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Microsatellite Typing of Catheter-Associated *Candida albicans* Strains

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1. Introduction

Candida albicans is the most common pathogenic fungus and occurs frequently in the digestive tract (Bernhardt, 1998; Doskey, 2004). Vaginal candidiasis (Mohanty et al. 2007; Paulitsch et al., 2006; Sobel, 2007) is also a wide spread problem. This species can become invasive, causing infections on many different sites in patients with severe underlying diseases (Marol & Yükesoy, 2008; Odds et al., 2007).

Catheter or shunt related infections caused by *C. albicans* (Pierce 2005) were reported e.g. by Sánchez-Portocarrero et al. (1994), David et al. (2005) and Tumbarello et al. (2007).

The classical picture of yeast cells as unicellular life forms is based on the pure-culture model of growth. In their natural habitat microorganisms including yeasts are mostly organized in biofilm ecosystems which are often ´multicultural´, made not only of yeasts but also of bacteria (El-Aziz et al., 2004; López-Ribot, 2005; Ramage et al., 2005; Nobile et al., 2006). The possibility to adhere to a surface is a very important factor for the development of fungal (Hogan, 2006; Verstrepen & Klis, 2006) and bacterial biofilms (Dolan, 2001).

Microsatellites, which are also known as short tandem repeats, are repeated nucleotide sequences with a length from 2 up to 7 base pairs. These polymorphic DNA loci are variable within a population and in this way multiple alleles are created for a single microsatellite locus. These different multilocus genotypes are used to distinguish strains within a single species (Applied Biosystems [AB], 2005). Microsatellite markers provide the possibility to discriminate strains of the same species and to trace their epidemiological pathways (Botterel et al., 2001; Sampaio et al., 2005).

For this study, pairs for three loci (CDC3, EF3, and HIS3) on three different chromosomes developed by Botterel et al. (2001) were used to compare the *C. albicans* strains which were found to produce a biofilm, with those strains which did not produce a biofilm on the investigated catheter material. The differentiation of biofilm and non-biofilm forming

strains was based on scanning electron microscopical findings (Paulitsch et al., 2009). Different primer pairs and also different combinations of primer pairs for the subtyping of *C. albicans* were reported elsewhere, see e.g. the works of Sampaio et al. (2005) or Fan et al. (2007).

For each marker and for a given isolate one or two bands were observed, and each observed band was assigned to an allele. Because *C. albicans* is diploid each strain can be characterized by six alleles with the method used.

The discriminatory power (DP) is a numerical index to describe the probability that two unrelated samples of a test group are placed in two different typing groups. The DP of EF3 is 0.86, the DP of CDC3 is 0.77, and the DP of HIS3 is 0.91 (Botterel et al., 2001). The combined DP of all three markers was 0.97. In order to get reliable results, this index has to be greater than 0.90 (Botterel et al., 2001).

2. Microsatellite typing

2.1 Material and methods

The 123 *C. albicans* (64 [52%] of them biofilm positive) strains for this study were collected during a study in biofilm forming abilities of yeast on indwelling devices (Paulitsch et al., 2009). The strains were stored at -70°C until examination.

Strains were subcultured on Sabouraud agar plates for 24 h at 35°C. For DNA extraction the PrepManTM Ultra Kit (Applied Biosystems [AB], Foster City, California) was used. For the microsatellite typing three different primer pairs were used (Botterel et al., 2001). The unmarked primers were HIS3R, CDC3R, and EF3 (Invitrogen, Lofer, Austria). The fluorescence labeling of the primers HIS3 (NEDTM, yellow), CDC3 (VICTM, green), and EF3R (6-FAMTM, blue) (all AB) was fitted to the DyeSet DS-33 (AB) which is recommended for 5-dye custom primer analyses. PCR was performed using the 96 well GeneAmp PCR System 9700 or the 96 well 2700 Thermal Cycler (both AB). PCR reactions were carried out as singleplex reactions for each primer pair. The samples were initially incubated for 2 minutes at 94°C to activate the Taq Polymerase (Eppendorf, Hamburg, Germany) and to denature the DNA. After thermal cycling (30 cycles; 94°C for 45 s, 48°C for 45 s, 68°C for 90 s) samples were kept at 68°C for another 5 minutes to complete partial polymerization.

