Medical Mycology July 2013, **51**, 483–492

informa
healthcare

Distribution, virulence attributes and antifungal susceptibility patterns of *Candida parapsilosis* complex strains isolated from clinical samples

ILKNUR TOSUN*, ZEYNEP AKYUZ*, NEJLA CEBECI GULER*, DOLUNAY GULMEZ†, GULCIN BAYRAMOGLU*, NESE KAKLIKKAYA*, SEVTAP ARIKAN-AKDAGLI† & FARUK AYDIN*

**Department of Medical Microbiology, Faculty of Medicine, Karadeniz Technical University, Trabzon, and †Department of Medical Microbiology, Hacettepe University Medical School, Ankara, Turkey*

It was recently proposed that *Candida parapsilosis* represents a complex composed of three closely related species, i.e., *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*. The aim of this study was to describe the distribution of *C. parapsilosis* complex isolates among clinical samples. We also evaluated antifungal susceptibility profiles, *in vitro* presence of lipase and secreted aspartyl proteinase, as well as their ability to grow in total parenteral nutrition (TPN) solution, and biofilm production. A total of 413 non-*C. albicans Candida* isolates were obtained from various clinical samples between 2010 and 2011 in a Turkish Tertiary Care Hospital. Of them, 42 were identified as members of the *C. parapsilosis* complex. Among these, 38 (90.5%) were *C. parapsilosis sensu stricto*, 3 (7.1%) *C. metapsilosis*, and 1 (2.4%) *C. orthopsilosis*. All isolates recovered from blood were found to be C. *parapsilosis sensu stricto* and *C. metapsilosis*. In phenotypic tests, all 42 isolates grew in TPN solution and, although 26.2% of *C. parapsilosis sensu stricto*-isolates were capable of forming biofilms *in vitro*, neither *C. orthopsilosis* nor *C. metapsilosis* isolates were able to do so. Acid proteinase activity was detected in 31% of isolates and lipase activity in 33%. All isolates were sensitive to voriconazole, caspofungin, and anidulafungin, with only a single *C. parapsilosis sensu stricto* isolate showing dose-dependent susceptible to fluconazole. While the number of *C. metapsilosis* and *C. orthopsilosis* isolates remained low, there were no significant differences in antifungal MIC as compared to *C. parapsilosis sensu stricto*.

Keywords *Candida parapsilosis*, *C. metapsilosis*, *C. orthopsilosis*, SAPP, virulence, antifungal susceptibility

Introduction

Candida parapsilosis accounts for a significant proportion of nosocomial infections, with an increasing prevalence in hospital settings. Earlier reports indicated that candidiasis caused by *C. parapsilosis* was associated with (a) the presence of indwelling devices, (b) the use of total parenteral nutrition (TPN) [1,2], and (c) the ability of this yeast to grow as biofilms [3].

Received 15 June 2012; Received in final revised form 12 October 2012; Accepted 30 October 2012.

Correspondence: Ilknur Tosun, Department of Clinical Microbiology, Faculty of Medicine, Karadeniz Technical University, Trabzon, 61080 Turkey. E-mail: ijune99@yahoo.com

Recently, three distinct groups of *C. parapsilosis* (groups I, II, and III) were described as new species, i.e., *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*, respectively [4]. The importance of *C. orthopsilosis* and *C. metapsilosis* as human pathogens remains unknown; however, studies point to their significant involvement in human candidiasis [5–8]. Interestingly, the incidence of *C. orthopsilosis* and *C. metapsilosis* infections may have increased since 2004 [6], with prevalence rates ranging from 2.3–9% and 0.9–6.9%, respectively, depending on the geographical area and clinical specimens analyzed.

In our hospital, as reported by the Infection Control Committee, *C. parapsilosis* became the second most common *Candida* species isolated from blood samples

DOI: 10.3109/13693786.2012.745953

(28.9%) between 2005 and 2009. We aimed to provide insight into the distribution of *C. parapsilosis sensu stricto*, *C. metapsilosis*, and *C. orthopsilosis*, their virulence properties and antifungal susceptibility profiles.

Materials and methods

Yeast strains

This study included 42 *C. parapsilosis* complex strains identified among 413 non-*Candida* isolates collected from February 2010 to January 2011 in Farabi Hospital in Trabzon, Turkey. The hospital is a 950-bed teaching facility affiliated with the Faculty of Medicine. Yeasts were initially observed on Gram-stained preparations, subcultured on Sabouraud glucose agar, and identified by germ tube tests, color changes on CHROMagar (CHROMagar, Paris, France), and carbohydrate assimilation reactions (20C AUX; bioMérieux, Marcy l'Etoile, France). Yeast isolates were stored in 20% (vol/vol) glycerol with Yeast Extract Peptone Dextrose (YEPD) at − 70°C until they were tested. *Candida albicans* SC5314 (as positive control for biofilm assay), *C. albicans* ATCC 10231 (as positive control in enzymatic evaluations), *C. parapsilosis* ATCC 22019 and C. *krusei* ATCC 6258 (for antifungal susceptibility testing controls) were included in the study. *C. parapsilosis* ATCC 22019, *C. parapsilosis* AM 2001/0013, *C. metapsilosis* J960161 and *C. orthopsilosis* J981226, kindly provided by Prof. Frank C. Odds and Dr Donna M. McCallum (University of Aberdeen, UK) were used as reference strains for molecular identification**.**

Sequence analysis of internal transcribed spacer (ITS) subunit rRNA regions

Yeast genomic DNA for PCR amplification was extracted using the High-Pure PCR Template Preparation Kit (Roche Diagnostic, Mannheim, Germany), according to the manufacturer's instructions. Sequencing of the 5.8S rRNA gene and the adjacent ITS1 and ITS2 regions was carried out. The identity of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* clinical isolates was confirmed by DNA sequencing of the ITS1 and ITS4 regions of the 28S rRNA gene. ITS1 (forward, 5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (reverse, 5′-TCC TCC GCT TAT TGA TAT GC-3′)-specific primers were used to amplify the ITS region, as described by White *et al*. [9]. The PCR was carried out under the following conditions: first cycle at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, at 60°C for 30 s, and at 72°C for 1 min, with a final extension step at 72°C for 10 min. The amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Both strands of purified amplicons were sequenced with an ABI Prism 3130 DNA analyzer (Applied Biosystems, Foster City, CA, USA) using primers for ITS1 and ITS4. The amplicons were sequenced and subjected to BLAST searches against the GenBank database (www.ncbi.nlm.nih.gov/genbank).

