Original Article

Designing a biochip following multiplex polymerase chain reaction for the detection of Salmonella serovars Typhimurium, Enteritidis, Infantis, Hadar, and Virchow in poultry products

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Abstract

Salmonella-contaminated foods, especially poultry-derived foods (eggs, chicken meat), are the major source of salmonellosis. Not only in the European Union (EU), but also in the United States, Japan, and other countries, has salmonellosis been an issue of concern for food safety control agencies. In 2005, EU regulation 1003/2005 set a target for the control and reduction of five target Salmonella enterica serovars—S. Typhimurium, S. Enteritidis, S. Infantis, S. Hadar, and S. Virchow—in breeding flocks. Thus, a simple biochip for the rapid detection of any of these five Salmonella serovars in poultry products may be required. The objectives of this study were to design S. Virchow-specific primers and to develop a biochip for the simultaneous identification of all or any of these five Salmonella serovars in poultry and poultry products. Experimentally, we designed novel polymerase chain reaction (PCR) primers for the specific detection of S. Virchow, S. Infantis, and S. Hadar. The specificity of all these primers and two known primer sets for S. Typhimurium and S. Enteritidis was then confirmed under the same PCR conditions using 57 target strains and 112 nontarget Salmonella strains as well as 103 non-Salmonella strains. Following multiplex PCR, strains of any of these five Salmonella serovars could be detected by a chromogenic biochip deployed with DNA probes specific to these five Salmonella serovars. In comparison with the multiplex PCR methods, the biochip assay could improve the detection limit of each of the Salmonella serovars from $N \times 10^3$ cfu/mL to $N \times 10^2$ cfu/mL sample in either the pure culture or the chicken meat samples. With an 8-hour enrichment step, the detection limit could reach up to $N \times 10^2$ cfu/mL.

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1. Introduction

Salmonellosis has been one of the major foodborne diseases worldwide. So far, more than 2600 different Salmonella serotypes have been identified [1]. According to a global survey from the World Health Organization, during the years 2001 to 2007, Salmonella Enteritidis and S. Typhimurium were the most common serotypes found in North America, Australia, New Zealand, and other countries [2]. These Salmonella serotypes are responsible for foodborne salmonellosis in humans followed by S. Infantis, S. Hadar, and S. Virchow [2,3]. S. Infantis has been ranked as the third most frequently found serovar infecting humans [4]. However, S. Virchow has recently become one of the predominant serovars in EU countries, ranking among the top five serovars [5]. This Salmonella serovar is known to associate with invasive infection in humans and is resistant to many antibiotics [6,7]. Regarding foodborne poisoning cases and outbreaks, consumption of undercooked beef, poultry, and eggs are most often associated with salmonellosis [8].

Salmonellosis also has been one of the major foodborne outbreaks and/or diseases within EU countries. It causes significant economic losses; for example, the European Food Safety Authority has estimated the economic burden of human salmonellosis to cost 3 billion euros a year [9]. Thus, EU regulation 1003/2005, amending regulation no. EU2160/2003 (European Parliament and European Council, 2005), sets regulatory targets for the control and the reduction of five target Salmonella serovars—S. Typhimurium, S. Enteritidis, S. Infantis, S. Hadar, and S. Virchow—in breeding flocks of Gallus gallus [10]. Accordingly, these five Salmonella serovars were prioritized by the EU for the control of poultry and poultry products entry, because of the significant risk they pose to public health [11,12]. Good monitoring and screening programs are thus required to prevent Salmonella infection.

In addition to the biotyping method for Salmonella identification [13], Salmonella serovars are determined by lipopolysaccharide (O antigen) and flagellar structures (H antigen) based on the Kauffmann–White scheme. All these steps are labor intensive and time consuming, costly, and complicated [14]. Thus, a rapid and accurate screening for the five Salmonella serovars in food samples is required. Over the past decades, polymerase chain reaction (PCR) has been frequently used for the detection of Salmonella and other foodborne pathogens [2,15,16]. A number of genus- and serovar-specific genes have been used for designing primers or probes specific for the detection of Salmonella serovars Enteritidis, Typhimurium, Typhi, Choleraesuis, Paratyphi, Hadar, and other pathogens [17–25]. For S. Infantis, based on fliB gene, specific PCR primers also have been designed [26]. However, to our knowledge, no primer set or probe has been reported for the specific detection of S. Virchow.

