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Identification of yeast species in the oral cavity of Iranian soldiers by disk diffusion method

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Abstract

Background: The disk diffusion method for identification of yeasts species was performed based on different but distinct susceptibilities of yeasts spp. to chemicals: janus green, ethidium bromide, 2,3,5-triphenyltetrazolium chloride, brilliant green, cycloheximide and rhodamine 6G.

Methods: A total of 568 Iranian soldiers went under study for isolation and identification of Yeast species from their oral cavity. A sterile swab was used for each individual and specimens were collected from the nasopharynx region, then inoculated to petri dishes containing Sabouraud Dextrose Agar and incubated for 48 hrs at 37 °C. All colonies were counted and stocked in distilled water and stored in a refrigerator for further analysis. The yeasts were identified by the "disk diffusion test" [6,8]. This is a simple, rapid, accurate, and inexpensive technique presented by Sobczak [8]. By this method we identified yeast species within 24-48 hrs.

Results: 51.4% of petri dishes were positive for yeast species and 318 strains were identified. Candida albicans, Candida kefyr, Candida tropicalis and Candida guilliermondii were the most common yeast species isolated from the oral cavity of soldiers.

Conclusion:We used this method because of its simplicity and other beneficial characteristics for rapid identification of large and numerous isolates and the results were compared with other morphological characters such as chlamydospore and germ tube production. In addition, we used some type strains (Candida parapsilosis: PTCC 5089, Candida tropicalis: PTCC 5028,Saccharomyces cerevisiae: PTCC 5052,Candida lipolytica: PTCC 5063,Candida lipolytica: PTCC 5064), and the results were acceptable.

Keywords:yeast identification, disk diffusion test, oral cavity, susceptibility testing, yeast isolation

Introduction

The aim of this study was to apply 6 different chemicals for identification of yeasts based on previous similar works in the literature.

The disk diffusion method for identification of yeasts species was developed based on different but distinct susceptibilities of yeasts spp. to chemicals: janus green, ethidium bromide, 2,3,5-triphenyltetrazolium chloride, brilliant green, cycloheximide and rhodamine 6G.

Sobczak [1] identified 594 of 623 routinely isolated yeasts by this method and compared the results with commercial API 20C aux-

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anogram tests and there was an agreement of 95.3%. He introduced the method as simple, inexpensive and technically straightforward for identification of yeasts.

Menon and Ponnuvel [2], in another study, identified 448 strains of Candida by the disk diffusion method. They also compared the results with conventional methods and the agreement between them was 98.8%. In addition to using 6 chemicals in Sobczak's study, they used one of the chemicals: fast green. We used 6 chemicals in our study.

Candida species are normal inhibitants of the human gastrointestinal tract and may be recovered from up to one-third of the mouths of normal individuals. The most common species associated with mucosal infection of the mouth is Candida albicans, although in certain circumstances, other species (Candida glabrata, C. tropicalis, C. parapsilosis, C. guilliermondii, C. kefyr, and C. krusei) have been isolated. Although C.albicans can be cultured from the mouths of noninfected normal individuals, it does not cause disease unless predisposing factors exist to allow infection to become established [3]. Candida species frequently isolated from this region include Candida albicans (75%), Candida tropicalis (8%), Candida krusei (3-6%) and Candida glabrata (2 – 6%)[4,5].

We know that Prior colonization with candida species is a significant risk factor for the acquisition of candidemia [6,7].

Methods

Specimens were collected from the nasopharynx region by sterile cotton swabs and samples inoculated on petri dishes containing Oxoid Sabouraud dextrose agar (which added 50mg/L chloramphenicol to it) and then incubated for 48 hrs at 37°C. Negative cultures were incubated for another 5 days and after 1 week, they were considered as negative cultures. Yeast colonies were counted and their morphological characteristics such as size, shape, and color were recorded, and then subcultured to small vials containing sterile distilled water until analysis at another time. Identification of yeasts by the simple disk diffusion test were performed. This is a simple, rapid, accurate, and inexpensive technique presented by Sobczak [1]. By this method we identified yeast species within 24-48 hrs. The principles and scientific base of method has been explained in detail in the literature [1]. Six chemical substances were used, including Janus green, Ethidium bromide, 2,3,5,Tri phenyl Tetrazolium Cloride (TTC), Brilliant green, Cycloheximide and Rhodamin 6G.

