

Characterization of clinically isolated *Ralstonia mannitolilytica* strains using random amplification of polymorphic DNA (RAPD) typing and antimicrobial sensitivity, and comparison of the classification efficacy of phenotypic and genotypic assays

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Ralstonia mannitolilytica strains isolated between February 2002 and March 2004 from 30 episodes of infection in 26 patients at Vienna University Hospital were characterized. Twenty-four of the episodes occurred within a 7 month period, suggesting they were outbreak-related, although no common source of infection was identified. The isolates were assayed using PCR to confirm species identification. Random amplification of polymorphic DNA (RAPD) typing classified the *R. mannitolilytica* isolates into four distinct genotypes: A/I, B/II, C/III and D/IV (15, 13, 1 and 1 isolates, respectively). API 20NE, VITEK Gram-negative Identification Card plus (GNI+) and VITEK Gram Negative Bacillus Identification (GNB) yielded negative or no acceptable biochemical profile for 4, 11 and 11 isolates, respectively. None of the isolates acidified D-arabitol or mannitol. Two isolates (7%) were positive for nitrate reduction. All 30 *R. mannitolilytica* isolates were resistant to desferrioxamine, and 29 were able to grow on BCSA. The most active compounds *in vitro* were ciprofloxacin and cefepime, whilst only the genotype D/IV isolate was sensitive to gentamicin and amikacin (the remaining 29 isolates being resistant to both).

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INTRODUCTION

The genus *Ralstonia*, established in 1995, includes 13 species of Gram-negative, non-fermentative bacilli (Yabuuchi *et al.*, 1995). *Ralstonia* species grow well in moist environments with minimal nutrient resources. The species most frequently encountered in human medicine is *Ralstonia pickettii* (formerly *Pseudomonas pickettii* and *Burkholderia pickettii*), which is most commonly isolated from the respiratory tract. Outbreaks of respiratory colonization have been described in association with contaminated solutions for patient care, but the presence of the pathogen in the respiratory tract is usually of no clinical significance (Labarca *et al.*, 1999; Yoneyama *et al.*, 2000). Pseudo-outbreaks, due to contaminated laboratory solutions, have been described (Boutros *et al.*, 2002). In addition, *Ralstonia paucula* and *Ralstonia gilardii* may be associated with human disease (Moissenet *et al.*, 2001; Vandamme *et al.*, 1999; Wauters *et al.*, 2001). The recently established species *Ralstonia mannitolilytica* (previously

named *Pseudomonas thomasi* and *R. pickettii* biovar 3/ 'thomasi') has been isolated from the respiratory tract of patients with cystic fibrosis, and was identified as the causative agent of recurrent meningitis and infection of a haematoperitoneum (Coenye *et al.*, 2002a; De Baere *et al.*, 2001; Vanechoutte *et al.*, 2001). We observed an increase in cases of *R. mannitolilytica* infection in our hospital in 2002.

Biochemical identification of *Ralstonia* species with commercially available tests is problematic. *R. pickettii* is easily confounded with *Burkholderia cepacia* and *Pseudomonas fluorescens* (Henry *et al.*, 2001; Kiska *et al.*, 1996). *R. mannitolilytica* shows similar biochemical properties to *R. pickettii*, but nitrate reduction (negative in *R. mannitolilytica*) and acidification of D-arabitol and mannitol (both negative in *R. pickettii*) have been reported to enable differentiation (De Baere *et al.*, 2001). In addition, it has been described that *R. mannitolilytica* strains are resistant to desferrioxamine, whereas *R. pickettii* strains are susceptible to this compound (Laffineur *et al.*, 2002). *R. pickettii* has been shown to grow on *Burkholderia cepacia* selective agar (BCSA) prepared according to Henry *et al.* (1997, 1999), but this has not been reported for other *Ralstonia* species. To date

Abbreviations: OF, oxidation-fermentation; PFGE, pulsed-field gel electrophoresis; RAPD, random amplification of polymorphic DNA.

