First report of *Methylobacterium radiotolerans* bacteraemia identified by MALDI-TOF mass spectrometry

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Abstract

Methylobacterium radiotolerans is a fastidious, pink-pigmented, obligate aerobic Gram-negative bacillus commonly isolated from various environmental sources, and only occasionally from clinical samples, mostly in immunocompromised patients or associated with intravascular devices and haemodialysis. It grows poorly on commonly used culture media and its identification is time-consuming by conventional means. In this study, we present a case of *M. radiotolerans* bacteraemia in an individual affected by end-stage renal failure, identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The species identification was further confirmed by biochemical and molecular methods. The susceptibility to various antimicrobial agents is also presented and discussed.

Keywords: Bacteraemia, MALDI-TOF MS, *Methylobacterium radiotolerans*, Sepsityper

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Introduction

The genus *Methylobacterium* comprises fastidious, pink-pigmented, facultative methylotrophe, obligate aerobic Gram-negative bacilli, and includes over 50 published species [1].

Methylobacteria are found in a wide range of environmental sources, including methane-rich environments [2,3]. Nitrogen-fixers, they have a beneficial function in plant development, but also in toxic pollutant biodegradation [4,5]. Occasionally, they are found in household biofilms [4]. Methylobacteria grow poorly on ordinary culture media, producing small, dry, pink-pigmented colonies after 4 or 5 days of incubation at 25–30° C. The best growth occurs on Sabouraud agar, but they grow also on modified Thayer–Martin, buffered charcoal–yeast extract and Middlebrook 7H11 agars [6]. At Gram-staining they appear as pleiomorphic, vacuolated, straight to slightly curved rods, that may resist decolouration, so exhibiting a Gram-variable appearance.

Methylobacteria (mainly *Methylobacterium mesophilicum*) have occasionally been reported in nosocomial infections, mostly in immunocompromised patients, and in association with intravascular devices [7–10]. *Methylobacterium radiotolerans* [11] is much less known. At the time of writing, only three reports of *M. radiotolerans* bacteraemia have been described [12–14] (Table 1).

Traditionally, identification of methylobacteria relies on either biochemical [6] or molecular (16S rRNA gene sequencing) [3,15] methods, but recently matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has demonstrated high effectiveness in the identification of known and novel species of *Methylobacterium* [16]. There are no guidelines for antimicrobial susceptibility testing and no defined breakpoints [17,18], but a broad resistance to β-lactams has been reported [3,8,19].

In this study, we present a case of bacteraemia caused by *M. radiotolerans* identified by MALDI-TOF MS directly from the positive blood-culture bottles. The strain was further identified as *M. radiotolerans* from the plate subculture, and the species identification was confirmed by DNA sequencing. The strain was isolated from an individual affected by end-stage renal failure who chronically developed a mild fever while undergoing haemodialysis. The bacteraemia was confirmed by three consecutive blood-culture samplings in a 2-week time-frame. To our best knowledge, this is the first report of an initial identification of a *Methylobacterium* strain by MALDI-TOF MS from a clinical sample.

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TABLE 1. Overview of clinical and microbiological features of *Methylobacterium radiotolerans* infections described until now in the literature

Authors, year, reference	Number of cases	Age	Sex	Underlying disease	Site of isolation	Antibiotic treatment	Catheter removal	Outcome
De Cal, 2009 [12]	37	Avg. 59.3 (26-88)	F = 15 M = 22	end-stage renal failure	peripheral blood *n* = 26 CVC *n* = 36	ciprofloxacin	yes	improved
Lai, 2011 [13]	2	1	F	end-stage renal failure	CVC	ciprofloxacin cefepime	yes	improved
		79	F	leukaemia	CVC	ciprofloxacin gentamycin	yes	improved
Li, 2015 bj [14]	4	59	F	leukaemia	CVC	levofloxacin	yes	improved
		12	M	leukaemia	CVC	levofloxacin clindamycin cefepime	yes	improved
		87	F	leukaemia	CVC	levofloxacin linezolid	yes	improved
		23	F	leukaemia	CVC	ciprofloxacin piperacillin/tazobactam	yes	improved
Our study	1	58	F	end-stage renal failure	perypheral blood and CVC	not known	not known	improved

Abbreviations: CVC, central venous catheter; F, female; M, male.

Methods and materials

The blood samples included and investigated in this study were collected during the febrile peak exhibited by the patient after the haemodialysis procedure, on 14, 21 and 28 February 2018. Each sampling comprised two draws from different body sites (from peripheral vein and from venous catheter), each one split into one aerobic and one anaerobic Bactec™ blood-culture media bottles (Plus Aerobic/F and Plus Anaerobic/F; Becton Dickinson, County Clare, Ireland). The samples were processed using the Bactec™ FX blood-culture system (Becton Dickinson). Once flagged as positive, the bottles underwent subculturing, Gram-staining and direct identification by MALDI Sepsityper® (Bruker Daltonik, Bremen, Germany).

