ISSN: 2395-6429

INTERNATIONAL JOURNAL OF CURRENT MEDICAL AND PHARMACEUTICAL RESEARCH

IJCMPR

Available Online at http://www.journalcmpr.com

DOI: http://dx.doi.org/10.24327/23956429.ijcmpr20170119

RESEARCH ARTICLE

DETECTION AND CHARACTERISATION OF *CANDIDA* SPECIES IN PATIENTS WITH CLINICAL CERVICO-VAGINAL INFECTIONS

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ARTICLE INFO

Article History:
Received 12th March, 2017
Received in revised form 27th April, 2017
Accepted 9th May, 2017
Published online 28th June, 2017

Key words:
Vulvovaginal candidiasis, *Candida species*, Vitek, Antifungal susceptibility

ABSTRACT

Introduction: Approximately 75% of all women suffer from VVC once during their lifetime with 5-8% of them suffering from recurrent episodes. Though *Candida albicans* is associated in 70-80% of cases, infection by non-albicans *Candida species* is on the rise. Emergence of more resistant species has stressed the importance of monitoring antifungal susceptibility of the various *Candida species.*

Objective: To determine the species distribution and antifungal susceptibility among *Candida* isolates from patients with VVC and to do a comparative analysis of different methods of detection of the *Candida* isolates.

Materials and Methods: The study was conducted over a period of 2 years and included 279 patients. The *Candida* isolates were identified using the Conventional technique, ChromAgar and Automated system (Vitek 2 Compact). Antifungal susceptibility was performed using the Vitek 2 ID cards.

Results: Of the 279 women, 122 had positive culture for *Candida* species. Of the 136 *Candida* species isolated, *C. albicans* was predominant (36%). The predominant Non-albicans species isolated was *C.glabrata* (31.6%), *C. tropicalis* (17.6%) and *C.lusitaniae* (7.3%). There was agreement between the Conventional, ChromAgar and automated system in the majority of samples. All of the isolates showed sensitivity to the different antifungal agents.

Conclusion: VVC is common, hampers normal life and propensity for recurrence highlights better understanding of its cause. Since non-albicans *Candida* species are emerging, identification through reliable and rapid techniques is essential for proper decision making. Rapid test using ChromAgar supplemented with an automated system may be a reliable method of detection of the different *Candida* species. The psychological, medical and socio-economic impact of VVC is underestimated, hence a complete mycological workup becomes mandatory.

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a common problem for women. Approximately 75% of all women suffer from VVC once during their lifetime with 5-8% of them suffering from recurrent episodes[1].

Symptoms generally include: Itching, burning, soreness, abnormal vaginal discharge, and dyspareunia[2,3].Many physicians believe that these signs and symptoms are suitable for diagnosis without any laboratory results, however studies have shown that approximately 10-50% patients with similar symptoms had no candidal vaginitis[3, 4].

Candida albicans accounts for 85-90% of cases of VVC worldwide[1, 5].Studies have reported an increase of non-albicans *Candida* species attributed to several factors such as an increase in over- the - counter use of antifungals to an increase in high-risk population groups thereby shifting the vaginal colonization and resulting in selection of more resistant species as *Candida glabrata*.[6-9]. This has stressed the importance of species identification and monitoring of antifungal susceptibility as non-albicans *Candida* often fail first line treatment.

This study was therefore undertaken to determine the species distribution and antifungal susceptibility among yeast isolates from patients with vulvovaginal candidiasis and to do a comparative analysis of different methods of detection of the Candida isolates up to their species level.

MATERIALS AND METHODS

The study was conducted over a period of 2 years from January 2013 to December 2015 and included 279 patients with signs and symptoms of vulvovaginal candidiasis. Married and sexually active women between 18-49 years, with history of curdy discharge attending Obstetrics and Gynaecology OPD and admitted at Obstetrics and Gynaecology ward at North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences (NEIGRIHMS) were included in this study. Pregnant women, women who were menstruating, never been sexually active and those on antifungals were excluded. High vaginal swab specimens were collected and were subjected to:

1. Direct Gram stain smear examination
2. 10% Potassium hydroxide (KOH) mount
3. Culture on Sabouraud dextrose agar (SDA) (Oxoid, UK) incubated at 37 °C for 24-48 h.

