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Original Article

Microbiologic and clinical characteristics of biofilm-forming *Candida parapsilosis* isolates associated with fungaemia and their impact on mortality☆

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ABSTRACT

Objectives: Biofilm formation (BF) by fungal isolates may dramatically complicate infection. We determined the ability of *Candida parapsilosis* isolates from single fungaemia episodes to form biofilms and we analysed biofilm subgroups for antifungal susceptibility and pathogenic potential. We then correlated BF with clinical characteristics and outcomes of the episodes.

Methods: BF was measured using the crystal violet biomass assay. Antifungal susceptibility of preformed biofilms was assessed, and virulence was studied using the *Galleria mellonella* model. A retrospective analysis of patients' clinical records was performed.

Results: Of 190 patient-unique isolates, 84, 38 and 68 were identified as having high BF (HBF), moderate BF (MBF) or low BF (LBF), respectively. Among 30 randomly selected isolates, nine (eight HBF and one MBF), six (all HBF) and one (HBF) isolates had elevated sessile minimum inhibitory concentrations to fluconazole, anidulafungin or amphotericin B; all HBF and MBF isolates had elevated voriconazole sessile minimum inhibitory concentrations. *G. mellonella* killing rates of HBF isolates were significantly greater than MBF (or LBF) isolates (50% vs. 20%, 2 days from infection). By comparing HBF/MBF (106 patients) and LBF (84 patients) groups, we found that HBF/MBF patients had more central venous catheter-related fungaemias (62/106 (58.5%) vs. 29/84 (34.5%), p 0.001) and were more likely to die at 30 days from fungaemia onset (61/106 (57.5%) vs. 28/84 (33.3%), p 0.01). In the HBF/MBF group, azole antifungal therapy and central venous catheter removal were significantly associated with a higher and lower 30-day mortality rate, respectively.

Conclusions: *C. parapsilosis* BF influences the clinical outcome in patients with fungaemia. **S. Soldini, Clin Microbiol Infect 2017;▪:1**

Introduction

In southern European surveys of *Candida* bloodstream infections (BSIs), *C. parapsilosis* was the second most commonly isolated species after *C. albicans* [1–4], and many isolates have been shown to exhibit decreased susceptibility to antifungal agents *in vitro* [5–8]. *Candida* BSIs are complicated by the propensity of isolates to adhere to and grow as biofilms on indwelling medical devices, such as intravascular catheters [9]. From these reservoirs, persister (antifungal tolerant) *Candida* cells [10,11] tend to migrate into the

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bloodstream [12]. Biofilm-forming *Candida* BSIs have been associated with the highest hospital mortality [13,14]. Thus, removal of the central venous catheter (CVC) is the only viable treatment option [15].

According to their different biofilm formation (BF) capabilities [16], clinical *C. parapsilosis* isolates feature distinct biofilm structures [17]. BF by *C. parapsilosis* should be considered within well-defined categories [18], especially when looking for clinical correlations with this phenotypic property [19]. Previously we found that hospital mortality was significantly greater in patients with biofilm-forming *Candida* BSI than in patients with non-biofilm-forming *Candida* BSI, but we reported only very restricted categories, i.e. biofilm formers and non-biofilm formers, of *Candida* isolates in our analysis of association with clinical outcomes [20].

The aim of the present study was to characterize BF by a large number of *C. parapsilosis* bloodstream isolates and subsequently analyse biofilm subgroups with respect to *in vitro* antifungal susceptibility and *in vivo Galleria mellonella* pathogenicity. Furthermore, a retrospective analysis of the relative cohort of patients was performed to determine if isolates' biofilm levels were related to clinical characteristics of fungaemias associated with these isolates.

Materials and methods

Study design and setting

We carried out a retrospective study of *C. parapsilosis* isolates consecutively recovered from BSIs at a 1200-bed tertiary care institution in Rome, Italy, between January 2005 and December 2015. All the first episodes of fungaemia were included in the study. Patients with polymicrobial fungaemia ($n = 10$) or with incomplete clinical data ($n = 2$) were excluded. Twenty-five patients and isolates have been previously studied [20]. For each patient, demographic and clinical information included hospital ward, underlying medical conditions, date of fungaemia onset (i.e. the day of the first positive blood culture), risk factors for fungaemia, time at risk (i.e. the number of hospital days from admission to the onset of fungaemia), details of antimicrobial therapy, length of hospital stay after the onset of fungaemia and 30-day outcome (i.e. assessed from the first positive blood culture until 30 days or death). The study protocol was approved by the institutional ethics committee (no. 1401/16), but informed consent from all patients was waived because of the observational nature of this study.

