

Genetic Structure of *Candida glabrata* Isolates from Hospitalized and Non-Hospitalized Patients in Sfax-Tunisia

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Received: May 12, 2015; Accepted: June 26, 2015; Published: July 15, 2015

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Abstract

Background: *Candida glabrata* has emerged as an important nosocomial pathogen, yet little is known about its epidemiology.

Aim: we aimed to analyze by polymorphic markers a collection of *C. Glabrata* isolates from hospital and non-hospital environment to look for inter-patients transmission at CHU Sfax hospital.

Methods: Two populations of *C. Glabrata* isolates were considered in our study. The first included 108 isolates from in-patients. The second population was collected from the out-patients and contain 37 isolates from vaginal and buccal sites.

Findings: A total of 145 *Candida glabrata* isolates were typed using three regular repeat units GLM4, GLM5 and MTI and irregular motif ERG3. Multilocus analyses resulted in the delineation of 62 genotypes and the identification of 8 new alleles and 33 new genotypes. Five genotypes G10, G42, G20, G13 and G22 were the most frequent and represented 51% of the entire population. Three genotypes G42, G10 and G20 predominated in both invasive and non-invasive population and in hospitalized and non-hospitalized. Moreover, the genotype G22 was absent in out-population and four patients infected with genotype G22 were hospitalized in the same intensive care unit and period.

Conclusion: We conclude that little variation exists between hospitalized and non hospitalized patients. The great differentiation was observed between buccal isolates and invasive population (2005-2008). Besides, dissemination to multiple patients from a single source (health care worker, equipment) could be an important factor in the transmission of this yeast in our hospital.

Keywords: *Candida glabrata*; Nosocomial Infection; Molecular Typing

Introduction

During the last decade, incidence of invasive candidiasis with non-*albicans* *Candida* species has increased [1,2]. *Candida glabrata* is the most significant species that has emerged and now regularly ranks number two, after *C. Albicans*, as the etiologic agent of superficial and invasive candidiasis occurring in adults [3,4]. Reasons for this change in species distribution remain uncertain but may be partially due to the natural resistance of

C. Glabrata to azole derivatives, widely used since the 1980s [5,6]. Currently, despite increasing clinical concern, the epidemiology of *C. Glabrata* remains poorly known compared to that of *C. Albicans*. While *C. Glabrata* is considered a commensal of the human digestive tract, its natural reservoir is still uncertain.

The use of molecular technique to determine genotype of clinical isolates can provide useful clues for estimating the reproductive mode, geographic prevalence's, and cross transmission of bloodstream infections between patients [7-12]. Possible transmission between patients has been suggested and clusters of invasive infections have been reported [13-15]. Molecular typing method would allow better understanding of the emergence of this species, notably in a hospital context. In the present study, we aimed to analyze by polymorphic markers a collection of *C. Glabrata* isolates from various sites in hospitalized and non hospitalized patients to look for association of variability with patient population.

Materials and Methods

Patients and isolates

A collection of *C. Glabrata* clinical isolates were collected in a prospective study between January 2005 and December 2011. The isolates were subdivided into outpatients and inpatients clinic populations of Habib Bourguiba Sfax-Hospital (Table 1). The first population included 108 isolates from in-patients and were subdivided as described in Table 1. Fifty nine invasive isolates from blood culture collected from different wards (intensive care unit, infectious disease unit, nephrology or endocrinology unit) of which 15 isolates were previously tested [3], and 49 isolates from urine samples. The second population contained 24 vaginal isolates and thirteen buccal isolates taken from oral cavities. All were collected from the outpatients at the same period [16]. All isolates were selected as only one isolate was considered for each patient and we were certain that patients from the outside hospital had never presented a positive culture from any site for *C. glabrata* and hadn't been hospitalized in the last two years.

Table 1: Number of isolates in subdivided population, MIC, Number of isolates with major multilocus type and Disequilibrium linkage analyses.

