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In vitro Susceptibility of Dermatophytes to Ketoconazole, Fluconazole and Tolnaftate

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The present study was undertaken with following aim and objectives. Isolation and identification of different aetiological agents causing dermatophytosis and to study antifungal susceptibility testing of isolated fungi. Isolation and identification was done by macroscopic, microscopic and biochemical tests.In-vitro drug susceptibility testing was done by broth macro dilution method The present study for isolation, identification and in-vitro drug susceptibility testing was done on 250 clinically diagnosed cases of dermatophytosis. Out of 250 cases of dermatophytosis, 138 cases (55.2%) were positive in direct microscopic examination (KOH) and total of 106 cases (42.4%) were positive in culture. 102cases (40.80%) were positive in direct examination (KOH).MIC range of ketoconazole for all the isolates was 0.25-8μg/ml.MIC range for all the isolates against fluconazole was 0.25-16μg/ml.MIC range for all the isolates against tolnaftate was 0.5->32μg/ml. This study highlighted that,in vitro susceptibility pattern indicates that dermatophytes are more susceptible to ketoconozole and fluconazole than tolnaftate.

Key words: Dermatophytosis, Dermatophytes,Tinea, *Trichophyton,* Drug susceptibility.

Dermatophytosis is a colonization by a dermatophytic fungus of the keratinized tissues the nails, the hair and the *stratum corneum* of the skin[1]. The degree of immunosuppression and the number of immunosuppressed patients are increasing at an unprecedented pace, the management of dermatophytoses would be a definite challenge to mankind in the years to come[2].

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In India which is a tropical country, the cause of dermatophytoses is adversely influenced by economic factors like poverty, poor hygiene and social conditions like overcrowding. Nature of dermatophytoses may change with passage of time, living population, evolution of preventive measures and hygienic conditions in society[3].

Dermatophytosis produce a dermal inflammatory response with intense itching and also of cosmetic importance[4]. Though various species of dermatophytes produce clinically characteristic lesions, but a single species may produce variety of lesions depending upon site of infection[5].

Dermatophytoses is a trivial disease but has lot of psychological effect and a costly disease in terms of treatment. Management depends on random selection of antifungal agents. But in-vitro

resistance has been encountered during the treatment of these lesions[6]. In order to predict the ability of a given antimycotic agent to eradicate dermatophytes, determination of the in-vitro susceptibility of dermatophytes may prove helpful[7]. So the present study was concerned with in-vitro drug susceptibility testing of dermatophytes.

MATERIALS AND METHODS

The study was done in the department of Microbiology Kasturba Medical College Mangalore over a period of two years.

Clinically suspected 250 cases of dermatophytosis attending the skin and venereal diseases in the out patient department of Kasturba Medical college hospital, Wenlock hospital and Lady Goschen Hospital were studied.

After history, clinical examination was done. The patient was made to sit in the goodlight and clinical examination of lesion was done. It includes number of lesions, types, presence of inflammatory margin, etc.

In vitro susceptibility of dermatophytes isolates obtained from the 250 clinically diagnosed cases of dermatophytosis, were studied using broth macro dilusion method.Total number of isolates tested were 106.

Drugs

Antifungal agents included commonly used anti fungal agents against dermatophytes both systemic and topical agents. Ketoconazole (Hi-media) Fluconazole (Hi-media) Tolnaftate (Hi-media).

Preparation of Stock Solution of Drugs

10mg each of ketoconazole, and Tolnaftate were dissolved in 1ml of dimethyl sulfoxide (DMSO). To this 9ml of sterile distilled water was added. Fluconazole was dissolved in 10ml of distilled water. Stock solution containing 1000µg/ml of the drug was stored by refrigeration. Fresh stock was prepared every month. Stock solution was further diluted with sterile distilled water to give appropriate dilutions[8].

