*doi:* 10.5958/0976-156X.2014.00013.6

# TECHNIQUES USED FOR ISOLATION AND IDENTIFICATION OF CANDIDA FROM THE ORAL CAVITY

[1] Deepa Mastammanavar [1]postgraduate
[2] Santosh Hunasgi [2] Professor
[3] Anila Koneru [3] Senior lecturer
[4] Vanishree M [4]Professor and Head
[5] Surekha R [5] Reader
[6]Vardendra M [6]Senior lecturer

[1-6] *Department of Oral and Maxillofacial Pathology*, *Navodaya Dental College, Raichur ,Karnataka, India*

**ABSTRACT:**. Mycotic infections have become a major cause of morbidity and mortality in clinically debilitated or immunocompromised patients. The co-existence of *Candida* species within humans either as commensals or pathogens has been subject of interest. The genus candida includes several species,C. albicans is by farthe most common species causing infections in humans. The emergence of non-albicans *Candida* species as significant pathogens has however been well recognized during the past decade. Although they are closely related they differ from each other with respect to epidemiology, virulence characteristics, and antifungal susceptibility. This review provides an overview of the reliable methods forcandidal isolation and identification of isolates from the oral cavity.

**KEYWORDS: Candida, isolation, identification, oral cavity**

## INTRODUCTION

The most common oral fungal infection in human beings is caused by the *Candida* species[1]. The term *Candida* originates from the Latin word candid, meaning white. The spores of *Candida* are a commensal, harmless form of adimorphic fungus[2]. Its prevalence in healthy human oral cavities, according to western studies, ranges from 40% to 60%[3]. However, when appropriate conditions such as local or systemic deficiencies in the host defenses supervene they become invasive and pathogenic pseudohyphae[2].

Mycotic infections have become a major cause of morbidity and mortality in clinically debilitated or immunocompromised patients. The co-existence of *Candida* species within humans either as commensals or pathogens has been subject of interest[4].

The genus candida includes several species **(Table 1);**C. albicans is by far the most common species causing infections in humans. The emergence of non-albicans *Candida* species as significant pathogens has however been well recognized during the past decade. Although they are closely related they differ from each other with respect to epidemiology, virulence characteristics, and antifungal susceptibility [5].

This review provides an overview of the reliable methods of candidal isolation and identification of isolates from the oral cavity.

**Methods of Isolation**

The techniques available for the isolation of *Candida* within the oral cavity include use of a smear, a plain swab, an imprint culture, collection of whole saliva, the concentrated oral rinse, and mucosal biopsy. Each methodhas particular advantages and disadvantages and the choice of sampling technique is primarily governed by the nature of the lesion to be investigated **(Table 2)** [2].

1. **Smear technique**: Scraping and smearing directly on the slide **(Fig.1)**
   - Merits: Simple and quick
   - Demerits: Low sensitivity [2,6].
2. **Plain swab:** Using cotton swab sample is collected from the lesional tissue **(Fig.2)**
   - Merits: simple
   - Demerits: Sample site selection is critical [2,6]
3. **Imprint technique**: Foam pads dipped in Sabourauds dextrose agar broth placed on a lesion **(Fig. 3)**
   - Merits: Sensitive and reliable, can discriminate between infected and carrier state.

