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The Prognostic and Diagnostic Markers of Invasive Candidiasis in Patients During Chemotherapy

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Abstract

The aim of the work was the early detection of *Candida* spp. in clinical samples of patients with carcinoma ovariorum undergoing chemotherapy by comparing three indicators of candidiasis: presence of mannan and yeast DNA in the bloodstream and colonization of mucosal membranes by *Candida* species as a prognostic marker of deep candidiasis. Thirty-one women with carcinoma ovariorum, during chemotherapy without symptoms of deep fungal infections, were examined twice over a six-day period. *C. albicans* was the dominant organism isolated from mucosal membranes. Two serum samples were positive for mannan on the first day of examination. All these patients were previously colonized by *Candida* spp. on mucous membranes. Four patients were positive on the last day of examination. Three of these patients were colonized by *Candida* spp. *C. albicans* infection was detected early in 4 out of 12 clinical samples by a combination of PCR and mannan-detecting methods. Colonization increases the risk of deep candidiasis. PCR and antigen detection are fast and reliable methods for early detection of *Candida* in bloodstream. For patients at risk, the clinical samples must be tested by at least two independent methods.

Key words: cancer, candidiasis, chemotherapy

Introduction

Severe fungal infections, especially candidiasis, represent a serious medical problem in immunocompromised patients during anticancer chemotherapy. The mortality rates among patients of risk for *Candida* infection remain high, ranging from 50–80%, despite the adequate treatment (Buchman *et al.*, 1990; Flahaut *et al.*, 1998; Pagno *et al.*, 1999; Richardson and Kokki, 1999).

Fungal infections appear mainly in people with dysfunctions of the immune system. In these patients the opportunistic and endogenous *Candida* strains that colonize superficial sites or non-sterile body fluids are the major etiologic agents of life-threatening infections. The factors predisposing to the systemic fungal infections are immunosuppresion, disturbance of residual bacterial flora by antibiotics, extensive surgical procedures, and AIDS. Systemic fungal infections are a great problem in patients receiving cancer therapy. In these patients profound neutropenia following chemotherapy is a serious risk factor for opportunistic fungal infections associated with high mortality risk (Holmes *et al.*, 1994; Füsle, 1997; Rüchel, 1997; Pfaller, 1995; Murray *et al.*, 1999). Disseminated candidiasis disturbs established schemes for chemotherapy and prolonging pause between courses reduces chances of successful treatment by about 20–30%. Thus, in patients of risk, the empiric antifungal therapy is usually recommended. On the other hand, this therapy may be unnecessarily toxic and costly, and it may play a part in spreading resistance among *Candida* species (Buchman *et al.*, 1990; Elie *et al.*, 1998; Shin *et al.*, 1997). High mortality rates in neutropenic patients treated for cancer may be reduced only by earliest possible diagnosis of systemic candidiasis with appropriate and effective treatment. Current methods for detection of candidiasis in clinical settings are poor at best.

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Too much time passes when employing microbiological cultures, while serological tests for antibody detection in immunocompromised patients are useless in clinical settings (Buchman *et al.*, 1990; Flahaut *et al.*, 1998; Richardson and Kokki, 1991; Shin *et al.*, 1999). The standard laboratory diagnostic approach to systemic candidiasis is based on fungal growth in culture, and is often delayed due to the relatively slow growth of yeasts and inadequate sensitivity of the method in early recognition of infection. Thus, a lot of effort is put into developing rapid and more sensitive diagnostic methods of systemic fungal infections and identifying the virulence determinants of isolated strains.

The invasive fungal infection occurs as a result of an increase in the biomass of the pathogenic fungi. The infection increases in aggressiveness as it spreads from an initial site of colonization, ultimately penetrating the host's protective barriers and damaging tissues. In the processes within bloodstream, there is an increase in fungal specific products that can potentially be utilised as markers of systemic infection. In this regard, techniques have been developed to detect yeast's proteins, metabolites, DNA and polysaccharides (Richardson and Kokki, 1999; Sendid *et al.*, 1999). The tests for the detection of antigens in sera-glucan, a major polysaccharide of the cell wall and the highly immunogenic mannan, of the *Candida* are commercially available for few years. Early detection of systemic fungal infection by these methods, or by PCR assays, should improve the survival time in patients at risk. The crucial step in these patients is the timely start of therapy with antimycotic drugs before presentation of clinical symptoms.

