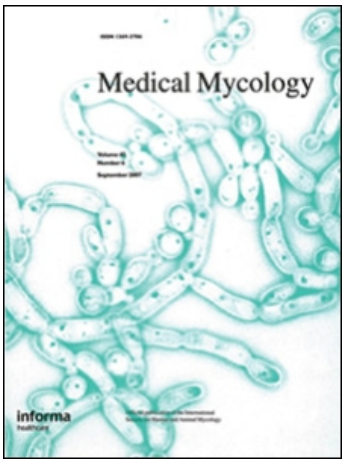

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Retrospective analysis of clinical yeast isolates in a hospital in the centre of Portugal: spectrum and revision of the identification procedures

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Retrospective analysis of clinical yeast isolates in a hospital in the centre of Portugal: spectrum and revision of the identification procedures

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We conducted a four-year (2003–2006) retrospective study of yeasts recovered in a hospital laboratory in the centre of Portugal to evaluate the epidemiology of yeast infections. Clinical isolates and data were gathered from 751 patients corresponding to 906 episodes of yeast infection. The isolates were first identified using classical and commercial methods, routinely employed at the hospital laboratory. We then re-identified the same isolates using RFLP of the ITS 5.8S rRNA gene and sequence of the D1/D2 domain of the 26S rRNA gene. *Candida parapsilosis* isolates were re-identified using the *Ban I* digestion of the *SADH* gene. *C. albicans* was the most frequently isolated of the yeasts found in the analysed specimens, with an overall incidence of 69.6% and then in deceasing order, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei*. *C. parapsilosis* was most frequently recovered from younger patients, decreasing with age, while *C. glabrata* occurrence increased with age. We found an increased number of cases of fungemia per 100,000 people per year, reaching a maximum of 4.4 during 2006.

Keywords Yeast infections, molecular yeast identification, risk factor, *Candida metapsilosis*, *Candida orthopsilosis*

Introduction

Yeasts are ubiquitous in the environment and are also part of the normal flora of the human body. Many yeasts, particularly members of the genus *Candida,* are opportunistic pathogens causing infections in individuals with immune diseases or with a weakened immune system. Bloodstream infections involving *Candida* spp. can be considered as emerging infections. The origin of the yeast infection can either be endogenous or exogenous and several studies point to either possibility [1–5]. Hedderwick and co-workers [6] carried out a 6-month surveillance of yeast colonization and infections in an adult intensive care unit, and found a low degree of cross-transmission for *Candida albicans*, but none for the other *Candida* species included in the investigation. Irrespective of this, the incidence of fungal infections has increased in the last three decades [7–10]. This increase may be the result of the greater the number of patients with dysfunctional immune systems [11] due to iatrogenic factors. In particular, the yeasts of the genus *Candida* are considered as the most common fungal agents of nosocomial invasive fungal infections, with an average mortality rate of 30% [10,12,13]. In recent years, the

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list of *Candida* species known as human pathogens has become more diverse, although the epidemiology of yeast infections is still ill-defined. *C. albicans* is the most frequent cause of candidemia, but *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. lusitaniae* and *C. tropicalis* have emerged as agents of human infections [9,10]. Moreover, these species have different susceptibility patterns toward antifungals [14]. Recent guidelines for the treatment of candidiosis point out that only through an accurate diagnosis of the etiologic agents can efficient therapy be designed [10,15,16].

The diagnosis of a yeast infection depends on fast and reliable identification methods of causative agents. Classical methods involving phenotypical analyses and serological tests [17] are time consuming and cause delays in the diagnoses and hamper the rapid choice of appropriate therapy. There has been an increasing interest in the application of molecular identification techniques because they are faster and more accurate [18], although they are more expensive and require advanced training for personnel.

The objective of this study was to characterize the occurence of yeast infections in a population in the centre of Portugal, attending a Hospital that serves a total population of 2,500,000 people. The accuracy of the more classic methods of yeast identification employed in the clinical centre was evaluated by the re-identification of the yeast isolates using two different molecular biology techniques (RFLP of the ITS 5.8S rRNA gene and sequencing of the domain D1/D2 of the 26S rRNA gene). A bank of clinical isolates was constructed, together with a retrospective study of the clinical data regarding the patients from whom the yeasts were isolated.

Materials and methods

This is a retrospective study including data from 2003 to 2006 concerning yeasts recovered as etiologic agents of human infections at the Centro Hospitalar de Coimbra (CHC). The hospital has a target population of 500,000 people (General Hospital and Maternity) and two million for the Paediatric Hospital.

Clinical data

Retrospectively, relevant clinical information of patients diagnosed with yeast infections obtained from the database of the Clinical Pathology Department of the CHC, were gathered and added to a data bank. The following risk factors/underlying diseases were considered; cystic fibrosis, immunodepression, cancer, diabetes, AIDS, catheter, post-operation status, pregnancy, antibiotic treatment, persistent fever (meaning persistent fever of unknown origin), solid organ transplant, admission in the reanimation unit, haemodialysis and burns. For every episode, the following data was recorded; age, gender, in- or outpatient status, yeast identification, collection date and specimen from which the yeast was isolated.

