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ORIGINAL ARTICLE

A sensitive and a rapid multiplex polymerase chain reaction for the identification of *Candida species* in concentrated oral rinse specimens in patients with diabetes

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ABSTRACT

Objectives: Oral candidiasis is being frequently recognized in patients with diabetes, and is associated with multiple pathogens including *Candida albicans, Candida parapsilosis, Candida glabrata* and *Candida tropicalis.* The aim of this study was to evaluate a usefulness of a Multiplex Polymerase Chain Reaction as a rapid diagnostic tool for identification of four oral Candida pathogens in patients with diabetes.

Materials and methods: A multiplex PCR was optimized to identify four *Candida species* in concentrated oral rinse samples. Common reverse primer, *ITS4* and four species-specific forward primers targeting *ITS1* and *ITS2* regions of yeast genome were used. Species-specific single amplicon were detected by agarose gel electrophoresis. Performance efficacy of multiplex PCR was compared with phenotypic identification.

Results: Out of 100 oral rinse samples, 72 were culture positive and of these 43 were at risk of oral Candida infection (>600cfu/ml). Multiple *Candida species* including *C. albicans, C. parapsilosis* and *C. tropicalis* were identified in 22 samples which had risk of oral Candida infection. In total, 85 patients were positive for Candida by multiplex PCR and of them 49 had multiple *Candida species*. All 43 colonized specimens were also positive by multiplex PCR. *C. albicans* was the most predominant organism (75/85) followed by *C. parapsilosis* (47/85), *C. tropicalis* (17/85) and *C. glabrata* (6/85). In specimens with multiple species, the two most common organisms were *C. albicans* and *C. parapsilosis.* Multiplex PCR yielded a sensitivity of 10 Candida cells/ml of oral rinse sample.

Conclusions: Multiplex PCR is found to be rapid, sensitive and specific than phenotypic identification methods in discriminating multiple *Candida species* in oral rinse specimens.

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Introduction

Candida species are commensals of the oral cavity, where *Candida albicans* is the predominant species, followed by *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata* and *Candida dubliniensis.*[1] Oral candidiasis is often associated with multiple pathogens predominantly *C. albicans, C. parapsilosis, C. glabrata and C. tropicalis.*[2] Oral candidiasis is a frequently observed opportunistic infection in patients with type 2 diabetes due to elevated glucose in their oral fluids and immune dysfunction.[3]

Rapid and accurate identification of *Candida species* is important for the better management of the patient due to the emergence of antifungal resistance.[4,5] While novel phenotypic identification techniques such as CHROMagar Candida medium are available, the results can be ambiguous because *Candida species* show high-degree strain variability and exhibit phenotypic switching.[6] Most of the other phenotypic tests such as API identification kits require prior pathogen isolation, which is time consuming and obviously with associated bias.[7] Molecular methods are proven to provide a more accurate and rapid identity especially in identifying polymicrobial Candida infections.[8,9]

As molecular methods provoke rapid, sensitive and specific identity of yeast species, different techniques including PCR, real time PCR, have being evaluated to identify multiple yeast species in clinical specimens.[9,10] PCRs based on *ITS*, 18S and 26S-28S rDNA regions have been used to identify *Candida species* in different clinical specimens including, saliva, blood, oral rinse, vaginal swabs, etc.[8–11] *ITS* region of the yeast is conserved compared to the large subunit rDNA gene,[12] and hence is a unique target for *Candida species* identification. Multiplex PCR has been evaluated to identify different *Candida species* in oral rinse specimens,[9] blood cultures [10] and vaginal specimens.[11] Different primer sets have been evaluated in these studies including universal yeast primers such as ITS1, ITS4 and species-specific primers such as CA, CT,[10,12] etc. However in most of these studies, researchers have used multiplex PCR to identify culture isolated yeasts while only limited studies have applied this technique to direct clinical specimens.[9,10,13] Multiplex PCR is

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useful in discriminating multiple yeast species based on unique banding patterns. However in some multiplex PCR studies, multiple bands were encountered for a single yeast pathogen.[9,13] Although, the studies have found multiplex PCR as a useful technique in identifying multiple *Candida species* in clinical specimens,[9,10] species identification based on more than a single band is less specific and difficult to interpret specially in clinical specimens having multiple yeast pathogens. In this study, we have evaluated the usefulness of a multiplex PCR which yields a unique single band for each yeast pathogen.

This study aimed to evaluate the usefulness of a rapid sensitive multiplex PCR to detect four important Candida pathogens in concentrated oral rinse samples where multiple *Candida species* may be present. Further, the study compared the performance of multiplex PCR with phenotypic yeast identification in identifying yeasts in concentrated oral rinse samples of patients with diabetes.

Materials and methods

Yeast strains

The yeast strains used to optimize the multiplex PCR were *C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, *C. parapsilosis* ATCC 22019 and *C. glabrata* ATCC 90030.

Concentrated oral rinse sample

Oral rinse samples were collected from 100 patients with type 2 diabetes. Ethical approval for the study was granted by the Ethics Review Committee, University of Sri Jayewardenepura, Sri Lanka (number 764/13). The experiments were undertaken after obtaining written consent of each subject in full accordance with ethical principles. Patients were given 10 ml of sterile phosphate-buffered saline in containers and advised to rinse their mouths for 60 s and spit into the same container provided. These oral rinse samples were kept at 4 °C and transported to the Department of Microbiology, for laboratory identification. The oral rinse sample was centrifuged at 6000 rpm for 10 min, and the supernatant was discarded. One milliliter of sterile phosphate-buffered saline was added to re-suspend the pellet and mixed well; 100 µl of the re-suspended specimens was used for culture in triplicate and remaining 700 µl was used to extract DNA.

