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COMPARATIVE STUDY OF PHENOTYPIC AND AUTOMATED METHODS IN IDENTIFICATION AND ANTIFUNGAL SUSCEPTIBILITY OF CANDIDA SPECIES ISOLATED FROM CLINICAL SAMPLES

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Introduction:

Candida, a fungus which was thought to be a harmless commensal a few decades ago has grown into a problem of enormous medical significance. It causes a plethora of diseases, ranging from superficial mycoses to deep seated invasive candidemia, which carries a mortality rate of above 50% in developing countries.[1]

To successfully treat candida infections and prevent unnecessary deaths,proper identification of candida species along with antifungal susceptibility testing(AST) is of paramount importance. Quickly commencing the correct antifungal treatment can double the chances of survival.[2][3][4]

Unfortunately, these are hardly carried out in resource poor countries, leading to treatment failures and selection of resistance strains[5] while at the same time subjecting the patients to the toxic side effects of inefficient antifungals. Few patients have mixed infection with 2 or more candida species,[6]and giving an empirical drug to which only 1 species is susceptible does not lead to complete cure and there is recurrence of the disease after cessation of the drug. Also the susceptibility of candida species to antifungals vary in different parts of the country[7] and resistance to newer class of drugs like echinocandins is not well known.[8] Lastly, ICMR recently released an advisory warning about the emergence of multi-drug resistant candida auris.[9]In all these cases, identification of species along with AST provides valuable epidemiological data which can pinpoint the source of an outbreak ,quickly identify and treat such vicious species, leading to reduced morbidity and mortality.

Traditionally the identification of species was carried out by phenotypic methods which were laborious and time consuming.[10]]The introduction of Chromagar[11] sought to alleviate these difficulties by rapidly identifying different species based on colour, while the disk diffusion method was used for AST. But they had their own share of problems, such as difficulty in differentiating between C.albicans and C.dubliniensis ,and giving incorrect AST results.[12] On the other hand, automated systems like Vitek 2 can also identify and do AST in a fraction of the time but their high cost, need of skilled personell, misindentifying one species for another and false MIC values[9]

prohibit their extensive use. In this study ,we compare the effectiveness and accuracy of both methods to find out which is more suited for our setting.

AIMS AND OBJECTIVES

1. To isolate candida from various clinical samples.
2. To identify the species and antifungal susceptibility using phenotypic and automated method.
3. To compare their results and find the cost to benefit ratio.

Subjects and Methods:

This is a prospective, cross sectional study which was conducted in our mycology laboratory for 2 months. The samples were collected from OPD and IPD patients. The samples which were used are skin scrapings, saliva, sputum, blood, bile, vaginal swabs and feces.

Inclusion criteria

1. Candida isolation from 2 consecutive samples
2. Less than 48 hours of hospital stay
3. Presence of pus cells in wet mount examination

Exclusion criteria

1. Use of corticosteroids,antibiotics or antifungals
2. Tobacco chewing,smoking and intraoral prostheses
3. Surgery

Procedure

1. *Using phenotypic methods*

a) **Gram stain**[15]

After gram staining,we looked for gram positive budding yeast cells with hypae and/or pseudohypae.This is to distinguish a candida infection from a bacterial infection.

b**) Isolation on Sabouraud dextrose agar**(SDA)[15]

Samples were cultured on 2 tubes of SDA,one of which contained cycloheximide ,at a temperature of 28'C and 45'C(being able to grow at both high and low temperatures will distinguish C.albicans from other species) in an aerobic environment for 48 hours.If there is no growth after 48 hours ,they were further inoculated for a week. The different species were identified crudely using the following colony characteristics.