Standard identification procedure for yeasts isolated in the hospital laboratory

The methodology used in the microbiology laboratory of the Clinical Pathology Department of the CHC starts with a direct microscopic examination of a smear of the specimen. This is followed by inoculating a portion of the specimen onto Sabouraud medium or blood culture bottle (BACTEC 9240 System with Aerobic Plus or F blood culture bottles (Becton Dickinson, The Netherlands). When the presence of yeast colonies was suspected, a rapid identification procedure was performed using the Bichro Latex albicans Fumouze (Fumouze Diagnostics, France), with a positive result indicating the identification of *C. albicans*. Alternatively, if the test result was negative, the Api® ID32C (bioMérieux, Portugal) was used in identifying the isolate.

Re-identification of yeasts by molecular biology methods

The yeast isolates were re-identified using RFLP of the 5.8S rRNA gene and of the adjacent ITS1 and ITS2, and by sequencing the D1/D2 contiguous fragments of the 26S rDNA. Total genomic DNA was extracted using the isopropanol method and specific gene amplification was obtained using the primers in Table 1. The identification by RFLP of the 5.8S and ITS1 and ITS2 region of the 18S rDNA was carried out as described previously [19–21]. Reference strains of *C. dubliniensis* and *C. lusitaniae* were obtained and, using the methodology described above, we determined the restriction patterns of these species (results not shown). The sequencing of the D1/D2 contiguous fragments of the

Table 1 Primers used in this study.

Primer name	Sequence	Reference
NL-1	5′- GCA TAT CAA TAA GCG GAG GAA AAG -3′	[26]
NL-4	5′- GGT CCG TGT TTC AAG ACG G -3′	
ITS-1	5′-TCC GTA GGT GAA CCT GCG G- 3′	[27]
ITS-2	5′-TCC TCC GCT TAT TGA TAT GC-3′	
S1F	5′- GTT GAT GCT GTT GGA TTG T-3′	[25]
SIR	5′- CAA TGC CAA ATC TCC CAA -3′	

26S rDNA was performed according to Kurtzman and Robnett [22], and assumed as the reference method. The sequences obtained were used to identify the yeasts by BLAST-search (GenBank; www.ncbi.nlm.nih.gov).

Using Tavanti and co-workers methods [23], the re-identification of the clinical isolates of *Candida parapsilosis* was performed by RFLP of the *SADH* gene. This method is based on the restriction of the SADH ('Secondary Alcohol Dehydrogenase') gene by the *Ban I* enzyme. This re-identification was performed on 50 different clinical isolates identified as *Candida parapsilosis* collected during this study.

Statistics

Descriptive statistics was used to summarize the data. Risk factors significantly associated with bloodstream infections in the univariate analysis that occurred in $>5\%$ of patients were evaluated in a stepwise logistic regression model.

Results

Demographic characteristics and risk factors

The total number of yeast infections diagnosed during the period in study was 906, although this number does not correspond to the number of patients since 155 suffered from repeat episodes. The total number of patients with at least one yeast infection was 751. The distribution of patients in age groups (Table 2) showed that the number of cases increased with age. During the 4-year period, there was a considerable increase in the number of cases diagnosed at the hospital laboratory each year. As for the gender, 52.3% of episodes occurred in men, and 47.7% in women (Table 2).

This study aimed at analyzing the spectrum of yeast infections and all the yeast isolates to which an aetiological role was attributed, independently of the place/tissue of infections. Consequently, they were recovered from different biological products (Table 2). Respiratory and urinary tract specimens were the most frequent source of yeasts. The number of blood isolates, corresponding to bloodstream infections (BSI) was 36, and the number of catheters from which yeasts were recovered was 60.

The clinical data of the patients from whom the isolates were obtained, either as in- or outpatients, was analyzed according to the underlying diseases and/or predisposing conditions, with the purpose of identifying the most important risk factors. A high number of episodes (248) did not have any associated risk factors. The highest numbers of episodes were associated with prolonged antibiotic treatment and persistent fever (Table 2).

Table 2 Characteristics of the patients' data and of the samples from which yeast isolates were obtained.

Data	Number of patients (%)	Number of episodes (%)
Year of study		
2003	139	153
2004	131	146
2005	197	237
2006	308	370
Sex		
Male	387 (51.5)	
Female	364 (48.5)	
Age groups (years) (n=754)		
≤ 0.5	28 (3.7)	38 (4.2)
0.5–3	49 (6.5)	60 (6.6)
3–16	63 (8.4)	75 (8.3)
16–40	113 (15.0)	129 (14.2)
40–65	116 (15.4)	159 (17.5)
≥ 65	385 (51.1)	445 (49.1)
Risk factor/underlying disease (n=1467)		
Antibiotics		560 (38.2)
Burns		1 (0.1)
Cancer		28 (1.9)
Catheter		131 (8.9)
Cystic fibrosis		5 (0.3)
Diabetes		19 (1.3)
Fever		457 (31.2)
Haemodialysis		3 (0.2)
HIV/AIDS		48 (3.3)
Immunodepression		30 (2.0)
Pregnancy		24 (1.6)
Post surgery		64 (4.4)
Reanimation unit		82 (5.6)
Solid organ transplant		15 (1.0)
Specimens (n=906)		
Respiratory		317 (35.0)
Urine		260 (28.7)
Catheter		60 (6.6)
Blood		36 (4.0)
Faeces		46 (5.1)
Vaginal		50 (5.5)
Skin		31 (3.4)
Other*		104 (11.5)

*Other specimens: gastric fluid (5), peritoneal fluid (28), male urethral fluid (4), cerebrospinal fluid (11), bile (14), unspecified tissue (2), unspecified fluid/pus (59), exsudate of the pharynx/tongue (20).