The aim of our work was the early detection of *Candida* spp. in clinical samples of patients with carcinoma ovariorum undergoing chemotherapy by comparing three indicators of candidiasis: 1) presence of mannan in the bloodstream; 2) presence of yeast DNA in the bloodstream; and 3) colonization of mucosal membranes by *Candida* species as a prognostic marker of deep candidiasis.

Experimental

Materials and Methods

Patients and clinical data. Thirty-one patients aged 34–54 years (mean 42,6 years) with confirmed carcinoma ovariorum were included in our study. The patients were qualified for chemotherapy and treated with the aggressive anti-cancer drug Taxol, and cisplatin according to Polish standards for carcinoma ovariorum treatment. Presently, Taxol and cisplatin are considered the most effective treatment for carcinoma ovariorum, with haematological toxicity from level II to IV (WHO scale). The treatment, comprising six courses, involved standard doses of Taxol and cisplatin at intervals of 21 days. The study was performed after 3, 4 course of chemotherapy.

Clinical samples and strains identification. In order to evaluate the colonization of mucosal membranes by *Candida* species, nasal, pharyngeal and vaginal swabs from 31 cancer patients were plated onto Sabouraud agar and CHROMagar Candida. The swabs were collected twice over a six-day period. The plated swabs were incubated at 35°C for 48–72 h and examined daily for evidence of growth. When a sample was found to be positive for *Candida*, the strain was identified. Isolates from CHROMagar Candida were identified according to the manufacturer's instructions. Isolates from Sabouraud agar were first identified microscopically by Gram staining to assess the cell morphology and then the germ tube test was performed. Isolates were also identified on the basis of biochemical features using the ID 32C (bioMérieux) test.

Detection of soluble antigen of *Candida* in serum. Sera for the serological tests comprised 62 samples from 31 cancer patients and 21 samples from 21 healthy donors.

Two procedures were used to detect the soluble antigen of *Candida* in serum. The first method was the immunoenzymatic test (ELISA) for detection of mannan in serum (Platelia Candida; BioRad). Before starting the test all tested sera and standards were heat-treated in the presence of EDTA to dissociate the immune complexes and to precipitate the serum proteins that may interfere with the test. The supernatants were tested after a final, clarifying centrifugation. Microplate wells were coated with monoclonal antibodies directed against β 1–5 oligomannosides of *Candida*. 50 µl of conjugate (peroxidase-labelled anti-mannan MAb) were simultaneously incubated with 50 µl of supernatant. After incubation, the wells were washed and the reaction was revealed by incubation in the dark with 200 µl chromogenic substrate. The enzymatic reaction was stopped by adding 1,5N H_2SO_4. The optical density was determined at 450 nm. The limit of detection of the assay is 0,25 ng of mannan per ml of serum tested. All reactions were performed in duplicate.

The second method was based on latex agglutination, using commercial test Pastorex Candida (BioRad). Briefly: 100 µl of treatment reagent was added to 300 µl of serum and placed on heat at 100°C for 3 minutes. After centrifugation at 10 000 g for 10 min., the supernatant was tested. 40 µl of supernatant fluid was deposited on the card, 10 µl of Candida latex was added and the card was shaked for 10 min. (160 rev./min.). A positive reaction was indicated by the agglutination of the latex particles.

PCR detection of *Candia albicans* DNA in human whole blood samples. Isolation of genomic DNA: Clinical specimens (n = 62) examined for yeasts' DNA presence were obtained from 31 cancer patients and included the whole blood samples. All samples were collected twice in a period of one week. In order to remove the erythrocytes from the blood specimens, 100 µl of the whole blood was added to 1 ml of sterile distilled water. Each sample was incubated on a shaker for 5 min. at RT. The lysate was centrifuged at 3,000 × g for 10 min. and the pellet was resuspended in 2 ml of sterile distilled water, vortexed and centrifuged again at 3,000 × g for 10 min. The erythrocyte-free pellet was washed in 2 ml of 20 mM Tris-HCl (pH 8.3), centrifuged for 10 min. at 3,000 × g, resuspended in 100 µl of RE lysis buffer (A&A Biotechnology) to which 10 µl of yeast lysis enzyme (ICN) was added.

