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Research Article

Simultaneous Detection of *Salmonella*, *Listeria Monocytogenes* and *Shigella* in Poultry Samples by Triplex PCR

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Abstract

Raw poultry is one of the important sources of major foodborne bacterial pathogens such as *Salmonella*, *Listeria monocytogenes* and *Shigella*. In this study, we developed and evaluated a triplex PCR method for simultaneous detection of three foodborne bacterial pathogens, *Salmonella*, *Listeria monocytogenes* and *Shigella*, with high specificity and sensitivity. A total of 502 samples were collected from chicken abattoirs, processing plants and retail vendors in Tai'an, Shandong Province of China, and used for the validation of a triplex PCR method. Results showed that *Salmonella, L. monocytogenes* and *Shigella* were detected in 26.9%, 6.2% and 5.2% of samples respectively. Our results demonstrate that the triplex PCR method can be used routinely to detect *Salmonella*, *L. monocytogenes* and *Shigella* during the poultry processing procedures in order to reduce the risk of foodborne disease outbreaks.

Keywords: Triplex PCR; Foodborne bacterial pathogens; *L. monocytogenes*; *Salmonella*; *Shigella*

Abbreviations

BHI; Brain Heart Infusion Medium; BPW; Buffered Peptone Water.

Introduction

Outbreaks and more than 250 known foodborne diseases could be caused by food contaminated with bacteria, viruses, parasites, and toxins, which continue to be a public health problem in the world [1-7]. In the United States, a total of 19,056 infections, 4,200 hospitalizations, and 80 deaths were reported in 2013 [8]. Raw poultry is considered to be one of the important sources of major foodborne bacterial pathogens such as *Salmonella, Listeria monocytogenes* and *Shigella,* with *Salmonella and Listeria monocytogenes* among the top five pathogens contributing to domestically acquired foodborne illnesses resulting in death in the United States [9]. The prevalence of bacteria pathogens in chickens have been well documented in many countries [10-13], however, the prevalence of bacterial pathogens in poultry products in Tai'an, Shandong Province of China has not been reported. The lack of microbiological monitoring systems in the poultry processing procedures in Shandong Province of China highlights the potential danger of cross-contamination of common bacterial pathogens from poultry products to humans.

The conventional methods for the detection of *Salmonella, Listeria monocytogenes* and *Shigella* are time-consuming and costly. Therefore, it is necessary to develop quick, simple, sensitive and cost-effective methods for simultaneous detection of these pathogens. Multiplex polymerase chain reaction (PCR) has been increasingly used for rapid detection of foodborne pathogens [14-20]. The objectives of the current study were to develop a triplex PCR method for simultaneous detection of *Listeria monocytogenes, Salmonella* and *Shigella*, also to evaluate the method in various samples collected from chicken abattoirs, processing plants and retail vendors in Tai'an, Shandong Province of China.

Materials and Methods

Bacterial strains

The *L. monocytogenes*, *Salmonella* enteric serotype Enteritidis and *Shigella dysenteriae* strains were obtained from the College of Animal Science and Veterinary Medicine, Shandong Agricultural University and used as representative target pathogens.

Sample collection and treatment

A total of 502 samples including chicken feces and feathers (60 each), chicken wash (92), chicken carcasses (50) from processing plants, fresh raw chickens (120), and frozen chickens (120) were collected in Tai'an, Shandong Province of China. Feces samples were collected immediately after arrival of the birds, placed into sterile polyethylene bags and processed. Briefly, 1 g of feces was diluted in 10 ml of 0.9% sterile saline solution and decanted for 5 min, and 1 ml of each sample was transferred to 5 ml of brain heart infusion (BHI) medium and incubated at 43°C for 12 h. Feather samples (25g) were added to 60 ml of PBS, and 1 ml was transferred to BHI medium

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and incubated at 43°C for 12 h. Chicken washes were collected by placing the carcass into sterile polyethylene bags containing 300 ml of 0.1% peptone water. The solution was then transferred to a sterilized glass flask (300 ml) and incubated at 43°C for 18–24 h. afterwards 1 ml of the solution was transferred to a tube containing 5 ml of BHI medium.

