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Molecular and experimental virulence of *Listeria monocytogenes* strains isolated from cases with invasive listeriosis and febrile gastroenteritis

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Abstract

We analyzed 27 *Listeria monocytogenes* strains of serotypes 1/2b and 4b, from invasive and gastroenteric listeriosis, for molecular and experimental virulence. Molecular virulence was tested by PCR for the presence of 8 major virulence-associated genes and genetic polymorphisms through restriction enzyme analysis; genomic DNA typing using pulsed-field gel electrophoresis was also performed. Experimental virulence was evaluated through intra-peritoneal and intra-gastric mouse virulence assays. Our results showed no significant differences in the virulence-related molecular properties of the strains analyzed. All strains were equally pathogenic following intra-peritoneal inoculation of mice. In mice inoculated intra-gastric with 4 representative strains of the 2 types of listeriosis, there were no significant differences in the bacterial count when comparing invasive and gastroenteric strains, suggesting that the strains were comparable in terms of mean oral infectivity.

Keywords: Listeriosis; Gastroenteritis; *Listeria monocytogenes*; PCR-RFLP; PFGE; Mouse virulence assay

1. Introduction

Foodborne listeriosis results from the ingestion of foods contaminated with the microbial pathogen *Listeria monocytogenes*. In adults, two main forms of the clinical syndrome have been described [1]: invasive listeriosis and gastroenteric listeriosis, the latter having been recognized only recently, following the identification of *L. monocytogenes* as the causative agent of several episodes of febrile gastroenteritis [2–10]. The two clinical forms differ in terms of clinical spectrum, target population, infectious dose, and incubation time [11].

Traditionally, the pathogenic mechanism of *L. monocytogenes* in invasive listeriosis has been described as a four-step process affecting either non-professional phagocytes (intestinal epithelial cells and hepatocytes) or professional phagocytes (macrophages), as follows: adherence and internalization; escape from the phagocytic vesicle into the host-cell cytoplasm; multiplication and movement through polymerizing actin filaments; and spread to the adjacent cells [1]. Each step is governed by one or more specific virulence factors produced by *L. monocytogenes*, which are encoded by distinct virulence-associated genes [12]. In susceptible individuals (e.g., immunocompromised individuals, the elderly, and pregnant women) even low quantities of cells ingested with contaminated food can cross the intestinal barrier, enter the circulation, multiply in the liver and spleen and, in the absence of an adequate immune

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response, spread to the target organs (i.e., the central nervous system and the placenta) [1]. In healthy individuals, extremely high quantities of cells cause invasion of the enterocytes in the intestinal tract, with consequent gastro-enteritis and fever, yet with no apparent progression to systemic infection [11]. It is well established that the altered immune response of people who get involved, or the high number of listeriae ingested play the major roles in the invasive or gastroenteric clinical outcomes, respectively. However, the possibility exists that differences in the virulence of strains might also contribute to the diverse clinical manifestations.

In vivo animal studies and cell cultures have shown that *L. monocytogenes* strains display variation in virulence [13–16]. Molecular analyses, including the detection of polymorphisms in known *L. monocytogenes* virulence-associated genes, and genomic DNA typing, also have some predictive value for *L. monocytogenes* pathogenicity, as demonstrated by the association found between subtypes and the virulence of strains [16,17].

In the present work, we analyzed 27 strains of serotypes 1/2b and 4b, from invasive and gastroenteric listeriosis, and 3 atypical non-human strains which lack expression of virulence, comparing the genetic polymorphisms in 8 major virulence-associated genes and the pulsed-field gel electrophoresis (PFGE) profiles of genomic DNA. To evaluate pathogenicity, all strains were tested with a mouse intra-peritoneal (i.p.) injection virulence assay. We also evaluated the oral infectivity of four *L. monocytogenes* isolates from invasive and gastroenteric listeriosis in A/J mice treated with pentobarbital, which have recently been shown to be a suitable murine model for orally acquired listeriosis [18,19].

2. Materials and methods

2.1. Bacterial isolates

Most of the *L. monocytogenes* isolates (Table 1) were from the culture collection of the Istituto Superiore della Sanità (ISS). Twenty-seven were of clinical origin, 9 of which were of serotype 1/2b and 18 of serotype 4b. All had previously been implicated in either sporadic cases or outbreaks of invasive or gastroenteric listeriosis. The isolates from gastroenteric listeriosis were from 5 distinct episodes, 2 of which occurred in Italy [2,7] and 1 each in Canada [6], Denmark [4] and the United States [3] (the latter strains were kindly provided by J.M. Farber, Health Canada, Ottawa, Canada; P. Gerner-Smidt, Statens Serum Institut, Copenhagen, Denmark; and B. Swaminathan, Centers for Disease Control and Prevention, Atlanta, GA). *L. monocytogenes* ScottA, from a large outbreak of invasive listeriosis [20], served as a virulent reference strain (provided by M.P. Doyle, University of Georgia, Griffin).

Three strains were of non-human origin: 2 were type strains from the ATCC collection and had originally been isolated from guinea pig (ATCC 43248) [21] and rabbit (ATCC 15313) [22], whereas the remaining strain (L 285) was isolated at the ISS from turkey meat imported from Brazil. Strain ATCC 43248 was of serotype 1/2a, whereas it was not possible to serotype the other 2 strains with the specific commercial kit used (Listeria Antiserum kit; Denka Seiken, Tokyo, Japan).

All isolates had previously been confirmed as *L. monocytogenes* on the basis of the sugar fermentation patterns (API Listeria; BioMérieux, Marcy l'Etoile, France), ELISA assay (Vidas *L. monocytogenes*; BioMérieux), and hemolysis on blood agar plates. However, the 3 strains of non-human origin were non-hemolytic. The atypical properties and lack of virulence of strains ATCC 43248 and ATCC 15313 had already been reported [23]: these two isolates were used as avirulent reference strains.

2.2. PCR amplification of virulence-associated genes and restriction-fragment length polymorphisms analysis

Extraction, purification and quantification of DNA from each strain were performed as previously described [24]. All of the primers used for specific PCR amplifications of the entire coding sequences of virulence-associated genes are reported in Table 2. PCR reactions with each primer pair were carried out separately in a programmable thermal cycler (M.J. Research, Waltham, MA). A final volume of 50 μl was used, containing 1 μl of DNA, dNTPs at a final concentration of 200 μM each, 1.25 U of Taq-polymerase (Perkin–Elmer, Foster City, CA), and the buffer provided by the manufacturer. Primers and $MgCl_2$ concentrations were optimized for each gene amplification. The cycling conditions were those described by Vines et al. [25]. Ten microlitre of the amplified products was separated by electrophoresis in 1% agarose gel containing ethidium bromide, and visualized under UV.

The remaining positive PCR reaction volumes were purified by the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and digested separately with 2 or 3 of the following endonucleases: *AcsI*, *ClaI*, *DraI*, *HaeII*, *HindIII*, *PstI*, *SacI*, *SspI* and *XhoII* (Roche Diagnostics, Penzberg, Germany). For each gene, the enzymes chosen were those, which according to the Webcutter 2.0 software (http://rna.lundberg.gu.se/cutter2/), were predicted to cut the sequence. The digested products were separated through electrophoresis in 2.5% agarose gel and subsequently visualized with ethidium bromide fluorescence.

2.3. PFGE typing

PFGE was performed according to the PulseNet standardized protocol [26]. DNA samples were cleaved