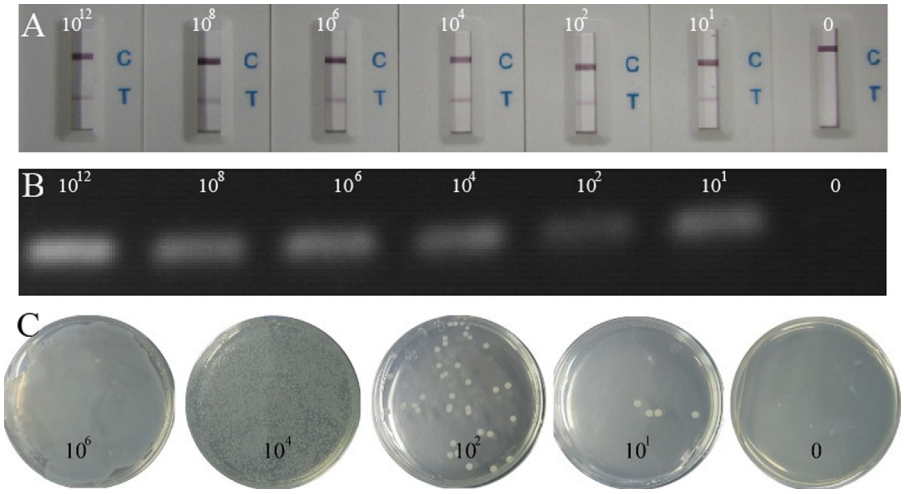

Fig. 3. Sensitivity of the biosensor. (A) Typical images of biosensor for different concentrations of *S. enteritidis* detection. From left to right, 10^{12} CFU to 0 CFU of *S. enteritidis* are used in the detection. (B) PCR confirmation of the *S. enteritidis*. (C) *S. enteritidis* culture plates with different concentrations. From left to right, the concentrations of *S. enteritidis* are 10^6 CFU/mL to 0 CFU/mL.

PCR amplification is 10^1 CFU/mL. The detection limit of PCR was the same as that of the biosensor. Parallelly prepared samples with the same concentration of *S. enteritidis* were also detected using conventional culture-based method. Numbers of colonies were formed in bacteria positive samples, and the colonies merged together when *S. enteritidis* researched 10^6 CFU/mL (Fig. 3C).

3.4. Selectivity of the biosensor for S. enteritidis detection

The selectivity of the biosensor was tested using various bacteria strains available for this study. *S. enteritidis*, *S. typhimurium*, *E. coli* O157, *Pseudomonas aeruginosa*, and *Citrobacterfreundi* at 10^4 CFU/mL were tested. As shown in Fig. 4A, only *S. enteritidis* showed a red test line, while non-target bacteria strains did not produce any visible test line. These results demonstrated the high specificity of the present biosensor.

Specificity of the biosensor was also investigated by introducing competitive c-aptamer without biotin modification. The unmodified c-aptamer can compete with the biotin-modified c-apatamer from adsorbing onto the streptavidin coated magnetic beads, decreasing the magnetic force during magnetic collection. As expected, intensities of the test lines decreased with the increment of unmodified c-aptamer (Fig. 4B), which further confirmed the high specificity of this biosensor.

3.5. Biosensor detection for S. enteritidis spiked samples

Commercially obtained milk, which confirmed to be *S. enteritidis*-negative, was spiked with *S. enteritidis* for the recovery analysis. Ten-fold serially diluted *S. enteritidis* was prepared in PBS. It was mixed with 1 mL of prepared milk right before detection. The ultimate concentrations of target bacteria were 0 CFU/mL, 10^1 CFU/mL, 10^2 CFU/mL, 10^4 CFU/mL, 10^6 CFU/mL, 10^{12} CFU/mL. All the *S. enteritidis* spiked samples were detected as positive results and the blank sample had non-detectable *S. enteritidis* (Fig. 5). The sensitivity of spiked sample detection was 10^1 CFU/mL. These results showed a promising method for the real food sample detection. At optimal conditions, a false positive rate less than 2% can be obtained.

While several novel and promising methods, such as PCR based methods, have been developed for pathogenic bacteria detection in foods and other types of samples, but specialized equipments and highly trained personnel are still required. Thus, point of care device, characterized by simplified handlings and reduced needs of laboratory instruments, which has a short assay time, emerges as an attractive way for in field test. Based on the lateral flow biosensor, we developed an aptamer based assay for simple and sensitive detection of food-borne bacteria. As low as 10^1 CFU *S. enteritidis* was visually detected in spiked food sample. The culture-based method also confirmed the present assay with high sensitivity and specificity.

The lateral flow biosensor we established presents several advantages over other methods. First, it is extremely simple to use and easy to interpret. This biosensor is comprised of a simple dipstick made of a porous membrane that contains capture-probe coated colloidal gold particles. They are immobilized on the test line in the presence of target DNA, which enables visual detection. No washing or manipulation is required, and the detection of amplified DNA can be completed within 10 min. While conventional PCR relies on the separation of PCR products by gel electrophoresis. It takes hours to analyze the resulting electrophoretic patterns.

