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Investigating the Prevalence, Antibiotic Resistance Pattern, and Serotyping of Shigella Isolates from Traditional and Industrial Olivier Salads

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ABSTRACT

Background: Food-borne diseases are one of the major problems in developing countries. This study aims to investigate the prevalence, antibiotic resistance pattern, and serotyping of *Shigella* isolates from Olivier salad. **Methods:** 150 samples of Olivier salad, including 50 industrial samples from 10 different available brands in Tehran's shops and 100 traditional samples from Tehran's southern regions, were randomly obtained between April to October of 2021. These samples were examined for bacterial isolation and identification, which was finally confirmed by the API-20E kit. Then, a serological reaction was used to confirm *Shigella* and determine the species. The antibiotic resistance pattern of isolates was evaluated according to Clinical and Laboratory Standards Institute (CLSI, 2021) instructions by the Kirby-Bauer method. **Results:** Among these samples, 10 isolates (6.6%) of *Shigella* were isolated, of which 6 (4%) belonged to serogroup D (*Shigella sonnei*), 3 (2%) to serogroup B (*Shigella flexneri*), and 1 isolate (0.66%) belonged to serogroup C (*Shigella boydii*). This study showed that all *Shigella* isolates were related to traditional Olivier salad and were not *Shigella* spp. isolated from the industrial salad. Other bacteria isolated from traditional salad included *Escherichia coli*, *Salmonella*, *Pseudomonas*, *Klebsiella*, and *Citrobacter*. Multidrug resistance (MDR) was not observed in all isolates, and among 10 isolates of *Shigella*, 40% showed complete resistance to ampicillin, but other isolates showed 60% intermediate resistance to this antibiotic. No resistance was observed for other tested antibiotics. **Conclusion:** The difference between the level of contamination in both traditional and industrial samples of salad well indicates familiarity with sanitary protocols and standards provided to reduce the microbial load.

Introduction

Foodborne diseases (FBD) are a major public health problem that costs billions of dollars annually, affecting millions of people worldwide and causing some to die or be hospitalized (Balaban and Rasooly, 2000, Peles *et al.*, 2007). There are many types of ready-to-eat foods in

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different countries according to their cultural and social contexts, which have increased the demand among the people due to their great variety, availability, lack of cooking time, and relatively low prices. Although these ready-to-eat foods have benefits, they can threaten public health (Mengistu *et al.*, 2022).

One of Iran's most consumed ready-to-eat foods is Olivier salad, usually produced in traditional and industrial ways. Due to the use of raw materials with a high risk of bacterial contamination, such as chicken, mayonnaise, and eggs, as well as high involvement of hands and devices in the production of this product, the nutritious environment of Olivier salad is suitable for bacterial growth (Ram *et al.*, 2019). Traditional production of this product in large volumes and exposure to unfavorable temperature conditions for a long time before mixing make it possible for contamination with a variety of pathogenic microorganisms, including *Shigella*. Therefore, there is a possibility of contamination through devices, especially the workforce, in this type of food. According to Iranian Standard No. 5272 (Institute of Standards and Industrial Research of Iran, 2007) , the total count of microorganisms with an allowable limit of 1×10^5 cfu/ml is acceptable, and for the count of coliforms, Iranian Standard No. 11166 is used, for which the acceptable number of microorganisms is 50 colonies (Institute of Standards and Industrial Research of Iran, 2008) .

Unlike other foods heated before consumption, ready-to-eat foods are becoming a growing concern because of direct consumption without heating (Osaili *et al.*, 2011). The cooling rate is an important factor in delaying food spoilage and inhibit-growth of many pathogens, including bacteria, but it is not enough to minimize the microbiological risk. Many bacteria can infect Olivier salad, which can have a coliform origin or be transmitted from other sources, such as the environment, or raw compounds such as vegetables, meat, and potatoes (Storrs, 2007). A human pathogenic bacterium, Shigella, is the main cause of bacillary diarrhea or shigellosis in humans. *Shigella* spp. belongs to the Enterobacteriaceae family, which is common in developing countries, especially in Asia (Nisa *et al.*, 2020). It is estimated that 125 million cases of shigellosis and 14,000 deaths occur annually in Asia (Balaban and Rasooly, 2000).

