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Evaluation of Vitik2 System for Clinical Identification of *Candida* species and Their Antifungal Exposure Test

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Abstract: The present study is an attempt for detection of *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida krusei* by conventional methods and multiplex methods as Vitik 2 system for these *Candida* species in vaginal swabs that collected from female patients clinically diagnosed with vulvovaginitis to find out the more specific, and sputum swab collected from male and female patients, sensitive and rapid method for diagnosis of this yeast. During the period from early December 2018 to the end May 2019, out of 110 samples a total of 110 vaginal swabs have been collected from female patients and sputum swab collected from male and female patients attending hospitals in Baghdad city (Al-Karkh and AL-Samarai Hospitals) and in Tikrit (General Tikrit Hospital) aged from 1-71 years. To sum up 72 out of 110 were detected for *Candida* species, To sum up 36 out of 72 sputum swabs were detected for *C. albicans*, 16 out of 72 sputum swabs were detected for *C. glabrata*, 7 out of 72 sputum swabs were detected for *C. tropicalis*, 10 out of 72 sputum swabs were detected for *C. krusi* and 3 out of 72 sputum swabs were detected for negative result.To sum up 38 out of 38 vaginal swabs were detected for *C. albicans*. A number of female from summation account 72 patient while summation of male 38 patient depending on the morphologic characteristics of these *Candida* species on the culture media including Sabouraud′s dextrose agar and CHROMagar, as well as the biochemical tests including automated biochemical tests such as VITEK 2 system. Then we performed a pharmacological sensitivity check for the following drugs using the Vitk2, *Candida albicans* which showed sensitivity to Voriconazole with Minimum inhibition concentration MIC (0.12), Flucytosine (1), Amphotericin B (0.5), Fluconazole (1), Micafungin (0.06),Caspofungin (0.25).Fluconazole ≤ 1.While, the results to the *candida* spp. as follows Fluconazole ≤ 1,voreconazole ≤ 0.12, caspofungin ≤ 0.25,Micafungin ≤ 0.06,Amphotricin B 1and flucytocine ≤ 1, which sensitive to all antifungals. CHROM agar *Candida* medium was shown to be extremely helpful in routine clinical mycology service, facilitating the detection of mixed cultures of yeasts and allowing directly identification of *C. albicans* as well as rapid pre summative identification of the yeast.

Keywords: yeast, Germ tube formation , CHROM Agar,SDA, Vitik2.

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Introduction

Candida albicans is the most important fungal opportunistic pathogen. It usually resides as a commensal in the gastrointestinal and genitourinary tracts and in the oral and conjunctiva flora (1). The fungal kingdom contains diverse organisms which make significant contributions to supporting life on our planet. Fungi are of vital importance to humanity as industrial manufacturers, play a key role in environmental nutrient recycling, and are model organisms for many eukaryotic processes. Our use of fungi throughout history has expanded in scope, from an edible agricultural

resource, to the production of food, beverages and are a valuable source of antimicrobial compounds. However, there are also many fungal species that are serious pathogenic threats to plant, animal, and human health(2) pathogen, it has recently been reported that, globally, fungal infections currently cause 1.5 million deaths per year(3) The risk of candidiasis is increased in patients with underlying malignancies and those undergoing chemotherapy, patients undergoing hematopoietic stem cell or solid organ transplantation, those with immunosuppressive diseases for example (HIV/AIDS), patients using broad-spectrum antibiotics or corticosteroids and using invasive medical interventions for example (central vascular catheters), and those with certain genetic risk factors(4) Nosocomial infections of candidiasis are becoming more common, and account for more than 85% of all invasive fungal infections in both Europe and thenUnited States(5). Fungi were diagnosed by germ tube formation. During the past two decades, the incidence of candidiasis has increased markedly with the advent of new diseases such as acquired immunes efficiency syndrome and the developmental of immuo suppressive therapy. Therefor, the present study was conducted for detection of *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida krusei* by conventional methods and multiplex methods as Vitik 2 system for these *Candida* species in vaginal sputum and sputum swabs.

