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Evaluation of semi-nested polymerase chain reaction (PCR) and mannan antigen detection compared to blood culture for diagnosis of candidemia

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Invasive *Candida* infections have emerged as an important pathogen in the last decade, especially in immunocompromized patients. The aim of the present study was to evaluate the detection of *Candida* species in blood samples from pediatric patients with sepsis by blood culture method versus antigen detection method by enzyme linked immunosorbent assay (ELISA) and molecular method by polymerase chain reaction (PCR). This cross-sectional study was carried out on children at Mansoura University Children Hospital (MUCH) with the presence of clinical signs suggesting sepsis with absence of prior antifungal therapy. Laboratory diagnosis included blood culture, mannan antigen detection of *Candida* species by ELISA and semi-nested PCR. *Candida* species were detected by blood culture in 15.6% of the children and was detected by PCR and antigen detection in 20% for each. Candidemia was more frequently detected among patients with central venous lines (38.5%), children with Diabetes Mellitus (DM) (23.1%) and children with frequent blood transfusions (15.4%). However, these risk factors were not statistically significant. Mortality rate among children with candidemia was 42.9%. The sensitivity, specificity, and accuracy of antigen detection method by ELISA compared to culture, were 71.4, 89.5 and 86.7%, respectively. The sensitivity, specificity and accuracy of semi-nested PCR compared to culture were 85.7, 92.1 and 91.1% respectively. It can be concluded from this study that *Candida* species is a frequent pathogen associated with pediatric sepsis. Blood culture though is a reliable laboratory method for its diagnosis may lack sensitivity and requires prolonged time. Semi-nested PCR for detection of candidemia is sensitive, specific and accurate method. Mannan antigen detection by ELISA is rapid and easy; however, it may lacks specificity; it can be used as a preliminary method for screening. Further studies are recommended to detect the appropriate laboratory algorithm for early diagnosis of candidemia to start antifungal therapy appropriately.

Key words: *Candida*, Mannan antigen, polymerase chain reaction (PCR), blood culture.

INTRODUCTION

Invasive *Candida* infections have emerged as an important pathogen in the last decade especially in immune-compromised patients. There are several factors associated with such increase like the use of broad

spectrum antibiotics, increase use of invasive devices like intravascular catheter and parenteral alimentation (Moran et al., 2009; Watson et al., 2003; Pappas et al., 2003).

Among susceptible patients for infection with *Candida* species are pediatric patients in intensive care units either neonatal (NICU) or pediatric intensive care unit (PICU) (Watson et al., 2003; Pappas et al., 2003). Previous study had demonstrated that infection rate with *Candida* species was increased around 10 folds among neonates in a five years duration (Dutta and Palazzi, 2011). The mortality rates due to invasive candidiasis are high and range from 20 to 30% (Kuzucu et al., 2008; Neu et al., 2009; Singhi and Deep, 2009) that can be increased up to 50% if there is a delay in appropriate laboratory diagnosis (Roilides et al., 2004; Vendettuoli et al., 2008).

Candida species in pediatric patients are usually associated with sepsis (Watson et al., 2003). However, there is a lack of diagnostic clinical signs and symptoms that indicates that the pathogen is *Candida* unless reported by the laboratory results. There is less practice of empirical antifungal treatment in pediatric patients compared to the adults (Zaoutis et al., 2005; Hegazi et al., 2014). Therefore an appropriate rapid laboratory diagnosis of candidemia children has utmost importance for appropriate antifungal therapy.

Blood culture considered as gold standard for candidemia diagnosis, takes several days for detection even with the use of automated blood culture systems. Moreover, phenotypic identification for species levels takes more time either by the use of chromogenic media or manual API identification system (Odds and Bernaerts, 1994; Latouche et al., 1997).

Another laboratory method for diagnosis of invasive candidiasis is serodiagnosis method. Serodiagnosis depends on the detection of cell wall mannan (mannoprotein); components of *Candida* species that is usually a soluble antigen marker detected transiently in blood during the infection (Yeo and Wong, 2002; Sendid et al., 2003).

Molecular method for direct identification of *Candida* species has proven to be an accurate and rapid method for detection of candidal infections (Roilides et al., 2004; Vendettuoli et al., 2008; Imran and Alshammry, 2016).

