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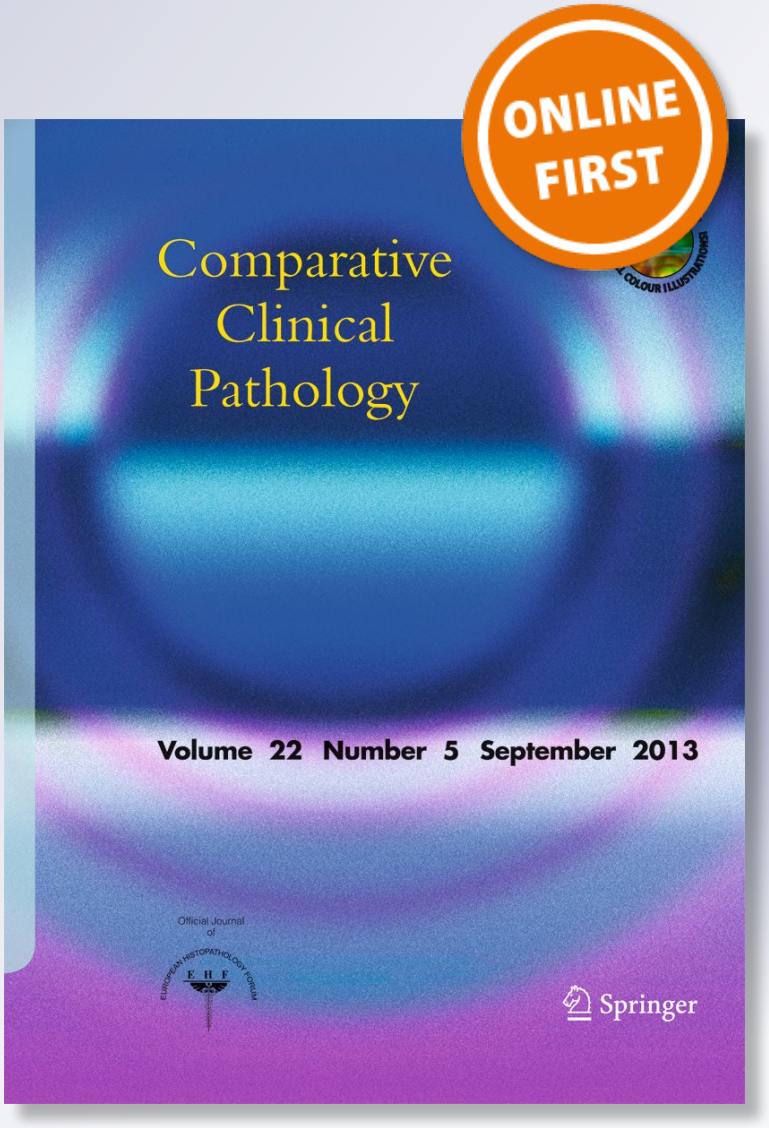

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ORIGINAL ARTICLE

Prevalence of *Listeria monocytogenes* in cerebrospinal fluid obtained from hospitalized patients

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Abstract
The aim of the present study was to determine the occurrence of *Listeria monocytogenes* in cerebrospinal fluid (CSF) samples obtained from patients with meningitis symptoms. A total of 102 CSF samples which were referred for bacterial culture to microbiology laboratory in Mashhad's Hospital were included in this study. All of the samples were cultured and positive ones confirmed by multiplex-PCR. Molecular serotyping and virulence characterization of isolates were performed. Random amplified polymorphic DNA-PCR using three set of primers was performed for evaluation of genetic diversity of these isolates. Five out of 102 samples were positive for *L. monocytogenes*. Three different serotypes were detected, and all of the isolates were potentially pathogenic. Low genetic diversity was determined among isolated *L. monocytogenes*.

