VIRULENCE FEATURES OF *L. MONOCYTOGENES* STRAINS ISOLATED FROM MEAT PRODUCTS

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Abstract

Listeria monocytogenes is an emerging bacterial foodborne pathogen responsible for listeriosis outbreaks. Frequently, listeriosis is transmitted through cured or processed meat, poultry, fish, seafood, dairy products, vegetables. This illness is characterized by septicaemia, meningitis, encephalitis and abortive disease (stillbirth or premature birth of the fetus). Listeria monocytogenes isolates from raw and processed meat were studied for the production of cell associated (adherence to HEp-2 cells) and enzymatic virulence factors, i.e.: pore forming toxins (hemolysine, lecithinase, lipase) and exoenzymes (gelatinase, amylase, caseinase, esculinase, DNase). The majority of the tested strains revealed adherece to HEp-2 cells with a predominant diffuse-aggregative pattern, as well as hemolysine, esculinase, caseinase and lipase. All L. monocytogenes strains harbored the hlyA gene. The presence of different virulence features in L. monocytogenes strains isolated from food products may explain the implication of these strains in the occurrence of severe illness.

Key words: *Listeria monocytogenes, meat products, virulence factors.*

INTRODUCTION

Taxonomically, *Listeria* genus is divided into ten species: *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. grayi* (McLauchlin, 2005), and the new described species *L. rocourtiae* (Leclerq et al., 2010), *L. marthii* (Graves et al., 2010), *L. fleischmanii* (Bertsch et al., 2013) and *L. weihenstephanensis* (Lang Halter et al., 2013). From them, only *L. monocytogenes* and *L. ivanovii* are pathogenic (Liu, 2006). *L. monocytogenes*, an important human and animal pathogen, is responsible for major outbreaks associated with food products. Although low-level *L. monocytogenes* contamination of meat products is relatively common, suggesting widespread exposure, foodborne listeriosis occurs in only a small proportion of susceptible individuals. In pathophysiology of

listeriosis, a main role is played by many virulence factors of this microorganism. In the present study, *L. monocytogenes* strains isolated from raw meat and meat products were tested for the presence of soluble virulence factors and ability to adhere to HEp-2 cells, as well as for the presence of *hlyA* gene.

MATERIALS AND METHODS

Bacterial strains

L. monocytogenes 1 (ATCC 19111) reference strain used in the study was obtained from NIRDMI Cantacuzino Bacterial Collection, Bucharest.

The experiment was performed on *L. monocytogenes* strains, collected from NIRDMI Cantacuzino *Zoonosis* Laboratory Collection, isolated from animal meat: 4 strains in raw meat - raw minced meat, pork and beef muscular tissues, poultry (carcass); 5 strains in meat products - sausages and other pork and beef preparations and 1 strain from boiled shell snails (Table 1).

For the detection and confirmation of *Listeria* spp. and *L. monocytogenes* there were used SR ISO 11290/2004 standards (Part 1 and Part 2). The following steps were followed: resuscitation, enrichment in demi-Fraser broth, selective enrichment in Fraser broth, isolation on 7% sheep blood agar and identification on PALCAM agar. Green colour colonies surrounded by a black zone on PALCAM agar plates were collected for further biochemical confirmation (CAMP test, carbohydrates use reactions), along with the reference strain *L. monocytogenes* type 1 ATCC 19111.

All the samples were subjected to serotyping tests performed with hiperimmune rabbit adsorbed sera against *L. monocytogenes* serotype 1a and *L. monocytogenes* serotype 4b (Table 1).

The stock cultures were maintained 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 experiment.

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

Samples	*B-hemo-lysis*	*CAMP Test*		*Acid from*				*L. monocytogenes Serological identification*	
		Staphylococcus aureus	*R. equi*	*D-Glucose*	*D-Manose*	*L-Rhamnose*	*D-Xylose*	*serotype 1a*	*serotype 4b*
Raw minced meat (pork, beef)	1	1	-	1	1	1	-	1	-
Pork muscular tissue	1	1	-	1	1	1	-	1	-
Beef muscular tissue	1	1	-	1	1	1	-	1	
Poultry carcass	1	1	-	1	1	1	-	-	1
Paste of Romanian sausages	2	2	-	2	2	2	-	2	-
Fresh sausages	2	2	-	2	2	2	-	2	-
Smocked bacon	1	1	-	1	1	1	-	1	-
Boiled shell snails	1	1	-	1	1	1	-	1	-

