



Phylogenic Relationship of Salmonella Serovars Isolated from Different Foodstuffs in Isfahan

Original Article

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ABSTRACT

Background and objectives: are divided into two species: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* has more than 2,500 serotypes. Serovars of *S. enterica* such as Typhimurium, Enteritidis, Paratyphi B, Paratyphi A and Newport are associated with human infections. Approximately 75% of human *Salmonella* infections have been associated with contaminated food such as eggs, chicken, beef, pork, dairy products, fruits and vegetables. The aim of this study was to determine the frequency of *Salmonella* strains isolated from various food sources in Isfahan, Iran.

Methods: Forty *Salmonella* strains were isolated from 450 suspected cases referred to the veterinary reference laboratory of Isfahan Province. The isolates were identified by differential and serotyping tests and then confirmed by PCR. A phylogenic tree was constructed with 34 sequences by neighbor-joining method using the MEGA7 software (version 7.1).

Results: Overall, 10 *Salmonella* serovars were isolated from 32 chicken meat, three beef and five egg shell samples. *S. enterica* serovar Ouakum (20%), *S. Enteritidis* (17.5%) and *S. Typhimurium* (17.5%) were the most common serovars, while *S. enterica* serovar Nitra (2.5%) was found as the least prevalent isolate.

Conclusion: In this study, *S. Typhimurium* species is placed in different clusters along with sequences reported from different parts of the world, indicating that the serovars are circulating all over the world.

Keywords: *Salmonella enterica*, Phylogenic tree, MEGA7 Software.

INTRODUCTION

Foodborne diseases are one of the most important health problems in the world, which are associated with a substantial economic and health burden (1). An important issue in food production is the growth of production proportional to supply and demand, and this supply must be hygienic and of high quality.

Food contamination can be caused by several agents including presence of bacteria and their byproducts in raw food materials or food products, which leads to culling. The presence of pathogenic bacteria such as *Salmonella*, *Escherichia coli*, *Campylobacter*, *Yersinia*, *Vibrio*, etc. in food products can have devastating effects on the health and economy of countries, which can be largely prevented by continuous surveillance (2). Salmonellosis is a zoonotic disease and one of the most common foodborne infections in the world. Each year, more than 1.4 million cases of *non-typhoid Salmonella* infection are reported worldwide (3). In the European Union, 52.3% and 23.3% of all salmonellosis cases are caused by *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium, respectively. *S. enterica* serovar Infantis has been also reported from Europe. According to the European Food Safety Authority, the overall economic burden of these infections in humans could be as high as 3 billion Euros per year (4). The clinical symptoms of human salmonellosis include subclinical gastroenteritis, severe bacteremia, fever, meningitis and other forms of extraintestinal infections (5). Classification of *Salmonella* species is difficult due to its high diversity. Today, more than 2,500 *Salmonella* serotypes have been identified that are categorized into groups A, B, C and D based on their virulence factors (6-8). *S. typhimurium*, *S. enterica* serovar Newport, *S. enterica* serovar Heidelberg and *S. enterica* serovar Javiana are associated with human infections in the US (9). *S. enterica* serovar Typhi is capable of surviving in harsh conditions and the human digestive tract. Biofilms formed by *Salmonella* in gallbladder can lead to chronic symptomless infection (typhoid carriers), which can evade the immune system. These bacteria can survive in cold environment (refrigerators)

but are sensitive to dryness, heat and disinfectants (10). Food associated to salmonellosis includes chicken meat, pork, beef, eggs, dairy products, seafood, parsley, coriander, broccoli, cauliflower, lettuce, spinach, etc. Contact with contaminated surfaces such as furniture, appliances and equipments is the main cause of cross contamination of chicken meat, beef and pork. Contamination in pre-prepared products made from meat and vegetables can also pose as a health threat (9). *S. Enteritidis* and *S. Typhimurium* that are considered as the main causes of human infections are commonly isolated from poultry, which is a major potential vehicle of *Salmonella enterica* serovar Kentucky ST198 clone. Therefore, presence of any new zoonotic serovar of *Salmonella* in poultry should be considered as a local and global public health threat (11).

This study aimed to determine of the frequency of *Salmonella* serovars from different sources and also to determine the phylogenetic correlation of these serovars referred to the veterinary reference laboratory of Isfahan Province, Iran.

