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Short communication

Oral candidiasis: a comparison between conventional methods and multiplex polymerase chain reaction for species identification

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Background/aim: Oral candidiasis is the most common fungal infection in dental practice, and is caused by yeasts that are normally present in the endogenous flora.
Methods: To evaluate a rapid diagnostic method for identification of *Candida* oral isolates, a multiplex polymerase chain reaction (PCR) was carried out on colonies and on oral rinse solutions from 95 subjects with suspected oral candidiasis and results were compared with those from seven commonly used phenotypic identification systems.
Results: Between four and nine species were characterized in the samples by the phenotypic methods. PCR identified the same species in 60 (74%) samples from both colony and oral rinse solutions. Statistical analysis, carried out only for the three most frequently isolated species (*Candida albicans*, *Candida glabrata*, and *Candida tropicalis*), showed good concordance in the comparison of multiplex PCR with API 20C AUX and with the Rapid Yeast Identification Panel; conversely, significant differences were registered in the comparison between the molecular method and other phenotypic systems, including four chromogenic media and the automated system Vitek2.
Discussion: Multiplex PCR was rapid and effective in the identification of *Candida* species and allowed the detection of more than one species in the same sample.

Key words: molecular diagnostic techniques; oral candidiasis; species identification

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Oral candidiasis is frequently seen in both dental and general practice (9, 10). It is mainly caused by *Candida albicans*, but the emergence of non-*albicans* species has been recently observed (7, 9).

Several factors, including iatrogenic, pathophysiological, and behavioral, may promote oral candidiasis generating a disturbance in the oral microbial ecosystem and causing an increase in the 'mycotic count' (3, 9).

Rapid and accurate identification of species is important to adequately assess the role of *Candida* spp. in clinical patterns and to choose adequate antifungal therapies, especially for critically ill patients (6). Various molecular methods have been developed for the identification of *Candida* spp. (2, 4). Among these, multiplex polymerase chain reaction (PCR) has emerged as a promising technology because of its simplicity, specificity, and sensitivity as well as its ability to potentially identify all *Candida* species in biological samples (1, 5).

To evaluate a quicker diagnostic approach to the diagnosis of oral candidiasis, we carried out a multiplex PCR for the identification of *Candida* spp. on colonies and on oral rinse solutions from subjects with suspected oral candidiasis and results were compared with those from seven commonly used phenotypic identification systems to assess their degree of concordance. Phenotypic tests included four chromogenic media, a microdilution panel, and two carbohydrate assimilation methods (manual and automated).

Material and methods

Ninety-five patients with clinically suspected oral candidiasis were recruited by the Laboratory of Bacteriology and Mycology, Hygiene Section of the Department of

Public Health and Clinical and Preventive Medicine, in the Ward for Diagnostics of Oral Mycoses of the Second University of Naples teaching hospital. Personal and clinical data were collected for all patients. Exclusion criteria were: use of antifungal agents, fasting in the previous 12 h, and oral hygiene carried out in the previous 6–8 h.

Samples were collected through the oral rinse solution technique and further dilutions were performed (5, 8). Mycotic count was determined by seeding sample dilutions (up to $1 : 10^{-3}$) on three Sabouraud–Dextrose agar plates supplemented with chloramphenicol. Total microbial count was measured by seeding oral rinse solutions (diluted up to $1 : 10^{-6}$) on six Plate Count Agar dishes. After incubation at 37°C for 24–48 h, the number of colony-forming units (CFU) was multiplied by the corresponding dilution factor to determine the most accurate number of mycotic/microbial cells in 1 ml saliva.

Each species identification was carried out with the following commercially available kits: API 20C AUX (bioMérieux Italia S.p.A., Rome, Italy); Vitek 2 card ID-YBC (bioMérieux Italia); Rapid Yeast Identification Panel (RYIP; Dade Behring Italia, Milan, Italy); CHROMagar *Candida* (AlfaWassermann Italia, Milan, Italy); Chromogenic *Candida* Agar (Oxoid Italia, Garbagnate Milanese, Milan, Italy); *Candida* Identification Agar (Biotest Italia, Trezzano s/n, Milan, Italy); and CandiSelect 4 (BIO RAD Italia, Segrate, Milan, Italy). All these tests were carried out following the respective manufacturer's instructions and by the same operators.

Multiplex PCR was first performed in duplicate directly on colonies, without preliminary DNA extraction, based on the method of Chang et al., as previously described (1, 5).