Molecular identification of C. parapsilosis *complex*

Identification of species comprising the *C. parapsilosis* group was achieved using PCR-restriction fragment length polymorphism (RFLP). Amplification of the secondary alcohol dehydrogenase-encoding (SADH) gene was performed by PCR with primers previously described by Tavanti *et al*. [4]. *Ban*I digestion patterns of the SADH PCR products were used to discriminate *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*. A 15 µl sample was loaded and separated by electrophoresis using a 7.5% acrylamide gel which was stained in ethidium bromide solution (0.5 µg/ml) and photographed under ultraviolet illumination. *C. parapsilosis* and *C. metapsilosis* were revealed by two and four band patterns, respectively, while *C. orthopsilosis* was detected by the presence of a non-digested band (716 bp) (Fig. 1).

Secretory aspartic proteinase (SAPP) gene analysis

The synthetic oligonucleotides used as primers were: 5′-AGT GGT CGT CAA ACC ACT CC-3′ and 5′-GAC GGA AGC AAG CGA AAT AG-3′ (*SAPP1*); 5′-TTA CTT GCC TGA CAG CAT-3′ and 5′-CGC ATA AGC GTG TCT CAA AA-3′ (*SAPP2*); and 5′-TAA TGC GTC TCA CTG GA-3′ and 5′-AGA CCC ATG ACC CCT AG-3′ (*SAPP3*) [4,10]. DNA amplification was performed using the following cycling parameters in a Perkin Elmer 9600

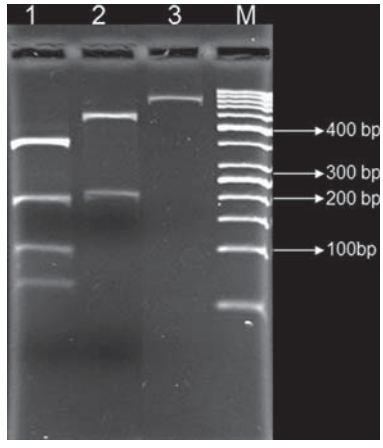

Fig. 1 Ban I restriction digestion of SADH-PCR product. Lane 1, *Candida metapsilosis*; Lane 2, *C. parapsilosis*; Lane 3. *C. orthopsilosis* type strain; Lane M, 50-bp ladder.

thermocycler (Perkin Elmer, Cetus, Norwalk, CT, USA): initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 54°C s for 50 s, and extension at 72°C for 50 s. A 7-min extension at 72°C was included at the end of the final cycles. Then, 10 µl PCR product was separated by electrophoresis using 2% (wt/vol) agarose. The gel was stained with ethidium bromide solution (0.5 µg/ml) and photographed under ultraviolet illumination.

Extracellular proteolytic activity

Candida parapsilosis isolates were analyzed for secretory proteolytic activity on solid medium as described previously [11], with minor modifications. The inducing medium contained 1.17% Yeast Carbon Base, 0.01% yeast extract, 0.2% bovine serum albumin (BSA), and 0.02 ppm bromphenol blue. The medium was prepared in citrate buffer (pH 4.5) and filter sterilized and then added to a solution of autoclaved 2% agar. Next, 10 µl of each yeast suspension (10^6 cells/ml) was inoculated onto BSA agar plates and incubated at 30°C for 7 days. Proteolysis was determined by clarification of the medium around the colony and the Pz values were calculated as described previously [12]. Briefly, proteinase activity (Pz) was expressed as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone. A Pz of 1.0 was evaluated as negative, 0.90–0.99 as weak, 0.80–0.89 as mild, and 0.70–0.79 as strong.

Determination of lipolytic activity

Lipolytic activity was examined according to the method of Gacser *et al*. [13], using colonies grown on rhodamine B plates (7 g/l Yeast Nitrogen Base [YNB], 20 ml/l olive oil, 50 ml/l fetal bovine serum [FBS], and 1 ml/l 1 M rhodamine B). Approximately 10 µl of each yeast suspension (10^6 cells/ml) was inoculated onto agar plates and incubated at 30°C for 7 days. Lipolytic activity was demonstrated by the presence of red-stained regions and lysis zones around the colonies.

Biofilm formation

Biofilm formation by *C. parapsilosis* complex isolates were quantified by metabolic activity as previously described [14]. Polystyrene 96-well plates (Greiner bio-one, Frickenhausen**,** Germany) were inoculated with 100 µl FBS overnight and washed twice with sterile phosphate-buffered saline (PBS). *C. parapsilosis* cells were collected by centrifugation, washed twice with PBS, suspended at 10^6 cells/ml in RPMI 1640 medium, and 100 µl suspensions of yeast were incubated at 37°C without shaking for 48 h. The wells were washed three times with 0.05% Tween-20 in PBS to remove non-adhered cells. Fungal cells that remained attached to the plastic surface were considered to have formed biofilm. A semiquantitative measurement of *C. parapsilosis* biofilm formation was obtained via the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay. The colorimetric change was measured using a microtiter reader at 492 nm. Biofilm production was considered as absent when the OD was equal and lower than values of wells containing XTT only.