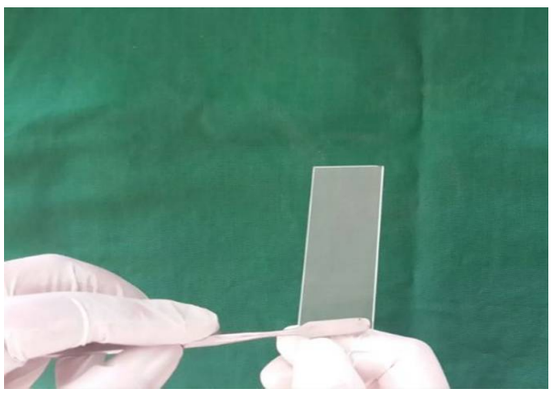

Fig.1.Smear Technique

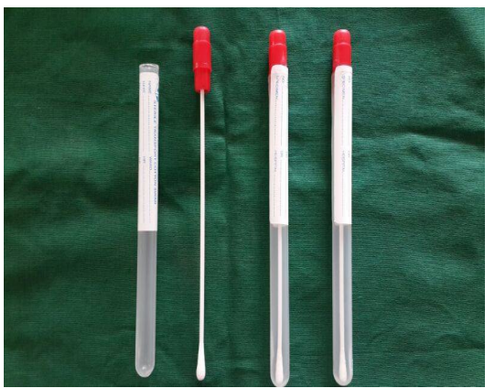

Fig.2.Plain Swab

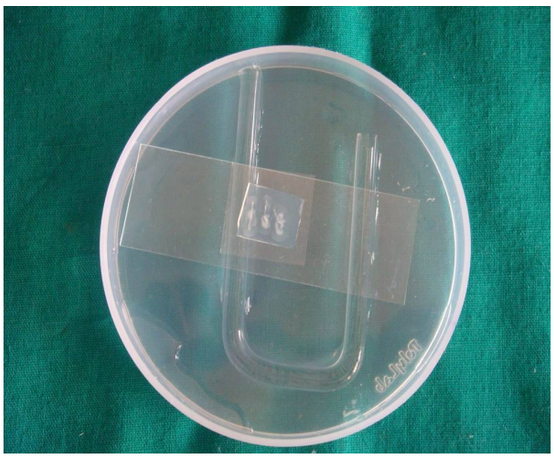

Fig.3.Imprint technique

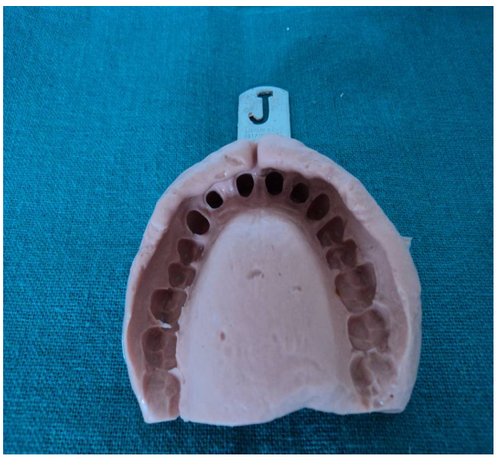


Fig.4.Impression culture technique

Fig.5.Concentrated Oral Rinse

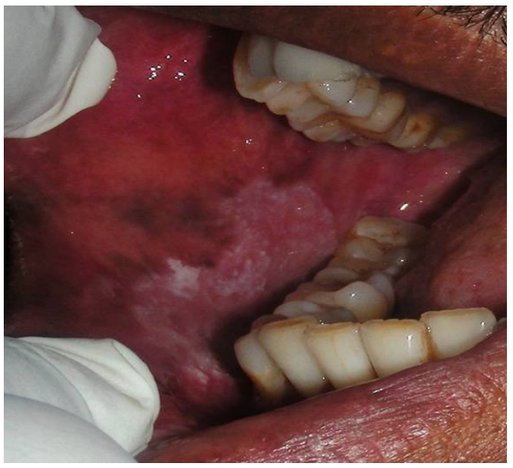

Fig.6. Biopsy

- Demerits: Readings above 50CFU/cm2 can be inaccurate
- Selection of site is difficult, if no clinical signs present [2,6]

4. **Impression culture technique:**Impression casting in agar fortified withbroth **(Fig. 4).**
   - Merits: To determine relative distribution ofyeasts.
   - Demerits: Useful mostly as research tool [2,6]
5. **Concentrated oral rinse:** 10 ml of sterile phosphate-buffered saline rinsed for 1minute. The solution is then concentrated (10-fold) by centrifugation and 50 ml, inoculated on an agar medium **(Fig. 5).**
   - Merits: Comparable in sensitivity with imprint method,simple method, better results if CFU>50/cm².
   - Demerits: Does not localize the site of infection [2, 6].
6. **Biopsy:**Biopsy is taken from the lesion **(Fig. 6).**
   - Merits: Confirmatory test.
   - Demerits: Invasive and inappropriate for majority of infections [2,6]

Where an accessible and defined lesion is evident, a direct sampling approach such as the use of a swab or an imprint is often preferred as this will provide information of the organismspresent at the lesion itself. In cases where there are no obvious lesions or in instances where the lesion is difficult to access, an indirect sample based on culturing saliva specimens or an oral rinse is more acceptable. Quantitative estimation of fungal load can be done using imprints, concentrated oral rinse, and culturing of oral rinse, as a means of differentiating between commensal carriage and pathogenic existence of oral *Candida*, with higher loads considered likely in the latter[2, 6]

**Identification**

**1. Direct microscopy**

Morphological features of candida species[7] **(Table 3)** need to be examined for identification. A smear is of value in differentiating between yeast and hyphal forms but is less sensitive than cultural methods[8]. Potassium hydroxide (KOH) preparation of the specimen reveals non-pigmented septate hyphae with characteristic dichotomous branching (at an angle of approximately 45°)(**Fig.7)**[9]. In KOH-Calcofluor fluorescent-stain method fungal characteristics like hyphae, yeast cells and other fungal elements will fluoresce [10]

A smear taken from the lesional site is fixed on to microscope slides and then stained either by the gram-stain or by the periodic acid Schiff (PAS) technique. Using these methods, candidal hyphae and yeasts appear either dark blue (Gram-stain) **(Fig. 8)** or red/purple (PAS) **(Fig. 9)** [11].