Materials and Methods

During the period from beginning December 2018 to the end May 2019, a total of 110 clinical samples were collected from patients hospitalized in Baghdad city (Kamal AL-Samarea and AL-Karkh hospitals), and General Tikrit hospital aged from 1-71 years. Different sources were collected from mouth, vaginal swabs the sputum specimens were collected in sterile containers. The samples were taken by sterile swabs with a transport medium and immediately transported to the laboratory of each hospital under aseptic conditions. Each specimen was streaked on selective media such as Sabouraud′s dextrose agar then sub culture on CHROM agar. It was incubated under aerobic conditions for 24 hrs at 37°C (6).

Culture Sabouraud′s Dextrose Agar

The medium was prepared according manufacture company by adding 65gm of Sabouraud′s dextrose agar in 900 ml of (D.W) and the mixture was brought to boil in water bath to dissolve all constituents completely, then the volume was adjusted up to (1L) by the (D.W). The pH was adjusted to (5.6) and then sterilized by autoclaving at 121C$^{\circ}$ under 15 p/anch^{2} for 15 minutes, and then the chloramphenicol was added to the Sabouraud′s dextrose agar with 50 µg/ml of medium as a final concentration(7).

Culture on CHROMagar

This medium was prepared according to instructions of the manufacture company by adding 56.3 gm of CHROM agar in 900 ml of (D.W), and then 1 ml of glycerol and 0.5 ml of Tween 60 were added. The volume was adjusted up to (1L) by (D.W) and the mixture was heated in

water bath and then sterilized by autoclaving at 121C° under 15 p/anch^2 for 15 minutes, after then the mixture was let cool at 45°C in water bath and poured on Petri dishes. (8).

Germ Tube Formation

This test included inoculation a small portion of an isolated colony in 0.5ml of human serum. The suspension was inoculated at 37ºC for 3hrs. Then a drop of this suspension was put on clean glass slide covered by cover-slip and examined under light microscope at 40 X (9).

Biochemical tests

VITEK 2 system

Vitik-2 system was used in this study in order to diagnose the *Candida. albicans, C. glabrata, C. tropicalis and C. krusei* isolates, which included several steps as follows:

I- Preparation of fungus suspension

A sterile swab was used to transfer a sufficient number of *C. albicans*, colonies of a pure culture and separately suspended in 3 ml of sterile saline in clear plastic test tube. The turbidity was adjusted up to 2.0 O.D.

II- Inoculation of identification card

Identification card was inoculated with C. albicans,. Isolates suspension using an integrated vacuum apparatus. A test tube containing the *Candida* suspension was placed into a special rack (cassette) and the identification card was placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The cassette can accommodate up to 10 tests or up to 15 tests. The filled cassette was placed either manually or transported automatically into a vacuum chamber station. After the vacuum was applied and air was re-introduced into the station, the Candida suspension was forced through the transfer tube into micro-channels that fill all the test wells

III- Card sealing and incubation

Inoculated card was passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. The carousel incubator can accommodate up to 30 or up to 60 cards. All card types are incubated on-line at 35.5 +_ 1.0 ºC. Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next read time. Data are collected at 15 minutes intervals during the entire incubation period (10).

Results and discussion

A total of 110 clinical specimens were collected from patients hospitalized in Baghdad city (Kamal Al-Samarea and Al-Karkh hospitals), and General Tikrit hospital aged from 1-71 years. A number of female from summation account 72 patient while summation of male 38 patient (Table 1). From 110 clinical specimens there were 107 (97.27%) positive for *Candida* spp. Were detected through aged from 1-71 years .Among this the highest percentage was 44(40.4%) aged from 21-39, whereas the lowest percentage was 3(2.72) aged 71 years (Table 2).

Table (1): Gender Wise Isolation of Candida Species (n=110)

Gender	Total No. of Isolates (n=110)	Percentage (%)
Female	72	65.45%
Male	38	34.55%
Total	110	100%
Chi-Square -χ^2 (P-value)	---	10.509 (0.0012)
** (P≤0.01).		

Table (2): Age wise Isolation of*Candida* Species (n=110)

Age Groups (year)	Total No.=84	Percentage (%)
1-20	27	24.54
21-39	44	40. 40
40-59	24	21.81
60-70	12	10.90
71	3	2. 72
Chi-Square -χ^2 (P-value)	---	4.272 (0.0001)
** (P≤0.01).		

