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Antifungal Susceptibility Pattern of Non - Dermatophytic Fungi Causing Onychomycosis

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Abstract

Introduction: Non-dermatophytic molds (NDM) are filamentous fungi or yeast, commonly found in nature as saprophytes and plant pathogens. The incidence of onychomycosis due to NDM is 1.45 – 16.6%. NDMs are usually resistant to conventional antifungal treatment.

Objective: To know the anti-fungal susceptibility pattern of non-dermatophyte fungi causing onychomycosis.

Materials and Methods: A prospective hospital based cross-sectional study was done on non - dermatophytic isolates from patients with clinical suspicion of onychomycosis. All non – dermatophytic isolates were subjected to anti-fungal susceptibility against terbinafine, itraconazole, fluconazole and griseofulvin by micro broth dilution method.

Results: NDM were isolated in 20.2% cases of clinically suspected onychomycosis, among which *Fusarium species* was the most common followed by *Aspergillus species* and *Candida species.* MIC50 (Mean Inhibitory Concentration) for overall non - dermatophytic isolates for terbinafine, itraconazole, fluconazole and griseofulvin was 0.25µg/mL, 0.5µg/mL, 32µg/mL and 2µg/mL respectively and the order of sensitivity was Itraconazole (74.7%) > terbinafine (68%) > Fluconazole (60%) > Griseofulvin (51.6%) of the study samples. For *Fusarium species*, the sensitivity for terbinafine was (73.5%) > itraconazole (67.6%) > fluconazole (64.7%) and griseofulvin (64.7%). For *Aspergillus species*, the sensitivity for itraconazole was 79.1% > fluconazole (58.3%) > terbinafine (54.1%) > griseofulvin (50%). For *Candida species*, the sensitivity was fluconazole (83.3%) > itraconazole (75%) > terbinafine (41.6%), while no candida species was found sensitive to griseofulvin.

Conclusion: Non-dermatophytes play a significant role in onychomycosis. On in vitro estimation, Itraconazole was the most sensitive drug, followed by terbinafine, fluconazole and griseofulvin.

Key words: Antifungal Agents; Fluconazole; Itraconazole; Onychomycosis; Terbinafine

Introduction

Onychomycosis is a fungal infection of nails caused by dermatophytes, yeasts, and non-dermatophytic molds.[1] For all the culture positive cases of onychomycosis, dermatophytes account for nearly 70%, non dermatophytic molds account for 1.45-16.60% and yeast account for 1-31% of the cases.[1,2] Few studies have shown a very high number of non – dermatophytes i.e. upto 68% of culture positive case of onychomycosis, it can be due to improved diagnostic techniques and increased awareness of non dermatophytic fungi as potential etiologic agents.[3,4] While non-dermatophytic onychomycosis respond to oral or topical antifungal therapy, poor or incomplete response might still be seen in some patients.[2,4]

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Resistance to anti-fungal drugs is a growing health crisis, fueled by widespread injudicious use of various antifungal drugs, which may be responsible for treatment failure. Hence, the targeted Antifungal therapy for the non- dermatophytic onychomycosis is of utmost importance.[5]

In vitro antifungal susceptibility tests are now mainly used for epidemiological surveys, determination of the degree of antifungal activity, and the prediction of clinical outcome based upon an optimization of antifungal therapy.[5] The data regarding the sensitivity pattern of various anti-fungals to NDM is scarce. Hence this study was conducted to determine the in vitro susceptibility of non dermatophytic fungi causing onychomycosis to Terbinafine, Fluconazole, Itraconazole, and Grieseofulvin by Broth microdilution technique.

Material and Methods - After obtaining ethical clearance from the Institutional Ethics Committee ,non-dermatophytic isolates on culture from the clinically suspected cases of onychomycosis, presenting to the Department of Dermatology, Shri Ram Murti Smarak Institute of Medical Sciences, Bhojipura, Bareilly from Nov 2016 to May 2018 were included. Further processing & anti fungal sensitivity study was done in Department of Microbiology. Figure.1 describes the study design.

For consideration of non-dermatophytes as a pathogen, three out of six criteria should be chosen. [4] The criteria taken in our study were-

1. Culture positivity for non dermatophytes
2. Absence of Dermatophytes in culture
3. Repeated isolation of the non dermatophytic molds on two separate occasions done on 2 to 4 weeks

Aspergillus niger ATCC 6275 and Candida albicans ATCC 10231 were used as quality control organisms.

