In vitro activity of 6 antifungal agents on candida species isolated as causative agents from vaginal and other clinical specimens

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ABSTRACT

Objective: To study the susceptibility pattern of candida species isolated from various clinical specimens to common usable antifungals in Libya.

Methods: Two hundred and four candida species were isolated from patients complaining of fungal infections gathered from vaginal swabs, nails, throat, hair and ear. Yeast isolates were identified to the species level by API 20C AUX Commercial system. The in vitro susceptibility to amphotericin B, nystatin, ketoconazole, miconazole, clotrimazole and econazole was determined using the macrodilution in broth method.

Results: *Candida albicans* was the most common isolated species from vaginal and *Candida tropicalis* from throat swabs. On the other hand *Candida parapsilosis* and *Candida guilliermondii* were more often isolated from the ear and hair. *Candida krusei* and *Candida fomata* were only isolated from vulvaginitis in this study. In vitro sensitivity showed that most of the isolates were inhibited at optimum ranges of minimal inhibitory concentration particularly with amphotericin B and nyastatin. On the other hand resistance strains of candida species were found against the 4 azoles antifungal agents.

Conclusion: *Candida albicans* and *Torulopsis glabrata* are among the most commmon cause of vaginitis among Libyan females. Amphotericin B, nystatin and clotrimazole were the most effective antifungals against most isolates in this study. Fungicidal effect was obtained with most antifungals at higher concentrations.

Keywords: Candida species, vaginitis, antifungal agents, susceptibility.

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The mycotic infections due to yeasts have increased during the last few years because of immunosuppressive therapies, prolonged antibiotic use and organ transplantation.[1] In this respect, infections caused by candida species out number all others mycoses apart from the infections caused by dermatophytes in which infections may range from superficial to deep-seated disease. A candida focus in the vaginal mucous membrane, in the intestinal or urinary tract of the patient, as well as in the genital tract of their partners, may contribute to unsuccessful therapy.[2] However during the past several years many antifungal agents have been produced and evaluated for use in the therapy of fungal infections.[3] Unfortunately resistant strains have emerged and have been reported by different workers from time to time.[4-5] For this reason the clinical laboratory is now gaining an important role in the selection and monitoring of antifungal chemotherapy. This work represents some aspects on the susceptibility of the most clinically important yeasts isolated from patients with candidosis to the most common usable antifungal in Libya.

Methods. ***Testing organism.*** Over a one year period a panel of 204 clinical isolates of pathogenic yeast were used in this study. The clinical isolates

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included 75 isolates of *Candida albicans (C.albicans)*, 39 *Candida tropicalis* (*C.tropicalis),* 30 isolates of *Candida parapsilosis* (*C.parapsilosis)*, 24 isolates of *Torulopsis glabrata (T.glabrata),* 18 isolates of *Candida guilliermondii* (*C.guilliermondii)* and 9 isolates each of *Candida krusei* (*C.krusei)* and *Candida fomata (C.fomata)*. Each isolate represented a unique isolate from a patient. Yeast isolates were identified to the species level by API 20C AUX Commercial System (BioMerieux, Marcy-l'Etoile, France). *Candida albicans* isolates were confirmed by germ tube production in human serum as well as for their chlamydospore formation on corn meal agar plus Tween 80.

Antifungal agents. The following 6 antifungal drugs were used: amphotericin B, nystatin, miconazole, ketoconazole, clotrimazole and econazole (Sigma Chemical Co., St. Louis, Missourii. USA). The 6 drugs were provided by the manufacturers as standard powders and stock solutions were prepared with the weight adjusted according to the potency of each drug. Stock solutions were prepared in 100% dimethylsulfoxide (Sigma) to obtain a stock solution of 5000mg/ml. A working solution containing 100μg/ml was prepared and stored in the refrigeration for approximately one week.

Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC). For MIC determination the macrodilution method in broth described by Shadomy and Pfaller, 1991[6] was used. Serial 2-fold dilution of the drugs ranging from 100μg/ml to 0.01μg/ml was prepared in tubes containing 2ml of yeast nitrogen base (Difco) supplemented with glucose (1%) and asparagine (0.15%). The test tubes were innoculated with a fresh overnight broth culture to provide a starting inoculum of ($1x10^4$ to $5x10^4$ cell/ml) and then incubated at 35°C for 24 hours. The MIC values were noted as the lowest concentration showing no visible growth. One loopful (0.01ml) of the broth tubes showing no visible growth was further subcultured onto a Sabouraud agar plate to determine the MFC. The plates were incubated at 30°C for 24 hours and then checked for viability. The concentration at which no growth occurred, or only a few colonies, upon subculture was considered to be the MFC.

