DIAGNOSIS of LISTERIA MONOCYTOGENES MENINGO-ENCEPHALITIS BY REAL-TIME PCR ON hly-GENE

Alban LE MONNIER1,2,3*, Eric ABACHIN1, Jean-Luc BERETTI1, Patrick BERCHE1 and Samer KAYAL1,4

1 Département de Microbiologie, Hôpital Necker-Enfants Malades, Assistance Publique-Hôpitaux de Paris, Université Paris Descartes, France
2 Centre National de Référence des Listeria, World Health Organization Collaborating Centre for Foodborne Listeriosis, Institut Pasteur, Paris, France.
3 Actual Address: Laboratoire de Microbiologie-Hygiène, Centre Hospitalier de Versailles, Le Chesnay, France
4 Actual Address: Département de Microbiologie, Hôpital Ponchaillou-Université Rennes1, Rennes, France

* Corresponding author and actual address: Dr Alban LE MONNIER
Laboratoire de microbiologie-Hygiène
Centre Hospitalier de Versailles
177 rue de Versailles
78157 Le Chesnay Cedex - FRANCE - EU
Tel: 33 1 39 63 91 07
Fax: 33 1 39 63 93 12
E-mail: a.lemonnier@ch-versailles.fr

Running title: Molecular diagnosis of Listeria meningo-encephalitis

Key words: Listeria monocytogenes, meningo-encephalitis, Real-Time PCR hly-gene, Clinical diagnosis, listeriosis
ABSTRACT

*L. monocytogenes* is a bacterial pathogen that can invade the central nervous system (CNS), causing meningo-encephalitis and brain abscesses. The diagnosis of CNS-listeriosis, based on the isolation of the bacteria in the cerebrospinal fluid (CSF), can be difficult because of previous antibiotic treatment and a low number of bacteria in CSF. To improve the sensitivity of microbiological diagnosis, we have developed a real-time PCR assay for detecting and quantifying *L. monocytogenes* DNA in the CSF. The designed primers specifically amplify the *L. monocytogenes* hly-gene, which encodes listeriolysin O, a pore-forming cytolysin. The PCR-hly assay provides reproducible quantitative results over a wide dynamic range of concentrations, and was highly sensitive while detecting a single gene copy/ml. By assaying a large panel of bacterial species, including species secreting pore forming cytolysin, we determined the specificity of the PCR-hly, which exclusively detects the *L. monocytogenes* DNA. We then analyzed 214 CSF samples from patients with suspicion of CNS-listeriosis. PCR-hly was positive in all cases in which *L. monocytogenes* was isolated by culture. Positive PCR-hly of the CSF was also obtained for five additional, and clinically confirmed CNS-listeriosis, and for which bacterial cultures were negative presumably due to previous treatment with antibiotics. As a complement to classical bacteriological CSF culture, our designed real-time PCR-hly assay proved to be valuable by enhancing the rapidity and the accuracy of the diagnosis of CNS infection by *L. monocytogenes*. In addition, the quantitative results provided may, in some instances, be useful for the follow-up of patients under treatment.
**INTRODUCTION**

*Listeria monocytogenes* is a facultative intracellular food-borne pathogen, widely found in the environment, which causes meningitis and meningo-encephalitis (22, 35). Despite an adequate antibiotic treatment, the overall mortality of CNS-infection is still high (25 to 30%) and neurological sequelae are frequent (22, 35). The apparent inefficacy of antibiotic therapy can be explained by the virulence of this facultative intracellular bacteria and because in many instances listeriosis occurs in immunocompromized hosts (22, 44, 46). Thus, the early initiation of an adequate antibiotic treatment is essential for the outcome. However, an adequate therapy might be delayed since the current first-line treatment for central nervous system (CNS) infections relies on third generation cephalosporins which are inactive against *L. monocytogenes* (44, 46).

CSF cellular abnormalities are usually not characteristic, and the diagnosis based on the detection of *L. monocytogenes* growth from the cerebrospinal fluid (CSF) requires usually 24 to 72 hours with additional time needed for confirming the identification. *L. monocytogenes* culturing in the CSF although specific, has unacceptable low sensitivity because of low number of bacteria within the CSF, low volumes of CSF, and/or previous treatment by an inadequate antibiotic regimen (35). Furthermore encephalitis or brain abscesses may occur without any meningitis, suggesting that *L. monocytogenes* can cross the blood-brain barrier at the level of brain parenchyma (29, 35).