Sample preparation for the injection in the 3100 Automatic Sequencer (AB) was done following the instructions. For analysis 1 µL of PCR product, 0.3 µL of size standard (GeneScanTM 500-LIZ®, AB) and 10 µL Hi-DiTM Formamide (AB) were mixed and transferred into a 96 well plate. The samples were denatured for 4 minutes at 94°C in a thermal cycler and immediately placed on ice. In every run three samples were used as internal control. The plate was transferred in the sequencer and processed using the Foundation Data Collection 3.0 software of the sequencer.

Data analysis was done with the GeneMapper® v3.7 software. Therefore it was necessary to set up the microsatellite analyses following the instructions of the manual (AB, 2005). The peaks were automatically detected (Auto Binning) with the created bin set, low quality data were checked manually and corrected. The results were exported in a Microsoft Excel sheet for documentation.

2.2 Results

Typing of 123 *C. albicans* strains was done with the above mentioned three primer pairs. Only from strain number 85 (sample W60) no data from the EF3 locus was producible. Although the DNA was isolated a second time and several PCR reactions were done for this locus, no peaks could be generated. In table 1 detailed information of all three loci for each strain is listed.

		CDC3/CDC3R		EF3/EF3R		HIS3/HIS3R	
	sample	**allele 1**	**allele 2**	**allele 1**	**allele 2**	**allele 1**	**allele 2**
1	*K7*	117	129	130	139	154	154
2	K10	125	125	133	133	174	186
3	*K11*	117	129	130	139	154	154
4	*K13*	125	125	126	133	166	182
5	K14	117	129	130	139	154	154
6	K15	125	125	123	123	174	182
7	*K16*	125	125	126	126	174	182
8	*K17*	117	129	130	139	154	154
9	K18	117	129	129	139	154	154
10	*K19*	117	125	120	129	162	218
11	*K21*	117	117	120	126	162	186
12	*K22*	117	125	120	129	162	162
13	K23	125	125	126	126	214	234
14	*K24*	117	125	120	129	162	214
15	*K25*	121	129	130	139	154	154
16	K26	117	129	130	139	154	154
17	*K27*	117	117	123	129	150	162
18	*K28*	125	129	123	123	154	166
19	*K29*	117	129	130	139	154	154
20	K30	117	117	130	139	154	154
21	K31	117	125	120	129	162	202
22	K32	117	121	126	129	162	182
23	*K35*	117	125	120	129	162	214
24	K36	117	125	120	129	162	166
25	K37	113	117	123	130	150	162
26	K38	121	125	123	137	154	166
27	*K39*	117	117	123	129	174	178
28	K40	125	125	123	137	158	158
29	K41	121	129	129	139	154	154
30	*K45*	125	125	123	137	154	166
31	K49	117	125	120	129	162	198
32	*K50*	117	125	120	129	162	198
33	K51	117	129	129	139	154	154
34	K53	117	129	129	139	146	154
35	K54	121	125	123	137	154	166