The purpose of this study was thus to design novel PCR primers specific for S. Virchow detection. In addition, new PCR primers specific for S. Infantis and S. Hadar were also designed. Together with the known primers for S. Typhimurium and S. Enteritidis, target genes for all these five Salmonella serovars could be amplified under the same PCR conditions. After hybridization of multiplex PCR (mPCR) products with species-specific probes deployed on the biochip, all or any of these five Salmonella serovars in poultry samples could be rapidly identified.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. These strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA); United States Department of Agriculture (USDA); Bioresource and Research Center (BCRC; Hsin-Chu, Taiwan); Centers for Disease Control (CDC; Taichung Branch, Taichung, Taiwan); Department of Veterinary, National Ping Tung University (Ping Tung, Taiwan); and Culture Collection of the University of Göteborg (CCUG; Göteborg, Sweden). The strains were stored in Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, MI, USA) containing 50% of glycerol at −80°C.

2.2. DNA preparation

One loopful of Salmonella strain was inoculated into 5 mL TSB and incubated at 37°C for 12 hours. Genomic DNA was extracted using Viogene, Blood & Tissue Genomic Mini kit (Viogene, Taipei, Taiwan). One milliliter of the culture was centrifuged for 5 minutes at 7000 rpm (Centrifuge 5424; Eppendorf, Hauppaug, NY, USA), and the pellet was washed by resuspension in 1 mL sterile water. After centrifugation again (5 minutes, 7000 rpm), the cell pellet was suspended in 250 μl lysostaphin buffer (2 mg/mL buffer; AMBI Products LLC, New York, NY, USA), 2.5 μl lysozyme (20 mg/mL; Sigma, St. Louis, MO, USA), and 20 μl RNase (20 mg/mL; Sigma). The mixture was incubated at 37°C for 1.5 hours, followed by addition of 200 μl EX Buffer (Viogene) and 25 μl proteinase K (10 mg/mL, Merck) and incubation at 60°C for 1 hour. After incubation at 70°C for another 30 minutes, total DNA was precipitated with alcohol and extracted according to the manufacturer’s manual (Viogene). Afterward, genomic DNA was eluted with 200 μL double deionized water.

2.3. Designing PCR primers and probes

Whole genome sequences of Salmonella serovars were obtained from the National Center for Biotechnology Information (https://ncbi.nlm.nih.gov). Open reading frames of serovars S. Infantis (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/SIN.dbs) and S. Hadar (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/HADAR.dbs) obtained from an FTP site of Sanger Institute were previously reported by Akiba et al [27]. Open reading frames of S. Hadar (Hadar-specific genomic region) and S. Infantis (Infantis-specific genomic region) were compared using basic local alignment search tool (http://
Table 1 – Bacteria strains used in this study.

