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## **Klebsiella MALDI TypeR: a web-based tool for Klebsiella identification based on MALDI-TOF mass spectrometry**

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1 ***Klebsiella* MALDI TypeR: a web-based tool for *Klebsiella***  
2 **identification based on MALDI-TOF mass spectrometry**

3  
4 **Running title: Web-based MALDI-TOF identification of *Klebsiella***

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## 16 **Abstract**

17 *Klebsiella* pathogens affect human and animal health and are widely distributed in the  
18 environment. Among these, the *Klebsiella pneumoniae* species complex, which includes  
19 seven phylogroups, is an important cause of community and hospital infections. The  
20 *Klebsiella oxytoca* species complex also causes hospital infections and antibiotic-associated  
21 haemorrhagic colitis. The unsuitability of currently used clinical microbiology methods to  
22 distinguish species within each of these species complexes leads to high rates of  
23 misidentifications that are masking the true clinical significance and potential epidemiological  
24 specificities of individual species.

25 We developed a web-based tool, *Klebsiella* MALDI TypeR, a platform-independent  
26 and user-friendly application that enables uploading MALDI-TOF mass spectrometry data in  
27 order to identify *Klebsiella* isolates at the species complex and phylogroup levels. The tool,  
28 available at <https://maldityper.pasteur.fr>, leverages a database of previously identified  
29 biomarkers that are specific for species complexes, individual phylogroups, or related  
30 phylogroups. We obtained 84% to 100% identification accuracy depending on phylogroup.  
31 Identification results are obtained in a few seconds from batches of uploaded spectral data.

32 *Klebsiella* MALDI TypeR enables fast and reliable identification of *Klebsiella* strains  
33 that are often misidentified with standard microbiological methods. This web-based  
34 identification tool may be extended in the future to other human bacterial pathogens.

35 **Keywords**

36 *Klebsiella*, mass spectrometry, software

## 37 Introduction

38 Bacteria of the genus *Klebsiella* are human and animal pathogens with a wide  
39 ecological distribution [1]. The genus *Klebsiella*, a member of the family *Enterobacteriaceae*,  
40 encompasses a huge diversity in terms of phylogenetic lineages, genomic content, pathogenic  
41 properties, and ecological distribution. Two major subdivisions of *Klebsiella* are the  
42 *Klebsiella pneumoniae* species complex (KpSC) and the *Klebsiella oxytoca* species complex  
43 (KoSC) [2]. The KpSC currently includes five taxonomic species, two of which comprise two  
44 subspecies. These taxa were initially defined as seven distinct phylogroups, Kp1 to Kp7 [3].  
45 *K. pneumoniae* (phylogroup Kp1) is one of the most problematic pathogens associated with  
46 antibiotic resistance worldwide. This species is phylogenetically closely related to *K.*  
47 *quasipneumoniae* [subsp. *quasipneumoniae* (Kp2) and subsp. *similipneumoniae* (Kp4)], *K.*  
48 *variicola* [subsp. *variicola* (Kp3) and subsp. *tropica* (Kp5)], *K. quasivariicola* (Kp6) and *K.*  
49 *africana* (Kp7). In turn, the KoSC comprises 6 species and 7 phylogroups [4]: *K.*  
50 *michiganensis* (Ko1), *K. oxytoca* (phylogroup Ko2), *K. spallanzanii* (Ko3), *K. pasteurii*  
51 (Ko4), *K. grimontii* (Ko6) and *K. huaxiensis* (Ko8). *K. oxytoca* is well known as a cause of  
52 opportunistic human infections and post-antibiotic hemorrhagic diarrhea [4,5].

53 Recent literature has brought into focus the difficulties that microbiology laboratories  
54 encounter in the identification of individual members of the *K. pneumoniae* and *K. oxytoca*  
55 species complexes [5–9]. Indeed, *Klebsiella* species are genetically similar and have few  
56 biochemical discriminatory biomarkers. As a consequence, the clinical significance of  
57 individual *Klebsiella* species and their potential epidemiological specificities are still not well  
58 defined. While Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF)

59 mass spectrometry can identify *Klebsiella* species through peak discrimination [4,5,10], the  
60 current software implementation is limited to identifying *K. pneumoniae*, *K. variicola* and *K.*  
61 *oxytoca* [9–11]. Here we present *Klebsiella* MALDI TypeR, a tool that leverages novel  
62 discriminant biomarkers in MALDI-TOF spectra to expand the number of identifiable  
63 *Klebsiella* species from MALDI-TOF data and improve identification accuracy, using a  
64 previously-published method [12].

