original article

Performance of the municipal wastewater treatment plant for removal of *Listeria monocytogenes*

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ABSTRACT

Aims: The aim of present study was determination of occurrence of Listeria Listeria spp. in various point of a municipal wastewater treatment plant.
Materials and Methods: The samples were collected of influent, effluent, raw sludge, stabilized sludge and dried sludge from north wastewater treatment plant Isfahan, Iran. The presence of *Listeria* spp. was determined using USDA procedure and enumerated by a three-tube most probable number assay using Fraser enrichment broth. Then, biochemically identified *Listeria monocytogenes* was further confirmed by PCR amplification.
Results: *L. monocytogenes, L. innocua* and *L. seeligeri* were isolated from 76.9%, 23.1% and 23.1% of influent, 38.5%, 46.2% and 7.7% of effluent, 84.6%, 69.2% and 46.2% of raw sludge, 69.2%, 76.9% and 0% of stabilized sludge and 46.2%, 7.7% and 0% of dried sludge samples, respectively. The efficiency of wastewater treatment processes, digester tank and drying bed in removal of *L. monocytogenes* were 69.6%, 64.7% and 73.4%, respectively. All phenotypically identified *L. monocytogenes* were further confirmed by PCR method.
Conclusion: Application of sewage sludge in agricultural farms as fertilizer may result in bacteria spreading in agriculture fields and contaminated foods with plant origin. This may cause a risk of spreading disease to human and animals. Using parameters such as BOD_5 is not sufficient standard for the elimination of pathogenic microorganisms.

Key words: Effluent, *Listeria monocytogenes*, PCR, sludge, wastewater treatment plant

INTRODUCTION

Listeriosis is essentially a foodborne disease caused by *L. monocytogenes* and to some extent *L. ivanovii*. The disease condition vary from that affect immunocompromised patients to febrile gastroenteritis and prenatal infections associated with fetal loss or abortion in humans and animals.[1] Although rare, the disease reported to have very high mortality rare (20-50%), thus making it of serious public health concern.[2]

L. monocytogenes commonly is a saprophytic organism living

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naturally in the plant-soil environment. The ubiquitous nature of *L. monocytogenes* results in contamination of numerous food products including meat, milk and dairy products, sea foods and vegetables.[3] A possible agricultural route of human exposure to *Listeria* is through the ingestion of uncooked food crops grown in the lands irrigated with contaminated water and/or fertilized with *Listeria*-contaminated biosolids.[4]

L. monocytogenes has been detected in wastewater,[5-12] sewage sludge[5,7,13-15] and *L. innocua* has been detected in compost and irrigation water.[16] Sewage sludge can be regarded as a reservoir of *L. monocytogenes* and the presence of this pathogenic bacterium in such fertilizer would increase the risk of crops contamination. Therefore, biological monitoring of the presence of pathogenic bacteria in the effluent and sludge of sewage treatment plants is of special importance.[14] In addition, numerous rules and regulations ratified for the existence of certain microorganism in effluent and sludge. For instance, US Environmental Protection Agency (EPA) has been setup an extensive regulation for reuse of effluent and sludge in agriculture lands.[17] However, recent studies have been indicated that the traditional standards are not effective on environmental dissemination of new emerging microorganisms.[18] In the other hand with reports of inadequate removal of *Listeria* from wastewater in developed world,[19,20] one can safely presume that wastewater treatment plants in Iran are insufficient at removing these pathogens from wastewater influents prior to reused for irrigating the farmlands.

The occurrences of *L. monocytogenes* in variety of foods including vegetables have been reported in Iran.[21,22] However, to our knowledge there are no study on the prevalence of *L. monocytogenes* and its removal by sewage treatment plants in Iran. Therefore, the present study examined the prevalence of *Listeria* spp. in various wastewater and sludge samples in Northern municipal wastewater treatment plant in Isfahan, Iran. We also evaluated the performance of this treatment plant on removal *Listeria* spp.