C.albicans	smooth , creamy , pasty , glistening
C. glabarata	Cream coloured, soft, glossy ,smooth colony
C. tropicalis	white to cream coloured colonies with peripheral fringe
C. parapsilosis	Soft, smooth, white sometimes lacy
C. krusei	Colonies are flat ,dry becoming dull, smooth or wrinkled
C.kefyr	Smooth , creamy appearance

c) **Germ tube test**[48]

Using a sterile loop,a colony of yeasts was transferred to a test tube containing 3 drops of human serum and emulsified.After incubating for 3 hours,a drop of solution was placed on a clean dry glass slide and covered with a cover slip.It was observed under 10 and 40x of microscope to see the presence or absence of germ tube.This a quick method to differentiate C.albicans from other species.

d) **Corn meal agar**[13]
Subcultures were made by furrowing the Corn meal agar plates with coverslips were applied on the streak line and incubated at 28 :C .After 2-5 days the plates were examined directly under a microscope to look for the following morphological characteristics of different candida species:-

C. albicans	Terminal and intercalary chlamydospores
C. glabarata	No pseudohyphae, only blastoconidia
C. tropicalis	Branching pseudohyphae and blastoconidia
C. parapsilosis	Curved pseudohyphae and blastconidia
C. krusei	Pseudohyphae and blastoconidia resembles crossed match stick
C. dublinensis	Terminal and intercalary chlamydospores

e)**CHROMagar**[49]
A single yeast colony was streaked on to the plate after which it was inoculated for 48-72 hours. After this time,the species were identified due to their characteristic colours:-

C. albicans	Light green
C. glabarata	Pink to purple
C. tropicalis	Blue with pink halo
C. parapsilosis	Cream to pale pink
C. krusei	Pink
C. dublinensis	Dark green

f) **Antifungal susceptibility test using disk diffusion**
It was carried out by disk diffusion method on using Mueller-hinton agar with 2% glucose and 0.5 µg/ml
Methylene blue for Fluconazole (25µg), Itraconazole(10 µg), Amphotericin B(100 U) and Nystatin(10µg).Each inoculum was standardized to 0.5 Mc Farland units and the zone break points were interpreted as per the following table[50].

Drug	Susceptible	Intermediate	Resistant
Fluconazole (25µg)	≥ 19 mm	15-18 mm	≤ 14mm
intraconazole(10 µg)	≥ 20mm	12- 19mm	≤ 11 mm
Amphotericin B(100 U)	>10mm	Not applicable	≤ 10mm

2. **Using automated methods for Identification**[42]
Vitek YST-ID cards were used. Cards were held at 35.5°C for 18 h inside the Vitek 2 instrument which will take optical density readings automatically at every 15 min. Based on these readings, the species was identified according to a specific algorithm.

Automated antifungal susceptibility test -
The AST was done using ASTYS01 and ASTYS06 cards. A standardized inoculum suspension was placed into a Vitek-2 cassette along with a sterile polystyrene test tube and an antifungal susceptibility test card for each organism. The Vitek-2 instrument diluted the inoculum , after which the cards were filled, incubated, and read automatically. The isolates were be considered resistant if they exhibit the following MICs[51].

Fluconazole	≥64 µg/ml
voriconazole	≥4 µg/ml
flucytosine	≥32 µg/ml
amphotericin-B	≥1 µg/ml
caspofungin	≥2 µg/ml