Re-identification of the clinical isolates by molecular biology

Our results showed that the methodology used by the microbiology laboratory of the Clinical Pathology Department of the CHC was very accurate and reliable, since only two isolates were misidentified when results were compared to those with the two different molecular techniques. One isolate, obtained from a urine

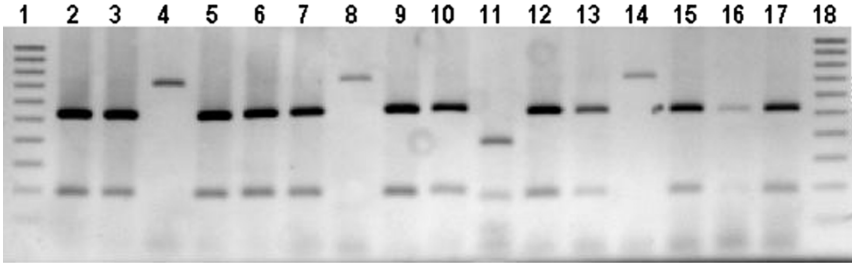

Fig. 1 Re-identification of clinical isolates of *Candida parapsilosis* by RFLP of the SADH gene by the enzyme BanI. The resulting fragments were separated by electrophoresis in a 1% agarose gel. Legend: Lane 11 – *C. metapsilosis*; Lanes 4, 8, 14 – *C. orthopsilosis*; Lanes 2, 3, 5, 6, 7, 9, 10, 12, 13, 15, 16, 17 – *C. parapsilosis*; Lanes 1, 18 – Molecular weight marker.

sample was first identified with the hospital routine procedures as *C. guilliermondii* and re-identified as *C. albicans* using the RFLP method. Surprisingly, when the D1/D2 domain of the 26S rDNA was sequenced, this same isolate was re-identified as *C. tropicalis*. Since we assumed this last procedure as the reference method, we decided to accept the last identification. Another isolate, first identified by classic methods as *C. intermedia* was re-identified by sequencing of the D1/D2 domains of the 26S gene as *C. tropicalis*.

Re-identification of C. parapsilosis isolates

The redefinition of the taxonomic group to which *C. parapsilosis* includes two additional species, *C. orthopsilosis* and *C. metapsilosis* [23], we reidentified the 50 clinical isolates indicated to be *C. parapsilosis*. All the *C. parapsilosis* isolates had been identified by classic methods, by RFLP of the ITS 5.8S rDNA and sequencing of the D1/D2 domains of the 26S gene. For the re-identification, we used the restriction pattern of the *SADH* gene with the *Ban I* enzyme [23]. Of the 50 isolates identified as *C. parapsilosis*, to which clinical pathologists originally attributed aetiological significance, five were re-identified as *C. metapsilosis* and six as *C. orthopsilosis*. The restriction patterns of some of the re-identified isolates are depicted in Fig. 1, along with the restriction pattern of *C. parapsilosis*.

In the group of yeasts re-identified as *C. orthopsilosis*, one was isolated from peritoneal fluid of a 25-day-old infant with an intestinal perforation admitted in the intensive care unit (ICU). One month later, *C. orthopsilosis* was again recovered from the blood of the same patient. At the time of blood collection and when admitted to the ICU, the patient was being medicated with antibiotics. A third isolate of the same species was obtained from a catheter in the same boy. Among the isolates re-identified as *C. metapsilosis*, a particular isolate was more thoroughly investigated since it had been recovered from peritoneal fluid of a liver transplant patient, who received three liver transplants. The first isolate was recovered from a drainage catheter after the third transplant. It was identified by the microbiology laboratory as *C. parapsilosis*. The same yeast was also obtained from peritoneal fluid and from a urinary catheter. A histopathological analysis of a liver biopsy, also from that period, revealed yeast cells.

Spectrum of agents

The five species most often recovered were, in decreasing order, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei* (Table 3). *C. albicans* accounted for 69.6% of the total number of yeast infections while non-C. *albicans Candida* species accounted for 28.3% of the yeast infections (Table 3). A total of 8 isolates of *Saccharomyces cerevisiae* were obtained. In regard to *Cryptococcus neoformans*, besides the systemic isolates, two were obtained from skin lesions (Table 3). From the analysis of the incidence of non-*C. albicans Candida* species in different age groups (Fig. 2), we conclude that *C. parapsilosis* is the predominant species in patients up to 16 years, whereas *C. glabrata* is more common in patients older than 16 years of age. Starting at the age group from 16 to 40 years, the relative incidence of *C. glabrata* increased progressively, whereas *C. parapsilosis* followed the opposite tendency.

Blood stream infections (BSI)

The number of blood cultures positive for yeasts obtained during the period considered in this study was 89. However, there were 2,153 blood cultures performed to detect fungi due to clinical indicators of infection. Of the total number of tests performed, only 24 (1.1%) yielded positive results using blood culture bottles. The other yeasts involved in candidemia, were isolated from patients with no clinical evidence of BSI, but with clinical evidence of a bacteremia, from which the blood cultures were found to be positive for yeasts.

