Original Article

Distribution of *TEM-1* Gene in *Salmonella enterica* Isolated from Poultry Carcasses in Iran

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Abstract

Salmonella is one of the most important bacteria isolated from poultry and a major source of bacterial infection to human. The aim of present research was to study *TEM*-1 beta-lactamase gene in serovars of *S. enterica subsp. enterica* isolated from poultry carcasses samples in Iran. A total of 600 samples of poultry carcasses were collected and *Salmonella* was isolated from the samples using bacterial culture methods and genomic DNA was extracted using DNA extraction kit. For the final approval of *Salmonella*, identification of *S. enterica* subsp. *enterica*, *S. enterica* serovars and *TEM-1* gene sequence-specific targets (*16S rRNA, fliC, rfbJ, fljB* and *TEM-1* genes) were amplified using the PCR assay. Finally, antimicrobial resistance determination was carried out by standard Bauer-Kirby disk diffusion method. Out of the 600 poultry carcasses samples collected from big cities of Iran, 287 samples were positive for *Salmonella* spp. The results showed that 41.5% of the *Salmonella* spp. were positive for *S. enterica* subsp. *enterica* and the highest frequency of serovars was related to ser. Typhimurium (41.2%). Furthermore, 73.1% of the serovars were positive for *TEM-1* gene. The results of present study showed a high prevalence of *TEM-1* gene in the isolated *Salmonella* from poultry carcasses, also the highest frequency of serovars and *TEM-1* gene significantly relating to S. Typhimurium (51.7%) and *S.* Enteritidis (31.0%). The highest rate of drug resistance and sensitivity were ampicillin (100%) and cefazolin (35%), respectively.

Keywords: poultry carcasses, *Salmonella enterica, TEM-1* gene

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Introduction

Salmonella is one of the most important infectious bacteria in human and animals worldwide (Pellegrini et al., 2015). *Salmonella* was reported as the agent of 450 deaths cases and 23,000 hospitalizations in the United States in 2013 and this pathogen is a significant public health concern (Bett et al., 2013). *Salmonella* is found almost everywhere in the environment, warm-blooded and cold-blooded animals. According to the Centers for Disease Control and Prevention (CDC), World Health Organization and Food and Agriculture Organization of the United Nations (2004), genus *Salmonella* is divided into two species: *Salmonella enteric*a (*S. enteric*a) and *Salmonella bongori* (*S. bongori*) (Lopez et al., 2012). Six subspecies (subsp) *S. enteric*a include 1) *S. enterica* subsp. *houtenae*, 2) *S. enterica* subsp. *Arizonae*, 3) *S. enterica* subsp. *enterica*, 4) *S. enterica* subsp. *Salamae*, 5) *S. enterica* subsp. *indica* and 6) *S. enterica* subsp. *Diarizonae* (Gong et al., 2014). The largest number of reported food-borne is known by *S. enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) in the USA (Doosti et al., 2015[a]; Jahid et al., 2015). *S.* Typhimurium causes economic burden, significant morbidity and mortality in human and animal populations (Vikram et al., 2011). In 2013, *Salmonella* was one of the most clinically important foodborne known in the USA (Jahid et al., 2015) and from 2008 to 2009, *Salmonella* infections were reported in over 46 states in the USA (Vikram et al., 2011). Basically this bacterial species on host range is limited including domestic fowl, livestock, humans, rodents, reptiles, and birds. *S.* Typhimurium infection causes nausea, diarrhea (mostly self-limiting) and vomiting. In addition, the clinical sign is characterized by headache, fever and abdominal pain. The emergence of this infection agent has been reported in developing countries (Hirose et al., 2002; Sharifzadeh et al., 2014).

Some gram-negative bacilli such as *salmonella* and *Escherichia coli* (*E. coli*) strains from particular types produce beta-lactamases. The Enterobacteriaceae family produces beta-lactamases, which are encoded by plasmids. For the first time, *TEM-1* was isolated from *E. coli* from a blood culture (Temonera) in Greece (Medeiros, 1984). One of the first reported beta-lactamases is *TEM-1*, which is encoded by plasmids. Almost 80 variants of the *TEM-1* penicillinases showed activities against extended-spectrum cephalosporins (Mulvey and Boyd, 2009). Today, reports indicate the prevalence of *TEM-1* beta-lactamase in certain parts of the world, suggesting that this type of beta-lactamase is a global problem (Shojapour et al., 2008; Black et al., 2010). The present study was done to investigate the frequency of *TEM-1* gene in serovars of *S. enterica subsp. enterica* isolated from poultry carcasses samples in Iran.