		CDC3/CDC3R		EF3/EF3R		HIS3/HIS3R	
	sample	**allele 1**	**allele 2**	**allele 1**	**allele 2**	**allele 1**	**allele 2**
36	K57	117	117	126	137	154	182
37	*K58*	117	117	129	133	150	170
38	*K59*	117	129	129	137	154	154
39	K60	117	129	130	139	154	154
40	K62	121	125	123	137	154	166
41	K63	117	129	129	139	143	154
42	K64	117	125	120	129	166	230
43	K65	111	117	123	130	154	198
44	K66	125	125	123	133	166	182
45	*K67*	117	117	130	139	154	158
46	*K68*	117	125	120	120	162	198
47	*K69*	117	129	130	139	154	158
48	K71	121	125	123	123	166	166
49	W2	113	117	123	129	150	162
50	W3	117	125	129	129	162	162
51	*W4*	117	117	126	139	154	186
52	*W5*	117	125	120	129	162	198
53	*W6*	117	129	130	139	154	154
54	*W8*	117	125	120	129	162	162
55	*W9*	117	125	120	120	162	206
56	W11	117	125	120	120	154	162
57	W13	117	121	129	129	150	150
58	W14	117	125	126	126	162	162
59	W15	125	125	126	133	166	182
60	W16	125	125	123	133	166	182
61	*W17*	117	125	129	133	186	206
62	W18	117	125	126	126	178	178
63	W19	117	125	120	129	162	198
64	*W20*	117	125	129	129	190	202
65	W21	125	125	126	126	186	222
66	*W22*	125	125	123	139	166	166
67	W25	117	125	120	129	206	210
68	*W26*	117	117	126	139	142	154
69	*W27*	109	117	129	139	154	154
70	W34	117	117	126	139	154	158
71	*W37*	117	125	126	126	162	186
72	*W38*	113	117	123	129	150	162
73	W39	117	125	126	126	162	186
74	W44	117	129	130	139	154	154
75	*W45*	117	129	130	139	154	154
76	W47	109	117	126	131	154	154
77	W48	117	125	130	133	154	162

		CDC3/CDC3R		EF3/EF3R		HIS3/HIS3R	
	sample	**allele 1**	**allele 2**	**allele 1**	**allele 2**	**allele 1**	**allele 2**
78	W51	117	125	126	133	166	166
79	*W52*	117	125	120	129	162	194
80	W53	125	129	130	139	166	166
81	W54	125	125	126	133	182	194
82	*W55*	117	125	120	120	162	214
83	*W56*	113	117	123	129	150	162
84	*W58*	121	129	139	139	154	154
85	*W60*	117	125			162	210
86	*W61*	117	129	130	141	154	154
87	W62	117	121	126	126	162	190
88	*W63*	125	125	133	133	194	198
89	*W64*	117	129	129	139	154	154
90	*W65*	109	117	126	137	154	214
91	*W67*	117	125	120	129	170	206
92	*W68*	121	121	120	129	162	202
93	*W69*	117	125	129	139	162	202
94	W70	117	129	129	139	154	154
95	W71	113	117	126	139	150	162
96	*W73*	117	117	126	137	154	186
97	*W74*	117	125	120	129	166	226
98	*W75*	109	117	126	129	162	186
99	*W76*	117	117	126	139	154	182
100	*W77*	117	129	130	130	154	186
101	*W78*	117	129	129	129	154	162
102	W79	121	129	130	141	154	154
103	W80	117	129	120	129	194	206
104	W82	117	117	123	129	150	150
105	*W83*	117	125	120	120	162	198
106	*W84*	113	117	129	129	162	162
107	*W85*	117	125	120	129	162	206
108	*W87*	117	117	120	129	162	178
109	*W91*	117	129	129	139	142	142
110	W92	117	125	123	133	166	186
111	W94	117	129	129	139	154	154
112	W96	113	117	129	129	150	150
113	W97	117	117	123	130	178	182
114	*W98*	121	129	130	139	154	154
115	*W101*	117	117	126	137	154	154
116	W102	125	125	123	133	166	182
117	W103	125	125	126	133	182	186
118	*W104*	117	129	130	139	154	154
119	*W106*	121	125	123	137	158	166

		CDC3/CDC3R		EF3/EF3R		HIS3/HIS3R	
	sample	**allele 1**	**allele 2**	**allele 1**	**allele 2**	**allele 1**	**allele 2**
120	W107	117	129	129	139	154	158
121	W108	117	129	123	133	162	202
122	*W110*	121	125	123	137	154	166
123	*W113*	117	125	129	133	178	178

(samples in italic letters: biofilm positive)

Table 1. Microsatellite data for 123 *C. albicans* strains.

A comparison of the results did not reveal information of typical microsatellite models for *C. albicans* strains which produced biofilms in this study. Only 41 of the investigated strains showed a similarity with one or up to six other strains (Table 2).

