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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

Yu-Cheng Chiang [a,*], *Hsien-Huang Wang* [b], *Latha Ramireddy* [a], *Hsin-Yen Chen* [a], *Chia-Ming Shih* [a], *Chien-Ku Lin* [a], *Hau-Yang Tsen* [a,*]

[a] *Department of Food Science and Technology, Hung Kuang University, Number 1018, Section 6, Taiwan Boulevard, Shalu District, Taichung City 43302, Taiwan, ROC*

[b] *DR. Chip Biotech, Science-Based Industrial Park, Chu-Nan, Miao-Li County 350, Taiwan, ROC*

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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 $N \times 10^0$ cfu/mL.

* *Corresponding authors*. Department of Food Science and Technology, Hung Kuang University, Number 34, Chung-Chi Road, Shalu, Taichung City 433, Taiwan, ROC.

E-mail addresses: honda224@gmail.com (Y.-C. Chiang), hytsen@sunrise.hk.edu.tw (H.-Y. Tsen).

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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. Typhimuruim, 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 Collection 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, Hauppauge, 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, SE07
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. Boecker*, *S. Bonn*, *S. Bouss*, *S.Bovismorbificans*, *S. California*, *S. Cerro*, *S. Chailey*, *S. Chester*, *S. Chittagong*, *S. Choleraesuis*, *S. Coleypark*, *S. Colindale*, *S. Colorado*, *S.Crossness*, *S. Derby*, *S. Djakarta*, *S. Dublin*, *S. Dugbe*, *S. Emek*, *S. Essen*, *S. Florida*, *S. Gera*, *S. Goerlitz*, *S. Haifa*, *S.Halmstad*, *S. Havana*, *S. Houten*, *S. Hvittingfoss*, *S. Indiana*, *S. Isangi*, *S. Java*, *S. Javiana*, *S. Johannesburg*, *S. Kedougou*, *S. Kentucky*, *S. Litchfield*, *S. Lexington*, *S. Limete*, *S. Livingstone* var. O14, *S. London*, *S. Massenya*, *S. Mbandaka*, *S. Meleagridis*, *S. Menhaden*, *S. Minnesota*, *S. Miami*, *S. Montevideo*, *S. Muenchen*, *S. Nchanga*, *S. Newington*, *S. Newpor*, *S. Nigeria*, *S. Ohio*, *S.Panama*, *S. Paratyphi* A, *S. Paratyphi B* var. *Java*, *S. Potsdam*, *S.* I6, 7: —, *S. Rubislaw*, *S. Salamae*, *S. Saintpaul*, *S. Sandiego*, *S. Schwarzengrund*, *S. Senftenberg*, *S. Seremban*, *S. Singapore*, *S. Srkansas*, *S. Stanley*, *S. Tambacounda*, *S. Tennessee*, *S. Thompson*, *S. Thomasville*, *S. Typhi*, *S. Uganda*, *S. Victoria*, *S. Vejle*, *S. Weltevreden*
Non-*Salmonella* strains	*Acinetobacter baumannii*, *A. calcoaceticus*, *A. johnsonii*, *A.* sp., *A. ursingii*, *Alcaligenes faecalis*, *Bacillus cereus*, *Brevibacterium linens*, *Citrobacter freundii*, *Clostridium acetobutylicum*, *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*, *Saccharomyces cerevisiae*,*Streptomyces filipinensis*, *Serratia ficaria*, *S. fonticola*, *S. odorifera*, *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 = Bioresources Collection and Research Center (Taiwan); CDC = Centers for Disease Control (R.O.C. Taiwan); CCUG = Culture Collection, University of Göteborg (Sweden); PT = 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′-CCTGATGAAAAGCGGACAAAG-3′ and the reverse primer was 5′-GCCTTGATAATGCCTGTGCAGAA-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