Analysis of yeasts in concentrated rinse sample by culture

Concentrated oral rinse sample (100 µl) was spread on Sabouraud's dextrose agar (SDA) plates in triplicates for semi quantitative analysis. After 24–48 h incubation at 37 °C, Colony Forming Units (cfu/ml) were recorded in the three plates, and the mean cfu/ml was calculated. Candida cell count of >600 per ml was considered as risk to oral Candida infection according to previous literature.[14–16] Presumptive identities of yeasts were done by germ tube test, sugar assimilation tests and chlamydospores formation.

DNA extraction using bead beater method

A concentrated oral rinse sample was used for DNA extraction using the bead beater method [17] with modifications. The 700 µl concentrated oral rinse sample was centrifuged (13000 rpm/10 min) and supernatant was discarded. Pellet was suspended in 100 µl STES buffer [200 mM Tris HCl (pH 7.6), 100 mM EDTA, 0.1% SDS] and 40 µl of TE buffer [10 mM Tris HCl (pH 8), 1 mM EDTA], 120 µl Phenol: Chloroform mixture (1:1 V/V) and 0.3 g sterile zirconium beads (0.1 mm diameter; Bio Spec-Products) were added. Then the sample was homogenized using mini bead beater (3110 BX, Biospec products, Bartlesville, OK) at 480 rpm for 5 min. The upper aqueous phase (100 µl) was transferred to a sterile micro centrifuge tube, and DNA was precipitated in the presence of 220 µl cold ethanol (100%) and 10 µl of 3 M sodium acetate at −20 °C for 18 h. The solution was centrifuged at 13000 rpm for 12 min, and the DNA pellet was air-dried and then dissolved in 30-µl TE buffer. Extracted DNA samples were stored at −20 °C.

Multiplex PCR

Multiplex PCR was carried out in 25 µl volume with 2 µl template DNA, 1x green GoTaq Flexi buffer (pH 8.5), 3 mM $MgCl_2$, 0.2 µM of each primer (ITS 4, CA, CT, CP, CGL) (Table 1), 0.2 mM Deoxy Nucleotide Triphosphate (dNTP) mix and 1.25 unit of Taq DNA polymerase (Promega, USA). A previously published universal primer *ITS4* [13] which targeted the *ITS* region of the yeast was used as the common reverse primer (Table 1) along with four other species-specific forward primers designated as CA, CT, CP and CGL (Table 1). These species-specific primers were previously published and targeted the *ITS1* and *ITS2* regions of *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata* [13] (Figure 1).

PCR amplification was done using GeneAmp PCR systems 9700 (Applied Bio Systems, USA). The PCR reaction was

Table 1. The primer sequences used for amplification of yeasts as described by Li et al.[10]

Target gene	Primer[a]	Primer sequence (5′–> 3′)	Amplicon size
26 S rDNA	ITS4	TCC TCC GCT TAT TGA TAT GC	–
ITS 1	CGL	CAC GAC TCG ACA CTT TCT AAT T	632 bp
ITS 1	CA	TCA ACT TGT CAC ACC AGA TTA TT	402 bp
ITS 2	CT	AAG AAT TTA ACG TGG AAA CTT A	149 bp
ITS 2	CP	GGC GGA GTA TAA ACT AAT GGA TAG	126 bp

[a]CGL, CA, CT and CP were forward primers while ITS 4 was common reverse primer.

Figure 1. Relative positions of various primers in the yeast genome which were used in the study.

initiated at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 S, 50 °C for 1 min, 72 °C for 1 min and a final elongation step at 72 °C for 5 min with final hold at 4 °C. All PCR experiments included a negative (no template) control and a positive control. Resulting PCR products were separated by electrophoresis using 1x TAE [40 mM Tris HCl (pH 8), 20 mM acetic acid, 1 mM EDTA] on a 3% (w/v) agarose gel, stained with ethidium bromide and viewed by UV trans-illuminator (Vilber Lourmat, QUANTUM ST5).

Optimization of multiplex PCR protocol

DNA extraction method

Two DNA extraction methods for extraction of yeast genomic DNA were compared: boiling method [9] and bead beater extraction method.[16] For this experiment, 10 ml of oral rinse sample was spiked with known cell concentrations of *Candida species* and divided into 5 ml aliquots and subjected to DNA extraction by both methods (boiling and bead beater method). Oral rinse samples were boiled, centrifuged and supernatant was used as template for direct multiplex PCR. But boiling method proved to be of low sensitivity possibly due to poor DNA extraction and presence of inhibitors in saliva. Therefore, a bead beater extraction method was further optimized to obtained reasonable yield of DNA.