| Species                         | Strain no.                                      |
|---------------------------------|-------------------------------------------------|
| **Salmonella Enteritidis**      | ATCC 13076, CF09.001, CF09.002, CF09.003, CF09.009, CF09.011, CF09.012, CF09.013, CF09.017, CF09.018, CF09.023, US, SD07 |
| **Salmonella Typhimurium**      | BCRC 10747, BCRC 12947, CF09.015, CF09.016, CF09.019, CF09.020, CF09.021, CF09.037, CF09.039, SD09.016, SD09.018, SD09.023 |
| **Salmonella Infantis**         | CC07.003, CC07.016, CC07.063, CC07.090, NQ08.053, SA08.050, SA08.059, SB08.005, SB08.038, SD08.109, US, SI20 |
| **Salmonella Virchow**          | CA08.075, CA08.116, CA08.135, CA08.158, CF08.016, CF08.030, CF08.039, CF08.041, CF08.050, CF08.054 |
| **Salmonella Hadar**            | CA08.102, CA08.106, CA08.124, CA08.129, CA08.157, CA08.159, CA08.160, CB09.011, CF09.010, US, SH12, US, SH13 |
| **Other Salmonella serovars**   | Salmonella abortusequi, S. Adelaide, S. Agona, S. Alachua, S. Alachua, S. Albany, S. Amager, S. Anatum, S. Augustenborg, S. Azteca, S. Bareilly, S. Berta, S. Blockley, S. Braenderup, S. Bredeny, S. Boer, S. Bonn, S. Bous, S. Boivin, S. Cerro, S. Cerro, S. Chatterjee, S. Chittering, S. Chittagong, S. Choleraesuis, S. Colepark, S. Colindale, S. Colorado, S. Crossness, S. Derby, S. Diakarta, S. Dublin, S. Duque, S. Emeke, S. Essen, S. Florida, S. Gera, S. Goerlitz, S. Hafsa, S. Halmstad, S. Havana, S. Houten, S. Hvittingfoss, S. Indiana, S. Isangi, S. Java, S. Javanica, S. Johannes Burg, S. Kedougou, S. Kentucky, S. Lichtfied, S. Lexington, S. Limete, S. Livingston var. O14, S. London, S. Masseny, S. Mbanda, S. Meleagridis, S. Menhadien, S. Minnesota, S. Miami, S. Montevideo, S. Muench, S. Nchanga, S. Nevington, S. Newpor, S. Nigeria, S. Ohio, S. Panama, S. Paratyphi A, S. Paratyphi B var. Java, S. Potsdam, S. 16, — — S. Rubislau, S. Salamae, S. Saintpaul, S. Sandiego, S. Schwarzengrund, S. Senftenberg, S. Seremban, S. Singapore, S. Sirkans, S. Stanley, S. Tambacouda, S. Tennesse, S. Thompson, S. Thomasville, S. Typhi, S. Uganda, S. Victoria, S. Veje, S. Weltevrede |
| **Non-Salmonella strains**      | Acinetobacter baumannii, A. calcoaceticus, A. johnsonii, Alcaligenes faecalis, Beilis cereus, Brevibacterium linens, Clostridium Freundii, Clostridium botulinum, C. difficile, C. haemolyticum, C. perfringens, Corynebacterium renale, Cronobacter sakazakii, Enterobacter aerogenes, E. cloacae, Erwinia carotovora, Escherichia coli, Hafnia alvei, Listeria innocua, L. monocytogenes, L. grayi, Moraxella catarrhalis, M. osloensis, Proteus vulgaris, Pseudomonas aeruginosa, P. fluorescens, P. mendocina, P. putida, Rahnella aquatilis, Sarccharomyces cerevisiae, Streptomyces filipinensis, Serratia ficaria, S. fonticola, S. odorfera, S. quinivorans, Shigella dysenteriae, S. sonnei, Staphylococcus sp., S. aureus, Streptococcus agalactiae, S. bovis, S. uberis, S. epidermidis, S. haemolyticus, S. hyicus, S. intermedius, S. saprophyticus, S. xylosus, Vibrio alginolyticus, V. parahaemolyticus, Yersinia enterocolitica |

ATCC = American Type Culture Collection (USA); BCRC = Biorepositories Collection and Research Center (R.O.C. Taiwan); CCUG = Culture Collection, University of Goteborg (Sweden); FT = National Ping Tung University (Pingtung, Taiwan); USDA = United States Department of Agriculture; US = City of New York Department of Health (New York, USA).

www.ncbi.nlm.nih.gov/BLAST). Serovar specific primers for S. Infantis and S. Hadar were then designed (Table 2).