Characterization of bacterial adherence to HEp-2 cells

Adherence tests were performed on HEp-2 cells, by using Cravioto's adapted method (Cravioto et al., 1979 and Lazar, 2003). The cells were cultured in Eagle Minimum Essential Medium (EMEM), supplemented with 10% bovine fetal serum, without antibiotics. The HEp-2 cells were grown to 70-80% confluent monolayers in tissue culture plates. The cell monolayers

were washed 3 times with phosphate buffered saline (PBS) and to each well was added 1 mL of fresh medium without antibiotics. Suspension from bacterial mid-logarithmic phase cultures grown in nutrient broth was adjusted to 10^8 CFU/mL and 1 mL was used for inoculating each well. The inoculated plates were incubated for 2 hrs at 37°C. After the incubation period, cells were washed 3 times with PBS, fixed with 70% methanol for 3 min, and stained with 10% Giemsa solution for 20 min. The plates were washed, dried at room temperature overnight, and examined by light microscopy (x2500) to evaluate the adherence patterns.

In vitro characterization of enzymatic virulence factors

Bacterial strains grown for 18 hrs incubation at 37°C, in nutrient broth, were cultivated on available media containing specific substrate for enzymes activity detection (Delcaru et al., 2012).

Lecithinase and **lipase** are enzymes implicated in pore production and bacterial invasion. Their activity was tested by spotting onto 2.5% yolk agar, respectively Tween 80 agar with a substrate at a final concentration of 1%. After incubation at 37ºC for 72 hrs, the reactions were read. A positive reaction was considered as a clearing zone surrounding the growth area for lecithinase activity, respectively an opaque (precipitation) area for lipase activity.

Caseinase and **gelatinase** are enzymes implicated in the tissue damage, evolution of infection process and rapid bacterial multiplication. For detection of these enzymes' production, the tested strains were spotted onto agar plate with 15% soluble casein, and respectively on agar with gelatin at 1% final concentration, after incubation at 37°C for 72 hrs, the reactions were examined. Proteolytic activity is showed by an opaque (precipitation) area for caseinase production, and respectively a transparent zone around the culture for gelatinase.

The **amylase** activity, an enzyme implicated in the polysaccharides hydrolysis, offering a nutritional competitive advantage to producing bacterial strains, was determined using starch as a substrate (1% final concentration in nutritive glucose). After 72 hrs incubation at 37°C the reaction was read. Starch hydrolysis appeared as a precipitation zone surrounding the culture spot.

DNase production, an enzyme that catalyses the hydrolytic cleavage of cellular DNA, with lesions in host cell, was studied by spotting the strains onto DNA agar medium. After incubation for 24 hrs at 37°C, a drop of HCl 1N solution was added to each spotted culture and the reactions were

examined. A clearing area surrounding the spot area was registered as a positive reaction.

PCR analysis

For molecular confirmation of the biochemically and serologically identified *L. monocytogenes* strains PCR was used for the detection of *hlyA* gene, which encodes for the main virulence factor of *L. monocytogenes*, listeriolysin O.

Preparation of bacterial DNA. Bacteria were cultured in 1 ml BHI overnight then centrifuged and resuspended in 1 ml of sterile water (2x) for washing. Genomic DNA was extracted from the isolates by boiling at 100^{o}C for 10 min followed by cooling at -20^{o}C. The primers used in this study are listed in Table 2.

Table 2. Primers used for the PCR assay

Primer	**Sequence**	**Gene**	**Source**
LL7 LL8	TTG CCA GGA ATG ACT AAT CAA G ATT CAC TGT AAG CCA TTT CGT C	*Hly A*	Amagliani, 2004

Amplification was performed in a total reaction volume of 25 µl. The reaction mixture contained 12.5 µl PCR Master Mix 2x, 0.5µl each primer, 1µl of template, and double-distilled H_2O was added to make a total volume of 25 µl. The parameters for the amplification cycles were as follows: denaturation for 15 s at 95^{o}C, annealing of primers for 20 s at 61^{o}C, and primer extension for 30 s at 72^{o}C (50x). Prior to the first cycle, the PCR mixture was incubated for 15 min at 95^{o}C. After the last cycle, the mixture was incubated for 5 min at 72^{o}C for the final elongation. The *L. monocytogenes* type 1 (ATCC 19111) strain was used as known positive strain in PCR analysis. The PCR products were analyzed by electrophoresis in 1.5% agarose gel.