Broth microdilution method - The CLSI M38-A2& M27-A3 guidelines were followed. The test was performed in microtiter plates with RPMI-1640 without bicarbonate and buffered to pH 7.0 with 3 [N-morpholino] propane sulfonic acid (MOPS).[6,7] For each drug six dilutions were used. Hundred microlitre of two fold drug dilutions were placed in wells with a multichannel pipette to yield twice the final strength required for the test i.e. 4-128 µg/ml for fluconazole, 0.25-8 µg/ml for griseofulvin, 0.125-4.0 µg/ml for itraconazole, and 0.015-0.50 µg/ml for terbinafine.

Stock solutions were prepared by dissolving the anti fungal powder in their specific solvent. Fluconazole was dissolved in distilled water in concentration of 1280 µg/ml, itraconazole in DMSO (Dimethyl sulfoxide) 400 µg/ml, terbinafine in DMSO 0.4 µg/ml and griseofulvin in DMSO 800 µg/ml.

Inoculum suspension of the fungi were prepared from 7-10 day old culture grown on PDA (Phosphate Dextrose Agar) at 28°C in a BOD (Biological Oxygen Demand) incubator. The resulting mixture of 0.5 mcfarland turbidity (0.5×10^4-5.0×10^4 spores/hyphae per ml) dilution of every isolated strain was made.

All the tests were performed in sterile, flat-bottomed, 96-well micro plates. For performing the susceptibility testing 100 µl of the RPMI 1640, was mixed with 100 µl antifungal drug inoculate in first well and total of six dilutions were prepared.

Then 100µl of the diluted inoculums suspension was added and brought the final dilution of drugs to 2.0-64.0µg/ml for fluconazole, 0.125-4.0µg/ml for griseofulvin, 0.062-2.0µg/ml for itraconazole, and 0.015-0.5 µg/ml for terbinafine. The micro titer plate contents were incubated at 28^0c, by avoiding desiccation of the wells and were read visually with the aid of an inverted mirror after 7 days of incubation. The range, GM Geometic Mean), MIC 50 (Minimum inhibitory concentration required to inhibit the growth of 50% of organisms) and MIC90 (Minimum inhibitory concentration required to inhibit the growth of 90 % of organisms) were determined for various isolated fungi.

Since, there is no established breakpoint for antifungals for non dermatophytes, MIC50 was assumed as a breakpoint. The strains showing the MIC value equal to or less than the MIC50 was considered sensitive and strains showing MIC value more than MIC50 was considered resistant.

Results

Total 470 patients were included in study, culture was positive in 276 patients, out of which 181 were dermatophytes and were excluded from the study while 95) non- dermatophytes were isolated (Figure-1. Among the non - dermatophytic isolates, the most common genra was *Fusarium species* in 34/95 i.e. 35.8% followed by *Aspergillus species* 24/95i.e. 25.3% and *Candida species* in 12/95 i.e 12.6% cases. Table 1 shows the number and the percentage of isolated non –dermatophytes.

The susceptibility patterns for the fungal isolates are tabulated in Table 2. In our study the MIC50 for various non-dermatophyte isolates for terbinafine, itraconazole, fluconazole and griseofulvin were 0.25µg/mL, 0.5µg/mL, 32µg/mL and 2µg/m, respectively. And MIC ranges were 0.03 - >0.5µg/mL, 0.062 - >2µg/mL, 2 - >64µg/mL and 0.125 - >4µg/mL, respectively, indicating that some isolates were resistant in vitro.

Table 3 shows the range, MIC50 and MIC90 of isolated non dermatophytes against the four drugs. The GM of MIC for fusarium species for terbinafine, itraconazole, fluconazole and griseofulvin were 0.24µg/mL, 0.63µg/mL, 29µg/mL and 2.1µg/mL, respectively. The Figure 4 shows the percentage of sensitive and resistant isolates against various antifungal drugs.

Since the breakpoint was determined on MIC50, on further analysis, *Fusarium species* had maximum sensitivity in vitro to terbinafine (73.5%) followed by itraconazole (67.6%), fluconazole (64.7%) and griseofulvin (64.7%). For *Aspergillus species*, maximum sensitivity was seen to itraconazole (79.1%), followed by fluconazole (58.3%), then terbinafine (54.1%) least to griseofulvin (50%). For *Candida species*, the maximum sensitivity was observed to fluconazole (83.3%), followed by itraconazole (75%) and terbinafine (41.6%), resistance in vitro was observed to griseofulvin. The percentages of sensitive isolates of various non dermatophytes according to the assumed breakpoint are tabulated in Table 4.

