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In vitro susceptibility testing of yeasts to nystatin – low minimum inhibitory concentrations suggest no indication of *in vitro* resistance of *Candida albicans*, Candida species or non-Candida yeast species to nystatin

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Abstract

In total, 14 yeast strains originating from patients with dermatomycoses, and 7 control strains (isolates from strain collections and collaborative ring trials) were investigated regarding their *in vitro* susceptibility to the polyene antifungal agent nystatin. Testing was performed using a broth microdilution assay based on the standardized method of susceptibility testing of yeasts per EUCAST (The European Committee on Antimicrobial Susceptibility Testing). Minimum inhibitory concentrations (MIC) for nystatin were measured. The reading of the MIC values was performed by both visual examination, and spectrophotometric measuring after 24 and 48 hours' incubation time at 36°C.

The visual read-out of growth inhibition revealed MICs for nystatin in a range from 3.7 to 7.4 IU/mL (0.625 to 1.25 µg/mL) for all *Candida* species tested. One of the *Candida* (*C.*) *albicans* strains, and both strains of *C. glabrata* and *C. tropicalis,* showed low MIC values of 3.7 IU/mL (0.625 µg/mL). *Geotrichum candidum* and *Trichosporon mucoides* were also inhibited by nystatin. The control strains (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. tropicalis)* confirmed the values which were found for the wild strains. The spectrophotometric measuring of the turbidimetry revealed slightly lower MIC values for *Candida* species. Spectrophotometric measurment of *Geotrichum candidum* and *Trichosporon mucoides* was unsuccessful or not possible; however, visual reading of the results was carried out effectively. Nystatin showed very good *in vitro* activity against these non-*Candida* yeast species. In conclusion, very good *in vitro* activity of nystatin against all tested yeast strains could be detected. The *in vitro* efficacy was independent of the origin of the strains, as both the wild strains isolated from patients in this study, and the control strains originating from strain collections, were inhibited.

Introduction

The common use of azole antifungal agents, in particular oral fluconazole, but also topical azoles, for the treatment of cutaneous and oral candidiasis, and possibly due to the use of azole fungicides in agricultural crop protection, has led to the emergence of azole and echinocandine resistance of clinical isolates of *Candida* (*C.*) *albicans* and other *Candida* species [1]. An alternative therapeutic approach is the use of topical polyene antifungals such as amphotericin B or nystatin. Only limited current data exists regarding *Candida* species which are resistant to polyene antifungals. Recently, a total of 201 clinical *C. albicans* isolates from Turkey investigated by Etest were found to be susceptible to amphotericin B [2]. A similar situation can be found for nystatin. However, *in vitro* susceptibility testing studies have shown a low percentage of *Candida* species isolated from HIV positive patients to be resistant to nystatin [3].

In this study, a total of 14 currently isolated yeast strains from dermatomycosis patients, and seven reference strains (controls), including isolates from the DSMZ strain collection (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and inter-laboratory ring tests of INSTAND e.V. (the German Society for Promoting Quality Assurance in Medical Laboratories, Düsseldorf, Germany), were tested for their *in vitro* susceptibility to the polyene antifungal agent nystatin. A broth microdilution assay which corresponded to the method of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), and allowed for determination of the minimal inhibitory concentration (MIC) of nystatin, was used for this purpose.

Materials and methods

Active substance

Nystatin: Pure nystatin from Dr. R. Pfleger GmbH, Bamberg, Germany was used in this study. Each ampoule contained: nystatin, lyophilized, approximately 195,000 International Units (IU); stored at refrigerator temperature of 4°C. The active substance content corresponded to 5916 IU nystatin per milligram.

Test strains

Sensitivity testing was performed using test strains from routine

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diagnostic testing at the Laboratory for Medical Microbiology in Mölbis (Table 1). Five reference strains (isolates from the DSMZ, Braunschweig, Germany) and two inter-laboratory ring test strains (INSTAND e. V., Düsseldorf, Germany) were used as controls (Table 1). The following 14 wild strains were investigated: *C. albicans* (4 strains), *C. glabrata* (2 strains), *C. krusei* (2 strains), *C. parapsilosis* (2 strains), *C. kefyr* (1 strain), *Geotrichum candidum* (1 strain), and *Trichosporon mucoides* (2 strains, Table 2).

