Listeria monocytogenes meningoencephalitis: molecular methods for diagnosis and for monitoring the response to chemotherapy

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Abstract

Background. *Listeria monocytogenes* is one of the most important human foodborne pathogens; it may be responsible for several disorders, like meningoencephalitis. Listerial isolation in cerebrospinal fluid (CSF) is often difficult using microbiologic traditional assays. The aim of this study is to evaluate the reliability of molecular techniques as an alternative tool in order to identify *Listeria monocytogenes* meningitis and in particular, to evaluate a real-time PCR and a conventional PCR for the target *hly*A gene.
Methods. In 2000-2004, 145 patients, without T-cell immunodeficiency, affected by meningoencephalitis of unknown origin were admitted to the Infectious Diseases Institute of Sassari, Italy; a lumbar puncture was performed at the time of hospital admission. Two different PCR techniques, i.e. RT-PCR and a conventional PCR, were performed in order to detect CNS listerial infection, in conjunction with traditional microbiologic assays.
Results. We identified fourteen patients affected by listerial meningitis using RT-PCR and conventional PCR. All but one of the CSF cultures were negative for *L. monocytogenes*. Molecular techniques were performed on the CSF samples collected during follow-up revealing that signal intensity decreased by 40%, 80% and 100% at day 15, 30 and 55 respectively, from the start of antibiotic treatment.
Conclusions. Considering the seriousness of CNS involvement caused by *L. monocytogenes* infection, prompt diagnosis is necessary in order to rapidly start specific treatment. Conventional PCR and RT-PCR are rapid assays for *L. monocytogenes* diagnosis and they might be useful for monitoring the efficacy of antibiotic therapy.

Key words: L. monocytogenes, meningitis, PCR real-time, conventional PCR

Introduction

The first identified foodborne disease outbreaks caused by *Listeria monocytogenes* (*L. monocytogenes*), described twenty-five years ago, have contributed greatly to the increase in interest in human listeriosis by scientific researchers.[1]

L. monocytogenes, one of the most important human foodborne pathogen, is a small, nonsporulating, mobile at ambient temperature, gram-positive bacillus; it grows on blood agar, producing hemolysis and at refrigerator temperature (4-8°C).

The organism is widespread in nature; in fact, it may be isolated from soil, drain water, decomposed vegetables, forages, foods, animals and humans.

There are 13 known serotypes to date, but only 1/2a, 1/2 b, 1/2c and 4b are pathogenic for humans.[2]

Ingestion of foods like fresh and soft cheese, meat, fish [3,4] may be the cause of sporadic cases or outbreaks.[3-6]

L. monocytogenes may be the etiologic agent of bacteremia or localized infections, such as cervicitis, ophthalmitis, regional lymph node involvement, dermatitis, endocarditis, meningitis and/or encephalitis, especially in young children.[7,8,9] During pregnancy, women are prone to develop bacteremia; it may precipitate abortion or fetal disseminated infection.[7-9]

Serious clinical syndromes are described in immunocompromised hosts, whereas asymptomatic infection is described in subjects without cell-mediated immunity impairment.

Although numerous authors have described foodbourne listeriosis,[3,9,10] the relationship between food and illness is not easily proven because the incubation period, which ranges from 1 to 90 days, does not allow for immediate food testing and a reliable medical history to be taken.

Moreover, isolation of *L. monocytogenes* in clinical samples, such as CSF, is often difficult, because of the low bacterial burden; this difficulty might be overcome by collecting at least 10 ml of CSF, but it is not always feasible due to the risk of a reduction in intracranial pressure.[11]

Molecular techniques may be useful in overcoming the difficulties in identifying listerial bacteria in clinical specimens and suspected foods; as immediate diagnosis allows antimicrobial therapy and preventive measures to be deployed.

