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Listeria monocytogenes isolated from food samples from a Romanian black market show distinct virulence profiles

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ABSTRACT

Listeria monocytogenes is a facultative intracellular foodborne pathogen responsible for listeriosis. In a recent study, in which we investigated neglected exogenous routes of transmission of foodborne pathogens into the European Union, we have isolated 15 *L. monocytogenes* strains in food products, which were imported from the Republic of Moldavia to Romania and illegally sold at a local market.

The aim of this study was to characterize the subtype and virulence potential of these 15 *L. monocytogenes* strains. Multilocus sequence typing revealed that these *L. monocytogenes* strains belong to six different sequence types (ST2, ST8, ST9, ST20, ST121 and ST155). In addition, *in vitro* virulence assays using human intestinal epithelial Caco2 and macrophage-like THP1 cells showed a high strain variability regarding the invasion efficiency in Caco2 cells (0.98–2.78%) and the intracellular growth rate in both cell types. Both ST121 strains and the ST9 isolate were unable to invade Caco2 cells, and all ST155 strains showed no proliferation inside macrophages and revealed low cytotoxicity. Furthermore we performed sequence analysis of three main virulence factors: PrfA, internalin A (InlA) and listeriolysin O (LLO). The Romanian food isolates showed a high diversity in the InlA and LLO amino acid sequences, whereas the amino acid sequence of PrfA of all strains was identical. Overall, the amino acid sequences of PrfA, InlA and LLO were identical for strains belonging to the same ST. We detected in total 30 different amino acid substitutions, resulting in seven different InlA variants, two of which have not yet been described. The three strains, which were unable to invade Caco2 cells, harboured a premature stop codon resulting in truncated InlA. Furthermore, we detected four different amino acid substitutions in the LLO sequence, which are present in four variants. The number of LLO mutations correlates negatively with intracellular growth in Caco2 and THP1 cells and subsequently with cytotoxicity.

In conclusion, we show that *L. monocytogenes* isolated from food samples from a Romanian black market show distinct virulence profiles, due to a high diversity in the amino acid sequence of main virulence factors.

1. Introduction

Listeria monocytogenes is a facultative intracellular pathogen responsible for severe illnesses in humans and animals including ruminants and birds. In healthy individuals listeriosis is usually restricted to a self-limiting febrile gastroenteritis, whereas in immunocompromised individuals and newborns an invasive and systemic infection can occur leading to meningitis, encephalitis and septicaemia with a high mortality rate of 25–30% (Ooi and Lorber, 2005; Allerberger and Wagner, 2010). In addition in pregnant women listeriosis can lead to infection of the foetus resulting in abortion (Poulsen and Czuprynski, 2013).

After consumption of contaminated food products, which is the main cause of human listeriosis (EFSA, 2012), *L. monocytogenes* multiplies in the intestinal lumen, crosses the intestinal barrier, enters the bloodstream and reaches the liver and spleen, where it proliferates. *L. monocytogenes* can escape the host immune system due to the ability to survive and proliferate inside macrophages (Goldfine and Wadsworth, 2002; Shaughnessy and Swanson, 2007). Furthermore, the bacteria can re-enter the bloodstream to cause fatal systemic or central nervous infection (Vazquez-Boland et al., 2001).

Most of the virulence factors, which play an essential role in the infection pathway of *L. monocytogenes*, are regulated by the transcriptional regulator PrfA (Freitag et al., 2009). A number of surface proteins including internalins are crucial for host cell invasion (Bierne et al., 2007). Internalin A (InlA), which interacts with E-cadherin present at the surface of the host cell, mediates the entry of *L. monocytogenes* into intestinal epithelial cells (Bonazzi et al., 2009). Several mutations in the *inlA* gene lead to a premature stop codon (PMSC) and subsequently result in a truncated and secreted InlA protein. These types of

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mutations, which are carried presumably by environmental strains, are associated with attenuated virulence (Nightingale et al., 2008; Van Stelten et al., 2010).

After *L. monocytogenes* enters the host cell, it escapes the vacuole, replicates intracellularly and spreads from cell to cell (Cossart, 2011). These processes are mainly mediated by the pore-forming toxin listeriolysin O (LLO) (Gedde et al., 2000; Hamon et al., 2012).

There is evidence for a high variability regarding the virulence potential and pathogenicity of different *L. monocytogenes* isolates. Strains of *L. monocytogenes* can be grouped into four evolutionary lineages (I–IV), and 13 serotypes. However, strains of only three serotypes (1/2a, lineage II; 1/2b and 4b, lineage I) have been associated with 98% of all human listeriosis cases (Orsi et al., 2011). Since conventional serotyping can only discriminate major categories of strains, which correlate strongly with lineages, molecular typing methods with higher discriminatory power are widely used for strain subtyping like pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). MLST is based in *L. monocytogenes* on seven housekeeping genes and enables inter-laboratory genotype comparison on an open accessible database. In recent years MLST has evolved to a reference method for global epidemiology and population biology (Maiden, 2006; Ragon et al., 2008).

In a recent project, which focused on neglected exogenous routes of transmission of foodborne pathogens into the European Union, we have isolated 15 *L. monocytogenes* strains from food products imported into the EU *via* the Romanian Moldavian border and illegally sold at a black market in Romania (Ciolacu et al., unpublished results). The aim of this study was to characterize the subtype and virulence potentials of these 15 *L. monocytogenes* strains. Therefore, we performed serotyping and MLST, and determined cytotoxicity and *in vitro* virulence potential using human intestinal epithelial and macrophage-like cells. In addition, we analyzed the full-length sequences of three main virulence genes (*prfA*, *inlA* and *hly*) to investigate correlations between the MLS type, amino acid sequence of main virulence factors and *in vitro* virulence potential.

