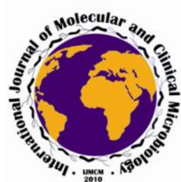

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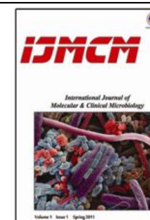

Molecular Identification of *Candida* Species in Clinical Isolates

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ABSTRACT

Candida exists as a normal flora on skin and mucosa. The prevalence of *Candida*-induced infections specially mucosal and vaginal infections have been raised in the world in last decade due to increase in predisposing factors. *C.albicans* is the major etiological agent of candidiasis, but infections due to other species have been increasing. The aim of this study was to determine *Candida* species by using polymerase chain reaction (PCR) in patient with oral and vulvovaginal candidiasis that referred to clinics of Babol University of Medical Sciences, Babol, Iran. Sixty clinical samples were collected from mouth (no= 29) and vagina (no= 31). Identification of *Candida* species was done by using a combination of conventional and PCR methods. Germ tube and vesicle (chlamidoconidia) were seen in 43(71.67%) and 42(75%) of *Candida* isolates, respectively. Assimilation of sugars and Gel electrophoresis of PCR products showed that 78.34% and 75% of isolates were *C.albicans*, respectively, followed by *C.parapsilosis, C.guilliermondii, C.tropicalis* and *C.glabrata*. According to our results, *C.albicans* was the major causative agent in mouth, and also in vulvovaginal candidiasis. We concluded that *Candida* spp. is prevalent in mouth and vagina, indicating the necessity for preventing candidiasis by using some antifungal drugs.

1. Introduction

The genus of *Candida* is considered as those yeasts which are colonized as normal flora on skin and mucosal surfaces (mouth cavity and vagina) in normal people (Abaci et al., 2011). These species under suitable conditions and in the case of providing factors cause superficial and systematic candidiasis of which mouth cavity and vaginal infections have the most frequency (Wahyuningsih et al., 2000; Taweechaisupapong et al., 2005). Nearly 75% of the women are afflicted to vaginal infections at least one time during their life (Tasic et al., 2002). Among 166 *Candida* species which are responsible for candidiasis, *C.albicans,*

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C.parapsilosis, C.tropicalis and *C.glabrata* are specified to those samples which are isolated in medical diagnostic laboratory (Selvarangan et al., 2003). In fact, *C.albicans* is responsible for 60-70% of the cases (Lim and Lee, 2000; Al-Abeid et al., 2004). In recent years the rate of non *Candida*-induced diseases which have low susceptibility to azoles are increasing dramatically (Mirhendi et al., 2006). In order to control nosocomial infections, identifying the species of genus *Candida* in laboratory is of vital importance (Elie et al., 1998; Ahmad et al., 2002). Common methods to identify *Candida* species includes examination of morphological and biochemical characteristics such as direct microscopic examination, to produce germ tube and vesicle (chlamydoconidia), assimilation test, to grow on CHROMagar and Oxoid Chromogenic *Candida* agar culture medium.

These clinical tests need several days to appear the results. Serological tests need high attention and are variable in susceptibility (Ahmad et al., 2002; Baquero et al., 2002; Baixench et al., 2006). Culture of samples is time consuming and the rate of success for some samples such as blood is less than 50% (Einsele et al., 1997). Although pathologic methods are susceptible and economical, but due to being invasive, they have special effects. Therefore, to consider to improve credible, susceptible and economical non invasive methods are increasing dramatically by which diagnosing lots of microorganisms in clinical samples are provided (Lim and Lee, 2000; Badiee et al., 2007).

Because genotyping methods are very rapid, precise and useable to determine most of the fungal diseases, they are more valuable compared to other methods and are considered to be suitable alternative for common techniques (Jrobi et al., 1999). At the moment standard techniques such as PCR and real-time PCR can facilitate rapid diagnosing of fungal diseases with the susceptibility more than 70%, even though the amount of fungal in a sample is very little (Maaroufi et al., 2003; Basková et al., 2007; Metwally et al., 2008). Because these methods are not standard and need lots of expensive devices and skilled experts and also cell wall of the fungal is rigid to release DNA of *Candida*, they are not considered as exact methods in routine affairs of medical laboratory (Maaroufi et al., 2004; Bretagne and Costa, 2005). However, using 5.8S, 18S and 28S regions of nuclear ribosomal among fungi and internal transcribed spacer (ITS) regions is helpful in this case (Chen et al., 2000; Wengenak and Binnicker, 2009).

Therefore, regarding ever-increasing prevalence of these diseases, this study is carried out with the aim of identifying species of *Candida* by using common molecular methods.