Primer validation for multiplex PCR

DNA extracted from standard *Candida species* (*C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, *C. parapsilosis* ATCC 22019 and *C. glabrata* ATCC 90030) were subjected to PCR using species-specific forward primers namely CA, CT, CP, CGL and common *ITS4* reverse primer. A product of 402 bp for *C. albicans*, 149 bp for *C. tropicalis*, 126 bp for *C. parapsilosis* and 632 bp for *C. glabrata* was considered as positive amplification of the particular *Candida species.*

Thermo cycling parameters

The annealing temperature for multiplex PCR was optimized using an annealing temperature between 45 and 55 °C. Different $MgCl_2$ concentrations (1–4 mM) were also tested. The PCR cycle numbers were varied and 30, 35, 40, 42 and 45 cycles were examined to determine optimal PCR amplification condition.

Determination of PCR inhibitors

In order to evaluate the inhibitory effects of saliva for this particular PCR amplification process, a comparative analysis was carried out using PBS as a control. A loopful of each *Candida species* was spiked into 10 ml of oral rinse solution and 10 ml of PBS solution in triplicate. The mixtures were then centrifuged (6000 rpm, 10 min), and supernatant was discarded. Pellet was suspended in 1 ml PBS and 700 µl was subjected to bead beater extraction followed by multiplex PCR.

PCR additives

Efficacy of PCR facilitators DMSO (1–5%), Formamide (0.25–1%) and BSA (0.2–0.6 mg/µl) to increase the sensitivity of the multiplex PCR was evaluated.

Sensitivity of the multiplex PCR

In order to determine the detection limit of Candida cells in concentrated oral rinse specimen by multiplex PCR, oral rinse specimen obtained from three healthy volunteers (confirmed as negative for Candida by culture and PCR methods) were pooled, and 10 ml each was aliquoted into three tubes containing 10 ml each. These were then spiked with 10^7 cfu/ml each *Candida species* (*C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata*). The spiked oral rinse samples were then serially diluted with above collected oral rinse specimen and were used for DNA extraction. These diluted samples were used for isolation of Candida DNA. Multiplex PCR amplification was then carried out using 2 µl of DNA as template to determine the sensitivity of the PCR.

Statistical analysis

Statistical analysis was carried out using 17.0 version of SPSS software. The chi-square test (X^2test) and Fisher's exact test were carried out to assess possible association. All these tests were two-sided. Significance level was specified as $p < 0.05$.

Results

Demographic data, selected risk factors and diabetes complications

All 100 patients included in the study were type 2 diabetes patients. The mean age of the patient population was 59.5 years and was between 35 and 85 years. Among them, 47 were males and 53 were females.

Oral *Candida species* were isolated from 28 males and 44 females. Of them, 14 males and 29 females had over 600 cfu/ml of Candida in their oral rinse specimens, indicating a risk for oral Candida infection. Female patients were found to be significantly predisposed to Candida carriage. Further being a female was found to be a risk factor for oral Candida infection ($p < 0.05$) (Table 2). Female gender also showed a statistically significant association with the presence of multiple *Candida species* in the oral cavity ($p < 0.05$) (Table 3).

Candida species were isolated from 11 out of 15 patients who claimed to chew betel-quid and 9 out of 16 patients who claimed to smoke. However, the habits of smoking and betel-quid chewing did not have a significant association with the presence of positive Candida by culture. Further these habits did not show a significant association with risk of oral Candida infection (>600 cfu/ml) (Table 2). Dentures were worn by 32 patients and of them 29 were positive for Candida by culture. *Candida species* >600 cfu/ml were detected in 25 patients and were at risk of oral infection. Of the 32 denture wearers, 31 were positive for Candida by PCR.

Table 2. Association between presence of oral yeast and risk of oral Candida infection (over 600 CFU) with patients' gender, habits such as smoking, betel chewing and denture-wearing condition in this study population of 100 diabetes patients.

		Presence of Candida by culture			Candida colony count			Presence of Candida by PCR		
Factor	N (%)	Positive N (%)	Negative N (%)	*p* value	≥600 CFU *N* (%)	≥ 600 CFU *N* (%)	*p* value	Positive *N* (%)	Negative *N* (%)	*p* value
Gender										
Male	47	28 (38.9)	19 (67.9)	0.009*	14 (32.6)	33 (57.9)	0.012*	37 (43.5)	10 (66.7)	0.098*
Female	53	44 (61.1)	09 (32.1)		29 (67.4)	24 (42.1)		48 (56.5)	05 (33.3)	
Smoking										
Yes	16	09 (12.5)	07 (65)	0.139**	06 (14)	10 (17.5)	0.628*	13 (15.3)	03 (20)	0.704**
No	84	63 (87.5)	21 (75)		37 (86)	47 (82.5)		72 (84.7)	12 (80)	
Betel Chewing										
Yes	15	11 (15.3)	04 (14.3)	1.000**	08 (18.6)	07 (12.3)	0.381*	15 (17.6)	00 (—)	–
No	85	61 (84.7)	24 (85.7)		35 (81.4)	50 (87.7)		70 (82.4)	15 (100)	
Denture wearing										
Yes	32	29 (40.3)	03 (10.7)	0.004*	25 (58.1)	07 (12.3)	0.000*	31 (36.5)	01 (6.7)	0.033**
No	68	43 (59.7)	25 (89.3)		18 (41.9)	50 (87.7)		54 (63.5)	14 (93.3)	

*p value taken from χ^2 test.
**p value taken from Fisher's exact test, –;p value cannot be calculated.

Table 3. Association between several facts and presence of multiple *Candida species*.