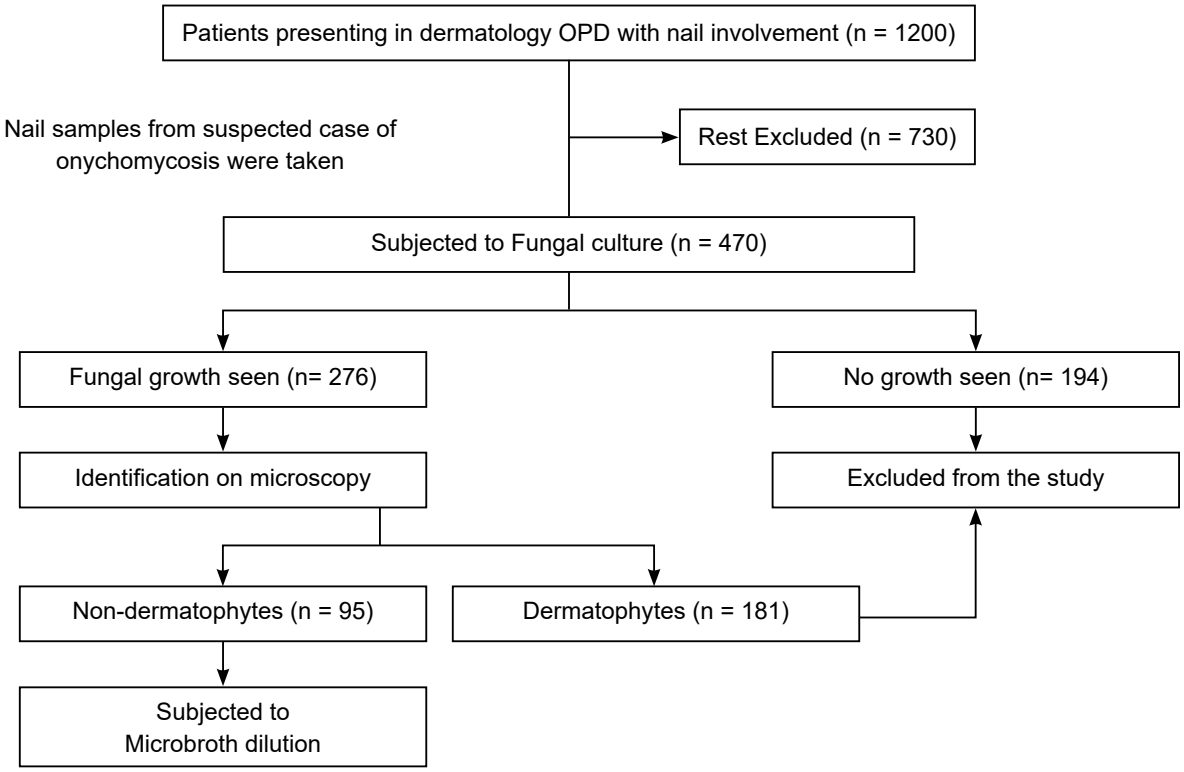

Figure 1: Study Design

Table 1: Number & percentage of fungal isolates

Species	Number	Percentage
Fusarium	34	35.8
Aspergillus	24	25.3
Candida	12	12.6
Neoscytalidium	6	6.3
Rhizopus	6	6.3
Alternaria	5	5.3
Penicillium	4	4.2
Bipolaris	2	2.1
Curvularia	2	2.1

Table 2: Antifungal susceptibility pattern of NDMs against 4 antifungal drugs

	Itraconazole (in µg/ml)	Terbinafine (in µg/ml)	Fluconazole (in µg/ml)	Griseofulvin (in µg/ml)
GM	0.57	0.27	32.85	2.45
MIC50	0.5	0.25	32	2
MIC90	1	0.5	>64	>4
Range	0.062->2	0.03->0.5	2->64	0.125->4

Table 3: Susceptibility Pattern of Fungal Isolates (Values in µg/ml)

