Prevalence, antimicrobial susceptibility and virulotyping of *Listeria* species and *Listeria monocytogenes* isolated from open-air fish markets

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**Abstract**

**Background:** The aim of this study was to investigate the prevalence and characterization of *Listeria* species and *Listeria monocytogenes* isolated from raw fish and open-air fish market environments. Eight hundred and sixty two samples including raw fish and fish market environments (samples from workers' hands, workers' knives, containers and work surface) were collected from the open-air fish markets in the Northern region of Iran.

**Results:** *Listeria* spp. was isolated from 104/488 (21.3 %) raw fish and 29/374 (7.8 %) of samples from open-air fish market environment. The isolates of *Listeria* spp. included *L. innocua* (35.3 %), *L. monocytogenes* (32.3 %), *L. seeligeri* (18 %), and *L. ivanovii* (14.3 %). Of the 43 *L. monocytogenes* isolates, 31 (72.1 %), 10 (23.3 %) and 2 (4.7 %) belonged to serovars 1/2a, 4b, and 1/2b, respectively. The *inlA, inlB, inlC, inlJ, actA, hlyA, iap, plcA,* and *prfA* virulence-associated genes were detected in almost all of the *L. monocytogenes* isolates. The *Listeria* spp. isolates showed high resistance against tetracycline (23.3 %), penicillin G, and cephalothin (each 16.5 %). Besides, we observed significant resistance level to tetracycline (27.9 %), ampicillin (20.9 %), cephalothin, penicillin G, and streptomycin (each 16.3 %) in the *L. monocytogenes* isolates. All of the isolates were susceptible to cefotaxime, gentamicin, kanamycin, and pefloxacin. We found that *tetM* (25.6 %), *tetA* (23.3 %), *ampC* (14 %), and *penA* (11.6 %) were the most prevalent antibiotic resistance genes in the *L. monocytogenes* isolates.

**Conclusions:** Recovery of potentially pathogenic *L. monocytogenes* from raw fish and environment of open-air fish market samples in this study is a convincing evidence for the zoonotic potential of listeriosis.

**Keywords:** *Listeria*, Seafood, Virulence genes, Serotyping, Antibiotic resistance, Resistance gene

**Background**

The genus *Listeria* includes facultatively anaerobic Gram-positive bacteria. *L. ivanovii* and *L. monocytogenes* from this genus are known as pathogenic species and have shown the ability to cause severe diseases in animals and both humans and animals, respectively. Listeriosis is a foodborne infection with high mortality rates which is caused by *L. monocytogenes* in humans. The pathogen is ubiquitous and has been isolated from animals, different types of foods, and environments worldwide [1–3].

There is a high possibility of *L. monocytogenes* contamination in the captured fish from contaminated waters and environments. The contamination could also happen during transportation and in the environment of fish markets. Although *L. monocytogenes* has been isolated from seafood, fish and fishery products, no major listeriosis outbreaks with these products has been reported so far [4]. However, contaminated fish and fish products are considered as the most frequent causes of a number of sporadic listeriosis cases [5].
**Methods**

**Sampling**

Between March 2012 and Jun 2014, 488 raw fish samples were purchased at the open-air fish market places in five major cities in Mazandaran Province, North of Iran. The purchased samples included *Ctenopharyngodon idella* (*n* = 135), *Rutilus kutum* (*n* = 124), *Liza auratus* (*n* = 120), and *Hypophthalmichthys molitrix* (*n* = 109). In addition, 374 environmental and workers swab samples from workers’ hands (*n* = 96), workers’ knives (*n* = 96), work surface (*n* = 92), and containers (*n* = 90) were collected from the open-air fish markets. All of the samples were transported in ice boxes to the laboratory within 3 h after sampling. All samples were obtained with the informed consent of the workers and ethics approval for this study was granted by the Islamic Azad University, Iran.

**Isolation and detection of *Listeria* spp.**

*Listeria* spp. were isolated and detected using ISO11290-1 method [22]. Briefly, samples were pre-enriched by half Fraser broth (Oxoid, Basingstoke, UK) and enriched by Fraser broth (Oxoid, Basingstoke, UK) for 48 and 24 h at 37 °C, respectively. Finally, the enriched Fraser broth-culture was streaked onto Palcam agar (Oxoid, Basingstoke, UK) and Oxford agar (Oxoid, Basingstoke, UK) followed by 24 to 48 h incubation at 37 °C. The presumed colonies were verified by biochemical tests and API *Listeria* (bioMérieux, Marcy l’Étoile, France). The isolates of *Listeria* spp. were then further confirmed by PCR [23].

