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Original Article

Detection of *Salmonella enterica* Serovar Typhimurium from Avians Using Multiplex-PCR

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Abstract

Salmonella enterica serovar Typhimurium and *S.enterica* serovar Enteritidis are the most frequently isolated serovars from food-borne diseases throughout the world. According to their antigenic profiles, *salmonella* shows different disease syndromes and host specificities. It is necessary and important to discriminate *salmonella* serovars from each other in order to ensure that each pathogen and its epidemiology are correctly recognized. Many PCR-based methods have been developed to identify *salmonella* serovars. The objective of present study was to identify *S.* Typhimurium in avians from different regions including: North, Northwest and capital city (Tehran) of Iran. Also in this research, the quality of CHROMagar™ Salmonella medium (CAS medium) in veterinary medicine was evaluated. The results of present study showed that out of 1870 intestine samples, fifty two *S.* Typhimurium including broiler (n=13), layer (n=12), duck (n=5), goose (n=5), sparrow (n=8), canary (n=3), pigeon (n=5) and African grey parrot (n=1) were identified using serotyping as well as multiplex-PCR. In conclusion, important measures must be taken on prevention and propagation of *S.* Typhimurium among avians. CHROMagar™ Salmonella medium has high levels of sensitivity and specificity and reduced the time to final identification of *salmonella spp.* in comparison with biochemical tests.

Key word: *Salmonella* Typhimurium, Avian, CAS medium, Multiplex-PCR, Iran

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Introduction

Salmonella are facultative anaerobe Gram-negative bacilli belonging to the family *Enterobacteriaceae.* The genus of *Salmonella* is very polymorphic and comprised of a number of genetically closely related serotypes, in which many of them are gastrointestinal pathogens in human and animals.[1]

The genus *Salmonella* consists of only two species: *S. enterica* and *S. bongori*. *Salmonella enterica* is divided into the following six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *Indica*.[2]

Nearly all-human and animal pathogen Salmonella are grouped under *S. enterica* subsp. *enterica*. *Salmonella enterica* subsp. *enterica* serovar Typhimurium is a broad-host-range serotype capable of causing systemic disease in a wide range of animals but is usually associated with gastroenteritis in a broad range of phylogenetically unrelated host species.[3]

Identification of *Salmonella* serotypes is important or critical for surveillance, improving prevention and control of food-borne diseases. It allows rapid detection, identification of sources, control of outbreaks, and also identification of emerging serotypes and new mechanisms of transmission.[4] Considering the importance of *S.* Typhimurium as a zoonotic organism; therefore it is important to discriminate it from other serovars of *S.*enterica .[5,6]

Phenotypic methods play an important role in the identification of *Salmonella* at the genus level. Serotyping according to Kaufmann-White scheme is widely used for primary typing of *Salmonella* based on the antigenic variability of the somatic (O), flagellar (H) antigens present in the cell wall of the organism, while phage typing and antibiogram are used for subdivision of serotypes.[7,8] However classical serotyping methods are time-consuming, labor intensive and require high quality of antisera and well-trained technicians.[9-11] Likewise, other drawbacks of serotyping such as the cross-reaction between serovars and the loss of serotype`s antigen have encouraged researchers to find rapid and simple methods based on molecular techniques.

To circumvent the problems associated traditional serotyping, PCR and similar nucleotide-based methods have made it possible to accelerate the identification of serotypes based upon the identification of unique genes or gene amplification and use as diagnostic tool.[10]

Multiplex PCR assay has been used to detect several pathogens including *Salmonella* in a single reaction.[12-15] Most of the multiplex PCR assays reported for *Salmonella enterica* have been used either for species identification, certain common and clinically important serotypes or some of the major serogroupings.[15-22] Molecular serotyping methods that are based on the detection of the same antigens as the Kauffmann-White Salmonella also enable the preservation of the epidemiological information.[17]

Since *S.* Typhimurium plays an important role in food-borne diseases, economic losses arising from this organism in poultry industry is not conniving and considering that there are few reports on the identification and molecular characterization of *S.* Typhimurium in poultry and avians close to poultry farm; thus, the objective of present study was to detect *S.* Typhimurium in poultry farms and free living birds near to them using multiplex-PCR method in North, Northwest and capital city (Tehran) of Iran.

