

Biochemical and molecular characteristics of *Listeria monocytogenes* isolates from a prosthetic mitral heart valve-bearing patient's blood cultures

Características bioquímicas e moleculares de *Listeria monocytogenes* isolados de hemoculturas de próteses de válvula mitral

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Abstract

Background: In Brazil, listeriosis is not a notifiable disease; thus, the incidence of Brazilian cases remains unknown. *Listeria monocytogenes* is not always included in automated systems, and its detection depends on the high skill level of microbiology laboratory professionals. This paper describes the characteristics of *L. monocytogenes* isolates fortuitously obtained from an endocarditis case in Recife, PE, Brazil. **Methods:** Six bacterial isolates obtained from six blood cultures from a 28-year-old male bearing a prosthetic mitral heart valve were analyzed by PCR using primers specific of *L. monocytogenes* to confirm a presumptive identification, determine the serotype and presence of the virulence genes (*inlA*, *inlB*, *inlC*, *inlJ*, *hly*, *plcA*, *actA*, *prfA*) in an attempt to determine the *Listeria* genotype by PCR-ribotyping. **Results:** The samples were identified as *L. monocytogenes* 4b. All investigated virulence genes were amplified by PCR, and the identity of the amplified segments was confirmed by sequencing. A deletion of 105 base pairs was detected in the *actA* gene. All of the samples generated the same PCR-ribotype pattern, clustered into a single ribotype, and were considered a single strain. **Conclusion:** *L. monocytogenes* infection should be considered in endocarditis differential diagnoses, especially among high-risk groups, due to its high pathogenicity and the environmental ubiquity.

Keywords: *Listeria*. Listeriosis. *Listeria monocytogenes*. Endocarditis. Virulence.

Resumo

Introdução: A listeriose não é uma doença de notificação obrigatória no Brasil e relatos de casos clínicos são escassos. *Listeria monocytogenes* não é rotineiramente incluída nos sistemas automatizados e sua detecção depende da habilidade dos profissionais dos laboratórios de microbiologia. Neste trabalho, descrevemos as características de isolados de *L. monocytogenes* obtidos de um caso de endocardite em Recife, PE, Brasil. **Métodos:** Seis isolados de seis hemoculturas obtidas de um paciente de 28 anos, portador de válvula cardíaca mitral protética foram analisados por PCR utilizando primers específicos para confirmar a identificação, determinar o sorotipo e a presença dos genes de virulência (*inlA*, *inlB*, *inlC*, *inlJ*, *hly*, *plcA*, *actA*, *prfA*) e para determinar o genótipo dos isolados por PCR-ribotipagem. **Resultados:** As amostras foram identificadas como *L. monocytogenes* 4b. Todos os genes de virulência investigados foram amplificados, e a identidade dos segmentos foi confirmada por sequenciamento. Uma deleção de 105 pares de bases foi detectada no gene *actA*. Todas as amostras revelaram padrão de amplificação similar por PCR-ribotipagem e foram consideradas uma única estirpe. **Conclusão:** Infecção por *L. monocytogenes* deve ser considerada em diagnósticos diferenciais de endocardite, especialmente entre os grupos de alto risco, devido à sua alta patogenicidade e ubiquidade ambiental.

Palavras-chave: *Listeria*. Listeriose. *Listeria monocytogenes*. Endocardite. Virulência.

BACKGROUND

Listeria monocytogenes is a rod-shaped gram-positive facultative intracellular pathogen that causes the disease so called listeriosis. It primarily affects pregnant women, newborns and immunocompromised adults, causing sepsis, abortion and infection of the central nervous system and resulting in high morbidity and mortality¹. Out of the 13 recognized *L. monocytogenes* serotypes, three (1/2a, 1/2b and 4b) appear to be more pathogenic; they are

epidemiologically associated with the most serious cases of human listeriosis².

In general, cardiovascular diseases, such as endocarditis and pericarditis, are associated with a poor prognosis and high mortality^{3,4,5}. Endocarditis caused by *L. monocytogenes* infection often occurs in patients with defective or prosthetic heart valves, especially aortic and mitral valves^{6,7}. Mortality

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Ethical issues: This work has not an ethical component. The bacteriological samples derived from spontaneous demand from the hospital microbiology laboratory and the patient's identity were confidentially maintained.

