Diversity and frequency of yeasts in the lower respiratory tract of TICU patients

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Preceding yeast colonisation is known to be a major risk factor for candidemia in special patient groups. Some yeasts are resistant to routinely used antifungals, biodiversity of the colonising strains is a critical point for therapy and the course of a possible infection. Diversity and frequency of yeasts in the respiratory tract of Trauma-Intensive-Care-Unit (TICU) patients without preventive antimycotic treatment were analysed in view of TICU stay duration and age groups. Two-hundred respiratory-tract-secretion samples (RTSS: sputum, tracheal and bronchial secretions, broncho-alveolar lavages) were serially taken from all TICU-patients stationed during a period of four months. RTSS were inoculated on a new semi-selective medium (SceSel+). Pure cultures (n = 493) from RTSS were identified by morphological and physiological means. Statistical analyses were conducted to determine correlations between patient age, duration of TICU stay, and patterns of colonising microorganisms. Of the RTSS, 108 were yeast-positive (54.0 %). Yeasts identified as *Candida albicans* occurred in 46.0 % of all RTSS, *C. glabrata* (10.0 %), *C. lusitaniae* (4.5 %), *C. tropicalis* (4.0 %), *C. dubliniensis* (2.5 %), *Rhodotorula* sp. (1.0 %), *C. famata,* and *C. kefyr* (0.5 %). In 84 samples (42.0 %), only strains of one species were isolated, 9.5 % contained two, while three or more were identified in 2.5 % of the samples. Colonisation by *C. glabrata* positively correlated with duration of the TICU stay. Duration of the TICU stay and patients' age are considered as main risk factors for multiple yeast colonisations shifting to therapy refractory yeasts. Yeast diversity increased significantly with age and duration of TICU stay. More than 50 % of patients younger than 30 years were almost exclusively colonised by *C. albicans* (22 out of 42 patients).

Keywords: epidemiology, *Candida* spp., intensive care, yeast consortia, risk factors

Candida albicans is known to cause severe opportunistic infections in predisposed patients such as immuno-compromised persons.

The number of *Candida* infections has been increasing due to the growing population of susceptible individuals (Perlroth *et al.* 2007). One of the major risk factors contributing to the development of candidemia in Intensive-Care-Unit (ICU) patients is a preceding colonisation by yeasts in the respiratory tract (Jorda-Marcos *et al.* 2007). Other risk factors include hospitalisation in an ICU, major surgery, and solid organ-transplantation (Perlroth *et al.* 2007). *Candida albicans*, the most frequent coloniser and agent of disseminated *Candida* infections, can be satisfactorily controlled by antimycotic therapy. Strains of *C. krusei* and *C. glabrata* are known to have an intrinsic resistance to fluconazole (Borg-von Zepelin *et al.* 1993) and *C. tropicalis* strains are known to be resistant to flucytosine (Fleck *et al.* 2007). Gray *et al.* (1988) described respiratory-tract-secretion samples (RTSS) as a reliable source for the detection of disseminating yeast infections.

Little is known concerning the diversity and frequency of different yeasts occurring in Trauma-Intensive-Care-Unit (TICU) patients (Wood *et al.* 2006). This study aims to provide appropriate data on colonising yeasts in the respiratory tract of TICU patients. A main focus was on the actual diversity of yeast consortia in RTSS of TICU patients including quantitative aspects.

Patients and Methods

A total of 200 RTS samples were taken, first at admission, and then, depending on conspicuous symptoms of respiratory infection (on average 3.4 samples per patient), over a four month period (October 2005 to February 2006) including 58 TICU patients (14 female [n = 21] and 44 male [n = 179]; age range 13 y to 84 y) at the Medical University Hospital Innsbruck, Austria. Most patients (95 %) were mechanically ventilated and received artificial nutrition support through internal tubes. The sampled patients suffered from multiple-traumata: according to our statistical data, 60 % of TICU patients have craniocerebral injuries, 50 % thorax injuries, 40 % abdomen or pelvic injuries, 20 % spinal injuries, and 70 % suffered from extremity injuries. Patients did not receive any preventive antimycotic therapy. The severity-code of injuries and the thereby resulting mortality rate for the TICU-inpatients is according to TRESS-methodology and SAPS2-24-methodology 20 % to 25 percent. In fact, the mortality rate at the TICU in Innsbruck is ten percent.