The mixture was incubated for 1h at 37°C. After this step, 200 µl of universal lysis buffer LT (A&A Biotechnology) and 20 µl of protease K (1.7 mg/ml; Qiagen) were added and the mixture was incubated 20 min. at 37°C and then the sample was heated at 75°C for 5 min. The sample was centrifuged at 10,000 rpm for 3min. and supernatant was applied to QiAmp spin column (Qiagen), centrifuged at 10,000 rmp for 1 min. and washed twice with 500 µl of washing buffer A1 (A&A Biotechnology). DNA was eluted with 100 µl of eluting buffer RE (10 mM TRIS, pH 8.5; A&A Biotechnology) preheated to 75°C. The sample was incubated at RT for 5 min. and centrifuged at 10,000 rpm for 1 min. The purified DNA preparation was kept at –20°C until PCR.

Primers and PCR amplification: Two oligonucleotides derived from *Candida albicans* rRNA fragments were used as the outer primers. The sequences of these oligonucleotides are the following:

camt1 5'-CACCCGATCCGCCTCCTACCGAAG-3'

camt2 5'cGTCTGCCCGATCCGTACCTCCGT-3'.

These primers amplify a 1039-bp fragment in the rRNA genes of *C. albicans.* The amplification was performed in a 50 µl reaction mixture containing: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 100 µl (each) deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP; A&A Biotechnology) and 1.25 U of Delta2 DNA polymerase (A&A Biotechnology) and 10 pmol (each) primer. A 2.5 µl aliquot of the extracted DNA was added to the mixture. PCR was performed in a thermocycler (Peqlab, Biotechnologie GmbH) as follows. The reaction was initiated by incubation at 95°C for 5 min. Then, the first cycle included 30 sec. of denaturation at 94°C, 1 min. of annealing at 64°C and 1 min. of elongation at 72°C. This first step was followed by 30 cycles. The PCR product was then analysed on a 2% agarose gel stained with ethidium bromide. The positive samples were tested by nested-PCR.

For nested-PCR, 1 µl of the PCR product from the first amplification and 10 pmol of *Candida albicans*-specific inner primers were mixed in a fresh reaction mixture. The sequences of these oligonucleotides are: camt3 5'-ATATATTAgTCTCCACCCGA-3' camt4 5'-atagAGTATAACCACCCGAT-3'. These generated PCR product of 317-bp of *Candida albicans*. The second amplification was performed in similar conditions with the exception of annealing temperature (47°C for 1 min.) and elongation temperature (72°C for 30 sec.). The amplified product was analysed on a 2% agarose gel stained with ethidium bromide.

Each reaction was carried out in duplicate. To avoid sample contamination, precautions suggested by Kwok and Higuchi (Kwok and Higuchi, 1989) were used. Cross-contamination by aerosols was reduced by physical separation of laboratory rooms used for reagent preparation, sample processing, and DNA amplification. Other precautions included UV irradiation for microcentrifuge tubes, racks, surfaces of laboratory benches, and instruments. Such laboratory procedures as autoclaving of buffers and distilled water, use of fresh lots of previously aliquoted reagents, combined use of positive-displacement pipetters and aerosol-resistant pipette tips, frequent changing of gloves, premixing reagents, addition of DNA as the last step, and testing negative controls, including omission of either the primer or the DNA template during PCR, were used. Appropriate negative controls were included in each test run, including controls omitting either the primer or the DNA template during PCR assay. In all experiments the negative controls always tested negative. Positive standard for PCR used 1ml of purified DNA for *Candida albicans*.

Results

Routine culture of the swabs from mucosal membranes on Sabouraud agar demonstrated, that 4 patients (13%) had *Candida spp*. on at least two sites (Pfaller, 1995; Yeo and Wong, 2002). *Candida* strains colonized pharynx (4 cases) and vagina (3 cases) most frequently. Nose swabs were positive in 2 cases. Moreover*, Candida* spp. strains were present in 6 other patients at only one site.