	Population	Sampling site	Susceptibility to FCZ		Total number of isolates	Number of multilocus genotypes	Number of isolates with Genotype					I_A	r_{BarD}	r_{BarS}	Proportion of compatible pairs of loci	Genotypic diversity
			S ^c	R ^d			G10	G26	G9	G13	G22					
Population from the inside hospital	CHU (2005-2008)	Blood culture	21	1	22	9	6	5	1	3	1	0.81	0.27	0.153	1	0.86
	CHU (2009-2011)	Blood culture	32	5	37	24	6	3	2	2	4	0.42	0.155	0.064	1	0.95
	CHU (2005-2011)	Urine	40	9	49	24	9	6	5	1	2	0.33	0.11	-0.14	0.33	0.94
Population from the outside hospital	Outpatient (2005-2011)	Buccal	13	0	13	13	0	0	0	0	0	-0.08	-0.031	0.26	0.33	1
	Outpatient (2005-2011)	Vaginal	24	0	24	11	6	7	3	2	0	0.11	0.04	-0.06	0.5	0.85
	Total		130	15	145	62	27	21	11	7	7	0.198 ^a	0.06 ^a	0.077	0	0.934

a: p value < 0.001 ; c: Sensitive isolates ; d: Resistant isolates

Identification and antifungal susceptibility test

For identification, isolates were plated onto Candiselect ID medium (Bio-rad, France) at 37°C for 48h. *C. Glabrata* was identified by its green color and this identification was confirmed by ID32C (Biomérieux, France) assimilation test and Glabrata RTT (Fumouze, Diagnostic, France). Fluconazole susceptibility testing was performed by measuring the Minimum Inhibitory Concentration (MIC) obtained through the use of the E-test (Biomérieux, France) as described by manufacture's protocol. A MIC $\geq$ 64 mg/L defined fluconazole resistance [17].

DNA extraction

Genomic DNA was prepared from cell pellets obtained from 5 ml of fresh overnight culture in Yeast Peptone Glucose (YPG) medium. Genomic DNA was extracted using the nucleospin™ Tissue method (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions.

C. glabrata typing

Molecular typing of invasive *C. Glabrata* isolates was performed as previously described by multiplex PCR using four microsatellite markers GLM4, GLM5, ERG3 and MTI [18,19]. Amplification was carried in 20 μ l volume containing 2 μ l of *C. Glabrata* DNA, 1X STR* buffer (Promega, USA), 0.2 mM deoxynucleoside triphosphate, 5 pmol each of the GLM4 and GLM5, 10 pmol of ERG3 primers, 20 pmol of the MTI primers and 1.25 U of Ampli Taq Gold DNA polymerase (Applied Biosystems, Courtaboeuf, France). After an initial step of 10 min at 95°C, the PCR included 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by an additional step of 5 min at 72°C. One microliter of the PCR mixture was then added to 24 μ l of

formamide containing 0.5 μ l of Genescan LIZ 500 marker (Applied Biosystems, Courtaboeuf, France) and denaturated for 2 min at 95°C. The PCR products were subjected to electrophoresis on an ABI 310 sequence analyzer and the data were analyzed with the Genescan software (Applied Biosystems, France).

Statistical analyses

The genetic differentiation between both populations was measured using Wright's Fixation Index (FST) computed with the Fstat V2.9.3 software developed by Goudet J. (<http://www2.unil.ch/popgen/softwares/Fstat293dist.exe>). FST is a measure of genetic divergence among populations [20]. The Fst value = 0.05 indicates little genetic divergence between populations while moderate genetic differentiation was considered as 0.05 < Fst < 0.15. A great genetic differentiation is defined as FST value ranged from 0.15 to 0.25.

Nei's genetic diversity was used as measure of gene diversity and differentiation among the populations [21]. To determine the extent of clonality and recombination in the different populations, the Index of Association (IA), r_{bard} , r_{bars} and Proportion of compatible pairs of loci were computed with Multilocus 1.3b software using 1000 randomizations tests to obtain reliable p-values and without considering repeated genotypes [22]. All test the null hypothesis of no linkage disequilibrium, which would indicate the absence of recombination. They are expected to be zero if populations are significantly freely recombining and greater than zero if there is association between alleles (clonality). The r_{bard} statistic takes into consideration the number of loci tested and is considered a more robust measure of association and r_{bars} test whether alleles tending in the same direction are positively or negatively associated. Prcompat should be 1 if all

pairs of loci are compatible with strict clonality.