Species	Drug	Range	GM	MIC 50	MIC 90
Fusarium (n=34)	Terbinafine	0.06 - >0.5	0.24	0.25	0.5
	Itraconazole	0.062 - >1	0.63	0.5	1
	Fluconazole	2 - > 64	29	32	>64
	Griseofulvin	0.125 – 4	2.1	2	4
Aspergillus (n=24)	Terbinafine	0.125 - > 0.5	0.31	0.25	>0.5
	Itraconazole	0.5 - >1	0.59	0.5	1
	Fluconazole	16 - >64	36.7	32	>64
	Griseofulvin	1 - >4	2.75	2	>4
Candida (n=12)	Terbinafine	0.25 - >0.5	0.39	0.5	0.5
	Itraconazole	0.25 – 1	0.46	0.5	0.5
	Fluconazole	8 - >64	31.27	32	64
	Griseofulvin	4 - >4	4	>4	>4
Rhizopus (n=6)	Terbinafine	0.03-0.25	0.175	0.18	NC
	Itraconazole	0.5 - >2	0.5	1	NC
	Fluconazole	64- >64	64	64	NC
	Griseofulvin	2-4	3	2	NC
Neoscytalidium (n=6)	Terbinafine	0.125->0.5	0.38	0.5	NC
	Itraconazole	0.25->2	0.65	0.5	NC
	Fluconazole	>64->64	>64	>64	NC
	Griseofulvin	2-4	3.67	4	NC
Alternaria (n=5)	Terbinafine	0.125-0.25	0.175	0.125	NC
	Itraconazole	0.5- 0.5	0.5	0.5	NC
	Fluconazole	16-32	28.8	32	NC
	Griseofulvin	1-2	1.6	2	NC
Penicillium (n=4)	Terbinafine	0.25-0.25	0.25	0.25	NC
	Itraconazole	0.5-1	0.63	0.5	NC
	Fluconazole	32->64	42.67	32	NC
	Griseofulvin	1-1	1	1	NC
Bipolaris (n=2)	Terbinafine	0.125-0.125	0.125	NC	NC
	Itraconazole	0.5-0.5	0.5	NC	NC
	Fluconazole	32-64	48	NC	NC
	Griseofulvin	2-4	3	NC	NC
Curvularia (n=2)	Terbinafine	0.25-0.25	0.25	NC	NC
	Itraconazole	0.5-0.5	0.5	NC	NC
	Fluconazole	32->64	N/A	NC	NC
	Griseofulvin	2-2	2	NC	NC

NC-Not calculated.*MIC 90 not calculated because of the smaller sample size (i.e. <10 strains in each category).

Table 4: Sensitivity pattern of isolates (values in percentage)

Species	Terbinafine (%)	Itraconazole (%)	Fluconazole (%)	Griseofulvin (%)
Fusarium	73.5	67.6	64.7	64.7
Aspergillus	54.1	79.1	58.3	50
Candida	41.6	75	83.3	0
Rhizopus	100	33.3	0	50
Neoscytalidium	66.6	50	0	16.6
Alternaria	100	100	100	100
Penicillium	100	75	50	100
Bipolaris	100	100	50	50
Curvularia	100	100	50	100

Table 5: Comparison of isolation rates of NDMs with the previous Indian studies

Studies	Kaur et al[8]	Grover et al[9]	Lone et al[10]	Lakshmanan et al[11]	Kaur et al[12]	Jesudanam et al[13]	Present study
YEAR	2013-2014	1999-2001	2010-11	2011-12	2000-05	1998-99	2016-19
PLACE	New Delhi (North india)	(North East India)	Kashmir (North india)	Tamil nadu (South India)	New Delhi (North India)	Vishkapatnam	Bareilly (North India)
Isolation rates	55 %	34 %	31.6 %	24.4 %	4.4 %	3.5 %	34.4%