Preparation of inocula

The isolates which were subcultured on sabourauds dextrose agar and incubated at room temperature for 10-14 days were taken. Growth was scraped with a flame sterilized thick bent wire, crushed and macerated thoroughly in a sterile mortar and pestle with sterile distilled water under aseptic conditions. Fungal suspension was further diluted with distilled water. Heavy particles of the suspension were allowed to settle for 3 to 5min. The final inoculums size was adjusted with a spectrophotometer at a wavelength of 530nm to a transmittance of 95%[8].

Determination of initial MIC

For each fungal strain tubes with the volume of 10ml, were prepared .Each antifungal agent (0.1ml) dilutions were added to tubes. The final concentration of drug achived in the tubes were in the range of 0.25,0.5,1,2,4,8,16 and 32µg/ml .Then 0.9ml of diluted fungal suspensions with RPMI 1640 was added to each tube.

One tube was taken which contained a 0.9ml volume of inoculums suspension and a 0.1ml volume of drug free medium (to assess the inhibitory effect of medium on fungal growth).

One tube was for (growth control) consisted of fungal suspensions with RPMI 1640.

One tube was for sterility control was run in parallel by including a 1ml volume of uninoculated drug free medium.

Tubes were incubated at 30°C for 48 hr. Growth control tubes were observed for the presence or absence of visible growth. When growth was visible, the growth in each tube was compared with that of the growth control tube. Optical density (OD) of each tube which was obtained from a spectrophotometer at a wavelength of 530nm was used to find the amount of reduction in turbidity as compared to that of the drug free control tube. MIC at which 50% of the isolates were inhibited (MIC_{-50}) was determined.[8]

RESULTS

The present study for isolation, identification and in-vitro drug susceptibility testing was done on 250 clinically diagnosed cases of dermatophytosis.

Out of 250 samples isolated 215(86%) were skin scraping, 24(9.6%) were nail clipping and 11 (4.4%) were hairs stubs. Out of 250 cases of dermatophytosis, 138 cases (55.2%) were positive in direct microscopic examination (KOH) and total of 106 cases (42.4%) were positive in culture.

Table 1. Results obtained in the direct microscopy and culture

	KOH Positive (n%)	KOH negative(n%)	Total(n%)
Culture positive	102 (40.80%)	4 (1.6%)	106 (42.4%)
Culture negative	36(14.4%)	108 (43.2%)	144 (57.6%)
	138 (55.2%)	112 (44.8%)	250 (100%)

Table 2. Incidence of various species of dermatophytes

Species	No. of isolates	Percentage
Trichophyton rubrum	69	65.09%
Trichophyton mentagrophyte	19	17.92%
Trichophyton violaceum	4	3.78%
Epidermophyton floccosum	9	8.49%
Microsporum audouinii	5	4.72%
	106	100%

Table 3. *In vitro* susceptibility of dermatophytes to ketoconazole

Organisms (Isolates)	Cumulative no. of isolates inhibited at indicated concentration (µg/ml)								MIC range (µg/ml)	MIC_{50} (µg/ml)
	0.25	0.5	1	2	4	8	16	32		
T. rubrum (69)	9	23	34	55	69	69	-	-	0.25-4	1
T.mentagrophyte (19)	-	4	6	9	17	19	-	-	0.5-8	2
T. violaceum (4)	-	2	3	3	4	4	-	-	0.5-4	-
E.floccosum (9)	-	3	4	6	7	9	-	-	0.5-8	-
M.audouinii(5)	-	-	5	5	5	5	-	-	1	-
106	9	32	52	79	102	106	-	-	0.25-8	1

MIC_{50} (50% of isolates inhibited) values taken for those species whose isolates numbered more than 10.

Table 4. *In vitro* susceptibility of dermatophytes to fluconazole

Organisms (Isolates)	Cumulative no. of isolates inhibited at indicated concentration (µg/ml)								MIC range (µg/ml)	MIC_{50} (µg/ml)
	0.25	0.5	1	2	4	8	16	32		
T. rubrum (69)	2	7	28	34	52	69	69	-	0.25-8	2
T.mentagrophyte (19)	-	4	7	10	16	17	19	-	0.5-16	2
T. violaceum (4)	-	1	2	2	3	4	4	-	0.5-8	-
E.floccosum (9)	-	3	4	4	6	9	9	-	0.5-8	-
M.audouinii(5)	-	1	2	2	5	5	5	-	0.5-4	-
106	2	18	42	52	82	104	106		0.25-16	2

MIC_{50} (50% of isolates inhibited) values taken for those species whose isolates numbered more than 10.

