

Original Article

Surveillance and characterization of *Candida* bloodstream infections in a Serbian tertiary care hospital

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Abstract

Introduction: *Candida* spp. frequently cause hospital-acquired bloodstream infections (BSI) with a high mortality rate (up to 70%). We analyzed the frequency, infection characteristics, potential predisposing factors, susceptibility to antifungal drugs, biofilm production and other virulence characteristics of *Candida* spp. isolates obtained from a tertiary care hospital in Niš, Serbia, during a one year period.

Methods: Medical histories, characteristics of isolated strains and drug susceptibility, as well as the effect on the function of isolated macrophages and other virulence features were evaluated. The obtained results were subjected to student's t-test and multivariate statistical analyzes.

Results: Herein we report an annual incidence of 3.65 cases of *C. albicans*, *C. lusitaniae* and *C. lipolytica* infections per 10⁵ population. Out of eight isolated strains, two (25%) were shown to be strong biofilm producers, one (12.5%) caused hemolysis on blood agar and in two (25%) cases macrophages were able to completely eliminate the yeast colonies. Chronic kidney disease, diabetes, malignant and other diseases were present in 37.5, 62.5, 50 and 75%, respectively, in the study group. All patients with *Candida* BSI received antifungal therapy (amphotericin B), however, hospital mortality was observed in 25% of patients.

Conclusions: Evaluation of local *Candida* epidemiology, antifungal susceptibility and virulence factors, as well as personalized patient risk factors are important for the surveillance of *Candida* BSI, especially in intensive care unit patients and may contribute to the improved options and outcome for patients with *Candida* BSI.

Key words: *Candida*; bloodstream infections; antifungal susceptibility; virulence factors; surveillance.

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Introduction

Over the last twenty years, nosocomial invasive infections caused by yeasts of the genus *Candida* represent a major medical problem, especially in intensive care unit (ICU) patients. The results of a recent research in European countries showed that fungi had a high prevalence of 17% of all hospital infections [1]. Prevalence of *Candida* caused fungemia is 8-15% of all hospital-acquired bloodstream infections (BSI) (4th place), making them one of the groups of most common infections in hospitalized patients. The epidemiological scenario of *Candida* BSI (CBSI) varies between different geographical regions. In Europe: i) the incidence of CBSI ranges from 3 to 8.6 cases per 100,000 population per year [2]; ii) the mortality of candidemia is 38% in hospitalized patients, and in

patients with malignancies up to 70% [3]. In Taiwan, CBSI are the second most common cause of hospital-acquired sepsis (after bacteremia by *Staphylococcus* species) [4]. In this region fungemia causes a very high rate (up to 70%) of mortality and a rise in price of treatment due to prolonged hospitalization and application of antifungal therapy. Clinical manifestations of CBSI are not specific (it can also be manifested as a fever of unknown origin) making diagnosis difficult [5]. Because of the existence of non-characteristic symptoms and clinical signs, it is very important to consider the risk factors and predisposing diseases in patients. Well-known risk factors include hematological and endocrine diseases, acquired and congenital immunodeficiency, use of broad-spectrum antibiotics and immunosuppressant drugs (steroids),

prolonged hospitalization, especially in ICUs, invasive medical procedures, parenteral nutrition, presence of a central venous catheter and hemodialysis [3,4]. CBSI are frequently associated with the formation of biofilms, growth of *Candida* organisms on medical devices such as venous or urinary catheters. These infections become more serious due to the fact that biofilm-forming *Candida* strains are frequently resistant to antifungal agents. Based on the results obtained in an investigation of an *in vitro* susceptibility of these yeasts to antifungal drugs, only amphotericin B and echinocandins showed efficacy against *Candida* biofilms [1,6]. This may be the reason why *Candida*-biofilm formation is associated with increased virulence and mortality of patients with CBSI [6]. The most prevalent cause of CBSI is *Candida albicans* [1], that is found in 50% of cases, followed by *C. parapsilosis*, *C. glabrata* and *C. tropicalis* [1,2]. In recent years, non-albicans species have become a more common cause of CBSI, primarily in patients with immunodeficiency and hematological diseases [1]. *Candida parapsilosis* predominates as a cause of CBSI in patients on hemodialysis with implanted catheters, in catheter-associated infections of neonates, which appears reasonable due to the fact that this species usually colonizes the skin [2,4]. Up to now there are no data concerning the incidence, characteristics of infections, risk factors and strain virulence characteristics of CBSI from the region of Niš, Serbia. The aim of the work was to analyze, for the first time, the cases of CBSI, occurring in a tertiary care hospital in Niš, Serbia, during a one-year period, and to indicate the characteristics of infections, potential predisposing factors for the occurrence of disease and death and resistance of *Candida* spp. isolates, as well as the importance of biofilm production and other virulence characteristics. This study was also designed to compare the obtained results with the known and generally accepted characteristics of *Candida* strains that were known to cause bloodstream infections.

Methodology

Source population

This prospective case-control study was done at the Institute of Public Health, Center for Microbiology, Niš, Serbia, during the period September 2014 - September 2015 (municipal areas of Niš, population 219108). During this period eight patients admitted to the Clinical Center of Niš, Serbia (850 beds, 19750 admissions annually, 25 medical wards) had at least one positive blood culture for the presence of *Candida* spp. The control group included patients who were

hospitalized in the same medical wards, during the same time period as the case patients, but who had not shown evidence of CBSI.

Study population

Only those patients for whom the complete data from their medical histories were available were included in this study. The data collected from the patients' medical records were: i) demographic characteristics (age, sex, body weight), ii) underlying diseases (chronic kidney disease (CKD), malignant disease, diabetes mellitus, etc.), iii) invasive procedures (central venous (CVC) and urinary catheterization (UC), parenteral nutrition, nasogastric tube (NGT)), iv) other associated conditions (previous bacteremia, previous infections, alcohol consumption, smoking, abdominal surgery, use of broad-spectrum antibiotics or immunosuppressive therapies (steroids, chemotherapy, etc.)), v) duration of hospitalization before the date of the first *Candida* spp.-positive blood culture (for the study group) or the total number of days spent in the hospital (for the control patients), hospital ward, admission to ICUs, vi) antifungal therapy, vii) disease outcome (mortality), cause of death, and viii) the serum level of creatinine, urea nitrogen, serum albumin and the total number of white blood cells. The predisposing events within one month prior to diagnosis and the laboratory data within seven days prior to the first positive *Candida* spp. blood culture were analyzed. The decision that a patient died directly from CBSI or an underlying illness was based on the cause of death stated in the death certificate of the patients.

Definitions

CBSI were considered to be nosocomial if blood cultures were positive >72 h after hospitalization and no evidence of *Candida* infections was found at the time of admission.

Incidence

Incidence rates were expressed as the number of cases of CBSI per 100,000 population and per 1,000 admissions.