Results. Table 1 lists the distribution of candida strains among clinical specimens. The results of the in vitro susceptibility of the 204 candida strains to the 6 antifungal are given in Table 2. The degree of activity varied over a wide range with most of the agents. For amphotericin B and clotrimazole the lowest MIC values were less than 0.01μg/ml and 0.02μg/ml, and the highest were more than 50μg/ml and 60μg/ml for miconazole and ketoconazole. Econazole showed activity from less than 0.08μg/ml to 20μg/ml. Minimal inhibitory concentration on *C.albicans* with nystatin varied between 0.3μg/ml and 12μg/ml. Against other candida species the best effect was observed with amphotericin B and nystatin followed by clotrimazole and econazole. Resistant strains were observed against miconazole and ketoconazole. Table 3 shows the MIC50, MIC90 and MFC50, MFC90 values. Amphotericin B showed considerable activity on all candida species and was followed by nystatin, clotrimazole, econazole, miconazole and finally ketoconazole. Killing effects were best achieved with amphotericin B and nystatin. The high MFC90 was read with most of the 6 antifungal agents.

Table 1 - Distribution of yeasts isolated from clinical specimens.

Candida spp	Specimen	n of strains	Total % of isolates
C. albicans	Vaginal swabs	30	(59)
T. glabrata	Vaginal swabs	24	(59)
C. tropicalis	Vaginal swabs	24	(59)
C. parapsilosis	Vaginal swabs	15	(59)
C. guilliermondii	Vaginal swabs	9	(59)
C. fomata	Vaginal swabs	9	(59)
C. krusei	Vaginal swabs	9	(59)
C. albicans	Nails	15	(59)
C. albicans	Throat swabs	15	(7)
C. tropicalis	Throat swabs	15	(15)
C. albicans	Ear swabs	10	(15)
C. parapsilosis	Ear swabs	15	(12)
C. albicans	Hair	5	(7)
C. guilliermondii	Hair	9	(7)
Total isolates 204 strains spp=species, C=candida, T=torulopsis, n=number			

Table 2 - In vitro activities of 6 antifungal agents against 204 Candida species MIC ranges (μg/ml).

Antifungal agent	C. albicans (75 strains)	Candida spp* (129 strains)	Candida total (204 strains)
Amphotericin B	> 0.01-2	> 0.01-2	> 0.01-2
Nystatin	0.3-12	0.15-1	0.15-12
Miconazole	0.04 > 50	< 0.02-> 50	< 0.02-> 50
Ketoconazole	0.15-60	< 0.15-> 60	< 0.15-> 60
Clotrimazole	< 0.02-25	0.15-12	< 0.02-> 25
Econazole	< 0.05-20	0.08-18	< 0.08-20
*39 *C.tropicalis*, 30 *C.parapsilosis*, 24 *T.glabrata*, 18 *C.guilliermondii* and 9 each of *C.fomata* and *C.krusei*. *C=Candida, T=torulopsis*			

Table 3 - Minimal inhibitory concentration (MIC) (50-90) and minimal fungicidal concentration (MFC) (50-90) values of candida strains against antifungal agents.

Antifungal agent	MIC_{50}	MIC_{90}	MFC_{50}	MFC_{90}
Amphotericin B				
C. albicans	0.2	0.78	0.78	6.25
Other Candida spp*	0.39	1.56	1.56	2.5
Total	**6.25**	**0.78**	**0.78**	**6.26**
Nystatin				
C.albicans	1.56	12.5	3.13	12.5
Other Candida spp*	0.78	3.13	3.13	12.5
Total	**1.56**	**6.25**	**3.13**	**12.5**
Clotrimazole				
C. albicans	1.56	25	25	> 100
Other Candida spp*	3.13	12.3	50	> 100
Total	**1.56**	**25**	**25**	**> 100**
Ketoconazole				
C. albicans	3.13	25	25	> 100
Other Candida spp*	0.78	> 100	1.56	> 100
Total	**3.13**	**25**	**25**	**> 100**
Econazole				
C. albicans	3.13	25	12.5	> 100
Other Candida spp*	6.25	> 100	25	> 100
Total	**3.13**	**25**	**12.5**	**> 100**
Miconazole				
C. albicans	3.13	25	12.5	> 100
Other Candida spp*	6.25	> 100	25	> 100
Total	**6.25**	**25**	**12.5**	**> 100**