To enhance the diagnosis accuracy, a serodiagnosis assay based on the detection of serum antibodies directed against the listeriolysine O (LLO) has been proposed (4, 16). But it allows only a retrospective diagnosis for listeriosis and lacks specificity since false-positive can be due to cross-reactions with hemolysins from other Gram-positive bacteria. In patients with severe immune deficiency, the serodiagnosis have somewhat lower sensitivity (4, 6, 16, 18).
Nucleic acid amplification testing by real time PCR assay is a rapid diagnosis procedure, and has been used successfully to diagnose a wide range of central nervous system infections (9, 14, 45). Several molecular methods have been developed in the food industry (12, 23, 26, 37, 43), based on the amplification of several specific genes of *L. monocytogenes* (*iap, hly, prfA, actA*) (27, 33, 36, 41). Some of these assays are sensitive enough to be valuable to the detection and quantification of *L. monocytogenes* in the environment and food products (23, 27, 33, 37, 41, 43). However, the results are highly dependent on the method used, the chosen target to be amplified, and the complexity of the food matrix product. Although a rapid, specific and sensitive detection of *L. monocytogenes* is important for medical diagnosis, only few works had described the application of these tests for the diagnosis of CNS-listeriosis in animals (24, 25, 28, 40) or in humans (24, 25, 28). Moreover, none of them were subjected to prospective clinical evaluation for the diagnostic and the follow-up of human listeriosis.

Our objective was first to design a highly specific and sensitive real-time PCR assay for the detection of *L. monocytogenes* by targeting the amplification of the *hly* gene, and then evaluate the PCR-hly assay for the detection of *L. monocytogenes* in CSF collected from patients suspected to have CNS-listeriosis.
MATERIALS AND METHODS

Bacterial strains

Reference bacteria and clinical isolates used in this study are listed in table 1. All bacteria were grown under aerobic or anaerobic conditions, at 37°C, on the appropriate culture medium: Brain Heart Infusion (BHI; Becton Dickinson, Le Pont-de-Claix, France) agar (Listeria spp), blood agar (Streptococcus spp, Staphylococcus spp, Clostridium spp, Bacillus spp), chocolate agar (Haemophilus influenzae, Neisseria meningitidis), and Löwenstein-Jensen (Mycobacterium tuberculosis) (bioMérieux, Marcy L’étoile, France).

Real time on hly gene (PCR-hly)

Probe and primer design for PCR-hly

The target DNA consisted of a well-conserved region of the single gene hly encoding for Listeriolysin O, a thiol-activated pore-forming cytolysin (31). The primers were designed with the Primer Express Software, version 1.5 (Perkin Elmer, Foster, CA, USA). The design of the primer was conducted after alignment of the sequences of various cytolysin published in GenBank with the hly-gene of L. monocytogenes: the pneumolysin (PLY) of Streptococcus pneumoniae, the perfringolysin of Clostridium perfringens (PFO), the streptolysin of Streptococcus pyogenes (SLO), the ivanolysin O of Listeria ivanovii (IVO) and the seeligeriolysin O of Listeria seeligeri (SLO) (NCBI sequence navigator). The forward primer LmH.172F (5’-TT TCA TCC ATG GCA CCA CC-3’) and the reverse primer LmH.242R (5’-ATC CGC GTG TTT CTT TTC GA-3’) were used to amplify a 71 bp fragment. The amplicon was detected with a TaqMan® internal oligonucleotide probe LmH.199T (FAM 5’-ATC CGC GTG TTT CTT TTC GA-3’) labeled with the reporter fluorescent dye 5-carboxyfluorescein (FAM) on the 5’ end, and a quencher molecule 6-carboxytetramethylrhodamine (TAMRA) covalently coupled on the 3’ end. Its hybridization temperature is 10°C below that of primer pair. This oligonucleotide probe was
unphosphorylated at the 3’ end to prevent probe elongation by the Taq DNA polymerase. The primer pairs and fluorescent probe were synthesized by Invitrogen (Cergy Pontoise, France).