Shigella infection occurs through food consumption (mainly potato salads, raw vegetables, meat, and milk) and poor hygiene at the production site (Liew *et al.*, 2014). The importance of shigellosis is due to the high prevalence of *Shigella* in the world and the high rate of infectivity of this bacterium, which the ingestion of as few as 10 organisms is enough to start the infection (DuPont *et al.*, 1989). *Shigella flexneri* (*Sh. flexneri*) accounts for about 66% of the *Shigella* species and *Shigella sonnei* (*Sh. sonnei*) for about 18% of the total *Shigella* isolated from food (Ahmed and Shimamoto, 2015). *Shigella* is acid-resistant, salt-tolerant, and can be survived on infectious levels in many types of foods such as fruits and vegetables, low-pH foods, prepared foods, and atmosphere-modified or vacuum-packed foods (Warren *et al.*, 2006). *Shigella* is an important factor in causing food outbreaks caused by consuming contaminated food in Iran (Saima *et al.*, 2018, Soltan Dalal *et al.*, 2005). Some studies have shown the presence of pathogenic bacteria such as *Shigella*, *Salmonella*, *Pseudomonas*, *Escherichia coli*, *Listeria*, and *Staphylococcus aureus* in prepared salads (Castro-Rosas *et al.*, 2012, Feroz *et al.*, 2013). This study aims to investigate the prevalence, antibiotic resistance pattern, and serotyping of *Shigella* isolates from Olivier salad.

Materials and Methods

Sampling

In this descriptive cross-sectional study, 150 samples of Olivier salad, including 50 industrial samples from 10 different available brands in Tehran's shops and 100 traditional samples from Tehran's southern regions, were randomly obtained between April to October of 2021. The samples were transferred to the laboratory under sterile conditions and a cold chain, where they were

examined for bacterial isolation and identification. According to different species in the *Shigella* genus, the biochemical characteristics of each isolate were identified and classified. Finally, the pattern of antibiotic resistance of the isolates was investigated.

Identification of *Shigella* strains

After complete uniformity of the samples under sterile conditions, to enrichment, 25 g of each sample was weighed and added to 225 CC of the Gram Negative Broth (GN Broth) medium. In order to evaluate the colorless *Shigella* colonies and their ability to ferment lactose, one tenth milliliter of the samples grown in the GN Broth medium was inoculated into the MacConkey (MAC) agar and Salmonella-Shigella agar and were incubated at 35 °C for 24 hours (Institute of Standards and Industrial Research of Iran, 2008). After this period, clear or colorless colonies suspected of *Shigella* were observed for identification by biochemical tests, such as Triple Sugar Iron (TSI), Methyl Red (MR) Voges-Proskauer (VP) tests, Lysine Iron agar (LIA), Sulfite Indol Motility (SIM), Simmon Citrate, and Urea broth. The results of differential media were examined with Enterobacteriaceae tables (Castillo *et al.*, 2006, Kimura *et al.*, 2004).

Finally, isolates were confirmed by API-20E kit (BioMerieux, France) to identify *Shigella* species. In order to test with API-20E, the bacterial colony was first cultured on a TSI medium. Then, a loop of a single colony was dissolved in physiological saline, and a concentration of half McFarland was prepared. Using a Pasteur pipette, the compartments were filled up (up to the brim) with the bacterial suspension and incubated for 24 hours. The next day, by adding reagents such as VP1, VP2, TDA, and Coax, the resulting discoloration was recorded and then read according to the codes introduced by the manufacturer.

Serotyping of isolates

Shigella serotype was identified by slide agglutination using antisera (Mast, England) based on manufacture instruments. Briefly, a Kligler Iron Agar (KIA) slant was inoculated with a single, well isolated, colony of *Shigella* spp. and was incubated at 35°C for 16-18 hours. Then, a drop of each of the antisera of *Shigella dysentery* (*Sh. dysenteriae*), *Sh. flexneri*, *Shigella boydii* (*Sh. boydii*), and *Sh. sonnei* was poured on the sterile glass slide, and for control, a drop of physiological serum was placed on another slide. Some of the suspected colonies (fresh culture for 16 to 18 hours) were placed on an antiserum and a controlled drop and mixed thoroughly. The slide was rotated, and the reaction was examined before 30 seconds. Specific clotting or complete agglutination during this period was considered a positive reaction without observing clots in the control drop.