Table 3 Yeast species isolated from different specimens

	Specimen								
	Respiratory*	Urine	Catheter	Blood	Faeces	Vaginal	Skin	Other	Total number (%)
C. albicans	235	177	36	22	31	42	23	65	631 (69.6)
C. catenulata	0	1	0	0	0	1	0	0	2 (0.2)
C. glabrata	24	25	1	1	5	6	1	6	69 (7.6)
C. guilliermondii	3	2	2	0	1	0	0	7	15 (1.7)
C. inconspicua	2	0	0	0	0	0	0	0	2 (0.2)
C. intermedia	2	2	1	0	0	0	0	2	7 (0.8)
C. krusei	6	8	3	0	3	0	0	3	23 (2.5)
C. lusitaniae	3	3	0	0	0	0	0	1	7 (0.8)
C. membranaefaciens	0	0	1	0	0	0	0	0	1 (0.1)
C. metapsilosis	1	0	0	0	0	0	0	1	2 (0.2)
C. orthopsilosis	1	0	1	1	0	0	0	1	4 (0.4)
C. parapsilosis	8	7	14	8	2	0	4	7	50 (5.5)
C. pelliculosa	0	0	0	1	0	0	0	0	1 (0.1)
C. pulcherrima	1	0	0	0	0	0	0	0	1 (0.1)
C. rugosa	1	0	0	0	0	0	0	0	1 (0.1)
C. sake	1	1	0	0	0	0	0	0	2 (0.2)
C. tropicalis	28	33	0	0	0	0	1	5	67 (7.4)
C. utilis	0	0	1	0	0	0	0	1	2 (0.2)
Cryptococcus neoformans	0	0	0	3	0	0	0	3	6 (0.7)
Saccharomyces cerevisiae	1	1	0	0	4	1	1	0	8 (0.9)
Other	3	0	0	0	0	0	0	2	5 (0.6)
Total (%)	320 (35.3)	260(28.7) (27.2)	60 (6.6)	36 (4.0)	46 (5.1)	50 (5.5)	30 (3.3)	104 (11.5)	906

*It includes bronchoalveolar lavage liquids, nasopharyngeal aspirates and expectorated suptum.

The low number of positive blood culture tests led us to estimate the number of episodes of candidemia per 100,000 people per year, between 2003 and 2006. For this calculation, only the data of patients older than 16 years were taken into account. We considered that this hospital has an catchment area of 500,000 people. From the total yeast isolates from the blood, and due to the retrospective nature of this study, we could only obtain patient's data from 36 cases. Of the total episodes of candidemia considered, about 95% were detected in in-patients. *Candida albicans* was the most frequent yeast species (61.1%) followed by *C. para-*

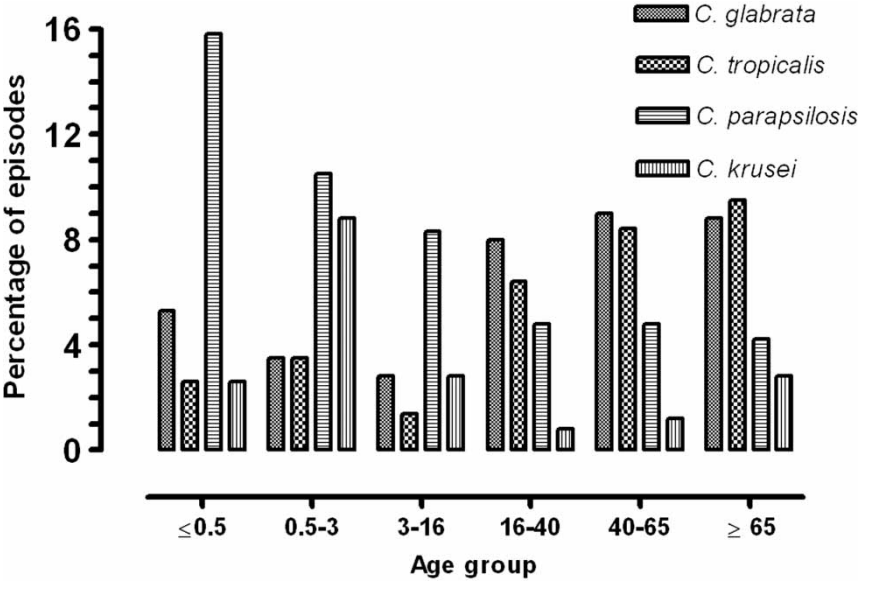

Fig. 2 Distribution of the most common non-*Candida albicans Candida* species per age group.

psilosis (22.2%), *Cryptococcus neoformans* (8.3%). One of the isolates first identified as *C. parapsilosis*, was re-identified as *C. orthopsilosis*. It is noteworthy that there was an absence of *C. tropicalis* and *C. krusei*, which are frequently found as pathogens in other sites such as the respiratory and urinary tracts. The results showed four episodes of *C. krusei* associated with catheters, but none in blood culture. A statistical study was performed in order to assess the probability of a patient developing a blood stream yeast infection. The results showed that the only variables with statistic impact was the presence of HIV (OR = 2.962; $P = 0.035$) or haemodialysis (OR = 12.411; $P = 0.050$). This last parameter shows a higher odds ratio when it is associated with patients who are over 65 years (OR = 16.920; $P = 0.048$). In addition, patients in which the clinician described persistent fevers of unknown origin have higher probability of a BSI (OR = 4.05; $P = 0.001$).