Details of the preparation of each of the chemicals are as follows:

Janus green:

5 grams of Janus green powder in one litre of Sabouraud dextrose broth dissolved and final pH adjusted to 8 by normal NaOH solution.This solution should be steriled by autoclave (15 min, 121°C).

Ethidium bromide;

1.25 grams of Ethidium bromide powder should be dissolved in one litre of distilled water and autoclaved at 121°C for 15 min.

2,3,5, Tri phenyl tetrazolium chloride:

5 grams of TTC powder should be dissolved in distilled water and autoclaved (15 min, 121°C). This solution must be kept in a dark place.

Brilliant green:

50 mg of Brilliant green powder should be dissolved in one litre of distilled water and autoclaved (15 min, 121°C).

Cycloheximide:

500mg of Cycloheximide (Actidione) should be dissolved in one litre of sterile distilled water.

Rhodamin 6G:

2 grams of Rhodamine 6G powder should be

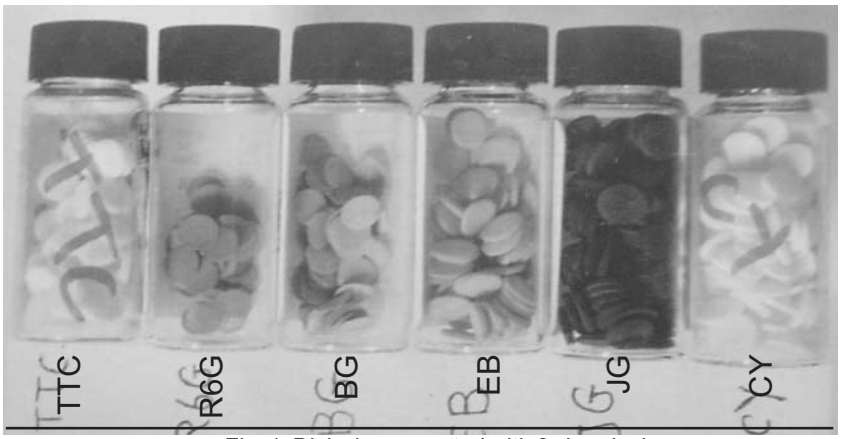

Fig. 1. Disks impregnated with 6 chemicals.

dissolved in one litre of ethanol and then must be autoclaved (15 min, 121°C).

All chemicals provided as mentioned, are stable for one year.

In the next step, we provided suitable paper disks from BBL (Sensi-blank disks 6 mm in diameter). These disks were placed in 6 mm diameter microplates in 6 rows and 30 microlitres of each chemical were added to them, then they were placed in a 37°C incubator for 42 hrs and stocked in a dry and dark place.These are stable for one year.

Final chemical concentration in paper disks was as below:

Janus green disks consisted of 150 µ gram, Ethidium bromide disks: 37.5 µ gram, TTC disks: 150 µ gram, Brilliant green disks: 1.5 µ gram, cycloheximide disks: 15µ gram and Rhodamin 6G disks consisted of 60 µ gram.

Method of disk diffusion test:

First of all, stock cultures were transferred and inoculated to petri dishes containing Sabouraud dextrose agar and incubated for 24 – 48 hrs at 37°C. A sterile cotton swab contaminated by yeast colony were transferred to 10 ml sterile saline and a clear and diluted solution was provided.

A whole surface culture in 8 centimeter sterile plastic petri dishes by wet and contaminated swab was performed and then 6 paper disks were inserted on surface of medium in certain arrangements, as shown in figure 2.