commercially available biochemical kits have rarely been updated in order to differentiate between *R. mannitolilytica* and *R. pickettii*. Recently, a PCR assay for identification of *R. mannitolilytica* targeting the 16S rRNA gene has been described (Coenye *et al.*, 2002b). This assay was shown to provide a sensitivity of 100 % and a specificity of 99 % (based on the examination of 34 *R. mannitolilytica* isolates and 118 control isolates). Random amplification of polymorphic DNA (RAPD) typing has previously been performed for *R. mannitolilytica* isolates using a primer set described for typing *B. cepacia* (Coenye *et al.*, 2002b; Chen *et al.*, 2001). Additional studies have applied this technique for typing *R. pickettii* (Boutros *et al.*, 2002; Maroye *et al.*, 2000). In the current work the *R. pickettii* RAPD primers P3 and P15 were employed for RAPD genotyping of 30 *R. mannitolilytica* clinical isolates.

The aims of this study were to characterize the strains using RAPD typing, to investigate the diagnostic accuracy of genotypic and phenotypic tests for species identification, and to determine the *in vitro* antimicrobial susceptibility pattern.

METHODS

Patients and isolates. *R. mannitolilytica* isolates from 26 patients of Vienna University Hospital (a 2200 bed University teaching hospital), isolated between February 2002 and March 2004, were included in this study [female, $n = 18$; male, $n = 8$; median age, 60 years (range, 42 to 85 years)]. Twenty-five patients were diagnosed with bacteraemia; if subsequent isolations of the same species were encountered within a 4 week period this was considered the same episode of bacteraemia, unless bacteraemia was diagnosed after readmission. Of the 26 patients studied, four experienced two episodes of bloodstream infection. The study isolates from patients with bloodstream infection were derived from peripheral blood ($n = 17$), central blood ($n = 3$), port-a-cath blood ($n = 7$) and intravascular catheter tips ($n = 2$). One patient was diagnosed with urinary tract infection. In this patient semi-quantitative urine culture yielded a count of 10^7 c.f.u. ml⁻¹.

The 30 episodes of *R. mannitolilytica* infection at our institution between February 2002 and March 2004 showed an unequal distribution over time. Twenty-two episodes were registered during the 7 month period from February 2002 to August 2002, whereas only eight episodes were counted from September 2002 to March 2004 (incidence of isolation of *R. mannitolilytica*: February 2002 to August 2002, 0.78 per 1000 admissions; September 2002 to March 2004, 0.15 per 1000 admissions; $p < 0.001$). Isolates from patients with cystic fibrosis were not included in the analysis.

Blood cultures were processed by the VITAL system (BioMérieux). Quantitative culture of urine samples was performed by Uricult plus (Orion Diagnostica). In the course of routine diagnosis, biochemical identification was performed by either API 20NE (BioMérieux) or one of the VITEK systems [Gram-negative Identification Card GNI plus (GNI+) or Gram-negative Bacillus Identification (GNB); BioMérieux].

From February 2002 to March 2004, all isolates of Gram-negative non-fermentative bacilli which could not be identified unambiguously or which were preliminarily identified as *Ralstonia* species were subjected to PCR for identification of *R. mannitolilytica* (Coenye *et al.*, 2002b). In addition, 16S rRNA gene sequencing for species identification was applied to seven of the *R. mannitolilytica* study isolates (MicroSeq 500 16S rDNA Bacterial Sequencing Kit; Applied Biosystems). Both the

BLAST database (National Centre for Biotechnology Information, USA) and the MicroSeq database provided by Applied Biosystems were used to identify similarities between these sequences and known sequences.

