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Identification of Pathogenic Fungi in Renal Transplant Patients by Conventional and Molecular Methods

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ABSTRACT

Opportunistic fungal infections due to the immune- compromised status of renal transplant patients are related to high rates of morbidity and mortality regardless of their minor incidence. Delayed in identification of invasive fungal infections (IFIs), will lead to delayed treatment and results in high mortality in those populations. The study aimed to assess the frequency of invasive fungal infection in kidney transplant recipients by conventional and molecular methods. This study included 100 kidney transplant recipients (KTR) (75 males, and 25 females), collected from the Centre of Kidney Diseases and Transplantation in the Medical City of Baghdad. Blood samples were collected during the period from June 2018 to April 2019. Twenty one out of 100 renal-transplanted patients were infected with pathogenic fungi, four of the patients were females and 17 were males. There is an observation of a high incidence of fungemia in patients with the abnormal value of blood urea according to PCR and culture results. Referring to fungal isolates the most prevalent was *Saccharomyces cerevisiae*, which account for 19 isolates out of 21 the other two isolates were *Zygosaccharomyces rouxii* and *Aspergillus flavus*. The results of the current study show significant correlation between PCR and culture methods at (P<0.0009).

1. Introduction

Since renal transplantation was first presented, thousands of patients with end-stage kidney failure have been significantly prolonged in their lives (Ezzatzadegan et al., 2012). Revolutions in the subject of immunosuppressive treatment enhanced the survival of the grafts and the recipients. Certainly, the occurrence of opportunistic infections has amplified because of this immune suppression. Fungi, in addition to bacterial and viral pathogens, have played a significant role in the high death and morbidity rates associated with opportunistic infections in kidney transplant recipients (Sanchez & Larsen, 2007). The risk of opportunistic infection in the kidney transplant recipient is verified by the relations between two elements (Pontes et al., 2020*)* the epidemiologic contacts of the individual encounters within the hospital and the community and a complicated function designated the total state of immunosuppression (Tolkoff-Rubin & Rubin, 1992).

Fungal species are normally disseminated in plant debris, soil and other organic substrates, and about 7 % (611,000 species) of all eukaryotic species on earth (Badiee & Hashemizadeh, 2014). Over 600 various fungi have been designated to infect humans, ranging from common to lethal infections (Brown et al., 2012*)*. The best method for the optimal administration of fungal infection is early recognition and identification of the causal agent, so that suitable treatment can be introduced as soon as possible, especially in immune-compromised patients *(*Badiee & Hashemizadeh, 2014*)*. Invasive fungal disease (IFD) is a life-threatening incident in immune-compromised patients, and there is a crucial need for dependable screening methods enabling rapid and broad discovery of pathogenic fungi (Landlinger et al., 2010*)*. Treatment needs initial suspicion

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and is difficult for the reason that only a few antifungal agents are available, most usually have side effects, and some organisms have established resistance (Badiee & Hashemizadeh, 2014).

Determine the frequency of fungal infection among renal transplant patients by conventional and molecular methods.

2. Materials and Method

This study included 100 kidney transplant recipients (KTR) (75 males, and 25 females), collected from the Center of Kidney Diseases and Transplantation in the Medical City of Baghdad. Blood samples collected during the period from June 2018 to April 2019. Collected blood was divided into 3 parts: One to three ml injected into a (BACT/ALERT culture media bottle and the positive one sub cultured into Sabouraud dextrose agar and then identified by the Vitek2 identification system. Two ml were mixed with EDTA and recruited for DNA extraction; and the other two ml injected into a plain tube for the assessment of the levels of blood creatinine, urea, as well as sugar. DNA was purified from EDTA blood samples, primers sequences (forward and reveres) used for the amplification analysis of ITS 2-Region rRNA (5,8SR 5‘- TCG ATG AAG AAC GCA GCG -3‘ and LR1 5‘- GGT TGG TTT CTT TTC CT –3‘Product's size(bp) > 400bp (Vilgalys & Hester, 1990), as a constitutive gene for fungal identification and conventional polymerase chain reaction (PCR) method was used. PCR products were run on gel electrophoresis, and final identification was performed using direct sequencing method (Sanger sequencing by ABI3730XL, automated DNA sequences (Macrogen Corporation – Korea).

