MOLECULAR IDENTIFICATION AND ANTIFUNGAL SUSCEPTIBILITY OF *CANDIDA TROPICALIS* AND *CANDIDA PARAPSILOSIS* ISOLATED FROM CANCER PATIENTS

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Abstract

Candida species cause systemic candidiasis in immunocompromised cancer patients. Currently, a large proportion of bloodstream infections are due to non-*albicans Candida* species (*Candida* species other than *C. albicans*), with *Candida tropicalis* and *Candida parapsilosis* being the most isolated *Candida* species from cancer patients. In this study, 52 *Candida* isolates were collected from cancer patient at the Apeksha Hospital, Maharagama, Sri Lanka. Molecular identification of isolated *Candida* samples employed a multiplex PCR technique utilizing specific primer pairs for two strains of both *Candida tropicalis* and *Candida parapsilosis*. Furthermore, to determine the susceptibility of the identified isolates, antifungal susceptibility testing was conducted using the disk diffusion method on Mueller-Hinton agar medium. Six antifungal drugs, including Fluconazole, Itraconazole, Clotrimazole, Miconazole, Ketoconazole, and Amphotericin B, were utilized in the susceptibility testing. In this study 38% of the strains were identified as *Candida tropicalis II* while, 31% were identified as *Candida parapsilosis II*. According to this study Fluconazole was the most susceptible drug against both species and Amphotericin B was the least susceptible drug. Ketoconazole, Clotrimazole, Itraconazole and Miconazole showed varying degrees of susceptibility patterns. The study concludes that multiplex PCR is a better approach for the identification of both *Candida tropicalis* and *Candida parapsilosis* for clinical and diagnostic purposes and Fluconazole is the best antifungal drug against *Candida parapsilosis*, while caution is advised when using Amphotericin B as a treatment option since its' low susceptible rates.
Keywords: cancer patients, *Candida tropicalis*, *Candida parapsilosis*, Multiplex PCR, anti-fungal susceptibility test

Introduction

Cancer patients suffer physically and psychologically from their disease condition. It intensifies when they contract infections given their immunocompromised nature. *Candida* is one of the main fungal type responsible for various infections in cancer patients, including candidemia or *Candida* blood infection.

Recently, a substantial fraction of bloodstream infections among cancer patients originates from *Candida* species other than *Candida albicans*. Among them, *Candida tropicalis* and *Candida parapsilosis* have emerged as major human pathogens, relentlessly causing bloodstream infections in these immunocompromised cancer patients.

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Candida tropicalis is one of the most important *Candida* species in terms of virulence and epidemiology. It can produce true hyphae and has also been considered a strong biofilm producer species and is highly adherent to epithelial and endothelial cells (Marcos-Zambrano et al. 2014). In addition, several cases reported the *C. tropicalis* resistant to the antifungal drugs currently available, such as the amphotericin B, azoles derivatives and echinocandins (Choi et al. 2016, Seneviratne et al. 2016).

C. parapsilosis is one of the leading causes of invasive candidal diseases particularly among immunocompromised individuals such as cancer patients. Secretion of hydrolytic enzymes, adhesion to prosthetics, and biofilm formation are the factors involved in disease pathogenesis of *C. parapsilosis* (Trofa et al. 2008).

The urgent need for a rapid and effective methodology for the identification and antifungal susceptibility testing of *Candida tropicalis* and *Candida parapsilosis* is evident. It allows clinicians to use the most appropriate antifungal agent in-order to decrease the mortality and morbidity rates of their cancer patients infected with *Candida tropicalis* and *Candida parapsilosis*.

This study aims to identify the *Candida tropicalis* and *Candida parapsilosis* among immunocompromised cancer patients at the Apeksha Hospital Maharagama, to assess their resistance to certain antifungal drugs, and to employ that knowledge in effective treatment in the future.

Materials and Methods

Sample collection and DNA extraction

Fifty-two *Candida* isolates collected from the Microbiology laboratory of the Apeksha Hospital Maharagama from September 2022 to February 2023 were used in the study.

The original 52 isolates cultured in a blood agar medium were brought to Molecular Laboratory, Medical Research Institute. All the isolates were subcultured on Sabouraud Dextrose Agar medium.

The 48- hour incubated cultures were stored at 4 °C to be used in further experiments.