Materials and Methods

Sample collection: Between June 2013 and August 2014, a total of 600 samples of poultry carcasses from suspected *Salmonella* infection cases were collected. Samples were collected carefully from poultry farms of nine provinces (Kohgiluyeh Va Boyer-Ahmad *n* = 65, Tehran *n* = 90, Esfahan *n* = 75, Shiraz *n* = 30, Khuzestan *n* = 175, Yazd *n* = 50, Chaharmahal Va Bakhtiari *n* = 40, Qom *n* = 35 and Mashhad *n* = 40) in Iran and transferred to Biotechnology Research Center, Islamic Azad University.

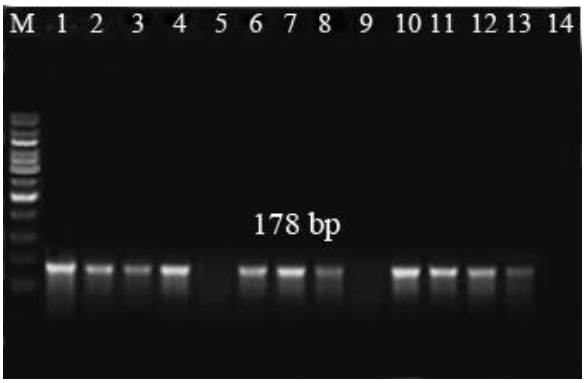

Figure 1 Gel electrophoresis for detection of *16S rRNA* gene from *Salmonella* spp. Lane M is 100 bp DNA ladder (Fermentas, Germany); lanes 1-4, 6-8 and 10-13 are positive samples; lanes 5 and 9 are negative samples; and lane 14 is negative control.

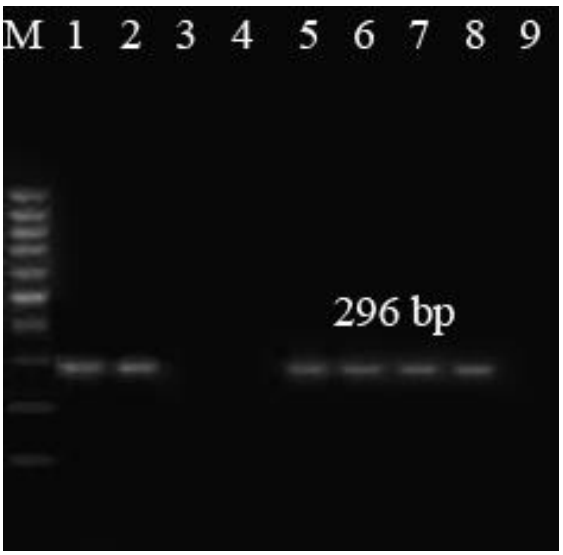

Figure 2 Gel electrophoresis for detection of *TEM-1* gene from *S. enterica* serovars. Lane M is 100 bp DNA ladder (Fermentas, Germany); lanes 1, 2 and 5-8 are positive samples; lanes 3 and 4 are negative samples; and lane 9 is negative control.

Salmonella culture: Initially, after removing the skin and before crushing, the surface of the carcasses were rinsed with distilled water. Then, twenty-gram meat of each carcass by separate scalpels was cut and was homogenized for 1 min in 225 ml. of buffered peptone water (BPW) in sterile conditions. Following overnight incubation at 37°C, 0.1 ml of the obtained suspension was inoculated in duplicate into tubes containing 10 ml Rappaport-Vassiliadis (RV) broth and incubated for 48 h at 42°C. Each of the RV broths was cultured on Brilliant Green Agar plates and incubated for 18-24 h at 37°C. Suspected colonies were confirmed by biochemical methods by inoculating into Lysine Decarboxylase Broth, Urea Broth and Triple Sugar Iron Agar and final confirmation was carried out using

specific *Salmonella* O and H agglutinating antisera (Whyte et al., 2002).

DNA Extraction: Genomic DNA for the PCR assay was extracted from bacterial colonies using DNP™ Kit (CinnaGen Co, Iran) according to the manufacturer's instructions. The quality of extracted DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm according to the method described by Sambrook and Russell (2001).

PCR assay for diagnosis of salmonella: PCR technique was used for definitive identification of *Salmonella*. Oligonucleotide primers for specific detection of *16S rRNA* gene of *Salmonella* spp. are described in Table 1, and were used to amplify 178 bp DNA fragment on agarose gel (Fig 1). All used primers in the present study were designed using Gene Runner software (Version 3.01). PCR was performed in final volume of 25 µl PCR reactions containing 50-100 ng of genomic DNA template, 5 µL of 10X PCR buffer, 1.5 mM MgCl2, 200µM dNTPS, 40 ng of each primer and one unit of Taq DNA polymerase enzyme. Thermal PCR conditions consisted of initial 5 min at 95°C and then 32 cycles of denaturing temperature at 94°C for 1 min, annealing temperature at 62°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min at a thermal cycler. The primers sequences for detection of *S. enterica* subsp. *enterica* and different serovars of subsp. *enterica* are described in Table 1.