The following samples are summarised under the term RTSS: sputum samples ('S' [n = 6]), tracheal secretions ('TS' [n =137]), bronchial secretions ('BS' [n = 5]), and broncho-alveolar lavages ('BAL' [n = 52]). Sampling methods depended on the condition of the patients. Sputum was sampled from patients that were not mechanically ventilated by using a sterile sputum tube (Seidel, Germany). TS were obtained via an orotracheal-suction-tube (Lo-Contour™ Murphy, Mallinckrodt, Ire-

land) or via a tracheotomy-suction-tube (Willy Rusch AG, Germany) from mechanically ventilated patients. TS of independently respiring patients were acquired via a nosotracheal-suction-tube (Dahlhausen, Germany). If TS was overly viscous, 10 mL of sterile physiological NaCl-solution was added to keep the sample sufficiently moist and fluid. BS was collected with a bronchoscope. BAL was sampled by the injection of NaCl-solution with a bronchoscope. The solution was re-absorbed with the sucker of the bronchoscope and was collected in a sterile vessel.

Fresh S, TS, and BS samples were homogenised by vortexing for 5 s to 10 s , and spread onto SceSel+ medium (Rainer *et al*. 2008) with a sterile cotton swab. BAL samples were centrifuged for 10 min at 2000 rpm, then the supernatant was discarded, and the resulting pellet spread onto SceSel+ medium with a sterile cotton-swab. All cultures were incubated at 37 °C for 7 days. From each petri dish, up to five pure cultures of single colonies (n = 493) were obtained and sub-cultured onto Sabouraud Glucose (2 %) Agar (SGA-2%).

Isolates of *Candida albicans* and *C. dubliniensis* were separated from all other yeast isolates using the chromogenic agar Candida-ID-2 (BioMérieux, France). Germ tube tests (Berardinelli and Opheim 1985) were applied when doubtful colouring appeared. To differentiate strains of *C. albicans* from *C. dubliniensis*, temperature tests were conducted at a temperature of 43 °C following the protocol of Pinjon *et al*. (2005). All other strains were identified with the help of API-C-20-AUX (BioMérieux, France). API results were confirmed by means of morphological and microscopic characteristics according to de Hoog *et al*. (2000) with a Nikon Optiphot 2 (Japan) with bright field and Nomarski Interference Contrast.

Chi-square tests and coefficient of contingency (C) were applied in order to detect significant dependences and correlations. Coefficients of contingency were categorised as follows: $C \leq 0.2$ indicates a very low correlation, $0.2 < C \leq 0.5$ indicates low correlation, $0.5 < C \leq 0.7$ indicates a medium correlation, $0.7 < C \leq 0.9$ indicates a high correlation and $0.9 < C$ a very high correlation. All reported p-values were 2-sided and a Type I error level of 5 % was used. Calculations were performed by using SPSS (version 14.0) software (SPSS GmbH Software).

Categories for statistic analyses: (1) "micro-organism positive" (samples containing fungi and/or bacteria), (2) "yeast-positive" (samples containing yeasts regardless of additional bacteria), (3) with the subcategories "exclusively yeasts", "exclusively bacteria", "yeasts and bacteria", (4) "abundance of individual *Candida* spp.", (5) "species diversity per RTSS".

Results

Overall, 54 % of the 200 RTSS were yeast-positive, 4.5 % were exclusively bacteria-positive, and in 41.5 % of the samples, neither

fungi nor bacteria were detected. Seven *Candida* species and one *Rhodotorula* species were identified from the 493 pure cultures obtained from the 200 original RTS samples.