3. Results and Disscation

3.1. Patients 'Descriptive Data

This study involved 100 kidney transplant recipients (KTR), collected from the Centre of Kidney Diseases and Transplantation in the Medical City of Baghdad. Among those recipients 75 (75%) were males, and 25 (25%) were females. The mean age was 37.8±12.74 years, ranging between 14 and 68 years. The majority of patients underwent kidney transplantation for the first time were 97 of 100 (97%), two patients for the second time (2%) and only 1 patient for the third time (1%). The mean post-transplantation period (PTP) was 12.72±7.04 months, ranging from one month to 24 months with highest percentage in PTP of (6-12months) which was 33% while the rest of the patients were distributed as the followings, 27% in 18-24months, 22% in <6 months and the lowest percent in 12-18months which was only 18% as shown in Figure 1.

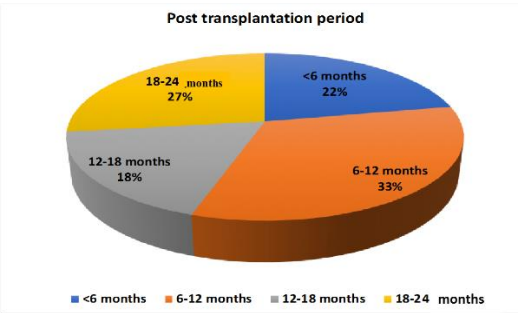

Fig. 1. Post-transplantation period (PTP) among kidney transplant recipients

3.2. Association of Fungemia with Patients' Descriptive Data

Fungemia in the current study was diagnosed in 21 out of 100 (21%) renal transplant recipients by both conventional and molecular methods (three by both culture and PCR technique and 18 by only PCR methods). PCR identification of fungal infections seems to be promising but still in its infancy (Sanchez & Larsen, 2007). The incidence of infections in renal transplant recipients had declined in the late 90's to 15%- 44% with a mortality rate of less than 5% (Khoury & Brennan, 2005). The frequency of IFIs ranges from 5% to 50% in kidney and liver transplants (Badiee & Alborzi, 2011). The ratios of fungal infection in renal transplant patients vary among research studies. Einollahi et al., (2008) reported that only 0.87% of kidney transplant patients had fungal infections. Ezzatzadegan, et al., (2012) found that 2.1% of patients developed fungal infection. Badiee and Alborzi (2011) conducted that fungal infections account for 5% of all infections in renal transplant recipients, while the incidents of fungal infection in this population account for 18%.

Out of 100 patients, 21 were infected with pathogenic fungi, four patients were females and 17 were males. The results show that there was no significant association between gender and PCR results. However, it is more prevalent in males (81%) in contrast to females (19%). There was no association between cultural results and sex factors despite the fact that all were positive cultures in males (100%). The results of the current study are in agreement with Einollahi, et al., (2008) who found that invasive fungal infections developed in 21 recipients (0.87%), 17 males and only 4 females. The male-to-female ratio for IFIs was 4.2 to 1.

3.3. Results of Conventional Methods

3.3.1. Culture Results

By considering the culture method as a gold standard for fungal isolation, the results of the current study revealed six samples were positive by BacT/ALERT® blood culture system, and only (3) out of these 6 were positive by Sabourauds dextrose agar (3%) as yeasts.

Most regular manual and automated blood culture systems are able to support the growth of yeasts such as *Candida spp.* Molds, and especially dimorphic fungi, often grow poorly in typical instrumented blood culture systems (Kirn & Weinstein, 2013). Breathnach and Evans (1995) reported that some fungi may be missed by the BacT/Alert system, either due to no growth in the medium or undiscovered slow growth, they found that out of (19) filamentous fungi only (11) species grew and were detected rapidly, (6) species grew slowly and (2) species did not grow. Badiee and Alborzi (2011) found that none of the patients with IFIs had a positive blood culture result.

3.3.2. Relation between Conventional Methods and Patients, Descriptive Data

The results of this study found that there were no relationships between cultures positive results and age factor (p value = 0.147), PTP (p value = 0.359), glucose level (p value = 0.964), urea level (p value = 0.483). Despite the fact that all patients with positive blood culture results showed abnormal values of blood urea level (Normal serum urea value is 20 – 40 mg/dL (Vaidyanathan, 2016), and creatinine level (p value = 0.700) as shown in Table 1.

