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Full Length Research Paper

Multiple molecular markers for diagnosis of conjunctivitis caused by *Candida* spp. in Iraq

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Candidiasis of the eye and its symptoms may lead to blindness in many patients. This study aimed to survey and diagnose ocular candidiasis by identification of the causative *Candida* spp. from 165 swabs samples collected from patients suffering from conjunctivitis (pink eye infection). A total of 35 clinical isolates of *Candida* spp. were isolated and preliminarily identified by CHROMagar technology. Based on the molecular diagnosis by specific polymerase chain reaction (PCR) primers and *Candida albicans* microsatellite (CAI), 8 of 24 isolates were identified as *C. albicans* and the rest as non-albicans. Ribotyping of 24 isolates was performed for 7 genotypes with a universal primer pair. *C. albicans* was detected with the highest frequency (33%; 8/24), followed by *C. famata* (29%) and C. *rugosa* (4.2%) with the lowest frequency. We concluded that ocular candidiasis is an important disease caused by *Candida* spp. as it was highly prevalent among the sampled swabs (approximately 21.2%; 35/165) from conjunctivitis patients. In addition, the use of molecular markers together with affirmable diagnostic tools for *Candida* isolates can produce coincident results at the molecular level. The elucidation of the results showed existence of type polymorphisms of CAI microsatellite as two bands of 297 and 240 bp.

Key words: Eyes candidiasis, molecular diagnosis, rDNA typing, CAI microsatellite polymorphism, Iraq.

INTRODUCTION

Over the past 10 years, the number of ocular candidiasis has significantly increased, particularly in poor countries (Bharathi et al., 2003; Saha and Das, 2006). Fungal keratitis is an important eyes disease, keratitis is the medical term for inflammation of the cornea, the most frequent cause of keratitis is bacteria, viruses, fungi and parasitic. More than 30 genera of filamentous fungi and yeasts were reported as the causative agents of keratitis. Predominantly saprophytic fungi such as *Fusarium*, *Aspergillus*, *Penicillium*, *Alternaria* and yeasts may cause eye infections in animal and humans (Rosa et al., 2003; Srinivasan, 2004; Thomas and Kaliamurthy, 2013).

Saha and Das (2006) implicated *Candida albicans* as a pathogen in 45.8% of keratitis cases. Callanan et al. (2006) mentioned that *Candida* spp. are opportunistic pathogens that cause mucosal infections such as keratitis, exogenous endophthalmitis infections in post-surgical patients, and other systematic mycoses, depending on the strain virulence and host vulnerability.

Candida is a genus of yeasts and is the most common cause of fungal infections worldwide. Many species are harmless commensalism or endosymbionts of hosts

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including humans; however, when mucosal barriers are disrupted or the immune system is compromised they can invade and cause disease. *C. albicans* is representative of the important pathogens causing exogenous fungal endophthalmitis, endogenous endophthalmitis and keratitis (Gregori et al., 2007). Several studies have been performed in the USA and other geographical areas to investigate the suffering of animals from eye keratitis (Moore et al., 1988; De Sousa et al., 2001). Oude Lashof et al. (2011) reported epidemic of ocular candidiasis in 370 patients, of which 49 had consistent findings with the diagnosis of positive ocular fungal infection; some of these fungi continue to inhabit the patient body as normal flora, while others behave as pathogens. Several reports refer to fungal eye infection as critical, as it accounts for 78% of all endophthalmitis infections (Aliyeva et al., 2004).

Although eyes have an inherent multi-defense mechanism against microbial infections, monitoring the increase of fungal eye infection needs to be synchronized with the increase in the number of patients with acquired immune deficiency (Matthews, 1994). Especially, individuals exposed to broad-spectrum antibiotics over a long period and those afflicted with AIDS are more disposed to fungal eye infection (Scalise, 1997).

Despite availability of reliable and rapid methods for yeasts identification, precise diagnosis of yeasts continues to remain controversial based on the phenotypic criteria such as chlamydospores abundance, germ tube formation, and colony colors on the CHROMagar candida medium and the difference in the colony colors between *C. albicans* and *C. dubliniensis* and others *Candida* spp. that show pink to white-pink color colonies, causing misidentification (Abaci et al., 2008). Most of the available biochemical tests are expensive, time consuming and do not always give rapid and precise results; on the other hand, identification of *Candida* isolates at the molecular level is crucial to assist in early diagnosis and for timely prescription of appropriate antifungal drugs (Imran and Al-Shukry, 2014).

