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*Original article*

# Invasiveness of *Listeria monocytogenes* strains isolated from animals in Poland

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## Abstract

Animals are important reservoir of *Listeria monocytogenes*, a pathogen causing serious infections in both humans and livestock. However, data on invasiveness of *L. monocytogenes* strains of animal origin is very scarce. Ability of 18 *L. monocytogenes* strains of animal origin to invade HT-29 cells was investigated. Plaque forming assay was used to assess invasiveness and ability of the pathogen to spread in the cell line. Almost 40% of *L. monocytogenes* strains were weakly invasive. It was shown that strains from serogroup 4b exhibited the highest invasiveness, whereas serogroup 1/2b consisted of strains of invasiveness below 0.0001%. Analysis of translated *inlA* and *inlB* gene sequences revealed no premature stop codons. Lineage-specific mutations in low invasive strains were identified within *inlA* and *inlB* sequences. Our results demonstrate high incidence of low invasive animal *L. monocytogenes* strains, which may be at least partly explained by unique point mutations in the InlA and InlB.

**Key words**: *Listeria monocytogenes*, animals, invasiveness

## Introduction

*Listeria monocytogenes* is a non-spore forming, facultative anaerobic Gram-positive bacterium, widespread in the environment (Farber and Peterkin 1991). The bacterium is a serious pathogen responsible annually for 1 662 invasive infections and 266 related deaths in the United States (Cartwright et al. 2013). *L. monocytogenes* was identified for the first time in 1926 as a cause of monocytosis in rodents. Then listeric infections were reported among several mammalian species, including feral and domestic ruminants, and monogastric animals (Gray and Killinger 1966). The main factor in disease transmission among animals is poor quality silage (Wesley et al. 2002). The species comprises 13 serotypes, which are classified into four genetically distant lineages (Ragon et al. 2008). Serotypes 1/2b and 4b (lineage I) have been mainly isolated from human food-borne epidemics, as well as sporadic cases in humans and animals.

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Table 1. *L. monocytogenes* strains used in the study.

| Strain | Source | Lineage | Genotype | | Serotype | Invasiveness [%] | Plaque size [mm] |
|---|---|---|---|---|---|---|---|
| | | | *actA* | RAPD | | | |
| 8z | pig (brain) | I | A1 | R1 | 1/2b (3b) | 2.75E-05 ± 7.78E-06 | 0.8 ± 0.09 |
| 9z | sheep (liver) | I | A1 | R2 | 1/2b (3b) | 7.46E-07 ± 1.26 | 0.37 ± 0.06 |
| 33z | goat (brain) | I | A1 | R2 | 1/2b (3b) | 7.2E-04 ± 3.2E-04 | 1.01 ± 0.12 |
| 34z | goat (brain) | I | A1 | R2 | 1/2b (3b) | 3.9E-06 ± 1.98E-06 | 1.05 ± 0.19 |
| 41z | sheep (liver) | I | A1 | R3 | 1/2b (3b) | 5.53E-07 ± 2.92E-07 | 1.03 ± 0.25 |
| 35z | sheep (liver) | I | A2 | R4 | 4b (d,e) | 20 ± 1.1 | 0.91 ± 0.15 |
| 36z | sheep (liver) | I | A2 | R4 | 4b (d,e) | 23.3 ± 9.3 | 1.16 ± 0.08 |
| 39z | sheep (liver) | I | A3 | R5 | 4b (d,e) | 12.6 ± 6.09 | 0.86 ± 0.12 |
| 40z | sheep (organs) | I | A3 | R6 | 4b (d,e) | 7.85 ± 3.6 | 0.8 ± 0.2 |
| 12z | pig (liver) | II | A6 | R7 | 1/2a (3a) | 5.2 ± 2.6 | 0.71 ± 0.17 |
| 13z | dog (spleen) | II | A6 | R7 | 1/2a (3a) | 2.6 ± 1.2 | 0.92 ± 0.16 |
| 42z | sheep (organs) | II | A6 | R8 | 1/2a (3a) | 2.75 ± 0.92 | 0.52 ± 0.12 |
| 43z | goat (brain) | II | A6 | R8 | 1/2a (3a) | 2.87 ± 1.41 | 0.65 ± 0.16 |
| 18z | sheep (brain) | II | A6 | R9 | 1/2a (3a) | 0.17 ± 0.08 | 0.84 ± 0.11 |
| 26z | sheep (spleen) | II | A5 | R10 | 1/2a (3a) | 0.43 ± 0.18 | 0.96 ±0.14 |
| 30z | sheep (brain) | II | A5 | R11 | 1/2a (3a) | 0.53 ± 0.31 | 0.7 ± 0.13 |
| 37z | sheep (foetus) | II | A4 | R12 | 1/2a (3a) | 0.0057 ± 0.0027 | 1.02 ± 0.17 |
| 38z | sheep (brain) | II | A4 | R13 | 1/2a (3a) | 0.073 ± 0.0038 | 1.09 ± 0.21 |