In case of chronic hyperplastic candidosis, a biopsy of the lesion is necessary for subsequent detection of invading candida by histological staining using either the PAS or Gomori'smethenamine silver stains**(Fig. 10).** Demonstration of fungal elements within tissues is done as they are dyed deeply by these stains. The presence of blastospores and hyphae or pseudohyphae may enable the histopathologist to identify the fungus as a species of candida and, given the presence of other histopathological features, make a diagnosis of chronic hyperplastic candidosis[12].

**2. Laboratory culture**

a. **Swab:** A swab of a lesional site is a relatively simple method of detecting growth and semi-quantitative estimation of candida can be obtained. The sampling approach involves gently rubbing a sterile cotton swab over the lesional tissue and then subsequently inoculating a primary isolation medium such as Sabouraud's dextrose agar (SDA) [13].
b. **Concentrated oral rinse:** The oral rinse technique involves the patient holding 10 ml of sterile phosphate-buffered saline (0.01 M, pH 7.2) in the mouth for 1minute. The solution is then concentrated (10-fold) by centrifugation and a known volume, usually 50 ml, inoculated on an agar medium using a spiral plating system. After 24–48 hrs incubation at 37°C, growth is assessed by enumeration of colonies and expressed as candidal colony forming units per ml (cfu ml-1) of rinse [8].
c. **Imprint culture:** The imprint method utilises a sterile foam-pad of known size (typically 2.5 cm2), previously dipped in an appropriate liquid medium, such as Sabouraud's broth, immediately before use. The pad is then placed on the target site (mucosa or intra-oral prosthesis) for 30 seconds and then transferred to an agar for culture[14].
d. **Culture media :**The most frequently used primary isolation medium for candida is SDA [7] which, although permitting growth of candida, suppresses the growth of many species of oral bacteria due to its low pH. Incorporation of antibiotics into SDA will further increase its selectivity[9]. Typically SDA is incubated aerobically at 37°C for 24–48 hrs. Candida develops as cream, smooth, pasty convex colonies on SDA( Fig. 11) and differentiation between species is rarely possible [9]. It is estimated that more than one candida species occurs in approximately 10% of oral samples and in recent years the ability to detect non-albicans species has become increasingly important[14].

In recent years, other differential media have been developed that allow identification of certain candida species based on colony appearance and colour following primary culture. The advantage of such media is that the presence of multiple candida species in a single infection can be determined which can be important in selecting subsequent treatment options[8]. Examples of these include Pagano-Levin agar **(Fig. 12)** or commercially available

