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Lineage and Serotype Identification of *Listeria monocytogenes* by Matrix-assisted Laser Desorption Ionization-time of Flight Mass Spectrometry

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Abstract

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The identification of *Listeria* species, lineages and serotypes remains a crucial issue not only in epidemic surveys, but also in monitoring of the diversity of bacteria in the food chain. The aim of this study was identification of *L. monocytogenes* strains at lineage and serotype level using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The performance of MALDI-TOF MS was tested to identify *L. monocytogenes* into two lineages (I and II) and four serotypes (1/2a, 1/2b, 1/2c and 4b) the most commonly found in humans and food. Total of 227 *L. monocytogenes* strains from different sources were subjected to the study. Some of strains (112) were used for main spectrum profile (MSP) library creation. Other strains of interest (115) were then correctly identified on the lineage level comparing with the library by MALDI-TOF MS analysis using Biotyper (90%) and ClinPro Tools (100%) software. The serotype identification with 55.7% (Biotyper) and 67.8% (ClinPro Tools) accuracy is rather a proof that under given conditions the method has not big potential to be used for serotyping. However, MALDI-TOF MS has a potential to identify lineages of *L. monocytogenes* of food and human origin.

Keywords: diversity; *Listeria*; MALDI-TOF MS; identification; serotyping

Listeria monocytogenes is a cause of foodborne disease primarily affecting pregnant women, newborns, elderly people (> 65 years old) and immunocompromised individuals. Recently, there is an increasing trend in occurrence of human listeriosis in the European Union (EU), including the Czech Republic. For the EU states, 2206 cases of listeriosis (0.46 cases per 100 000 inhabitants) were reported in 2015 with an incidence of 0.34 cases per 100 000 inhabitants in the Czech Republic (EFSA & ECDC 2016).

There are at least four evolutionary lineages (I, II, III and IV) of *L. monocytogenes* with different but overlapping ecological niches. Most isolates of *L. monocytogenes* belong to lineage I and II which include serotypes 1/2a, 1/2c (lineage II) and 1/2b, 4b (lineage I) the most commonly associated with human cases of listeriosis (Dumas *et al.* 2009). It seems that lineage II strains are better adapted to the saprophytic and environmental life cycle compared to lineage I strains which are often involved in human listeriosis outbreaks (Cabrita *et al.* 2010). On the

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other hand, strains of lineages III and IV are more frequently isolated from animals (Orsi *et al.* 2011). Lineages III and IV include serotype 4a and 4c strains but also some strains of serotype 4b which are typically associated with lineage I (Tsai *et al.* 2011).

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF MS) is rapid, reliable and sensitive procedure representing an alternative approach to molecular biological methods (Nomura 2015). The high throughput and speed associated with complete automation has made MALDI-TOF MS an emerging technology applicable for microbiological identification (Singhal *et al.* 2015). MALDI-TOF MS is commonly used for bacteria identification (Randall *et al.* 2015; Angeletti 2016) and bacteria subtyping (Nakano *et al.* 2015) or detection of antibiotic resistance (Hrabák *et al.* 2012; Weiser *et al.* 2012).

Identification of bacteria below the species level enables both characterization of epidemic surveys and monitoring the diversity and spread of bacteria in the environment and food processing plants as well (Trémoulet *et al.* 2002; Dreyer *et al.* 2015). Serotyping of *L. monocytogenes* represents the first step of listeria typing. The aim of this study was to develop a method for rapid identification of *L. monocytogenes* lineages and serotypes employing MALDI-TOF MS.

MATERIAL AND METHODS

Bacterial strains. Total of 227 strains of *L. monocytogenes* of human (61 strains from sporadic cases of listeriosis) and food origin (166) were subjected to the study. According to the specific-*Listeria* O and H antigen slide agglutination and multiplex polymerase chain reaction (PCR) method (Doumith et al. 2004), strains were classified into lineage I (n = 113) including serotype 1/2b (n = 57) and serotype 4b (n = 56), and lineage II (n = 114) including serotype 1/2a (n = 57) and serotype 1/2c (n = 57).