Culture media

In accordance with EUCAST specifications [EUCAST Definitive Document EDef 7.2 Revision], fully synthetic Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (Sigma-Aldrich Chemie GmbH, Schnelldorf, product R8758-1L, Germany), supplemented with glucose to a final concentration of 20 g/l (2%), was used for susceptibility testing [4]. 3-Morpholinopropanesulfonic acid (MOPS, Sigma-Aldrich Chemie GmbH, Schnelldorf, product no. M3183-100G, Germany), adjusted to a final concentration of 0.165 mol/l and a pH of 7.0, was used as the buffer for the RPMI 1640 medium.

Procedure for testing the antifungal sensitivities of *Candida* and other yeast species to nystatin by broth microdilution assay according to EUCAST

The broth microdilution assay according to EUCAST [Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts, EUCAST DEFINITIVE DOCUMENT EDef 7.2 Revision] was used to assess the antifungal activity of nystatin against the specified yeasts *in vitro*. This method allows for determination of the minimum inhibitory concentration for each strain in IU/mL (and µg/mL). RPMI 1640 medium was used in sensitivity testing.

Table 1. Control strains.

5 isolates from the DSMZ (DSM) strain collection (Braunschweig, Germany)
Candida albicans
https://www.dsmz.de/catalogues/details/culture/DSM-28719.html
Candida tropicalis
https://www.dsmz.de/catalogues/details/culture/DSM-28720.html
Candida glabrata
https://www.dsmz.de/catalogues/details/culture/DSM-28718.html
Candida krusei
https://www.dsmz.de/catalogues/details/culture/DSM-28721.html
Candida parapsilosis
https://www.dsmz.de/catalogues/details/culture/DSM-28722.html
2 strains from INSTAND e.V. Mycology Ring Trials (Düsseldorf, Germany)
Candida tropicalis INSTAND e.V. Mycology Ring Trial 09/2008, Strain 3
Candida lusitaniae INSTAND e.V. Mycology Ring Trial 1/2005, Strain 4

Table 2. Clinical isolates and sources of the yeast strains investigated in this *in vitro* study.

Yeast	Origin
Candida albicans 115370/2015	Oral mucosa
Candida albicans 803972/2015	Stool / intestine
Candida albicans 115323/2015	Urethral swab
Candida albicans 115369/2015	Skin at corner of mouth
Candida glabrata 703950/2015	Urine / genital area
Candida parapsilosis 113753/2014	Throat swab
Candida parapsilosis 216209/2015	Fungal nail clippings, first digit (both sides)
Candida krusei 703658/2015	Urine
Candida krusei 113204/2015	Vaginal swab
Candida kefyr 803965/2015	Stool
Geotrichum candidum 803545/2015	Stool
Trichosporon mucoides 215610/2015	Toe nail
Trichosporon mucoides 215470/2015	Toe nail

Table 3. Method of serial dilution of nystatin and final concentration of the antifungal agent in the serial dilution test.

No.	Nystatin Working solution		Nystatin Final concentration	
	IU/ml	µg/mL	IU/ml per well	µg/mL per well
I	11832	2000	59.16	10
II	5916	1000	29.58	5.0
III	2958	500	14.79	2.5
IV	1479	250	7.4	1.25
V	740	125	3.7	0.625
VI	370	62.5	1.85	0.312
VII	185	31.25	0.92	0.156
VIII	92	15.55	0.46	0.078
IX	46	7.77	0.23	0.039
X	23	3.88	0.115	0.0195
XI	Growth control			
XII	Sterility control			

Serial dilution of nystatin

Nystatin was serially diluted with phosphate buffer pH 6.0 to 10 different concentrations (Table 3). Nystatin stock solution was prepared by mixing the pure substance with the solvent N, N-dimethylformamide (DMF, CNR C95257) to yield a solution with a nystatin concentration of 11832 IU/mL (2000 µg/mL). The stock solution with an antimicrobial concentration of 11832 U/mL was then serially diluted with phosphate buffer pH 6.0 (CNR C99070) at a ratio of 1:2 to yield a total of 10 different test concentrations. The serial 1:2 dilution pattern yielded a geometric dilution series of working solutions with nystatin concentrations ranging from 11832 IU/ml (2000 µg/mL, highest concentration) to 23 IU/ml (3,88 µg/mL, lowest concentration).