**DNA extraction for PCR**

Before the extraction of the bacterial DNA of each indicated strain (Table 1), the bacteria, grown overnight at 37°C in adequate culture broth (BHI, BD) and blood culture bottles (BacT/Alert, bioMerieux), were collected, centrifuged at 5,000 g for 10 min at 4°C, then washed twice and re-suspended in saline isotonic solution and frozen at -80°C in 1 ml aliquots. Titration of these aliquots was systematically performed by serial 10-fold dilutions on appropriate media agar plates. These aliquots were thawed and diluted to obtain samples artificially infected at a calibrated inoculum (1x10^6 CFU/ml) for each indicated strain. 200 µl were used for the DNA extraction by the QI Amp, DNA mini Kit N° 51306 (Quiagen S.A., Courtaboeuf, France) according to the manufacturer’s recommendations. PCR-hly was then performed in triplicate for each strain. DNA was also extracted from CSF samples, previously stored at -20°C when necessary, by use of the MagNA Pure Compact System (Roche Diagnostics).

**Quantitative Real-time PCR conditions**

PCR reactions were performed in a total volume of 50 µL contained 1X TaqMan® Universal PCR Master Mix (Applied Biosystems), 500 nM of each primer, 100 nM probe and 15 µL of the DNA extract. All the amplification steps were carried out in a Thermocycler coupled to the ABI Prism 7700 Sequence Detector® system (Applied Biosystems) with the following protocol: first heating (95°C for 10 min) followed by 45 amplification cycles including denaturation (95°C for 15 sec) and hybridization combined with elongation (60°C for 1 minute). The DNA of an indicated amount of *L. monocytogenes* EGD-e was extracted and used to generate a standard curve. Quantitative results of the real-time PCR assay were expressed as a fractional cycle number and then we determined the Cycle Threshold (CT)
value, which marked the cycle when the fluorescence of a given sample significantly exceeded the baseline signal. In the absence of a signal beyond 45 cycles, the detection of hly-gene is considered as negative. PCR inhibitors were systematically checked, using an external control corresponding of exogenous extracted DNA of *Mycoplasma pneumoniae* amplified in the same conditions. Negative and positive (*L. monocytogenes* DNA) controls were also processed in parallel of each series.

**Studied population**

*Patients and cerebrospinal fluid (CSF) collection*

From January 2000 to December 2006, all the CSF collected from patients hospitalized in our institution or other hospitals and suspected of having a CNS-listeriosis, were analyzed by PCR-hly. DNA was extracted from at least 200 µl of the sampled CSF (as indicated above). CSF specimens were stored at -80°C before the PCR-hly were performed routinely once a week in our laboratory. In parallel standard bacterial culture of the CSF were performed by each laboratory on blood agar (bioMérieux) incubated 2 to 5 days in aerobic and anaerobic atmosphere and chocolate agar (bioMérieux) incubated 2 to 5 days in CO2 5% atmosphere associated to an enrichment step in BHI broth (Difco) during 48h and sub-cultured in the same conditions for 2 to 5 days. Serology was performed for the detection of anti-LLO antibodies as a retrospective diagnosis (4). A definitive diagnosis of CNS-listeriosis was retained when the patient had a neuro meningeal syndrome and/or a rhombencephalitis associated with: i) *L. monocytogenes* isolate from CSF, ii) *L. monocytogenes* isolate from blood culture, and/or iii) Conversion of the anti-LLO-serology when standard microbiological methods failed to isolate *L. monocytogenes* (4, 19).

Patients with clinical and microbiological documented listeriosis but no sign of CNS-infections (materno-neonatal infection, isolated bacteriemia, cutaneous focal infections) and for which CSF was systematically collected, were included in this study. Finally, normal CSF
and those recovered for other known etiological meningitis (inflammatory, viral, bacterial and non listerial) were used as control samples (see characteristics of each groups in supplemental data, Table S2).

**Statistical analysis**

The difference in means of Threshold Cycle Number was analyzed using non parametric Kruskall-Wallis test. P values of <0.05 were considered to be statistically significant.