CDC3/CDC3R		EF3/EF3R		HIS3/HIS3R		n		
allele 1	**allele 2**	**allele 1**	**allele 2**	**allele 1**	**allele 2**	**+**	**-**	**total**
113	117	123	129	150	162	2		2
117	129	129	139	154	154	1	4	5
117	129	130	139	154	154	7	4	11
121	129	130	139	154	154	2		2
121	125	123	137	154	166	1	3	4
117	125	120	129	162	162	2		2
117	125	126	126	162	186	1	1	2
117	125	120	120	162	198	2		2
117	125	120	129	162	198	2	2	4
117	125	120	129	162	214	2		2
125	125	123	133	166	182		3	3
125	125	126	133	166	182	1	1	2
						23	18	41

(+: biofilm positive; -: biofilm negative)

Table 2. Microsatellite models.

The most convergent data were generated with the CDC3 primer pair, only 12 different allele pairs were; found, with the EF3 primer pair 25 different pairs were located, and HIS3 primers provided 50 different pairs of alleles.

From six patients two strains were available, each of them originated from different samples and showed *C. albicans* infections in routine diagnostics. Both samples from one patient were biofilm positive, from another patient both samples were negative. The microsatellite data of these catheters are listed in table 3. When only HIS3 and CDC3 alleles were compared, five out of the six patients showed the same strain two times, when they were also compared with EF3 primer alleles, only one patient had the same strain two times.

The comparison of the genotyping of biofilm forming *C. albicans* strains (e.g. see figure 1) with non-biofilm forming *C. albicans* species shows also a consistent distribution of genotypes.

