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Epidemiology and Molecular Typing of *Candida krusei* Based on PCR-RFLP of the ITS rDNA Regions

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Abstract

Purpose: *Candida krusei* strains are intrinsically resistant for the first choice antifungal. Fast identification of *C. krusei* as an infectious agent will decrease the risk of choice of not correct therapy. The aim of the present work was to study the epidemiology of *Candida krusei* infections during 10 years. We also attempt to study the phylogeny of these isolates by PCR- RFLP.

Methods: Two hundred five cases of *C. krusei* candidiasis were referred to laboratory of parasitology mycology, UH Habib Bourguiba of Sfax-Tunisia during 10 years (2006 to 2016). Identification of our strains was performed by conventional methods and by PCR-ITS amplification followed by a digestion with three restriction enzymes MspI, HinfI and HincII.

Result: The mean frequency of cases of *C. krusei* candidiasis was 17.08 per year. Invasive infection represented 10.24%. The superficial infections with *C. krusei* represented 89.76% of cases. Analysis of the phylogeny tree allowed us to deduce that there is a great diversity in *C. krusei* strains. No particular genotype has been associated with the sampling site, or department or year of infection. We noted that patient P4 was hosted by three strains with the same genotype.

Conclusion: The modification in epidemiology of candidiasis emphasizes the necessity to monitor local incidence, species distribution and susceptibility in order to optimize therapy and outcome. Molecular methods are essential for correct identification of the Candida species in order to obtain clues regarding the source of infection and to apply the correct therapy for the infected individual.

Keywords: *Candida krusei*; ITS regions; PCR-RFLP; Phylogeny

Introduction

Climatic variability, as a primary expression of the climate change, is the most significant environmental problem that humanity will face in the next years [1]. It is playing an important role in the increase of Influenza circulation, contributing to the burden of Acute Respiratory Infections (ARIs) [2]. ARIs are the main cause of morbidity and one of the leading causes of death at world level [3]. Influenza is considered the most contagious of the ARIs and the causative agent is influenza virus [4]. This infection affects people of all ages and spreads easily and in schools, community homes and workplaces with a negative social and economic impact by income loss though the size reduction of the workforce and the productivity, increases of the absenteeism, and interruption of the economic activity [5]. The clinical spectrum can range from Influenza like Illness (ILI) of mild course to a Severe Acute Respiratory Infection (SARIs) that usually requires hospitalization in Intensive Care Units and can lead to death. The World Health Organization (WHO) estimates that seasonal Influenza viruses cause approximately 3-5 million cases, 250,000-500,000 deaths and 200,000 hospitalizations annually [6]. In Americas Region 80000 deaths due to Influenza are estimated annually [7]. In the most serious Influenza pandemic occurred in 1918 caused by influenza A (H1N1) with an estimated of more than 20 million deaths in two years [8,9].

Influenza viruses are classified in: influenza type A, B and C. Being types A and B the most important in terms of human disease [10]. Both show a similar genetic and structure, but differ in their biological, evolutionary and epidemiological characteristics [11,12]. The annual Influenza burden differs unpredictably from year to year, between age groups and from region to region [13-15]. There are several factors that can influence the seasonality of Influenza viruses such as the antigenic drift and antigenic change, the host immune response, social and climatic factors and solar radiation [16-18]. Information on seasonal patterns remains limited in the large regions of Central America [19]. Further studies should link the latitudinal gradients of the seasonality of influenza epidemics with the climatic elements [20]. Influenza viruses have a well-defined seasonal pattern [21,22] in countries with a temperate climate, with epidemics during the winter season (northern hemisphere: December-April and southern hemisphere: June-September) [23-26]. Humid and rainy conditions favor the viral activity in the tropical regions [23,27,28]. Three patterns have been observed: 1) Infections that occur throughout the year with peaks related to the rainy season, 2) Infections that occur throughout the year with biannual peaks associated with the rainy season, 3) Infections that occur without a clear seasonality [29-32].

In Cuba, influenza and pneumonia constitute the fourth cause of death and the first cause death by infectious diseases [33,34]. Since 2000, the Cuban Ministry of Public Health (MINSAP) has

implemented a comprehensive ARI Care and Control Program, for the prevention and control of these infections [35]. The vaccination campaign is the main strategy to reduce the burden of influenza disease and vaccination is started before the seasonal months [36]. Therefore, the surveillance of climate variability that seasonal influenza can have as a prerequisite for pandemic preparedness and response is of vital importance. For this reason, knowing the epidemiology, the seasonal pattern of influenza viruses and the influence by climate is important in the planning of treatment and control strategies and allows defining when to vaccinate and which vaccine formulation to apply [22].

