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# Molecular identification of fungal infections in Children with Intermittent Peritoneal dialysis

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**Abstract**

**Background:** Fungi is a major cause of peritonitis infections and it is very important to have a reliable test to detect these bacteria in peritoneal fluid by molecular methods .

**Aims of the study:** Detection of the most common Fungi in peritoneal fluid from pediatric patients with acute and chronic renal failure who undergo peritoneal dialysis, through molecular diagnostic methods.

**Methods**: One hundred clinical peritoneal fluid isolates were submitted to molecular methods (Polymerase chain reaction (PCR) and DNA sequencing) for detection of fungi.

**Results**: By molecular methods according to PCR technique, the following Fugi were identified: *C. tropicalis* 6 (8.2%), *C. albicanis* 3 (4.1%), *C. parpsilosis* 2 (2.7%) and *C. glebrata* 1 (1.4%). According to DNA sequencing technique, the following fungi were identified: *C. tropicalis* 8 (11.0%), *C. albicanis* 5 (6.8%), *C. parpsilosis* 1 (1.4%), *Penicillium chrysogenum* 10 (13.7%), *Aspergillus fumigatus* 12 (16.4%), *Chaetomium globosum* 5 (6.8%) , Neurospora spp. 5(6.8%), Trichoderma spp. 3 (4.1%), *Clavispora lusitaniae* 8 (11.0%) and *Rhodotorula mucilaginosa* 6 (8.2%)

**Conclusion:** The most common fungi were the best.pathogene responsible for peritonitis were *C. tropicalis*, *C. albicanis* and *Aspergillus fumigates.*

**Key words:** peritonitis, Molecular detection of fungal peritonitis, peritoneal dialysis infections.

## INTRODUCTION

Kidney failure, is a medical condition in which the kidneys fail to adequately filter waste products from the blood. The two main forms are acute kidney injury, which is often reversible with adequate treatment, and chronic kidney disease, which is often not reversible. In both cases, there is usually an underlying cause.

Peritoneal dialysis (PD) is a treatment for patients with severe acute and chronic kidney disease. Peritonitis is one of the most frequent complications of peritoneal dialysis (PD) and 1% – 15% of episodes are caused by fungal infections. The mortality rate of fungal peritonitis (FP) varies from 5% to 53%; failure to resume PD occurs in up to 40% of patients [1]. The majority of these FP episodes are caused by Candida species, *Candida albicans* and Aspergillus spp. historically been reported to be the most common cause of (PD) [2]. Peritonitis still the leading cause of technique failure in continuous ambulatory peritoneal dialysis (CAPD) patients. Incidence of peritonitis depends on factors such as age, race, educational background, environment, and type of dialysis system used, while the outcome depends on the organisms isolated. Culture method, remains the gold standard for diagnosis of fungal infections becomes positive at 35 °C (± 2 °C) for 48 hours, after sampling [3], but the development of rapid diagnostic methods has been identified as an important medical need to supplement conventional culture diagnostics and molecular techniques have potential to fulfill this need. Nucleic acid based diagnostic systems, including polymerase chain reaction (PCR) and gene Sequencing methods as well as the application of DNA is well known sensitive techniques for a more rapid detection and the specific identification of pathogens [4, 5].

## SUBJECTS AND METHODS

### The Study Population

A total of one hundred peritoneal fluid samples, from pediatric patients who admitted to Children Welfare Teaching Hospital and Central Teaching Hospital of pediatric suffering from renal diseases. Samples collection was done at the last day (day three) of peritoneal dialysis, in a period from May 2016 to February 2017. The age range was between 1days and 12 years. The whole work was done at Microbiology Department - College of Medicine/Al-Nahrain University, except DNA sequencing which was done in Macrogen Corporation – Korea.

### Sample Collection and Processing

Five ml of peritoneal dialysis for each 100 patiants were collected in separated tube, sealed with parafilm and stored in a deep freez at (-20)C°. for molecular examination [6].

### Molecular method for the diagnosis of fungi

### DNA extraction

DNA was extracted according to(Vogelstein and Gillespie,1979) [7]adopted by the manufacturer of DNA extraction kit . DNA was extracted from each peritoneal fluid sample using a freezing–thawing technique [8] for cell lysis, and the QIAamp® DNA Mini Kit to purify DNA.

### Polymerase Chain Reaction (PCR) for Fungi

### Oligonucleotide primers

Primers sequences were used for the amplification of Candida species, genes were selected according to Nabil S. Harmal *et al*., [9].While the C. *krusei* primers sequences amplification was selected according to [10] and primers sequences of ITS gene as universal gene for all fungal species according to [11]. The general properties of these primers were checked by using Oligonucleotide Properties Calculator program, as shown in table (1).