Growth in TPN

Approximately 10^4 cells of each test microorganism were added to 10 ml of a commercial TPN solution in sterile plastic tubes. Tubes were incubated at 30°C and sampled at 0, 24, and 48 h. Each sample was inoculated onto Sabouraud dextrose agar (SDA) plates and the numbers of colony forming units (CFU) were counted at each time point.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed by broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) M27-A3 guidelines [15]. Of 42 strains, 40 (36 *C. parapsilosis sensu stricto*, three *C. metapsilosis* and one *C. orthopsilosis*) retained viability at the time of antifungal susceptibility testing and were included in the assay. Fluconazole (Pfizer Inc., Groton, Connecticut, USA), voriconazole (Pfizer Inc., Sandwich, Kent, UK), caspofungin (Merck & Co., White House Station, New Jersey, USA), anidulafungin (Pfizer Inc., Groton, Connecticut, USA) and amphotericin B (Bristol-Myers Squibb Co., Middlesex, UK) were provided in standard powder forms by the respective manufacturers. As recommended by CLSI, antifungal stock solutions were prepared in water for fluconazole and caspofungin and in dimethylsulphoxide (DMSO) for anidulafungin, voriconazole, and amphotericin B. Antifungal concentration ranges were as follows: fluconazole and anidulafungin 64–0.125 µg/ml; voriconazole and amphotericin B 8–0.015 µg/ml; and caspofungin 16–0.03 µg/ml. The minimum inhibitory concentrations (MICs) were determined by visual reading at 24 and 48 h of incubation at 35°C. The MIC recorded was the lowest antifungal concentration reducing turbidity by approximately 50% (MIC-2) for all antifungals, with the exception of amphotericin B [15]. For amphotericin B, the lowest drug concentration that prevented any discernible growth (MIC-0) was recorded as the MIC [15]. *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were included as quality controls in each test run [16]. Clinical breakpoints proposed for

C. parapsilosis by Pfaller *et al*. were used for interpretation of the MIC results obtained for flucanozole, voriconazole, caspofungin and anidulafungin [17]. Since amphotericin B clinical breakpoints have not been validated, only MIC distributions were depicted.

Results

Of 42 *C. parapsilosis* complex isolates, three were identified as *C. metapsilosis* and one as *C. orthopsilosis*. The distribution of the three species in different clinical samples is detailed in Table 1 and as noted most were isolated from urine (47.6%) and blood (21.4%) specimens. The distribution of the three species among nine blood isolates was 77.8% (7/9) *C. parapsilosis sensu stricto* and 22.2% (2/9) *C. metapsilosis*, with no isolates of *C. orthopsilosis* recovered from these samples. *C. parapsilosis sensu stricto* was isolated from patients of all age groups, whereas *C. orthopsilosis* and *C. metapsilosis* were isolated from elderly patients.

The sequences of ITS region of three *C. metapsilosis* isolates (length of analyzed sequences: 403–530 nts) and one *C. orthopsilosis* strain (length of analyzed sequence: 442 nts) were identical and showed 100% identity with the corresponding sequences. The ITS region sequences of all strains showed similarities with the corresponding sequences of the reference *C. parapsilosis* (FJ872015.1), *C. orthopsilosis* (EU564208), and *C. metapsilosis* (EU564207.1) strains. Similar results were found by SADH-RFLP.

All isolates grew in TPN, with some *C. parapsilosis* and one *C. metapsilosis* strain reaching their maximum concentration within 48 h. Colony counts of the *C. orthopsilosis* strain, two of the three *C. metapsilosis* strains, and nine of the 39 *C. parapsilosis sensu stricto* strains did not increase prominently after 24 h (Table 2).

A total of 11 (26.2%) of the 42 *C. parapsilosis* complex isolates were biofilm-positive (Table 2), all of which were *C. parapsilosis sensu stricto*. Urine isolates were more likely to form biofilms than isolates from other sources.

Table 1 Distribution of *Candida parapsilosis* complex isolates.

Site of isolation	*C. parapsilosis sensu stricto*	*C. metapsilosis*	*C. orthopsilosis*	Total
Urine	20	–	–	20
Blood	7	2	–	9
Respiratory tract	3	1	–	4
Ear	3	–	1	4
Skin	3	–	–	3
Mucosal surface	2	–	–	2
Total	38	3	1	42

Lipase activity was detected in 33.3% (14/42) of isolates (Table 2). Urine-derived isolates had the most lipase activity. A total of 13/42 (31%) *C. parapsilosis* complex isolates were found to produce acid proteinase, six (46%) of which had strong activity (Table 2). Isolates from specimens other than blood possessed strong proteinase activity. None of the *C. metapsilosis* or *C. orthopsilosis* isolates exhibited proteinase activity, although all isolates possessed genes encoding the enzymes (*SAPP1–3*; Fig. 2).

MIC results obtained for isolates of *C. parapsilosis sensu stricto* are shown in Table 3a and those recorded for *C. metapsilosis* or *C. orthopsilosis* are depicted in Table 3b. The isolates of *C. parapsilosis sensu stricto* appeared highly susceptible to antifungal drugs in general, only one isolate being dose-dependent susceptible to fluconazole. All remained susceptible to voriconazole, caspofungin, and anidulafungin (Table 3a). In general, the MICs obtained for the *C. metapsilosis* and *C. orthopsilosis* isolates did not display differences as compared to those for *C. parapsilosis sensu stricto* (Table 3b).

Discussion

Candida parapsilosis is a ubiquitous microorganism in the natural environment. Its virulence is associated with its capacity to adhere to plasic surfaces, and consequently, to the development of candidemia related to catheters [18,19]. Recently, Tavanti *et al*. [4] described two new species belonging to the *C. parapsilosis* complex, *C. metapsilosis* and *C. orthopsilosis*. *C. metapsilosis* was less virulent *in vitro* as compared to *C. orthopsilosis* and *C. parapsilosis sensu stricto* [20,21]. These two new species may inhabit important niches in certain patient populations, thus stressing the need for continued surveillance in each country to monitor the prevalence and distribution of the members of the complex [22]. Defining the virulence determinants of this new species will help to understand their pathogenic characteristics.

After SADH PCR-RFLP identification of *C. parapsilosis* complex isolates, the identity of all isolates was confirmed by PCR-based sequencing using primers for ITS1 and ITS4. We found that 2/3 *C. metapsilosis* were recovered from blood, and only one *C. orthopsilosis* was obtained from an ear sample. Lockhart *et al*. collected isolates of *C. parapsilosis* between 2001 and 2006 as part of the ARTEMIS Global Surveillance study [6]. *C. parapsilosis* accounted for 91.3% of the isolates, while 6.1% were *C. orthopsilosis* and 1.8% were *C. metapsilosis*. In addition, *C. orthopsilosis* increased longitudinally during this surveillance study. A population-based study from Spain found that *C. metapsilosis* comprised 6.9% and *C. orthopsilosis* 5.7% of all *C. parapsilosis* complex isolates collected between 2002 and 2003 [8]. Silva *et al*. reported

Table 2 Phenotypic properties of *Candida parapsilosis* complex isolates.