## 65 **Material and methods**

66           **Bacterial strains and reference identification by genomic sequencing.** A total of  
67 341 strains were included. A first set consisted of 78 clinical isolates (50 belonging to the  
68 *Klebsiella pneumoniae* species complex, 28 to the *Klebsiella oxytoca* species complex),  
69 selected to be representative of the phylogenetic diversity of these taxa. These isolates have  
70 been used in previous studies [4,5]. Three and 24 spectra per isolate were recorded for the  
71 KpSC and for the KoSC, respectively, as described previously [4,5]. A second set of 262  
72 isolates belonging to the KpSC was collected in Australia from humans and animals. Isolate  
73 genomes were sequenced using Illumina paired-end technology and assembled using SPAdes.  
74 Species identification was performed with Kleborate [13,14], which compares each genome  
75 to a curated set of species references using Mash distances; these species calls were used as  
76 reference in subsequent analyses.

77           **Preparation of bacterial samples and MALDI-TOF MS analysis.** An overnight  
78 culture on Luria-Bertani agar (37°C, 18 h) was used to prepare the samples with the  
79 ethanol/formic acid extraction procedure following the manufacturer recommendations  
80 (Bruker Daltonics, Bremen, Germany). Samples (1 µL) were spotted onto an MBT Biotarget  
81 96 target plate, air dried and overlaid with 1 µL of a saturated  $\alpha$ -cyano-4-hydroxycinnamic  
82 acid matrix solution in 50% of acetonitrile and 2.5% of trifluoroacetic acid. External  
83 calibration of the mass spectra was performed using Bruker Bacterial Test Standard. For the  
84 assessment of the direct colony picking protocol, a single bacterial colony was picked with a  
85 sterile toothpick and directly deposited onto a 96-spot polished steel target and processed as  
86 the sample spot previously mentioned. In addition, the same two protocols were followed

87 using blood agar medium (Columbia agar plus 5% sheep blood). A MALDI Biotyper  
88 Microflex LT controlled by Compass flexControl software (version 3.4; Bruker Daltonics,  
89 Billerica, Massachusetts, USA) was used to generate mass spectra for all isolates. Mass  
90 spectra were acquired using automatic mode and default settings (2000 to 20 000 Da; linear  
91 positive mode; 240 laser shots).

92       **Identification of phylogroups based on MALDI-TOF data in *Klebsiella* MALDI**  
93 **TypeR.** The KpSC and the KoSC encompass species and subspecies that are defined as  
94 phylogroups. As such, we aimed to distinguish them on the basis of MALDI-TOF data, using  
95 information from the spectra, defined as peak shifts [15]. Identification of phylogroups is  
96 therefore based on a small selection of biomarkers for which one (or more) protein weight  
97 modifications have been previously reported [4,5]. In fact, each phylogroup could be seen as  
98 comprising MALDI-Types, as described by Bridel *et al.* 2020 [12]: a specific combination of  
99 protein  $m/z$  values of these biomarkers. *Klebsiella* MALDI TypeR was run on a 4 CPUs 16-  
100 GB RAM CentOS Linux 7 (Core) virtual machine.

## 101 **Results and Discussion**

102       **Development of *Klebsiella* MALDI TypeR and its web interface.** *Klebsiella*  
103 MALDI TypeR was developed as a web application with R (version 3.4.0) and R Shiny  
104 (version 1.4). A user can upload either a single spectrum or multiple spectra (a maximum  
105 number of spectra is not defined, but we recommend working with batches of up to 100  
106 spectra per analysis). The uploaded spectra must be in zip archive format. The zip archive  
107 must contain the raw data from any spectrometer. This includes the tree structure, which must

108 include spectra in the expected format (txt, csv, fid, ciphergen, mzXML, mzML, imzML,  
109 analyze, cdf or msd – see MALDIquant Foreign documentation for further details). This  
110 application has been tested with spectra (raw data folders) from Bruker spectrometer only but  
111 could theoretically handle any proper archive containing one of the previously mentioned file  
112 formats. Supplementary Figure 1 describes each step to reach one raw data folder. Each  
113 archive may correspond either to a single raw spectrum or several raw spectra of the same  
114 isolate (technical replicates). For better results, we recommend using at least 3 technical  
115 replicates for each isolate.