Serotypes 1/2a, 1/2c and 3a (lineage II) have been associated with sporadic cases of listeriosis, whereas serotypes 4a and 4c are generally isolated from animals (Vhzquez-Boland et al. 2001). It has been shown that serotypes 1/2a, 1/2b, 1/2c and 4b are most frequently isolated from the liver and spleen of experimentally infected mouse (Barbour et al. 2001).

Serotype 4b strains of human and food origin have been shown to present the highest invasion efficiency among *L. monocytogenes* isolates (Wałecka--Zacharska et al. 2013). Nonetheless, little is known about invasiveness of *L. monocytogenes* strains of animal origin.

Food contamination usually occurs by cross-contamination of the final product by *L. monocytogenes* present in food processing plant environments. Despite infected animals are infrequently linked to human infections, animal-derived food products not processed before consumption, as well as raw food of plant origin contaminated by manure from infected or shedding animals can directly cause human listeriosis (Nightingale et al. 2004).

Our aim was to determine the ability to invade and spread within HT-29 cells of 18 *L. monocytogenes* strains isolated from animals.

## Materials and Methods

### Growth of L. monocytogenes

The study was conducted on 18 *L. monocytogenes* strains isolated from independent listeriosis cases in animals (Table 1). All strains were classified into serogroups using multiplex PCR (Doumith et al. 2004). Assignment to lineages was based on *actA* gene partial sequence analysis (Zhou et al. 2005). RAPD-PCR was used to further differentiate the strains (Bania et al. 2009). Single colonies of *L. monocytogenes* were seeded into 5 ml of BHI and grown at 37°C with shaking at 180 rev $min^{-1}$ for 6 hours. Then 50-μl aliquots from these cultures were used to inoculate 5 ml of fresh BHI and the bacteria were grown at 37° with shaking at 230 rev $min^{-1}$ for 18 hours. Two to eight log CFU of bacteria were taken for infection of HT-29 and plated in duplicate onto BHI agar.

### Cell line and culture conditions

The human adenocarcinoma cell line HT-29 (Institute of Immunology and Experimental Therapy, Wrocław, Poland) was cultured in DMEM (Dulbecco's modified Eagle's medium; Sigma-Aldrich, Poznań, Poland) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Warsaw, Poland), 2 mmol $l^{-1}$ glutamine, 100 IU $ml^{-1}$ penicillin, and 100 μg $ml^{-1}$ streptomycin (Sigma-Aldrich) at 37°C in 5% $CO_2$.

### Plaque-Forming Assay (PFA)

HT-29 cells were seeded onto 6-well plates and grown to obtain almost confluent monolayers. Twenty-four hours before infection, the standard

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culture medium was replaced by DMEM without antibiotics. HT-29 cells were infected with 2 to 8 log CFU of bacteria for 2 h, then the medium was replaced by DMEM containing 100 µg/mL gentamicin (Sigma-Aldrich) and incubated for 1·5 h. Each well was then overlaid with DMEM containing 10 µg $ml^{-1}$ gentamicin and 1·0 % low-melting-point agarose (Prona, Gdańsk, Poland). After 3 days of culture the number of plaques and plaque size were determined. Diameters of a minimum 10 randomly selected plaques per well were measured on digital images. Each assay was performed in duplicate and repeated at least three times. Invasiveness was expressed as the number of plaques per number of log CFU deposited.

