Different scenarios for *Candida parapsilosis* fungaemia reveal high numbers of mixed *C. parapsilosis* and *Candida orthopsilosis* infections

Leonardo Silva Barbedo,[1] Catarina Vaz,[2] Célia Pais,[2] Maria Helena Galdino Figueiredo-Carvalho,[1] Mauro de Medeiros Muniz,[1] Rosely Maria Zancope-Oliveira[1]† and Paula Sampaio[1]†

Correspondence
Paula Sampaio
psampaio@bio.uminho.pt

[1]Laboratório de Micologia, Instituto Nacional de Infectologia Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil

[2]Centro de Biologia Molecular e Ambiental (CBMA), Departamento de Biologia, Universidade do Minho, Braga, Portugal

Nosocomial fungal bloodstream infections (BSI) are increasing significantly in hospitalized patients and *Candida parapsilosis* has emerged as an important pathogen responsible for numerous outbreaks. The objective of this study was to evaluate *C. parapsilosis sensu lato* infection scenarios, regarding species distribution and strain relatedness. One hundred isolates of *C. parapsilosis sensu lato* derived from blood cultures and catheter tips were analysed by multiplex microsatellite typing and by sequencing D1/D2 regions of the ribosomal DNA. Our results indicate that 9.5 % of patients presented infections due to *C. parapsilosis* and *Candida orthopsilosis,* 57.1 % due to *C. parapsilosis*, 28.3 % due to *C. orthopsilosis* and 4.8 % due to *Candida metapsilosis*. Eighty per cent of the *C. parapsilosis* BSIs were due to a single strain that was also identified in the catheter, but in 10 % of the cases *C. parasilosis* was identified in the catheter but the BSI was due to *C. orthopsilosis*. There is a significant probability that *C. parapsilosis* isolates collected from the same patient at more than 3 months interval are of different strains (P=0.0179). Moreover, several isolates were identified persistently in the same hospital, infecting six different patients. The incidence of polyfungal BSI infections with *C. parapsilosis* and *C. orthopsilosis* is reported herein for the first time, emphasizing the fact that the species identified in the catheter is not always responsible for the BSI, thus impacting the treatment strategy. The observation that strains can remain in the hospital environment for years highlights the possible existence of reservoirs and reinforces the need for accurate genotyping tools, such as the markers used for elucidating epidemiological associations and detecting outbreaks.

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INTRODUCTION

The incidence of candidaemia continues to increase, particularly in hospitalized patients. Due to the substantial morbidity and mortality associated with these infections it is clear that fungal diseases have emerged as important public health problems (Pfaller & Diekema, 2007). While *Candida albicans* remains the most common causative agent of candidaemia, the incidence of *Candida parapsilosis* has increased significantly, outranking *C. albicans* in some studies, depending on the period and geographical area (Aittakorpi *et al.*, 2012; Lagrou *et al.*, 2007; Lockhart *et al.*, 2012; Maganti *et al.*, 2011; Nucci *et al.*, 2013; Parmeland *et al.*, 2013; Tragiannidis *et al.*, 2012; Wu *et al.*, 2011; Xess *et al.*, 2007). Candidaemia risk factors include the use of broad-spectrum antibiotics, cancer chemotherapy, immunosuppressive agents and indwelling medical devices (Tumbarello *et al.*, 2007). Nosocomial fungaemia due to *C. parapsilosis* is mainly associated with the presence of a central venous catheter, and with the use of parenteral nutrition (Barchiesi *et al.*, 2004). Although the primary reservoir in the hospital setting is unknown, *C. parapsilosis* carriage on the skin of healthy individuals, particularly on the hands of health care workers, and hospital environmental surfaces has been consistently observed (Lupetti *et al.*, 2002; Sabino *et al.*, 2011; Vaz *et al.*, 2011). Although frequently associated with neonates and the use of

†These authors contributed equally to this work

Abbreviations: BSI, bloodstream infection; DP, discriminatory power; HSE, Hospital dos Servidores do Estado; HUPE, Hospital Universitário Pedro Ernesto; SAM, Hospital Samaritano.

The GenBank/EMBL/DDBJ accession numbers for the D1/D2 region of the 28S rRNA gene sequence of the isolates described in this study are KJ817066 to KJ817165.

parenteral nutrition (Dizbay *et al.*, 2008), *C. parapsilosis* has also been frequently identified in adult intensive care units, surgery and internal medicine departments (Diab-Elschahawi *et al.*, 2012). The discrimination of *C. parapsilosis* strains is fundamental not only for the rapid identification of the strains involved in the infection but also to clarify nosocomial cross-transmission and possible routes of transmission in hospital settings (van Asbeck *et al.*, 2009). Since the description of a microsatellite multiplex strategy for *C. parapsilosis* strain differentiation (Sabino *et al.*, 2010), this method has been applied in several studies mainly regarding outbreaks (Diab-Elschahawi *et al.*, 2012; Romeo *et al.*, 2013; Vaz *et al.*, 2011) and is described as the most discriminatory method for *C. parapsilosis* strain differentiation. Herein, we used this method to genotype presumed *C. parapsilosis* isolates involved in fungaemia episodes from three hospitals in Brazil and to determine the patterns of relatedness of the isolates, including the pair identified in the bloodstream/catheter.

METHODS

Strains. During the period August 2002 to April 2006 at Hospital dos Servidores do Estado (HSE), 76 strains were isolated from 42 patients and were identified as *C. parapsilosis sensu lato*. For comparison, an additional 20 strains from Hospital Universitário Pedro Ernesto (HUPE) isolated before the year 2000 were also studied, although no patient's information was available, as well as four isolates from Hospital Samaritano (SAM). In total, 100 isolates of *C. parapsilosis sensu lato* derived from blood cultures and catheter tips from patients admitted at three different hospitals in Rio de Janeiro, Brazil, between 1998 and 2006, were analysed (Table 1). These isolates were stored in 40 % (v/v) glycerol at −80 °C at the yeast stock collection of the Laboratório de Micologia do Instituto Nacional de Infectologia (INI) da Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil.