After insertion of disks, all of the plates were incubated at 37°C for 24-48 hrs. Then, the zone of inhibition around each disk was measured accurately. Resistance to each chemical was indicated by full growth of colonies around the disks and any sensitivity to each chemical was indicated by different sizes of inhibitory zones (area of no growing yeasts) around related disks. A code system was used in order to simplify the results of the recordings, numerals (1 to 6) as mentioned above were used for each of the disks and a 6 digit number made a certain code. For example 123456 means sensitivity to all 6 chemicals, and 000000 means resistance to all 6 chemicals, and 103406 means sensitivity to disks 1(JG), 3(TTC), 4(BG), and 6 (R6G) and resistance to disks 2(EB), and 5(CY). These 3 examples are related to C. tropicalis, Geotrichum sp. and C. albicans (variety stellatoidea) respectively (Table 1).

Results

A total of five-hundred and sixty-eight oral swabs were cultured. 51.4% of petri dishes were positive for yeast species and three hundred and eighteen strains were identified by the disk diffusion method (Tables 2 and 3). Candida albicans, Candida kefyr, Candida tropicalis and Candida guilliermondii were the

Species	*JG*	*EB*	*TTC*	*BG*	*CY*	*R6G*	*Code*
C. albicans	+	-	-	+	-	+	100406
	+	+	-	+	-	+	120406
	-	-	-	+	-	+	000406
C. guilliermondii	+	+	-	+	+	+	120456
C. krusei	+	+	+	+	+	+	123456
	-	+	+	+	+	+	023456
C. lipolytica	-	+	-	+	-	-	020400
C. parapsilosis	+	+	-	+	+	-	120450
	-	-	-	+	+	-	000450
	-	+	-	+	+	-	020450
C. kefyr	+	+	+	+	-	+	123406
C. albicans(var.stellatoidea)	+	-	+	+	-	+	103406
C. tropicalis	+	+	+	+	+	+	123456
Geotrichum sp.	-	-	-	-	-	-	000000
	-	+	-	+	-	-	020400
R. rubra	+	+	-	+	-	+	120406
T. candida	-	+	-	+	+	+	020456
C. glabrata	-	+	+	-	+	-	023050
	-	-	+	-	+	-	003050
	-	+	+	+	+	-	023450
	+	+	+	+	+	-	123450
	+	+	+	-	+	-	123050
	-	-	+	+	+	-	003450

Table 1. Sensitivity of different yeast strains to chemicals.

more frequent yeast species isolated from the oral cavity of soldiers (Table 3).

In some yeasts, which sensitivity to each of the chemicals had been the same, we used other characteristics of yeasts e.g size and shape of colony, germ tube test and chlamydospore production in corn meal plus tween 80 agar for differentiating.

Five standard strains provided by Iranian Type Culture Collection (PTCC) went under examination, along with the isolated yeasts, and we achieved significant results. These standard

Species	*JG*	*EB*	*TTC*	*BG*	*CY*	*R6G*	*Code*
G. candidun	-	-	-	-	-	-	000000
C. rugosa	-	-	-	+	+	-	000450
Cr. laurenti	-	+	-	-	+	-	020050
C. lipolytica	-	+	-	+	-	-	020400
C. parapsilosis	-	+	-	+	+	-	020450
T. candida	-	+	-	+	+	+	020456
C. glabrata	-	+	+	-	+	-	023050
C. krusei	-	+	+	+	+	+	023456
C. albicans	+	-	-	+	-	+	100406
C. albicans(var.stellatoidea)	+	-	+	+	-	+	103406
R. rubra	+	+	-	+	-	+	120406
Cr. neoformans	+	+	-	+	+	-	120450
C. guilliermondii	+	+	-	+	+	+	120456
C. kefyr	+	+	+	+	-	+	123406
S. cerevisiae	+	+	+	+	+	-	123450
Cr. albidus	+	+	+	+	+	-	123450
C. tropicalis	+	+	+	+	+	+	123456

Table 2. Identification of isolated yeasts from clinical specimens using the disk diffusion test.