DNA was extracted from *Candida* isolates, grown in Sabouraud Dextrose broth medium using the Qiagen DNeasy Blood and Tissue Extraction Kit (Quiagen 2020).

Multiplex PCR of DNA samples

Four primer pairs specific to *C. tropicalis* and *C. parapsilosis* (Table 1) were categorized into two Primer mixes {Ps I and Ps II (Table 2)} to be used in multiplex PCR. This study used previously designed specific primers by Kato et al., 2001 to the DNA topoisomerase II genes of *C. tropicalis* and *C. parapsiolosis*. 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 strains of *C. tropicalis* (*C. tropicalis I* / *C. tropicalis II*) and *C. parapsilosis* (*C. parapsilosis I* / *C. parapsilosis II*).

DNA samples were amplified in 50 µL reactions containing 10 µL genomic DNA (10–30 µg/mL), 10 µL 10× PCR buffer, 5 µL MgCl2 (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 primer pairs.

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 1. Primers used in this study for PCR amplification for *C. tropicalis* and *C. parapsilosis* and their oligonucleotide sequences (Kanbe et al. 2002)

Target species	Primer	Direction (F/R)	Sequence
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. 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 2. Primer mixes and the size of multiplex PCR for *C. tropicalis* and *C. parapsilosis* (Kanbe et al. 2002)

Primer Mix	Target species	Forward primer	Reverse primer	Size of the PCR product (bp)
Ps I	*C. parapsilosis I*	CPPIF41	CPPIR122	837
	C. parapsilosis II	CPPIIF41	CPPIIR69	310
Ps II	*C. tropicalis I*	CTPIF36	CTPIR68	318
	C. tropicalis II	CTPIIF36	CTPIIR121	860

Visualization of Multiplex PCR products

All PCR products and restriction fragments 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.

Antifungal susceptibility testing using the disc diffusion method.

Antifungal susceptibility testing by disk diffusion methods was performed using Mueller-Hinton agar medium according to the CLSI guidelines (CLSI document M44-A) and manufacturer's instructions with six antifungal drugs (Fluconazole, Itraconazole, Clotrimazole, Miconazole, Ketoconazole and Amphotericin B). The media and antifungal disks used in the tests were from HiMedia Laboratories 2021.

The colonies of identified *C. tropicalis* and *C. parapsilosis* isolates were suspended in 5 mL of sterile 0.85% saline, and the turbidity was adjusted to yield 1×10^5 -1×10^6 cells/mL (0.5 McFarland standard). *Candida* suspensions were spread on the medium using sterile cotton swabs according to the manufacturer's instructions. 25 antifungal disks were placed on the inoculated Mueller-Hinton agar plates using the dispenser provided with each disc set, and the plates were incubated at 37 ^{0}C. The zones of inhibition were recorded after 24 hours. Zone diameters were read manually with a calliper to determine inhibition zones.
The interpretive criteria for antifungal susceptibility testing were those published by the HiMedia Laboratory's instructions.

Results

Multiplex PCR amplification

Out of 52 samples 20 samples were identified as *C. tropicalis II* strain (38%) and 16 samples (31%) were identified as *C. parapsilosis* strain. *C. tropicalis* strains I / II and *C. parapsilosis* strains I / II can be differentiated using multiplex PCR (Figures 1, 2).
C. tropicalis I and *C. parapsilosis I* strains were not recorded in this study.