PCR for identification of *R. mannitolilytica*. PCR was regarded as the gold standard for identification of the pathogen at species level. One isolate from each episode ($n = 30$) was included in the present study. DNA from pure culture was extracted by the Qiaamp DNeasy kit (Qiagen) according to the manufacturer's recommendations. PCR was performed as described previously using the primers Rm-F1 (5'-GGG AAA GCT TGC TTT CCT GCC-3') and Rm-R1 (5'-TCC GGG TAT TAA CCA GAG CCA T-3') (Coenye *et al.*, 2002b). The PCR mixture included a Ready-To-Go (RTG) PCR bead (Amersham Biosciences), 2 µl (50 pmol) of each primer (VBC genomics), 3 µl of DNA solution and sterile distilled water to a final volume of 25 µl. The amplification conditions included an initial denaturation (94 °C for 2 min), 30 cycles (94 °C for 1 min, 57 °C for 1 min, 72 °C for 90 s) and a final extension (72 °C for 10 min). The PCR products were analysed by electrophoresis in a 2 % agarose gel (Agarose MP, Roche Diagnostics) for 1.5 h (5 V cm⁻¹) and ethidium bromide staining. In-house sequenced strains of *R. pickettii* and *Pseudomonas huttiensis* (MicroSeq 500 16S rDNA Bacterial Sequencing Kit) were used as negative controls.

Comparison of commercially available identification systems.

Three different commercially available identification systems were applied to the 30 isolates: API 20NE, VITEK GNI+ and VITEK GNB. The assays were performed according to the manufacturer's recommendations.

Key reactions for differentiation of *R. mannitolilytica* from *R. pickettii*.

Three biochemical reactions that have been shown to discriminate between *R. mannitolilytica* (negative for nitrate reduction) and *R. pickettii* (negative for acidification of D-arabitol and mannitol) were investigated (De Baere *et al.*, 2001). Acidification of D-arabitol and mannitol were determined by oxidation-fermentation (OF)-tests (basic medium: Merck). Nitrate reduction was investigated by cultivation of the isolates in nitrate reduction broth (Fluka) and addition of NIT 1 (sulfanilic acid, acetic acid) and NIT 2 (N,N-dimethyl-1-naphthylamine) after 48 h of incubation at 35 °C. In addition, a commercially available test for nitrate reduction (Nitrati Test, Liofilchem) was applied. Susceptibility to desferrioxamine was determined using 6 mm (diameter) paper discs loaded with 250 µg of desferrioxamine. An in-house sequenced strain of *R. pickettii*, and *Staphylococcus epidermidis* DSM 20044 (organisms which have been reported to be susceptible to desferrioxamine), as well as *Klebsiella oxytoca* ATCC 700324 (*Klebsiella* species are not inhibited by desferrioxamine) were used as control organisms (Laffineur *et al.*, 2002; Lindsay & Riley, 1991).

In vitro susceptibility testing.

In vitro susceptibility testing was performed by Kirby-Bauer disc diffusion test on Mueller-Hinton agar according to NCCLS guidelines. The following compounds were tested (required diameters for resistance, intermediate susceptibility, and susceptibility are given in parentheses): piperacillin-tazobactam (≤ 17 , 18–20, ≥ 21), ceftazidime (≤ 14 , 15–17, ≥ 18), cefepime (≤ 14 , 15–17, ≥ 18), imipenem (≤ 13 , 14–15, ≥ 16), gentamicin (≤ 12 , 13–14, ≥ 15), amikacin (≤ 14 , 15–16, ≥ 17) and ciprofloxacin (≤ 15 , 16–20, ≥ 21).

Growth on *Burkholderia cepacia* selective agar (BCSA).

BCSA plates were prepared as described by Henry *et al.* (1997). Strains from overnight cultures on MacConkey agar were subcultured on BCSA. The presence or absence of bacterial growth, and the colours of colonies and haloes, were documented after 24 and 48 h of incubation at 35 °C. In addition to *R. mannitolilytica*, isolates of *Stenotrophomonas maltophilia* ($n = 91$), *R. pickettii* ($n = 26$) and *Alcaligenes (Achromobacter) xylosoxidans* ($n = 19$) were investigated; species identification of the *R. pickettii*

isolates was performed by PCR as described by Coenye *et al.* (2002b). Four *B. cepacia* isolates were used as positive controls.

