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SENSITIVITY OF YEASTS TO AMPHOTERICIN B AND 5-FLUOROCYTOSINE

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Summary

One hundred and forty-four clinical yeast isolates were tested for antifungal susceptibility to Amphotericin B (AMB) and 5-Flurocytosine (5FC). 61% (88 of 144) of the total yeast isolates were *C. albicans*. Yeasts were most frequently isolated from high vaginal swabs. High vaginal swabs constituted 64% (92 of 144) of the total number of specimens. Antifungal susceptibility testing of yeasts was conducted by employing an agar dilution technique. 76% (67 of 88) of *C. albicans* demonstrated MIC values of $\leq$ 1.0 ug/ml to 5FC. All yeasts tested against AMB demonstrated MICs of $\leq$ 0.25 ug/ml. Resistance to 5FC and AMB was defined as any isolate demonstrating an MIC of $>$ 16 ug/ml and MIC $\geq$ 2 ug/ml respectively. Based on this definition approximately 6% of total yeasts and 8% of *C. albicans* were resistant to 5FC. All yeasts tested were sensitive to AMB.

Keywords: Fungus, susceptibility testing, Amphotericin B, 5-Fluorocytosine.

INTRODUCTION

During the past two decades, *Candida* has been increasingly noted as a cause of systemic fungal infections in the immuno-compromised host.[1] The first reliable agent for the treatment of life threatening deep mycosis which has been in use for more than thirty years is amphotericin B (AMB).[2] This was followed by the formulation of 5-fluorocytosine (5FC) in 1964[3] and imidazoles like miconazole in 1969 and ketoconazole in 1979. Despite the introduction of newer imidazoles, AMB and 5FC remain as the mainstay of treatment for systemic yeast infections.[4] Resistance to 5FC is of major concern. In yeasts, resistance to 5FC is commonly reported for *Candida albicans*[5,6] and for other species of *Candida*.[7,8] The resistance seen is either innate or acquired in nature.[1] In contrast to 5FC, the incidence of resistance of fungal isolates to AMB has been rarer; resistance been largely confined to the yeasts. Amphotericin B resistance has been documented in a number of *Candida* species recovered from patients with neutrophil defects.[11,12] *Candida lusitaniae* has been reported to be more resistant to AMB than other species of *Candida*.[12] The present study addresses the above concern by studying the occurrence of resistance to AMB and 5FC in clinical yeast isolates.

MATERIALS AND METHODS

Strains

A total of 144 yeasts isolated from 144 clinical specimens were studied. The yeasts which were identified by a conventional method,[13] comprised of 88 strains of *C. albicans*, 18 of *Candida tropicalis*, 2 of *Candida krusei*, 2 of *Candida parapsilosis*, of *Candida pseudotropicalis*, 2 of *Candida stellatoidea*, 1 of *Candida guillermondii*, 1 of *Candida rugosa* and 22 of *Torulopsis glabrata*.

Media

Yeast Nitrogen Base (YNB) (Difco) and Antibiotic Medium No-3 (M-3) (Difco) were utilized for testing 5FC and AMB respectively. A 10 strength broth of YNB was prepared by dissolving 6.7g of YNB, 10g of glucose and 1.5g of L-asparagine in 100 ml of distilled water. A 10 strength broth of M-3 was prepared by dissolving 17.5g of M-3 (Difco) in 100 ml of distilled water. Yeast Nitrogen Base and M-3 were filtered, sterilized and stored at 4°C until use. Yeast Nitrogen Base and M-3 agar were made by doing a 1:10 dilution in a previously prepared sterile Base Agar which constituted of 20 g of Oxoid Agar No. 1 in 900 ml of water.

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Yeast Inoculum

A 24–48 h culture of yeast grown on YNB agar was suspended in normal sterile saline (0.85%) and its turbidity adjusted to an absorbance of 0.15 (+ 0.02) measured at 540 nm (Spectronic 20, Bausch and Lomb). This procedure resulted in yeast concentration of 10^5 cfu/ml. Inoculation of 5 ul of the yeast suspension in the agar dilution technique resulted in a final concentration of 5×10^3 cfu/ml.

Antifungal Agents

A 1280 ug/ml stock solution of 5FC (Sigma Chemical Company, St. Louis, USA) was prepared by dissolving 64 mg of 5FC in 50 ml of sterile distilled water. Stock solutions of AMB (Fungizone; E.R. Squibb and Sons Inc. Princeton, NJ, USA) of 5000 ug/ml was prepared by dissolving 50 mg of AMB in 10 ml of sterile distilled water. Stock solutions of 5FC was aliquoted and stored at –70°C until use. Stock solutions of AMB was aliquoted, stored in the dark at –70°C and used within 2 months.