Identification of yeast

The isolates obtained from fungal culture were identified as Candida species by Colony morphology and Gram staining.

Speciation of Candida species:

Conventional method

1. Germ tube test was performed for presumptive identification of *Candida albicans*.
2. The isolates were then subjected for further identification on the basis of growth on Corn meal agar, Temperature studies, Sugar fermentation and Sugar assimilation tests. Sugar assimilation was performed using Yeast Nitrogen Base and the following carbohydrate disks glucose, raffinose, galactose, lactose, maltose, cellobiose, inositol, sucrose, dulcitol and xylose were used. The growth of Candida sp around the carbohydrate disk indicates a positive sugar assimilation test.

A. Chromagar media(HiCrome Candida Differential Agar, Hi-media): The identity of the yeast was also analysed by subculturing in Chromagar media and kept for 48 hours incubation at 35-37° C. *C.albicans, C.tropicalis, C.krusei, C.glabrata* and *C.parapsilosis* were identified by the specific color of colonies on CHROMagar based on the pigmentation of the developing colonies.This medium shows different colour colonies for *C. albicans* (light green), *C. tropicalis* (metallic blue to purple), *C. krusei* (purple, fuzzy), *C glabrata* (cream to white), *C parapsilosis* (cream to white, may have mauve centre) and *C kefyr* (Cream to white, beige/yellow)

B. The yeast isolates were also subjected to identification by Vitek2 ID cards (Biomerieux).

Antifungal susceptibility test

1. Performed using Vitek2 cards (AST - YS01 cards) (Biomerieux) according to manufacturer's instructions.
2. Disc diffusion method using glucose methylene blue Mueller Hinton agar (0.5 µgm/ml) for Fluconazole (25µgm) was also performed for all Candida species with the exception of *C. krusei*, in accordance with Clinical Laboratory Standards Institute (CLSI) M-44A guidelines. The agar surface was inoculated by using a swab dipped in a cell suspension adjusted to the turbidity of 0.5 McFarland standard.

Quality control was assessed by testing the CLSI recommended quality control strains *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 22019 and *Candida krusei* ATCC 6259. The data was analysed statistically. Kohen's kappa coefficient is interpreted as follows: (0.01-0.20 slight agreement, 0.21-0.40 fair agreement, 0.41-0.60 moderate agreement, 0.61-0.80 substantial agreement, 0.81-1.00 almost perfect or perfect agreement)

RESULTS

A total of 279 women with signs and symptoms of vulvovaginal candidiasis were included in this study. The women had a mean age of 28 years.

KOH for yeast cells through direct microscopy were positive in 117 of cases (41.9%). 27 samples were KOH negative but positive by culture and 22 were KOH positive but negative by culture as shown in Table 1.

Table 1 Correlation between KOH examination and culture of the isolates

	KOH positive	KOH negative	Total
Culture positive	95	27	122 (43.7%)
Culture negative	22	135	157
Total	117 (41.9%)	162	279

Of the 279 women, 122 of them had positive culture for Candida species (Figure 1 and 2). A total number of 136 Candida species was isolated from these patients.

As per the conventional methods using CMA (Figure 3), temperature studies, sugar assimilation (Figure 4) and sugar fermentation techniques, *C. albicans* was the most isolated species (36%).

Figure 1 Growth of *C.krusei* on SDA

Figure 2 Growth of *C.albicans* on SDA

The predominant non-albicans species isolated was *C. glabrata* (31.6%) followed by *C. tropicalis* (17.6%), *C. lusitaniae* (7.3%), *C. parapsilosis* (2.9%), *C. krusei* (2.2%), *C. dubliniensis* (1.4%) and *C. guilliermondi* (0.7%) as shown in Table 2. Mixed infection with multiple species of Candida was observed in 14 cases. The majority of the mixed cultures yielded *C. albicans* and *C. tropicalis*.