Discussion

The main goal of this study was to understand the incidence of yeast infections in a population in the centre of Portugal and to assess the accuracy of the identification procedures used. It was also our aim to identify groups of patients at higher risk of acquiring a BSI.

The main risk factors contributing to yeast infections and their classification in order of importance have been the subject of several studies [10,24]. Suggestions have been made of the usefulness of assessing the need for early therapy or even prophylaxis against fungal infections, now that the most important risk factors are known [10] such as catheterized patients [25], those receiving prolonged antibiotic treatment [26], pregnancy [27], patients with uncontrolled diabetes [28], solid organ transplant recipients [29], patients with AIDS [30] and cancer patients undergoing cytotoxic chemotherapy [29]. In the population here studied, the majority of yeast infections occurred in patients with all of these risk factors.

Age is also a critical factor and was considered in our study. As expected, the age group where the most yeast infections were diagnosed was in patients over 65 years. The latter tend to present signs of immunosenescence, a gradual reduction of the effectiveness of the immune system due to the natural ageing process [31]. Additionally, the ageing of the mucosa and the skin weakens nonspecific defences against invading microorganisms [32]. Also, the patients in this age group have increased probability of suffering from diseases which are risk factors for yeast infections, such as diabetes, renal insufficiency (haemodialysis) and cancer (chemotherapy) [26,29,33,34]. Accordingly, the data we obtained showed that patients older than 65 years under haemodialysis had the highest risk of developing a BSI.

The observation that a high number of episodes was not associated with a risk factor clearly indicates that either the clinical data of the clinical pathology database are not complete and/or there are clinical features that clinicians do not identify as being related to fungal infections. However, it is important to note that the data was collected from the database of the Clinical Pathology Department of the CHC and not from the complete clinical file of the patients. It is likely that the transfer of clinical information between the assisting physician and the clinical pathologist might be incomplete. The retrospective nature of the study, coupled to the exiguous data transfer to the clinical pathologist, hypothetically led to the omission of important risk factors. This limitation has been described in other epidemiological studies based in hospital laboratories and the clinical data associated with it [35].

One of the most serious yeast infections is that of the bloodstream, which is associated with a high mortality [10]. In addition to the increased frequency of this type of infection, there is also a tendency for the emergence of new pathogenic species. A study recently published in the Portuguese population showed that the incidence of fungemia is 2.7 per 1,000 hospital admissions with a mortality rate of 39.3% [12].

The diagnosis of fungemia is sometimes hindered by the methods used to establish it. Blood culture methods may often yield false negative results at an early stage of infection [36]. According to Pfaller and Diekema [10], the frequency with which blood culture tests are requested and the type of culture employed can affect the fungemia incidence rates in epidemiological studies based on laboratory results. A study concerning the efficiency of the BACTEC 9240, the blood culture bottle used in the investigation, showed that it only spiked with 100 CFU/ml of different yeasts. Therefore, about 10% of the aerobic culture bottles gave false negative results after a 21-day incubation. Furthermore, the percentage of false negatives increased when the incubation time was reduced to 5 days [37]. Considering that in many cases of bloodstream infection the concentration of yeast cells can be less than 10 CFU/ml [38], we can infer that the clinical practice, using a 5-day incubation period, may be expected to yield an even larger percentage of false negative results. The number of candidemia episodes obtained during this study is close to the incidence found in other European countries like Norway (2 and 3 per 100,000 inhabitants) and Spain (4.9 per 100,000 inhabitants).

However, the incidence of candidemia in this investigation is considerably lower than that found in North American studies (between 6 and 24 cases per 100,000 inhabitants, per year) [10]. A study recently published showed that in Scotland the incidence of candidemia is 4.8 cases per 100,000 population per year [35]. Nevertheless, we believe that the number of laboratory diagnosed bloodstream infections is certainly below the real incidence. In many cases in which there was a strong clinical suspicion of a fungal infection, antifungal medication was administered with a positive outcome even when the blood culture test turned negative. A reliable, highly efficient method for detecting yeast blood infections is required.

Although this study included a wide range of clinical isolates, from skin to systemic candidiosis, we believe that at the clinical setting level, it is more important to recognize the probability of a patient to develop a systemic yeast infection, than a localized yeast infection. Others have pointed that this is especially true if certain risk factors are present, for example if gastrointestinal pathologies are present [12,35].

A conclusion drawn from this study is that the combination of methods used in the microbiology laboratory of the clinical pathology service of CHC are excellent since only two yeast isolates were re-identified by molecular biology as different species. We did not consider for this calculation the re-identification of the isolates of *C. parapsilosis*, since *C. metapsilosis* and *C. orthopsilosis* are new species that did not yet exist in the databases of the clinical routine identification methods. The combination of several methods certainly contributes to the quality and efficacy of the identification procedure routinely used at the hospital laboratory.

In recent years there has been a growing interest in using and developing new identification methods based on molecular biology, like real-time PCR [39], sequencing and/or RFLP of different regions of the genome [18,21,40,41]. These techniques take advantage of regions of the genome, which, in addition to being conserved in all yeast species, have a great interspecies variability. These methods provide more reliable results in a shorter period of time. However, the cost of the equipment and need for highly trained staff may be disadvantages to their use.

