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MALDI-TOF mass spectrometry-based identification of *Listeria* species in surveillance: a prospective study

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ABSTRACT

This study aimed to evaluate MALDI-TOF MS for species discrimination of *Listeria* in the context of routine surveillance. MALDI-TOF MS yielded 100% accuracy for the identification of *L. monocytogenes, L. innocua, L. ivanovii, L. fleischmannii, L. grayi, L. seeligeri, L. weihenstephanensis* and *L. welshimeri*, as confirmed by whole genome analyses.

Highlights

- MALDI-TOF MS correctly identifies human pathogenic *Listeria* species.
- Prospective collected isolates (*n*=1,201) were identified with 100% of accuracy.
- Reliable identification is linked to a good quality reference library of MALDI-TOF MS spectra.
- MALDI-TOF MS is a fast and cost-effective tool for routine *Listeria* identification.

Keywords

Listeria identification, MALDI-TOF, mass spectrometry, food, public health surveillance.

Listeria monocytogenes is a Gram-positive bacillus and the cause of listeriosis, a severe human foodborne infection (Vazquez-Boland et al., 2001). It belongs to the genus *Listeria*, which currently includes 17 species: *L. aquatica, L. booriae, L. cornellensis, L. fleischmannii, L. floridensis, L. grandensis, L. grayi, L. innocua, L. ivanovii, L. marthii, L. monocytogenes, L. newyorkensis, L. riparia, L. rocourtiae, L. seeligeri, L. weihenstephanensis* and *L. welshimeri* (Orsi and Wiedmann, 2016). Of these, five are routinely isolated from food: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*, but only *L. monocytogenes*, and rarely *L. ivanovii*, are of medical concern (Guillet et al., 2010). In the context of *Listeria* surveillance, it is therefore critical to use an accurate, simple, rapid and cost-effective method to distinguish between these species. Current identification tools, such as API-*Listeria* (bioMérieux, Marcy l'Etoile, France) (Bille et al., 1992), PCR serogrouping (Doumith et al., 2004) and 16S rRNA gene sequencing (Sontakke et al., 2009) are expensive and time-consuming.

Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) has become a reliable tool for bacterial identification in clinical microbiology (Angeletti, 2016). The identification accuracy, low cost of reagents/consumables per sample after purchasing and maintenance of equipment, and speed of data acquisition are the most prominent advantages of this technology. MALDI-TOF MS has been successfully used for the detection of *L. monocytogenes* from selective enrichment broth (Jadhav et al., 2014), rapid identification of *Listeria* species (Barbuddhe et al., 2008; Rychert et al., 2013; Hsueh et al., 2014; Jadhav et al., 2015; Ojima-Kato et al., 2016), discrimination of lineages (Ojima-Kato et al., 2016) and subtyping of *Listeria monocytogenes* (Barbuddhe et al., 2008; Jadhav et al., 2015; Ojima-Kato et al., 2016). Previous studies used the Andromas MALDI-TOF MS system (Paris, France) (Farfour et al., 2012), the Shimadzu Corporation MALDI-TOF MS system (Kyoto, Japan) (Jadhav et al., 2014, 2015; Ojima-Kato et al., 2016) or the Vitek MALDI-TOF MS system (bioMérieux, Marcy

l'Etoile, France) (Rychert et al., 2013), and did not exhibit high species identification accuracy. The Bruker Daltonics (Bremen, Germany) MALDI-TOF MS system has been used with success, but on a limited number of phenotypically identified strains and to establish mass spectral fingerprints of some reference strains (Barbuddheet al., 2008; Hsueh et al., 2014).

In the context of a National Reference Centre for *Listeria*, that receives around 1,800 *Listeria* isolates per year from clinical (20%) and food (80%) origins, we evaluated the Bruker Daltonics system of MALDI-TOF MS Biotyper as a method for rapid identification of *L. monocytogenes* and the 16 other *Listeria* spp. described until now. We also compared the results to phenotypic-based identification. We first performed a validation study, and then used this system prospectively in the context of *Listeria* surveillance in France. Its accuracy was confirmed by bacterial identification using average nucleotide identity BLAST (ANIb) determined from whole genome sequences (Goris et al., 2007).