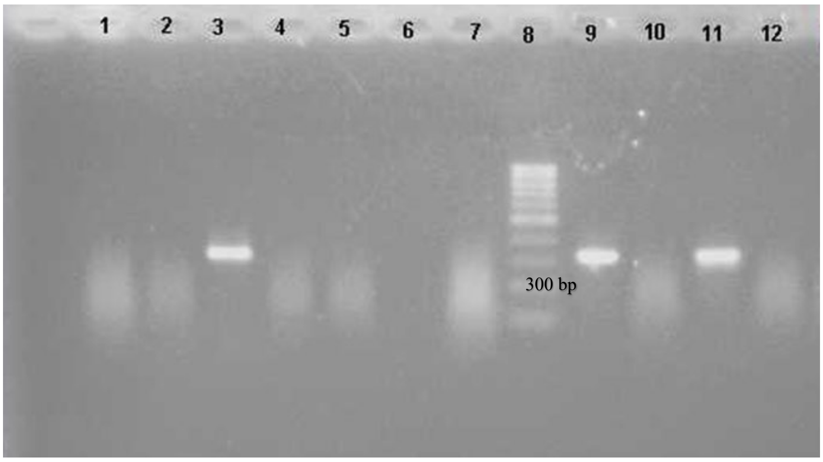

Figure 1. Multiplex PCR products of C. *parapsilosis II,* separated on 0.8% agarose gel.
Lane 1: Negative control, Lane 8 – 1000 bp ladder, Lane 3,9 and 11 - Amplified products of different *C. parapsilosis II* (isolate number 3,8 and 9 respectively)

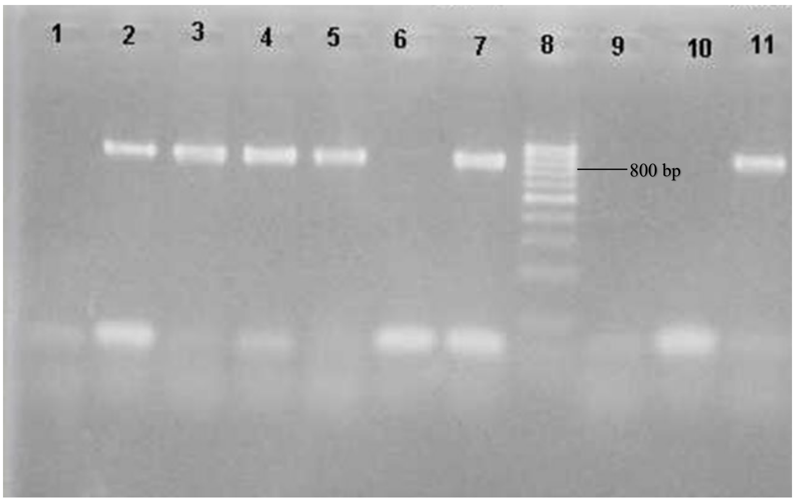

Figure 2. Multiplex PCR products for *C. tropicalis II*, separated on 0.8% agarose gel.
Lane 1: Negative control, Lane 8 – 1000 bp ladder, Lane 2,3,4,5,7 and 11 - Amplified products of *Candida tropicalis II* (isolate number 2,4,5,7,10 and 11 respectively).

Antifungal susceptibility testing.

Antifungal susceptibility testing was conducted on a total of 20 samples identified as *C. tropicalis II* and 16 samples identified as *C. parapsilosis II.*

Among tested six antifungal drugs Fluconazole was the most susceptible drug against *C. tropicalis* and *C. parapsilosis* and Amphotericin B was the most resistant drug. Ketoconazole, Clotrimazole, Itraconazole and Miconazole showed varying degrees of susceptibility patterns.

Out of 20 *C. tropicalis II* isolates, 18 were susceptible to Fluconazole (90%) and two were resistant (10%) to Fluconazole. Six were susceptible (30%), 13 were intermediate (65%) and one were resistant (5%) to Itraconazole. Two were susceptible (10%), 10 were intermediate (50%) and 8 were resistant (40%) to Clotrimazole. Out of 20 *C. tropicalis* isolates, 15 were susceptible (75%), three were intermediate (15%) and two were resistant (10%) to Miconazole. Nine were intermediate (45%) and 11 were resistant (55%) and none of isolates were susceptible to Ketoconazole. Finally, to Amphotericin B, 17 were resistant (85%), three were intermediate (15%) and none of isolates were susceptible to Amphotericin B.

Out of 16 *C. parapsilosis II*, all 16 were susceptible to Fluconazole (100%). Ten were susceptible (62.5%), six were intermediate (37.5%) and none of isolates were resistant to Itraconazole. Seven were susceptible (43.75%), seven were intermediate (43.75%) and two were resistant (12.5%) to Clotrimazole. Out of 16 isolates 12 were susceptible (75%) and four were resistant (25%) to Miconazole. Nine were susceptible (56.25%), five were intermediate (31.25%) and two were resistant (12.5%) to Ketoconazole. Finally, to Amphotericin B, 12 were resistant (75%), four were intermediate (25%) and none of isolates were susceptible to Amphotericin B (Table 3).