Different molecular methods for detection of candidia infection, have been used such as real time PCR (Liguori et al., 2007; Li et al., 2003), Multiplex PCR (Sampath et al., 2017) and nested PCR (Ahmad et al., 2002; Çerikçioglu et al., 2010).

PCRs based on amplifications of 18S and 28S rDNA regions have been used to identify different *Candida* species in clinical specimens, including blood. 18SrRNA gene is a highly conserved region in Fungi and is the primary target transcript used for the detection of *Candida* by PCR (Weerasekera et al., 20013; Makene, 2014).

Thus the aim of the present study was to evaluate the detection of *Candida* in blood samples from pediatric patients with sepsis by blood culture method versus semi-nested PCR and mannan antigen detection method by enzyme linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

This cross-sectional study was carried out in Mansoura University Children hospital, Egypt from March 2016 till March 2017. The study was approved by Mansoura Faculty of Medicine ethical committee. Written approval consent was obtained from the parents of the studied children. The inclusion criteria for children were the presence of clinical signs suggesting sepsis with the absence of prior antifungal therapy. Children with underlying malignancy or neutropenia and receiving antifungal empirical therapy were excluded from the study.

Sample collection

Each child participating in the study was subjected to full medical history taking and clinical examination. Candidemia was defined as positive blood culture for *Candida* species either in one or two blood cultures (Neu et al., 2009; Lagrou et al., 2007). Six milliliters blood samples were obtained from children under complete aseptic techniques, divided as follows; two milliliters sample in plain vacutainer for serum separation for ELISA, two milliliters over EDTA for PCR and two milliliters was inoculated into blood culture Bact/Alert system.

Candida identification

Blood cultures were incubated for 7 days at 37°C and cultured on Sabouraud dextrose agar (SDA) with chloramphenicol and blood agar. *Candida* colonies were identified by colony morphology on SDA, germ tube formation and chlamydospore production. Further identification to species level was performed by API *Candida* (bioMérieux, France) (Ahmad et al., 2002).

Mannan antigen detection by ELISA

Mannan antigen was determined using Platelia *Candida* Antigen kits Platelia™ *Candida* (Bio-Rad, France) following the manufacturer's recommendations, is a one-stage immunoenzymatic sandwich microplate assay for quantitative detection of soluble mannan antigen in human blood. The absorbance (optical density) of samples and calibrator were determined with a spectrophotometer set at 450/620 nm wavelength. Each experiment included a calibration curve, which was made with a pool of normal human serum supplemented with known concentrations of mannan ranging from 0.1 to 2 ng/ml.

For mannanemia levels exceeding 2 ng/ml (above the range of the calibration curve) was further diluted and retested to obtain a precise concentration (Sendid et al., 2003).

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Semi-nested PCR for detection of *Candida*

Two sets of primers were used to increase these sensitivity and specificity of the assay. DNA was extracted from ethylenediamine tetraacetic acid (EDTA) blood using QIAamp DNA blood Mini Kit supplied by QIAGEN companies. Then 10 µl of extracted DNA was subjected to the first round of amplification using ready to go PCR beads which contain all the reagents necessary for PCR reaction except primers and the template. In the first round of amplification the Panfungal primers targeting 18S rRNA gene were used

F: 5` AGGGATGTATTTATTAGATAAAAAATCAA 3`.
R : 5 CGCAGTAGTTAGTCTTCAGTAAATC 3`.

All tubes were transferred to the thermal cycler where they were subjected to initial one cycle of denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 20 s, and final one cycle of extension at 72°C for 7 min. Three microliter of the amplified panfungal products were used in semi-nested PCR for further amplification using the *Candida* genus specific primer by the same method of amplification with the exception of annealing temperature, which was 66°C.

***Candida* genus specific primers**

F: 5´ GGGAGGTAGTGACAATAAATAAC 3´.
R: 5´CGTCCCTATTAATCATTACGAT 3´ (Jaeger et al., 2000)

In every PCR run, a negative control (distilled water) and a positive control (Purified *Candida* DNA) were included.