On this basis, the aim of this study is to evaluate the reliability of molecular techniques as an alternative tool in order to identify listerial meningitis as opposed to standard microbiologic techniques, often inadequate for diagnosis. In particular we aim to evaluate a real-time PCR (RT-PCR) and a conventional PCR, using the *hlyA* gene, a highly conserved genomic region and codifying for Listeriolysin O.

Methods

Patients and Cerebrospinal fluid (CSF) samples

In 2000-2004, 145 patients affected by meningoencephalitis of unknown origin were admitted to Infectious Diseases Institute of Sassari, Italy.

After the first clinical evaluation, all of them underwent a lumbar puncture and started empirical antibiotic treatment with ampicillin 3.0 gm q6h IV plus chloramphenicol 1.0 gm q12h IV and antiviral therapy with acyclovir 10 mg/Kg q8h IV; this therapy was switched when results of the CSF laboratory tests, such as agglutination test for bacterial antigens (Slidex Meningitis-Kit, bio-Mérieux), bacterial culture and PCR for *M. tuberculosis*, *L. monocytogenes* and neurotropic viruses indicated a specific pathogen.

In order to evaluate central nervous system (CNS) listerial infections two different PCR techniques were performed, i.e. a RT-PCR and a conventional PCR for the *hlyA* gene.

Oligonucleotides

Software Beacon Designer 2.0 (BioRad) was utilized in order to create primers for the RT-PCR (Biosense Srl), amplifying a 64 bp fragment on the basis of the listerial *hlyA* gene (GenBank accession n. M24199).The primer and probe sequences were:
*Hly*Sense
5'–GAGGTTCCGCAAAAGATGAAGTTC-3'
*Hly*antiSense
5'-AGGAAGTTTGTTGTATAAGCAATGGG-3'
*Hly*TaqMan probe
5'-FAM-ACGGCAACCTCGGAGACTTACGC-Black Hole 1-3'

Oligonucleotides, specific for the *hlyA* gene (5'-CGGAGGTTCCGCAAAAGATG-3' and 5'-CCTCCAGAGTGATCGATGTT-3'), codifying for *Listeriolysin* O and amplifying a 234 bp fragment, were used for the conventional PCR.[12]

Real-time PCR

One µl bacterial DNA, 1 X PCR TaqMan buffer, 6 mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 50 nM primers, 100 nM probe, 1 U Ampli*Taq* Gold DNA polymerase and 0.2 U AmpErase uracil N-glycosylase were added to the reaction mixture (20µl final volume).

Amplification and detection were performed using a real-time iCyclerTM PCR (Biorad), supplied with an optical unit for fluorescence reading.

Cycling parameters were: 50°C for 2 minutes, 95°C for 10 minutes, 50 cycles at 95°C for 15 seconds and 63°C for 1 minute.[13]

A standard curve, obtained by plotting threshold cycle against Colony Forming Units –CFUs- (log), was calculated by the iCycler iQ Real-Time PCR Detection System Software Version 3.1 (Biorad).

Conventional PCR

Amplification steps (40 cycles) were: denaturation – 92°C for 30 seconds, annealing – 53 °C for 30 seconds, extension – 72°C for 30 seconds.

Agarose gel electrophoresis separated amplified fragment, stained in a 0.5 mg/ml ethidium bromide solution. Electrophoretic bands were analyzed using VDS-ImageMaster (Amersham Pharmacia) and GelCompare II software (GelCompare II -Bionumerics), comparing their position with a marker (100 bp ladder), volume and amount of DNA.

Each sample in duplicate and one negative control were analyzed both for real-time PCR and conventional PCR in order to rule out false positive results due to contamination.