Species identification. The isolates were phenotypically identified *as C. parapsilosis sensu lato* at the hospital of origin using VITEK 2 and API 20 C AUX systems (bioMérieux) following the manufacturer's instructions. Molecular identification at the species level was performed by sequencing the D1/D2 region of the 28S rRNA gene (Asadzadeh *et al.*, 2009). Sequences were edited with the Sequencer version 4.9 software package (Genes Codes Corporation), aligned with MEGA version 4.0.2 software and compared by BLAST with sequences available from NCBI GenBank.

Microsatellite multilocus PCR amplification and fragment size determination. Isolates identified as *C. parapsilosis* were further discriminated by multilocus microsatellite amplification. Cultures were grown at 30 °C for 2 days on YPD agar plates. Cells for colony PCR were prepared as described by Vaz *et al.* (2011). The primers, PCR amplification conditions and the allele size determination by GENESCAN (version 3.7) analysis after a run on an ABI PRISM 310 genetic analyser (Applied Biosystems) were as described previously (Sabino *et al.*, 2010) with some modifications as described by Vaz *et al.* (2011), namely the redesign of one primer. Fragment sizes were determined automatically using GENESCAN 3.5 analysis software. The discriminatory power (DP) of the method was calculated as described by Hunter & Gaston (1988).

Data analysis. A distance matrix was generated using the Cavalli-Sforza & Edwards method (Cavalli-Sforza & Edwards, 1967) with Populations software (version 1.2.28) and the dendrogram was constructed by the unweighted pair group method in NTSYSpc software (version 2.02; Applied Biostatistics). Categorical data were analysed using Fisher's exact test, unless stated in the text. Results were considered statistically significant when *P*-values were lower than 0.05.

RESULTS

Identification of *C. parapsilosis sensu lato* isolates and epidemiology

A total of 100 isolates from *C. parapsilosis sensu lato* fungaemia were analysed in this study. The majority of the isolates were derived from the HSE (76 %) while 20 % were derived from the HUPE and only 4 % from the SAM. Isolates included 83 from blood cultures and 17 from catheter tips (Table 1). Genotyping isolates with the microsatellite multiplex failed to amplify all markers in 39 isolates. Since this multiplex strategy is specific to *C. parapsilosis sensu stricto* (Sabino *et al.*, 2010), all the isolates were sequenced. Sequencing showed that 37 strains were *Candida orthopsilosis*, two were *Candida metapsilosis* and the remaining 61 were *C. parapsilosis* (Table 1). All sequences showed 100 % identity. The majority of the *C. orthopsilosis* isolates (29 isolates) derived from the HSE, while the eight isolates remaining were obtained from patients attending the HUPE. These isolates were obtained from 19 adult patients and seven children. All *C. metapsilosis* isolates were identified in patients from the HSE. Thus, considering all isolates, 61 % were *C. parapsilosis*, 37 % were *C. orthopsilosis* and 2 % were *C. metapsilosis*.

Considering only the isolates from the HSE, we determined that 57.1 % (24 from 42) of patients presented fungaemia due to *C. parapsilosis*, 28.6 % (12 from 42) due to *C. orthopsilosis* and 4.8 % (2 from 42) due to *C. metapsilosis*. Curiously, in the HSE *C. parapsilosis* and *C. orthopsilosis* were isolated from the same patient in 9.5 % (4 from 42) of patients. All cases of polyfungal infection were observed in male patients, but no significant difference ($P>0.05$) in species distribution was observed between the two sexes. Three of these polyfungal infections occurred in children and one in an adult patient. Likewise, no significant difference was observed ($P>0.05$) regarding species isolation considering the different years of isolation (from August 2002 to April 2006). It was not possible to perform these analyses with isolates from the HUPE since patient information was incomplete. All patients under 18 years old (nine children) were from the HUPE and 78 % were infected with *C. orthopsilosis* (Table 1).

Microsatellite multiplex genotyping of *C. parapsilosis* isolates

Genotyping of all *C. parapsilosis sensu stricto* isolates with the microsatellite markers identified 39 multilocus genotypes of which 24 were observed only once (Table 1). The most prevalent genotype, MG1, was found in five isolates