Of all *Candida* species isolated from patients, 10 were identified as *C. albicans.* These strains were isolated from 6 patients. 5 strains of *C. glabrata* were isolated from 4 patients. One strain of *C. kruzei* was isolated from 1 patient (Table I).

Table I
The positive culture results on the first day of examination

No of occupied sites	Patients	Site of *Candida* isolation		
		vagina	nose	pharynx
3or 2 sites	4	*C. albicans*	*C. albicans*	*C. albicans*
	1	*C. albicans*	–	*C. albicans*
	3	*C. glabrata*	–	*C. glabrata*
	6	–	*C. albicans*	*C. albicans*
1 site	2	–	*C. glabrata*	–
	5	–	*C. albicans*	–
	7	–	–	*C. glabrata*
	8	*C. albicans*	–	–
	9	–	*C. albicans*	–
	10	*C. glabrata*	–	–
	11	–	*C. kruzei*	–
none	12–31	–	–	–

Table II
Comparative identification of *Candida spp.* by routine phenotypic identification methods versus antigen detection and PCR/nested PCR method

No of occupied sites	Patients' no	First examination				Second examination		
		Routine phenotypic identification	antigen conc. by ELISA (ng/ml)	latex agglutin.	PCR/nested PCR	antigen conc. by ELISA (ng/ml)	latex agglutin.	PCR/nested PCR
3 or 2 sites	4	*C. albicans*	< 0.25	–	+/+	1	–	+/+
	1	*C. albicans*	1.5	+	+/+	1.5	+	+/+
	3	*C. glabrata*	>1	+/–	–	1	–	–
	6	*C. albicans*	0	–	–	0	–	–
1 site	2	*C. glabrata*	< 0.25	–	–	1	–	–
	5	*C. albicans*	< 0.25	–	–	< 0.25	–	–
	7	*C. glabrata*	0	–	–	0	–	–
	8	*C. albicans*	0	–	–	0	–	–
	9	*C. albicans*	0	–	–	< 0.25	–	–
	10	*C. glabrata*	0	–	–	0	–	–
	11	*C. kruzei*	0	–	–	0	–	–
None	12–31	None	0	–	–	0	–	–

Thirty-one patients were classified into 3 groups according to the number of sites occupied by *Candida* spp. The first group (patients 4, 1, 3 and 6; 12.9%) comprised patients colonized by yeasts on two or three sites. The second group (patients 2, 5, 7, 8, 9, 10 and 11; 22.5%) corresponds to patients colonized at only one site by *Candida* spp. The third group (patients 12–31; 70.9%) comprised patients with no signs of *Candida* present on mucosal membranes (Table I). On the sixth day of examination we did not observe any other cases of yeast's colonization on mucosal membranes.

Each ELISA experiment for circulating mannan detection in sera was performed in duplicate. The repeatability of the optical density (OD) values on a single microtiter plate corresponded to a coefficient of variation of < 10%. The sensitivity of detection was 0.25 ng of mannan per ml. In this study we considered > 0.25 ng/ml to be the cutoff level since 100% of the healthy controls had mannanemia values that were equall or less than this value.

The results obtained for each of the 31 individual patients are summarized in Table II. Table II compares the methods used to identify markers of candidiasis: routine culture indicating mucous membranes colonization, circulating mannan detection and detection of *C. albicans* DNA as markers of disseminated candidiasis. All patients have been classified into 3 groups according to the number of sites occupied by *Candida* spp. The patients' classification was based on the mucosal membranes occupation by *Candida* spp., because colonization is regarded as one of the prognostic markers of deep candidiasis.

In patients no 1 and 3 from the first group, a high mannan concentration was detected in the serum samples early (on the first day of examination). Mannnan concentration was above 1 ng/ml in both patients, while the latex agglutination method gave positive (no 1) and questionable (no 3) results. One of these patients (no 1) was colonized by *C. albicans*. For this patient PCR identification confirmed disseminated candidiasis caused by endogenous species. The phenotypic identification of the strain that colonized the mucosal membranes (*C. albicans*) matched the PCR reaction.