Chicken carcasses taken from broiler processing plants were picked up randomly from the final wash tanks at the abattoirs. Each carcass was placed in a sterile plastic bag containing 500 ml of PBS and shaken for 2 min. The carcass was suspended to allow the diluents to drain back in the plastic bag, and then returned to the wash tank. Frozen as well as fresh carcasses were obtained from abattoirs and street vendors, but only fresh carcasses were obtained from street vendors. Live chickens were sacrificed and plucked upon purchase. Carcasses were transported to the laboratory in separate containers on ice. Frozen carcasses were thawed overnight at room temperature [10], while fresh carcasses were processed within 3 h after collection. Each carcass was placed in a large, heavy-duty plastic bag with 225 ml of buffered peptone water (BPW) containing 1% Tween 80 and 0.05% sodium thiosulphate. Each carcass was massaged inside the bag for 1 min. The rinse was decanted and 50 ml portions were used for the detection of *Salmonella*, *L. monocytogenes* and *Shigella*.

DNA extraction

One milliliter of cell suspension was centrifuged at 8,000×*g* for 2 min. The cell pellet was washed twice with 400 µl of STE Buffer (100 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and centrifuged at 8000×*g* for 2 min. The pellet was resuspended in 200 µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and 100 µl of Tris-saturated phenol (pH 8.0) were added followed by a vortex-mixing step of 60s. The cell suspension was subsequently centrifuged at 13,000×*g* for 5 min at 4°C to separate the aqueous phase from the organic phase and 160 µl of the upper aqueous phase was transferred to a clean 1.5 ml tube containing 40 µl of TE buffer. The solution was mixed with 100 µl of chloroform and centrifuged for 5 min at 13,000×*g* at 4°C. The aqueous phase (160 µl) was transferred to a clean 1.5 ml tube containing 40 µl of TE and 2 µl of RNase (10 mg/ml), and incubated at 37°C for 10 min to digest RNA. Chloroform (100 µl) was added to the tube, and the solution was mixed and centrifuged for 5 min at 13,000×*g* at 4°C. The upper aqueous phase (150 µl), which contained purified DNA, was transferred to a clean 1.5 ml tube and used directly for the subsequent experiments or stored at -20°C.

Primer design for triplex PCR

The primer pairs were selected based on their specificities reported in the literature. To find the best combination of primers for the triplex PCR, the computer simulation of different combinations of all the primer pairs considered was performed with the aid of software Web Primer available online (http://www.yeastgenome.org/cgi-bin/web-primer). The selected target genes were *invA* gene [21] for *Salmonella*, *inlB* gene [22] for *L. monocytogenes*, *IpaH* gene [4,23] for *Shigella*, and 16S rRNA gene [24] for the internal control. The name and sequence of selected primer sets for the triplex PCR are listed in Table 1.

PCR amplification and restriction enzyme digestion

Triplex PCR amplification was performed in 50 µl reaction mixture containing 5 µl 10×PCR reaction buffer, 75 pmol $MgCl_2$, 10 pmol of dNTPs, 20 pmol inlB primers, 5 pmol invA primers, 5 pmol IpaH primers, 1 pmol 16S-rRNA primers, 5U *Taq* DNA polymerase (Takara), and 4 µl DNA template (20-200ng/µl). Amplification was carried out in a thermal cycler (Applied Biosystems 2720) using the following conditions: an initial denaturation at 94°C for 3 min followed by 28 cycles consisting denaturation at 94°C for 30s, primer annealing at 57°C for 1 min and extension at 72°C for 1 min 30s; and a final extension step at 72°C for 10min. DNA purified from reference strains was used as the positive control for triplex PCR reactions. PCR products were applied to 3% agarose gels in the TAE running buffer (40 mM Tris-acetate, 1 mM EDTA, pH7.5) containing 0.5 µg/ml of ethidium bromide for electrophoresis. The amplified DNA fragments were visualized and photographed under the UV light. PCR amplified products (6 µl) for *invA*, *inlB*, *ipaH*, and 16S - rRNA genes were digested with 2 U of restriction enzyme *KpnI* (Gibco BRL), and digested DNA products were analyzed by electrophoresis on 3% agarose gels stained with ethidium bromide. Two DNA markers, 100 bp and 2K bp ladders, were used as size standards.