Statistical analyses were performed using Stata 9 (Statacorp, College Station, TX).
RESULTS

Development of Real Time PCR-hly

Sensitivity of the PCR-hly

We first studied the sensitivity of the PCR-hly to detect an amount of pure DNA extracted from *L. monocytogenes* EGD-e strain with predetermined bacterial concentrations covering six logs ranging from 1x10⁷ to 10 CFU/ml. As few as one copy per reaction could be detected between the 36 and the 37th cycles. The calculated coefficients of correlation of the resulting standard curves of *L. monocytogenes* ($r^2 = 0.995$) demonstrated the linearity of the quantification over a range of seven logs. Similar results were obtained using the *L. monocytogenes* LO28 (serovar 1/2C) and a clinical strain (serovar 4b) (data not shown). PCR-hly performed in quadruplicate for the same extract is highly repeatable (Fig. 1). We further controlled the reproducibility of the quantitative PCR-hly on five aliquots of *L. monocytogenes* EGD-e suspension extracted independently and showed the same analytical sensitivities.

Specificity of the PCR-hly

The specificity of designed primers to amplify exclusively the hly-gene of *L. monocytogenes* was tested in a variety of different *Listeria* species including (*L. seligerii, L. ivanovii, and L. innocua*) and a large panel of other Gram-positive or Gram-negative bacteria as showed in Table 1. All the *L. monocytogenes* species tested gave a positive amplification. In contrast, the PCR-hly failed to amplify any product of *L. monocytogenes* EGD deleted for the hly-gene (EGD Δhly (5) demonstrating that the primers are specifically directed to the hly-gene (data not shown). We then tested within *Listeria* genus the species that are able to secrete an hortolog for hly-gene: *L. seeligerii* and *L. ivanovii* secreting seeligerolysin (SLO) and ivanolysin (ILO), respectively. The PCR-hly failed to amplify any product in these bacteria as it did with...
L. innocua, a non hemolytic and non virulent species used as a negative control (Table 1). As expected PCR-hly failed to amplify any PCR product with bacterial pathogens that also secrete a pore forming cytolysin such as perfringolysin of Clostridium perfringens (PFO), pneumolysin of Streptococcus pneumoniae (PLY), streptolysin of Streptococcus pyogenes and S. dysgalactiae (SLO), or with bacterial pathogens from other genus usually encountered in meningitis (Table 1). In order to decipher whether the PCR-hly results are not influenced by the biological abnormalities of CSF samples, we tested 55 clinical CSF collected from patients hospitalized in our institution and for which the diagnosis was known (non listerial meningitis including viral etiologies and systematic samples of non infectious meningitis) (Supplemental data Table S1). With all the tested samples, no PCR inhibition was detected and the PCR-hly amplification never gave a false positive result.

Altogether, the results showed the absence of any cross-amplification between L. monocytogenes and other species within the same genus, or other bacterial genus as well as when CSF were biologically abnormal. We thus clearly demonstrated that the designed PCR-hly is highly specific to amplifying the hly-gene of L. monocytogenes virulent strains.

Prospective clinical evaluation

Detection of L. monocytogenes in cerebrospinal fluid (CSF)

Based on these preliminary in vitro results, we aimed at prospectively evaluating the performance of the real-time quantitative PCR-hly carried out with DNA extracted from CSF samples collected prospectively over a 7-year period from patients suspected of having a CNS-listeriosis. We analyzed 313 CSF samples (corresponding to 304 patients) originating from Necker-Enfants Malades hospital (18%) and from other French hospitals (82%). Although negative after PCR amplification, ninety-nine of the 313 CSF were not considered for the final evaluation of our PCR-hly assay, because at least one of the diagnosis criteria for CNS-listeriosis (see material and method for the diagnostic criteria) lacked.
The median age and the sex ratio of the population studied were 40 years (range: 1 day to 93 years) and 1.3 respectively. Nineteen percent were children (< 15 years) with a median age of 2.7 years (range: 0 to 13.4) and 81% were adults with a median age of 48 years (range: 16.1 to 93.0). Among the 205 patients included in the study (214 eligible CSF), we identified 30 cases of listeriosis for which we analyzed 39 CSF: in six cases CSF was collected twice, and in one case it was collected four times for controlling infection under antibiotic treatment.

