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Identification of *Candida* Isolates from Cancer Patients Using Multiplex PCR

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Abstract
Aim of this study was to identify the *Candida* isolates from Cancer Hospital Maharagama, Sri Lanka by using Multiplex PCR (polymerase chain reaction) method. In this study, Multiplex PCR was used to identify *Candida* isolates from cancer patients at Apeksha Hospital, Sri Lanka. In multiplex PCR, 18 specific primers (nine primer pairs) were separated into three groups for multiplex PCR (PS I, PS II and PS III). Different sized PCR products corresponding to each *Candida* species were produced by PCR using the primer mixes. Among 52 *Candida* isolates, 49 were identified using the multiplex PCR procedure. *Candida tropicalis* was found to be the most prevalent species (38%), followed by *Candida parapsilosis* (31%), *Candida albicans* (13%), *Candida glabrata* (8%) and *Candida krusei* (4%). The study concludes that multiplex PCR is a better approach for the identification of *Candida* species and recommends the use of it for clinical and diagnostic purposes and for research purposes.

Keywords: Multiplex PCR, Cancer Patients, *Candida*

1. Introduction

Candida species are the most common opportunistic pathogens in debilitated or immunocompromised hosts and cause systemic candidiasis, which has high rates of morbidity and mortality. Immunocompromised individuals, such as those getting chemotherapeutic treatment for cancer are susceptible to a multitude of life-threatening opportunistic infections caused by *Candida* (Chi-Yang Lin et al., 2007). In recent years antifungal drug resistance of *Candida* species has become a major problem in the effective management of cancer patients in Sri Lanka. Identification of drug-resistant *Candida* strains has become an important supporting tool for physicians faced with making difficult decisions regarding treatment of patient with *Candida* infection. The first step in effective antifungal treatment is the identification of the pathogenic *Candida* species. Identification methods could be of various forms, such as molecular biochemical or morphological.

In earlier investigations on the epidemiology of *Candida* sp. various phenotypic characteristics were utilized to determine, but the methods lacked resolving capacity and were also frequently erroneous and time-consuming.

With the advent of molecular genetics, new, more powerful DNA-based typing methods have emerged as the "gold standard" for epidemiological studies.

Multiplex PCR is a rapid diagnostic test that mixes primers from numerous distinct species in a single PCR tube. Consequently, it might be used to concurrently identify multiple species in a specimen (Mahmoudi Rad et al., 2012). But when using Multiplex PCR, it is critical to design and use accurate primers according to the study that conduct.

In Sri Lanka, any reports of using multiplex PCR on identification of *Candida* species have not been published up to date so this study will be helpful to establish a proper molecular identification facility in Apeksha Hospital, Sri Lanka.

The study aims to investigate the molecular identification of *Candida* isolates through multiplex PCR. The first objective is to utilize multiplex PCR for the molecular identification of *Candida* isolates. Secondly, the study aims to evaluate effectiveness of this method in identifying *Candida* species. By achieving these objectives, the study seeks to contribute to the understanding of *Candida* species distribution and improve diagnostic approaches for identifying *Candida* in cancer healthcare settings.

2. Methodology

2.1 Sample Collection

Candida isolates were collected from the Microbiology laboratory of the Apeksha Hospital Maharagama Sri Lanka. The original samples were cultured in a blood agar medium, and for experimental purposes, they were again sub-cultured in Sabouraud Dextrose Agar medium and Sabouraud Dextrose Broth medium.

2.2 DNA Extraction

DNA was extracted using the Qiagen DNeasy Blood and Tissue Extraction Kit (Quiagen India 2019).

2.3 Multiplex PCR of DNA samples

Nine primer pairs specific to *Candida* species (Table 2) were categorized into three Primer mixes. (Ps I, Ps II and Ps III (Table 3)) to use in multiplex PCR. Initially all the DNA samples extracted from *Candida* isolates were amplified using the primers in Ps I group and visualized. The DNA samples that tested negative to the primers in Ps I was subjected to amplification using Ps II primers. The remaining DNA samples that tested negative to both Ps I and Ps II were amplified using the primers in Ps III.