Statistical analysis

Statistical analysis was performed using the software Primer 5.0 System and Fisher's exact test (Analyse-it Software, Ltd., Leeds, England).

Results

The target genes specific for three common bacterial pathogens were *invA* (287bp amplicon for *Salmonella*, NC_003197.1), *inlB* (148bp amplicon for *L. monocytogenes*, NC_003210.1) and *ipaH* (600bp amplicon for *Shigella*, NC_008258.1). Each primer set was tested individually by using the DNA sample purified from the reference strain as a template in PCR to ensure its specificity. Results showed that each of the pathogen-specific primer sets generated a single band at the expected size (Fig. 1A). Sequencing results

Table 1: Microbial pathogen-specific genes and primers for triplex PCR.

Pathogen	Infection dose (N)	Target gene (accession No.)	Primers	Sequence (5'-3')	Product size (bp)
Salmonella spp.	10^6	invA [21]	InvA-F	GTGAAATTATCGCCACGTTCGGG	285
		(NC_003197.1)	InvA-R	TCATCGCACCGTCAAAGGAAC	
L. monocytogenes	<1000	inlB [22]	InlB-F	AAAGCACGATTTCATGGGAG	148
		(NC_003210.1)	InlB-R	ACATAGCCTTGTTTGGTCGG	
Shigella spp.	<10	ipaH [[4,23]	IpaH-F	GTTCCTTGACCGCCTTTCCGATACCGTC	600
		(NC_00825.1)	IpaH-R	GCCGGTCAGCCACCCTCTGAGAGTAC	
Bacterial DNA (internal control)		16S rRNA [24] (J01859)	16S-F 16S-R	CCTACGGGAGGCAGCAGT CGTTTACGGCGTGGACTAC	475

confirmed that PCR products were 100% identical to those listed in the GenBank (data not shown). The 16S rRNA gene of foodborne bacterial pathogens was used as the internal control, which contained a conserved fragment flanking the variable regions 1 (V1) to 3 (V3) of the gene (475bp) and was designed previously for the oligonucleotide array [24]. The use of the internal control facilitates the interpretation of the amplification results, since a control gene product should always be amplified even when there is no target sequence present, and can thus indicate whether the PCR reaction works or not. This is particularly important for PCR-based diagnostic methods performed with complex samples such as meat products. PCR results showed that the internal control gene and target genes were amplified simultaneously (Figure. 1).

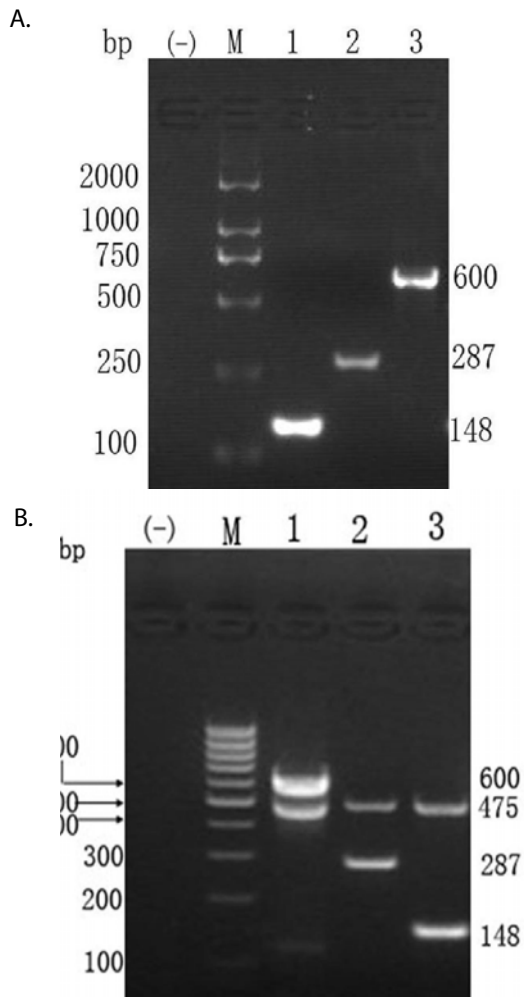

Figure 1: PCR products obtained by using single primer set specific for one of the three target genes (A) and two primer sets specific one target gene and one internal control (16S rRNA). M, 2K DNA Marker; 1, *inlB* gene fragment (148bp) amplified from *L.monocytogens*; 2, *invA* gene fragment (287bp) amplified from *Salmonella* spp.; 3, *ipaH* gene fragment (600bp) amplified from *Shigella*; (-), negative control. (B) M, 100bp ladder Marker; 1, *ipaH* gene fragment (600bp) and internal control (475bp) from *Shigella*; 2, *invA* gene fragment (287bp) and internal control from *Salmonella* spp.; 3, *inlB* gene fragment (148bp) and internal control from *L. monocytogens*; (-), negative control.