The final PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide. Generated PCR products of 728 and 402 bp for panfungal and *Candida* species; respectively. Negative control was included in each amplification set consisting of sterile distilled water. Positive control was extracted DNA from *Candida* isolated from clinical sample in the laboratory. The process was performed under entirely sterile conditions for PCR to avoid contamination (Van Burik et al., 1998).

Statistical analysis

The data were analyzed using Statistical Package for Social Science software computer program version 17 (SPSS, Inc., Chicago, IL, USA). Quantitative non parametric data were presented in median and interquartile range (IQR). Mann-Whitney U test was used for comparing quantitative no- parametric data. Chi-square "$\chi 2$" or Fischer's exact tests, as indicated, were used to compare the qualitative data. *P* value less than 0.05 was considered statistically significant

The sensitivity, specificity, positive predictive value, accuracy of antigen and PCR to differentiate between positive and negative *Candida* culture were calculated.

The sensitivity and specificity of antigen (quantitative) to differentiate between positive and negative *Candida* culture was examined at different cutoff points using ROC curve analysis to determine the best cutoff point as well as the diagnostic power of each test.

RESULTS

The present study included 90 children affected with sepsis. They were 51.1% males and 48.9% females. The risk factors for the development of sepsis were presence of central venous line in 34.8%, urinary catheter in10.1%, low birth weight preterm newborn 19.1%, DM type 1 24.7%. The outcome of the studied children ranged from death 24.7% to discharge with complete recovery in 74.2%. The majority of the studied children were on ampicillin+sulbactam (unasyn) (75%) and/or Cefotaxime (65.5%) as empirical antibiotics therapy (Table 1).

Candida species were detected by blood culture in 15.6% of the children and was detected by PCR and antigen detection in 20% for each (Table 1).

Species identification by API *Candida* showed eight isolates of *Candida albicans*, three isolates *of Candida tropicalis* and three isolates of *Candida parapsilosis* (Figure 1).

Candidemia was more frequently detected among patients with central venous lines (38.5%), children with DM type 1(23.1%), low birth weight (15.4%) and children with frequent blood transfusions (15.4%). However, these risk factors were not statistically significant. The mortality rate among children with candidemia was 42.9% (Table 2).

Mannan antigen detection by ELISA was associated with a positive blood culture in 10 patients while it was negative in 4 patients with positive culture. Moreover, antigen detection by ELISA was positive in 8 patients with negative culture. The sensitivity, specificity, and accuracy of antigen detection method by ELISA 71.4, 89.5 and 86.7%, respectively (Tables 3 and 4).

In comparison between detection of *Candida* by blood culture and PCR, PCR was positive in association with culture in 12 patients (66.7%) while it was negative in 2 positive cultures and positive in 6 cases with negative blood culture. The sensitivity, specificity and accuracy of PCR were 85.7, 92.1 and 91.1%, respectively (Tables 5 and 6).

The cutoff value of *Candida* antigen by ELISA was 49.5 pg/ml with sensitivity 88.9% and specificity 54.2% (Figure 2).

DISCUSSION

Invasive infection by *Candida* species is common among critically ill children. Blood culture is the gold standard method for diagnosis of sepsis. Nevertheless, the time between sampling and obtaining the results of culture may be as long as 7 to 10 days, which may be too long for the introduction of effective treatment. Moreover, the sensitivity of this method for the isolation of fungi is low (Badiee et al., 2014; Sherman-Weber et al., 2004). *Candida* species was detected by blood culture in 15.6%. The most common species detected by API were *C. albicans* followed by *C. tropicalis* and *C. parapsilosis*. This is similar to results reported previously by Taira et al. (2014).

The non-*Candida albicans* species isolated from the present study were *C. tropicalis* and *C. parapsilosis,* which were recorded previously to be common fungal species among children (Colombo et al., 2013; Santolaya et al., 2014).

The mortality rate among children with candidemia was

Table 1. Demographic, clinical and microbiological results of the studied patients.