No.	Strain	Isolation site	Species	Growth in TPN		Lipase	Proteinase	biofilm
				24 h	48 h			
1	C-38	blood	*C. parapsilosis*	2.2×10^6	5.3×10^7	+	0.94[b]	+
2	C-39	urine	*C. parapsilosis*	1.2×10^7	3.7×10^7	−	0.94	−
3	C-40	urine	*C. parapsilosis*	2.2×10^6	1.9×10^7	+	0.78	−
4	C-41	urine	*C. parapsilosis*	2.8×10^6	7×10^6	+	1.0	−
5	C-52	urine	*C. parapsilosis*	3×10^6	1.3×10^7	−	1.0	−
6	C-64	urine	*C. parapsilosis*	7.5×10^6	1.2×10^7	+	0.76	−
7	C-68	urine	*C. parapsilosis*	1.5×10^6	8.6×10^7	+	1.0	−
8	C-73	urine	*C. parapsilosis*	1.5×10^6	4.5×10^7	+	1.0	+
9	C-81	Oral swab	*C. parapsilosis*	3.4×10^6	5.6×10^6	+	1.0	−
10	C-120	urine	*C. parapsilosis*	1.2×10^7	9.1×10^7	−	1.0	+
11	C-121	blood	*C. parapsilosis*	3.1×10^7	1.3×10^8	−	1.0	+
12	C-139	urine	*C. parapsilosis*	1.5×10^7	6×10^7	−	1.0	−
13	C-141	urine	*C. parapsilosis*	1.1×10^7	1.3×10^7	+	0.72	+
14	C-170	blood	*C. parapsilosis*	9.3×10^6	1×10^7	−	1.0	−
15	C-171	blood	*C. parapsilosis*	2.6×10^6	5.7×10^6	−	1.0	−
16	C-174	urine	*C. parapsilosis*	1.5×10^6	4.6×10^7	−	1.0	+
17	C-177	urine	*C. parapsilosis*	4.5×10^6	6.2×10^6	−	1.0	−
18	C-196	skin	*C. parapsilosis*	8.4×10^6	5.4×10^7	−	1.0	−
19	C-203	blood	*C. parapsilosis*	4.5×10^6	1.3×10^7	−	1.0	−
20	C-231	urine	*C. parapsilosis*	4.8×10^6	6.4×10^7	−	1.0	+
21	C-232	skin	*C. parapsilosis*	4.6×10^6	4.8×10^7	−	1.0	−
22	C-249	ETA[a]	*C. parapsilosis*	1×10^7	1.8×10^7	−	0.78	−
23	C-269	urine	*C. parapsilosis*	9.8×10^6	5.8×10^7	−	1.0	−
24	C-275	urine	*C. parapsilosis*	1.2×10^7	1.6×10^7	−	1.0	−
25	C-276	blood	*C. parapsilosis*	6.7×10^6	9.8×10^7	+	1.0	+
26	C-282	skin	*C. parapsilosis*	6.7×10^6	1.1×10^8	−	1.0	−
27	C-287	blood	*C. parapsilosis*	3.9×10^6	7.2×10^7	+	0.90	−
28	C-292	urine	*C. parapsilosis*	6.2×10^6	1.4×10^7	−	0.87	−
29	C-308	urine	*C. parapsilosis*	3×10^6	5.1×10^7	−	1.0	+
30	C-311	urine	*C. parapsilosis*	6.3×10^6	2.3×10^7	+	0.74	+
31	C-328	ETA[a]	*C. metapsilosis*	4.1×10^6	5.6×10^6	−	1.0	−
32	C-339	ear	*C. parapsilosis*	7.7×10^6	1.3×10^7	+	1.0	−
33	C-348	urine	*C. parapsilosis*	8.1×10^6	9.3×10^6	−	1.0	−
34	C-351	ear	*C. orthopsilosis*	4.7×10^6	4.5×10^6	−	1.0	−
35	C-352	ETA	*C. parapsilosis*	6.1×10^6	4.3×10^7	+	0.87	−
36	C-357	blood	*C. metapsilosis*	5.3×10^6	1.4×10^7	−	1.0	−
37	C-359	ear	*C. parapsilosis*	7.3×10^6	9.6×10^7	+	0.74	+
38	C-362	ear	*C. parapsilosis*	4.8×10^6	4.5×10^7	−	0.96	−
39	C-394	ETA	*C. parapsilosis*	9×10^6	1×10^7	−	1.0	−
40	C-396	urine	*C. parapsilosis*	7.6×10^6	4.9×10^7	−	1.0	−
41	C-397	blood	*C. metapsilosis*	2.6×10^6	9.4×10^6	−	1.0	−
42	C-419	Oral swab	*C. parapsilosis*	6.3×10^6	7.4×10^7	−	0.81	−

TPN, total parenteral nutrition solution; [a]ETA, endotracheal aspirate; [b]Radius of proteolysis. Proteinase activity (Pz) was considered as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone, Pz = 1; negative, Pz, 0.90–0.99 as weak, 0.80–0.89 as mild, < 0.79 as strong.

91.4% *C. parapsilosis*, 2.3% *C. orthopsilosis*, and 2.9% *C. metapsilosis* in their study [22]. New species were found in biological samples other than blood. Canton *et al*. identified *C. parapsilosis* (90.7%), *C. orthopsilosis* (8.2%), and *C. metapsilosis* (1.1%) in candidemia patients [23]. Most candidemias (*C. parapsilosis*, 76.4%; *C. orthopsilosis*, 70.0%; *C. metapsilosis*, 100%) were observed in adults.