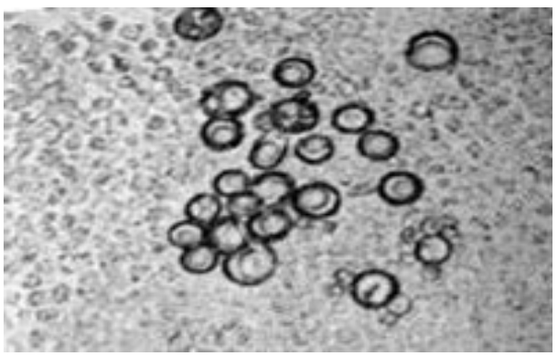

**Fig.7.Potassium hydroxide**

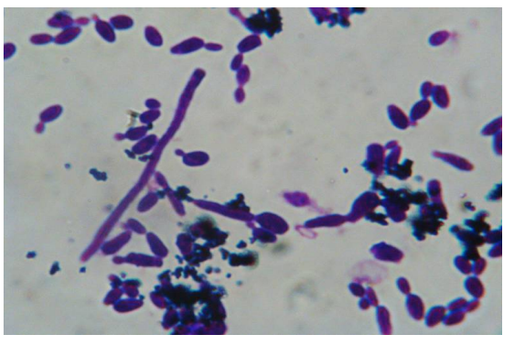

**Fig.8.Grams stain**

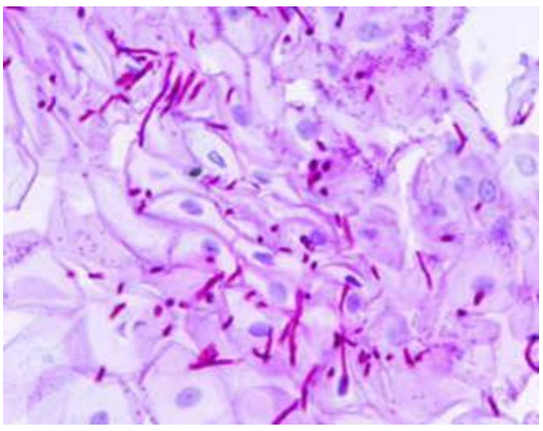

**Fig.9.Periodic acid schiffs stain**

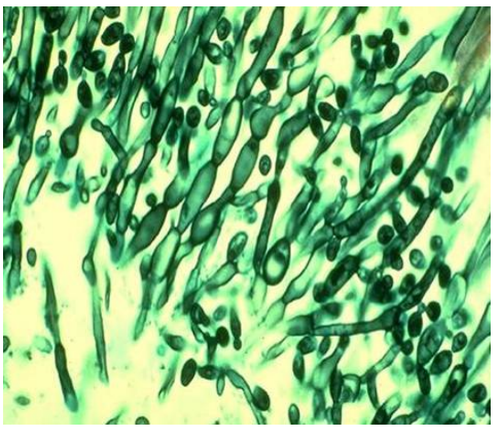

**Fig.10.Gorcott'sMethanamine silver stain**

**Fig.11.Sabourauds Dextrose Agar**

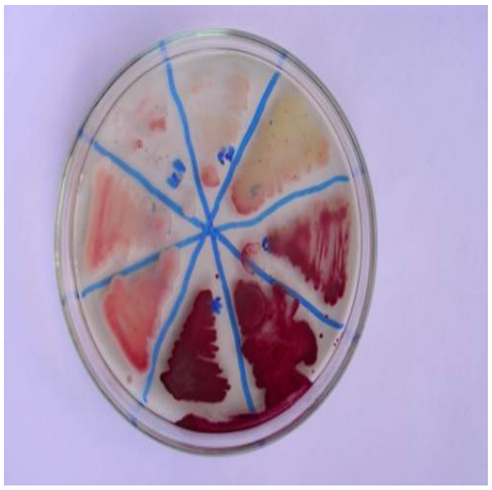

**Fig.12.Pagano-Levin agar**

chromogenic agars namely, CHROMagar Candida, Albicans ID, Fluroplate or Candichrom albicans[8].

Pagano-Levin agar distinguishes between candida species based on reduction of triphenyltetrazolium chloride. The medium produces pale coloured-colonies of C. albicans, whilst colonies of other candida species exhibit varying degrees of pink coloration. Pagano-Levin agar has a similar sensitivity to SDA but is superior for the detection of more than one species in the sample[17]. CHROMagar Candida identifies C. albicans, C. tropicalis and C. krusei based on colony colour and appearance **(Fig. 13)**[18], whilst Albicans ID and Fluroplate have proven beneficial for the presumptive identification of C. albicans[19]. The specificity of identification is reported to be 95% for CHROMagar Candida[20] and 98.6% for Albicans ID and Fluroplate agars[19]. The use of CHROMagar Candida as a primary isolation agar has been cited as an approach that permits discrimination of the newly described C. dubliniensis [21] from C. albicans. On CHROMagar Candida, C. dubliniensis reportedly develops as darker green colonies compared with those of C. albicans[22]. However, discrimination between these two species using CHROMagar appears to decline upon subculture and storage of isolates. Failure of C. dubliniensis to grow on agar media at the elevated incubation temperature of 45°C has recently been suggested as an alternative test to discriminate between these two species [23].

**e. Candidal speciation ;** Identification of yeasts based on primary culture media can be confirmed through a variety of supplemental tests traditionally based on morphological and physiological characteristics of the isolates.

**Morphological criteria**

The germ-tube test is the standard laboratory method for identifying C. albicans. The test involves the induction of hyphal outgrowths (germ-tubes) **(Fig. 14)** when subcultured in horse serum at 37 °C for 2-4 hours. Approximately 95% of *C.* albicans isolates produce germ tubes, a property also shared by C. stellatoidea, and C. dubliniensis [14].

