Antimicrobial susceptibilities of *Listeria monocytogenes* human strains isolated from 1970 to 2008 in Brazil

Susceptibilidade antimicrobiana de cepas humanas de *Listeria monocytogenes* isoladas no período de 1970 a 2008 no Brasil

Cristhiane Moura Falavina dos Reis¹, André Victor Barbosa¹, Leonardo Alves Rusak¹, Deyse Christina Vallim¹ and Ernesto Hofer¹

ABSTRACT

**Introduction**: *Listeria monocytogenes* is the causative agent of listeriosis, a foodborne illness that affects mainly pregnant women, the elderly and immunocompromised patients. The primary treatment is a combination of ampicillin with an aminoglycoside, in addition to a second-choice drug represented by chloramphenicol, erythromycin, tetracycline and rifampicin. The aim of this study was to analyze the antimicrobial susceptibility profile of strains isolated from human sources in the last four decades. **Methods**: Sixty-eight strains were selected from the culture collection of the Laboratory of Bacterial Zoonoses/LABZOO/FIOCRUZ isolated in different regions of Brazil from 1970 to 2008 and primarily isolated from cerebrospinal fluid and blood culture. Susceptibility tests to antimicrobial drugs were evaluated using the criteria established by Soussy using the Kirby-Bauer method and E-Test strips were used to determine the minimum inhibitory concentration (MIC). **Results**: Among the strains tested, serovar L4b (60.3%) was the most prevalent, followed by serovar 1/2a (20.6%), 1/2b (13.2%) and the more uncommon serovars 1/2c, 3b and 4ab (5.9%). All strains were susceptible to ampicillin, cephalothin, erythromycin, gentamicin, teicoplanin and vancomycin. Only one strain (1.5%) showed resistance to rifampin, and two (3%) were resistant to trimethoprim-sulfamethoxazole. MICs with values up to 2μg/ml reinforce the need for microbiological surveillance. **Conclusions**: The study demonstrated low prevalence of strains resistant to the antimicrobial drugs indicated in the treatment of human listeriosis. Monitoring antimicrobial resistance profile is still very important to determine adequate treatment, especially in immunocompromised patients.

Keywords: *Listeria monocytogenes*. Antimicrobial susceptibilities. Listeriosis.

RESUMO

**Introdução**: *Listeria monocytogenes* é o agente etiológico da listeriose, doença de origem alimentar que acomete principalmente grávidas, pacientes immunoempridos e idosos. O tratamento primário é a associação de ampicilina a um aminoglicosídeo além de outros, em segunda escolha, representados por cloranfenicol, eritromicina, tetraciclina e rifampicina. O presente estudo teve como objetivo analisar o perfil de susceptibilidade aos antimicrobianos de amostras de origem humana isoladas nas últimas quatro décadas. **Métodos**: Foram selecionadas 68 cepas provenientes de casos clínicos humanos ocorridos em diferentes regiões do país no período de 1970-2008. A susceptibilidade aos antimicrobianos testados foi determinada através dos critérios estabelecidos por Soussy pelo método de Kirby-Bauer e a concentração mínima inibitória realizada através do E-Test. **Resultados**: A amostragem constituiu-se de 68 cepas, isoladas principalmente de líquido cefalorraquidiano, e hemocultura no período, pertencentes ao Laboratório de Zoonoses Bacterianas/LABZOO/FIOCRUZ. O sorovar L4b (60,3%) foi o mais prevalente, seguido do sorovar 1/2a (20,6%), 1/2b (13,2%) e aqueles mais raros representados por 1/2c, 3b e 4ab (5,9%). Todas as cepas foram sensíveis à ampicilina, cefalotina, eritromicina, gentamicina, teicoplanina e vancomicina. Apenas uma cepa (1,5%) apresentou resistência à rifampicina, enquanto duas (3%) foram resistentes à associação de sulfametoxazol-trimetoprim. **Conclusões**: A prevalência de cepas resistentes aos antibacterianos indicados para o tratamento de lista,eriose humana, em casos clínicos e em hemocultura, tem sido muito baixa. **Palavras-chaves**: *Listeria monocytogenes*. Susceptibilidade antimicrobiana. Listeriose.

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**Received in**: 27/10/2010

**Accepted in**: 15/12/2010

INTRODUCTION

*Listeria monocytogenes* is a gram positive, facultative anaerobe, intracellular bacterium and the etiologic agent of human and animal listeriosis. The disease affects primarily pregnant women, newborns and patients with degenerative diseases and/or immunocompromised patients, is clinically manifested as meningitis and septicemia, has a high mortality rate, between 20 and 30% of cases, and causes neurological sequelae in some cases1-3.