**Serotyping of *L. monocytogenes* isolates**

The detected *L. monocytogenes* isolates were serotyped using the commercially prepared *Listeria* antisera against somatic (O) and flagellar (H) antigens according to the manufacturer (Denka-Seiken Co. Ltd., Tokyo, Japan).

**Detection of virulence genes by multiplex-PCR**

Two multiplex-PCR were used for detection of nine virulence-associated genes. Detection of *inlA, inlB, inlC,* and *inlJ* genes was performed by a multiplex-PCR using primers and cycling conditions as described by D Liu, ML Lawrence, FW Austin and AJ Ainsworth [13], and H Jamali and KL Thong [24]. The PCR assay was done as previously described for the *actA, hlyA, iap, plcA,* and *prfA* genes in *L. monocytogenes* isolates [25].

**Phenotypic detection of antimicrobial resistance in *Listeria* spp. isolates**

Antibiotic susceptibility testing was done using the Kirby–Bauer disc diffusion method on Mueller Hinton agar (Oxoid, Basingstoke, UK) supplemented with 5 % defibrinated sheep blood [26]. Ampicillin (30 μg), chloramphenicol (30 μg), cephalothin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefuroxime (30 μg), erythromycin (15 μg), florfenicol (30 μg), gentamycin (10 μg), kanamycin (30 μg), pefloxacin (5 μg), penicillin G (10 unit), rifampicin (5 μg), streptomycin (30 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), and vancomycin (30 μg) were applied as antibiotic agents. As CLSI breakpoints for *Listeria* species only include a few antimicrobial agents such as sulfamethoxazole–trimethoprim, ampicillin, and penicillin; therefore CLSI [26] breakpoints for *Enterococcus* were used for the other antimicrobial agents as recommended by M Conter,
D Paludi, E Zanardi, S Ghidini, A Vergara and A Ianieri [27], and Q Li, J Sherwood and C Logue [28].

Antimicrobial resistance genes profiling of L. monocytogenes isolates

The tetracycline resistance genes (tetA, tetB, tetC, tetL, tetM, and tetS), ampicillin resistance gene (ampC), vancomycin resistance gene (vanA and vanB), erythromycin resistance gene (ermB), florfenicol resistance gene (floR), chloramphenicol resistance gene (cmlA), and streptomycin resistance gene (strA) were detected using PCR as previously described [29–38].

Results and discussion

Although the people in the Northern region of Iran rely on fish as their primary animal protein source, there is still no study done on the prevalence and characterization of L. monocytogenes in fish and environment of fish markets. The prevalence of Listeria spp. in raw fish and fish markets environments in this study is presented in Table 1. Out of the 862 tested samples, 133 (15.4 %) were contaminated with Listeria spp., out of which 104 (78.2 %) and 29 (21.8 %) were isolated from raw fish and fish market environments, respectively. The Listeria spp. isolates included L. monocytogenes (n = 43, 32.3 %), L. innocua (n = 47, 35.3 %), L. seeligeri (n = 24, 18 %) and L. ivanovii (n = 19, 14.3 %). All 43 isolates of L. monocytogenes, identified by biochemical tests, were also confirmed using PCR.

Among the 862 samples, 32 Ctenopharyngodon idella (23.7 %), 27 Rutilus kutum (21.8 %), 24 Hypophthalmichthys molitrix (22 %), and 21 Liza auratus (17.5 %) were naturally contaminated with Listeria spp. In addition, 12 work surface (13 %), 11 workers’ hands (11.5 %), and 3 containers (3.3 %), and 3 workers’ knives (3.1 %) harboured Listeria spp. Several studies on the prevalence of Listeria spp. in fish, fish products and environments have been performed worldwide [39, 40]. The prevalence of Listeria spp. isolated from raw fish and fish markets in this study concurred with the earlier findings in Iran and other countries [41, 42]. However, our findings showed higher prevalence of Listeria spp. compared with the previous studies [43, 44].

In the present study, L. monocytogenes was isolated from 37/488 raw fish (7.6 %) and 6/374 environments of fish markets (1.6 %). Previous reports by H Momtaz and S Yadollahi [43] and VS Parihar, S Barbu...
All 43 isolates of L. monocytogenes were tested for the presence/absence of virulence genes inlA, inlB, inlC, and inlJ. Although, inlA, inlB, and inlC genes were observed in all of the L. monocytogenes, inlJ gene was detected in 42/43 (97.7 %) of the L. monocytogenes isolates. The surface-associated internalin is alleged to play a role in the pathogenesis of listeriosis [53]. This is the first study, to the best of our knowledge, to examine L. monocytogenes isolates from raw fish and environment of fish markets for the presence of four main internalin genes (inlA, inlB, inlC, and inlJ). Similar results were obtained in the previous studies, where the tested internalin genes were present in almost all of the examined L. monocytogenes isolates from animals [17], human listeriosis [54], different kinds of foods [15, 18, 24] and environmental samples [55].