Keywords *L. monocytogenes* · Serotyping · CSF · RAPD

Introduction

Listeria monocytogenes (*L. monocytogenes*) is a gram-positive bacterium, which is motile at ambient temperature (Vazquez-Boland et al. 2001). Transmission of *Listeria monocytogenes* to humans is occurred via direct contact with animals, cross-infection of newborn babies in hospital, and food-borne infection. The latter two ways were the cause of majority of human listeriosis. Listeriosis is an uncommon but a serious food-borne disease that can be life-threatening to compromised patients and newborn babies (Frye et al. 2002) and has a high mortality rate (30% or higher) (Vazquez-Boland et al. 2001).

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L. monocytogenes is a widespread microorganism and could be isolated from any types of food. The ability of *L. monocytogenes* to grow at refrigerator temperature arose some problems with ready-to-eat (RTE) food products (Liu 2006). Human listeriosis began with some unspecific flu-like symptoms such as chills, headache and musculoskeletal pain, and gastroenteritis. But, it can be developed into septicemia, meningitis, encephalitis, abortion, and in some cases even death without appropriate antibiotic therapy (Vazquez-Boland et al. 2001). Therefore, the accurate diagnosis of disease is very essential. Moreover, virulence properties of *L. monocytogenes* strains may also influence on the course of infection and clinical outcome (Swaminathan and Gerner-Smidt 2007).

1/2a, 1/2b, and 1/2c are the three most frequently isolated serotypes of *L. monocytogenes* from food and environment (Vazquez-Boland et al. 2001). However, 1/2a, 1/2b, and 4b serotypes are the causes of more than 95% of outbreaks in humans. Listeriosis outbreaks are mostly caused by 4b serotype of *L. monocytogenes* (Swaminathan and Gerner-Smidt 2007).

Moreover, *L. monocytogenes* present in low numbers in clinical sample such as CSF; then, isolation of bacteria is very difficult. Use of molecular techniques including polymerase chain reaction (PCR) may be helpful in discovering listeria in these clinical specimens. Several specific genes of *Listeria* species could serve as good targets in PCR assay (Doumith et al. 2004; Liu 2006; Liu et al. 2007).

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The *prs* gene is one of them that encodes the putative phosphoribosyl pyrophosphate synthetase. Moreover, *prfA* gene of *Listeria monocytogenes* is another good target for detection of *L. monocytogenes*. Therefore, the *prs* and *prfA* genes have the potential of being appropriate targets for detection of *L. monocytogenes* by m-PCR.

Although there are some reports of *L. monocytogenes* from foods of animal origin and clinical cases in animals (Fallah et al. 2012; Safarpoor Dehkordi et al. 2013) but, no epidemiological data on human listeriosis were available in Iran. The present study describes the detection of *L. monocytogenes* in CSF of patients whom admitted to Mashhad's hospitals. Serotyping and virulence potential determination of the isolates were performed. Also, genetic relatedness of the isolates were determined by random amplified polymorphic DNA-PCR (RAPD-PCR).

Materials and methods

Samples

A total of 102 CSF samples were collected from bacteriology laboratory of three main hospitals in Mashhad. CSF samples were obtained via lumbar puncture and were submitted for microbiological analysis. They were taken from patients with different ages and sex, whom hospitalized because of meningitis symptoms.

Patient characteristics

Medical records were reviewed for data regarding age, sex, and results of culture of CSF for all specimens. In the positive samples, associated illnesses and kind of antimicrobial therapy were also investigated. On the basis of age, samples were categorized in three groups. "Neonate and infant group" consisted of samples from 0 day to 2 years of age, "Children and adults group" consisted of 2- to 65-year-old patients, and "Elderly group" consisted of patients with age of more than 65 years.

Isolation of *Listeria monocytogenes*

One milliliter of each CSF sample was added to 9 ml of Listeria Enrichment Broth (LEB, Merck, Germany), and isolation of bacteria was done according to Zeinali et al. (2015a).

Multiplex-PCR for *Listeria monocytogenes* confirmation

DNA extraction from suspected colonies was performed using boiling method (Adzitey et al. 2013). Confirmation of *Listeria* spp. and *Listeria monocytogenes* in multiplex-PCR assay was performed by *Prs* and *LM lip1* primers, respectively according to Zeinali et al. (2015a). The sequences of primers and predicted size of the amplified products are shown in Table 1.

