



Potential Use of DNA Aptamer-Magnetic Bead Separation-PCR Assay for *Salmonella* Detection in Food

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HIGHLIGHTS

- Limit of detection of *Salmonella* spp. for Aptamer-Magnetic bead Separation-Polymerase Chain Reaction (AMS-PCR) method was 10^2 CFU/ml.
- AMS-PCR was 10 times more sensitive than conventional PCR.
- In comparison with the culture method, AMS reduced the pre-enrichment and enrichment times.
- Combining AMS with PCR is cost-effective, time-saving, and highly specific for monitoring of *Salmonella* spp. in foods.

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Acronyms and abbreviations

AMS=Aptamer-Magnetic bead Separation
PCR=Polymerase Chain Reaction
CFU=Colony Forming Unit
IMS=Immunomagnetic Separation
PBS=Phosphate Buffered Saline

ABSTRACT

Background: *Salmonella* is one of the most common food-borne pathogens that can cause illness. In this study, the sensitivity and the specificity of Aptamer-Magnetic bead Separation-Polymerase Chain Reaction (AMS-PCR) method were determined for *Salmonella* spp. detection.

Methods: Different concentrations of *Salmonella enterica* were mixed with streptavidin-magnetic beads coated with biotinylated DNA aptamer. The bound bacteria were eluted and tested with PCR targeting the *invA* gene of *Salmonella*. Ten different serovars of *Salmonella enterica* and four non-*Salmonella* were tested to determine the specificity of the DNA aptamer. For field application, 14 different food samples were tested and compared with the culture method.

Results: The limit of detection of AMS-PCR method was 10^2 CFU/ml which was 10 times more sensitive than conventional PCR without AMS (10^3 CFU/ml). The AMS-PCR assay showed high specificity as it detected ten different serovars of *Salmonella enterica* with no cross-reactivity with other food-borne pathogens. AMS-PCR reduced the analytical duration from 6 to 7 h instead of 4 days by the culture method.

Conclusion: In comparison with the culture method, AMS helped to improve the upstream sample preparation in reducing the pre-enrichment and enrichment times. So, it seems that combining AMS with PCR is cost-effective and time-saving. In addition, it is highly specific for monitoring of *Salmonella* spp. in food chain.

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Introduction

Salmonella is one of the most common food-borne pathogens that can cause severe illness in human beings (Carrasco et al., 2012; Robinson, 2014). Salmonellosis is typically a zoonotic disease which its bacterial agent is

widely distributed in foods, such as poultry, raw food, vegetables, eggs, unpasteurized milk, as well as juice (Mukhopadhyay and Ramaswamy, 2012; Ricke et al., 2015). Ingestion of food contaminated with *Salmonella*

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results in salmonellosis with clinical symptoms of abdominal cramp, diarrhea, vomiting, nausea, and fever. Salmonellosis can also result in severe illness in immune-compromised individuals that can lead to life-threatening septicemia (Kunwar et al., 2013).

Development of an accurate, rapid, and specific detection tool for detection of *Salmonella* in foods is crucial as an early step of the disease control. Although culture method is the gold standard for bacterial detection, it is laborious and time-consuming requiring 5-7 days for positive confirmation (Lee et al., 2015). Hence, intensive research has been conducted to develop rapid, sensitive, and specific detection tools, including molecular, immunological, and biosensor approaches (Zhao et al., 2014). However, enrichment culture is still needed for pre-analytical sample preparation which remains the main challenge for rapid detection (Suh and Jaykus, 2013).

The food inhibitors, such as salts, preservatives, and diversity of microbiota may interfere the detection steps. To increase the sensitivity, specificity, and rapidity of detection method, the pre-analytical sample preparation step is important to separate, concentrate, and purify the target bacteria from the food samples (Brehm-stecher et al., 2009; Stevens and Jaykus, 2004). Immuno Magnetic Separation (IMS) that involves the use of specific antibody for coating the surface of magnetic beads has been widely used for pre-analytical sample preparation of complex food matrix (Jeníková et al., 2000; Romero et al., 2016; Wang et al., 2013, 2014; Xiong et al., 2014). However, antibody is relatively unstable, with short shelf-life, expensive, and difficult to produce (Famulok and Mayer, 2011; Jayasena, 1999).