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

Strains	Target gene [a]	Primer	Size	Tm	Ref.
S. Enteritidis	*Prot6e*	ENTE-F: 5′-ATATCGTCGTTGCTGCTTCC-3′ ENTE-R: 5′-CATTGTTCCACCGTCACTTTG-3′ Probe/TTTTTTTTTTTTTTTTTTAGGCGCTCATCGGTCCTGCTGT	206	59	Malorny et al [29]
S. Typhimuruim	*MDH*	TYPH-F: 5′-CGCATTCCACCACGCCCTTC-3′ TYPH-R: 5′-TGCCAACGGAAGTTGAAGTG-3′ Probe/TTTTTTTTTTTTTTTTTTTTGAAGGGCGTGGTGGAATGCG	261	59	Lin and Tsen [28]
S. Hadar	*HSR3*	HADA-F: 5′-CCTCAAATTAATGCCAGAGAG-3′ HADA-R: 5′–5′-GTAGCAATTTATGGCTTACTACA-3′ Probe/AACCGAAACTTTACTGCGAGAGAGGTTTTTTTTTTTTTTT	427	59	This study
S. Infantis	*ISR2–ISR3*	INFA1-F: 5′-GACGCTATCAATTCAAGCAGAC-3′ In-Vi Uni-R: 5′-ATACGATACTACAATACCCGACG-3′ INFA2-F: 5′-TGAGGGCGAGAGGTATTGTTAT-3′ In-Vi Uni-R: 5′-ATACGATACTACAATACCCGACG-3′ Probe/TACGTCTGCTTGAATTGATAGCGTCTTTTTTTTTTTTTTT	268 240	59	This study
S. Virchow	Hypothetical protein	VIRC1-F: 5′-ATTGTTTATGCATAGGCCGAC-3′ In-Vi Uni-R: 5′-ATACGATACTACAATACCCGACG-3′ VIRC2-F: 5′-GTCAACCGATACTGTAGTAGTGCA-3′ In-Vi Uni-R: ATACGATACTACAATACCCGACG-3′ Probe/TTTTTTTTTTTTTTTTTTTGTCGGCCTATGCATAAACAAT	273 234	59	This study

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

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, 72°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

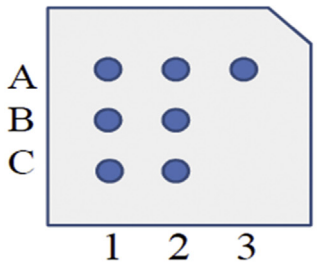
Chicken meat samples purchased from local food markets (Taichung, Taiwan) were used to determine the detection limit of each *Salmonella* species. The samples were prepared according to Chiang et al [30]. Briefly, 25 g of raw or sterilized (121°C for 15 minutes) chicken meat samples were homogenized 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 0.1mL of *Salmonella* cells with known cell counts, i.e., 0 cfu/mL and $N \times 10^0$–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, 25μL of each oligonucleotide probe (10–20μM) 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 primers 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 CROSSLINKER 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.

(A)



A1	*Salmonella* Enteritidis
A2	*Salmonella* Typhimuruim
A3	*Salmonella* Infantis
B1	*Salmonella* Virchow
B2	*Salmonella* Hadar
C1	Hybridization positive control
C2	Hybridization negative control

(B)