A characterization of yeast infections requires the analysis of the isolated species. Although most epidemiological studies in the literature concern BSI, *C. albicans* would be expected to be the most frequently isolated yeast. In spite of that, it is noteworthy that we found a great variety of non-C. *albicans Candida* species. In the literature, there are several reports indicating the tendency for diversification of species of yeast pathogenic to humans [10,42]. This can be due to the increased number of individuals with greater susceptibility to this kind of infectious agents, as well as to the selection of several species due to differences in their intrinsic susceptibilities to some of the most frequently employed antifungals [10,43]. The distribution of infections within the age group of this study was similar to those obtained in an investigation in the USA concerning systemic infections [10]. Studies conducted in neonatal ICUs concluded that neonates became colonized by *C. parapsilosis* acquired from the ICU setting and not from their mothers [44–46]. This yeast species was also the most commonly isolated from the hands of healthcare workers, due to the ability of higher persistence on the hands [47]. However, no link was found between this and patient acquisition of the infection/colonization [6].

Saccharomyces cerevisiae is an uncommon pathogen. Disease caused by this 'friendly' yeast is associated with severe immunosuppressed patients [48]. During the four-year period of this study, none of the eight isolates were recovered from immunocompetent patients. In the majority of circumstances the patients were very debilitated or immunodepressed.

C. dubliniensis is usually associated with HIV-positive patients although some cases have been reported in other patient populations [49]. *C. dubliniensis* may be easily misidentified as *C. albicans* due to their close phenotypic resemblance [50]. The re-identification of *C. albicans* isolates by sequencing of the D1/D2 domains of the 26S rRNA gene confirmed that in the period considered in this study no *C. dubliniensis* strains were isolated. In a one-year prevalence study in a Dutch hospital, 0.8% of the germ tube positive yeasts were *C. dubliniensis* [49]. An American study mentioned an incidence of 0.9% for this species [51], with most isolates recovered from oropharyngeal and fecal samples. Given these previous reports of low incidence, and considering that our investigation did not include oropharyngeal specimens and only includes a total of 38 *C. albicans* isolated from fecal specimens, this finding would be expected. Nevertheless, a report using a peptide nucleic acid fluorescent *in situ* hybridization analysis showed incidences of 7% in blood specimens [52].

The new yeast species belonging to the *C. parapsilosis* group, *C. metapsilosis* and *C. orthopsilosis*, were described during 2005 [23]. *C. metapsilosis*, was previously classified as *C. parapsilosis* group III and was considered a non-pathogen [53,55]. The species was thought to be best adapted to a non-mammalian environment, as opposed to *C. parapsilosis* and

C. orthopsilosis [23]. Additionally, the same authors considered the possibility of the latter species to be the evolutional ancestor of *C. parapsilosis* and *C. orthopsilosis*. An *in vitro* study compared the virulence of the three species in human reconstituted epidermis and oral epithelium, indicating that *C. metapsilosis* is less virulent than *C. parapsilosis* and *C. orthopsilosis* [54]. Nevertheless, Kocsubé and co-workers reported the isolation of a *C. metapsilosis* from a BSI [55]. And, recently it was reported in Spain that the prevalence of *C. metapsilosis* is 1.7% and 1.4% for *C. orthopsilosis*, as agents of candidemia [56]. Here we described one case of systemic infection/colonization by *C. metapsilosis* from a very critical patient. During the 4-year period of this study, the number of BSI due to *C. orthopsilosis* was one.

Final remarks

This study intended to analyze yeasts isolated from every possible biological specimen. In the literature, we could not find studies with a similar objective and the majority of available studies focus on BSI. The presence and isolation of yeasts from normally sterile sites, such as blood and cerebrospinal fluid, is sufficient to make a diagnosis of infection, while yeasts recovered from wounds, urine, sputum or faeces have a reserved etiological significance as they could be commensals and/or yeast contaminants. The diagnosis of diseases caused by yeasts has to be based on clinical data (fever, skin lesions) and epidemiologic indicators (risk factors), rather than just on the isolation of the fungus [57]. However, even if a yeast isolate is not considered as an agent of infection at one site, its presence may be the basis for the development of a secondary pathology. Colonization is a factor of increased risk for many fungal infections. Because of their opportunistic nature, they may continue as colonizers until the host enters a clinical state that is favourable for proliferation and invasion of tissue [58,59].