Isolates of *Candida albicans* were most common (46.0 % of the RTSS), followed by *C. glabrata* (10.0 %), *C. lusitaniae* (4.5 %), *C. tropicalis* (4.0 %), C. *dubliniensis* (2.5 %), *Rhodotorula* sp. (1.0 %), *C. famata* (0.5 %), and *C. kefyr* (0.5 %).

Of the 108 yeast-positive samples (= 100 %), 84 samples (77.8 %) contained strains belonging to a single species whereas 19 (17.6 %) belonged to two species, and three or more species were identified in the remaining five samples (4.6 %).

Correlations were found between the duration of the TICU stay and (i) positive samples with microorganisms according to "category 3" ($p < 0.001$, $C = 0.550$), (ii) colonisation with *C. glabrata* ($p < 0.001$, C= 393), and (iii) colonisation with *C. tropicalis* ($p < 0.001$, $C = 0.437$).

The simultaneous occurrence of *C. dubliniensis* and *C. lusitaniae* was significant ($p < 0.001$; $C = 0.386$) and also that of *C. glabrata* and *C. tropicalis* ($p < 0.001$, C= 0.527).

Significant correlations were found between patients' age and the abundance of microorganisms in general ("category 1") ($p = 0.004$, $C = 0.297$), colonisation with yeasts ("category 2") ($p < 0.001$, $C = 0.331$), colonisation types according to "category 3" ($p = 0.002$, $C = 0.406$), colonisation with *C. lusitaniae* ($p < 0.001$, $C = 0.580$), *C. albicans* ($p = 0.001$, $C = 0.325$), *C. glabrata* ($p = 0.001$, $C = 0.318$), *C. dubliniensis* ($p = 0.008$, $C = 0.282$), *C. tropicalis* ($p = 0.024$, $C = 0.261$) and the number of yeast species per sample (category 5) ($p < 0.001$, $C = 0.510$). *Candida lusitaniae* was isolated exclusively from the respiratory tract of 31 to 40 year old patients. *Candida glabrata* was found with the highest frequency in patients older than 70 years and was not found in patients younger than 41 years. Most *Candida tropicalis* isolates (87.5 %) originated from patients older than 70 years. The respiratory tract of patients younger than 30 years was yeast-negative or was colonised by strains of a single species, whereas in RTSS from patients older than 31 years up to four yeast species could be identified (Table 1).

Correlations were detected for the diversity per RTSS ("category 5"). So, *C. albicans* and *C. glabrata* were most frequently found as single colonisers ($p < 0.001$, $C = 0.654$; $p < 0.001$, $C = 0.519$). *Candida lusitaniae* ($p < 0.001$, $C = 0.532$), *C. dubliniensis* ($p < 0.001$, $C = 0.509$), and *C. tropicalis* ($p < 0.001$, $C = 0.430$) tended to grow in communities with other yeasts.

Discussion

This study was intended as a 'descriptive study' aiming to elucidate the fungal diversity and the corresponding colonisation rates in respiratory tract secretion samples (RTSS). All patients stationed at

the TICU within the four month period were sampled despite their individual case history and hence do not necessarily represent a homogenous patient group; following up the latter, another shortcoming of this study is that information on subsequently developed and proven candidemia are not provided, because this was not within the bounds of possibility for this study.

Although the frequency of systemic fungal infections in Intensive-Care patients has increased in recent years, only few studies exist on the diversity and frequency of yeasts (Perlroth *et al.* 2007). *Candida* infections are responsible for 10.1 % of all nosocomial infections at Intensive Care Units (Jarvis and Martone 1992). The mortality rate of ICU patients correlates significantly with *Candida* infections (Chen *et al.* 2000) and may reach 60 % (Pfaller *et al.* 1999). In our study, 68.9 % of the 58 patients were permanently or transiently colonised by yeasts. Risk factors for candidemia such as *Candida* colonisation of the respiratory tract have been described previously (Gray *et al.* 1988). Whether or not *Candida*-positive BAL or BS samples represent strong evidence for fungal and/or bacterial infection of the lower respiratory tract (Gray *et al.* 1988) seems to be still a matter of controversy (pers. comm. Anonymous 2009).