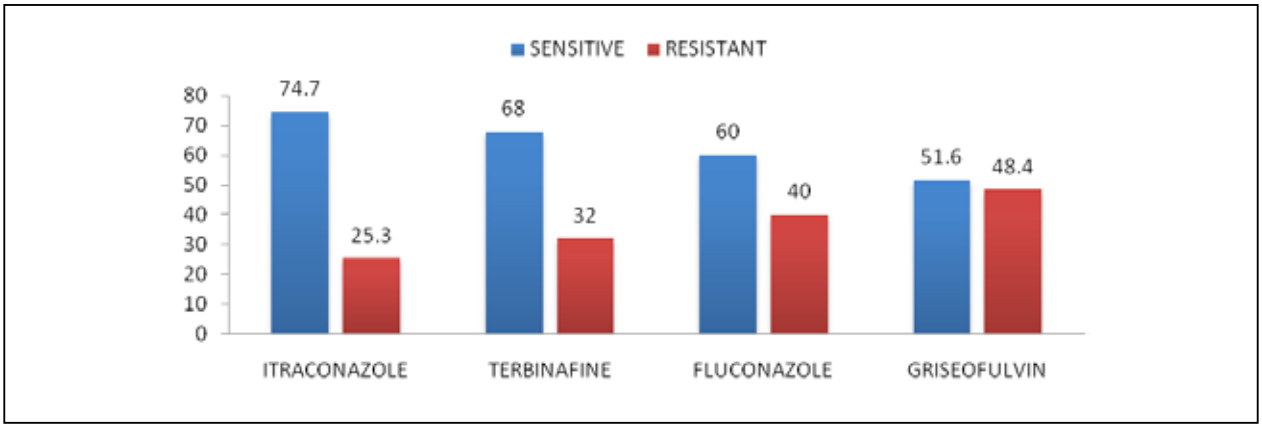

Figure 2: Percentage of sensitive and resistant non-dermatophytic fungal isolates against various antifungal drugs

Discussion

In the present study, the isolation rate of the fungus from the suspected cases of onychomycosis was 58.7%, out of all the fungal isolates 34.4% were non-dermatophytes. The isolation rates in the previous studies conducted from various parts of India are given in Table 5. This variation in the isolation rate can be attributed to varied climatic or geographic differences & incomplete antifungal treatment. This table also helps us conclude that there is rise in NDMs.

In the present study, *Fusarium species* comprised 35% of NDMs isolated followed by *Aspergillus species* in 25% of NDMs and *Candida species* was seen in 12% cases. This is in contrast to studies by Attal et al, Bassiri-Jahromi et al, and Adhikari et al who have shown Aspergillus species more common than Fusarium species.[14-16] While according to Biradar et al, Fusarium species was most commonly isolated followed by Aspergillus species.[17]

For non – dermatophytes, the order of in-vitro activity was terbinafine (GM - 0.27µg/mL) >itraconazole (GM - 0.57µg/mL) > griseofulvin (GM – 2.45µg/mL) > fluconazole (GM - 32.85µg/mL). This results were in concordance with the study by Gupta et al where the order of in vitro activity was terbinafine > itraconazole > fluconazole.[18] In same study by Gupta et al, MIC range, MIC50 and MIC 90 of non- dermatophytes for

itraconazole was 0.06 - >8µg/mL, 4µg/mL and >8µg/mL, for terbinafine it was 0.06 - >2µg/mL, 2µg/mL, >2µg/mL and for fluconazole, 1 - >64µg/mL, 64µg/mL and >64µg/mL , respectively.[18] Biancalana et al also concluded that for NDM, the MIC range and median for terbinafine was 0.008–4.10 µg/mL and 2.05µg/mL and for itraconazole was 0.05–8.0 µg/mL and 0.25 µg/mL.[19] In the past study of Garcia-Effron et al, terbinafine exhibited a good activity in vitro with a geometric mean (GM) of MICs of 1.57µg/mL. However, MIC values ranged between 0.03 and >16 µg/mL.[20]

According to the present study, in case of *Fusarium species*, the mean MIC of terbinafine, itraconazole, fluconazole and griseofulvin was 0.24µg/mL, 0.63µg/mL, 29µg/mL and 2.1µg/mL, respectively. According to Ghannoum et al, for *Fusarium species*, the mean MIC of terbinafine, itraconazole, fluconazole and griseofulvin were >16µg/mL, 6µg/mL, >64µg/mL and 64µg/mL, respectively.[21] In a study by Alastruey – Izquierdo et al, for MIC range for terbinafine and itraconazole was 0.25-32µg/mL and 1-16µg/mL, respectively.[22]

In our study, for *Aspergillus species*, the mean MIC of terbinafine, itraconazole, fluconazole and griseofulvin was 0.31µg/mL, 0.59µg/mL, 36.7µg/mL and 2.75µg/mL. respectively. Ghannoum et al reported that for *Aspergillus species*, the mean MIC of terbinafine, itraconazole, fluconazole and griseofulvin was 0.53µg/mL, 0.375µg/mL, >64µg/mL and >64µg/mL, resepectively.[21] In a past study by Lalitha et al, the MIC 50 of itraconazole for overall NDM, *Aspergillus species* and *Fusarium species* was 0.5 µg/mL, 0.125 µg/mL and >8 µg/mL, respectively.[23]