Although, the actA, hlyA, and iap genes were detected in all of the L. monocytogenes isolates, plcA and prfA genes were observed in 41 (95.3 %) and 42 (97.7 %) of the isolates, respectively. The prevalence rate of these five virulence genes in this study is in concurrence with the earlier studies in Iran [43] and India [56] where the actA, hlyA, iap, plcA, and prfA genes were observed in all the L. monocytogenes isolates recovered from seafood samples. The presence of nine virulence-associated genes in almost all of the L. monocytogenes isolates suggests that these isolates could be potentially virulent.

In total, 53 and 57 Listeria spp. isolates (39.8 and 42.9 %) were resistant to one and two antibiotics, respectively (Table 3). In addition, nine isolates of Listeria spp. (6.8 %) were multi-drug resistant. The most frequent antibiotic-resistance was resistance to tetracycline (23.3 %), followed by penicillin G, cephalothin (each 16.5 %), streptomycin (15.8 %), florfenicol (15 %), erythromycin (14.3 %), ampicillin (12 %), trimethoprim-sulfamethoxazole (12.5 %), ceftazidime (8.3 %), vancomycin (6 %), rifampicin (3.8 %), and chloramphenicol (1.5 %).

### Table 2 Prevalence of L. monocytogenes serovars in raw fish and environmental samples

| Serovars of L. monocytogenes | 1/2a | 4b | 1/2c |
|-----------------------------|------|----|------|
| Ctenopharyngodon idella     | 6 (60 %) | 3 (30 %) | 0 |
| Rutilus kutum               | 9 (64.3 %) | 4 (28.6 %) | 1 (7.1 %) |
| Liza auratus                | 6 (85.7 %) | 1 (14.3 %) | 0 |
| Hypophthalmichthys molitrix| 4 (57.1 %) | 2 (28.6 %) | 1 (7.1 %) |
| Workers’ hands              | 2 (100 %) | 0 | 0 |
| Workers’ knives             | 1 (100 %) | 0 | 0 |
| Work surface                | 2 (100 %) | 0 | 0 |
| Containers                  | 1 (100 %) | 0 | 0 |
| Total                       | 31 (72.1 %) | 10 (23.3 %) | 2 (4.7 %) |

### Table 3 Resistance profiles of Listeria spp. isolated from raw fish and environmental samples

| Listeria spp. (n = 133) | L. monocytogenes (n = 43) | L. innocua (n = 47) | L. seeligeri (n = 24) | L. ivanovii (n = 19) |
|--------------------------|---------------------------|---------------------|-----------------------|----------------------|
| Ampicillin               | 16 (12 %)                 | 9 (20.9 %)          | 5 (10.6 %)            | 2 (8.3 %)            |
| Chloramphenicol          | 2 (1.5 %)                 | 1 (2.3 %)           | 1 (2.1 %)             | 0                    |
| Cefotaxime               | 0                         | 0                   | 0                     | 0                    |
| Ceftazidime              | 11 (8.3 %)                | 6 (14 %)            | 4 (8.5 %)             | 0                    |
| Cefuroxime               | 1 (1.2 %)                 | 0                   | 1 (3.6 %)             | 0                    |
| Cephalothin              | 22 (16.5 %)               | 7 (16.3 %)          | 11 (23.4 %)           | 3 (12.5 %)           |
| Erythromycin             | 19 (14.3 %)               | 6 (14 %)            | 9 (19.1 %)            | 3 (12.5 %)           |
| Florfenicol              | 20 (15 %)                 | 6 (14 %)            | 11 (23.4 %)           | 2 (8.3 %)            |
| Gentamycin               | 0                         | 0                   | 0                     | 0                    |
| Kanamycin                | 0                         | 0                   | 0                     | 0                    |
| Pefloxacin               | 0                         | 0                   | 0                     | 0                    |
| Penicillin G             | 22 (16.5 %)               | 7 (16.3 %)          | 9 (19.1 %)            | 2 (8.3 %)            |
| Rifampicin               | 5 (3.8 %)                 | 1 (2.3 %)           | 2 (4.3 %)             | 2 (8.3 %)            |
| Streptomycin             | 21 (15.8 %)               | 7 (16.3 %)          | 8 (17 %)              | 3 (12.5 %)           |
| Tetracycline             | 31 (23.3 %)               | 12 (27.9 %)         | 11 (23.4 %)           | 5 (20.8 %)           |
| Trimethoprim-sulfamethoxazole | 14 (10.5 %)  | 5 (11.6 %)          | 5 (10.6 %)            | 3 (12.5 %)           |
| Vancomycin               | 8 (6 %)                   | 3 (7 %)             | 4 (8.5 %)             | 1 (4.2 %)            |
| Resistant to 1 antibiotic| 53 (39.8 %)               | 15 (34.9 %)         | 14 (29.8 %)           | 10 (41.7 %)          |
| Resistant to 2 antibiotics| 57 (42.9 %)               | 20 (46.5 %)         | 27 (57.4 %)           | 8 (33.3 %)           |
| Resistant to > 2 antibiotics| 9 (6.8 %)               | 6 (14 %)            | 3 (6.4 %)             | 0                    |
and cefuroxime (1.2 %). Out of 43 L. monocytogenes isolates, 15 (34.9 %), 20 (46.5 %) and 6 (6.4 %) were resistant to one, two and more than two antibiotics. The L. monocytogenes isolates indicated high resistance to tetracycline (27.9 %), ampicillin (20.9 %), cephalothin, penicillin G, and streptomycin (each 16.3 %). All of the Listeria spp. isolates were sensitive to cefotaxime, gentamicin, kanamycin, and pefloxacin.