To sum up 36 out of 72 sputum swabs were detected for *C. albicans*, 16 out of 72 sputum swabs were detected for *C. glabrata*, 7 out of 72 sputum swabs were detected for *C. tropicalis*, 10 out of 72 sputum swabs were detected for *C. krusi* and 3 out of 72 sputum swabs were detected for negative result. While 38 out of 38 vaginal swabs were detected for *C. albicans*. The result of *Candida* species isolation showed that the first most common isolated species was *C. albicans* (74 isolates) 67.27%,the second isolated species was *C. glabrata* (16 isolates)19.04%,the third isolated species was *C. tropicalis* (7 isolates) 8.33%, the fourth isolated species was *C. Krusi* (10 isolates) 11.9%. and 3 isolates swabs were detected for negative result (Table 3). The result of this study in agreement with results of different worldwide studies which conducted on *Candida* species that referred the first and second most common isolated species were *C. albicans*and *C. glabrata* (11). Conventional diagnosis of *C. albicans, C. tropicalis, C. glabrata and C. krusi.*

Table(3): Isolation of *Candida* species

Candida Species	Total No. of Isolates n=110	Percentage of Isolates%
Candida albicans	74	67.27
Candida glabrta	16	19.04
Candida tropical	7	8.33
Candida Krusi	10	11.9
Chi-Square -χ^2 (P-value)	---	112.85 (0.0001)
** (P≤0.01).		

Culture Ccharacteristics

Culture on Sabouraud′s Dextrose Agar

Colonies of *C. albicans, C. tropicalis, C. glabrata and c. krusi* isolates on Sabouraud′s dextrose agar appeared white to creamy colony shing glabrous and smooth as show in Figure 1 ,2,3 and 4, respectively.

Figure (1): Creamy colonies of *Candida albicans* isolate on SDA after 48hr incubation at 37^{0}C.

Figure (2): Creamy colonies of *Candida glabrata* isolate on SDA after 48hr incubation at 37^{0}C.

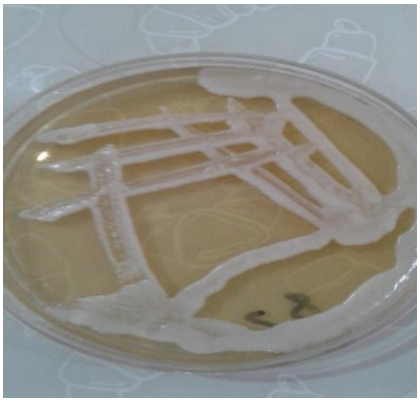

Figure (3): Creamy colonies of *Candida* on SDA after 48hr incubation at at 37^{0}C

Figure (4): Creamy colonies of *Candida tropicalis* isolate *krusi* isolate on SDA after 48hr incubation at 37^{0}C

In this study, the morphological characteristics of *C. albicans, C. glabrata, C. tropicalis and C. krusi*isolates on plates of the SDA containing chloramphenicol (50µg/ml) were matching with the morphological characteristics of *Candida* obtained by (12,13), they referred that the colonies of *Candida*, on the routinely used SDA, are cream to yellow in color, and depending on the *Candida* species, colony texture may be smooth, glistening or dry, or wrinkled and dull. Any growth obtained was further identified by its temperature requirement, rate of growth, colony morphology and lactophenol cotton blue mounts. Other study exhibit that the yeast identification was done on the basis germ tube production, and colour production on CHROM agar(14) Furthermore, the results of current study

were consistent with the findings of (15).

Culture on CHROM Agar

All the positive cultures of *C. albicans, C. glabrata, C. tropicalis* and *C. krusi* isolates were sub cultured again on CHROM agar and the morphological characteristics of these growing isolates were determined. The results showed grow of blue colonies of *C. albicans* isolates (Figure 5), whereas Pink-purple colonies of *C. glabrata* Isolates (Figure 6), and dark blue colonies of *C. tropicalis* isolates (Figure 7) and the *C. krusi* isolates gave white colonies (Figure 8).

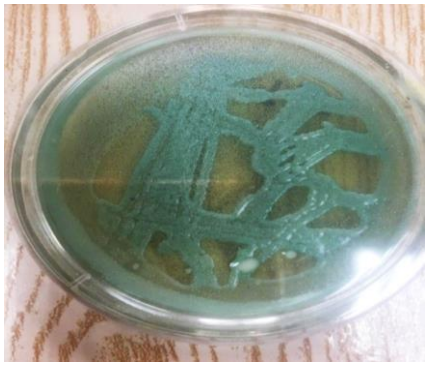

Figure (6): Green colonies of *Candida albican* isolate grow on plate of the CHROM agar After 72hr incubation at 37^0C.

