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Original article

Simultaneous Detection of *invA*, *STM4497*, and *fliC*183 Genes in *Salmonella typhimurium* by Multiplex PCR Method in Poultry Meat Samples in Zanjan, Iran

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Background: *Salmonella typhimurium* is one of the most important species of Salmonella that is intracellular parasite and attacks host mucus membrane. These bacteria can cause gastroenteritis, and their main transmission route is water, poultry, meat, egg, and raw food. The aim of this study was to detect three virulence genes associated with *S. typhimurium* named *invA*, *STM4497*, and *fliC183* genes by Multiplex PCR method.
Materials and Methods: 183 samples of poultry were collected from food products in Zanjan (Iran) and cultured in BPW (Buffered Peptone Water) for 18 hr and at 37°C, and in RVS broth (Rappaport Vassiliadis Soya) for 6 hr at 41.5°C. After amplification of genomic DNA by Multiplex PCR method, occurrence of pathogen contamination was checked and compared with standard strain.
Results: From the total of 183 collected samples, 52(28.4%) samples were positive for *S. typhimurium*. The frequency of *STM4497*, *fliC183*, and *invA* genes were 49 (27%), 3 (2%), and 53 (29%), respectively.
Conclusion: Simultaneous detection of *invA*, *STM4497*, and *fliC*183 genes were recognized as a key for detection of *S. typhimurium* by Multiplex PCR method.

Keywords: *Salmonella typhimurium*, Food-borne pathogen, Multiplex PCR

1. Background

Microbial food-borne pathogens are considered as the most extensive ones, and millions of the human illnesses are caused annually by them in different areas, which will lead to the emergence of important public health issues and remarkable economic responsibilities (1). *Salmonella spp*., *Escherichia coli O157:H*, and *Listeria monocyto* genes are considered as the most important pathogens among the most recognized microbial food-borne pathogens (2). Salmonellosis is a major public health problem, so recognition of its serotypes can improve prevention and control of food-borne diseases (3, 4).

Salmonella is a kind of non-spore forming rod, a gram negative and facultative anaerobe that can ferment glucose belonging to the *Enterobacteriaceae* family (5). Related products to poultry are usually contaminated with Salmonella, and diseases can be transmitted to the humans through consumption or handling of the products (6, 7). Salmonella infections are widespread globally in both developed and developing countries and are affective reasons in the morbidity and economic costs (8).

Several virulence genes such as *invA*, *STM4497*, and *fliC*183 have been used to detect *Salmonella typhimurium* in food samples (1, 9, and 10). The *invA* gene has been recognized as an international standard for detection of Salmonella genus and has been proved as a sufficient PCR target with potential diagnostic applications (11, 12). The *STM4497* gene transcribes a putative cytoplasmic protein. The region including STM4497 gene houses a putative Type II restriction enzyme that is only present in serovar Typhimurium (13 ,14). The *invA* gene codes for protein in inner membrane of bacteria, which is essential to invade epithelial cells (8). The *fliC* gene has a conserved The *fliC* gene has a well-maintained terminal region and a variable central region, which controls the antigenic particularity. The flagellin gene *fliC* encrypts the major component of the flagellum in *Salmonella enterica serovar typhimurium* (15).

Methods of Salmonella detection traditionally need to 5-7 days and should have several steps and biochemical and serological tests. Also, faster approaches at DNA level are investigated (13). PCR is considered as an alternative to the tedious time consuming procedure of Salmonella identification (9). The Polymerase Chain Reaction (PCR) is a reliable method to identify food-borne pathogens like Salmonella (8). In Multiplex PCR (mPCR) method, several specific primers are mixed in a single PCR assay for the simultaneous and concurrent increase in more than one target DNA sequence (16).

2. Objectives

The purpose of this study was to identify three virulence genes associated with *S. typhimurium* named *invA*, *fliC183*, and *STM4497* genes by Multiplex PCR method.

3. Materials and Methods

3.1. Bacterial strains and DNA extraction

Standard bacterial strain of *S. typhimurium* (ATCC 14028) was obtained from Research Center, Shahid Beheshti University of Medical Sciences in Tehran, Iran. *S. typhimurium* (ATCC 14028) was used for optimization of the test as a positive control. Isolates were grown overnight at 37°C on Tryptic Soy Agar (TSA), and a single colony was picked and inoculated into 5 mL of Tryptic Soy Broth (TSB) and grown at 37°C with shaking. Bacterial genomic DNA was extracted from pure bacterial culture using a commercial

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Cinna Pure-DNA (Gram Negative Bacteria) according to the manufacturer's instructions. Final DNA concentrations were determined using a bio photometer (9).

3.2. Artificial contamination of poultry

Twenty five grams of each poultry sample were homogenized with 225 mL of TSBYE (Tryptic Soy Broth Yeast Extract) and artificially contaminated individually with *S. typhimurium* (ATCC 14028) at 7 levels of contamination (10^1,10^2,10^3,10^4,10^5,10^6,10^7cfu.25^{-1} g). Bacterial genomic DNA was extracted from 1 mL of overnight cultures using a commercial Cinna Pure-DNA (Gram Negative Bacteria) according to the manufacturer's instructions. Final DNA concentrations were determined using a spectrophotometer. Each of the levels of *S. typhimurium* serial contamination in a plate of TSA was cultured.