Table 1 Sequence of primers

Primer	Sequence (5′-3′)	Reference
D8635	*GAGCGGCCAAAGGGAGCAGAC*	Cocolin et al. (2005)
HLWL74	ACG TAT CTG C	Park et al. (2012)
OPM01	GTT GGT GGC T	Lawrence et al. (1993)
Prs	GCT GAA GAG ATT GCG AAA GAA G	Doumith et al. (2004)
LM lip1	CAA AGA AAC CTT GGA TTT GCG G GAT ACA GAA ACA TCG GTT GGC	Wernar et al. 1992
lmo0737	GTG TAA TCT TGA TGC CAT CAG AGGGCTTCAAGGACTTACCC	Doumith et al. (2004)
lmo1118	ACGATTTCTGCTTGCCATTC AGGGGTCTTAAATCCTGGAA	Doumith et al. (2004)
ORF2819	CGGCTTGTTCGGCATACTTA AGCAAAATGCCAAAACTCGT CATCACTAAAGCCTCCCATTG	Doumith et al. (2004)
ORF2110	AGTGGACAATTGATTGGTGAA	Doumith et al. (2004)
inlC	CATCCATCCCTTACTTTGGAC AATTCCCACAGGACACAACC	Liu et al. (2007)
InlJ	CGGGAATGCAATTTTTCACTA TGTAACCCCGCTTACACAGTT	Liu et al. (2007)
hlyA	AGCGGCTTGGCAGTCTAATA GCAGTTGCAAGCGCTTGGAGTGAA GCAACGTATCCTCCAGAGTGATCG	Doijad et al. (2011)

Listeria monocytogenes (ATCC 7644) and deionized distilled water were used as positive control and negative control, respectively. Presence of 274-bp and 370-bp bands was considered to be a positive result for *Listeria monocytogenes*. The reliability of this PCR assay has been investigated previously (Wernar et al. 1992; Doumith et al. 2004). Also, the presence of PCR inhibitors and/or degradation of DNA in samples were examined through spiking negative specimens with *L. monocytogenes* ATCC 7644. It would minimize the potential for false negative assay results.

Multiplex-PCR for serogroup identification

A multiplex-PCR assay was carried out to separate the major *L. monocytogenes* serovars (1/2a, 1/2b, 1/2c, and 4b) into distinct serogroups (Zeinali et al. 2015a). The marker genes selected, according to previous studies (Doumith et al. 2004), were *lmo0737*, *lmo1118*, *ORF2819*, and *ORF2110*. The *prs* gene, specific for *Listeria* spp., was used as an internal amplification control. Target genes, primer sequence, and PCR products size are listed in Table 1.

Reference strains ATCC 7644 and IBRC 10671 (which is equivalent to strain ATCC 13932) were used as positive controls for each amplification assay and deionized distilled water as negative control. Finally, 4 μl of the PCR products was separated on a 1.5% agarose gel (Merck, Germany) and visualized on a transilluminator after green viewer staining.

Detection of virulence genes

Detection of *inlC* (517 bp), *InlJ* (238 bp), and *hlyA* (456 bp) virulence genes of *Listeria monocytogenes* isolates was carried out according to Zeinali et al. (2015a).

Random amplified polymorphic DNA analysis

The five isolates were analyzed using RAPD-PCR with three different primers, including D8635 (Cocolin et al. 2005), HLWL74 (Park et al. 2012), and OPM01 (Lawrence et al. 1993), which had been previously published according to Zeinali et al. (2015b). Table 1 shows the sequence of these primers. Table 2 shows the amplification conditions of these three primers.

Interpretation of PCR fingerprint images

Scanned images were analyzed using Photocap software. Bands were assigned on a presence-absence basis. The software estimated band sizes for RAPD-PCR data. The data were analyzed using SPSS software. Because of binary data, Jaccard distance matrix and Ward's hierarchical cluster technique were used; isolates were clustered and displayed in dendrogram form. Also, in order to declare the effect of sex and age on the occurrence of *L. monocytogenes*, chi-square test by using SPSS software (version 16) was performed.