Agar Dilution Test

The minimum inhibitry concentration (MIC) for AMB and 5FC were determined by the agar dilution technique. The protocol for 5FC MIC determination was reported by a Working Group.[14] Briefly, stock solutions of 5FC were serially diluted 2 fold in 10 strength YNB broth to obtain an initial concentration ranging from 0.08 – 640 ug/ml. In order to obtain solidified media, each concentration of 5FC in YNB broth was diluted 1:10 with Base Agar, mixed well and left to solidify in petri dishes. The final 5FC concentration ranged from 0.008 – 64 ug/ml. Fourteen different yeasts, inclusive of a control strain were tested simultaneously by pipetting 5 ul of each suspension onto pre designated sites on the agar surface. Plates were incubated at 37°C and inspected for growth at 24 and 48 h. The minimum inhibitory values were determined at 48 h. The MIC is defined as the lowest concentration of drug in which no visible growth is observed. Inoculum and medium controls consisted of media free from drugs which were inoculated and uninoculated respectively. The control strain used for 5FC and AMB testing was a laboratory isolate of *C. tropicalis* (M9/88), found to be sensitive to AMB (MIC < 0.008 ug/ml) and 5FC (MIC < 0.008 ug/ml). The MIC to AMB was determined by the same procedure, except that the media used was M-3 and the concentration of AMB ranged from 0.008 – 2 ug/ml. The temperature of incubation was 30°C.

RESULTS

Source and types of yeast isolates

Candida albicans was found to be the yeast most frequently isolated. *Candida albicans* represented 61% (88 of 144) of the total yeast isolates. The next most frequently isolated yeast was *T. glabrata* which represented 15% (22 of 144) of total yeast isolates (Table 1). The specimen from which yeasts were most frequently isolated was high vaginal swabs which constituted 64% (92 of 144) of the total number of specimens received. 72% (63 of 88) of *C. albicans* and 81% (18 of 22) of *T. glabrata* were recovered from high vaginal swabs.

Antifungal susceptibility testing

The MIC value of *C. albicans* to 5FC ranged from 0.008 – 64 ug/ml. However, 76% (67 of 88) of *C. albicans* demonstrated MICs of $\leqslant$ 1.0 ug/ml. 100% (18 of 18) of *C. tropicalis* and 77% (17 of 22) of *T. glabrata* also demonstrated MICs $\leqslant$ 1.0 ug/ml (Table 2). All species of yeasts tested against AMB demonstrated MICs $\leqslant$ 0.25 ug/ml. The mode AMB MIC value which was 0.032 ug/ml was represented by 61% (88 of 143) of total yeasts tested (Table 3).

DISCUSSION

A total of 144 clinical yeast isolates were studied for their antifungal susceptibility to AMB and 5FC. *Candida albicans* was the most highly recovered species, representing 61% (88 of 144) of the total yeast isolates. Speller and Davies[9] who studied 778 yeasts for antifungal susceptibilities reported similiar initial isolation rates for *C. albicans* and *T. glabrata*. The yeasts which were studied were routine laboratory isolates. Hence, it is not known whether these yeasts were mere colonizers or involved in a disease process in patients from which they were isolated. It is probable that some of the yeasts could have been involved in pathogenesis. Additionally, colonizers may become invaders when local or general defense mechanisms are impaired.[15,16]

Amphotericin B and 5FC susceptibility testing is influenced by the type of media chosen for conducting the test.[17,18] Activity of 5FC is abrogated in complex media like Sabourad Dextrose Agar and Brain Heart Infusion Agar which contain competing purines and pyrimidines.[19,20] A defined media like YNB is usually used in studies of fungal susceptibility to 5FC.[21,22] In our study, YNB proved to be excellent medium for 5FC testing. In preliminary studies for a selection of a media suitable for AMB testing, 4 different media, namely, phosphate buffered YNB (pH 7.0) (Difco), SDA (pH 5.6) (Difco),

TABLE 1
SOURCES AND TYPES OF CLINICAL YEAST ISOLATES

Species	Sputum	Urine	HVS*	Pus	Mouth/Throat swab	Peritoneal aspirate	Vitreous tap	Skin scrapping	Blood	Total No. Species
C. albicans	2	9	63	3	3	3		5		88
C. tropicalis		4	7	2	1	2	1		1	18
C. krusei		4	3		1					8
C. stellatoidea	1	1								2
C. pseudotropicalis		2								2
C. parapsilosis				1					1	2
C. guillermondii			1							1
C. rugosa									1	1
T. glabrata		1	18		2	1				22
Total no. specimens	3	21	92	6	7	6	1	5	3	144

* HVS = High Vaginal Swab.