Table 1. Characterization of all isolates analysed in this study

Patient no.	Isolate	Hospital	Origin	Gender	Age classification	Collection date	Species identification	GenBank accession no.	Microsatellite fragment (bp)				MG	Dendrogram code
									CP1 all1 : all2	CP4a all1 : all2	CP6 all1 : all2	B all1 : all2		
1	072	HSE	Blood	M	Adult	13/1/2003	*C. orthopsilosis*	KJ817137	NA	NA	NA	NA	–	–
	074	HSE	Blood			13/1/2003	*C. orthopsilosis*	KJ817139	NA	NA	NA	NA	–	–
	075	HSE	Blood			9/2/2004	*C. orthopsilosis*	KJ817140	NA	NA	NA	NA	–	–
2	095	HSE	Blood	F		29/10/2005	*C. parapsilosis*	KJ817160	219 : 261	251 : 251	252 : 288	147 : 149	MG-6	095HSEb02
3	088	HSE	Blood	F	Adult	10/1/2005	*C. parapsilosis*	KJ817153	237 : 243	299 : 299	297 : 327	129 : 129	MG-9	088HSEb03
4	083	HSE	Blood	M	Adult	9/6/2004	*C. parapsilosis*	KJ817148	240 : 243	NA	273 : 303	127 : 139	MG-16	083HSEb04
	084	HSE	Blood			1/7/2004	*C. parapsilosis*	KJ817149	240 : 243	NA	273 : 303	127 : 139	MG-16	084HSEb04
5	001	HSE	Blood	F	Adult	19/12/2002	*C. parapsilosis*	KJ817066	189 : 240	236 : 236	315 : 315	165 : 167	MG-1	001HSEb05
	002	HSE	Blood			19/12/2002	*C. parapsilosis*	KJ817067	189 : 240	236 : 236	315 : 315	165 : 167	MG-2	002HSEb05
6	013	HSE	Catheter	F	Adult	6/11/2002	*C. parapsilosis*	KJ817078	189 : 240	236 : 236	315 : 315	165 : 167	MG-1	013HSEc06
	057	HSE	Blood			28/10/2002	*C. parapsilosis*	KJ817122	189 : 240	236 : 236	315 : 315	165 : 167	MG-1	057HSEb06
	058	HSE	Blood			6/11/2002	*C. parapsilosis*	KJ817123	189 : 240	236 : 236	315 : 315	165 : 167	MG-1	058HSEb06
7	089	HSE	Blood	M	Adult	10/1/2005	*C. orthopsilosis*	KJ817154	NA	NA	NA	NA	–	–
8	012	HSE	Catheter	M	Adult	11/9/2002	*C. parapsilosis*	KJ817077	240 : 240	239 : 239	294 : 294	121 : 121	MG-12	012HSEc08
9	078	HSE	Blood	M	Child	5/5/2004	*C. orthopsilosis*	KJ817143	NA	NA	NA	NA	–	–
	079	HSE	Blood			6/5/2004	*C. orthopsilosis*	KJ817144	NA	NA	NA	NA	–	–
10	077	HSE	Blood	M	Child	9/3/2004	*C. parapsilosis*	KJ817142	243 : 243	236 : 236	300 : 300	111 : 111	MG-28	077HSEb10
11	027	HSE	Catheter	M	Child	29/10/2005	*C. parapsilosis*	KJ817092	243 : 261	242 : 242	354 : 360	129 : 129	MG-37	027HSEc11
	096	HSE	Blood			21/11/2005	*C. orthopsilosis*	KJ817161	NA	NA	NA	NA	–	–
	097	HSE	Blood			5/12/2005	*C. orthopsilosis*	KJ817162	NA	NA	NA	NA	–	–
	098	HSE	Blood			1/2/2006	*C. parapsilosis*	KJ817163	240 : 240	236 : 236	264 : 267	145 : 145	MG-10	098HSEb11
12	006	HSE	Blood	M	Child	14/4/2003	*C. orthopsilosis*	KJ817071	NA	NA	NA	NA	–	–
	017	HSE	Catheter			14/4/2003	*C. orthopsilosis*	KJ817082	NA	NA	NA	NA	–	–
13	024	HSE	Catheter	M	Child	8/8/2005	*C. parapsilosis*	KJ817089	243 : 246	299 : 302	285 : 291	127 : 129	MG-35	024HSEc13
	092	HSE	Blood			8/8/2005	*C. orthopsilosis*	KJ817157	NA	NA	NA	NA	–	–
	093	HSE	Blood			23/8/2005	*C. parapsilosis*	KJ817158	243 : 246	299 : 302	285 : 291	127 : 129	MG-35	093HSEb13
14	023	HSE	Catheter	M	Child	28/7/2005	*C. parapsilosis*	KJ817088	240 : 240	236 : 236	264 : 267	145 : 145	MG-10	023HSEc14
	091	HSE	Blood			28/7/2005	*C. orthopsilosis*	KJ817156	NA	NA	NA	NA	–	–
15	010	HSE	Blood	M	Child	1/2/2006	*C. parapsilosis*	KJ817075	240 : 243	332 : 341	270 : 303	127 : 139	MG-23	010HSEb15
	011	HSE	Blood			1/2/2006	*C. parapsilosis*	KJ817076	240 : 243	332 : 341	270 : 303	127 : 139	MG-23	011HSEb15
16	056	HSE	Blood	M	Child	29/8/2002	*C. orthopsilosis*	KJ817121	NA	NA	NA	NA	–	–
17	018	HSE	Catheter	M	Child	2/1/2004	*C. orthopsilosis*	KJ817083	NA	NA	NA	NA	–	–
	067	HSE	Blood			26/12/2003	*C. orthopsilosis*	KJ817132	NA	NA	NA	NA	–	–
	068	HSE	Blood			11/2/2004	*C. orthopsilosis*	KJ817133	NA	NA	NA	NA	–	–
	071	HSE	Blood			2/1/2004	*C. orthopsilosis*	KJ817136	NA	NA	NA	NA	–	–
18	020	HSE	Catheter	M	Child	12/8/2004	*C. parapsilosis*	KJ817085	243 : 243	236 : 236	291 : 294	131 : 131	MG-27	020HSEc18
	085	HSE	Blood			26/7/2004	*C. parapsilosis*	KJ817150	243 : 243	236 : 236	291 : 294	131 : 131	MG-27	085HSEb18

Table 1. cont.