Candida albicans and C. dubliniensis can also be identified from other species based on their ability to produce morphological features known as chlamydospores. Chlamydospores are refractile, spherical structures generated at the termini of hyphae following culture of isolates on a nutritionally poor medium such as cornmeal agar **(Fig. 15).** Isolates are inoculated in a cross-hatch pattern on the agar and overlaid with a sterile coverslip. Agars are incubated for 24-48 hours at 37°C and then examined microscopically for chlamydospore presence [14].

**Physiological criteria/biochemical identification**

Biochemical identification of candida species is largely based on carbohydrate utilization. Traditional testing would have involved culture of test isolates on a basal agar lacking a carbon source. Carbohydrate solutions would then be placed within wells of the seeded agar or upon filter paper discs located on the agar surface. Growth in the vicinity of the carbon source would indicate utilization. Commercial systems are based on the same principle but test carbohydrates are housed in plastic wells located on a test strip. Growth in each well is read by changes in turbidity or colour changes in certain kit systems. Numerical codes obtained from the test results are used to identify the test organism based on database comparison (Fig. 16 )[24].

**f. Serology:** Serological tests are frequently used to ascertain the clinical significance of candida species isolates. Rising titers of IgG antibodies to candida albicans may reflect invasive candidiasis in immunocompetent individuals. The detection of IgA and IgM antibodies is important to identify an acute infection. Immunosuppressed individuals often show variability in antibody production and in such a case the use of an antigen detection test is recommended. Tests like enzyme linked immunosorbent assay (ELISA) and radiao immuno assay (RIA) for detection of candidial antigen, either cell-wall mannan or cytoplasmic constituents are now available in developed countries **(Fig. 17)** [25].

Serological diagnosis is often delayed and the tests still lack sensitivity and specificity. Furthermore, antibody production in immunocompromised patients is variable, making diagnosis complicated[26]. This is due to the fact that fungal antigens and metabolites are often cleared rapidly from the circulation and the presence of antibodies does not always imply a candida infection, especially in patients with serious underlying disease or who are taking immunosuppressive drugs [27]. Serologic tests are normally not a diagnostic tool for oral candidosis. However, such tests may be a prognostic instrument in patients with severe oral candidosis who respond poorly to antimycotic therapy [28].

**g. Molecular-based identification methods;**Identification by analysis of genetic variability is a more stable approach than using methods based on phenotypic criteria. For the identification of candida based on genetic variation are analyses of electrophoretic karyotype differences and restriction fragment length polymorphisms (RFLPs) using gel electrophoresis or DNA–DNA hybridization[8].

Species-specific PCR approaches have also been used for candida species identification. Several target genes have been reported for candida species

**Table 1.Species of *Candida***

| |
|---|
| *Candida albicans* |
| *Candida glabrata* |
| *Candida dubliniensis* |
| *Candida guilliermondii* |
| *Candida krusei* |
| *Candida lusitaniae* |
| *Candida parapsilosis* |
| *Candida tropicalis* |
| *Candida kefyr* |

**Table 2.Methods of obtaining *Candida* from the oral cavity.**

| Sl.no | Isolation methods | Advantages | Disadvantages |
|---|---|---|---|
| 1. | Culture of whole saliva | Sensitive; viable organisms isolated | Problems may occur with collection of sample; |
| 2. | Concentrated oral rinse | Quantitative; viable cells isolated | Some patients have difficulty in using rinse; not site specific |
| 3. | Swab | Simple to use; viable cells isolated; site specific | Difficult to standardize |
| 4. | Smear | Simple to use; not reliant on culture | Viable cells not determined; species identity notreadily confirmed |
| 5. | Imprint culture | Quantitative; viable cells isolated; site specific | Some sites difficult to sample |
| 6. | Biopsy | Essential for chronic hyperplastic candidosis | Invasive; not appropriate for other forms of candidosis |

**Table 3. Morphological characteristics of *Candida* species.**

| Sl.no | Features | |
|---|---|---|
| 1. | Size (*μ*m) | 3–6 |
| 2. | Shape | Spherical or oval |
| 3. | Number of buds | Single; chains |
| 4. | Attachment of buds | Narrow |
| 5. | Thickness | Thin |
| 6. | Pseudohyphae &/or hyphae | Characteristic |
| 7. | Number of nuclei | Single |