Results

Prevalence of *L. monocytogenes*

Five out of 102 samples showed colonies with aesculin hydrolysis on Oxford agar. These colonies were also catalase positive and showed umbrella form motility at 25 °C. In the m-PCR assay, these five samples had the 370 bp of *prs* and 274 bp of *prfA* genes and confirmed as *L. monocytogenes*. These positive samples were obtained from patients who were referred to hospital with complaints of fever, nausea, weakness, and lethargy. All of them were suspected to bacterial meningitis accompanied by other symptoms. 19.2% of patients were neonates and infants. 50.5 and 30.3% of patients were grouped as adults and elderly age group, respectively. 39.8% of samples were taken from women and 60.2% of them belonged to male patients.

The demographic characteristics, associated illnesses, and data regarding antimicrobial therapy for the positive samples are summarized in Table 3. In the routine microbiological testing in microbiology laboratory of hospital, only in one sample, the CSF culture was positive. It was the sample which received no antibiotic before lumbar puncture. In statistical analysis, Fisher's exact test did not show any significant effect ($P < 0.05$) of sex

Table 2 Amplification conditions of RAPD primers

Primer	Initial denaturation	Amplification reaction	Final extension
D8635	94 °C for 4 min	35 cycles, 1 min at 94 °C, annealing at 39 °C for 45 s; and extension at 72 °C for 1 min	–
HLWL74	95 °C for 4 min	45 cycles, 1 min at 95 °C, 2 min at 35 °C, and 1 min at 72 °C	72 °C for 10 min
OPM01	–	44 cycles, 94 °C for 1 min, 2 min at 30 °C, and 72 °C for 2 min	Final cycle, 94 °C for 1 min, 30 °C for 2 min, and 72 °C for 10 min

Table 3 Demographic characteristics of positive samples

	1	2	3	4	5
Age	No data	Neonates	45 years	82 years	24 years
Sex	No data	No data	Man	Woman	Man
Receipt of antibiotics before lumbar puncture	No data	No data	Ceftriaxone and clindamycin	Vancomycin and cefeprime	–
Discharge from hospital while receiving antibiotic therapy	No data	No data	Azithromycin	Co-amoxyclav and theophylline	Vancomycin and Ceftriaxone
Primary diagnosis	No data	Meningitis	Fever + encephalitis + pneumonia	Fever, nausea, vomiting, pneumonia + meningitis	Fever + headache + nausea

and age on the occurrence of *L. monocytogenes* in CSF. It may due to low number of positive samples.

Serogrouping of *L. monocytogenes* and investigation of virulence genes

Among these five isolates, two of them recognized as 4b and one of them as 1/2b serogroup. The other two isolates were recognized as 1.2a/1.2c serogroup. All of the five human isolates of *L. monocytogenes* showed the presence of *inlC*, *inlJ*, and *hlyA* virulence genes.

RAPD-PCR

All of the primers produced amplified bands. Two primers (D8635 and OPM01) have produced 18 different bands whereas HLWL74 primer produced 17 bands. Although the number of isolates was low, but among these three primers, D8635 had the best discriminatory power due to producing most polymorph bands. Totally, 53 different bands with sizes ranging from 150 to 3300 base pair (bp) were produced. Twenty-eight bands were polymorph. According to our results, 52.83% of polymorphisms were detected among these isolates. Otherwise, genetic diversity was low among these five isolates.

In our study, among five human isolates of *L. monocytogenes*, 20 bands were detected as monomorph (which were seen in one isolate) and five bands as unique bands (which were seen in four isolates). Similarity matrix was constructed based on the presence or absence of a band for each isolate which was scored as 1 and 0, respectively. The most similarity was observed between isolate Nos. 4 and 5, which were isolated from one hospital. In this study, isolates of *Listeria* were classified into three clusters (designed as I to III) (Fig. 1). Isolates 1 and 2 were associated with cluster I and II, respectively. Three other isolates (3, 4, and 5)