Clinical presentations are presented in Table 2: i) CNS-listeriosis (n=24), ii) Non CNS-listeriosis including materno-neonatal infections (n=3), isolated bacteraemia with sepsis (n=2), and cutaneous focal infection (n=1). For all the other patients (n=175) the diagnosis of listeriosis has been ruled out according to the criteria previously defined or because other bacterial etiologies were finally identified (n=13) (Table 2).

CNS-infections were divided into two distinct groups according to the characteristics of the CSF sent to our laboratory for analysis, notably the delay between the time when the CSF was collected and the onset of the disease. We observed 14 cases (12 adult and 2 neonates) for which the CSF was early collected within the two first days and 10 cases for which the CSF was collected between 5 and 10 days after the onset of the disease. In these ten latter cases, all patients were already under active antibiotic treatment, however, clinical presentation, cellular and biochemical disorders of the CSF, and LLO-serology conversion were strongly suggestive for the diagnosis of CNS listeriosis. Thus, the diagnosis of CNS-listeriosis was retained although *L. monocytogenes* could not be isolated from all the CSF collected or from other clinical specimens.

**Result of standard microbiological tests performed on CSF**

Standard bacterial culture on the first collected CSF yields *L. monocytogenes* in nine cases (64%) (Table 2). Among these nine cases, three were positive only after using the enrichment method (patients 1, 6, and 9, Supplemental data Table S2).
Nine additional CSF (corresponding to 7 patients) were collected several days after the start of antibiotic therapy for controlling treatment efficacy. Among these nine CSF, only two specimens from patient 5 (Supplemental data Table S2) were positive in culture after using the enrichment method.

Direct examination of CSF retrieved evocative Gram-positive bacilli in only four cases. All of these cases corresponded to positive culture. This highlights the low number of bacteria present in the CSF specimens possibly due to an antibiotic treatment started before CSF collection.

**Result of quantitative PCR-hly**

Among all the analyzed cases, PCR-hly did not provide false negative result when *L. monocytogenes* was isolated by standard culture of CSF. There was no positive PCR-hly for patients with listeriosis without CNS involvement (materno-neonatal infections, bacteriemia, and cutaneous focal infection). PCR-hly remained negative in all the cases for which the diagnosis of listeriosis was definitely excluded (Table 2).

However, PCR-hly was positive in five additional CSF for which *L. monocytogenes* was not retrieved by culture even after enrichment technique (patients 2, 3, 7, 8 and 14, Supplemental data Table S2), thus completing the diagnosis of CNS-listeriosis. In all cases, discrepancies between culture and PCR could be explained by the administration of antibiotics started between 1 to 5 days before CSF collection.

The analysis of the quantitative results of PCR-hly showed the presence of a significantly lower amount of *L. monocytogenes*-DNA when previous administration of effective antibiotics (Supplemental data Table S2). Similarly, low bacterial inoculum in the CSF, evidenced by the low amount of bacterial DNA, made the standard bacterial techniques, like culture and the direct examination for observation of specific Gram-positive bacilli, less sensitive (Fig. 2).
Concerning CSF collected for control under antibiotic treatment, PCR-hly was positive for four specimens including the two positive CSF in culture (collected two and five days after effective antibiotic treatment was initiated). In all cases, the CT values were lower than those obtained for the first collected CSF.
The development of a molecular test for the detection of *L. monocytogenes* in clinical specimens was motivated by the need for a rapid and reliable test for the diagnosis of CNS-listeriosis, complementary to the culture. Indeed, the rapid detection and identification of causative pathogens in bacterial meningitis is critical for a rapid adaptation of antibiotic treatment (7). Moreover, this test should be highly sensitive, because CNS-listeriosis is often linked to the presence of low number of bacteria in CSF.

The *hly* gene, part of the genome of all of the species *L. monocytogenes*, was chosen as a target for the detection and the quantification by real-time PCR of *L. monocytogenes* infection. It is a 1590 bp single copy gene coding for a thiol-activated pore-forming cytolysin, the Listeriolysin O (LLO) a major and specific virulent factor for *L. monocytogenes* which was necessary for the invasiveness of the bacteria (31). Thus, *hly*-gene appeared to be a reliable and highly conserved (about 99%) candidate target already used for specific detection of *L. monocytogenes* in food products (23, 24, 36, 37). However, various sequences of *hly*-gene have been tested, leading to various results in terms of specificity (23, 24, 36-38).