Sample preparation for MALDI-TOF MS. Standard procedure based on ethanol-formic acid extraction was adapted (Freiwald & Sauer 2009). After overnight incubation on sheep blood agar (LabMediaServis, Czech Republic), a loopful fresh culture was suspended in 300 µl of deionized water. Then 900 µl of ethanol (Sigma Aldrich, USA) was added and the suspension was mixed for 15 seconds. After centrifugation at 15 000 *g* for 2 min, supernatant was removed and the bacterial pellet was used for protein extraction. Prior to 15 s vortex mixing, 40 µl of 70% formic acid (Sigma Aldrich) was added to disrupt bacterial cell wall. Then 40 µl of acetonitrile (Sigma-Aldrich) was added and the suspension was vortexed for 15 s and centrifuged at 15 000 *g* for 2 minutes. One microliter of the supernatant yielding protein extract was spotted onto a polished steel target plate (Bruker Daltonics, USA). Dried spots were overlaid by 1 µl of the saturated α-cyano-4-hydroxycinnamic acid (HCCA; Sigma Aldrich) solution in acetonitrile, water and trifluoracetic acid (Sigma Aldrich) (50 : 47.5 : 2.5, v/v/v) mixture and allowed to air dry. Each sample was tested only once. Only in case, when log score is < 2000 we would repeat the analysis, but it did not happen in any case in this study.

MALDI-TOF MS data acquisition. Mass spectral analyses were carried out using Autoflex speed instrument (Bruker Daltonics) operated in the linear positive mode using Flex Control 3.4 software (Bruker Daltonics). Standard instrument setting with laser frequency of 1 kHz, mass range of 2000–20 000 *m/z*, ion source 1 and 2 of 19.5 and 18.3 kHz was used, respectively. For each series of strains, external calibration of the mass spectra was performed by Bacterial Test Standard (Bruker Daltonics) containing *Escherichia coli* DH5α reference strain along with RNase A and myoglobin. The sum spectra comprising 2000 laser shots were acquired within an individual spot. According to the pre-defined lattice raster, 200 to 400 shots were obtained out of each of the ten raster position.

MALDI-TOF MS data analysis

Biotyper identification. Mass spectra were processed by Biotyper (software version 3.1, Bruker Daltonics) standard procedure. Results of strain genus and species identification using MALDI Biotyper (MBT) 6903 Main Spectrum Profile (MSP) Library (Bruker Daltonics) were expressed as log score value according to recommendation of Bruker Daltonics (0.000–1.699 indicated as not reliable identification, 1.7–1.999 indicated as probable genus identification, 2–2.299 indicated secure genus identification and probable species identification, 2.3–3 indicated highly probable species identification).

Library creation and validation using Biotyper and ClinPro Tools. Biotyper and ClinPro Tools (version 3.0; Bruker Daltonics) software

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was used to create reference library to identify *L. monocytogenes* strains on lineage and serotype level. The strains used for library creation were selected on the basis of different pulsotypes, sources and time of isolation to cover diversity among *L. monocytogenes* strains. Library creation was based on the value of log score (> 2.300 indicating highly probable species identification), strain quantity (n = 56 strains per lineage, n = 28 strains per serotype), 70% peak frequency minimum and statistical evaluation.

Using Biotyper, mass spectra were processed by MSP Creation Standard Method modified by peak frequency minimum of 70% to create lineage MSPs (I and II) and serotype MSPs (1/2a, 1/2b, 1/2c and 4b). Individual spectra used for creation of corresponding MSP were compared to this MSP yielding log scores. Statistical analysis of log scores was performed by Grubb's test to detect outlier. If outlier was detected, spectrum was removed and the new MSP was created. Library creation was not accompanied by any outlier values.

Automated analysis of mass spectra was performed by ClinPro Tools (version 3.0) through spectra pretreatment, model generation and spectra classification (Ketterlinus *et al.* 2005). To process spectra, mass range of 2 000–20 000 *m/z,* top hat baseline subtraction, spectrum resolution of 800, recalibration with 0.5‰ maximal peak shift and 30% match to calibration peaks of the standard, and average peak list calculation with signal to noise ratio of 5 was used. Models used for lineage (I and II) and serotype (1/2a, 1/2b, 1/2c and 4b) creation were generated using genetic algorithm (used parameters: maximal number of best peaks – 40, automatic detection of initial number of peak combinations – true, maximal number of generation – 50, mutation rate – 0.2, crossover rate – 0.5, use varying random seed – false, number of neighbors – 3). For each model, the reliability and the accuracy were assessed through recognition of capability and cross validation values.

Identification and evaluation of sample cohort using Biotyper and ClinPro Tools. Sample cohort of 115 strains of *L. monocytogenes* included lineage I (n = 57) with serotype 1/2b (n = 29) and serotype 4b (n = 28), and lineage II (n = 58) with serotype 1/2a (n = 29) and serotype 1/2c (n = 29). These samples represented strains which were not used for Biotyper libraries introduction and for ClinPro Tools models creation. For the lineage or serotype identification using Biotyper, the best-match of MSP representing the highest log score of sample strain against lineage or serotype library was considered as result (the score limit was > 2.000). For ClinPro Tools, external validation was carried out through lineage or serotype models based on genetic algorithm to identify lineage or serotype of sample strains. The output parameters and parameters for evaluation were the same as were used for creating and validation of lineage and serotype library using ClinPro Tools and these are described in previous paragraph.