Antimicrobial susceptibility testing

The antimicrobial susceptibility test of isolates was performed by Kirby–Bauer disk diffusion method on Mueller Hinton's medium as recommended by the Clinical Laboratory Standards Institute guidelines (Cockerill, 2013). The following antibiotic disks (Mast, England) were used: gentamicin (10 µg), ceftriaxone (30 µg), ciprofloxacin (15 µg), cotrimoxazole (10 µg), ampicillin (30 µg), nalidixic acid (µg), and cephalothin (2 µg). The diameter of the no-growth halo was compared with the CLSI standard, and the results were reported as sensitive (S), intermediate (I), and resistant (R) (Cockerill, 2013).

Ethical considerations

This article was the result of a research grant approved by Food Microbiology Research Center, in Tehran University of Medical Sciences with the ethics code IR.TUMS.SPH.REC.1399.299.

Results

Identification of *Shigella*

From 150 samples, 10 (6.66%) *Shigella* were isolated, which 6 (4%) *Sh. sonnei*, 3 (2%) *Sh. flexneri*, and 1 (0.66%) *Sh. boydii* isolates were identified. None of the isolates were recognized as *Sh. dysentery*. This study was conducted in 6 districts of Tehran, including districts 10, 11,16, 17, 19, and 20, and the contamination rates of *Shigella* from these districts were 20%, 30%, 20%, 10%, 10% , and 10%, respectively.

Other bacteria isolated from present study included *Salmonella*, *Pseudomonas*, *Escherichia coli* (*E. coli*), *Klebsiella*, *Citrobacter*, *Proteus*, *Povidencia*, *Hafnia*, and *Enterobacter*. It should be noted that most of these bacteria were present in traditional olivier salads and as shown in **Figure 1**, the highest rate was related to *Citrobacter* (60.6%), and the lowest was related to *Povidencia* species (0.6%). In this study, 20 *E. coli* (13.3%) were isolated, of which 18 (90%) were related to traditional Olivier salad, and 2 (10%) isolates were related to industrial Olivier salad. Five samples (5%) of traditional olivier salads were simultaneously infected with *Shigella* and *E. coli*.

For the total count of microorganisms, national standard No. 5272 was used, which the acceptable limit was 1×10^5 cfu/ml. Also, for counting coliforms, national standard No. 11166 was used, which the acceptable limit was 50 colonies. Based on the total count of bacteria, all (100%) samples of traditional Olivier salad were found unusable, but in terms of the presence of coliforms, this amount was 57%, in which the samples were unusable due to the presence of higher than the allowable limit. However, these numbers were 2% and 0% for industrial Olivier salads, respectively. These results determinated that the level of contamination in industrial Olivier salad was very low compared to traditional Olivier salad. The following diagram compared bacterial contamination between traditional and industrial Olivier salad samples (**Figure 2**).

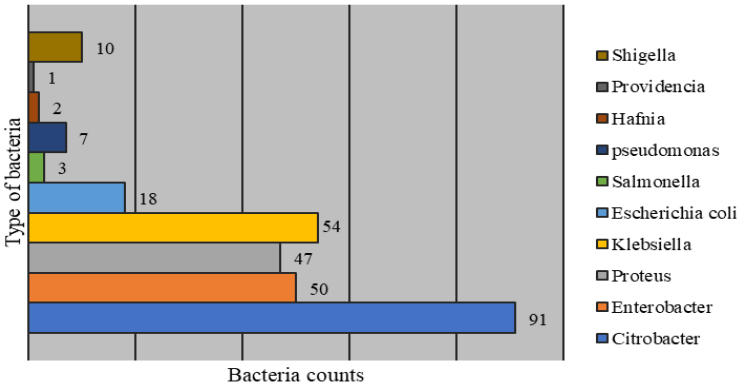

Figure 1. Number of isolated different bacteria from 100 traditional Olivier salad samples in the current study.