Despite important similarities between *hly*-gene and genes encoding for proteins belonging to the same pore-forming cytolysin family, especially the ivanolysin O (77% identity) (2, 31), the targeted region was found to be specific for *L. monocytogenes*. Moreover, since the PCR-*hly* does not cross with other bacteria usually responsible for meningitis, this test could be included in the scheme of multiplex-PCR for identifying the major etiologies of bacterial meningitis (7, 9, 45).

Some teams described the use of amplification of the universal bacterial 16S rDNA gene for detecting *L. monocytogenes* in clinical samples (3, 8, 25, 39, 42). However, the broad-range PCR 16S rDNA presents some drawbacks like less sensitivity and the requirement for sequencing the amplicons provides a delaying response that does not match our objective of
rapid etiological diagnosis (10, 32, 48). In order to enhance the rapidity of the diagnosis, we privileged real-time PCR which appears to be highly sensitive as theoretical threshold detection was as low as one gene copy/ml (7, 9, 13, 14, 45). Turn-around-time of this real-time PCR-hly is two hours, thus expected time-to-result of this test, when performed on-demand, could be less than 24 hours. Rapid and accurate results are helpful for rapid adaption of the empiric antibiotic treatment, usually based on high dose of third generation cephalosporins inactive against *L. monocytogenes*, adding or removing ampicillin (44).

Nevertheless, technical improvements should enhance the efficacy of DNA extraction and avoid inhibitors in order to optimize molecular detection of *hly*-gene in other clinical samples like blood, placenta and feces but also environmental and food samples (1, 30). The sensitivity of PCR-hly could be further improved by targeting genes with multiple copies in the *L. monocytogenes* genome.

According to the results of the prospective clinical evaluation, PCR-hly appears to be more sensitive than standard bacterial culture and direct examination. PCR-hly rescues the diagnosis of CNS-listeriosis when culture remains sterile or the isolation of the bacterium has been delayed because of the need of an enrichment step. It should also be emphasized that discrepancies between culture and PCR corresponded to unusual clinical presentations like ventriculo-peritoneal shunt infection (patient 5, Supplemental Table 2) (Le Monnier et al. submitted for publication), exclusive encephalitis presentation (patient 8, Supplemental Table 2) or previous administration of antibiotics active against *L. monocytogenes* (patient 2, 4, 5, 7, 8, and 14, Supplemental Table 2) as already described by Fayol et al. for instance (patient 14, Supplemental Table 2) (15). In all of these cases, the quantitative value of PCR-hly confirmed the low number of bacteria present in the CSF specimens.

Moreover, in the case of patient 5, the results of quantitative PCR-hly were used for demonstrating the persistence of infection from a medical device despite effective antibiotic
therapy. Our results suggest a quantitative indication of the PCR-hly for monitoring treatment efficacy (Le Monnier et al. submitted for publication).

For the ten probable cases not microbiologically documented, the anamnesis and LLO-serology conversion reported do not definitively make the evidence of evolutive CNS-listeriosis despite evocative CSF anomalies. Moreover, the lack of sensitivity of both culture and PCR-hly is reinforced when CSF specimens were sampled late after the onset under effective antibiotic treatment. However, in at least two cases (patient 26, and 30, Supplemental Table 2), PCR-hly failed to confirm the diagnosis despite high suspicion of CNS-listeriosis according to the clinical presentation, the radiological findings evocative of bacterial abscess localized in the rhombencephal area by MRI, the LLO-serology conversion, and the favorable evolution under antibiotic treatment active against *L. monocytogenes*. The exclusive encephalitis presentation without meningitis constitutes a potential source of false negative results. Indeed, the bacterium could be located exclusively in the brain parenchyma with few or no bacterial releasing in the CSF at the early times (34, 35). In these cases, culture is, however, no more contributive. LLO-serology conversion appears as an interesting alternative but only for retrospective diagnosis when culture and PCR remains non contributive (4, 16, 18).

