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INVESTIGATION OF *L. MONOCYTOGENES* – HEP-2 CELLS RELATIONSHIPS BY CULTURE BASED AND MICROSCOPY TOOLS

Marius Eduard CAPLAN[1], Lorena Andreea MATEESCU[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, 011464, Bucharest, Romania
[2]University of Bucharest, Faculty of Biology, 1-3 Portocalelor Ale., District 6, 60101, Bucharest, Romania

Corresponding author email: eduardcaplan@yahoo.com

Abstract

The first step of the infection process must triggered by virulent Listeria monocytogenes strains is the attachment to, and invasion of, the gastrointestinal epithelium. The purpose of this paper was to investigate the ability of L. monocytognes species isolated from different clinical specimens to adhere, invade and multiply in HEp-2 cells. All investigated L. monocytogenes strains exhibited the ability to adhere, invade and multiply in the eukaryotic HEp-2 cells, the internalization being followed by the activation of cellular signaling pathways, leading to the release of thin, long cytoplasmic extensions, vacuolization and apoptosis. The ability of L. monocytogenes strains to survive inside the host cell could explain their implication in chronic recurrent diseases and long-term colonization, the internalization process providing effective protectionagainst host defenses and antibiotic treatment.

Key words: *Listeria monocytogenes, apoptosis, fluorescence, invasion, HEp-2 cells.*

INTRODUCTION

Listeria monocytogenes is a fascinating bacterial pathogen able to survive in a saprophytic environment and to induce disease in mammalian hosts (Pizzaro-Cerda et al., 2012). The potential of the saprophytic *L. monocytogenes* to cause listeriosis, especially in newborns and immunocompromised individuals is correlated with its capacity to survive within macrophages, to invade nonphagocytic cells and replicate therein (Allerberger and Wagner, 2010). The first step of infection must be attachment to, and invasion of, the gastrointestinal epithelium by virulent *L. monocytogenes* (Schlech et al, 1994; Carnejo et al., 2011). The purpose of this paper was to investigate the ability of *L. monocytognes* species isolated from different clinical specimens to adhere, invade and multiply in HEp-2 cells.

MATERIALS AND METHODS

Bacterial strains

The experiment was performed on *L. monocytogenes* strains, collected from NIRDMI Cantacuzino *Zoonosis* Laboratory Collection, isolated from different clinical specimens (Table 1).

Table 1. Source of isolation and identification/confirmation of L. monocytogenes from the investigated strains (No.)

Year of isolation **Clinical specimen**	**2010**	**2011**	**2012**
Blood culture Septicemia	2		1
Cerebrospinal fluid Meningitis	1	5	1
Cerebrospinal fluid Meningo-encephalitis	1		
Total	4	5	2

The analyzed strains have been identified using classical cultural, biochemical and serological tests and were preserved at-80ºC in Brain Heart Infusion (BHI) broth (Oxoid) with 20% glycerol and next there were streaked on 7% blood agar plates at 37ºC for 24 hrs, prior the experiments (McLauchlin, 2005).

Study of the adherence and invasion capacity to the cellular substrate represented by HEp-2 cells (Cravioto's adapted method) (Cravioto et al., 1979; Lazar et al., 2002; Chifiriuc et al., 2010).

In this purpose, HEp-2 cells were routinely grown in Eagle's minimal essential medium (Eagle MEM) supplemented with 10% heat inactivated (30 min at 56°C) foetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), and 0.5 ml of gentamycin (50 µg/ml) (Gibco BRL) and incubated in a 5% CO_2 humidified atmosphere, at 37°C for 24 hrs (Kalliomaki et al., 2001). HEp-2 cell monolayers grown in 6 multi-well plastic plates were used at 80-100% confluence. Bacterial strains from an overnight culture on 2% nutrient agar were diluted at 107 CFU/ml in Eagle MEM with no antibiotics. The HEp-2 cell monolayers were washed 3 times with Phosphate Buffered Saline (PBS) and 2 ml from the bacterial suspension were inoculated in each well. The inoculated plates were incubated for 3 hrs at 37°C. After incubation, the monolayers were washed 3 times with PBS, briefly fixed in cold ethanol (3 min), stained with Giemsa stain solution (1:20) (Merck, Darmstadt, Germany) and incubated for 30 min. The plates were washed, dried at room temperature overnight, examined microscopically (magnification, ×2500) with the immersion objective (IO) and photographed with a Contax camera (Company, City, Country) adapted for Zeiss (Axiolab 459306) microscope (Zeiss, City, Country). For the quantitative assay of adhesion and invasion capacity, the infection step was performed in duplicates for each strain, and after 3 hrs incubation of the HEp-2 monolayer in the presence of microbial strains, the first well plates were washed four times in PBS, the cells were permeabilized by Triton X 1% (Sigma) and incubated for 5 min at 37°C for the release of intracellular invasive bacteria. Thereafter, serial ten-fold dilutions in saline solution were performed and 20 µl from each dilution was spotted in triplicates on solid media; in the second plate, after 2 hrs of incubation the monolayer was washed 4 times in PBS and 1 ml of 100 mg/ml gentamycin solution was added; the plates were further incubated for 1 h, in order to kill all adherent extra-cellular bacteria. Thereafter, the second plate was treated as the first one. After incubation at 37°C for 24 hrs, we counted the bacterial colonies in each spot and the results were expressed by CFU/ml.