Quality control

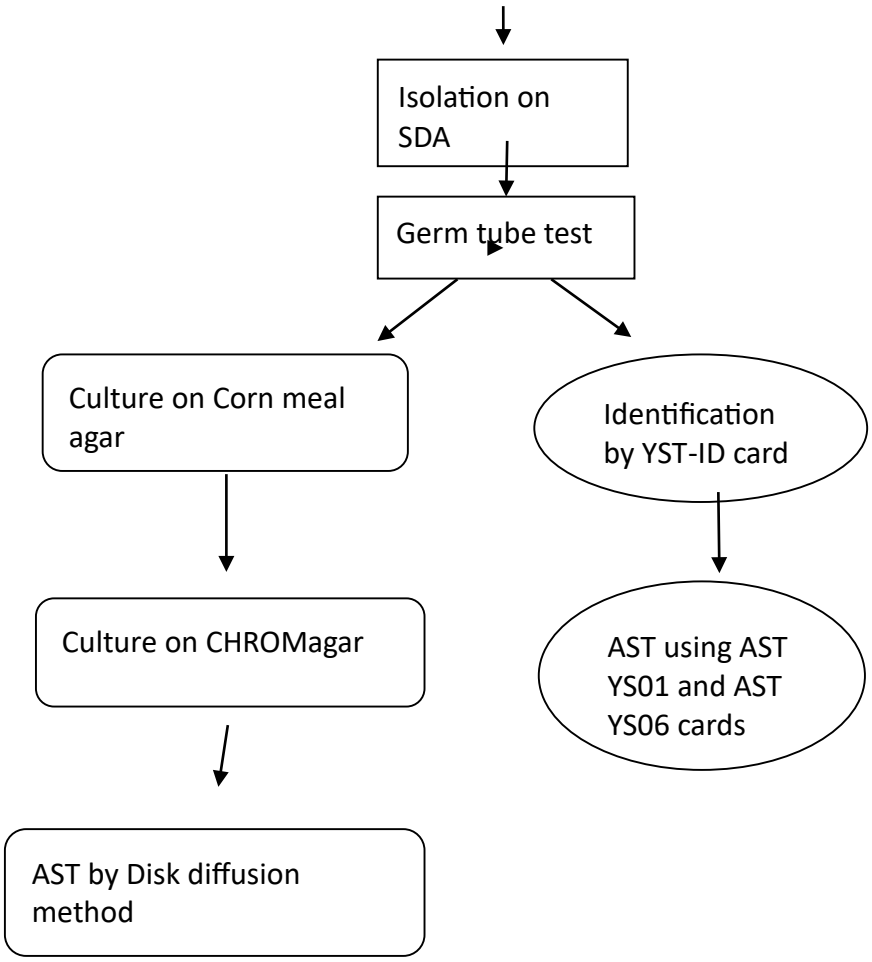
Following standard strains were tested each time to ensure quality control:-
Candida Albicans ATCC 10321 , Candia parapsilosis ATCC 22019, Candida tropicalis ATCC 750, Candida Krusei ATCC 6258.

Statistical analysis

It was done using SPSS software version 25.Chi square test was done to compare the significance of the results from the two methods.

Flowchart showing step by step method for the comparison of phenotypic and automated method

Gram stain
Gram stain



Results:

A total of 39 isolates of Candida species were obtained from various clinical samples over a 2 month period from both outpatient and inpatient department.The isolates were grown in Candida CHROMAGAR and Dalmau plate culture was done on cornmeal agar for species identification.Candida species identification and Antifungal susceptibility testing was done in VITEK-2 from colonies grown on 5 % Sheep blood agar. Antifungal testing was also done by disk diffusion method as per CLSI recommended method. The results were analyzed.

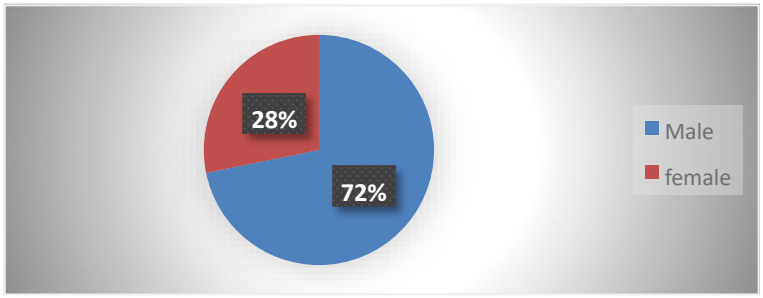
In our study, non Albicans Candida species were predominant (Table-1). Candida albicans isolates were 7.69% of total isolates. Among non Albicans Candida species, Candida tropicalis (38.46%) was the predominant species isolated, followed by Candida glabrata(23.07%) ,Candida guilliermondi (12.82%), Candida parapsilosis(10.25%) , Candida krusei (5.14%) and Candida kefyr (2.58%)

Table 1-Percentage distribution of Candida species by VITEK-2

Species	Number of isolates	Percentage
C.albicans	5	7.69%
C.glabrata	9	23.07%
C.tropicalis	13	38.46%
C.parapsilosis	4	10.25%
C.krusei	2	5.14%
C.kefyr	1	2.58%

Among the 39 isolates, there were 28 isolates from men and 11 isolates from women (Chart 1).