Materials and Methods

Collection of Samples. A total of 1870 samples from intestine contents of avians (broiler, layer, sparrow, duck, goose, pigeon, canary and African grey parrot) collected from different geographical regions including North, Northwest and

capital city (Tehran) of Iran, between June 2009 and June 2010. All samples were taken to microbiology laboratory of the Faculty of Veterinary Medicine (Urmia University) under refrigeration to be processed at the same day and in sterile condition.

Isolation of *Salmonella*. Each sample (1-2 gram) was enriched in Selenit broth (Merck, Germany) tubes and incubated overnight at 37°C. A loopful of each tube was streaked onto CHROMagar™ Salmonella medium (CAS medium) (CHROMagar Microbiology, Paris, France) plates incubated at 37°C for 24 h. Suspected colonies, typical mauve colonies on CAS medium were gram-stained and were tested by biochemical tests. Pure cultures were obtained from Salmonella-Shigella agar medium (Laboratories CONDA, Spain). Lactose and urease negative bacteria was tested by indole, methyl red, voges proskauer and citrate (IMViC) biochemical tests.[23]

All pure isolates were maintained on MacConkey agar (Merck, Germany) plates. For longer storage, isolates grown in Leuria-Bertani broth (Merck, Germany) were mixed with glycerol to obtain final concentration of 15% glycerol and kept at -80°C.[24]

Serotyping of isolates. All isolates presumptively identified as *Salmonella*, were serotyped at Razi vaccine and serum research institute (Iran) using slide agglutination test and *S.* Typhimurium isolates were identified according to their serotyping formula: 1,4,5,12:i:1,2. To confirm the results of serotyping all isolates tested by multiplex-PCR.

Genomic DNA extraction. DNA extraction of all *Salmonella* isolates were performed from overnight culture in buffered pepton water (Scharlau Microbiology, Spain) by Genomic DNA purification kit (Fermentas, Germany) with some modifications including the use of phenol-chloroform-isoamylalcohol (25:24:1) instead of chloroform, which was indicated in DNA extraction protocol by manufacturer. Purity of DNA was tested spectrophotometrically at wavelength of 260 and 280 nm (Eppendorf biophotometer plus 6132, Germany). Extracted DNAs were diluted to give a final concentration of 50 ng (5 µl).

Multiplex-PCR reaction for isolates. All isolates tested by multiplex-PCR described previously [6] with some modifications.[25] Four sets of primers used in this study (Table 1), include: Rfbj, FljB, FliC and ST-139 and ST-141 which their target genes encode O4 antigen (663bp), H2:1, 2 (526bp), H1: i (183bp) and InvA (284bp) respectively. In present study *S.* Typhimurium ATCC 1730 used as positive control. Multiplex-PCR was performed in a final volume of 25 µl containing: PCR buffer (10mM Tris-HCl, 50mM KCl, 1.5 mM $MgCl_2$, pH 8.7), dNTP (200µM), each primer (1µM) and Smartaq™ DNA polymerase (1U) (Cinnagen, Iran) and template DNA (50ng). For negative control, sterile water added instead of nucleic acid. PCR reaction was performed in a DNA thermocycler (Model CP2-003, Corbett, Australia) as follows: An initial denaturation at 95°C for 5min., 35 cycles of denaturation at 95°C for 1min, annealing at 65°C for 1min, elongation at 72°C for 30s and final 7min extension period at 72°C. Amplified products were separated by 2% agarose gel electrophoresis at 80 V for 1 h and photographed under UV illuminator (Model DOC-008, EEC).

Results

Identification of *S.* Tyhpimurium. Out of 1870 samples, 181 (9.6%) isolated as *Salmonella* and 52 identified as *S.* Tyhpimurium including: broiler (n=13), layer (n=12) duck (n=5), goose (n=5), sparrow (n=8), canary (n=3), pigeon (n=5) and African grey parrot (n=1). Distributions of *Salmonella* and *S.* Typhimurium among different regions of Iran are shown in Table 2.

The results of serotyping were confirmed with multiplex-PCR. Primers listed in table 1 for multiplex-PCR could

Table 1. Primers` characteristics used in this study

Primer	Target gene	Primer length (bp)	Sequence	Amplified fragment size (bp)	Reference
Flic-s	*fliC*	24	3'- CCCCCTTGACCATTCTACCGATA	183	Lim et al. (2003)
Flic-as		24	3'- CCGTATAGGACATTGTCAACGTCG		
ST-139	*invA*	26	3'- AACGGGCTTGCACCGCTATTAAAGTG	284	Zahraei et al. (2007)
ST-141		22	3'- CCAAGGAAACTGCCACGCTACT		
FljB-s	*fljB*	24	3'- CCAATGTCTTCGGCATGGTAAGCA	526	Lim et al. (2003)
Flj-as		24	3'- GGCTTCAGCAATGATAGCTGCCAT		
Rfbj-s	*rfbJ*	24	3'- CATAGTTCAACCTTGACCACGACC	663	Lim et al. (2003)
Rfbj-as		24	3'- ACGAATGGTTATTTCGGCCTTCGG		

successfully amplify the expected sizes: 183, 284, 526 and 663 bp from *fliC*, *invA*, *fljB* and *rfbJ* genes, respectively (Fig. 1). From negative control, no PCR product was obtained.