Table 2 Distribution of the Candida isolates (Conventional method)

Species	Total (%)
C. albicans	49 (36%)
C.glabrata	43(31.6%)
C. tropicalis	24 (17.6%)
C.lusitaniae	10(7.3%)
C.parapsilosis	4 (2.9%)
C.krusei	3 (2.2%)
C.dubliniensis	2 (1.4%)
C.guilliermondi	1 (0.7%)
	136

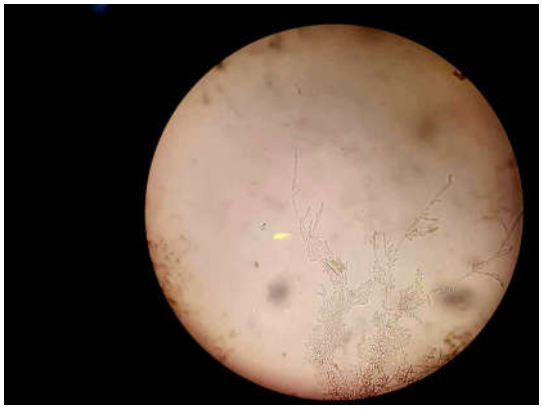

Figure 3 Growth of *C.krusei* on CMA

Figure 4 Sugar assimilation test

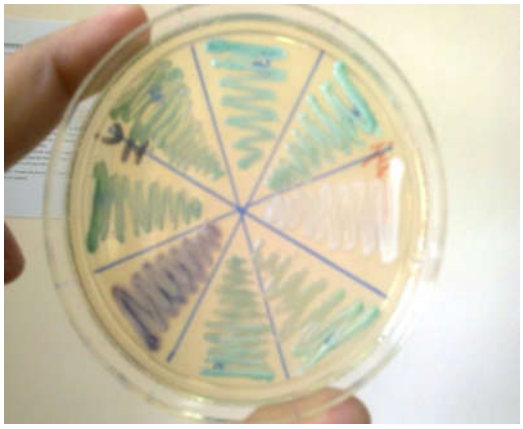

Figure 5 Growth of *Candida species* on Chrom Agar

In the identification using the Vitek system of identification, only those isolates which were identified with a probability of > 90% were included. The ones where 2 or three probable species were identified or in which entirely different yeast other than Candida sp was identified and those with low discrimination were excluded. As shown in Table 3, identical results by all the three methods, CMA, Vitek and Chromogenic medium was seen for 49 of *C. albicans* species and all species of *C. glabrata, C. parapsilosis and C. krusei*. The two species identified as *C. dubliniensis* with the CMA were identified as *C. albicans* and 2 of the Candida identified as *C. lusitaneae* by the conventional method were identified as *C. tropicalis* and *C. albicans* using Vitek. The one isolate of *C. guilliermondi* was identified by Vitek as *C. albicans.* 2 of the isolates thought to be *C. lusitaneae* were reported as *C. guilliermondii* using Vitek. Four isolates not properly discriminated were excluded. Antifungal susceptibility to Fluconazole by agar diffusion method (Figure 5), as performed in accordance with CLSI M-44A guidelines showed that 70% of the isolates were sensitive to Fluconazole, 3.7% were susceptible dose dependant isolates and 26.3% were resistant to fluconazole (Table 4).

Most of the isolates of *C.glabrata* (62.8%) were resistant to Fluconazole. Fluconazole resistance was not tested for *C. krusei* which is intrinsically resistant to fluconazole.

The antifungal susceptibility using Vitek antifungal susceptibility system (AST YSO1) for Amphotericin B, Fluconazole, Flucytocine and Voriconazole was done for the 132 isolates detected by Vitek 2 ID (Table 5).

AST-YS01 Vitek 2 card contain Amphotericin B (range, 0.03 to 16 µg/ml), Flucytosine (range, 0.125 to 64 µg/ml), Fluconazole (range, 1 to 64 µg/ml), and Voriconazole (range, 0.125 to 16 µg/ml). For fluconazole, MIC values ≤ 8 µg/mL was considered susceptible (S), 16-32 µg/mL was considered as susceptible dose-dependent (SDD/I), and ≥64 µg/mL as resistant (R). For Amphotericin, MICs ≤ 1 µg/mL were considered to be S and ≥1 µg/mL was R. For Flucytosine, MICs ≤ 4 µg/mL were considered to be S, 8-16 µg/mL was I, and ≥32 µg/mL was R. For Voriconazole, MICs ≤ 0.125µg/mL were S and ≥16 µg/mL were considered resistant.