Chart 1 – Sex distribution

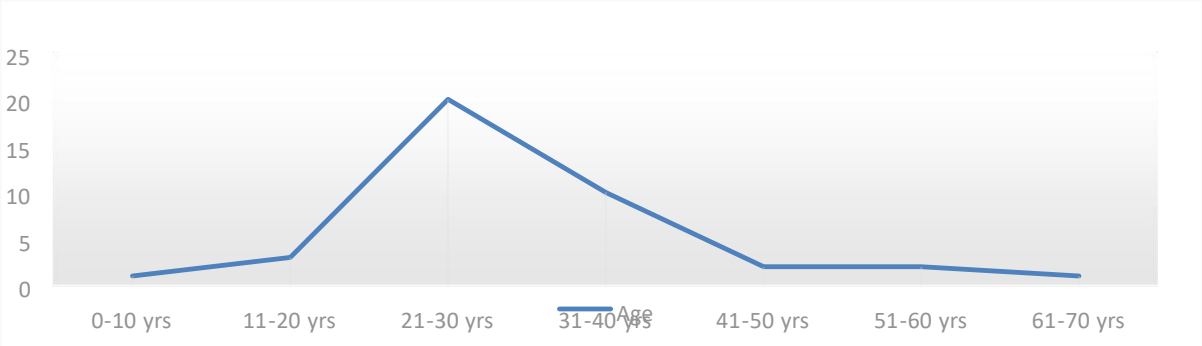


Maximum number of samples were isolated from patients of age 21-30 years,followed by 31-40 years (Table-2,Chart 2).

Table 2 -Age distribution of Candida isolates

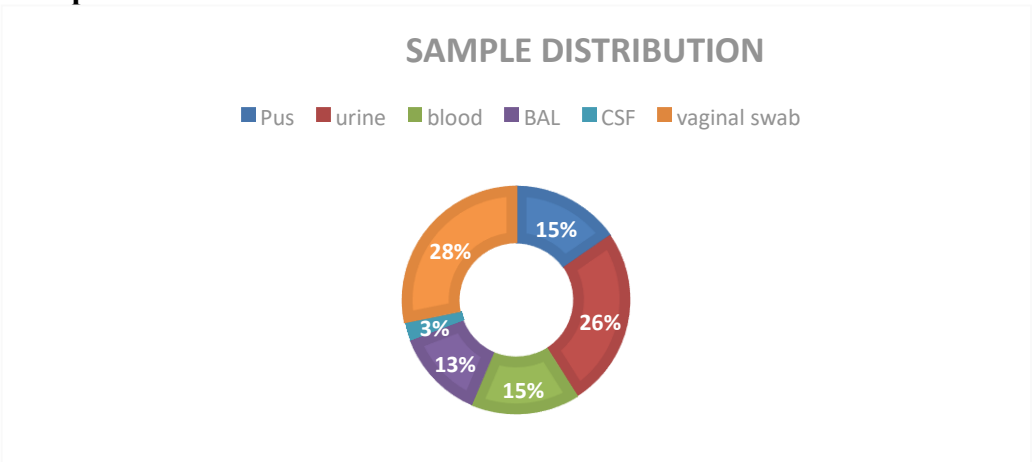
Age range (in years)	Number of isolates	Percentage
0-10	1	2.56%
11-20	3	7.69%
21-30	20	51.28%
31-40	10	25.64%
41-50	2	5.12%
51-60	2	5.12%
61-70	1	2.56%

Chart 2 – Age distribution of Candida isolates



In our study, Candida was isolated mainly from high vaginal swabs (29%) followed by urine samples (26%). (Chart 3)