Patient no.	Isolate	Hospital	Origin	Gender	Age classification	Collection date	Species identification	GenBank accession no.	Microsatellite fragment (bp)				MG	Dendrogram code
									CP1 all1 : all2	CP4a all1 : all2	CP6 all1 : all2	B all1 : all2		
19	025	HSE	Catheter	M	Adult	23/8/2004	*C. parapsilosis*	KJ817090	240 : 243	332 : 341	270 : 303	127 : 139	MG-23	025HSEc19
	094	HSE	Blood			23/8/2005	*C. orthopsilosis*	KJ817159	NA	NA	NA	NA	–	–
20	021	HSE	Catheter	F	Adult	12/8/2004	*C. parapsilosis*	KJ817086	207 : 240	239 : 239	312 : 312	129 : 131	MG-4	021HSEc20
21	029	SAM	Blood	M	Adult	6/9/2005	*C. parapsilosis*	KJ817094	207 : 240	236 : 236	282 : 282	131 : 133	MG-3	029SAMb21
22	065	HSE	Blood	M	Adult	10/11/2003	*C. orthopsilosis*	KJ817130	NA	NA	NA	NA	–	–
23	087	HSE	Blood	M	Adult	24/9/2004	*C. orthopsilosis*	KJ817152	NA	NA	NA	NA	–	–
24	064	HSE	Blood	F	Adult	17/6/2003	*C. metapsilosis*	KJ817129	NA	NA	NA	NA	–	–
25	059	HSE	Blood	F	Adult	3/2/2003	*C. orthopsilosis*	KJ817124	NA	NA	NA	NA	–	–
26	099	HSE	Blood	F	Adult	3/4/2006	*C. parapsilosis*	KJ817164	243 : 243	296 : 296	264 : 324	129 : 157	MG-31	099HSEb26
27	003	HSE	Blood	M	Adult	20/2/2003	*C. parapsilosis*	KJ817068	243 : 243	299 : 299	264 : 342	129 : 157	MG-32	003HSEb27
	014	HSE	Catheter			20/2/2003	*C. parapsilosis*	KJ817079	243 : 243	299 : 299	264 : 342	129 : 157	MG-32	014HSEc27
	054	HSE	Blood			29/8/2002	*C. parapsilosis*	KJ817119	240 : 240	272 : 272	273 : 273	103 : 103	MG-14	054HSEb27
	055	HSE	Blood			29/8/2002	*C. parapsilosis*	KJ817120	240 : 240	272 : 272	273 : 273	103 : 103	MG-14	055HSEb27
28	080	HSE	Blood	M	Adult	20/5/2004	*C. parapsilosis*	KJ817145	240 : 243	NA	267 : 270	143 : 145	MG-15	080HSEb28
29	061	HSE	Blood	M	Adult	28/2/2003	*C. parapsilosis*	KJ817126	240 : 243	269 : 302	270 : 303	127 : 127	MG-20	061HSEb29
	063	HSE	Blood			12/5/2003	*C. parapsilosis*	KJ817128	243 : 243	236 : 236	273 : 291	131 : 131	MG-26	063HSEb29
30	032	SAM	Blood	F	Adult	7/10/2005	*C. parapsilosis*	KJ817097	207 : 240	236 : 236	276 : 282	131 : 133	MG-2	032SAMb30
31	066	HSE	Blood	F	Adult	14/11/2003	*C. orthopsilosis*	KJ817131	NA	NA	NA	NA	–	–
32	009	HSE	Blood	F	Adult	1/2/2006	*C. parapsilosis*	KJ817074	240 : 243	332 : 332	270 : 303	127 : 139	MG-22	009HSEb32
33	019	HSE	Catheter	F	Adult	27/2/2004	*C. parapsilosis*	KJ817084	240 : 240	239 : 239	279 : 312	109 : 109	MG-11	019HSEc33
	076	HSE	Blood			27/2/2004	*C. parapsilosis*	KJ817141	240 : 240	239 : 239	294 : 312	109 : 109	MG-13	076HSEb33
34	081	HSE	Blood	F	Adult	2/6/2004	*C. parapsilosis*	KJ817146	243 : 243	290 : 290	291 : 291	131 : 131	MG-30	081HSEb34
35	004	HSE	Blood	M	Adult	20/2/2003	*C. orthopsilosis*	KJ817069	NA	NA	NA	NA	–	–
	016	HSE	Catheter			28/2/2003	*C. orthopsilosis*	KJ817081	NA	NA	NA	NA	–	–
	060	HSE	Blood			20/2/2003	*C. orthopsilosis*	KJ817125	NA	NA	NA	NA	–	–
	062	HSE	Blood			28/2/2003	*C. orthopsilosis*	KJ817127	NA	NA	NA	NA	–	–
36	005	HSE	Blood	M	Adult	28/2/2003	*C. parapsilosis*	KJ817070	243 : 243	365 : 365	210 : 306	129 : 129	MG-34	005HSEb36
	015	HSE	Catheter			28/2/2003	*C. parapsilosis*	KJ817080	243 : 243	365 : 365	210 : 306	129 : 129	MG-34	015HSEc36
37	082	HSE	Blood	F	Adult	3/6/2004	*C. parapsilosis*	KJ817147	264 : 264	236 : 236	273 : 273	143 : 143	MG-39	082HSEb37
38	100	HSE	Blood	M	Adult	29/10/2005	*C. parapsilosis*	KJ817165	207 : 240	281 : 311	282 : 312	133 : 133	MG-5	100HSEb38
39	007	HSE	Blood	F	Adult	29/9/2005	*C. parapsilosis*	KJ817072	234 : 243	287 : 296	297 : 297	131 : 131	MG-8	007HSEb39
	008	HSE	Blood			29/9/2005	*C. parapsilosis*	KJ817073	234 : 243	287 : 296	297 : 297	131 : 131	MG-8	008HSEb39
	026	HSE	Catheter			29/9/2005	*C. parapsilosis*	KJ817091	234 : 243	287 : 290	297 : 297	131 : 131	MG-7	026HSEc39
40	028	HSE	Blood	F	Adult	19/12/2002	*C. metapsilosis*	KJ817093	NA	NA	NA	NA	–	–
41	022	HSE	Catheter	F	Adult	24/9/2004	*C. parapsilosis*	KJ817087	240 : 243	341 : 341	270 : 303	127 : 139	MG-24	022HSEc41
42	030	SAM	Blood	M	Adult	6/9/2005	*C. parapsilosis*	KJ817095	240 : 243	329 : 329	270 : 303	127 : 139	MG-21	030SAMb42
	031	SAM	Catheter			6/9/2005	*C. parapsilosis*	KJ817096	240 : 243	332 : 332	270 : 303	127 : 139	MG-22	031SAMc42
43	053	HSE	Blood	M	Adult	29/8/2002	*C. orthopsilosis*	KJ817118	NA	NA	NA	NA	–	–

Table 1. cont.