Besides the identification of the relationships between climatic variables and viruses, it is also necessary to understand and explain the mechanisms of biotropic responses of influenza viruses to several climatic conditions (interacting all climate-forming variables) described as climatic indexes of Bultó (BIs). Our study intends to understand the effects of climate changes and climatic conditions that favor the circulation of influenza in a tropical country such as Cuba, which allows anticipating their behavior, making maps of risk and formulate prediction models according climatic conditions. At the same time, the results may constitute valuable scientific information to update and refine the National Program for the Prevention and Control of ARI and regional and global influenza surveillance programs.

Materials and Methods

C. krusei isolates

Our work is a retrospective study on *C. krusei* candidiasis (superficial and deep) diagnosed in the laboratory of parasitology mycology, UH Habib Bourguiba of Sfax-Tunisia for a period of 10 years from 2006 to 2016. Samples were systematiquely cultured on Sabouraud-Thiophenicole (ST) at 37°C. Identification of the colony morphology and microscopic features of the cultures was done after cultivation on Candi SelectTM 4 and using API ID 32 C (bioMérieux, Paris, France).

DNA extraction

Molecular study was conducted on 53 strains of *C. krusei* collected from 49 patients and from different clinical sites: urinary (37), hemoculture (5), vaginal (6), bronchoalveolar lavage (1), nasal (1), sputum (1), auricular (1), toe (1), collected from the Habib Bourguiba Hospital from 2006 to 2016 (Table1). The genomic DNA was extracted using epicentre Kit (MasterPure Yeast DNA Purification Kit) as indicated by the manufacturer's instruction, and eluted with 50 µl of sterile water.

Patients	ID	Date	Clinical Site	Service
Patient 1	U1P1	2006	Urine	Urology
Patient 2	P2U2	2007	Urine	Infectious diseases
Patient 3	P3U3	2007	Urine	Nephrology
Patient 4	P4U5	2007	Urine	Neurology
	P4U4	2007	Urine	Neurology
	P4U6	2007	Urine	Neurology
Patient 5	P5U7	2008	Urine	Infectious diseases
Patient 6	P6HC3	2011	Blood	Intensive care unit
Patient 7	P7V1	2012	Vaginal	Gynecology
Patient 8	P8V6	2012	Vaginal	Endocrinology
Patient 9	P9U8	2012	Urine	Gynecology
Patient 10	P10U9	2012	Urine	Gynecology
Patient 11	P11U10	2012	Urine	Urology
Patient 12	P12U11	2012	Urine	Urology
Patient 13	P13GO1	2013	Toe	Orthopedy
Patient 14	P14C1	2013	Sputum	Urology
Patient 15	P15U12	2013	Urine	Infectious diseases
Patient 16	P16U13	2013	Urine	Pneumology
Patient 17	P17U14	2013	Urine	Urology
Patient 18	P18HC4	2013	Blood	Hematology

Patient 19	P19HC5	2014	Blood	Infectious diseases
	P19HC1	2014	Blood	Endocrinology
Patient 20	P20LBA1	2014	BAL	Pneumology
Patient 21	P21N1	2014	Nasal	Intensive care unit
Patient 22	P22U15	2014	Urine	Endocrinology
Patient 23	P23U16	2014	Urine	Infectious diseases
Patient 24	P24U17	2014	Urine	General surgery
Patient 25	P25U18	2014	Urine	Urology
Patient 26	P26U19	2014	Urine	Urology
Patient 27	P27U20	2015	Urine	Urology
Patient 28	P28U21	2015	Urine	Intensive care unit
Patient 29	P29U22	2015	Urine	Urology
Patient 30	P30U23	2015	Urine	Urology
Patient 31	P31U24	2015	Urine	Infectious diseases
Patient 32	P32U25	2015	Urine	Urology
Patient 33	P33U26	2015	Urine	Urology
Patient 34	P34U27	2015	Urine	General surgery
Patient 35	P35V2	2015	Vaginal	Gynecology
Patient 36	P36A1	2016	Auricular	Infectious diseases
Patient 37	P37HC2	2016	Blood	Intensive care unit
Patient 38	P38U28	2016	Urine	Intensive care unit
Patient 39	P39U29	2016	Urine	Infectious diseases
Patient 40	P40U30	2016	Urine	Urology
Patient 41	P41U32	2016	Urine	Infectious diseases
	P41U31	2016	Urine	Infectious diseases
Patient 42	P42U33	2016	Urine	Urology
Patient 43	P43U34	2016	Urine	Endocrinology
Patient 44	P44U35	2016	Urine	Gynecology
Patient 45	P45U36	2016	Urine	Nephrology
Patient 46	P46U37	2016	Urine	Urology
Patient 47	P47V3	2016	Vaginal	Gynecology
Patient 48	P48V4	2016	Vaginal	Gynecology
Patient 49	P49V5	2016	Vaginal	Gynecology