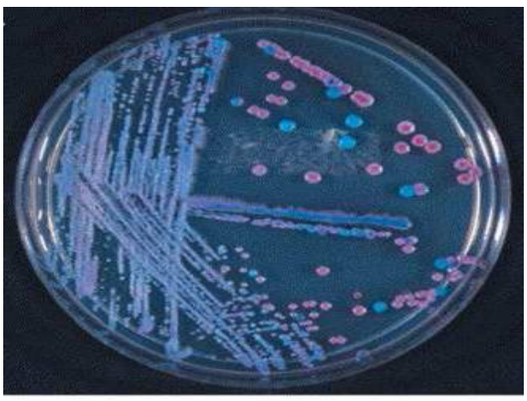

Fig.13.CHROMagar

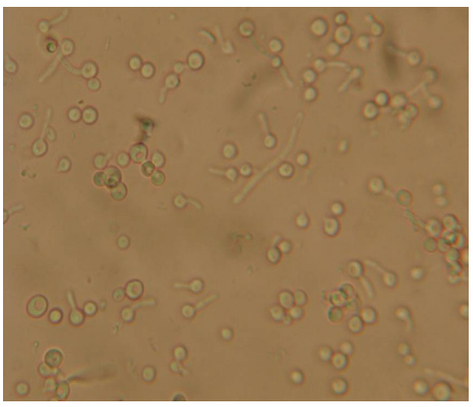

Fig.14.Germ-tube test

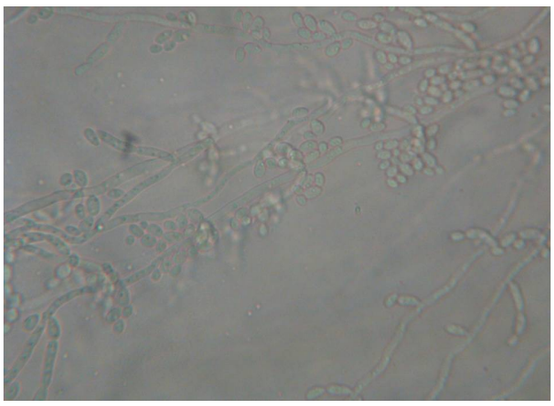

Fig.15.Chlamydospores

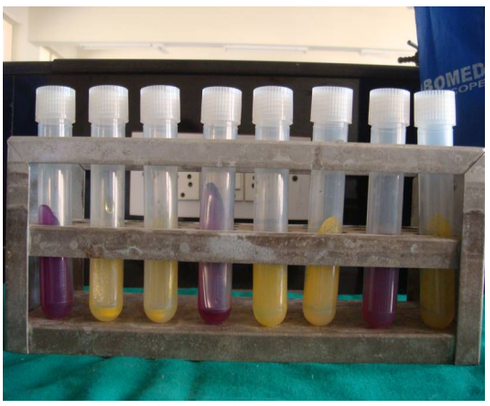

Fig.16.Carbohydrate Assimilation Test

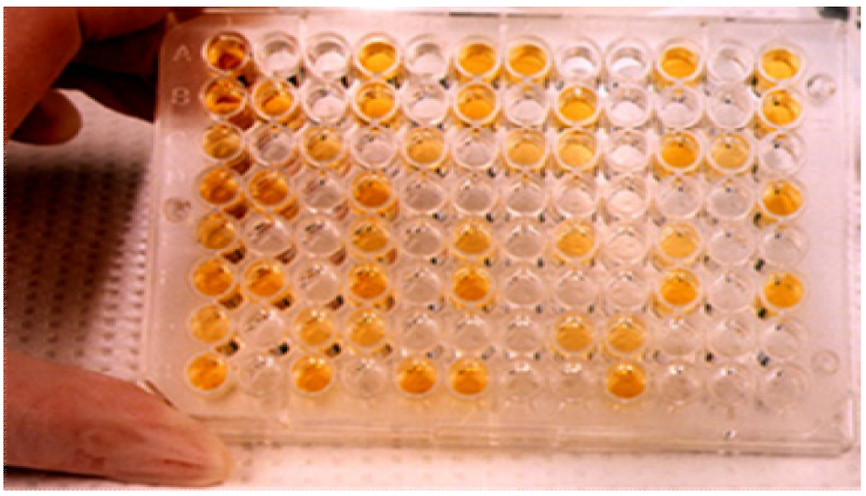

Fig.17.Serological tests

discrimination, although those most frequently amplified are the sequences of the ribosomal RNA operon. Identification can be obtained based on PCR product sizes obtained following gel electrophoresis resolution, or PCR product sequence variation determined either by direct sequencing or through the use of restriction fragment analysis following cutting of PCR sequences with restriction endonucleases [14].

Fluorescence in situ hybridization with peptide nucleic acid method (PNA Fish) is a new detection technique which targets highly conserved species-specific sequences in the abundant rRNA of living C. albicans. Individual cells can be detected directly without the need for amplification [29]. This technique achieves a sensitivity of 98.7–100%, with a specificity of 100%, allowing for the discrimination of C. albicans from the phenotypically similar C. dubliniensis [30].