For S. Virchow-specific primers, as S. Virchow was relatively common and consists of genes similar to those of S. Infantis [26], by comparison of the S. Infantis genome sequence with the partial sequences available for S. Virchow, primers that allowed the amplification of S. Virchow genome were designed using Primer premier 5.0 software (PREMIER Biosoft, Palo Alto, CA, USA). The sequence of the forward primer was 5′-CCTGATGAAACGCGACAGAAG-3′ and the reverse primer was 5′-GCCCTTATATCGCCTGCAAGA-3′, corresponding to S. Infantis (Acc. No: NZ_LN649235.1) positions 3710138 to 3710158 and positions 3713529 to 3713507, respectively. These primers generate a PCR product from S. Virchow gene with a molecular size of approximately 3000 bp. This DNA fragment was purified with PCR-M cleanup system (Viogene) and sequenced. We then compared the sequence of this PCR product with the sequence of S. Infantis using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). Species-specific primers for S. Virchow were designed (Table 2). For S. Typhimurium and S. Enteritidis, primers previously reported to be specific to these two serovars were used [28,29]. These primers were synthesized by MDBio Inc. (Taipei, Taiwan). DNAs from all these five Salmonella serovars were then PCR assayed using thermal cycler 2720 (Perkin-Elmer Corporation, Norwalk, CT, USA).

2.4. PCR assay

The specificity of all primer sets was tested with DNA isolated from bacterial strains listed in Table 1. The PCR reactions were performed in a total volume of 25 μL mixture containing 200μM of each deoxynucleoside triphosphate (PRO tech Technology Enterprise Co., Ltd., Taipei, Taiwan), 1 × PCR buffer (PRO tech Technology Ent. Co.), 0.2μM each primer, 0.6 units of Prozyme (PRO tech Technology Ent. Co.), and 2 μL of

www.ncbi.nlm.nih.gov/BLAST). Serovar specific primers for S. Infantis and S. Hadar were then designed (Table 2).

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each target DNA (150–200 ng). The PCR conditions were as follows: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and a final extension at 72°C for 7 minutes. PCR products were visualized by 2% agarose gel electrophoresis.

2.5. PCR detection limit for Salmonella

One loop of Salmonella Enteritidis ATCC 13076, S. Typhimurium BCRC 10747, S. Hadar CA08.102, S. Infantis CC07.016, and S. Virchow CA08.158, respectively, was cultured in 5 mL TSB followed by incubation at 37°C for 18 hours in a rotating shaker at 150 rpm. One hundred microliters of the cell suspension was then 10-fold serially diluted with sterile water. All dilutions (0.1 mL) were then plated on Tryptic Soy Agar, and colony-forming units (cfu/mL) were counted. In addition, DNA extracts from these cell dilutions were PCR assayed according to the conditions described earlier.

2.6. Detection of Salmonella in chicken meat

Chicken meat samples purchased from local food markets (Taichung, Taiwan) were used to determine the detection limit of each Salmonella species. The samples were prepared according to Chiang et al [30]. Briefly, 25 g of raw or sterilized (121°C for 15 minutes) chicken meat samples were homogenized with 225 mL of 1% Bacto Peptone water using a Stomacher 400 (Seward, London, UK) at high speed for 1 minute. Then, 1 mL of the homogenized broth was mixed with 8.9 mL TSB, then 0.1 mL of Salmonella cells with known cell counts, i.e., 0 cfu/mL and N × 10^0–10^7 cfu/mL (N = 1–9) were inoculated to the mixture followed by incubation at 37°C for 0 hour or 8 hours. DNA was then extracted from each of these samples and assayed by PCR under the same PCR conditions.

2.7. Biochip detection of Salmonella

Five sets of primers including one of the two primer sets for S. Infantis and S. Virchow, respectively, were used for mPCR followed by biochip assay. These primers were ENTE-F/ENTE-R (206 bp), TYPH-F/TYPH-R (261 bp), INFA1-F/InVi-R (268 bp), VIRC1-F/InVi-R (273 bp), and HADA-F/HADA-R (427 bp). Primers and probe sequences are listed in Table 2. In addition, primers at the 5’-end were labeled with biotin and subjected to PCR assay. These biotin-labeled PCR amplicons were used for the biochip assay.