The analysis of other evolutive clinical presentations of listeriosis highlights the fact that *L. monocytogenes* is not systematically linked to CNS-infection, although some physicians systematically include the research of a possible infra clinical meningitis in the investigation and management of such severe infections.

Until now, the definitive diagnosis of listeriosis is exclusively based on the isolation of *L. monocytogenes* in clinical samples. Cases of listeriosis diagnosed by the detection of *L. monocytogenes* DNA are currently not included in the data of epidemiological surveillance because of the lack of prospective evaluation of molecular assays.
Although the trend shows an increasing number of cases of listeriosis in France and throughout the world (21), listeriosis remains a rare disease. During the period studied, between 200 and 250 cases of listeriosis were diagnosed yearly in France (i.e.: incidence of 3 to 4 cases per million inhabitants). Less than one third of these cases consisted in CNS-listeriosis whether in adult or more rarely in newborns (Data from the annual report of the French National Reference Centre for *Listeria*). Although being the second cause of bacterial meningo-encephalitis in France behind *Mycobacterium tuberculosis* (34), meningo-encephalitis due to *L. monocytogenes* is an even rarer clinical presentation. Only 12 cases were diagnosed in 2007 during a one-year study collecting encephalitis cases in France (34). Thus, national recruitment of cases was an absolute requirement to conduct such studies but led inevitably to several missing data and inconclusive cases because of a loss of information. Despite some problems of methodology, this study is the first prospective evaluation of the diagnostic value of molecular assay for the diagnosis of CNS-listeriosis. We are aware that molecular diagnostic testing must not replace culture, the only technique that allows antimicrobial susceptibility testing and epidemiological and microbiological surveillance of emerging strains including investigation of clusters of cases (17, 21). However, altogether our results suggest that the definition of listeriosis should include cases diagnosed by molecular tests as with other infectious diseases (7, 11, 14, 47, 48).

In conclusion, we have demonstrated that as a routine test, real-time PCR-hly, provides rapid and reliable information allowing antibiotic therapy to be quickly adapted. This study also showed, in our studied population, that *Listeria* culture can be insensitive when the patient has been previously treated with antibiotics, while PCR is still highly sensitive. In our institution, we perform PCR-hly as a complementary test to standard culture for rapid or retrospective diagnosis of *Listeria* meningo-encephalitis. We also used the quantitative results for the follow-up of a patient receiving antibiotic treatment until CSF sterilization.
We are grateful to all colleagues who sent CSF samples and clinical data. This work was supported by the Institut Pasteur, the Institut de Veille Sanitaire and the Assistance Publique Hôpitaux de Paris (APHP).
REFERENCES


FIGURE LEGEND

FIG. 1. Result of real-time quantitative PCR-hly assay.
Plot of the Individual CT values on four time repetition assay against the input target quantity of *L. monocytogenes* EGD-e expressed in Log10 (colony forming unit/ml) (common log scale) showing the linearity of the quantification over a range of seven logs from dilutions of 1 to 1x10^7 CFU/ml. The computer calculated correlation coefficient is 0.995.

FIG. 2. Comparison of the quantitative results of PCR-hly according to previous antibiotic regimen and the result of direct examination and culture performed on CSF.
ATB: previous antibiotics were given (Yes) or not (No) before CSF were taken.
Dir Ex: direct examination of CSF retrieved (Pos) or not (Neg) gram-positive rods evocative of *Listeria monocytogenes*.
CSF cult: standard culture of CSF yielded (Pos) or not (Neg) *Listeria monocytogenes*, as well after an enrichment step or not.
Difference in means of Threshold Cycle Number was analyzed using Kruskall-Wallis test. P values of <0.05 were considered to be statistically significant.
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Reference</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Listeria genus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em> EGD-e 1/2a (LLO)</td>
<td>Sequenced strain EGD-e (20)</td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em> LO28 1/2c (LLO)</td>
<td>Sequenced strain L028</td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 1/2a (LLO)</td>
<td>Serovar reference strain CLIP 74902</td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 1/2b (LLO)</td>
<td>Serovar reference strain CLIP 74903 POSITIVE</td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 1/2c (LLO)</td>
<td>Serovar reference strain CLIP 74904</td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 4b (LLO)</td>
<td>Serovar reference strain CLIP 74910</td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 4b (LLO)</td>
<td>Clinical strain</td>
<td></td>
</tr>
<tr>
<td><em>L. ivanovii</em> 5 (IVO)</td>
<td>Reference strain CIP 12229, CIP 7842</td>
<td>Negative</td>
</tr>
<tr>
<td><em>L. seligerii</em> 1/2b (LSO)</td>
<td>Reference strain CIP 73021</td>
<td>Negative</td>
</tr>
<tr>
<td><em>L. innocua</em> 6a</td>
<td>Sequenced strain, CIP 11262 (20)</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Gram positive bacteria secreting a pore forming cytolysin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em> (PLY)</td>
<td>Clinical strain</td>
<td>Negative</td>
</tr>
<tr>
<td><em>S. pyogenes</em> (SLO)</td>
<td>Clinical strain</td>
<td>Negative</td>
</tr>
<tr>
<td><em>S. dysgalactiae subsp dysgalactiae</em> (SLO)</td>
<td>Clinical strain</td>
<td>Negative</td>
</tr>
</tbody>
</table>
### Legend Table 1