### Gell Electrophoresis

PCR product was subjected to 1% (wt/vol) agarose gel electrophoresis with ethidium bromide (0.5 µg /ml). PCR products electrophoresis amplicon visualization was performed using an UV light transilluminator according to (Sambrook and Russell,2001) [12]

### Gene Sequencing:

PCR product for ITS of fungi and 16s RNA of bacteria was sent for Sanger sequencing using ABI3730XL, automated DNA

sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using genious software.

Table (1): Primers sequences with their relevant product size.

| *Candida species* | Primer ID | Sequence 5'--3' | Annealing temperature | Product length |
|---|---|---|---|---|
| *C.albicans* | CAF CAR | TTGTGTTGCTACATCA CCAAC TTTGCTGGCAACTTGA TTACC | 63°C | 538 bp |
| *C.glabrata* | CgF CgR | TCTCACACTCCATTGT CTCA AGCAGGTTTACCATCA GAA | 50°C | 404 bp |
| *C.parapsilosis* | CPF CPR | TCCATCGACGAATTGA TTG ACCGTTTTGAGACCTC AAG | 60°C | 252 bp |
| *C. krusei* | CkF CkR | CAT TGG CCG TTT CCA TTG TGT TC CAT CAA ACC AAG CGT GAT TCT TGC | 65°C | 359bp |
| *C.tropicalis* | CTF CTR | CCCATACGATTTATGG AAT CCATTGACACAAGCA TTTAC | 53°C | 501 bp |
| ITS gene | CTF CTR | TCC GTA GGT GAA CCT GCG G TCC TCC GCT TAT TGA TAT GC | 55°C | 599 bp |

Procedure of Polymerase Chain Reaction (PCR) for Fungi According to [11].tables(2, 3).

Table (2): PCR reaction mixture Composition used for amplification of *C.albicans, C.tropicalis, C.parapsilosis, C.glabrata, krusei* and ITS gene (Convetional PCR).

| Reagents | (Volume /µl ) |
|---|---|
| Forwad Primer | 1 |
| Reverse Primer | 1 |
| DNA template | 5 |
| PCR master mix | 12.5 |
| (DNAse free) water | 5.5 |
| Total volume | 25 |

Table (3): The PCR thermocycler program for fungal genes.

| Steps | Temperature | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 95°C | 4 min | |
| Denaturation<br>Annealing<br>Elongation | 95°c<br>55°c<br>72°c | 30 sec<br>30 sec<br>1 min | 30 |
| Final extension<br>Hold | 72°c<br>4°c | 10 min | |

## RESULTS

### Fungi identified by PCR and DNA sequencing techniques

According to PCR, the following fungi were identified: *C. tropicalis* 6 (8.2%), *C. albicanis* 3 (4.1%), *C. parpsilosis* 2 (2.7%) and *C. glebrata* 1 (1.4%), table (4), figures (1, 2). According to DNA sequencing technique, the following fungi were identified: *C. tropicalis* 8 (11.0%), *C. albicanis* 5 (6.8%), *C. parpsilosis* 1 (1.4%), *Penicillium chrysogenum* 10 (13.7%), *Aspergillus fumigatus* 12 (16.4%), *Chaetomium globosum* 5 (6.8%) , Neurospora spp. 5(6.8%), Trichoderma spp. 3 (4.1%), *Clavispora lusitaniae* 8 (11.0%) and *Rhodotorula mucilaginosa* 6 (8.2%), table (5).

**Table (4):** Fungal isolates identified by PCR technique.

| Fungus | No. | % |
|---|---|---|
| *C.tropicalis* | 6 | 8.2 |
| *C.albicanis* | 3 | 4.1 |
| *C.parpsilosis* | 2 | 2.7 |
| *C.glebrata* | 1 | 1.4 |

**Table (5):** Fungal isolate productes by DNA sequencing technique.

| | No. | % |
|---|---|---|
| *C.tropicalis* | 8 | 11.0 |
| *C.albicanis* | 5 | 6.8 |
| *C.parpsilosis* | 1 | 1.4 |
| *Penicillium chrysogenum* | 10 | 13.7 |
| *Aspergillus fumigatus* | 12 | 16.4 |
| *Chaetomium globosum* | 5 | 6.8 |
| Neurospora spp. | 5 | 6.8 |
| Trichoderma spp. | 3 | 4.1 |
| *Clavispora lusitaniae* | 8 | 11.0 |
| *Rhodotorula mucilaginosa* | 6 | 8.2 |