Age (Median -IQR)		0.30	0.10-4.00
Sex	Male	46	51.1%
	Female	44	48.9%
Outcome	Death	22	24.7%
	Discharge without improvement	2	2.2%
	Discharge with improvement	66	74.2%
Risk factors	Central line	31	34.8%
	Urinary catheter	9	10.1%
	Frequent blood transfusion	10	11.2%
	Low birth weight	17	19.1%
	DM	22	24.7%
Culture of *Candida*	P	14	15.6%
	N	76	84.4%
Antigen	P	18	20.0%
	N	72	80.0%
PCR	Yes	18	20.0%
	No	72	80.0%
Unasyn	Yes	66	75.0%
	No	22	25.0%
Cefotaxime	Yes	57	65.5%
	No	30	34.5%
Amikacin	Yes	18	20.5%
	No	70	79.5%

Data expressed as median(IQR) or as frequency (Number-percent); IQR: interquartile range; P: positive; N: Negative; DM: Diabetes Mellitus.

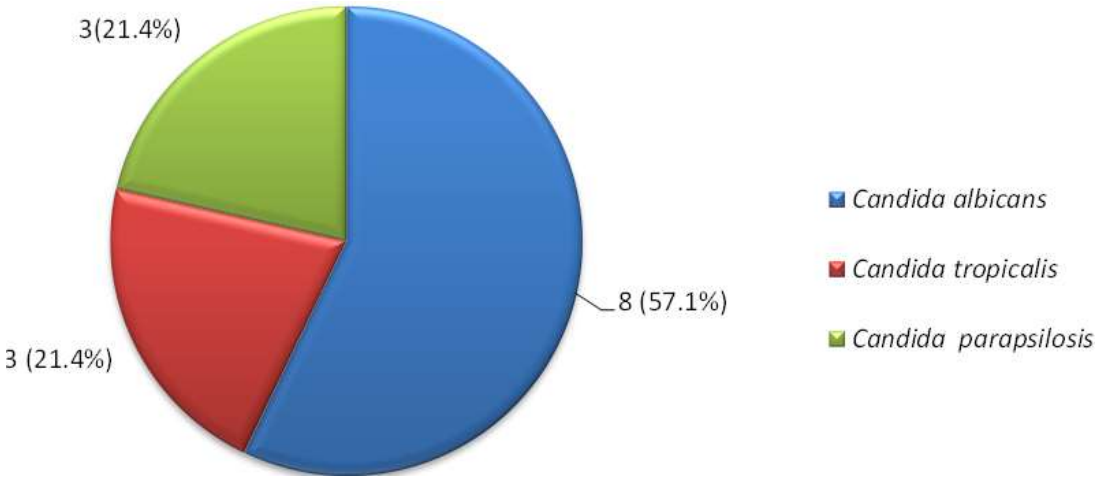


Figure 1. Distribution of isolated *Candida* species by API *Candida.*

42.9%. This rate was similar to study reported previously by Hegazi et al. (2014) and Santolaya et al. (2014).

Table 2. Association between *Candida* culture positive and some clinical risk factors.

Parameter		Culture of *Candida*				P
		P		N		
		No.	%	No.	%	
Risk factors	Central line	5	38.5	26	34.2	
	Urinary catheter	1	7.7	8	10.5	
	Frequent blood transfusion	2	15.4	8	10.5	0.97
	Low birth weight	2	15.4	15	19.7	
	DM type 1	3	23.1	19	25.0	
Outcome	Death	6	42.9	16	21.3	
	Discharge without improvement	0	0.0	1	1.3	0.2
	Discharge with improvement	8	57.1	58	77.3	

Data expressed as median (IQR) or as frequency (number-percent), IQR: Interquartile range; P: Probability; significance <0.05; DM: Diabetes Mellitus.

Table 3. Association between *Candida* culture and antigen detection.

		Antigen					
		P		N		Total	
		No.	%	No.	%	No.	%
***Candida* culture**	P	10	71.4	4	28.6	14	15.6
	N	8	10.5	68	89.5	76	84.4
	Total	18	20.0	72	80.0	90	100.0

Table 4. Sensitivity, specificity, PPV, NPV and accuracy of antigen detection compared to culture.

Mannan antigen detection	True positive	False negative	True negative	False positive	Sensitivity	Specificity	PPV	NPV	Accuracy
	10	4	68	8	71.4%	89.5%	55.6%	94.4%	86.70%

PPV: Positive predictive value; NPV: Negative predictive value.