Random amplification of polymorphic DNA (RAPD). A previously described RAPD primer set (P3: 5'–AGA CGT CCA C–3', and P15: 5'–AAT GGC GCA G–3') was applied (Maroye *et al.*, 2000). DNA from pure culture was extracted by the Qiaamp DNeasy kit (Qiagen) according to the manufacturer's recommendations. The PCR mixture included a Ready-To-Go (RTG) RAPD Analysis bead (Amersham Biosciences), 2 µl (50 pmol) of one of the primers (VBC Genomics), 3 µl of DNA solution and sterile distilled water to a final volume of 25 µl. The amplification conditions included an initial denaturation (94 °C for 5 min), 40 cycles (94 °C for 30 s, 36 °C for 30 s, 72 °C for 90 s). PCR was performed in a TGradient thermocycler (Biometra). RAPD products were analysed by electrophoresis in 2% NuSieve 3:1 agarose gel (Cambrex) for 2.5 h (2.5 V cm⁻¹) and ethidium bromide staining. The patterns were interpreted visually by two researchers; single-band differences were ignored (Renders *et al.*, 1996).

RESULTS

RAPD using the primer P3 revealed four clusters of isolates with different genotypes. RAPD using primer P15 yielded corresponding results (Fig. 1). According to RAPD using P3/P15, most isolates belonged to two major clusters, A/I and B/II, which consisted of 15 and 13 isolates, respectively, while two strains had distinct genotypes and individually formed C/III and D/IV. The genotype of each of the 30 isolates is indicated in Table 1.

Comparison of commercially available identification systems and biochemical reactions

Four, 11 and 11 isolates were negative or yielded no acceptable biochemical profile using API 20NE, VITEK GNI+ and VITEK GNB, respectively. Fourteen, 16 and 15 isolates, respectively, were identified as *R. pickettii* using the same systems. The most frequent misidentification of *R. mannitolilytica* by the VITEK programs was as *B. cepacia* (two isolates each), while API 20NE classified 11 isolates (37%) as *Pseudomonas fluorescens*. The detailed results of these tests for the 30 isolates are shown in Table 1.

None of the isolates acidified D-arabitol or mannitol. Two isolates (7%) were positive for nitrate reduction (Table 1). All of the *R. mannitolilytica* isolates and *K. oxytoca* ATCC 700324 were resistant to desferrioxamine (no inhibition zone). The in-house sequenced *R. pickettii* strain and *S. epidermidis* DSM 20044 showed inhibition diameters of 25 and 28 mm, respectively.

Growth on *Burkholderia cepacia* selective agar (BCSA)

All of the *R. mannitolilytica* isolates except one (genotype D/IV) grew on BCSA agar (97%), showing transparent colonies with red haloes after 24 h, and blue colonies with red haloes after 48 h of incubation at 35 °C. All the *B. cepacia* and *R. pickettii* isolates grew on BCSA, but only one of the 91 *S. maltophilia* isolates (1%) and none of the *A. xylooxidans* isolates did.