2. Material and Methods

2.1. Clinical samples

The samples were collected from mouth and vagina at Mycology Laboratory, Department of Parasitology and Mycology, Babol Medical Science University in 2011-2012. The average of ages for two group were 15-45 years and mean ages and SD for vaginal samples and mouth samples were 31.5 ± 0.3 and 24.61 ± 5.2 years, respectively.

They were preserved in glycerol 50% and at -70°C. The samples were cultured on Sabouraud dextrose agar supplemented with 50 mg/L chloramphnicol as antibacterial agent.

2.2. Production of germ tube

Suspension of fungal colonies was prepared in 0.5 ml of pooled serum (prepared from Shahid Beheshti Hospital, Babol, Iran) and was incubated at 37°C for 2 hours. Afterwards, a drop of suspension was placed on a microscopic slide to observe germ tube.

2.3. Production of vesicle (chlamydoconidia)

Little amounts of fungal colonies were cultured on corn meal agar supplemented with Tween 80 at 30°C for 72 hours. Then, it was examined microscopically to determine terminal and lateral characteristic vesicles (chlamydoconidia).

2.4. Carbohydrate assimilation test

After 3 times consecutive passage of yeasts on Beef Extract Agar medium to free them from

sugars, assimilation test was carried out by disk diffusion method on Yeast Nitrogen Base (YNB) medium according to the manufacturer instructions.

2.5. Molecular methods

DNA extraction: glass bead method was performed for DNA extraction. Briefly, 200 micro liters of lysing buffer, 200 micro liters of phenol chloroform solution and 200 micro liters of 0.5 mm diameter glass beads were added to fungal colony. After vigorous shaking and centrifugation in 10000 rpm, the supernatant were isolated and DNA was precipitated by 0.1 volume 3M sodium acetate (pH 5.2) and 2.5 volume cold isopropanol, centrifuged and washed by 70% ethanol. The pellet re-suspended in 100 micro liters TE buffer and stored at -20˚C until used (Mirhendi, 2006).

PCR: A molecular kit was used which was planned with the aim to identify main species of identified *Candida*. Also, this kit could amplify nucleic acid of rDNA region with the help of PCR and polymorphism of restriction enzymes (RFLP)) Mirhendi, Isfahan, Iran (MSP1 restriction enzyme was used for digestion of product.

Products of PCR-RFLP were subjected to electrophoresis for about 45 minutes to 1 hour at 80-100 voltage. One of the wells was specialized to the DNA size marker, in each time. After staining of the gel, it was photographed using gel document system and regarding formed bands, they were compared with ladder marker and species of *Candida* were identified.

2.6. Statistics analysis

Qualitative variables were compared using the chi-square and those with p value <0.05 were considered as significant difference.

3. Results

In this study 60 clinical samples included 31 vagina and 29 mouth samples were examined. Germ tube produced in 43 samples (71.67%) in pooled serum, which most of them were related to mouth isolates; however the difference among them was not significant. Forty samples (70%) produced vesicle in corn meal agar medium with Tween 80. Production of vesicle in mouth samples were more than vaginal samples, but the difference among them was not significant (Table 1).

Assimilation test displayed that *Candida parapsilosis* and *Candida guilliermondii* are located after *Candida albicans*. *Candida tropicalis* was determined just in vagina samples (Table 2 and Figure 1).

Results of molecular detection based on PCR-RFLP showed that among 5 species identified, 75% of the samples were *Candida albicans* followed by *Candida parapsilosis*, *Candida guilliermondii*, *Candida tropicalis* and *Candida glabrata* (Table 3 and Figure 1).

Table 1. Germ tube and vesicle formation by *Candida* species isolated from vagina and mouth

Test	Result	Source of isolation		Total (%)
		vagina	Mouth	
Germ tube	Positive	18	25	43 (71.67)
	Negative	13	4	17 (28.33)
	Total	31	29	60 (100)
Vesicle (Chlamidoconidia)	Positive	19	23	42 (70)
	Negative	12	6	18 (30)
	Total	31	29	60 (100)

Table 2. Identification of *Candida* species by carbohydrate assimilation test

Candida species	Vaginal samples (%)	Mouth samples (%)	Total (%)
C. albicans	21 (67.74)	36 (89.65)	47 (78.34)
C. parapsilosis	6 (19.35)	0	6 (10.0)
C. guilliermondii	1 (3.23)	2 (6.9)	3 (5.0)
C. tropicalis	2 (6.45)	0	2 (3.33)
C. glabrata	1 (3.23)	1 (3.45)	2 (3.33)
Total	31 (51.67)	29 (48.33)	60 (100)

