Journal of Mycology Research. Vol 1, No 1: Page 43-53, September 2014

Role of denture-wearing on colonization and antifungal resistance of oral *Candida albicans* isolates in healthy people

Ghasem Vahedi[1*], Golnaz Sharafi[1], Ali Vahedi[2], Sahra Vahedi[3], Tabassom Mohajer[1], Teimur Abbasi[2]

[1]Mycology Research Center , Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

[2]Faculty of Dentistry, Tabriz University of Medical Science, Tabriz, Iran

[3] Faculty of Nursing and Midwifery, Tabriz University of Medical Science, Tabriz, Iran

*** Corresponding Author: Email: g_vahedi@yahoo.com, Tel: +98(935) 3339777**

(Received: 10 May 2014, Accepted: 22 July 2014)

Abstract:

The primary focus of the present study was to evaluate the occurrence of *Candida albicans* isolates in the oral cavity and its probable correlation with dental implant applications and prosthesis. We collected oral swabs from patients who had attended private dentistry clinics, followed by stringent and controlled antifungal susceptibility testing and calculation of colony forming units (CFUs). Amphotericin B, Fluconazole and Itraconazole were three antifungals tested by CLSI M27-A2 broth microdilution protocol. The MIC ranges for three tested antifungals in *Candida albicans* isolates were obtained as 0.0625-1, 0.125-16 and 0.0313-0.5 µg/ml, respectively. Additionally, in non-*Candida albicans* isolates, MIC ranges for three antifungals were achieved as 0.25-1, 0.125-16 and 0.0313-0.125 µg/ml, respectively. MIC50 values of both tested azoles in the *Candida albicans* group were higher than related values in the non-*Candida albicans* group. Moreover, CFU counts for denture-acquired people were higher than for participants not wearing denture applications, indicating the proposal that the surface of dentures or any other synthetic implants in the oral cavity may result in providing an appropriate environment for the colonization of yeasts.

Keywords: antifungal, resistance, CFU, denture, Albicans.

Introduction

The oral cavity comprises a large and highly diverse population of opportunistic and pathogenic microorganisms. *Candida* and other yeast species are the main fungal flora in the oral cavity (Akpan and Morgan 2002). However, there are complex interrelationships among oral microbiota, which determines their population size and opportunistic behaviour. Generally, these microorganisms exhibit commensal and symbiotic attributes; however, in particular situations, for example, in the case of compromised patients, they can also exhibit a more disadvantageous side. Moreover, some unwelcome changes in the natural oral environment, such as denture settings, can result in the undesirable alteration of oral microscopic inhabitants. This may facilitate the emergence of resistant pathogenic microorganisms in the oral cavity of the host (Fidel et al. 1999).

Candida species are the predominant cause of fungal infection in humans. In most cases, the source of the infection is endogenous commensal flora from the mucosal surfaces. In addition, *Candida* species are a major cause of oral candidiasis. The most common form of oral candidiasis is denture-related stomatitis (DRS) with *Candida albicans* (*C. albicans*) as the main cause (Figueiral et al. 2007). Moreover, denture-plaques containing *Candida* spp. are one of main subsequent challenges for patients with DRS (Nikawa et al. 1998). However, generally, the defence mechanisms of a healthy host consistently limit and control the microbiota populations present on oral surfaces. On the other hand, the microbes have to react against host defence to survive (Boix and Nogués 2007). In this mean, the

adherence ability of microbes on mucosal surfaces is a key feature. Furthermore, the unsafe use and inappropriate hygienic care of external and internal dental prosthetic devices such as partial or complete dentures and dental implants may cause these settings to act as a microbial reservoir and adherence within the oral environment (Allen et al. 1999).

In recent years, more attention has been focused on understanding the roots of antifungal resistance as a result of increasing reports of resistance to antifungal drugs. Polyenes and azoles are two most-often prescribed antifungal derivatives which drug resistance to these groups of antifungals is usually reported by researchers (Sanglard and Odds 2002). Among *Candida* species, acquired resistance to Amphotericin B (AmB) as a main known polyene is infrequent. Some *Candida* species, such as *Candida krusei*, has emerged as intrinsically resistant to azoles (Kanafani and Perfect 2008). This resistance to azoles has been stated by many research teams and more frequently than a resistance to AmB, possibly due to the more common administration and fungi-static nature of these antifungal agents (White et al. 1998). Furthermore, one recent scientifically-discussed important predisposing factor for antifungal resistance emergence in *Candida* spp. is the formation of biofilms. Biofilms are primarily a mix of yeast and bacteria species on appropriate synthetic or natural surfaces such as catheters and dentures (Chandra et al. 2001). Due to the complex and usually impenetrable structure of biofilms and the high expression of resistance-related genes in yeasts present in biofilms, they demonstrate higher levels of resistance to antifungals (García-Sánchez et al. 2004).

The aim of the present study was to clarify the possible impact of denture-wearing on colonization and antifungal resistance of oral *Candida albicans* and non-*Candida albicans* isolates. We expect the possibility of a higher colonization count and resistance of yeast isolates from healthy denture-wearing volunteers compared to healthy but non-denture-wearing individuals.

Materials and Methods

Specimen collection and growth conditions: Twenty-five individuals referred to private dental clinics in Miandoab, West Azerbaijan Province, Iran, participated voluntarily in this study. The demographic conditions of both denture-wearing and non-wearing groups were roughly similar. Twelve patients wore denture(s) and 13 did not own any dentures. The detailed history of wearing dentures and other external medicinal interventions, as well as any lesions or scars present in the oral cavity was documented. A questionnaire was designed for recording valuable data including information regarding age, sex, oral lesions, any history of health problems, drug usage, blood type and smoking. Samples were collected from the oral cavity by sterile swab from the upper and lower gingival, or in individuals with dentures, from the surface of upper and lower dentures, and in the case of oral lesions or ulcers, from the lesion or ulcer surfaces. Swabs were cultured on Sabouraud dextrose agar (Merck, Germany) and supplemented with 0.5% Chloramphenicol (Sigma-Aldrich Inc., USA) at 35°C for seven days.