MIC=minimal inhibitory concentration, MFC=minimal fungicidal concentration, C=candida, *=39 *C. tropicalis*, 30 *C. parapsilosis,* 24 *T. glabrata*, 18 *C. guilliermondii* and 9 each of *C. fomata* and *C. krusei*. C=candida T=torulopsis

Discussion. In this paper we are representing some aspects on the prevalence and in vitro susceptibility testing of major candida species isolated from various body sites to the most common usable antifungal drugs in Libya. *Candida albicans* was the most common isolated yeast from patients with vaginal candidosis in this study, but also other candida species especially *T. glabrata* may emerge as the 2nd most important pathogen. In addition the following yeast species were isolated from patients with vaginitis, *C.tropicalis, C.parapsilosis, C. gilliermondii*. Although the extent of microbiology services provided varies considerably from one laboratory to another, however most clinical microbiology laboratories provide a full range of services in bacteriology, but their services in others are quiet variable. In this consensus, services provided by many laboratories in mycology are still limited to direct microscopic examination, culture of fungi and identification of yeast, but not for susceptibility or any filamentous fungi that grows in cultures that may need to be sent to a reference laboratory for identification. These different services are recognized by national laboratory inspection and accreditation agencies. Further, more research in medical mycology is still considered less important by many practitioners and health organizers in many 3rd world countries. This may reflect the reason for few published studies in medical mycology by some countries such as Libya. Although there are recently approved methods for susceptibility testing of yeast by the National Committee for Clinical Laboratory Standard (NCCLS)[7] as well as references control, which were not available at our laboratory to be followed. However the macrobroth dilution method that has been used in this study was suitable for in vitro testing with isolates of *C.albicans* and other yeast-like fungi against amphotericin B and other azoles[6] used in this study. However in spite of that, the clinical response of the test results remain to be established. The test method provides a uniform and reproducible means of assessing the relative in vitro activities, of commonly used antifungal agents against clinical isolates. This is due to the key technical problems conducted by the NCCLS Subcommittee such as preparation of innoculate, composition of medium and conditions for incubation which have been incorporated in the test.[8] The interpretation of our in vitro susceptibility data is difficult to assess, not because of the method which has been used or lack of control strains. This is because even with the broth dilution tests proposed by the NCCLS[9] as the standard method which was more highly developed by 1997,[7] the improved interlaboratory reproducible results from the use of NCCLS guidelines has only lead to the establishment of interpretive breakpoints for fluconazole and itraconazole.[9] Although the NCCLS M27-A consensus document for in vitro susceptibility testing of candida and cryptococcus is a good attempt to find the correlation between microbiological and clinical resistance and failure, but not between in vitro susceptibility and therapeutic success.[10] Furthermore, the breakpoint (MIC) that can be applied to the susceptibility tests are established based more on the resistance limits than on susceptibility. This is due to the fact that clinical infections are a complex and dynamic biological process that only a modest degree of correlation should be expected between MIC endpoint and clinical response to therapy. As shown in Table 2, amphotericin B has been to be the most effective drug among both polyene and azole antifungal drugs. This is because although, amphotericin B has been used frequently to treat fungal infections caused by yeasts, primary resistance or the development of resistance to this antifungal agent has not been a significant problem. In respect to azole antifungals, clotrimazole and econazole had a lower MIC range (<0.02->20mg/ml) than others. Minimal inhibitory concentration values ranged between (<0.15->60 and <0.02->50mg/ml) in the case of ketoconazole and miconazole. There are important differences among results reported by

several investigators in the literature.[11-12] Although we can not interpret that one drug is superior to another used in this study, we conclude that amphotericin B, nystatin and clotrimazole remain the best to be used in the absence of new antifungal drugs. These findings also may be useful in determining susceptibility ranges of candida species and resistance to more common usable antifungal agents in Libya, particularly in failure of drug therapy in patients with serious candida infections.

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