Members of the genus *Listeria* are widely distributed in nature and can be detected in the environment (soil, vegetables, silage and water) and in the intestinal tract of humans and animals2. The species is a significant food-borne pathogen4.

*Listeria monocytogenes* presents uniform antimicrobial susceptibility, including drugs commonly used for treating human listeriosis, such as ampicillin or in association with an aminoglycoside (e.g. gentamicin), and other second-choice antimicrobials represented by chloramphenicol, erythromycin, tetracycline and rifampicin5-6. However, clinical strains resistant to chloramphenicol, erythromycin, streptomycin, tetracycline, vancomycin and trimethoprim have been recently described7. The widespread distribution of epidemiologically serotypes of *L. monocytogenes* and their resistance to commonly used antibiotics indicate a potential public health risk. Given this situation, it is assumed that the system for monitoring antimicrobial resistance profile is extremely important to determine the appropriate treatment of human listeriosis. Therefore, the main goal of this study was to analyze the profile of antimicrobial resistance in strains isolated from humans in different regions of Brazil during the last four decades.

METHODS

Bacterial strains

Sixty-eight strains isolated from 1970 to 2008 were selected, including human clinical cases occurring in different regions of the country. The samples belong to the collection of Bacteriological...
Culture Collection Laboratory, Bacterial Zoonoses of the Oswaldo Cruz Institute/LABZOO/IOC/FIOCRUZ (Table 1), were maintained in tryptose agar semi-solid at 4°C throughout the study period and stored at -20°C in BHI plus 20% glycerol.

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<td>14</td>
<td>15</td>
<td>12</td>
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</table>

CFS: cerebrospinal fluid.

Phenotypic identification was performed in accordance with methods described by Rocourt & Seeliger. For the identification of serogroups/serovars, the technique of slide agglutination test was used, with poly and monovalent somatic and flagellar antisera prepared by LABZOO, according to the technical guidance of Seeliger & Höhne.

**Genotypic analysis by PCR**

The extraction of bacterial chromosomal DNA was performed using the Blood & Tissue Dneasy Kit (Qiagen), in accordance with the manufacturer’s specifications.

To determine the strains detected, primers targeting the 23S rRNA genes (marker of genus), \( hly \) (marker of the species \( L.\) monocytogenes) and the markers D1 and D2 to were used confirm the identification of serogroups/serovars, according to literature (Table 2).

The amplification reactions were performed in volumes of 25µl with a primer concentration of 50pmol/µl, 1U Taq polymerase, 0.2mM of each deoxynucleotide triphosphate, 2.5mM MgCl2 and 50ng of DNA. For the PCR, the PX2 thermal cycler equipment (Thermo Fisher Scientific Inc. Waltham, MA, USA) was used under the following conditions (D1 + D2 primers): an initial step of 95°C for 3min followed by 25 cycles at 95°C/30s, 56°C/30s, 72°C/1min and a final extension at 72°C for 10min. For amplification with primers 23S rRNA + \( hly \) 95°C/5min followed by 40 cycles of 95°C/1min, 62°C/1min and 72°C/1min, followed by a final extension at 72°C for 8min. All PCR products were determined by gel electrophoresis on 1% agarose 0.5 X TBE buffer and visualized under UV light after staining with ethidium bromide. As molecular weight markers, the 2-log DNA ladders were used (New England BioLabs Inc.).

**Antimicrobial susceptibility**

Antimicrobial resistance was analyzed using the disk diffusion method, in accordance with the CLSI, and was performed with standard discs (Oxoid) indicated for infections caused by Gram-positive bacteria: ampicillin (10µg), cephalothin (30µg), chloramphenicol (30µg), erythromycin (15µg), gentamicin (10mg), norfloxacinc (10µg), rifampicin (5µg), sulfamethoxazole/trimethoprimg (25µg), teicoplanin (30µg), tetracycline (30µg) and vancomycin (30µg). To maintain quality control of performance and reliability of the results, the standard strains of Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923 were used.

The size of the inhibition zone was determined according to CLSI guidelines, 2009, for Staphylococcus spp. Ampicillin and vancomycin were determined using the criteria established for Listeria spp. by Soussy et al. According to their behavior before the use of antibiotics, the strains were classified as sensitive, intermediate and resistant.