For biochip construction, 25 µL of each oligonucleotide probe (10–20 µM) was mixed with 25 µL of 2× probe solution (DR. Chip Biotech, Hsin-Chu, Taiwan). It was then spotted onto the plastic chip (DR. Chip Biotech) by using the Micro-Arrayer Ezspot SR-A300 (Shuai Ran Precision, Taoyuan, Taiwan). For positive control, only the biotin-labeled probes were spotted, whereas for negative control, none of the probes (buffer only) were spotted on the chip (Figure 1). The amplified DNA was then cross linked onto the plastic chip by ultraviolet irradiation with UV CROSSLINER CL-508.S (UVItec, Cambridge, England, UK) at 254 nm/0.6–1.2 J. For hybridization, 25 µL of each biotin-labeled PCR products was heated at 95°C for 10 minutes and immediately cooled in ice-water bath for 5 minutes. Next, 200 µL of DR. Hyb Buffer (DR. Chip Biotech) and the denatured PCR products were mixed and loaded into a chip chamber well. Hybridization was carried out for 30 minutes at 50°C with gentle vibration in the DR. Mini Oven (DR. Chip Biotech). Wash steps and chromogenic reaction were carried out as previously described [30]. Next, the hybridization signals were determined by scanning with DR. AiM Reader (DR. Chip Biotech). To determine the detection limit and specificity of the biochip for Salmonella in pure culture or in raw or sterilized chicken meat samples, similar steps as described earlier were followed.

Table 2 – Sequences of primers and probes and the sizes of PCR products.

| Strains          | Target gene   | Primer                          | Size | Tm  | Ref.          |
|------------------|---------------|---------------------------------|------|-----|---------------|
| S. Enteritidis   | Prot6e        | ENTE-F: 5'-ATATCGCTTTGTGCCTCTCC-3' | 206  | 59  | Malorny et al [29] |
|                  |               | ENTE-R: 5'-CATTGGCACCCTTTTTGTTTGC-3' |      |     |               |
| S. Typhimurium   | MDH           | TYPH-F: 5'-CGGATCCACACCCGGCTCTC-3' | 261  | 59  | Lin and Tsen [28] |
| S. Hadar         | HSR3          | TYPH-R: 5'-TGGCAGGGAAGTTGTGAATGC-3' | 427  | 59  | This study     |
| S. Infantis      | ISR2–ISR3     | HADA-F: 5'-CCCTAAAATTCGACGAGAG-3' | 268  | 59  | This study     |
| S. Virchow       | Hypothetical  | In-Vi Uni-R: 5'-ATACGATACGAAATGCCGAGG-3' | 240  |     |               |
| protein          |               | In-Vi Uni-R: 5'-TGGGCGGAGGTTTTGATAT-3' |      |     |               |
|                  |               | HADA-R: 5'-TGGGCGGAGGTTTTGATAT-3' |      |     |               |
|                  |               | TYPH-F: 5'-ATACGATACGAAATGCCGAGG-3' | 234  |     |               |
|                  |               | In-Vi Uni-R: 5'-TGGGCGGAGGTTTTGATAT-3' |      |     |               |

HSR = Hadar-specific genomic region; ISR = Infantis-specific genomic region; MDH = malate dehydrogenase; PCR = polymerase chain reaction. *Prot6e: S. Enteritidis-specific gene (fimbrial biosynthesis).
3. Results

3.1. Test specificity of the primer sets

The specificity of S. Typhimuruim, S. Enteritidis, S. Virchow, S. Hadar, and S. Infantis primers was tested by PCR assay against 12 S. Typhimuruim, 12 S. Enteritidis, 10 S. Virchow, 11 S. Hadar, 11 S. Infantis, 182 nontarget Salmonella bacteria strains, and 103 non-Salmonella strains under the same PCR conditions with an annealing temperature of 59°C (Table 3). The amplification size was 206 bp for S. Enteritidis and 261 bp for S. Typhimuruim. Two primer sets for S. Infantis and two sets for S. Virchow were designed, and their specificity was checked by PCR (Table 2). All these four primer sets share the same reverse primer, i.e., In-Vi Uni-R (Table 2). For S. Infantis, two amplified products, i.e., 268 bp and 240 bp, and for S. Virchow two products, i.e., 273 bp and 234 bp, could be generated. For S. Hadar-specific primers, S. Hadar strains generated PCR products with a size of 427 bp. All primers generated positive results to their target strains, whereas nontarget Salmonella strains and strains other than Salmonella generated negative results (Table 3).