Colony counting and species identification: To deny observer error and increase accuracy, we took digital photographs of the SDA culture's front surfaces and saved them on computer; then, yeast colonies per swab (CFUs/swab) were counted from the saved digital images using OpenCFU software version 3.8.4 (Geissmann 2013). This was followed by a microscope examination using lactophenol cotton blue staining. *Candida albicans* isolates were distinguished from other yeasts by a series of specific tests including germ tube formation, incubation at 45°C, culture on CHROMagar™ *Candida* (CHROMagar, France), culture on cornmeal agar containing tween 80 media (Sigma-Aldrich Inc. USA) and Sabouraud dextrose agar containing 6.5% NaCl (Merck, Germany) (salt tolerance test); additionally, RapID™ YEAST PLUS System (Remel, USA) tests and a PCR assay were conducted. All the tests were conducted according to the instructions documented by their manufactures or were based on published protocols. The salt tolerance test and

incubation at 45°C was conducted to distinguish between suspected *Candida albicans* and *Candida dubliniensis* isolates Swabs were cultured on Sabouraud dextrose agar (Merck, Germany) and supplemented with 0.5% Chloramphenicol (Sigma-Aldrich Inc., USA) and incubated at 35°C for seven days.

PCR identification: All yeast isolates were incorporated in a PCR assay using two pairs of *Candida albicans*- and *Candida dubliniensis*-specific primers from a series of published Multiplex PCR primer pairs (Lim and Lee 2002) to establish a standard PCR assay. *Candida albicans*-specific primer sequences read as follows: forward: AAGCTCTGATACCTACACTAGCGA; reverse: GTTAGGTCTAAAGTCGAAGTCATC; these sequences are highly specific for the *Candida albicans* integrin-like protein coding gene (αINT1). *Candida dubliniensis*-specific primer sequences read as follows: forward: GCATTTGGTACCGTAAGGATACCA; reverse: CACTAGATGATTCCGGTGTTTTGG; these sequences are highly specific for the *Candida dubliniensis* agglutinin-like sequence protein coding gene (ALS). DNA extraction was done based on glass bead disruption and the phenol-chloroform extraction method (Amberg et al. 2006). The PCR program was also adjusted according to instructions recommended by the authors of the previously mentioned multiplex PCR article (Lim and Lee 2002), with slight modifications.

In vitro antifungal susceptibility testing: Antifungal agents including Itraconazole, Fluconazole and Amphotericin B were used in this study. All drugs were purchased as standard powders from Sigma-Aldrich Inc., Germany. A stock solution was prepared at concentrations 100 times higher than the final concentration and stored at -70°C until use, according to manufacturer instructions. For the susceptibility testing of antifungal agents, RPMI 1640 medium (with L-glutamine and without sodium bicarbonate) (Gibco) was dissolved in morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich Inc., Germany) buffers. All stages of preparation and susceptibility testing were conducted according to the M27-A2 standard protocol of the Clinical and Laboratory Standards Institute (CLSI). A 100-fold dilution of each Itraconazole and Fluconazole antifungals and a 10 times dilution of Amphotericin B were prepared in dimethyl sulphoxide (DMSO) and normal saline, respectively. Itraconazole and Amphotericin B in a ratio of 1:50 and Fluconazole in a ratio of 1:5 were added to the RPMI solution. Thus, 10 dilutions of Itraconazole and Amphotericin B at concentrations of 1600 µg/ml to 3.13µg/ml and 10 dilutions of Fluconazole at concentrations of 640 µg/ml to 1.25 µg/ml were prepared. To prepare the yeast suspension, first, from all yeast isolates, fresh 24-hours cultures were prepared at 35ºC. Then, a standard solution of McFarland 0.5 was prepared and absorbance was adjusted to 0.1 at a 530 nm wavelength using a spectrophotometer. The McFarland 0.5 suspension was employed to visually assess the concentration of yeast suspension tubes. The number of yeasts in the suspension tubes prepared by the method described above was between $1x10^6$ -$5x10^6$ yeasts/ml. The next step was preparing dilutions of yeast suspensions in a ratio of 1:50 using distilled water or normal saline, which for the concentration of the tubes would be between $2x10^4$ - $1x10^5$ yeasts/ml. Additionally, further dilution was required, at which the yeast suspensions were diluted at a ratio of 1:20 using the RPMI stock solution, at which point the concentration of tubes reach $1x10^3$-$5x10^3$ yeasts/ml. The resulting suspensions of yeast were employed as inoculum suspensions in the 96-well flat-bottomed sterile plastic microtiter plates. In the final step, the broth microdilution method was performed using the 96-well flat-bottomed sterile plastic microtiter plates. The inoculation was conducted in a clean environment using a standard microbiological hood; 100 µl of all prepared dilutions of antifungal agents were dispensed in the first 10 wells of a 96-well plate, followed by 100 µl of yeast inoculation suspension. In each row, one well served as a control without the drug and another as a control

without yeast. Standard ATCC strains as well, according to M27-A2 standard guidelines, as quality control, like other yeast isolates, in related wells, were inoculated and all testing was conducted in duplicates. Following on, all 96 inoculated well-plates were incubated for 48 hours at 35ºC. The minimum inhibitory concentrations (MICs) of test wells were visually characterized and compared to the growth rate of control wells.

Statistical analysis: We performed statistical tests using MedCalc software version 13 (Schoonjans et al. 1995); because distribution of our dependent continuous data regarding CFU counts were not normal at a P value of less than 0.05, according to the Kolmogorov-Smirnov test of normality, we chose the Kruskal-Wallis test as a non-parametric equivalent to ANOVA to test the significance of the difference in CFUs counted for denture-wearing and non-wearing groups. To determine any difference in antifungal susceptibility, we interpreted our results according to various groups of participants and thereby accomplished our aim through a series of chi-square tests conducted on our independent categorical variables (groups and species). Moreover, we performed a proportion test for revealing possible differences between the proportion of subgroups in the Fluconazole's MIC interpretations and the Intraconazole's MIC interpretations. According to the predefined factors of the performed tests, P values of less than 0.05 were assumed as significant. Additionally, because of the low number of each non-*Candida albicans* species and for achieving a convenient sample size and more reliable statistical results with a minimum bias, we assumed all non-*Candida albicans* isolates into one group.

