The persistence of multifocal colonisation by a single ABC genotype of *Candida albicans* may predict the transition from commensalism to infection

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Candida albicans *is a common member of the human microbiota and may cause invasive disease in susceptible populations. Several risk factors have been proposed for candidaemia acquisition. Previous* Candida *multifocal colonisation among hospitalised patients may be crucial for the successful establishment of candidaemia. Nevertheless, it is still not clear whether the persistence or replacement of a single clone of* C. albicans *in multiple anatomical sites of the organism may represent an additional risk for candidaemia acquisition. Therefore, we prospectively evaluated the dynamics of the colonising strains of* C. albicans *for two groups of seven critically ill patients: group I included patients colonised by* C. albicans *in multiple sites who did not develop candidaemia and group II included patients who were colonised and who developed candidaemia. ABC and microsatellite genotyping of 51 strains of* C. albicans *revealed that patients who did not develop candidaemia were multiply colonised by at least two ABC genotypes of* C. albicans*, whereas candidaemic patients had highly related microsatellites and the same ABC genotype in colonising and bloodstream isolates that were probably present in different body sites before the onset of candidaemia.*

Key words: *Candida albicans* - genotyping - colonisation - bloodstream infection - strain maintenance or replacement

Candida species have been implicated in a variety of human diseases. *Candida albicans* is the most prevalent species isolated from yeast bloodstream infections and is associated with high rates of morbidity and mortality (Wey et al. 1988, Gudlaugsson et al. 2003). The risk factors for developing candidaemia include prematurity, azotaemia, central venous catheter use, chemotherapy, mucous membrane barrier breakage, broad spectrum antibiotics, neutropaenia, total parenteral nutrition, steroids and surgery. Specific patient groups vulnerable to candidaemia include critically ill intensive care unit (ICU) patients, neonates and patients with haematological or solid malignancies (Colombo & Guimarães 2003, Almirante et al. 2005, Eggimann et al. 2005, Colombo et al. 2006, Otrosky-Zeichner et al. 2011).

Candida spp colonisation is an independent risk factor for candidaemia, but it is well established that a minority of colonised patients will acquire invasive diseases. Despite the fact that 3-60% of patients may be colonised by *Candida* spp during hospitalisation, less than 5% of them will develop candidaemia (Pittet et al. 1994, Cornwell et al. 1995, Borzotta & Beardsley 1999, Godoy et al. 2003, Marchetti et al. 2004, Leon et al. 2006).

Previous authors have suggested that the risk of candidaemia increases with the number of body sites colonised by *Candida*. The intensity of *Candida* colonisation in critically ill patients may predict subsequent infections with identical strains (Pittet et al. 1994, Vincent et al. 1998). Recently, Leon et al. (2006) developed a scoring system named the "*Candida* score" and proved that patients with *Candida* multifocal colonisation had higher overall mortality rates compared to those patients with unifocal colonisation by this yeast. The calculation of a *Candida* score was based on the variables generated from a logistic regression model that evaluated the risk factors independently associated with candidaemia. The authors rounded the score for patients with exposure to total parenteral nutrition, surgery or multifocal *Candida* species colonisation up to 1 and the weight for clinically severe sepsis up to 2 and established a cut-off value of 2.5. Therefore, critically ill patients with candidaemia who had a score of 2.5 were considered to be 7.75 times more likely to have a proven infection than patients with a *Candida* score up to 2.5.

It is clear that the number of body sites colonised by the *Candida* species impacts the risk of acquiring candidaemia; however, whether the persistence or replacement of a single clone of *C. albicans* in multiple anatomical sites of the organism may represent an additional risk in the transition from commensal to invasive candidiasis has not been largely investigated.

By evaluating the genotypes of 165 *C. albicans* isolates using multilocus sequence typing (MLST) and ABC genotyping, Odds et al. (2006) have shown that in cases of candidaemia, strain maintenance, predominant clonality and microvariation occur in isolates obtained from

Financial support: FAPESP, CNPq (475338/2006-5)
GMC received a postdoctoral fellowship from FAPESP (2005/04442-1) and FPS a Master's degree studentship from FAPESP (2007/05880-8).
GMC and FPS contributed equally to this work.
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Received 23 May 2011
Accepted 27 October 2011

non-sterile sites cultured before the onset of *Candida* bloodstream infection. These results strongly suggest that candidaemic patients are usually infected with their own commensal isolates that exhibit multiple colonisation and clonal dissemination as previously described (Reagan et al. 1990, Pittet et al. 1991, 1994, Caugant & Sandven 1993, Voss et al. 1994, Marr et al. 2001, Verma et al. 2003).