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References

1 Voss A, Hollis RJ, Pfaller MA, Wenzel RP, Doebbeling BN. Investigation of the sequence of colonization and candidemia in nonneutropenic patients. *J Clin Microbiol* 1994; **32**: 975–980.
2 Reagan DR, Pfaller MA, Hollis RJ, Wenzel RP. Characterization of the sequence of colonization and nosocomial candidemia using DNA fingerprinting and a DNA probe. *J Clin Microbiol* 1990; **28**: 2733–2738.
3 Burnie JP, Odds FC, Lee W, Webster C, Williams JD. Outbreak of systemic *Candida albicans* in an intensive care unit caused by cross infection. *British Med J* 1985; **290**: 746–748.
4 Betremieux P, Chevrier S, Quindos G, *et al*. Use of DNA fingerprinting and biotyping methods to study a *Candida albicans* outbreak in a neonatal intensive care unit. *Pediatr Infect Dis J* 1994; **13**: 899–905.
5 Vaudry WL, Tierney AJ, Wenman WM. Investigation of a cluster of *Candida albicans* in a neonatal intensive care unit. *J Infect Dis* 1988; **158**: 1376–1379.
6 Hedderwick SA, Lyons MJ, Liu M, Vazquez JA, Kauffman CA. Epidemiology of yeast colonization in the intensive care unit. *Eur J Clin Microbiol Infect Dis* 2000; **19**: 663–670.
7 Eggimann P, Garbino J, Pittet D. Epidemiology of *Candida* species in critically ill non-immunosuppressed patients. *Lancet Infect Dis* 2003; **3**: 685–702.
8 Richardson MD. Changing patterns and trends in systemic fungal infections. *J Antimicrob Chemother* 2005; **56** (Suppl. 1): i5–i11.
9 Sendid B, Cotteau A, François N, *et al*. Candidaemia and antifungal therapy in a French university hospital: Rough trends over a decade and possible links. *BMC Infect Dis* 2006; **6**: 80–89.
10 Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 2007; **20**: 133–163.
11 Walsh TJ, Groll A, Hiemenz J, *et al*. Infections due to emerging and uncommon medically important fungal pathogens. *Clin Microbiol Infec* 2004; **10**: 48–66.
12 Costa-de-Oliveira S, Pina-Vaz C, Mendonça D, Gonçalves-Rodrigues A. A first Portuguese epidemiological survey of fungaemia in a university hospital. *Eur J Clin Microbiol Infect Dis* 2008; **24**: 23–30.
13 Pappas PG, Rex JH, Lee J, *et al*. Prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin Infect Dis* 2003; **37**: 634–643.
14 Sanglard D, Odds FC. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect Dis* 2002; **2**: 73–85.
15 Buchner T, Fegeler W, Bernhardt H, *et al*. Treatment of severe *Candida* infections in high-risk patients in Germany: consensus formed by a panel of interdisciplinary investigators. *Eur J Clin Microbiol Infect Dis* 2002; **21**: 337–352.
16 Pappas PG, Rex JH, Sobel JD, *et al*. Guidelines for the treatment of candidiasis. *Clin Infect Dis* 2004; **38**: 161–189.
17 Hoppe JE, Frey P. Evaluation of six commercial tests and the germ-tube test for presumptive identification of *Candida albicans*. *Eur J Clin Microbiol* 1999; **18**: 188–191.

18 Borman AM, Linton CJ, Miles SJ, Johnson EM. Molecular identification of pathogenic fungi. *J Antimicrob Chemother* 2008; **61**: i7–i12.

19 Guillamón JM, Sabaté J, Barrio E, Cano J, Querol A. Rapid identification of wine yeast species based on RFLP analysis of the ribosomal ITS regions. *Arch Microbiol* 1998; **169**: 387–392.

20 Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int J Syst Bacteriol* 1999; **49**: 329–337.

21 Llanos-Frutos R, Fernández-Espinar MT, Querol A. Identification of species of the genus *Candida* by analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Anton Leeuw Int J G* 2004; **85**: 175–185.

22 Kurtzman CP, Robnett CJ. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Anton Leeuw Int J G* 1998; **73**: 331–371.

23 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.

24 León C, Ruiz-Santana S, Saavedra P, *et al*. EPCAN Study Group. A bedside scoring system ("*Candida* score") for early antifungal treatment in nonneutropenic critically ill patients with *Candida* colonization. *Crit Care Med* 2006; **34**: 730–737.

25 Thomas DP, Bachmann SP, Lopez-Ribot JL. Proteomics for the analysis of the *Candida albicans* biofilm lifestyle. *Proteomics* 2006; **6**: 5795–5804.

26 Nucci M, Marr KA. Emerging fungal diseases. *Clin Infect Dis* 2005; **41**: 521–526.

27 Essman M, Larsen B. Protective effect of the selective estrogen receptor modulator LY117018 on rat vaginal *Candida albicans* colonization. *Gynecol Obstet Invest* 2000; **49**:57–61.

28 Peleg AY, Weerarathna T, McCarthy JS, Davis TM. Common infections in diabetes: pathogenesis, management and relationship to glycaemic control. *Diabetes Metab Res Rev* 2007; **23**: 3–13.

29 Ostrosky-Zeichner L, Sable C, Sobel J, *et al.* Multicenter retrospective development and validation of a clinical prediction rule for nosocomial invasive candidiasis in the intensive care setting. *Eur J Clin Microbiol Infect Dis* 2007; **26**: 271–276.

30 Fidel PL. *Candida*-host interactions in HIV disease: Relationships in oropharyngeal candidiasis. *Adv Dent Res* 2006; **19**: 80–84.

31 Aw D, Silva AB, Palmer DB. Immunosenescence: emerging challenges for an ageing population. *Immunology* 2007; **120**: 435–446.

32 Laube S. Skin infections and ageing. *Ageing Res Rev* 2004; **3**: 69–89.

33 Brawner DL, Cutler JE. Oral *Candida albcans* isolates from non-hospitalized normal carriers, immunocompetent hospitalized patients, and immunocompromised patients with or without acquired immunodeficiency syndrome. *J Clin Microbiol* 1989; **27**: 1335–1341.