Lockhart *et al*. [6] identified, among 75 yeast isolates from Turkey, one *C. ortopsilosis* and two *C. metapsilosis* strains, but there was no information regarding the clinical samples from which they were recovered or regional distribution. In our previous study, between October 2005 and July 2009, 98.5% (67/68) of the isolates we identified were *C. parapsilosis sensu stricto*, and 1.5% (1/68) was identifed as *C. orthopsilosis*, whereas no *C. metapsilosis*

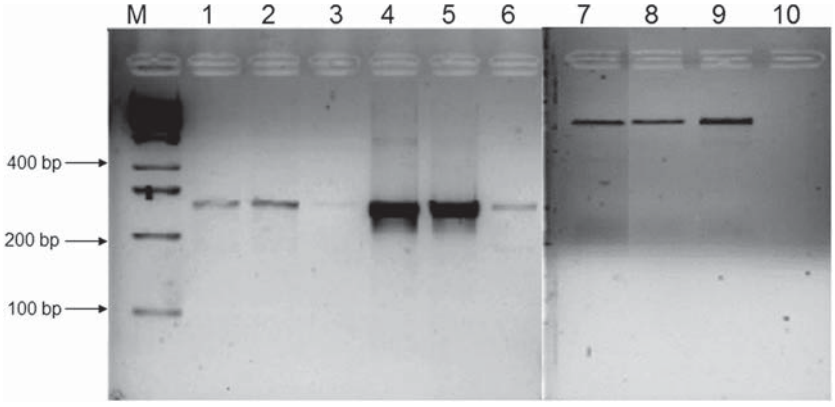

Fig. 2 *SAPP* gene amplification product of *Candida parapsilosis* complex. Lane M, 100-bp ladder; Lane 1 *C. parapsilosis SAPP1*; Lane 2 *C. orthopsilosis SAPP1*; Lane 3 *C. metapsilosis SAPP1*; Lane 4 *C. parapsilosis SAPP2*; Lane 5 *C. orhtopsilosis SAPP2*; Lane 6 *C. metapsilosis SAPP2*; Lane 7 *C. parapsilosis SAPP3*; Lane 8 *C. orthopsilosis SAPP3*; Lane 9 *C. metapsilosis SAPP3*.

isolates were detected. The strain identified as *C. orthopsilosis* was from a urine specimen, and all the blood isolates were *C. parapsilosis sensu stricto* [24]. *C. metapsilosis* has been reported to be recovered from clinical samples only rarely [25]. Interestingly, *C. metapsilosis* was increasingly found during the study period in our hospital. These new species are ubiquitous in nature, and their potential for infection is not well known. Ghannoum *et al*. [26] isolated *C. metapsilosis* from oral swabs, suggesting that this species could also be a human commensal microorganism. Ge *et al*. [12] reported a high frequency of isolation of *C. metapsilosis* (35.5%) from skin samples. Whether the isolation of new species reveals geographic regional differences in various clinical samples will be evaluated by future studies. In light of these findings, it is likely that new species will increasingly become the agents of future infections.

C. parapsilosis can be a member of the human microbiome, but possesses the ability to grow in parenteral nutrition solutions, to form biofilms on catheters, to spread by hand carriage, and to persist in the nosocomial environment [3]. Although *C. metapsilosis* is less virulent than *C. parapsilosis sensu stricto* and *C. orthopsilosis*, some authors have reported the isolation of this species from bloodstream infections (BSI) [8,27,28]. The ability of

Table 3 MIC values and antifungal susceptibility profiles of *Candida parapsilosis* (a), *C. metapsilosis*, and *C. orthopsilosis* (b) strains included in the study*.

a)

Species/Antifungal drug *Candida parapsilosis sensu stricto* ($n = 36$)	MIC_{50}	MIC_{90}	MIC range	S%	S-DD%	R%
Fluconazole						
24 h	0.5	1	≤0.125–4	97.2	2.8	0
48 h	0.5	1	≤0.125–4	97.2	2.8	0
Voriconazole						
24 h	≤0.015	≤0.015	≤0.015–0.03	100	0	0
48 h	≤0.015	0.03	≤0.015–0.06	100	0	0
Amphotericin B						
24 h	1	1	0.5–2	ND	ND	ND
48 h	2	2	1–2	ND	ND	ND
Caspofungin						
24 h	1	1	0.5–1	100	0	0
48 h	1	1	1	100	0	0
Anidulafungin						
24 h	2	2	0.5–2	100	0	0
48 h	2	2	0.5–2	100	0	0

**In vitro* susceptibility categories were determined using the currently proposed CLSI clinical breakpoints for *C. parapsilosis* (17). ND, Not determined due to lack of validated clinical breakpoints; S, susceptible; S-DD, dose-dependent susceptible; R, resistant.

b)

Species	MIC range (μg/ml)									
	FLC		VRC		AMB		CAS		ANID	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
C. metapsilosis ($n = 3$)	0.25–1	0.5–1	≤0.015	≤0.015	1	2	0.5	0.5	0.25–0.5	0.25–0.5
C. orthopsilosis ($n = 1$)	1	1	≤0.015	≤0.015	1	2	0.5	0.5	1	1

FLC, fluconazole; VRC, voriconazole; AMB, amphotericin B; CAS, caspofungin; ANID, anidulafungin.

C. albicans and other pathogens to grow in parenteral nutrition formulations is known and is considered to be a risk factor for the development of catheter-related BSI in patients receiving parenteral nutrition [29]. Swindel *et al*. [2] showed that parenteral lipid emulsion induced germination and supported the growth of *C. albicans*, increasing biofilm formation on medical catheter surfaces. In this study, viable counts of all strains increased at least two log in TPN after 24 h incubation. After 48 h, slight reduction was seen in colony counts of the *C. orthopsilosis* strain, but viable counts of *C. parapsilosis sensu stricto* and *C. metapsilosis* strains increased. *C. orthopsilosis* was isolated from an ear-derived sample. These findings should be confirmed, but the characteristics of biofilm formation on catheter materials and growth in TPN could be virulence factors of *C. orthopsilosis* and *C. metapsilosis*, as much as *C. parapsilosis sensu stricto*.

C. parapsilosis biofilms tend to be thinner, less structured, and consist almost exclusively of aggregated blastospores [30]. Interestingly, biofilm formation by *C. parapsilosis* has been described as being strongly strain-dependent [31,32]. Additionally, two newly identified species of the *C. parapsilosis* complex, *C. metapsilosis and C. orthopsilosis*, have also been shown to be capable of forming biofilms on silicone elastomer discs [33]. Biofilms are readily formed by *C. parapsilosis* cells grown in media containing high glucose and lipid concentrations, which is analogous to BSI in patients receiving parenteral nutrition. Notably, biofilm-deficient *C. parapsilosis* lipase mutants were less virulent in tissue culture infection models and also in mice [20]. A total of 11 (26.2%) of the 42 *C. parapsilosis* complex isolates were biofilm-positive. *C. parapsilosis sensu stricto* was the only species to form biofilms in some studies [7,25,34]. In contrast, Lattif *et al*. [33] showed that all three species were able to form biofilms using an *in vitro* model of silicone catheter discs. Discrepancies between these studies may be due to the use of different strains and methods.