DISCUSSION

About three quarter of all women suffer at least one episode of vulvo vaginitis infection during their life time. The incidence of this condition varies as reported in different studies from 9.5% by Mendiratta *et al* (1992)[9] to 30.4% by Kikani KM *et al* (2010)[10]. In this study the incidence is 43.7%.*Candida albicans* (36%) is the commonest isolate followed by *Candida glabrata* (31.6%) as seen in most studies[8,9]. Worldwide, rates of the isolation of *C. albicans* in cases of VVC ranged between 47% and 89% as seen in studies from Nicaragua, Australia, Turkey, Iran, Nigeria and India. [11] The percentage of Non-albicans Candida species was 64.2% comparable to a study done by Mohanty S *et al* (2007)[12]. Many studies have also reported an increase rate of isolation of Non-albicans Candida species which could be the result of inappropriate and over-the-counter use of antifungal medications. This could be a cause of concern in this area considering the higher level of resistance of Non-albicans Candida species to the commonly used azole agents. Among the Non- albicans Candida species, **C. glabrata** is the most common isolate in this study.

prior to inoculation[14]. So although a KOH mount could give an

Table 3 Comparison between the different identification methods

Species	Conventional	Vitek	% Kappa coefficient (Conventional and Vitek)	Interpretation	Chromogenic medium with CMA
C. albicans	49	53	0.94	Perfect agreement	49
C.glabrata	43	43	1	Perfect agreement	43
C. tropicalis	24	21	0.92	Perfect agreement	24
C.lusitaniae	10	6	0.74	Substantial agreement	0
C.parapsilosis	4	4	1	Perfect agreement	4
C.krusei	3	3	1	Perfect agreement	3
C.dubliniensis	2	0	0	No agreement	-
C.guilliermondii	1	2	0.66	Substantial agreement	-
Total	136	132			123

Table 4 Antifungal susceptibility to Fluconazole by Agar Diffusion method

	Susceptible(S)	Susceptible Dose Dependant (SDD)	Resistant (R)	Total
C. albicans	43(87.8%)	3(6.1%)	3 (6.1%)	49
C.glabrata	13(30.2)	3(0.7%)	27(62.8%)	43
C. tropicalis	24(100%)	0	0	24
C.lusitaniae	8(80%)	1(10%)	1(10%)	10
C.parapsilosis	4(100%)	0	0	4
C.dubliniensis	2(100%)	0	0	2
C.guilliermondii	1(100%)	0	0	1
Total	93 (70%)	5(3.7%)	35(26.3%)	133

Table 5 Antifungal susceptibility using Vitek antifungal susceptibility system (AST YSO1)

		Susceptible (S)	Intermediate (I)	Resistant (R)	Total
C. albicans	Fluconazole	46	2	5	53
	Amphotericin B	46	0	0	
	Flucytosine	53	0	0	
	Voriconazole	49	2	1	
C.glabrata	Fluconazole	14	2	27	43
	Amphotericin B	43	0	0	
	Flucytosine	42	1	0	
	Voriconazole	30	13	0	
C.tropicalis	Fluconazole	21	0	0	21
	Amphotericin B	21	0	0	
	Flucytosine	21	0	0	
	Voriconazole	19	2	0	
C.lusitaniae	Fluconazole	5	1	0	06
	Amphotericin B	6	0	0	
	Flucytosine	6	0	0	
	Voriconazole	6	0	0	
C.parapsilosis	Fluconazole	4	0	0	4
	Amphotericin B	4	0	0	
	Flucytosine	4	0	0	
	Voriconazole	2	2	0	
C.krusei	Fluconazole	0	0	3	3
	Amphotericin B	3	0	0	
	Flucytosine	1	2	0	
	Voriconazole	1	1	1	
C.guilliermondii	Fluconazole	2	0	0	2
	Amphotericin B	2	0	0	
	Flucytosine	2	0	0	
	Voriconazole	2	0	0	
Total					132

This highlights the importance of determining Candida spp. and their susceptibility pattern in women with VVC so effective treatment can be implemented.