The evaluation of CAS medium in comparison with diagnostic biochemical tests. All biochemical tests used in this study confirmed that suspected colonies obtained from CAS medium were *salmonella*. No difference between identified colonies as *salmonella* on CAS medium and diagnostic biochemical tests was observed.

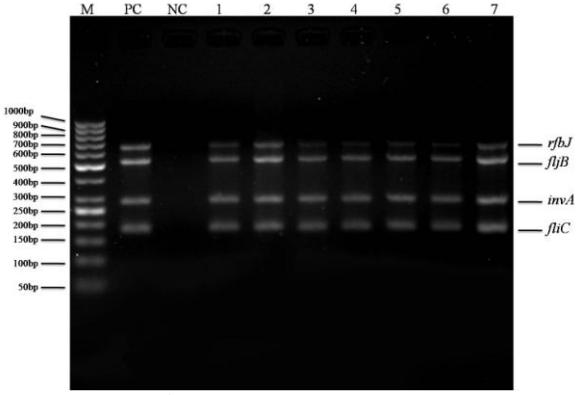

Fig 1. The results of multiplex-PCR assay. Lane M: 50bp DNA Ladder (Fermentas, Germany); Lane PC: Positive control; Lane NC: Negative control; Lane 1 to 7: *S. enterica* serovar Typhimurium

Discussion

Salmonellosis continues to be the major problem for food industries and public health system.[26] Poultry, free living birds and poultry products have been incriminated in the majority of traceable food-borne illnesses by this bacteria.[27,28] *Salmonella* infection in food-producing animals is often clinically not apparent.[29]

One of the most frequent causes of infection by *Salmonella* reported in humans has been through the handling of raw poultry carcasses and products, together with the consumption of undercooked poultry meat.[30] The usual causative agent of disease is *S.* Typhimurium or the emerging pathogen *S.* Enteritidis.[31] Conventional culture methods used for the isolation of *Salmonella* include, non-selective pre-enrichment followed by selective enrichment and plating on selective and differential agars. Suspect colonies are then confirmed biochemically and serologically.[30] CHROMagar Salmonella medium is a newly designed selective chromogenic medium and purports to detect salmonellae as mauve colonies at 18 to 24h of incubation, which other members of the family *Enterobacteriaceae* appearing as blue or uncolored colonies.[32] Conventional selective media for *Salmonella* isolation have very poor specificity, and the numerous false-positive results necessitate time-consuming complementary tests.[33]

In present study, isolation and identification of *S.* Typhimurium with CAS medium was performed and followed by biochemical tests. The results of present study showed that the use of plating on CAS medium demonstrated high levels of sensitivity and specificity and reduced the time to final identification of *Salmonella spp.*, resulting substantial cost saving. Results of present study are in agreement with previous studies, which recommended the use of CAS medium for primary plating and single media of choice

Table 2. Distribution of *Salmonella* and *S*. Typhimurium among different regions of Iran

Avians	Northwest			North			Tehran		
	Samples	***Salmonella***	***S*. Typhimurium**	**Samples**	***Salmonella***	***S*. Typhimurium**	**Samples**	***Salmonella***	***S*. Typhimurium**
Broiler	331	32	7	282	25	5	44	10	1
Layer	285	23	6	210	12	5	28	7	1
Sparrow	183	8	4	120	6	3	79	5	1
Duck	17	3	1	13	8	3	15	2	1
Goose	20	4	2	11	10	2	15	1	1
Pigeon	90	9	3	83	6	1	38	6	1
Canary	3	1	1	---	---	---	2	2	2
African grey parrot	---	---	---	---	---	---	1	1	1
Total	929	80	24	719	67	19	222	34	9

for detection and presumptive identification of *Salmonella* in stools.[32-34]

The results of present study showed that all colonies which identified as *Salmonella* on CAS medium, had biochemical profiles related to *Salmonella*. Thus, no false-positive or false-negative results were observed.