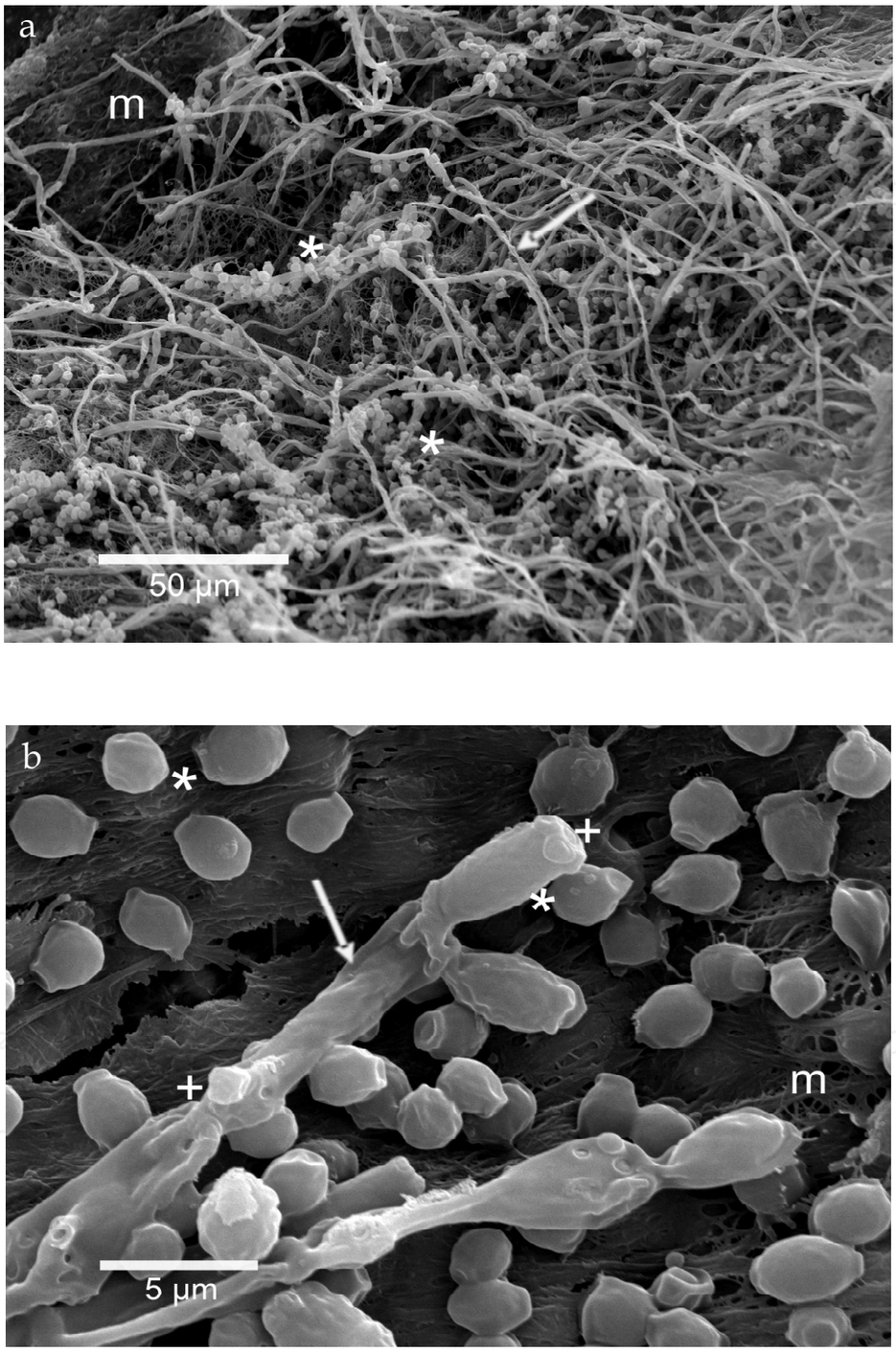

Fig. 1. (a) Biofilm of *C. albicans* (W65) in catheter lumen. (b) Biofilm detail of *C. albicans* (W91). *: yeast cells; +: bud scars; m: matrix material; arrow: hyphae. SEM micrographs were taken with a Philips XL30 ESEM scanning electron microscope using the high vacuum mode (emission electrons detection, acceleration voltage 20 kV, operating distance 10 mm).

			CDC3/CDC3R		EF3/EF3R		HIS3/HIS3R	
Patient	**sample**	**biofilm**	**allele 1**	**allele 2**	**allele 1**	**allele 2**	**allele 1**	**allele 2**
1	K15	-	125		126	133	166	182
	K16	+	125		123	133	166	182
2	K17	+	117	129	130	139	154	
	K18	-	117	129	129	139	154	
3	K49	-	117	125	120	129	162	198
	K50	+	117	125	120	129	162	198
4	K59	+	117	129	129	137	154	
	K60	-	117	129	130	139	154	
5	K67	+	117		130	139	154	158
	K69	+	117	129	130	139	154	158
6	W15	-	125		126	133	166	182
	W16	-	125		123	133	166	182

(+: biofilm positive; -: biofilm negative)

Table 3. Microsatellite models of 12 strains from six patients (two strains each).

3. Discussion

The catheters which were investigated in this study originated from many different stations of mainly two hospitals. The analyses of the genotypes of 123 *C. albicans* strains collected from these samples give many interesting points to think about. The comparison of the CDC3, EF3, and HIS3 genotyping results from the two hospitals (data not shown) did not provide suitable data for distinguishing the epidemiological distribution of *C. albicans*. The contribution of the genotypes was consistent within the University Hospital of Graz compared with the AKH Vienna hospital. This was also true for the aggregation of the data, no significantly dominant genotype was detected, only a group of 11 (8.9%) strains (Table 2) was found to be the most frequent genotype with the multilocus genotype characterised by CDC3: 117-129, EF3: 130-139, and HIS3 154-154. All other groups within this study consist of at most 5 strains. These results are comparable to those of Eloy et al. (2006) who studied the genotypes of *C. albicans* in two different hospitals using the CDC3, EF3, and HIS3 typing system. An overall number of 67 isolates were tested and 50 different genotypes were found. Eight patients shared the same genotype in one hospital; the same genotype was also present in 3 strains in the second hospital. Botterel et al. (2001) tested 100 isolates for their microsatellite profile. They detected 5, 12, and 18 alleles in the CDC3, EF3, and HIS3 system, respectively. The different associations of this alleles led to 10 CDC3, 22 EF3, and 25 HIS3 allele associations within this system. A group of 17 isolates was found to share the genotype.