Direct microscopy using KOH preparation plays an important role in diagnosing fungal infections however culture gives a definitive diagnosis. In this study, 27 of the culture positive samples showed no fungal elements on direct KOH mount. This could be because the fungus could have been in an inactive sporulating phase difficult to be seen by microscopy but able to grow in appropriate media[13].Of the culture negative cases, 22 showed fungal elements on KOH mount but failed to grow in culture. This could be due to non-viability of the fungi be relied upon as a screening method for exclusion of a specimen. Culture has to be performed for all the specimens.

Although there are different methods of identification of yeasts such as the germ tube test, conventional and automated methods, chromogenic culture media, and molecular techniques, yet unfortunately, these methods also have their limitations. Identification of Candida to the species level using the conventional phenotypic method is time consuming and labour intensive. The turnaround time using the conventional method for diagnosis was several days. Results from ChromAgar (Figure 4) was faster as compared to the conventional method and has the ability to detect the presence of mixed cultures as well but the ChromAgar used in this study

had limitations of being able to identify only a few species as per manufacturer's instructions and interpretation of *C.glabrata, C parapsilosis and C.kefyr* (cream to white colonies) had to be correlated with growth on corn meal agar plate. Hence only 123 of the isolates were identified to the species level by Chromagar media. The Vitek 2 system on the other hand has the advantage of being fully automated and can determine yeast growth spectrophotometrically. In this study, there was perfect agreement for the majority of the Candida isolates when compared with the Conventional method. It also has the advantage of a faster turnaround time. Hence in our laboratory, for faster results, a chromogenic media supported with an automated method such as the Vitek system would be ideal for species identification and susceptibility testing of the isolates. The same was expressed by Neetu J *et al* (2012)[15] in a similar study done at a tertiary care centre in India.

More rapid and accurate alternatives such as molecular methods to enable better identification of the different species have been recommended by certain authors,[3, 5] yet there are disadvantages such as the requirement for a molecular laboratory set up, requirements for technical skills, being expensive and also the chances of contamination.[15-17] More importantly, the use of PCR although rapid and reliable has its limitations for use on direct clinical sample.

The antifungal testing by disk diffusion for Fluconazole revealed that 70% of the isolates were susceptible, 3.7% of the isolates were Susceptible Dose Dependant (SDD) and 26.3% were resistant to Fluconazole. 62.8% of *C. glabrata* were resistant to Fluconazole. Since *C. glabrata* was the most common isolate among the Non-albicans Candida species, antifungal susceptibility testing is recommended to avoid treatment failures.

In this study, having performed the Vitek 2 automated system for identification and antifungal susceptibility, we found the system to be advantageous in the faster turnaround time for identification and the ability of the system to interpret the MIC values as well. In other studies, this system has also exhibited a high level of reproducibility and agreement with the CLSI broth dilution reference method[18, 19]. Our study is limited by the fact that we had not performed the Broth dilution method by which we could have correlated the MIC values with results obtained with the Vitek automated system. However it can still be concluded that this method is reliable since treatment options can be made available faster in case of resistance to the commonly used azole agents. Furthermore, empiric treatment guidelines can accordingly be formulated conforming to this area based on the antifungal susceptibility testing.

CONCLUSION

Vulvovaginal candidiasis is exceedingly common, hampers normal life and propensity for recurrence highlights better understanding of its cause. Identifying *Candida* up the species level is of importance considering the different species exhibiting virulence characteristics and antifungal susceptibilities. It is important that clinicians make a definitive diagnosis for candidiasis and that microbiology laboratories identify organisms to species level, for those women with recurrent symptoms or those failing initial treatment, so that appropriate antifungal medications are prescribed.

Conventional methods for identification of Candida species are time-consuming and may lead to misdiagnosis among closely related species. Since non-albicans *Candida* species are emerging, identification through reliable and rapid techniques is essential to aid the clinician in proper decision making. A rapid manual test such as a Chromagar supplemented with an automated system may be an accurate and reliable method of detection of the different species of *Candida.* Majority of isolates recovered in this study was *C. albicans* which were susceptible to azoles. Thus continued use of azole agents can be implemented for empirical therapy of uncomplicated candidal vulvovaginitis. Since the psychological, medical and socio-economic impact of vaginal candidiasis may be underestimated, a complete mycological workup becomes mandatory.

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