



Molecular Detection of *Salmonella enterica* Serovar Typhimurium in Ready-to-Eat Vegetable Salads Consumed in Restaurants of Tabriz, North-West of Iran

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HIGHLIGHTS

- *Salmonella enterica* serovar Typhimurium was found in 4.44% of the ready-to-eat vegetable salads in Tabriz, Iran.
- There was no significant difference between contamination rate and various areas.
- Vegetable salads consumed in Tabriz may have health risk for consumers.

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Acronyms and abbreviations

REVS=Ready-to-Eat Vegetable Salad
PCR=Polymerase Chain Reaction

ABSTRACT

Background: In recent years, food-borne outbreaks have been increased by consumption of raw fruits and vegetables contaminated with bacterial pathogens like *Salmonella* spp. in many countries. This study was designed in order to molecular detection of *Salmonella* in Ready-to-Eat Vegetable Salad (REVS) consumed in restaurants of Tabriz, North-West of Iran.

Methods: In this cross-sectional study, 90 REVS samples were randomly collected from five different areas of the Tabriz, Iran from February to June 2016. The presence of *S. enterica* serovar Typhimurium isolates was assessed using real-time polymerase chain reaction technique. Data analysis was performed using SPSS software version 19.0.

Results: Out of 90 REVS samples, 4 (4.44%) were found to be positive with *S. enterica* serovar Typhimurium. There was no significant difference between *S. enterica* serovar Typhimurium prevalence in various areas ($p>0.05$).

Conclusion: This survey showed that REVS consumed in restaurants of Tabriz, Iran may have public health risk in terms of presence of *S. enterica* serovar Typhimurium; so, it is required to improve the food safety standards in this area. Next investigations should be done to find the antimicrobial susceptibility of the identified isolates.

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Introduction

In recent years, food-borne outbreaks have been increased by consumption of raw fruits and vegetables contaminated with bacterial pathogens like *Salmonella* spp. in many countries (Khalil et al., 2015; Kochakkhani

et al., 2017; Kotzekidou, 2013). To date, more than 2500 *Salmonella* serotypes have been identified which half of them belongs to *Salmonella enterica* serovar Typhimurium. The main sources of transmission of *Sal-*

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monella spp. are poultry, ready-to-eat products, dairy products, fruits, and vegetables (Caponigro et al., 2010; Hur et al., 2012; Keithlin et al., 2015). Several *Salmonella* outbreaks have been reported which are related to the consumption of vegetables. For instance, in Brazil from 2000 to 2011, about 1.33% of outbreaks have been reported to be associated with the consumption of leafy green vegetables (Brandão et al., 2014). It has also been stated that in England and Wales from 1992 to 2006, 4% of infectious intestinal diseases outbreaks were related with the consumption of prepared salads (Little and Gillespie, 2008).

Isolation and identification of bacteria by biochemical standard methods are laborious and time-consuming. However, Polymerase Chain Reaction (PCR)-based techniques are high specificity and sensitivity approach in order to detection and identification of food-borne pathogenic agents (Kochakkhani et al., 2016; Kotzekidou, 2013). There is limited information about incidence of *Salmonella* in Ready-to-Eat Vegetable Salad (REVS) consumed in Iran especially in Tabriz city. Thus, this study was designed in order to molecular detection of *Salmonella* spp. in REVS consumed in restaurants of Tabriz, North-West of Iran.

Materials and methods

Study area

This study was conducted in Tabriz, North-West of Iran which is the fifth largest city of the country with population of about 1.5 million. The geographical location of Tabriz is schematically illustrated in Figure 1.

Sampling

In this cross-sectional study, 90 REVS samples were randomly collected from five different areas of the city, including North (n=15), South (n=24), West (n=15), East (n=21), and Center (n=15) from February to June 2016. The samples consisted of mixed salads containing raw vegetables such as lettuce, cabbage, tomato, cucumber, and carrot. The average weight of each REVS sample was about 100 g at the time of purchase. Samples were placed in sterile bags and were transported to the Food Microbiology Laboratory of Faculty of Veterinary Medicine, Tabriz University and stored at 4 °C until next analysis.