Microbiological testing

For microbiological analyses, peripheral blood (10 ml from adults and 3-5 ml from pediatric patients) was collected (by venipuncture, no longer than one minute after a previous disinfection of the puncture site) and processed using a media for fungi (Bactec Mycosis media, Becton Dickinson) in a BACTEC 9120 system (Automated Blood Culture Systems Becton Dickinson,

Instrument Diagnostic System, Sparks, MD, USA), applying a standard protocol (incubation during 14 days - 336 hours, at 35 °C). Positive samples, detected by the system computer, were immediately subcultured on a Sabouraud dextrose agar (SDA) (Hi Media Pvt Ltd, Mumbai, India).

Identification of isolates

After the initial detection, as described above, *Candida* spp. were isolated using standard mycological procedures and a chromogenic medium (Liofilchem Chromatic Candida, Liofilchem/Bacteriology products, Italy) in the laboratory for mycological analyses of the Public Health Institute of Niš. The identifications were done by the National Reference Medical Mycology Laboratory, Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, based on: (i) culturing, based on colored colony morphology (HiCrome Candida Differential Agar (Hi Media Pvt Ltd, Mumbai, India), (ii) commercialized biochemical systems for clinical yeast identification (Fungifast, ELITech France), and (iii) matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed using Micronaut-AM MHK-2 plates (Merlin Diagnostika, Bornheim, Germany) that contained serial twofold dilutions of amphotericin B (AmB) (0.03-16 µg/ml), fluconazole (FLUC) (0.125-128 µg/ml), 5-fluorocytosine (5-FLU) (0.06-32 µg/ml), itraconazole (ITR) (0.03-4 µg/ml), posaconazole (POS) (0.015-16 µg/ml), voriconazole (VOR) (0.015-16 µg/ml), micafungin (MCF) (0.01-16 µg/ml), caspofungin (CAS) (0.01-16 µg/ml) and anidulafungin (ADF) (0.01-16 µg/ml). The susceptibility testing was performed according to the manufacturer's instructions. The reference strains *Candida albicans* ATCC 24433 and *C. krusei* ATCC 6258 (*Issatchenkia orientalis* Kudrjanzev ATCC 6258) were used as the quality controls. The results were read visually, and the minimum inhibitory concentration (MIC) end points were defined as the lowest concentration that produced a prominent decrease in turbidity (a 50% growth reduction) compared with the drug-free growth control. Interpretation of the obtained MIC values was based on the EUCAST criteria [7].

Biofilm, germ tube and hemolysin production assays

The isolated *Candida* spp. strains were stored on SDA slants at 4 °C until analyses. For the analyses, all

Candida strains were cultured for 18 h (early exponential stage) on SDA plates at 37 °C. Overnight cultures were used for the preparation of cell suspensions used in further experiments, and these suspensions corresponded to 0.5 McFarland turbidity. Suspensions were made in sterile phosphate buffered saline (PBS), adjusted to the desired turbidity using a McFarland densitometer (DEN-1 McFarland Densitometer, Biosan). The *Candida* isolates were screened and quantified for their ability to form biofilms under static conditions in 96-well microtiter plates by a crystal violet method as described previously [8]. Briefly, biofilm formation was monitored after 48 h at 37°C in RPMI (Roswell Park Memorial Institute) medium, supplemented with 8% glucose ($1-2 \times 10^6$ cfu/mL). After the incubation period, the medium was gently aspirated, the plates washed, dried (45 min at 37°C) and stained with 0.4% (w/v) crystal violet solution. After the removal of excess dye, the wells were filled with 200 µL of 96% (v/v) ethanol and 45 min later, 100 µL of the resulting solutions was transferred into new microtiter plates. The absorbance was read at 595 nm on an ELISA reader (Multiscan Ascent, Labsystems, Finland). All experiments were done in triplicate and repeated three times. The biofilm producing abilities were differentiated into the following groups: non-, weak, moderate and strong biofilm producers, according to the criteria given by Stepanović *et al.* [8]. To assess the germ tube-producing capability of the isolates, the strain suspensions adjusted to 0.5 McFarland turbidity in PBS were cultured in two cultivating media – tryptic soy broth (TSB) and TSB supplemented with pooled human serum (10%, v/v). The final number of cells corresponded to $1-2 \times 10^7$ in each tube. After two hours of incubation at 37°C, samples of all isolated strains were placed on microscopic slides and observed under a light microscope ($\times 40$ magnification) for germ tube production ability. Cells were counted in 5 fields per slide, afterwards the percentage of cells that formed germ tubes was calculated. All experiments were done in triplicate and repeated three times [9,10]. Hemolytic activity of *Candida* isolates was screened on blood agar plates by the method described by Manns *et al.* [11]. Blood agar plates were spot inoculated with 10 µL of suspension having 0.5 McFarland turbidity ($\sim 10^7$ cells/mL). The culture plates were incubated at 37°C for 48 h. The presence of a zone of hemolysis around the colony indicated hemolysin production. Hemolytic activity was calculated in terms of the ratio of the diameter of the colony to that of the translucent zone of

hemolysis (mm). All experiments were done in triplicate and repeated three times.

Animal housing and cell isolation

Male Wistar rats aged 3 months were obtained from the Vivarium of the Institute of Biomedical Research, Faculty of Medicine, University of Niš, Niš, Serbia. Animals were kept under standard laboratory conditions at 22 ± 2 °C and 60% humidity, with food and water available *ad libitum*. All experimental procedures with the animals were conducted in compliance with the declaration of Helsinki and European Community guidelines for the ethical handling of laboratory animals (EU Directive of 2010; 2010/63/EU) and were also approved by the local Ethics Committee. The cells were harvested by peritoneal lavage with PBS seven days following an intraperitoneal injection of a thioglycolate medium (Institute of Virology, Vaccines and Sera” Torlak”, Belgrade, Serbia), when the majority of the cells in peritoneal exudates were macrophages. The peritoneal lavages were centrifuged (1200 rpm, 10 min at 4 °C) and the resulting cell pellets were washed. After this procedure, cell viability was determined to be >95% by the trypan blue dye exclusion method and the concentration of individual cell suspensions was adjusted to 2.5×10^6 cells per ml and used as such in macrophage viability and candidacidal activity of macrophages assays, as well as for myeloperoxidase (MPO) enzyme measurement [12]. For the *Candida* phagocytosis assay cell concentration was adjusted to 1×10^6 cell/ml.

