original article

Prevalence of *Listeria monocytogenes* in the river receiving the effluent of municipal wastewater treatment plant

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ABSTRACT

Aims: The objective of this study was to evaluate the prevalence of *Listeria* spp. in the river water before and after discharge of the effluent of the municipal wastewater treatment plant (WWTP) in Isfahan, Iran.

Materials and Methods: A total of 66 samples were collected bi-weekly over 4 months from eleven discrete sampling locations in Zayandehrood River, Iran. Three sampling sites were located above the discharge point and five sites were located after the discharge point of WWTP. Samples were also collected from the influent and the effluent of WWTP. *Listeria* spp. were isolated using a selective enrichment procedure and a subculture onto polymyxin-acriflavine-lithium chloride-ceftazidime-esculin-mannitol Agar. All isolates were subjected to standard biochemical tests.

Results: *L. monocytogenes* was isolated from influent (83%), effluent (50%) and (18.5%) river water. *Listeria* spp. was not found before the discharge point in river water. However, *L. monocytogenes* was isolated in samples collected from 200 m (33%), 500 m (33%), 2 km (16.5%), 5 km (16.5%) and 10 km (16.5%) downstream from the WWTP. *Listeria innocua* (9%) and *Listeria seeligeri* (10%) were the second most frequently isolated species.

Conclusion: During the wastewater treatment, *Listeria* spp. is not removed completely. *L. monocytogenes* is widely distributed in the Zayandehrood river. *L. monocytogenes* released into surface water demonstrates a potential risk for public health. These results indicate the need for appropriate water management in order to reduce human and animal exposure to such pathogens.

Key words: *Listeria monocytogenes*, river, wastewater treatment plant

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INTRODUCTION

Listeria spp. are an important intracellular, facultative anaerobic, Gram-positive, non-spore forming and motile when the bacteria is grown between 20°C and 25°C. These are catalase positive and psychrotrophic bacteria capable of growing in low temperature. *Listeria* spp. are widely found in natural environments such as soil, water, sewage, vegetables, wastewater sludge, excrements and

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animal feces.[1,2] The genus *Listeria* contains seven species of *Listeria*. Six of them (*Listeria grayi*, *Listeria innocua*, *Listeria ivanovii*, *Listeria welshimeri*, *Listeria murrayi* and *Listeria seeligeri*) are not usually pathogens for humans while only *Listeria monocytogenes* is considered one of the most important foodborne pathogens that can causes listeriosis in human and animals. As a consequence, *L. monocytogenes* is now considered one of the major public health concern of the food industry world-wide.[3,4] Listeriosis may cause 20-50% mortality among susceptible people such as infants, pregnant women, elderly, patients with cancer and immuno-compromised persons.[1,2,5] In general, milder forms of listeriosis may cause symptoms such as cramps, diarrhea, headache, mild flu-like illness (e.g., chills, fatigue, muscle and joint pain), nausea, persistent fever and vomiting. However, the severe foodborne listeriosis may lead to meningitis, meningoencephalitis, septicemia and spontaneous abortion. There are 13 serovars of *L. monocytogenes*; however, the majority of human listeriosis caused by *L. monocytogenes*, serovars ½a, ½b and 4b.[1] Humans are affected with this disease through consumption of milk, meat, vegetables and generally food and water contaminated with these bacteria. They may also be infected by inhaling the bacterium suspended in the air, insects, direct contact with excrements of humans carrying the *Listeria* and contaminated animal products.[3,4] Control of *Listeria* spp. contamination is particularly difficult since the pathogen is ubiquitous in the environment and capable of growth in diverse conditions including of temperatures between 1°C and 45°C, a pH of 4 to 9 with a high concentration of sodium chloride and reduced water activity (aw 0.90-0.92; 11.5% NaCl).[1,6-8] As this microorganism multiplies in low temperatures, it has become an increasing concern as one of the main foodborne pathogens and the cause of 28% of all deaths related to foodborne diseases in the world.[9] *Listeria* spp. are very common and can be found almost anywhere in the environment. The pathogenic *L. monocytogenes* is also commonly distributed in various environments and can be found in wastewater at a high level.[5] *L. monocytogenes* has also been isolated from the soil, surface water, sewage, vegetation, fecal matter, animal feed, agricultural ecosystem and domestic environments.[1,10]