Briefly, the following conditions were used. The reaction mix (50 µl) comprised 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 3.2 µM primers, 1.25 U *Taq* DNA polymerase (Roche Diagnostics, Milan, Italy). Predenaturation was performed at 95°C for 10 min, followed by 40 amplification cycles (1 min at 95°C, 1 min at 50°C, 1 min at 72°C) and a final extension at 72°C for 10 min. Genus-specific and species-specific ribosomal RNA regions were chosen as the targets for primers, as previously reported (5). The analysis of the amplification products was carried out by electrophoresis on 2% agarose gel with ethidium bromide (0.5 µg/ml).

All these identification methods were performed on the same colony isolated from a pure culture for each sample. For analysis of oral rinse solutions, 5 ml from each sample was centrifuged at 20,000 ***g*** for 5 min. The supernatant was discarded and the pellet was resuspended in 100 µl sterile distilled water; 20 µl was used for the multiplex PCR using the protocol described above. The following American Type Culture Collection (ATCC) strains were analyzed with all the methods: *C. albicans* 90028, *C. albicans* 36082, *Candida parapsilosis* 22017, *Candida krusei* 6258.

McNemar's test was carried out to assess concordance among each method and multiplex PCR on colonies. Significance level was specified as 0.05.

Results

Eighty-one (85.3%) of the 95 oral rinse solutions analyzed gave positive results with both cultural and molecular detection methods, whereas no *Candida* species were found in the remaining 14 samples (14.7%). Mycotic counts and microbial counts ranged between 10 and 5.5×10^6 CFU/ml and 9×10^4 and 15.3×10^7 CFU/ml, respectively. Between a minimum of four and a maximum of nine species were identified through the nine employed methods (Table 1).

PCR identified the same species in 60 (74%) samples from both colony or oral rinse solutions; results were discordant in 16 (19.8%) samples while a mixed flora was found in five oral rinse solutions (6.2%). In particular, three oral rinse solutions were positive for *C. albicans* and *Candida glabrata*, one for *Candida guilliermondii* and *C. glabrata*, and one for *C. guilliermondii* and *Candida tropicalis*. Control strains were correctly identified by all the methods.

Statistical analysis was only possible for the three species that had sufficient frequencies of detection (*C. albicans*, *C. glabrata*, and *C. tropicalis*). Significant differences were observed between PCR analysis on colonies and the four chromogenic media ($P < 0.05$). Similar differences were observed in the case of Vitek 2; conversely, no discordance was seen with the API system, nor with RYIP.

As previously reported (5), PCR analysis without preliminary DNA extraction detected yeasts even when mycotic counts were as low as 10 CFU/ml and in samples

Table 1. Number of strains identified for each species through multiplex polymerase chain reaction and the seven phenotypic systems

	Multiplex PCR					Chromogenic substrates			
Species	Colony	Oral rinse[1]	API 20C AUX	Vitek 2	RYIP	CHROMagar *Candida*	OCCA	*Candida* identification agar	CandiSelect 4
C. albicans	68	65	64	62	64	63	61	62	63
C. glabrata	8	9	6	6	5	7	7	9	10
C. tropicalis	2	2	5	6	4	6	8	4	7
C. krusei		2	1	1	2			1	1
Saccharomyces cerevisiae			2	2	1	2			
C. parapsilosis	1	1	1	1	2	2	2		
C. guilliermondii		3			1				
C. kefyr	1	2	1		1	1	1		
C. famata	1		1						
C. dubliniensis		2		1					
Pichia farinosa					1				
C. norvegensis				1					
C. lusitaniae				1					
No identification							2	5	
n of species identified	6	8	8	9	9	6	5	4	4

[1]In five samples there was a mixed flora; total identified strains = 86.
OCCA, Oxoid Chromogenic *Candida* Agar; PCR, polymerase chain reaction; RYIP, Rapid Yeast Identification Panel.

that were heavily contaminated by bacteria (total microbial count $>3 \times 10^6$).

Discussion

In this study, 14.7% of samples from subjects with clinically suspected oral candidiasis were negative for yeast detection. This stresses the importance of laboratory diagnosis for oral candidiasis.

Conventional techniques may require several days for completion. PCR has emerged as a valid alternative to the traditional methods, because it significantly shortens identification times, particularly in cases of slow-growing and difficult-to-culture strains. However, nucleic acid extraction may sometimes be laborious and time-consuming.

On the basis of previous experiences, we evaluated here a multiplex PCR protocol for the identification of the main species responsible for oral candidiasis both from colonies and oral rinse solutions, without the need for preliminary DNA extraction and purification. Results obtained from colony analyses were compared with those yielded by seven commonly used commercially available kits.