DNA samples were amplified in 50 µL reactions containing 10 µL genomic DNA (10–30 µg/mL), 10 µL 10× PCR buffer, 5 µL $MgCl_2$ (25mM), 2.5 µL dNTPs (10 mM), 1.5 µL forward primer (100 pmol/uL), 1.5 µL reverse primer (100 pmol/uL) (total volume of the all forward and reverse primers varies from mix to mix.) with and 1 µL *Taq* DNA polymerase (5 units /µL) (Kanbe *et al*., 2002).

Final volume was adjusted to 50 µL using PCR grade water depending on the total volume of respective primer set.

The PCR was performed, preheating at 96 °C for three min, then 30 cycles of 90 °C for 45 s, 55 °C for 30 s, 72 °C for 90 s with final extension for 10 min at 72 °C.

Table 2: Primers used in this study for PCR amplification and their oligonucleotide sequences. (Kanbe et al., 2002)

Target species	Primer	Direction (F/R)	Sequence
C. albicans	CABF59	F	5'-TTGAACATCTCCAGTTTCAAAGGT-3'
	CADBR125	R	5'-GTTGGCGTTGGCAATAGCTCTG-3'
C. parapsilosis I	CPPIF41	F	5'-TGACAATATGACAAAGGTTGGTA-3'
	CPPIIR122	R	5'-TGTCAAGATCAACGTACATTTAGT-3'
C. parapsilosis II	CPPIIF41	F	5'-GGACAACATGACAAAAGTCGGCA-3'
	CPPIIR69	R	5' -TTGTGGTGTAATTCTTGGGAG-3'
C. dubliniensis	CDBF28	F	5'-AAATGGGTTTGGTGCCAAATTA-3'
	CDBR110	R	5'-GTTGGCATTGGCAATAGCTCTA-3'
C. krusiei	CKSF35	F	5'-GAGCCACGGTAAAGAATACACA-3'
	CKSR57	R	5' -TTTAAAGTGACCCGGATACC-3'
C. kefyr	CKFF35	F	5' -CTTCCAAAGGTCAGAAGTATGTCC-3'
	CKFR85	R	5'-CTTCAAACGGTCTGAAACCT-3'
C. glabrata	CGBF35	F	5' -CCCAAAAATGGCCGTAAGTATG-3'
	CGBR103	R	5' -ATAGTCGCTACTAATATCACACC-3'
C. tropicalis I	CTPIF36	F	5' -GTTGTACAAGCAGACATGGACTG-3'
	CTPIR68	R	5' -CAAGGTGCCGTCTTCGGCTAAT-3'
C. tropicalis II	CTPIIF36	F	5' -CTGGGAAATTATATAAGCAAGTT-3'
	CTPIIR121	R	5' -TCAATGTACAATTATGACCGAGTT-3'

Table 3: Primer mixes and specific primers in each of the mixes (Kanbe et al., 2002)

Primer Mix	Target species	Forward primer	Reverse primer	Size of the PCR product (bp)
Ps I	*C. albicans*	CABF59	CADBR125	665
	C. parapsilosis I	CPPIF41	CPPIR122	837
	C. parapsilosis II	CPPIIF41	CPPIIR69	310
Ps II	*C. dubliniensis*	CDBF28	CDBR110	816
	C. krusei	CKSF35	CKSR57	227
	C. kefyr	CKFF35	CKFR85	532
	C. glabrata	CGBF35	CGBR103	674
Ps III	*C. tropicalis I*	CTPIF36	CTPIR68	318
	C. tropicalis II	CTPIIF36	CTPIIR121	860

2.4 Visualization of PCR products

All PCR products were visualized by running gel electrophoresis at 100 V for 30 min, using 0.8% agarose gels stained with ethidium bromide, under UV light. The gels were photographed using the BioRad GelDoc system.