116 The spectra can be visually inspected after upload (**Figure 1**). A panel shows each  
117 uploaded spectrum for quick visual inspection (*e.g.*, intensity checking) and a table highlights  
118 the presence (if any) of empty spectra.

119 For identification, the tool was designed with a 2-step process. The first step (**Figure**  
120 **2, panel B1**) identifies isolates at the species complex level using two sets of biomarkers,  
121 each set corresponding to reference biomarkers for (i) the KpSC (29 biomarkers, **Table S1**) or  
122 (ii) the KoSC (57 biomarkers, **Table S1**). This step can be performed, based on user choice,  
123 with one of two alternative peak detection algorithms. The first one is based on the R package  
124 MALDIquant [16] and is set by default, as it runs faster. The second one uses the  
125 MassSpecWavelet package [17] and generally detects more significant peaks, but is slower.  
126 Species complex identification is based on the ratio of common peaks between the sample  
127 peak list and a reference peak list, to the number of peaks in the corresponding reference (this  
128 ratio is called “Identification Value”). The input sample is assigned to the species complex  
129 with the highest Identification Value.

130 The second step consists in the identification of the specific phylogroup  
131 (corresponding to a species or subspecies) within the defined species complex (**Figure 2,**  
132 **panel B2**). The raw spectrum is processed again by the Multi Peak Shift Typing (MPST)  
133 method [12], but this time using the set of biomarkers corresponding to the assigned species  
134 complex, which allows recalibration of  $m/z$  values. The biomarkers used for the two MPST  
135 schemes (one for each species complex, see **Tables 1 to 4**) are based on previous work [4,5].  
136 Briefly, an MPST scheme is analogous to a multi-locus sequence typing (MLST) scheme  
137 where an allele type (AT) corresponds to an isoform (IF) for one protein (each IF is defined  
138 by a specific mass – or a  $m/z$  value on the spectrum) and a MALDI-Type (MT) corresponds to  
139 a sequence type (ST). Each MT was observed within a single phylogroup. The tool attempts  
140 to define the IF for each biomarker, and then to attribute the spectrum to the phylogroup  
141 corresponding to the relevant MT (**Figure 2, panel B3**). The only exception to this process  
142 was introduced for the discrimination of *K. oxytoca* phylogroups Ko1, Ko4 and Ko6 (*K.*  
143 *oxytoca*, *K. pasteurii* and *K. grimontii*, respectively). These three species form a phylogenetic  
144 branch of closely related species within the *K. oxytoca* species complex [4], and their spectra  
145 are very similar such that they share the same MT. In order to distinguish among these three  
146 phylogroups, we added 3 previously described biomarkers [4], for which the tool checks for  
147 presence/absence within the spectra (*i.e.*, these 3 biomarkers do not correspond to peak  
148 shifts). If any of the above biomarkers (peak shifts or presence/absence) is not found,  
149 automatic identification is not considered possible, and the result is reported to the user as NT  
150 (not typable). In this case, manual analysis or data re-acquisition are needed.

151 To help resolve these cases and allow users to further explore the data, we included a  
152 feature enabling to interactively explore the spectra. The plot window (**Figure 3**) shows the

153 sample spectra overlaid on the reference spectra (1 to 3 spectra for each phylogroup).  
154 Predefined windows (one for each biomarker) allow the user to directly investigate specific  
155 spectral regions. This feature could also enable the detection of new isoforms or biomarkers.  
156 It also contains a customizable option that allows the user to select any region of the spectra.

157 The *Klebsiella* MALDI TypeR tool was made available at <https://maldityper.pasteur.fr>  
158 . A single isolate analysis required approximately 10 seconds to complete and the analysis of  
159 10 isolates takes about 35 seconds. The tool does not have a limit to the number of isolates  
160 that can be analyzed simultaneously, and it performed well with batches of 100 isolates that  
161 were uploaded at once.