Table 1: Characteristics of patients included in the molecular studies.

PCR-RFLP

Amplification of 18S (Partial sequence), ITS1, 5, 8 S, ITS2 (Total sequence) and 28 S (Partial sequence) of rDNA were performed with primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') as designated by White et al. [17]. Amplification reactions were performed in final volumes of 50 µL containing 0.5 µg of template DNA, 10 µL of 5X reaction buffer (pH

8.5), 25 mM $MgCl_2$, 0.2 mM (each) dATP, dCTP, dGTP, and dTTP (Promega), 30 pmol of each primer and 5 U of GOTaq® DNA polymerase (Promega). PCR was performed in a thermocycler (Biorad), it consisted of an initial denaturation at: 94°C for 3 minutes, followed by 35 cycles of 30 seconds at 94°C, 1 minute at 60°C, and 30 seconds at 72°C and a final extension at 72°C for 10 minutes. PCR products were separated in 1.5% agarose gel, stained with ethidium bromide and visualized with an UV transilluminator and photographed.

The amplicons [20 µL of each PCR sample were digested with three restrictions enzymes: MspI [C/CGG], HinfI [G/ANTC] and HincII [GTC/RAC] for 2 hours, 6 hours and 16 hours, respectively, with agitation and then electrophoresed in a 3% agarose gel, stained with ethidium bromide, and observed under UV light. The digestion profiles obtained were repeated twice to ensure reproducibility of the technique and certitude of results.

Phylogenetic analysis

For the RFLP analysis the degree of similarity was calculated by applying the Dice coefficient test. This was performed using NTSYS-PC numerical taxonomy and multivariate analysis system, Version 2.1. (Exeter Software, Setauket, NY). A dendrogram was generated using UPGMA methods.

Results

During our study, we collected 205 cases of *C. krusei* candidiasis from 2006 to 2016. The mean frequency of cases of *C. krusei* candidiasis was 17.08% per year, from 11 to 28 cases per year. An increasing of the incidence of candidiasis infections caused by *C. Krusei* was noted, with a maximum number of cases (28) was reported in 2016. We also found a female predominance (65%) compared to men (35%). The superficial infections with *C. krusei* accounted for 184 cases (89.76%). Candiduria was the most frequent (57.56%) followed by vulvo-vaginal candidiasis (12.19%) and oral candidiasis (5.37%). Peripheral specimens (axillary, nasal, inguinal, anal ...) represented 30 cases (14.64%). The deep infections represented 10.24% (21 cases). Systemic candidiasis represented 8% of *C. krusei* invasive candidiasis infections followed by broncho-alveolar lavage (2.24%). PCR amplifications of the ITS rDNA fragment revealed a product of 500 bp for 50 strains. In three cases we detected a co-infetion *C. albicans (700 pb)* - *C. krusei* (500 pb) (Figure 1). Digestion of the ITS regions by MspI showed two patterns: A (250 bp) (94.3%) and B (500 bp) (5.7%). Using HinfI, two patterns were obtained: C (137-145-219 bp) (96.2%) and D (500 bp) (3.8%). HincII revealed also two patterns: E (396-105 bp) (94.3%) and F (500 bp) (5.7%) (Figure 2).

A dendrogram based on the RFLP profiles allowed the separation of the *C. Krusei* isolates into genetic similarity clusters. The total of 53 isolates could be allocated into four clusters, with the similarity index between the isolates within the cluster being 80% or higher (Figure 3). Analysis of the phylogeny tree allowed us to deduce that there is a great diversity in *C. krusei* strains. No particular genotype that has been associated with the sampling site, department or years of infection. However, we noticed that the patient P4 was hosted by three strains with the same genotype.