In contrast, few authors have suggested that multiple strain types may coexist in the same clinical sample from a unique patient (Takasuka et al. 1998, Kam & Xu 2002, Samaranayake et al. 2003). By investigating different *C. albicans* colonies obtained from healthy volunteers and patients with oral and vaginal candidiasis, Jacobsen et al. (2008) reported a higher prevalence of strain microevolution and substitution among healthy volunteers compared to infected patients. The authors inferred that superficial infection may result from the selective overgrowth of a single subtype or fewer subtypes before invasive infection.

By comparing the concordance among MLST and ABC genotyping, Odds et al. (2006) stated that the latest technique is reliable when strains differ in more than one single nucleotide polymorphism. However, ABC genotyping should not be used as a unique method for *C. albicans* typing. First described in 1999 by McCullough et al., ABC genotyping was developed on the basis of the presence or absence of an insert in the DNA that encodes for the 25S ribosomal RNA gene among different *C. albicans* genotypes (McCullough et al. 1999). Microsatellite typing using primer M13, which amplifies short tandem repeats, was first employed to type *Cryptococcus* strains in Brazil (Casali et al. 2003) and has been extensively used to type this encapsulated yeast (Loperena-Alvarez et al. 2010, Pedroso et al. 2010, Ferreira-Paim 2011). Nevertheless, only a few studies have employed the M13 primer to type *C. albicans* (Bartie et al. 2001).

It seems reasonable to hypothesise that the proliferation of a single *C. albicans* subtype may be a predictor for candidaemia. However, there is a lack of prospective studies in the medical literature that have determined whether the dissemination of a single clone of *C. albicans* to multiple anatomical sites occurs in patients who remain colonised by this yeast, but do not develop candidaemia during hospitalisation. In this study, we prospectively evaluated the dynamics of the persistence or replacement of *C. albicans* genotypes for two well established groups of critically ill patients: group I included three patients colonised by *C. albicans* in multiple sites who did not develop candidaemia during hospitalisation and group II included four patients who were colonised by *C. albicans* and who developed candidaemia.

SUBJECTS, MATERIALS AND METHODS

Strains - We selected a total of 51 isolates of *C. albicans* that were prospectively collected from the different anatomical sites of six patients admitted at a tertiary care hospital in São Paulo, Brazil. In addition, we included a patient (patient 5) who had two positive urine cultures for *C. albicans* collected at a seven-day interval and who developed candidaemia. These isolates were obtained from patients sequentially admitted to the ICU who agreed to participate in a surveillance confidential study. Only patients who required more than 72 h of ICU admission were enrolled in this study. Samples were systematically collected from urine, rectal swabs, skin surrounding the catheter entry site, surgery wounds, tracheal secretions and blood until the onset of candidaemia or discharge from the ICU (Table). The clinical isolates were collected during the study over a period of two years. The isolates was stored on YPD glycerol (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose and 3% glycerol) at -70ºC. The *C. albicans* strains ATCC90028 and SC5314 were used as external control strains for the phenotypic and molecular identification and typing of our isolates.

Procedures for species identification - The isolates were plated on CHROMagar Candida® (CHROMagar Microbiology, Paris, France) to check for purity, viability and screening for green colonies. The production of chlamydoconidia on cornmeal agar and green colour on CHROMagar Candida® were the presumptive tests adopted for the initial identification of *C. albicans*. The final identification of the strains was confirmed by the sequencing of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) region (White et al. 1990).

C. albicans genotyping methods - To establish the genetic relatedness of the colonising and bloodstream isolates of *C. albicans*, all of the strains were typed by microsatellite and ABC genotyping (based on the amplification of an intron present in the 26S rDNA gene).

DNA extraction - The isolates were grown in Falcon tubes that contained 2 mL YPD medium for 16 h at 30ºC at 200 rpm in a gyratory shaker for 48 h. DNA was extracted using the fast small scale isolation protocol previously described (Melo et al. 1998).