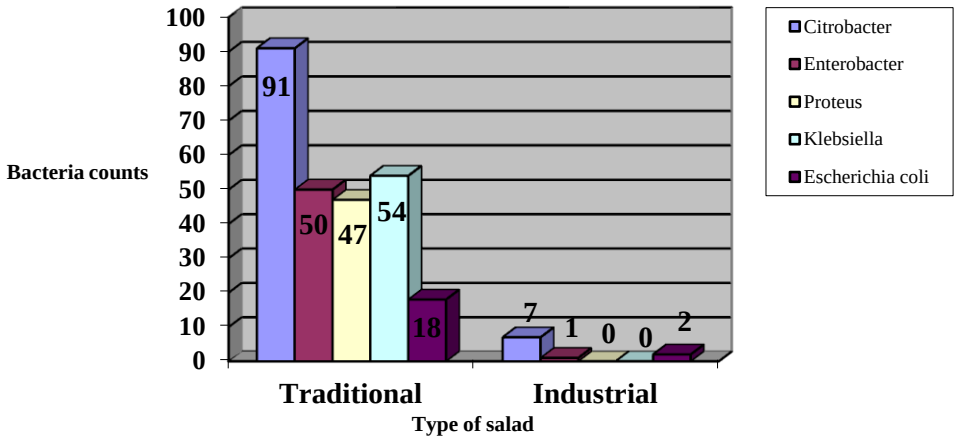

Figure 2. Comparison of isolated bacteria with the highest frequency between industrial (50) and traditional Olivier salad (100) samples.

Antibiotic resistance phenotypes

The results of antibiotic resistance test, among 10 isolates of *Shigella*, 4 (40%) isolates showed complete resistance to ampicillin, but other isolates (6 isolates, 60%) showed intermediate resistance to this antibiotic. No resistance was observed for other tested antibiotics. All isolates of *Shigella* were sensitive to gentamicin, nalidixic acid, and cotrimoxazole. In the case of ceftazidime, cephalothin, and ciprofloxacin antibiotics, all isolates showed sensitivity of some 95% to these antibiotics.

Discussion

Salad is one of the most widely consumed and popular foods that are often eaten as a main course or side dish. Being rich in nutrients has led to the preparation of salad not only at home but also on the menu of restaurants, fast foods, and hotels. Since salad is consumed raw, its contamination with various pathogenic microbes causes infection or poisoning in consumers (Mengistu *et al.*, 2022, Mritunjay and Kumar, 2015). High contamination in traditional salads may be due to the fact that most of the process of preparation and production of Olivier salad in Iran is manual, and the possibility of contamination through contaminated hands is high. Also, salad ingredients may be exposed to unsuitable temperature conditions for a long time before mixing(Jalali M and K, 2007, Ram *et al.*, 2019). Due to the preparation and processing process of industrial Olivier salad, it is expected to be less contaminated with bacteria. Also, in the industrial sample, after production, it is packed in a closed container, and the possibility of contamination is minimized. The findings of the current study showed that the frequency of bacteria isolated from industrial Olivier salad was much lower than traditional Olivier salad. The same was true of the diversity of isolated bacteria, as only three bacterial species, *Citrobacter*, *E. coli*, and *Enterobacter*, were isolated from industrial Olivier salad samples, and other members of the *Enterobacteriaceae* family were not identified. In addition, the frequency of these three bacterial species was very low in industrial Olivier salad samples, which showed the importance of following hygienic protocols to reduce the microbial load. However, in the samples of traditional Olivier salad, clearly not observing the hygienic standards of people and the workplace had caused high microbial contamination in such samples (Shahin *et al.*, 2019). *Shigella spp.*, as the causative agent of bacillary dysentery estimated 55,000 and 110,000 deaths and hospitalizations.Also, it was involved in several foodborne outbreaks (Ben Braïek *et al.*, 2018).

Limited studies investigated the incidence and antimicrobial susceptibility of Shigella isolated from food products. In Isfahan, presence of *Shigella* in different food samples was studied; 1400 food samples were examined, and 11 *Sh. sonnei* and 8 *Sh. flexneri* isolates were detected. This finding was consistent with the results of this study since the highest frequency was related to *Sh. sonnei* and *Sh. flexneri* serogroups (Shahin *et al.*, 2019). Previous studies showed that cold storage by refrigeration can not be used as the sole agent to control bacterial growth. A study by Hwang On egg and pasta salads demonstrated that *Listeria monocytogenes*, as a psychrotolerant bacterium, could grow at temperatures of 4 to 12 °C, and it was also found that *Listeria* can grow in the pH range of 4.7 to 5.2 (Hwang and Marmer, 2007) . The use of mayonnaise sauce due to its pH and the presence of citric acid can be used as an inhibitor of microbial growth. However, it is important to note that adding vegetables such as carrots or cabbage can absorb the available citric acid to vegetables and reduce its effective concentration in Olivier salad (Brocklehurst, 1994). In a study conducted by Soltan Dallal *et al.*, a total of 1055 cases of 249 foodborne diseases were reported in 20 provinces of Iran118 of which (11.2%) contained *Shigella* (Soltan Dallal *et al.*, 2020).

