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Molecular identification of various dermatophytic infections attending a tertiary care hospital by using conventional PCR and comparing with conventional methods, Kanchipuram

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Abstract

Dermatophytic fungi affecting keratinized tissues (skin,hair,nails) of humans tend to grow at slower pace in conventional culture methods such as SDA .For their rapid and accurate species identification molecular methods like conventional PCR,RT-PCR etc ., are useful. By using random primers the nucleic acids of dermatophytes isolated from various dermatophytic infections are amplified and identified in a precise manner. PCR can be used as conventional diagnostic method in routine diagnostic laboratories as it has capacity to diagnose dermatophytic infections rapidly and accurately without need for time taking biochemical and cultural identification.

Key-Words: Dermatophytes, SDA, PCR, Keratinised tissue

Introduction

Dermatophytic infections are commonest superficial infections.Recent statastics showed that 20-25% of world population had suffered from one of this infections[1]The geographic distribution tend to depend on following factors such as climate,environmental or socioeconomic,immigration etc.,commonest species being Trichophyton rubrum,Microsporum canis, Trichophyton interdigitale; Microsporum audouinii and Trichophyton violaceum and Trichophyton tonsurans. [2,3]Accurate diagnosis of dermatophytic infections is essential for epidemiological purpose,for initiating infection control measures ,for specific antifungal therapy,preventing transmission to others[4,5]

In all routine laboratories the identification is by culture on SDA or DTM (dermatophyte mediumor by various biochemical reactions [6]

.In recent years the focus has been shifted to molecular methods for accurate species identification after detailed toxonomical classification.They have advantage of rapidity,less handilng of culture,increased sensitivity as well as specificity when compared to conventional methods.[7]

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Material and Methods

The material (skin scrapings,infected nails,infected hair with intact root)is placed in 10 – 20% KOH preparation for few minutes .For nail sample 20%KOH is used and the sample in 20%KOH is warmed under Bunsen flame before being examined.In present study only one case of T.manuum was sent for dermatophyte culture.

Dermatophyte medium

Dermatophyte medium composition was of papaic digest of soybean meal,dextrose,cyclohexamide,phenol red,chloramphenicol,Agar at final Ph 5.6 +/- 0.2 and the media is poured on to a petridish /test tube .The inoculated media is incubated at 25 degree C. Observed for growth after 24 hours every day upto 7 days. Interpretation of results : Red colour around the colony suggests positive for dermatophyte and growth without colour change indicates non-dermatophyte

SDA medium Sabourauds dextrose agar

RPeptone, water, Dextrose with PH 5.6

PCR technique 11 out of35 positive samples including Trichophytonrubrum, T.mentagrophyte, T.tonsurans, M. audouinii were further studied by PCR - RFLP.[8]

.For DNA extraction a small portion of colony was taken into a 1.5 ml tube with 300ml of lysate buffer(100Mm Tris – HCl. PH 7.5,10 ml EDTA ,0.5 % w/v SDS,100 mM NaCl),300 mL of phenol/chloroform.,300ml of glass beads (0.5mm in diameter).The entire suspention was vortexed for 5 mts,centrifuged at 5000rpm for 5 minutes and the suspention was transferred to a new tube and again

extracted by using chloroform.By using 2-proponol and 0.1 volume of 3 M sodium acetate (pH 5.2) in equal proportion the DNA was precipitated.and subjected to 208^0c for 20 minutes.The pellet was washed with 300 mL of 70% ethanol,air dried and finally the DNA was resuspended in 50 ml sterile distilled water.For PCR amplification ITS1-5.8S-ITS2 rDNA was amplifiedfor each sample by using the universal primers ITS1 (50TCCGTAGGTGAACCTGCGG-30) and ITS4 (50-TCCTCCGCTTATTGATATGC-30) in a 25 mL reaction mixture, containing 12.5 mL of premix (Ampliqon, Denmark), 2 mL of DNA template,and 0.5 mM of each forward and reverse primer [9]. PCR cycle parameters were preheating at 94 8C for6 min; 35 cycles of 30 s at 94 8C, 30 s at 58 8C, and 1 min at72 8C; the final extension time was 10 min at 72 8C.PCR products were identified by RFLP.8 ml of PCR product was treated with 0.5 ml of enzyme,1.5 ml of 10X buffer and 5 ml of water at 37 8 C for 20 minutes.PCR ampliconsand digested products were analysed by gel electrophoresis. (Tris 0.09 M, Boric acid 0.09 M,EDTA 2 mM) buffer at 100 V for approximately 60 min in 1.5%and 2% agarose gels, respectively).For identification of species size of the fragments were compared with the profile obtained previously.