Table 3. Identified *Candida* species from mouth and vagina by PCR-RFLP

Candida species	Vagina samples (%)	Mouth samples (%)	Total (%)	Size (s)
C. albicans	19 (61.29)	26 (89.65)	45 (75)	238, 297
C. parapsilosis	7 (22.58)	0	7 (11.67)	520
C. guilliermondii	1 (3.23)	2 (6.9)	3 (5.0)	82, 155, 371
C. tropicalis	2 (6.45)	1 (3.45)	3 (5.0)	184, 340
C. glabrata	2 (6.45)	0	2 (3.33)	314, 557
Total	31 (51.67)	29 (48.33)	60 (100)	--

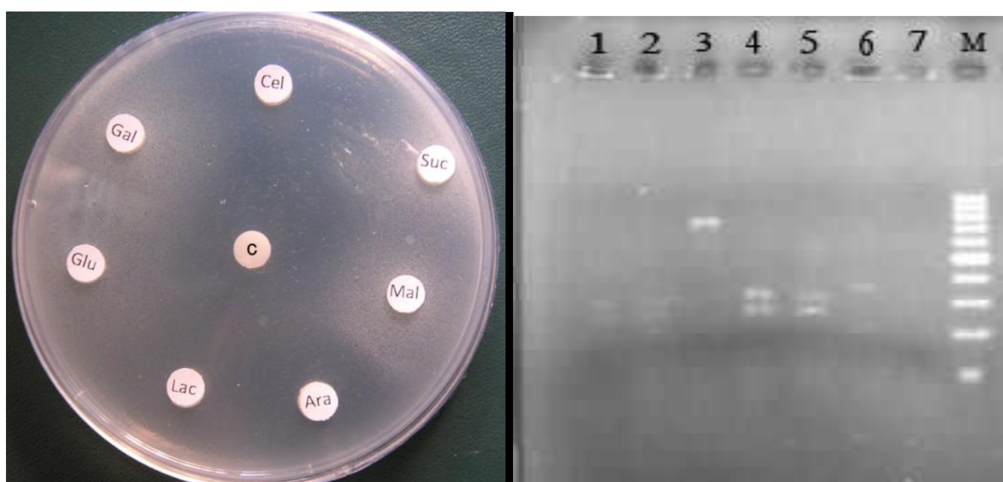

Figure 1. Assimilation of some sugar and RFLP results in gel documentation of *Candida* species. a. Glu: Glucose, Gal: Galactose, Cel: Cellobiose, Suc: Sucrose, Mal: Maltose, Ara: L-Arabinose and Lac: Lactose (prepared in 20% dilution), C: Blank. b. Lanes 1 and 2: *C.albicans*, lanes 4 and 5: *C.krusei*, lane 6: *C.guilliermondii*, line 7: Negative control and lane M: 100 bp ladder molecular size marker.

4. Discussion

In this survey, 78.33% of isolates identified as *C.albicans*; and others were *C.parapsilosis, C.guilliermondii, C.tropicalis* and *C.glabrata*. Emission of germ tube is a primary method to isolate *C.albicans* (Zavalza-Stiker et al., 2006). In this study only in 95.56% of *C.albicans* isolates was seen that are in contrast with results in other studies. In one study all the isolates have produced germ tube (Ahmad et al., 2002). So, in one study which was carried out on 115 species of *Candida* isolated from mouth and vagina, 37% and 42% of them could emit germ tube during 2 hours respectively, which increased to 93% and 83% after 4 hours (Vidoto et al., 1999). In this study most of the production of germ tube belonged to vagina isolates. Different surveys indicated that various medium cultures from the point of stimulating yeasts produced germ tube with different speed (Vardar-Ünlü, 1998). Corn meal broth medium is of those in which 100% of *C.albicans* isolates can highly produce germ tube, so that, 94.1% of 111 *C.albicans* isolates produce germ tube after 2 hours. It was shown that there was no difference between production of germ tube in this medium culture and in pooled serum (Nakamoto, 1998).

In the present survey, 93.33% of *C.albicans* isolates produce vesicle (chlamydoconidia). There are some differences and similarities between results of this study and other ones. In one study all of the 215 *C.albicans* isolates could produce vesicle (Syverson, 1981) and this difference may be due to the amount of oxygen, pH and elements of medium culture; so that, adding milk to the medium can be helpful to increase production of vesicle (Van-Deventer et al., 1995). In another study, all of 25 clinical isolates of *C.albicans* produced vesicle on corn meal agar medium in which milk was added at 28 degree of centigrade with rotating movement 150 rpm during 16 hours, so that ,this proportion was less than 66% in other samples (Zavalza-Stiker et al., 2006). Although corn meal agar medium is a suitable one to produce vesicle but, production of vesicle to identify *C.albicans* needs 48-72 hours and it is problematic (Nakamoto, 1998).