Preparation of microdilution plates

Testing was performed using sterile, flat-bottom 96-good microdilution plates with a total volume of approximately 300 µl. 100 µl of each respective nystatin working solution was diluted with 9.9 ml [2X RPMI / 2% glucose], equivalent to a ratio of 1:100. Next, 100 µl of the respective nystatin solution was pipetted into the respective wells numbered 1 to 10. Thus, each well contained 100 µl of the respective nystatin working solution and 2X concentrated RPMI 1640 / 2% glucose medium with 1% solvent. By subsequent 1:2 dilution with inoculum, the final concentration of nystatin in the test wells was adjusted to 59.16 IU/ml (10 µg/mL) to 0.115 IU/ml (0.0195 µg/mL), respectively (Table 3). Column 11, the growth control, contained antifungal-free [2X RPMI 1640 / 2% glucose] medium. Column 12, the sterility control, contained antifungal-free RPMI medium (with no nystatin); 100 µl distilled water was added instead of inoculum.

Inoculum

The inoculum was prepared using 18- to 48-hour-old yeast cultures grown on Sabouraud´s 4% dextrose agar (under aerobic conditions at 35 °C). The yeast suspensions were prepared using distilled water. The densities of the test cell suspensions ranged from 0.5 to 2.5 × 10^5 colony-forming units (CFU) per milliliter [CFU × ml^{-1}]. This was accomplished by comparison with a McFarland standard (bioMérieux SA, Marcy l'Etoile, France): McFarland standard no. 0.5 = 1-5 × 10^6 CFU/mL. A working suspension with 1-5 x 10^5 CFU/mL was achieved by subsequent preparation of a 1:10 dilution (with distilled water).

In each case, the yeast cell density was checked and confirmed by transferring cells to Sabouraud´s dextrose agar and subsequently counting the colonies (after 24 or 48 hours of incubation).

A MultiPipette was used to dispense the yeast suspensions onto the plates (100 µl inoculum per well, columns 1 to 11). Subsequent 1:2 dilution with nystatin/[RPMI 1640 / 2% glucose] working solution was performed to yield the aforementioned test yeast cell suspension concentrations ranging from 0.5 to 2.5 × 10^5 CFU/ml in the microplates.

Incubation was performed under aerobic conditions at 36 °C. Visual readings were obtained after 24 and 48 hours of incubation. Turbidity i.e. growth inhibition on the microplates was also determined spectrophotometrically at 450 nm after 24 and 48 hours of incubation. The absorbance measured with the blank sample (background absorbance) was deducted from the absorbance values obtained with the test samples. The MIC was defined as the lowest concentration of the active substance that inhibited growth of the microorganisms.

Growth controls in antifungal-free culture medium (RPMI 1640, column 11) and sterility controls containing no inoculum (column 12) were run with each MIC determination.

The MIC_{50}, which represents the MIC that inhibits 50% of the isolates, and the MIC_{90} which represents the MIC which inhibits 90% of the isolates of the species tested, was calculated.

Results

In each case, the MIC values were read visually and spectrophotometrically (at 450 nm), after 24 and, and 48 hours of incubation at 36°C. All yeast strains were tested in duplicate.

Visual reading

After 24 hours of incubation, the growth of the vast majority of studied yeast strains allowed MIC reading which could be considered as conclusive. All *Candida* species could be fully evaluated after 24 hours of incubation (Tables 4a,4b and 4c). The *Geotrichum candidum* strain and the two *Trichosporon mucoides* strains grew slower *per se* and thus could not be evaluated until the second day (Table 4b).

Table 4a. Minimum inhibitory concentrations (MIC) of nystatin against various yeast strains (*Candida albicans*, *Candida glabrata* and *Candida parapsilosis*) as determined by broth microdilution assay according to EUCAST. MIC values of strains (double determination) were read after 24 and 48 hours of incubation at 36°C. Visual reading.

