Genotyping and virulence factors of *Listeria monocytogenes* in terms of food safety

TEREZA GELBÍČOVÁ – IVANA KOLÁČKOVÁ – RENÁTA KARPÍŠKOVÁ

Summary

The present study was designed to assess heterogeneity of virulence factors among strains of *Listeria monocytogenes* isolated from the food chain and humans in the Czech Republic. The virulence characteristics, presence of *Listeria* pathogenicity island 1 (LIPI-1) as well as *inlA*, *inlB, inlC* and *inlJ* genes of tested strains of *L. monocytogenes* from foods and food processing plants were comparable with human strains independently of particular serotypes. Restriction polymorphism of *inlA* gene confirmed a correlation between the restriction fragment length polymorphism (RFLP) profiles and the serotypes. Strains of serotype 1/2a and 1/2c with RFLP profiles, which may be characterized by production of a truncated internalin A, were detected not only in food strains, but also in 44% of strains isolated from clinical cases of listeriosis. Premature stop codon (PMSC mutation type 3) in the *inlA* gene associated with the production of truncated internalin A was detected in one *L. monocytogenes* strain from a ready-to-eat fish product. Considering food safety, all tested food strains should still be considered as pathogenic to humans even though some of these showed reduced virulence on the basis of genotyping results.

Keywords

food; virulence; internalin A; premature stop codon

Human listeriosis is a relatively rare (0.32 cases per 100 000 population in European Union) but serious zoonotic disease, with high morbidity, hospitalization and mortality rate in susceptible human population. *L. monocytogenes* is a ubiquitous bacterium and can be found in the environment but also in ready-to-eat foods, which constitute the main route of transmission to humans [1]. Regulations regarding the presence of *L. monocytogenes* in ready-to-eat foods range from zero tolerance in the USA to 10^2 CFU·g^{-1} in the retail market in EU countries according to European Commission 2073/2005 [2] and correspond with the severe character of human listeriosis.

A number of studies refer to different virulence of *L. monocytogenes* strains of particular genetic lineages, with regard to distribution or different genotypic characteristics of certain serotypes [3, 4]. Currently, the strains of *L. monocytogenes* are divided into four genetic lineages for which different pathogenic potential is assumed [5, 6]. According to published studies, strains of lineage I (serotypes 1/2b, 3b, 4b, 4d, 4e, 4ab and 7), in particular strains of serotype 4b, are mainly responsible for human outbreak-related listeriosis. Lineage II strains (serotypes 1/2a, 3a, 1/2c and 3c) are usually associated with foods, environment, animals and sporadic cases of listeriosis [3–5]. Lineages III and IV include not only strains of serotypes 4a and 4c, but also strains of serotype 4b, which are typically associated with lineage I [7]. Strains of lineages III and IV occur infrequently, mostly in ruminants, and they cause listeriosis in animals and humans only rarely [3].

Current knowledge of genetic mechanisms responsible for heterogeneity in virulence among *L. monocytogenes* strains of different serotypes, subtypes and origin is, however, still limited. The major virulence cluster of *L. monocytogenes* harbours six virulence genes (*prfA*, *hlyA*, *plcA*, *plcB*,

Tereza Gelbíčová, Department of Experimental Biology, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic.
Ivana Koláčková, Renáta Karpíšková, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic.

Correspondence author:
Tereza Gelbíčová, e-mail: terezag@sci.muni.cz, tel.: +420 549 496 254

mpl, *actA*) that encode proteins critical for its intracellular life cycle. Further virulence factors are internalin A and internalin B, which mediate entry of the pathogen into host cells, or a group of genes such as *sigB*, *hpt*, *lisRK*, *svp* and *clp* encoding proteins that help bacteria survive in the host environment [8]. Some authors described strains with deletions of one or more genes encoding the key virulence factors [9, 10] or premature stop codon (PMSC) mutations of *inlA* gene associated with attenuated mammalian virulence [11, 12]. Numerous studies showed that *L. monocytogenes* strains isolated from foods more often carry mutations leading to the release of truncated internalin A than strains isolated from humans [10–13]. Mutations leading to PMSC were detected in 45% of isolates originating from ready-to-eat foods in USA [13]. The likely cause of this finding is the predominant occurrence of serotypes 1/2a and 1/2c in foods because these mutations prevail among lineage II strains [13].

Information about differences in virulence of *L. monocytogenes* could help to predict possible risks for humans through consumption of contaminated foods. The aims of the present study were: (a) to detect and compare the frequency of key virulence genes in *L. monocytogenes* strains originating from foods, food processing plants and humans; (b) to evaluate the prevalence of potentially invasive and non-invasive strains of *L. monocytogenes* from the food chain and clinical cases of listeriosis using restriction fragment length polymorphism (RFLP) analysis, and (c) to evaluate the methods for characterization of virulence factors in *L. monocytogenes* isolated from the food chain.