A validation set of 386 *Listeria* strains representative of *Listeria* genus and *L. monocytogenes* diversity (Chenal-Francisque et al., 2015) was first analyzed (Table 1). A particular focus was given to *L. monocytogenes* (*n*=353), to fully cover the diversity of this species, which is the main common pathogenic *Listeria* isolated in clinical and food microbiological laboratories and responsible for human listeriosis. In addition, a set of 34 non-*Listeria* isolates corresponding to frequent contaminants often phenotypically misidentified as *Listeria* were used as negative controls (Table 2). A prospective analysis was then carried out for all the *Listeria* strains received at the French National Reference Center of *Listeria* (NRCL) in 2016 (*n*=1,201, Table 3).

Strains were grown overnight (18h) on Brain Heart Infusion (BHI; Becton Dickinson Difco, Franklin Lakes, USA) agar plates at 35°C. The samples were prepared by full protein extraction and analysed according to the Bruker Daltonics instructions. As described in previous studies (Barbuddhe et al., 2008; Hsueh et al., 2014; Capocefalo et al., 2016,), this full protein extraction

was crucial to obtain reliable *Listeria* identification to the species level (data not shown). Briefly, colonies were transferred into a 2-ml screw-cap extraction tube (Eppendorf, Germany) containing 300 µl of distilled water and then mixed with 900 µl of absolute ethanol by pipetting. The suspension was pelleted by centrifugation at 13,000 rpm for 2 min, air dried, and then reconstituted with 50 µl of 70% formic acid (Sigma-Adlrich, Darmstadt, Germany) before the addition of 50 µl of acetonitrile (Sigma-Aldrich) to the mixture (Bizzini et al., 2010). The mixture was then centrifuged at 13,000 rpm for 2 min. Next, 1 µL of the supernatant was deposited in duplicate spots onto a polished steel MALDI-TOF MS target plates (Bruker Daltonics, Bremen, Germany) and air dried at room temperature. As quality control system, 1 µL of Bruker Bacterial Test Standard (BTS; Bruker Daltonics) was also used at the beginning and end of every series of samples. Dried spots were overlaid with 1 µl of a HCCA matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in acetonitrile 50%, water 47.5% and trifluoroacetic acid 2.5%; Bruker Daltonics) and let air dry again at room temperature according to manufacturer's instructions (Bruker Daltonics). The MALDI-plate was inserted into the source of a microFlex LT/SH MALDI-TOF MS instrument (Bruker Daltonics) with a 60-Hz nitrogen laser (337 nm wavelength) and automatically read. The spectra were collected in the linear positive mode in a mass range covering m/z 1,960–20,132 in the FlexControl v3.4 software (Bruker Daltonics). All spectra ranging from the mass-to-charge ratio (m/z) 2,000–20,000, corresponding to the resulting ribosomal protein profiles, were analyzed using the MALDI BioTyper (MBT) compass explorer software v4.1.60 (Bruker Daltonics) and compared to reference spectra of the MALDI BioTyper Reference library (database [DB-5989] MS; Bruker Daltonics) for automatic identification. The whole treatment from processing acquired spectra to identification was performed automatically without user intervention using the integrated pattern matching algorithm of the software (Barbuddhe et al., 2008). Scores of ≥2.000 indicated species-level identification, scores of 1.700–

1.999 indicated genus-level identification, and scores of <1.699 indicated no reliable identification. For each isolate, 2 spots were submitted to two analyses to obtain at least 4 identical and reliable scores results (2.0 ≤ score ≤ 3.0). Automatized bacterial identification were valid if the BTS was identified as *Escherichia coli* DH5-alpha. All isolates with discrepant identification results between the molecular and Bruker Biotyper methods were tested twice.