Listeria spp. are widely dispersed in the natural environment and are able to survive in secondary and physical treatments of wastewater. Therefore, environmental authorities have been recommended a new standard for the presence of this bacterium in the wastewater sludge and municipal wastewater.[11] This microorganism can enter the food chain, through reuse of treated wastewater like the sludge used in agriculture land (as a fertilizer) or irrigation.[1,5,10,11] Transmission of *Listeria* to humans through consumption of vegetables/fruits irrigated with effluent of wastewater has been also reported.[2] Therefore, continuous monitoring of water resources for the presences of *Listeria* is of essential to control the disease in humans and animals. Various studies have been reported the presence of *Listeria* spp. in raw and treated wastewater as well as in raw and digested sludge.[3,10,11] This bacterium has also been isolated from surface waters in Europe and USA.[2,12] We have reported the prevalence of *Listeria* spp. in various foods including plant origin foods in Isfahan, Iran.[13] We have also reported the presence of bacterium in the wastewater treatment plant (WWTP) in the same region.[14] However, to our knowledge, there are no data on the presence of *Listeria* in the surface water in Iran. Therefore, the aim of this study was to determine the prevalence of *Listeria* spp. in the river and assess the role of South Isfahan municipal sewage treatment plant as a source of river contamination.

MATERIALS AND METHODS

Sampling site description

Zayandehrood river water supplies drinking water and irrigation of agricultural lands. The South Isfahan Sewage Treatment Plant WWTP is the largest plant in Isfahan covering a population of 700,000 people. The WWTP under investigation in this study applied for municipal wastewater treatment and located in Southeast of Isfahan, Iran. The different sampling sites along the river and influent and effluent of the WWTP are depicted in Figure 1.

Water sampling

A total of 66 samples were collected in a 4 month period from August to November 2009. Water samples were collected from nine sampling sites in 18.5 km along the Zayandehrood river. Sites 1, 2 and 3 were located at 8.5 km, 1 km and 200 m upstream of the effluent discharge area (site 4) of Isfahan South WWTP respectively. Sites 5, 6, 7, 8 and 9 were located at 200 m, 500 m, 2 km, 5 km and 10 km of the downstream discharge area respectively. Furthermore, samples were taken from the influent and the effluent of the WWTP. Samples were collected in sterile wide-mouth 500 ml plastic containers and transferred to the School of Health, Isfahan University of Medical Sciences, Isfahan, Iran in portable insulated cool boxes and were analyzed after transporting to the laboratory immediately.[15]

Isolation of *Listeria* spp.

Isolation of *Listeria* spp. from water and wastewater samples were carried out using the modified selective enrichment and

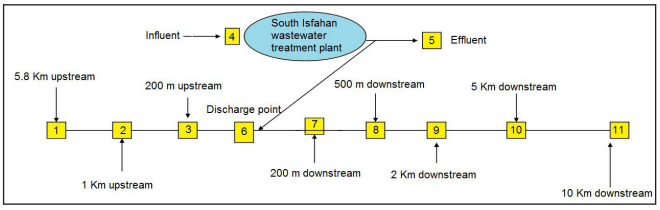

Figure 1: The sampling site numbers along the river and influent and effluent of the wastewater treatment plant

isolation protocol according to the United States Department of Agriculture method.[16,17] Briefly, 100 ml of each sample was filtrated through a 0.45 µ filter in sterile conditions. Then the filter was transferred to 9 ml of *Listeria* enrichment broth, University of Vermont Media (UVMI) culture medium (Merck, Germany). For the wastewater samples, 10 g was added to 100 ml of UVMI and all primary enrichment broths were incubated for 24-48 h at 35°C. 0.1 ml of the primary medium was added to 9.9 ml of the Fraser broth (Merck, Germany) enrichment medium and incubated for 48 h at 30°C. Secondary enrichment broth was then enrichment streaked onto polymyxin-acriflavin-lithium chloride-ceftazidime-esculin-mannitol (PALCAM) Agar (Merck, Germany) supplemented with PALCAM selective supplement (HC784958 Merck; Germany) and incubated at 37°C for 24-48 h. The plates were examined for typical *Listeria* colonies (black colonies with black sunken) and at least 3-5 typical colonies were sub-cultured on Tryptone Soy Agar supplemented with 0.6% of yeast extract and incubated at 37°C for 24 h. All isolates were subjected to gram staining, catalase and motility test at 25°C and 37°C, acid production from glucose, manitol, rhamnose, xylose, α-methyl-D-mamoside, nitrate reduction, hydrolysis of esculin and Methyl Red-Voges Proskauer test. Furthermore, for further confirmations of *Listeria* spp., all isolates subjected to other biochemical reactions, ß-hemolytic activity and the Christie-Atkins-Munch-Peterson (CAMP) test.[18] The CAMP test was performed by a streak of hemolytic *Staphylococcus aureus* and a *Rhodococcus equi* culture in parallel and diametrically opposite to each other on a sheep blood agar plate. Then, several test cultures were streaked parallel to one another, but at right angles to and between the *S. aureus* and *R. equi* streaks. After incubation at 35°C for 24-48 h, the plates were examined for hemolysis reaction.[18] *L. monocytogenes* 4a (IRTCC1293) strain used as a positive control for all biochemical tests.

