

# DISK DIFFUSION METHOD IN ENRICHED MUELLER HINTON AGAR FOR DETERMINING SUSCEPTIBILITY OF CANDIDA ISOLATES FROM VARIOUS CLINICAL SPECIMENS

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## Abstract

**Background:** *Candida* species are responsible for various clinical infections ranging from mucocutaneous infection to life threatening invasive diseases. Recently there is a serious concern with increased resistance of antifungal drugs and its consequences. Thus, identification of *Candida* and its antifungal susceptibility testing has a paramount significance in the management of *Candidal* infections. The aim of the study was to determine antifungal susceptibility pattern of *Candida* by Mueller-Hinton agar media supplemented with glucose and methylene blue for disk diffusion testing of fluconazole, miconazole, clotrimazole, amphotericin B and nystatin.

**Methods:** A total of 35 *Candida* species was isolated from 2000 clinical specimens over 6 month's period from July 2016 to December 2016. Growths on Blood agar and chromogenic agar were evaluated for colony appearance and microscopic examination. Antifungal susceptibility testing was performed by disk diffusion using Mueller-Hinton agar supplemented with glucose and methylene blue.

**Results:** *Candida* species were more sensitive to clotrimazole (88.58%) and amphotericin B (88.58%) followed by nystatin ((77.14%), miconazole (74.29%) whereas fluconazole showed the highest level of resistance (60%).

**Conclusions:** The increase in resistance to fluconazole is of serious concern as it is the most commonly used azole for candidiasis. The sensitivity profile of *Candida* isolates will be helpful to choose appropriate antifungal agents, thus decreasing patient's morbidity and mortality.

**Keywords:** *Candida*, Antifungal susceptibility, Mueller-Hinton agar.

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## Introduction:

*Candida* species is a normal commensal flora of human body inhabiting skin, mucous membranes and gastrointestinal tract but may be associated with superficial and deep seated fungal infections.<sup>1</sup> The switch of *Candida* species from commensal to a potent pathogen is facilitated by various virulence factors such as adherence to host tissues, medical devices, biofilm formation, and secretion of extracellular hydrolytic enzymes.<sup>2</sup> Recently, the commonly

used antifungal drugs fluconazole, voriconazole, ketoconazole, econazole, itraconazole, miconazole, clotrimazole, amphotericin B, caspofungin, nystatin etc. are showing significant variation in the susceptibility pattern of *Candida*. The drug resistance scenario has been increasing during last decades due to over growing use of random antifungal agents.<sup>3</sup> Several previous studies reported the emergence of drug resistance *Candida* species in global scenario.<sup>4,5</sup> Therefore, the change in drug

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susceptibility pattern of *Candida* and introduction of newer antifungal agents has made the *in vitro* susceptibility testing of antifungal agents more relevant for using specific and sensitive drugs. Thus, the isolation and susceptibility testing of *Candida* isolates in clinical specimens have become increasingly important for management of fungal infections.<sup>3</sup>

At present the broth macro- and microdilution methods are considered to be the reference antifungal susceptibility tests for yeasts.<sup>6,7</sup> However, clinical laboratories consider these methods to be too complex and laborious for routine use. The Clinical and Laboratory Standards Institute (CLSI) validated the disk-diffusion (DD) method,<sup>8</sup> which is very attractive for routine clinical use because it is simple and inexpensive. It has some limitations such as it does not provide the minimum inhibitory concentration (MIC) of the antifungals tested because it uses a fixed concentration of each antifungal. Therefore, the yeasts are classified qualitatively as susceptible/sensitive (S), susceptible-dose dependent (SDD)/Intermediate (I), or resistant (R).<sup>9</sup> The agar disk diffusion test for fluconazole that has been proposed by the National Committee for Clinical Laboratory Standards (NCCLS)<sup>10</sup> was developed by Meis *et al.*<sup>11</sup> and refined by Barry and colleagues.<sup>12</sup> This method has been expanded to include voriconazole.<sup>13,14</sup> The proposed NCCLS method employs Mueller-Hinton agar (MHA) supplemented with 2% glucose and 0.5 µg of methylene blue (GMB) per ml.<sup>10</sup> At present time, there is no commercial source of MHA supplemented with GMB (MH-GMB); however, MHA may be prepared and supplemented with GMB in the laboratory, or the surfaces of commercially available MHA plates may be flooded with a GMB solution. Although prepared and flooded plates have been shown to perform equally well in testing both fluconazole and voriconazole.<sup>15,16</sup> However, the shelf life of the prepared MH-GMB plates has not been established. Currently, it is recommended that both prepared and flooded plates should be used within 24 hours of preparation.<sup>10,15,16</sup>