Macrophage assays

The cell suspensions in RPMI medium were added into 96-well microtiter plates and cultured at 37 °C for 1 h under 5% (v/v) CO₂. After the initial incubation period, the non-adherent cells were removed from all wells, filled with 200 µL of *Candida* suspensions in RPMI medium adjusted to $1-2 \times 10^6$ cfu/mL. The plates were incubated for additional 24 h at 37 °C under 5% (v/v) CO₂. After this period, the content of all wells was aspirated and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (5 mg/ml) was added. After the incubation period the formed formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The content of each well was transferred into new plates and the absorbance read at 540 nm (Multiscan Ascent, Labsystems, Finland). The results are expressed as % viability relative to untreated control cells. All experiments were done in triplicate and repeated three times. In another set of wells MPO

activity in macrophages cultivated with *Candida* suspensions was measured following a standard procedure that utilizes 1,2-diaminobenzene as the chromogen [12]. Candidacidal activity of peritoneal macrophages was done 1 h after the incubation of *Candida* suspensions with adherent cells. After this time elapsed 50 µL from each well was used for the preparation of a serial dilution (diluting factor 10x) in sterile saline. The samples from each dilution and also undiluted samples (directly from wells) were seeded on SDA plates. After 24 h of incubation at 37°C, viable counting of survivor cells (number of colonies) was performed. The results are expressed as hundreds of cells per ml. The phagocytosis assay was done on microscopic slides with previously adhered macrophages (from 100 µL of the suspension containing 1×10^6 macrophages/mL) following a previously described method [13]. The slides were covered with heat inactivated yeast cell suspensions $1-2 \times 10^6$ cfu/mL and incubated for 90 minutes at 25°C. Afterwards, the slides were washed with PBS, fixed in methanol and stained with Giemsa. The phagocytic index was determined by calculating the average number of yeast cells engulfed by or adhering to 100 macrophages. The percentage phagocytosis and the phagocytic index were calculated following the formula: percentage phagocytosis = number of positive cells per 100 cells observed x phagocytic index - average number of yeast cells engulfed by a positive cell.

Statistical analysis

Agglomerative hierarchical clustering (AHC) and principal component analysis (PCA) were performed using the Excel program plug-in XLSTAT version 2011.3.02. The method was applied utilizing the risk factors, clinical characteristics and strain virulence data as the original variables (the values were taken to be 1 if positive and 0 if negative). AHC was determined using Pearson dissimilarity, where the aggregation criterion was simple linkage, unweighted pair-group average and complete linkage, and Euclidean distance, where the aggregation criterion were weighted pair-group average, unweighted pair-group average, and Ward’s method. The magnitudes of Pearson correlation coefficients were treated as follows: very weak (0.0-0.19), weak (0.2-0.39), moderate (0.4-0.59), strong (0.6-0.79) and very strong (>0.8) [14]. Student’s t-test analysis was done on mean values of the case and control groups using GraphPad Prism version 5.03 (GraphPad Software Inc., San Diego, CA, USA).

Table 1. Identification and minimal inhibitory concentrations (MIC) of systemic antifungals for the investigated *Candida* spp.

Strain (MALDI-TOF identification)	AmB	FLUZ	ITR	VOR	5-FLU	POS	MCF	CAS	ADF
1. <i>Candida lusitanae</i>	0.25	1	0.25	0.125	<0.06	0.03	0.125	0.5	0.125
2. <i>Candida albicans</i>	1	0.125	0.03	0.01	0.06	0.01	0.01	0.06	1
3. <i>Candida albicans</i>	1	0.125	0.03	0.01	0.06	0.01	0.01	0.01	0.03
4. <i>Candida albicans</i>	1	0.25	0.06	0.01	0.06	0.01	0.03	0.125	0.03
5. <i>Candida lusitanae</i>	1	0.125	0.03	0.01	0.06	0.01	0.125	0.5	0.125
6. <i>Candida albicans</i>	0.5	8 ^a	2 ^a	2 ^a	<0.06	16 ^a	0.01	0.06	0.25
7. <i>Candida lipolytica</i>	0.5	0.25	0.06	0.03	0.06	0.01	0.05	1	1
8. <i>Candida albicans</i>	0.5	4 ^a	4 ^a	>16 ^a	<0.06	>16 ^a	0.01	0.06	0.01

* a – resistant;

Probability values (*p*) less than 0.05 were considered to be statistically significant.

Results

During the study period, a total of 829 blood cultures were investigated, taken from 369 patients. A positive finding of blood infections was confirmed in 56 (15.18%) patients, of which 8 (2.17%) were *Candida* spp. positive.

Incidence rates

We identified 8 episodes of CBSI, yielding an annual incidence of 3.65 cases/100,000 population and 0.4/1,000 admissions.

Microbiological characteristics of the isolated *Candida* spp.

Candida albicans was the most commonly isolated species (62.5%, 5 patients), whereas other *Candida* species isolated included *C. lusitanae* (25%, 2 patients) and *C. lipolytica* (12.5%, 1 patient) (Table 1). In the case of all *Candida* isolates, *in vitro* antifungal susceptibility testing showed a satisfactory candidacidal effectiveness of 5-fluorocytosine, amphotericin B, micafungin, caspofungin and anidulafungin (Table 1). Isolates No. 6 and 8 were resistant to fluconazole, itraconazole, voriconazole and posaconazole. The following can be concluded from an assessment of the virulence factors of the isolates (Table 2). In static conditions, among the isolated *Candida* spp.: i) only two strains (*C. albicans* and *C. lusitanae*) turned out to be strong biofilm producers (25%); ii) one strain (*C. albicans*) showed a complete absence of biofilm-forming ability (12.5%); iii) one strain of *C. lipolytica* and *C. lusitanae* displayed a weak biofilm-producing ability (25%). As for the germ tube formation: all tested strains (*C. albicans*) exhibited a relatively low number of produced germ tubes, while strain No. 6 did not produce filaments at all during the

incubation period (2 h). The testing of hemolytic activity showed that only one strain (*C. lipolytica*) caused hemolysis on blood agar plates, producing a transparent halo around colonies.

Five *Candida* strains were able to reduce macrophage viability up to ca. 70% (74-77%), whereas macrophages were capable, in two cases, to completely eliminate yeast colonies (Table 2). Activity of MPO varied significantly between the strains (from 354 to 777, OD ×1,000). The phagocytic activity expressed as the index of phagocytosis differed among the strains and ranged from 5 to 27 (Table 2).

Patients' characteristics

General patient characteristics are shown in Table s1 (Supplementary material). The age of patients was from 15 days to 83 years, and 10 days to 72 years, with body weight of 2.65 to 87, and 2.75 to 92 kg in patients with CBSI and in the control group, respectively. More than a half of them in both groups were males (50.0 and 62.5%, respectively). The hospitalized patients originated from five different clinics belonging to the Clinical center Niš (more than 60% were situated in ICUs) where they spent 60 and 49 days on average in the case and control groups, respectively (Table s1). The data concerning patient concomitant diseases and hospitalization characteristics are presented in Table s2. CKD, diabetes, malignant and other diseases were present in 37.5, 62.5, 50.0 and 75.0% of the case group patients, while in the control group, the frequency of their occurrence was 25.0, 31.2, 25.0 and 81.2%, respectively. The percent of patients from the case group that underwent catheterization, CVC or UC, was 75% (for both types of catheters), whereas, in the control group, CVC was applied in 43.8% and UC in 37.5%. As part of CBSI treatment, all 6 patients with CVC from the case group underwent removal of the catheter. The total percentage of incidence of parenteral food intake and NGT, in the case group, was 62.5 and

Table 2. Virulence characteristics of the isolated *Candida* strains.