Polymerase chain reaction (PCR) assay and ITS region sequencing - The primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATAT-3′) were used to amplify the ITS region (White et al. 1990). The samples were amplified in a Thermocycler (model 9600) (Applied Biosystems, Foster City, CA, USA) using the following cycling parameters: one initial cycle of 94ºC for 3 min followed by 30 cycles of 1 min at 94ºC, 1 min at 57ºC, 1 min at 72ºC and a final cycle of 5 min at 72ºC. The PCR products were size-separated by agarose gel electrophoresis and the gel was stained in a 0.5 µg/mL ethidium bromide buffer solution (1 x tris-acetate-ethylenediamine tetraacetic acid). The purified PCR products were sequenced using the dideoxynucleotide method in an ABI PRISM 3100 automated sequencer (Applied Biosystems, CA, USA). The sequencing reaction included each primer separately and the BigDyeTM Terminator reagent kit (Applied Biosystems) and it was performed according to the manufacturer's instructions. The nucleotide sequences were submitted for BLAST analysis (blastn) at the National Center for Biotechnology Information (ncbi.nlm.nih.gov) website for species identification.

Microsatellite typing PCR and ABC genotyping PCR - Microsatellite typing was performed using the primer M13 (5'-GAGGGTGGCGGTTCT--3') (IDT) as

previously described (Casali et al. 2003). ABC genotyping was performed with the following primers: CA-INT-L (5′-ATAAGGGAAGTCGGCAAAATAGATCCGTAA-3′) and CA-INT-R (5′-CCTTGGCTGTGGTTTCGCTAGATAGTAGAT-3′) (McCullough et al. 1999). Briefly, 1.0 µL of DNA 40 ng/µL, 2.5 µL of 10 x PCR buffer (100 mM tris-HCl, pH 8.3, 500 mM KCl, 3.5 mM MgCl2), 5 µL of dNTPmix (100 mM each dNTP), 1.0 µL of each primer (50 pmol/µL), 0.13 µL of Tween 20 and 1.0 unit of Taq DNA polymerase were added to a final volume of 25 µL. Forty-five cycles of amplification were performed using the same cycling parameters described previously, except the annealing temperature was 36ºC for the microsatellite typing.

Computer-assisted microsatellite data analysis - Gel images were analysed with the GelCompar II software, version 4.5 and BioNumerics (Applied Maths, Kortrijk, Belgium). The similarities between the profiles were calculated using the Dice coefficient to generate the matrixes of similarity coefficients to dendrogram constructions. For profile clustering, the unweighted pair-group method with arithmetic averages was used with a tolerance of 2%.

RESULTS

All of the isolates that were phenotypically identified as *C. albicans* had their identification confirmed with rDNA ITS region sequencing. Notably, the patients included in this analysis were not on antifungal therapy or prophylaxis at the time of enrolment. Patient demographic data and underlying conditions are described in Table.

The genomic DNA of all of the isolates was successfully amplified with the M13 primer, which targets genome repetitive sequences. The DNA amplification generated well-defined band patterns, ranging from 500 pb-2 KB and the microsatellite technique showed sufficient discriminatory power for recognising intraspecific variation (Figure). As expected, the external control strains of *C. albicans,* SC5314 and ATCC90028, were placed in completely different clusters by the dendrogram analysis performed using GelCompar II, proving the efficiency of the technique in separating strains isolated from different geographic areas (Figure).

C. albicans microsatellite typing results of colonised vs. candidaemic patients - The results obtained using microsatellite typing suggest that each patient was colonised by a group of specific strains. Isolates with some degree of genetic variability were obtained from group I patients (who did not develop candidaemia). This finding suggests that these isolates underwent microevolution or were completely replaced during the patient's hospitalisation. The majority of the genotypic profiles of the sequential isolates from patient 1 were identical (100% similarity). Isolates 904 C1 (catheter tip), 904 A (rectal swab) and 904 B (urine) were placed within different clusters. Patient 2 was colonised in three different body sites. Strains 143 A (rectal swab) and 143 C (surgery wound) showed 100% similarity, whereas an isolate from tracheal secretion (138 C) had a very different genotypic profile compared to the two other strains and was placed in a different cluster after the dendrogram analysis (84% similarity). This trend was still more remarkable among the isolates from patient 3. Some of the isolates presented only 60% similarity and were placed in very distinct clusters, except for two strains that were considered identical (170 A and 184 A1 both from rectal swabs collected on different days) (Figure).