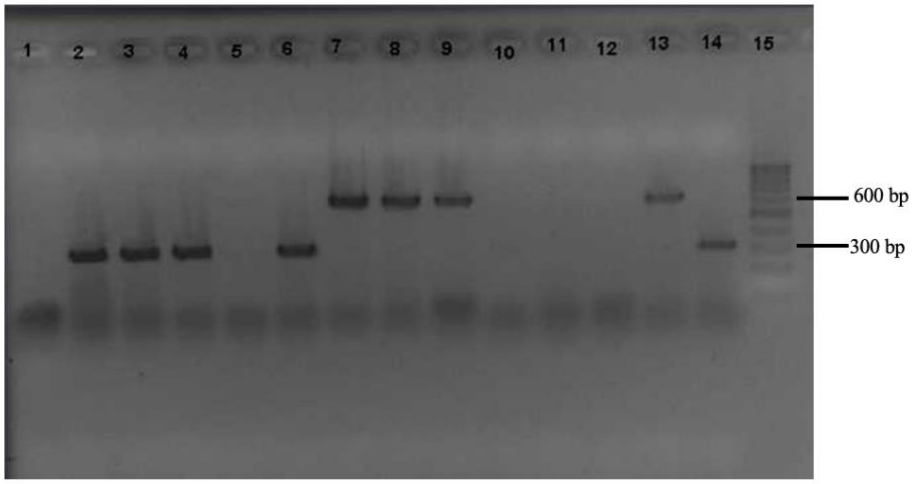
3. Results

Out of the 52 *Candida* isolates,49 isolates were identified using the multiplex PCR procedure.

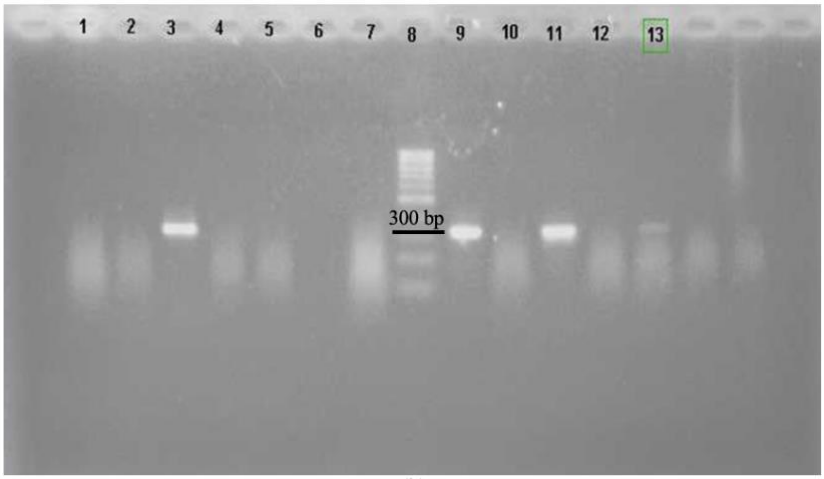
Out of 49 *Candida* isolates 20 were *C. tropicalis II* (38%), followed by 16 *C. parapsilosis* II (31%), seven *C. albicans* (13%), four *C. glabrata* (8% and two *C. krusei* (4%).

C. tropicalis strains I / II and *C. parapsilosis* strains I / II can be differentiated using multiplex PCR primers that used in this study. Out of 52 samples all 20 *C. tropicalis* samples were identified as *C. tropicalis II (*38%) and all

16 *C. parapsilosis* samples were identified as *C. parapsilosis II* (31%) and *C. tropicalis* I and *C. parapsilosis* I were not recorded.



(a)

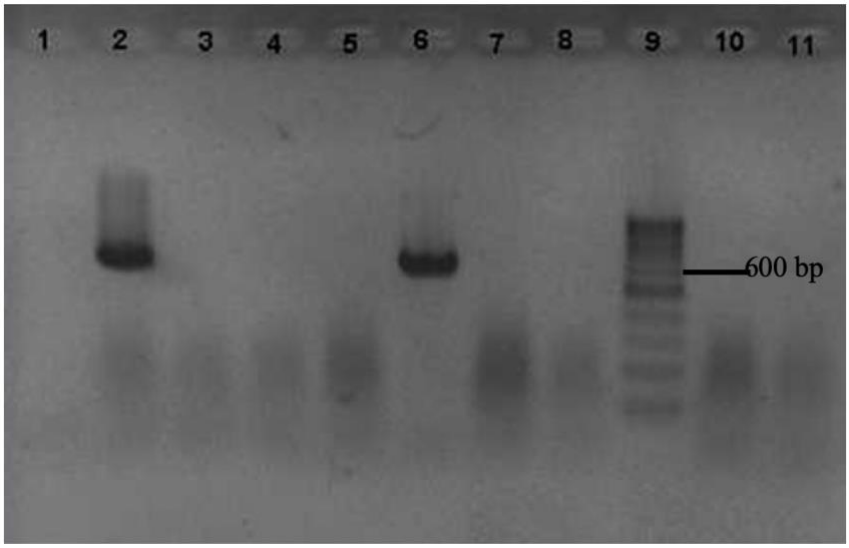


(b)

