Rapid and cost-effective identification of *Bartonella* species using mass spectrometry

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Bacteria of the genus *Bartonella* are emerging zoonotic bacteria recognized in a variety of human diseases. Due to their poor chemical reactivity, these fastidious bacteria are poorly characterized using routine phenotypic laboratory tests. Identification is usually achieved using molecular techniques that are time-consuming, expensive and technically demanding. Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a new technique for bacterial species identification. This study evaluated the use of MALDI-TOF MS for rapid genus and species identification of *Bartonella* species. Reference strains representing 17 recognized *Bartonella* species were studied. For each species, MS spectra for four colonies were analysed. The consensus spectrum obtained for each species was unique among spectra obtained for 2843 bacteria within the Bruker database, including 109 alphaproteobacteria. Thirty-nine additional blind-coded *Bartonella* strains were correctly identified at the species level, including 36 with a significant score. Altogether, these data demonstrate that MS is an accurate and reproducible tool for rapid and inexpensive identification of *Bartonella* species.

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INTRODUCTION

Bartonella species are fastidious, Gram-negative bacteria belonging to the *Alphaproteobacteria* which are associated with mammals. These bacteria are considered as emerging pathogens. They possess a natural cycle that involves persistent intra-erythrocytic infection in a reservoir host. Arthropod vectors transmit the bacteria between reservoirs and disease-susceptible hosts, including humans (Breitschwerdt & Kordick, 2000). Of the 19 officially recognized species (<http://www.bacterio.cict.fr/b/bartonella.html>), the three most common pathogens for humans are *Bartonella bacilliformis* (the agent of bartonellosis or Carrion's disease), *Bartonella quintana* (which causes trench fever) and *Bartonella henselae* (the agent of cat-scratch disease). The latter two species can also cause endocarditis, bacillary angiomatosis and other infections in immunocompromised patients (Welch, 2005). Other *Bartonella* species have been reported to cause disease in humans, but their role is less well defined. These include *Bartonella alsatica* (Raoult *et al.*, 2006), *Bartonella elizabethae* (Daly *et al.*, 1993), *Bartonella koehlerae* (Avidor *et al.*, 2004), *Bartonella vinsonii* subsp. *arupensis*

(Welch *et al.*, 1999) and *Bartonella vinsonii* subsp. *berkhoffii* (Roux *et al.*, 2000), which have been associated with endocarditis, and *Bartonella grahamii*, which has been associated with cases of neuroretinitis (Kerkhoff *et al.*, 1999). Other species have been isolated from the blood of vertebrate animals, although their pathogenicity in humans remains unknown (Birtles *et al.*, 1995; Heller *et al.*, 1998).

Bacteria from the genus *Bartonella* grow slowly, regardless of the technique used, typically requiring from 7 days to 6 weeks before growth can be detected (Welch, 2005). In addition, these micro-organisms exhibit limited biochemical reactivity, and thus are poorly identified by routine phenotypic methods. Over the past 10 years, amplification-based techniques have been increasingly used to identify *Bartonella* species. Various molecular targets, including the 16S rRNA gene, 16S–23S rRNA spacer, citrate synthase (*gltA*), β -subunit of the RNA polymerase (*rpoB*), riboflavin synthase (*ribC*), cell division protein (*ftsZ*), GroEL chaperonin (*groEL*) and 17 kDa antigen (Bereswill *et al.*, 1999; Houpijian & Raoult, 2001; Sweger *et al.*, 2000; Zeaiter *et al.*, 2002a, b) have been assessed. However, despite their specificity, molecular techniques are expensive, time-consuming and technically demanding.

Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has

Abbreviation: MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

emerged as a new technique for bacterial species identification (Hsieh *et al.*, 2008). MALDI-TOF MS has been applied with success to *Burkholderia cepacia* (Vanlaere *et al.*, 2008), *Clostridium* species (Grosse-Herrenthey *et al.*, 2008), *Erwinia* species (Sauer *et al.*, 2008), *Escherichia coli* (Parisi *et al.*, 2008), *Listeria* species (Barbuddhe *et al.*, 2008), non-fermenting bacteria (Degand *et al.*, 2008; Mellmann *et al.*, 2008), oral anaerobic bacteria (Stingu *et al.*, 2008) and *Yersinia enterocolitica* (Parisi *et al.*, 2008). Via rapid and reproducible analysis of the peptidic and proteic content, MALDI-TOF MS provides a protein fingerprint specific to a given bacterial strain. However, this technique has not yet been applied to *Bartonella* species, despite their implication in a variety of animal hosts, arthropod vectors and human diseases. In the present study, we performed MS analysis of representative strains of all recognized *Bartonella* species that have been cultured.

METHODS

Bartonella strains. Type strains from 17 recognized *Bartonella* species were included in our study. *Bartonella peromysci* and *Bartonella talpae*, which cannot be cultured, were not evaluated. For *B. henselae*, we included a type strain for each of the two genotypes, Houston and Marseille. For *B. vinsonii*, a type strain for each of the three recognized subspecies (*vinsonii*, *arupensis* and *berkhoffii*) was assessed. All strains were grown on Columbia blood agar containing 5 % whole sheep blood (bioMérieux). The strains were incubated at 37 °C in a humidified CO₂-enriched environment, except for *B. bacilliformis*, which was incubated at 28 °C. The *Bartonella* species and strains studied are detailed in Table 1. For each species, a type strain was used. In addition, in order to estimate the specificity of MALDI-TOF MS, 39 additional *Bartonella* strains were blind-tested by MS. These included 13 *B. henselae*, four *B. quintana*, 20 *Bartonella bovis*, one *Bartonella schoenbuchensis* and one *Bartonella clarridgeiae* strain (Table 1). The *B. henselae* test strains comprised six and seven strains of the Marseille and Houston genotypes, respectively. Identification of these strains was confirmed by PCR amplification and sequencing of the 16S–23S rRNA spacer, as described previously (Houpikian & Raoult, 2001).

MALDI-TOF MS. For each strain, four isolated colonies were harvested in 20 µl sterile water. One microlitre of each mixture was deposited on a target plate (Bruker Daltonics) in separate sample positions and allowed to dry at room temperature. Two microlitres of matrix solution composed of α -cyano-4-hydroxycinnamic acid (Sigma) saturated with 2.5 % trifluoroacetic acid and 50 % acetonitrile was then added and allowed to co-crystallize with the sample. Samples were processed in an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics) using Flex Control software (Bruker Daltonics). Positive ions were extracted with an accelerating voltage of 20 kV in linear mode. Each spectrum indicates the sum of the ions obtained from 200 laser shots performed in five different regions of the same sample position. Laser shots exhibiting a resolution of <500 were rejected. The spectra were analysed in an *m/z* range of 2000–20 000, and the analysis was performed with Flex Analysis and BioTyper software. The spectrometer was calibrated every week using the Bruker bacterial test standard (Bruker Daltonics). The data obtained from four replicates were added to minimize random effects. The presence and absence of peaks were considered as fingerprints for a particular isolate. A maximum of 100 peaks with a signal-to-noise ratio of 3 was selected in the range of

3000–15 000 Da. The main spectra were then generated as a reference using all spectra obtained for a single micro-organism. In general, 75 peaks were picked automatically, which occurred for at least 25 % of the spectra and with a mass deviation of 200 p.p.m. From these data, a reference spectrum was determined for each species. The profiles were analysed and compared using ClinPro Tools version 2.1 software (Bruker Daltonics). Scores ≥ 2.0 were considered significant, as recommended by the manufacturer (Bruker Daltonics). For the blind test, the eight tested strains were compared with the complete Bruker database, along with reference spectra obtained for all 20 *Bartonella* species tested. Species identification was obtained when a test strain demonstrated a best match with a spectrum in the database, with a log score greater than 2.