Subculturing was carried out on horse blood agar, Sabouraud-dextrose agar and CHROMagar Orientation (Meus, Piove di Sacco, VE, Italy) at 35°C in aerobic conditions (as only the aerobic bottles turned positive), until bacterial growth was observed (72 h).

Direct identification by Sepsityper was performed following the manufacturer's instructions. Briefly, bacterial cells were obtained by treatment of 1 mL of positive blood culture with a lysis buffer. After washing of the bacterial pellet with the respective buffer, the cells were extracted according to the manufacturer's standard protocol. One microlitre of the extract was spotted onto a MALDI target and overlaid with matrix solution. After drying, species identification was performed using the MALDI Biotyper Compass 4.1 software (Bruker Daltonik).

Species identification and Gram staining were repeated from the plate culture.

Susceptibility testing was performed determining MIC values by the gradient diffusion method (MIC Evaluator Strips; Oxoid, Basingstoke, UK), testing all the routine antimicrobial agents potentially active against Gram-negative bacteria (Table 2). Susceptibility to nitrofurantoin, tobramycin, minocycline and piperacillin/tazobactam was carried out using disc diffusion methodology (these molecules are not available in the format required for the gradient diffusion method in our laboratory). The EUCAST procedure and the interpretative criteria used for standard susceptibility testing of *Pseudomonas aeruginosa* were adopted (bacterial suspension prepared at a turbidity of 0.5 McF, Mueller–Hinton agar medium (Meus, Piove di Sacco, Italy), and incubation at 37°C in aerobic conditions) [17]. In parallel, the antimicrobial susceptibility test was performed also applying an incubation temperature of 32°C, which is the optimal growth temperature for methylobacteria. To achieve

TABLE 2. Results of antimicrobial susceptibility test

Agent	MIC (test strips) or inhibition zone diameter (disc-diffusion)
Amikacin	0.25 mg/L
Gentamycin	0.064 mg/L
Tobramycin (10 mg/L)	41 mm
Nitrofurantoin (100 mg/L)	no inhibition zone
Levofloxacin	0.125 mg/L
Ciprofloxacin	1 mg/L
Erythromycin	4 mg/L
Azithromycin	64 mg/L
Tetracycline	0.5 mg/L
Minocycline (30 mg/L)	55 mm
Clindamycin	64 mg/L
Fosfomycin	>256 mg/L
Trimethoprim/sulphamethoxazole	>32 mg/L
Rifampicin	0.064 mg/L
Ceftriaxone	2 mg/L
Cefotaxime	8 mg/L
Cefepime	2 mg/L
Amoxicillin/clavulanate	1 mg/L
Piperacillin/tazobactam (30/6 mg/L)	29 mm
Meropenem	4 mg/L
Imipenem	0.25 mg/L
Aztreonam	>64 mg/L
Colistin	64 mg/L

sufficient bacterial growth to read the MICs, incubation had to be prolonged to 72 h.(See Table 3)

Biochemical characterization was performed using API 20NE and API 20E systems (bioMérieux, Marcy L'Etoile, France) according to the manufacturer's instructions, in order to get a more comprehensive characterization of the isolate.

For molecular confirmation of the species identification, the sequence of the 5′ part of the 16S rRNA gene was determined by standard Sanger sequencing. The obtained DNA sequence was compared with the uncurated NCBI and curated EzTaxon databases.

Organism identification at species level was further confirmed by sequencing the 5′ part of the 16S rRNA gene, using the bacterial universal forward primer SSU-bact-27f (5′-AGAGTTTGATCCTGGCTCAG-3′) [20]. The sequence obtained (471 bp) was compared with the published sequences in the GenBank uncurated database using the Basic Local Alignment Search tool (BLAST) algorithm [21] and with the curated published sequences in the EzTaxon databases [22].

Results

All three consecutive blood-culture samples collected between 14 and 28 February 2018 gave positive results in a 2-week time frame. Only the aerobic bottles of the blood-sample sets turned positive, after an average incubation time of 32 h (28–36 h).

Gram-staining revealed large, partially discoloured, elongated rods. Preliminary MALDI-TOF identification after Sepsityper preparation delivered *M. radiotolerans* as the result, with a high confidence level (ID log score >1.8).

Plates showed no growth at 24 and 48 h. At 72 h, small, salmon-pigmented colonies were observed only on CHROMagar Orientation and Sabouraud agar. These colonies exhibited the same Gram-staining pattern observed in the direct smear from the positive bottle, and were identified by MALDI Biotyper as *M. radiotolerans* (ID log score >2.0).

Results of biochemical characterization are reported on Table 1, and were coherent with the expected features of the species. Both the API systems were readable after 72 h of incubation.

The 16SrDNA sequence analysis identified the strains as *M. radiotolerans* with 100% sequence homology over 471 bp to the type strain JCM 2831 (GenBank accession numbers CO001001, NR074244.1).