Molecular-based technology can also be used to identify strains of candida species although the use of techniques such as Pulsed Field Gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD) analysis and repeat sequence amplification PCR (REP) are largely reserved for epidemiological investigations in research of oral candidosis [14].

**CONCLUSION**

*Candida*is the resident microflora, appropriate isolation methods are required to ascertain the presence in the mouth along with their number. It is also important to identify the infecting 6 ISRN Dentistry strains of *Candida* because isolates of *Candida* species differ widely, both in their ability to cause infection and also intheir susceptibility to antifungal agents. Various phenotypic techniques are available for identifying isolated *Candida*including using morphological culture tests, differential agar media, and biochemical assimilation tests. These methods are supplemented with recent molecular techniques largely reserved for epidemiological investigations.

## References


1. Jainkittivong, Kuvatanasuchati, Pipattanagovit P andSinheng W. Candida in oral lichen planus patients undergoing topical steroid therapy.OOOE 2007;104:61-6.
2. Raju S B and RajappaS.Isolation and Identification of Candida from the Oral Cavity:Review Article.ISRN Dentistry 2011;1-7. http://dx.doi.org/10.5402/2011/487921
3. SamaranayakeL.Commensal oral candida in Asian cohorts. Mini Review.Int J Oral Sci 2009;1(1):2-5. http://dx.doi.org/10.4248/ijos.08006
4. Kumar S R, S M GanvirandHazarey V K.Candida and Calcofluorwhite: Study in precancer and cancer. JOMFP.2009;13(1):1-8.
5. Species identification of candida isolates obtained from oral lesions of hospitalized and non-hospitalized patients with oral Candidiasis
6. Sitheeque M.A.M. Chronic hyperplastic candidosis/candidiasis (candidal leukoplakia).Crit rev oral biol med 2003; 14(4):253-267. http://dx.doi.org/10.1177/154411130301400403
7. OkungbowaF. IDede., A. P. O, IsikhuemhenO. S.Cellmorphology variations and budding patterns in Candida isolates. Advances in Natural and Applied Sciences, 2009; 3(2):192–195.
8. D. W. Williams and M. A. O. Lewis, "Isolation and identification of Candida from the oral cavity" Oral Diseases 2000;6(1):3–11. http://dx.doi.org/10.1111/j.1601-0825.2000.tb00314.x
9. C. Baveja, "Medicalmycology," in Text Book ofMicrobiology for Dental Students, 2010;3rd edition, Arya Publications, Delhi, India: p.322–323.
10. B. J. Harrington and G. J. Hageage."Calcofluor white: tips for improving its use," Clinical Microbiology Newsletter1991;13(1):3–5. http://dx.doi.org/10.1016/0196-4399(91)90048-Z
11. J. C. Davenport and J. M. A. Wilton."Incidence of immediate and delayed hypersensitivity to Candida albicans in denture stomatitis," Journal of Dental Research. 1971;50(4):892– 896. http://dx.doi.org/10.1177/00220345710500041701
12. A. Nassar, M. Zapata, J. V. Little, andM. T. Siddiqui, "Utility of reflex gomorimethenamine silver staining for Pneumocystis jirovecii on bronchoalveolar lavage cytologic specimens: a review" Diagnostic Cytopathology2006;34(11):719–723. http://dx.doi.org/10.1002/dc.20540
13. T. Ax'ell, T. Simonsson, D. Birkhed, J. Rosenborg, and S. Edwardsson, "Evaluation of a simplified diagnostic aid (Oricult-N) for detection of oral candidoses," Scandinavian Journal of Dental Research. 1985;93(1):52–55.
14. P. D. Marsh and M. Martin, "Oral fungal infections," in Oral Microbiology, 2009;, Churchill Livingstone, Edinburgh, UK, p. 166–179.
15. F. C. Odds, "Sabouraud('s) agar," Journal of Medical and Veterinary Mycology, 1991; 29; p.355–359. http://dx.doi.org/10.1080/02681219180000581
16. L. P. Samaranayake, T. W. MacFarlane, and M. I. Williamson,"Comparison of Sabouraud dextrose and Pagano-Levin agar media for detection and isolation of yeasts from oral samples," Journal of Clinical Microbiology.1987;25(1);162–164.
17. L. P. Samaranayake, T. W. MacFarlane, P.-J. Lamey, and M. M. Ferguson, "A comparison of oral rinse and imprint sampling techniques for the detection of yeast, coliform and Staphylococcus aureus carriage in the oral cavity," Journal of Oral Pathology, vol. 15, no. 7, pp. 386–388, 1986. http://dx.doi.org/10.1111/j.1600-0714.1986.tb00646.x
18. D. Beighton, R. Ludford, D. T. Clark et al., "Use of CHROMagarCandida medium for isolation of yeasts from dental samples," Journal of Clinical Microbiology, vol. 33, no. 11, pp.3025–3027, 1995.
19. P. Rousselle, A. M. Freydiere, P. J. Couillerot, H. De Montclos, and Y. Gille, "Rapid identification of Candida albicans by using albicans ID and fluoroplate agar plates," Journal of Clinical Microbiology, vol. 32, no. 12, pp. 3034–3036, 1994.