RESULTS

Prevalence of *Listeria* spp. and *L. monocytogenes* in the WWTP and river water samples are presented in Table 1. Results of the study showed that from a total of 66 samples, positive samples for *Listeria* spp. and *L. monocytogenes* were 20 (30.30%) and 18 (27.27%) respectively. The prevalence of *L. monocytogenes* in samples collected from influent, effluent and river water were 5 (83.33%), 3 (50%) and 10 (18%), respectively (Figure 2). Comparison of results related to *L. monocytogenes* and *Listeria* spp. showed that in most samples *L. monocytogenes* was present with other species. Other species of *Listeria* included in this study was *L. innocua* and *L. seeligeri*.*L. seeligeri* and *L. innocua* were detected in 10.06% and 9.09% of all samples. In Figure 3, the percentage of the presence of different species of *Listeria* spp. in various sites is shown.

Table 1: The prevalence of *Listeria spp*. and *L. monocytogenes* in Zayandehrood river water and influent and effluent of Isfahan, Iran, South wastewater treatment plant

Sampling sites	Total	*Listeria* spp. no (%)	*L. monocytogenes* no (%)
Influent	6	5 (83.3)	5 (83.33)
Effluent	6	4 (66.66)	3 (50)
River water	54	11 (20.73)	10 (18.51)
Total	66	20 (30.30)	18 (27.27)

DISCUSSION

In the present study, the prevalence of *L. monocytogenes* in the Zayandehrood river before and after the effluent discharge of the municipal WWTP was investigated. A total of 66 samples were taken from 11 sites in order to assess the impact of WWTP discharge to the river. Sampling along the river clearly showed the presences of *Listeria* were related to the discharge of municipal wastewater. Despite the discharge of industrial wastewater to the river, no *Listeria* was found in the river water upstream from the WWTP. Whereas, *L. monocytogenes* was found in 50% of samples collected downstream from the plant [Figure 3].

Results of this study indicated that the presence of *L. monocytogenes* in the Zayandehrood river was due to

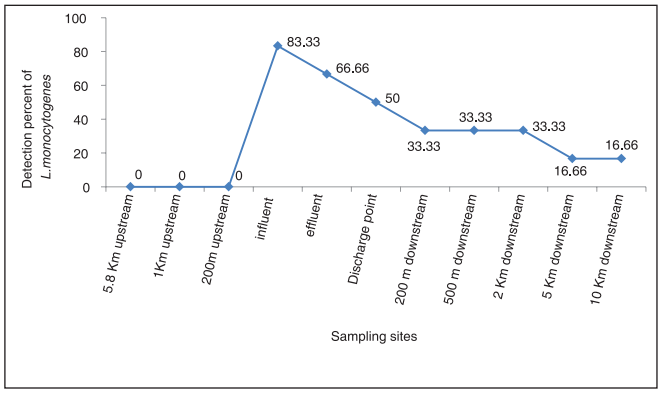

Figure 2: Detection of *Listeria monocytogenes* in nine sampling sites of the study area