An aptamer may be a proper alternative element to be used as a ligand or biological factor for fast, specific, and accurate detection of bacteria. An aptamer is a synthetic single strand DNA (ssDNA) or RNA that can be selected from the nucleic acid library using systemic ligand by exponential enrichment process (Ellington and Szostak, 1990; Tuerk and Gold, 1990). An aptamer can form a functional specific 3D structure with high affinity to a wide range of targets (Song et al., 2012) comparable with monoclonal antibodies, therefore making it a suitable alternative for diagnostics (Famulok et al., 2007). Other advantages of the aptamer are long-term stability, high affinity, specificity, easy to use, and low cost to production (Amaya-González et al., 2013; Song et al., 2012).

In this study, we used a biotinylated DNA aptamer bound to streptavidin-magnetic beads as a biological element to separate *Salmonella* cells from a complex food matrix, followed by PCR as subsequent detection method. Sensitivity and specificity of Aptamer-Magnetic bead Separation-Polymerase Chain Reaction (AMS-PCR) method were determined. We further evaluated this

approach with naturally contaminated food samples. Our method of AMS-PCR was compared with the culture method to determine its performance in terms of detection time.

Materials and methods

Preparation of aptamer

The sequence of the ssDNA aptamer (5'-T ATG GCG GCG TCA CCC GAC GGG GAC TTG ACA TTA TGA CAG-3') was used that previously reported by Joshi et al. (2009) and modified with biotin at the 5' end by a commercial company (Integrated DNA Technologies, USA).

Preparation of bacterial cultures

An overnight cell culture of *Salmonella enterica* was centrifuged at 1844 × g for 10 min, and the cell pellet was washed twice with 1 ml of 1 × Phosphate Buffered Saline (PBS; 0.1 M, pH 7.4). The cell density was adjusted to 0.5 McFarland standard in 1 × PBS by using the DEN-1 densitometer (Biosan, Latvia) and the corresponding Colony Forming Unit (CFU) were determined by plate counting method on the Luria-Bertani (LB) agar (Oxoid, UK).

The strains of 10 different serovars of *Salmonella enterica* previously isolated from food or clinical sources were used for evaluation, including *S. Typhimurium*, *S. Enteritidis*, *S. Typhi*, *S. Paratyphi B*, *S. Paratyphi A*, *S. Corvalis*, *S. Indiana*, *S. Pullorum*, *S. Albany*, and *S. Branderup*. Each strain of non-*Salmonella* bacteria comprised *Escherichia coli*, *Shigella flexneri*, *Vibrio parahaemolyticus*, and *Staphylococcus aureus*. These bacterial strains as the common food-borne pathogens were prepared from the Laboratory Culture Collection of Laboratory of Biomedical Science, University of Malaya, Malaysia.

Preparation of AMS

Streptavidin Magnesphere® Paramagnetic Particles (Promega, USA) or magnetic beads were washed with 1 × PBS and prepared as per manufacturer's instruction. Briefly, 600 µl of the magnetic beads were washed three times with 1 ml of 1 × PBS. After magnetic separation by using Polyattract® System 100 Magnetic Separation Stand (Promega, USA), the beads were resuspended in 1 ml of 1 × PBS (final concentration of 10 mg/ml). Four µl of 0.4 nmol biotinylated aptamer was coupled to 2.5 mg of washed magnetic beads in 1 × PBS at ambient room temperature. The aptamer-conjugated magnetic beads were used within 30 min.

Sensitivity test

To determine the sensitivity of AMS-PCR, 1 ml of each serially diluted *Salmonella enterica* suspension (10^0 – 10^7 CFU/ml) was mixed separately with aptamer-conjugated magnetic beads and incubated for 30 min at ambient temperature with gentle shaking. Then, the bound bacteria-aptamer-conjugated magnetic beads were recovered by using the magnetic separation stand and washed four times with $1 \times$ PBS-5% Tween 20 buffer, with a final wash in 200 μ l of $1 \times$ PBS. The bacterial cells were separated from the magnetic beads on the magnetic stand and eluted in nuclease-free water. Crude DNA was extracted from boiled cells. Briefly, bacterial cells were heated at 99 °C for 5 min and snapped cooled on ice. After a brief centrifugation at $13000 \times g$ for 5 min, the supernatant was transferred into another sterile microtube and used as DNA template for PCR analysis. The experiment was repeated with bare magnetic beads without aptamer as negative control.