The preference for using combinations of identification tools (phenotypic and molecular tools) in the identification processes is still controversial among researchers: Campbell et al. (1998) reported that ApI *Candida* (ApI= carbohydrate assimilation patterns by commercial test kit gave equivocal results) and Auxacolor tests require supplemental biochemical tests and morphological assessment for accurate taxonomic judgment, while Kanbe et al. (2002) preferred molecular assessment combined with conventional tests. Liguori et al. (2010) considered multiplex polymerase chain reaction (PCR) assays as an alternative to the conventional techniques for the identification of *Candida* spp.

The use of molecular markers for diagnosis of *C. albicans* is an efficient tool with high discriminatory power, including typing of PCR products based on the amplification of the target regions with specific primers like CABF59/CABR110, rDNA typing (Kanbe et al.,2002) and *C. albicans* microsatellite (CAI microsatellite) polymorphism. CAI microsatellite (short tandem locus on chromosome 4), CAA separated by trinucleotides (CTG), and the repeat sequence $(CAA)_n$ $CTG(CAA)_n$ have been increasingly used as markers for genetic analysis. Sampaio et al. (2003) reported the PCR product of CAI microsatellite to be 252 bp in size. Costa et al. (2010) and Dalle et al. (2000) reported the importance of microsatellites loci numbers and the variations in microsatellites lengths in the high level of polymorphisms, suggesting microsatellites (known as simple sequence repeats or short tandem repeats, are repeating sequences of 2-5 base pairs of DNA) as reliable markers for typing *Candida* isolates. PCR analysis of microsatellites was found to be a highly efficient molecular tool for rapid and accurate identification of *C. albicans* strains (Hennequin et al., 2001; Sampaio et al., 2005; Spampinato and Leonardi, 2013). Microsatellite length polymorphisms (MLP) analysis has also recently emerged. Botterel et al. (2001) and Sampaio et al. (2003) reported that microsatellite CAI, located on the non-coding region, imparted discriminatory power and was correlated with high polymorphism.

This study aimed to survey and diagnose *Candida* spp. infecting the human eyes by using the CHROMagar medium and multiple molecular markers such as specific PCR primers for *C. albicans*, rDNA typing and CAI microsatellite polymorphisms.

MATERIALS AND METHODS

Patient samples

A total of 165 swabs of infected human eyes were collected from different age (1 month to 70 years) and gender of patients attending the outpatient clinics in the main hospital of Karbala province, Iraq, between June 2012 and May 2013. All samples were collected using sterile swabs. The swabs were directly diluted in 1 mL sterile distilled water, and a loop full of this suspension was streaked on Sabouraud dextrose agar (SDA) medium plate supplemented with chloramphenicol and streptomycin (50:50 µg/mL). The cultures were incubated for 24-48 h at 37°C (Oliveira et al., 2011).

CHROMagar culturing

Purified single colonies from SDA were streaked on CHROMagar and incubated for additional? 24 - 48 h at 30°C. *Candida* isolates were classified according to their colors on the CHROMagar medium based on the color key described by Nadeem et al. (2010).

Extraction of genomic DNA

A total of 24 isolates of *Candida* spp. were subjected to DNA extraction and PCR analysis. Loopful of single colony suspension of each *Candida* isolates was mixed with lyses buffer [200 mM Tris-HCl, 20 mM ethylenediamine-tetraacetic acid (EDTA), 150 mM NaCl and 0.5% sodium dodecyl sulfate (SDS)] for 10 min by

Table 1. The targets, primer sequences, PCR products and references.

Primer or target region	Name of primer	Sequence (5'-3')	Amplified fragment size (bp)	Reference
Specific primer	CABF59	TTGAACATCTCCAGTTTCAAAGGT	515	Kanbe et al., 2002
	CABR110	GTTGGCGTTGGCAATAGCTCTG		
CAI microsatellite	Forward	ATGCCATTGAGTGGAATTGG	252	Sampaio et al., 2005
	Reverse	AGTGGCTTGTGTTGGGTTTT		
ITS1-5.8S-ITS2	ITS1	TCCGTAGGTGAACCTGCGG	450-650	Tamura et al., 2001
	ITS4	TCCTCCGCTTATTGATATGC		

vortexing. Then, 200 µL of phenol:chloroform was added to the suspension and mixed by centrifugation at 7000 rpm for 2 min. The supernatant was transferred into fresh sterile tubes, to which 500 µL isopropanol was added with mixing by centrifugation at 13000 rpm for 12 min for DNA precipitation. Then, isopropanol was decanted and the pellet was washed twice with 70% ethyl alcohol and centrifuged again at 7000 rpm for 2 min. Next, ethanol was decanted and the pellet DNA was dried and re-suspended in 50 µL Tris-EDTA (TE) buffer and preserved at −20°C until use (Iwata et al., 2006).