Regarding the patients who were colonised by *C. albicans* and who developed candidaemia (group II), bloodstream isolates were indistinguishable or highly related to other strains of the same patient, although some degree of genetic variability was observed among previously colonising strains. Patient 4 had colonising strains that exhibited genetic variability ranging from 85-100% similarity, suggesting that strain microevolution occurred during the patient's ICU hospitalisation. Furthermore, isolate 731 D from blood was considered indistinguishable from isolate 704 B (100% similarity), a strain recovered from urine before the onset of candidaemia. Patient 5 was included in the analysis because of two positive urine cultures that were collected within a week of developing candidaemia. These strains (19 B and 15 B1) were considered highly related (above 97% similarity) to the 15 D bloodstream isolate. Patient 6 showed some degree of difference among the colonising strains of *C. albicans* (86-100% similarity), possibly due to microvariations and similarity of 93% among a *C. albicans* bloodstream isolate (757) and the previous colonising strains. Finally, patient 7 had a blood isolate (997, 5 G) that was 100% similar to the colonising isolates of different body sites, such as urine (1002 B) and surgery wounds (982 E) and 97% similarity among all of the isolates, proving that the strains were highly related.

C. albicans ABC genotyping results of colonised vs. candidaemic patients - When ABC genotyping was used, we observed that at least two different A, B or C genotypes were observed within the colonising strains of *C. albicans* from group I (patients who did not develop candidaemia). Nevertheless, patients who were colonised and developed candidaemia (group II) due to *C. albicans* had a single A or B genotype strain at the beginning of colonisation through the onset of candidaemia (Table).

Regarding group I, patient 1 was colonised by several genotype C strains, but a genotype A strain was also isolated. This isolate (904 C1; skin surrounding the catheter entry site) was placed in a different cluster from the majority of the isolates from the same patient when the microsatellite technique was used. Patient 2 had two genotype B strains that were also considered to be indistinguishable (143 A, rectal swab; 143 C, surgery wound) and a genotype C strain (138 C; tracheal secretion) that was again placed in a different cluster when the microsatellite method was used. Interestingly, patient 3 was colonised by a combination of A and B genotypes (Table).

In contrast, group II patients were solely colonised by a single A or B genotype of *C. albicans* in all anatomical sites. Patients 4 and 7 were only colonised by genotype A strains, whereas patients 5 and 6 were only colonised by genotype B strains (Table). None of the strains isolated from the candidaemic patients belonged to the C genotype.

TABLE

Microbiological and clinical data for seven patients colonised and/or infected by Candida albicans

Patient	Isolate number	Site of isolation	ABC typing	Apache II score	Age (years)	Gender	Associated conditions	Clinical outcome
1 (group I)	868 A1	Rectal swab	C	22	58	Female	Cerebral vascular accident	Discharged
	881 A	Rectal swab	C	-	-	-	-	-
	893 A	Rectal swab	C	-	-	-	-	-
	904 A	Rectal swab	C	-	-	-	-	-
	904 B	Urine	C	-	-	-	-	-
	904 C1	Catheter	A	-	-	-	-	-
	914 A	Rectal swab	C	-	-	-	-	-
	914 B	Urine	C	-	-	-	-	-
2 (group I)	138 C	Tracheal secretion	C	21	61	Male	Cranioencephalic trauma	Discharged
	143 A	Rectal swab	B	-	-	-	-	-
	143 C	Surgery wound	B	-	-	-	-	-
3 (group I)	133 C1	Tracheal secretion	B	20	59	Male	Cardiovascular disease	Discharged
	170 A	Rectal swab	A	-	-	-	-	-
	170 D1	Skin surrounding catheter entry	B	-	-	-	-	-
	176 A	Rectal swab	B	-	-	-	-	-
	184 A1	Rectal swab	A	-	-	-	-	-
	184 C	Skin surrounding catheter entry	A	-	-	-	-	-
4 (group II)	624 A	Rectal swab	A	25	47	Female	Cardiovascular disease	Candidaemia (discharged)
	645 A	Rectal swab	A	-	-	-	-	-
	657 A	Rectal swab	A	-	-	-	-	-
	657 B	Urine	A	-	-	-	-	-
	672 A	Rectal swab	A	-	-	-	-	-
	672 B	Urine	A	-	-	-	-	-
	672 D	Skin surrounding catheter entry	A	-	-	-	-	-
	687 A1	Rectal swab	A	-	-	-	-	-
	687 B	Urine	A	-	-	-	-	-
	687 C	Skin surrounding catheter entry	A	-	-	-	-	-
	704 A	Rectal swab	A	-	-	-	-	-
	704 B	Urine	A	-	-	-	-	-
	731 A	Rectal swab	A	-	-	-	-	-
	731 D	Blood	A	-	-	-	-	-
5 (group II)	15 B1	Urine	B	20	62	Male	Cardiovascular disease, benign gastrointestinal disease	Candidaemia (discharged)
	15 D	Blood	B	-	-	-	-	-
	19 B	Urine	B	-	-	-	-	-