In the present study, we explored the susceptibility pattern of *Candida* isolates from

clinical specimens by Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg of methylene blue per ml (flooded plates) for disk diffusion testing of Fluconazole, Miconazole, Clotrimazole, Amphotericin B and Nystatin which are commonly used antifungal in our country.

#### **Methodology:**

A laboratory based cross sectional study was carried out in the Microbiology Laboratory of Popular Diagnostic Centre Ltd, Dhanmondi, Dhaka from July 2016 to December 2016.

#### **Specimen collection**

A total of 35 *Candida* species was isolated from 2000 different clinical specimens (High vaginal swab, blood, tracheal aspirate, sputum, ear swab, wound swab) that were submitted for laboratory investigation.

#### **Specimen processing**

The examination of specimens (High vaginal swab, tracheal aspirate, sputum, ear swab, wound swab) were performed by wet mount, Gram stain, culture on Blood agar (Oxoid, UK), MacConkey agar (Oxoid, UK) and chromogenic agar (Himedia, India). Inoculated Blood agar, MacConkey agar and chromogenic agar plates were aerobically incubated at 37°C for 24–48 hours. The other various organisms grown in these plates were not included in the present study. Only *Candida* isolates were proceeded for further investigation. From an isolated colony, microscopic examination (wet film and Gram staining) was performed. The small, creamy white colonies on blood agar that showed Gram positive budding yeast cells with pseudohyphae on microscopic examination were processed for antifungal susceptibility of *Candida* isolates.

For blood culture, estimations were carried out by BACTEC 9120 Automated Blood Culture Analyzer (USA). Blood cultures that entered into automated, continuous-monitoring protocols were incubated for 5 days. Once blood cultures became positive for growth by signaling from automated systems, a Gram stain was performed. Subcultures were performed at blood agar and MacConkey agar media. These allowed the identification of *Candida* over the next 24–48 hours.

**Antifungal susceptibility testing**

The Candida isolates were tested by disk diffusion method using Muller-Hinton agar supplemented with 2% glucose and 0.5µg of methylene blue/ml.<sup>10</sup> The interpretive criteria for the fluconazole were those published by Barry and colleagues<sup>15</sup> and the CLSI M44-A.<sup>8</sup> The response to the other antifungal agents, for which there is no standardized method and no interpretive breakpoints, were interpreted according to the manufacturers’ instructions (Mast group limited, UK) and were adopted from published studies (Table- 1).<sup>9,17,18</sup>

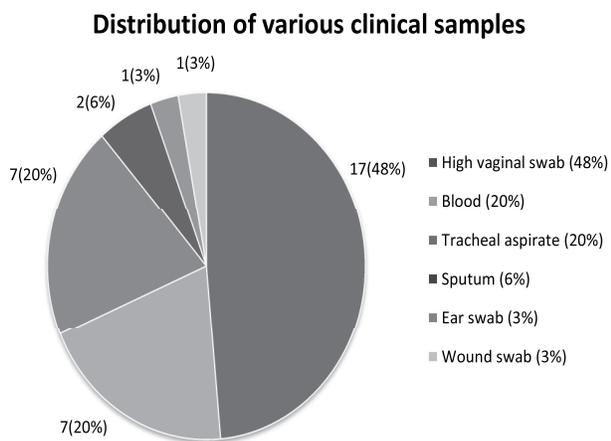
**Flooding procedure**

Flooded plates were prepared according to Barry *et al.*<sup>15</sup> A stock solution of methylene blue (5mg/ml) was prepared and refrigerated at 2 to 8°C. In 100 ml of 40% glucose solution, 200µl of the stock methylene blue was added to give 10µg of methylene blue per ml of 40% glucose (GMB solution). The GMB solution was dispensed into