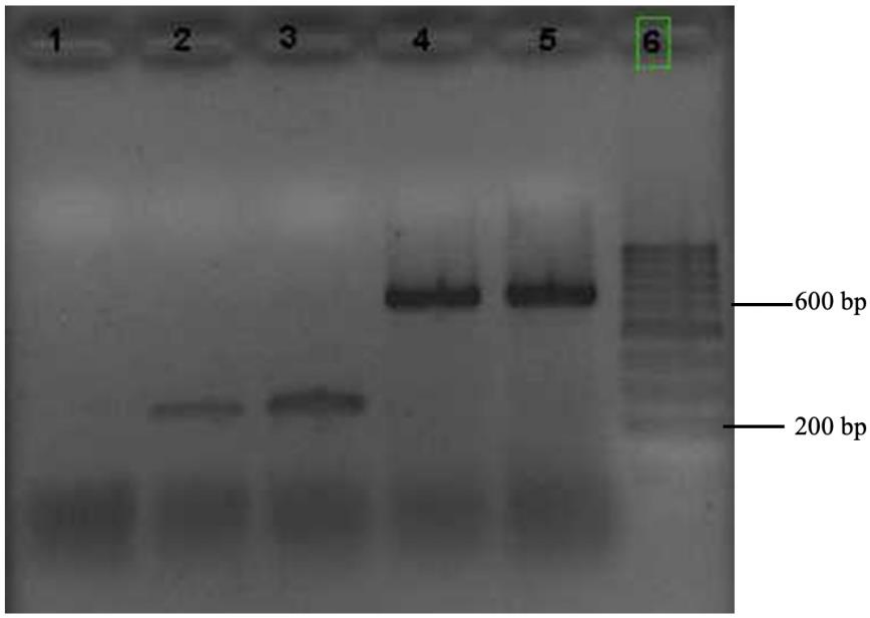
Figure 1: Multiplex PCR products of *Candida* isolates with the Ps I primer mix separated on 0.8% agarose gel.

(a) Lane 1: negative control; lane 2,3,4,6,14: *C. parapsilosis II*; lanes 7,8,9,13: *C. albicans*; lane 15: Molecular marker.

(b) Lane 1 and 2: negative control; lane 3,9,11: *C. parapsilosis II*



(a)



(b)

Figure 2: Multiplex PCR products for the Ps II primer mix separated on 0.8% agarose gel

(a) Lane 1: negative control; lane 2 and 6: *C. glabrata*; lane 9: Molecular marker.

(b) Lane 1: negative control; lane 2 and 3: *C. krusei*; lane 4 and 5: *C. galbrata* ; Lane 6 : Molecular marker.

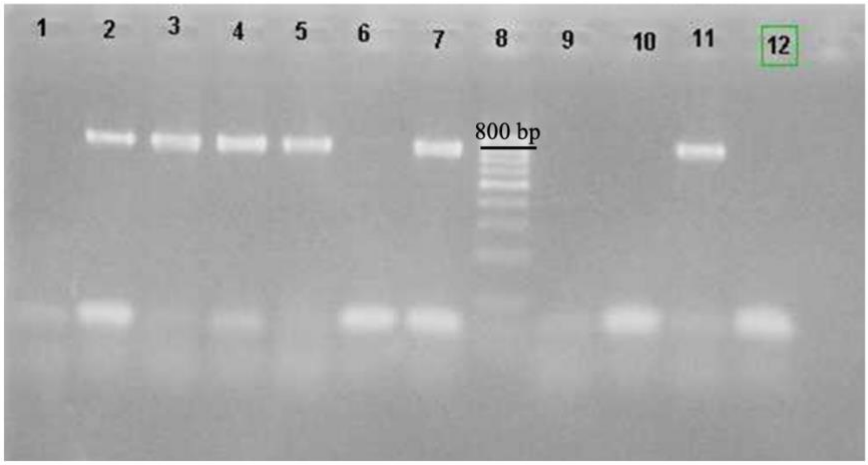


Figure 3: Multiplex PCR products for the Ps III primer mix separated on 0.8% agarose gel.
Lane 1: negative control; lanes 2,3,4,5,7 and 11: *C. tropicalis II*; Lane 8: Molecular marker.