3.2. Multiplex PCR detection of Salmonella serovars

The annealing temperature used for all five primer sets shown in Table 2 was similar, i.e., 59°C. Thus, these five primer sets could be used in a multiplex PCR (mPCR) assay under the same PCR conditions. This mPCR system allowed the amplification of each of the five target Salmonella serovars as shown in Figure 2. Because of the closely similar
sizes of some PCR products, the mPCR assays were performed with two combination groups, e.g., two serovars (Figure 2A) and multiple serovars (Figure 2B). However, because the molecular sizes of the PCR products for S. Typhimurium (261 bp), S. Infantis (268 bp), and S. Virchow (273 bp) were closely similar, only two or three bands on agarose gel could be observed. For example, in lanes 6–9 of Figure 2B, only three bands among all four or five Salmonella serovars could be observed. In addition, some PCR products might generate faint bands. Under such PCR conditions, such PCR system did not allow us to discriminate among these three Salmonella serovars. In addition, the detection limit of each of the primer sets to Salmonella cells in PBS or in raw or sterilized chicken homogenate samples was $N \times 10^3$ cfu/mL. With an 8-hour preenrichment step, the detection limit for each of these five Salmonella serovars could reach $N \times 10^3$ cfu/mL either in PBS or in chicken meat samples (Table 4).

### Table 3 - Specificity of the PCR primers* for the detection of Salmonella Enteritidis, S. Typhimurium, S. Infantis, S. Virchow, and S. Hadar.

| Bacteria species                | Total no. of strainsa | Salmonella Enteritidis | Salmonella Hadar | Salmonella Typhimurium | Salmonella Infantis | Salmonella Virchow |
|--------------------------------|-----------------------|------------------------|------------------|------------------------|---------------------|-------------------|
| Salmonella Enteritidis          | 12                    | 12+                    | —                | —                      | —                   | —                 |
| Salmonella Hadar               | 11                    | —                      | 11+              | —                      | —                   | —                 |
| Salmonella Typhimurium         | 12                    | —                      | —                | 12+                    | —                   | —                 |
| Salmonella Infantis            | 11                    | —                      | —                | 11+                    | 11+                 | —                 |
| Salmonella Virchow             | 10                    | —                      | —                | 10+                    | —                   | —                 |
| Nontarget Salmonella strains   | 182                   | —                      | —                | —                      | —                   | —                 |
| Non-Salmonella strains         | 103                   | —                      | —                | —                      | —                   | —                 |

PCR = polymerase chain reaction.  
* Assay conditions were as those described in Materials and methods. For each assay, single primer sets specific for each Salmonella serovar was used.  
b The name and source of the strains are shown in Table 1.

3.3. Use of biochip following mPCR for the discrimination of all five Salmonella serovars

Because the mPCR method was unable to discriminate all five Salmonella serovars simultaneously, we thus designed a biochip following this mPCR step. The biochip was constructed by spotting the oligonucleotide probes listed in Table 2 for the detection of five Salmonella serovars. Allocation of the probes on the biochip is demonstrated in Figure 1. All positive control generated positive signals, whereas all negative control generated negative results. Figure 1 shows the hybridization patterns for multiple Salmonella targets artificially spiked in chicken samples. The spiked cells for each Salmonella serovar in chicken homogenate was $N \times 10^4$ cfu/g sample. For either the raw or the sterilized sample, similar results could be obtained. All combinations of Salmonella serovar generated the expected hybridization patterns, and no false hybridization signal was observed.