3.3. Sample collection and Simultaneous direct detection of S. typhimurium in pre-enriched poultry samples by Multiplex PCR method

One hundred and eighty three samples of poultry were collected from different supermarkets at random in Zanjan, Iran between April 2014 and October 2014. Each sample package was placed in sterile container and transferred to laboratory with dried ice within 30 minutes after purchase.

Twenty five grams of each poultry sample (chest) were incubated in BPW (Buffered Peptone Water) at 37°C for 18hr, followed by incubation in RVS Broth (Rappaport Vassiliadis Soya) at 41.5°C for 6 hr (17). The samples centrifuged at 4000 rpm for 5 min. The DNA from the enriched culture was obtained by using a DNA extraction kit (Cinna Pure-DNA /Gram Negative Bacteria), and the purified DNA was used as a template for the PCR assay. Forward and reverse primer pairs were designed for *invA*, *STM4497*, and *fliC*183 genes by Gene Runner software after obtaining gene sequences from Gene Bank (http://www.ncbi.nlm.nih.gov). The oligonucleotide primers used in this study are presented in Table 1. The primers were analyzed for melting temperature (Tm), hairpin structures, and dimers by Oligo Analyzer software.

Table 1. Primers used in this study.

Gene	total number (183)	Z-amount	p-value	Result
invA	53(29%)	-	-	-
STM4497	49(27%)	-	-	-
FliC	3(2%)	-	-	-
invA/ STM4497	49(27%)	0.47	0.641	Not acceptable
invA/ FliC	3(2%)	7.85	0.000	Acceptable
STM4497/ FliC	0	7.38	0.000	Acceptable
invA/ STM4497/ FliC	0	7.38	0.000	Acceptable

The multiplex PCR for *invA*, *flic*, and *STM4497* genes were optimized in a single master tube. PCR amplification was carried in 50 µL reaction mixture which contained:1X PCR Buffer, 1 mM of dNTP, 1 unit of Taq polymerase ,20 ng of DNA template, and 0.5 µM of each forward and reverse primer, and $MgCl_2$ was standardized for each primer between 2, 2.5, and 3 mM concentration by PCR gradient thermocycler model (Eppendorf, Germany). PCR amplifications were performed as follows: the first denaturation cycle of DNA at 95°C for 5 min followed by 30 cycles, each consisting of 45 s denaturation at 95°C, 1 min annealing at 60°C for *flic*, 61°Cfor *invA*, 63°C for *STM4497*, and 1 min extension at 72°C, with the final extension cycle of 72°C for 5 min, and sample were kept at 22°C until processed (18). The PCR products were further analyzed by agarose gel electrophoresis (3% agarose), stained with DNA safe stain and visualized by a UV trans-illuminator.

4. Results

To achieve particularity, PCR primers was designed which were unique to a single region of the target genes of *S. typhimurium*. The sequences of forward and reverse primers are summarized in Table1. An adequate amount of the pure total DNA is basic in order to extract them in PCR based detection. The optimal concentration of $MgCl_2$ observed, was 2.5 mM. In evaluating PCR for the detection of microorganisms, two important criteria must be satisfactory: Specificity and sensitivity. The sensitivity of the detection depends on the condition of the PCR reaction such as primer annealing temperature, primer concentration, Mg^{2+} concentration, extension time and characteristics of DNA polymerase used (19).

The results of multiple PCR for simultaneous amplification of three genes are shown in Figure 1.The expected sizes were 291, 357, and 743 bp from *invA*, *STM4497*, and *fliC*183 genes, respectively. Furthermore, the accuracy of the methods after artificial inoculation was evaluated to be 10^1 CFU.mL^{-1}.

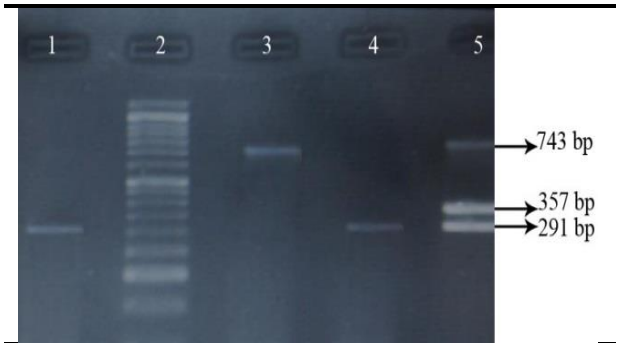

Figure 1. Simultaneous detection of three genes of *Salmonella typhimurium*. Line 1: *STM4497* (357bp), Line 2:50 bp DNA ladder, Line 3: *fliC183* (743bp), Line 4: *invA* (291bp), Line 5: Multiplex PCR products: *fliC183* (743bp) , *STM4497*(357bp), *invA* (291bp).