Sample preparation

From each REVS sample, 50 g of representative section was transferred into a sterile plastic pouch, containing 450 µl of 0.1% sterile peptone water homogenized

for 60 s using a pulsifier (Microgen, UK) at room temperature. The solution was centrifuged for 5 min at 8000 rpm in sterile tube. After that, the pellet was washed three times with 0.1% sterile peptone water and the centrifugation was repeated. Peptone water was then added to the precipitate in the last stage and stored at -20 °C until used for DNA extraction (Kochakkhani et al., 2016).

DNA extraction

DNA extraction was carried out using Accuprep Genomic DNA Kit (Bioneer No.k-3032, Daejeon, South Korea) according to the manufacturer's instruction with some modifications. Briefly, 500 µl of each REVS sample was mixed by 200 µl of tissue lysis buffer in a sterile microtube. Then, 30 µl proteinase K solution (20 mg/ml; Thermo Fisher Scientific, USA) was added, mixed, and incubated at 60 °C for 1 h. Then, 200 µl binding buffer was added, mixed, and incubated at 60 °C for 10 min, and then 100 µl isopropanol was added. The solution was transferred into a new sterile 1.5 ml microtube containing silica filter. Centrifuge was done at 12000 rpm for 2 min and the flow through was discarded. Each sample was washed two times with ethanol 70 and 95%, respectively. Each microtube was air dried by centrifugation at 13000 rpm for 2 min. Binded DNA was eluted by 100 µl Tris-EDTA buffer. All extracted DNA were stored at -20 °C until next step. The quality and quantity of the extracted DNA were analyzed by agarose gel electrophoresis (Akhtarian, Iran) and Bio photometer (Eppendorf, Biophotometer plus, Germany), respectively.

SYBR Green I real-time PCR assay

SYBR Green I real-time PCR was performed using Rotor GeneQ- 5 PLEX, (QIAGEN, USA). Each reaction was performed in a final volume of 10 µl contained 5 µl of 2x SYBR Green I master mix (Bioneer, South Korea), 0.5 µl of each primer (end concentration of 200 nM), 1 µl template DNA (100 ng/µl) and 3 µl ddH₂O. The genus specific primer pair named sefA was used in order to detection of *S. enterica* serovar Typhimurium (Table 1). The real-time PCR procedure was carried out under the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 40 s, annealing at 58 °C for 40 s, and elongation at 72 °C for 40 s; and also the final extension at 72 °C for about 10 min.

Genomic DNA of *S. enterica* serovar Typhimurium as control sample was used for preparation of standard curve (Elizaquível et al., 2011). Liquid culture of *S. enterica* serovar Typhimurium SA2380, ATCC35987 was used for DNA extraction and spiking steps. DNA was extracted from a dilution series with concen-

tration ranging from 10^2 to 10^5 CFU/ml in 10 fold dilutions of bacterial suspension, grown in tryptic soy broth. A non-spiked sample was used as negative control (Figure 2). Real-time PCR and analysis were performed as reported by Elizaguível et al (2011).

Statistical analysis

The analysis of Chi square test was used to determine the statistical differences between the areas of *S. enterica* serovar Typhimurium vegetable salads samples using SPSS Inc., Chicago, IL (v. 19.0). A probability level of

$p < 0.05$ was considered as statistically significant.

Results

Out of 90 REVS samples, 4 (4.44%) were found to be positive with *S. enterica* serovar Typhimurium. As shown in Table 2, there was no significant difference between *S. enterica* serovar Typhimurium prevalence in various areas ($p > 0.05$). Based on the results of quantitative PCR, bacterial loads in contaminated samples ranges from 6.4×10^2 to 9.17×10^4 (Table 3).