	Multiple *Candida species* from multiplex PCR		
Fact(s)	Presence *N* (%)	Absence *N* (%)	*p* value
Gender			
Male	14 (29.2)	33 (63.5)	0.001*
Female	34 (70.8)	19 (36.5)	
Smoking			
Yes	06 (12.5)	10 (19.2)	0.359*
No	42 (87.5)	42 (80.8)	
Betel chewing			
Yes	09 (18.8)	06 (11.5)	0.303*
No	39 (81.2)	46 (88.5)	
Denture wearing			
Yes	23 (47.9)	09 (17.3)	0.001*
No	25 (52.1)	43 (82.7)	
Duration of diabetes			
<5 yrs	16 (33.3)	17 (32.7)	0.849*
5–10 yrs	09 (18.8)	13 (25.0)	
11–15 yrs	06 (12.5)	07 (13.5)	
>15 years	17 (35.4)	15 (28.8)	

*p value taken from χ^2 test.

There was a statistically significant association between denture wearing and presence of Candida by culture and PCR ($p < 0.05$). Further a statistical significance was observed between denture wearing and risk of oral Candida infection and denture wearing and presence of multiple species (Table 3).

Out of 100 patients, 33 had been diagnosed with diabetes within the last 5 years, 22 had diabetes for a duration of 5–10 years, 13 for 10–15 years and 32 for >15 years. In this study population, the patients who were positive for Candida by culture and PCR, as well as the patients who had >600 cfu/ml, showed no significant association with the duration of diabetes ($p > 0.05$).

Out of 100 patients with diabetes, 62 had complications including retinopathy, peripheral neuropathy, diabetes nephropathy and diabetes foot ulcers. However, there was no statistically significant association between these complications and risk to the Candida infection ($p > 0.05$) (Table 4). In the study group, 14% had xerostomia, 13% had taste alteration and 6% had halitosis. No statistical significance was found with these clinical presentations and risk of Candida infection (Table 5).

Optimization of the multiplex PCR

Conventional glass bead extraction method was found to be more effective to obtain high DNA yields (up to 1200 ng/μl) compared to the boiling method. The purity of the DNA was also higher using bead extraction method. Saliva had an inhibitory effect on multiplex PCR as described in Figure 2. Therefore, several PCR facilitators (BSA, Formamide and DMSO) were tested to enhance the PCR reaction, and a significant improvement of multiplex PCR was observed in the presence of BSA as a PCR additive. Annealing temperature 50 °C gave sharp bands for all four *Candida species* in multiplex PCR and non-specific bands were not visible. The optimal concentration of $MgCl_2$ for PCR was 3 mM. When considering the multiplex PCR cycle numbers, a sensitivity of 10 cells/ml was obtained with 35 PCR cycles. Although PCRs with 40 and 42 cycles gave more intense PCR bands, these were avoided due to the possibility in obtaining multiple bands due to non-specific amplification resulting in multiple bands.

Culture identification of Candida species

Out of the 100 concentrated oral rinse samples, 72 were culture positive. *C. albicans* was the predominant organism (64/72) followed by *C. parapsilosis* (21/72), *C. tropicalis* (12/72), *Candida krusei* (5/72) and *C. glabrata* (4/72). Multiple yeast species were isolated in 38 out of 72 positive oral rinse specimens (Table 6).

Of the 72 culture-positive oral rinse samples, 43 had a Candida colony count of >600 cfu/ml and were at risk of developing oral Candida infection. Of them, majority had *C. albicans* (39/43) followed by *C. parapsilosis* (10/43), *C. tropicalis* (7/43), *C. glabrata* (4/43) and *C. krusei* (3/43). Multiple *Candida species* were identified in 22 of the samples out of 43 which had a yeast cfu of >600/ml. Among the patients who had multiple species, *C. albicans* and *C. parapsilosis* were seen in 7, *C. tropicalis* and *C. albicans* in 4, C. *albicans* and *C. glabrata* in 3, C. *tropicalis and C. parapsilosis* in 1, *C. albicans and C. krusei* in 2, *C. albicans* and unidentified yeast species in 3 and *C. parapsilosis* and unidentified yeasts in 1 sample. Three species together were detected in one specimen (*C. albicans*, *C. tropicalis* and *C. parapsilosis*).

Table 4. Association between presence of oral Candida, risk of oral Candida infection and complications of diabetes in the study group.

Complications	No of patients ($N = 100$)	Presence of Candida by culture: Positive *N* (%)	Presence of Candida by culture: Negative *N* (%)	*p* value	Colony count >600 CFU/ml: Positive *N* (%)	Colony count >600 CFU/ml: Negative *N* (%)	*p* value	Presence of Candida by PCR: Positive *N* (%)	Presence of Candida by PCR: Negative *N* (%)	*p* value
Retinopathy	42	31 (43.1)	11 (39.3)	0.732*	19 (44.2)	23 (40.4)	0.700*	39 (45.9)	03 (20)	0.061*
Peripheral neuropathy	28	22 (31)	06 (21.4)	0.342*	13 (30.2)	15 (26.3)	0.666*	25 (29.4)	03 (20)	0.548**
Diabetes nephropathy	11	06 (8.3)	05 (17.9)	0.283**	04 (9.3)	07 (12.3)	0.753**	09 (10.6)	02 (13.3)	0.669**
Diabetes foot ulcers	02	02 (2.8)	00 (-)	–	00 (-)	02 (3.5)	–	01 (1.2)	01 (6.7)	–

*p value taken from χ^2 test.
**p value taken from Fisher's exact test.
¯p values cannot be calculated.