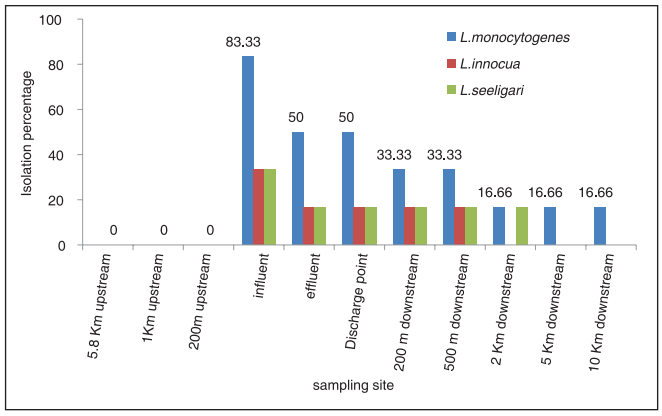

Figure 3: Prevalence of *Listeria monocytogenes*, *Listeria innocua* and *Listeria seeligeri* along the river in the study area

discharge of wastewater from the WWTP and significantly increased after wastewater discharged (P value < 0.05). Furthermore, this species seem highly resilient in water as it was transferred by the river to distances over 10 km downstream from the WWTP. However, non-pathogenic species, *L. seeligeri* and *L. innocua* was not found in a distance of over 2-5 km below the effluent discharge to the river.

Only limited studies have been conducted on river water contamination concerning *Listeria* world-wide. Due to the different methods of isolation used, variation in the sampling plan and other limitations it is difficult to compare the results of the studies. For example, Arvanitidou *et al*. (1997) studied the prevalence of *Listeria* and *Salmonella* in surface waters of Northern Greece. These authors reported low level of 3.9% contamination for *L. monocytogenes* compared to the current study (50%). Furthermore, logarithmic mean values of the standard bacterial index did not show any significant difference between *L. monocytogenes* positive and negative samples.[19] Luppi *et al*. (1986) also reported a lower level of prevalence in Italy. They isolated different types of *Listeria* from 22% of samples taken from the PO river in Italy.[20] In contrast, two previous studies, in Canada and Spain reported a higher prevalence of river water contamination of *Listeria*.[2,21] Lyautey *et al*. (2007) examined the spread of *L. monocytogenes* near the Ontario river. This river overlooks various urban and rural areas and 314 samples were taken from 22 sampling sites. The Canadian researcher reported 64% and 10% prevalence for *Listeria* spp. and *L. monocytogenes* respectively. In addition, they have found a significant correlation between the presence of *L. monocytogenes* and animal farming and agricultural lands in the vicinity of the river.[2] Bernagozzi *et al*. (1994) assessed the spread of *Listeria* spp. in surface water in 94 different samples of river, brackish water and municipal sewage. These authors reported the prevalence of 74.4% for *L. monocytogenes* in river water.[21] In another Spanish study, Combarro *et al*. (1997) examined *Listeria* spp. spread in the river receiving the final effluent of a WWTP, using extended aeration method in Santiago de Compostela. They have reported 92% of the mean efficiency of the treatment plant for elimination of *Listeria* spp. and prevalence of 44.3% for *L. monocytogenes* in river water.[12] The results of the present study are in agreement with those obtained by Combarro *et al*. (1997)[12] as we also found a high load of *Listeria* below the discharge of wastewater. Both study clearly demonstrated that treatment to purify the wastewater in Santiago de Compotela and Isfahan does not impede the release of *Listeria* into the effluent, which increase the level of *Listeria* population in river receiving the effluent.

It should be remembered that *L. monocytogenes* is a serious pathogen for human and animals and its release into the environment implies a significant health risk that must not be ignored. The Zayandehrood river water is mostly used for the irrigation of agricultural lands in the vicinity of the river, it is possible that contaminated water be regarded as a reservoir of *Listeria* via contamination of vegetable and crops grown in the land located downstream of the treatment plant. This may increase the human and animal exposure to *Listeria* with a consequential risk to public health. The greater concern is that the contamination of vegetables has been reported in the same area.[13] Further study need to establish the root of transmission of *Listeria* to human and animal in the region. Application of appropriate molecular typing methods (*pulsed-field gel electrophoresis*, Ribotyping, *restriction fragment length polymorphism*) will provide valuable information on molecular epidemiology listeriosis in the region. Furthermore, these methods can demonstrate genetic link between human, animal, crop and vegetable (irrigated by contaminated water) isolates and river water. On the other hand, it is essential to improve the efficiency of the removal of pathogenic bacteria including *Listeria* in the WWTP. Meanwhile, the most-effective accomplished method of control of dissemination of *Listeria* is through an environmental monitoring program, which includes frequent testing. Controlling the presence of *Listeria* spp. in the water used for vegetable irrigation is also one the most effective means of reducing the likelihood of product contamination. Therefore, continual monitoring of river water and local plant origin foods is an essential control measure of spreading disease.

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