**Determination of minimum inhibitory concentration**

After examination of the susceptibility by disk diffusion method in agar, 43 strains were randomly selected to determine the minimum inhibitory concentration (MIC) to ampicillin (0.016-256µg/ml), tetracycline (0.016-256µg/ml) and rifampicin (0.016-256µg/ml) by the E-test method, in accordance with the manufacturer’s instructions (AB Biodisk). The MIC values were defined as the lowest concentration of antibiotic able to inhibit growth and the rate of change of MIC50 (where 50% of bacteria were inhibited) and MIC90 was calculated to specify the antimicrobial activity.

**RESULTS**

Of the 68 strains analyzed, 37 (53%) were isolated from cerebrospinal fluid (CSF), 26 (41%) were isolated from blood and the remaining 6% were isolated from one of the following samples: placental tissue, vaginal secretion, cervical lymphadenitis and peritoneal fluid. The strains showed absolute consistency in the results obtained from phenotypic and genotypic analyzes. Antigenic characterization of \( L.\) monocytogenes permitted the identification of five serovars, with the highest frequency determined for serovar 4b (n = 41, 60.3%), followed by 1/2a (n = 14, 20.6%) and 1/2b (n = 9, 13.2%), and the more uncommon serovars, 1/2c (n = 2, 2.9%), 3b (n = 1, 1.4%) and 4ab (n = 1, 1.4%). The temporal relations...
of the serotypes isolated are shown in Table 3. Currently, there is no criterion recommended by the CLSI for the interpretation of Listeria susceptibility, except for penicillin and ampicillin breakpoint, hence the breakpoints recommended for the interpretive criteria for Staphylococcus spp. were applied. All 68 strains analyzed were also susceptible to ampicillin, gentamicin, erythromycin, cephalothin, teicoplanin and vancomycin. Over the last four decades, a slight variation in the number of strains showing resistance to certain antimicrobials has been observed. In the 70s, only one strain of the serovar 1/2a (1.5%) was resistant to rifampicin isolated from CSF, and two serovar 4b (3%) samples isolated during the 1990s from blood were resistant to the association of trimethoprim-sulfamethoxazole. In contrast, in the 1980s and from 2000 to 2008, no resistance observed has been observed. A total of 29 (42.6%) strains have shown intermediate resistance profile for antimicrobials: chloramphenicol (7.4%), norfloxacin (27.9%), tetracycline (5.9%) and rifampicin (1.5%), distributed over the last four decades.

All 43 strains tested against antimicrobial agents (rifampicin, ampicillin and tetracycline) using the E-test were sensitive. Rifampicin had the lowest MIC90 (0.25µg/ml), indicating its effective activity against Listeria. The values for ampicillin and tetracycline ranged from 0.25 to 4µg/ml and showed a level of MIC90 of 2µg/ml (Table 4).

### DISCUSSION

Among the 13 serotypes of L. monocytogenes in the literature, serovar 4b is primarily responsible for most of the outbreaks in humans while the serovar 1/2a prevails in food and in some regions of the world where it is more common in human cases.

In relation to serovars of L. monocytogenes identified in this study, a higher incidence of serovar 4b (60.3%) was observed, which is in agreement with research by Hofer et al. and reports dating back to the 1970s. The frequency of serovar 4b was also demonstrated by Hofer et al. in renal transplant recipients from the same hospital in São Paulo. In the same state, Lemes-Marques et al. observed the incidence of the same serovar in clinical isolates from 1990 to 2005. Hofer et al. performed phenotypic analysis of strains of L. monocytogenes isolated from clinical material from 1969 to 2000 in different regions of the country, noting the higher incidence of serotype 4b followed by 1/2a, in agreement with the results obtained in this study. In the aforementioned study, the prevalence of serotype 4b in CSF samples compared to blood isolates was also evident, which is consistent with the results obtained in this work, particularly for the 1970s. It is important to emphasize that all the strains tested were susceptible to ampicillin, which incidentally is the principal drug of choice for the treatment of listeriosis. It association with gentamicin has also been indicated and used successfully in the treatment of listeriosis, a situation supported by this study, since all strains were susceptible to gentamicin. The discrete level of rifampicin resistance in this study, another drug of choice for treatment is in agreement with the findings of Hofer & Oliveira, and Pore-Gluckowska & Markiewicz, who reported resistance in clinical strains from different parts of the world. It appears that virtually the same profile has been identified over the years and in different countries. In relation to tetracycline, this research highlighted the extreme sensitivity of the 68 strains to this drug, contrasting with the emergence of clinical strains resistant to tetracycline, related to the gene tetM.

### ACKNOWLEDGMENTS

The authors would like to thank Evaldo Soares da Silva for his technical collaboration.

### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

### FINANCIAL SUPPORT

IOC/FIOCRUZ, CNPq (Proc. 301545/2006-5).

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