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NON – CULTURE - BASED METHODS FOR DIAGNOSIS OF INVASIVE CANDIDA INFECTION

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ABSTRACT

The purpose of this study was to detect the abilities of non culture based methods (Enzyme-linked immunosorbent assay and real-time PCR) in the diagnosis of invasive Candida infection. Subjects and Methods : Forty five patients with hematological malignancies who had febrile neutropenia and failed to respond to broad spectrum antibiotic therapy were included in the study, they were subjected to blood cultures using BD BACTEC™ Mycosis- IC/F culture vials in BACTEC brand fluorescent series instrument, then identification of Candida species in positive samples was done by culture on sabaraud's dextrose agar and the isolated colonies were identified morphologically and biochemically. The Candida mannan antigen was detected by ELISA technique and Multiplex, real time PCR was used for detection of DNA of the common five Candida species in blood. Results: The study revealed 4 out of 45 blood cultures were positive for Candida, otherwise 13 out of 45 serum samples were positive for Candida antigen by ELISA technique. On using Multiplex, real time PCR test, 14 out of 45 whole EDTA blood samples were positive for Candida (50% C. albicans, 14.3% C tropicalis, 14.3% C glabrata , 14.3 % C parapsilosis and 7.1 % C krusei). There was very good agreement between Multiplex, real time PCR and Ag detection by ELISA (Kappa=0.94) for the diagnosis of Candidemia among the patients with hematological malignancy, while there was fair agreement between blood culture technique and both Multiplex, real time PCR and Ag detection by ELISA (Kappa=0.35 and 0.38 respectively). As regarding the time consumed to diagnose candidemia , blood culture method took the most time to detect it (up to 15 days) while Multiplex, real time PCR took the least time after extraction procedures (less than 1 hour). Conclusion: Multiplex, real time PCR is superior to conventional methods for the diagnosis of candidemia , being accurate, reproducible, rapid method and it could differentiate Candida species.

INTRODUCTION

Advances in medical intervention provide a growing number of immunocompromised patients who are at risk from opportunistic fungal infection and now it is widely published and accepted that invasive fungal infections are a major cause of mortality and morbidity in the immunocompromised states such as some neutropenic patients with hematological malignancies, chemotherapy, organ transplantation, AIDS and other disease that can affect the immune system[10].

Candida and Aspergillus species account for the vast majority of fungal infections, but other less commonly recognized fungi can cause life-threatening infection in these hosts as well [31]. Candida species are the fourth most commonly encountered hospital acquired pathogens in blood stream infections and it is associated with mortality rates as high as 60%. So early initiation of antifungal therapy is paramount in reducing the high mortality rates associated with fungaemia and this is dependent on early detection of fungal infection[25].

The current "gold standard" for detection of systemic infection is blood culture, but this is believed to lack sensitivity and has been shown to be positive in less than 50% of patient with chronic disseminated candidiasis, also it is time-consuming taking up to 3 weeks, and this is an unacceptable period for the treatment of fungaemia [11]. This lack of reliable early diagnosis may lead to the unnecessary empirical treatment of patients who do not have fungal infection. The end result is unacceptable toxicity in many patients, massive expense and resistance to commonly used antifungal agents [20].

The limitations associated with classic culture techniques for the diagnosis of invasive fungal infections have lead to the emergence of many non-culture-based methods, with superior sensitivities and quicker turn around times [37]. Methods have been developed for the detection of both circulating antibodies and antigens, but

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the usefulness of antibody detection may be limited when the patients under investigation are immunosuppressed and /or heavily colonized but uninfected [18].

Among the various antigens present in the blood of patients suffering from invasive fungal infection, carbohydrates, particularly cell wall components; are favored for the diagnosis of the infection. For the detection of Candida, the polysaccharide mannan is a major marker, as it contributes over 7% of the dry weight of the yeast C. albicans. It is non covalently bound to the cell wall and is extremely immunogenic [10].

The use of PCR in the detection of systemic fungal infection has been extensively published and provides potential in terms of sensitivity and specificity. A variety of post amplification methods have been used to exploit the variable regions within the rRNA amplicons and identify the genus or species causing infection. The major drawback with these techniques is that they require post amplification handling and so increase the time to result and more importantly, the chance of contamination [37].