Primers and PCR assays

Simple PCR method was performed with a specific pair of primers (Table 1) (CABF59 and CABR110) (Kanbe et al., 2002) and CAI microsatellite pair primer for *C. albicans* (Hennequin et al., 2001), ITS1-ITS4 pair primer for ribotyping and diagnosis of *Candida* spp. (Ahmad et al., 2010). In detail, a total of 0.7 µL DNA for each one of the 24 *Candida* isolates was mixed with the PCR mixture consisting of 12.5 µL 20X Master Mix (Promega), 2 µL of each primer (10 pemole), and 8 µL deionized water made up to 25 µL with molecular-grade water reaction volume; each primer pair set was used in a single PCR reaction, using the Thermal Cycler System (Labenat, USA).

The PCR conditions were designated for all primer pairs: first denaturized at 95°C for 5 min, followed by 30 cycles of initial denaturation at 95°C for 30 s, annealing at 56°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

The PCR products were analyzed by 1.2% agarose gel (Bio Basic Canada Inc.) electrophoresis performed at 100 V using Tris-borate-EDTA (TBE) buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA bands were detected by using the Desktop Gel imager scope 21 ultraviolet transilluminator (Korea Comp.)

RESULTS

The phenotypic tests

A total of 165 swabs were examined from 165 patients; of which, 93/165 (56.4%) were positive for fungal infection. In addition, 53/93 (57%) isolates of filamentous fungi were isolated. The most frequent species were *Aspergillus flavus*, *Aspergillus niger*, *Fusarium solani*, *Acrimonum* sp., and *Blastomyces*. Moreover, 35/93 (37.63%) *Candida* strains were isolated, 8 of which were classified as *C. albicans* (green color colony on CHROMagar) and the others as non-albicans isolates (pink to white-pink color colony on CHROMagar). Due to imprecise distinction between pink and white-pink colors, all non-albicans isolates have been mentioned to show pink color (Table 2).

Molecular diagnosis of *C. albicans* by specific primer pair

A specific primer pair (Table 1) was found to be the best primer for diagnosis of *C. albicans* by successful amplification of the target region for *C. albicans*, producing PCR product of approximately 515-bp size (lanes 1-2, 5, 9-10, 14-15,21), but it did not yield amplification products with non-albican isolates (Figure 1). *C. albicans* was detected with the highest frequency (33%; 8/24), followed by *C. famata* (29%) and C. *rugosa* (4.2%) with the lowest frequency (Table 2).

Ribotyping of ITS1-5.8S-ITS2 region

Amplification of the ITS1-5.8S-ITS2 region of 24 isolates of *Candida* spp. with the universal primer pair ITS1 and ITS4 (standard ITS1+ITS4 primers used by most labs for amplified The ITS region for most widely sequenced DNA region in fungi) yielded PCR products ranging from 419 to 650 bps in length; some PCR products showed size species-specific differences: *C. albicans* was about 530 bp; *Candida famata* 650 bp; *Candida saitoana* 600 bp, and *Candida rugosa* 419 bp (Table 2, Figure 2). The pink color of non-albican isolates on the CHROMagar medium made it difficult to identify these isolates in comparison with molecular diagnosis with universal primer pair ITS1 and ITS4 (Table 2).

The ribotyping rDNA of the ITS1-5.8S-ITS2 region for 24 isolates of *Candida* spp. revealed 7 genotypes: the *C. albicans* showed a high-frequency percentage (33%; 8/24), followed by *C. famata* (29%), and a lower frequency for C. *rugosa* (4.2%) (Table 2). Ribotyping rDNA of *Candida* species elucidate no intraspecies variability among 24 isolates of *Candida* of interest (Figure 2).

Table 2. The colony color on CHROMagar medium, frequency percentage based on ribotyping, and PCR products of the set of specific, universal and microsatellite primers pairs.