Yeast strain	Nystatin MIC in IU/ml		Nystatin MIC in µg/ml	
	24-hour reading	48-hour reading	24-hour reading	48-hour reading
Candida albicans 115370/2015	3.7	7.4	0.625	1.25
	3.7	7.4	0.625	1.25
Candida albicans 803972/2015	7.4	7.4	1.25	1.25
	7.4	7.4	1.25	1.25
Candida albicans 115323/2015	7.4	7.4	1.25	1.25
	7.4	7.4	1.25	1.25
Candida albicans 115369/2015	7.4	7.4	1.25	1.25
	7.4	7.4	1.25	1.25
Candida glabrata 703950/2015	3.7	7.4	0.625	1.25
	3.7	7.4	0.625	1.25
Candida glabrata Routine strain, Mycology Course Cologne 2015	3.7	7.4	0.625	1.25
	7.4	7.4	1.25	1.25
Candida parapsilosis 113753/2014	7.4	14.79	1.25	2.5
	7.4	14.79	1.25	2.5
Candida parapsilosis 216209/2015	7.4	14.79	1.25	2.5
	7.4	7.4	1.25	1.25
MIC_{50}	7.4	7.4	1.25	1.25
MIC_{90}	7.4	14.79	1.25	2.5

Table 4b. Minimum inhibitory concentrations (MIC) of nystatin against various yeast strains (*Candida krusei*, *Candida tropicalis*, *Candida lusitaniae*, *Candida kefyr*, *Geotrichum candidum* and *Trichosporon mucoides*) as determined by broth microdilution assay according to EUCAST. MIC readings (double determination) were taken after 24 and 48 hours of incubation at 36 °C. Visual reading

Yeast strain	Nystatin MIC in IU/ml		Nystatin MIC in µg/ml	
	24-hour reading	24-hour reading	24-hour reading	48-hour reading
Candida krusei 703658/2015	7.4	7.4	1.25	1.25
	7.4	7.4	1.25	1.25
Candida krusei 113204/2015	7.4	14.79	1.25	2.5
	7.4	14.79	1.25	2.5
Candida tropicalis Ring Trial 09/2008 Strain 3	3.7	7.4	0.625	1.25
	3.7	7.4	0.625	1.25
Candida lusitaniae Ring Trial 1/2005 Strain 4	7.4	7.4	1.25	1.25
	3.7	7.4	0.625	1.25
Candida kefyr 803965/2015	7.4	7.4	1.25	1.25
	7.4	7.4	1.25	1.25
Geotrichum candidum 803545/2015	Not evaluable	Not evaluable	Not evaluable	Not evaluable
	Not evaluable	7.4	Not evaluable	1.25
Trichosporon mucoides 215610/2015	Not evaluable	7.4	Not evaluable	1.25
	Not evaluable	7.4	Not evaluable	1.25
Trichosporon mucoides 215470/2015	Not evaluable	7.4	Not evaluable	1.25
	Not evaluable	7.4	Not evaluable	1.25
MIC_{50}	7.4	7.4	1.25	1.25
MIC_{90}	7.4	14.79	1.25	2.5

Table 4c. Minimum inhibitory concentrations (MIC) of nystatin against various control strains (reference strains/collection strains of *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei* and *Candida tropicalis*) as determined by broth microdilution assay according to EUCAST. MIC readings (double determination) were taken after 24 and 48 hours of incubation at 36 °C. Visual reading

Yeast strain	Nystatin MIC in IU/ml		Nystatin MIC in µg/ml	
	24-hour reading	48-hour reading	24-hour reading	48-hour reading
Candida albicans DSM 28719	7.4	7.4	1.25	1.25
	7.4	7.4	1.25	1.25
Candida glabrata DSM 28718	7.4	7.4	1.25	1.25
	7.4	7.4	1.25	1.25
Candida parapsilosis DSM 28722	7.4	14.79	1.25	2.5
	7.4	7.4	1.25	1.25
Candida krusei (*Pichia kudriavzevii*) DSM 28721	7.4	7.4	1.25	1.25
	7.4	7.4	1.25	1.25
Candida tropicalis DSM 28720	3.7	7.4	0.625	1.25
	3.7	7.4	0.625	1.25
MIC_{50}	7.4	7.4	1.25	1.25
MIC_{90}	7.4	7.4	1.25	1.25