The complete set of reference spectra obtained in the present study is available in the URMS database online (http://ifr48.timone.univ-mrs.fr/portail2/index.php?option=com_content&task=view&id=97&Itemid=54).

RESULTS

Using a standard sample preparation and analytical procedure, colony sampling from the agar plate to species identification took 10 min per strain. For multiple strains, we obtained spectra at a rate of 15 strains h⁻¹ at a total cost of 1.65 euros per strain (including personnel costs). Only those peaks with a relative intensity above 0.1 that were constantly present in all four sets of data obtained for a given strain were retained. The standard deviation for each conserved peak did not exceed the 6 *m/z* value. The set of peaks thus determined was specific to each strain (Fig. 1). When compared with the entire Bruker database, which contains spectra from 2843 bacteria, including 109 alphaproteobacteria but no *Bartonella* species, the reference spectra obtained for all 20 tested strains were unique, with scores of less than 1.7 for the four spectra obtained for each strain. Within the *B. vinsonii* species, each subspecies exhibited specific peaks: *B. vinsonii* subsp. *berkhoffii* displayed specific peaks at 4500 and 7500 Da, *B. vinsonii* subsp. *vinsonii* had specific peaks at 10 200 Da and *B. vinsonii* subsp. *arupensis* demonstrated specific peaks at 5300, 5800, 6800, 8000, 11 700 and 12 700 Da. Among the *B. henselae* species, the spectra were very similar, with the exception of two peaks in the 5000–5500 Da range that were present in the Houston strain but absent in the Marseille strain (Fig. 2). These spectra were added to our URMS MALDI-TOF MS database and are available on the website given in Methods.

Subsequently, spectra from the 39 blind-tested *Bartonella* strains were unambiguously identified at the species level with identification scores ≥ 2.0 when compared with the complete database, with the exception of *B. clarridgeiae* (1.88) and two *B. bovis* strains (1.2 and 1.0, respectively). However, in each of these three cases, the highest score obtained indicated the correct species. Reference spectra from these 39 strains were then added to our database. Spectra from all *B. quintana* strains were identical. Among *B. henselae*, each Houston and Marseille strain exhibited a score >2.0 against both genotypes, with the score obtained

