Detection and pathogenicity of *Listeria monocytogenes* in common carp (*Cyprinus carpio*) fish in Baghdad, Iraq

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Abstract

A few reports are available for detection of *L. monocytogenes* in fish in Iraq, however, the current study was undertaken to investigate the potential role of Listeria spp. in common carp fish in Baghdad province, Iraq. A total of fresh thirty raw common carp (*Cyprinus carpio*) were purchased from fish sellers of various local markets in Baghdad city from (December 2017 to March 2018) The viscera was removed aseptically, the bacterial isolation and identification was conducted by a conventional culture method using Listeria selective media, biochemical tests and Vitek 2 for gram-positive. Pathogenicity of isolates was studied in vivo by inoculating mice with bacterium. Targeting virulence associated genes was used to detect the virulence and to confirm the *L. monocytogenes* isolates. The isolates were tested for antimicrobial susceptibility by disk diffusion method for 12 antibiotics. The results revealed that 6.66% of *L. monocytogenes* were identified from common carp fish viscera and the isolates were pathogenic in mice. *L. monocytogenes* virulence associated genes were detected in both isolates, while *L. innocua* virulence associated gene (Lin0372) was detected in one of the two isolates. The isolates were resistant to 7 out of 12 antibacterial drugs including tetracycline, ampicillin, methicillin, cefixime, oxacillin, cefotaxime and penicillin G. The results suggest that presence of *L. monocytogenes* in fish may have a serious role in public health hygienic in humans.

Keywords: *L. monocytogenes*, Lin0372, Antimicrobial susceptibility, PCR, Fish

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كشف وإمراضية *L. monocytogenes* في اسماك الكارب الشائع (*Cyprinus carpio*) في مدينة بغداد، العراق

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الخلاصة

لا توجد دراسات وافيه عن وجود جراثيم *L. monocytogenes* في الأسماك في العراق، فقد أجريت الدراسة الحالية للكشف عن جراثيم Listeria في أسماك الكارب الشائع في محافظة بغداد / العراق. تم شراء ثلاثين من اسماك الكارب الشائع *Cyprinus carpio* من بائعي الأسماك من مختلف الأسواق في مدينة بغداد للفترة من كانون الأول ٢٠١٧- أذار ٢٠١٨. تمت إزالة الأحشاء بطريقة معقمة. اجري العزل الجرثومي باستخدام الوسائط الانتقائية الخاصة بجراثيم Listeria والاختبارات الكيميائية الحيوية و Vitek 2 موجبة لصبغة كرام. تم إجراء فحص الإمراضية بحقن العزلات في الفئران، استخدام جينات ضراوة للكشف عن ضراوة وتأكيد عزلة *L. monocytogenes*. تم اختبار عزلات *L. monocytogenes* لمعرفة مدى حساسيتها للمضادات الحيوية باستخدام اثني عشر من المضادات الحيوية. كشفت النتائج عن عزل عزلتان من *L. monocytogenes* ٦،٦٦% من سمك الكارب الشائع وكانت العزلات ضارية في الفئران. وان كلا العزلتين يحملان جينات الضراوة الخاصة *L. monocytogenes* إلا أن واحده من العزلات كانت حامله للجين

Lin0372 الخاص بال *L. innocua*. إضافة الى ذلك وجدت أن العزلات كانت مقاومة لسبعة من أصل أثني عشر الأدوية المضادة للبكتيريا التأليه التتراسيكلين، الأمبيسلين، الميثيسيلين، السيفإكسيم، الأكسنسلين والسيفوتاكسين والبنسلين G. تشير النتائج إلى أن وجود *L. monocytogenes* في الأسماك لها دور على الصحة العامة.

Introduction

Listeria spp. are distributed in nature, soil, sewage, dust and water, and isolated from poultry, dairy products and domestic and wild animals. *L. monocytogenes* is considered emerged and an important foodborne pathogen causing severe diseases in humans especially in low immune system patients, including the pregnant women, elderly and newborns (1,2). Listeriosis infection has increased with increasing of ready-to-eat, heat-and-eat food product consumption. Also, *Listeria* spp. were isolated from food products and several studies have shown that the seafood such as fish and fish products could be contaminated with *L. monocytogenes.* Thus, it is considered as the important cause of a number of sporadic listeriosis caused by consumption of fish and its products (1-6). Many virulence factors have been used to detect the pathogenicity of *L. monocytogenes*, these include listeriolysin O (*hly*A), intenrnalins (*inl*A, *inl*B), phosphatidyl-inositol-phospholipase C (PI-PLC, plcA), actin (actA), virulence regulator (*prf*A) and Iap (invasion associated protein) (7,8). *L. monocytogenes* infections in human are caused by serotypes 1/2a, 1/2b, 1/2c and 4b strains, and 4b serovar is responsible for all major outbreaks of invasive listeriosis (7,9). Differentiation of *L. monocytogenes* from other *Listeria* spp. may not be distinguished using conventional techniques such as selective and differential media and standard biochemical tests for differentiation of *L. monocytogenes*, *L. innocua*, *L. ivanovii* and *L. seeligeri* strains that show similar results to *L. monocytogenes* in these tests (10,11). It was found that 1.4% of *L. monocytogenes* isolates were misidentified as *L. innocua* using Vitek 2 (12). Therefore, *Listeria* spp. confirmation must be performed by molecular methods such as polymerase chain reaction (PCR) and sequencing (12,13).