34 Kullberg BJ, Oude-Lashof AML. Epidemiology of opportunistic invasive mycoses. *Eur J Med Res* 2002; **7**: 183–191.

35 Odds FC, Hanson MF, Davidson AD, *et al*. One year prospective survey of *Candida* bloodstream infections in Scotland. *J Med Microbiol* 2007; **56**: 1066–1075.

36 Ellepola AN, Morrison CJ. Laboratory diagnosis of invasive candidiasis. *J Microbiol* 2005; **43**: 65–84.

37 Horvath LL, Hospenthal DR, Murray CK, Dooley DP. Detection of simulated candidemia by the BACTEC 9240 system with Plus Aerobic/F and Anaerobic/F Blood Culture Bottles. *J Clin Microbiol* 2003; **41**: 4714–4717.

38 Maaroufi Y, De Bruyne JM, Duchateau V, Georgala A, Crokaert F. Early detection and identification of commonly encountered *Candida* species from simulated blood cultures by using a real-time PCR-based assay. *J Molec Diagn* 2004; **41**: 3293–3298.

39 White PL, Barton R, Guiver M, *et al*. Consensus on fungal polymerase chain reaction diagnosis? A United-Kingdom-Ireland evaluation of polymerase chain reaction methods for detection of systemic fungal infections. *J Mol Diagn* 2006; **8**: 376–384.

40 White TJ, Bruns T, Lee S, Taylor J. 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*. San Diego, CA: Academic Press, 1990: 315–322.

41 Trost A, Graf B, Eucker J, *et al*. Identification of clinically relevant yeasts by PCR/RFLP. *J Microb Meth* 2003; **56**: 201–211.

42 Fridkin SK. The changing face of fungal infections in health care settings. *Clin Infect Dis* 2005; **41**: 1455–1460.

43 García-Martos P, Domínguez I, Marin P, *et al*. Antifungal susceptibility of emerging yeast pathogens. *Enferm Infec Microbiol Clin* 2001; **19**: 249–256.

44 Ruiz-Diez B, Martinez V, Alvarez M, Rodriguez-Tudela JL, Martinez-Suarez JV. Molecular tracking of *Candida albicans* in a neonatal intensive care unit: long-term colonizations versus catether related infections. *J Clin Microbiol* 1997; **35**: 3032–3036.

45 Reef SE, Lasker BA, Butcher DS, *et al*. Nonperinatal nosocomial transmission of *Candida albicans* in a neonatal intensive care unit: prospective study. *J Clin Microbiol* 1998; **36**: 1255–1259.

46 Waggoner-Fountain LA, Walker MW, Hollis RJ, *et al*. Vertical and horizontal transmission of unique *Candida* species to premature newborns *Clin Infect Dis* 1996; **22**: 803–808.

47 Rangel-Frausto MS, Houston AK, Bale MJ, Fu C, Wenzel RP. An experimental model for study of *Candida* survival and transmission in human volunteers. *Eur J Clin Microbiol Infect Dis* 1994; **13**: 590–595.

48 de Llanos-Frutos R, Fernandez-Espinar MT, Querol A. A comparison of clinical and food *Saccharomyces cerevisiae* isolates on the basis of the potential virulence factors. *Anton Leeuw Int J G* 2006; **90**: 221–231.

49 Meis JF, Lunel FM, Verweij PE, Voss A. One-year prevalence of *Candida dubliniensis* in a Dutch university hospital. *J Clin Microbiol* 2000; **38**: 3139–3140.

50 Mosaid A, Sullivan D, Salkin IF, Shanley S, Coleman DC. Differentiation of *Candida dubliniensis* from *Candida albicans* on Staib Agar and Caffeic Acid-Ferric Citrate Agar. *J Clin Microbiol* 2001; **39**: 323–327.

51 Jabra RM, Baqui AA, Kelley JA, *et al*. Identification of *Candida dubliniensis* in a prospective study of patients in the United States. *J Clin Microbiol* 1999; **37**: 321–326.

52 Jabra-Rizk MA, Johnson JK, Forrest G, *et al*. Prevalence of *Candida dubliniensis* fungemia at a large teaching hospital. *Clin Infect Dis* 2005; **41**: 1064–1067

53 Clark TA, Morgan J, Brandt M, *et al*. Hospital outbreak of *Candida parapsilosis* bloodstream infections – Mississippi, 2001. Proceedings of the 42nd Intersci Conf Antimicrob Agents Chemother, San Diego, California. 2002: M-880.

54 Gácser A, Schäfer 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.

55 Kocsubé S, Tóth M, Vágvölgyi C, *et al*. Occurrence and genetic variability of *Candida parapsilosis sensu lato* in Hungary. *J Med Microbiol* 2007; **56**: 190–195.
56 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.
57 Ascioglu S, Rex JH, de Pauw B, *et al*. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin Infect Dis* 2002; **34**: 7–14.
58 O'Donnell K. Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Curr Genet* 1992; **22**: 213–220.
59 Agvald-Öhman C, Klingspor L, Hjelmqvist H, Edlund C. Invasive candidiasis in long-term patients at a multidisciplinary intensive care unit: *Candida* colonization index, risk factors, treatment and outcome. *Scand J Infect Dis* 2008; **40**: 145–153.