PCR assay

The forward and reverse primers were designed in-house (5'-ATC CCT TTG CGA ATA ACA TCC T-3' and 5'-GGG CGC CAA GAG AAA AAG A-3') to target the invasive A gene (*invA*) of *Salmonella*. Each 25 μ l PCR mixture contained $1 \times$ PCR buffer, 1.8 mM $MgCl_2$, 0.12 mM dNTPs, 0.8 μ M for each forward and reverse *invA* primer, 0.06 U GoTaq Flexi DNA polymerase (Promega, USA), and 5 μ l of DNA (approx. 25 ng/ μ l) as the template. PCR conditions consisted of initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 45 s. The final extension was done at 72 °C for 10 min. The PCR products were electrophoresed on 2% agarose gel in $0.5 \times$ Tris Borated EDTA (TBE) buffer. The gel was stained in GelRed™ (Biotium, USA) and visualized by Gel Doc™XR (Bio-Rad, USA) imaging system. The experiment was repeated three times. Sterile distilled water was used as negative controls.

Specificity test

To determine the specificity of the DNA aptamer, ten different *Salmonella enterica* serovars, including *S. Typhi*, *S. Albany*, *S. Braenderup*, *S. Corvallis*, *S. Paratyphi A*, *S. Paratyphi B*, *S. Enteritidis*, *S. Pullorum*, *S. Typhimurium*, as well as *S. Indiana* were subjected to AMS followed by PCR as described above.

Non-*Salmonella* cells, including *V. parahaemolyticus*, *E. coli*, *S. aureus*, and *Sh. flexneri* were mixed together with *Salmonella* to form a bacterial cocktail before they were tested by AMS. To ensure the aptamer did not target any non-*Salmonella*, the eluted bacteria were tested

with PCR using specific in-house primers (sequences not shown) targeting *V. parahaemolyticus*, *E. coli*, *S. aureus*, and *Sh. flexneri*, respectively. The experiment was repeated twice. Bare magnetic beads without any aptamer was used as a negative control.

Detection of *Salmonella* in foods

The AMS-PCR method was evaluated with 14 food samples, including chicken (n=4), vegetables (n=8), and beef (n=2) purchased from different retail markets in Kuala Lumpur. The scheme of evaluation for food testing is illustrated in Figure 1. After homogenization, the samples were pre-enriched for 3 h in Buffered Peptone Water (BPW; Merck, USA) to revive any sublethally injured bacteria. Then, an aliquot of 1 ml of the pre-enriched broth-culture was mixed with the aptamer-conjugated magnetic beads (AMS) followed by direct PCR (approach A) while another 1 ml was directly processed for DNA extraction without AMS step (approach B, as a negative control). In approach C, 1 ml of the pre-enriched broth-culture was transferred and incubated in the selective enrichment broth media included Rappaport-Vassiliadis Soya broth (RVS; Oxoid, UK), Peptone broth (Oxoid, UK) for 24 h at 37 °C or Selenite Cystine (SC) broth (Oxoid, UK) for 12 h at 42 °C. An aliquot of these selective enrichment broth media were then processed for DNA extraction, followed by PCR. In approach D, aliquots of the overnight selective broth cultures (RVS or SC) were streaked onto selected media, Brilliance™ *Salmonella* agar (Oxoid, UK) for *Salmonella* isolation. Presumptive *Salmonella* colonies (purple color) were picked and purified on LB agar followed by confirmation with PCR. Approach A and D (culture as gold standard) were compared and the sensitivity and specificity percentage were calculated as described by Parikh et al. (2008).

Results

For sensitivity test, different concentrations of bacterial cell suspension (10^0 - 10^7 CFU/ml) were subjected to the AMS initial separation, followed by PCR. The experiment was repeated three times and reproducible results were obtained (data not shown). Using the AMS-PCR approach, the limit of detection was 10^2 CFU/ml (Figure 2a, lane 8). No PCR amplicon was observed when tested with bare magnetic beads without any aptamer (Figure 2a, lane 6). This value was 10 times more sensitive than the method without AMS step (10^3 CFU/ml; Figure 2b, lane 3).