Phylogenetic relationships were estimated based on multilocus genotypes using the Neighbor-Joining method as implemented in <http://pubmlst.org/>. The effect of covariates on the cluster distribution was tested with the Chi-squared test.

Results

Genotyping analysis

In the present study, a total of 145 *Candida glabrata*

isolates from inpatients and outpatients were typed using polymorphic markers (MTI, ERG3, GLM4 and GLM5). From hospitalized and non hospitalized patients, three and two separated subgroups according to site and time of isolation were characterized respectively (Table 2). Multilocus analyses resulted in the delineation of 62 genotypes (Table 2). Forty eight of the multilocus genotypes, corresponding to 33.1% of the total number of isolates, were represented by single isolate.

C. Glabrata genotyping were performed by only four

Table 2: Genotype distribution of multilocus type identified in our study.

Genotypes	MTI	ERG3	GLM4	GLM5	Isolates number (%)
G1	246	190	267	262	3(2)
G2	240	204	276	259	1(0.7)
G3	239	227	261	262	1(0.7)
G4	227	181	273	301	1(0.7)
G5	238	197	270	298	2(1.3)
G6	238	200	291	271	1(0.7)*
G7	238	197	273	262	3(2)
G8	238	197	273	304	2(1.3)
G9	238	197	288	271	11(7.5)
G10	238	197	273	298	27 (18.6)
G11	238	197	267	262	3(2)
G12	238	227	258	301	1(0.7)*
G13	238	197	273	301	8(5.51)
G14	238	197	270	271	1(0.7)
G15	238	197	273	259	1(0.7)
G16	238	198	273	298	1(0.7)
G17	238	198	288	271	1(0.7)*
G18	240	181	261	265	1(0.7)*
G19	240	200	273	274	1(0.7)*
G20	227	227	276	262	1(0.7)
G21	225	200	270	259	1(0.7)*
G22	239	260	276	259	7(4.8)
G23	239	260	276	262	1(0.7)
G24	238	197	273	271	1(0.7)*
G25	239	190	264	259	1(0.7)
G26	240	204	264	259	21(14.4)
G27	239	183	270	259	1(0.7)*
G28	240	197	285	271	1(0.7)*
G29	240	204	285	268	1(0.7)*
G30	239	197	273	298	2(1.3)
G31	238	227	270	298	1(0.7)
G32	239	227	267	259	1(0.7)
G33	239	227	264	259	1(0.7)*
G34	238	190	261	268	1(0.7)*
G35	239	227	276	262	4(2.7)

G36	240	190	264	271	1(0.7)*
G37	239	181	273	274	1(0.7)*
G38	238	197	276	271	1(0.7)*
G39	238	197	276	259	1(0.7)*
G40	238	198	261	274	1(0.7)*
G41	240	200	273	265	1(0.7)*
G42	240	204	267	259	1(0.7)*
G43	240	239	288	268	1(0.7)*
G44	240	204	273	307	1(0.7)*
G45	238	197	276	262	1(0.7)
G46	238	227	273	298	2(1.3)*
G47	240	227	276	262	1(0.7)*
G48	225	183	273	265	1(0.7)*
G49	240	205	264	259	1(0.7)
G50	225	183	276	265	1(0.7)*
G51	240	260	273	259	1(0.7)*
G52	238	197	246	271	1(0.7)*
G53	225	181	276	265	1(0.7)*
G54	225	204	267	265	1(0.7)*
G55	238	227	276	262	1(0.7)*
G56	238	198	276	301	1(0.7)*
G57	240	197	264	259	2(1.3)*
G58	240	198	273	274	1(0.7)*
G59	239	183	270	259	1(0.7)*
G60	240	204	246	259	1(0.7)*
G61	240	205	270	259	1(0.7)*
G62	238	197	246	259	1(0.7)*

*: New genotypes not previously described

polymorphic markers instead of six and allowed the identification of 8 new alleles (locus GLM4, alleles 246, 285 and 291; locus GLM5, alleles 307 and 268; locus ERG3, alleles 239 and 183; locus MTI, alleles 225) and 37 new genotypes (Table 2) in addition to 30 alleles and 37 genotypes previously described [14].