### Sequencing of inlA and inlB genes

Conventional PCR was performed. Briefly, 1 µl of appropriate DNA, obtained using DNA extraction kit (Eurx, Gdańsk, Poland), was added to a mix containing 450 nmol $l^{-1}$ of primers 200 mmol $l^{-1}$ dNTPs, 10 mol $l^{-1}$ buffer Tris-HCl of pH 8·3, 50 mnol $l^{-1}$ KCl, 1·5 mmol $l^{-1}$ $MgCl_2$, 0·1% Triton X-100, and 2U of RED-Taq genomic polymerase (Sigma). Primers for *inlA* were from Kovačević et al. (2013).

Primers for *inlB*-for: GTTTTCGGACTATATC-TAGC and for *inlB*-rev: TTAT-TTCTGTGCCCTTAAATTAGCTGC were designed based on known *inlB* gene sequences using Molecular Beacon software (BioRad). The protocol was as follows: initial denaturation at 95°C for 3 min., followed by 32 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 2 min. Then PCR product was sequenced using the BigDyeTM Terminator Ready Reaction Cycle Sequencing kit (Genomed,Warsaw, Poland). Sequences were translated into proteins using BioEdit software (http:// http://www.mbio.ncsu.edu/bioedit/bioedit.html) and the presence of mutations was determined by comparing InlA and InlB sequences to *L. monocytogenes* EGDe strain (Glaser et al. 2001).

## Statistical analysis

Each experiment was performed at least in triplicate. The mean values were used for statistical analysis (StatSoft, Krakow, Poland). Kruskal-Wallis test was used to assess differences in invasiveness and plaque size between lineages and serogroups. Significance was set at $p<0.05$.

## Results

### Invasiveness and ability to spread in HT-29 cells of L. monocytogenes strains

The 18 *L. monocytogenes* strains were classified into three serogroups: 1/2a (3a) (9 strains), 1/2b (3b) (5 strains) and 4b (4d,e) (4 strains). Determination of partial *actA* gene sequence revealed 6 different *actA* genotypes, i.e. *actA* I-VI, and allowed strain assignment to lineage I (9 strains) and lineage II (9 strains). Thirteen different RAPD genotypes (R1-R13) were determined. No significant difference in the ability to spread in HT-29 cell monolayer was found between three serogroups and two lineages. Mean plaque size diameters were 0.85 ± 0.29 mm, 0.93 ± 0.15 mm and 0.82 ± 0.19 mm in serogroup 1/2b(3b), 4b(d,e) and 1/2a(3a), respectively. Analysis of *L. monocytogenes* ability to invade HT-29 cells revealed no significant differences of invasiveness between lineage I and II (invasiveness of lineage I was 7.08 ± 9.43%, invasiveness of lineage II was 1.62 ± 1.81%). Difference of invasiveness was noted between the serogroups. The most invasive were serogroup 4b(d,e) strains (15.4 ± 7.01%). Strains of invasiveness ranging from 5.2 ± 2.6% – 0.0057 ± 0.0027% were found in the serogroup 1/2a(3a). The serogroup 1/2b(3b) consisted of strains of invasiveness below 0.0001% (*actA* I type, RAPD genotypes R1, R2, R3).

### Analysis of InlA and InlB sequences

Analysis of complete *inlA* gene sequences reveled that all *L. monocytognes* strains should produce full-length InlA protein, since no premature stop codons were detected in translated nucleotide *inlA* sequences. Twenty-nine amino-acid point mutations, from which seven were lineage I-specific, were found in the translated InlA protein sequence, as compared to InlA of EGDe strain (Table 2). Low invasive strains from lineage I (8z, 9z, 33z, 34z, 41z) possessed unique mutation Asn32Ser in the InlA sequence. In turn, the hallmark of lineage II low invasive strains were four amino acid substitutions in InlA, namely Ile157Leu, Asn546Asp, Val644Ile, Ala652Thr.