MATERIALS AND METHODS

Plant description

The current investigation was conducted at the Northern municipal wastewater treatment plant in Isfahan, Iran. The Isfahan city has three large wastewater treatment plants in North, South and East with 1,200,000, 800,000 and 250,000 people of nominal capacity, respectively. The Northern treatment plant is the largest one in which treated a part of sewage of the city with the inlet flow rate of 1.6 m^3/sec currently. The flow diagram of the plant is shown in Figure 1.

Sample collection

Samples were collected on a monthly basis from the influent, effluent, raw sludge, stabilized sludge and dried sludge between December 2010 and January 2011. As shown in Figure 1, various samples were collected from five spots of this sewage treatment plant based on the standard method.[23] Each site was sampled 13 times and in total 65 samples obtained. Sewage and sludge samples collected in the plastic containers (500 ml) and plastic bags, respectively and transported in cooler boxes containing ice packs to the School of Health Laboratory, Isfahan University of Medical Sciences for analysis.

Sample analysis

Samples were analyzed for the presence of *Listeria* spp. using cultivation methodology recommended by United States Department of Agriculture (USDA).[23] Briefly, a 10g of sample was transferred to 90 ml of first enrichment medium UVM I (University of Vermont Media formula) (Merck, Germany) incubated in 30°C for 24 h. 0.1 ml of primary enrichments were transferred to 10 ml of UVM II (Fraser broth) (Merck, Germany) and incubated at 35-37°C for 48 h. Secondary enrichments were streaked on PALCAM agar supplemented with selective supplement HC784958 (Merck, Germany). The plates were examined for typical *Listeria* colonies. At least 3 to 5 typical colonies were selected and purified on the TSAYE culture medium. All isolates were subjected to standard biochemical tests such as Gram staining, carbohydrate fermentation (L-rhamnose, D-xylose, glucose, D-manitol), catalase test, MR/VP test, motility test at 25°C and CAMP test.[24,25]

In order to quantified *L. monocytogenes*, 3-tube most probable number (MPN) method was used in supplemented Fraser broth culture medium. The wastewater and sludge samples were cultivated by the amounts of 10 ml, 1 ml and 0.1 ml in each tube.[14,20] The samples of raw sludge, stabilized sludge and the dried sludge were diluted up to 10^{-1} and 10^{-2} respectively and MPN of samples was determined

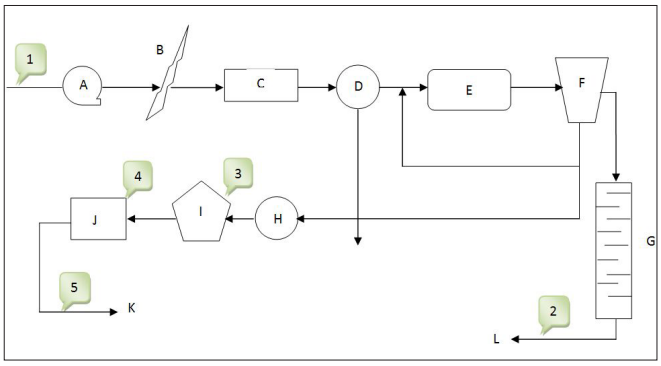

Figure 1: Flow diagram of Northern Isfahan, Iran sewage treatment plant, A: Pumping station, B: Screening, C: grit removal, D: Primary sedimentation tank, E: Aeration tank, F: secondary sedimentation tank, G: Chlorination basin, H: Thickening sludge, I: Anaerobic digester, J: Sludge drying bed, K: Amendment soil, L: Effluent. The numbers indicate the sampling spots. 1) Influent, 2) Effluent, 3) raw sludge (primary mixed sludge + secondary sludge), 4) stabilized sludge (raw sludge processed by the digester), 5) dried (final) sludge