Isolate collection and identification

C. parapsilosis isolates from patients' blood cultures were identified as described elsewhere [5]. Isolates were kept frozen in glycerol and were subcultured at the time of this study. Before testing, isolates were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [21] to include only isolates ($n = 190$) identified as *C. parapsilosis sensu stricto* (hereafter referred to as *C. parapsilosis*). Isolates ($n = 9$) identified as *C. orthopsilosis*, which together with *C. metapsilosis* belongs to the *C. parapsilosis* species complex [22], were excluded.

BF and antifungal susceptibility testing

All *C. parapsilosis* isolates were tested for BF according to Marcos-Zambrano et al. [18]. Briefly, 100 μL of each isolate's cell suspension standardized to 1×10^6 cells/mL in RPMI 1640 broth medium (Sigma-Aldrich, St. Louis, MO, USA) was allowed to grow as biofilms in 96-well microtitre plates at 37°C for 24 hours. After BF, the wells were washed with 100 μL of a phosphate-buffered saline (PBS) solution to discard nonadhered cells. The biomass level of each isolate was quantified using the crystal violet assay [23]. Isolates were then categorized using optical density at 540 nm ($OD_{540\ nm}$) cutoff values (<0.44, 0.44–1.17 and >1.17), recently proposed to classify *Candida* species isolates [18], as having low BF (LBF), moderate BF (MBF) and high BF (HBF), respectively.

Isolates with LBF ($n = 10$), MBF ($n = 10$) and HBF ($n = 10$), which were chosen randomly among others, were tested for antifungal susceptibility using 24-hour-old biofilms individually treated with each antifungal agent (fluconazole, voriconazole, anidulafungin or amphotericin B) for 24 hours in 96-well microtitre plates. All antifungal agents were prepared according to the Clinical and Laboratory Standards Institute (CLSI) [24] and were used at drug concentration ranging 0.03 to 64 mg/L. Sessile minimum inhibitory concentrations (SMICs) were assessed on the basis of the metabolic reduction of 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide [25]. The SMICs were expressed as the lowest drug concentrations at which a 50% decrease in absorbance was detected compared to that of the biofilms formed in the absence of drug [26]. The minimum inhibitory concentrations (MICs) for planktonic cells were determined by the CLSI reference method and were defined as the lowest drug concentrations that caused either an approximately 50% (for fluconazole, voriconazole and anidulafungin) or 100% (for amphotericin B) growth inhibition compared to that of the drug-free growth control [24].

In vivo killing assay using the G. mellonella model

Killing assays in *G. mellonella* were performed to assess the virulence of randomly selected LBF ($n = 3$), MBF ($n = 3$) and HBF ($n = 3$) *C. parapsilosis* isolates [19,27,28]. Briefly, sixth-instar larvae (300–350 mg in weight; RedBug.it, Milan, Italy) were inoculated within 24 hours of receipt. A Hamilton syringe fitted with a 26-gauge blunt needle was used to inject 10 μL of each isolate's suspension in sterile PBS (5×10^5 cells per larva) into the larval haemocoel. At least ten larvae were inoculated per isolate per experiment; experiments used three independent isolates of each group. Control groups of larvae receiving 10 μL of sterile PBS in exactly the same manner or mock-inoculated larvae pierced on the proleg with a sterile needle (noninjected larvae) were also included in each experiment. Larvae were incubated at 37°C. Survival was recorded at 24-hour intervals for 7 days. Larvae were considered dead when they displayed no movement in response to touch together with a dark discoloration of the cuticle. According to previous studies [29,30], histologic analysis of the larvae after infection was performed by killing representative larvae from each group at 24, 48 and 72 hours after infection. Briefly, the larvae were inoculated with buffered formalin and processed to obtain transverse-cut sections for microscopic evaluation. Tissue sections were stained with periodic acid–Schiff and examined by a technician and a pathologist. Image acquisition was performed by the NanoZoomer-XR C12000 series (Hamamatsu Photonics, Hamamatsu City, Japan).

Definitions of clinical data and therapeutic measures

An episode of fungaemia was defined as the isolation of *C. parapsilosis* in one or more blood cultures obtained from a peripheral vein of a patient with consistent clinical manifestations. The episode was defined as catheter related if the same *Candida* species was also isolated from a catheter tip [31]. In most episodes, the differential time of positivity was also used as an indicator of catheter-related fungaemia [32]. Fungaemia was defined as nosocomial if it occurred more than 48 hours after admission to the hospital and if no signs or symptoms of infection were noted at admission (https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/0512-TED-PPS-HAI-antimicrobial-use-protocol.pdf).

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