Real-time PCR greatly simplifies amplicon recognition by providing the means to monitor the accumulation of specific products continuously during cycling . This removes the need for post- amplification handling, reducing both turnaround time and the potential for contamination, and provides a species/ genus level of identification[11].

The aim of this study was to detect the abilities of non culture based methods (Enzyme-linked immunosorbent assay and real-time PCR) in the diagnosis of invasive Candida infection.

SUBJECTS AND METHODS

This study was conducted at Clinical Pathology Department of Banha University Hospital and Clinical Hematology Unit, Ain-Shams University Hospitals on forty five patients with hematological malignancies who had febrile neutropenia (< 1000 neutrophile/ mL with a temperature of > 38 °C) that had failed to respond to broad spectrum antibiotic therapy after 2 days of treatment during the period from April to October 2006. They were 28 males and 17 females, their age ranged from 15 to 74 years with mean age of 43.3 ± 11.2 years.

Ten ml of blood was collected by a venipuncture needle under complete aseptic conditions and was used as follows: Two ml of blood was put in a vacutte tube containing EDTA for doing real-time PCR, two ml of blood was left to clot then the serum was stored in a sterile plastic epindorf tubes at- 20 °C till the time of ELISA Candida antigen detection and six ml of blood was used for inoculating BD BACTECTM Mycosis –IC/F blood culture vials.

Blood Culture: Using BD BACTEC™ Mycosis- IC/F culture vials (Becton, Dickinson and Company, Sparks, Maryland 21152 USA) which are selective media for yeast and fungi; and they are designed for use with BACTEC brand fluorescent series instruments. If yeasts and fungi are present in the test sample inoculated into the BACTEC vial, CO_2 will be produced when the micro-organisms metabolize the substrate present in the vial. Increase in the fluorescence of the vial sensor is monitored by the BACTEC fluorescent series instrument. Analysis of the rate and the amount of CO_2 increase enables the instrument to determine if the vial is postitive, Identification of Candida species was done by culture on sabaraud's dextrose agar (oxoid) and the isolated colonies were identified morphologicaly by Gram stain and Germ tube test and biochemically by Auxacolor ™ 2 test; (Bio - Rad Sanofi pasture, FRANCE).

Detection of Candida mannan antigen in serum by Enzyme linked immunosorbent assay (Platelia Candida Ag test - Bio-Rad Sanofi pasture, FRANCE)

It is a one- stage, sandwich, microplate immunoenzymatic assay that allows detection of mannan in human serum. It uses the monoclonal rat EBCA-1 antibody directed against the β 1-5 oligomannosides of Candida [32]. By using the quantitative mode of this method, 4 range points were used (0.25, 0.50, 1.0 and 2.0 ng/ml) to establish a standard curve, from which the concentration of the Ag was obtained. Serum samples with a concentration less than 0.25 ng/ml were considered "negative". Serum samples with a concentration between 0.25 and 0.5 ng/ml were

considered "intermediate" for the presence of mannan antigen, while serum samples with a concentration greater than or equal to 0.5 ng/ml were considered "positive."

Multiplex, real- time PCR :

Multiplex,real-time PCR was used for detection and identification of the common five species of Candida in blood which are (Candida albicans, Candida tropicalis, Candida glabrata, Candida krusei and Candida parapsilosis).This was done by LightCycler 1.5 (Roche) system using LightCycler- DNA Master SYBR Green I PCR Kit (Roche Diagnostics), which is a ready-to – use reaction mix, containing the SYBR Green I dye as detection format. SYBR Green I is a DNA double- strand specific dye, and its fluorescence is greatly enhanced by its binding to dsDNA. During each phase of DNA synthesis, the SYBR Green I dye that is already included in the reaction mix, binds to the amplified PCR products, and the amplicon can be detected by its fluorescence [35].

Candida DNA isolation was done using Puregene DNA purification Kit (Gentra . Minneapolis , Minnesota 55441 USA) according to the manufacturer's instructions.

Primers design: The primers targeted the sequences between 18S and 28S rDNA is distinct for each Candida species.