The MIC values of nystatin were in the range of 3.7 to 7.4 IU/mL (0.625 to 1.25 µg/mL) for all *Candida* species (Tables 4a,4b and 4c). Specifically, the MICs for one *C. albicans* strain, the two *C. glabrata* strains and *C. tropicalis* were in the low range of 3.7 IU/mL (0.625 µg/mL). The two non-*Candida* species, *Geotrichum candidum* and *Trichosporon mucoides,* were also inhibited well by nystatin; the MIC values for these yeasts remained constant at 7.4 IU/mL (1.25 µg/mL, Table 4b). The five reference strains from the DSMZ collection (Braunschweig, Germany) *(C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. tropicalis)* confirmed the MIC values obtained for

the wild strains. The minimum inhibitory concentrations for these DSM *Candida* strains also ranged from 3.7 IU/mL (0.625 µg/mL, *C. tropicalis*) to 7.4 IU/mL (1.25 µg/mL, Table 4c).

Spectrophotometric measurement and determination of MIC

The minimum inhibitory concentrations obtained by spectrophotometric absorbance measurement and optical density determination based on turbidity due to growth of the investigated yeast strains are presented in Tables 5 a-c. Compared to the visual readings, the spectrophotometrically determined MIC values for the clinical isolates of *C. albicans* and *C. glabrata* were slightly lower: 3.7 IU/mL (0.625 µg/mL) at 24 hours. Spectrophotometric readings at 48 hours were also lower: in contrast to the visual readings, only a few strains had a photometric MIC value of 7.4 IU/mL (1.25 µg/mL, Tables 5a, 5b and 5c).

C. parapsilosis: After 24 hours of incubation, spectrophotometric determination of the MIC for this slowly growing yeast was not possible, but visual reading was feasible (Table 4a, Table 5a). After 48 hours, the MIC values determined by spectrophotometry were in the range of 7.4 IU/mL (1.25 µg/mL), which is slightly lower than the visual readings (Table 5a).

C. krusei, *C. lusitaniae*, *C. tropicalis* and *C. kefyr* were well measurable. The spectrophotometrically and visually determined minimum inhibitory concentrations for these yeasts were comparable, but those determined by spectrophotometry tended to be slightly lower (Table 5b).

Geotrichum candidum (known as the "cheese mold") and *Trichosporon mucoides* are slow-growing yeasts that do not tend to develop homogeneous growth. *Geotrichum candidum*, in particular, tends to develop relatively large single colonies at the bottom of microplate wells. Consequently, photometric measurement is problematic and inferior to visual reading in these cases. When determined by spectrophotometry, the measured optical density values are not in the evaluable range and are generally too low (Table 5b). This does not apply to the visual readout method, with which (as described above) a good inhibitory effect of nystatin against the two arthrospore-forming yeast species was observed.

Table 5a. Minimum inhibitory concentrations (MIC) of nystatin against various yeast strains (Candida albicans, Candida glabrata and Candida parapsilosis) as determined by broth microdilution assay according to EUCAST. MIC readings (double determination) were taken after 24 and 48 hours of incubation at 36 °C. Spectrophotometric readings were taken at 450 nm. The cut-off was defined as an optical density (OD) value of 0.300. All OD values > 0.300 were interpreted as positive for yeast growth.