Strain	Biofilm formation	Hyphal transformation assay (%)	Hemolytic activity	Macrophage killing activity (%)	MPO activity (ODx1000)	Candidical activity of macrophages (x 10 ² cells)	Macrophage phagocytosis assay (index)
1.	weak	-	-	76	364	26	27
2.	moderate	23	-	74	354	0	11
3.	moderate	17	-	77	604	17	13.5
4.	moderate	6	-	81	777	8.3	6
5.	strong	-	-	76	605	21	5
6.	strong	0	-	78	427	19	5
7.	weak	-	+	100	624	0	9.5
8.	-	9	-	100	609	30	14

* Determined activity was present (+) or absent (-).

50.0%, respectively. All patients with CBSI received AmB; 75% of them received this antifungal alone, whereas the remaining 25% received dual therapy, i.e. AmB with fluconazole or 5-fluorocytosine as the second drug (Table s3). Out of the total of 8 patients with CBSI analyzed, hospital mortality was observed in 25% (death occurred due to CBSI) (Table s3). Both antibiotic and immunosuppressive therapies applied prior or during the hospitalization of patients were found in 75.0% and 50.0% of patients from the case group, and in 37.5% and 37.5% from the control group,

respectively. The data regarding previous operations and bacteremia findings for the patients are shown in Table s3. Serum biochemical parameters are also shown in Table s3.

Statistical analysis

The number of white blood cells (WBC) varied from 4,500 to 17,000 cells/mm³. The mean value of WBC count in the case group was higher ($p=0.0043$) than the WBC count for the patients from the control group. Mean values of other variables did not differ

Table 3. Statistical analysis of case and control groups.

Variable	Case group (n=8)		Control group (n=16)		p value
	n*	% or mean ± S.D	n*	% or mean ± S.D	
Age	/	55.7 ± 14	/	52 ± 11.8	0.503
Sex (male)	5	62.5	8	50	0.67
Alcohol	2	25	6	37.5	0.66
Smoker	2	25	2	12.5	0.57
Body weight (kg)	/	74 ± 8.6	/	73 ± 5.1	0.722
Hospitalization spent in intensive care unit	4	75	10	62.5	0.88
Number of hospital days	/	60 ± 25	/	46 ± 13.5	0.085
Presence of CKD	3	37.5	4	25	0.64
Diabetes	5	62.5	5	31.3	0.20
Malignant diseases	4	50	4	25	0.36
Other diseases	6	75	13	81.3	1.0
Previous operations	4	50	3	18.8	0.16
Total parenteral food intake	5	62.5	5	31.3	0.20
CVC	6	75	7	43.8	0.21
UC	6	75	6	37.5	0.19
NGT	4	50	4	25	0.36
Disease outcome death	2	25	2	12.5	0.57
Other antibiotics	6	75	6	37.5	0.19
Immunosuppressive therapy	4	50	6	37.5	0.67
Previous infections	6	75	6	37.5	0.19
Previous bacteremia	6	75	8	50	0.38
White blood cells	/	11806 ± 4890	/	7538 ± 1710	0.004
Albumin (g/dL)	/	2.96 ± 0.49	/	3 ± 0.4	0.832
Urea (mmol/L)	/	7.3 ± 2.6	/	6 ± 1.7	0.153
Creatinine (µmol/L)	/	108 ± 39.6	/	88 ± 32.9	0.202

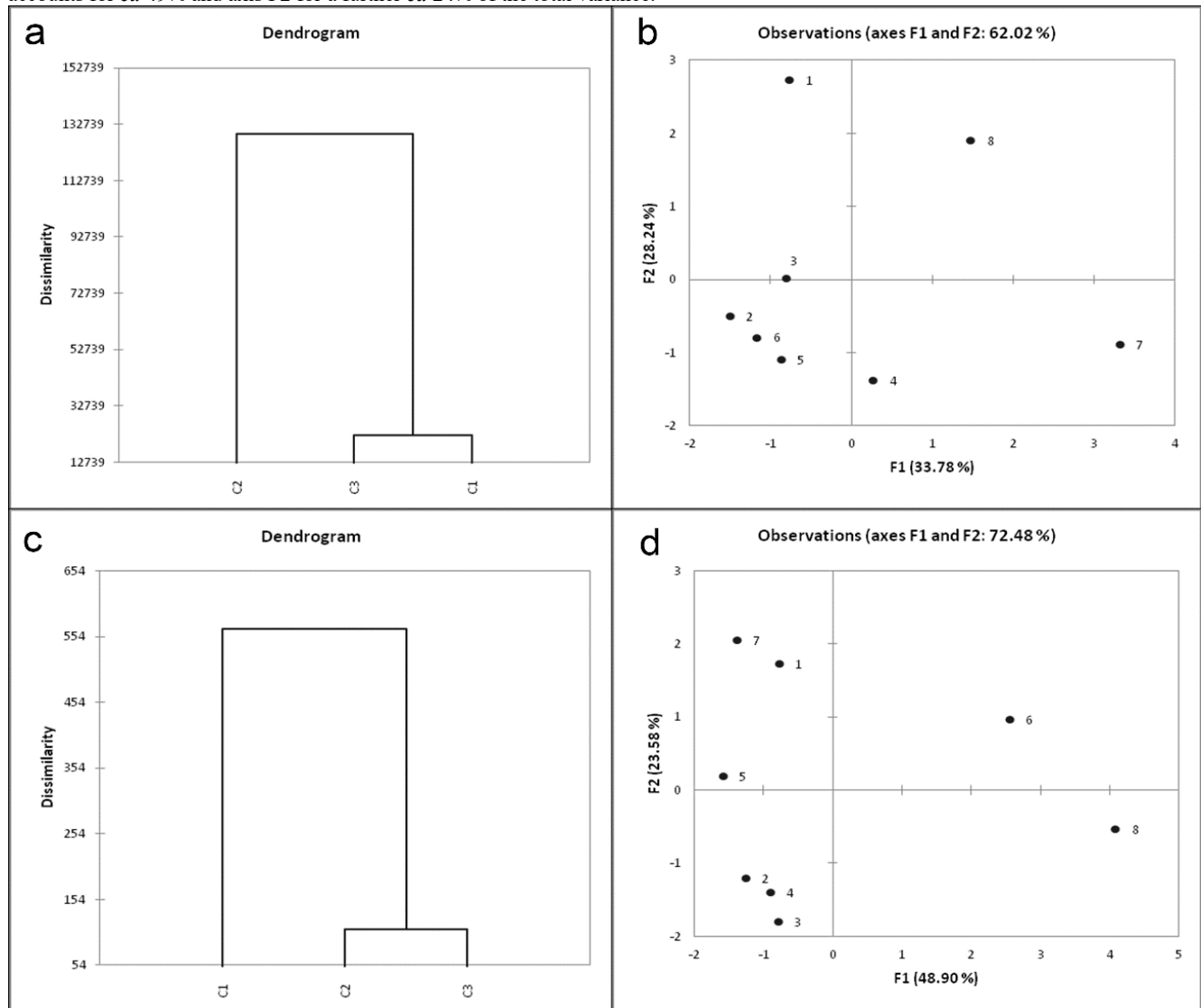
* Number of positive variables (in case of patients sex the value is for male patients); in italic font are p values that represent a statistically significant difference between two groups.