MATERIALS AND METHODS

From June 2016 to June 2017, a total of 40 *Salmonella* strains were isolated from 450 suspected *Salmonella* spp. cases that were referred to the *veterinary reference laboratory* of Isfahan Province, Iran. Swabs samples pre-soaked in sterile peptone broth were used to swab the egg shell surface. The swab samples were inoculated into peptone broth (Merck, Germany) and incubated for 18 h at 37 °C (12). Twenty-five grams of beef or chicken meat were homogenized and mixed with 250 ml of buffered peptone water (Merck, Germany). After incubation for 18 h at 37 °C, 0.1 ml of the pre-enriched cultures were inoculated into 10 ml of selenite F-broth (Merck, Germany) (13). After incubation at 37 °C for 18 h, the samples were subcultured on *Salmonella-Shigella* agar (Merck, Germany) and *Xylose Lysine Deoxycholate* agar (Biolife, USA). After incubation at 37 °C for 24 h, colonies with typical morphology of *Salmonella* were subjected to differential tests including MR-VP, TSI, SIM and Simon-citrate (Biolife, USA)(14).

Agglutination test was performed with Baharafshan kit (Tehran, Iran) to detect serogroups A-D based on presence of O and H

antigens according to the manufacturer's guidelines.

The standard *S. Typhimurium* ATCC 14028 and the isolated strains were grown in Brain Heart Infusion (BHI) broth (Merck, Germany) and incubated at 37 °C overnight. The bacteria were then subcultured on BHI agar. After overnight incubation at 37 °C, single colonies were collected and whole genomic DNA was extracted by the boiling method. The *invA* gene sequence was amplified by PCR using *invA* gene primers (forward: 5'-GTGAAATTATCGCCACGTTCGGGCAA-3', reverse: 5'-CATCGCACCGTCAAAGGAACC-3')

(Sinaclon Iran) as described previously (15). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, denaturation at 94 °C for one min, annealing at 61 °C for one min, 30 cycles of extension at 72 °C for one min and final extension at 72 °C for 10 min. The purified PCR products (Figure 1) were sequenced by the Sanger dideoxy method (Seqlab, Germany). The obtained sequences were confirmed by alignment for *invA* gene using the DNA Databank of Japan. A nucleotide BLAST search of the sequences against the National Center of Biotechnology Information (NCBI) nucleotide sequence database was performed. Using neighbor-joining method, a phylogenetic tree with 1,000 bootstraps was constructed in MEGA7 (Molecular Evolutionary Genetic Analysis Software, version 7.1).

RESULTS

The *Salmonella* serovars isolated from different food sources in Isfahan are listed in table 1. The frequency of isolates in beef, chicken and egg samples was 9.7%, 8.5% and 8%, respectively. Moreover, *S. Ouakam* and *S. Enteritidis* were the most prevalent isolates from chicken (22.5%), while *S. Newport* was isolated only from beef.

Overall, 10 *Salmonella* serotypes were identified. In the NCBI's BLAST search, 97-99% sequence identities were observed with sequences of *Salmonella* serovars reported from other countries. The phylogenetic tree was constructed using 34 sequences of *invA* genes obtained in this study and 15 sequences from studies in the US, China, Switzerland, Egypt, United Kingdom, Taiwan, South Africa, Republic of Korea and Canada. *Yersinia enterocolitica* strain AF542975.1 was used as an outgroup.

In the phylogenetic construct (Figure 2), our sequences for *S. Newport* isolated from beef were placed in the same cluster with already reported sequences from USA. All *S. Typhimurium* isolates were placed in four separate clusters. Sequences reported from Korea and China along with our sequence of *S. Typhimurium* SS4 were placed in one cluster. However, *S. Typhimurium* SS1 (obtained from our study) was placed in another cluster along with sequences from the USA and Switzerland. *S. Typhimurium* SOA1 was placed in a cluster with previously reported sequences from Australia, the United Kingdom, Taiwan and the USA. Two of our sequences related to *S. Typhimurium* SS11 and *S. Typhimurium* SS22 along with a sequence from USA were placed in the fourth cluster. All *S. enterica* serovar Anatum sequences were placed in the same cluster, which were similar to another cluster containing sequences reported from the USA. Our *S. Ouakam* sequences were placed in two distant clusters that each was along with sequences reported from China or the USA. Our only *S. Nitra* sequence was placed in a single cluster along with a sequence reported from Canada. *Salmonella enterica* Egy reported from Egypt was placed in a separate cluster.