However, one of the main differences between the salads is the method of their preparation. In the traditional salad, all the steps are done by hand and workforce, but in industrial Olivier salad samples, part of the work is done by machine

without manual intervention, which can justify the leading cause of contamination, which is the manipulation of food by workforce (Vahabi Anaraki N and Abbasvali, 2016).

In a study conducted by Tajbakhsh *et al*. in 2015 on 50 samples of Olivier salad (30 industrial, and 20 traditional samples), *Salmonella* contamination was found in 5 samples (55.6%) and 4 samples (44.4%) of industrial and traditional Olivier salads, respectively (Tajbakhsh and Moumeni, 2015). However, these results were not in agreement with the findings which showed the rate of *salmonella* contamination was 2%. These differences can be attributed in part to the method of isolation of *Salmonella*. Due to the presence of *Salmonella* species in animals' excreta, livestock industries that produce meat or milk are a main factor in the continued presence of this human pathogen in the food chain. Therefore, such animal products can be considered as a potential agent of transmission of this disease. In 2022, a study was undertaken by Soltan Dallal *et al.* to examine the frequency of water and foodborne illnesses in the province of Kurdistan. The findings indicated that the occurrence of *Shigella* was 3% (Soltan Dallal *et al.*, 2024).

The high contamination in traditional salads compared to industrial ones can be due to the fact that the steps of preparation and production of Olivier salad are manual, and the components of the salad may be subjected to inappropriate temperature conditions for a long time before being mixed (Jalali M and K, 2007). In a study conducted by Soltan Dallal *et al.* to investigate the prevalence of foodborne diseases in Semnan province, *Shigella*, and *Salmonella* with 2.9% bacterial frequency (Soltan Dallal *et al.*, 2020).

One interesting finding in the current study was that *Shigella* isolates showed high sensitivity to nalidixic acid. In addition, high resistance to ampicillin was observed, and no multiple resistances were observed in any of the *Shigella* isolates. These results were inconsistent with Ahmed *et al*.'s findings, which investigated the antibiotic resistance of *Shigella* isolates from 1600 food samples (800 meat products and 800 dairy products). In their finding, 24 out of 27 *Shigella* isolates (88.9%) showed multidrug resistance phenotypes to at least three classes of antibiotics. *Shigella* species that were multidrug resistant included *Sh. flexneri* (66.7%), *Sh. sonnei* (18.5%), and *Sh. dysenteriae* (3.7%) (Ahmed and Shimamoto, 2015). Concerning recommended antimicrobial drugs for *Shigella*, the prevalence of resistance was the highest for streptomycin (100.0%), followed by kanamycin (95.8%), nalidixic acid (95.8%), tetracycline (95.8%), spectinomycin (93.6%), ampicillin (87.5%) and sulfamethoxazole-trimethoprim (87.5%). But, as far as the authors know, no study was done to investigate the antibiotic resistance of *Shigella* species isolated from Olivier salad.

Conclusion

The difference between the level of contamination in both traditional and industrial samples of salad well indicates familiarity with sanitary protocols and standards provided to reduce the microbial load. Therefore, in industrial Olivier salad, samples with strict observance of this process, the microbial load has reached a minimum; but, in traditional Olivier salad samples, rates are much higher due to a lack of familiarity with these health tips. In general, it is recommended that lack of hygiene during production, contamination of raw materials, and elevated storage temperature are among the key influential factors in the increased contamination of Olivier salad. Therefore, training the personnel for preparing this type of food and controling and monitoring of the food chain must be prioritized to reduce the possibility of microbial contamination.

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Conflict of interest

All the authors declared no conflict of interests.

Authors' contribution

Conceptualization was done by MM Soltan Dallal, formal analysis by A Rahimi Foroushani, investigation by S Jahanbakhshi, methodology by MM Soltan Dallal, project administration by MM Soltan Dallal, K Samimi-Rad, supervision by MM Soltan Dallal, writing of original draft by S Jahanbakhshi, A Naser and MR Mohammadi, and writing, review, and editing were done by A Naser, SZ Mirbagheri, MR Mohammadi, and MM Soltan Dallal.

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