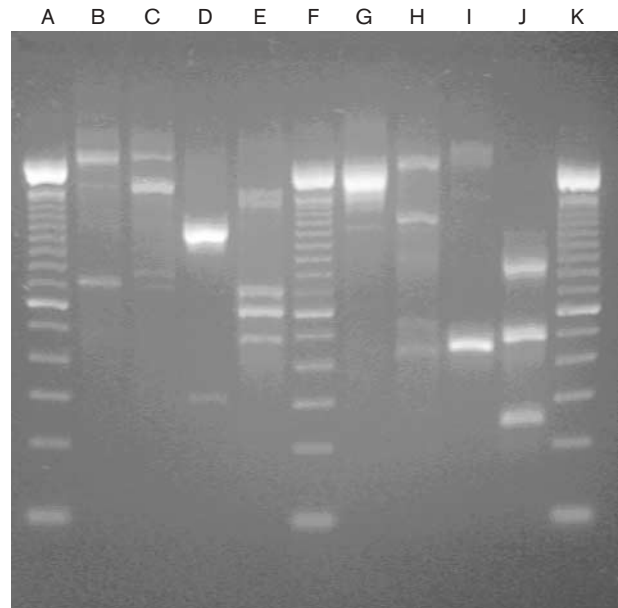


Fig. 1. RAPD profiles of clinical *R. mannitolilytica* isolates obtained using primers P3 and P15, respectively. Lanes: A, 100 bp ladder (Invitrogen); B to E, P3 genotypes (A, B, C and D, respectively); F, 100 bp ladder; G to J, P15 genotypes (I, II, III and IV); K, 100 bp ladder. The genotypes A, B, C and D (P3) corresponded to the genotypes I, II, III and IV (P15), respectively. The isolates chosen as representative strains are indicated in Table 1.

In vitro susceptibility testing

The genotype D/IV isolate was sensitive to all seven antimicrobial agents tested. All the *R. mannitolilytica* isolates of genotypes A/I, B/II and C/III were resistant to gentamicin and amikacin, and the genotype C/III isolate was also resistant to ceftazidime. Six genotype A/I and three genotype B/II isolates were sensitive to ceftazidime (as was the genotype D isolate), whilst the remaining 19 isolates (9 A/I and 10 B/II) displayed intermediate susceptibility to ceftazidime. With the exception of one B/II isolate, all 30 *R. mannitolilytica* isolates were sensitive to ciprofloxacin and cefepime. Ten isolates (9 A/I and 1 D/IV) were sensitive, 8 (4 A/I, 3 B/II and 1 C/III) showed intermediate susceptibility and 12 (2 A/I and 10 B/II) were resistant to imipenem. Susceptibility to piperacillin-tazobactam was more varied, with 7 genotype A/I isolates sensitive, 5 intermediate and 3 resistant; 2 genotype B/II isolates sensitive, 5 intermediate and 6 resistant; the C/III isolate intermediate; and the D/IV isolate sensitive (Table 2).

DISCUSSION

RAPD detected only four different genotypes among the 30 *R. mannitolilytica* isolates. However, RAPD was not resolved by pulsed-field gel electrophoresis (PFGE), which is still the gold standard in molecular typing. Therefore, the sensitivity

Table 1. Characterization of 30 clinical *R. mannitolilytica* isolates using phenotypic and genotypic assays

According to the manufacturer, the quality of identification by VITEK GNB is graded into excellent (E), very good (VG), good (G), acceptable (A) and low (L). API 20NE results with < 75 % probability were indicated as 'low selectivity'. NA, Not applicable; VNFGNB, various non-fermenting Gram-negative bacilli.