Results

Twelve denture-wearing and 13 non-denture-wearing healthy individuals participated in the study, in which a total of 35 yeasts were isolated. In total, 12 (34.3%) isolates were from non- denture-wearing individuals and 23 (65.7%) isolates were from denture-wearing volunteers, roughly

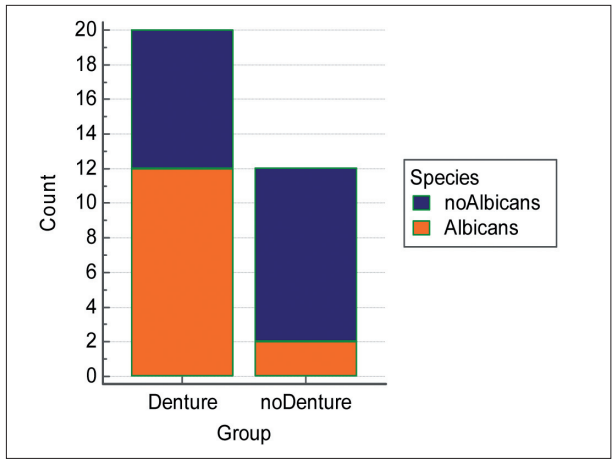

Fig. 1. Number of yeast species isolates from denture wearing (Denture) and not wearing (noDenture) groups indicated by orange color for the *Candida albicans* isolates and Blue color for non-albicans yeast isolates within the bars.

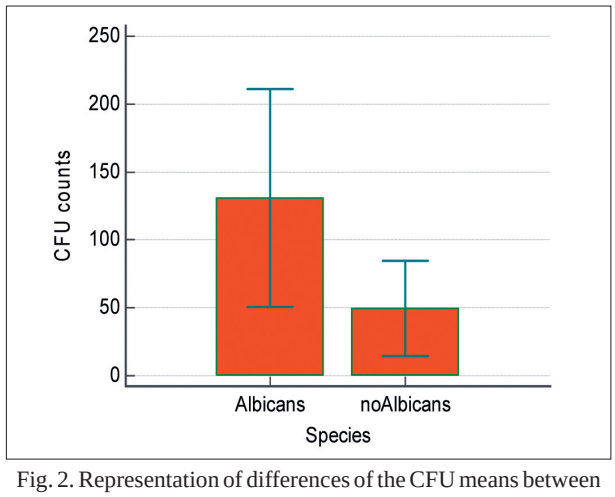

Fig. 2. Representation of differences of the CFU means between species groups including *C. albicans* and non-albicans indicated by Albicans and noAlbicans under the bars, respectively.

Table 1. Distribution of yeast identified species isolated from denture wearing and not wearing healthy people.

Yeast species	Number (%)
Candida albicans	14 (40%)
Candida glabrata	6 (17.1%)
Candida dubliniensis	6 (17.1%)
Candida tropicalis	4 (11.4%)
Candida parapsilosis	2 (5.7%)
Unknown yeast	3 (8.7%)
Total	35 (100%)

implying a two-fold increase in yeast isolation counts in the denture-wearing group. From a total of 25 participants, 16 (64%) had been colonized by yeast species. Moreover, 11 (91.7%) denture-positive patients out of 12 were colonized by

Table 2. MIC50, MIC90, Range and number of resistant and non-resistant isolates in species groups including *C. albicans* and non-albicans groups. [a]Resistant isolates of yeast species. [b]Non-resistant isolates of yeast species.

Yeast species	Antifungal agents	MIC µg/ml			R[a] N (%)	non-R[b] N (%)
		50%	90%	Range		
C. albicans	Amphotericin B	0.5	1	0.0625-1	0(0%)	14(100%)
	Fluconazole	16	16	0.125-16	0(0%)	14(100%)
	Itraconazole	0.5	0.5	0.0313-0.5	0(0%)	14(100%)
non-*albicans*	Amphotericin B	0.5	1	0.25-1	0(0%)	18(100%)
	Fluconazole	4	16	0.125-16	0(0%)	18(100%)
	Itraconazole	0.25	0.5	0.0313-0.125	0(0%)	18(100%)

Table 3. Differences in number of resistant and non-resistant yeast isolates tested against three antifungal agents in comparison of two denture wearing and not wearing groups. [a]Resistant isolates of yeast species. [b]Non-resistant isolates of yeast species.

Antifungal agents	Denture wearing		No denture wearing	
	R[a] N (%)	non-R[b] N (%)	R[a] N (%)	non-R[b] N (%)
Amphotericin B	0 (0%)	20 (100%)	0 (0%)	12 (100%)
Fluconazole	0 (0%)	20 (100%)	0 (0%)	12 (100%)
Itraconazole	0 (0%)	20 (100%)	0 (0%)	12 (100%)

yeasts, whereas only five (38.5%) denture-negative participants out of a total of 13 had been colonized by yeasts; as such, the difference between the two groups were significant (P = 0.0187, chi-square test). The *C. albicans* isolates number in the denture-wearing group was 12 compared to two in the non-denture wearing group, while non-albicans yeast isolates were counted as 8 in the denture-wearing group, compared to 10 for the non-denture-wearing group (Please see Fig. 1). The difference between groups were significant (P = 0.0430, chi-square test). From a total of 35 yeast isolates we were able to identify, *C. albicans*, *C. glabrata*, *C. dubliniensis*, *Candida tropicalis* and *Candida* parapsilosis, with frequencies of 14, 6, 6, 4 and 2, respectively. Three remaining unknown isolates were defined as missing data, because of their erroneous identification and antifungal susceptibility testing results (Please see Table 1).

Moreover, differences between the means of CFU counts in the denture-present group compared to the denture-absent group was highly significant (P= 0.001730, Kruskal-Wallis test); in other words, CFUs/swab means in the denture-positive group were significantly higher than in the denture-negative group. Furthermore, scrutinizing the possible differences in CFU counts by assuming each yeast species to have been present in the statistical test resulted in even more highly significant values in CFUs/swab count means comparison between *C. albicans* and non-albicans yeast groups. CFU counts level in the *C. albicans* group was significantly higher than in the non-albicans yeasts group (P= 0.000099, Kruskal-Wallis test) (Please see Fig. 2).

Antifungal susceptibility testing results showed a notable drop in the MIC50 and MIC90 rates of azoles in the non-albicans group compared to the *C. albicans* group. MIC50 values of fluconazole susceptibility testing in the non-albicans group were lower than related values in the *C. albicans* group. Additionally, MIC50 of Itraconazole in the non-albicans group was 0.25 in comparison to 0.5 in the *C. albicans* group (Please see Table 2).

We did not observe any significant difference between the resistant and non-resistant (by assuming susceptible and susceptible dose-dependent as non-resistant group) frequencies of each antifungal drug across the species groups (please see Table 2).