Even if yeasts other than *Candida albicans* were increasingly more often identified over the past years, *C. albicans* is still the most common fungus isolated from critically ill patients (Perlroth *et al.* 2007, Pfaller *et al.* 1999). In our study, *C. albicans* was the predominant colonising yeast, but with 46.0 %, it mirrored a world-wide trend of decreasing frequency (Perlroth *et al.* 2007). This observation may be explained also by the improved differentiation between *C. albicans* and *C. dubliniensis*; the latter was first described in 1995 (Sullivan *et al.* 1995). *Candida glabrata w*as the second most common yeast, confirming previous results (Perlroth *et al.* 2007, Maesaki *et al.* 1993). In contrast, Pfaller *et al.* (1999) reported *C. parapsilosis* as the second most frequent yeast in Europe, Canada and Latin-America. In our study, the absence of *C. parapsilosis* was unlikely to be caused by the isolation procedure with SceSel+, as this yeast has been reported not to be sensitive to dichloran or benomyl (Deak *et al.* 2001, Summerbell 1993), which are two major selective components of SceSel+. Additional growth tests on SceSel+ (data not shown) revealed, however, that *C. parapsilosis* was able to grow on this medium, though slower than other yeasts. To date, *C. parapsilosis* has rarely been encountered in the Medical University Hospital Innsbruck. Other investigations reported a frequency of *C. lusitaniae* of one to two percent in ICU-patients (Maesaki *et al.* 1993, Ahmad *et al.* 2003)]. *Candida lusitaniae* presents problems in patients receiving cytotoxic chemotherapy or broad-spectrum antibiotics, as well as in patients with major surgery or in transplant recipients (Alomar *et al.* 2003, Dilorenzo *et al.* 1997).

During this study, up to four yeast species per patient were identified. TICU patients older than 41 years were more frequently colonised by yeast consortia (Table 1) or, in the case of colonisation by yeasts of a single species other than *C. albicans,* with *C. dubliniensis*, *C. glabrata*, or *C. tropicalis*. This may be due to a less effective natural clearance of the respiratory tract (Wilson 2005). It could be speculated that the number of respiratory tract colonisers may have an impact on the risk of the development of intrinsic or acquired antimycotic resistances of non-*albicans Candida* strains (Bassetti *et al.* 2006). A positive practical impact of this finding is that for patients younger than 41 years, medication problems should not be expected (Table 1).

There is evidence that different kinds of yeasts grow in communities, having a predilection for different parts of the respiratory tract. According to Azoulay *et al.* (2006), single colonisers are *C. albicans, C. glabrata,* and *C. tropicalis*, whereas *Candida parapsilosis, C. lusitaniae,* and *C. krusei* were usually isolated together with other *Candida* representatives. We also detected a positive correlation for the co-occurrence of *C. dubliniensis* and *C. lusitaniae*. All *C. lusitaniae* and most *C. dubliniensis* strains (83.3 %) were found together with at least one other yeast. *Candida glabrata* (30 % of all *Candida glabrata* strains) was the most frequent single coloniser after *C. albicans* (81.5 %). When growing in communities, *C. glabrata* was mainly associated with *C. tropicalis*. Colonisation and infection by *C. albicans* is thought to be endogenous, inhibiting the growth of less competitive yeasts like *C. dubliniensis* (Perlroth *et al.* 2007).

Our data support the results of Azoulay *et al.* (2006) who reported that the colonisation of the respiratory tract by yeasts increases with the time of ICU-stay. At admission to the TICU, 19.5 % of our patients had yeast-positive RTSS. After 15 days of TICU-stay, the percentage increased to more than 30 %. At the day of admission to TICU, approximately 56 % of RTSS were "microorganism-positive" (category 1: samples with yeasts and/or bacteria). After 21 days, 86 % of RTSS were microorganism-positive; after six weeks the lower respiratory tract of all sampled patients was colonised by microorganisms.