Secretion of hydrolytic enzymes, such as aspartic proteinases, phospolipases, and lipases has been associated with *C. parapsilosis* virulence. Gacser and his colleagues [20] showed that the reduction in damage to reconstituted human tissues (RHT) by ebelactone B and pepstatin A indicated that lipases and aspartic proteinases are involved in the pathogenesis of disease caused by *C. parapsilosis*. It was also observed that *C. orthopsilosis* induced a high level of macrophage damage, similar to *C. parapsilosis sensu stricto* isolates, while *C. metapsilosis* exhibited the lowest cytotoxicity.

Lipases are involved in both the hydrolysis and synthesis of triacylgycerols. For *C. parapsilosis*, the presence of the CpLIP1 and CpLIP2 genes has been reported, with the latter known to encode for an active protein [33]. Recently, Gacser *et al*. [20] demonstrated that a lipase inhibitor significantly reduced tissue damage during *C. parapsilosis* infection of RHT, and that CpLIP1 and CpLIP2 mutants formed thinner, less complex biofilms and had reduced growth in lipid-rich media. In our study, lipase activity was detected in 33.3% of isolates. Lipase enzyme activity of the strains of these new species is needed to be studied in a large number of strains.

In *C. parapsilosis*, three orthologous genes have been found to-date, i.e., *SAPP1–3*. Of the respective proteins, only Sapp1p and Sapp2p have been studied extensively on an enzymological level. The *SAPP3* gene product has not yet been isolated or cloned [36]. In addition, Horváth *et al*. [37] demostrated that the *SAPP1* gene has two copies in the *C. parapsilosis* genome (*SAPP1a and SAPP1b*). They showed that the *C. parapsilosis* Δ Δsapp1a- Δ Δsapp1b mutant was unable to escape human immune system components. Silva *et al*. [31] showed that the expression of SAP genes by *C. parapsilosis* was strain-dependent, with *SAPP1* being expressed by all isolates causing infections. In our study, 31% of the *C. parapsilosis sensu stricto* isolates were found to be acid proteinase producers, with urine-derived isolates having higher SAP activities. None of the *C. metapsilosis* or *C. orthopsilosis* strains demonstrated SAP enzyme activity. Lin *et al*. [38] reported that group I isolates showed strong or moderately strong proteinase activity, whereas groups II and III isolates had low proteinase activity. Tavanti *et al*. [39] determined that 33.9% of *C. parapsilosis sensu stricto* strains produced proteinase, the higher producers being recovered from blood and mucus specimens. In addition, expression of some virulence factors varied among strains isolated from different geographical

regions. Sabino *et al*. [40] showed that *C. orthopsilosis* were SAP producers, whereas *C. metapsilosis* were not. Hensgens *et al*. [41] found five isolates (40%) of *C. metapsilosis* isolates were proteinase producers. In another study, [12] some non-blood *C. parapsilosis* and *C. metapsilosis* isolates exibited higher proteinase activities. Reasons for the variability between our study and others may be due to method or strains isolated from different geographical samples. All isolates had *SAPP1–3* genes. The *in vivo* expression of these genes in new species should be evaluated in infection models.

We also investigated the antifungal susceptibility profiles of our strains. With the exception of one fluconazole-dose-dependent susceptible strain of *C. parapsilosis sensu stricto*, all isolates exhibited low *in vitro* MICs and were susceptible to voriconazole, caspofungin, and anidulafungin using *C. parapsilosis*-specific CLSI interpretive MIC breakpoints [17]. We also compared the susceptibility profiles of *C. parapsilosis sensu stricto* isolates to those of *C. metapsilosis* and *C. orthopsilosis* and found that their MICs were not significantly different as compared to those generated for *C. parapsilosis sensu stricto*. Moreover, using the breakpoints proposed for *C. parapsilosis* for interpretation of the results of *C. metapsilosis* and *C. orthopsilosis*, these two species also appear susceptible to the noted antifungal drugs. However, our data remain inadequate for justification of any comparative conclusion on *in vitro* susceptibility patterns since the number of the *C. metapsilosis* and *C. orthopsilosis* strains included in the study is very small (n-3 and 1, respectively).

Antifungal susceptibility profiles of *C. parapsilosis* complex strains have been previously investigated in various studies [42–45], but the results in most of these [44,45] refer to *C. parapsilosis* complex rather than individual species of *C. parapsilosis* complex. In these studies, including ARTEMIS disk antifungal surveillance program (comprising strains from 124 medical centers worldwide), high levels of susceptibility to both fluconazole and voriconazole, as well as relatively low MICs of caspofungin, micafungin, and anidulafungin (≤ 2 μg/ml) have been reported, suggesting that *C. parapsilosis* strains are, in generally, highly susceptible to azoles and echinocandins [42–45]. These results are in accordance with the high levels of azole and echinocandin susceptibility that we have detected for our strains.

There are limited antifungal susceptibility data published for isolates of the individual species that comprise the *C. parapsilosis* complex [22,23,27,44,46]. Furthermore, the recent modifications in species-specific MIC breakpoints render the resistance rates reported in different studies difficult to compare. In general, reduced susceptibility and relatively high MICs of fluconazole (≥ 8 μg/ml) have been described for some but a few isolates of species of *C. metapsilosis* [27,41] and *C. orthopsilosis* [25,47]. High fluconazole and echinocandin MICs (≥ 8 μg/ml) have been detected in a low number of strains of *C. parapsilosis sensu stricto* as well (46). The azole and echinocandin MICs generated in our hands for our strains belonging to *C. metapsilosis* and *C. orthopsilosis* are relatively low and similar to those obtained for *C. parapsilosis sensu stricto.* However and as noted previously, testing more isolates is required for determination of virtual rates of resistance among the strains of the two former species.