Overall, multilocus analysis of the 145 isolates showed that genotypes G10, G26, G9, G13 and G22 were the most frequent and represented respectively 18.6%, 14.48%, 7.58%, 5.51% and 4.8%. These multilocus genotypes together represented 51% of the entire population and were identified in all studied isolation sites except buccal site which has been characterized by unique genotypes (G41, G43, G51, G53-G60). These five genotypes represented 72.7% of the invasive candidiasis population (2005-2008), 70.8% of the vaginal population (2005-2011), 45.9% of invasive candidiasis population (2009-2011) and urinary isolates (2005-2011) each.

When comparing isolates from inside and outside hospital, we noted that each population was dominated by the same genotypes and shared alleles and frequency in most cases. The

frequency of some genotypes varied from one population to another although it was not significantly associated ($p \geq 0.05$), i.e G22 was more common in invasive population (5.1%) and completely absent in outside of the hospital.

Population genetic analysis

In order to assess differentiation among populations, Wright Fixation Index (Fst) was calculated between hospitalized and non hospitalized population (Fst = 0.0274). This result indicated a little genetic differentiation suggesting a non specific genetic structure of *C. Glabrata* population from inside or outside the hospital.

Wright fixation index was calculated for all pairwise combinations of the five sub-populations (Table 3). Invasive population (2005-2008) had a very similar structure to vaginal population and invasive population (2009-2011) was similar to urinary population with an Fst value 0.004 and 0.002, respectively. However, moderate genetic differentiation ($0.05 < Fst < 0.15$) was observed between vaginal isolates (2005-2011) vs invasive candidemia (2009-2011). A moderate genetic differentiation

exist also between invasive candidemia (2005-2008), urine and vaginal isolates versus buccal samples (Table 3).

Genetic diversity varied from 0.85 for vaginal population to 0.95 in invasive isolates (2009-2011) and buccal samples (ID = 1) (Table 3). Buccal populations were considered the most divergent followed by invasive candidemia (2009-2011). In addition, there were a higher number of strains with a unique genotype within 13 among 13 isolates of buccal population than invasives isolates (2009-2011) (15 among 37 isolates), invasives isolates (2005-2008) (3 among 22) and vaginal isolates (2005-2011) (5 among 24 isolates).

Neighbor-joining tree was constructed for the entire population based on the polymorphism of the four polymorphic markers as shown in Figure 1. There was no unique topology for intra or extra-hospital isolates. Isolates from different anatomic location were subdivided into different genotypes (Figure 1).

A neighbor-joining tree was constructed to show genetic relationship among 62 observed multilocus types in our combined population (Figure 2). Our population was subdivided into four major groups. The first group contains a unique multilocus type G30 from urinary population, the second contain G8 from vaginal population, the third contain the G10 which was the most frequent genotype and the fourth contains all other types including four of the major multilocus types G10, G26, G13 and G22.

To determine the extent of clonality and recombination in different populations, we used four measures of associations (IA, r_{bard}, r_{bars} and Proportion of compatible pairs of loci) [18]. For all studied sub-populations, a randomization test for linkage disequilibrium measures presumably rejected the null hypothesis of recombination as well as the total isolates considered to be a single population although proportion of compatible pairs of loci

reveals some degree of recombination (prcompat < 1) (Table 1).

The antifungal susceptibility to fluconazole of these strains was not different between hospitalized and non hospitalized patients (Table 1). Among 59 invasive *C. Glabrata* isolates and 86 non invasive isolates, only 15 isolates were resistant to fluconazole (10.34%). Genotypic analysis of these isolates revealed that they had different genotypes and were subdivided into 8 multilocus types (Figure 1) and the resistant phenotype was not significantly associated with a population or another ($p \geq 0.05$).