162 **Evaluation of the identification tool.** We first tested fifty KpSC isolates grown  
163 overnight on lysogeny broth (LB) agar (37°C, 18h) and subjected to ethanol/formic acid  
164 extraction following the manufacturer recommendations (Bruker Daltonics, Bremen,  
165 Germany). For each strain, a total of 3 spectra from the same spot were acquired (Bruker  
166 Microflex LT mass spectrometer, Paris). This dataset yielded correct assignments to species  
167 complex for 50/50 (100%) isolates, 47 (94%) of which had an identification value >50%  
168 (**Tables S2 and S3**). The same samples prepared using different experimental conditions (two  
169 media: LB and Columbia agar plus 5% sheep blood; each with both extraction and direct  
170 application in the MALDI-TOF target) also yielded correct complex assignments for all  
171 isolates, albeit with lower confidence (**Table S2**). Regarding phylogroup assignment, using  
172 LB-extraction 90% of samples were correctly identified at phylogroup level (**Table S3**), while  
173 the other sample preparation methods yielded better results overall (**Table S4**). To validate  
174 robustness of the tool to instrument and user variation, we tested an additional dataset

175 comprising 248 KpSC isolates prepared using the LB-extraction method (176 isolates with a  
176 single spectrum, 26 in duplicate and 68 in triplicate); these spectra were generated on a  
177 different instrument (Bruker MALDI Biotyper microflex LT-SH) in a different laboratory  
178 (Melbourne, Australia). 244/248 isolates (98%) were assigned to the correct species complex,  
179 and 219/244 (90%) were correctly identified at the phylogroup level (84% to 100% for each  
180 phylogroup, see **Table S5**).

181 The KoSC assignments were tested using 28 isolates prepared using LB-extraction and  
182 triplicate spectra generated on the Bruker Microflex LT mass spectrometer instrument (Paris).  
183 All isolates were correctly assigned to complex level and most (89%) were assigned to the  
184 correct MALDI-Type (**Tables S6 and S7**). However, discrimination of MALDI-Type MT1  
185 (n=16, 57%) into phylogroups Ko1, Ko4 and Ko6 was poor (**Table S8**) because the three  
186 additional biomarkers intended to discriminate between these phylogroups were captured only  
187 rarely.

## 188 **Conclusions**

189 We developed the *Klebsiella* MALDI TypeR web tool for identification of *Klebsiella*  
190 species based on MALDI-TOF MS. Our tool allows the fast analysis of single or multiple  
191 spectra, including their visualization. We show that MALDI-TOF MS leads to accurate  
192 identification for *Klebsiella* phylogroups of the KpSC, the major *Klebsiella* species complex  
193 found in clinical samples. *Klebsiella* MALDI TypeR therefore represents an important  
194 resource for the identification of the KpSC members, which are currently difficult to identify  
195 in routine microbiology practice. For the KoSC, we found promising data, but further

196 validation based on a larger dataset would be required. The current biomarker approach does  
197 not appear to be suitable for accurate discrimination of Ko1, Ko4 and Ko6. In the future, the  
198 website may be updated to integrate additional methods for identification and typing, such as  
199 supervised learning.

## 200 **Declarations**

### 201 **Ethics approval and consent to participate**

202 Not applicable

203

### 204 **Consent for publication**

205 Not applicable

206

### 207 **Availability of data and materials**

208 The datasets used and/or analyzed during the current study are available from the  
209 corresponding author on reasonable request.

210

### 211 **Competing interests**

212 The authors declare that they have no competing interests.

213

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218 **Authors' contributions**

219 LMJ, TH, CR and VP performed the MALDI-TOF data acquisition. SBridel, SCW and CR  
220 analyzed the MALDI-TOF data. SBridel designed and programmed the initial version of the  
221 *Klebsiella* MALDI TypeR tool and website. SCW, CR, KEH and SBrise provided input in  
222 the choice of data processing algorithms and web site design. SBridel wrote the original draft.  
223 All authors contributed to and approved the final version of the manuscript.

224

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