20. M. A. Pfaller, A. Houston, and S. Coffmann, "Application of CHROMagarCandida for rapid screening of clinical specimens for Candida albicans, Candida tropicalis, Candida krusei, and Candida (Torulopsis) glabrata," Journal of Clinical Microbiology, vol. 34, no. 1, pp. 58–61, 1996.
21. D. J. Sullivan, T. J. Westerneng, K. A. Haynes, D. E. Bennett, andD. C. Coleman, "Candida dubliniensissp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals," Microbiology, vol. 141, no. 7, pp. 1507–1521, 1995. http://dx.doi.org/10.1099/13500872-141-7-1507
22. A. Schoofs, F. C. Odds, R. Colebunders, M. Ieven, and H. Goossens, "Use of specialised isolation media for recognition and identification of Candida dubliniensisisolates from HIVinfected patients," European Journal of Clinical Microbiology and Infectious Diseases, vol. 16, no. 4, pp. 296–300, 1997. http://dx.doi.org/10.1007/BF01695634
23. E. Pinjon, D. Sullivan, I. Salkin, D. Shanley, and D. Coleman, "Simple, inexpensive, reliable method for differentiation of Candida dubliniensisfrom Candida albicans," Journal of Clinical Microbiology, vol. 36, no. 7, pp. 2093–2095, 1998.
24. A. N. B. Ellepola and C. J. Morrison, "Laboratory diagnosis of invasive candidiasis," The Journal of Microbiology, vol. 43, pp. 65–84, 2005.
25. D. Aubert, D. Puygauthier-Toubas, P. Leon et al., "Characterization of specific anti-Candida IgM, IgA and IgE: diagnostic value in deep-seated infections," Mycoses, vol. 39, no. 5-6, pp.169–176, 1996. http://dx.doi.org/10.1111/j.1439-0507.1996.tb00121.x
26. R. Wahyuningsih, H. J. Freisleben, H. G. Sonntag, and P. Schnitzler, "Simple and rapid detection of Candida albicans DNA in serum by PCR for diagnosis of invasive candidiasis," Journal of Clinical Microbiology, vol. 38, no. 8, pp. 3016–3021, 2000.
27. S. F. Yeo and B.Wong, "Current status of nonculture methods for diagnosis of invasive fungal infections," Clinical Microbiology Reviews, vol. 15, no. 3, pp. 465–484, 2002. http://dx.doi.org/10.1128/CMR.15.3.465-484.2002
28. E. Budtz-J¨orgensen, "Histopathology, immunology, and serology of oral yeast infections. Diagnosis of oral candidosis," ActaOdontologicaScandinavica, vol. 48, no. 1, pp. 37–43, 1990. http://dx.doi.org/10.3109/00016359009012732
29. J. R. Shepard, R. M. Addison, B. D. Alexander et al., "Multicenter evaluation of the Candida albicans/Candida glabratapeptide nucleic acid fluorescent in situ hybridization method for simultaneous dual-color identification of C. albicans and C. glabrata directly from blood culture bottles," Journal of Clinical Microbiology, vol. 46, no. 1, pp. 50–55, 2008. http://dx.doi.org/10.1128/JCM.01385-07
30. J. Trnovsky, W. Merz, P. Della-Latta, F. Wu, M. C. Arendrup, and H. Stender, "Rapid and accurate identification of Candida albicans isolates by use of PNA FISHFlow," Journal of Clinical Microbiology, vol. 46, no. 4, pp. 1537–1540, 2008. http://dx.doi.org/10.1128/JCM.00030-08


.


**Corresponding Author**

Dr. **Deepa Mastammanavar**
Post graduate student,
Department of Oral and Maxillofacial Pathology,
Navodaya Dental College and Hospital,
Raichur, 584101
Karnataka
Phone No; 07795583994
Email: dr.deepa4186@gmail.com