Candida sp.	Isolate no.	CHROMagar color	PCR product (bp)			Frequency (%)
			ITS	CABC	CAI	
Candida albicans	1	Green	530	515	240&297	33.3
C. albicans	2	Green	530	515	240&297	33.3
C. famata	3	Pink	650	-	-	29.2
C. rugosa	4	Pink	419	-	-	4.2
C. albicans	5	Green	530	515	240&297	33.3
C. famata	6	Pink	650	-	-	29.2
C. famata	7	Pink	650	-	-	29.2
C. famata	8	Pink	650	-	-	29.2
C. albicans	9	Green	530	515	240&297	33.3
C. albicans	10	Green	530	515	240&297	33.3
C. famata	11	Pink	650	-	-	29.2
C. famata	12	Pink	650	-	-	29.2
C. utilis	13	Pink	560	-	-	12.5
C. albicans	14	Green	530	515	240&297	33.3
C. albicans	15	Green	530	515	240&297	33.3
C. famata	16	Pink	650	-	-	29.2
C. saitoana	17	Pink	600	-	-	12.5
C. saitoana	18	Pink	600	-	-	12.5
C. inconspicua	19	Pink	503	-	-	4.2
C. saitoana	20	Pink	600	-	-	12.5
C. albicans	21	Green	530	515	240&297	33.3
C. *guilliermondii*	22	Pink	603	-	-	4.2
C. utilis	23	Pink	560	-	-	12.5
C. utilis	24	Pink	560	-	-	12.5

Figure 1. Gel electrophoresis of PCR products of amplified target regions for primers CABF59 and CABR110. Bands in lanes (1-2, 5, 9-10, 15-16, 21) are for *C. albicans* (515 bp); others lanes are for non-albicans isolates (no target region). M = molecular marker, 100 bp.

Polymorphism of CAI microsatellite of *C. albicans* isolates

The amplification of repeated CAI microsatellite identified 8 isolates as *C. albicans* from 24 isolates. CAI microsatellite pair primer was successfully amplified by CAI microsatellite for all *C. albicans* and produced two bands of approximately 240 and 297 bp sizes, but it could not amplify for non-albicans isolates (Table 2 and Figure 3).

DISCUSSION

Over the past 10 years, the number of ocular candidiasis has significantly increased, particularly in poor countries (Saha and Das, 2006). The study presented herein showed a high prevalence of ocular candidiasis, 93 (56.4% patients samples) of 165 patients with fungi caused by *Candida* were positive for fungal infection (21.2% patients samples) affecting the human eye health and aesthetics of the patient (Oude Lashof et al., 2011).

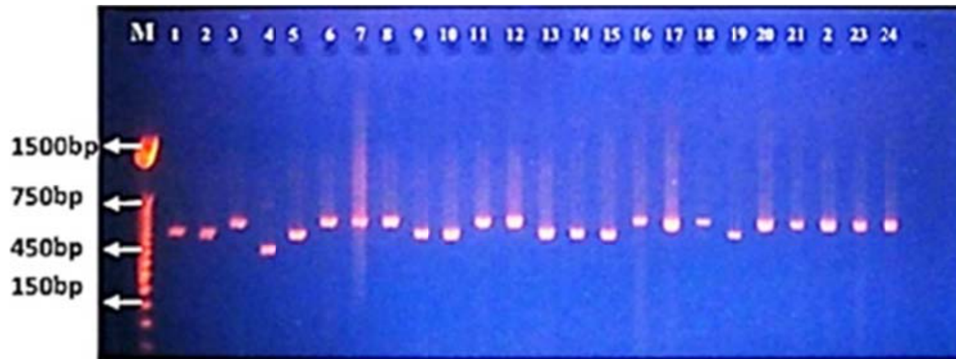

Figure 2. Gel electrophoresis of PCR product of the amplified target region for the primer ITS1-5.8S-ITS2. Bands in lanes 1-2, 5, 9-10, 14-15, and 21 are for *C. albicans* (530 bp); 4 for *C. rugosa* (419 bp); 3,6-8,11-12, and 16 for *C. famata* (650 bp); 22 for C. *guilliermondii* (603 bp); 13, 23-24 for *C. utilis* (560 bp); 17, 18, 20 for *C. saitoana* (600 bp); 19 for *C. inconspicua* (503 bp). M = molecular marker, 100 bp.

Figure 3. Gel electrophoresis of PCR product of the amplified target region for primers in CAI microsatellite. Bands in lanes 1-2, 5, 9-10, 15-16, and 21 are for *C. albicans* (240&297 bp); others lanes are for non-albicans isolate s(no target region). M = molecular marker, 100 bp.

C. albicans was the most frequent cause of ocular candidiasis, followed by *C. famat*, *C. saitoana* and *C. regosa*, respectively. This result is coincident with earlier studies of Durand (2013) and who mentioned a high prevalence of *C. albicans* as the most common cause of ocular candidiasis, accounting for 92% of the cases in this review.