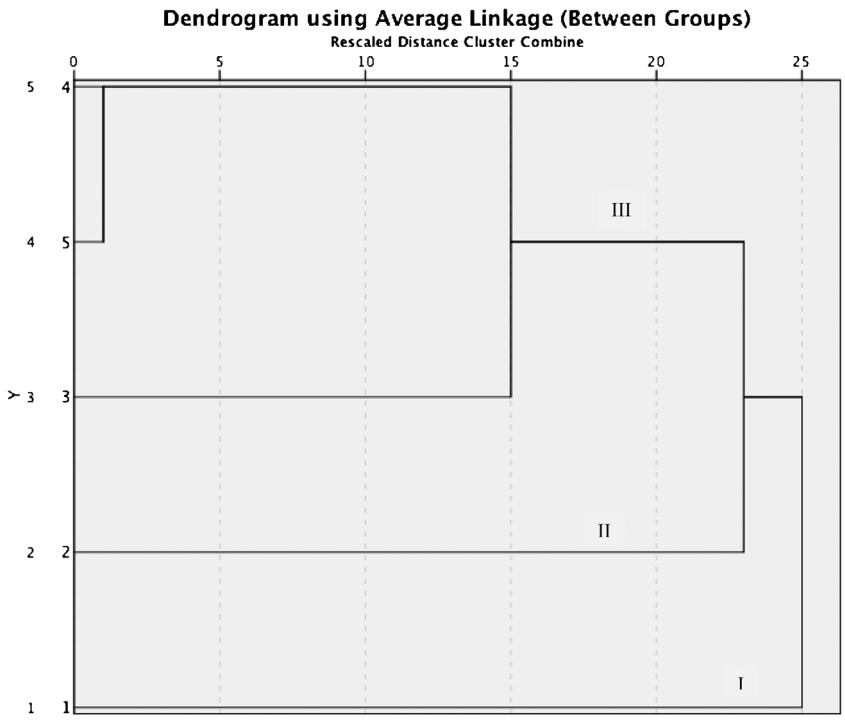

Fig. 1 Dendrogram of *Listeria monocytogenes* isolates with different clusters

were grouped into cluster III. Every cluster consisted of the isolates of one hospital uniquely. Cluster III contained isolates which all of these patients had similar signs while admitted to hospital. Isolate No. 2 which constituted cluster II was isolated from a neonate. Control assays, which contained no cell lysate, yielded no detectable amplified product. RAPD-PCR showed more than 95% repeatability.

Discussion

In the present study, *L. monocytogenes* was recovered from five out of 102 CSF samples. Adults with the age of 65 and older constitute more than half (58%) of *Listeria*-infected persons. Risk of *Listeria* infections in this group is four times greater than that in general population (CDC 2013). In other studies, different samples and different rate of isolation were reported; Amaya-Villar et al. (2010) reported *L. monocytogenes* meningitis in 46 out of 278 cases of acute community-acquired bacterial meningitis (Ac-ABM) (Amaya-Villar et al. 2010).

Our findings, which show the role of *L. monocytogenes* as a causative agent of human meningitis and meningoencephalitis, are in agreement with the earlier reports on the isolation of *L. monocytogenes* from 4.2% of blood samples (Tabatabaie et al. 2012) and CSF of patients with CNS listeriosis (Monnier et al. 2011). Reports of listeriosis from humans in Iran are rare, either because of failure to identify the isolates due to poor isolation techniques or low incidence rate. In another study, among 145 patients, 14 of them were diagnosed as meningitis due to *L. monocytogenes* by using conventional PCR. In this study, all but one of the CSF cultures were negative for *L. monocytogenes* (Piana et al. 2005).

In the present study, five isolates of *L. monocytogenes* were recovered from CSF of patients. Three isolates were detected as highly pathogenic serotypes (4b and 1.2b), and two isolates were detected as low pathogenic serotype (1.2a); one pathogenic 4b isolate of *L. monocytogenes* (isolate 4) was recovered from an old woman. The history of the patient revealed that she was admitted in hospital with complaints of fever, nausea, vomiting, weakness, general malaise, and lethargy. She had a history of Alzheimer from 2 years ago and lived at an elderly healthcare center. She was admitted with suspicious of meningitis. Another 4b serotype of *L. monocytogenes* (isolate 3) was recovered from a 45-year-old man with a history of unconsciousness, speechless, seizure, and paresis of left side of the body. At the clinical examination, fever and other signs of encephalitis were observed. Among patients suffering from meningoencephalitis, the rate of isolation of serotype 4b is rather higher than in patients suffering from blood stream infection (Swaminathan and Gerner-Smidt 2007).