↳

Patient	Isolate number	Site of isolation	ABC typing	Apache II score	Age (years)	Gender	Associated conditions	Clinical outcome
6 (group II)	680 A	Rectal swab	B	23	44	Male	Central nervous system disease	Candidaemia (discharged)
	690 A	Swab real	B	-	-	-	-	-
	690 C	Tracheal secretion	B	-	-	-	-	-
	724 A	Rectal swab	B	-	-	-	-	-
	763 A	Rectal swab	B	-	-	-	-	-
	763 B	Urine	B	-	-	-	-	-
	777 A	Rectal swab	B	-	-	-	-	-
	777 B	Urine	B	-	-	-	-	-
	791 B	Urine	B	-	-	-	-	-
	757	Blood	B	-	-	-	-	-
7 (group II)	965 A	Rectal swab	A	11	55	Male	Cardiovascular disease	Candidaemia (death)
	965 C1	Tracheal secretion	A	-	-	-	-	-
	974 C	Tracheal secretion	A	-	-	-	-	-
	974 D	Skin surrounding catheter entry	A	-	-	-	-	-
	982 E	Skin surrounding catheter entry	A	-	-	-	-	-
	1002 B	Urine	A	-	-	-	-	-
	997,5 G	blood	A	-	-	-	-	-

DISCUSSION

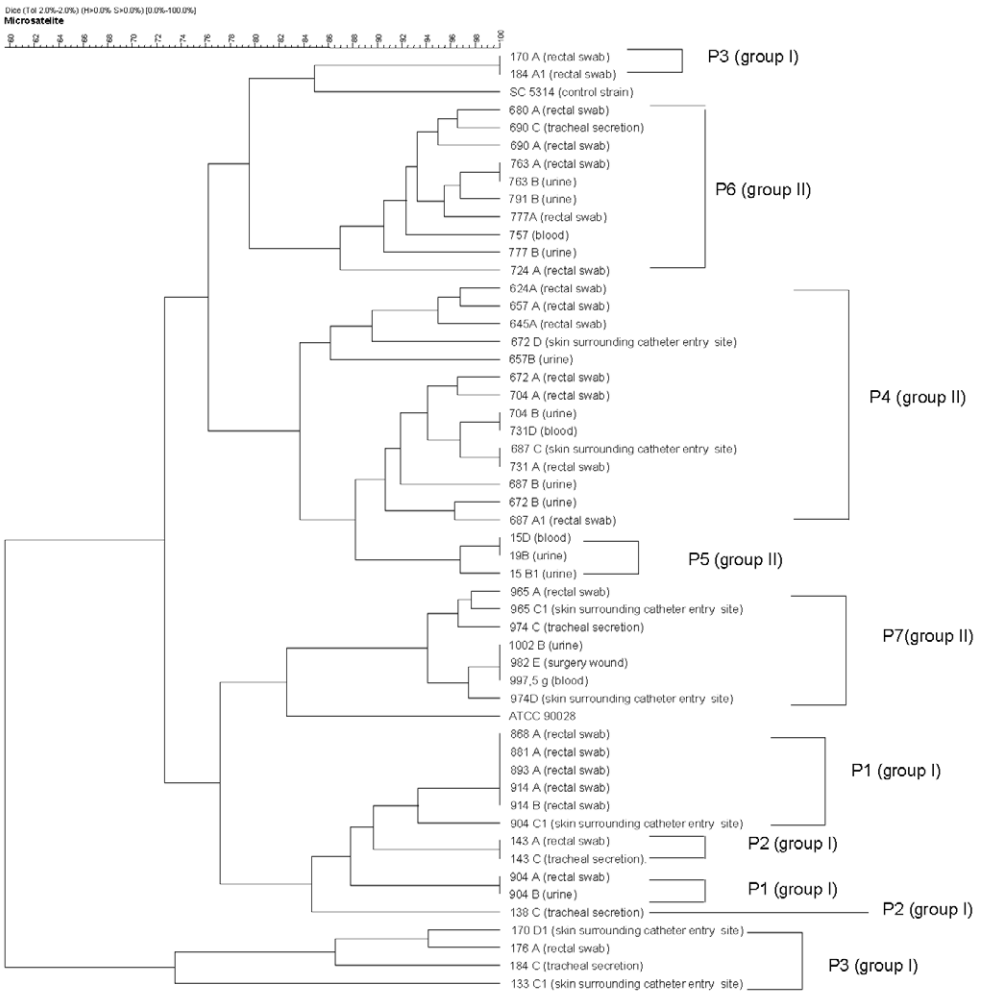
In this study, we prospectively evaluated the genotypes of the colonising and infecting strains of *C. albicans* from patients sequentially admitted for more than 72 h in the ICU. Overall, we found a great diversity among the different colonising strains of *C. albicans* obtained from various anatomical sites of patients who did not develop candidaemia despite previous multifocal colonisation, whereas the persistence of a single A or B genotype was documented in patients who developed candidaemia. These data support the hypothesis that the presence of a single, well-adapted dominant strain in non-sterile sites obtained from patients with candidaemia may increase the risk of transition from commensalism to infection.