Candida albicans

CALB1 TTTATCAACTTGTCACACCAGA

CALB2 ATCCCGCCTTACCACTACCG

Candida glabrata

CGL1 TTATCACACGACTCGACACT ,

CGL2 CCCACATACTGATATGGCCTACAA

Candida krusei

CKRU1 GCATCGATGAAGAACGCAGC

CKRU2AAAAGTCTAGTTCGCTCGGGCC

Candida parapsilosis

CPA1 GCCAGAGATTAAACTCAACCAA

CPA2 CCTATCCATTAGTTTATACTCCGC

Candida tropicalis

CTR1 CAATCCTACCGCCAGAGGTTAT

CTR2 TGGCCACTAGCAAAATAAGCGT

Then the described protocol consists of four programmes:

Program 1: Denaturation of the template DNA

Program 2: Amplification of the target DNA

Program 3: Melting curve analysis for product identification.

Program 4: Cooling the rotor and thermal chamber and subsequent setting of the fluorescence parameters.

Melting curve analysis : Melting curve analysis was used to check the specificity of an amplified product. Each Candida species in this study has its melting temperature which was specific for the sequences between 18S and 28S rDNA . The melting temperatures were 85.71 ± 0.13 for Candida albicans, 84.02 ± 0.14 for Candida glabrata, 90.32 ± 0.09 for Candida krusei, 84.43 ± 0.16 for Candida parapsilosis and 84.03 ± 0.13 for Candida tropicalis

RESULTS

In the present study only 4 patients out of the 45 enrolled were positive for Candida by blood culture technique with percentage of 8.9%. Two of these 4 Candida isolates were Candida albicans, both were isolated from patients with AML, one was Candida tropicalis and was isolated from patient with ALL and the last one was Candida krusei and it was isolated from patient with Lymphoma.

Our study found that 13 patients (28.9%) out of the 45 enrolled were positive by Platelia™ Candida Ag detection test. Candida antigen could be detected in all the blood culture positive cases (100%) and in 9 of the blood culture negative cases (21.9%).

Multiplex,real time PCR was successful in detecting Candida in all positive blood culture cases (100%) and in 10 cases out of 41 negative blood culture patients (24.4%).

It was found that C.albicans represented the most commonly isolated Candida species 50% (7 out of 14) followed by C.tropicalis,

C.glabrata,C.parapsilosis with equal percentages of 14.3% (2 of 14) for each one of them and lastly came C.krusei with the lowest percentage 7.1% (1 out of 14).

In the diagnosis of Candidemia there was fair agreement between the results of blood culture and Ag detection methods (kappa= 0.38), and between the results of blood culture and multiplex, real-time PCR (kappa= 0.35), however there is very good agreement between the results of Ag detection test and multiplex, real-time PCR (kappa= 0.94).

The diagnostic validity test was done for Ag detection test and blood culture method considering Multiplex,real-time PCR as the gold standard method. The specificity of blood culture method and Ag detection test was 100%, while the sensitivity of Ag detection test was 92.9% and that of blood culture was 28.6% as it failed in identification of 10 cases detected positive by Multiplex, real-time PCR.

Regarding the time taken to diagnose Candidemia, it was found that blood culture method took the most time (3-15 day) ,while Multiplex,real-time PCR took the least time (60 minutes) after extraction procedures.

Table (1): Results of different methods used for diagnosis of Candidemia.

No of examined cases	Positive cases detected by different methods					
	Blood culture		Ag detection		Multiplex, Real-ime PCR	
45 (100%)	No	%	No	%	No	%
	4	8.9%	13	28.9%	14	31.1%

Table (2) : Agreement between blood culture technique, Ag detection test and multiplex, real-time PCR for diagnosis of Candidemia.

Test method	Blood culture versus Ag detection test.	Blood culture versus multiplex, real-time PCR.	Ag detection versus multiplex, real-time PCR
Agreement	30.8%	28.6%	92.9%
Kappa	0.38	0.35	0.94

value of K	Strength of agreement
0.2	poor
o.2-0.4	fair
0.41-0.6	moderate
0.61-0.8	good
0.81-1.00	very good

Table (3): Specificity, Sensitivity, PPV, NPV, False+ve and False-ve results of Ag detection test and blood culture methods for diagnosis of Candidemia taking Multiplex,real-time PCR as gold standard.