Although *L. monocytogenes* has been reported sensitive to many antibiotics, on the other hand, it has been reported as multi-drug resistant in other studies (7,14). Accordingly, this study aimed to detect and pathogenicity of *L. monocytogenes* in fish in Baghdad city, Iraq.

Materials and methods

Ethical approval

The study was carried out in the laboratory of Zoonotic Diseases unit, College of Veterinary Medicine, University of Baghdad, from Dec 2017-March 2018, and approved by the Animal Care and Use Committee (Approval No. 1788/25 Nov 2017).

Isolation of *Listeria*

A total of 30 fresh raw common carp (*Cyprinus carpio*) fish purchased from fish sellers of various local markets in Baghdad city from December 2017-March 2018were subjected to cultural and PCR methods to detect of *L. monocytogenes.* Isolation was carried out according to (15,16). By using 70% ethanol and povidone-iodine fish skins were cleaned and disinfected, after that fish was opened with a sterile scalpel blade and viscera was removed to a sterile mortar and pestle containing sterile normal saline and well ground. Five ml of suspension were inoculated into 15ml Listeria enrichment broth (LEB) (Himedia) and incubated at 37ºC for 24 hrs. Then a loopful from LEB was streaked on to PALCAM agar (Himedia and incubated at 37ºC for 24 hrs. Colonies surrounded by a black zone on PALCAM agar plates were picked and inoculated onto HiChrome *Listeria* agar (Himedia) then a bluish green colony were selected and stained with Grams' stain. Further confirmation was applied by Vitek 2 Gram-positive identification card.

Pathogenicity in mice

The pathogenicity of *Listeria* isolates in mice was investigated according to Menudier and his co-worker method (17). Briefly, the isolates were grown on trypticase soya agar plate at 37°C for 24 hrs. Then, 3-5 colonies were picked and homogenized with sterile normal saline and the of bacterial suspension was adjusted to McFarland tube No. 0.5 that's equal to 10^8 CFU/ml . To each isolate, three Balb/c mice weighing 18-20g were injected intraperitoneally (I/p) with approximately 5×10^7 CFU in 0.5 ml. Control animals were injected with 0.5 ml (I/p) with normal saline. The animals were observed for 7days.

Molecular identification

The genomic DNA of the isolates was extracted according to the protocol of a commercial Wizard Genomic DNA purification Kit, Promega. PCR on 16S rRNA was applied using 27F primer (F-GAG TTT GAT CTT GGC TCA G) and 1492R primer R-TAC GGT TAC CTT GTT ACG ACT T) yielding about 1,300 bp (18).

The PCR reaction mixture volume of 25 µl included: (PCRpremix12.5µl, forward primer 1 µl, revers primer1 µl, nuclease free water 8.5 µl and finally DNA 2 µl). After that the amplification was done inside thermo-cycler (BioRad, USA) using the following program: initial denaturation at 95ºC for 5 min and1cyle; denaturation at 95ºC for 30 sec and 30 cycles; annealing at 60ºC for 45 Sec and 30 cycles and extension at 72ºC for 1 min and 30 cycles and final

extension at 72ºC for 7 min and 1 cycle hold at 4 for 10 min and 1 cycle. To confirm the presence of the amplified DNA fragments, 1% agarose gel electrophoresis was performed. For sequencing, the PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer by Macrogen Corporation, Korea. The similarity was applied using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) for analyzing the results.

PCR targeting virulence genes

A listed of specific primers (Table 1) were used for the detection of virulence genes and to distinguish *L. monocytogenes* from *L. innocua* using PCR *L. innocua* associated genes (Lin0372, Lin0454-lin0455) and *L. monocytogenes* associated genes (*prf*A, actA, *hly*A) were detected. The PCR reaction mixture containing a total volume of 20 µl, included: PCR (premix10 µl, 1 µl of each forward primer and reverse primers, nuclease free water 6 µl and DNA 2 µl).The amplification was transported to thermo-cycler (BioRad, USA) to start the Program: initial denaturation 2 min at 95 ºC, 1 cycles; denaturation for 30sec at 95 ºC, 35 cycles; annealing for 45sec at 57 ºC for Lin0372, Lin0454-lin0455 and *prf*A, and annealing of actA and *hly*A 60 ºC for 45sec, 35 cycles; extension 1min at 72 ºC, 1cycle; final extension 7min at 72 ºC, 1cycle;hold at 10°C for 10 min,1cycle.After PCR amplifycation,1%agarosegel electrophoresis was adopted to detect the presence of amplification.