For *Candida species*, the mean MIC of terbinafine, itraconazole, fluconazole and griseofulvin was 0.39µg/mL, 0.46µg/mL, 31.27µg/mL and 4µg/mL respectively. For Candida albicans, the GM of terbinafine, itraconazole, fluconazole and griseofulvin were 0.43µg/mL, 0.54µg/mL, 26.3µg/mL and 4µg/mL respectively. For Non albicans candida, the GM of terbinafine, itraconazole, fluconazole and griseofulvin were 0.35µg/mL, 0.55µg/mL, 40µg/mL and >4µg/mL respectively. In a past study by Bueno et al, in case of C. albicans, the mean MIC for terbinafine, itraconazole and fluconazole were 0.69µg/mL, 0.097µg/mL and 0.65µg/mL, respectively.[24] While in case of C.parapsilosis, the mean MIC for terbinafine, itraconazole and fluconazole were 0.67µg/mL, 0.083µg/mL and 0.98µg/mL, respectively. This higher mean MIC for candida species to fluconazole in our study can be due to emerging or growing resistance to fluconazole causing higher dose requirement. According to Ryder et al, the MIC50 of terbinafine for C. albicans and C. parapsilosis was 1µg/mL and 0.06µg/mL, respectively. The MIC50 of fluconazole for C. albicans and C.parapsilosis was 0.5µg/mL and 1µg/mL, respectively.[25] In our study, slight high MICs obtained for Candida species with terbinafine are consistent with the differential fungistatic or fungicidal activity that has been previously reported by Gupta et al, therefore, we would recommend cautious use of terbinafine against different Candida strains.[26] Ghannoum et al also observed a wide range in the MICs of the non-dermatophyte molds and yeasts, with Itraconazole and terbinafine showed greater activity against the filamentous molds and fluconazole higher antifungal activity against the yeasts.[21] However, in our study mean MIC for fluconazole was higher both for filamentous fungi and yeasts as compared to other anti - fungal, that can be attributed to the fact that fluconazole being most commonly used and misused antifungal drug for the fungal infection, making genera resistant to them or requirement of higher dose of fluconazole for Non dermatophytic fungi. This result is in concordance to Zisova et al, who recommended that higher weekly doses (300–450 mg) of fluconazole is required when the offending agent is a NDM.[27]

Various interlaboratory variation in antifungal MIC data can be due to the batch of growth medium, performance of the medium from different manufacturers, the pH and even the solvent used to prepare antifungal stock solutions, in addition to long-established sources of variation such as inoculum size, incubation time, end-point criterion and, in the case of azole antifungals, the 'trailing growth' effect.[28-33] Laboratories in USA prefers the use of microdilution plates with U-bottomed wells for antifungal susceptibility testing, and method M27-A stipulates the use of such plates, yet in Europe flat-bottomed wells are the commonly used. There are various unpublished anecdotes of, like the use of CO_2 versus air incubators and sealed versus unsealed microdilution plates, according to judgement, availability or laboratory habit, indicate further possible sources of variation in test outcomes.

Shortcoming of the study was that Differentiation in species for non - dermatophytic fungi was not done in our study, it plays an important role in antifungal susceptibility pattern of non-dermatophyte. Different species in same genus show different pattern of anti-fungal susceptibility as well as different pattern of prevalence with varied geographical areas. Higher dilutions of the drugs are required to find the Minimum inhibitory concentration of drugs against those isolates that showed in vitro resistance to drug.

MIC breakpoints have not yet been established for onychomycosis, but it still remains unclear whether the in vitro activity of antifungal drugs is predictive of the clinical outcome.

Conclusion

Fusarium species were the most common non-dermatophyte isolated followed by Aspergillus species. Maximum sensitivity for overall non dermatophytes were seen with Itraconazole > Terbinafine > Fluconazole > Griseofulvin. Fusarium species was most sensitive to terbinafine and least to griseofulvin. Aspergillus species was most sensitive to itraconazole and least to griseofulvin. Candida species was most sensitive to fluconazole and resistant to griseofulvin. Rhizopus species and Neoscytalidium species were most sensitive to terbinafine and resistant to fluconazole species. Penicillium species was most sensitive to terbinafine and griseofulvin and least to fluconazole. Alternaria species was sensitive to all the four drugs. Bipolaris species was most sensitive to terbinafine and itraconazole, and least to fluconazole and griseofulvin. Curvularia species was most sensitive to terbinafine and least sensitive to fluconazole.

Due to the paucity of the literature about the MIC for individual antifungal drugs for non-dermatophytes, it was difficult to standardise the MIC of various drugs for non – dermatophytes. Further studies are required in this field to standardise the fungal susceptibility procedure, to prevent the variation in testing procedure hence causing variation in the results. The standardised technique will help which further reduce the development of resistance to various antifungal agents by initiation of target - centered treatment.

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