Table 1
Relation between Culture Positive Rresults and Patients' Descriptive Data

	Culture				P value
	Positive		Negative		
	Mean	SD	Mean	SD	
Age	48	21.07	37.44	12.06	0.147
PTP	9.33	5.03	13.16	7.13	0.359
Glucose	114.33	71	113.23	40.46	0.964
Urea	85.33	48.22	54.58	75.03	0.483
Creatinine	1.23	0.15	1.59	1.6	0.700

SD=stranded deviation, PTP=post-transplant period

Elevated blood urea level in fungal infection conducted in other researches, Mohanty and Sahu (2014) revealed a case report of fungal infection with *Fusarium species* in a kidney transplant patient, the laboratory investigations showed impaired renal function, with elevated serum creatinine levels (2.8 mg/dl) and blood urea levels (87 mg/dl) (Mohanty & Sahu, 2014). Tobon, et al., (2003) also found abnormal rises in blood urea (50 mg/dL) in patient with diabetes who established a severe invasive fungal infection due to *Rhizopus species* post- operatively after a heart/kidney transplantation consequent intensive immunosuppressive therapy.

3.4. Molecular Method Results

3.4.1. PCR Technique

The PCR product was checked by gel electrophoresis in a concentration of 1.5% agarose gel, the band of PCR products was 400 bp as shown in Figure 2.

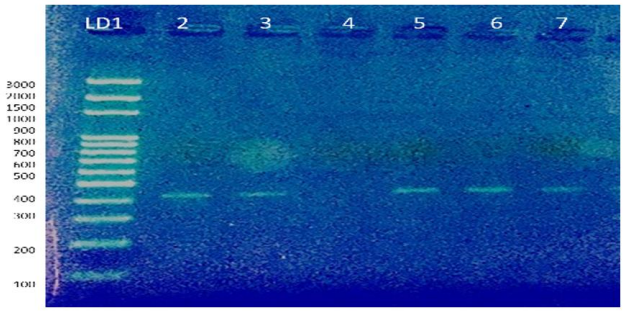

Fig. 2. Gel Electrophoresis (1.5% agarose) of ITS2 gene (400bp) using 100bp DNA ladder and Diamond™ Nucleic Acid Dyeto (LD1=DNA ladder; Lane (2, 3, 5, 6, and 7) =positive results; Lane 4=negative result (100 volt for one hour)

Most researches focus on either the ITS1 (Angebault et al., 2020) or ITS2 (Heeger et al., 2019). Angebault, et al., (2020) mentioned that ITS2 consented to the detection of a larger number of minor taxa compared with ITS1. Using ITS-2 for metabarcoding could potentially increase the comparative studies among fungi and plants, a variety of molecular methods are increasingly becoming valuable devices in all aspects of fungal diagnostics.

3.4.2. Relation between Molecular Methods and Patients, Descriptive Data

The results of the current study found that there were no relationships between PCR results and age factor (*p* value = 0. 999), PTP (*p* value = 0. 061), glucose level (*p* value = 0. 411), urea level (*p* value = 0. 720), and creatinine level (*p* value = 0. 096) as shown in Table 2.

Table 2
Relation between PCR results and patients' descriptive data

	PCR				P value
	Positive		Negative		
	Mean	SD	Mean	SD	
Age	37.76	13.54	37.76	12.15	0.999
PTP	10.48	6.61	13.73	7.09	0.061
Glucose	106.67	33.71	115.01	42.88	0.411
Urea	50.29	26.17	56.89	82.7	0.720
Creatinine	1.3	0.34	1.66	1.76	0.096

3.5. Relation between Conventional and Molecular Methods

The results of the current study showed a direct correlation coefficient (0.320) between PCR and culture methods and are highly associated at (P<0.0009) as shown in Table 3. Only three (3%) samples were positive in both PCR and culture methods, versus 21 (21%) samples detected by PCR.

Table 3
Correlation coefficients between culture and PCR result

	Culture		PCR results
	Positive	Negative	
79%	0	79	Negative
21%	3	21	Positive

Chi-squared=10.945, Significance level=P<0.009

This agrees with Gosiewski, et al., (2014**)** who mentioned that the use of the advanced methodology, gave a ratio of positive results of 69.6% compared to 18.6% acquired with the method of blood culture in the monitored culture system. According to the findings of a few studies that looked especially at the prevalence and etiology of IFD following kidney transplant, there may be significant geographic differences in the epidemiology of IFD in this population. The results of this study show that *A.flavus* isolate is related to abnormal values (100%) of both creatinine and blood urea. The *Z.rouxii* isolate is related to the abnormal value of blood urea (100%) and the normal value of creatinine (100%). On the other hand, the isolates of *S.cerevisiae* were related to the abnormal value of blood urea (94.7%) as shown in Table 4**.**

Table 4
Positive and negative results of fungemia by conventional and molecular methods with fungal species according to blood urea values