To check the specificity of the AMS-PCR, 10 different *Salmonella* serovars and four different bacteria spp. (*S. aureus*, *E. coli*, *Sh. Flexneri*, and *V. parahaemolyticus*)

were tested. All the *Salmonella* serovars were amplified and showed a distinctive 149 bp band (Figure 3). No amplicon was detected when the elute was tested for four mentioned non-*Salmonella* bacteria. These results indicated that aptamer was able to select and distinguish *Salmonella* serovars from other species. We also tested the bare magnetic beads without aptamer as a negative control (data not shown).

Out of 14 samples tested with the AMS-PCR, 5 were

tested as true positives and 7 were true negatives (Table 1). When the same food samples were analyzed without the initial AMS step (approach B), 13 out of 14 samples were tested negative, i.e. no *Salmonella* was detected. Even though, approach B could reduce the time of detection to one h as compared to AMS and decreased the sensitivity of the detection. The AMS-PCR step (approach A) took 6-7 h while the conventional culture method took 2 days (approach C) to 3 days (approach D).

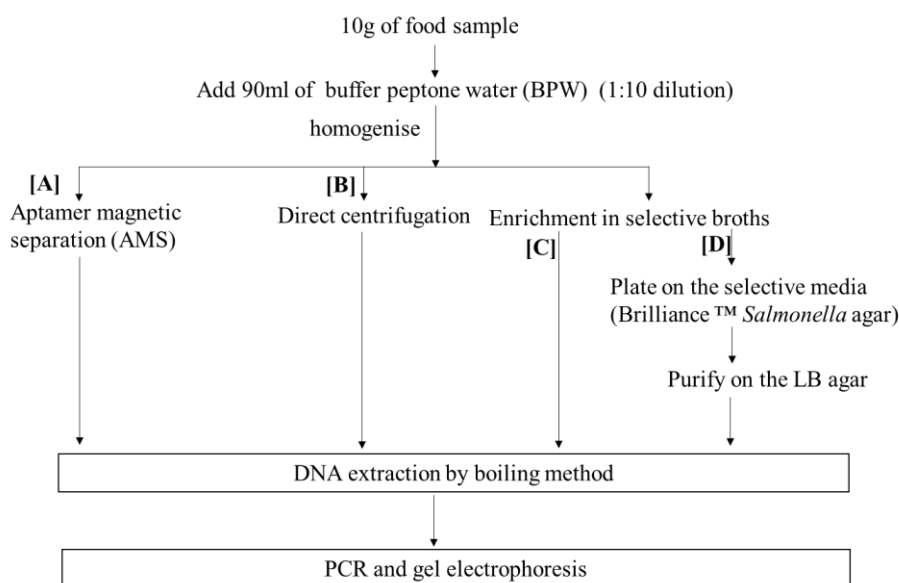


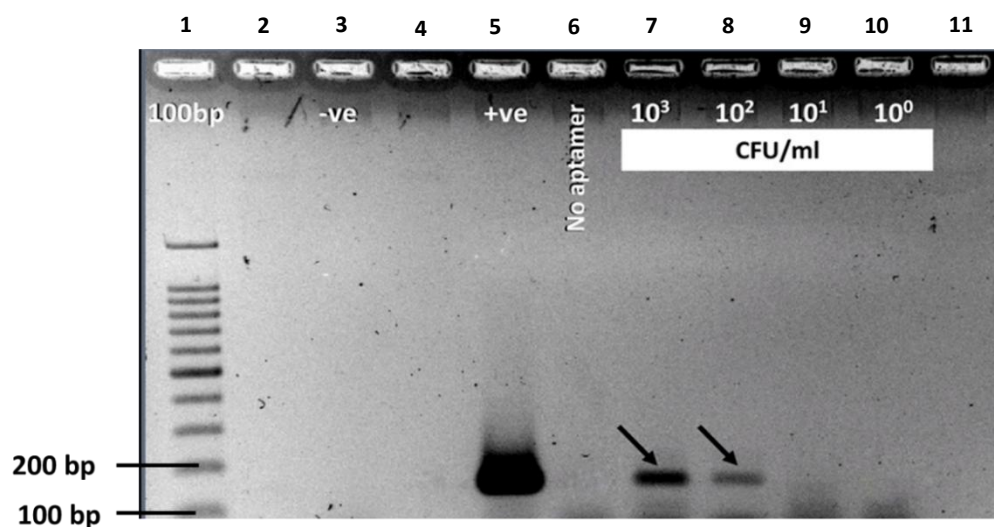
Figure 1: The workflow for AMS-PCR detection for *Salmonella* detection by comparing four different approaches namely [A], [B], [C], and [D]. All of these procedures were conducted on each food sample