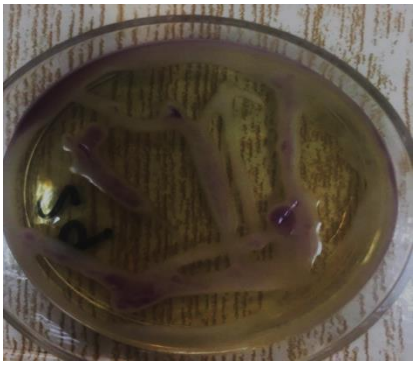

Figure (7): Pink colonies of *Candida glabrata* isolate grow on plate of the CHROM agar After 72hr incubation at 37^0C.

Figure (8): Dark blue colonies of *Candida tropicalis* isolate grow on plate of the CHROM agar after 72hr incubation at 37^0C.

Figure (9): White colonies of *Candida krusei* isolate grow on the plate of CHROM agar after 72hr incubation at 37^0C.

In current study, the observed phenotypic characteristic which refer to the color of growing colonies of *C. albicans, C. glabrata, C. tropicalis*and *C. krusi*isolates on the CHROM agar was similar to colors of colonies of these *Candida* species on the same medium that characterized by (16).

Germ tube formation

Elongated daughter cells from the round mother cell without constriction at their origin are referred to as true germ tubes while constricted hyphae as pseudohyphae. The *C. albicans isolates* were showed positive results and the

formation of germ tubes was seen as long tube like progections extending from the yeast cells (Figure 9). The germ tubes were formed within two hours of incubation and this is a unique diagnosis characteristics of *C. albicans* differation it from other *albicans* species. Germ tube test is very cost effective as compared to phenotypic methods and PCR-RFLP. The turnaround time for other phenotypic methods is 2-4 working days and for PCR-RFLP it is 2 working days while as for germ tube test it is only 1-2 hours.

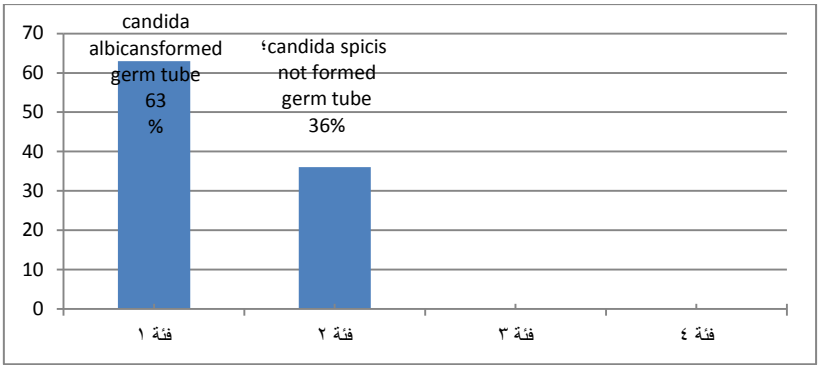

Figure (9): Germ tube formation.

Out of 74 test strains of *C. albicans*, 63(85%) formed true germ tubes after 1 h at 37◦C while the remaining grew as a yeast form. All the 36(100%) test strains of *C. species* grew as a yeast form (Figure 10).

Conventionally germ tube test in human serum at 37°C for 2–3 h is used to differentiate between *C. albicans* from other non- *albicans* species but both *C. albicansand C. dubliniensis* produce germ tube by this method (17). The results of this study were agree with this of (18) when mentioned that all *C. albicans* strains were germ tube test(GTT) positive when tested directly from the colony, and a *non-albicans* were GTT negative when tested directly from the colony.

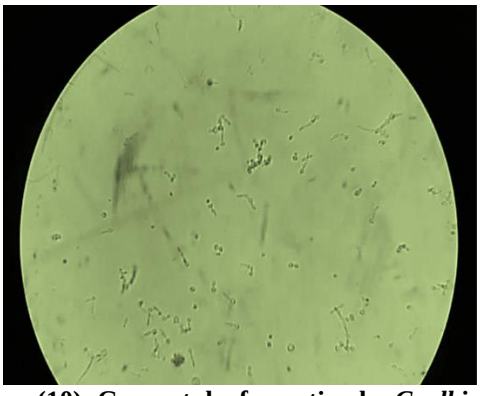

Figure (10): Germe tube formation by *C. albicans.*

Biochemical Tests

For further diagnosis of *C. albicans, C. glabrata, C. tropicalis and C. krusi*isolates of the present study, considered as confirmative tests and to decrease the diagnosis time of these

four *Candida* species isolates by the biochemical tests, the VITEK 2 system.