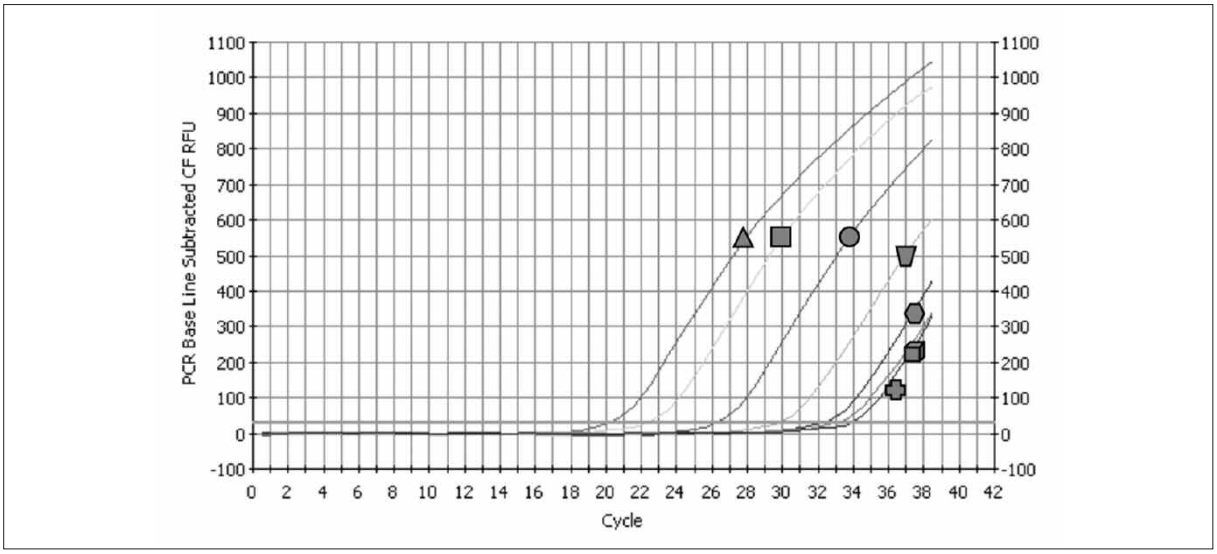
A standard curve, obtained by plotting DNA amount against band volume, was calculated by using the software GelCompare II.

Sensitivity and specificity assessment

In order to evaluate the specificity of the molecular techniques, ATCC strains or *L. monocytogenes*, *L. grayi*, *L. seeligeri*, *L. ivanovii*, *L. innocua* collections and organisms that most frequently cause bacterial meningitis or produce hemolysins like Listeriolysin O, such as *S. pneumoniae*, *S. pyogenes*, *H. influenzae*, *S. aureus*, were subjected to amplification; *Campylobacter jejuni* subsp. *jejuni*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Escherichia coli* O157:H7, *Salmonella enterica* serovar Enteritidis, *Salmonella Enterica* serovar Typhimurium and *Yersinia enterocolitica* were used as negative controls.

In order to evaluate the sensitivity and the cut-off for detection, serial dilutions of *L. monocytogenes*, cultured overnight, were prepared; they were

Figure 1. RT-PCR detection and amplification of hly sequences. Serial dilutions of L. monocytogenes genomic DNA, equivalent to 3 x 105 (▲), 3 x 104 (■), 3 x 103 (●), 3 x 102 (▼), 60 (⬟), 30 (❒), 15 (✚), CFU.



equivalent to 3×10^5, 3×10^4, 3×10^3, 3×10^2, 60, 30, 15, 8, 4 and1 CFUs.

A genomic DNA molecule, corresponding to 2.94 fg DNA on the basis of *L. monocytogenes* genome, was measured using a GeneQuant *pro* RNA/DNA Calculator (Amersham Pharmacia).

Statistical analysis

Paired Student's *t*-Test was used in order to show difference between the paired CFUs (log) obtained from RT-PCR and conventional PCR (p value: 0.05).

Results

Specificity and sensitivity of RT-PCR and conventional PCR

The sensitivity and specificity of RT-PCR and conventional PCR were evaluated using *L. monocytogenes* suspensions in sterile Milli Q water.