Secondly, the sample pretreatment of this assay is simpler than that of PCR based methods, while comparable level of sensitivity is

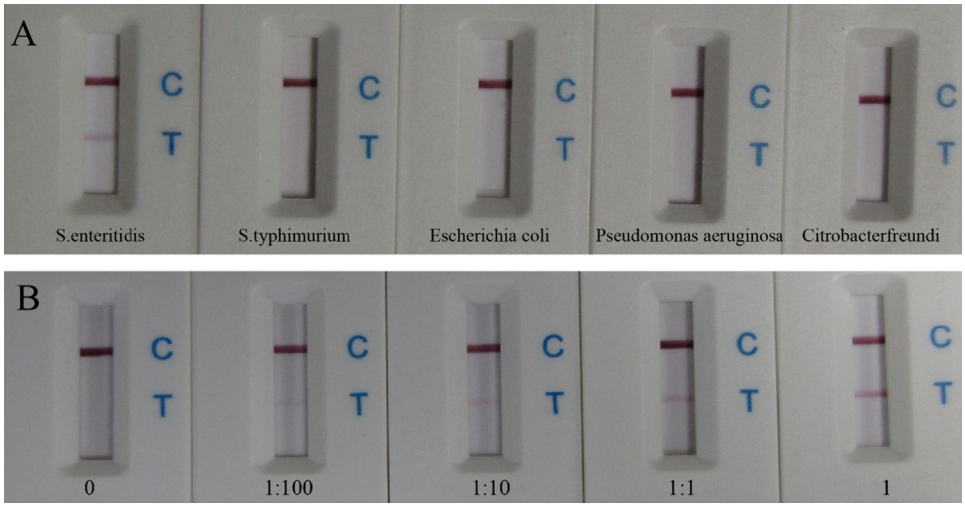

Fig. 4. Selectivity of the biosensor. (A) Typical images of the biosensor for the detection of different strains of pathogens. (B) Typical images of biosensor for *S. enteritidis* detection with different concentrations of unmodified c-aptamer. From left to right, the ratios of modified/unmodified c-aptamer are 0:1, 1:100, 1:10, 1:1, and 1:0.

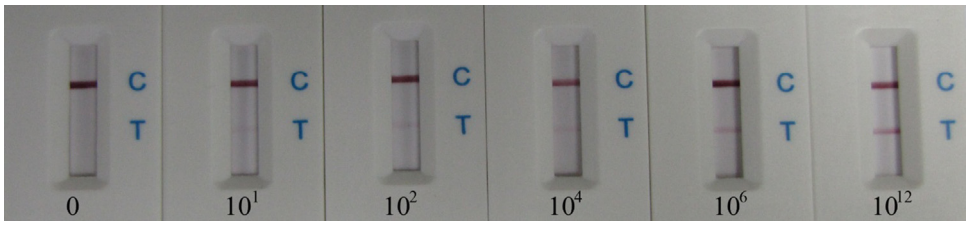

Fig. 5. Typical images of the biosensor for *S. enteritidis* spiked sample detection. From left to right, the concentrations of *S. enteritidis* are 0 CFU/mL, 10^1 CFU/mL, 10^2 CFU/mL, 10^4 CFU/mL, 10^6 CFU/mL and 10^{12} CFU/mL.

achieved. The simplicity of sample processing is an important fact for in field tests, which is one of the most challenging problems for the nucleic acid based assays. In the present study, the tedious cell lysis and DNA/RNA extraction was avoided. Using two bacterium-specific aptamers, the separated and concentrated specific bacterium can be directly added into the reaction buffer for isothermal amplification. Rather than bacterium DNA, the amplification-aptamer which acts as bacterium-tag and signal amplifier is finally detected by the biosensor. No specialized instrumentation such as thermal cycler or centrifuge is needed, which facilitates the fast in field test.

Thirdly, the specificity of the present assay is relative higher than that of nucleic acid based method, and is comparable to that of antibody based method. As DNA can persist in sample for a long time, positive results still can be encountered even the bacteria have broken down in nucleic acid based method. Furthermore, the specificity needs to be confirmed by sequencing the amplified fragment. Unlike previous methods, the present assay depends on the integrity of pathogen, and no DNA/RNA origin from bacteria is engaged in the detection. The amplified DNA is further confirmed by the sandwich hybridization on the test zone. The result has better response to the viable pathogens in the sample.

4. Conclusion

In conclusion, we developed an aptamer based biosensor for simple and sensitive detection of food-borne bacteria. It combined different rapid methods, including antibody-free enrichment of specific bacteria, and lateral flow biosensor based DNA detection. Furthermore, tedious DNA extraction and purification was avoided in this SDA-based isothermal amplification. Therefore, only the captured amplification-aptamer on cell membrane is amplified, limitations of conventional DNA amplification based method such as false-positive can be largely reduced. With reduced handling and simpler equipment, this assay provided simple and rapid alternatives to conventional methods. Moreover, this method could be applied to detect other bacteria by using different bacterium specific aptamers.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.01.015.

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