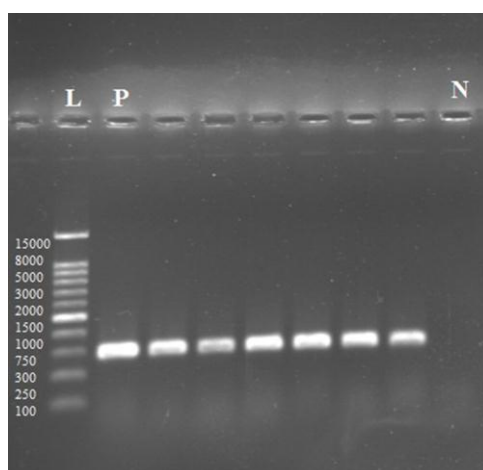


Figure 1. Electrophoresis results of *invA* gene PCR. L: 100bp DNA Ladder, P: positive control, N: negative control.

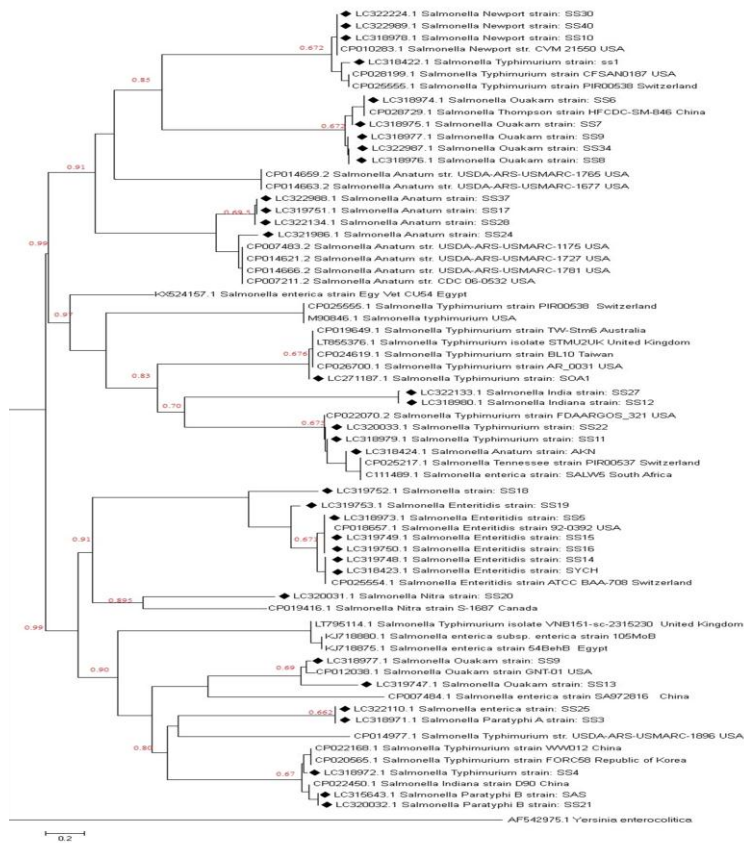


Figure 2. Phylogenetic tree of various *Salmonella* serovars from different parts of the world. ♦ indicates *Salmonella* strains isolated in this study.

Table 1. Frequency of *Salmonella* serovars isolated from different sources

Source	Number	%	Serovar/	serogroup	Number of isolates	%
Broiler chicken	32	79.5	<i>Salmonella</i> Enteritidis	D	7	21.87
			<i>Salmonella</i> Ouakam		7	
			<i>Salmonella</i> India	*	5	21.87
			<i>Salmonella</i> Anatum		5	
			<i>Salmonella</i> Paratyphi B	*	2	15.62
			<i>Salmonella</i> Paratyphi A		1	
			<i>Salmonella</i> Typhimurium	*	3	15.62
			<i>Salmonella</i> Enterica		1	
			<i>Salmonella</i> Nitra	B	1	6.25
				A		
				B		3.125
				*		9.375
Egg	5	12.8	<i>Salmonella</i> Typhimurium	B	4	80
			<i>Salmonella</i> Ouakam		1	
				*		20
Beef	3	7.7	<i>Salmonella</i> Newport	C	3	100