The highest discrimination power was shown by the Vitek 2 automated system and RYIP. Using the RYIP, difficulties arose in the interpretation of results. Automated reading of the RYIP gave a quicker and more accurate result for yeast identification compared with manual systems. Automation would, however, increase the cost over manual methods, which was also the case for the automated Vitek2.

API 20C AUX was accurate in species identification, easy to perform, and not expensive. However, precision is required during standardization and inoculum seeding, correct interpretation of the turbidity level requires skill, and readout times can be up to 72 h.

The chromogenic media were easy to use and inexpensive. Their main limitations were the low discrimination power and a chromatic gradient, which was not always easy to interpret. Their use may be suggested for primary cultures, for screening, or for preliminary tests.

Multiplex PCR was rapid and effective. Eliminating the requirement for DNA extraction avoided the use of potentially hazardous or expensive chemicals. When used on oral rinse solutions, molecular analysis was particularly advantageous because of its rapidity (5 h), its efficiency (even in cases of minimal counts and heavy contamination), and its ability to identify several species simultaneously, which is very difficult with methods based on the cultural isolation of a single colony. Results from the two PCR techniques were not statistically different ($P > 0.05$). However, the lower number of *C. tropicalis* identifications that was obtained with multiplex PCR compared with the other methods could be the result of the high similarity of amplicon size between *C. albicans* and *C. tropicalis*. Visual interpretation of results should therefore be considered as a critical point. Moreover, the number of primers should be increased to extend the identification power of this method to other *Candida* species.

Real-time multiplex PCR, which has been successfully applied to oral rinse samples (11), could be useful in determining the mycotic count index. This will allow the quantification of the whole oral mycotic population and the establishment of the pathogenic roles of different species, with consequent improvements in the choice of the best therapy. Automation of the process could also eliminate the possibility of operator mistakes.

In conclusion, the choice of identification method for *Candida* spp. should be carefully considered based on the different performances of each single method, to select that which is most convenient. Availability of funds and of trained personnel, especially when automated systems are not available, are other significant issues that will affect this choice.

Multiplex PCR is an accurate, inexpensive, and easy-to-perform technique, so it could be considered as a valid alternative to traditional phenotypic methods, at least in laboratories that are already equipped with molecular biology tools. The direct application of this technique to oral rinse samples without nucleic acid extraction can be useful in large epidemiological studies. However, some critical points regarding its discrimination power need further investigations and improvements.

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References

1. Chang HC, Leaw SN, Huang AH, Wu TL, Chang TC. Rapid identification of yeast in positive blood cultures by a Multiplex PCR method. J Clin Microbiol 2001: **39**: 3466–3471.
2. Chen SC, Halliday CL, Meyer W. A review of nucleic acid-based diagnostic tests for systemic mycoses with an emphasis on polymerase chain reaction-based assays. Med Mycol 2002: **40**: 333–357.
3. Epstein JB, Pearsall NN, Truelove EL. Quantitative relationships between *Candida albicans* in saliva and the clinical status of human subjects. J Clin Microbiol 1980: **12**: 475–476.
4. Liguori G. Molecular epidemiology and prevention of nosocomial infections by *Candida* spp. In: Giammanco G, De Flora S, eds. Molecular methods in public health. Torino: Centro Scientifico Editore, 2004: 131–138.
5. Liguori G, Lucariello A, Colella G, Marinelli P, De Luca A. Rapid identification of *Candida* species in oral rinse solutions by PCR. J Clin Pathol 2007: **60**: 1035–1039.
6. Perlroth J, Choi B, Spellberg B. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Med Mycol 2007: **45**: 321–346.
7. Ruhnke M. Epidemiology of *Candida albicans* infections and role of non-*Candida albicans*. Yeasts Curr Drug Targets 2006: **7**: 495–504.
8. Samaranayake LP, Macfarlane TW, Lamey PJ, Ferguson MM. A comparison of oral rinse and imprint sampling techniques for the detection of yeast, coliform and *Staphylococcus aureus* carriage in the oral cavity. J Oral Pathol 1986: **15**: 386–388.
9. Serpico R, Liguori G. La candidosi orale: clinica, diagnosi e terapia. Padova: Piccin Nuova Libraria, 2000.
10. Sherman RG, Prusinski L, Ravenel MC, Joralmon RA. Oral candidiasis. Quintessence Int 2002: **33**: 521–532.
11. White PL, Williams DW, Kuriyama T, Samad SA, Lewis MA, Barnes RA. Detection of *Candida* in concentrated oral rinse cultures by real-time PCR. J Clin Microbiol 2004: **42**: 2101–2107.