Investigating the antifungal susceptibility testing values across the denture situation again demonstrated no significant differences when comparing antifungal resistance across the denture-wearing and non-denture-wearing groups (Please see Table 3). However, for the Fluconazole group, we had 16 (50%) SDD isolates, whereas in the Itraconazole group, there were only 8 (25%) SDD isolates. The difference was not significant but considerable (P = 0.0707, test of proportion).

Discussion

Some of the yeast species are natural inhabitants of the oral cavity. Based on published reports, they are present in the oral cavity of about 60-80% of healthy people (Cannon et al. 1995; Sanchez-Vargas et al. 2005). *Candida albicans* is one of the major yeast flora on human mucosal surfaces and can colonize 40-50% of healthy people; it is the fourth most prevailing cause of nosocomial blood infections. Despite its pathogenic attributes and severe virulence factors, *C. albicans* in healthy people can commensally live beside other natural human microbiomes and can help to regulate immune responses by consistent stimulation of the immune system (Pérez and Johnson 2013).

Dentures are generally fabricated with metal or thermosetting acrylic resins as a base. The base is routinely equipped with plastic or porcelain teeth. In this regard, there is evidence indicating the impact of medical devices such as central venous catheters or dentures on the microbial profile of the host organ. Yeast species can easily colonize the surface of catheters or the acrylic resin surfaces of dentures (Verran and Maryan 1997). These microbial colonies and filamentous networks on an appropriate and generally artificial field are known as biofilms. Biofilms on dentures or catheters are mainly a blend of both bacterial and fungal species. Additionally, because of their filamentous and impenetrable structure, in addition to their altered gene expression profile, they are typically resistant to most of the standard antimicrobial therapies (Allen et al. 2010).

Alongside environmental stimuli and location condition, each fungal species has different characteristics in terms of colonization and antimicrobial resistance abilities. For instance, *C. dubliniensis* is known to be more sensitive than *C. albicans* to antifungal therapies (Sanglard and Odds 2002), while *C. glabrata* and C. krusei have shown evidence of higher antifungal MICs or intrinsic antifungal resistance (Vanden Bossche et al. 1994). In our study, none of the 32 yeasts tested were Amphotericin B (AmB)-resistant. However, the MIC range of AmB in the denture-wearing group was 0.25-1 µg/ml, whereas in the non-denture-wearing group it was 0.625-1 µg/ml (data not shown). Furthermore, both MIC50 and MIC90 of AmB in the denture-wearing group were 1 µg/ml, while related values for the non-denture-wearing group were 0.25 µg/ml and 0.5 µg/ml, respectively, indicating higher frequencies of higher values of MICs for AmB in the denture-positive group. However, the obtained MICs from AmB testing did not appear to be very high (the highest tested MIC concentration was 16 µg/ml). These results indicated that denture-wearing may affect the AmB-resistance of oral yeasts, particularly *C. albicans* species, but this influence was somewhat average. Furthermore, it is now clear that the sterol-binding feature is not the only mode of action of AmB (Gray et al. 2012). Oxidative-dependent stimulation of the immune system's polymorphonuclear (PMN) cells, a hypoxic environment, reactive oxygen species and some extracellular factors like secreted catalase, extracellular scavengers and pro-oxidants are also involved in the lethal action of AmB. Catalase, which is produced by some bacteria and fungi (e.g., *C. albicans*) can break down oxygen free radicals and has a vital role in the survival of many pathogens in stressed conditions. It has been observed that catalase can inhibit the lethal action of AmB (Brajtburg et al. 1990). The oral cavity, because of its connection to the outside environment and the transition of nutritional materials, is generally disposed to being affected by many external factors. By scrutinizing patient medical records, we found a history of smoking, denture-related lesions (also termed denture-related stomatitis and *Candida*-associated denture induced stomatitis) and poor hygienic care of dentures for most of the participants in which we isolated yeasts with higher AmB MICs, compared to MICs in non-denture-wearing individuals (Pereira-Cenci et al. 2008). In our study, seven (35%) isolates with AmB MICs of 1 µg/ml from the denture-wearing group were *C. albicans*, while only two (16.7%) isolates with MICs of 1 µg/ml were *C. albicans* in the non-denture-wearing group. Smoking can affect pH in the oral cavity and

as a result, the local denture environment can turn acidic; In addition, hypoxic environment can be created as a result of denture-wearing (Visvanathan and Nix 2010). An acidic extracellular environment favours *C. albicans*' higher enzymatic activity and can also cause changes in the oral microbiota population, resulting the dominance of *C. albicans* species. Furthermore, it has been observed that when AmB is present in a hypoxic environment, up to an 80% reduction in lysis rates of *C. albicans* protoplasts can occur compared to when incubations occur in the air (Sokol-Anderson et al. 1986). High occurrence of yeasts, showing high MIC values of AmB (which in the current study were mostly isolated from denture-wearing individuals) may be the result of some unwelcome changes in hypoxic situations, reactive oxygen species production amount, catalases produced by oral bacteria or yeasts and the yeast population in the oral environment as a result of using dentures. We did not evaluate each mentioned factors independently, so we would not be eligible to propose the exact factors and an acquired resistance occurrence is even rarer (Conly et al. 1992). The results of the current study might therefore direct us to the claim that isolated yeasts with high MIC values to AmB in the denture-wearing group emerged due to denture-induced environmental changes. However, further studies are required to clarify the main factor(s) and mechanism(s) involved.

In the current study, we also evaluated the antifungal resistance levels of our yeast isolates to two azoles. We could not establish any proof in terms of the differences in antifungal resistance levels of yeast isolates when comparing the denture-wearing and non-denture-wearing patients. In fact, in the case of AmB, MICs were determined as the lowest drug concentration for preventing any discernible growth. For azoles, MIC was defined as a drug concentration for which a 50% reduction in turbidity was observed in comparison with a drug-free control (Alexander et al. 2007). This phenomenon, which was observed in a series of tandem wells in a 96-well-plate row, is known as the trailing effect, a known attribute of azoles (Espinel-Ingroff et al. 2007). All of our participants were medically-approved to be healthy and as such, none of them had been receiving antifungal therapies at the time of specimen collection. Additionally, there was no history of antifungal administrations among participants. Despite the AmB, for which acquired resistance is rare and which is known for its fungicidal properties, in the case of azoles - which are known for their fungi-static attributes - acquired resistance occurs frequently, mostly during azole-antifungal therapy (Marichal and Bossche 1995). In this respect, where AmB was concerned, MICs were classified as similarly susceptible or resistant. On the other hand, MIC values for azoles were defined as susceptible, susceptible dose-dependent and resistant. Thus, considering the generally relative and acquired nature of resistance to azoles, the absence of any azole-resistant isolate in our denture-wearing and non-denture-wearing healthy participants may have been due to their naïve nature regarding exposure experience to azoles, as none of our isolates were known antifungal intrinsic-resistant species.