Experimental results using specific primer sets and reference strains showed that three target genes were successfully amplified without nonspecific bands in the triplex PCR, demonstrating the specificity of this method (Figure. 2). The sensitivity of the triplex PCR was then assessed by testing the mixture of DNAs extracted from the serial dilutions of the target pathogens (10^5, 10^4, 10^3, 10^2, and <10 cells/ml) in the bacterial culture. The detection of three pathogens were achieved at the concentration of bacterial pathogens as low as 10^2 cells/ml (data not shown).

To validate the triplex PCR method, a total of 502 samples were collected from chicken abattoirs, processing plants and retail vendors in Tai'an, Shandong Province of China, and used for simultaneous detection of three foodborne pathogens. The results showed that *Salmonella* was detected in 26.9% (135/502) of various samples collected in Tai'an, China (Table 2). The highest rate of contamination by *Salmonella spp*. was detected in fecal samples (26/60, 43.3%) followed by the fresh raw chicken (46/120, 38.3%), frozen chicken (33/120, 27.5%), feather samples (10/60, 16.7%), chicken washes (14/92, 15.2%), and the processing plant in Tai'an (6/50, 12%).

The overall contamination rates of *L.monocytogens* and *Shigella spp*. for various samples collected in Tai'an were 5.2% (26/502) and 6.2% (31/502) respectively. However, *Shigella spp*. and *L.monocytogens* were not detected in samples collected from feces and feathers. Among samples collected from other four sources (chicken washes, processing plant, fresh raw chicken and frozen chicken), 2.17%~18% were contaminated by *Shigella spp*. and 4% ~ 10.8% were contaminated by *L. monocytogens* (Table 2).

One or more of the bacterial pathogens were detected in 178 of 502 (35.5%) samples, with one pathogen detected in 164 (32.7%) samples, two pathogens detected in 11 (2.2%) samples, and three pathogens detected in 3 (0.6%) samples. In samples collected from frozen chickens, 55 out of 120 (45.8%) were contaminated by three common bacterial pathogens. No significant differences were observed among samples collected from fresh raw chickens (42.5%) and frozen chickens (45.8%). Twenty one samples from chicken washes (22.8%) and 15 samples from the processing plant (30%) were contaminated by three bacterial pathogens.

Discussion

In this study, a cost-effective triplex PCR method was developed for the simultaneous detection of three foodborne bacterial pathogens

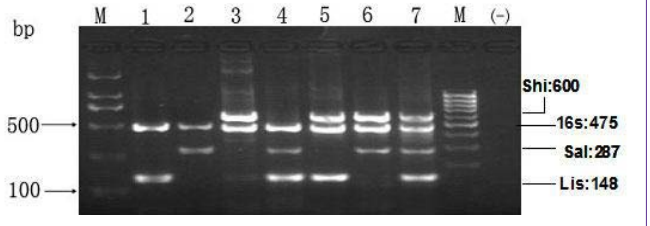

Figure 2: Amplification products obtained by multiplex PCR. M, 100 Ladder Marker and 2K Marker; 1, Multiplex PCR with *L.monocytogens*; 2, Multiplex PCR with *Salmonella spp*.; 3, Multiplex PCR with *Shigella spp*.; 4, Multiplex PCR with *L.monocytogens* and *Salmonella*; 5. Multiplex PCR with *L.monocytogens* and *Shigella*; 6, Multiplex PCR with *Salmonella spp* and *Shigella*; 7, Multiplex PCR with *L.monocytogens*, *Salmonella* and *Shigella*; (-), negative control.