RESULTS AND DISCUSSIONS

During this study we have studied the virulence feature of *L. monocytogenes* strains isolated from different food products. The tested strains formed β - haemolytic colonies on blood agar plate, while on PALCAM agar they have shown green colour colonies surrounded by a black zone, indicating the presence of esculinase, responsible for esculine

hydrolysis, resulting esculetol, which could act as an iron chelating agent, providing iron even in limited conditions. When tested for fermentation reactions, *L. monocytogenes* strains were positive for D-Glucose, D-Manose, L-Rhamnose and negative for D-Xylose (Table 1). CAMP test was positive, with an arrow head shape haemolysis in the presence of *Staphylococcus aureus* (Figure 1).

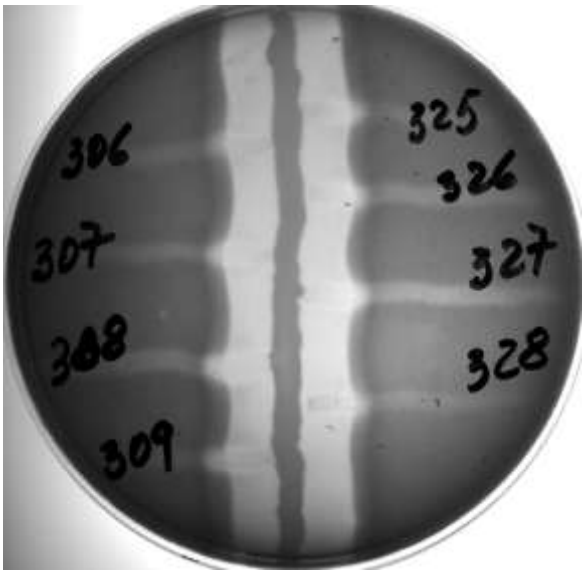

Figure 1. Positive CAMP Test for *L. monocytogenes* strains in the presence of *S. aureus* strains

The microbial adherence to different substrata, specially to epithelial cells, represents a *sine qua non* condition for the colonization of the host organism and the progression of the infectious process.

The *L. monocytogenes* strains exhibited three distinct adherence patterns to HEp-2 cells, i.e.: localized adherence, in which bacteria form characteristic microcolonies adhered on the surface on the host cell (Figure 2a), diffuse adherence, in which isolated *L. monocytogenes* cells adhere uniformly to the whole surface of the eukaryotic cell (Figure 2b), and aggregative adherence, in which large bacterial aggregates adhere both to the host cell surface and between them in a stacked brick appearance (Figure 2c). Some mixed patterns, i.e.: diffuse-localized, aggregative-localized and diffuse-aggregative have been also observed.

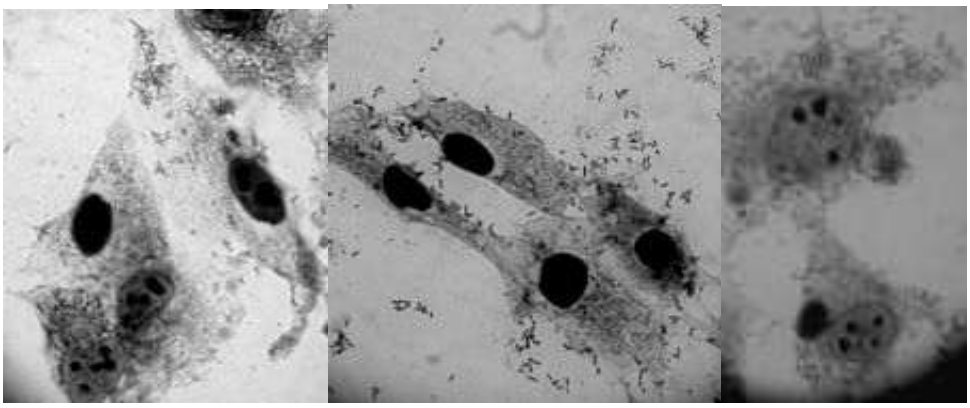

Figure 2. a. Localized adherence - characteristic microcolonies on the surface of HEp-2 cells. b. Diffuse adherence – bacterial cells dispersed over the cell surface. c. *L. monocytogenes* diffuse-aggregative adherence - both to the host-cell surface and between them in a stacked brick appearance (x2500).