Table 1: Primers used for the detection of *Salmonella enterica* serovar Typhimurium

Target gene	Length (bp)	Primer sequence (5'-3')	Product size (bp)	Reference
sefA	20	GCAGCGGTTACTATTGCAGC	131	This Study
	20	TTATGTGGACCAGTAGCAGT		

Table 2: Prevalence of *Salmonella enterica* serovar Typhimurium in vegetable salads sampled from various areas of Tabriz, Iran

Sampling area	No. of samples	No. of positive samples (%)
North	15	2 (13.33)
West	15	0 (0.00)
Central	15	0 (0.00)
East	21	1 (4.76)
South	24	1 (4.16)
Total	90	4 (4.44)

Table 3: Ct values and bacterial load measurements for spiked-control samples (SCS) and some REVS samples

Spiked-control samples and REVS samples	Ct	Bacterial load (CFU/ml)
SCS1	19.69	10^5
SCS2	23.25	10^4
SCS3	26.58	10^3
SCS4	29.26	10^2
REVS1	23.59	9.17×10^4
REVS2	26.48	10.13×10^3
REVS3	27.70	6.51×10^2
REVS4	27.83	6.40×10^2

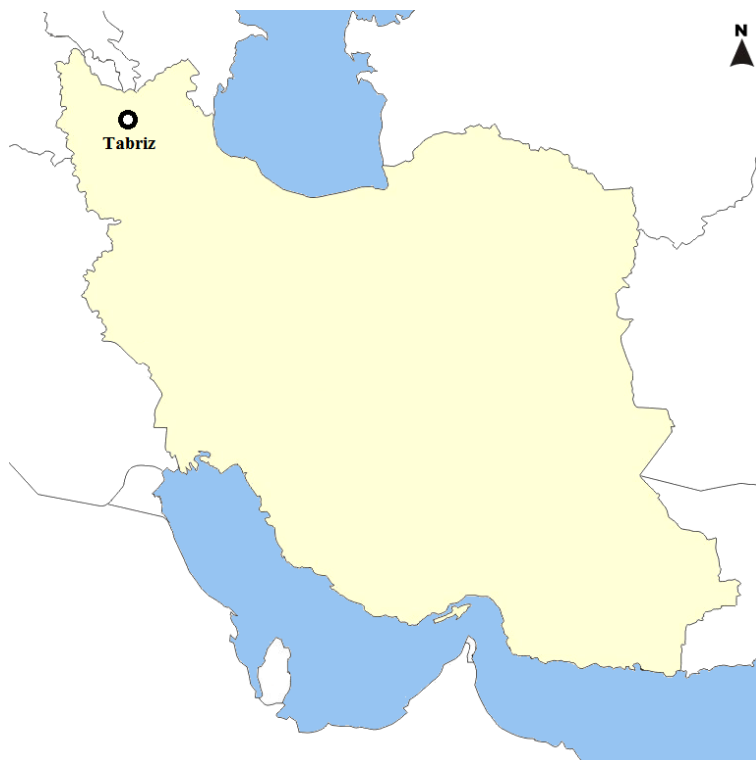


Figure 1: Geographical location of Tabriz city in schematic map of Iran

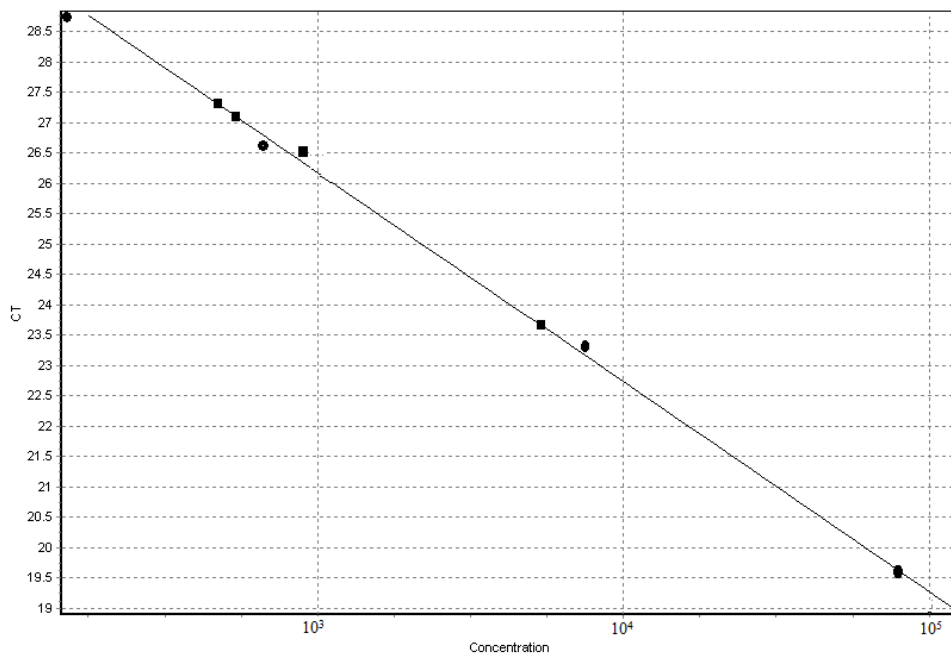


Figure 2: Standard curve for the SYBR green I real-time PCR amplification of *S. enterica* serovar Typhimurium DNA. A plot of Ct value against different concentrations of extracted DNA from spiked-control samples (circles) and REVS samples (squares) are indicated

Discussion

This is the first report on contamination of REVS with *S. enterica* serovar Typhimurium in Tabriz city of Iran. According to the Iran national standards, *Salmonella* must not be detected in 25 g of REVS in order to be acceptable for human consumption (ISIRI, 2009; 2014). This study showed that some of REVS served in restaurants located in Tabriz had unsatisfactory health conditions due to the presence of *S. enterica* serovar Typhimurium. Since REVS receives minimal processing and it is often eaten raw, bacterial contamination can represent serious health risk. Vegetables used in salads could be contaminated with pathogenic bacteria during the production, processing, packaging, and distribution. This contamination can arise from environmental, animal, or human sources (Akoachere et al., 2018; De Giusti et al., 2010; Klingbeil et al., 2016). The contamination rate of *S. enterica* serovar Typhimurium found in this study was similar to the same bacterial contamination in raw vegetable salads obtained from Abidjan, Côte d'Ivoire as 2.6% (Toe et al., 2017). Low contamination rate in raw vegetables have previously been reported in the Czech Republic (0.3%) by Vojkowska et al. (2017) and in Italy (0.75%) by De Giusti et al. (2010). However, Abakari et al. (2018) showed 73.3% contamination rate of *Salmonella* spp. in REVS samples vended in Ghana which was too higher than what we found in our research. Such