Table 1: Primers used in this study

Primer	Sequences	Product size bp
Lin0372-F	5`-CGTTGTTTTGGCGTTGTCGATTGTTATTG-3`	1675 (10)
Lin0372-R	5`-TACACGATGATCTCCTGTTGCTGGTAG-3`	
Lin0454-lin0455-F	5`-GGATTTGGTAAATTATACAAAGGTTTTAAG-3`	849 (10)
Lin0454-lin0455-R	5`-TGCTTCTTGGCATTTTAGTAATCTTTC-3`	
*prf*A-F	5`-GTATTTTTCTATATGATGGTATCACAAAGCTC-3`	540 (10)
*Prf*A-R	5`-CATATCTTTTGAGATAATCAAGATTTTGTAC-3`	
actA-F	5`-CGCCGCGGAAATTAAAAAAAGA-3`	839 (7,16)
actA-R	5`-ACGAAGGAACCGGGCTGCTAG-3`	
*hly*A-F	5`-GCAGTTGCAAGCGCTTGGAGTGAA-3`	456 (3,16)
*hly*A-R	5`-GCAACGTATCCTCCAGAGTGATCG-3`	

Antimicrobial susceptibility test

The *L. monocytogenes* isolates from carp fish were tested for susceptibility to antimicrobial drugs using disk diffusion method. Mueller-Hinton (MH) agar was used as described by Ling-ling and his co-worker method (19). Twelve antibiotics disk were used including: oxacillin (Ox,1µg), penicillin G (p,10µg), Ampicillin (AMP, 25 µg), chloramphenicol (C, 10 µg), Methicillin (ME, 5 µg), Imipenem (IPM, 10 µg), cefixime (cFm, 5 µg), cefotaxime (CTx,30µg), Ciprofloxacin (CIP,10µg), Tetracycline (TE, 10 µg), Amikacin (AK, 30 µg) and Ofloxacin (Ofx, 10 µg).

Results

Isolation and identification

The results revealed the isolation of only two isolate of *Listeria* spp. 6.66%. On HiCrome Listeria agar, a bluish green colony were observed with ß hemolysis on blood agar. Gram positive rods, catalase positive, ferment lactose and L-rhamnose and showed CAMP test with *Staphylococcus aureus* giving 97% *L. monocytogenes* by Vitek 2.

Isolates pathogenicity in mice

The results of pathogenicity indicated that the two isolates were found to be pathogenic to mice, as all mice were dead within 72 hrs. *L. monocytogenes* was isolated from liver and spleen of dead animals, while control animals remained alive.

Molecular identification

The results obtained using 16S rRNA partial sequencing and analyzed using GenBank databases, showed that the first isolate had100% homology with *L. monocytogenes.* While the second isolate had 99.3% homology with the corresponding genes of *L. innocua* strain ATCC 33090 in (NCBI).

Targeting virulence associated genes

Targeting virulence associated genes were used to detect the virulence of the isolates and to confirm isolates of *L. monocytogenes.* The first *L. monocytogenes* isolate had *L. monocytogenes* virulence-associated genes and lacked *L. innocua* virulence-associated genes that confirmed it was *L. monocytogenes.* While second isolate also had *L. monocytogenes* virulence genes and lacked *L. innocua* virulence genes except harboring to Lin0372 gene

as shown in (Figure 1), so this confirms that the second isolate was also *L. monocytogenes.*

Sequencing

Partial sequences of the 16SrRNA gene of second *L. monocytogenes* strain was deposited in GenBank, with the accession numbers MH092995.1.

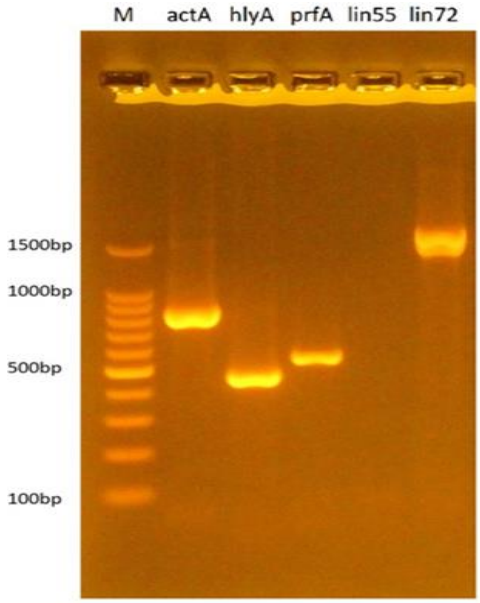

Figure 1: Agarose gel electrophoresis shows the presence of *L. monocytogenes* and *L. innocua* virulence associated genes of second *L. monocytogenes* isolate was fractionated on 1% agarose gel stained with Eth.Br. Lane M:100bp DNA marker. actA shows 839pb; *hly* 456 pb; *prf*A 540pb; Lin0372 (Lin72) 1675pb; Lin0454-lin0455 (Lin55) 849pb (not present).