The lower limit for the detection of RT-PCR was 15 CFUs; a fluorescent signal, equivalent to 3×10^5 CFUs, was present after 18 amplification cycles (Figure 1). The lower limit for detection of conventional PCR was 100 CFUs, using the same dilutions.

In order to identify the presence of CSF inhibitors of PCR amplification, a *L. monocytogenes* suspension in CSF was prepared, at a bacterial concentration of 3×10^5. The results were15 CFUs and 100 CFUs for real-time PCR and conventional PCR, respectively. Two different techniques were used to extract DNA from the CSF samples: temperature exposure (98 °C for 15 minutes) and QIAamp Tissue extraction kit (Qiagen). No differences were identified.

On this basis, standard curves were generated; the curve, obtained by RT-PCR results, was characterized by a R^2 coefficient of 0.994 (i.e. high linearity; y = -3.343 x + 41.744) (Figure 2); this molecular assay was characterized by an elevated efficiency of 99.1% (Figure 2).

A standard curve was obtained from conventional

Figure 2. Representative standard curve generated from the amplification data.

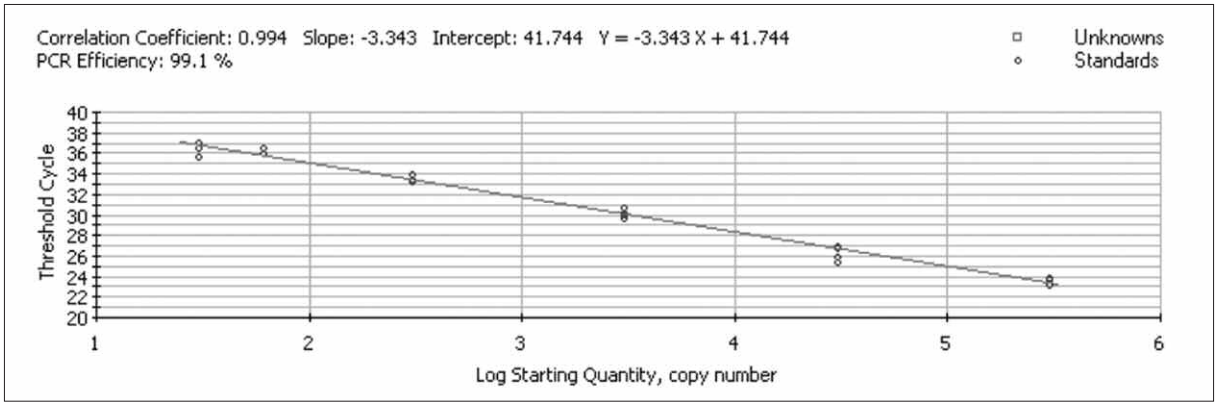
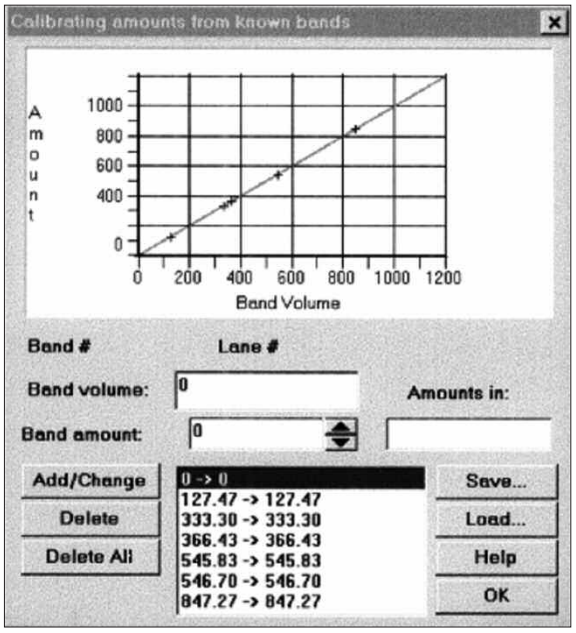


Figure 3. Conventional PCR: standard curve plotting bands volume against DNA amount.