Nucleic acid of *C. albicans* was also detected early by the PCR technique in the blood sample of patient no 4 six days before the appearance of mannan in the patient's serum. It is worthy to note that in this case *C. albicans* colonized three anatomical sites. In the case of patient no 6, who was colonized by *C. albicans* on two sites, no other markers of candidiasis were detected on the first or the second day of examination.

In the second group of patients it was not possible to detect antigenemia or *C. albicans* DNA in blood samples on the first day of examination. We observed one case of positive case of mannanemia six days later (patient no 2).

In patients from the third group, we did not observe signs of *Candida* present on mucosal membranes or other markers of candidiasis in serum or blood samples.

Disseminated *C. albicans* infection was detected early in 4 out of 12 (33%) clinical samples, by a combination of PCR and mannan-detecting ELISA methods. Moreover, the PCR method allowed us to detect 4 positive cases of deep *C. albicans* infections, whereas positive mannanemias were identified in 3 cases

Table III
Results of mannan detection versus *Candida albicans* DNA detection in clinical samples

Candida spp./no of patients	No of clinical samples*	No of clinical samples positive for the following:			
		Mannanemia of ≥ 1 ng/ml		PCR identification	
		I examin.	II examin.	I examin.	II examin.
C. albicans/6	12	1 (8%)	2 (16%)	2 (16%)	2 (16%)
C. glabrata/4	8	1 (12.5%)	2 (25%)	0 (0%)	0 (0%)
C. kruzei/1	2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
0/ 20	40	0 (0%)	0 (0%)	0 (0%)	0 (0%)

* the clinical samples from each patient were collected twice

(Table III). All patients were at the higher risk of the candidiasis as the routine culture indicated fungal colonization of the mucosal membranes. In four patients with *C. glabrata* present on mucosal membranes, an increase in mannan concentration was observed in 2 clinical samples. PCR method did not identify DNA of *C. albicans* in the blood of these subjects.

When microbiological and serological investigations were positive, antifungal treatment at standard dosages was performed.

Discussion

Infections due to *Candida spp*. remain the most frequent complications in cancer patients despite an increase in systemic fungal infections over the last few years. Candidiasis is the most prominent infection with a high mortality rate in patients at risk (Pagno *et al.*, 1999).

For many years *Candida albicans* was regarded as the most frequent etiologic agent involved in almost all cases of candidiasis, but recent reports indicate an increase in candidiasis sustained by non-*albicans* species: mainly *Candida krusei*, *Candida tropicalis* and *Candida glabrata* (Pagno *et al.*, 1999; Shin *et al.*, 1997; Yamamura *et al.*, 1999; Abbas *et al.*, 2000). Our study confirms this finding: *Candida albicans* was the predominant species isolated from mucosal membranes, but other non-*albicans* species were also present.

Along with the appearance of life-threatening *Candida* non-*albicans* strains, the strains resistant to antifungal drugs appeared. It may be a direct result of the widespread use of antifungal agents for the prophylaxis and treatment of candidiasis (Pfaller, 1995; Yamamura *et al.*, 1999; Morgenstern *et al.*, 1999). In many oncological hospitals over the world, antimycotic prophylaxis is routinely prescribed and administrated at the start of chemotherapy, also in the absence of positive culture results.

In order to reduce the mortality rate among the group of patients at risk there is a need for rapid, sensitive and specific tests for appropriate antifungal therapy before presentation of clinical symptoms. Nowadays patient evaluation, mycological cultures, diagnostic imaging, and biopsies are the standard clinical and laboratory approaches in diagnosis and therapeutic monitoring of systemic candidiasis. Unfortunately, these methods very often lack sensitivity in the early recognition of infection and are imprecise as markers of complete eradication (Richardson and Kokki, 1999). In the context of these disadvantages, new approaches to early diagnosis of disseminated candidiasis are being developed.

In our work we compared traditional diagnostic methods with novel methods – serological and molecular. The routine culture allowed us to detect mucosal membranes colonization in 4 patients undergoing chemotherapy. Although the importance of colonization is unclear, in most instances precedes fungemia and is regarded as an independent risk factor for systemic fungal infection (Safdar *et al.*, 2001).