Chart 3 - Sample distribution



Patients with Diabetes Mellitus, followed by pregnancy had the highest risk of candidiasis(table 3)

Table 3 – Predisposing risk factors for candidiasis

Risk factors	**Percentage**
Diabetes mellitus	55%
Pregnancy	20%
Indwelling catheter	14%
No identifiable risk factors	11%

Candida species identification was carried out by two methods- VITEK-2 YST card and conventional methods-morphology on corn meal agar with Tween 80 (Hi Media, India), HiCrome *Candida* agar morphology (Hi Media, India), VITEK -2 YST card method was taken as gold standard in our study and was compared with conventional methods (Table-4,Table-5,Chart -4)

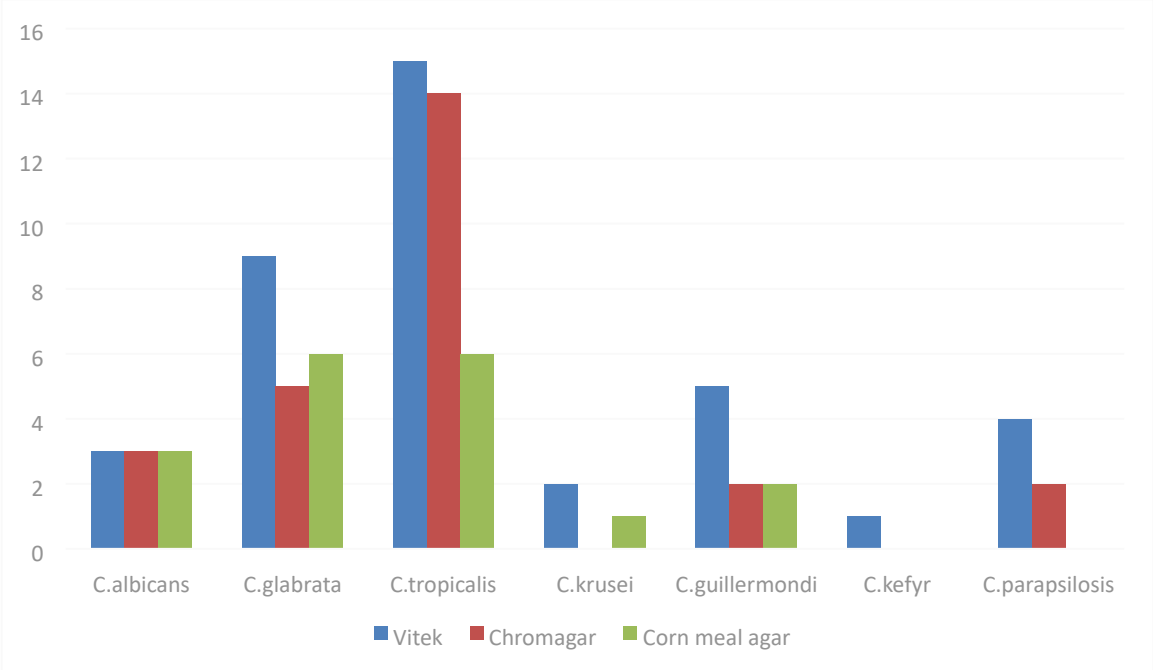
Table 4-Percentage correlation of Candida Chromagar in species identification

Species	Vitek	Chromagar	% correlation chromagar of
C.albicans	3	3	100%
C.glabrata	9	5	55%
C.tropicalis	15	14	93%
C.krusei	2	0	0%
C.guillermondi	5	2	40%
C.kefyr	1	0	0%
C.parapsilosis	4	2	50%

Table 5 - Percentage correlation of Cornmeal agar in species identification

Species	Vitek	Corn meal agar	% accuracy of corn meal agar
C.albicans	3	3	100%
C.glabrata	9	8	88.9%
C.tropicalis	15	12	80%
C.krusei	2	1	50%
C.guillermondi	5	2	40%
C.kefyr	1	0	0%
C.parapsilosis	4	4	100%

Chart 4-Comparative chart of species identification by Chromagar and Cornmeal agar with VITEK-2



Candida chromagar showed good correlation with Vitek-2 for identifying Candida albicans (100%)andtropicalis (93%) only. Cornmeal agar method showed good correlation for species identification with VITEK-2 for albicans(100%), parapsilosis(100%), tropicalis(93.3%) and glabrata(88.9%).(p value <0.05).