Conventional identification was carried out by API-*Listeria* (bioMérieux) combined with hemolysis test on horse blood agar according to manufacturer's instructions (Bille et al., 1992). PCR serogrouping of *Listeria* isolates was determined by a multiplex PCR and interpreted as described by Doumith et al. (2004) and Leclercq et al. (2011). Genome sequences were obtained after DNA extraction (DNeasy Blood & Tissue kit, Qiagen, Denmark), library preparation (Nextera XT DNA Sample kit, Illumina, California, USA) and sequencing using NextSeq 500 (2 x 150 bp) platform (Illumina, California, USA), according to the manufacturer's protocol. Sequence assemblies were built using CLC Assembly Cell 4.3.0. (Qiagen, Hilden, Germany) from FqCleaner filtered reads with an average read coverage higher than 40X, as described previously (Moura et al., 2017). Genome-based species identification was determined by average nucleotide identity BLAST (ANIb) analysis against the *Listeria* species reference genomes deposited at NCBI database (https://www.ncbi.nlm.nih.gov), as described previously (Goris et al., 2007). The congruence between MALDI identification results and those obtained by classical reference methods and whole genome sequencing was evaluated.

The accuracy of MALDI-TOF identification results is shown in Table 1. Unambiguous identification at the species level was obtained for the 8 most frequently isolated species: *L. monocytogenes* (353/353), *L. welshimeri* (3/3), *L. innocua* (4/4), *L. ivanovii* (4/4), *L. fleischmannii* (3/3), *L. grayi* (2/2), *L. seeligeri* (4/4), and *L. weihenstephanensis* (1/1). For strains with subspecies variants (*L. ivanovii*, *L. fleischmannii* and *L. grayi*), MALDI-TOF MS provided a

correct species identification, but not at the sub-species level. As expected, since the corresponding reference profiles are absent from the current version of MALDI Biotyper reference library, unreliable identification was obtained for *L. grandensis*, *L. rocourtiae*, *L. aquatica*, *L. cornellensis*, *L. riparia*, *L. floridensis*, *L. booriae* and *L. newyorkensis*. These last species could not be identified by API-*Listeria*. Of note, these species have never been isolated in the context of *Listeria* surveillance in France. Finally, *L. marthii*, a rarely isolated species, was always misidentified as *L. monocytogenes* with acceptable scores, also due to the lack of reference profile for this species in the database. None of the negative controls were misidentified as *Listeria* species (Table 2).

We then applied this method for the prospective identification of 1,201 strains received in the context of *Listeria* national surveillance in France from April 2016 to December 2016 (Table 3). These strains were isolated from human (n=299), food (n=731), environmental (n=155), animal (n=2) or unknown origin from owncheck food manufacturers (n=14) samples. In total, 5 species were correctly identified at the species level and included *L. monocytogenes* (n=1,112), *L. innocua* (n=65), *L. welshimeri* (n=20), *L. seeligeri* (n=3) and *L. ivanovii* (n=1) isolates. All isolates were correctly identified by MALDI-TOF MS at the species level, as confirmed by ANIb identification from whole genome sequence analysis.

This work demonstrates the complete reliability of MALDI-TOF mass spectrometry as a rapid (less than 40 minutes) approach for the identification of *Listeria* in human, animal, food, and environmental microbiology, with 100% of accuracy for *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. fleischmannii*, *L. grayi*, *L. seeligeri*, *L. weihenstephanensis* and *L. welshimeri* identification. This study also underlines the importance of a good quality and continuously updated reference MBT library for accurate identification of *Listeria*.

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Conflict of interest: none

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Table 1

Results of species and subspecies identification by applying the MALDI-TOF MS method on a validation set of 386 *Listeria* isolates [a].