Yeast strain	Nystatin MIC in IU/ml		Nystatin MIC in µg/ml	
	Measurement: 24 hours	Measurement: 48 hours	Measurement: 24 hours	Measurement: 48 hours
Candida albicans 115370/2015	3.7	3.7	0.625	0.625
	3.7	3.7	0.625	0.625
Candida albicans 803972/2015	3.7	7.4	0.625	1.25
	3.7	7.4	0.625	1.25
Candida albicans 115323/2015	3.7	7.4	0.625	1.25
	3.7	3.7	0.625	0.625
Candida albicans 115369/2015	3.7	7.4	0.625	1.25
	3.7	7.4	0.625	1.25
Candida glabrata 703950/2015	3.7	7.4	0.625	1.25
	3.7	7.4	0.625	1.25
Candida glabrata Routine strain, Mycology Course Cologne 2015	3.7	3.7	0.625	0.625
	3.7	3.7	0.625	0.625
Candida parapsilosis 113753/2014	Not evaluable	7.4	Not evaluable	1.25
	Not evaluable	7.4	Not evaluable	1.25
Candida parapsilosis 216209/2015	Not evaluable	7.4	Not evaluable	1.25
	Not evaluable	3.7	Not evaluable	0.625
MIC_{50}	3.7	7.4	0.625	1.25
MIC_{90}	3.7	7.4	0.625	1.25

In the spectrophotometric evaluation, the *in vitro* susceptibility of the reference strains from the DSMZ Braunschweig corresponded to that of the wild strains. *C. parapsilosis* and *C. krusei*: Spectrophotometric determination of the MICs for these strains was only possible after 48 hours of incubation (Table 5c). The spectrophotometrically determined MIC values for the other yeasts were generally comparable and, in isolated cases, slightly lower than the corresponding visual readings.

Discussion

Nystatin is a polyene antifungal agent which therefore belongs to the same group of antifungal substances as amphotericin B and natamycin. Structurally, nystatin belongs to the family of polyene macrolide antibiotics and was first successfully isolated in 1950 from the bacterium *Streptomyces noursei*. The drug has subsequently been available as a "classical" topical antifungal agent which is used to treat fungal diseases of the skin and mucous membranes. Nystatin is a yellowish powder which is practically insoluble in water but soluble in propylene glycol and dimethylformamide [5]. Nystatin undergoes slow degradation in aqueous suspensions. The drug is sensitive to light, heat, oxygen and pH shifts below pH 3 and above pH 9.

The mechanism of action of nystatin is based on binding of the polyene to sterols in the yeast plasma membrane resulting in a change in their permeability. Consequently, the fungal cells lose potassium, sugar and phosphate ions, which leads to the impairment of glycolysis and cellular respiration. Nystatin has a fungistatic effect but can also show fungicidal activity at high concentrations.

The primary activity of nystatin both *in vitro* as well as *in vivo* in patients involves mainly yeasts. *C. albicans* and other *Candida* species are significantly inhibited by nystatin. Nystatin is also known to have an inhibitory effect on some molds.

Nystatin is used for the topical treatment of cutaneous and mucosal fungal infections caused by *C. albicans* and other yeast species. Examples of these infections include cutaneous candidiasis involving intertriginous areas (e.g. groin or between fingers) and the free skin. *Candida* infections of the orointestinal tract, as well as infections of the mucous membranes of the genital tract caused by *C. albicans* and other *Candida* species, can be effectively treated using nystatin preparations [6].

Film-coated tablets for the treatment of orointestinal candidiasis contain, for example, 500,000 IU nystatin. One gram of nystatin oral gel contains, for example, 100,000 IU nystatin. Since nystatin is hardly absorbed from the gut it is not expected to cause systemic effects following oral administration.

Previously, it has been stated that no nystatin resistant yeasts exist, and in particular that there is no *C. albicans* which is resistant to nystatin.

In India, an *in vitro* study of antifungal drug susceptibility of *Candida* species from human immunodeficiency virus (HIV) positive and HIV negative patients with and without oropharyngeal candidiasis has recently been published [3]. *Candida* isolates from HIV negative

Table 5b. Minimum inhibitory concentrations (MIC) of nystatin against various yeast strains (Candida krusei, Candida tropicalis, Candida lusitaniae, Candida kefyr, Geotrichum candidum and Trichosporon mucoides) as determined by broth microdilution assay according to EUCAST. MIC values (double determination) were read after 24 and 48 hours of incubation at 36 °C. Spectrophotometric readings were taken at 450 nm. The cut-off was defined as an optical density (OD) value of 0.300. All OD values > 0.300 were interpreted as positive for yeast growth.