Forty-four point mutations were found in translated InlB protein sequence, including 12 lineage I-specific (Table 3). Low invasive strains from lineage I (8z, 9z, 33z, 34z, 41z) were distinguished by three amino acid substitutions, namely Asn76Asp, Asn371Asp, Ala390Val.

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Table 2. Amino-acid residues that differ among the *L. monocytogenes* strains, based on the analysis of *inlA* gene.

| Strain | Amino acid residue | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 32 | 51 | 94 | 118 | 142 | 157 | 187 | 416 | 420 | 426 | 454 | 461 | 474 | 476 | 494 | 495 | 500 |
| EGDe, 30z | N | T | V | N | T | I | S | A | A | A | T | N | S | P | L | A | V |
| 8z,9z,33z,34z,41z | S | · | L | D | · | · | · | E | P | V | A | · | N | S | · | · | A |
| 36z | · | · | L | D | S | · | · | E | P | V | A | · | N | S | N | V | A |
| 35z,39z,40z | · | · | L | D | S | · | · | E | P | V | A | · | N | S | · | · | A |
| 12z,13z,18z,26z,42z | · | A | L | D | · | · | N | · | · | · | A | · | · | · | · | · | A |
| 37z,38z | · | A | · | · | · | L | · | · | · | · | · | · | · | · | · | · | · |
| 43z | · | A | L | D | · | · | N | · | · | · | A | Y | · | · | · | · | A |

| Strain | Amino acid residue | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 530 | 546 | 558 | 573 | 593 | 645 | 648 | 652 | 664 | 738 | 781 | 790 |
| EGDe, 30z | H | N | N | D | A | V | S | A | A | D | L | M |
| 8z,9z,33z,34z,41z | Y | · | · | · | · | · | T | · | T | N | I | V |
| 36z | Y | · | D | · | · | · | T | · | T | N | I | V |
| 35z,39z,40z | Y | · | D | · | · | · | T | · | T | N | I | V |
| 12z,13z,18z,26z,42z | · | · | · | E | P | · | · | · | · | · | · | · |
| 37z,38z | · | D | D | E | P | I | T | P | · | · | · | · |
| 43z | · | · | · | E | P | · | · | · | · | · | · | · |

Table 3. Amino-acid residues that differ among the *L. monocytogenes* strains, based on the analysis of *inlB* gene.

| Strain | Amino acid residue | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 41 | 49 | 69 | 73 | 76 | 91 | 117 | 132 | 138 | 164 | 176 | 181 | 197 | 205 | 246 | 251 | 262 |
| EGDe, 30z | P | S | A | N | N | V | A | V | L | P | I | V | Q | A | P | S | T |
| 8z,9z,33z,34z,41z | · | P | L | S | D | I | · | I | · | L | L | I | E | S | S | T | I |
| 35z, 36z | · | P | L | S | · | I | · | I | · | L | L | I | E | S | S | T | I |
| 39z,40z | S | P | L | S | · | · | · | I | I | · | L | I | E | S | S | T | I |
| 12z,13z,18z,26z,42z,43z | S | P | · | · | · | · | T | I | · | · | · | · | · | · | · | · | · |
| 37z,38z | · | · | · | · | · | · | T | I | · | · | · | · | · | · | · | · | · |

| Strain | Amino acid residue | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 291 | 304 | 371 | 373 | 387 | 390 | 396 | 445 | 446 | 470 | 482 | 486 | 489 | 501 | 502 | 533 | 555 |
| EGDe, 30z | T | I | N | N | V | A | A | G | K | M | R | S | S | K | H | G | K |
| 8z,9z,33z,34z,41z | I | V | D | S | M | V | · | · | · | I | K | L | A | · | · | · | · |
| 35z, 36z | I | V | · | S | M | · | · | · | E | I | K | L | A | T | L | D | I |
| 39z,40z | I | V | · | S | M | · | · | S | E | I | K | L | A | T | L | D | I |
| 12z,13z,18z,26z,42z,43z | · | · | · | · | · | · | T | · | · | · | · | · | · | · | · | · | · |
| 37z,38z | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |

| Strain | Amino acid residue | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 557 | 558 | 578 | 580 | 582 | 584 | 594 | 599 | 600 |
| EGDe, 30z | T | R | A | N | V | R | K | C | Q |
| 8z,9z,33z,34z,41z | · | · | · | · | · | · | · | · | · |
| 35z, 36z | I | Q | V | S | I | W | T | R | T |
| 39z,40z | I | Q | V | S | I | W | T | R | T |
| 12z,13z,18z,26z,42z,43z | · | · | · | · | · | · | · | · | · |
| 37z,38z | · | · | · | · | · | · | · | · | · |