Figure S1a. Dendrogram of AHC obtained by agglomerative hierarchical clustering using the original variables (virulence factors) and representing the dissimilarity relationships of 8 isolated *Candida* strains (observations). As dissimilarity metric, the Euclidian distance was used (dissimilarity within the interval [0, 132739], using Wards method as aggregation criterion). Three statistically different groups of strains were found (C1–C3).

Figure S1b. Principal component analysis of 8 isolated *Candida* strains (variables-virulence factors): ordination of strains (designations defined in Table 1). Axes (F1 and F2 factors: first and second principal components) refer to the ordination scores obtained for the samples. Axis F1 accounts for ca 34% and axis F2 for a further ca 28% of the total variance.

Figure S1c. Dendrogram of AHC obtained by agglomerative hierarchical clustering using the original variables (MIC values) and representing the dissimilarity relationships of 8 isolated *Candida* strains (observations). As dissimilarity metric, the Euclidian distance was used (dissimilarity within the interval [0, 554], using Wards method as aggregation criterion). Three statistically different groups of strains were found (C1–C3).

Figure S1d. Principal component analysis of 8 isolated *Candida* strains (variables-MIC values): ordination of strains (designations defined in Table 1). Axes (F1 and F2 factors: first and second principal components) refer to the ordination scores obtained for the samples. Axis F1 accounts for ca 49% and axis F2 for a further ca 24% of the total variance.



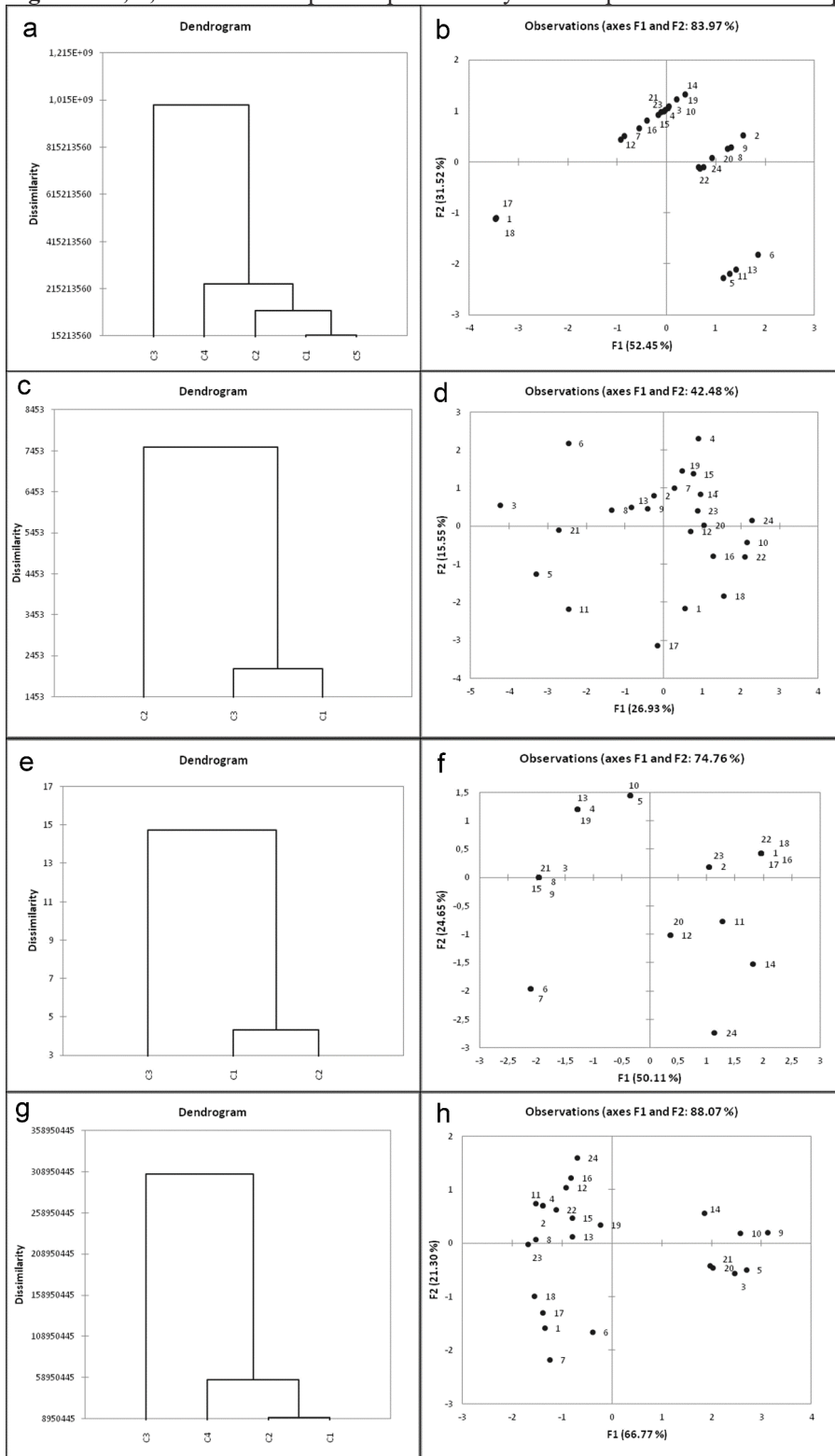
significantly for the two groups (Student’s t-test) (Table 3).

Detailed AHC and PCA results are given in the supplementary material of this paper (Table s4-s7 and Figure s1-s3). AHC analysis of MIC values of standard

antimycotics segregated three groups of *Candida* strains (Figure s1a and b), whereas this analysis divided the eight isolates into three groups according to virulence characteristics of the isolated strains (Figure s1c and d).