Results and Discussion

In our present study all clinically diagnosed cases of superficial mycotic infections starting from October 2014 to August 2015 attending a dermatology OP in a tertiary care hospital were included.Among 220 clinically diagnosed cases of superficial mycotic infections 132 cases were clinically diagnosed as dermatophytic infections.Rest of them (88cases)were due to various other superficial mycotic infections such as P.versicolar ,P.rosea,Piedra etc., that are included under exclusion criteria in our study.(table1),fig 1

Table 1

	Number of cases	Percentage
Clinically diagnosed superficial mycotic infections due to dermatophytes	132	60%
Other infections such as P.versicolar ,P.rosea,Piedra etc	88	40%
Total number of cases	220	

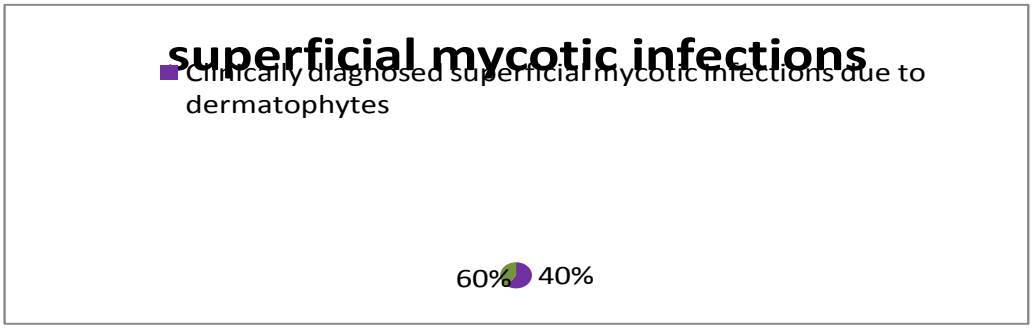


Figure 1

Table 2: Age distribution of various clinically diagnosed dermatophytic infections

S/no.	Age	Number of cases
1.	0-10	4(3.03%)
2.	11-20	16(12.12%)
3.	21-30	35(26.51%)
4.	31 – 40	37(28.03%)
5.	41 – 50	24(18.18%)
6.	51 – 60	11(8.3%)
7.	Above 60	5 (3.7%)
Total	132	

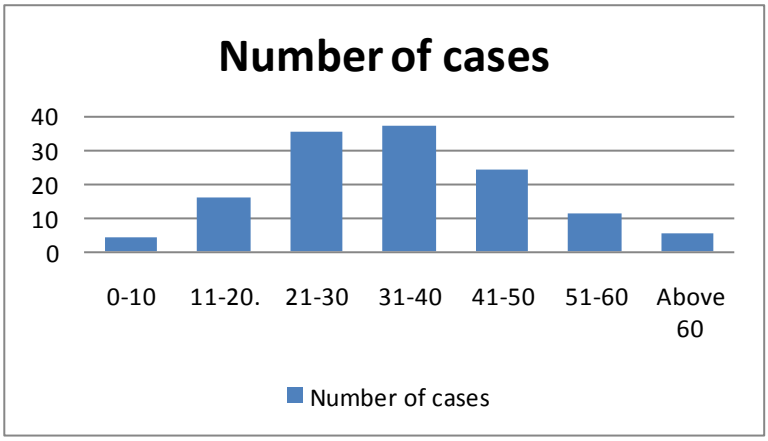


Fig 2

Out of 220 cases 132 cases were due to different dermatophytic infections and 88 cases were due to pytiriasis versicolar. In present study only dermatophytic infections are included. Among 132 cases 88 (66.6%)cases were male patients and 44(33.3%) cases were female patients.(fig 3)