Discussion

In this study, we evaluated the potential use of the DNA aptamer as an alternative element for upstream preparation in food analysis specifically to separate and concentrate *Salmonella* spp. in the food matrix. The aptamer was modified with biotin at 5' end to complement the streptavidin coated magnetic beads via non-covalent bonds. By a magnetic stand, this complex was attracted and the unbound particles were separated. The washing process involved 1×PBS–5% Tween 20 buffer that helped to disrupt the hydrophobic and electrostatic interaction between bacteria and the food surface (Goulter et al., 2009; Ukuku and Fett, 2002). This helps to concentrate the bacterial cells from the complex food matrix and facilitate bacterial elution for separation process. The subsequent detection was conducted by using conventional PCR which is a rapid, simple, and low-cost method that can amplify small amount of target DNA with high throughput.

Typically, the infectious dose of *Salmonella* in human infection is 10^2 to 10^5 organisms (D'Aoust, 1985), even though it may be varied depending on infected population, i.e., immune status, age, and pathogenicity of the bacteria (Hara-Kudo and Takatori, 2011). The high affinity of the aptamer was able to concentrate the targeted *Salmonella* cells which contributed the higher sensitivity of detection and decreased the loss of the bacteria in the sample. In a study, the sensitivity of detection could be increased to 10^1 - 10^2 CFU/9 ml of the *Salmonella* culture when real-time PCR was used as the detection method (Joshi et al., 2009). In another research, bacteriophage coupled with PCR was applied to separate *E. coli* that showed similar sensitivity with our study (Wang et al., 2016). Based on Suh and Jaykus (2013), the limit of detection of aptamer magnetic assay with real-time PCR was 10^1 - 10^2 CFU/500 μ l for *Listeria monocytogenes*; whereas it was 10^1 - 10^2 CFU/ml for *Campylobacter*

(a)



(b)