		PCR		Culture		Species			
		Positive	Negative	Positive	Negative	*A.flavus*	*S.cerevisiae*	*Z.rouxii*	Negative
Urea	Abnormal	20 95.2%	70 88.6%	3 100.0%	87 89.7%	1 100.0%	18 94.7%	1 100.0%	70 88.6%
	Normal	1 4.8%	9 11.4%	0 0.0%	10 10.3%	0 0.0%	1 5.3%	0 0.0%	9 11.4%
	Total	21 100%	79 100%	3 100%	97 100%	1 100%	19 100%	1 100%	79 100%
P value		0.333 NS		0.727 NS			0.834 NS		

Frequency was statistically not significant according to the age groups among positive and negative patients with fungemia although it's more prevalent in age group (21-30 years) which was (38.1%) using PCR technique while it was equally distributed (33.3%) in the age groups (21-30, 4-50 and >50 years) as shown in Table 5.

Table 5
Frequency of fungemia among renal transplant recipients according to age groups by conventional and molecular methods

		PCR		Culture		Species			
		Positive	Negative	Positive	Negative	*A.flavus*	*S.cerevisiae*	*Z.rouxii*	Negative
Age groups	<= 20 years	1	5	0	6	0	1	0	5
	%	4.80%	6.30%	0.00%	6.20%	0.00%	5.30%	0.00%	6.30%
	21-30 years	8	20	1	27	0	8	0	20
	%	38.10%	25.30%	33.30%	27.80%	0.00%	42.10%	0.00%	25.30%
	31-40 years	4	23	0	27	0	3	1	23
	%	19.00%	29.10%	0.00%	27.80%	0.00%	15.80%	100.00%	29.10%
	41-50 years	6	22	1	27	1	5	0	22
	%	28.60%	27.80%	33.30%	27.80%	100.00%	26.30%	0.00%	27.80%
	years> 50	2	9	1	10	0	2	0	9
	%	9.50%	11.40%	33.30%	10.30%	0.00%	10.50%	0.00%	11.40%
Total		21	79	3	97	1	19	1	1
		100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%
P value		0.788 NS		0.647 NS		0.792 NS			

This is in close to other researchers, Einollahi, et al., (2008) in Iran who reported that the main age of invasive fungal infections after kidney transplantation was 48±10 years. Magda M. Azab, et al., (2008) also reported that the mean age of patients with fungema was 48.1±11.8y, with the highest incident in age >52 years old. According to fungal species (42.1%) of *S., cerevisiae* isolates were found in the age group (21-30years) while the isolates of *Z.rouxii and A.flavus* were in age groups (31-40years) and (41-50years) respectively, as shown in Table 5.

Over 10% of the isolates linked to invasive aspergillosis in transplant recipients were discovered to be cryptic species, necessitating the use of molecular identification approaches to differentiate these species. Gorton et al., (2013) mentioned the limits of current conventional diagnostic procedures for speciation of novel pathogenic *Candida spp.*, and highlighted the need for alternate diagnostic methodologies such as ribosomal rRNA sequencing. Baškova et al., 2007) determined the nucleotide sequences of the ITS2 region for 13 more Candida species, in addition to those previously described. They discovered that within the ITS2 region, no two Candida species had similar sequences. Garner et al., (2010) discovered that enough variation occurred within this region to construct a database containing the ITS2 length and sequence polymorphism for these isolates after conducting a study to examine 34 yeast species from diverse genera for ITS2 sequence polymorphism. The investigators also tested over 400 clinical yeast isolates to validate the database in this study. The ITS region includes two sections (ITS1 and ITS2) that border the conserved 5.8S region (Badotti et al., 2017; Bengtsson-Palme et al., 2013). The effectiveness of these sub-regions in the recognition of species in many fungal isolates has been estimated, and some authors declare that ITS1 is more variable than ITS2 (Kõljalg et al., 2013; Zaher, 2015). Others have found opposite results (Mbareche et al., 2020; Yao et al., 2010) or that both the sub-regions are suitable as metabarcoding markers (Mello et al., 2011).

At the species level, and even within species, the ITS region of nuclear DNA (rDNA) has become the most sequenced region for fungal taxonomic identification.

4. Conclusions

In Conclusion, the current study showed that:

- The percentage of fungemia among renal transplant patients was 21%, which is considered a risk factor for mortality.
- PCR technique and ITS2 rDNA gene sequencing offers accurate identification information about the genes of fungi, which can be used for research purposes

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Competing Interests

The authors have declared that no competing interests exist.

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