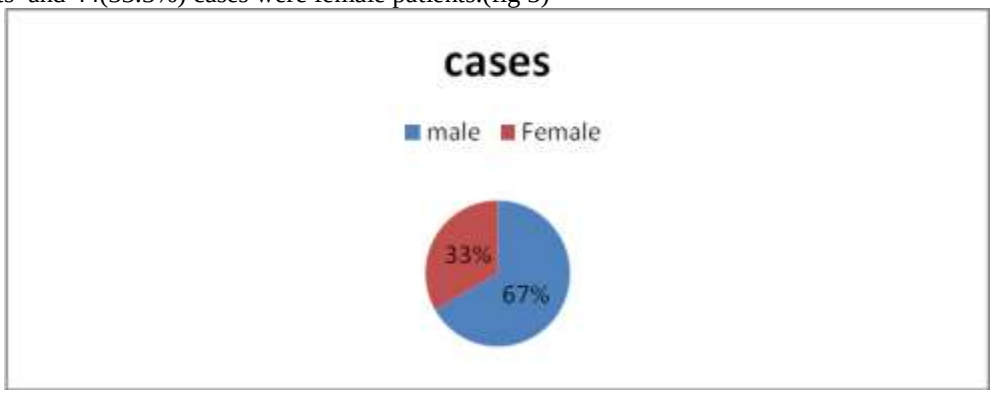
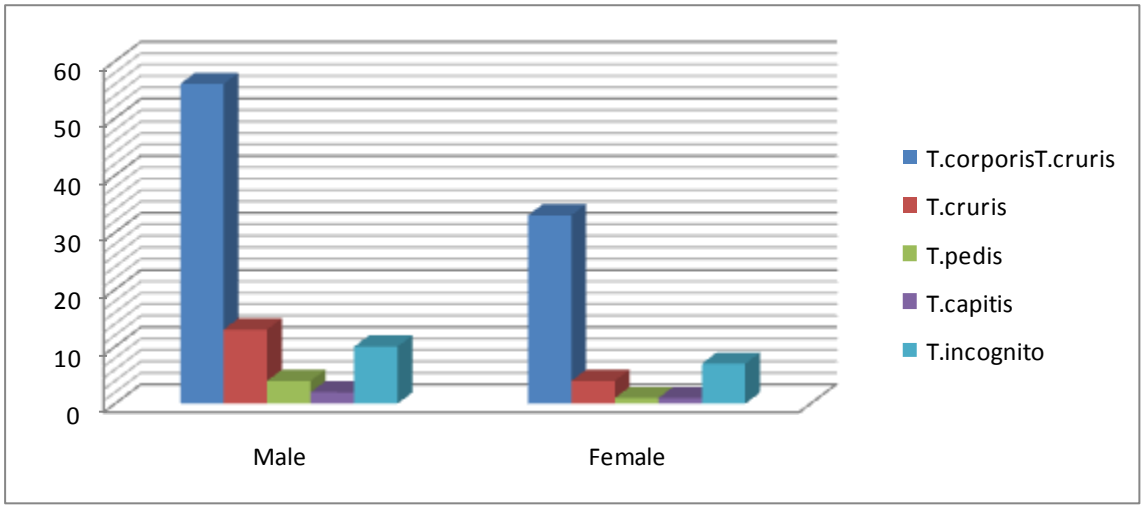


Fig 3

Table-3: SEX DISTRIBUTION OF DIFFERENT CLINICAL TYPES OF DERMATOPHYTES

Tinea corporis infections were found to be the most common

Clinical types	Male	Female	Total
T.corporis	56	33	89
T.cruris	13	4	17
T.pedis	4	1	5
T.capitis	2	1	3
T.incognito	10	7	17
Total			132