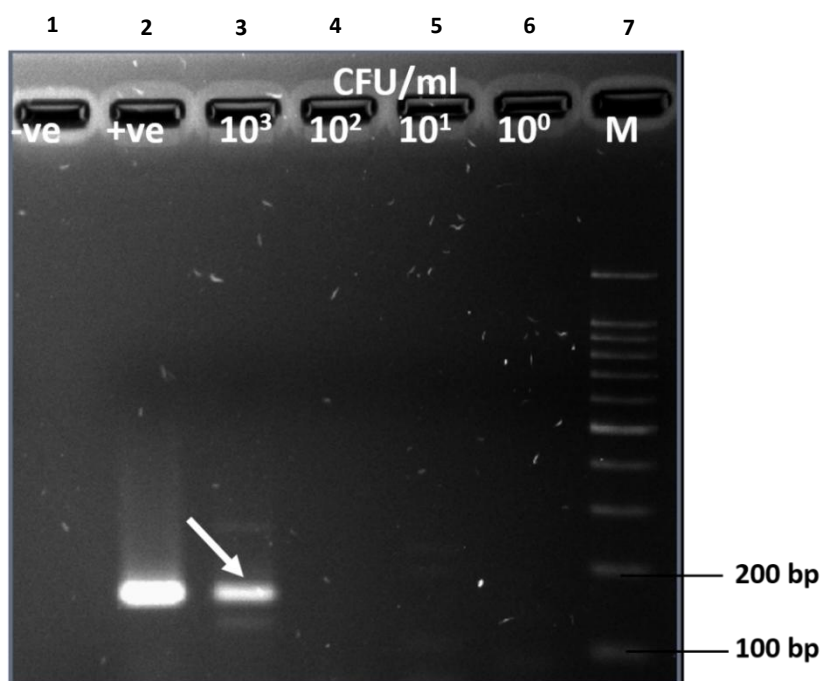


Figure 2: The sensitivity results of AMS-PCR (a) and without AMS (b) for *Salmonella* detection. (a) Lane 1: 100 bp DNA ladder; lanes 2, 4, and 11: empty; lane 3: negative control; lane 5: positive control; lane 6: no aptamer; lane 7: 10^3 CFU/ml; lane 8: 10^2 CFU/ml; lane 9: 10^1 CFU/ml; lane 10: 10^0 CFU/ml. The limit of detection of AMS-PCR was 10^2 CFU/ml. (b) Lane 1: negative control; lane 2: positive control; lane 3: 10^3 CFU/ml; lane 4: 10^2 CFU/ml; lane 5: 10^1 CFU/ml; lane 6: 10^0 CFU/ml; lane 7: 100 bp DNA ladder. Arrows indicate the size of the expected amplicon (149 bp). Magnetic beads without aptamer was used as negative control

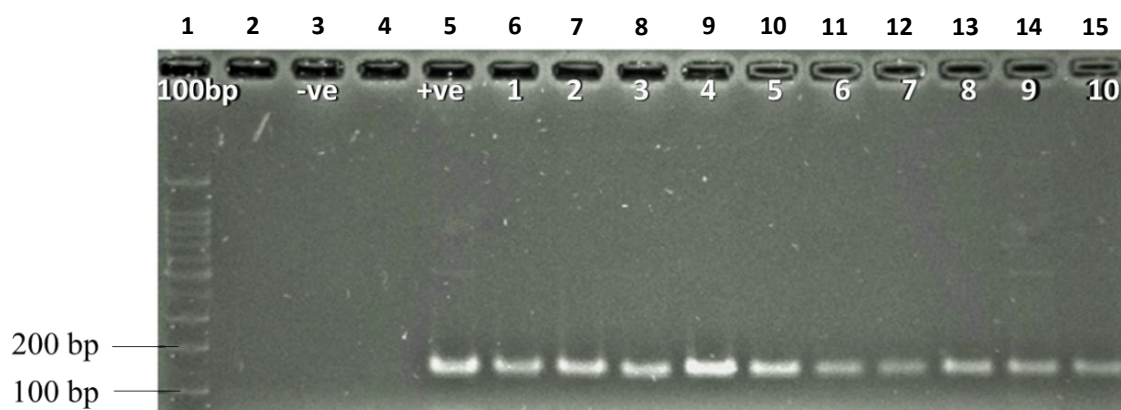


Figure 3: The specificity test of aptamer magnetic beads with ten different *Salmonella enterica* serovars. Lane 1: 100 bp DNA ladder; lanes 2 and 4: empty lanes; lane 3: negative control; lane 5: positive control; lane 6: *S. Typhi*; lane 7: *S. Albany*; lane 8: *S. Braenderup*; lane 9: *S. Corvallis*; lane 10: *S. Paratyphi A*; lane 11: *S. Paratyphi B*; lane 12: *S. Enteritidis*; lane 13: *S. Pullorum*; lane 14: *S. Typhimurium*; lane 15: *S. Indiana*. The PCR product is 149 bp in length

Table 1: Summary of the PCR results of *Salmonella* detection in naturally contaminated food samples by four approaches of [A], [B], [C], and [D] (see footnote)

Types of food	Sample number	PCR results (149 bp)				Interpretation
		[A]	[B]	[C]	[D]	
Chicken	1	-	-	+	+	False negative
	2	+	-	+	+	True positive
	3	+	-	+	+	True positive
	4	+	-	+	+	True positive
Vegetable	5	-	-	-	-	True negative
	6	-	-	-	-	True negative
	7	+	-	+	+	True positive
	8	+	+	+	+	True positive
	9	-	-	-	-	True negative
	10	-	-	-	-	True negative
	11	-	-	-	-	True negative
	12	-	-	-	-	True negative
Beef	13	-	-	+	+	False negative
	14	-	-	-	-	True negative

*The '+' and '-' indicate the presence and absence of *Salmonella* DNA, respectively