Antifungal susceptibility testing was carried out by two methods-Vitek-2 and disk diffusion using Mueller-hinton agar with 2% glucose and 0.5 µg/ml Methylene blue. Species wise Antifungal susceptibility pattern by disk diffusion method and VITEK are given in table 6 and 7 respectively.

Table 6-Species wise Antifungal Sensitivity as per Disk diffusion method

Species	**Fluconazole**			**Itraconazole**			**Amphotericin B**		
	S	**SDD**	R	S	**SDD**	R	S	**SDD**	R
C.albicans	3(100%)	0	0	3(100%)	0	0	3(100%)	0	0
C.glabrata	0	0	9(100%)	9(100%)	0	0	9(100%)	0	0
C.tropicalis	9(60%)	0	6(30%)	15(100%)	0	0	15(100%)	0	0
C.krusei	0	0	2(100%)	2(100%)	0	0	2(100%)	0	0
C.guillermondi	0	0	5(100%)	5(100%)	0	0	5(100%)	0	0
C.kefyr	1(100%)	0	0	1(100%)	0	0	1(100%)	0	0
C.parapsilosis	4(100%)	0	0	4(100%)	0	0	4(100%)	0	0
Percentage	43.58%	0	56.41%	100%	0	0	100%	0	0

Table 7-Species wise Antifungal Sensitivity as per Vitek

Species	**Fluconazole**			**Voriconazole**			**Amphotericin B**			**Capsofungin**		
	S	**SDD**	**R**	**S**	**SDD**	**R**	**S**	**SDD**	**R**	**S**	**SDD**	**R**
C.albicans	3 (100 %)	0	0	3(100 %)	0	0	3(100 %)	0	0	3(100 %)	0	0
C.glabrata	0	0	9	9(100 %)	0	0	9(100 %)	0	0	9(100 %)	0	0
C.tropicalis	15	0	0	15(100 %)	0	0	15(100 %)	0	0	15(100 %)	0	0
C.krusei	0	0	2(100 %)	2(100 %)	0	0	1(50 %)	0	1(50 %)	1(50 %)	1(50 %)	0
C.guilliermondi	0	0	5(100 %)	5(100 %)	0	0	5(100 %)	0	0	5(100 %)	0	0
C.kefyr	1(100 %)	0	0	1(100 %)	0	0	1(100 %)	0	0	0	0	1(100 %)
C.parapsilosis	4(100 %)	0	0	4(100 %)	0	0	4(100 %)	0	0	4(100 %)	0	0
Percentage	58.9%	0	41.02 %	87.17 %	0	12.82 %	97.43 %	0	2.56 %	94.87%	2.56 %	2.56 %

S-Susceptible SDD- Susceptible dose dependent R-Resistant

Comparison of AFT was done for Fluconazole and Amphotericin B for different species by both methods are given in charts 5 and 6 respectively

Chart 5- Comparison between Vitek-2 and Disk diffusion for Fluconazole susceptibility