Table 3. Results of antifungal susceptibility testing (AFST) of *C. tropicalis II* and *C. parapsilosis II strains* against six common antifungals

Species	Antifungal agent	Potency/ mg	Number of isolates	% S	% I	% R
C. tropicalis II			20			
	FLC	20		90	0	10
	IT	25		30	65	5
	CC	30		10	50	40
	MIC	10		75	15	10
	KT	30		0	45	55
	AMP	30		0	15	85
C. parapsilosis II			16			
	FLC	20		100	0	0
	IT	25		62.50	37.50	0
	CC	30		43.75	43.75	12.50
	MIC	10		75	0	25
	KT	30		56.25	31.25	12.50
	AMP	30		0	25	75

FLC - Fluconazole, IT - Itraconazole, CC - Clotrimazole, MIC - Miconazole, KT -Ketoconazole, AMP - Amphotericin B.

S - Susceptible, I - Intermediate, R - Resistant.

Discussions

Candida bloodstream infections are responsible for as high as 50% mortality rate among the infected patients. Non-*albicans Candida* spp. like *C. tropicalis* and *C. parapsilosis* infections have increased significantly among cancer patients in recent years. Therefore, identification and antifungal susceptibility testing of *C. tropicalis* and *C. parapsilosis* play a critical role in managing infected cancer patients.

The study site for this research is the Apeksha Hospital in Maharagama, Sri Lanka. It is the largest hospital in the country dedicated to cancer treatment. Apeksha Hospital receives patients from all around the country, and the samples collected were not filtered. It can be assumed that the *Candida* species diversity observed during the study period could be a good indicator of the pathogenic *Candida* diversity.

Multiplex PCR is one of the most accurate methods to identify *Candida* species and it offers a higher level of resolution by differentiating specific strains within the identified *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).

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 (Keller et al. 1997). 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.

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), *C. parapsilosis* is an emerging major human pathogen that has dramatically increased in significance and prevalence.

In the past, *C. 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.

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 results of this study *C. tropicalis II* strain and *C. parapsilosis II* strain predominate the studied sample population.
This study employed multiplex PCR technique to identify *C. tropicalis* and *C. parapsilosis*. However, to further enhance the accuracy and resolution of results, future investigations can incorporate sequencing methods.
It is evident that Fluconazole emerges as a consistently effective drug against both species. According to this study Fluconazole could be a reliable treatment option for *Candida* infections caused by these species. Cha and Sobel (2004), also state exceptional therapeutic record of Fluconazole for various *Candida* infections including, candidemia.
Miconazole also demonstrates notable efficacy, ranking as the second most susceptible drug against *C. tropicalis* and *C. parapsilosis*. Therefore, Miconazole can be used as an alternative treatment option, especially when Fluconazole may not be suitable or available. This finding aligns with the study conducted by Barasch and Griffin (2008). They have reviewed recent evidence on the efficacy of Miconazole, and its effect on *Candida* infections.
Amphotericin B exhibits a concerning trend of resistance among both species studied. Therefore, clinicians should be caution when considering Amphotericin B as a therapeutic option against *C. tropicalis* and *C. parapsilosis*. According to Centres for Disease Control and prevention (CDC) up to one-third of *Candida* species are resistant to the Amphotericin B (www.cdc.gov 2020).
Both species exhibit varying susceptibility profiles to the remaining antifungal drugs, Itraconazole, Clotrimazole, and Ketoconazole. So, it highlights the importance of considering species-specific treatment strategies when dealing with *Candida* infections. Choosing of antifungal drug based on the susceptibility profile of the specific *Candida* species could lead to improved treatment outcomes in cancer patients.
The presence of intermediate response rates observed for some antifungal drugs, implies the need for further investigation and careful consideration of these drugs' efficacy against *Candida* infections in cancer patients. Intermediate responses may arise due to reduced effectiveness or potential development of resistance over time.

Conclusions

C. tropicalis and *C. parapsilosis* species are the more prominent *Candida* species that caused infections among studied cancer patient population.
Our findings suggest that Fluconazole is the most reliable treatment option for both species. Miconazole also demonstrates notable efficacy, but Amphotericin B demonstrates a disturbing trend of resistance among the *C. tropicalis* and *C. parapsilosis*.
Finally, this study led to the conclusion that multiplex PCR can be used for precise and rapid species identification for both research and diagnostic application.

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