102cases (40.80%) were positive in direct examination (KOH) as well as culture. In 4 cases (1.6%) direct examination was negative but they were culture positive. 108cases (43.2%) were negative in both direct examination and culture.Sensitivity was 73.9%, Specificity was 96.4%, Positive predictive value was 96.2%, Negative predictive value was 75%. (Table 1)

Out of total 250 clinical isolates 106 cases(42.4%) were culture positive. *Trichophyton rubrum* were the commonest isolates 69(65.09%) other isolates were *Trichophyton mentagrophytes* 19(17.92%), *Trichophyton violaceum* 4(3.78%), *Epidermophyton floccosum* 9 (8.49%), *Microsporum audouinii* 59(4.72%). (Table 2)

All the isolates were inhibited within a concentration of 8µg/ml.32 isolates were inhibited within 0.5µg/ml.MIC_{50} for *T. rubrum* isolates were 1µg/ml and *T.mentagrophyte* were 2µg/ml.MIC range for *T. rubrum* was within 0.25-4µg/ml.MIC range for all the isolates against ketoconazole were distributed over a range of 0.25-8µg/ml.MIC_{50} for all isolates against ketoconazole was at 1µg/ml. (Table 3)

All the isolates were inhibited within a concentration of 16µg/ml.18 isolates were inhibited within 0.5µg/ml.MIC_{50} for *T. rubrum* isolates were 2µg/ml and *T.mentagrophyte* were 2µg/ml.MIC range for *T. rubrum* was within 0.25-8µg/ml.MIC range for all the isolates against fluconazole were distributed over a range of 0.25-16µg/ml.MIC_{50} for all isolates against fluconazole was at 2µg/ml. (Table 4)

101 isolates were inhibited within a concentration of 32µg/ml.53 isolates were inhibited within 8µg/ml.MIC_{50} for *T. rubrum* isolates were 8µg/ml and *T.mentagrophyte* were 8µg/ml.MIC range for *T. rubrum* was within 2->32µg/ml.MIC range for all the isolates against Tolnaftate were distributed over a range of 0.5->32µg/ml.MIC_{50} for all isolates against Tolnaftate was at 8µg/ml. (Table 5)

MIC range of ketoconazole for all the isolates was 0.25-8µg/ml.MIC range for all the isolates against fluconazole was 0.25-16µg/ml.MIC range for all the isolates against tolnaftate was 0.5->32µg/ml. (Table 6)

Table 5. *In vitro* susceptibility of dermatophytes to tolnaftate

Organisms (Isolates)	Cumulative no. of isolates inhibited at indicated concentration (µg/ml)								MIC range (µg/ml)	MIC_{50} (µg/ml)
	0.25	0.5	1	2	4	8	16	32		
T. rubrum (69)	-	-	-	4	12	36	43	66	2->32	8
T.mentagrophyte (19)	-	-	-	2	4	9	12	17	2->32	8
T. violaceum (4)	-	1	1	1	2	2	4	4	0.5-16	-
E.floccosum (9)	-	-	1	3	3	4	9	9	1-16	-
M.audouinii(5)	-	-	1	2	2	2	5	5	1-16	-
106		1	3	12	23	53	73	101	0.5->32	8

MIC_{50} (50% of isolates inhibited) values taken for those species whose isolates numbered more than 10.