From 183 poultry meat samples, 53(29%) were positive with *invA* gene. Amplification of *invA* gene nowaday has been recognized as an international standard for detection of Salmonella genus (20). Therefore, 29% of the samples were contaminated with salmonella genus. From 53 poultry meat samples positive with *invA* gene, only 3(2%) of the samples with *fliC* gene and 49(27%) of the samples with *STM4497* gene were positive. The *fliC* and *STM4497* genes have been recognized for detection of *S. typhimurium* (9, 10). According to the results, from the total of 183 collected samples, 52(28.4%) samples were positive for *S. typhimurium*. Out of 53 poultry meat samples positive with *invA* gene, just one (1%) sample was shown for *invA* gene without any prevalence of *fliC* and *STM4497* genes, therefore, just one sample was contaminated with other Salmonella genus. As the results, 3 and 49 samples were positive for *fliC* and *STM4497* genes, respectively, which shown 28.4% of the samples were contaminated with *S. typhimurium* (table 2).

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Table 2. *InvA, STM4497 and FliC* genes frequencies in all samples and Comparative test results.

Gene	Primer sequence (5'-3')	Tm	Amplicon size (bp)
invA	F:GATCCCCGCATTGTTGATTG	67.3	291
	R:ACGACGACCCTTCTTTTTCCT	64.8	
STM4497	F:CAGGTTCAGAGCCGCATTAGC	68	357
	R:GCCAGGCGTTACCCATTCC	68.6	
FliC	F:ATGCCGATACTACGATTGCTTT	63.4	743
	R:TTTACGGTGTTGCCCAGGTT	66.2	

5. Discussion

Most of the infections in humans and animals result from Salmonellosis (21, 22). *S. typhimurium* is important in the laboratory since it helps to the establishment of the connection between clinical diseases and probable sources of infection (3). PCR methods are more sensitive compared to conventional culture techniques (20). In vitro amplification of DNA by PCR method is seen as a powerful and strong device in microbiological diagnostics and shows a type of high sensitivity and specificity in detection of special pathogenic bacteria in different food structures (23). Since Multiplex PCR can detect *Salmonella spp*., some measures can be taken by industries and producers to avoid contamination of their products (24). In Multiplex PCR, the primer pairs should be specific types for target genes, and the PCR products should be in different sizes (16). Guo and colleagues (1999) used *invA* gene to identify salmonella from turkeys (25). Ferretti and colleagues (2001) reported a fast method for *invA* gene, which could detect salmonella serotypes within a maximum of 12 hours (26).

Several surveys have displayed that *invA, STM4497, andfliC*183 genes are present in a majority of isolates of *S. typhimurium* (1, 9, 25). In present study, three virulence genes were selected from *S. typhimurium* as bacterial pathogen: the *invA, STM4497, and fliC*183 genes (1, 9, 10, 11, and 27). Oliveria reported that the *invA* and *fliC*183 genes were 100% specific for identification of *S. typhimurium* from poultry samples by Multiplex PCR assay (20).

According to the results of our study, 52 out of 183 samples from Zanjan city (Iran) were positive for *S. typhimurium* by Multiplex PCR method. In many studies, there were increases in the frequency of S. typhimurium in poultry meat samples in different areas. Jamshidi and colleagues (2009) showed that 8.3 and 1.6% of the poultry carcasses were contaminated with *Salmonella spp*. and *S. typhimurium*, respectively in Mashhad province by m-PCR method (20). Zahraei-Salehi and colleagues (2005) reported that the *invA* gene was present in 15.6% of poultry meat contaminated with Salmonellae in Shiraz province by traditional culturing, which then was confirmed by PCR method (8).

Although in our study, the prevalence of *STM4497* and *fliC183* genes were 27 and 2%, respectively, but *invA* gene was present in 29% of the poultry meat. Aseel and colleagues (2011) used PCR methods to identify *S. typhimurium* in south of Iraq, and the total percentage of *invA* gene was 30.4% (22). Moreover, in the present study, the prevalence of *invA* gene decreased. The difference in the results may be attributed to diversity in sampling methods. In a study by O'Regan and colleagues (2008), the real-time multiplex PCR assay was performed as sensitive as the traditional cultural method in detecting Salmonella in contaminated chicken samples (17). According to Saeki (2013), the mPCR assays showed high specificity for the simultaneous detection of *S. Typhimurium* in chicken meat (28).

The present study supports the ability of multiplex PCR-based assay to confirm the isolates as salmonella. The results of this survey identify the usefulness of the Multiplex PCR method for rapid detection of *S. typhimurium* from poultry meat. The inspection of chicken meat for *S. typhimurium* should be supervised by Public Health and Veterinary Authorities to make sure about the detection of zoonosis. It also helps to find the reasons of its prevalence among human beings. Also, observations show that sanitary standards need to be improved in poultry meat.

6. Conclusion

The presence of *invA*, *fliC183*, and *STM4497* genes can be useful in the identification of *S. typhimurium* by Multiplex PCR method in food testing laboratories.

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors' Contribution

All authors contributed extensively to the work presented in this paper

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