PCR plotting of the optical density of electrophoretic bands, i.e. the amount of DNA derived from amplification of bacterial dilutions (Figure 3).

Standard curves for both RT-PCR and conventional PCR were repeated for every sample of CSF.

Specificity was 100% because the negative controls were not amplified.

Patients

Fourteen patients (14/145 = 9.7%; male : female *ratio* was 5 : 9; mean age was 30.2 years, range 14-67), admitted to the Infectious Diseases Institute of Sassari for meningoencephalitis of unknown origin, were affected by listerial meningitis, diagnosed using molecular assays; a specific clinical pattern was not identified at the time of hospital admission (Table 1). They were not immunocompromised; a possible source of listerial infection was identified in only three of the fourteen subjects admitted to the infectious diseases institute (one subject worked at a cheese factory, one ate a soft cheese and one, a fishmonger, was stung by the first dorsal fin of a large fish).

There was no evidence of a specific pattern of biochemical alteration of the CSF (Table 2).

The agglutination test for bacterial antigens, from the organisms that most frequently cause meningitis, was negative (*H. influenzae, S. pneumoniae, N. meningitidis, E. coli K1)*; all but one of the CSF cultures were negative for *L. monocytogenes*: small colonies, characterized by a translucid aspect and producing beta hemolysis, were detected using Columbia agar supplemented with 5% sheeps blood (bioMérieux). Listerial detection was confirmed by the Api Listeria system test (bioMérieux).

Antibiotic sensitivity was determined by Kirby-Bauer disc diffusion method; the isolated strain was resistant to ceftriaxone and sensitive to penicillin, chloramphenicol, ampicillin, meropenem, amoxicillin, amoxicillin as well as clavulanate potassium and imipenem.

Results of the molecular techniques used for *M. tuberculosis* and neurotropic viruses were negative, while CSF samples were positive for *L. monocytogenes* using conventional PCR and RT-PCR (positivity rate of molecular assays was 100% *vs*. 7.1% of cultural method).

Listerial CFU logs of the 14 CSF samples, derived by the number of threshold cycles of RT-PCR,

Table 1. Clinical characteristics of fourteen patients affected by meningitis.

PATIENT	FEVER (°C)	HEADACHE	NUCAL RIGIDITY	VOMITING *	MENTAL STATUS
1	39.6	Severe	Moderate	Absent	Normal
2	38.5	Severe	Severe	Absent	Normal
3	38.4	Absent	Absent	Occasional	Normal
4	39.3	Severe	Absent	Moderate	Normal
5	38.5	Moderate	Absent	Absent	Normal
6	39	Severe	Moderate	Moderate	Confused
7	39.5	Severe	Mild	Moderate	Normal
8	37.4	Severe	Absent	Moderate	Normal
9	37	Severe	Severe	Frequent	Normal
10	40	Severe	Severe	Absent	Normal
11	37.5	Moderate	Severe	Absent	Normal
12	37.6	Mild	Mild	Frequent	Confused
13	Absent	Severe	Severe	Absent	Normal
14	40.2	Absent	Severe	Absent	Confused

** Vomiting: Occasional: ‹ 2 bouts/day; Moderate: 2 - 5 bouts/day; Frequent : › 5 bouts/day*

Table 2. Fourteen patients affected by meningitis: CSF abnormalities and RT-PCR results.