Detection of TEM-1 gene: Specific oligonucleotides for detection of *TEM-1* gene of subsp. *enterica* are described in Table 1, and were used to amplify 296 bp DNA fragment on agarose gel (Fig 2). The reaction was carried out as before with annealing temperature of 59°C.

The PCR products were detected in 1.5% ethidium bromide (EtBr)-stained agarose Gel Electrophoresis (AGE). Constant voltage of 80 V for 30 min was used for PCR product separation. A 100 bp DNA ladder was used to determine the amplicon sizes as a size marker. The gels viewed on UV transilluminator and photographed were obtained in UVI doc gel documentation systems. Then, the 10 PCR-amplified products were extracted from agarose gel using a DNA extraction gel kit according to the manufacturer's protocol and finally were subjected to DNA sequencing and sequence similarity was checked using nucleotide BLAST analysis at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.

Antimicrobial susceptibility test: Antimicrobial susceptibility from 87 *TEM-1* positive samples was determined using the standard Kirby-Bauer disk diffusion method according to the guidelines of Clinical and Laboratory Standard Institute (CLSI). Antimicrobial agents tested were cefazolin (30 µg), amoxicillin (20 µg), ampicillin (10 µg) and ticarcillin (10 µg). These antimicrobial agents were from the family of the beta-lactam antibiotics and were chosen on the basis of their importance in treating human or animal salmonellose. Susceptibility of the isolates to each antimicrobial agent was measured and results were interpreted in accordance with interpretive criteria provided by CLSI (2014).

Statistical analysis: All data were analyzed by MS Excel 2013 and SPSS software, and the p value was calculated using Chi-square and Fisher's exact tests to find any significant relationship. A p value of <0.05 was considered statistically significant.

Table 1 Profile of oligonucleotide primers used in the present study

Name	Sequence length (bp)	Annealing	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
16S rRNA	178	62	CGGGGAGGAAGGTGTTGTG	GAGCCCGGGGATTTCACATC
***S. enterica* subsp.**	214	63	TGCTATTTTGCCCTGTACACTGC	TTCGGGGGAGACTATACCTACAG
fliC	183	64	ATAGCCATCTTTACCAGTTCCCCC	GCTGCAACTGTTACAGGATATGCC
rfbJ	663	65	CCAGCACCAGTTCCAACTTGATAC	GGCTTCCGGCTTTATTGGTAAGCA
fljB	526	65	ACGAATGGTACGGCTTCTGTAACC	TACCGTCGATAGTAACGACTTCGG
TEM-1	296	59	TCCGCTCATGAGACAATAACC	ATAATACCGCACCACATAGCAG

Results

The results of bacterial culture and PCR assay showed that 287 of 600 (47.8%) samples were positive for *Salmonella* spp. In addition, it was found that 119 of 287 (41.5%) *Salmonella* spp. were positive for *S. enterica* subsp. *enterica*. To differentiate between *Salmonella* serovars presence of *fliC*, *rfbj* and *fljb* genes was determined (Table 2). Positive samples were isolated from all 9 study areas of Iran (Table 3). The highest frequency of serovars was related to ser. Typhimurium (41.2%) and ser. Enteritidis (26.9%) and the lowest frequency was related to ser. Landau (2.5%) and ser. Newport (3.4%).

Only 87 out of 119 samples (73.1%) were positive for *TEM-1* gene (ser. Landau $n = 0$, ser. Saintpaul $n = 2$, ser. Agona $n = 2$, ser. Enteritidis $n = 27$, ser. Kentucky $n = 2$, ser. Newport $n = 1$, ser. Typhimurium $n = 45$, and other ser. $n = 8$). The highest rate of *TEM-1* gene was related to ser. Typhimurium (51.7%) and ser. Enteritidis (31.0%) and the lowest rate was related to ser. Landau (0.0%) and ser. Newport (1.1%), respectively.

The resistance pattern of isolates to 4 antimicrobial agents tested in this study is shown in Figure 3. The highest rate of drug resistance and sensitivity were ampicillin (100%) and cefazolin (35%), respectively.