In this study, one of the isolates which had been diagnosed as 1.2a serotype was recovered from a 24-year-old man (isolate 5). He was admitted to hospital with complaints of fever, headache, and nausea without vomiting. At the examination, signs of meningitis were observed. Isolate of 1.2b serotype was recovered from a neonate. During an outbreak of febrile gastroenteritis, *Listeria monocytogenes* was isolated from one blood specimen and from 87.23% of stool specimens (Aureli et al. 2000). All *listeria* isolates were serotype 4b and were found to be identical on DNA analysis (Aureli et al. 2000). Kiss et al. (2006) evaluated the incidence of human listeriosis in Hungary during 2004. Three perinatal and 14 nonperinatal human listeriosis cases were diagnosed. Serotype 4b was the most common serotype accompanied by these diseases as found in 52.8% of cases. At the second-line serotype, 1/2a occurred in 23.5% of the cases. In another study, among the nine isolates of *L. monocytogenes* from blood and CSF of patients with clinical listeriosis, most prevalent serotype was 1/2b (4) and then 1/2a (3) serotype. Two of the isolates were recognized as 4d serotype (Nappi et al. 2005).

In our study, all of the five isolates of *L. monocytogenes* showed the presence of *inlC*, *inlJ*, and *hlyA* virulence genes. Cabrita et al. (2010) showed that LLO was always present in 1/2a, 4b, and 4c serovars, but *inlC* was only present in virulent strains (serovars 4b and 1/2a) and was absent in low virulent strain (serovar 4d/4e/4c) (Cabrita et al. 2010).

In the present study, five isolates of *L. monocytogenes* were analyzed by three primers in RAPD-PCR. Cluster III contained three isolates, in which two of the isolates were 4b serotype and one of them was 1.2a serotype. O'Donoghue et al. (1995) observed that the 14 epidemiologically related serovar 4b isolates gave indistinguishable profiles with the exception of one isolate. Each cluster of I and II contained one isolate which was serotype 1.2a and 1.2b, respectively. In our study, RAPD-PCR could differentiate isolates from one serogroup. Similar findings were observed in other studies (O'Donoghue et al. 1995; Mazurier and Wernars 1992; Macgowan et al. 1993). Franciosa et al. (2001) reported the clinical isolate of serotype 1/2a, clustered with the 1/2b isolates, while using RAPD.

Macgowan et al. (1993) showed that serotyping has less discriminatory ability than RAPD as at least six different RAPD profiles were obtained from eight 1/2a serotypes of *L. monocytogenes*. Also, three profiles were obtained from the eight serovars of 1/2b isolates of *L. monocytogenes*, and six profiles from ten serovars of 4b isolates. Inoue et al. (2001) reported that 1/2b and 4b serotypes of *L. monocytogenes* were distinguished from 1/2a and 1/2c serotypes in the RAPD. Moreover, serovar 4b was distinguished from serovar 1/2b by a difference in the RAPD sub-cluster category.

Conclusion

Our results emphasize that listerial meningitis is not uncommon, and listeriosis should be considered in the etiologic

diagnosis of fever and gastrointestinal disease. However, we believe that m-PCR may be useful for excluding the diagnosis of listerial meningitis and may assist in the decision to discontinue antibiotic therapy.

RAPD analysis with multiple primers is a low-cost test with high discriminatory power. We have shown that serogroup l/2 of *L. monocytogenes* strains is a more diverse group than serovar 4b strains, and RAPD-PCR will provide a technique of considerable value in typing of *L. monocytogenes*.

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Compliance with ethical standards All samples were taken according to international procedures.

Ethical approval This article contains no studies involving animal participants.

Conflict of interest The authors declare that they have no conflicts of interest.

Informed consent Informed consent was obtained from all individual participants included in this study.

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