Table 5. Association between presence of oral Candida, risk of oral Candida infection and oral complications of diabetes in the study group.

Oral Complications	No of patients ($N = 100$)	Presence of Candida by culture: Positive *N* (%)	Presence of Candida by culture: Negative *N* (%)	*p* value	Colony count >600 CFU/ml: Positive *N* (%)	Colony count >600 CFU/ml: Negative *N* (%)	*p* value	Presence of Candida by PCR: Positive *N* (%)	Presence of Candida by PCR: Negative *N* (%)	*p* value
Xerostomia	14	09 (12.5)	05 (17.9)	0.527**	05 (11.6)	09 (15.8)	0.553*	12 (14.1)	02 (13.3)	1.000**
Taste alteration	13	10 (13.9)	03 (10.7)	1.000**	05 (11.6)	08 (14)	0.723*	12 (14.1)	01 (6.7)	0.685**
Halitosis	06	05 (6.9)	01 (3.6)	–	03 (7.0)	03 (5.3)	–	06 (7.1)	00 (–)	0.587**

*p value taken from χ^2 test.
**p value taken from Fisher's exact test; –, p values cannot be calculated.

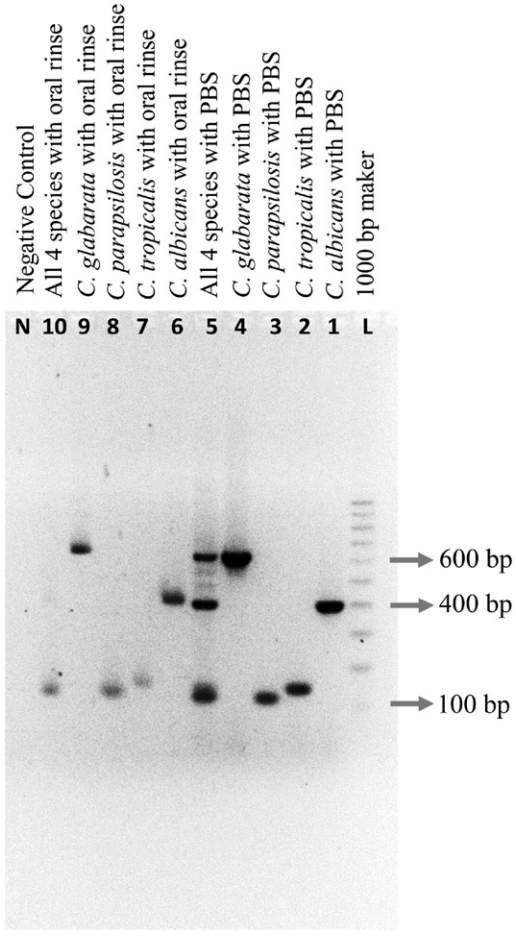

Figure 2. Inhibitory effect of saliva on multiplex PCR. The figure shows the banding patterns for Candida multiplex PCR with template DNA extracted from spiked PBS and spiked oral rinse solution. Lanes 1–5 are the PCR products obtained from *Candida species* in PBS. Lanes 6–10 show PCR products of *Candida species* spiked in oral rinse solution.

Single *Candida species* were identified in 21 out of 43 patients. The predominant species was *C. albicans* (20/21) followed by *C. glabrata* (1/21).

Out of 72 patients, 29 were found to be not at risk of developing oral Candida infection (>600 cfu/ml). Of the 29 specimens, 17 were having multiple species while 12 specimens had single species. Among the patients having multiple species, *C. albicans* and *C. parapsilosis* were seen in 7, *C. albicans* and C. *tropicalis* in 4, *C. albicans* and *C. krusei* in 2 specimens. Further, one single specimen yielded *C. albicans* and other unidentified yeasts, while another single specimen yielded *C. parapsilosis* and other unidentified yeasts. Three yeast isolates together were seen in a one specimen (*C. albicans*, *C. parapsilosis* and *C. krusei*) while *C. albicans*, *C. parapsilosis* and other unidentified yeast were detected in another specimen (1/15). Single species were detected in 12 patients where *C. albicans* was present in 10, whilst one specimen each had *C. parapsilosis* and *C. tropicalis*, respectively.

Multiplex PCR analysis of yeast species

In 100 concentrated oral rinse specimens which were subjected to multiplex PCR, 85 were positive for *Candida species*. Multiplex PCR was able to identify *C. albicans* (75/85), *C. tropicalis* (17/85), *C. parapsilosis* (47/85) and *C. glabrata* (6/85) based on the amplicon sizes (Table 6). Figure 3 shows the PCR gel electrophoresis results of six representative oral rinse specimens, where four showed multiple *Candida species*.

Out of the 85 PCR positive specimens, 49 had multiple *Candida species* whereas 36 had single *Candida species*. Out of 85, 43 had a colony count >600 cfu/ml and were found to be at risk of oral Candida infection. Of these, 15 had single species and 28 had multiple species.

Table 6. Detailed description of identification of different *Candida species* using culture and multiplex PCR.