screw cap tubes (1.5 ml for 100-mm-diameter plates), and that solution was then sterilized by autoclaving. The day before testing, refrigerated tubes containing the GMB solution were allowed to warm to room temperature and at the same time MH agar plates were dried. The dried agar surfaces were then flooded with the GMB solution, and the liquid was allowed to adsorb overnight at room temperature on a flat surface. Disk diffusion tests were performed by preparing a saline suspension of freshly isolated colonies that was then adjusted to match the turbidity of a McFarland 0.5 standard. A sterile applicator swab was used to inoculate the surface of each agar plate. Antifungal discs containing fluconazole (25ig), clotrimazole (10ig), and miconazole (10ig), amphotericin B (20 ig) and nystatin (100ig) (Mast group limited, UK) were placed on the inoculated media. The plates were then incubated at 37°C for 24 hours.

**Table-I**  
*Interpretive break points of different antifungal drug*

Antifungal Drug	Zone Diameter Interpretive Standards [mm]		
	Sensitive (S)	Intermediate (I)	Resistant (R)
Fluconazole (25µg)	≥19	15-18	≤14
Miconazole (10µg)	>20	10-20	<10
Clotrimazole (10µg)	≥20	11-19	≤10
Amphotericin B (20µg)	>10	-	≤10
Nystatin (100µg)	>10	-	≤10



**Fig.-1:** *Distribution of various clinical samples*

**Statistical analysis**

The information collected was reviewed and inconsistencies was investigated and clarified. Data were statistically described in terms of frequencies and percentages. All statistical calculations were done using computer program Statistical Package for the Social Science (SPSS, USA) version 20 for Microsoft Windows.

**Results**

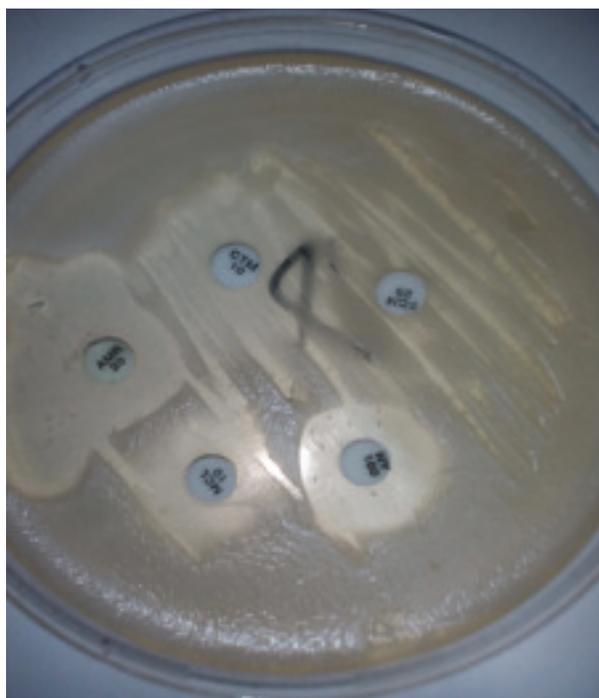
Among the 35 Candida species, highest 17(48%) isolates was found from High vaginal swab (figure-1).

Two thousand (2000) clinical specimens were analyzed and 1.75% was culture positivity. In Table-II, among the five antifungal agents, the

**Table-II**  
Sensitivity of *Candida* species against several Antifungal Drugs

Antifungal Drug	Total number (%) N=35		
	Sensitive (S)	Intermediate (I)	Resistant (R)
Fluconazole	11(31.43%)	3(8.57%)	21(60%)
Miconazole	26(74.29%)	3(8.57%)	6(17.14%)
Clotrimazole	31(88.58%)	2(5.71%)	2(5.71%)
Amphotericin B	31(88.58%)	2(5.71%)	2(5.71%)
Nystatin	27(77.14%)	3(8.57%)	3(8.57%)

highest level of sensitivity was observed 31(88.58%) in both clotrimazole and amphotericin B, whereas fluconazole showed the highest 21(60%) level of resistance.