The diversity of isolated *Candida* strains has been changing with the duration of TICU-stay. At admission to TICU, most patients were colonised by *C. albicans* only. During TICU-stay, the abundance of *C. albicans* decreased significantly, whereas the abundance of *C. glabrata* increased, and the frequency of *C. tropicalis* remained almost stable. So far, we have no causal explanation for these phenomena. Comparable results have also been found by Borg-von Zepelin *et al.* (1993).

An important finding of the present study was the detection of age-dependent risk groups for yeast colonisation: a positive correlation of the frequency of microorganisms (category 1) and patient's age; exclusively patients older than 70 years were colonised by yeasts and bacteria (subcategory of category 3). The bacteria in these samples

Tab. 1. – Diversity of yeasts and corresponding colonisation rates in respiratory tract secretion samples (RTSS) of TICU patients (n = 58; 13 females, 45 males) hospitalised at the Medical University Hospital Innsbruck during the sample period (four months), according to age groups. Values refer to the number of positive samples; [n] is the respective sample size.

Patients	**Samples**	**RTSS**[1]				**Diversity and Frequency of *Candida* strains**[2]					**Species per RTSS**				
Age groups	[n]	Y	Y & B	B	S	*C. albicans*	*C. dubliniensis*	*C. glabrata*	*C. tropicalis*	*C. lusitaniae*	**0**	**1**	**2**	**3**	**4**
≤ 20 y	13	2	0	2	9	2	0	0	0	0	11	2	0	0	0
21 – 30 y	29	21	0	0	8	20	0	0	1	0	8	21	0	0	0
31 – 40 y	17	13	0	0	4	11	3	0	0	9	4	4	6	2	1
41 – 50 y	31	12	1	1	17	11	0	3	0	0	18	12	1	0	0
51 – 60 y	12	10	0	0	2	10	0	2	0	0	2	8	2	0	0
61 – 70 y	40	16	0	1	23	16	0	1	0	0	24	15	1	0	0
≥ 71 y	58	29	4	5	20	22	3	14	7	0	25	21	10	2	0
Total	**200**	**103**	**5**	**9**	**83**	**92**	**6**	**20**	**8**	**9**	92	83	20	4	1

[1] Y = RTS samples with exclusively yeasts; Y & B = yeasts and bacteria; B = exclusively bacteria; S = sterile samples

[2] The horizontal checksums do not equal the respective numbers of yeast positive RTSS because of multiple colonisations; compare 'Species per sample'.

must be considered as antibiotic resistant, because the isolation medium (SceSel+) contained chloramphenicol, streptomycin, and ciprofloxacin.

We found that older patients, especially patients over 70 years, are distinctly more often colonised by yeasts other than *C. albicans*. Positive age-dependent correlations were found for the abundance of *C. dubliniensis* (patients 31–40 years, > 70 years), *C. glabrata* (patients > 41 years), and *C. lusitaniae* (patients 31–40 years), whereas the abundance of *C. albicans* decreased with patients' age. The frequency of the relatively rarely isolated *C. tropicalis* (4.0 % of all 200 RTSS) was mostly found in patients older than 70 years.

A similar phenomenon has been observed in bloodstream infections (Bassetti *et al.* 2006). Analyses revealed a correlation between the abundance of yeast consortia (diversity per sample) and the age of patients. Most yeast-negative samples originated from patients younger than 21 years. In samples from patients younger than 31 years, no more than one species was identified, which proved to be *C. albicans* in 95 % of the samples. Patients older than 31 years were significantly more often colonised by different yeasts simultaneously, thus the choice of the most efficient antifungal agent may be critical because the risk for ineffective medications increases with the diversity of yeasts.

From this study we have determined that the period of stay at the TICU coincides with the diversity and abundance of yeasts in the lower respiratory tract of patients. Similarly, there is evidence that the age of patients is a predisposing factor for yeast colonization, particularly for colonization by multi-species consortia. As this was a descriptive study, an important issue for future research has to be the clarification of the impact of individual patients histories on the correlations and colonisation patterns reported here.

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