Discussion

We studied a genetic population structure of *C. Glabrata* isolates from inside and outside of the CHU hospital in Sfax (Tunisia). Polymorphic markers analysis revealed that all loci showed a high degree of diversity and notably each locus represented 6 -11 alleles (Table 2). Most of them were common to the majority of isolates but few were less common and were identified in only one or at least two isolates.

Genotypic distribution remains similar to that described in Tunisia in previous study when analyzing 85 unrelated isolates from different anatomical sites by six microsatellites [18]. In Tunisian population, the same genotypes (G10, G26 and G9) dominated. G10 was the most frequent type which was observed in 27 clinical isolates. The latter type was identified in phylogenetic tree as a single branch which seems to be stable and did not undergo natural evolution process. The overall abundance of G10 but also G26 and G9 suggest that at least some isolates with identical types may be clonally related by descent. In addition, we have also provided that there was a significant clonal component for the entire population (IA = 0.198).

In our report, we revealed that the inpatient population was not significantly differentiated from out-patient population ($F_{st} <$

Table 3: Fst differentiation for subdivided populations.

Populations	No. of isolates	Fst
CHU (2005-2008) vs CHU (2009-2011) invasive candidemia	59	0.0138
CHU (2005-2011) urine vs CHU (2005-2008) invasive candidemia	71	-0.0054
CHU (2005-2011) urine vs CHU (2009-2011) invasive candidemia	86	0.002
Vaginal isolates (2005-2011) vs CHU (2005-2011) urine isolates	73	0.0305
Vaginal isolates (2005-2011) vs CHU (2005-2008) invasive candidemia	46	0.004
Vaginal isolates (2005-2011) vs CHU (2009-2011) invasive candidemia	61	0.0524
Invasive candidemia (2005-2011) vs non-invasive candidemia (buccal-urine-vaginale)	145	0.002
Inpatients vs outpatients populations	145	0.0274
CHU (2005-2008) invasive candidemia vs buccal samples	37	0.1202
CHU (2009-2011) invasive candidemia vs buccal samples	50	0.048
CHU (2005-2011) urine vs buccal samples	62	0.0817
Vaginal isolates (2005-2011) vs buccal samples	37	0.0955