Table 6. *In vitro* activity of three antifungal drugs

Dermatophyte (species)	MIC RANGE AGAINST THE DERUGS (µg/ml)			
	No. of isolates	Ketaconazole	Fluconazole	Tolnaftate
T. rubrum	69	0.25-4	0.25-8	2->32
T.mentagrophyte	19	0.5-8	0.5-16	2- >32
T.violaceum	4	0.5-4	0.5-8	0.5 – 16
E.floccosum	9	0.5 – 8	0.5 – 8	1 – 16
M.audouinii	5	1	0.5 – 4	1 – 16
	106	0.25 – 8	0.25 -16	0.5 – >32

DISCUSSION

Among the various fungal infections of human beings dematophytes is a most common infection of the world.[2]

The results of the in-vitro sensitivity tests of the dermatophyte isolates against the three antifungal agents tested in the present study showed difference in sensitivity in MIC and MIC_{50} values. Among the drugs tested ketoconazole and fluconazole showed to be more effective followed by tolnaftate. In the present study ketoconazole inhibited 50% of the isolates (MIC_{50}) at a concentration of 1µg/ml. MIC range for ketoconazole was within 0.25 - 8µg/ml.

In the study done by Venugopal V.P., Venugopal in 1992 MIC range was 0.01-5µg/ml and ketoconazole inhibited 50% of the isolates (MIC_{50}) at the concentration of 1 µg/ml.[9] Hossein Nowrozi, Grolrokh Nazeri in 2008, reported that ketoconzole MIC range against dermatophyte isolated was 0.5-0.32 µg/ml.[8] Zafer Chetinkay Nrui Kiraz reported in 2005 MIC range for ketoconazole against dermatophytes 0.25-64 µg/ml.[7] Some isolates showed inhibition in higher concentration of drug.

Crystiane R.A. Karla C.M. et al reported in 2009 that MIC range for ketoconazole against dermatophytic isolates was 0.03- 4 µg/ml.[10] Fluconazole in the present study MIC range 0.25-16 µg/ml and inhibition of 50% of isolates(MIC-$_{50}$)at 2 µg/ml. Study done by Hossein Nowrozi, Golrokh Nazeri in 2008, reported higher MIC range 24-48 µg/ml.[8] Zafer chetinkay, Nuri Kiraz reported in 2005 MIC range for fluconazole was 0.25-64 µg/ml[7]. DA Santos, MES Barros, JS, Hamda in 2006 reported MIC range for fluconazole 0.125->64 µg/ml against dermatophyte isolates.[11] Cystiane R. A. Karla C.M. in 2009 reported in this study that MIC range of fluconaozole against isolates 2-32 µg/ml.[10]

In the present study MIC for tolnaftate ranged 0.5->32 µg/ml three isolates of *T. rubrum* were not inhibited at 32 µg/ml which was the highest concentration tested. Two isolates of *T. mentagrophyte* was not inhibited at 32µg/ml which was the highest concentration tested. MIC_{50} was 8 µg/ml.

In study done by MN Sumana & V. Rajgopal in 2002 MIC range is form 2->16 µg/ml. In this study 47.22% isolates showed inhibition at the highest concentration tested 16µg/ml.[6]

Present study also showed the development of resistance in dermatophyte against tolnaftate. The difference in MIC and sensitivity pattern could be attributed to the variable susceptibility pattern of the indigenous isolates and above all the test conditions. Test conditions have been known to have a major influence on the MIC values in antifungal sensitivity tests .Test medium varied in most of the studies. Preparation of inoculums and inoculums density also differed. Test results may also have been influenced by the use of oral drugs in the present study.

Considering the high MIC values of some of the strains tested it may be observed that those strains might not be open to eradication in clinical terms with conventional treatment protocol.

CONCLUSION

In-vitro susceptibility test of the dermatophyte isolates were done against three antifungal agents by the broth macrodilution method. The sensitivity pattern (MIC and MIC_{50}) in this study indicated that the tested isolates were more susceptible to ketoconazole and fluconazole than tolnaftate. High MIC value of some isolates suggested that those strains may not be amenable to eradication in the usual treatment protocol.Dermatophytoses is a trivial disease but has lot of psychological effect and a costly disease in terms of treatment. Management depends on random selection of antifungal agents. But in-vitro resistance has been encountered during the treatment of these lesions. In order to predict the ability of a given antimycotic agent to eradicate dermatophytes, determination of the in-vitro susceptibility of dermatophytes may prove helpful.

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