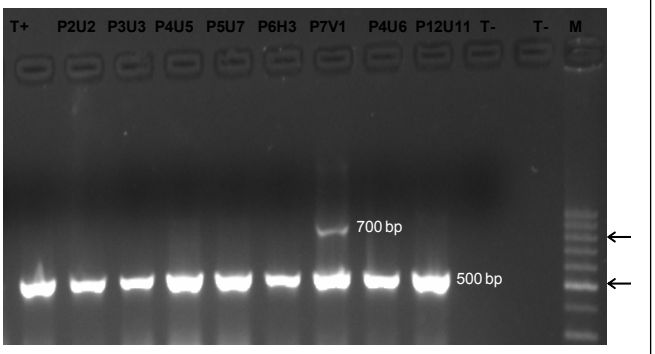

Figure 1: PCR amplification profile of ITS 1, 5.8 S and ITS2 regions of *C. krusei* strains collected from clinical specimens; P7V1: *C. krusei*-C. *albicans*; M: size marker 100 bp; T-: negative control, T+: positive control.

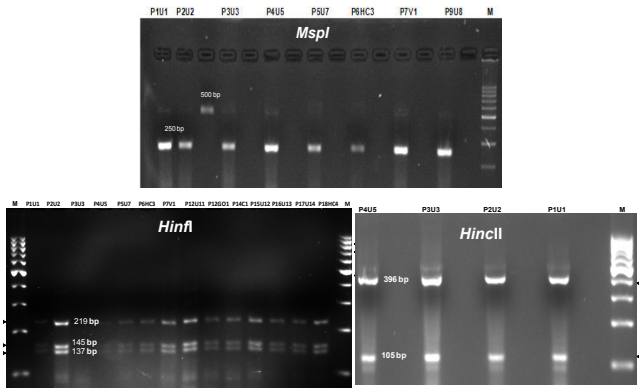

Figure 2: MspI, HinfI and HincII restrictions profile after PCR amplification of the ITS1-ITS2 rDNA on 3% agarose gel (M: size marker 100 bp).

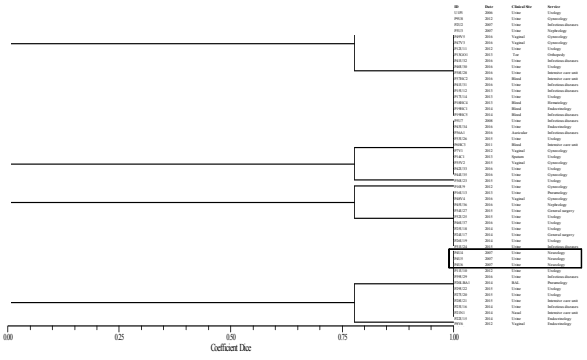

Figure 3: UPGMA dendogram based on the Dice similarity coefficient upon analysis of three restrictions enzymes in 53 *C. krusei* isolates. The bordered strains represent the patient P4 hosted by three strains with the same genotype.

Discussion

C. krusei strains are intrinsically resistant for the first choice antifungal. Fast identification of *C. krusei* as an infectious agent will decrease the risk of choice of not correct therapy. During our study, the mean frequency of cases of *C. krusei* candidiasis was 17.08% per year. According to Krcmery et al. [19] *C. krusei* is responsible for 2% to 25% of the fungal infections with a higher incidence in neutropenic patients with leukemia (13%-25%) [18,19]. Infections caused by *C. krusei* are also common in bone marrow transplant patients. However, it is rare in intensive care units and in neonatology [20]. Some studies reported a clear predominance of the male sex [21-23]. However, we found a female predominance of 65%. In the present study, urinary tract infection accounted for 57.56% of *C. krusei* infection. *C. albicans* was the most involved species in urinary candidiasis (41.5%) and *C. krusei* (1.4%) was rare [24]. Other studies showed also that *C. krusei* had a low frequency in the urinary tract [25,26].