[A]: An aliquot of food homogenate → mixed AMS → elute → extract DNA → PCR

[B]: An aliquot of food homogenate → centrifuged and wash → extract DNA → PCR

[C]: An aliquot of food homogenate → selective enrichment broth → aliquot for DNA extraction → PCR

[D]: An aliquot of food homogenate → selective enrichment broth → streaked on selective medium → picked presumptive colonies, purify on the LB → DNA extraction → PCR

jejuni when tested with mixed microbiota as reported by Suh et al. (2014). In addition, IMS-PCR and IMS-ELISA has been applied to detect *Alicyclobacillus* strains in apple juice with a sensitivity of 10^1 and 10^5 CFU/ml, respectively (Wang et al., 2013; 2014). Even though similar IMS technique was used in both studies, downstream application is equally important to increase the sensitivity level. The results of this study also showed that the bare magnetic beads alone did not influence detection of *Salmonella*. This indicates the important role and specificity of the aptamer for detection by using AMS.

Our studied aptamer showed high specificity with no binding to non-*Salmonella* cells and had the ability to select and distinguish *Salmonella* serovars from the other species. Similar specificity results were found by Ma et al. (2014) and Yuan et al. (2014) using the same aptamer sequence with some modifications.

Based on the results of this study, AMS technique was comparable with the culture conventional assay in respect of *Salmonella* detection in foodstuffs. One of the main concerns of food-borne pathogens detection is the ability to eliminate the food inhibitors in complex

food matrix which may influence the sensitivity and ability of the detection assay (Jeníková et al., 2000). The targeted food-borne pathogens need to be separated and concentrated from complex mixtures. Using AMS in naturally contaminated food sample, targeted bacteria are expected to be separated from the complex environment of food, including non-target microbiota and food ingredients, such as fats, protein, divalent cations, and phenolic compounds that may act as inhibitors (Brehm-stecher et al., 2009). This will decrease the time of detection from days to hours which are important in food-borne outbreaks investigation.

We showed that AMS-PCR (approach A) showed comparable results with the culture method (approach D) as the gold standard, demonstrating its reliability to detect *Salmonella*. This method was compared with the PCR results using DNA extracted from pre-enriched broth culture (approach B) and selective broth culture (approach C). As the AMS concentrated, the targeted bacteria in the initial separation process prior to detection helped to reduce the time of detection from 4 days in culture method to 6-7 h. Usually, in the culture method, the pre-analytical sample processing step takes longer time to enrich microbiota present on a food matrix. To some extent, the sensitivity of any advanced detection tools to detect food-borne pathogens is limited by the preanalytical steps in food analysis prior to detection (Robinson, 2014; Suh et al., 2013). The application of AMS prior to detection step is an alternative method to decrease detection time and increase the selectivity as this method involves interaction between bio-recognition element (aptamer) and its target (*Salmonella*). In addition, as culture method is too laborious and involves multiple steps, AMS-PCR offers a simple detection with high selectivity.

In this study, we noted false negative results for two meat samples. This could be attributed to the nature of the food matrix itself. For instance, high fat content in the meat caused difficult separation process of the bacteria from the sample food matrix in comparison with vegetables samples (Robinson, 2014). We observed a high amount of the food particles in the meat being stuck to the magnetic beads and that might have caused the failure of the magnetic beads recovery. This carry-over particles could have affected the efficacy of the PCR amplification due to food inhibitors (Stevens and Jaykus, 2004). All the vegetable samples showed true positive results probably because the environment of the vegetables was not as complex as meat, i.e. no fats that might contribute the disruptions of the magnetic beads surface-aptamer. The magnetic beads were able to concentrate the bacteria in the vegetables without interference of its food matrix.

Conclusion

To the best of our knowledge, this is the first study used AMS-PCR for *Salmonella* detection in various naturally contaminated food samples. In comparison with the culture method, AMS helped to improve the upstream sample preparation in reducing the pre-enrichment and enrichment times. So, it seems that combining AMS with PCR is cost-effective and time-saving with highly specific for monitoring of *Salmonella* spp. in foods. Therefore, this could be a proper alternative approach instead of the conventional culture method of *Salmonella* detection.

Author contributions

A.N.Z. conducted the experimental work. A.N.Z. and K.L.T. designed the study, analyzed the data and wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

All the authors declared that they have no conflicts of interest.

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