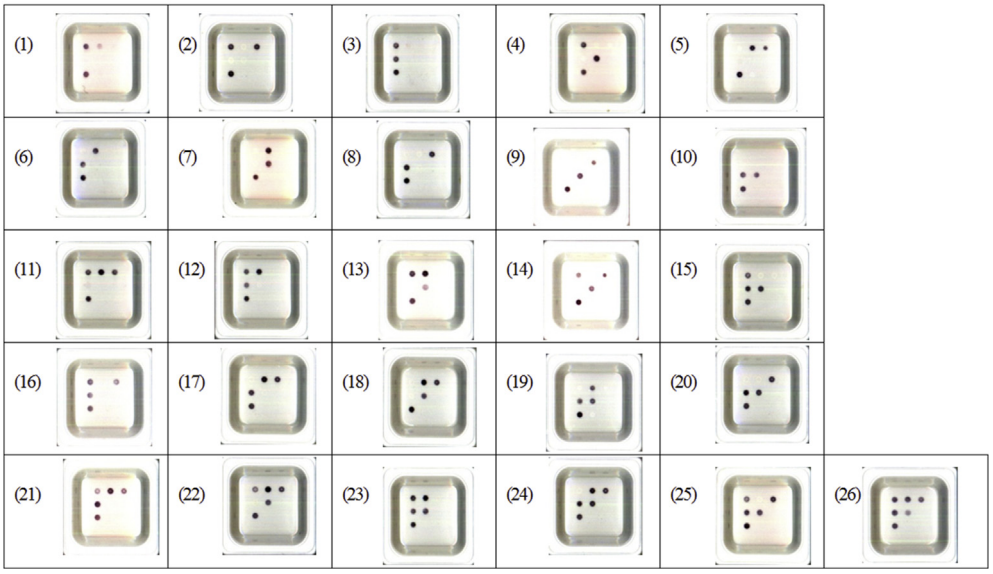


Figure 1 – Detection of the *Salmonella* strains using the biochip. Conditions for the biochip hybridization were described in Materials and methods. (A) Allocation of specific oligonucleotide probes on the biochip. Sequences of probes are listed in Table 2. (B) Simultaneous detection of different combinations of *Salmonella* serovars in chicken meat samples by biochip (1–26). The *Salmonella* counts spiked for each serovar were 10^4 cfu/mL of chicken homogenate. (1) E, T; (2) E, I; (3) E, V; (4) E, H; (5) T; (6) T, H; (7) T, H; (8) I, V; (9) I, H; (10) V, H; (11) E, T, I; (12) E, T, V; (13) E, T, H; (14) E, I, H; (15) E, V, H; (16) E, I, V; (17) T, I, V; (18) T, I, H; (19) T, V, H; (20) I, V, H; (21) E, T, I, V; (22) E, T, I, H; (23) E, T, V, H; (24) T, I, V, H; (25) E, I, V, H; (26) E, T, I, V, H. E = Enteritidis, H = Hadar, I = Infantis; V = Virchow; T = Typhimurium.

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

Table 3 – Specificity of the PCR primers[a] for the detection of *Salmonella* Enteritidis, S. Typhimuruim, S. Infantis, S. Virchow, and S. Hadar.

		PCR result with						
		Salmonella Enteritidis	*Salmonella* Hadar	*Salmonella* Typhimurium	*Salmonella* Infantis		*Salmonella* Virchow	
Bacteria species	Total no. of strains[b]	ENT-F/ ENT-R	HAD-F/ HAD-R	TYP-F/ TYP-R	INF1-F/ InVi-R	INF2-F/ InVi-R	VIR1-F/ InVi-R	VIR2 F/ InVi-R
Salmonella Enteritidis	12	12+	—	—	—	—	—	—
Salmonella Hadar	11	—	11+	—	—	—	—	—
Salmonella Typhimurium	12	—	—	12+	—	—	—	—
Salmonella Infantis	11	—	—	—	11+	11+	—	—
Salmonella Virchow	10	—	—	—	—	—	10+	10+
Nontarget *Salmonella* strains	182	—	—	—	—	—	—	—
Non-Salmonella strains	103	—	—	—	—	—	—	—

PCR = polymerase chain reaction.
[a] 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.

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. Typhimuruim (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 $N \times 10^0$ cfu/mL either in PBS or in chicken meat samples (Table 4).

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.