and the dilution coefficients were taken into consideration. The positive tubes (blackened) were confirmed using biochemical and PCR methods.[25] The pair of primers 234 (5′-CATCGACGGCAACCTCGGAGA-3′) and primer 319 (5′-ATCAATTACCGTTCTCCACCATT-3′) were used to amplify a 417 bp internal fragment of the *bly*A gene.[26] PCR was achieved as described by Fitter *et al.*, and 16 µl of the PCR amplified reaction mixtures were subjected to horizontal gel electrophoresis in 1.8% agarose gels run in 1× Tris-borate (TBE). PCR products were visualized using ethidium bromide staining (1 µg/ml) and photographed under UV light. *L. monocytogenes* serotype 4a (IRTCC 1293) provided from Razi Institute in Iran used as a positive control.

Statistical analysis

Calculation of mean and standard deviations were performed using Microsoft Excel office 2007 version. Test of significance (independent T-test) were performed using SPSS 10 version. Independent *t*-test was used to compare differences in the *L. monocytogenes* MPN mean between influent and effluent, raw sludge and stabilized sludge, and stabilized sludge and dried sludge. All tests of significance were considered statistically significant at *P* value ≤ 0.05.

RESULTS

The prevalence of *Listeria* spp. in different samples collected in various stages of Northern Isfahan's sewage treatment plant is shown in Figure 2. In the current study 52/65 (80%) of all samples were positive for *Listeria* spp. and, *L. monocytogenes*, *L. innocua*, *L. seeligeri* were isolated from 41/65 (63.1%), 30/65 (44.6%), 10/65 (15.4%) of the tested samples, respectively. The mean MPN of *Listeria* spp. in different sewage samples are shown in and Table 1. The independent *t*-test showed a significant difference in mean of MPN of *L. monocytogenes* between the influent and effluent samples (*P* value = 0.036), the raw sludge and stabilized sludge (*P* value = 0.025), and also the stabilized sludge and the dried sludge (*P* value = 0.027). The removal percentage of *L. monocytogenes* in sewage treatment process, the digester tank, and sludge drying media was determined as 69.6%, 64.7%, and 73.4%, respectively. All *L. monocytogenes* isolates also confirmed by PCR method and amplified a 417 bp internal fragment of the *bly*A gene [Figure 2].

DISCUSSIONS

Listeria monocytogenes is considered a ubiquitous foodborne pathogen which can lead serious infections in human. One of the potential sources of this bacterium is municipal wastewater treatment plants. Sludge and effluent of sewage treatment plants are using in agricultural farms as a fertilizer in Iran. Therefore, the bacteria may spread on agriculture land and other environment. This may in particular, contaminate the foods of plant origin and cause a risk of spreading listeriosis

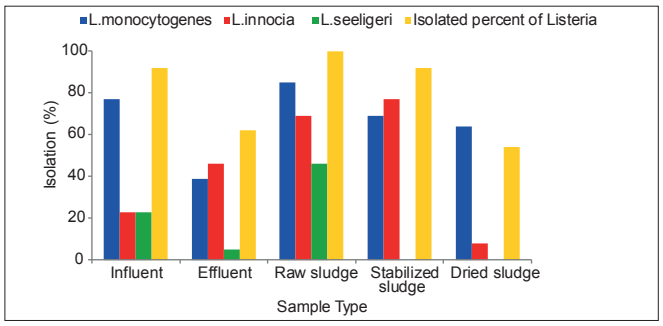

Figure 2: Percentage of *Listerias spp*. isolation in various samples collected from different spots of Northern Isfahan's, Iran Sewage Treatment

to human and animals. Despite the isolation of *Listeria* spp. from various foods and vegetables in Iran,[21] there are no data on occurrence of bacterium in municipal sewage so far. Therefore, this study was conducted to isolate and enumerate *L. monocytogenes* in the sewage treatment plant in Northern Isfahan, Iran. The provided data can be used to develop appropriate control strategies of disease in human and animal.