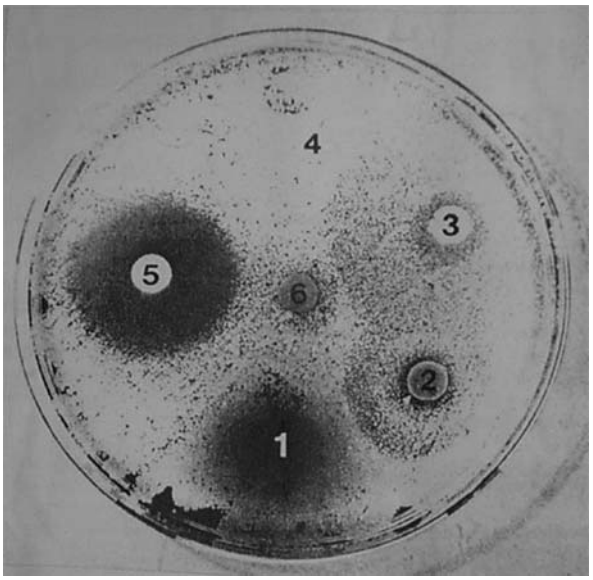

Fig. 2. Each of the disks were placed in certain and specific places. Disk no 1: JG, disk no 2: EB, disk no 3: TTC, disk no 4: BG, disk no 5; CY and disk no 6: R6G (Candida glabrata).

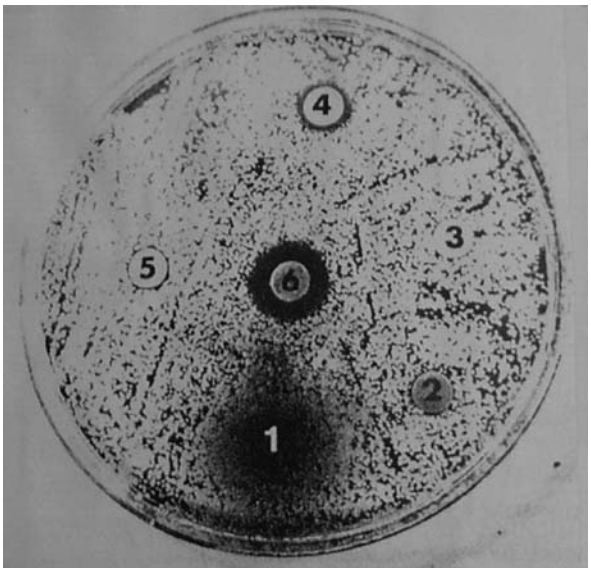

Fig. 3. Disk diffusion test : Candida albicans.

strains include: Candida parapsilosis (PTCC 5089), Candida tropicalis (PTCC 5028), Saccharomyces cerevisiae (PTCC 5052), Candida lipolytica (PTCC 5063), Candida lipolytica (PTCC 5064).

Discussion

Although there are different phenotypic methods used for identification of Candida species - such as germ tube production, chlamydospore production, assimilation and fermentation tests, culture on CHROMagar Candida, Candida ID2 agar, API 20C and others-the disk diffusion susceptibility test is a simple, practical, inexpensive and relatively rapid technique for the identification of clinically important yeast species. Until now, there are at least two studies in the literature which used this method

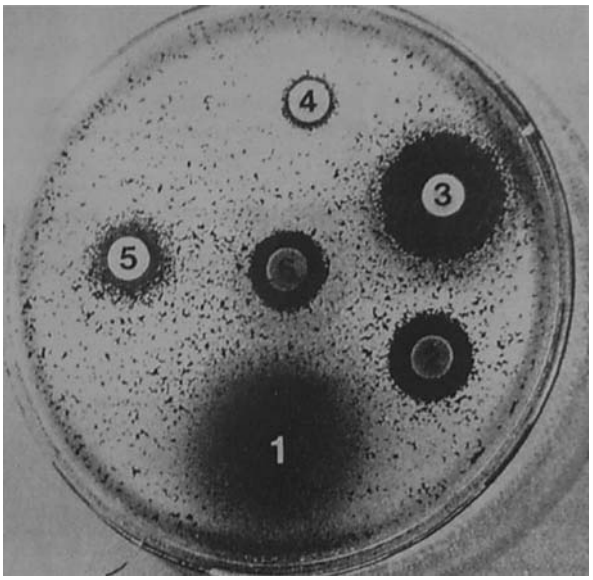

Fig. 4. Disk diffusion test: Candida tropicalis.

