

Multiplex-PCR serotyping of *Listeria monocytogenes* isolated from human clinical specimens

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The genus Listeria is composed of six species of which Listeria monocytogenes is considered the single pathogenic species that causes listeriosis in humans. Of the 13 serovars of L. monocytogenes, 1/2a, 1/2b and 4b are responsible for the majority of clinical cases. The aim of this work was to detect L. monocytogenes in the cerebrospinal fluid sample of premature newborns and to characterize this sample using biotyping, serotyping and molecular typing. The results indicated the presence of L. monocytogenes in the clinical sample studied. Moreover, the isolate was identified as the 4b serovar that was characterized by the presence of a unique 691 bp band after analysis using the Multiplex-PCR technique. The results of repeated Multiplex-PCR and sequencing have indicated that the L. monocytogenes isolate was an atypical 4b serovar, which is the first time this finding has been reported.

Key words: *Listeria monocytogenes* - listeriosis - Multiplex-PCR - cerebrospinal fluid

The genus *Listeria* is composed of six species including *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri* and *Listeria grayi*. Only two of these species, namely *L. monocytogenes* in humans and *L. ivanovii* in other mammals, are considered pathogenic (Gasanov et al. 2005).

L. monocytogenes can survive and grow under various ambient conditions and is known as the pathogen that causes listeriosis. *L. monocytogenes* is a gram-positive bacterium that is mobile, non-spore-forming, and is characterized by a rod-shaped morphology. *L. monocytogenes* belongs to the facultative intracellular bacteria that invade, replicate and multiply in a variety of cells. Neonates, pregnant women, the elderly and the immunocompromised populations are particularly at risk of infection. The disease can result in an abortion, in addition to stillbirths, septicemia, meningitis, encephalitis and possibly death. The presence of this bacterium in the processing of foods and its natural distribution within the environment coupled with its inherent resistance and ability to grow in some foods, makes it difficult to control and regulate (Bubert et al. 1999, Nelson et al. 2004, Hofer & Hofer 2005, Gandhi & Chikindas 2007).

L. monocytogenes can be found in a wide variety of raw and processed foods. Dairy products such as soft cheeses have been associated with *Listeria* contamination and have been implicated in severe outbreaks (Hofer et al. 2006).

L. monocytogenes strains are serotyped according to their somatic and flagellar antigens. Although 13 serovars have been described, only three of these serovars, specifically 1/2a, 1/2b and 4b, account for the majority of clinical cases. Doumith et al. (2004a) affirms that at least 95% of strains isolated from contaminated foods and infected patients were of serotypes 1/2a, 1/2b, 1/2c and 4b. Interestingly, while serovar 1/2a is the most frequently isolated strain from contaminated foods, the majority of epidemic listerioses were caused by the type 4b strain.

Multiplex-PCR is a variant of traditional PCR techniques that introduces two or more sets of primer pairs with specificity for different genes or gene regions. For *L. monocytogenes* serotyping, a PCR-based method was developed to probe four gene targets. The four major serotypes 1/2a, 1/2b, 1/2c and 4b can produce four distinct PCR profiles with properly designed primer pairs. This technique can be easily adapted to different laboratories, is quick and reproducible (Doumith et al. 2005). The Multiplex-PCR analysis is utilized to detect the presence of virulence-associated genes of *L. monocytogenes* (Kaur et al. 2007). It was developed for rapid speciation and virulence determination of *L. monocytogenes* (Liu et al. 2007).

The aim of this work was to identify the species of *Listeria* that is present in a particular clinical sample and to determine the serovar through biotyping, serotyping and molecular typing using the Multiplex-PCR method, as described by Doumith et al. (2004a).

SUBJECTS, MATERIALS AND METHODS

The clinical strain was isolated from a sample of the cerebrospinal fluid (CSF) from a newborn whose birth occurred at the 7th month of gestation. The newborn's mother reported a fever and inertia after consuming gorgonzola cheese. The strain that was isolated from the CSF sample was cultured for 16 h in nutrient-rich broth and the enriched culture was spread onto a chromogenic

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agar plate (Oxoid) that is specific for *L. monocytogenes* (Scotter et al. 2001). Five presumptive colonies were selected and biochemical identification of *L. monocytogenes* was performed in accordance with methods described by Scotter et al. (2001). The isolated colonies were serotyped on the basis of somatic (O) and flagellar (H) antigens, according to Seeliger and Höhne (1979) in the Bacterial Zoonosis Lab. of Oswaldo Cruz Institute/Fiocruz. The Multiplex-PCRs were performed using four primer pairs that were specific for *L. monocytogenes* in addition to one primer pair that was specific for *Listeria* spp. (Doumith et al. 2004a). The amplified fragments were electrophoresed in a 2% agarose gel (in TBE buffer), stained with ethidium bromide solution (10 mg/mL), visualized with a UV transilluminator and photographed.