Date of isolation (dd/mm/yyyy)	Patient	VITEK (GNI+)		VITEK (GNB)		API 20NE			RAPD-type		Nitrate reduction
		Species	Identification (%)	Species	Identification	Species	API 20NE code	ID (%)	P3	P15	
26/02/2002	A	<i>R. pickettii</i>	98	Negative	NA	<i>P. fluorescens</i>	55555	98.2	A	I	No
09/04/2002	B	<i>B. cepacia</i>	94	Negative	NA	Low selectivity	44555	NA	A	I	No
19/03/2002	C	Negative	NA	<i>R. pickettii</i>	E	<i>R. pickettii</i>	45455	84.1	A	I	No
27/03/2002	D	<i>R. pickettii</i>	94	<i>R. pickettii</i>	VG	<i>R. pickettii</i>	45455	84.1	A	I	No
14/04/2002	E	Negative	NA	<i>R. pickettii</i>	G	<i>P. fluorescens</i>	55555	98.2	A	I	No
03/05/2002	F	<i>R. pickettii</i>	99	Negative	NA	<i>P. fluorescens</i>	55555	98.2	A	I	No
24/05/2002	G	Negative	NA	<i>R. pickettii</i>	E	<i>R. pickettii</i>	45455	84.1	A	I	No
17/06/2002*	G	Negative	NA	Negative	NA	<i>R. pickettii</i>	45455	84.1	A	I	No
06/06/2002	H	Negative	NA	<i>Y. enterocolitica</i>	A	<i>R. pickettii</i>	45455	84.1	A	I	No
06/06/2002	I	Negative	NA	Negative	NA	<i>R. pickettii</i>	45455	84.1	B	II	No
03/07/2002	J	Negative	NA	<i>R. pickettii</i>	A	<i>R. pickettii</i>	45455	84.1	A	I	No
09/12/2002	K	<i>B. cepacia</i>	96	<i>R. pickettii</i>	L	<i>R. pickettii</i>	45455	84.1	A	I	No
29/03/2002	L	<i>R. pickettii</i>	99	<i>B. cepacia</i>	L	<i>P. fluorescens</i>	55555	98.2	B	II	No
30/08/2002*	L	<i>R. pickettii</i>	99	<i>R. pickettii</i>	VG	Low selectivity	57775	NA	B	II	No
03/04/2002	M	<i>R. pickettii</i>	99	<i>R. pickettii</i>	G	<i>P. fluorescens</i>	55555	98.2	B	II	No
29/05/2002	M	<i>R. pickettii</i>	99	<i>R. pickettii</i>	G	<i>P. fluorescens</i>	55555	98.2	B	II	No
04/04/2002	N	<i>R. pickettii</i>	99	<i>R. pickettii</i>	E	<i>P. fluorescens</i>	55555	98.2	B	II	No
13/04/2002	O	<i>R. pickettii</i>	99	<i>B. cepacia</i>	L	<i>P. fluorescens</i>	55555	98.2	B	II	Yes
27/04/2002	P	<i>R. pickettii</i>	99	Negative	NA	<i>P. fluorescens</i>	55555	98.2	B	II	No
05/06/2002	Q	<i>R. pickettii</i>	98	<i>R. pickettii</i>	G	<i>P. fluorescens</i>	55555	98.2	B	II	Yes
14/08/2002	R	<i>R. pickettii</i>	98	<i>R. pickettii</i>	E	<i>R. pickettii</i>	45455	84.1	B	II	No
09/03/2002	S	<i>R. pickettii</i>	99	<i>R. pickettii</i>	G	<i>R. pickettii</i>	1041475	99.7	B	II	No
21/05/2002*	S	<i>R. pickettii</i>	98	<i>R. pickettii</i>	L	<i>P. fluorescens</i>	55555	98.2	C	III	No
28/11/2002	T	<i>R. pickettii</i>	98	Negative	NA	<i>R. pickettii</i>	45455	84.1	B	II	No
10/12/2002	U	<i>R. pickettii</i>	99	Negative	NA	Low selectivity	55575	NA	B	II	No
31/01/2003	V	Negative	NA	<i>R. pickettii</i>	G	Low selectivity	43135	NA	A	I	No
30/04/2003	W	Negative	NA	Negative	NA	<i>R. pickettii</i>	45455	84.1	A	I	No
09/01/2004*	X	<i>Pseudomonas</i> sp.	87	VNFGNB	L	<i>P. putida</i>	142455	98.2	D	IV	No
12/01/2004	Y	Negative	NA	Negative	NA	<i>R. pickettii</i>	45455	84.1	A	I	No
13/02/2004	Z	Negative	NA	Negative	NA	<i>R. pickettii</i>	45455	84.1	A	I	No

*Isolates which are shown as representative strains in Fig. 1.