The final primary evaluated factor in our study was colony count, which was compared between the two denture groups, as well as for two yeast groups; both comparisons resulted in highly significant differences. These results were comparable to similar studies that have indicated higher colonization when complete or partial dentures are present in the oral cavity (Darwazeh et al. 2001; BarBeau et al. 2003). The acrylic resin surface of dentures may facilitate this phenomenon (Henriques et al. 2004). Moreover, most colonized patients in our study were those who had been challenged by *Candida*-associated denture-induced stomatitis (CADS), which is the most prevalent form of oral candidiasis. In 90% of cases, *Candida* species are the predominant cause of CADS and among *Candida* species, *C. albicans* is primarily responsible for CADS (Salerno et al. 2011). Thus, elevated rates of CFUs in the denture-wearing group, as well as its higher incidence in the

C. albicans group could be justified.

Results from other studies mainly support our observations; however, some contradictory evidence also exists. Colonization of the oral cavity by *Candida* yeasts in elderly people were the subject of a study that resulted in 67% colonization of total cases by *Candida* spp., for which *C. albicans* is the most frequently occurring species. However, this study could not show any relation between denture-wearing and the occurrence of oral candidiasis, while there was a highly significant relationship between denture-wearing and colonization by *Candida* spp. (Grimoud et al. 2003). In a similar study, the incidence rate of *Candida* spp. in no denture wearers was higher compared to-denture-wearing participants. Again, *C. albicans* was the dominant isolate but there were no significant differences between adults with and without dentures in the isolation frequency of *C. albicans* (Zaremba et al. 2005).

Authors of another related study have found a link between denture-induced stomatitis, yeast presence and denture hygienic care (Kulak-Ozkan et al. 2002). Other notable research was conducted on two groups of denture-wearers, including patients suffering from oral candidiasis and a control group without oral candidiasis to investigate changes in antifungal resistance. However, the results did not show any differences, possibly due to the similar denture status of the two studied groups (Koga-Ito et al. 2006). Additionally, an evaluation of the isolation frequencies of *Candida* spp. in complete denture wearers showed that 60% of people involved in the study had been colonized by *Candida* spp. (Darwazeh et al. 2001). Another group of researchers found that there was no significant relation between oral *Candida* carriage and denture-wearing status (Martins et al. 2010). However, another research team stated that they had been able to isolate *C. albicans* species in 66.7% of denture-positive people, compared to only 28.9% *C. albicans* isolation in non-denture-wearing people, showing a highly significant difference (Daniluk et al. 2005). In a study that is to some degree comparable to the present research, antifungal susceptibility also was the subject, where the authors tested several antifungals, including three azoles (but not AmB), in healthy denture-wearing people. No evidence of significant differences in antifungal susceptibility between healthy denture-wearing group and healthy non-denture-wearing group were found (Lyon et al. 2008). This finding is in agreement with our azole antifungal susceptibility testing results. An interesting retrospective study was also conducted to investigate the antifungal susceptibility and yeast colony numbers in people with full or partial dentures over a period of five years. The results demonstrated that mean colony number in full denture wearers was about two-fold the related number present in a partial denture group. *C. albicans* was the most frequent isolated yeast, but its frequency dropped during the five studied years, whereas other yeast species such as *C. glabrata* showed an increasing frequency during the study's five-year period (Loster et al. 2012). We could not find any identical or comparable studies to our own in the literature, which was conducted in Iran, except for two reports that evaluated only the yeast colonization levels in denture-wearing and non-denture-wearing people. In more recent research, the participants were elderly people with heart conditions attending a hospital in Tehran, the capital city of Iran. Results showed a higher percentage of oral colonization by yeasts in a denture-positive group compared to a denture- negative group and this difference was highly significant. Mean CFU counts of *C. albicans* in denture-acquired people was higher than that for people with natural teeth and the difference was highly significant (Taheri Sarvtin et al. 2014). In another report performed in Iran, the authors again evaluated the colonization of dentures by yeasts and identified them according to species level. *C. albicans* was the most often isolated yeast, while the authors also stated that they had been able to isolate *C. dubliniensis* species at a considerable frequency (Zomorodian et al. 2011). A high prevalence of *C. dubliniensis* was also observed in our study, which might direct us to the proposal of a possible connection between denture-wearing and colonization by *C.*

dubliniensis in healthy people.

Considering the above mentioned literature sources, our study is possibly the first to primarily focus on a comparison of three antifungal susceptibility testing values, as well as colony counts between healthy denture wearers and non-denture wearers in Iran. Other studies focusing on oral mycoflora in Iran have mainly employed immunocompromised patients, particularly HIV positive individuals. However, there are some complications to such studies, including difficult sampling procedures, no standardized or general antifungal susceptibility testing protocols for all yeast species, observer error when visually interpreting MIC results, the presence of both intrinsic antifungal-resistant and non-resistant isolates belonging to one species and the possibility of errors in microdilution broth protocols, due to extensive hand manipulation and human involvement during the testing procedures.

Conclusion

Wearing dentures may lead to an increase in the colonization of oral yeasts. Moreover, wearing dentures may cause slight changes in antifungal susceptibility values, although these changes are not significant. The changes may occur in specific situations, for example, if dentures are not regularly cleaned or other predisposing factors consequently occurred with denture-wearing, such as smoking. Further precise studies are required to gain a clear understanding of the exact effect of each factor on oral fungal inhabitants.