Table 2 Diagnosis patterns of different serovars of *S. enterica* subsp. *enterica*

Serovars	PCR results		
	fliC	*rfbJ*	*fljB*
Ser. Typhimurium	+	+	+
Ser. Landau	+	-	+
Ser. Saintpaul	-	+	+
Ser. Agona	-	+	-
Ser. Enteritidis	-	-	-
Ser. Kentucky	+	-	-
Ser. Newport	-	-	+

Table 3 Distribution of *S. enterica* serovars in different provinces of study area

Salmonella serovars	Province									
	Tehran	Esfahan	Shiraz	Khuzestan	Yazd	Chaharmahal Va Bakhtiari	Kohgiluyeh va Boyer-Ahmad	Qom	Mashhad	Total
Ser. Landau	0	1	0	1	0	1	0	0	0	3
Ser. Saintpaul	0	1	1	2	0	0	0	1	0	5
Ser. Agona	2	2	0	1	0	0	0	1	1	7
Ser. Enteritidis	8	3	2	4	3	3	5	1	3	32
Ser. Kentucky	1	1	0	0	0	1	1	1	0	5
Ser. Newport	1	1	0	2	0	0	0	0	0	4
Ser. Typhimurium	10	3	5	8	6	3	4	4	6	49
Other Ser.	2	2	0	3	1	2	2	1	1	14
Total	23	14	8	21	10	10	12	9	12	119

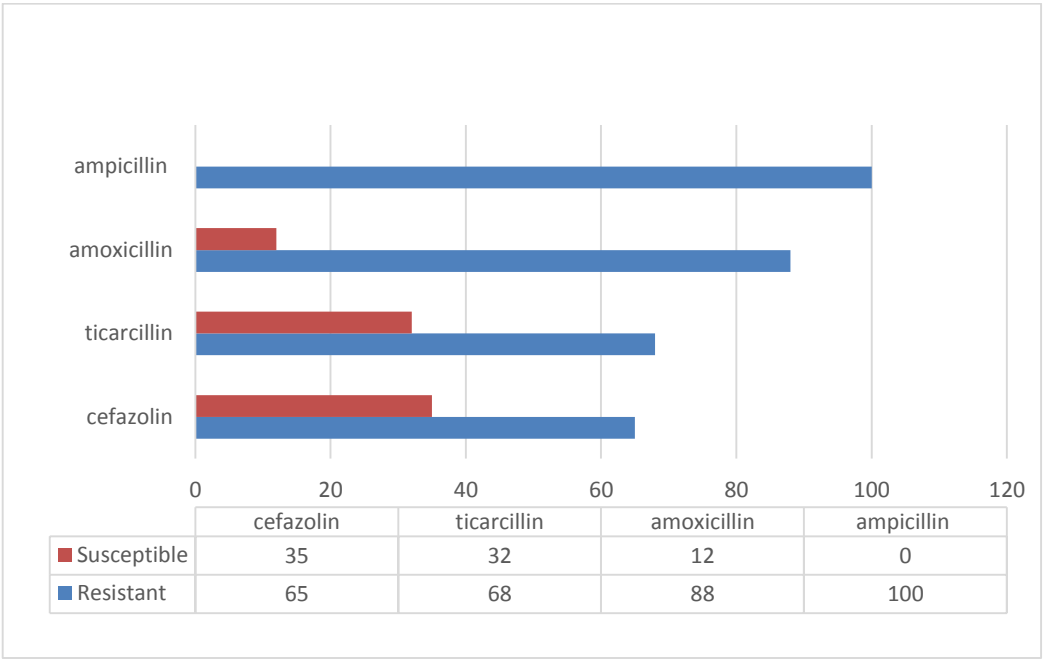

Figure 3 Percentages of antimicrobial resistance detected among *TEM-1* positive samples

Discussion

Salmonella is an important pathogenic bacterium predominantly found in animals and human. *Salmonella* is known with more than 2500 serovars of *enterica* subsp worldwide (Torpdahl et al., 2013). Salmonellosis has been reported as one of the most important diseases in the USA (Narayanan and Edelmann, 2014). A complete genome sequence of this bacterium on chromosome is 4,857 kbp (Mcclelland et al., 2001). Development of antibiotic resistance in *S.* Typhimurium has been considered as a public health problem throughout the world and recent reports have shown the association of *Salmonella* in the development of human and animal illness. This present study observed relatively high prevalence of *Salmonella* in egg shells, and also a high prevalence of *S. enterica* subsp. *enterica*, and Typhimurium and Enteritidis serovars. There are some studies that have examined *Salmonella* infection in poultry and the prevalence of antibiotic resistance genes in this bacterium. The study of El-Aziz (2013) showed a high prevalence of *S.* Typhimurium in retail chicken meat and chicken giblets and indicated that most infection was related to

chicken liver, meat and heart. In the investigation of Gong et al. (2014) in China between 2006 and 2012, a total of 323 samples of *Salmonella* were isolated from 51 poultry farms in seven regions of 12 provinces. They reported that the infection rate was about 10% in chicken samples; this reported rate is very low compared to the result of our study and other studies. The study of Mohamed et al. (2014) from July 2004 to June 2005 in the USA reported a high relative prevalence of *Salmonella* infection (45.2%) in broiler carcasses samples, which is similar to the present study's results.