Figure S2a, c, e and g. Dendrograms of AHC obtained by agglomerative hierarchical clustering using the original variables and representing the dissimilarity relationships of 24 patients (observations)

Figure S2b, d, f and h. Principal component analysis of 24 patients: ordination of patients



⊞ **Figure S3a, c and e.** Dendrograms of AHC obtained by agglomerative hierarchical clustering using the original variables and representing the dissimilarity relationships of 8 isolated *Candida* strains (observations)

Figure S3b, d and f. Principal component analysis of 8 isolated *Candida* strains: ordination of strains

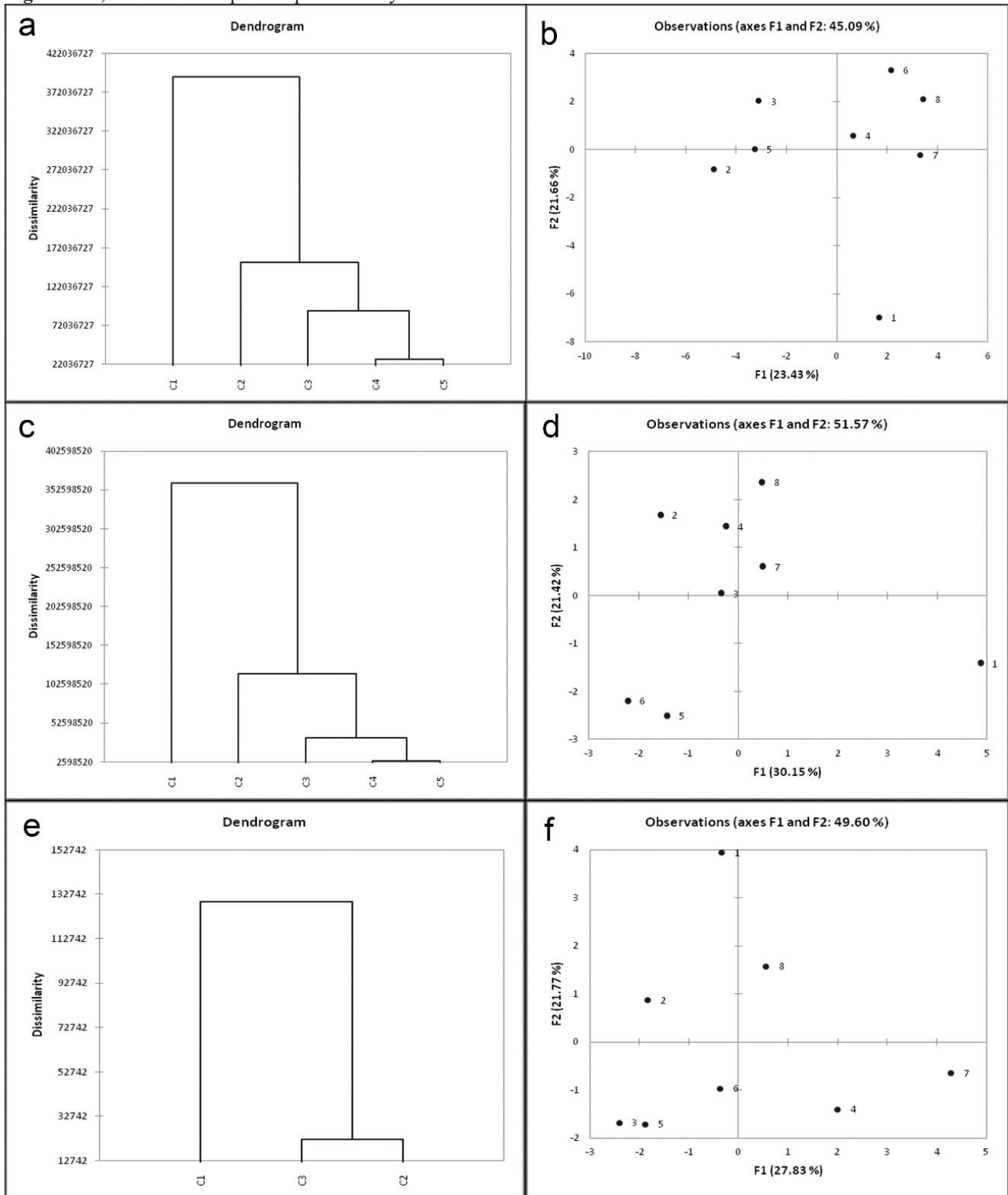
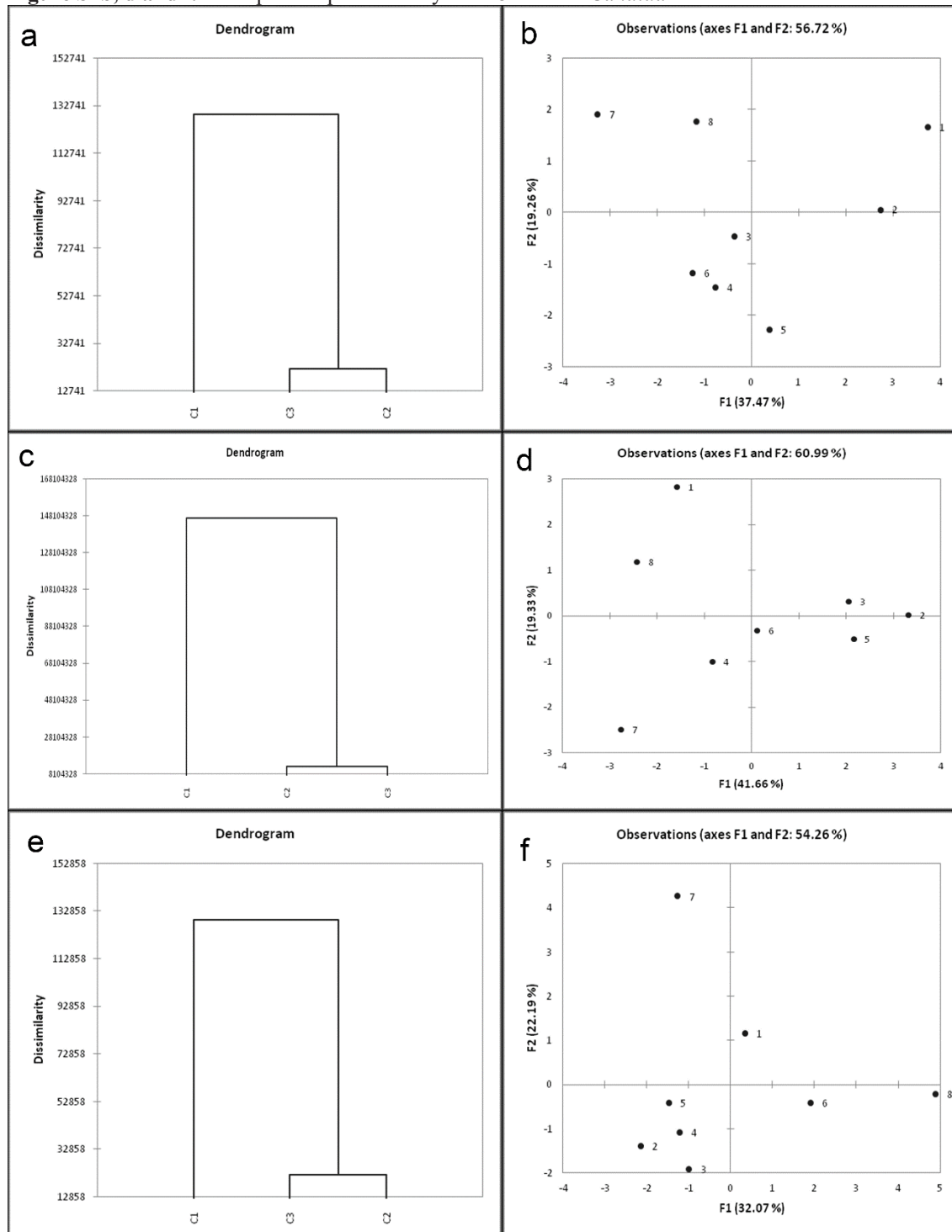