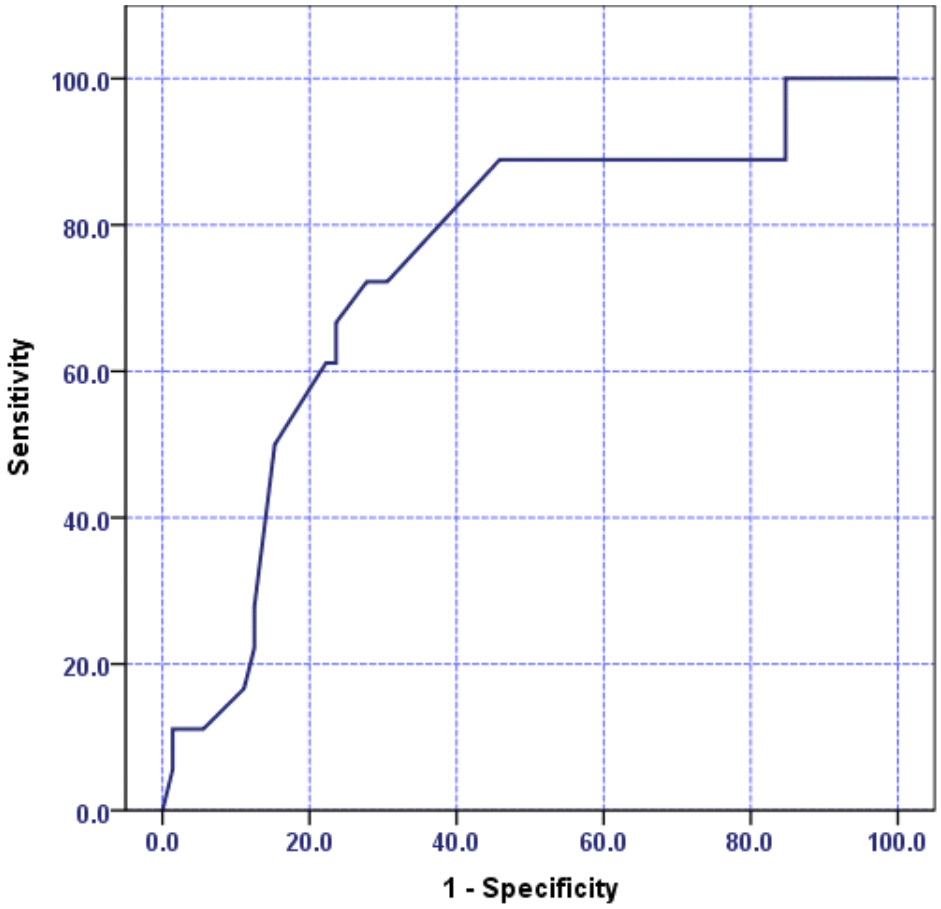
Table 5. Association between *Candida* culture and PCR.

		PCR					
		P		N		Total	
		No.	%	No.	%	No.	%
***Candida* culture**	P	12	66.7	2	2.8	14	15.6
	N	6	33.3	70	97.2	76	84.4
	Total	18	100	72	100	90	100.0

Table 6. Sensitivity, specificity, PPV, NPV and accuracy of PCR compared to culture.

PCR	True positive	False negative	True negative	False positive	Sensitivity	Specificity	PPV	NPV	Accuracy
	12	2	70	6	85.7%	92.1%	66.6%	97.2%	91.11%

PPV: Positive predictive value; NPV: Negative predictive value.



	AUC (95-CI%)	P value	Cutoff value	Sensitivity	Specificity	PPV	NPV	Accuracy
Antigen	74.4 %(61.7%-87.1%)	<0.001*	49.50	88.9%	54.2%	32.65%	95.12	61.11%

Figure 2. ROC curve of mannan antigen versus culture. ROC: Receiver operating characteristic; AUC: Area under the curve; CI: Confidence interval; PPV: Positive predictive value; NPV: Negative predictive value.