MATERIALS AND METHODS

Bacterial strains

A total of 410 strains obtained in 2007–2012 were screened within the activities of the Czech National Reference Laboratory (NRL) for listeria, Brno, Czech Republic. The strains were isolated from foods and swabs from food processing plants (337) and clinical cases of human listeriosis (73).

Serotyping

Serotyping was performed by the slide agglutination method using antisera (Denka Seiken, Tokyo, Japan) and subsequently confirmed by multiplex polymerase chain reaction (PCR) for all strains [14, 15].

Detection of virulence genes

For all strains, four PCR assays were performed to detect the following virulence genes:

1. *prfA* [16] and *plcA* [17];
2. *hlyA* [18] and *actA* [17];
3. *plcB* [17];
4. *inlA, inlC, inlJ* [19] and *inlB* [17].

In PCR assays, primers synthesized by Generi Biotech (Hradec Králové, Czech Republic), PPP polymerase (Top-Bio, Praha, Czech Republic) or a Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany) were used.

Detection of potentially invasive and non-invasive strains of *L. monocytogenes* by PCR-RFLP of *inlA* gene

A total of 181 randomly selected strains of *L. monocytogenes* representing various serotypes isolated from diverse sources (Tab. 1) were analysed. Polymorphism of *inlA* gene in *L. monocytogenes* strains was investigated by PCR-RFLP using primers seq01 and seq02 [20]. The amplified product of *inlA* (733 bp) was cleaved by restriction endonuclease *Alu*I (New England BioLabs, Ipswich, Massachusetts, USA). PCR-RFLP fragments were analysed by electrophoresis in 3.5% agarose gel (Serva, Heidelberg, Germany) with subsequent staining in a solution of ethidium bromide and visualisation under UV light.

Detection of inlA PMSC mutation type 3 using PCR-RFLP

A group of 49 strains with RFLP profiles 1 and 4 were screened for the presence of *inlA* PMSC mutation type 3. The strains had been isolated from foods (40) and humans (9) and were of serotype 1/2a (34 strains of RFLP profiles 1 and 4) and serotype 1/2c (15 strains of RFLP profile 4). The *inlA* PMSC mutation type 3 was detected with PCR-RFLP using primers inlA trun F and inlA trun R [11]. The amplified product *inlA* (180 bp) was cleaved by restriction endonuclease *Rsa*I (New England BioLabs). PCR-RFLP fragments were separated by electrophoresis in 3.0% agarose gel (Serva) with subsequent staining in a solution of ethidium bromide and visualisation under UV light.

RESULTS AND DISCUSSION

In this study, 410 strains of *L. monocytogenes* isolated from foods, food processing plants and humans were found to be mostly of serotype 1/2a (Tab. 1). The second most common serotype was 1/2b (64 strains), followed by serotypes 4b (48 strains), 1/2c (33 strains), and 4d (3 strains).

Tab. 1. Distribution of virulence genes and RFLP profiles in *L. monocytogenes* strains of different origin and serotype.

Strain origin	Serotype	Number of strains (percentage)	Percentage of strains positive for			RFLP profile	Number of strains tested
			prfA, *hlyA*, *plcA*, *plcB*, *actA*	*inlA*, *inlB*	*inlC*, *inlJ*		
Human	1/2a	49 (67.1%)	100%	100%	100%	1	13
						3	7
						4	5
						5	1
	1/2b	11 (15.1%)	100%	100%	100%	2	8
	1/2c	2 (2.7%)	100%	100%	100%	4	2
	4b	11 (15.1%)	100%	100%	100%	2	9
Foods and the environment of food processing plants	1/2a	213 (63.2%)	100%	100%	100%	1	17
						3	14
						4	18
						5	6
	1/2b	53 (15.7%)	100%	100%	100%	2	30
	1/2c	31 (9.2%)	100%	100%	100%	4	27
	4b	37 (11.0%)	100%	100%	100%	2	21
	4d	3 (0.9%)	100%	100%	100%	2	3
Total		410					181

However, other studies reported *L. monocytogenes* serotype 4b to be the most common in humans, being implicated also in the majority of human listeriosis outbreaks [21, 22]. The predominance of serotype 1/2a in humans [23, 24] and the potential of serotype 1/2a strains to cause epidemic cases of listeriosis was demonstrated not only by an outbreak in the Czech Republic in late 2006 and early 2007 [25], but also by other studies [26, 27]. The differences can be explained by the predominance of a certain serotype in the food chain in a given area. A significant feature of the bacterium *L. monocytogenes* from the aspect of food safety is its ability to persist in the environment of food processing plants. Ward et al. [28] in their study explained that the low prevalence of lineage III strains in the human population is not associated with attenuated virulence of serotypes of this lineage, but with its low prevalence in foods.