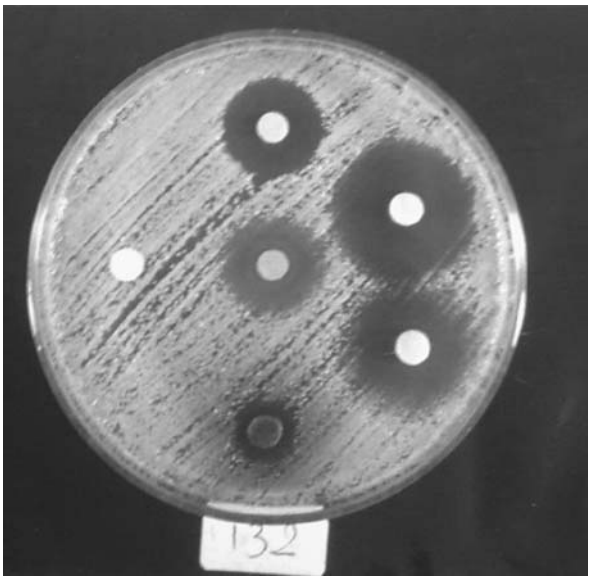

Fig. 5. Disk diffusion test : Candida kefyr.

Isolated & identified yeasts	*Number of colonies*	*Relative frequency (%)*
Candida albicans	163	51.25
C. kefyr	69	21.69
C. tropicalis	29	9.12
C. guilliermondii	19	5.97
C. albicans(var.stellatoidea)	13	4.1
C. parapsilosis	6	1.88
C. krusei	4	1.25
S. cerevisiae	4	1.25
C. rugosa	3	0.94
C. glabrata	3	0.94
C. lipolytica	3	0.94
T. candida	1	0.31
Rhodotorula sp.	1	0.31
Total	318	100.00

Table 3. Identified yeast species in this study.

for rapid identification of clinically isolated yeasts: H. Sobczak in 1985 [1] identified 594 of 623 routinely isolated yeasts by this method and compared the results with commercial API 20C auxanogram tests and there was an agreement of 95.3%. He introduced the method as simple, inexpensive and technically straightforward for identification of yeasts.

Menon and Ponnuvel, in another study [2], identified 448 strains of Candida by the disk diffusion method.They also compared the results with conventional methods and the agreement between them was 98.8%. In addition to using 6 chemicals in Sobczak s study, they used one of the chemicals: fast green.

We used 6 chemicals in our study. The results presented here reveal that the disk diffusion test identified almost all 318 clinical isolates of Candida. Only 0.8% of clinically isolated yeasts could not be differentiated by this method [1, 8, 9].

The disk diffusion method described offers, therefore, a reliable means for the identification of Candida species isolated from clinical specimens.

There are numerous studies in the literature about yeast flora of human oral cavity which were carried out in different geographical areas, but the main purpose of this study was not the comparison of such issues.

References

1. Sobczak H. A simple disk diffusion test for differentiation of yeast species. J Med Microbiol 1985; 20 (3): 307-16

2. Menon T, Ponnuvel KM. Disc diffusion test in the identification of Candida species. Mycoses 2000; 43:165-168

3. Anaissie EJ, Michael R, Michael AP. Clinical Mycology. 1st ed. NY: Churchill Livingstone; 2003. pp. 195-201

4. Appleton SS. Candidiasis: pathogenesis, clinical characteristics, and treatment. J Calif Dent Assoc 2000; 28:942

5. Bodey GP, Fainstein V. Candidiasis. New York: Raven Press; 1985. pp.55-9.

6. Calderone RA. Candida and Candidiasis. ASM Press. Washington DC. 2002. pp: 328

7. Wenzel RP. Nosocomial candidemia: risk factors and attributable mortality. Clin Infect Dis 1995; 20:1531-1534

8. Hazen KC. New and emerging yeast pathogens. Clin Microbiol Rev. 1995; 8:462

9. Pittet D, Monod M, Suter PM, Frenk E, Auckenthaler R. Candida colonization and subsequent infections in critically ill surgical patients. Ann Surg 1994; 220:751-758