Antibacterial susceptibility test

The *L. monocytogenes* isolates showed resistance to 7 out of 12 antibiotic drugs including: oxacillin, penicillin g, ampicillin, cefotaxine, cefixime, methicillin and tetracycline, while sensitive to amikacin, chloramphenicol, imipenem, ciprofloxacin and ofloxacin.

Discussion

L. monocytogenes has been reported from food products in Iraq, including meat and meat products, milk and dairy products (20-22). In fish, few reports, particularly on the molecular level are available. Our results showed that the detection of *L. monocytogenes* in carp fish was 6.66%. Compared with other reports, *Listeria* spp. was isolated from 13% of frozen fish, and *L. monocytogenes* represented 5% in Basra city of Iraq (23). In Iran, *L. monocytogenes* isolate was represented 7.6% and 7.72% reported by Jamali et al and Momtaz and Yadollahi (4,24). A higher percentage reported 37.65% in fresh fish in Nigeria by Lennox *et al*. (25). *L. monocytogenes* is considered as an important human pathogen, and our results revealed that the fresh common carp contain *L. monocytogenes* in their viscera due to infection or normally found. Therefore, the present of *L. monocytogenes* in viscera of fish may lead to contaminate fish meat and then might be a source of foodborne pathogens during the processing procedure. According to culture, biochemical tests, ß hemolysis on blood agar, catalase positive, fermentation of both L-rhamnose and lactose and vitek 2, in addition to pathogenicity study in mice indicated that isolates were *L. monocytogenes.*

The results of 16S rRNA partial sequencing showed that the second isolate had a similarity of 99.3% with *L. innocua* ATCC 33090.After detecting the specific genes, this isolate had *L. monocytogenes* genes and lacked *L. innocua* genes Lin0454-lin0455 but had Lin0372 gene. High similarities of 99.5% and 99.9% exist between *L. monocytegenes, L. innocua, L. ivanovii, L. seeligeri,* and *L. welshimeri.* Genetic similarity is found between *L. monocyto*-genes serovar 4a strains and *L. innocua* that possesses *L. innocua*-specific genes lin0372 and lin1073. In contrast, *L. innocua* ATCC 33090 has no mortality in the murine intraperitoneal infection (26). Thus, this isolate is not *L. innocua* ATCC 33090 but *L. monocytogenes* and may be serovar 4a. In addition, these isolates are pathogenic due to the presence of virulence genes and causing mortality in mice. Similar results have been reported, *L. monocytogenes* harboring virulence genes actA, *hly*, *prf*A and *plcA* and generally pathogenic in vivo model of infections (16,27,28).

The *L. monocytogenes* isolates show resistance to oxacillin, penicillin g, ampicillin, cefotaxime, cefixime, methicillin and tetracycline. *L. monocytogenes* isolates have been detected as multidrug resistant such as tetracycline, ampicillin, erythromycin, gentamicin, trimethoprim-sulfa-methoxazole, chloramphenicol, rifampin and streptomycin (4,29,30).

Other studies reported *L. monocytogenes* sensitivity to ampicillin, chloramphenicol, rifampin, tetracycline, gentamicin, erythromycin, vancomycin, penicillin and imipenem (31,32). Ampicillin is commonly used in the treatment of *Listeria* infections, and a high antibiotics resistance of *L. monocytogenes* strain, with broad used of antibiotics, thus multidrug resistant *L. monocytogenes* have been isolated from various food samples. The presence of multidrug resistant *L. monocytogenes* will have a negative impact on the public health and safety of food (18). This study indicated the presence of *L. monocytogenes* in viscera of raw common carp fish. Therefore, for public health hygiene, viscera should be removed immediately from the fish after harvesting to prevent contamination other tissues.

Hygienic methods should be carried out during transportation and handling processing of fish and its products. In addition to conventional methods, it is necessary to use the virulence associated genes to distinguish between the *Listeria* spp. and to detect the pathogenic strains.

Conclusion

Our results suggest that *L. monocytogenes* isolated from fish have the potential role in public health hygienic in humans.

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Conflict of interest

None, there is any conflict of interest.

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