Table 2. *In vitro* antimicrobial susceptibility of 30 clinical *R. mannitolilytica* isolates determined by Kirby–Bauer disc diffusion test, using NCCLS criteria

Antimicrobial agent	Susceptibility*		
	Sensitive	Intermediate	Resistant
Piperacillin-tazobactam	10 (7, 2, 0, 1)	11 (5, 5, 1, 0)	9 (3, 6, 0, 0)
Ceftazidime	10 (6, 3, 0, 1)	19 (9, 10, 0, 0)	1 (0, 0, 1, 0)
Cefepime	29 (15, 12, 1, 1)	0	1 (0, 1, 0, 0)
Imipenem	10 (9, 0, 0, 1)	8 (4, 3, 1, 0)	12 (2, 10, 0, 0)
Ciprofloxacin	29 (15, 12, 1, 1)	0	1 (0, 1, 0, 0)
Gentamicin	1 (0, 0, 0, 1)	0	29 (15, 13, 1, 0)
Amikacin	1 (0, 0, 0, 1)	0	29 (15, 13, 1, 0)

*Data are displayed as number of isolates, with numbers from each genotype (A/I, B/II, C/III and D/IV, respectively) presented in parentheses.

of RAPD for typing *Ralstonia* species may be questioned. Previously published findings indicate that RAPD using the primer set P3/P15 is equal to PFGE for small-scale epidemiological investigations, although it should be noted that the isolates in this previous study by Boutros *et al.* (2002) were closely related, which hindered the comparison of one method against the other. In general, RAPD is an adequate typing method for Gram-negative non-fermentative rods (Mahenthalingam *et al.*, 1996). A large-scale study on the comparative accuracy of RAPD and PFGE for typing the emerging pathogens *Ralstonia* species is warranted.

Recently described clinical manifestations of *R. mannitolilytica* include respiratory colonization in cystic fibrosis patients, recurrent meningitis and infection of a haemoperitoneum (Coenye *et al.*, 2002a; De Baere *et al.*, 2001; Vanechoutte *et al.*, 2001). Before the establishment of the species *R. mannitolilytica* in 2001, bacteraemia and bacteriuria due to *P. thomasi* had been reported (Phillips *et al.*, 1972). All patients with *R. mannitolilytica* bacteraemia in the present study had fever when the positive blood culture(s) were drawn. Two patients (7% of episodes) died. One patient in the present study, who had undergone recent renal transplantation, was diagnosed with a urinary tract infection due to *R. mannitolilytica*.

Most previously described cases of *Ralstonia* species infections were outbreak-related. In 1972, a *P. thomasi* outbreak involving 25 patients (bacteraemia and bacteriuria) was traced to parenteral fluids prepared with contaminated deionized water (Phillips *et al.*, 1972). Pan *et al.* (1992) reported that from 24 patients, 39 isolates were recovered which were preliminarily identified as *R. pickettii*, of which 23 actually belonged to *P. thomasi*. This outbreak was traced to contaminated saline solution prepared by the hospital pharmacy. In the present situation, 24 episodes of infection with *R. mannitolilytica* within 7 months represented an unusually high incidence, which was confirmed by continued surveillance of the pathogen until March 2004. Although the patients were treated at different wards located all over the hospital area, a common source of infection (related to

therapeutic or diagnostic measures performed in the hospital) was considered. As *Ralstonia* species are known to cause outbreaks related to contaminated solutions for patient care, the first step of outbreak investigation was a review of the intravenous-therapy regimens of the patients. However, no single solution was identified to have been administered to all infected patients, so intravenous-therapy was excluded as the common source of infection. Comprehensive environmental screening, including approximately 250 samples (e.g. distilled water, saline solution, Ringer lactate, heparin solution, solvents, soaps and surfaces), was performed, but no *Ralstonia* species were isolated. Due to the lack of a plausible source of infection, no infection control measures were implemented. The prolonged outbreak stopped in autumn 2002.