VITEK 2 System

The biochemical tests of VITEK 2 system were used to diagnosis the *C. albicans, C. glabrata, C. tropicalis*and *C. krusi* isolates of this study. At the end of the initial incubation period, VITEK 2 system was identified presence of *C. albicans* in 74 samples (67.27%), *C. glabrata* in 16 samples (19.04%), *C. tropicalis* in 7 samples (8.33%) and *C. krusi* in 10 samples (11.9%) out of 107 positive culture samples of vaginal swabs and sputum swab respectively, out of 3 negative culture sample. Because relatively some of the phenotypic identification procedures are based on colorimetric or pH-based changes and usually require 18 to 24 h to identify organisms and some of them are based on changes in preformed enzymes, shortening the time necessary to make identification, the instrument is designed to decrease the turnaround time for the identification of microorganisms and use more conventional processes remains (19).

In fact, the erroneously elevated MICs by the Vitek 2 automated reading method not only may lead to inappropriate selection of antifungal therapy but also depict false rates of high antifungal resistance in epidemiological studies (20). Furthermore the study conducted by (21) showed that all the germ tube negative *Candida* spp. were specialize by the automated system (Biomerieux, Vitek 2 C) using YST identification, as well as AST, determined by YST-AST-01 card. Also the result of this study goes together with results of study conducted by (22) to identify different *Candida* species (*C. albicans, C. glabrata*and *C. parapsilosis*) attributed to systemic candidiasis by cornmeal agar supplemented with tween-80, germ tube formation in serum.

Antifungal Susceptibility Testing (AST)

All twenty type strain isolates clinical isolates demonstrated sufficient growth at 15 h of incubation, and the species could be determined by the VITEK 2 system at different incubation periods. The VITEK 2 system met the desired reproducibility criteria (100% correct) for the identification of all strains at the species level Following additional tests, as recommended by the manufacturer, all 20 challenge strains were successfully identified by the VITEK 2 system. No isolates failed to grow in the VITEK 2 system in any of the replicates in any test all 20 type strain isolates were correctly identified at the species level by the VITEK 2 system. To assess the performance of the VITEK 2 system for the determination of MICs, we evaluated 20 clinical isolates. The VITEK 2 MIC results were produced after 14 to 27 h of incubation for all *Candida* spp. The mean time-to-result for the VITEK 2 system was 15 h for amphotericin B (with a range of 11 to 27.8 h) and fluconazole (with a range of 9 to 24.2 h), 13.1 h for flucytosine (with a range of 13.0 to 27 h), and 12.4 h for voriconazole (with a range of 8.1 to 25.1 h). All except 7 of the 32 clinical isolates (5 isolates of *C. krusei*, *C. kefyr*, and *C. pelliculosa)* demonstrated sufficient growth at 24 h of incubation for the CLSI BMD method, allowing the majority of the MICs to be determined at this time point.

Tables 4 and 5 summarizes the *in vitro* susceptibilities of 74 clinical isolates of *C. albicans* and 36 clinical isolates *Candida* spp. respectively, to amphotericin B, 5-flucytosine, fluconazole ,voriconazole, Caspofungin and Micafungin, as determined by the VITEK 2 system and by the two reference BMD methods (CLSI and EUCAST). For the CLSI method, the 24-h results are presented for all but seven isolates, which did not exhibit sufficient growth at this point; for these isolates, the results from 48 h are presented.

Table (4): *In vitro* susceptibility of *C. albicans* to antifungal using VITEK 2 system.