All genes playing a key role in pathogenesis of *L. monocytogenes* (*prfA*, *hlyA*, *plcA*, *plcB*, *actA*, *inlA*, *inlB*) were detected in all strains not only from clinical cases of listeriosis, but also from foods and swabs from food processing plants. Differences in the incidence of virulence genes belonging to LIPI-1, and the *inlA*, *inlB*, *inlC* and *inlJ* genes, were not found even among the serotypes in the present study (Tab. 1). Results of the present study are in agreement with previous studies demonstrating that genes of the pathogenicity island LIPI-1, and the *inlA* and *inlB* genes, are invariable parts of the genome of *L. monocytogenes* [4, 17, 19]. According to Liu et al. [19], application of a technique to detect *inlC* and *inlJ* genes could open a new way to rapid differentiation between virulent and avirulent *L. monocytogenes* strains. In our study, the *inlC* and *inlJ* genes were invariably found in *L. monocytogenes*, which was in agreement with previous studies [9, 24, 29]. This fact demonstrates that the detection of *inlC* and *inlJ* genes alone may not serve as an efficient indicator of the pathogenic potential of *L. monocytogenes.* This opinion is consistent with other authors [29].

Using a PCR-RFLP method [20] to identify potentially non-invasive strains of *L. monocytogenes,* five different profiles were detected in 181 human and food strains (Tab. 1). RFLP profiles 2 and 3, in which functional internalin A has been invariably found by authors from another study [20], were detected in 51% of the investigated strains. Strains of serotype 1/2a, providing RFLP profile 5, in which the expression of full-length internalin A is also assumed, were detected only rarely (4%), which is consistent with other studies [20, 30, 31]. Strains of RFLP profiles 1 and 4 had been previously characterized by production of a trun-

cated internalin A and a reduced capability of invading Caco-2 epithelial cells [20, 30]. However, in the present study, these profiles were detected not only in food strains, but also in 44% of strains isolated from clinical cases of listeriosis. In contrast with our results (Tab. 1), TAMBURRO et al. [31], using RFLP analysis, demonstrated the expression of potentially truncated internalin A in all food-derived strains (serotypes 1/2a, 1/2c and 3a) except for seven strains of serotype 1/2b.

Our results corroborate the existence of a relationship between different RFLP profiles and serotypes, which is consistent with previous studies [30, 31]. RFLP profile 2 was detected only in strains of serotypes 1/2b, 4b and 4d. The strains of *L. monocytogenes* serotype 1/2a belonged not only to RFLP profiles 3 and 5, but also to profiles 1 and 4, specific to potentially non-invasive strains. Interestingly, all tested strains of serotype 1/2c were found to be of RFLP profile 4, which is in accordance with the study of ROUSSEAUX et al. [20].

Results obtained by RFLP analysis do not unequivocally show whether the examined strains actually produce internalin A with molecular weight of 80 kDa, or whether other factors also play a role in their invasiveness [20]. To date, 18 naturally occurring mutations leading to PMSC, which result in expression of truncated internalin A, have been identified in the *inlA* gene [13]. Products of PCR amplification do not carry all possible point mutations leading to the production of truncated internalin A.

In USA, PMSC mutations type 1 (in strains of lineage I) together with types 3 and 4 (in strains of line II) represent more than 90% of PMSC mutations, with type 3 being the most common [13]. Strains potentially harbouring point mutations leading to the expression of a truncated internalin A were purposely selected for screening for the presence of *inlA* PMSC mutation type 3. In our study, PMSC mutation type 3 was detected by RFLP [11] in one *L. monocytogenes* strain isolated from a ready-to-eat fish product. The majority of mutations leading to PMSC (except for mutations types 6 and 12) are typical for a country where they were originally described [13]. The results of our study demonstrate that strains carrying PMSC mutation type 3 can also be detected in the Czech Republic. However, for an examination of prevalence of this mutation in the Czech Republic, the study of a larger number of strains would be needed.

Evaluation of the pathogenic potential of *L. monocytogenes* by characterization of virulence genes using PCR-based methods may represent a fast screening technique. However, the virulence potential of *L. monocytogenes* (with the exception of clinical isolates) must be definitely confirmed by gene expression methods, using tissue cultures or biological experiments. PCR detection of key virulence genes alone or RFLP analysis of *inlA* gene does not reflect real virulence of *L. monocytogenes*, but can represent an additional typing method. Moreover, previous studies demonstrated that *L. monocytogenes* strains carrying mutations leading to PMSC in the *inlA* gene can be also implicated in human listeriosis [13] or cross the placental barrier after oral exposure of pregnant mice and guinea pigs [32]. All *L. monocytogenes* strains should be considered as pathogenic with regard to compliance of acceptable levels of *L. monocytogenes* in ready-to-eat foods.

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