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Journal of Mycology Research. Vol 1, No 1: Page 55-62, September 2014

Rolling Circle Amplification (RCA): an approach for quick detection and identification of fungal species

Sahar Javaheri Tehrani[1], Mansour Aliabadian[1.2], Abdolmajid Fata[3], Mohammad Javad Najafzadeh[4*]

[1]Department of Biology, Ferdowsi University of Mashhad, Mashhad, Iran

[2]Research Department of Zoological Innovations, Institute of Applied Zoology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

[3]Department of Parasitology and Mycology, Research Center for Cutaneous Leishmaniasis, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

[4]Department of Parasitology and Mycology and Cancer Molecular Pathology Research Center, Ghaem Hospital, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

*** Corresponding Author: Email: najafzadehmj@mums.ac.ir, Tel: +98(513) 84031401**

(Received: 20 July 2014, Accepted: 30 August 2014)

Abstract:

Conventional methods for fungal identification in the clinical laboratory rely on morphological and physiological tests. These tests often need several days or weeks to complete and are frequently unspecific. Molecular identification mostly implies sequencing, which is relatively expensive and time-consuming, as well as impractical for large numbers of isolates. The Rolling Circle Amplification approach, known as RCA, is a quick, critical and economic method for fungal species' identification. Despite its high speed, this method is highly sensitive, and it has been widely used for the detection of pathogenic fungi. The specific probes are designed based on the differences in the nucleotide regions of the target gene for the target species. The amplification product can be visualized by agarose gel electrophoresis, but can also be visualized in gel-free systems using fluorescence staining of the amplified product by SYBR Green in combination with a UV transilluminator. Thus, the simplicity, sensitivity, robustness and low costs make RCA an attractive technique for the reliable identification of sibling species and other closely related fungi.

Keywords: Padlock probes, Rolling Circle Amplification (RCA), Single nucleotide polymorphisms.

Introduction

It seems fair to say that many systematists agree that fungi species are real, important and sometimes extremely difficult to identify. There is also a growing sense that the virtually limitless empirical data available from emerging genomic databases may help solve the problem of delimiting difficult, recently derived species (Shaffer and Thomson, 2007). By creating a gene and protein bank, the researchers have found some methods that utilize the help of the new science of Bioinformatics to provide more accurate, specific and faster techniques than the conventional molecular techniques, which are very expensive and require skilled professionals in Diagnostic Sciences (Brown, 2010; Zhou et al., 2008). Today, some of the methods which have been receiving more attention from researchers are the Isothermal methods, in which DNA replication is made possible without the need to apply a thermal cycler. RCA is one such method, which has received most attention because of its ultra-high specificity. In RCA, a Padlock probe is used to identify the SNPs (Single-nucleotide polymorphisms) of the genome and since it creates a closed form in binding to a target DNA, it almost completely removes the risk of non-specific sequences replication (Gusev et al., 2001; Moradi et al., 2008; Schweitzer and Kingsmore, 2001; Yoshida et al., 2005).

RCA: Rolling Circle Amplification (RCA) is a sensitive, specific and reproducible isothermal

DNA amplification technique used for rapid molecular identification of microorganisms (Najafzadeh et al., 2013) which has gained great attention over the past decade (Wang et al., 2014b; Kuhn et al.; 2002). RCA technology, which has an intrinsically wide dynamic range, involves a robust and simple procedure that can provide a universal platform for the localization of a wide variety of molecules as a function of either antigenicity or nucleic acid sequence (Gusev et al., 2001). RCA is particularly useful to discriminate closely related species or genotypes within species, which may differ by only single nucleotide differences (Sun et al., 2011). RCA is based on the rolling replication of short single-stranded DNA (ssDNA) circular molecules (Lizardi et al., 1998; Fire and Xu, 1995; Najafzadeh et al., 2011) by certain DNA polymerases at a constant temperature, which only requires a simple platform, such as heating blocks or a water bath (Tsui et al., 2011a). This process discovered in the mid-1990s (Nilsson, 2006; Kobori and Takahashi, 2014; Demidov, 2005). The development of RCA probes to distinguish single species or groups of species relies on the presence of sufficient sequence data and useful species-specific polymorphisms in the genes of correctly identified species (Davari et al., 2012). The ligation allows efficient distinction among sequence variants and can efficiently be utilized for detection of single nucleotide polymorphisms, as DNA ligase will ligate the two ends of the probes only in cases of a perfect match with the target (Jehan and Lakhanpaul, 2006). Furthermore, the ligation reaction is sensitive to mismatches between the probes and the target (Wang et al., 2005; Kaocharoen et al., 2008a; Zhou et al., 2008; Tsui et al., 2010a; Tsui et al., 2013). RCA uses a strand-displacing DNA polymerase to continuously amplify a circular DNA template at a constant low temperature, producing a long DNA molecule with tandem repeats of the circular template (Asiello and Baeumner, 2011). Deoxynucleotides (dNTPs) are added to extend a primer bound to a single-stranded circular template, by DNA polymerases possessing strand displacement activity. This gives rise to a long single-stranded fragment of DNA comprising concatemers of the original circular template (Pang et al., 2007). RCA involves an initial forward primer that binds to the padlock probe and initializes RCA, and a second primer that targets the repeated ssDNA sequence of the primary RCA product, finally generating large numbers of copies of the DNA fragments. This is called hyperbranching RCA (H-RCA) (Tsui et al., 2011a; Pang et al., 2007; Lizardi et al., 1998; Tsui et al., 2013). Accordingly, the geometric RCA is more potent, as compared with its linear alternative, yielding 109 or more copies of a circular sequence in about an hour (Demidov, 2005). Non-circularized probes are removed by exonuclease treatment, while the circularized ones may be amplified by using universal primers (Szemes et al., 2005). By increasing the hybridization temperature and shortening the 3' arm (below the reaction temperature), the discrimination of SNP can be further improved (Faruqi et al., 2001; Tsui et al., 2011a) and precise amounts of RCA product can be generated that are dependent upon the quantity of dNTPs incorporated into the reaction mixture (Pang et al., 2007). Due to the drastic signal amplification power, RCA has been widely employed in various sensing schemes for the analyses of proteins and nucleic acids (Li et al., 2008; Wang et al., 2014a). The RCA reactions have been run on the single-stranded DNA and RNA targets, and also, with the aid of PNA openers, these reactions can be performed with dsDNA (Demidov, 2005). The duration of the RCA assay was two hours (Najafzadeh et al., 2013), however, a positive signal was usually evident within 15 minutes after onset of RCA reaction when performed by real time PCR (Sun et al., 2011). The entire process, including DNA extraction, PCR amplification, ligation of padlock probes, exonucleolysis, RCA itself and gel electrophoresis could be finished within one working day (Sun et al., 2011).

Materials and Methods

DNA extraction and amplification: DNA extraction protocols vary with the samples used.

The ITS region is widely used as a target sequence for the identification of pathogenic fungi. However, whereas many fungi show insufficient diversity in ITS, hypervariable partial genes and introns can be used, such as tubulin, actin, translation elongation factor1-α. The amplicons were generated with commercial primers following the manufacturer's instructions.