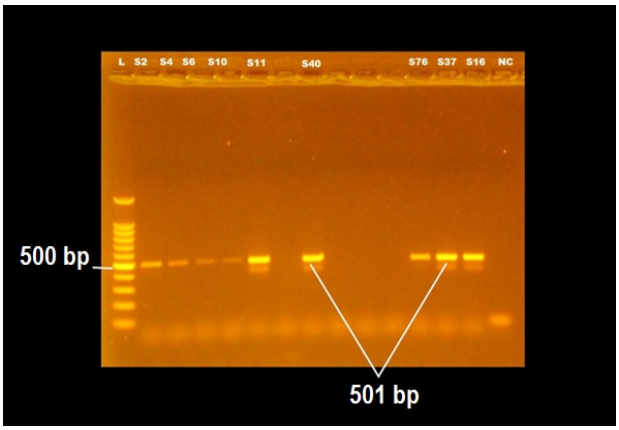


**Figure (1):** Gel electrophoresis of conventional PCR products of *C.tropicalis* (501 bp); negative control; MW, 2000 pb ladder; (2% agarose,5v/cm (70)1hr).

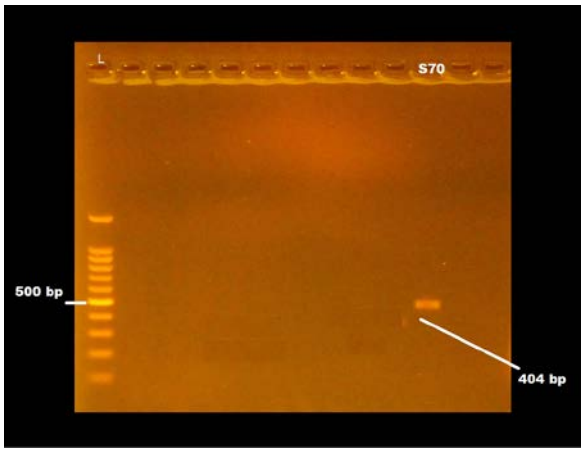


**Figure (2) :**Gel electrophoresis of conventional PCR products of *C.glebrata* (404 bp); negative control; MW, 1000 pb ladder; (2% agarose,5v/cm (70)1hr).

## DISCUSSION

### Fungal Isolates by PCR and DNA sequencing technique

### PCR teachnique

The use of PCR, in the present study, permitted the isolation of the following fungal agents: *C. tropicalis, C. albicanis, C.*

*parpsilosis and C. glebrata*. The low detection rate of PCR in this study returns to the use of Candida species praimers only, and this agree with the findings of (Klingspor and Jalal, 2006)[13] who stated that in patients with positive PCR results for fungal infection, verification with conventional methods was possible (50%) of cases only. (Klingspor and Jalal, 2006)(13) found that out of 50 samples of peritoneal fluid, all were PCR-negative for Aspergillus and cultures were negative for Aspergillus; the most common fungal agent according to PCR, and in accordance with the current results was *Candida* spp. In addition, antifungal therapy may reduce the fungal load and may also alter the fungal target by damaging the fungal cell wall/membrane, releasing the DNA and providing a free-circulating rather than a cell-associated DNA source (White & Barnes, 2009)[14]. If free DNA is targeted, then uncomplicated commercial nucleic acid purification systems can be used, whereas if the DNA source is cell-associated, steps to lyse the fungal cell must be applied (White & Barnes, 2009)[14] .

**DNA sequencing**

By DNA sequencing technique the followings fungi had been diagnosed: *C. tropicalis* 8(11.0%), *C. albicanis* 5(6.8%), *C. parpsilosis* 1(1.4%), *Penicillium chrysogenum* 10(13.7%), *Aspergillus fumigates* 12(16.4%), *Chaetomium globosum* 5(6.8%), *Neurospora spp.* 5(6.8%), *Trichoderma* spp. 3(4.1%), *Clavispora lusitaniae* 8(11.0%), *and Rhodotorula mucilaginosa* 6(8.2%).