It is important to emphasise that the patients who did not develop candidaemia were colonised by different *C. albicans* genotypes and strain replacement (as supported by 2 different techniques) occurred even among *Candida* strains isolated from the same anatomical site at two different time points of collection. Patient 3 was sequentially colonised by a combination of genotype A and B strains within the same anatomical site. According to Jacobsen et al. (2008), a combination of different genotypes is expected more among colonised individuals compared to patients exhibiting superficial infections with *C. albicans*. Therefore, it is reasonable to hypothesise that an adapted genotype succeeded during disease development, whereas strain replacement may have occurred in patients who did not develop candidaemia during hospitalisation.

It is very unlikely that commensal strain replacement occurs in patients who develop candidaemia (Reagan et al. 1990, Pittet et al. 1991, 1994, Caugant & Sandven 1993, Voss et al. 1994, Marr et al. 2001, Verma et al. 2003, Odds 2010). By evaluating 30 hospitalised patients with multifocal colonisation by *C. albicans* who developed candidaemia, Marco et al. (1999) found highly similar or identical genetic patterns between colonising and infecting strains typed with a Ca3 probe. Odds et al. (2006) demonstrated a high genetic relatedness within the colonising strains from multiple sites and the bloodstream isolates obtained from 11 different sets of strains of a patient admitted to the ICU.

Several authors have demonstrated that candidaemic patients are previously colonised in multiple non-sterile sites by *Candida* strains of clonal origin; however, we did not find any prospective study that evaluated whether this phenomenon occurs in critically ill patients with multifocal colonisation who do not develop candidaemia. Our findings suggest that *C. albicans* strain replacement may occur in colonised patients who do not progress to candidaemia.

Despite being a controversial issue, several authors have recommended serial fungal surveillance cultures in critically ill patients to better identify individuals who should be treated with empirical antifungal therapy when a substantial degree of fungal colonisation is observed (Eggimann et al. 2005, Leon et al. 2006). According to our data, ABC genotyping could help clinicians predict whether or not a critically ill patient who is multicolonised by *C. albicans* would develop candidaemia.



Unweighted pair-group method with arithmetic averages dendrogram with 2% of tolerance of 51 strains of *Candida albicans* clinical isolates collected from different body sites.

In conclusion, as demonstrated by ABC and microsatellite genotyping, we suggest that in addition to the number of body sites colonised by *Candida*, multifocal colonisation by a single genotype may increase the risk of developing candidaemia in colonised patients. We demonstrated that a patient who was colonised in a single anatomical site by a persistent *C. albicans* genotype B (isolated from urine) progressed to invasive infection (patient 5). The limitations of our study are represented by the small number of patients evaluated in the present series and by the fact that strain virulence factors were not included in our analysis. Further studies are necessary to confirm whether our findings may be extrapolated to larger populations.

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