Method	Specificity	Sensitivity	PPV	NPV	False+ ve	False - ve
Ag detection	100%	92.9%	100%	96.9%	0	1
Blood culture	100%	28.6%	100%	75.6%	0	10

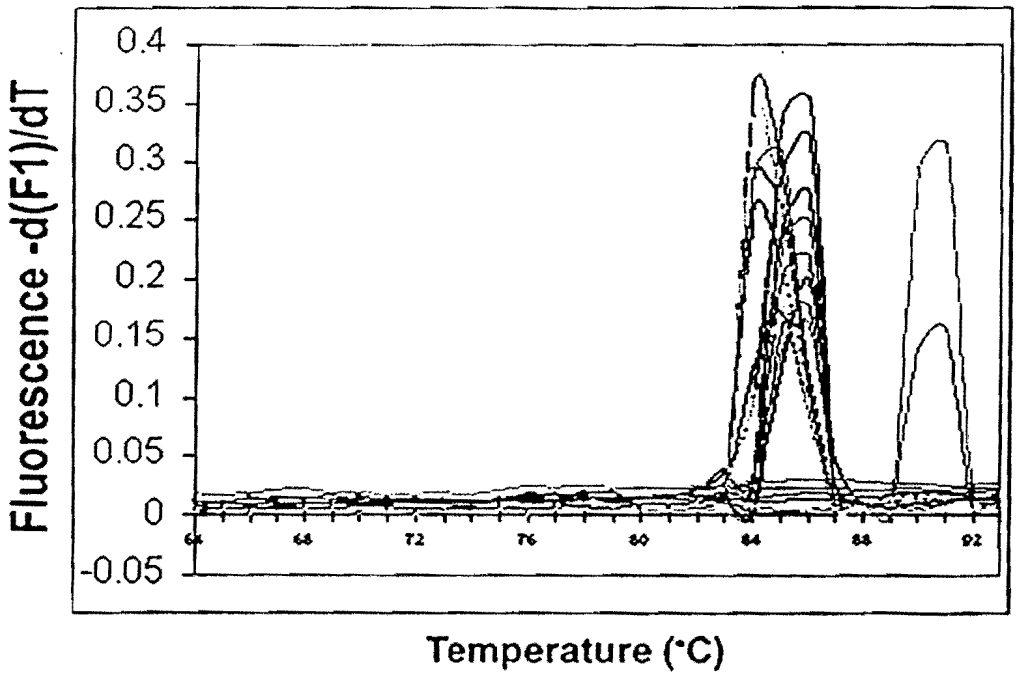

Fig. (1) : Melting temperature curves for different Candida species

DISCUSSION

The incidence of invasive fungal infections has increased dramatically in recent decades, especially among immunocompromised patients. However, the diagnosis of these infections in a timely fashion is often very difficult. Conventional microbiologic and histopathologic approaches generally are neither sensitive nor specific, and they often do not detect invasive fungal infection until late in the course of the disease [40].

Candidiasis frequently occur in patients with hematological malignancies , who experience periods of prolonged granulocytopenia caused by intensive cytostatic chemotherapy . In these patients, colonization of mucosal surfaces by endogenous Candida species is often followed by invasion of the vascular space which carries a high risk of disseminated Candidiasis . For this reason most neutropenic patients with fever that fails to respond to single or multiple broad-spectrum antibiotic therapy are empirically treated with amphotericin B (AmB) which has many adverse side effects [3].

Alternatively, azoles which are fungistatic agents have been developed, for example: Fluconazole, which is one of azoles antifungals has a low level of toxicity and it is as effective as amphotericin B for the treatment of candidemia, however C. glabrata and C. krusei are innately more resistant to fluconazole. The prophylactic use of azole antifungal drugs has led to the emergence of an increasing number of azole-resistance fungal strains[9]. As more and more alternative antifungal agents with various spectra of activities are developed and become available , treatment according to accurate diagnosis has become even more important . Therefore , rapid species identification will be more critical for effective disease therapy and control[28]. It could improve the survival of patients with hematological malignancy by allowing the initiation of specific antifungal treatment while the fungal biomass is still low[27].

This study was designed to evaluate the abilities of non-culture based methods (Enzyme linked immunosorbent assay for detection of Candida antigen mannan and Multiplex,real-time PCR for detection and identification of the five common species of Candida in blood) in the diagnosis of invasive candidiasis in patients with hematological malignancy and to compare their results with those obtained by conventional methods in order to devise a rapid & accurate method for detection and identification of Candida species.

Blood cultures are the current gold standard