Negative: *hly*-gene was not detected after 45 CT; CIP: Collection of Institut Pasteur, CLIP: Collection of Listeria Institut Pasteur. When the bacteria secreted a thiol-activated pore forming cytolysin, its abbreviations is indicated in parenthesis: CLY: Cereolysin, IVO: Ivanolysin O, LLO: Listeriolysin O, LSO: Seeligerilysin O, PFO: Perfrengolysin, PLY: Pneumolysin, SLO: Streptolysin O.

\( ^a \) Strains of *S. epidermidis* lacking (CIP 68.21) or carrying (CIP 105.777) the *ica* operon which is essential for biofilm production.

<table>
<thead>
<tr>
<th></th>
<th>Clinical strain</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. dysgalactiae subsp equisimilis</strong> (SLO)</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Clostridium perfringens</strong> (PFO)</td>
<td>Clinical strain</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Bacillus cereus</strong> (CLY)</td>
<td></td>
<td>Negative</td>
</tr>
</tbody>
</table>

**Other bacterial strains frequently associated with community or nosocomial CNS infections**

<table>
<thead>
<tr>
<th></th>
<th>Clinical strain</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neisseria meningitidis</strong></td>
<td>Sequenced strain Z2491</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Streptococcus agalactiae</strong> (group B)</td>
<td>Sequenced strain Nem316</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Haemophilus influenzae</strong> (group B)</td>
<td>Clinical strain</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Escherichia coli antigen K1</strong></td>
<td>Clinical strain</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>Clinical strain</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Staphylococcus epidermidis</strong> (<em>ica -</em>)(^a)</td>
<td>CIP 68.21</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Staphylococcus epidermidis</strong> (<em>ica +</em>)(^a)</td>
<td>CIP 105.777</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td>Clinical strain</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Mycobacterium tuberculosis</strong></td>
<td>Clinical strain</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Table 2. Result of standard microbiological tests and PCR-hly performed on the first collected CSF according to different clinical presentation

<table>
<thead>
<tr>
<th></th>
<th>Listeriosis</th>
<th>Non Listeria meningitis&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CNS infection</td>
<td>CNS infection</td>
</tr>
<tr>
<td></td>
<td>n=24</td>
<td>n=6</td>
</tr>
<tr>
<td>Cult+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cult-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR-hly positive</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>PCR-hly negative</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>162</td>
</tr>
</tbody>
</table>

Legend Table 2

Cult+ or Cult- are related to positive or negative bacterial culture of the first collected CSF respectively.

<sup>a</sup> CNS-listeriosis was ruled out according to criteria defined in material and methods

<sup>b</sup> Bacteria isolated in non Listeria meningitis: Streptococcus agalactiae (3), Escherichia coli (2), Cryptococcus neoformans (2), Staphylococcus epidermidis (2), Staphylococcus pneumoniae (2), Neisseria meningitidis (1), Streptococcus pyogenes (1).
$Y = 36.6 - 3.35 \cdot X$

$r^2 = 0.995$

Log CFU of EGD-e/ml

FIG. 1