One of the first beta-lactamases is *TEM-1*, which is plasmid encoded. In addition to *Salmonella*, other bacteria such as *Vibrio cholerae*, *Pseudomonas aeruginosa* and *Neisseria* also have the ability to produce this enzyme. Many reports indicated that the prevalence of *TEM-1* beta-lactamase is a global problem, especially in many parts of the world (Shebani et al., 2010; Zamanzad et al., 2008). A new study of Doosti et al. (2015[b]) reported a prevalence rate of 62% for *TEM-1* gene and a high drug resistance in *Klebsiella pneumoniae* isolated from cockroaches from hospitals which is somewhat similar to the present study's results.

Since very few studies have been done on the prevalence of *TEM-1* gene in *S. enterica* serovars, this study cannot conclude the rate of frequency of this gene and also drug resistance in this bacterium. However, in comparison to other studies, the frequency of *TEM-1* gene and also drug resistance in *S.* Typhimurium and *S.* Enteritidis isolated from poultry carcasses in the present study was high. Furthermore, the results of the present study showed that PCR as a highly sensitive and specific method could be used for the diagnosis of different serovars of *Salmonella* subsp. The present study showed the high prevalence of *TEM-1* gene in isolated *Salmonella* from poultry carcasses, therefore it seems that control and eradication programs are necessary to prevent and reduce its negative effects on health and economy. Moreover, it is essential to screen all regions regularly to prevent the spread of this pathogen.

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บทคัดย่อ

การกระจายตัวของยีน TEM-1 ของเชื้อ *Salmonella enterica* ที่แยกได้จากซากไก่ในประเทศอิหร่าน

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Salmonella เป็นเชื้อแบคทีเรียชนิดหนึ่งที่มีความสำคัญมากที่สุดที่แยกได้จากไก่ และเป็นแหล่งหลักของการติดเชื้อแบคทีเรียในคน วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษายีน TEM-1 beta-lactamase ใน serovars ของ S. enterica subsp. enterica ที่แยกได้จากตัวอย่างซากไกในประเทศอิหร่าน ตัวอย่างซากไก่ทั้งสิ้นจำนวน 600 ตัวอย่างได้ถูกเก็บ และเชื้อ Salmonella ได้ถูกแยกจากตัวอย่างโดยใช้การเพาะเชื้อแบคทีเรีย genomic DNA ได้ถูกสกัดโดยใช้ชุดสกัด สำหรับการยืนยันเชื้อ Salmonella การจำแนกชนิดของ S. enterica subsp. enterica, S. enterica serovars และเป้าหมายของลำดับยีน TEM-1 ที่จำเพาะ (16S rRNA, ยีน fliC, rfbJ, fljB **และ** *TEM-1) ได้ถูกเพิ่มจำนวนโดยใช้เทคนิค PCR ในที่สุด การดื้อยาได้ถูกวัดด้วยวิธีมาตรฐาน Bauer-Kirby disk diffusion จากตัวอย่างซากไก่จำนวน 600 ตัวอย่าง ที่เก็บได้จากเมืองใหญ่ของประเทศอิหร่าน พบว่า 287 ตัวอย่างให้ผลบวกต่อ Salmonella spp. พบว่า 41.5% ของ Salmonella spp. ให้ผลบวกต่อ S. enterica subsp. enterica และความถี่สูงสุดของ serovars มีความสัมพันธ์กับ ser. Typhimurium (41.2%) นอกจากนั้น 73.1% ของ serovars ให้ผลบวกต่อยีน TEM-1 การศึกษานี้ได้แสดงให้เห็นว่ามีความชุกของยีน TEM-1 สูงสุดจากเชื้อ Salmonella ที่แยกได้จากตัวอย่างซากไก่ และความถี่สูงสุดของ serovars และยีน TEM-1 มีความสัมพันธ์อย่างมีนัยสำคัญกับ S. Typhimurium (51.7%) และ S. Enteritidis (31.0%) อัตราการดื้อยาและความไวสูงสุด คือ ampicillin (100%) และ cefazolin (35%)*

คำสำคัญ: ซากไก่ *Salmonella enterica*, ยีน *TEM-1*

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