Laboratory characterization of *S*. Typhimurium is epidemiologically important as it helps establish the connection between clinical cases and possible sources of infection. Knowing the disadvantages of serotyping, alternative methods such as PCR-based assays have been described for the identification of specific *Salmonella* serotypes.[35-37]
The primary advantages of PCR tests are increased sensitivity and less time required to process samples in the laboratory when compared to standard culture methods.[30]

Herrera-Leon et al. (2004) developed a multiplex PCR assays which could distinguish first-phase flagellar antigens commonly expressed by *Salmonella* strains.[38] Echeita et al. (2002) described a multiplex-PCR specific for the second-phase flagellar antigen.[39] Sequencing and characterization of *fliC* showed that short read sequence assays could be used to identify *fliC* alleles in approximately 97% of the 50 medically most important *Salmonella*.[40]

To date, many other PCR-based techniques were developed to screen *S. enterica* serovars.[9,10,41,42]

Shanmugasundaram and Radhika (2009) developed simple and multiplex-PCR assays for the detection of *S*. Typhimurium by selective amplification of *fliC*, *fljB*, *iroB*, *invA*, *rfbJ*, STM2755 and STM4497 genes. The results of their study showed that, the multiplex-PCR using *iroB*, *invA*, STM4497 and STM2755 primers promises to be better alternative.[43] Lim et al. (2003) developed a multiplex-PCR using *rfbJ*, *fliC* and *fljB* which proved to be capable of identifying of *S*. Typhimurium specifically and differentiating it from other *Salmonella* serovars in addition to non-*Salmonella* enteric pathogens.[21] Zahraei et al. (2007) used Lim et al. (2003) method with some modifications including the use of *invA* gene primers (ST139-141) as general primers for *Salmonella*. The results of their study showed that detection of *Salmonella* at genus level with universal primers and identification of *S.*

Typhimurium by using specific primers of O4, H:1,2 and H:i antigens can potentially permit to more readily evaluate fecal and other types of samples for the presence of these organism. In their study 22 out of 33 *Salmonella* isolates obtained from bovine diarrheic fecal specimens identified as *S.* Typhimurium by multiplex-PCR.[25] This method seems to be more trustable and widely used in recent years. This method allowed for making a specific identification of O4, H:1,2 and H:i antigens properties, because, only *S.* Typhimurium has the antigenic structure combination of O4, H:1,2 and H:i among all other *Salmonella* serovars.

In another study, 13 *Salmonella* serovars out of 58 were positive for *S.* Typhimurium using the same primers described by Zahraei et al. (2007) in poultry farms of East Azerbayjan.[44] Mirzaei et al. (2010) showed that 9 out of 18 samples obtained from house sparrows in Tehran were positive for *rfbJ*, *fljB*, *invA* and *fliC* genes based on multiplex-PCR assay.[45]

In present study, 52 out of 1870 samples from different regions including North, Northwest and capital city (Tehran) of Iran were positive for *S.* Typhimurium using the method described by Lim et al. (2003) and modified by Zahraei et al. (2007). All isolates were serotyped too, and multiplex-PCR could confirm the results of serotyping successfully.

Salmonella detection has been reported from poultry and free-living birds by other researchers. In a study, the prevalence of *Salmonella* was 6% in house sparrows collected around poultry houses in Tehran.[45] Results of another study carried out with carcasses of 779 wild birds in Great Britain between 1995 and 2003 showed that *S.* Typhimurium was the most predominant serovar in wild birds.[46] Kapperud et al. (1998) showed that serovar Typhimurium has established a reservoir in avian wild life in Norway, and epidemiological and bacteriological evidence indicate that wild birds may transmit the infection to humans and to poultry. These reports indicate that strains of *Salmonella spp.* in wild birds could correlate with strains isolated from domestic animals and chickens.[47]

In conclusion, the detection of *S.* Typhimurium in poultry farms and free living birds showed that measures must be taken on prevention and propagation of *S.* Typhimurium among avians and humans. Involved poultry may prepare the way for the incidence of other diseases which would lead to not only economic losses in poultry industry but also would threaten public health. To develop vaccine against, *S.* Typhimurium might be a possible option. CHROMagar™ Salmonella medium can be used as a choice medium for identification of *Salmonella* in fecal samples in veterinary diagnostic laboratories as an alternative where PCR-based techniques is not readily available for use.

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