Yeast strain	Nystatin MIC in IU/ml		Nystatin MIC in µg/ml	
	Measurement: 24 hours	Measurement: 48 hours	Measurement: 24 hours	Measurement: 48 hours
Candida krusei 703658/2015	7.4	7.4	1.25	1.25
	7.4	7.4	1.25	1.25
Candida krusei 113204/2015	7.4	7.4	1.25	1.25
	7.4	7.4	1.25	1.25
Candida tropicalis Ring Trial 09/2008 Strain 3	3.7	3.7	0.625	0.625
	3.7	3.7	0.625	0.625
Candida lusitaniae Ring Trial 1/2005 Strain 4	7.4	7.4	1.25	1.25
	3.7	7.4	0.625	1.25
Candida kefyr 803965/2015	3.7	1,85	0.625	0.3125
	3.7	3.7	0.625	0.625
Geotrichum candidum 803545/2015	Not evaluable	Not evaluable / No growth	Not evaluable	Not evaluable / No growth
	Not evaluable	Not evaluable, but good growth of large individual colonies visually evaluable	Not evaluable	Not evaluable, but good growth of large individual colonies visually evaluable
Trichosporon mucoides 215610/2015	Not evaluable	Not evaluable, but good growth of large individual colonies visually evaluable	Not evaluable	Not evaluable, but good growth of large individual colonies visually evaluable
	Not evaluable	Not evaluable, but good growth of large individual colonies visually evaluable	Not evaluable	Not evaluable, but good growth of large individual colonies visually evaluable
Trichosporon mucoides 215470/2015	Not evaluable	Not evaluable, but good growth of large individual colonies visually evaluable	Not evaluable	Not evaluable, but good growth of large individual colonies visually evaluable
	Not evaluable	Not evaluable, but good growth of large individual colonies visually evaluable	Not evaluable	Not evaluable, but good growth of large individual colonies visually evaluable
MIC_{50}	7.4	7.4	1.25	1.25
MIC_{90}	7.4	7.4	1.25	1.25

Table 5c. Minimum inhibitory concentrations (MIC) of nystatin against various control strains (reference strains/collection strains of Candida albicans, Candida glabrata, Candida parapsilosis, Candida krusei and Candida tropicalis) as determined by broth microdilution assay according to EUCAST. MIC values (double determination) were read after 24 and 48 hours of incubation at 36 °C. Spectrophotometric readings were taken at 450 nm. The cutoff was defined as an optical density (OD) value of 0.300. All OD values > 0.300 were interpreted as positive for yeast growth.

Yeast strain	Nystatin MIC in IU/ml		Nystatin MIC in µg/ml	
	Measurement: 24 hours	Measurement: 48 hours	Measurement: 24 hours	Measurement: 48 hours
Candida albicans DSM 28719	3.7	7.4	0.625	1.25
	3.7	7.4	0.625	1.25
Candida glabrata DSM 28718	3.7	7.4	0.625	1.25
	3.7	7.4	0.625	1.25
Candida parapsilosis DSM 28722	Not evaluable	7.4	Not evaluable	1.25
	Not evaluable	7.4	Not evaluable	1.25
Candida krusei (Pichia kudriavzevii) DSM 28721	Not evaluable	7.4	Not evaluable	1.25
	Not evaluable	7.4	Not evaluable	1.25
Candida tropicalis DSM 28720	1.85	7.4	0.3125	1.25
	1.85	7.4	0.3125	1.25
MIC_{50}	3.7	7.4	0.625	1.25
MIC_{50}	3.7	7.4	0.625	1.25

patients were much more susceptible to antifungals when compared to those which were HIV positive. *Candida* species from HIV patients were susceptible to fluconazole in 86.1% of cases, and dose-dependent susceptible in 13.9%. Resistance to fluconazole was not found. HIV negative patients showed susceptibility, dose dependent susceptibility and resistance to fluconazole in 94.1%, 2.9% and 2.9% of isolates. For amphotericin B in HIV positive patients, 66.7% of *Candida* species were susceptible, and 22.2% resistant. HIV negative individuals showed 85.3% susceptibility to amphotericin B, 5.9% dose dependent susceptibility, and 8.8% resistance. In HIV positive patients 61.1% of *Candida* isolates were susceptible to nystatin, 36.1% were dose dependent susceptible, and 2.8% were resistant to nystatin. Otherwise, HIV negative individuals showed nystatin susceptibility in 91.2% of isolates, dose dependent susceptibility in 8.8%, and there were no nystatin resistant isolates.