RESULTS

In the chromogenic agar, formation of blue halo colonies characteristic of *L. monocytogenes* was observed. The isolated colonies were positive for glucose, maltose and rhamnose, negative for manitol and xilose. They were catalase-positive, small gram positive bacteria that were rod-shaped, and capable of producing a narrow beta-hemolysis zone on sheep blood agar. In addition, results from a CAMP test using beta hemolysin-producing *Staphylococcus aureus* were positive and a tumbling motility test at 30°C indicated that the bacteria were motile. The conclusive identification of *L. monocytogenes* serovar 4b, genus 23S, hly +, was performed according to the guidelines reported by Rocourt et al. (1983). Analysis of the Multiplex-PCR data in accordance with Doumith et al. (2004a) indicated a variety of bands, including a 370 bp band (*Listeria* spp.), a 471 bp band (*L. monocytogenes* serovar 1/2b, 3b, 4b, 4d, 4e), a 597 bp band (*L. monocytogenes* serovar 4b, 4d, 4e) and a 691

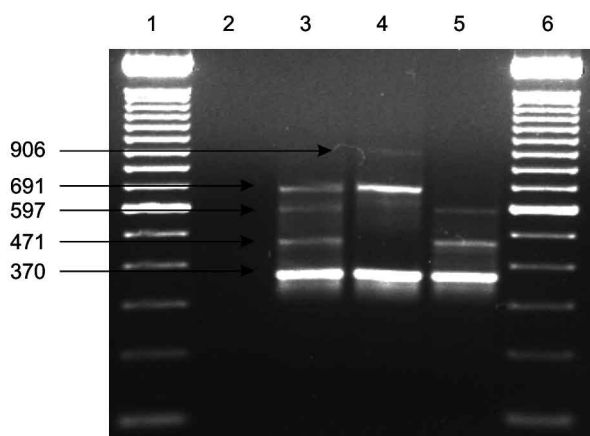
bp band (*L. monocytogenes* serovar 1/2a, 1/2c, 3a, 3c) (Figure). The strains were identified as belonging to the *L. monocytogenes* serovar 4b. The reproducibility of the 691 bp fragment was ratified by a repeated Multiplex-PCR assay and a Simplex-PCR. Direct sequencing of this band proved that this fragment is the gene *lmo0737* (NCBI Access: EU980451).

The results of biotyping, serotyping and molecular typing confirmed the presence of *L. monocytogenes*. However, the appearance of the 691 bp fragment in the Multiplex-PCR analysis was not characteristic of the 4b serovar. Studies from Doumith et al. (2004b) report a relationship between the two larger genomic divisions of species that are represented for serovars strains 1/2a and 4b, suggesting a possible horizontal gene transfer event. This result suggests that the strain identified in this work appears to be a unique 4b serovar that has undergone a gene transfer event. In accordance with the work of Chen and Knabel (2007), the lineage I, containing serovars 1/2b, 3b, 3c and 4b of the *L. monocytogenes* and its isolates, include major epidemic clones of *L. monocytogenes* that cause a large number of human listeriosis cases. Previous molecular subtyping studies have identified four major epidemic clones of *L. monocytogenes*, where three of these major epidemic clones (ECI, ECII and ECIV) belong to serovar 4b. Among the 13 serovars of *L. monocytogenes*, isolates from serovars 1/2a and 4b account for most of the major listeriosis outbreaks and, interestingly, all identified epidemic clones of *L. monocytogenes* belong to these two serotypes. The serovars 1/2a and 4b are characterized by the specific markers *lmo0737* and *ORF2110*, respectively. Studies from Doumith et al. (2004a) shows that the marker *lmo0737* produces a 691 bp fragment in 1/2a isolates.

DISCUSSION

In agreement with a report by Ericsson et al. (1995), the majority of the cases of human listeriosis over the past decade have been caused by the *L. monocytogenes* serovar 4b strain. Results from Kathariou (2002) also affirms that the serovar 4b has been responsible for numerous sporadic cases. Furthermore, Gray et al. (2004) reported that the majority of cases of human listeriosis outbreaks have been caused by serovar 4b. However, only in rare occasions does a non-4b serovar strain of listeriosis account for numerous outbreaks. Jacquet et al. (2004) reports that most of the major food borne outbreaks due to invasive listeriosis in both Europe and North America have been caused by *L. monocytogenes* strains belonging to serovar 4b. Doumith et al. (2004b) reported that the majority of sporadic cases of foodborne outbreaks have been caused by serovar 4b strains, suggesting that these strains may possess unique virulence properties. Upon relating the serovar distribution with its geographic origins, Hofer et al. (2006) observed a predominance of the 4b serovar occurring in all regions of Brazil during the periods of 1969 and 1972-2000. Ramaswamy et al. (2007) reports that the most invasive outbreaks were caused by 4b serovar.

The isolate described in this study demonstrated that a new bacterial strain can emerge with variations that result



Representative Multiplex-PCR amplification patterns of clinical isolate of *Listeria monocytogenes* analyzed by 2% agarose gel electrophoresis. Lanes 1, 6: molecular weight marker (100 bp, Invitrogen); 2: Multiplex-PCR negative control; 3: *L. monocytogenes* isolated (370, 471, 597, 691 bp); 4: positive control 1, ATCC 7644 (INCQS 00266) (370, 691, 906 bp; Serovars: 1/2c and 3c); 5: positive control 2, ATCC 19117 (INCQS 00327) (370, 471, 597 bp; Serovars: 4b, 4d and 4e).

from horizontal gene transfer. This is an interesting and significant result considering that the serovar 1/2a is the most frequently isolated food borne pathogen, although the majority of epidemic listeriosis is caused by type 4b.

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