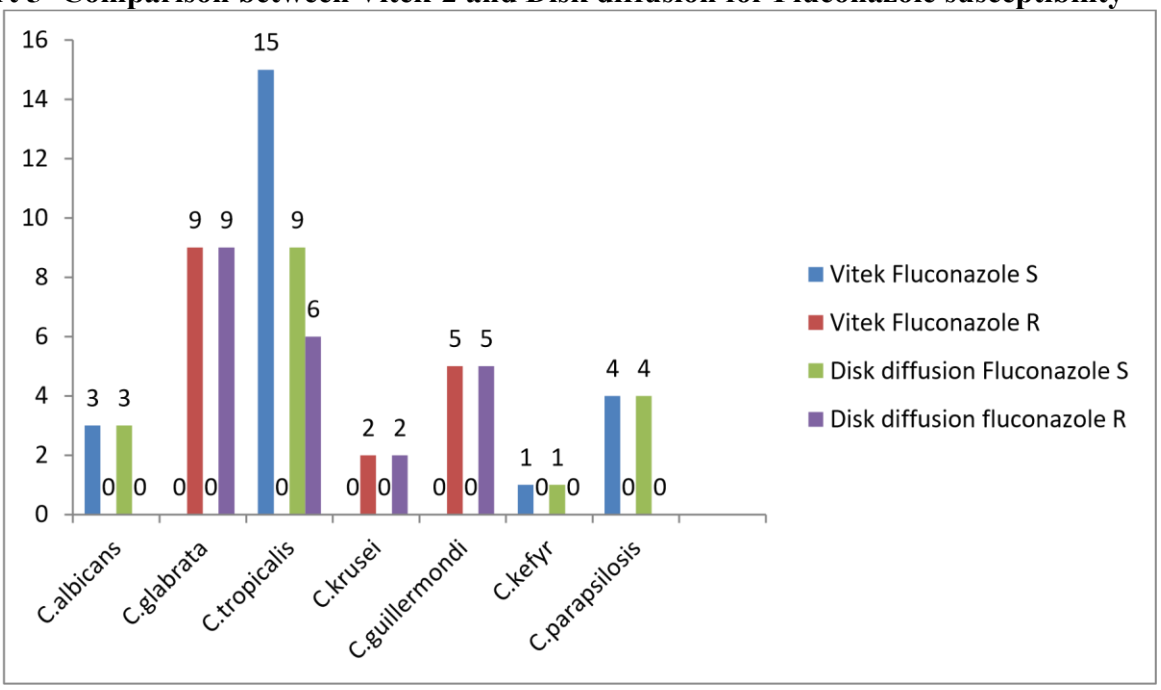
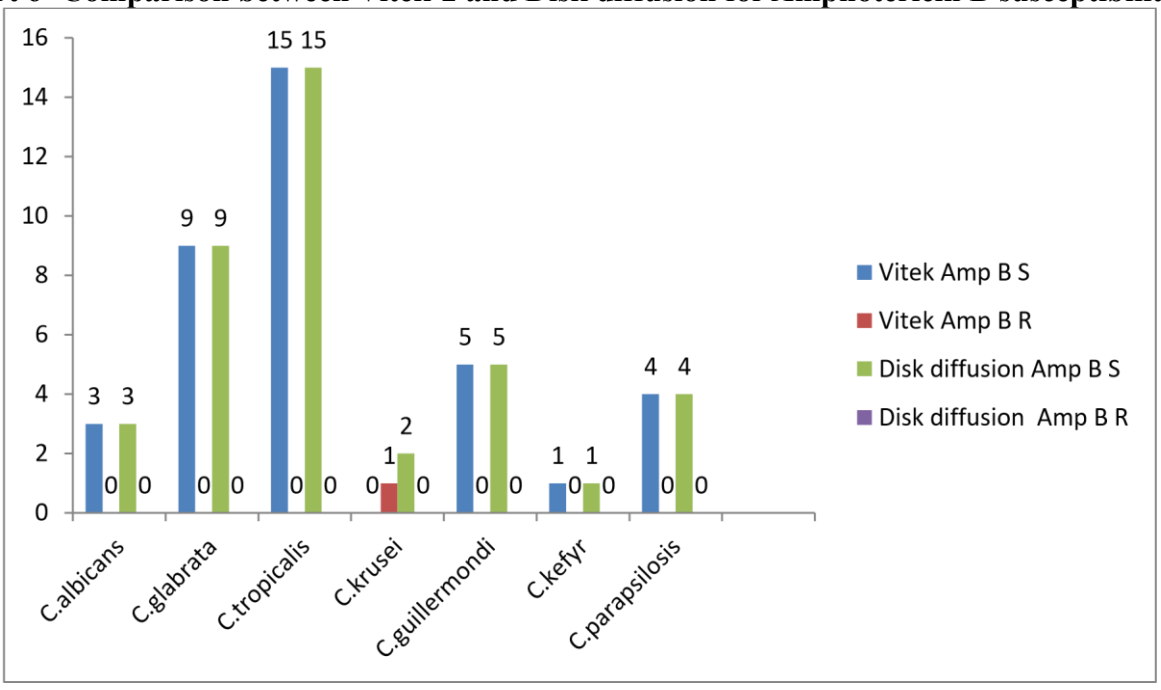