In this study, the majority of the tested strains adhered to HEp-2 cells with a predominant diffuse-aggregative pattern (Table 3).

Table 3. Adherence patterns of *L. monocytogenes* strains isolated from meat

Adherence patterns		***L. monocytogenes (No.)***	
		Serotype 1a	***Serotype 4b***
Localized adherence		1	-
Diffuse adherence		2	-
Aggregative adherence		1	-
Mixed pattern	*diffuse-localized*	1	-
	aggregative-localized	1	-
	diffuse-aggregative	3	-
Negative		-	1

The secretion of soluble virulence factors is very important for the evolution of *L. monocytogenes* infectious process. The expression of the tested soluble virulence factors is shown in Table 4.

Lipase, acting as pore-forming toxin, was present in the majority (9 from 10) of *L. monocytogenes* strains isolated from meat products.

Caseinase, which increases by its activity the progression of the infectious process, was revealed at all *L. monocytogenes* tested strains.

Lecithinase, gelatinase, amylase and DNase were absent in this food isolates.

Table 4. Presence of soluble virulence factors in *L. monocytogenes* strains isolated from meat.

Virulence factors	***Tested strains (Nr.)***	***Positive strains (Nr.)***
Lipase	10	9
Lecithinase	10	0
Caseinase	10	10
Gelatinase	10	0
Amylase	10	0
DNase	10	0
Esculinase	10	10
Listeriolysin O	10	10

All the analyzed strains have been confirmed by the PCR analysis of the hlyA gene (Figure 3).

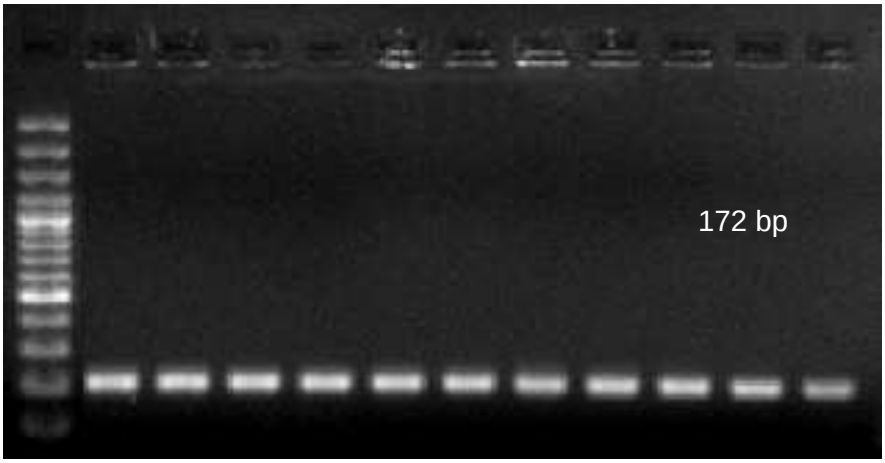

Figure 3. Agarose gel electrophoresis (1.5%) of *hlyA* gene amplification product: 1 = Ladder 100bp; 2–10 = *L. monocytogenes* 1a; 11 = *L. monocytogenes* 4b; 12 = *L. monocytogenes* ATCC 19111.

CONCLUSIONS

All investigated strains were confirmed as *L. monocytogenes* by biochemical activity and detection of *hlyA* gene, encoding listeriolysin O.
The majority of *L. monocytogenes* strains presented soluble virulence factors: lipase, caseinase, esculinase, and listeriolysin.
The adherence assay reveals correlations between the virulent character of tested strains and their source of isolation, the majority of them being with diffuse-aggregative pattern.
These tests can be used like *screening* methods for identification of pathogenic *L. monocytogenes* strains.

AKNOWLEDGEMENTS

This work was supported by the strategic grant POSDRU / ID 76888, Project "Doctoral program for training scientific researchers" cofinanced by the European Social Found within the Sectorial Operational Program Human Resources Development 2007-2013.

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