Patient no.	Isolate	Hospital	Origin	Gender	Age classification	Collection date	Species identification	GenBank accession no.	Microsatellite fragment (bp)				MG	Dendrogram code
									CP1 all1 : all2	CP4a all1 : all2	CP6 all1 : all2	B all1 : all2		
44	069	HSE	Blood	M	Adult	29/12/2003	*C. parapsilosis*	KJ817134	243 : 243	251 : 251	252 : 252	145 : 161	MG-29	069HSEb44
	070	HSE	Blood			30/12/2003	*C. parapsilosis*	KJ817135	243 : 243	251 : 251	252 : 252	145 : 161	MG-29	070HSEb44
	073	HSE	Blood			13/1/2004	*C. parapsilosis*	KJ817138	243 : 243	251 : 251	252 : 252	145 : 161	MG-29	073HSEb44
–	086	HSE	Blood	–	Adult	–	*C. orthopsilosis*	KJ817151	NA	NA	NA	NA	–	–
–	090	HSE	Blood	–	Adult	–	*C. orthopsilosis*	KJ817155	NA	NA	NA	NA	–	–
–	033	HUPE	Blood	–	Adult	–/–/1998	*C. orthopsilosis*	KJ817098	NA	NA	NA	NA	–	–
–	034	HUPE	Blood	–	Adult	2/9/1999	*C. orthopsilosis*	KJ817099	NA	NA	NA	NA	–	–
–	035	HUPE	Blood	–	Adult	–/–/1998	*C. parapsilosis*	KJ817100	246 : 246	299 : 299	243 : 303	129 : 147	MG-38	035HUPb00
–	036	HUPE	Blood	–	Adult	–/–/1998	*C. orthopsilosis*	KJ817101	NA	NA	NA	NA	–	–
–	037	HUPE	Blood	–	Adult	–/–/1998	*C. orthopsilosis*	KJ817102	NA	NA	NA	NA	–	–
–	038	HUPE	Blood	–	Adult	–/–/1998	*C. parapsilosis*	KJ817103	240 : 243	245 : 272	273 : 273	133 : 133	MG-19	038HUPb00
–	039	HUPE	Blood	–	Adult	23/9/1999	*C. parapsilosis*	KJ817104	240 : 243	233 : 233	255 : 255	127 : 127	MG-17	039HUPb00
–	040	HUPE	Blood	–	Adult	23/9/1999	*C. parapsilosis*	KJ817105	240 : 243	233 : 254	255 : 255	127 : 127	MG-18	040HUPb00
–	041	HUPE	Blood	–	Adult	2/9/1999	*C. parapsilosis*	KJ817106	240 : 243	233 : 254	255 : 255	127 : 127	MG-18	041HUPb00
–	042	HUPE	Blood	–	Adult	23/9/1999	*C. parapsilosis*	KJ817107	243 : 249	299 : 302	288 : 303	105 : 127	MG-36	042HUPb00
–	043	HUPE	Blood	–	Adult	2/9/1999	*C. parapsilosis*	KJ817108	243 : 249	299 : 302	288 : 303	105 : 127	MG-36	043HUPb00
–	044	HUPE	Blood	–	Adult	2/9/1999	*C. parapsilosis*	KJ817109	243 : 243	329 : 329	285 : 306	129 : 129	MG-33	044HUPb00
–	045	HUPE	Blood	–	Adult	–/–/1998	*C. orthopsilosis*	KJ817110	NA	NA	NA	NA	–	–
–	046	HUPE	Blood	–	Adult	–/–/1998	*C. orthopsilosis*	KJ817111	NA	NA	NA	NA	–	–
–	047	HUPE	Blood	–	Adult	–/–/1998	*C. orthopsilosis*	KJ817112	NA	NA	NA	NA	–	–
–	048	HUPE	Blood	–	Adult	–/–/1998	*C. parapsilosis*	KJ817113	243 : 243	212 : 236	303 : 303	111 : 111	MG-25	048HUPb00
–	049	HUPE	Blood	–	Adult	–/–/1998	*C. parapsilosis*	KJ817114	243 : 243	212 : 236	303 : 303	111 : 111	MG-25	049HUPb00
–	050	HUPE	Blood	–	Adult	–/–/1998	*C. parapsilosis*	KJ817115	243 : 243	212 : 236	303 : 303	111 : 111	MG-25	050HUPb00
–	051	HUPE	Blood	–	Adult	–/–/1998	*C. parapsilosis*	KJ817116	243 : 243	212 : 236	303 : 303	111 : 111	MG-25	051HUPb00
–	052	HUPE	Blood	–	Adult	–/–/1998	*C. orthopsilosis*	KJ817117	NA	NA	NA	NA	–	–

–, No information available; all1, allele 1; all2, allele 2; MG, multilocus genotype; NA, no amplification; adult, >18 years old; child, <18 years old.

and was retrieved from two patients. Considering only independent isolates (the first *C. parapsilosis* strain isolated from each patient), the combined DP of the multiplex was 0.99, which is in agreement with previous studies (Diab-Elschahawi *et al.*, 2012; Sabino *et al.*, 2010). In this study, isolates with the same allelic combination at all microsatellite markers correspond to genetically similar strains and were defined as identical, while isolates with distinct allelic combinations at more than one locus correspond to genetically dissimilar strains and were considered different strains. The remaining cases, with minor changes in only one locus, were considered as micro-variations.

In order to analyse strain relatedness, the multiple isolates collected from the same patient were grouped (Table 2). First, we observed that 12 of these patients were infected exclusively with *C. parapsilosis*, five with *C. orthopsilosis* and four with both *C. parapsilosis* and *C. orthopsilosis*. These two species were isolated on the same day in all four patients except patient 11, so the majority of these infections may be considered polyfungic. Curiously, in patients infected only with *C. orthopsilosis* (patients 12, 17 and 35), this species was also isolated from the catheter; however, when *C. parapsilosis* was also present (patients 11, 13, 14 and 19) the species isolated from the catheter was always *C. parapsilosis*.

Analysing *C. parapsilosis* strain relatedness from patients with catheter and blood cultures isolates, we observed that five patients showed identical bloodstream and catheter strains (patients 6, 13, 18, 27 and 36), three showed micro-variations in the pair bloodstream/catheter strains (patients 33, 39 and 42) and two presented different strains (patients 11 and 27). If the identical and microvariant cases are grouped, 80 % of *C. parapsilosis* bloodstream/catheter pairs showed similar strains. Indeed, there is a statistical probability ($\chi^2=4.00$, $P=0.04$) that the strain observed in the catheter will also be present in the blood culture. However, when Yates' correction was applied this association was no longer significant ($\chi^2=1.78$, $P=0.18$). Although the cases of microvariation observed in this study were associated with the presence of a catheter, the isolation dates were the same so it was impossible to determine whether microvariation occurred in the catheter strain.