Table 1. Strains used in this study

<i>Bartonella</i> species	Strain name*	References in culture collections†
<i>B. alsatica</i>	IBS 382 ^T	CSUR B4, CCUG 45774
<i>B. bacilliformis</i>	KC583 ^T	CSUR B620, ATCC 35685
<i>B. birtlesii</i>	IBS 325 ^T	CSUR B5, CCUG 44360
<i>B. bovis</i>	91-4 ^T	CSUR B6, CCUG 43828
	URBBCiNHV319‡	CSUR B7
	URBBCiNHV320‡	CSUR B8
	URBBCiNHV321‡	CSUR B9
	URBBCiNHV345‡	CSUR B10
	URBBCiNHV346‡	CSUR B11
	URBBCiNHV347‡	CSUR B12
	URBBCiNHV348‡	CSUR B13
	URBBCiNHV349‡	CSUR B14
	URBBCiNHV350‡	CSUR B15
	URBBCiNHV351‡	CSUR B16
	URBBCiNHV353‡	CSUR B17
	URBBCiNHV359‡	CSUR B18
	URBBGaNHV360‡	CSUR B19
	URBBKNHV372‡	CSUR B20
	URBWM134‡	CSUR B604
	URBWM135‡	CSUR B605
	URBWM136‡	CSUR B606
	URBWM137‡	CSUR B607
	BVG9-1‡	CSUR B621
	BVG9-2‡	CSUR B622
<i>B. capreoli</i>	IBS 193 ^T	CSUR B21, CCUG 43827
<i>B. chomelii</i>	A828 ^T	CSUR B22, CCUG 47497
<i>B. clarridgeiae</i>	73	CSUR B23, CCUG 45776
	URBCMNH27‡	CSUR B24
<i>B. doshiae</i>	R18 ^T	CSUR B50, ATCC 700133
<i>B. elizabethae</i>	F9251 ^T	CSUR B51, ATCC 49927
<i>B. grahamii</i>	V2 ^T	CSUR B52, ATCC 700132
<i>B. henselae</i>	Houston-1 ^T	CSUR B53, ATCC 49882
	URBHLLY8§	CSUR B54, CCUG 50772
	URBHLE9‡§	CSUR B55
	URBHMNHC57M‡§	CSUR B68
	URBHMNHC77M‡§	CSUR B73
	URBHMNHC78M‡§	CSUR B74
	URBHMNHC81M‡§	CSUR B77
	URBHMNHC82M‡§	CSUR B78
	URBHMNHC50H‡	CSUR B62
	URBHMNHC51H‡	CSUR B63
	URBHMNHC52H‡	CSUR B64
	URBHMNHC54H‡	CSUR B65
	URBHMNHC55H‡	CSUR B66
	URBHMNHC56H‡	CSUR B67
	URBHMNHC58H‡	CSUR B69
<i>B. koehlerae</i>	C-29 ^T	CSUR B131, ATCC 700693
<i>B. quintana</i>	Fuller ^T	CSUR B133, ATCC VR-358
	Toulouse‡	CSUR B134, CCUG 45777
	URBQPIEH2‡	CSUR B135
	URBQGBAA3‡	CSUR B136
	URBQMLY4‡	CSUR B137
<i>B. schoenbuchensis</i>	R1 ^T	CSUR B560, CCUG 50783
	URBSMNH224‡	CSUR B561
<i>B. taylorii</i>	M6 ^T	CSUR B577, CCUG 50774
<i>B. tribocorum</i>	IBS 506 ^T	CSUR B578, CCUG 45778
<i>B. vinsonii</i> subsp. <i>vinsonii</i>	Baker ^T	CSUR B601, ATCC VR-152

Table 1. cont.

<i>Bartonella</i> species	Strain name*	References in culture collections†
<i>B. vinsonii</i> subsp. <i>berkhoffii</i>	93-C01 ^T	CSUR B602, ATCC 51672
<i>B. vinsonii</i> subsp. <i>arupensis</i>	94-513 ^T	CSUR B603, ATCC 700727

*Type strains are indicated.

†CSUR, Collection de souches de l'Unité des Rickettsies; CCUG, Culture Collection, University of Göteborg; ATCC, American Type Culture Collection.

‡Strains used for the blind test.

§*B. henselae* Marseille strains.

against their homologous genotype being higher than that against the heterologous genotype.

DISCUSSION

We have demonstrated that MALDI-TOF MS is a reliable tool for the identification of *Bartonella* strains, and we created the URMS database of spectra available online. To the best of our knowledge, this is the first attempt to identify members of the genus *Bartonella* using MS. Although it contains 109 reference spectra from alphaproteobacteria, the Bruker database includes no *Bartonella* fingerprints.

The ability of *Bartonella* to cause zoonosis has stimulated investigation of an increasing number of potential animal hosts. This has led to the discovery of many novel *Bartonella* species in the past decade, including human pathogens. However, *Bartonella* organisms remain inert in most biochemical tests, and current identification in clinical laboratories is based mostly on gene amplification. In the present study, we obtained specific spectra for each of the currently recognized *Bartonella* species using MALDI-TOF MS, and demonstrated in a blind test that it reliably identified 39 strains at the species level. It should be emphasized that, in addition to providing accurate identification, MALDI-TOF MS permitted a substantial gain in terms of time prior to identification. In fact, using this method, identification of a given strain was obtained within 15 min after colonies were detected on agar plates, with a mean output of 15 strains h⁻¹. This reduces the delay prior to identification associated with molecular methods by at least 24 h. Moreover, the method was cost-effective, with a total cost of 1.65 euros per strain. This cost was mostly due to personnel charges, as the reagent costs for MALDI-TOF MS are only about €0.02 per strain.