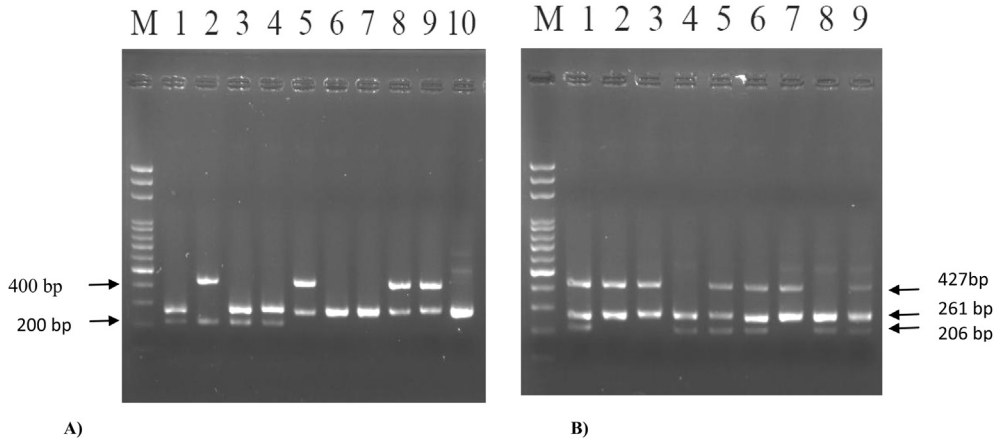


Figure 2 – Detection of the *Salmonella* strains by mPCR using ENTE-F/ENTE-R (206 bp), TYPH-F/TYPH-R (261 bp), INFA1-F/ InVi-R (268 bp), VIRC1-F/InVi-R (273 bp), HADA-F/HADA-R (427 bp) primers. (A) Lane M: 100 bp ladder; lane 1: E, T; lane 2: E, H; lane 3: E, I; lane 4: E, V; lane 5: T, H; lane 6: T, 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, H. E = Enteritidis; H = Hadar; I = Infantis; T = Typhimurium; V = Virchow.

Table 4 – Detection limit for poultry meat samples by multiplex PCR and biochip.

Strain	Multiplex PCR				Biochip			
	RAw chicken meat (CFU/mL)		Sterilized chicken meat (CFU/mL)		Raw chicken meat (CFU/mL)		Sterilized chicken meat (CFU/mL)	
	Without enrichment	With enrichment [b]	Without enrichment	With enrichment [b]	Without enrichment	With enrichment [b]	Without enrichment	With enrichment [b]
Salmonella Enteritidis	$N \times 10^3$	N[a] $\times 10^0$	$N \times 10^3$	$N \times 10^0$	$N \times 10^2$	$N \times 10^0$	$N \times 10^2$	$N \times 10^0$
Salmonella Typhimuruim	$N \times 10^3$	$N \times 10^0$	$N \times 10^3$	$N \times 10^0$	$N \times 10^2$	$N \times 10^0$	$N \times 10^2$	$N \times 10^0$
Salmonella Infantis	$N \times 10^3$	$N \times 10^0$	$N \times 10^3$	$N \times 10^0$	$N \times 10^2$	$N \times 10^0$	$N \times 10^2$	$N \times 10^0$
Salmonella Virchow	$N \times 10^3$	$N \times 10^0$	$N \times 10^3$	$N \times 10^0$	$N \times 10^2$	$N \times 10^0$	$N \times 10^2$	$N \times 10^0$
Salmonella Hadar	$N \times 10^3$	$N \times 10^0$	$N \times 10^3$	$N \times 10^0$	$N \times 10^2$	$N \times 10^0$	$N \times 10^2$	$N \times 10^0$

Under the multiplex PCR conditions five primer sets were used but single *Salmonella* serovar was used for each assay.
PCR = polymerase chain reaction.
[a] $N = 1–9$.
[b] Eight-hour enrichment.

When the detection limit of the chip was evaluated with raw and sterilized chicken homogenate spiked with *Salmonella* cells, the detection limit was $N \times 10^2$ cfu/mL for each *Salmonella* serovar. In comparison with the detection limit of mPCR, the detection limit could be improved from $N \times 10^3$ 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 $N \times 10^0$ 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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