In the current study 80% of all samples were positive for *Listeria* spp. and, *L. monocytogenes*, *L. innocua*, *L. seeligeri* were isolated from 63.1%, 44.6% and 15.4% of the tested samples, respectively. *L. monocytogenes*, *L. innocua* and *L. seeligeri* were isolated from 76.9%, 23.1% and 23.1% of influent, 38.5%, 46.2% and 7.7% of effluent, 84.6%, 69.2% and 46.2% of raw sludge, 69.2%, 76.9% and 0% of stabilized sludge and 46.2%, 7.7% and 0% of dried sludge samples, respectively. Considering a long detention time (20 days for anaerobic digesters and 2-3 month for sludge drying beds) and suitable temperature for the growth of mesophilic bacteria in sludge digesters (In order to stabilize the organic material, reduce the volatile organics and pathogenic bacteria population and improvement of the sludge dewatering characteristics) the above results show that *L. monocytogenes* was dominant species in all samples whereas, *L. innocua* was more common in effluent samples. Also, *L. seeligeri* was not found in the raw and stabilized sludge and all isolated species from dried sludge were (except one for *L. innocua*) *L. monocytogenes*. These findings indicate that other species are more sensitive in compared to *L. monocytogenes* to the environmental factors during treating process. This is in agreement with the report of Spanish study[15] that indicated *L. seeligeri* and *L. innocua* were more destructed during sewage treatment. It seems that the pathogenic species of *L. monocytogenes* was able to survive even after digestion and sludge drying beds. That may indicate the importance of monitoring of this species in the sludge and sewage treatment plants.

Similar to our finding, most of the environmental study reported high rate of contamination. For example, a study in 2005 in France,[20] isolated *Listeria* spp. from 84.4% of effluent and 89.2% raw sludge. In other study, Garrec *et al.*, (2003) also isolated *Listeria* spp. and *L. monocytogenes* in 87% and

Table 1: Mean MPN of *Listeria spp*. in the sewage and sludge of northern isfahan's sewage treatment plant

Listeria spp.	Sample type	Unit	Mean MPN	Maximum	Standard deviation
L. monocytogenes	Influent	MPN/100mL	6.08	28	7.5
	Effluent	MPN/100mL	1.85	11	3.16
	Raw sludge	MPN/g dry matter	57	233	60
	stabilized sludge	MPN/g dry matter	20.12	88.46	24.2
	Dried sludge (Final)	MPN/g dry matte	5.53	23.9	7.3
L. innocua	Influent	MPN/100mL	1	6	2
	Effluent	MPN/100mL	3.92	15	6.6
	Raw sludge	MPN/g dry matter	25	58.3	21.16
	stabilized sludge	MPN/g dry matter	15.7	42.3	13.55
	Dried sludge (Final)	MPN/g dry matte	0.5	6.52	1.8
L. seeligeri	Influent	MPN/100mL	0.69	3	1.32
	Effluent	MPN/100mL	0.46	6	1.66
	Raw sludge	MPN/g dry matter	14.75	58.33	18.68
	stabilized sludge	MPN/g dry matter	0	0	0
	Dried (Final) sludge	MPN/g dry matte	0	0	0

73% of dewatered sludge and in 96% and 80% of sludge stored in tanks respectively. They found the number of *L. monocytogenes* in dewatering sludge and digested sludge as 0.15-20 MPN/g and 1-240 MPN/g, respectively.[14] This result is slightly higher than what has been found in current study. Also Odjajar *et al*., in South Africa reported the detection of free-living *Listeria* species and associated-planktons in 96% and 58-67% of final effluents of a rural wastewater treatment facility samples, respectively.[12] This research group, in other work reported that the *Listeria* population of 2.9×10^0 to 1.2×10^5 cfu/ml in municipal wastewater effluents.[27]

A study in Poland, reported the isolation rate of *Listeria* in mechanical and biological treatment plant samples as 90% *L. monocytogenes*, 5% *L. seeligeri* and 5% *L. grayi* from the point of municipal sewage delivery to the treatment plant.[19] Also a study in Sweden reported significantly lower rate of isolation of *L. monocytogenes* from 8/64 (12%) of collected raw sludge samples and 1/69 (2%) from treated sludge samples.[28]