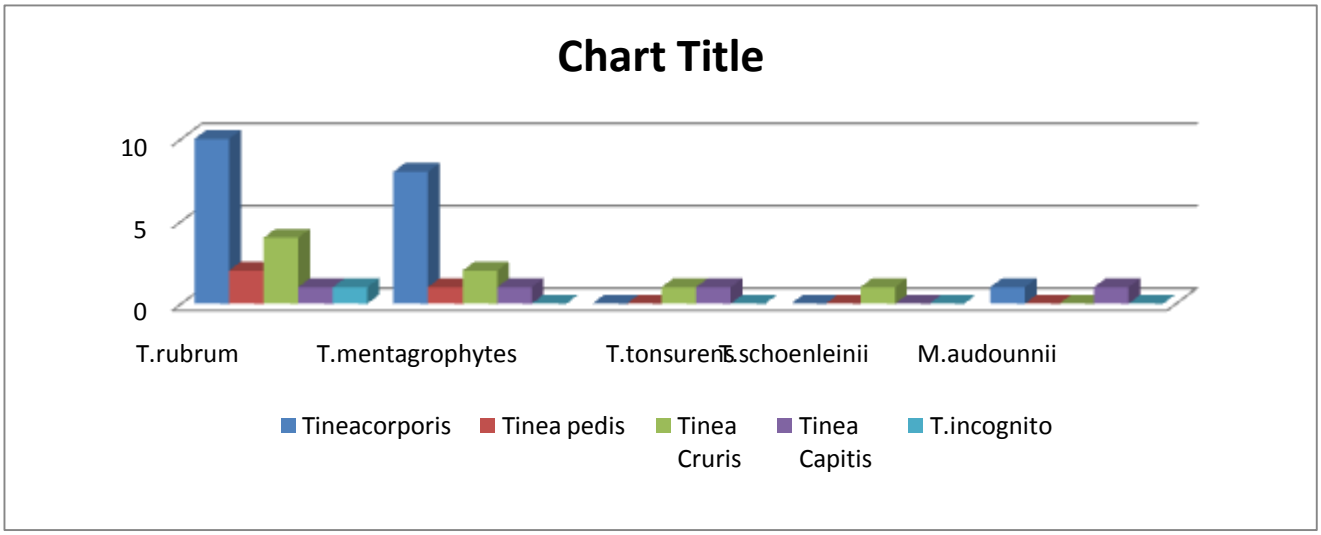
Out of 46 samples collected upon request from dermatologist for 10% KOH mount and fungal culture 35 samples(76%) have given rise to different fungal isolates that include Trichophyton rubrum,T.mentagrophytes T. schoenleinii ,M. audounnii ,M.gypseum. 11 samples were culture negative. (24%).Among culture negative cases many were from cases of T.incognito.out of 46 samples 25 samples (54.34%) showed the presence of fungal elements.21 samples(45.66%) were negative for 10%KOH mount.

Dermatophytes isolated	Tinea corporis	Tinea pedis	Tinea Cruris	Tinea Capitis	T.incognito	Total
T.rubrum	10	2	4	1	1	18(51.45%)
T.mentagrophytes	8	1	2	1	--	12(34.28%)
T.tonsurens	-	0	1	1	-	2(5.7%)
T.schoenleinii	-	0	1		-	1(2.8%)
M.audounnii	1	0	-	1	-	2(5.7%)
Total	19(54.28%)	3(8.5%)	8(22.8%)	4(11.4%)	1(2.8%)	35

Comparison of culture positivity and KOH positivity

	KOH positive	KOH negative	Culture positive	Culture negative
Total number of samples processed 46	25	21	35	11
Culture positivity in KOH positive cases	25	-	25	nil
Culture positivity in KOH negative cases	-	21	10	11

Total dermatophytes isolated - 35



The two sets of SDA and DTM were inoculated with properly collected sample from growing edge of the lesion and incubated at 25°C and 37°C.The growth in DTM had resulted in colour change to red colour because of the presence of phenol red indicator and due to production of alkaline biproducts.Th e growth was further studied for morphology of colony, rate of growth, typical microscopic morphology,(macroconidia.microconidia). Further morphology is confirmed by slide culture.speciation is done based on microscopic morphology and hair perforation test, urease test

Christesen urease medium with 1% glucose were inoculated with growth obtained from primary isolation media for 7-14 days incubated at 25°C.

Hair perforation test

The hair were inoculated sterilised and placed in distilled water with sterile yeast nitrogen base.The colonies of dermatophytes are inoculated by touching the hair and incubated at room temparature for 14 – 20 days T.mentagrophyte were positive for urease test and hair perforation test.