Table 2: Incidences of *Salmonella*, *Shigella spp* and *L.monocytogens* in samples collected from sources.

Type of samples	Number		Pathogen-positive samples		
		Overall No. (%)	*Salmonella spp.* No. (%)	*Shigella spp.* No. (%)	*L.monocytogenes* No. (%)
Feces	60	26 (43.3)	26 (43.3)	0 (0)	0 (0)
Feathers	60	10 (16.7)	10 (16.7)	0 (0)	0 (0)
Chicken washes	92	21 (22.8)	14 (15.2)	2 (2.17)	9 (9.8)
Processing plant	50	15 (30)	6 (12)	9 (18)	2 (4)
Fresh raw chicken	120	51 (42.5)	46 (38.3)	3 (2.5)	7 (5.8)
Frozen chicken	120	55 (45.8)	33 (27.5)	12 (10)	13 (10.8)
Total	502	178 (35.5)	135 (26.9)	26 (5.2)	31 (6.2)

(*Salmonella, Listeria monocytogenes* and *Shigella*). This PCR-based method is highly specific and sensitive, which can be used routinely in resource-limited locations to detect these pathogens simultaneously. The possibility of false positive results was minimized by using the internal amplification control, and could be identified when the internal control was not amplified. Based on our experience, the primer concentration for the 16S-RNA gene in the triplex PCR is a critical aspect, since low concentrations of 16S-RNA primers could lead to substantial variations in the 16S-RNA gene amplification while high concentrations could negatively affect the PCR sensitivity. Therefore, the initial 16S-RNA primer concentration in the PCR should be reproducibly tested, and kept as low as possible to avoid the inhibition of the specific target amplification. In order to determine the optimal primer concentration for the 16S-RNA gene, tenfold dilution series of the 16S-RNA primers were made and tested in the triplex PCR as described in the Materials and Methods section. Results indicated that 1pmol of 16S-RNA primers could be used in the triplex PCR without significantly affecting the amplification of the target genes (data not shown). The competitive effect of 16S-RNA primers on the specific amplification of *invA*, *inlB* and *ipaH* genes was negligible.

Furthermore, this triplex PCR method was successfully used to investigate the risk of potential foodborne bacterial contaminations in 502 samples collected from many sources in Tai'an, Shandong Province of China. Our data showed that poultry samples appear to be prominent reservoirs of *Salmonella* and the prevalence of *Salmonella* in total samples (26.9%) was four or five times higher than that of other two pathogens. Fecal samples and feather samples showed high rates of contamination by *Salmonella*, but no contamination by *L.monocytogens* and *Shigella*, suggesting that they were unlikely the source of *L.monocytogens* and *Shigella*. The extent of *Salmonella* presence from the fresh raw chicken and frozen chicken were similar; however, significantly more samples were contaminated with *Salmonella* compared to other categories. The prevalence of *Salmonella* in the UK poultry was found to be 25~29% using the cultural methods [25]. Using the multiplex-PCR method, *Salmonella typhimurium and Salmonella* enteritidis strains were detected in 30.6% of environmental swabs of poultry houses in Ploufragan, France [26], and 2.9% of the fish samples in Iran [27]. Thus, the incidences of *Salmonella* in Ta'ian, China were higher than those reported for the European poultry. The rates of contamination for the other two pathogens were much lower than that of *Salmonella*. The level of *L. monocytogens* contamination in frozen samples was significantly higher than that in fresh samples (Table 2). This is consistent with observations that *L. monocytogens* can grow over a wide range of environmental conditions such as refrigeration temperatures [13], which allows it to overcome the food preservation and safety barriers, and creates a potential risk to human health. The prevalence of *L.monocytogenes* in retail poultry in Leon, Spain, was 32% [12], while the organism was isolated from 62% of broilers tested at abattoirs, processing plants and retail outlets in Finland [13].

In conclusion, we have developed an easy and rapid triplex PCR method for the simultaneous detection of *Salmonella*, *Listeria monocytogenes* and *Shigella* with high specificity and sensitivity. Our results demonstrate that three common foodborne bacterial pathogens are prevalent in raw poultry products in Tai'an, China, and indicate the necessity to screen bacterial pathogens routinely during the poultry processing procedures in order to reduce the potential risk of foodborne disease outbreaks.

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