![Figure 2](image_url)  
Detection of the Salmonella strains by mPCR using ENTE-F/ENTE-R (206 bp), TYPH-F/TYPH-R (261 bp), INFA1-F/InVi-R (268 bp), VIRC1-F/InVi-R (273 bp), HADA-F/HADA-R (427 bp) primers. (A) Lane M: 100 bp ladder; lane 1: E, T; lane 2: E, H; lane 3: E, I; lane 4: E, V; lane 5: T, H; lane 6: T, I; lane 7: T, V; lane 8: H, I; lane 9: V, H; lane 10: I, V. (B) Lane M: 100 bp ladder; lane 1: E, T, H; lane 2: T, I, H; lane 3: I, V, H; lane 4: E, I, V; lane 5: E, V, H; lane 6: E, T, I, H; lane 7: T, I, V, H; lane 8: E, T, I, V; lane 9: E, T, I, V. E = Enteritidis; H = Hadar; I = Infantis; T = Typhimurium; V = Virchow.
When the detection limit of the chip was evaluated with raw and sterilized chicken homogenate spiked with Salmonella cells, the detection limit was $N \times 10^3$ cfu/mL for each Salmonella serovar. In comparison with the detection limit of mPCR, the detection limit could be improved from $N \times 10^7$ cfu/mL to $N \times 10^2$ cfu/mL by following the use of the biochip. With an 8-hour enrichment step, the detection limit also reached $N \times 10^2$ cfu/mL (Table 4). These results demonstrate that the biochip may serve for specific and highly sensitive detection of these five Salmonella serovars.

4. Discussion

For rapid and simultaneous detection of different Salmonella serovars, mPCR with multiple species-specific primers has been used [29]. However, in some cases, some PCR products may generate faint signals or may be undiscriminated because of the closely similar molecular sizes. For example, Akiba et al [27] developed an mPCR system to identify seven major serovars of Salmonella, i.e., Typhimurium, Choleraesuis, Infantis, Hadar, Enteritidis, Dublin, and Gallinarum. However, S. Virchow was not included in the serovars investigated, and few false-positive results were observed in the mPCR assays used to identify Typhimurium, Choleraesuis, Enteritidis, and Dublin. Moreover, because of the closely similar sizes of some PCR products, not all of the seven Salmonella serovars could be simultaneously detected. In this study, we developed an mPCR method to amplify the DNAs from five target Salmonella serovars. Following mPCR, we used a biochip that was capable of simultaneous and specific detection of all or any of these five Salmonella serovars (Figure 1). To the best of our knowledge, this is the first biochip designed for the simultaneous detection of all of these five Salmonella serovars. Compared to the use of only mPCR, this biochip allows users to double check the detection specificity of these Salmonella serovars because all the primers used for mPCR and the probes deployed on the chip are specific to these five Salmonella serovars. Furthermore, this mPCR–biochip system could further improve the strain discrimination and the detection limit as compared to only the use of mPCR.

Use of DNA biochip following PCR using either universal primers or multiplex primers offers a powerful tool for simultaneous detection of multiple targets in microbial diagnosis. For example, McCabe et al [31] used 16S rRNA-based universal primers to amplify template DNA in filter spots containing boiled bacteria followed by hybridization with species-specific probes and identified 14 clinical isolates. Wang et al [32] also used 16S rRNA gene-based universal primers to amplify the variable region of bacterial 16S rRNA gene, followed by reverse hybridization of the products to species-specific probes on a chip. As for the use of multiplex PCR to amplify the target bacteria genes followed by the use of biochips, such techniques have been used for the rapid detection of different bacteria genus or species based on different genes [19,29,30,33], and different virulence genes, such as the enterotoxin types, of Staphylococcus aureus [34]. Ideally, many different pathogenic bacteria species, toxin, or virulence genes could be simultaneously detected as long as the quantity of each target DNA is enough for PCR and the biochip used is highly sensitive. However, because the purpose of this study was to offer a simple and convenient method for the rapid detection of the five Salmonella serovars set by EU regulation, only five Salmonella serovars were set as targets of this biochip.

In conclusion, in this study, we designed a biochip for five major Salmonella serovars. Because salmonellosis has been a global issue of concern among food control agencies, the biochip we developed may offer a rapid and reliable method to monitor these salmonella cells in not only in poultry or poultry products, but also in other food samples as well as clinical samples. Finally, a reminder should be made that before this biochip can be recognized as an official method (e.g., Association of Official Analytical Chemists method in the United States), more studies designed according to the guidelines of official methods may be needed.
Conflicts of interest

All authors declare that they have no conflicts of interest.

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