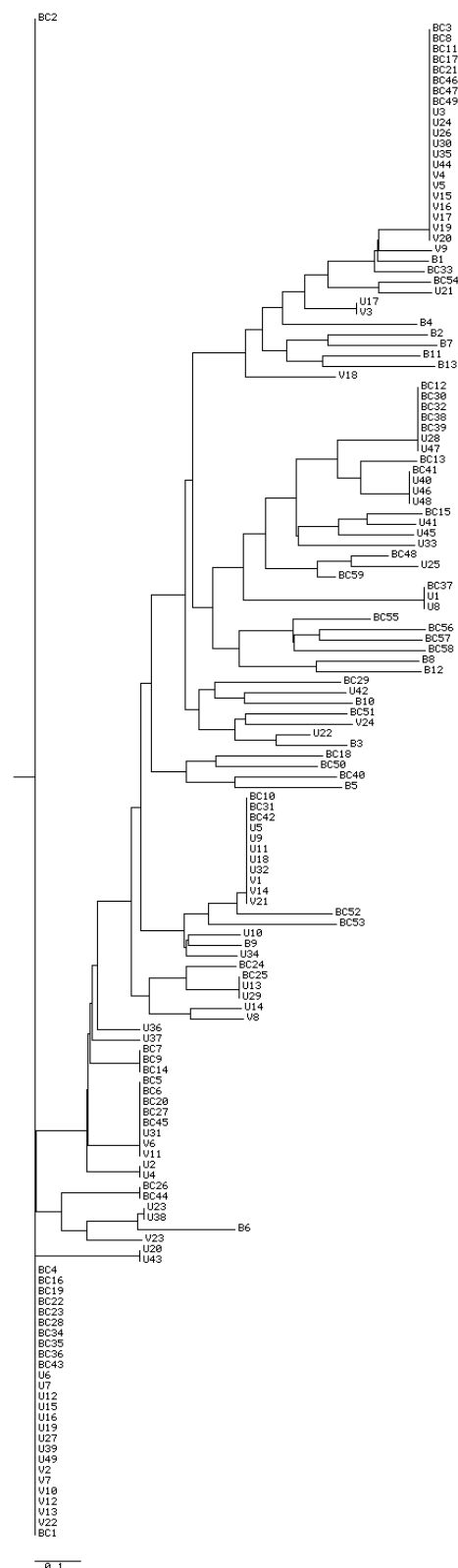


Figure 1: Genetic relationship between the different isolates of *Candida glabrata* grouped according to (i) Anatomic location (U: urine; V: vaginal; B : buccal and BC: blood culture) their origin (ii) (POP1: BC1-22; POP2: BC23-59; POP3: U1-U49; POP4: V1-V24; POP5: B1-B13). Identical isolates from intensive care units from 2009 were framed and isolates from outpatients were arrounded.

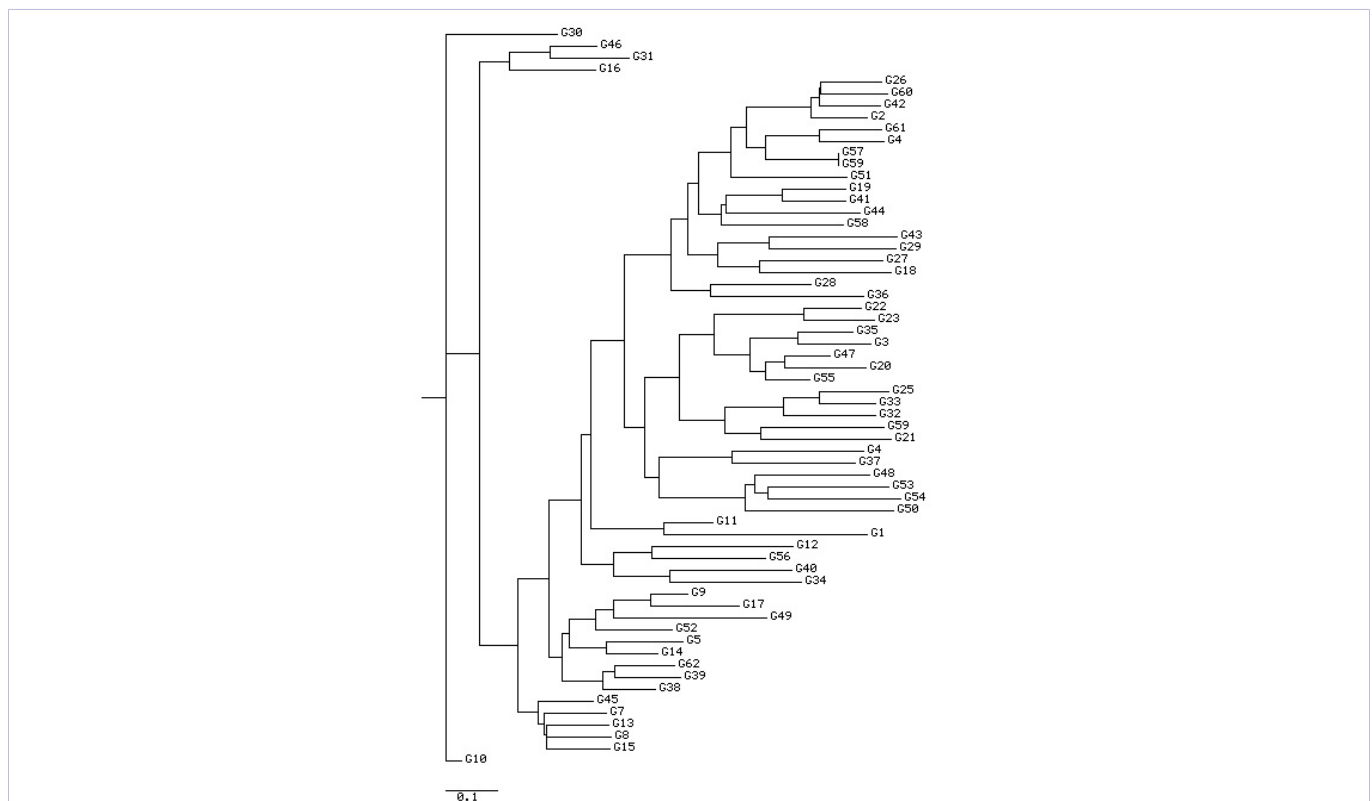


Figure 2: Neighbor-joining dendrogram for the 62 genotypes identified in this study. Major groups previously identified are indicated.

