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Research Article

Multiplex polymerase chain reaction identification of *Candida* species colonized sputum of patients suffering from various respiratory tract disorders in Duhok, Iraq

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ABSTRACT

Background: Candida species are part of the body normal flora. Under certain conditions, these opportunistic microorganisms may lead to infection. The purpose of this study was to identify Candida species isolated from sputum from patients suffering from respiratory tract disorders.
Methods: A total of 59 sputum samples taken from patients attending Azadi hospital at Duhok province, Kurdistan Region/Iraq. For primary isolation, sputum samples were cultured on sabouraud dextrose agar (SDA). Suspected colonies of Candida isolates were then sub cultured on chromogenic Candida agar for presumptive identification. Genomic DNA extraction was performed using a genomic DNA extraction kit. For rapid identification of Candida spp, specific primers based on the genomic sequence of DNA topoisomerase 11 of *C. albicans, C. parapsilosis I, C. parapsilosis II, C. guilliermondi, C. dubliniensis, C. krusei, C. kefyr* and *C. glabrata, C. tropicalis I, C. tropicalis II, C. lusitaniae* were used. The Multiplex PCR products were separated by electrophoresis in 1.5% agarose gel, visualized by staining with ethidium bromide, and photographed.
Results: Three Candida species namely *C. albicans, C. glabrata* and *C. tropicalis* were differentiated by their colour produced on Chromogenic Candida agar. PCR with the primer mixes yielded 4 different sized of PCR products corresponding to *C. albicans, C. glabrata, C. Keyfer and C. tropicalis II, C. glabrata* was the most common species (33.33%), followed by *C. albicans* (16.66%). The highest rate of isolation of Candida species was between the ages of 36 to 45.
Conclusion: This study concluded that phenotypic characteristics on selective agar medium such as chromogenic Candida agar are useful for presumptive identification of Candiada spp with the support of molecular method such as multiplex PCR.

Keywords: Candida species, Multiplex PCR, Sputum, Chromogenic candida agar, Iraq

INTRODUCTION

Candida species are prevalent in the oral cavity. C.albicans is the most common species isolated from oral cavity in both healthy and those suffering from various respiratory tract disorders.[1,2] In a special condition Candida becomes an opportunistic human pathogen and cause candidiasis, which colonizes in several histological special sites, including the skin, oral cavity, esophagus, digestive tract, vagina and blood vessels of humans.[2,3] The non- albicans Candida species increased during the last few decades by replacing *C. albicans* in many clinical samples like bloodstream infection.[2] The medically important non-albicans Candida (NAC) species

includes: *Candida glabrata, Candida tropicalis, Candida parapsilosis, Candida krusei, Candida guilliermondii, Candida lusitaniae, Candida kefyr,* and *Candida dubliniensis*, but they exhibits lower isolation rates, and there are also other species which are non-pathogenic.[4] Several studies dealt with the identification and characterized Candida species isolated from sputum in HIV positive patients[5-7] and from pulmonary tuberculosis patients.[8,9] In Iraq, however, eight Candida species namely *C. albicans, C. curvata, C. glabrata, C. kefyr, C. krusei, C. paropsiloses, C. tropicalis and C. utilis* were detected in sputa of patients with pulmonary tuberculosis in Basrah, Iraq.[8] *C. albicans, C. krusei and C. tropicalis* were also identified in sputa of patients with lower respiratory tract infection in Mosul, Iraq.[10] Traditionally, the identification and classification of Candida species were done by time consuming and unreliable methods such as serotyping.[11] Colony morphotyping,[12] conventional culture techniques, and morphological and biochemical analysis.[13] Nonetheless, the improvement in molecular assay technology for identifying Candida species, such as randomly amplified polymorphic DNA analysis (RAPD), has overcome these limitations during the last couple of years. However, methods such as single and direct PCR or Multiplex PCR have not been used extensively despite being highly sensitive and specific with a shorter turn-around time.[14-17] Multiplex PCR is a rapid diagnostic assay which combines many specific species primers in one PCR tube. Hence, it could be used to identify more than one species in a specimen simultaneously.[18]

The aim of this study is directed to isolate and identify Candida species in sputa from patients suffering from different respiratory tract disorders attending Azadi hospital, Duhok province, Kurdistan Region, Iraq using Multiplex PCR method.

METHODS

Sputum samples were taken from 59 patients suffering from various respiratory tract disorders attending the Azadi hospital, Duhok city, Kurdistan region, Iraq during a period from October, 2013 to March 2014.