PATIENT	GLUCOSE (mg/dl)	PROTEINS (mg/dl)	CHLORIDE(mEq/L) (mEq/L)	CELL COUNT [mmc(% cell type)]	RT-PCR	
					C_T Value	CFUs Log
1	45	110	119	480 (90 neutrophils)	32,2	3,21
2	50	103	114	250 (70 monocytes)	30,4	3,74
3	57	185	108	100 (80 monocytes)	35	3,36
4	59	118	N.A.	1500 (53 neutrophils)	32	3,26
5	48	151	116	negative	29,8	3,92
6	63	900	122	240 (N.A.)	31,3	3,48
7	42	190	N.A.	200 (N.A.)	35,9	2,98
8	44	162	120	96 (N.A.)	30,5	3,71
9	57	203	115	› 100000 (85 neutrophils)	33,3	2,86
10	51	68	118	400 (N.A.)	32,8	3,01
11	53	119	114	420 (N.A.)	30,9	3,6
12	74	291	N.A.	› 50000 (neutrophils)	32,3	3,15
13	48	132	117	240 (60 monocytes)	29,2	4,1
14	72	71	120	2444 (90 monocytes)	30,9	3,57

ranged from 2.86 to 4.1 (Figure 2), while those, derived from the amount of DNA detected by conventional PCR, ranged from 2.76 to 4.16.

The Paired Student's *t*-Test showed no significant difference between CFUs (log) obtained from RT-PCR and conventional PCR (p value: 0.71).

Lumbar puncture was repeated at different times (after 15, 30 and 55 day from the beginning of antibiotic therapy: ampicillin 3.0 gm q6h IV plus chloramphenicol 1.0 gm q12h IV) in order to monitor the infections response to therapy. Conventional PCR and RT-PCR performed on CSFs collected during follow-up revealed that the signal intensity decreased by 40%, 80% and 100% at day 15, 30 and 55 respectively, from the start of antibiotic treatment.

Discussion

Listerial meningoencephalitis is not frequent but it is characterized by high fatality.[14]

Diagnosis of CNS involvement is difficult because of clinical (signs and symptoms) and laboratory (CSF protein level, opening pressure, and CSF-to-serum glucose ratio) variability, as evidenced by numerous authors;[11,14,15] moreover, CSF cultures are often negative because of the low bacterial burden at the site of infection and at least 10 ml of CSF should be collected and seeded in order to enhance the probability of isolating listeria.[11]

Considering the seriousness of CNS involvement, prompt diagnosis is necessary in order to start rapidly specific therapy; *L. monocytogenes* has a relatively high natural resistance to cephalosporins, and third generation cephalosporins are commonly used as first-line empirical treatment for bacterial meningitis according to international guidelines.[16]

The high sensitivity and specificity of molecular techniques might be useful for identifying CNS listerial infection, even when the CSF bacterial load is low, and for monitoring the response to antimicrobial therapy (Figure 1).

RT-PCR demonstrated high diagnostic efficacy, mainly in relation to the correct selection of primers and probes. Following amplification, manipulations are not necessary, and, consequently, carry-over was eliminated; results may be obtained rapidly allowing for real time monitoring of the molecular process.

Although our primary goal was to evaluate the presence of *L. monocytogenes* in the CSF, RT-PCR was useful in order to evaluate the microbial burden and, therefore, the response to antimicrobial drugs, by measuring the intensity of the fluorescence, a direct marker of the amplicon concentration, related to the amount of DNA; in fact a 40% and 80% reduction in the intensity of the amplification signal was evident in our samples in relation to the reduced quantity of DNA, after 15 and 30 days of therapy, respectively.

Conventional PCR, a rapid, simple, low-cost assay, indicated for every laboratory, is useful for detecting the presence of *L. monocytogenes* in CSF.

Although this analytical assay is characterized by numerous biases, our data demonstrated that conventional PCR can also be used for quantitative analysis [17] and for monitoring the efficacy of antibiotic therapy.[18]

Molecular assays have improved the possibility of identifying the etiologic agents of meningitis of unknown origin, such as viral meningitis. The usefulness of molecular techniques, like RT-PCR

and conventional PCR, specific for listerial diagnosis, might finally allow us to understand the real incidence of *L. monocytogenes* meningitis.

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