2. Materials and methods

2.1. Bacterial strains, culture conditions and DNA isolation

All *L. monocytogenes* strains used in this study were isolated within the framework of the FP7 EU project PROMISE from food products imported from the Republic of Moldavia to Romania and illegally sold at a local market in Galati (Ciolacu et al., unpublished results). We analyzed 200 food samples between July 2012 and February 2013 and isolated 15 *L. monocytogenes* strains using the ISO 11290-1:1996 standard method. Nine isolates were recovered from raw or processed fish, four strains from meat products and one strain from dairy products (Table 1). The type strain EGDe (ATCC® BAA-679) was used as a reference strain.

Bacterial cultures were maintained at −80 °C in tryptic soy broth supplemented with yeast (TSB-Y) containing 40% glycerol. Isolates were cultivated on tryptic soy agar supplemented with yeast extract (TSA-Y, Merck, Germany) at 37 °C for 24 h.

For DNA isolation strains were grown overnight on TSA-Y plates at 37 °C and DNA was extracted using 25 g/l Chelex® 100 Resin (Bio-Rad Laboratories) according to Gill et al. (1992). For the *in vitro* virulence assays single colonies were inoculated in 8 ml brain heart infusion (BHI, Merck) and incubated overnight at 25 °C. The cultures were diluted 1:20 (OD_{600} of 0.2, approx. 10^8 CFU/ml) in a minimal growth medium consisting of RPMI-1640 supplemented with 1% L-glutamine (both PAA, Pasching, Austria) and 0.08 mg/ml ferric citrate (Merck) and incubated for 2 h at 25 °C.

2.2. Serotyping and multi-locus sequence typing

A multiplex PCR targeting *lmo0737*, *lmo1118*, *ORF2819*, *ORF2110* and *prs* was performed to determine serovar-specific groups (1/2a, 3a; 1/2c, 3c; 4b, 4d, 4e; 1/2b, 3b, 7; and 4a, 4c) according to Doumith et al. (2004).

MLST, which is based on seven housekeeping genes (*abcZ* (ABC transporter), *bglA* (beta glucosidase), *cat* (catalase), *dapE* (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase), *lhkA* (histidine kinase)) was performed according to Salcedo et al. (2003). PCR products were sequenced (LGC Genomics) and for each housekeeping gene an allele number was assigned. Sequence type (ST) numbers were assigned for each distinctive allele combination using the Institute Pasteur *L. monocytogenes* MLST database. A detailed protocol of the MLST procedure including primers, PCR conditions, and allelic type is available at the Institute Pasteur website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.html).

2.3. In vitro virulence assays

For the *in vitro* virulence assay human intestinal epithelial cells (Caco2, ATCC® HTB-37™) and human macrophage-like cells (THP1, ATCC® TIB-202™) were used. The cells were cultivated in Eagle's minimum essential medium (MEM for Caco2) or RPMI-1640 (for THP1) containing 2 mM L-glutamine, 10% foetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin sulfate, 0.25 mg/ml amphotericin B and 1% non-essential amino acids (all PAA) at 37 °C in a humidified atmosphere (95% relative humidity) containing 5% CO_2. Prior to the experiments, THP1 cells were activated for 24 h with 40 ng/ml phorbol 12-myristate 13-acetate (Sigma Aldrich). The virulence assays were performed as previously described by Pricope et al. (2013) with minor modification. Briefly, cell monolayers were infected with *L. monocytogenes* strains in the mid-logarithmic growth phase at a multiplicity of infection of 25 for 1 h at 37 °C. The cell monolayers were washed with Dulbecco's Phosphate Buffered Saline (PBS; PAA) and incubated in MEM or RPMI-1640 and 10% foetal bovine serum containing gentamicin (100 μg/ml, PAA) for 45 min (invasion) and 4 h (intracellular growth), respectively. The cells were lysed with 1 ml 0.1% Triton X-100 (Merck) and colony forming units (CFU)were determined by plating on TSA-Y agar. The invasion efficiency (%) was calculated as mean CFU recovered after 45 min of gentamicin treatment divided by CFU of the inoculum. The intracellular growth coefficient (IGC) was calculated as follows: IGC = (intracellular $bacteria_{4\ h}$ − intracellular $bacteria_{45\ min}$) / intracellular $bacteria_{45\ min}$. Each experiment was performed in triplicate and repeated at least 3 times.

Table 1
L. monocytogenes strains used in this study.

Strain	Source	Isolation date	Serotype[a]	Sequence type
EGDe	Animal tissue	1924	1/2a	35
RO01	Mackerel	27.07.2012	1/2a (3a)	20
RO02	Marinated fish	14.09.2012	1/2a (3a)	8
RO03	Smoked herring	11.02.2013	1/2a (3a)	20
RO04	Raw-dried salami	10.12.2012	1/2a (3a)	20
RO05	Smoked salmon	10.12.2012	1/2a (3a)	155
RO06	Anchovy	10.12.2012	1/2a (3a)	20
RO07	Fish with spices	10.12.2012	1/2a (3a)	155
RO08	Smoked sprat	11.02.2013	1/2a (3a)	20
RO09	Fish in oil with herbs	06.11.2012	1/2a (3a)	155
RO10	Butter	15.01.2013	1/2a (3a)	121
RO11	Poultry	15.01.2013	1/2a (3a)	121
RO12	Lard	22.01.2013	1/2a (3a)	8
RO13	Herring with spices	07.02.2013	1/2a (3a)	155
RO14	Pork rind	22.01.2013	1/2c (3c)	9
RO15	Herring with spices	15.01.2013	4b (4d, 4e)	2

[a] Serovar-specific groups were determined by multiplex PCR according to Doumith et al. (2004).