In addition, we demonstrated that the discriminatory power of MALDI-TOF MS extended beyond the species level for the genus *Bartonella*. Within the *B. vinsonii* species, each of the three recognized subspecies (*vinsonii*, *berkhoffii* and *arupensis*) exhibited specific spectra. Within the *B. henselae* species, representative strains of the Marseille and Houston genotypes initially identified using 16S rRNA gene sequencing (Drancourt *et al.*, 1996) were also differentiated with accuracy, with two peaks in the

5000–5500 Da range specific for the latter genotype (Fig. 2). Six Marseille and seven Houston strains previously identified using 16S rRNA gene PCR and sequencing were correctly discriminated in our blind test, thus demonstrating the accuracy of MS for classifying *B. henselae* strains. The distribution of these genotypes in cats and humans remains puzzling, with the simultaneous presence of two copies of the 16S rRNA gene in some strains (Viezens & Arvand, 2008) incurring even greater complexity. Therefore, reliable discrimination of *B. henselae* genotypes using MS might facilitate a better understanding of the population dynamics of this bacterium. In addition to *B. henselae*, MS was able to identify *B. clarridgeiae*, a species that is also associated with cats. MS thus appears suited for the rapid identification of *Bartonella* strains isolated from cats.

In a period when medical costs should be reduced as much as possible, the rapidity, reproducibility and low cost of MALDI-TOF MS demonstrate a major advantage over the usual identification methods. However, we acknowledge the fact that MALDI-TOF MS instruments such as the Autoflex II may not currently be affordable for most clinical microbiology laboratories. Simpler and cheaper MALDI-TOF MS instruments, such as the bench top Microflex (Bruker Daltonics), may soon be used routinely in laboratories without loss of data quality. Thus, not only is MALDI-TOF MS highly species specific for studying genetic variability, but the results obtained are unequivocal and transferable. Another major advantage of MS is that a laboratory technician without any spectrometric background can easily use this method. After depositing the sample and matrix as described in Methods, the spectrometer can be programmed such that the laser automatically impacts the entire surface of the matrix, with no human intervention, in order for the software to perform species identification. In addition, MS spectra are easily stored and compared. To date, however, only proprietary databases are available. To make it an identification tool of interest for the scientific community and allow researchers worldwide to share and compare their MS fingerprints, it might be useful to create a free and autonomous database with dedicated search tools (such as the GenBank database and the BLAST software for nucleotide and amino acid sequences) for MS spectra (Benson *et al.*, 2003; Tatusova & Madden, 1999).

Fig. 1. Virtual gel view of MS reference spectra from the 20 tested *Bartonella* species using the ClinPro Tools database (Bruker Daltonics). 1, *B. alsatica*; 2, *B. bacilliformis*; 3, *B. bovis*; 4, *B. birtlesii*; 5, *B. capreoli*; 6, *B. chomelii*; 7, *B. clarridgeiae*; 8, *B. doshiae*; 9, *B. elizabethae*; 10, *B. grahamii*; 11, *B. henselae* Houston; 12, *B. henselae* Marseille; 13, *B. koehlerae*; 14, *B. quintana*; 15, *B. schonbuchensis*; 16, *B. taylorii*; 17, *B. tribocorum*; 18, *B. vinsonii* subsp. *vinsonii*; 19, *B. vinsonii* subsp. *arupensis*; 20, *B. vinsonii* subsp. *berkhoffii*.

Fig. 2. Comparison of reference mass spectra from *B. henselae* strains Houston and Marseille.

In conclusion, we determined reference MS spectra for all cultivated and recognized *Bartonella* species. We also demonstrated that MALDI-TOF MS presents several advantages over conventional molecular identification for these bacteria, including rapidity, reliability, reproducibility and low cost. We developed a database comprising spectra for *Bartonella* strains which any scientist may use to compare a newly isolated strain. We acknowledge that this database may not enable identification of novel species, but will continue to supplement it with spectra obtained from newly described species.

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