The probability of deep candidiasis was evaluated by serological methods detecting circulating mannan in patients' blood. We tested 62 serum samples drawn from cancer patients, with detection of mannan in 6 cases. Subjects with mannanemia also showed signs of *Candida* sp. on mucosal membranes. Mannan antigenemia detection for the immunodiagnosis is now one of the most widely studied antigens in patients with candidiasis. Many investigators (Richardson and Kokki, 1999; Sendid *et al.*, 1999; Yeo and Wong, 2002) suggest that positive mannan results may correlate with invasive candidiasis. Furthermore, studies have also shown a correlation between detectable mannanemia and tissue invasive by *Candida* spp. in

patients' with candidemia (Yeo and Wong, 2002). In our study, we used two tests to detect mannan in serum samples of examined persons.

The latex agglutination test has been widely used as the first commercially available antigen detection test. The latex agglutination test has shown good specificity, but poor sensitivity, due to the rapid clearance of the antigen from patient's sera and a low amount of circulating antigen (Richardson and Kokki, 1999; Füsle, 1997; Sendid *et al.*, 1999).

By using the ELISA method, the detection limit has been improved up to 0,25 ng of mannan per ml. This resulted in an increase in sensitivity, and allowed detection of mannanemia in 13% of patients at risk. However, these results are still disappointing because in 5% of patients with negative antigen detection, the symptoms of disseminated infection were clinically observed (data not shown). This illustrates one of the major limitations of the fungemia detection tests: the transient character of mannan circulation. Several mechanisms have been proposed to account for this observation, including the quick degradation of mannose oligomers by serum mannosidases, the binding of the mannose oligomers to soluble serum proteins (mannose binding protein C3) or membranous receptors of phagocytes (Sendid *et al.*, 1999). As a consequence, sensitivity rises with the number of serum samples available from each patient. The performance of mannan detection in serum varies with the frequency of testing in a given patient. Regular antigen monitoring of high-risk patients is recommended as a means of increasing the sensitivity of the test and advancing the date of the first positive result (Yeo and Wong, 2002).

The doubling time for yeasts is long – an hour or more, compared to 20 min. for bacteria. Furthermore, most clinical specimens are contaminated by bacteria that compete for nutrients and thereby can add to doubling times. For this reason, specimens that are believed to contain yeasts are generally plated out onto a selective medium that contains antibacterial agents. The specific identification of fungus requires 3 to 5 days (Buchman *et al.*, 1990). With the PCR-based amplification procedure, contamination by the bacterial and human cells is avoided and because of the high sensitivity of the method it was possible to detect a small amount of yeast DNA in blood.

In our work, 62 clinical samples from cancer patients undergoing chemotherapy were tested for the presence of *C. albicans* nucleic acid in blood. 33% (4 samples) were PCR positive and 25% (3 samples) were mannan-positive. All patients with disseminated *Candida albicans* fungemia were colonized by the same species. The PCR method appears to be more sensitive than mannan detecting immunoenzymatic method. In our work the difference in the sensitivity of both tests was not significant, but other authors (Buchman *et al.*, 1990; Richardson and Kokki, 1999, Shin *et al.*, 1999; Yeo and Wong, 2002) indicate the higher sensitivity of PCR based methods compared with other non-culture tests.

In the presented work, we did not detect other than *Candida albicans* species by PCR. However, the high specificity of species-specific primers demonstrated by many investigators (Buchman *et al.*, 1990; Holmes *et al.*, 1994; Elie *et al.*, 1998; Shin *et al.*, 1997; Shin *et al.*, 1999) confirm their value in early detection and differentiation of *Candida* species present in clinical materials.

The conclusions from our work are: (i) colonization increases the risk of disseminated candidiasis occurrence and may be used as its prognostic marker; (ii) PCR and mannan detection are fast and reliable methods for early detection of *Candida* in bloodstream; (iii) in order to increase the rate of detection of systemic fungal infections, the clinical samples should be collected at least twice; (iv) for patients at risk, the clinical samples must be tested by at least two independent methods.

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