Susceptibility of *C. parapsilosis* complex strains to amphotericin B has also been studied by various investigators [22,23,25,27,41,45]. However, interpretive criteria for assessment of *in vitro* susceptibility test results for amphotericin B has been problematic so far against all *Candida* spp., including *C. parapsilosis* and clinical MIC breakpoints are not yet available. Using the epidemiological cutoff values, strains with amphotericin B MICs of ≤ 2 μg/ml are now classified as wild-type strains [17] and all of our strains fall into this category (Table 3). Amphotericin B MICs reported by other investigators of isolates of *C. parapsilosis* complex also appear, in general, to fall within the category of wild-type in general [22,23,25,27,41,45].

In the present study, *C. parapsilosis sensu stricto* was recovered from all types of clinical samples, but interestingly, we isolated more *C. metapsilosis* strains as compared to those of *C. orthopsilosis* which differs from other studies [6,23,46]. To the best of our knowledge, this is the first study where lipase activity has been evaluated for the *C. parapsilosis* complex. Differences in virulence factors between species could not be determined at a statistically significant level because of the small numbers of the newly described isolates. It is not yet clear whether these species pose a special risk for some patient groups or if there are regional disparities. The virulence properties of these new species should be tested in a wider collection of isolates. Virulence characterization of the new species will guide treatment options against them.

Acknowledgements

We thank Professor Frank Odds and Dr Donna MacCallum (School of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, UK) for providing us the J960161 (*C. metapsilosis*), J981226 (*C. orthopsilosis*), and AM2001/0013 (*C. parapsilosis*) strains.

Declaration of interest: This work was supported by the University of Karadeniz Technical Research Fund (2009.114.001.10), which was awarded to I.T. S.A.A. does not have any potential conflicts of interests related particularly to this paper. Otherwise, she has received

investigator initiated research grant support from Pfizer and speaker honoraria from Merck and Pfizer. She has been at the Advisory Board for Pfizer-Turkey.

References

1 Shin JH, Kee SJ, Shin MG, *et al*. Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. *J Clin Microbiol* 2002; **40**: 1244–12488.

2 Swindell K, Abdul A, Jyotsna C, Mukherjee PK, Ghannoum MA. Parenteral lipid emulsion induces germination of *Candida albicans* and increases biofilm formation on medical catheter surfaces. *J Infect Dis* 2009; **200**: 473–480.

3 Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 2007; **20**: 133–163.

4 Tavanti A, Davidson AD, Gow NAR, Maiden MCJ, Odds FC. *Candida orthopsilosis* and *Candida metapsilosis spp*. nov. to replace Candida parapsilosis groups II and III. *J Clin Microbiol* 2005; **43**: 284–292.

5 Cassone A, De BF, Pontieri E, *et al*. Biotype diversity of *Candida parapsilosis* and its relationship to the clinical source and experimental pathogenicity. *J Infect Dis* 1995; **171**: 967–975.

6 Lockhart SR, Messer SA, Pfaller MA, *et al*. Geographic distribution and antifungal susceptibility of the newly described species *Candida orthopsilosis* and *Candida metapsilosis* in comparison to the closely related species *Candida parapsilosis*. *J Clin Microbiol* 2008; **46**: 2659–2664.

7 De Toro M, Torres MJ, Maite R, *et al*. Characterization of *Candida parapsilosis* complex isolates. *Clin Microbiol Infect* 2011; **17**: 418–424.

8 Gomez-Lopez A, Alastruey-Izquierdo A, Rodriguez D, *et al*. Prevalence and susceptibility profile of *Candida metapsilosis* and *Candida orthopsilosis*: results from population-based surveillance of candidemia in Spain. *Antimicrob Agents Chemother* 2008; **52**: 1506–1509.

9 White TJ, Bruns T, Lee S, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds). *PCR Protocols*: *A Guide to Methods and Applications*, New York: Academic Press, 1990: 315–322.

10 Silva S, Henriques M, Oliveira R, *et al*. Characterization of *Candida parapsilosis* infection of an *in vitro*-reconstituted human oral epithelium. *Eur J Oral Sci* 2009; **117**: 669–675.

11 Dostal J, Hamal P, Pavlickova L, *et al*. Simple method for screening *Candida* species isolates for the presence of secreted proteinases: a tool for the prediction of successful inhibitory treatment. *J Clin Microbiol* 2003; **41**: 712–716.

12 Ge YP, Lu GX, Shen YN, Liu WD. *In vitro* evaluation of phospholipase, proteinase, and esterase activities of *Candida parapsilosis* and *Candida metapsilosis*. *Mycopathologia* 2011; **172**: 429–438.

13 Gacser A, Trofa D, Schafer W, Nosanchuk JD. Targeted gene deletion in *Candida parapsilosis* demonstrates the role of secreted lipase in virulence. *J Clin Invest* 2007; **117**: 3049–3058.

14 Pierce CG, Uppuluri P, Tristan AR, *et al*. A simple and reproducible 96 well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat Protoc* 2008; **3**: 1494–1500.

15 Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*, 3rd ed., approved standard M27-A3. Wayne, PA: Clinical and Laboratory Standards Institute, 2008.

16 Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*, 3rd suppl., M27-S3. Wayne, PA: Clinical and Laboratory Standards Institute, 2008.

17 Pfaller MA, Diekema DJ. Progress in antifungal susceptibility testing of *Candida* spp. using Clinical and Laboratory Standards Institute Broth Microdilution Methods, 2010–2012. *J Clin Microbiol* 2012; **50**: 2846–2856.

18 Bernardis F, Mondello F, Millan RS, Ponton J, cassone A. Biotyping and virulence properties of skin isolates of *Candida parapsilosis*. *J Clin Microbiol* 1999; **37**: 3481–3486.

19 Douglas LJ. Candida biofilms and their role in infection. *Trends Microbiol* 2003; **11**: 30–36.

20 Gacser A, Schafer W, Nosanchuk JS, Salomon S, Nosanchuk JD. Virulence of *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* in reconstituted human tissue models. *Fungal Genet Biol* 2007; **44**: 1336–1341.

21 Orsi CF, Colombari B, Blasi E. *Candida metapsilosis* as the least virulent member of the "*C. parapsilosis*" complex. *Med Mycol* 2010; **48**: 1024–1033.