The susceptibility test was readable only after 72 h, at 32°C but not at 37°C. MIC results are reported in Table 2. Given the lack of breakpoints, the results were interpreted accordingly with the criteria used for *P. aeruginosa* (for the molecules for which breakpoints exist), and with an extrapolation of the interpretative category from the mere magnitude of the MIC values for the others.

The patient had a favourable outcome, and none of the blood-culture samples subsequent to the reports included in this study were positive for *M. radiotolerans*.

TABLE 3. Results of biochemical characterization

Reaction	Result	Expected result
Glucose (GLU)	—	—
Mannitol (MAN)	—	—
Mannose (MNE)	—	—
Sorbitol (SOR)	—	—
Rhamnose (RHA)	—	—
Saccharose (SAC)	—	—
Melibiose (MEL)	—	—
Amygdaline (AMY)	—	—
Arabinose (ARA)	+	+
Inositol (INO)	—	—
N-acetyl-glucosamine (NAG)	—	—
Maltose (MAL)	—	—
Gluconate (GNT)	+	+
Ornithine (ODC)	—	—
Arginine (ADH)	—	—
Lysine (LDC)	—	—
Tryptophan (deaminase) (TDA)	—	—
Tryptophan (indol production) (IND)	—	—
Urease (URE)	+	+
Ortho-nitro-phenyl-galactosidase (ONPG)	—	—
Para-nitro-phenyl-β-D-galactopyranoside (PNPG)	—	—
H_2S	—	—
Esculin (ESC)	—	—
Gelatine (GEL)	—	—
Citrate (CIT)	—	—
Caprate (CAP)	—	—
Adipate (ADI)	+	—
Malate (MLT)	+	+
Phenylacetate (PAC)	—	—
Acetoin production (Voges–Proskauer; VP)	—	—
Nitrate (NO_3)	—	—
Nitrite (NO_2)	—	—
Oxidase (OX)	+	+
Catalase (CAT)	+	+

Discussion

Methylobacterium radiotolerans is an environmental species [2,3], only very occasionally isolated from clinical samples [7–10]. Due to its poor or even absent growth on all conventional media at 35 ± 2°C, cultivation requires peculiar adjustments deviating from standard routine workflow. Traditionally, laboratory methods for species identification and antibiotic susceptibility testing are challenging, laborious and slow [6].

In this study, we present the first case of an initial identification at species level of *M. radiotolerans* by MALDI-TOF MS (an identification by this technology was attempted in the paper by Li et al. [14], in which two out of the four strains investigated, initially identified by molecular methods, were subsequently identified by the Vitek MS instrument from the colony as 'unvalidated' results, while two were not identified). The species identification was first obtained directly from the positive blood-culture bottle, after a quick extraction of the bacterial

cells from the blood culture by a commercially available system, and later from the plate subcultures. Antimicrobial susceptibility testing was performed following the criteria defined for *Pseudomonas* adjusted to the growth requirements of methylobacteria, as no specific guidelines are currently available. The *M. radiotolerans* strain was susceptible to gentamycin (in discordance with the previous Italian outbreak [12]), and it showed a broad reduced susceptibility to β-lactam agents (cephalosporins, meropenem, aztreonam), and resistance to trimethoprim/sulphamethoxazole, both consistent with the existing literature [3,8,12,19].

MALDI-TOF MS represents a reliable and rapid routine identification method for methylobacteria at species level [16], that can be applied directly on the positive blood-culture flasks. In this study, the identification at species level of *M. radiotolerans* from the plate subculture by MALDI-TOF MS enabled at least 3 full days to be saved for the time to report, in comparison with biochemical methods. The prompt identification enabled by Sepsityper directly from the positive blood-culture bottles shortened the time to report a further 3 days. The adjustment of the growth conditions to set up the susceptibility testing, enabled by the prompt species identification, was crucial to achieve the determination of MIC values, as no growth was observed on Mueller–Hinton agar at 37°C. The strains showed reduced susceptibility to many of the agents widely used in empirical therapy, so the early MALDI-TOF identification of *M. radiotolerans* enabled the prompt optimization of treatment.

The possibility of achieving reliable species identification directly from the positive flask is especially relevant in cases such as the one reported in this study, when growth on solid medium is unusually slow, or even fails completely, because a correlation between a faster reporting time and a better clinical outcome for the patient has been widely reported [23,24]. Opota et al. found that a pathogen rarely found in severe infections like bloodstream infections, *Aerococcus urinae*, could be more frequently detected after introduction of MALDI-TOF MS into clinical routine diagnostics [25]. It is unclear if broad-range PCR, as found in the case of *Aerococcus urinae*, would further increase the detection rate of *M. radiotolerans* infections. But the ability to detect the rare pathogen directly from blood cultures as demonstrated in our laboratory seems to be a suitable, more rapid and cost-effective alternative for micro-organisms that are difficult to culture on solid media.

Conflict of interest

Dr. Kostrzewa is an employee of Bruker Daltonik, the manufacturer of the MALDI-TOF system used in this study. All other authors declare no conflicts of interest.

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