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occurs in 100% of untreated cases; however, appropriate treatment reduces this rate to 20-30%. This mortality rate is still higher than for endocarditis caused by another bacteria, which is typically 14% lethal⁴. *L. monocytogenes* endocarditis is clinically diagnosed by symptoms associated with bacteremia, and treatment of infections is usually accomplished through the administration of antibiotics, often ampicillin and an aminoglycoside³. Early identification of *L. monocytogenes* in endocarditis cases and the determination of its antimicrobial sensitivity are crucial for the treatment of affected patients. However, *Listeria* is seldom suspected as the causative agent of endocarditis, and patient treatment is typically tailored for bacteria that are more frequently involved in endocarditis such as *Staphylococcus aureus*, *Streptococcus viridans*, *Enterococcus* and coagulase negative *Staphylococcus* (CoNS)⁸. Therefore, the establishment of appropriate treatments may be delayed, aggravating the patient's condition and possibly leading to death as a result of the high pathogenicity of *L. monocytogenes*⁹.

This study analyzed six *L. monocytogenes* cultures as obtained from a prosthetic mitral heart valve-bearing patient who presented it at an emergency cardiology facility. The presumptive *Listeria* identification was confirmed by PCR at the genus, species and serotype levels. The genomic profile of the organism was analyzed by PCR-ribotyping, *Listeria* virulence genes involved in the infection pathway of the main host cells were detected, and the antimicrobial susceptibility of the strains was assessed.

METHODS

Bacteria isolation, presumptive identification and antimicrobial susceptibility assessment

Six blood cultures were obtained from a 28-year-old male bearing a bovine pericardial mitral heart valve who presented himself in a cardiology emergency facility in Recife, PE, Brazil. Following clinical evaluation, three blood cultures were collected at 30-minute intervals; however, endocarditis or *Listeria* infection was not suspected, and the patient was released before the blood culture results were completed. Four days later, the patient entered the intensive care unit after his condition had deteriorated. A second series of blood cultures was collected, and the patient was successfully diagnosed and treated, resulting in a full recovery.

The bacterial growth after 24 hours of incubation was initially identified as *Listeria* spp. by routine screening using standard bacteriological tests (colony morphology on blood agar plates, Gram staining and motility in semisolid medium)¹⁰. Antimicrobial susceptibility was determined by disk diffusion on agar plates according to CLSI guidelines for *Staphylococcus* spp.¹¹ and Soussy et al. (1994)¹² for ampicillin and vancomycin.

Four reference strains from the Collection of the Pasteur Institute (CLIP), the Centers for Disease Control (CDC), and the American Type Culture Collection were used: CLIP12612 (*L. innocua*, negative control), CDC F4561 (*L. monocytogenes* 1/2a), CDC F4976 (*L. monocytogenes* 1/2b), and ATCC 19115 (*L. monocytogenes* 4b, positive control) were accounted for.

DNA extraction

DNA samples were extracted following a protocol based on Ausubel et al.¹³, with minor modifications. Briefly, 1 ml of each bacterial culture in brain heart infusion broth (BHI, Biobras) was centrifuged for 10 minutes at 14,000 rpm, the resulting pellet was washed with 500 µL of Tris:EDTA (10:1), and 10 µL of 1% lysozyme and 10 µL of 0.5% Proteinase K were added. The samples were incubated at 60°C for 20 minutes, then 100 µL of STE (2.5% SDS, 0.25 M EDTA, 10 mM Tris pH 8.0) was added. The samples were then incubated at 60°C for 15 minutes, at room temperature for 5 minutes and in an ice bath for 5 minutes. Next, 130 µL of 7.5 M sodium acetate was added, and the samples were re-incubated in an ice bath for 15 minutes and centrifuged for 3 minutes at 14,000 rpm. At this point, 700 µL of the supernatant was transferred to a new tube, 420 µL of isopropanol was added, and the samples were then incubated at -80°C for 30 minutes and centrifuged at 14,000 rpm for 10 minutes. The supernatant was discarded, and the pellet DNA was vacuum dried and resuspended in 10 µL of 0.02% RNase. DNA yield was quantified by comparison with known amounts of λ *Hind*III DNA (Sigma).