TABLE 2
SENSITIVITY OF 144 CLINICAL YEAST ISOLATES TO 5-FLUOROCYTOSINE

MIC	*C. albicans* (88) a		*C. tropicalis* (18)		*T. glabrata* (22)		*C. krusei* (8)		Others c. (8)		All Yeasts (144)	
	No.	(%) b	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
0.008	4	5	5	27			1	13	2	25	12	8
0.016	7	13			5	23	1	25			13	7
0.032	5	18	2	39	1	27				37	9	24
0.063	14	34	4	61	9	68	1	38	1	50	29	44
0.125	21	83	4	83					2	75	27	63
0.25	12	72	3	100	2	77					17	74
0.5	4	76					1	50			5	78
1.0	1	77					2	75			3	80
2	1	78									1	81
4	10	90			4	95	2	100			16	92
8	2	92									2	93
16	0	92			1	100					1	94
32	4	96							2	100	6	98
64	3	100									3	100

a Number of yeasts tested
b Cumulative percentage
c *C. stellatoidea* (2), *C. pseudotropicalis* (2), *C. parapsilosis* (2), *C. guillermondii* (1), *C. rugosa* (1).

TABLE 3
SENSITIVITY OF CLINICAL YEAST ISOLATES TO AMPHOTERICIN B.

MIC	*C. albicans* (88) a		*C. tropicalis* (17)		*T. glabrata* (22)		*C. krusei* (8)		Others c. (8)		All Yeasts (143)	
	No.	(%) b	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
0.008	8	9	3	18	2	9	1	13	1	13	15	10
0.016	5	15	2	29			1	25	1	25	9	17
0.032	60	83	10	88	12	64	1	38	4	75	87	78
0.063	10	94	1	94	8	100			2	100	21	92
0.125	3	96					5	100			8	98
0.25	2	100	1	100							3	100

a Number of yeasts tested
b Cumulative percentage
c *C. stellatoidea* (2), *C. pseudotropicalis* (2), *C. parapsilosis* (2), *C. guillermondii* (1), *C. rugosa* (1).

Diagnostic Sensitvity Testing agar (pH 7–7.4) (Oxoid) and M-3 (pH 7.0) were tested.

The activity of AMB on SDA after 48 h of incubation was drastically reduced. Other workers[23,24] have reported that an acidic pH has an adverse effect on AMB activity. Antibiotic Medium No.3 was found to be the best medium for AMB testing, showing the biggest zones of inhibition on disk diffusion tests. This medium has also been the medium of choice by several workers for AMB antifungal susceptibility testing.[18,25]

The inoculum size is an important factor which influences susceptibility test results of 5FC. In general MIC increases as a function of inoculum size.[8,26] In view of this, yeast concentrations used in our study were standardized objectively by using a spectrophotommeter. The final yeast concentration was 5 x 10^3 cfu/ml. Other workers, who have employed the agar dilution technique for 5FC antifungal susceptibility, have used similiar inoculum concentration as ours.[17,27]

76% (67 of 88) of *C. albicans* in our study demonstrated MICs $\leq$ 1.0 ug/ml to 5FC. This finding is similiar to that of other workers who had employed the agar dilution technique to determine the MICs of yeasts to 5FC.[5,8,28] Bergan and Vangdal[28] reported that 79.6% of the 127 strains of *C. albicans* tested demonstrated MICs $\leq$ 1.0 ug/ml to 5FC. In our study, resistance to 5FC was defined as an isolate demonstrating an MIC > 16 ug/ml.

Based on this definition, the resistance rate of *C. albicans* and all yeasts to 5FC was 8% and 6% respectively. Studies have indicated that *C. albicans* serotype B rather than A is generally associated with resistance.[5,6] Correlation studies between serotype and susceptibility was not attempted in our study. Scholer[10] demonstrated an average prevalence of 5FC resistance in 10% of primary yeast isolates. The low resistance rate to 5FC in our yeast isolates supports our contention that these yeasts were most probably pretreatment isolates. Resistance to AMB was defined as any isolate demonstrating an MIC $\geq$? ug/ml. All yeasts tested in our were sentitive to AMB, demonstrating MICs $\leq$ 0.25 ug/ml. Yeasts which are resistant to AMB are usually isolated from patients who are receiving cytotoxie chemotherapy, patients who are granulocytopenic or who are on long-term treatment with AMB.[11,12,29] It is probable that the patients from whom the strains were isolated, did not have the above predisposing factors.

The present study reports the antifungal susceptibility of clinical yeast isolates to AMB and 5FC. Further studies could include association between 5FC susceptibility and *C. albicans* serotype. Such studies would be of epidemiologic and clinical value.

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