**Fig.-2:** Antibiotics susceptibility of *Candida* isolate estimated by agar disc diffusion method.

Symbols: FCN— Fluconazole, MCL — Miconazole, CTM- Clotrimazole, AMB— Amphotericin B, NY — Nystatin

### Discussion

Incidence of fungal infections especially candidiasis has been increasing during last two to three decades.<sup>19-20</sup> Infections with these yeasts have a direct impact on the choice of antifungal therapy and clinical outcome.<sup>19</sup>

In this study, *Candida* species were more susceptible to clotrimazole (88.58%) and amphotericin B (88.58%) followed by nystatin (77.14%), miconazole (74.29%). Fluconazole showed the highest level of resistance (60%). Similar findings were found in a study where *Candida* species were found to be more susceptible to clotrimazole (82%) followed by miconazole (44%) respectively whereas 20% of total isolates were found to be resistant to fluconazole by disc diffusion method.<sup>21</sup> In previous studies the *Candida* spp was sensitive to antifungal agents. In Jordan, Al-Abeid *et al.* (2004) showed that all tested *Candida* were susceptible to nystatin, miconazole, ketoconazole and fluconazole.<sup>22</sup> In different countries resistant pattern against fluconazole varied among studies. Quindos *et al.* (1999),<sup>23</sup> Maroszyńska *et al.* (2013)<sup>18</sup> and Salehei *et al.* (2012)<sup>24</sup> showed that 9.8%, 32% and 85.1% respectively of isolates of *Candida* species were resistant to fluconazole. Unfortunately it is shown that resistance to antifungal azoles has increased.<sup>18,24</sup> The increase in resistance to fluconazole is of serious health concern as it is the most commonly used azole for superficial as well as deep candidiasis. The results of present study are in accordance with the results of other studies in the respect of amphotericin B and fluconazole susceptibility. Different studies also have shown different resistance pattern for other antifungals. In one study they showed that the most sensitive antifungal drug against *Candida* was amphotericin B, with almost all *Candida* species being resistance to fluconazole.<sup>25</sup> It was shown in another study that most of the isolates were sensitive to amphotericin-B and nystatin, but where highly

resistance to fluconazole.<sup>26</sup> Kothari and Sagar *et al.* (2008), from North India reported the susceptibility profile of *Candida* isolates as 92% sensitive to amphotericin B and 36% to fluconazole.<sup>27</sup>

Prolonged therapy and increased use of antifungals for recurrent candidiasis are the most common risk factors for azoles resistance among *Candida* isolates.<sup>28</sup> Fluconazole became available for use by clinicians in 1990 and provided many advantages over other antifungals.<sup>29</sup> Azoles have the advantage of being taken orally, which increase their potency.<sup>28</sup> Due to its good pharmacokinetic properties as well as its broad spectrum of activity fluconazole was the gold-standard treatment of fungal infections during the 1990s.<sup>30</sup> Unfortunately, the over prescription of this drug by physicians for prophylaxis or treatment led to an increase in resistance to azole drugs. In a study of Dhaka, Bangladesh, Uddin *et al.* (2011) revealed that majority (79.8%) of the patients were taking fluconazole without any specific indication. In addition the study showed that 68.9% patients were prescribed or dispensed fluconazole by the drug seller or village-doctors (quack) that may be destructive to public health in every consideration.<sup>31</sup> Therefore, the inappropriate use of antifungal drugs and introduction of over-the-counter antimycotics in some countries worldwide including Bangladesh predispose to development of antifungal resistance.<sup>31-33</sup>

### Conclusion

Among commonly used antifungal drugs clotrimazole, amphotericin B, miconazole and nystatin demonstrated a high rate of sensitivities while fluconazole was the least effective. Mueller-Hinton agar supplemented with glucose and methylene blue for disk diffusion testing is an easy, simple, rapid and reliable and inexpensive method to determine antifungal susceptibility pattern of *Candida* isolate especially in the laboratory with limited resources. The present study showed the increase in the resistance especially to azoles is a major concern. Therefore the species level identification of *Candida* isolates and its sensitivity profile is necessary and this will be

helpful to choose appropriate antifungal agents, thus decreasing patient's morbidity and mortality. Common antifungal drugs should be chosen after careful analysis or confirmatory diagnosis.

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### Conflict of interests

The author(s) declare that they have no conflict of interests.

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