Positive blood culture for *Candida* was more frequently detected among patients with central venous lines (38.5%), children with DM type 1 (23.1%) and children with frequent blood transfusions (15.4%). However, these risk factors were not statistically significant. The presence of invasive devices, immunocompromized conditions and frequent blood transfusion are known risk factors for invasive *Candida* infections (Celebi et al., 2007; Baldesi et al., 2017; Chapman et al., 2017).

Modern technologies have been developed for rapid detection and identification within two hours of low concentration of *Candida* species in bloodstream infections approximating 1 CFU/ml such as magnetic resonance-based technology T2*Candida* ® (Lau et al., 2010). Other tests that could eventually be used such as the β-1,3 glucan and mannan tests (Montagna et al., 2009; Nguyen et al., 2012).

The sensitivity, specificity, and accuracy of the antigen detection method by ELISA were 71.4, 89.5 and 86.7% respectively. The sensitivity and of *Candida* antigen detection in the present study was similar to previous study (Alam et al., 2007) and it was less than that reported by Kurita et al. (2009). While the specificity was higher than that previously reported (Badiee et al., 2014). The difference in sensitivity may be attributed to the fact that mannan antigen of *Candida* species is rapidly cleared from the patient's blood (Kurita et al., 2009)

The study tried to detect the best cutoff value for quantitative detection of mannan antigen. This increases the sensitivity of the test with lowering the specificity. The cutoff value of *Candida* antigen by ELISA was 49.5 pg/ml with sensitivity 88.9% and specificity 54.2%. Thus detection of mannan antigen by ELISA can be used as a promising screening test for detection of candidemia (Duettmann et al., 2016). However, it has to be confirmed by more specific laboratory methods before the start of

antifungal therapy.

In a comparison between detection of *Candida* by blood culture and PCR, PCR was positive in association with culture in 12 patients (66.7%) while it was negative in 2 positive cultures. Similarly, Taira et al. (2014) reported that PCR was positive in blood samples with negative culture. This can be attributed to the presence of transient episodes of candidemia that leads to systemic infection in those patients.

In the present study, PCR was positive in 6 cases with negative blood culture. There are some concerns about the comparison of PCR to blood culture as gold standard technique due to lack of blood culture sensitivity to detect all positive cases with candidemia (Avni et al., 2011). It has been estimated that a single blood culture can miss from 25 up to 50% of disseminated candidiasis (Olaechea et al., 2004). Thus the reporting of positive PCR results in negative blood culture samples may not be reported as false positive as these patients may actually have a *Candida* bloodstream infection that was not detected by a single blood culture. Moreover, these cases may represent nonviable organisms, free *Candida* DNA in the blood, or persistence of a low-level infection. Several studies have previously demonstrated that molecular techniques perform better than culture methods (Ahmad et al., 2002; Lau et al., 2008; Avni et al., 2011).

The overall accuracy of semi-nested PCR was 91.1% with good sensitivity 85.7% and specificity 92.1%. Thus, this method can be applied as a specific rapid laboratory diagnosis associated with appropriate clinical evaluation to start antifungal therapy in suspected patients (Tirodker et al., 2003; Wellinghausen et al., 2009; Ruppenthal et al., 2005). Similar conclusion was made by a previous study from Egypt with PCR for 16 srRNA (Hassan et al., 2014).

On the other hand other studies with higher number of patients are required to evaluate its role as a screening method for highly susceptible patients. The advantage of PCR as rapid and specific method for accurate diagnosis of candidemia is extremely important in rapid introduction of antifungal therapy, improvement of candidemia outcomes, reducing the costs of unnecessary antibiotics therapy and reducing the hospital costs (Zilberberg et al., 2010; Kaufman, 2010).

It can be concluded from this study that *Candida* species is a frequent pathogen associated with pediatric sepsis. Blood culture though is a reliable laboratory method for its diagnosis may lack sensitivity and requires prolonged time. Semi-nested PCR for detection of candidemia is sensitive, specific and accurate method. Mannan antigen detection by ELISA is rapid and easy; however, it may lacks specificity can be used as preliminary method for screening. Further studies are recommended to detect the appropriate laboratory algorithm for diagnosis of *Candida* sepsis to start antifungal therapy appropriately and the clinical outcome associated with earlier diagnosis achieved with direct PCR

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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