	Culture										Multiplex PCR									
Candida species	Positive ($N = 72$/100)	Single Spp. (34)	Multiple Spp. (38)	*p* value	<600 CFU ($N = 29$/72)	Single Spp. (12)	Multiple Spp. (17)	*p* value	>600 CFU ($N = 43$/72)	Single Spp. (21)	Multiple Spp. (22)	*p* value	Positive ($N = 85$/100)	Single spp. (36)	Multiple Spp. (49)	*p* value	<600 CFU ($N = 42$/85)	Single Spp. (21)	Multiple Spp. (21)	*p* value	>600 CFU ($N = 43$/85)	Single Spp. (15)	Multiple Spp. (28)	*p* value
C. albicans	64	30 (88.2)	34 (89.5)	–	25	10 (76.9)	15 (93.8)	–	39	20 (95.2)	19 (86.4)	–	75	27 (75)	48 (98)	0.002**	34	13 (61.9)	21 (100)	–	41	14 (93.3)	27 (96.4)	–
C. parapsilosis	21	01 (2.9)	20 (52.6)	0.000*	11	01 (7.7)	10 (62.5)	0.006**	10	00 (-)	10 (45.5)	0.001**	47	08 (22.2)	39 (79.6)	0.000*	27	07 (33.3)	20 (95.2)	0.000*	20	01 (6.7)	19 (67.9)	0.000*
C. tropicalis	12	01 (2.9)	11 (28.9)	0.003*	05	01 (7.7)	04 (25)	–	07	00 (-)	07 (31.8)	–	17	01 (2.8)	16 (32.7)	0.001*	04	01 (4.8)	03 (14.3)	–	13	00 (-)	13 (46.4)	0.002**
C. glabrata	04	01 (2.9)	03 (7.9)	–	00	00 (-)	00 (-)	–	04	01 (4.8)	03 (13.6)	–	06	00 (-)	06 (12.2)	–	03	00 (-)	03 (14.3)	–	03	00 (-)	03 (10.7)	–
Other	12	01 (2.9)	11 (28.9)	0.003*	06	00 (-)	06 (27.3)	–	06	00 (-)	06 (28.6)	–	–	–	–	–	–	–	–	–	–	–	–	–

*p values taken from χ^2 test.
**p values taken from Fisher's exact test.
p value cannot be calculated.

Figure 3. Multiplex PCR with patient samples. M – 100bp ladder, Lane 1 – positive for *C. albicans*, *C. parapsilosis* and *C. tropicalis*. Lane 2 – *C.albicans*. Lane 3 – *C. tropicalis*. Lane 4 – *C. glabrata*, *C. albicans* and *C. parapsilosis*. Lane 5 – *C. albicans* and *C. parapsilosis*. Lane 6 – *C. parapsilosis*. Lane N – negative control.

Of the 43 specimens which showed a colony count >600 cfu/ml, majority had *C. albicans* (41/43) followed by *C. parapsilosis* (20/43), *C. tropicalis* (13/43) and *C. glabrata* (3/43). Multiple *Candida species* were identified in 28 of these specimens which had a yeast >600 cfu/ml. Of them, *C. albicans* and *C. parapsilosis* were seen in 13, *C. tropicalis* and *C. albicans* in 7 while *C. albicans and C. glabrata* and *C. tropicalis* and *C. parapsilosis* were detected in 1 patient each. Three species together were detected in four specimens (*C. albicans*, *C. tropicalis* and *C. parapsilosis*), while C. *albicans*, *C. tropicalis* and *C. glabrata* and *C. albicans, C. parapsilosis* and *C. glabrata* were seen in one specimen each. Single *Candida species* were identified in 15 out of 43 patients. Of them, the predominant species was *C. albicans* (14/15) followed by *C. parapsilosis* (1/15).

Of 85 patients, 42 were found to be not at risk of developing oral Candida infection (<600 cfu/ml). Of the 42 patients, 21 had multiple species whilst remaining 21 patients had single species. *C. albicans* and *C. parapsilosis* together were seen in 15 while *C. albicans* and *C. tropicalis* were seen in one specimen. Three yeasts (*C. albicans*, *C. parapsilosis* and *C. glabrata*) together were seen in three specimens, while two specimens had *C. albicans*, *C. tropicalis* and C. *parapsilosis*. Single species were detected in 21 specimens where *C. albicans* was detected in 13, *C. parapsilosis* in 7 and *C. tropicalis* in 1 specimen.

Comparison of culture and multiplex PCR for identification of Candida species

All 43 oral rinse specimens which had >600 yeast cfu/ml were also positive by multiplex PCR. Table 6 describes the comparison between different *Candida species* identified using multiplex PCR in comparison to culture.

Using both culture and multiplex PCR, *C. albicans* was found to be the predominant oral yeast species (64/72 by culture and 75/85 by multiplex PCR). Out of the 43 patients who were at risk of Candida infection (>600 cfu/ml), 39 specimens isolated *C. albicans*. Of these 39, 20 had single *C. albicans species* while in 19 specimens *C. albicans* was associated with another yeast species by culture isolation. Based on multiplex PCR results of 43 specimens, *C. albicans* was detected in 41 specimens. Of the 41, 14 had single *C. albicans species* while in 27 specimens, *C. albicans* was associated with another yeast species by multiplex PCR.

C. parapsilosis was the second most common oral yeast found in this population using both the methods (21/72 by culture and 47/85 by multiplex PCR). Out of the 43 patients who were at risk of Candida infection (>600 cfu/ml), 10 were found positive for *C. parapsilosis* by culture. All 10 of these *C. parapsilosis* isolates were found together with another *Candida species*. When analyzed using PCR, 20 specimens detected *C. parapsilosis*. Out of the 20 specimens, 19 were detected together with another *Candida species*.