The 43 L. monocytogenes were examined for the presence of resistance genes. Six of 43 L. monocytogenes isolates (14 %) harbored more than one antimicrobial resistance gene. Among the evaluated serovars of L. monocytogenes isolates, a higher prevalence of antimicrobial resistance genes was detected in serovar 1/2a (81.5 %), followed by serovar 4b (18.5 %). However, the resistance genes were not found in serovar 1/2c isolates.

For tetracycline resistance, the tetM and tetA genes were present in 91.7 and 83.3 % of the tetracycline-resistant isolates, respectively and 71.4 % of the penicillin-resistant isolates harboured penA gene. Out of 7 streptomycin-resistant isolates, 42.9 and 14.3 % isolates contained strA, and strB, respectively. Furthermore, the ampC and vanA resistance genes were found in 66.7 and 33.3 % of the ampC- and vancomycin-resistant isolates, respectively. However, the tetB, tetC, tetL, tetS folB, cmlA, and vanB were not detected in the examined L. monocytogenes isolates from raw fish and open-air fish market environments. The prevalence rate of the tetM, tetA, penA and strA genes in the present study is in agreement with earlier investigations, in which a high frequency of these resistance genes in L. monocytogenes isolates was reported by C Poyart-Salmeron, P Trieu-Cuot, C Carlier, A MacGowan, J McLauchlin and P Courvalin [57], and V Srinivasan, H Nam, L Nguyen, B Tamilselvam, S Murinda and S Oliver [29].

In the current study, the phenotypic resistance profiles of the L. monocytogenes isolates were not confirmed by detection of resistance genes. For instance, 2 of 6 vancomycin-resistant isolates which showed phenotypic resistance to vancomycin, harbored vanA gene and none of them contained vanB. Likewise, out of 43 L. monocytogenes isolates, 7 (16.3 %) were phenotypically resistant to penicillin G, however, only 6 of the isolates (11.6 %) carried penA resistance gene. The same results were reported in earlier studies [29]. This inconsistency suggests that mutation in ribosomal protein gene or decreased outer membrane permeability can contribute to antimicrobial resistance phenotypes [58, 59].

A high resistance of L. monocytogenes to tetracycline and penicillin G was observed in the present study. Our findings were in agreement with a previous investigation, in which a high resistance of L. monocytogenes to tetracycline and penicillin G was also reported by AA Fallah, SS Saei-Dehkordi and M Mahzounieh [47], and O Rodas-Suárez, J Flores-Pedroche, J Betancourt-Rule, EJ Quiñones-Ramírez and C Vázquez-Salinas [39]. Tetracycline and fluoroquinolones are widely applied as growth supplement and therapeutic agents in Iranian fish farms, respectively. The presence of antibiotic-resistant L. monocytogenes as well as multi-drug resistant isolates in fish on the one hand and transmission of the pathogen through contaminated fish on the other hand, clarify major public health concerns associated with this pathogen.

Conclusions

In conclusion, recovery of potentially pathogenic L. monocytogenes from raw fish and environment of open-air fish market samples evidences the zoonotic potential of listeriosis. Hence, further surveillance of the prevalence of L. monocytogenes and also of emerging antibiotic resistance is required to enable the recognition of the contaminated foods, as well as ensure the effective antibiotic treatment.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

BR designed the study. HJ, MP and BR were responsible for isolation, antimicrobial susceptibility test, serotyping, virulotyping of the strains and drafted the manuscript. CYL, WFW, SI, and AA contributed ideas and edited the manuscript. All authors read, commented on, and approved the final manuscript.

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