Padlock probe design: In order to design a padlock probe, at first we selected a gene with enough resolution as the target for the padlock probe design (Tsui et al., 2013). To ensure the efficiency of the padlock probe binding, the padlock probes were designed with minimum secondary structure and with the Tm of the 5' end probe binding arm close to or above the ligation temperature (63 °C) (Feng et al., 2013; Najafzadeh et al., 2013; Najafzadeh et al., 2011). To increase its discriminative specificity, the 3' end binding arm was designed with a Tm 10°C-15°C below ligation temperature (Najafzadeh et al., 2013; Najafzadeh et al., 2011) and specificity can be increased by selecting polymorphisms in the 3' end binding arm (Najafzadeh et al., 2013). The genetic linker region was also carefully designed to minimize any similarity to potentially cross-reacting sequences after BLAST search. The specificity of the probes was confirmed by BLAST analysis in GenBank (Sun et al., 2011).

A padlock probe refers to long oligonucleotides (about 100 bp) (Tsui et al., 2011a), comprising (i) a 5´-phosphorylated end, (ii) a "backbone" containing binding sites for the RCA primers (RCA primers 1 and 2, respectively; designated by bold uppercase letters) as well as the nonspecific linker regions (designated by bold lowercase letters), and (iii) a 3´ end. The 5´ and 3´ ends of the probe are complementary to the 5´ and 3´ termini of the target sequence in reverse (Zhou et al., 2008; Tong et al., 2007). Phosphate groups were added at the 5' ends of the molecules as required for enzymatic ligation (Nilsson et al., 1994). The basic structure of a padlock probe is depicted in Fig. 1.

Ligation of the padlock probe: One microliter of gene amplicon was mixed with 2 U pfu DNA ligase (Epicentre Biotechnologies, Madison, WI, USA) and 0.1 µmol lµ1 padlock probe in 20 mmol lµ1 Tris-HCl (pH 7.5), 20 mmol lµ1 Cl, 10 mmol lµ1 MgCl2, 0.1% Igepal, 0.01 mmol lµ1 rATP, and 1 mmol lµ1 DTT, with a total reaction volume of 10 µl. Padlock probe ligation was conducted with one cycle of denaturation for five minutes at 94°C, followed by five cycles of 94°C for 30 seconds and four minutes of ligation at 63°C.

Exonucleolysis: Exonucleolysis is required to remove an unligated padlock probe and template PCR product and thus reduces subsequent ligation-independent amplification events. This was performed in a 20-µl vol by addition of 10 U each of exonuclease I and III (New England Biolabs, Hitchin, UK) to the ligation mixture and incubation at 37°C for 30 minutes, followed by 94°C for three minutes to deactivate the exonuclease reaction.

Rolling Circle Amplification (RCA) reaction: Two microliters of ligation product were used as a template for RCA. The total volume was 50 µl containing 8 U Bst DNA polymerase (New England Biolabs), 400 µmol lµ1 deoxynucleoside triphosphate mix, and 10 pmol of each RCA primer in distilled water. Probe signals were amplified by incubation at 65°C for 60 minutes.

Data Analysis: The RCA amplicons can be detected using several methods, such as fluorescence (Szemes et al., 2005), radiolabeling (Banér et al., 1998), UV absorbance (Kuhn et al., 2002), and gel electrophoresis (Sun et al., 2011), by using either direct incorporation of various labels into the RCA products (Banér et al., 1998) or label-decorated amplicons (Schweitzer and Kingsmore, 2001) and colorimetrically (Ali and Li, 2009). The simplest method is gel electrophoresis on a 1% agarose gel to verify the specificity of probe-template binding. Positive reactions showed a ladder-like pattern, whereas negative reactions showed a clean background.

Applications of RCA: The RCA technology is promising for molecular diagnostic and pharmacogenomic use (Kuhn et al., 2002). To date, RCA has mainly been used for the detection of viruses (Wang et al., 2005, Schubert et al., 2007) and bacteria (Tong et al., 2007). The RCA technique has successfully been applied to

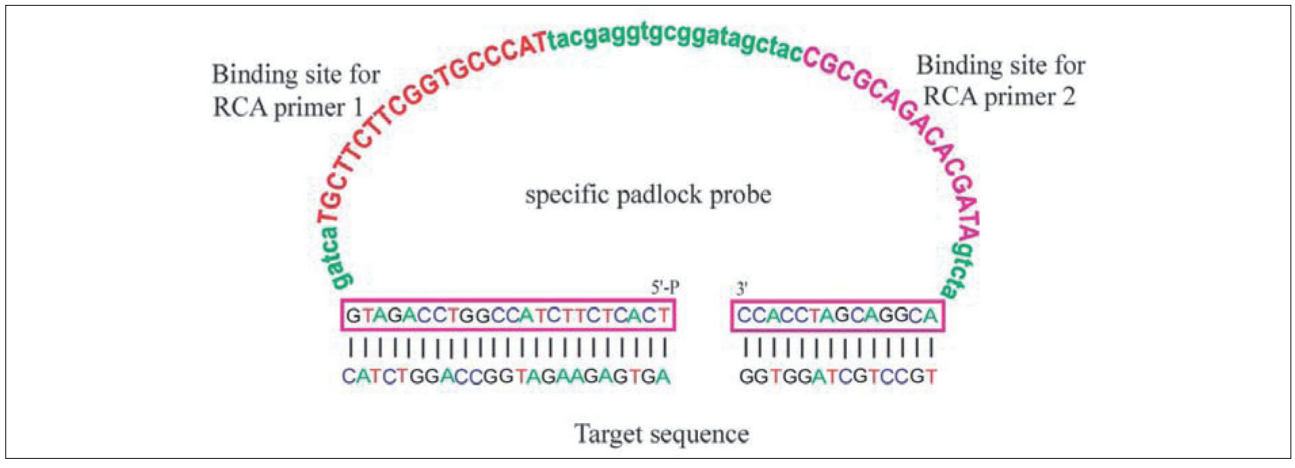

Fig. 1. Schematic representation of a padlock probe.