C. tropicalis was the third most common oral *Candida species* found in this patient population. Twelve isolates (12/72) were identified as *C. tropicalis* using culture, and 17 (17/85) were detected as *C. tropicalis* using multiplex PCR. Out of the 43 patients who were at risk of oral Candida infection, 7 had *C. tropicalis* by culture whilst 13 were detected using multiplex PCR. All identified *C. tropicalis* isolates were found to be co-habited with another *Candida species*.

C. glabrata was less commonly identified in the study population (4/72 by culture, 6/85 by PCR). Of the culture-positive four samples, all four were found to be at risk of infection (>600 cfu/ml) whilst 3/6 which were identified by PCR were at risk of developing Candida infection. One which was positive as *C. glabrata* by culture gave a negative result when subjected to multiplex PCR. Three samples were found together with other yeasts whilst one sample had single *C. glabrata species* by culture identification. All three *C. glabrata* PCR-positive specimens were at risk of infection. Further they were co-habited with another *Candida species*.

Out of the 28 culture negative oral rinse specimens, 16 were positive by multiplex PCR, of them majority had *C. parapsilosis* (13/16) followed by *C. albicans* (10/16), *C. tropicalis* (2/16) and *C. glabrata* (2/16). Out of 16, 8 samples had multiple *Candida species* by multiplex PCR.

Three oral rinse specimens that were positive by culture were negative by multiplex PCR. Out of these three, two had single species, of which one had only *C. parapsilosis,* and the other had yeast which was not identified using culture methods. In one specimen, two species were detected (*C. parapsilosis* and unidentified yeast species).

Considering PCR as the gold standard, culture method had a sensitivity of 80%, specificity of 73.3%, positive predictive value (PPV) of 94.4% and negative predictive value (NPV) of 39.3%. When culture was used as the gold standard, PCR had a sensitivity of 94.4%, specificity 39.3%, PPV of 80% and NPV of 72%.

Discussion

Multiplex PCR described in this study enabled the simultaneous identification of four important multiple oral yeast pathogens. Using this method which yielded species-specific single amplicons provided higher discrimination than reported previously.[9,13] Further it was found to be a rapid, sensitive and simple technique which could be directly applied to clinical specimens containing multiple *Candida species*. This study describes the use of the multiplex PCR for the identification of *Candida species* from direct oral rinse specimens. Its application can be extended to other clinical specimens including blood specimens which can have an important clinical impact for early and rapid identification.

Universal primer *ITS4* [12] and species-specific primers CA, CT, CGL and CP [10] were used successfully in this study for amplification of four pathogenic *Candida species* from oral rinse specimens. Li et al. had used eight species-specific primers including CA, CT, CGL and CP to identify eight *Candida species* from blood culture.[10] However, the discriminatory power of this PCR was less compared to the current study. The multiplex PCR reported in this study takes the advantage of the high-copy number of rRNA genes and the differences in the length of *ITS* regions. In the fungal genome, *ITS* region is known to be highly variable and is a good target for species identification.[18]

The multiplex PCR enabled the simultaneous identification of four *Candida species*: *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata*. It yielded species-specific single amplicons. However following agarose gel electrophoresis, *C. parapsilosis* and *C. tropicalis* bands were located close together due to the resulting amplicon base pair sizes: 126 bp and 149 bp. Although these two bands sizes were close to each other, in this study these were well separated with the 3% agarose gel. A polyacrylamide gel could be used to obtain better discrimination for these two bands. However, polyacrylamide gel electrophoresis needs technical expertise and is associated with higher cost and toxicity.[19]

The PCR parameters reported in this study had a detection limit of 10 cells/ml in the oral rinse specimen. This high sensitivity in the oral rinse specimen is a marked improvement over other reported single round yeast PCRs with a sensitivity of 100 cells/0.5 ml.[20] In another study by Tarini et al. in 2010, a detection limit of 26–29 cells/ml was reported using five culture isolated *Candida species*.[21] The lack of possible PCR inhibitors in isolated colonies may be useful in improving the sensitivity of the multiplex PCR.[22] Further, a study done by Taira et al. in 2014 among a group of paediatric Candidemia patients demonstrated a sensitivity of 4 Candida cells/ml of blood.[22] However, as the oral cavity harbours a vast variety of microorganisms and saliva has an inhibitory

effect against PCR,[23] the detection limit of 10 Candida cells/ml reported in this study is an improvement.

In the present study, Candida was identified in 85% of oral rinse specimens by multiplex PCR and more than half of the positive specimens had multiple *Candida species*. Among the culture-positive patient population >50% had a colony count of >600 cfu/ml and was considered to be at risk for Candida infection. The samples which were at risk of infection had a significant association with multiple *Candida species* by PCR ($p < 0.05$). It is known that uncontrolled glycemic levels in patients' predispose to candidiasis.[3,24] In this study too, the observation of significant multiple species involvement is in agreement with previous findings and indicates a predisposition to oral complications such as periodontitis and oral thrush.