This genotype was the same as reported by Eloy et al. (2006) in the group of 11 genotype identical strains. Both authors reported the multilocus genotype characterised by CDC3: 117-125, EF3: 126-135, and HIS3 162-162 for their most common strains.

Totally different data were provided from Shi et al. (2007) who collected isolates by female and male patients with genital infection, rectal and oral samples. The authors reported 54.9% of the strains investigated to show the same multilocus genotype, these results were clearly different from all other studies.

The CDC3 locus showed 12 different allele pairs, the EF3 locus 25 allele pairs, and the HIS3 locus 50 allele pairs. This is convergent with the data within the three loci and leads to 94 multilocus genotypes. When compared with the results of Botterel et al. (2001) who reported 65 different multilocus genotypes with different allele associations of 10 for CDC3, 22 for EF3, and 25 for HIS3, it is obvious that the HIS3 locus was clearly more divergent within the current study. However, it remains unclear whether this variation is typical for *C. albicans* strains collected from BSI, or if the discriminatory power (DP) of the HIS3 locus (0.91) is not strong enough. The calculated overall DP for the CDC3, EF3, and HIS3 multilocus genotyping was 0.97. It is worth noting that the DP of HIS3 alone was the highest of the three loci (CDC3: 0.77, EF3: 0.86) (Botterel et al., 2001). Nevertheless, a comparison of the typing information without the HIS3 locus showed that the groups of strains sharing the same genotype do not increase significantly (data not shown).

The comparison of the genotyping of biofilm forming *C. albicans* strains with non-biofilm forming *C. albicans* species shows also a consistent distribution of genotypes. There is no literature to compare these specific results with, but as aforementioned, a consistent contribution of genotype data collected with the CDC3, EF3, and HIS3 multilocus genotyping system seems to be normal for *C. albicans* strains.

The collected information about strains from the same patients are worth a closer look: Only one patient out of six showed 2 strains sharing the multilocus genotype. Using the same typing system, Beretta et al. (2006) investigated 14 isolates of eight patients and reported 4 strains with the same genotype for one patient out of three. Another patient had 2 of 3 strains sharing the genotypes (Beretta et al., 2006). When only HIS3 and CDC3 alleles were compared, five out of the six patients in the current study show the same strain twice. Because of these findings, the typing was done without EF3 locus information, and as it is mentioned above for the typing without HIS3 allele information, no significant increase in the numbers of strains sharing the same multilocus genotype could be seen (data not shown).

Recapitulating the multilocus genotyping with the CDC3, EF3, and HIS3 system during this study, the data presented here is in good agreement with the authors mentioned above.

4. Conclusion

The multilocus genotyping with the CDC3, EF3, and HIS3 system during this study did work well and provided data comparable to former studies. Therefore it is strongly indicated that the genotyping of *C. albicans* strains should be continued in future studies. Aditionally the results give possible evidence that genotypes do not matter in the connection to biofilm forming abilities, so that potentially all *C. albicans* strains are able to

form such ecosystems. In that case, studies like the recent one can only give evidence of epidemiological behavior of the species investigated.

Another set of microsatellite markers is likely to give more information about those strains which are able to form biofilms on indwelling devices or about the epidemiological behavior of clinically important strains.

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This book represents an overview on the diverse threads of epidemiological research, brings together the expertise and enthusiasm of an international panel of leading researchers to provide a state-of-the art overview of the field. Topics include the epidemiology of dermatomycoses and Candida spp. infections, the epidemiology molecular of methicillin-resistant Staphylococcus aureus (MRSA) isolated from humans and animals, the epidemiology of varied manifestations neuro-psychiatric, virology and epidemiology, epidemiology of wildlife tuberculosis, epidemiologic approaches to the study of microbial quality of milk and milk products, Cox proportional hazards model, epidemiology of lymphoid malignancy, epidemiology of primary immunodeficiency diseases and genetic epidemiology family-based. Written by experts from around the globe, this book is reading for clinicians, researchers and students, who intend to address these issues.

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