different fungal species like *Candida*, *Aspergillus*, and *Scedosporium* spp., *Cyphellophora* and relatives, *Fonsecaea* spp., *Exophiala* spp., *Trichophyton* spp., *Penicillium marneffei*, *Cladophialophora* carrionii, Pneumocystis jirovecii, *Fusarium* spp.and *Cryptococcus* species (Zhou et al., 2008; Najafzadeh et al., 2011; Sun et al., 2011; Kong et al., 2008; Tong et al., 2007; Feng et al., 2013; Lackner et al., 2012; Hamzehei et al., 2013; Tsui et al., 2010b; Chen and Kong, 2007; Tsui et al., 2011b; Kaocharoen et al., 2008b; Eriksson et al., 2009). RCA provides a powerful tool for a rapid and specific identification of fungi in the clinical laboratory and offers significant potential for ecological studies (Sun et al., 2011). The RCA potential to identify nucleic acid targets, antibodies and antigens in clinical samples has recently been demonstrated in several feasibility studies (Demidov, 2005). RCA-mediated multiplex profiling of cytokines on microarrays with femtomolar sensitivity offers an advantageous approach for proteomic surveys (Demidov, 2005; Li et al., 2008; Banér et al., 1998). RCA can be used for mitochondrial DNA visualization in cells immobilized on a glass substrate (Kobori and Takahashi, 2014). RCA can also be used for accurate and sensitive detection of allergens in food, which is imperative for eliminating potential health hazards triggered by food allergies (Kobori and Takahashi, 2014). Combining RCA with electrical DNA detection produces results in readout with a very high signal-to-noise ratio, an essential feature for sensitive and specific detection assays (Russell et al., 2014). RCA could enhance the use of markers of current interest, as well as permit the integration of emerging information from genomics and proteomics into cell- and tissue-based analyses (Gusev et al., 2001). This technique has been employed for the detection of single nucleotide polymorphisms (SNPs) within DNA fragments, forming the basis of diagnosis for numerous disease states (Pang et al., 2007). The method has been applied for amplified detection of viral RNA from tissue samples and for preparative in vitro synthesis of catalytic antisense RNA (Banér et al., 1998). RCA has also been combined with magnetic beads and reporter DNA probes in a sandwich assay to detect viral DNA (Asiello and Baeumner, 2011).

Advantages and limitations: (RCA has several substantial advantages over other amplification techniques, as follows): RCA is sensitive (Demidov, 2005; Feng et al., 2013; Najafzadeh et al., 2013; Pang et al., 2007; Davari et al., 2012; Tsui et al., 2010a; Kong et al., 2008; Kuhn et al., 2002), specific (Najafzadeh et al., 2013; Kong et al., 2008; Feng et al., 2013; Russell et al., 2014; Najafzadeh et al., 2011), rapid (Feng et al., 2013; Najafzadeh et al., 2013; Tsui et al., 2010a; Kong et al., 2008; Wang et al., 2014a; Najafzadeh et al., 2011), inexpensive (Najafzadeh et al., 2013; Demidov, 2005), cost effective (Feng et al., 2013; Davari et al., 2012), easily operated (Feng et al., 2013; Wang et al., 2014b), contamination resistant (Kobori and Takahshi, 2014), highly efficient (Wang et al., 2014a), flexible (Kong et al., 2008)

and more error-proof (compared to PCR) (Demidov, 2005). RCA does not require highly specialized equipment, while it is relatively easily expanded to multiple or routine identifications (Davari et al., 2012). RCA is reproducible, thus reducing the chance of false positives (Najafzadeh et al., 2013; Demidov, 2005; Tsui et al., 2010a; Kong et al., 2008; Najafzadeh et al., 2011). RCA can also be performed under isothermal conditions and does not require thermal cycling (Kobori and Takahashi, 2014; Asiello and Baeumner, 2011; Pang et al., 2007; Demidov, 2005; Li et al., 2008) and makes RCA readily adaptable to routine clinical use with fewer issues concerning quality control of the instrument (Li et al., 2008). RCA can be performed by a larger variety of DNA polymerases compared to PCR, which only relies on thermostable enzymes (Demidov, 2005). Interpretation of the results is straightforward and is based on a simple positive or negative result (Tsui et al., 2010a). The method's simplicity, large multiplex potential, immunity to false positives/ cross-contamination and easy compatibility with other detection/ imaging techniques are key advantages (Kuhn et al., 2002). Furthermore, the RCA technology provides a faster, more sensitive and economical option to the currently available PCR-based methods (Wang et al., 2005). The most distinguished feature of RCA is that it can be easily carried out on a chip for high-throughput detections (Feng et al., 2013; Wang et al., 2014b). Arguably the main advantage of RCA is that it can be performed under isothermal conditions with minimal reagents and that it avoids the generation of false-positive results, a problem that is frequently encountered in PCR-based assays (Wang et al., 2005). All these unique properties of RCA facilitate its application in different research and molecular diagnosis areas like in situ detection, microarray, immunoassay, SNP, etc. (Li et al., 2008). The shortcoming of supersensitive RCA assays is that they require certain caution to avoid possible contamination/false positives (Demidov, 2005).

Conclusion

Rapid and accurate detection and identification of fungal pathogens at the species and subspecies Level both in the clinical setting (Szemes et al., 2005) and natural environment, or on plant materials, are the keys to proper patient treatment and disease/pathogen surveillance, containment and eradication. However, many fungal pathogens exist as species-complexes or they have very low abundance in the clinical specimen and natural environment (Tsui et al., 2013). Furthermore, sensitive and selective detection of sequence-specific DNA has become increasingly important in modern life science owing to its potential applicability, ranging from genetic research of diseases to clinical diagnosis and therapy (Wang et al., 2014a). In addition, the genome information of many species has been revealed; DNA-based analyses have been crucial in many biotechnology industries in the medical- and food-related sectors (Kobori and Takahashi, 2014). Furthermore, as a result of the padlock probes being used as a means of combining pathogen-specific molecular recognition and universal amplification, increasing sensitivity and multiplexing capabilities without limiting the range of potential target organisms has been achieved (Szemes et al., 2005). Due to these robustness and simplicity characteristics, the RCA-based assays hold a distinct position in the area of molecular diagnostics among other single-temperature amplification techniques (Demidov, 2005). Therefore, it is recommended to use the RCA technique as an easy and practical method with a distinct position among isothermal techniques, for DNA diagnostics as a very practical identification method (Najafzadeh et al., 2011).

Acknowledgments

This study was financially supported by the Office of Research Affairs, Ferdowsi University of Mashhad, Iran (Project no. 2/24432) and by the Deputy of Research, Mashhad University of

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