PCR identification: genus, species and serotyping

To confirm the identification of the culture, specific primers were used in PCR reactions for amplification of the 23S rRNA gene from the *Listeria* genus¹⁴ and *lmo* 2243 from *L. monocytogenes* species¹⁵. The serotype was assessed using primers specifically directed to genes from the three most pathogenic serotypes: *lmo* 0737 for 1/2a¹⁵, *gltB* for 1/2b¹⁶ and ORF2110 for 4b¹⁷. The reaction mixtures were prepared in 25 µL volume and included 20 ng of genomic DNA, 50 mM KCl, 10 mM TrisHCl (pH 8.0), 200 mM dNTP (Invitrogen), 15 mM MgCl₂, 20 pM of each primer and 1U of *Taq* DNA polymerase (Promega). Amplifications were performed in a Biometra thermocycler programmed for 30 cycles of 1 min at 92°C, 1 min at 55°C and 1 min at 72°C followed by a 7 min final extension at 72°C. As a negative control, a tube containing all reagents but genomic DNA was used in each reaction.

Detection of virulence genes

The presence of virulence genes involved in the three stages of infection was assessed by PCR under the conditions

described above. Specific primers were used to detect *inlA*¹⁸, *inlB*¹⁹, *inlC*²⁰ and *inlJ*²⁰, which are involved in host cell adhesion and invasion; *hly*¹⁴ and *plcA*²¹, which are involved in vacuole escape; *actA*²², which is involved in intracellular propagation; and *prfA*²¹, which acts as a virulence gene regulator.

Each amplified gene segment was purified and sequenced in an automated ABI3100 sequencer (Applied Biosystems, USA). The consensus sequence for each gene was compared with published sequences available in GenBank (<http://www.ncbi.nlm.nih.gov>) using the Basic Local Alignment Tool (BLASTn). Multiple alignments were performed by Mega⁴²³.

PCR-ribotyping

Ribotyping was performed by PCR using specific primers for amplification of the 16S-23S rDNA intergenic spacer region as described²⁴.

RESULTS

Antimicrobial susceptibility

All samples revealed sensitivity to ampicillin, cephalothin, ciprofloxacin, chloramphenicol, erythromycin, gentamicin, penicillin, rifampin, tetracycline, trimethoprim/sulfamethoxazole and vancomycin and resistance to cefoxitin by agar disk diffusion.

Determination of the genus, species and serotype by PCR

Segments of the expected size for the 23S rRNA gene specific to the *Listeria* genus (239 base pairs = bp), the *lmo2234* gene specific to *L. monocytogenes* species (420 bp) and the ORF2110 gene specific to serotype 4b (597 bp) were amplified in all samples (data not shown), and the samples were thus determined to be *L. monocytogenes* serotype 4b. *lmo 0737* for 1/2a and *gltB* for 1/2b did not amplify.

Virulence genes

Segments of the expected size were amplified in all samples for the *inlA*, *inlB*, *inlC* and *inlJ* genes, which are involved in the bacterium adhesion and host cells invasion; the *hly* and *plcA* genes, which are involved in vacuole escape; and the *prfA* gene, which regulates virulence genes, (data not shown). Comparisons of the amplified segment sequences with published sequences revealed 98 to 100% identity (data not shown).

A 432 bp segment was detected instead of the expected 537 bp segment, indicating a 105 bp deletion. This amplicon was present in all samples for the *actA* gene, which is involved in the ability to spread cell-to-cell (Figure 1).

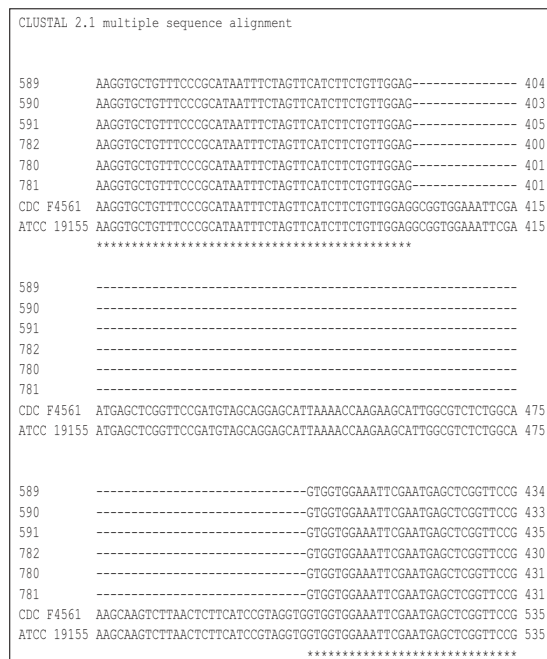


Figure 1. Multiple alignments of partial sequences showing the 105 bp deletion in the *actA* gene in samples from blood cultures compared with reference strain sequences.