Figure S4a, c and e. Dendrograms of AHC obtained by agglomerative hierarchical clustering using the original variables and representing the dissimilarity relationships of 8 isolated *Candida* strains (observations)

Figure S4b, d and f. Principal component analysis of 8 isolated *Candida* strains: ordination of strains



The statistical treatment of the data (AHC), including all patient characteristics (Tables s1-s3), placed the patients into seven groups where two groups, with one patient per group (groups 3 and 4), were with CBSI (Figure s1). Data analysis of the patient

concomitant diseases and hospitalization characteristics (Table s2) divided the patients into three groups (Figure s2c and s2d) with *Candida* strains present in each group (predominantly in the second group with the longest hospitalization period). The analysis of data concerning

medication usage and disease outcome segregated one group with a number of patients with CBSI, where all of the patients had consumed both antibiotics and immunosuppressive therapy and had the history of previous infections and bacteremia. The statistical treatment of the data regarding blood and serum biochemical parameters (Table s3) set up four groups of patients, however, patients with *Candida* infections were present in all of them (Figure s3g and s3h).

Clustering analysis that included all patient and *Candida* virulence data segregated the patients into 5 groups (Figure s3 and s4). A separate AHC analysis on selected patient data (Tables 1-2 and s1-s3) and *Candida* virulence data resulted in various grouping patterns. Almost all of these analyses placed strain No. 4 in a separate clade (Figure s4c and s4d) pointing out a specificity of strain No. 4.

Principal component analysis (PCA) of MIC values of standard antimycotics (with 5-fluorocytosine excluded due to the fact that it did not contribute to this analysis; all values were the same) revealed a high positive Pearson correlation among the values MIC of triazol derivatives (Table s5).

When PCA was done on a large data set of patient (case and control group) parameters, a number of both positive and negative Pearson correlations were observed among the variables (Table s6). Urinary catheter correlated positively with a previous application of antibiotics and existence of infections, while NGT correlated with previous bacteremia (Table s6). PCA of data from patients with CBSI (Table s1-s4) and *Candida* virulence characteristics (Tables 1 and 2) revealed additional large positive and negative Pearson correlations (Table s7).

Discussion

Invasive mycoses, including CBSI, are life-threatening infections that have significantly become more frequent in recent years. Having in mind that the Clinical Center of Niš receives around 350 patients daily, it was quite interesting to establish that only 8 *Candida* strains were isolated from blood cultures over a period of one year (Table 1). Despite the recent reports that non-albicans *Candida* spp. are more common in serious fungal infections our results showed exactly the opposite, i.e. *C. albicans* was the dominant cause (62.5%) of CBSI [15,16]. CBSI caused by *C. lipolytica* and *C. lusitaniae* were rarely reported and accounted for only 7% of all detected CBSI [17,18].

CBSI incidence in Niš area was 3.65 cases/10⁵ population, that is in agreement with the data obtained from northern and central European countries. A higher

incidence was recorded in Spain and USA (6-8/10⁵ population), while the highest was noted in Atlanta and Baltimore (USA) where it amounted to 13.3 and 26.2 /10⁵ population, respectively [2,19,20]. These variations in the geographical distribution of CBSI incidence is most probably due to demographic differences or dissimilar patient management.

Candida spp. are opportunistic yeasts that colonize the skin, mucosal surfaces and the gastrointestinal tract (GIT) and it has been shown these opportunistic strains are important sources of CBSI [21]. Patients that have cell-mediated immune deficiency or are receiving immunosuppressive agents (such as steroids) are at high risk to develop CBSI [22]. It is believed that Th1 cell responses characterize the carriage of saprophytic yeast and the resistance to disease in healthy individuals, whereas on the other side Th2 responses are predominantly connected with the pathology observed in immunocompromised hosts. Serious primary diseases, such as diabetes, chronic renal failure, malignant diseases and SLE, could lead to the depression of humoral and cellular immunity, which are the basic defensive mechanisms preventing *Candida* systemic/invasive infections [3]. In our survey, all patients with CBSI suffered from serious primary diseases, conditions or displayed other risk factors for the development of CBSI (Table s2). Multivariate statistical analyses (MVA, Figure s4a and s4b), which included the analyses of concomitant diseases and hospitalization characteristics of patients, showed that immunosuppressant therapy was a significant predisposing factor for various infections of the bloodstream. Also, the AHC analysis of the data concerning medication usage and disease outcome revealed that patients in group C1 (four patients out of eight with CBSI) were placed there mainly due to their usage of antibiotics and immunosuppressive therapy, as well as the history of previous infections and bacteremia (Table s3, Figure s2e and s2f). This pointed to the fact that CBSI in these patients were probably a consequence of the presently analyzed risk factors.

Based on the molecular identification of *Candida* genotypes, it is known that the source of *C. albicans* CBSI is the gastro-intestinal tract (GIT), and of non-albicans species causing CBSI are probably contaminated catheters [23]. Indirectly, the treatment with broad-spectrum antibiotics that influences *Candida*-colonization of GIT, followed by a translocation of the fungi into the blood, could be a risk-factor for candidemia [3]. On the other hand, contaminated catheters enable a direct inoculation of *Candida* spp. in the blood. Evans *et al.* showed that 67%

of patients with CBSI had fungal colonization of catheters [14]. Among others, *C. albicans* CBSI predisposing factors include: previous bacterial urinary tract infections, parenteral nutrition and leukocytosis $\geq 15000 \text{ mm}^3$. For non-albicans CBSI, the risk factors are: age <65 years, neutropenia, thrombocytopenia and chemotherapy of a primary malignancy [5]. Arendrup *et al.* [24] demonstrated that two thirds of patients had received abdominal surgery or intensive care treatment prior to the development of fungemia. In our study, one strain was isolated from a patient that underwent abdominal surgery (No. 1, Table s1). *Candida lipolytica* BSI have been associated with catheterization, immunodeficiency and severe underlying diseases [17]. *Candida lusitanae* BSI have been linked with prolonged hospitalization, taking of broad-spectrum antibiotics and immunosuppressive therapy [18]. When an AHC analysis was done on the data concerning the biochemical parameters, a clear division into four groups could be seen, where *Candida* strains No. 1, 6 and 7 were grouped together (Figure s3g). This is probably due to a high WBC number in these patients ($>15,000/\text{mm}^3$), which points to the significance of this predisposing factor for CBSI. One group of patients was probably separated according to high levels of urea and creatinine and a low number of WBC, among which the majority had CRD (62.5%; Table s2 and s3), but only one patient with an isolated *Candida* strain (No. 2) was placed into that group (Figure s3g). Also, there are publications that underlined the presence of CRD as a possible risk factor in patients with non-biofilm-forming *Candida* strains [6]. Among the herein reported strains, three of them (No. 2, 3 and 5) were isolated from the material of patients with CRD (Table s3) but none of them had a non-biofilm-forming strain (Table 2). In animal models, it was observed that during and after CBSI, the kidneys suffer a great burden making it a primary target organ [25], which is in accordance with the increased kidney serum parameters found in these patients (Table s3).