## Discussion

Virulence of *L. monocytognes* population has been shown to be heterogenic. Eight to 21% of strains are estimated to be avirulent or weakly virulent (Roche et al. 2005). There is little information on pathogenicity of *L. monocytogenes* strains of animal origin. Therefore, in our study we used plaque-forming assay to describe ability of 18 *L. monocytogenes* strains isolated from animals to invade and spread in the human adenocarcinoma cell line.

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Almost 40% of strains studied here were weakly invasive. In contrast to previous studies no differences were found between invasiveness values of isolates assigned to *L. monocytogenes* lineages I and II (Wiedmann et al. 1997, Zhou et al. 2005). Both lineage I and lineage II comprised invasive and weakly invasive strains, which varied in the ability to invade HT-29 cells up to 7.6 and almost 3 orders of magnitude, respectively. The most invasive were serogroup 4b(d,e) strains, which agrees with the results of our previous study (Wałecka-Zacharska et al. 2013).

Little is known about the molecular basis of virulence heterogeneity in *L. monocytogenes* population. Internalin InlA and InlB are the key surface proteins containing N-terminal leucine-rich repeats (LRRs) domain that interact with their respective host-cell receptors, leading to bacterial internalization (Bierne et al. 2007). It has been shown that loss of *inlA* and *inlB* genes seriously hamper *L. monocytogenes* ability to invade epithelial cells (Kim et al. 2005). A number of mutations in *inlA* gene, leading to premature stop codons (PMSCs), have been observed in *L. monocytogenes*. Such mutations result in production of truncated InlA, contributing to attenuated invasion efficiency of the bacteria (Nightingale et al. 2005, Nightingale et al. 2008). However, recent study by Roche et al. (2012) reports on low virulent *L. monocytogenes* strains expressing full length InlA. In our study, 7 out of 18 strains exhibited invasiveness under 0.1%, from which five had invasiveness below 0.0001%. Analysis of the translated InlA sequence revealed no PMSCs in all strains. Instead we have found a number of point mutations in the InlA (Table 2). Some of them were earlier reported to be lineage-specific (Ragon et al. 2008). Low invasive strains from lineage I possessed unique mutation (Asn32Ser) in the signal peptide of the *inlA* gene, whereas lineage II-strains were characterized by one amino acid substitution in LRR region (Ile157Leu) and three mutations in the domain B of InlA (Asn546Asp, Val644Ile, Ala652Thr).

Two mutations in LRR domain of InlB, namely Ala117Thr and Val132Ile, have been described. Since this region is crucial for interaction with the InlB receptor (Met) and Ala117Thr substitution leads to change of hydrophobicity of amino acid, the role of this mutation in the protein inactivation has been postulated (Temoin et al. 2008, Roche et al. 2012). We have identified 44 mutations in the InlB sequence (Table 3). We have found one mutation in the LRR domain (Asn76Asp) and two in the C-terminal region (Asn371Asp; Ala390Val) in low invasive lineage I strains.

Remaining mutations, including Ala117Thr, however, appeared in both invasive and weakly invasive strains. Whether mutations reported here play role in *L. monocytogenes* ability to invade HT-29 cells merits further confirmation.

Comparison of results presented here with our previous data on *L. monocytogenes* strains from human clinical cases (n=14) and food (n= 27) demonstrates that low invasive strains are more prevalent among animal population than human and food isolates (Wałecka-Zacharska et al. 2013).

In summary, results presented here demonstrate high incidence of low invasive *L. monocytogenes* strains of animal origin which are characterized by unique point mutations in InlA and InlB. Importance of these mutations requires further investigation.

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