Vulvovagininal candidiasis caused by *C. krusei* accounted for 12% in our study. However, *C. krusei* is a rare cause of vaginitis [27,28]. Estimated annual incidence of *C. krusei* vaginitis varies from 1% to 5% of all fungal vaginitis [28-30]. They are generally chronic, refractory and resistant to conventional antifungal treatments [28]. In our study, peripheral specimens accounted for 14.64% of *C. krusei* superficial candidiasis. Pfaller et al. [3] showed that *C. krusei* is isolated in 2.2% of the skin and soft tissue [4]. In parallel with the increased incidence of superficial candidiasis, there has been a significant increase in the incidence of systemic candidiasis [31,32]. In the present study, *C. krusei* invasive candidiasis accounted for 10.24% of all infections due to *C. krusei*. Other studies have shown that *C. krusei* accounted for only 2% of these infections [33]. In western France, *C. krusei* was isolated in 4.1% of the cases [34]. In Spain, 2.3% of candidemia cases were due to *C. krusei* between 1988 and 2003 [35]. A multicentre study in Europe showed that 12% of candidemia in hematology patients were due to *C. krusei* with a mortality of 20% to 67% [36]. This discrepancy in the incidence of *C. krusei* between superficial and deep infections can be explained by the difference in risk factors, underlying pathologies and the influence of broad-spectrum antibiotics. *C. krusei* was predominant in neutropenic patients with leukemia, which increases the frequency of dissemination and mortality (93% for *C. krusei* versus 16% for *C. albicans*) [21]. Indeed, infections caused by *C. krusei* are opportunistic infections that are multiresistant and this may explain the increase in deep infections. As epidemiology, virulence and antifungal susceptibility often vary among strains, a rapid and accurate identification of the species causing the disease is crucial for treatment and epidemiological studies [37]. Early diagnosis of invasive fungal infections is essential to reduce the mortality rates. Given the many limitations of phenotyping methods, molecular biology methods have been adapted because of their ability to detect extremely earlier detection of fungal pathogens, allowing earlier initiation of antifungal therapy and perhaps improved chance of survival [38]. Polymerase chain reaction technology can directly detect the presence of fungi with high level of sensitivity and specificity [39]. In this study, we used universal primers ITS1 and ITS4 for the amplification of ITS1 and ITS2 regions, including 5.8S subunit genes and digested it with three restriction enzymes. PCR amplification of ITS region can easily identify *C. krusei*. We also identified three cases of co-infection (*C. krusei- C. albicans*). These results were confirmed by others [40-42].

Fujita *et al.* [43] described *Hin*fI restriction patterns as exhibiting a superior discriminatory power among distinct *Candida* isolates compared to patterns obtained with other enzymes such as *Eco*RI or *Msp*I [43]. Additionally, Sancak *et al.* [44] compared HinfI restriction endonuclease-based analysis of genomic DNA with a PCR-based method for molecular typing of 90 *Candida krusei*. They established a correspondence of almost 100% between the results obtained with *the two* methodologies and confirm that these methods are accurate for the typing of *C. krusei* isolates to clarify the epidemiology of nosocomial infections [44]. In the present study, a great diversity of *C. krusei* isolates was found by using PCR-RFLP with three restrictions enzymes. No particular genotype that has been associated with the sampling site, or department or year of infection. Most certainly, patient P4 harbors a reservoir of *C. krusei* and was colonized throughout a long period of time, as described similarly for *Acinetobacter* [45] and *Pseudomonas* [46].

In conclusion, this study showed a significant increase in the frequency of *C. krusei* species, which has become a problematic for clinicians in the recent years. The modification in epidemiology emphasizes the necessity to monitor local incidence, species distribution and susceptibility in order to optimize therapy and outcome. Diagnostic and epidemiological research aimed at determining the source and means of transmission of candidiasis requires not only species identification of isolates but also differentiation within species. With severe infections and having antifungal susceptibility differences, identification of pathogen *Candida* has a significant importance. Many methods are available to identify *Candida species*. These methods can be divided into two main groups: phenotypic and genotypic methods. Molecular methods provide an excellent identification of the species especially when it is mixed infections not detected by the phenotypic methods. Interestingly, molecular methods assure a direct identification of the sample without passing by the culture, which is faster and reduces the risk of contamination. In addition, genetic discrimination among *C. krusei* isolates may offer some important clues to understanding transmission and pathogenesis. Therefore, we recommend PCR-RFLP because it is a simple and easy method to identify *C. krusei* in medical mycology laboratories.

Disclosure of Potential Conflicts of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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