Chart 6- Comparison between Vitek-2 and Disk diffusion for Amphotericin B susceptibility



Discussion:

Candida species are usually present as normal commensals in the human body, i.e. on skin, mouth, large intestines, urinary and reproductive systems. The most common aetiology of many fungal infections especially among diabetic, HIV infection, hospital admitted and immunocompromised patients is known to be Candida species[52].With the overuse of antibacterial agents, immunosuppressive agents, cytotoxins and steroids, a new category of systemic mycoses has emerged. The emergence of non-albicans Candida species as significant pathogens has been well recognized now. Also many Non albicans Candida species are intrinsically drug resistant and can cause treatment failure leading to the need of identification and Antifungal susceptibility testing for Candida isolates. In our study most of the patients were in the age range of 21–30 years (51.28%)

which is similar to Nelson et al which noted a high infection rate in the 20–29 age group (Table-2)[53]. Vulvovaginal candidiasis prevalence was 28%.This correlated with 26% as per Ibadan et al[54].This was followed by Urine (26%).

Among Species Candida tropicalis was the predominant species (38.46%) followed by Candida glabrata (23.07%),Candida parapsilosis (10.25%). Candida albicans was fourth at just (7.69%). These findings correlate with other studies like Goel et al[45]and Iman et al [55]which indicate the emergence of non alicans Candida species as leading cause for Candida infections. Identification of Candida species is important as non-albicans is more resistant to azoles as compared to C. albicans. C. krusei and C. glabrata is intrinsically resistant to fluconazole.

Antifungal susceptibility pattern by VITEK-2 showed that Candida isolates were more susceptible to Amphotericin B(97.43%) than fluconazole (58.9%) as in the study of Manikandan et al[56]and Goel et al[45]. Caspofungin (94.87%) and Voriconazole also showed high sensitivity (87.17%).

In Comparison of Chromagar for species identification with Vitek-2, it showed good percentage correlation for albicans and tropicalis. Chromagar is useful in rapidly identifying some species like Albicans, tropicalis but it fails to identify properly other non albicans Candida species which is very important considering the fact that some species are intrinsically resistant to some antifungal agents(like glabrata to Fluconazole) which may ultimately may lead to treatment failure. In case of Cornmeal agar, good correlation was found to albicans(100%), parapsilosis(100%), tropicalis (80%) and glabrata(88.9%). Cornmeal agar was found to be better alternative to chomagar for identifying more non albicans species compared to chromagar in our study. Also Hicrome agar falsely identified C.parapsilosis as C.glabrata similar to findings of Sagar et al [57]

On comparison of VITEK-2 with Disk diffusion method for Fluconazole sensitivity, 100% concordance was shown for albicans, parapsilosis, glabrata, guilliermondi,kefyr and krusei. Only for tropicalis, results were discordant, Vitek showing all 15 isolates as sensitive whereas Disk diffusion showed 9 isolates as sensitive similar to findings of Wadha Alfouzan et al[58]which also found discordance for tropicalis. For amphotericin B all species showed concordant results except for Krusei where 2 isolates showed resistance by disk diffusion compared to 1 isolate by Vitek-2. These findings of our study indicates that Disk diffusion can be a more economical and suitable alternative to Vitek-2 Susceptibility testing for Fluconazole and amphotericin B for both albicans and non abicans candida species

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