Regarding the relatedness of *C. parapsilosis* strains isolated at different collection dates, regardless of the presence or absence of a catheter in patients that maintained the infecting strain (patients 4, 6, 13, 18 and 44), the collection dates were always within 1 month (Table 2). On the contrary, in all cases in which a strain replacement was observed (patients 11, 27 and 29) the collection dates were always greater than 3 months. We observed that the probability of strain replacement within 3 to 6 months was statistically significant ($P=0.0179$), suggesting that in these infections *C. parapsilosis* strains were mainly acquired from the environment. One example was patient 11: a *C. parapsilosis*-positive catheter culture was collected on day 1 with no positive blood culture, in the following 2-month period two blood cultures were positive for *C. orthopsilosis* and a third blood culture was positive for a completely different *C. parapsilosis* strain; these two *C. parapsilosis* strains were collected 3 months apart. The presence of *C. orthopsilosis* (patients 11 and 13), did not affect this correlation.

Clustering of *C. parapsilosis* isolates

When strains were compared regardless of their origin, we observed that different patients shared similar strains (Fig. 1). Patient 6 presented a positive blood culture with isolate 057 and one month later the same strain was present in the catheter and again in the blood (Table 1). More than a month after this episode, patient 5, from the same hospital, was infected with a similar strain (001). Other patients from the same hospital, patients 14 and 11, also presented strains with the same multilocus genotype (MG-10), collected approximately 7 months apart (023 and 098). Similarly, patients 19 and 15 from the same hospital shared strains with MG-23 (010 and 025), collected almost 18 months apart (23 August 2004 and 1 February 2006). Curiously, patients 42 and 32, from different hospitals, also showed strains with the same multilocus genotype (MG-22; isolates 009 and 031) collected 5 months apart (6 September 2005 and 1 Febuary 2006). Interestingly, MG-22 and MG-23 differ only at locus CP4a and are considered microvariants. In addition, MG-24 observed in isolate 022 from patient 41 and MG-21 in isolate 030 from patient 42 may also be considered microvariants of MG-22 and MG-23 (Table 1). These observations suggest that isolates with multilocus genotypes similar to isolate 25 (MG-23), the first to be isolated, have been persistently isolated in hospital settings over the period August 2004 to February 2006, affecting six different patients (Fig. 1). Similar cases of micro-variation in different patients from the same hospital were observed: isolate 029 from patient 21 (MG-3) and one month later in isolate 032 from patient 30 (MG-2), and isolate 062 from patient 29 (MG-26) and 14 months later the microvariant MG-27 in isolate 085 from patient 18. Considering that the patients in this study are not related, we cannot exclude the hypothesis of cross-contamination of patients by the hands of healthcare personnel, as extensively reported in other studies (Lupetti *et al.*, 2002; Vaz *et al.*, 2011). Isolates 039 and 040 or 041 are also considered microvariants. However, due to the lack of information regarding patients' identity these may not be considered as possible cross-contaminants.

DISCUSSION

The incidence of *Candida* in bloodstream infections has been reported to be higher in Brazil than in the USA or Europe, where *C. parapsilosis* has now become, respectively, the second and third most common aetiological agent of these infections after *C. albicans* (Colombo *et al.*, 1999; Dizbay *et al.*, 2008; Hajjeh *et al.*, 2004). However, more recently some European regions, including southern

Table 2. Genotypes obtained with microsatellites CP1, CP4a, CP6 and B in *C. parapsilosis* isolates from blood cultures and catheters

Patient	Hospital	Origin	Collection date	MG or species identified	*C. parapsilosis* multilocus genotype (bp) CP1, CP4a, CP6 and B (all1 : all2)
Patient with catheter and bloodstream isolates					
6	HSE	Blood	28/10/2002	MG-1	189 : 240 236 : 236 315 : 315 165 : 167
		Catheter	06/11/2002	MG-1	189 : 240 236 : 236 315 : 315 165 : 167
		Blood	06/11/2002	MG-1	189 : 240 236 : 236 315 : 315 165 : 167
11	HSE	Catheter	29/10/2005	MG-37	243 : 261 242 : 242 354 : 360 129 : 129
		Blood	21/11/2005	*C. orthopsilosis*	ND
		Blood	05/12/2005	*C. orthopsilosis*	ND
		Blood	01/02/2006	MG-10	240 : 240 236 : 236 264 : 267 145 : 145
12	HSE	Catheter	14/04/2003	*C. orthopsilosis*	ND
		Blood	14/04/2003	*C. orthopsilosis*	ND
13	HSE	Catheter	08/08/2005	MG-35	243 : 246 299 : 302 285 : 291 127 : 129
		Blood	08/08/2005	*C. orthopsilosis*	ND
		Blood	23/08/2005	MG-35	243 : 246 299 : 302 285 : 291 127 : 129
14	HSE	Catheter	28/07/2005	MG-10	240 : 240 236 : 236 264 : 267 145 : 145
		Blood	28/07/2005	*C. orthopsilosis*	ND
17	HSE	Blood	26/12/2003	*C. orthopsilosis*	ND
		Catheter	02/01/2004	*C. orthopsilosis*	ND
		Blood	02/01/2004	*C. orthopsilosis*	ND
		Blood	11/02/2004	*C. orthopsilosis*	ND
18	HSE	Blood	26/07/2004	MG-27	243 : 243 236 : 236 291 : 294 131 : 131
		Catheter	12/08/2004	MG-27	243 : 243 236 : 236 291 : 294 131 : 131
19	HSE	Catheter	23/08/2004	MG-23	240 : 243 332 : 341 270 : 303 127 : 139
		Blood	23/08/2004	*C. orthopsilosis*	ND
27	HSE	Blood	29/08/2002	MG-14	240 : 240 272 : 272 273 : 273 103 : 103
		Catheter	20/02/2003	MG-32	243 : 243 299 : 299 264 : 342 129 : 157
		Blood	20/02/2003	MG-32	243 : 243 299 : 299 264 : 342 129 : 157
33	HSE	Catheter	27/02/2004	MG-11	240 : 240 239 : 239 279 : 312 109 : 109
		Blood	27/02/2004	MG-13	240 : 240 239 : 239 294 : 312 109 : 109
35	HSE	Blood	20/02/2003	*C. orthopsilosis*	ND
		Catheter	28/02/2003	*C. orthopsilosis*	ND
		Blood	28/02/2003	*C. orthopsilosis*	ND
36	HSE	Catheter	28/02/2003	MG-34	243 : 243 365 : 365 210 : 306 129 : 129
		Blood	28/02/2003	MG-34	243 : 243 365 : 365 210 : 306 129 : 129
39	HSE	Catheter	29/09/2005	MG-7	234 : 243 287 : 290 297 : 297 131 : 131
		Blood	29/09/2005	MG-8	234 : 243 287 : 296 297 : 297 131 : 131
42	SAM	Catheter	06/09/2005	MG-22	240 : 243 332 : 332 270 : 303 127 : 139
		Blood	06/09/2005	MG-21	240 : 243 329 : 329 270 : 303 127 : 139
Patients with only bloodstream isolates					
1		Blood	13/01/2003	*C. orthopsilosis*	ND
		Blood	09/02/2004	*C. orthopsilosis*	ND
4		Blood	09/06/2004	MG-16	240 : 243 000 : 000 273 : 303 127 : 139
		Blood	01/07/2004	MG-16	240 : 243 000 : 000 273 : 303 127 : 139
5		Blood	19/12/2002	MG-1	189 : 240 236 : 236 315 : 315 165 : 167
9		Blood	05/05/2004	*C. orthopsilosis*	ND
		Blood	06/05/2004	*C. orthopsilosis*	ND
15		Blood	01/02/2006	MG-23	240 : 243 332 : 341 270 : 303 127 : 139
29		Blood	28/02/2003	MG-20	240 : 243 269 : 302 270 : 303 127 : 127
		Blood	12/05/2003	MG-26	243 : 243 236 : 236 273 : 291 131 : 131
44		Blood	29/12/2003	MG-29	243 : 243 251 : 251 252 : 252 145 : 161
		Blood	30/12/2003	MG-29	243 : 243 251 : 251 252 : 252 145 : 161
		Blood	13/01/2003	MG-29	243 : 243 251 : 251 252 : 252 145 : 161