It should be noted, however, that nystatin-resistant isolates of *C. albicans* have repeatedly been reported. In a recent study in Uganda, for example, nystatin resistance of *C. albicans* isolated from women with vulvovaginal candidiasis was observed in 0.61% of patients [7]. The fact that sensitivity testing in the Ugandan study was performed using a simple agar diffusion test can be regarded as a point of criticism. Conformity with recognized methods such as the EUCAST broth microdilution method was not evident.

Recently, Diaz *et al.* [8] determined the *in vitro* susceptibility of vaginal *Candida* isolates to fluconazole, clotrimazole and nystatin by M27-A3 microdilution method. Among the 145 isolates were 126 *C. albicans*, 16 *C. glabrata*, 2 *C. parapsilosis*, and one *C. tropicalis*. Five isolates of *C. albicans* and one isolate of *C. tropicalis* were *in vitro* resistant to fluconazole. Five *C. glabrata* and 1 *C. tropicalis* were *in vitro* resistant to clotrimazole.

The spectrum and *in vitro* antifungal susceptibility pattern of yeast isolates were investigated in Ethiopian HIV patients with oropharyngeal candidiasis [9]. The authors found that *C. albicans* was the most frequent species followed by *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. kefyr*,

Cryptococcus laurentii, and *Rhodotorula* species. In total, 5.8%, 5.8%, 12.3%, 8.4%, and 0.6% of the isolates were resistant to amphotericin B, clotrimazole, fluconazole, ketoconazole, and miconazole, respectively. 1.3% isolates were resistant to nystatin. Interestingly, the resistance was higher for amphotericin B when compared with nystatin. Not surprisingly, the azole antifungals also exhibited higher resistance levels than nystatin.

In Iran, 120 clinical isolates of *C. parapsilosis* from blood stream infections were tested for *in vitro* resistance [10]. Only three (2.5%) *C. parapsilosis* strains were resistant to fluconazole, three (2.5%) were resistant to itraconazole, and two (1.7%) were amphotericin B resistant. In this study, nystatin was not investigated.

The present study found low MIC values for nystatin against yeasts such as *C. albicans*, for which the MIC of nystatin was in the range of 3.7 IU/ ml (0.625 µg/mL, 24 hour spectrophotometric reading). The various other yeast species were also well inhibited by nystatin *in vitro* at comparable minimum inhibitory concentrations, which ranged from 1.85 to 7.4 IU/ml (0.3125 to 1.25 µg/mL) and were predominantly at a focal point of 3.7 IU/ml (0.625 µg/mL). Even slow-growing *Candida* species such as *C. parapsilosis* were also inhibited well by nystatin *in vitro*. *C. glabrata* and *C. krusei,* two species known to frequently develop resistance to fluconazole, and the latter which has intrinsic resistance to fluconazole, were also inhibited well by nystatin *in vitro*, as shown by the MIC values of 3.7 and 7.5 IU/ml (0.625 to 1.25 µg/mL), respectively.

The fact that nystatin was also shown to have very good *in vitro* activity against non-*Candida* species, such as *Geotrichum candidum* and *Trichosporon mucoides*, is worth mentioning. However, it was necessary to resort to visual read-out of growth inhibition test results as optical density measurement is error-prone due to the slow and inhomogeneous growth of these species.

Conclusions

In summary, nystatin exhibited very good *in vitro* activity against all of the tested yeast strains, *C. albicans* and non-*Candida albicans* species as well as *Geotrichum candidum* and *Trichosporon mucoides,* regardless of whether they were wild strains or control strains from culture collections. There was no evidence of *in vitro* resistance by *C. albicans*, other *Candida* species, or non-*Candida* yeast species to nystatin, despite the long time period in which it has been used as a topical antifungal agent.

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