RESULTS AND DISCUSSION

Validation of lineage and serotype library using Biotyper and ClinPro Tools. Based on the slide agglutination and multiplex PCR, 112 strains classified as *L. monocytogenes* were divided to lineages (I and II) and serotypes (1/2a, 1/2c, 1/2b and 4b). MALDI-TOF

Table 1. Validation of lineages using Biotyper and ClinPro Tools

	Target isolate classification			Off-target isolate classification			SEN	SPE	ACC
	Total	TP	FN	Total	TN	FP	(%)		
Biotyper lineage MSP library									
Lineage I	56	52	4	56	52	4	92.9	92.9	92.9
Lineage II	56	52	4	56	52	4	92.9	92.9	92.9
ClinPro Tools lineage model									
Lineage I	56	56	0	56	56	0	100.0	100.0	100.0
Lineage II	56	56	0	56	56	0	100.0	100.0	100.0

TP – true positive (correctly identified); FN – false negative (incorrectly identified); TN – true negative (correctly rejected); FP – false positive (incorrectly rejected); SEN – sensitivity; SPE – specificity; ACC – accuracy

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Table 2. Validation of serotypes using Biotyper and ClinPro Tools

	Target isolate classification			Off-target isolate classification			SEN	SPE	ACC
	Total	TP	FN	Total	TN	FP		(%)	
Biotyper serotype MSP library									
Serotype 1/2a	28	19	9	84	63	21	67.9	75.0	73.2
Serotype 1/2c	28	21	7	84	61	23	75.0	72.6	73.2
Serotype 1/2b	28	18	10	84	64	20	64.3	76.2	73.2
Serotype 4b	28	24	4	84	58	26	85.7	69.1	73.2
ClinPro Tools serotype model									
Serotype 1/2a	28	26	2	84	79	5	92.9	94.1	93.8
Serotype 1/2c	28	28	0	84	77	7	100.0	91.7	93.8
Serotype 1/2b	28	27	1	84	78	6	96.4	92.9	93.8
Serotype 4b	28	24	4	84	81	3	85.7	96.4	93.8

*for abbreviations see Table 1

MS analysis correctly identified these 112 strains as *L. monocytogenes* with log scores over 2.3 value. To create lineage and serotype MALDI-TOF MS-based library/model, acquired mass spectra were arranged, processed and validated using Biotyper and ClinPro Tools (Table 1 and 2). Results of lineage validation showed similar characteristics for both Biotyper and ClinPro Tools (accuracy of 92.9 and 100%, respectively). Using ClinPro Tools, all serotypes showed higher values of sensitivity and specificity when compared to the values obtained by Biotyper (Table 2). The corresponding accuracy of 93.8% was determined for ClinPro Tools and 73.2% for Biotyper, respectively.

Identification and evaluation of cohort samples using Biotyper and ClinPro Tools. Lineage and serotype identification of 115 *L. monocytogenes* sample strains was provided by MALDI-TOF MS using previously created Biotyper MSP libraries and ClinPro Tools models. Results of identification obtained by Biotyper and ClinPro Tools are summarized in Table 3 and 4. Lineage identification of samples showed different results depending on used software. The values of sensitivity and specificity that were determined by ClinPro Tools (i.e., 94.8 and 96.5% for lineage II, 96.5 and 94.8% for lineage I) were higher when compared to those obtained by Biotyper (i.e, 65.5 and 77.2% for lineage II, 77.2 and 65.5% for lineage I). Similarly, the accuracy of 95.7% was obtained by ClinPro Tools and 71.3% for Biotyper, respectively. Identification of serotypes resulted in generation of heterogeneous data. However, the similar trend showing higher values of sensitivity, specificity and accuracy for ClinPro Tools was observed. In detail, serotype-based identification using ClinPro Tools showed sensitivity and specificity in range of 82.8–50 and 73.6–62.8%, respectively. Equally, sensitivity and specificity determined by Biotyper were found to be lower, having range of 62.1–48.2 and 58.1–53.5%, respectively. The accuracy of determination corresponding to 67.8% for ClinPro Tools and to 55.7% for Biotyper was obtained.

The potential of MALDI-TOF MS for typing of lineages (I and II) and serotypes (1/2a, 1/2c, 1/2b and 4b) was tested on 227 strains of well-characterized *Listeria monocytogenes*. Ethanol-formic acid extraction enables purification of bacterial culture and concentration of ribosomal proteins predominantly detected by MALDI-TOF MS, thus, spectra of high quality are achieved (Stevenson *et al.* 2010). Therefore, extraction procedure was preferred and resulted in correct *L. monocytogenes* species identification of 227 strains used in this study. In detail, log score exceeded 2.3 and 2 value for 190 and 37 strains, respectively.