MG, multilocus genotype; all1, allele 1; all2, allele 2; ND, not determined.

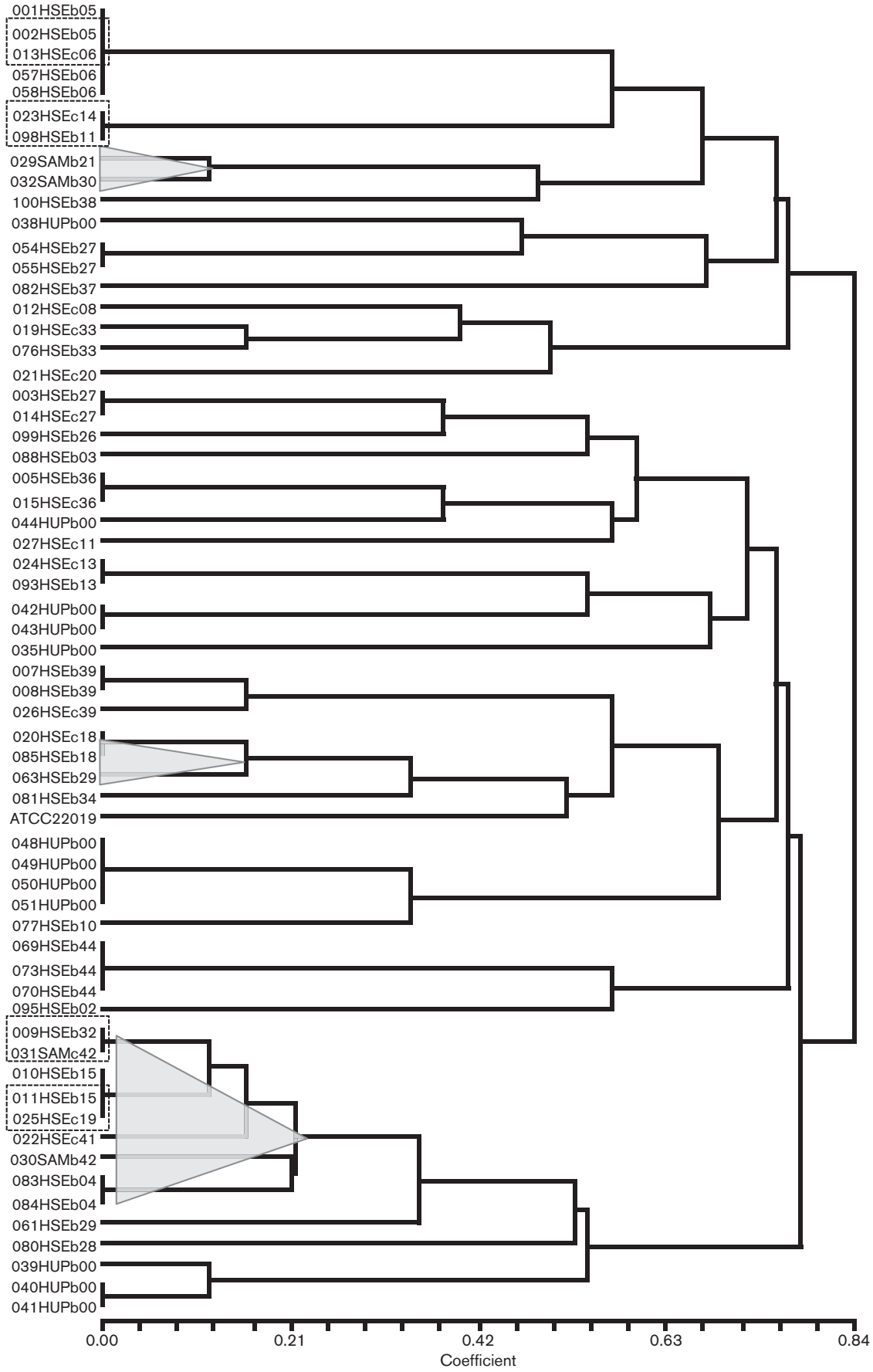

Fig. 1. Dendrogram showing relationships among 61 *Candida parapsilosis* isolates based on multilocus microsatellite genotypes. Dashed boxes represent strains from different patients sharing the same multilocus genotypes, and triangles represent microvariant strains from different patients. The isolates are represented by a code that includes the isolate number (000–100), followed by the hospital of origin (HSE, HUP or SAM), by the letter b (for blood) or c (for catheter), and finally the patient number (01–44; 00 denotes no information available).