Species/subspecies	PCR serogroups[b]	Hemolysis	Correct MALDI-TOF identification (%)
L. monocytogenes	IIa, IIb, IIc, IVb, IVb-v1, L	+	353/353 (100)
L. innocua	L	-	4/4 (100)
L. ivanovii	L	+	4/4 (100)
L. ivanovii subsp. *ivanovii*	L	+	3/3 (100)
L. ivanovii subsp. *londoniensis*	L	+	1/1 (100)
L. welshimeri	L	-	3/3 (100)
L. seeligeri	L	+	4/4 (100)
L. grayi	L	-	2/2 (100)
L. gayi subsp. *grayi*	L	-	1/1 (100)
L. grayi subsp. *murrayi*	L	-	1/1 (100)
L. fleischmannii	L	-	3/3 (100)
L. fleischmannii subsp. *coloradensis*	L	-	1/1 (100)
L. fleischmannii subsp. *fleischmannii*	L	-	2/2 (100)
L. weihenstephanensis	/	-	1/1 (100)
L. marthii	L	-	0/4 (0)[c]
L. aquatica	L	-	0/1 (0)[d]
L. booriae	/	-	0/1 (0)[d]
L. cornellensis	/	-	0/1 (0)[d]
L. floridensis	L	-	0/1 (0)[d]
L. grandensis	/	-	0/1 (0)[d]
L. newyorkensis	/	-	0/1 (0)[d]
L. riparia	/	-	0/1 (0)[d]
L. rocourtiae	/	-	0/1 (0)[d]

[a] Isolates were selected to represent the diversity of *Listeria* species. The second and third columns indicate, for each species, the PCR serogroups and the hemolysis results on horse blood

agar plates (bioMérieux), respectively. Proportions of correctly identified isolates by MALDI-TOF MS are given in the fourth column relatively to API-*Listeria* identification and genome-based identification results by average nucleotide identity BLAST (ANIb).

[b] according to Doumith et al. (2004) and Leclercq et al. (2011). PCR serogroup L comprised strains of serovars 4a, 4ab, 4c of *L. monocytogenes* and other species, except *L. booriae, L. cornellensis, L. grandensis, L. newyorkensis, L. riparia, L. rocourtiae,* and *L. weihenstephanensis*.

[c] Misidentified species

[d] Non-identified species or low scores of identification (scores ≤ 1.99)

TABLE 2.

Results of species identification by applying the MALDI-TOF MS method on a negative controls set of 34 non *Listeria* isolates.

Species	MALDI-TOF score	Correct MALDI-TOF identification [a]
Achromobacter xylosoxidans	2.148	1/1 (100%)
Bacillus cereus	1.86-2.13	3/3 (100%)
Bacillus circulans	1.91	1/1 (100%)
Bacillus pumilus	1.85	1/1 (100%)
Carnobacterium maltaromaticum	2.15	1/1 (100%)
Enterococcus faecalis	2.22-2.62	17/17 (100%)
Erysipelothrix rhusiopathiae	2.18	1/1 (100%)
Microbacterium arborescens	2.22	1/1 (100%)
Staphylococcus epidermidis	1.81-2.04	7/7 (100%)
Staphylocccus warneri	2.1	1/1 (100%)

[a] compared with genome-based species identification using the average nucleotide identity BLAST (ANIb) analyses

Table 3.

Results of species identification by applying the MALDI-TOF MS method on a validation set of 1201 *Listeria* isolates.

Species	PCR serogroups[a]	Hemolysis	Correct MALDI-TOF MS identification[b]
L. innocua	L	-	65/65 (100%)
L. ivanovii	L	+	1/1 (100%)
L. monocytogenes	IIa, IIb, IIc, IVb, IVb-v1	+	1112/1112 (100%)
L. seeligeri	L	+	3/3 (100%)
L. welshimeri	L	-	20/20 (100%)

[a] according to Doumith et al. (2004) and Leclercq et al. (2011). PCR serogroup L comprised strains of serovars 4a, 4ab, 4c of *L. monocytogenes* and other species, except *L. booriae, L. cornellensis, L. grandensis, L. newyorkensis, L. riparia, L. rocourtiae,* and *L. weihenstephanensis*.

[b] Compared with genome-based species identification using the average nucleotide identity BLAST (ANIb) analyses.