0.05). Moreover, little differentiation was conducted for all pair wise population except buccal population which was considered moderately differentiated especially from invasive candidemia population. This latter result may be due to the specific virulence and resistance capacities of *C. Glabrata* in oral mucosa and digestive tract isolates [23,24].

Despite a lack of differentiation between the two populations ($F_{st} = 0.0274$), each of them included some genotypes which were not found in the other (Figure 1). Indeed, the inside hospital population was more divergent and showed the inclusion of 38 multilocus types. On the other hand, the outside hospital population was less divergent but showed the inclusion of 18 multilocus types not described in inside hospital.

In our setting, when analyzing 108 isolates from hospitalized patients and 37 from non hospitalized patients, we identified one multilocus type for 2.5 isolates for hospital environment and one multilocus type for 1.6 isolates in outpatients. Other studies, when analyzing intra-population diversity by Multilocus Sequence Type (MLST) established one multilocus type for every 4.8 patients in Baltimore in 2008 and one multilocus type for every 2.4 isolates in Atlanta from 1992 to 1993 [10,11,25]. These results demonstrated that our method may be more discriminative in examining population structure.

Most of *C. glabrata* fungemia are thought to originate from the endogenous flora of the host when host factors or environmental conditions allow them to switch from a commensal state to a pathologic state. In order to predominate as a pathogen, an

isolate must first predominate as a commensal. In our study, three genotypes G10, G26 and G9 predominated in both invasive and non-invasive population and in hospitalized and non hospitalized patients. It means that invasive population was not differentiated from colonizing strains and indicated that any clone can be pathogenic in any infection site. This suggests also that endogenous flora of the host was the main source of infection and excludes the hypothesis that inter-patient transfer plays a role in the prevalence of at least most popular genotypes.

Moreover, some multilocus genotype G22 and G13 were more prevalent in invasive population. The genotype G22 were almost absent in out-population (vaginal and buccal samples). Our results suggested that some multilocus type may be stronger pathogen or that horizontal transfers can explain their highest prevalence inside the hospital. Furthermore, we notice that four patients (among 7 patients) infected with genotype G22 were hospitalized in 2009 in the same intensive care unit in the same period. This made that second hypothesis was more available and can be confirmed when analyzing a more large collection of isolates within and outside of the hospital.

It is also important to note that antifungal pressure exerted by some resistant genotypes can play a role in determining the population structure inside the hospital. It had been previously shown that there was no association between antifungal susceptibility and particular genotypes in previous study in Tunisia [3]. Similarly, Lott *et al.* [11] affirmed that fluconazole resistance profiles from invasive and non invasive population

were not significantly different and were not associated with a particular sequence type. In our study, we didn't found an emergence of any of the major multilocus type and fluconazole resistance in inside or in the outside population hospital except for isolates collected in 2009 (4 isolates) in which two of them showed a resistant phenotype (Figure 1). Eleven percent of *C. Glabrata* isolates in this study were fluconazole resistant. Among them, 80% were isolated from hospitalized patients ($p \geq 0.05$). Selection pressure exerted in patients inside the hospital perhaps due to antifungal treatment may be responsible.

We can conclude that very little differentiation exists between both hospitalized and non hospitalized patients, invasive and colonizing population and the dissemination to multiple patients from a single source (health care worker, equipment) could be an important factor in the transmission of this yeast in our hospital.

Acknowledgement

This study was financially supported by the Tunisian ministry of high education. No conflict of interest and approval states were not required. No specific funding was received for this study.

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