In the present work, the efficiency of wastewater treatment processes, digester tank and drying bed in removal of *L. monocytogenes* were 69.6%, 64.7% and 73.4%, respectively. In contrast, a study in Spain, reported the higher average efficiency of 92% removal of *Listeria* species in the sewage treatment plant.[15]

Alghazali *et al*., in Iraq, reported higher prevalence of *Listeria* spp. of 100% in treated wastewater effluent but lower densities of 3-28 MPN/ml compare to this study. They have also reported higher efficiency rate of a municipal sewage treatment plant of removal of 85-99.7% for *Listeria* species.[7] Similar to the current study, Paillard *et al*., reported 84.4% prevalence of *Listeria* spp. in treated wastewater in France with population ranging from 0.3 to 21 MPN/ml.[20] Also, in Holland *L. monocytogenes* isolated from 90% and *L. seeligeri* and *L. graee* both from 5% of income untreated sewage.[19]

The number of biochemical test which has been used to differentiate species within the genus *Listeria*, is limited.[29,30] The main distinctive criteria between *L. monocytogenes* and *L. innocua* based on the haemolytic activity of the former is CAMP test.[31] However, this test is not reliable method for characterization *Listeria* species and distinction between *L. monocytogenes* and *L. innocua*.[32] Therefore, in this study, all of *L. monocytogenes* isolates also confirmed by PCR method [Figure 3].

In the current study, the *L. monocytogenes* species was detected as a dominant species in all samples of sewage and sludges except in the final effluent and stabilized (that *L. innacua* was dominant species). This finding is in agreement with the most of the previous studies. The raw sludge had the highest MPN of *L. monocytogenes*. This result indicated that *L. monocytogenes* is more resistant compare to other *Listeria* species. As it was survived after the digestion process and sludge drying bed. The efficiency of the Northern Isfahan's Sewage Treatment Plant to remove the bacterium in the effluent and dried sludge was 69.6% and 90.2%, respectively. As shown in Figure 2, *L. monocytogenes* isolated from 38.5% and 46.2% of the final effluent and sludge samples, respectively. These results have shown that *L. monocytogenes* were present in wastewater treatment plant effluents and sludge at high level. There are no regulation on reuse of effluent for irrigation of farmlands and the dried sludge as a fertilizer in Iran concerning *L. monocytogenes*, so the *L. monocytogenes* population may increase in soil, surface waters, and the nature. In this respect, the reuse of effluent and sludge may spread the bacterium in the environment and infect humans and animals. Therefore, it is recommended to monitor regularly *L. monocytogenes* contamination in soil and the agricultural products of the lands irrigated with effluent of the treatment plant. In addition, the use of sewage sludge as a fertilizer need to be precisely reconsiders by regulatory authorities.

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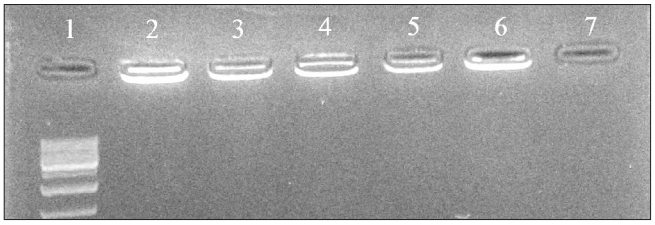

Figure 3: Agarose gel showing the amplified PCR product of *L. monocytogenes* isolated from sewage and sludge samples; Lane 1:100bp DNA ladder; Lane 2: positive control, *L. monocytogenes* serotype 4a (IRTCC 1293) provided from Razi Institute in Iran; Lane 3: *L. monocytogenes* isolated from the effluent; Lane 4: *L. monocytogenes* isolated from raw sludge; Lane 5: *L. monocytogenes* isolated from the stabilized sludge; Lane 6: negative control

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