Each sample was homogenized and diluted in screw capped vials (28 ml.) Containing 3-5 ml of sterilized distilled water and small sterilized glass beads and shacked with the help of test tube shaker.[8,19] The diluted homogenized sputum sample then inoculated onto Sabouraud dextrose agar (SDA) (Lab. M Limited Co.Uk) amended with 0.250 mg/L Chloramphenicol for primary isolation of Candida species. Plates were incubated at 37˚C and examined after 2-3 days. Suspected Candida colonies with white to cream coloured were sub cultured onto fresh SDA plates and incubated for 24-48 hours.[4,20] For presumptive identification, Candida isolates were sub cultured onto chromogenic candida agar (Rapid labs Ltd,Essex,UK) which differentiate Candida species on the basis of colour change.[21] According to the manufacturer's instructions the colors of the colonies are as follows: *Candida albicans* (Emerald, with metallic shine), *C. dublinensis* (Dark green, matt), *C. glabrata* (White, shiny), *C. krusei* (Light pink), *C. tropicalis* (Red purple).

Genomic DNA extraction and purification were performed using a Genomic DNA Extraction kit (provided by Jena Bioscience GmbH/ Germany) based on the guidelines.

For Multiplex PCR using primer mixes each one of the primer pairs were designed for a species of Candida was grouped, based on the following criteria: (a) no primers in a group form dimers and /or interrupt PCR amplification; (b) the number of the group should be as small as possible;(c) each primer pairs yields one major PCR product, and each species of Candida should clearly be distinguished by the size (bp) of the PCR products, these primers were described by Kanbe et al. for sequencing of the Candida DNA topoisomerase II genes.[22] In this study, three sets of primer groups were prepared. These groups were designated S1, S11 and S111, and referred to as a 'primer mix 'in this study. S1was composed of four specific primer pairs for identification of *C. albicans, C. parapsilosis I, C. parapsilosis II* and *C. guilliermondii*; S11 was for *C. dubliniensis, C. krusei, C. kefyr* and *C. glabrata*; and S111 was for *C. tropicalis I, C. tropicalis II, C. lusitaniae*. For the set S1 and S11 primers the PCR was performed in 25 µl reaction mixture consisting of approximately 5 µl of template DNA, 10 µl of Taq DNA Polymerase 2X Ready Mix, 1 µl of forward and reverse primers (1 µl) for each one and 2µl of PCR-grade water all of these kept in a single tube. While for set S111 primers the amount of water was increased to the 4 µl in the reaction mixture. The species-specific primer pairs used in each mix and calculated sizes of PCR products generated by each set of the mixes are listed in Table 1.

The PCR cycle parameters were as follows; one cycle of initial denaturationat 95˚C for 5 min; then 35 cycles of denaturation at 95˚C for30 sec, annealing at 57˚C for 30 sec, and extension at 72˚C for 1 min; and followed by one cycle of final extension at 72˚C for 2 min. PCR products were analysed by agarose gel electrophoresis in 1X TBE buffer at 100 V for 120 min in gel composed of 1.5% agarose. 100bp ladder DNA Marker was run with PCR products for sizing of the bands. Gels were stained with ethidium bromide solution (concentration of 0.5 µg/ml) for 30 min, then visualized with a UV transilluminater and photographed.

RESULTS

Out of 59 sputum samples obtained from patients suffering from respiratory tract disorders and cultured on sabouraud dextrose agar, 18 samples (30.50%) were positive for Candida species. The highest positive Candida isolates were obtained from samples taken from patients >46 years (Table 2).

The isolates which were cultured on Sabouraud dextrose agar and showed positive results were re-cultured on chromogenic candida agar plate (Rapid Labs Ltd., Essex, U.K.) and incubated at 37°C for 48 hr. Out of 18 samples 13 isolates were identified to their species level on chromogenic candida agar medium (Figure 1). These included *C. albicans* (Emerald with metallic shine) (5/18) 27.78%, *C. glabrata* (White shiny) (6/18) 33.33% and *C. tropicalis* (Red purple) (2/18) 11.11%. Five isolates were not diagnosed by chromogenic Candida agar medium, so, 72.22% of the isolates were only identified (Table 3).

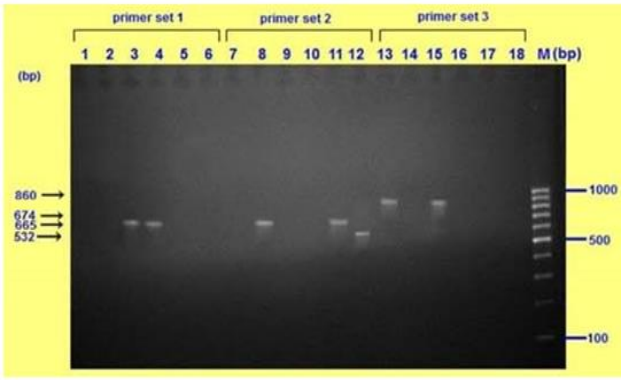
Table 1: Primers for PCR amplification used in this study and their sequences.