* Not detected

DISCUSSION

The contamination of foodstuff with *Salmonella* spp. and their transfer to humans are global health concerns (16). The marked increase in global consumption of poultry may subsequently increase the risk of poultry-associated foodborne diseases (17). In Canada, *S. enterica* serovar Hadar, *Salmonella* Kentucky and *S. enterica* serovar Heidelberg were the most prevalent serotypes in chicken meat (18), while in Vietnam, *Salmonella* Anatum followed by *S. enterica* serovar Infantis and *S. enterica* serovar Emek were the most prevalent isolates (19). Moreover, *S. Typhimurium* was reported as the most prevalent (10%) serotype in Iraq (20). These findings show that various *Salmonella* serotypes are present in foodstuff from different parts of the world. In the present study, 8.5% of chicken meat samples were contaminated with *Salmonella* spp., mainly with *S. Ouakam* or *S. Enteritidis*. Previous studies in Poland (4) and the USA (18) also reported a high rate of *S. Enteritidis* and *S. Ouakam* contamination (21). In a study in Brazil, only 12% of the isolates were *S. Enteritidis* (22). In a study in Egypt on 450 cloacal, 400 chicken egg and 250 chicken meat samples, the highest rate of contamination (2% with *S. Enteritidis*) was related to chicken meat (23). The high rate of *Salmonella* contamination in chicken meat samples could be associated with breeding, transport, slaughtering and packaging stages. Due to the high risk of human infection, culling of the contaminated flocks and sophisticated sanitary procedures are recommended. Another source of infection with *Salmonella* spp. is contaminated eggs. The prevalence of egg contamination varies in different geographical locations. In the present study, 8% of the tested eggs were contaminated with *Salmonella* spp. [*S. Typhimurium* (80%) and *S. Ouakam* (20%)].

In India, the rate of egg contamination with *S. Typhimurium* (24) and *S. Enteritidis* (25) was reported to be 5% and 10%, respectively. However, a study in South Korea found no *Salmonella* contamination among 135 chicken eggs (26). Egg shells can be contaminated with *Salmonella* spp. during laying by contact with stool or other contaminated eggs during collection (27). Indeed, implementing proper procedures to prevent contamination of eggs during these processes could reduce the risk of

Salmonella infections in humans.

S. enterica serovar Dublin, *S. Enteritidis*, *S. Typhimurium* and *S. Newport* are the most prevalent *Salmonella* spp. isolated from cows and calves (28, 29). In the present study, of 31 beef samples, three cases (9.7%) were contaminated with *S. Newport*. In a study in Denmark on 2,985 retail pork and chicken meat samples, *S. Newport* was isolated only from two samples (30). In Mexico City, except for *S. Typhimurium* and *S. Anatum*, 14.7% of chicken, pork and beef ground meat were contaminated with *S. Newport* (31). Studies in Canada, England and China reported other *Salmonella* spp. including *S. Enteritidis*, *S. enterica* serovar Indiana, *S. Heidelberg* and *S. Typhimurium* as the major contaminants of beef, chicken meat and retail meats (32-34). Inconsistent with our study, the mentioned studies found no *S. Newport* contamination. The high rate of *S. Newport* contamination in our study might be due to the fact that we tested already suspected samples delivered to the veterinary laboratory.

Based on the results, *S. Ouakam*, *S. Enteritidis* and *S. Typhimurium* were the most prevalent serovars of *Salmonella* in the foodstuff samples from Isfahan during the study period. Given the diversity of *Salmonella* species, morphological and biochemical tests cannot identify every species; hence, molecular techniques are employed for a more specific identification of these species. In this study, 40 sequences from the *invA* gene of *Salmonella* spp. isolated from different food sources were compared to previously reported sequences by phylogenetic analysis. This showed that *S. Ouakam* species circulating in the region shares the same ancestor with the ones reported from the USA. This may be due to importation of animal food ingredients from the US, migration of wild birds (35), etc.

CONCLUSION

In this study, *S. Typhimurium* species is placed in different clusters along with sequences reported from different parts of the world, indicating that the serovars are circulating all over the world.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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