PCR-ribotyping

All samples generated a similar amplification pattern of the 16S-23S rDNA intergenic spacer region and therefore clustered into the same ribotype. Control serotypes 1/2a and 1/2b displayed a similar amplification pattern, clustering into the same ribotype (named R1), while serotype 4b generated a different amplification pattern and therefore clustered in a separate ribotype (named R2). The six blood culture samples clustered into ribotype 2 (Figure 2).

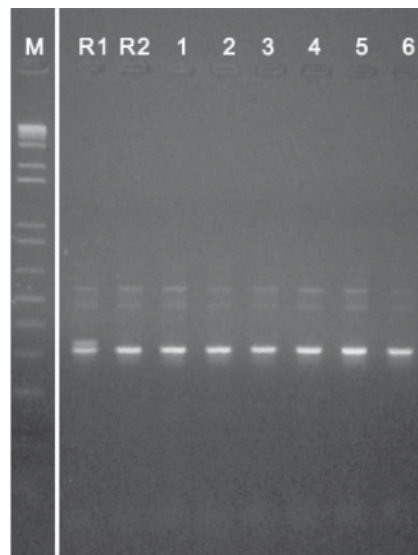


Figure 2. PCR ribotyping profile. Lines: M: 100bp Molecular marker; R1: *Listeria monocytogenes* ATCC 19115, serotype 4b; R2: *Listeria monocytogenes* CDC F4976, serotype 1/2b; 1-6: samples from the study.

DISCUSSION

L. monocytogenes can be misidentified by gram-stain-determined morphology as *Corynebacterium* spp, which is often regarded as a contaminant, especially when the colonies grown from the clinical sample are scarce. In our region, *L. monocytogenes* is not always included in automated systems and is not often an expected cause of infection because of its low clinical frequency. Therefore, its detection depends on a high level of skill among microbiology laboratory professionals. In Brazil, listeriosis is not a notifiable disease, so the number of Brazilian cases remains unknown²⁵. However, the severity of this life-threatening disease demands rapid treatment to avoid a fatal outcome.

Most clinical *L. monocytogenes* isolates are susceptible to a wide range of antibiotics^{26,27}; however, resistance to chloramphenicol, erythromycin, streptomycin, tetracycline, vancomycin and trimethoprim has been reported²⁸. In our region, the various automated identification systems widely employed in clinical microbiology laboratories, although easy and quick, they are not always programmed for low-frequency organisms like *L. monocytogenes*. The need for accurate antimicrobial susceptibility determination of the microorganisms from clinical material is crucial to avoid ineffective drug use, which can slow the treatment process, aggravate the patient's clinical condition, and possibly lead to a fatal outcome. In this study, antimicrobial susceptibility tests were carried out through disk diffusion on agar, and the cultures were resistant only to cefoxitin.

The six cultures were classified as serotype 4b, the most frequent causative agent of human listeriosis, and clustered into the same ribotype, indicating a single strain. All of the cultures harbored the virulence genes involved in the different stages of the infection. Surprisingly, the *actA* gene, which is involved in actin-dependent intracellular motility, displayed a 105 bp deletion. Others have reported a decrease in lethality in mice and cell-to-cell spreading in plaque formation

assays of serotype 4b strains carrying a similar deletion²⁹. A substitution of alanine for proline at position 267 in the *actA* gene, or the absence of other genes, such as *inlC* and *inlJ*, may be responsible for the reduced intracellular propagation capacity³⁰. However, the ability to spread to other cells and lethality in mice were not significantly reduced by the same 105 bp deletion in the *actA* gene in serotype 1/2b³¹. In our study, in spite of the 105 bp deletion in the *actA* gene, the strain remained pathogenic and capable of invading the blood stream. It is possible that other virulence-related genes are involved, or that the *actA* gene is not needed for infection in highly susceptible individuals.

L. monocytogenes endocarditis is rather infrequent, with barely over 70 cases published in the world³². Individuals with a history of rheumatic heart disease, hypertrophic cardiomyopathy, mitral valve prolapse or ischemic cardiomyopathy are particularly susceptible^{4,32}. The average age of *L. monocytogenes* endocarditis patients increased from 47.1 years during the period from 1955 to 1984 to 65.5 years from 1985 to 2000⁴. The patient from our study was the youngest (28 years) among those patients published literature available.

CONCLUSION

Although uncommon in our region, *L. monocytogenes* infection should always be considered in the endocarditis differential diagnosis due to its high pathogenicity and broad environmental distribution, which may increase the frequency of contamination by this organism.

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