In this study 5 species of *Candida* identified with assimilation test which is not comparable with some studies. In one study, 215 isolates of *C.albicans* was identified by using 11 different kinds of sugars. Results of survey indicate that some of the strains cannot absorb many sugars (Syverson, 1981). Non *C.albicans* species have a relatively high abundance which is being considered in a study (Akpan and Morgan, 2002). Importance of these species is mostly because of the high resistance against azoles (Selvarangan et al., 2003). Problems of using this method and long time of incubation (3-5 days) have made scientists looking for novel methods and other medium cultures (Nakamoto, 1998; Mirhendi et al., 2006; Abaci et al., 2011). One of these methods is to use Vitek or API kits which are usually expensive (Chen et al., 2000; Ahmad et al., 2002). Temperature and incubation period play a role to make the results of various methods different, so, the rate of error in identification is 60-100% (Syverson, 1981; Jrobi et al., 1999; Ahmad et al., 2002).

In present study most of the identified species with molecular detection are *C.albicans* which is similar to some studies (Einsele et al., 1997; Jrobi et al., 1999; Ahmad et al., 2002). *C.parapsilosis* which is the second one in this study play an important role in nosocomial infections (Elie et al., 1998; Schbereiter-Gurtner et al., 2007). Translatable region (ITS1) which encodes 5.8S and 18S, plays an important role to identify the main species of medicine (Selvarangan et al., 2003; Mirhendi et al., 2006; Klingspor and Jalal, 2006). Although in the past time, susceptibility of this method was low but, consecutively it has been increased (Klingspor and Jalal, 2006). Thus, some studies have introduced its susceptibility up to 100% (Shin et al., 1997; Maaroufi et al., 2003). It has the ability to distinguish the species of *Candida* and isolation of *Candida* from clinical samples (Nakamoto, 1998; Baquero et al., 2002). Some researchers admit these methods in addition to be susceptible are simple and do not need complicated stages (Shin et al., 1997; Mirhendi et al., 2006; Fattahi et al., 2007). On the other hand, examination of normal people samples for *Candida* is negative using this method, also it is time saving. So that, identifying fungal species would be available during 2.5-10 hours (Van-Deventer et al., 1995; Vidoto et al., 1999; Metwally et al., 2008). This method can make results positive before appearing clinical signs so, it can be a proper alternative to diagnosing infections of *Candida* fast (Mirhendi et al.,

2006; Klingspor and Jalal, 2006; Badiee et al., 2007; Lau et al., 2010). Indeed, susceptibility of isolation and identification of fungal by molecular test depend on time and temperature and in clinical samples depend on the kind of sample, temperature and infection rate (Lau et al., 2010). This method can isolate some species of *Candida* from various sources simultaneously is simple (Mirhendi et al., 2006; Badiee et al., 2007).

Although using universal primers make primary identification of fungal easy, specific primers to identify the species of *Candida* are essential (Ahmad et al., 2002). In this method firstly, PCR is carried out by using universal primers afterwards, it is possible to identify major species of *Candida* with digesting products of PCR by restriction enzyme such as *MSPI* to differentiate among species (Mirhendi et al., 2006; Klingspor and Jalal, 2006). One or more than one enzyme is used to distinguish among species in some studies and in this study one enzyme is used. Using this method cannot isolate *C.albicans* from *C.dubliniensis* (Mirhendi et al., 2006). Thus it is probable that some of the species of *C.albicans* belong to *C.dubliniensis.* In this study harmony between molecular method and assimilation test was 90%. In one study the number of 112 and 107 isolates from 7 species of *Candida* were determined by phenotyping and genotyping methods, respectively; although there was no significant difference among them but, 3 cases were identified incorrectly (Jrobi et al., 1999). Usually there is nearly 90-100% harmony between results of molecular methods and general methods (Ahmad et al., 2002; Fattahi et al., 2007). So, molecular method has been accepted as a standard method (Ahmad et al., 2002; Bretagne and Costa, 2005). Thus, in this study results achieved by PCR has been considered as a final method to identify species.

Results indicate high percentage of the species are included non *C.albicans* ones which are usually more resistant against antifungal drugs. So, molecular methods can be used to identify *Candida* species faster along with common methods. Further works are needed to make usage of this method in the laboratory.

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