Italy, have reported bloodstream incidences for *C. parapsilosis* infection of around 60 %, similar to those observed in Brazil (Delfino *et al.*, 2014). Moreover, the distribution of *C. orthopsilosis* and *C. metapsilosis* as agents of invasive infections has marked variability. In this study, we report an overall prevalence of 37 % for *C. orthopsilosis* and 2 % for *C. metapsilosis* among *C. parapsilosis sensu lato* isolates. The majority of the patients analysed in this study were from the HSE and in this hospital the incidence was 28.6 % for *C. orthopsilosis* and 4.8 % for *C. metapsilosis*. These values are higher than those described in a multi-centre surveillance study conducted in Brazil between 2003 and 2004 (*C. orthopsilosis* was 9 % and *C. metapsilosis* was 3 %; Gonçalves *et al.*, 2010), and those in a Spanish multi-centre study that reported 8.2 % of *C. orthopsilosis* and 1.1 % of *C. metapsilosis* (Cantón *et al.*, 2011). Our values are relatively similar to those recorded in a Spanish study in which 23.5 % of *C. orthopsilosis* and 2.1 % of *C. metapsilosis* were found (Garcia-Effron *et al.*, 2012). Nevertheless, it is known that the incidence of *C. parapsilosis sensu lato* infections varies according to multiple factors, such as the age and underlying disease of patients, geographical location of the hospitals studied and climatic and socio-economic conditions.

A significant finding of this study was the observation that the same patient can simultaneously have *C. parapsilosis* and *C. orthopsilosis*. Indeed in 9.5 % of patients from the HSE, both *C. parapsilosis* and *C. orthopsilosis* were isolated and only in one case were the two species isolated on different dates. To our knowledge, there are few studies reporting polyfungic infections involving *C. parapsilosis sensu lato* species, and none from systemic infections. It has previously been reported that in superficial infections, 7.5 % those that identified *C. parapsilosis* complex species were polyfungal (Feng *et al.*, 2012). However, all combined *C. parapsilosis* and *C. albicans* with *Candida tropicalis* or *Candida guilliermondii*. A previous study reported two cases of polyfungal infections (*C. albicans* and *Candida glabrata*) in 20 hospitalized patients with catheter-related candidaemia; however, none of the *C. parapsilosis* complex species was involved in polyfungal infections (Escribano *et al.*, 2014). It has been reported that polymicrobial BSIs are strongly associated with increased mortality (Kim *et al.*, 2013). Although there is no study to date regarding the survival of patients infected with *C. parapsilosis* and *C. orthopsilosis*, the identification of BSI due to more than one species is of extreme importance.

C. parapsilosis is notorious for the ability to form biofilms on catheters and other implanted devices (Tumbarello *et al.*, 2007), more so than the closely related species *C. orthopsilosis* and *C. metapsilosis* (Lattif *et al.*, 2010). This may explain why in all cases of polyfungal infection the species identified in the catheter was *C. parapsilosis* and never *C. orthopsilosis*. This was not due to the fact that *C. orthopsilosis* was not able to form biolfim, because when *C. orthopsilosis* was the only species present, in both blood culture and catheter, biofilm was identified in the catheter. This highlights the fact that the species identified in the catheter is not always that responsible for the BSI. In the last decade, antifungal susceptibility among *C. parapsilosis sensu lato* strains has been considered a matter of concern worldwide. The identification of one species in the catheter and another in the bloodstream gives a different perspective on the evaluation and use of antifungals. This may have been the case for our patient 11 that showed multiple BSIs due to *C. orthopsilosis* and *C. parapsilosis*, but the catheter was initially colonized with *C. parapsilosis*.

In the case where multiple isolates from the same patient were recovered, we observed that 80 % of *C. parapsilosis* bloodstream/catheter pairs showed similar strains (identical and microvariants strains), indicating a high probability that the *C. parapsilosis* strain observed in the catheter will also be present in the blood culture. The occurrence of genetic changes in a strain has been described in catheter-colonizing isolates rather than in blood isolates, for *C. parapsilosis* (Romeo *et al.*, 2013) as well as for *C. albicans* (Shin *et al.*, 2004). However, regardless of their origin (blood or catheter), there was a statistically significant correlation between the identity of the isolate and the date of collection (P=0.0179). In samples that were collected less than 1 month apart the isolates were genetically related (identical or microvariants), while samples collected at more than 3 months′ interval were genetically distinct. These observations suggest that *C. parapsilosis* BSI strains are mainly acquired from the environment. The identification in this study of genetically related isolates, persistently isolated from BSI occurring in different patients in the same hospital, sometimes over several years, further reinforces the environmental acquisition of these infections. The temporal persistence of single *C. parapsilosis* strains over long periods of time in the same hospital or ward has been described in other studies, and was also associated with outbreaks (Ásmundsdóttir *et al.*, 2008; Romeo *et al.*, 2013; Viviani *et al.*, 2006).

In conclusion, taking all our results together, five main scenarios for *C. parapsilosis* fungaemia were identified in this study: (i) monofungal infections due to all three species of the complex; (ii) polyfungal, always including *C. parapsilosis*; (iii) maintenance of the *C. parapsilosis* infecting strain when collection dates are less than three months apart;

(iv) replacement of the *C. parapsilosis* infecting strain when collection dates are more than three months apart; and (v) micro-variation in the pair bloodstream/catheter *C. parapsilosis* strains.

Although the detection of BSIs with *C. parapsilosis* should include alerts regarding security breaks in catheter care and infection control procedures, as previously reported, the identification of polyfungal infections and their consequences for treatment should also be considered an alert. The observation that strains can remain in the hospital environment for years is also an alerts for the possible existence of specific reservoirs and reinforces the need for accurate genotyping tools, such as the microsatellite markers used, for both elucidating epidemiological associations and the detection of outbreaks.

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