In a study carried out by Giacobino *et al*. (2016)[15] using DNA sequencing on patients with peritonitis, the following agents were identified: *Candida parapsilosis* was the leading species (9/23), followed by *Candida albicans* (5/23), *Candida orthopsilosis* (4/23), *Candida tropicalis* (3/23), *Candida guilliermondii* (1/23), and *Kodamaea ohmeri* (1/23). Some of these fungal isolates are similar to that isolated in the present study such as *Candida albicans* and *Candida tropicalis*; however, different agents were isolated in the current study such as *C.parpsilosis, Penicillium chrysogenum, Aspergillus fumigates, Chaetomium globosum, Neurospora spp., Trichoderma spp., Clavispora lusitaniae, and Rhodotorula mucilaginosa.*

On the other hand, in the present study, DNA sequencing permitted identification of agents that were not shown by routine culture method and this may be attributable to that some of these diagnosed fungi by DNA sequencing may be come from technique contamination or DNA extraction method for example.

Thorough a long search of published literature to compare the current results regarding to reliability of DNA sequencing for fungal peritonitis it was no such puplishments, so the current results can be considered the first concerning DNA sequencing. The low sensitivity of this method makes it suitable as an adjuvant method to culture and not as substituant for it.

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**Contribution Details**

Samples selections, design, acquisition of data and molecular methods diagnosis. Drafting the article and revising it critically for important intellectual content.

**Conflict Of Interest**

The author declares that they have no competing interests.

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**REFERENCES**

1- Prasad N[1], Gupta A. Fungal peritonitis in peritoneal dialysis patients. Perit Dial Int. (2005) May-Jun;25(3):207-22.

2- Aydin U, Ismail K, *et al*. Fungal peritonitis in peritoneal dialysis: an analysis of 21 cases Received: 30 March 2010 / Accepted: 8 May 2010 / Published online: 6 June 2011, 10.1007/s11255-010-9763-2.

3- Heimo.W, Andreas.H. A multiplex real-time PCR assay for rapid detection ad differentiation of 25 bacterial and fungal pathogens from whole blood samples.Article in Medical Microbiology and Immunology.September 2008 Impact Factor: 3.04 · DOI: 10.1007/s00430-007-0063-0 ·PubMed.

4- Struelens MJ, de Mendonca R. The emerging power of molecular diagnostics: towards improved management of life threatening infection. (2001) Intensive Care Med 27(11):1696–1698.

5- Davis TE, Fuller DD. Direct identification of bacterial isolates in blood cultures by using a DNA probe. J Clin Microbiol (1991) 29(10):2193–2196.

6- Sayyada G. Nadeem, Shazia T. Hakim, Shahana U. Kazmit. Use of CHROMagar Candida for the presumptive identification of Candida species directly from clinical specimens in resource-limited settings. Libyan J Med (2010); 5: 2144:1-4. doi: 10.3402/ljm.v5i0.2144

7- Vogelstein, B. and Gillespie, D. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. (1979). 76 (2): 615 619.

8- Reischl U, Pulz M, Ehret W, Wolf H. PCR-based detection of *Mycobacteria* in sputum samples using a simple and reliable DNA extraction protocol. Biotechniques (1994) Nov (5):844–5.

9- Nabil, S. Harmal, Alireza Khodavandi, Mohammed. A. Alshawsh *et al.* Identification and differentiation of *Candida species* using specific polymerase chain reaction (PCR) amplification of the phospholipase B gene. African Journal of Microbiology Research. 2013;7(20): 2159-2166. DOI: 10.5897/AJMR2013.2501

10- Anna Br-D and Aleksandra Si. Molecular detection of *Candida krusei* International Research Journal of Microbiology, (2012) Vol. 3(8) pp. 275-27.

11- White, T.J., T.Bruns, S.lee, and S.Tailor. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics.(1990) in M. A. Innins, D.H. Gelfand, J.J. Sninsky, and T.J.White(ed.),PCR protocols. Aguide to methods and applications. Academic Press,Inc.,San Diego, Calif p.315-322.

12- Sambrook, J. and Russell, D. W. Molecular Cloning: A Laboratory Manual Cold Spring Harbor. New York, USA, Cold Spring Harbor Laboratory Press, N.Y. (2001).

13- Klingspor L, Jalal S. Molecular detection and identification of Candida and Aspergillus spp from clinical samples using real-time PCR. Clin Microbiol Infect. 2006;12(8):745–53.

14- Barnes RA, White PL, Bygrave C, Evans N, Healy B & Kell J. Clinical impact of enhanced diagnosis of invasive fungal disease in high-risk haematology and stem cell transplant patients. J Clin Pathol (2009) 62: 64–69. doi: 10.1136/jcp.2008.058354.

15- Giacobino J, Montelli AC, Barretti P, et al. Fungal peritonitis in patients undergoing peritoneal dialysis (PD) in Brazil: molecular identification, biofilm production and antifungal susceptibility of the agents. *Med Mycol*. 2016;54:725–32.