We believe emphasize that the rate of such infection is higher among patients in Iraq who receive lesser care and inadequate and imprecise diagnosis. Fungal keratitis is most common in tropical regions and developing countries, where it constitutes over 50% of keratitis (Galarreta et al., 2007). Our results show that 37.63% of eyes candidiasis caused by *Candida* spp. And in accordance with earlier studies in South India, about 44% of corneal ulcers are caused by fungi. 17% in Nepal, 36% in Bangladesh, 38% in Ghana and 35% in south Florida in the US (Upadhyay et al., 1991; Hagan et al.,1995). For efficient treatment with antifungals, early diagnosis of invasive *Candida* infection and accurate identifications of the causative agents by molecular markers are necessary and also accelerates clinical administration for the better treatment decision and increases the chance of successful treatment and recovery of the patients; moreover, several of the conventional diagnosis methods are not very efficient.

Our results based on use multiple molecular markers for diagnosis of conjunctivitis caused by *Candida* highlight the demerits of the routine conventional assay such as CHROMagar candida medium used, which is in agreement with the report of Ahmed et al. (2010) who asserts that phenotypic tests may not always provide clear identification for most *Candida* spp.; as 16 isolates of *Candida* appear as pink or white-pink color colony, which could be delusive because several *Candida* spp. produce approximately the same reaction color on CHROMagar (Beighoton et al., 1995) (Table 2). Therefore, molecular methods, especially PCR, are being increasingly used for the rapid detection of fungal spp. (Tamura et al., 2001; Alfonso, 2008; Ferrer and Ali, 2011). However, the use of a single molecular marker may not be sufficient for precise diagnosis of all *Candida*

infections because of microevolutionary changes among fungal species (Sampaio et al., 2005).

On the other hand, the use of specific primers for *C. albicans* revealed PCR product to be of approximately 530 bp in length in this study. Our PCR results confirmed that specific primers could not amplify the target DNA of non-albicans isolates (Figure 1), as also reported by Kanbe et al. (2002). The use of specific primers for each *Candida* spp. is costly and often impractical, but use of a simple method such as ribotyping with universal primers allows cheaper and more reliable typing of a large number of isolates.

Ribotyping method showed 7 genotypes of *Candida* spp. based on the use of universal primers ITS1 and ITS4. The ribotyping rDNA of *Candida* sp. elucidated no intraspecies variability among 24 isolates of *Candida* of interest (Figure 2). These results are coincident with that of Fujita et al. (2001) within the range of PCR profile (419 - 650 bp). We thus believe that ribotyping is a rapid, easy and reliable method as it produces specific patterns and can often not require the use of specific primers for individual *Candida* sp. Ribotyping methods was used with numerous clinical and environmental isolates (Isogal et al., 2010); similar results were also observed by Katiraee et al. (2010). Shokohi et al. (2010, 2011) emphasised that factors such as personal hygiene, culture and internal immunity defect may play a role in incidence of eyes infections among the Iranian communities.

Our CAI microsatellite results showed two bands of 240 and 297 bp sizes (Figure 3). This result can be attributed to the existence of two loci of CAI microsatellite in *C. albicans*, leading to the production of two bands or may arise from multiple repetition of CAI microsatellite. These results conflict with those of Sampaio et al. (2005) who obtained PCR product of 252 bp size.

Spampinato and Leonardi (2013) suggested a more complex scenario of 27 repeated units of CAA and one unit of CAG in a secondary structure; the changes in the microsatellite length could be due to the insertion of trinucleotide. Insertion in the first band of 297 bp size could be due to 27-times repeated CAA unit, while the second band of 240 bp size could be due to the low repeated CAA unit.

However, our results were coincident with those of Spampinato and Leonardi (2013), who reported that the PCR analysis of microsatellite is a highly efficient molecular tool for the rapid and accurate identification of *C. albicans*. The length of variation with a high level of polymorphisms of the PCR product makes them reliable markers for the detection of *Candida* sp. (Pupko and Graur, 1999; Sampaio et al., 2010). The analysis of PCR products of *C. albicans* CAI alleles revealed them to be a microsatellite composed of variable units of CAA and CAG. The differences in the products were mainly due to the variable number of CAA repeated units (Katiraee et al., 2010).

We thus conclude that ocular candidiasis is an important disease in Iraq, caused by *C. albicans* and *C. famata* the most common pathogens associated with as first risk, with a prevalence of 21.2% in conjunctivitis patients, making it important to be diagnosed in a timely manner. Multiple molecular markers is recommended for the diagnosis and identification of the causative agents of these infections to achieve a conformable data. CAI microsatellites are a highly efficient polymorphism assay that can be used as a diagnostic and differentiating tool for *Candida* sp. to facilitate identification of *Candida* spp. in clinical samples. The result of this study is very importance to the populations of Iraq because it showed many common fungi associated with conjunctivitis patients in Iraq, Hopefully, this will lead to an increase in research initiatives on this complex problem.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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