Fluorescent actin staining (FAS) (Knutton et al., 1991; Chifiriuc et al., 2008). Bacterial suspensions in nutrient broth prepared from cultures of 24 hrs on agar plates were used for being inoculated into the subconfluent, HEp-2 monolayers of 24 hrs cultivated in 6-multiwell plates with coverslips. After 3 hrs of incubation at 37°C, the plates were washed 3 times in PBS and the cells were briefly fixed by glutaraldehyde and permeabilized with PBS-Sap-BSA. The coverslips were removed, stained with DAPI or PI (propidium iodide), mounted in glycerol-PBS and examined by incident-light fluorescence using an Olympus Bx40 fluorescence microscope with adequate filters.

RESULTS AND DISCUSSIONS

Our results revealed that *L. monocytogenes* tested strains exhibited different adherence abilities for colonizing the HEp-2 cells, as demonstrated by different adherence patterns and rates (Table 2).

The invasion assay revealed that 27.3% of the analyzed strains were highly invasive, 27.3% were moderately invasive and 45.4% were classified as low invasive (Table 2).

The low invasion rate of some of the tested strains is reflecting a reduced ability to internalize and multiply inside the eukaryotic cells following the initial adherence step. The examination of fluorescent labeled HEp-2 cells infected with *L. monocytogenes* confirmed the results obtained in the quantitative assay of viable, internalized cells.

Table 2. The adherence and invasion percentages of the tested cells on HEp-2 cells

Tested parameter	**(%) of positive strains**
Adherence to the cellular substratum	**100%**
Localized adherence	9%
Diffuse adherence	27%
Aggregative adherence	37%
Mixed adherence patterns	27%
Invasion of the HEp-2 cells substratum	**100%**
Highly invasive 3	27.3%
Moderately invasive 3	27.3%
Low invasive 5	45.4%

The invasion ability was present in all tested strains (Figure 1).

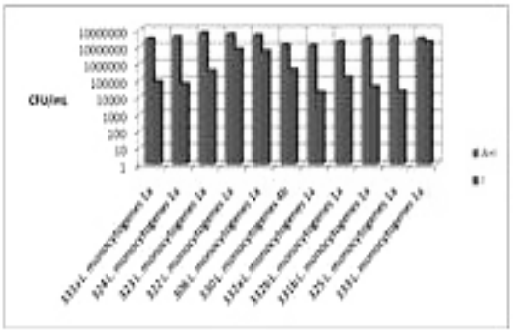


Figure 1. Graphic representation of the invasion and adherence rates of different L. monocytogenes strains

Thus the fluorescence microscopy aspects showed the presence of single bacterial cells or associated in microcolonies inside the mammalian cells, embedded in cytoplasmic vacuoles (Figure 2).

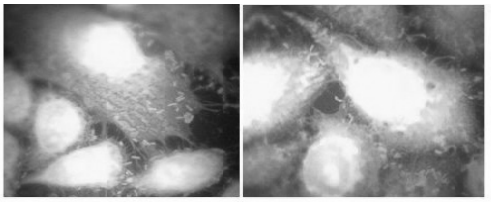


Figure 2. PI stained HEp-2 cells infected with *L. monocytogenes*, showing single bacterial cells or associated in microcolonies inside the mammalian cells. a. Bacterial cells adhered to HEp-2 cells; b. Cytoplasmic vacuoles

As the quantitative assay of the invasive ability is based on the counting of viable cells, it is possible that the internalized bacterial cells be metabolically active, playing an important role in the intracellular survival and antibiotic resistance, factors favoring the persistence of these infections.

The bacterial cell interaction with the host cell induced changes in the epithelial cell membrane, which exhibited long and thin membrane elongations, aspect demonstrating an endocytic process triggered by the bacterial cells (Figure 3).

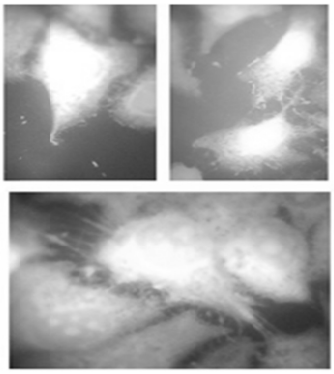
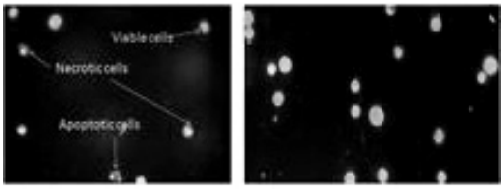


Figure 3. PI stained HEp-2 cells infected with *L. monocytogenes,* showing long and thin membrane extensions (x 100)

Once present in the cytosol of HEp-2 cells, *L. monocytogenes* could induce the apoptosis of the host cell, as revealed in Figure 4.



a. Infected HEp-2 cells; b. Control cells

Figure 2. FAS staining of HEp-2 cells infected with *L. monocytogenes* (DAPI staining, x40)

CONCLUSIONS

All investigated *L. monocytogenes* strains exhibited the ability to adhere, invade and multiply in the eukaryotic HEp-2 cells, the internalization being followed by the activation of cellular signaling pathways, leading to the release of thin, long cytoplasmic extensions, vacuolization and apoptosis. The ability of *L. monocytogenes* strains to survive inside the host cell could explain their implication in chronic recurrent diseases and long-term colonization, the internalization process providing effective protection against host defenses and antibiotic treatment.

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