Candida biofilm production is a significant risk-factor for CBSI, especially in diabetic patients and those with catheters, due to hyperglycemic conditions and the fact that a urine catheter allows the adaptation of *Candida* organisms to a biofilm lifestyle. In our work, 4 of CBSI patients with *Candida* biofilm-producing strains had diabetes mellitus and CVC and/or UC reference. Tumbarello *et al.* [6] confirmed that the use of CVC and parenteral nutrition are highly associated with the development of *Candida* biofilm in CBSI patients. Also, *Candida* biofilm-forming species can cause an infection on the surface of the epithelium

without the presence of biomaterials. All these findings make it difficult to determine the role of biofilm in the pathogenesis of CBSI. Our AHC analysis of biofilm-formation pathogenicity data (Table s2 and Figure s2c and s2d) separated only one *Candida* strain into a separate group (C3 group) and this strain was characterized as a weak biofilm producer and did not have the ability to undergo hyphal transformation (Table 2). These findings additionally support the relevance of biofilm formation ability as an important risk-factor.

CBSI is a serious disease whose clinical course often has a lethal outcome with high mortality in the period of 30 days [2]. Previous studies have shown that there is no significant difference in the mortality caused by different *Candida* species [2,5,6]. However, some studies suggested an association between high rates of mortality of CBSI and cultivation of biofilm-forming *C. albicans* and *C. parapsilosis* from the blood, which confirms that biofilm formation significantly affects the virulence of *C. albicans* or enhances pathogenic potential of *C. parapsilosis* [6]. Delays in the initiation of antifungal therapy, longer than 12–15 h after the identification of positive blood cultures, correlated with an increased risk of death [26]. The type of the administered antifungal therapy could also have a major impact on the outcome of CBSI. The use of initial therapy with fluconazole was a significant protective factor, reducing mortality by approximately 30% [27]. In the course of our study, mortality of 25% was noted. Our findings concerning disease outcome and biofilm production are the expected ones. One of the strains that caused death was a strong, while the other was a weak biofilm producer (Tables 2 and s3). We could only presume that the weak biofilm-producing strain (No. 7) probably caused a bloodstream infection and led to death via a mechanism that does not include biofilm formation. This strain was completely sensitive to innate immune system cells (Table 2), however, the presence of acute leukemia and the treatment with immunosuppressive therapy (Tables s2 and s3) were probably the predisposing factors for the death outcome observed in this patient.

Many investigations showed that pathogenic microorganisms are associated with the isolation and characterization of strains, while there is not enough information about the effects of the isolated strains on the activity of host cells (for example, macrophages) and their mutual interaction. Even though the pathogenic microorganisms interact with many host cell types, the interaction with a single cell type that can be achieved in *in vitro* conditions allows the observation

of specific interactions among them [28]. In our study the effect of *Candida* isolates on the activity of cells of the innate immunity and their viability was determined. In addition to this, virulence factors, such as hyphal transition, biofilm formation and hemolytic activity were investigated (Table 2). Macrophage viability estimated by the MTT assay revealed that some strains were more capable in inducing macrophage death than others. Phagocytosis and the killing of pathogens represent an early mechanism of host defense. Although the sensitivity to the attack of macrophage is well correlated with the reduced virulence *in vivo*, a resistance to macrophages does not ensure virulence in an *in vivo* infection model [28].

When the pathogen is ingested, the activity of MPO raises and increases the production of reactive oxygen, nitrogen and chlorine species [29]. This enzyme is also released during phagocytosis into the extracellular environment where it carries out both its cytotoxic and immunoregulatory (on macrophages) activity [30]. MPO activity seems to be important in strain grouping, since the MVA treatment of data from all groups of patient characteristics (Tables s2-3 and 3) and the virulence characteristics segregated one group that consisted of only one strain, No. 4 (Figure s1). The increase in enzyme activity by this strain was the highest among the isolates (Table 2), but when considering other virulence characteristics this strain could be classified as a moderate-to-low virulent strain (Table 2).

One of the most important virulence factors of *C. albicans*, that enables tissue penetration and invasion, is the ability to change cellular morphology from yeast to hypha, the so-called dimorphic transition [21]. The dimorphic transition can develop intracellularly, although slowly, causing the rupture of vacuolar membrane allowing the phagocytosed pathogen to escape [29]. Almost no correlation (0.042) was found between the hyphal transition and phagocytic activity but since our study included a small number of cases, this should be taken with caution (Table s5). Phagocytosis was found to positively correlate with antibiotics usage and previous infections existence in CBSI patients, suggesting that the isolated strains might be more prone to phagocytosis due to an effect of antibiotics on them. Another virulence characteristic of *Candida* strains that could be taken as a possible risk factor is their ability to produce hemolysins (hemolytic activity). The activity of this enzyme is developed in microorganisms that acquire iron for their survival and the ability to establish infection within the mammalian host. Up to date, there is no data reporting production of hemolysin by *C.*

lipolytica and this is the first recorded report on this subject. The summarized data on the virulence characteristics of *Candida* strain showed that the strains responsible for CBSI displayed different virulence factors.

There are doubts when considering the type, dose and duration of antifungal therapy applied in the treatment of CBSI. Over the last five years susceptibility of *Candida* isolates to fluconazole has been reduced (less than 80%), i.e. there is an emerging resistance to standard antifungals [2,27]. In our study some of the isolated strains were resistant (Table 1) to the effect of triazole group of systemic antimycotics, such as fluconazole, itraconazole, voriconazole and posaconazole. CBSI caused by *Candida* spp., that are frequently associated with biofilm formation on various medical devices such as catheters (venous and urinary), are known to be refractory to commonly applied antifungal agents (such as fluconazole) but *in vitro* analyses revealed that AmB and echinocandins were efficient against these strains [6]. Our PCA revealed that biofilm production had a negative and a positive correlation with MIC values of azole derivatives and amphotericin B, respectively (Table s7). This finding is in accordance with the up to now suggested emergence of resistance towards azole derivatives. Resistance occurrence is still not clear and it can be the consequence of several processes such as appearance of resistant variants from a common genotype, selection of strains from mixed population and reinfection with a resistant subtype [15].

Conclusion

CBSI are severe diseases associated with high morbidity and mortality. Evaluation of local *Candida* epidemiology, antifungal susceptibility and virulence factors, as well as personalized patient risk-factors are important for surveillance of CBSI, especially in ICU patients and may contribute to the improved options and outcome for patients with CBSI.

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