Selected Organism	Antimicrobial	MIC	Interpretation
Candida albicans	Fluconazole	≤ 1	S
	Voriconazole	≤ 0.12	S
	Caspofungin	≤ 0.25	S
	Micafungin	≤ 0.06	S
	Amphotericin B	0.5	S
	Flucytosine	≤ 1	S
	P-value	0.0366 *	100% S
*** (P≤0.05).**			

Table (5): *In vitro* susceptibility of *Candida* spp. to antifungal using VITEK 2

Selected Organism	Antimicrobial	MIC	Interpretation
***Candida* spp.**	Fluconazole	16	S
	Voriconazole	<= 0.12	S
	Caspofungin	<= 0.25	S
	Micafungin	<= 0.12	S
	Amphotericin B	0.5	S
	Flucytosine	16	S
	P-value	0.0001 **	100% S
**** (P≤0.01).**			

In our study out of 110 isolates isolated, 67.27% were *Candida albicans*and 33.73% were Non-*Candida albicans*. Non-*Candida albicans*were isolated more in numbers than *C. albicans*. Mehta *et al*.(23), has reported that Candida albicans was the commonest species isolated (40.9%). The isolation of Non-*Candida albicans* (59.1%) predominated over *Candida albicans* (40.9%), their study disagrement with our study. In our study out of 110 isolates, isolated *Candida albicans* were 67.27%. "Among 33.73% of Non-*Candida albicans*, the *Candida tropicalis* were 8.33%, *Candida krusei* were 11.9%, *Candida glabrata*, 19.04 has reported that the commonest Candida isolate was *C. albicans* (31.42%) followed by *C. tropicalis* (26.66%). Other species isolated were *C. glabrata* (19.04%), C. parapsilosis (10.47%), C. krusei (5.71%), C. kefyr (4.76%) and *C. guilliermondii* (1.9%)(24). The most common Non-*Candida albicans* isolates were *C. tropicalis,C. Krusei* and *C. guilliermondii*. This study correlates with our findings.

*Candida tropicalis*species has emerged as the major *Candida* non-*albicans* species (33.73%) in our study. Our study correlates with the study by (25) (40.9%), and (26) (46.25%) who have also reported *Candida tropicalis* as the most common *Candida non-*

albicans species. In our study, out of isolates Candida albicans which showed sensitivity to Voriconazole were show minimam inhibition concentration MIC (0.12), Flucytosine were(1), Amphotericin B were(0.5), Fluconazole were (1), Micafungin were(0.06), Caspofungin were(0.25). Yenisehirli *et al*.(27)has reported that all the tested *C. albicans* isolates were found to be susceptible to amphotericin B. 85% of the isolates were classified as showing susceptibility to caspofungin. The sensitivity rates of *C. albicans* isolates to fluconazole and voriconazole were 74% and 86% respectively. The *Candida albicans* showed sensitivity to Amphotericin B, Fluconazole, Voriconazole .This study bears a good correlation with our study. In our study, out of isolates not *Candida albicans* showed sensitivity to Voriconazole were (0.12), Amphotericin B were(1), Flucytosine were(≤ 1), Micafungin were (≤0.06), Fluconazole were(≤ 1) and Caspofungin were(≤0 . 25). Pasqale T *et. al*.(28) has reported that C. tropicalis is usually susceptible to the Voriconazole, Flucytosine and amphotericin B. In our study not *Candida albicans* showed high sensitivity to Voriconazole, amphotericin B and Flucytosine. Thus, our study has similar findings.In our study, out of 7 isolates *Candida krusei* showed sensitivity to Voriconazole, Caspofungin, Micafungin and Amphotericin B were 85% each, Flucytosine and Fluconazole were 15% each. Orozco *et al* .(29)has reported that C. krusei is intrinsically resistant to fluconazole . Our study correlates well their study.

In this study, out of 5 isolates *C. guilliermondii* showed sensitivity to Voriconazole, Caspofungin and Micafungin were 60%, Amphotericin B, Flucytosine and Fluconazole were 80% each. Girmenia *et al*.(30) has reported that *C. guilliermondii* is an uncommon *Candida* species. This species has reduced susceptibility to Fluconazole. However, *C. guilliermondii* is usually susceptible to Amphotericin B. The conventional methods of identification of *Candida* species are time consuming and difficult to perform. *Candida glabrata* was identified as the most common *Candida non-albicans* species. *Candida albicans* showed high sensitivity to Voriconazole, Amphotericin B capsifungin and micafungin.

In conclusion, in spite of great cost of CHROM agar *Candida* medium identification of *C. albicans* colonies as well as the pre summative identification of the other most common yeasts, and the each recognition of association of multiple yeast species. Thus this easy-to- work and time saving medium appears to be well studied for routine us in clinical mycology laboratories.

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