MALDI-TOF MS has been reported to classify *L. monocytogenes* into lineages (Barbuddhe *et al.* 2008; Hsueh *et al.* 2014) or to typing of *L. monocytogenes* during the investigation of contamination sources in food processing environments (Jadhav *et al.* 2015). Unfortunately, these experiments involved limited number of *L. monocytogenes* strains differing in serotypes. Another obstacle represents reliable detection of specific biomarkers based on ribosomal

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Table 3. Lineage identification using Biotyper and ClinPro Tools

	Target isolate classificationh			Off-target isolate classification			SEN	SPE	ACC
	Total	TP	FN	Total	TN	FP		(%)	
Biotyper lineage identification									
Lineage I	58	38	20	57	44	13	65.5	77.2	71.3
Lineage II	57	44	13	58	38	20	77.2	65.5	71.3
ClinPro Tools lineage identification									
Lineage I	57	55	2	58	55	3	96.5	94.8	95.7
Lineage II	58	55	3	57	55	2	94.8	96.5	95.7

*for abbreviations see Table 1

proteins that are usually difficult to reproduce (Jadhav *et al.* 2014; Ojima-Kato *et al.* 2016). MALDI TOF MS generally detects housekeeping proteins such as ribosomal proteins in range usually of 3000–15 000 *m/z*. In this study, capsular polysaccharides or protein antigens (e.g., the specific-*Listeria* O and H ones) were not detected. The main reason for the absence of the polysaccharide signals results from the using of the sample preparation method suitable for protein detection. Protein antigen signals are not detected due to MALDI suppression effect. Therefore, sub-identification could be attributable to the differences in housekeeping proteins associated with lineages and serotypes. Strains of variable origin and different pulsotypes were selected to reduce the close relationship among strains in lineages or serotypes and to enhance the sensitivity of sub-identification. However, it has been observed that lineages showed superior identification over serotyping. Two lineages (each created of 56 strains) and four serotypes (each created of 28 strains) were used for *L. monocytogenes* subtyping. For serotyping, lower amount of strains per serotype might have contributed to higher rate of misidentification results.

Combination of 2D electrophoresis followed by peptide mass fingerprinting using MALDI-TOF MS has been used to identify *L. monocytogenes* protein patterns (Trémoulet *et al.* 2002). However, processing of sample strains including gel electrophoresis and MALDI-TOF MS is a time-consuming method for routine lineage and serotype identification (Phan-Thanh & Mahouin 1999). It should be mentioned that simple use of Biotyper without any additional procedures after automatic spectra acquisition can predetermine this software applicable for routine sub-identification. Contrary, ClinPro Tools requires

Table 4. Serotype identification using Biotyper and ClinPro Tools

	Target isolate classification			Off-target isolate classification			SEN	SPE	ACC
	Total	TP	FN	Total	TN	FP		(%)	
Biotyper serotype identification									
Serotype 1/2a	29	14	15	86	50	36	48.2	58.1	55.7
Serotype 1/2c	29	15	14	86	49	37	51.7	57.0	55.7
Serotype 1/2b	29	18	11	86	46	40	62.1	53.5	55.7
Serotype 4b	28	17	11	87	47	40	60.7	54.0	55.7
ClinPro Tools serotype identification									
Serotype 1/2a	29	24	5	86	54	32	82.8	62.8	67.8
Serotype 1/2c	29	18	11	86	60	26	62.1	69.8	67.8
Serotype 1/2b	29	22	7	86	56	30	75.9	65.1	67.8
Serotype 4b	28	14	14	87	64	23	50.0	73.6	67.8

*for abbreviations see Table 1

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additional time to evaluate data reaching results with enhanced discrimination. However, this software is not commonly included in MALDI-TOF MS software package and require additional cost.

CONCLUSIONS

Slide agglutination method and multiplex PCR (for serogroups detection) present suitable sensitive methods for serotyping of *Listeria monocytogenes*. In this study, new approach based on Biotyper and ClinPro Tools creating MSP libraries and CPT models to identify lineage (I and II) and serotype (1/2a, 1/2b, 1/2c and 4b) in *L. monocytogenes* was designed. MALDI-TOF MS of sample strains showed applicable lineage identification with accuracy of 95.7% using ClinPro Tools and 71.3% for using Biotyper, respectively. However, ClinPro Tools and also Biotyper showed limited accuracy of 67.8 and 55.7% for tested serotypes. Ethanol-formic acid extraction yielded spectra of high quality applicable for lineage and serotype typing of *L. monocytogenes*. Results demonstrate MALDI-TOF MS as possible tool for first step in routine sub-identification of *L. monocytogenes* of food and human origin.

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