Set No.	No	Target species	Forward primer	Reverse primer	Sizes of PCR product (bp)
Set S1	**1**	***C. albicans***	CABF59 (5-TTGAACATCTCCAGTTTCAAAGGT-3)	CADBR125 (5-AGCTAAATTCATAGCAGAAAGC-3)	665
	2	***C. parapsilosis I***	CPPIF41 (5-TGACAATATGACAAAGGTTGGTA-3)	CPPIR122 (5-TGTCAAGATCAACGTACATTTAGT-3)	837
	3	***C. parapsilosis II***	CPPIIF41 (5-GGACAACATGACAAAAGTCGGCA-3)	CPPIIR69 (5-TTGTGGTGTAATTCTTGGGAG-3)	310
	4	***C. guilliermond***	CGLF41 (5-CCCAAAATCACAAAGCTCAAGT-3)	CGLR61 (5-TACGACTTGAAGTTGCGAATTG-3)	205
Set S11	**1**	***C. dubliniensis***	CDBF28 (5-AAATGGGTTTGGTGCCAAATTA-3)	CDBR110 (5-GTTGGCATTGGCAATAGCTCTA-3)	816
	2	***C. glabrata***	CGBF35 (5-CCCAAAAATGGCCGTAAGTATG-3)	CGBR103 (5-ATAGTCGCTACTAATATCACACC-3)	674
	3	***C. kefyr***	CKFF35 (5-CTTCCAAAGGTCAGAAGTATGTCC-3)	CKFR85 (5-CTTCAAACGGTCTGAAACCT-3)	532
	4	***C. krusei***	CKSF35 (5-GAGCCACGGTAAAGAATACACA-3)	CKSR57 (5-TTTAAAGTGACCCGGATACC-3)	227
Set 111	**1**	***C. tropicalis I***	CTPIF36 (5-GTTGTACAAGCAGACATGGACTG-3)	CTPIR68 (5-CAAGGTGCCGTCTTCGGCTAAT-3)	318
	2	***C. tropicalis II***	CTPIIF36 (5-CTGGGAAATTATATAAGCAAGTT-3)	CTPIIR121 (5-TCAATGTACAATTATGACCGAGTT-3)	860
	3	***C. lusitaniae***	CLTF39 (5-CATGTCGAAATGCAACCCCCCG-3)	CLTR119 (5-GCGTACACTTGTGGCCATCTTTA-3)	799

Based on PCR results, four of primer pairs amplified species-specific DNA fragments from genomic DNA template, the size of PCR products was from 532 to 860 bp and they were of a specific size corresponding to each species of Candida (Figure 2 and 3). The DNA of *Candida albicans*, *C. tropicalis II*, *C. glabrata* and *C. kefyrwas* amplified by PCR and generated DNA fragments of 665, 860, 674 and 532 bp, respectively. These were the exact sizes corresponding to each species (Table 1).

Among sputum samples, 83.34% contained only one species of Candida and 16.66% contained more than one species of Candida (Table 5). The results of the analysis of PCR method showed that C. glabrata was the most frequently isolated species 33.33%, followed by *C. albicans* 16.66%, *C. tropicalis II* 11.11%, *C. kefyr* 11.11%, *C. albicans* + *C. tropicalis*II 11.11%, and *C. glabrata* + *C. tropicalis II* 5.55%.

Figure 1: Chromogenic Candida agar plate showing chromogenic color change for A: *Candida albicans* (Emerald, with metallic shine). B: *Candida glabrata* (White, shiny), and C: *Candida tropicalis* (Red purple).



Lanes (3): *C. albicans* (665 bp) and lane (15), *C. tropicalis* II (860 bp), this patient has dual Candida species infection by *C. albicans* plus *C. tropicalis* II. Lane (4): *C. albicans* (665 bp). Lanes (8, 11): *C. glabrata* (674 bp). Lane (12): *C. kefyr* (532 bp). Lanes (13): *C. tropicalis* II (860 bp).

Figure 2: Agarose gel (1.5%) of PCR products obtained with species-specific primers. Lane (M) indicates 100-bp DNA marker.

Lane (1, 2): *C. albicans* (665 bp). The sample in lanes (3, 9, and 15): did not show any result. The sample in lanes (4, 10, and 16): did not show any result. Lanes (11): *C. glabrata* (674 bp) and lane (17): *C. tropicalis* II (860 bp), this patient has dual Candidaspeceis infection by *C. glabrata* plus *C. tropicalis* II. Lane (12): *C. glabrata* (674 bp).