There appear to be two possible explanations for this epidemiological constellation, taking into account the results of molecular typing: (i) an unidentified common source containing several strains of *R. mannitolilytica*, and (ii) two distinctive epidemiological events (each with an as-yet-unidentified common source) related to the common genotypes (A/I and B/II), with genotype D/IV reflecting an isolated incident. Genotype C/III may similarly reflect an isolated incident or may represent bacterial succession (genotypic adaptation) of the initial infectious agent. An environmental source (distilled water) harbouring five distinct strains of *R. pickettii* has previously been described (Maroye *et al.*, 2000). A similar constellation may be suspected in the present case, at least with regard to genotypes A/I and B/II. For three of the four patients with recurrent bacteraemia, the same strain was isolated during both episodes of bacteraemia. In these cases colonization with the causative strain is biologically plausible.

A true epidemic is most plausible in the present situation, due to: (1) the patients were treated in 15 different wards, (2) the *R. mannitolilytica* isolates were recovered and processed on different days, and (3) in 12 of the 30 episodes of bacteraemia more than one blood culture was positive for *R. mannitolilytica*. In addition, four patients experienced two

episodes of bacteraemia. Given that an average of 16 000 sets of blood culture bottles are processed per year in our hospital, it is very unlikely that a second blood culture set from the same patient became contaminated with *R. mannitolilytica* randomly.

Correct identification of *Ralstonia* species is important because misidentification can highly compromise infection control measures. In addition, misidentification of *Ralstonia* species as *B. cepacia* can have a tremendous impact on the life of cystic fibrosis patients (Saiman & Siegel, 2004). As previously reported for *R. pickettii*, *R. mannitolilytica* was most frequently confounded with *P. fluorescens* and *B. cepacia* in the present study (Henry *et al.*, 2001; Kiska *et al.*, 1996). It has to be noted that the three commercially available identification systems tested in this study were not updated to identify *R. mannitolilytica*. However, given that the biochemical profile of *R. mannitolilytica* is most similar to that of *R. pickettii*, VITEK GNI+ showed the best performance, followed by VITEK GNB. The PCR assay for identification of *R. mannitolilytica* described by Coenye *et al.* (2002b) is a valuable tool for the identification of this species.

With regard to the biochemical differentiation of *R. mannitolilytica* from *R. pickettii*, the present data indicate that nitrate reduction is variable in *R. mannitolilytica*, although most isolates appear to be negative for this reaction. Mannitol has been described to be both assimilated and acidified by *R. mannitolilytica* (Vanechoutte *et al.*, 2001). None of the *R. mannitolilytica* isolates in this study acidified mannitol, as determined by OF test. In contrast, the reaction 'mannitol' contained in API 20NE was positive for all isolates. The OF test determines acid production from mannitol, whilst API 20NE investigates assimilation (i.e. growth by utilization) of the respective sugar.

In addition to *Burkholderia* species, growth on *Burkholderia cepacia* selective agar (BCSA) has essentially been described for *Pseudomonas* species, *Flavobacterium indologenes*, *A. xylosoxidans* and yeasts, but growth of these organisms was only observed in a minority of isolates (Henry *et al.*, 1997, 1999; Wright *et al.*, 2001). Growth of *R. pickettii* on this medium has been described previously, but this has not previously been studied for *R. mannitolilytica*. Additional experiments, in the course of the present study, showed that *R. pickettii* is more sensitive to elevated concentrations of crystal violet (>0.2 g l⁻¹) than is *R. mannitolilytica*, but higher concentrations of crystal violet also led to slower growth of the latter organism.

The results of *in vitro* susceptibility testing against commonly used antibiotics were variable within genotypes A/I and B/II. This may be due to secondary acquisition of resistance. As an alternative explanation, the disc diffusion test may be inadequate and poorly reproducible for testing *Ralstonia* species, as has been shown for other Gram-negative non-fermentative bacilli (Denton & Kerr, 1998). Further studies, with a higher number of isolates and incorporating additional methods (such as agar dilution, broth microdilution or Etest) are required to determine valid *in vitro* resistance

rates of the pathogen. However, previous observations that antibiotyping (at least by disc diffusion test) is helpful in the initial discrimination of outbreak-related isolates were not confirmed in the present study (Boutros *et al.*, 2002; Maroye *et al.*, 2000).

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