4. Discussion

Candida infections have increased significantly in recent years which may be due to the aggressive use of immunosuppressants among patients such as cancer patients. Rapid and accurate *Candida* identification plays a critical role in effective management of *Candida* infections.

The DNA topoisomerase II gene is present in all eukaryotes, and its nucleotide sequence is composed of highly conserved regions separated by species-specific regions. The sequence analysis of the DNA topoisomerase II (DNA gyrase) gene was applied not only to understand phylogenetic relationships but also for development of a diagnostic identification system of broad species of medically important microorganisms by PCR (Huang, 1996). Based on the characteristics of the *Candida* DNA topoisomerase II genes, Kato *et al*., 2001 suggested that they were suitable as targets for PCR based identification of several *Candida* species. This study used previously designed specific primers by Kato *et al*., 2001 to the DNA topoisomerase II genes of *Candida* species by using Multiplex PCR.

49 isolates out of 52 (87.03%) were identified using the multiplex PCR procedure. The Multiplex PCR analysis provided further insights into the specific strains within *C. tropicalis* and *C. parapsilosis*, distinguishing between strain I and strain II. This level of strain identification can have implications for understanding strain-related virulence factors, genetic diversity, and potential differences in antifungal resistance profiles. According to Arastehfar *et al*., 2018 multiplex PCR procedure is one of the most accurate methods to identify *Candida* species. However, for the multiplex PCR procedure, species-specific primers are required. This process could be expensive, and the primer sets should be customized according to the species estimated to be identified.

In this study *C. tropicalis* was the most prominent species, constituting 38% of the isolates. This finding suggests that *C. tropicalis* infections may be more prevalent in the studied population. This finding aligns with some previously conducted research studies. According to Kumar, *2018 C. tropicalis* has also emerged as an important opportunistic fungal pathogen. That study concludes that incidents of *C. tropicalis* infections have risen all over the world in past two decades.

C. parapsilosis was the second most prominent species, accounting for 31% of the isolates. This finding aligns with previous research indicating that *C. parapsilosis* is a significant cause of candidemia and other infections, particularly in healthcare settings. According to Trofa et al., 2008., *Candida parapsilosis* is an emerging major human pathogen that has dramatically increased in significance and prevalence.

In the past, *Candida albicans* was considered the most prevalent species causing *Candida* infections. However, according to some studies in recent years, there has been an increase in the prevalence of non-*albicans Candida*

species such as *C. tropicalis* and *C. parapsilosis*. In a study conducted by Deorukhkar et al., 2014, non-*albicans Candida* species were the predominant pathogens isolated. These findings are like this study because, during the study period, *C. tropicalis* and *C. parapsilosis* were most prominent. A recent study conducted by Sigera et al., 2019 in Sri Lanka has also demonstrated that non albicans species are more prominent than *C. albicans* in Sri Lanka. Their finding on *C. tropicalis* and *C. parapsilosis* being the most prominent species agrees with the findings of this study.

Even though *C. glabrata and C. krusei* may not be as prevalent as C. tropicalis or C. parapsilosis, these species have been known to cause infections with unique characteristics and varying antifungal susceptibility patterns. Further investigation is warranted to understand the specific risk factors and clinical implications associated with these less common *Candida* species.

This study employed multiplex PCR techniques to identify *Candida isolates*. However, to further enhance the accuracy and resolution of results, future investigations can incorporate sequencing methods.

Multiplex PCR offers a higher level of resolution by differentiating specific strains within the identified *Candida* species. And Multiplex PCR is one of the most accurate methods to identify *Candida* species. Researchers interested in understanding the molecular epidemiology of *Candida* infections or investigating specific strain characteristics may find Multiplex PCR to be a more suitable choice (Arastehfar *et al*., 2018).

Multiplex PCR, with its ability to differentiate species and specific strains within identified species, is more valuable for research purposes (such as studying genetic diversity, strain-related characteristics, and molecular epidemiology) and in clinical diagnosis.

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