Figure 3: Agarose gel (1.5%) of PCR products obtained with species-specific primers. Lane (m) indicates 100-bp DNA marker.

Table 2: Distribution of Candida among patients suffering from respiratory infection, and their relation to sex and age.

Gender	Age (years)	Number samples examined	No. positive	Culture on Sabouraud's dextrose agar %
Male	15-25	0	0	(0/0) 0%
	26-35	0	0	(0/0) 0%
	36-45	14	4	(4/14) 28.5%
	>46	40	12	(12/40) 30%
Total male		(54/59)91.52%	16	(16/54) 29.62 %
Total from all infection				(16/18) 88.88 %
Female	15-25.	0	0	(0/0) 0%
	26-35.	0	0	(0/0) 0%
	36-45.	0	0	(0/0) 0%
	>46	5	2	(2/5) 40%
Total female		(5/59)8.47%		(2/5)40 %
Total from all infection				(2/18) 11.11 %
Total		59		(18/59) 30.50%

Table 3: Identification of Candida species using chromogenic Candida Agar.

Candida species	Culture on chromogenic Candida agar
	Sputum
C. albicans	(5/18) 27.78%
C. dubliniensis	-----
C. glabrata	(6/18) 33.33%
C. krusei	-----
C. tropicalis	(2/18) 11.11%
Not detected on chromogenic agar	(5/18) 27.78%
Candida spp. identification	(13/18)72.22%

Table 4: Distribution and identification of Candida species using multiplex PCR from sputum swabs.

Candida species	PCR-based identification
	Sputum
C. albicans	(3/18) 16.66%
C. albicans + C.krusei	-----
C. albicans + C. tropicalis II	(2/18) 11.11%
C. dubliniensis	-----
C. glabrata	(6/18) 33.33%
C. glabrata + C. tropicalis II	(1/18) 5.55%
C. krusei	-----
C. tropicalis II	(2/18) 11.11%
C. kefyr	(2/18) 11.11%
C. kefyr + C. tropicalis II	-----
C. guilliermondii	-----
Not identification	(2/18) 11.11 %
PCR-based for identification *Candida species*	(16/18) 88.8%

DISCUSSION

Colonization and contamination of sputum samples by Candida species in patients with various respiratory tract disorders is very common, although the clinical relevance is unclear. However, several studies showed the regular detection of Candida species in sputa of patients with lower respiratory tract infection, cystic fibrosis, pulmonary tuberculosis and HIV infected patients.[5-9]

High isolation rate for Candida species from sputum samples were reported from patients at the age above 50 year in Nepal and India.[23,24] These results are in a agreement with our finding. However, the positive *C. albicans* in the present study was less than those reported by Lathaet al.[24] whereas, the results of the current study for non-albicans Candida species was higher.

Similarly, our study was in coincidence with the results of Kali et al.[25] in term of *C. glabrata* prevalence which was obtained from sputum, with the result of Jhaet al.[23] regarding *C. tropicalis* prevalence and with Latha et al.[24] in terms of *C. albicans* with *C. tropicalis II* and *C. albicans* with *C. krusei* prevalence.

The use of PCR system with the species- specific primer mixes is rapid and it simplifies the assay further than that of PCR using a single pair of primers, because it is possible to identify unambiguously 10 species of Candida from just three sets of primers.[22]

In this study, the colony color and morphology of Candida isolates on chromogenic candida agar were examined and it allowed the presumptive identification of only three Candida spp. namely *Candida albicans* which showed emerald color with metallic shine colony, Candida galbrata showed white shiny colony and *Candida tropicalis* showed red purple colony but it was difficult to differentiate other Candida spp.

The results of the present study indicate that three major species of Candida were isolated from clinical samples assigned to the species while *C. kefyr*, *C. dubliniensis* and *C. guilliermondii* were not identified and distinguished from other species using phenotypic method. This finding is in agreement with previous study.[26,27] Phenotypic characterization on the selective agar medium such as chromogenic candida agar is useful to determine Candida species with support of Molecular diagnosis. The Identification of Candida species with Multiplex PCR is a practical and reliable method, and it is useful for the identification of some of clinically isolated Candida species, such as *Candida kefyr*, *C. dubliniensis* and *C. guilliermondii* which could not be identified by chromogenic candia agar.

CONCLUSION

The result of this study showed that *C. glabrata* was the most common species colonized sputum of patients suffering from various respiratory tract disorders with the prevalence 33.33% followed by *C. albicans* 16.66%. The highest rate of isolation of Candida species was between the ages of 36 to 45.

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