differences can be due to variation of sanitary conditions during cultivation, harvesting, packaging, distribution, and preparation of the vegetables in the studied regions. It has shown that different hygienic practices may affect the prevalence rate of bacterial contamination in the vegetable samples such as food vendors and also the sources of cultivation (Abakari et al., 2018; Gurler et al., 2015; León et al., 2013). However, we found no significant relation with the *Salmonella* contamination rate and the area from which our vegetables were obtained.

Contamination of REVS by *S. enterica* serovar Typhimurium is of major concern for public health, because this bacterium leads annually millions cases of enteric diseases worldwide (Dunkley et al., 2009). *Salmonella* is mainly originated from mammalian feces; so, it is assumed that untreated waste water may be used in irrigation of cultivating farms in our studied area. Similar inference has been announced by Muinde and Kuria (2005), who stated that bacterial contamination of vegetables is mostly due to using grey water generated from kitchen, bathroom, and laundry during cultivation. However, to prove this hypothesis, it is necessary to do further researches in order to determine the presence of *Escherichia coli*, as a fecal indicator, in the raw vegetables vended in Tabriz.

It is known that the presence of *Salmonella* spp. in food stuff is mainly related to cross-contamination during handling process. Bacterial contamination of the vegetables can be controlled by appropriate hygienic measures such as prevention of cross-contamination. Proper implementation of the Hazard Analysis and Critical Control Point (HACCP) system in the restaurants can help to prevent cross-contamination (Abakari et al., 2018). The presence of *Salmonella* spp. found in the current survey may be attributed to poor hygiene practices in the personnels who work in the restaurants of Tabriz. In this regard, direct bacterial culture sampling from workers hands is recommended in future to find the precise origin of the contamination in REVS.

Conclusion

This survey showed that REVS consumed in restaurants of Tabriz, Iran may have public health risk in terms of presence of *S. enterica* serovar Typhimurium; so, it is required to improve the food safety standards in this area. Next investigations should be done to find the antimicrobial susceptibility of the identified isolates.

Author contributions

P.D. and M.H.M. designed the study; H.K. conducted the experimental work; H.K. and P.D. analyzed the data; K.H., P.D., and M.H.M. wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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