Diabetes mellitus predisposes the patient to many oral and non-oral infections due to the uncontrolled glycemic index [24] and immunocompromised state.[25] Prevalence of oral *Candida species* in patients with type 2 diabetes has been documented as 13–64%.[26] Although no significant association was observed, in the 42 patients with retinopathy Candida was positive by PCR in a higher proportion. However, the patients who were at risk of infection (>600 cfu/ml) had no significant association. Due to the unavailability of the HbA_1C data for all the patients included in this study, the status of the diabetes control and the role of Candida in uncontrolled diabetes could not be assessed. Further, no association was detected with presence of oral *Candida species*, risk of oral Candida infection with the duration of diabetes in this study. However, other studies have reported a significant association with oral carriage of yeast and duration of diabetes.[27] In the present study, gender and denture wearing had a significant association with presence of Candida and risk of Candida infection. Denture wearing is a known predisposing factor for oral Candidiasis.[14] Dentures are known to give good surfaces for Candida adherence and microcolony formation which results in biofilm formation. In the present study, majority of the females had a risk of oral Candida infections. Of the 32 denture wearers in this study, 23 were female. Majority of the female patients were more than 50 years of age (58.9 years). The observed higher oral Candida carriage and the significant association with female gender seen in this study may be due to the hormonal changes seen with menopause. It has been reported in previous studies that menopause is associated with changes in the oral and vaginal mucosa with increased predisposition to candidiasis.[28] Further, poor oral hygiene is known to contribute towards predisposition to candidiasis. Although other studies reported an association between smoking and betel-quid chewing with Candida colonization but in the present study, such an association was not detected.

The sensitivity (94.4%) and NPV (72%) of PCR are higher compared to the culture method, whereas the specificity (73.3%) and PPV (94.4%) of culture method were higher than PCR. The present multiplex PCR could detect 10 Candida cells/ml of sample which includes both live and dead cells. Whereas in the culture, only live cells are detected. The high sensitivity of multiplex PCR indicates its value in identification of yeasts over culture. The reason for the lesser specificity seen in this PCR may be due to amplification of only four *Candida species*. Using culture methods, all viable yeast species were isolated out of which most were identified to the species level while only eight were identified as *Candida species*. However, if the targeted four species were not present in the sample, the PCR will become negative which is a disadvantage of this multiplex PCR. In the present study, *C. krusei* could also be identified using culture method.

Several studies have previously demonstrated that discriminatory ability of molecular techniques in identifying *Candida species* is remarkably high compared to culture identification methods.[8,29,30] However a limitation in the present study was the use of a conventional multiplex PCR which could not quantify the yeast population. A real-time multiplex PCR could have been more valuable as a quantitative as well as a qualitative technique to identify multiple oral yeasts species. The application of a real-time PCR for routine diagnostic use is limited due to the high cost and complexity of the technology. Melting curve analysis in real-time PCR facilitates rapid and more sensitive identification of multiple yeast species as described by Fricke et al. [31,32] In Alnuaimi et al. study, eight yeast species were identified using a real-time PCR melting curve analysis in yeast culture isolates. In the present study, extracted DNA from the direct clinical specimens was subjected to multiplex PCR which can give more rapid and clinically valuable results. In application of clinical specimens to PCR, several factors could contribute to the sensitivity such as presence of PCR inhibitors, presence of pathogenic and host-derived DNA and presence of several sets of primers. This could result in false positives and false negatives. Therefore in the present study, we have targeted only four common oral yeast pathogens in direct oral rinse specimens. Based on our results, multiplex PCR in combination with culture analysis is more valuable.

The comparison of multiplex PCR and culture analysis applied to the total yeast community in oral rinse samples illustrated the strengths and weakness of each technique. Using a concentrated oral rinse sample was found to be effective in determining the total yeast community compared to swab sampling, imprint sampling or a saliva sample.[33] When determining the total oral yeast population, cultivable yeasts can be identified based on their phenotypic properties. However, the procedure will take at least 2–3 days, whereas multiplex PCR will generate results within 3–4 hours. Multiplex PCR reported in this study could identify only four important Candida pathogens, which is a limitation. These four Candida pathogens *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. tropicalis* are responsible for >80% of the oral yeast infections.[34,35] CHROMagar Candida is a good alternative to the conventional culture identification due to the direct identification ability based on the colony colour and the relatively shorter time required (24–48 h).[36] In the present study, three culture-positive patients were negative by multiplex PCR indicating the presence of other yeast species. Further two yeast species could not be identified to species level using phenotypic methods which was a limitation of using phenotypic methods alone for identification.

In this study using both culture and PCR, *C. albicans* was found to be the predominant oral yeast associated with

Diabetes mellitus. Several studies have reported this finding previously.[24] Further non-albicans species were found to be associated with *C. albicans* as reported in previous studies.[24] Non-albicans *Candida species* are becoming equally important and an emerging causes for oral non-oral Candida infections.[37] Further, the development of resistance to azole antifungals indicates their importance as emerging pathogens causing Candidiasis.[37] Therefore, the identification of non-albicans *Candida species* can no longer be ignored.

Conclusions

In conclusion, multiplex PCR can be used for the identification of *C. albicans*, *C. tropicalis, C. glabrata* and *C. parapsilosis* in oral rinse specimens in a single-step PCR. It is a promising, effective and relatively fast method to identify multiple Candida pathogens directly from clinical specimens. Denture wearing and female gender had a higher predisposition for risk of infection. *Candida albicans* was the predominant pathogen followed by *C. parapsilosis*.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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