22 Silva AP, Miranda IM, Lisboa C, Pina-Vaz C, Rodrigues AG. Prevalence, distribution and antifungal susceptibility profiles of *Candida parapsilosis, C. orthopsilosis,* and C. metapsilosis in a tertiary care hospital. *J Clin Microbiol* 2009; **47**: 2392–2397.

23 Canton E, Peman J, Quindos G, *et al*. Prospective multicenter study of the epidemiology, molecular identification, and antifungal susceptibility of *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* isolated from patients with candidemia. *Antimicrob Agents Chemother* 2011; **55**: 5590–5596.

24 Cebeci Guler N, Tosun I, Bayramoğlu G, Buruk K, Aydın F. Genotypic identification and distribution patterns of *Candida parapsilosis* complex species (*C. parapsilosis* sensu stricto, *C. metapsilosis* and *C. orthopsilosis*) isolated from clinical samples. *Mikrobiyol Bul* 2011; **45**: 723–728.

25 Tavanti A, Hensgens LA, Ghelardi E, Campa M, Senesi S. Genotyping of *Candida orthopsilosis* clinical isolates by amplification fragment length polymorphism reveals genetic diversity among independent isolates and strain maintenance within patients. *J Clin Microbiol* 2007; **45**: 1455–1462.

26 Ghannoum MA, Jurevic RJ, Mukherjee PK, *et al*. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PloS Pathog* 2010; **6**: e1000713.

27 Miranda-Zapico I, Eraso E, Hernández-Almaraz JL. Prevalence and antifungal susceptibility patterns of new cryptic species inside the species complexes *Candida parapsilosis* and *Candida glabrata* among blood isolates from a Spanish tertiary hospital. *J Antimicrob Chemother* 2011; **66**: 2315–2322.

28 Kocsube S, Toth M, Vaguölgyi C, *et al*. Occurrence and genetic variability of *Candida parapsilosis* sensu lato in Hungary. *J Med Microbiol* 2007; **56**: 190–195.

29 O'Grady NP, Alexander M, Dellinger EP, *et al*. Guidelines for the prevention of intravascular catheter-related infections. *Pediatrics* 2002; **110**: e51

30 Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immun* 2002; **7**: 878–888.

31 Silva S, Henriques M, Martins A, *et al*. Biofilms of non-*Candida albicans Candida* species: quantification, structure and matrix composition. *Med Mycol* 2009; **47**: 681–689.

32 Silva S, Henriques M, Oliveira R, Williams D, Azeredo J. *In vitro* biofilm activity of non-*Candida albicans Candida* species. *Curr Microbiol* 2010; **61**: 534–540.

33 Lattif AA, Mukherjee PK, Chandra J, *et al*. Characterization of biofilms formed by *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis*. *Int J Med Microbiol* 2010; **300**: 265–270.
34 Song JW, Shin JH, Shint DH, *et al*. Differences in biofilms production by three genotypes of *Candida parapsilosis* from clinical sources. *Med Mycol* 2005; **43**: 657–661.
35 Neugnot V, Moulin G, Dubreucq E, Bigey F. The lipase/acyltransferase from *Candida parapsilosis*: molecular cloning and characterization of purified recombinant enzymes. *Eur J Biochem* 2002; **269**: 1734–1745.
36 Heidingsfeldova-Hruskova O, Dostal J, Majer F, *et al*. Two aspartic proteinases secreted by the pathogenic yeast *Candida parapsilosis* differ in expression pattern and catalytic properties. *Biol Chem* 2009; **390**: 259–268.
37 Horváth P, Nosanchuk JD, Hamari Z, Vágvölgyi C, Gácser A. The identification of gene duplication and the role of secreted aspartyl proteinase 1 in *Candida parapsilosis* virulence. *J Infect Dis* 2012; **205**: 923–393.
38 Lin D, Wu LC, Rinaldi MG, Lehmann PF. Three distinct genotypes within *Candida parapsilosis* from clinical sources. *J Clin Microbiol* 1995, **33**: 1815–1821.
39 Tavanti A, Hensgens LA, Mogavero S, *et al*. Genotypic and phenotypic properties of *Candida parapsilosis sensu stricto* strains isolated from different geographic regions and body sites. *BMC Microbiol* 2010; **10**: 203–214.
40 Sabino R, Sampaio P, Carneiro C, Rosado L, Pais C. Isolates from hospital environments are the most virulent of the *Candida parapsilosis* complex. *BMC Microbiol* 2011; **11**: 180–189.

This paper was first published online on Early Online on 6 December 2012.

41 Hensgens LAM, Tavanti A, Mogavero S, Ghelardi E, Senesi S. AFLP genotyping of *C. metapsilosis* clinical isolates: evidence for recombination. *Fungal Genet Biol* 2009; **46**: 750–758.
42 Pfaller MA, Diekema DJ, Gibbs DL, *et al*. Geographic and temporal trends in isolation and antifungal susceptibility of *Candida parapsilosis*: a global assessment from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. *J Clin Microbiol* 2008; **46**: 842–849.
43 Pfaller MA, Chaturvedi V, Diekema DJ, *et al*. Comparison of the Sensititre YeastOne colorimetric antifungal panel with CLSI microdilution for antifungal susceptibility testing of the echinocandins agaist *Candida* spp. using new clinical breakpoints epidemiological cutoff values. *Diagn Microbiol Infect Dis* 2012; **73**: 365–368.
44 Pfaller MA, Woosley LN, Messer SA, Jones RN, Castanheira M. Significance of molecular identification and antifungal susceptibility of clinically significant yeasts and moulds in a global antifungal surveillance programme. *Mycopathologia* 2012; **174**: 259–271.
45 Cordoba S, Vivot W, Bosco-Borgeat ME, *et al*. Species distribution and susceptibility profile of yeasts isolated from blood cultures: results of a multicenter active laboratory-based surveillance study in Argentina. *Rev Argentina Microbiol* 2011; **43**: 176–185.
46 Treviño-Rangel RD, Garza-González E, González JG, *et al*. Molecular characterization and antifungal susceptibility of the *Candida parapsilosis* species complex of clinical isolates from Monterrey, Mexico. *Med Mycol* 2012; **50**: 781–784.