COMPARISON OFNUMBER OF CULTURE POSITIVES ON DTM AND SDA

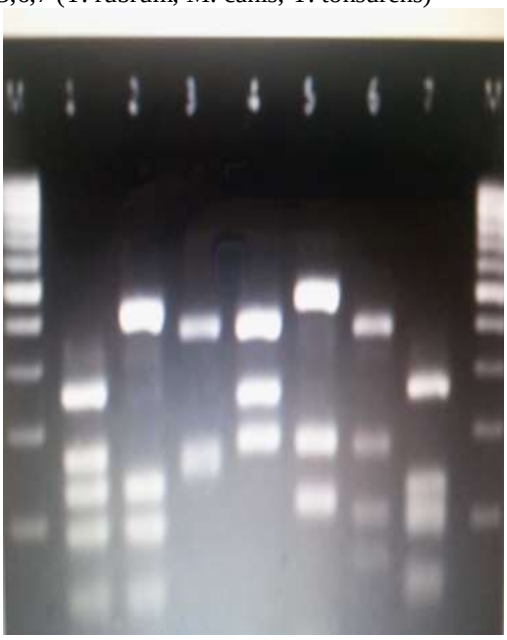
	Positive cases	Percent age
Total number of cultures	46	-
Total number of dermatophytes	35	76.08%
Cutures positive by DTS	35	76.08%
cultures positive by SDA	33	71.73%

Out of 46 culture for isolating dermatophytes 35 were culture positive and were isolated on DTM (76.08%)where as 11 were culture negative.whereas on SDA 71.73% i.e.,33 cases were isolated .

Identification of germatophytes by PCR METHOD

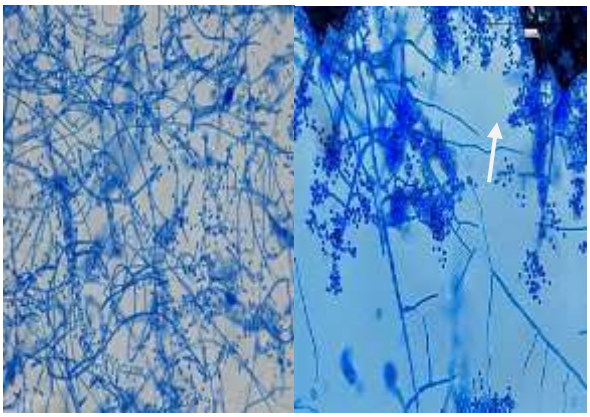
Among 11 isolates that were T.rubrum were identified as T.rubrum even by PCR where as two T.rubrum by conventional culture was found to be T.interdigitale .T.mentagrophytes were identified both by culture and PCR,Microsporum canis was identified by PCR,T.violaceum was also identified by PCR which were misdiagnosed by culture on SDA.

LANE 1 AND LANE2 (T.interdigitale), LANE 3,4,5,6,7 (T. rubrum, M. canis, T. tonsurens)



Different dermatophytes isolated on special dermatophyte media showing colour change

T. rubrum on DTM Growth of M.canis and T. mentagrophytes on SDA



T. rubrum showing microconidia
T. mentagrophytes with microconidia

Most of the dermatophytic infections are usually diagnosed and treated based on clinical presentations alone , as it takes long time to obtain culture report Microscopic examination of the sample often needs expertise to identify fungal elements with dermatophyte morphology.However it offers painless and rapid results. As many of the non-infective conditions often present with similar clinical presentation , culture identification of deramtopthytic infections are usually recommended for those cases that require prolonged treatment with antifungal agents [(10).] In our present study also out of 132 cases clinically diagnosed as dermatophytic infections only 46 cases were requested for culture confirmation for dermatophytes and for 10% KOH mount..Among 132 clinically diagnosed cases male to female ratio was 1:2 i.e., 88 :44.This finding is correlating with many studies [(11,12)] The age distribution is also correlating to several studies.i.e., more common among 30-40 years age group.[(13,14).]Seasonal distribution more common during October to December months.out of 46 cases 35 cases were culture positive i.e., 76% and 24% were culture negative on special dermatophyte media whereas 25 cases (54.34%)were positive for 10% KOH mount and 21 cases were negative .(45.66%).This is correlating with other studies [(15,16,17)] 59.20%,53.3%,49% ,however some of the studies show that 10%KOH preparation is more sensitive [(18,19,20)].The commonest infections were Tinea corporis(54.28%) and the commonest causative organism was Trichophyton rubrum(51.45%).This is correlating with many other studies in India [(21)]In present study the 10%KOH mount showed 54.34% positive i.e.,25 positives out of 46 samples where as culture positive were 35 out of 46 clinical cases(76%).All 10%KOH positive cases were positive even for culture .This can be attributable to personel collection of sufficient samples from active growing edge of the lesion& their proper transportation .According to some studies 10%KOH is more sensitive than fungal culture[(22)] .however in our study fungal culture was more sentive(76%) this is correlating other studies where culture positive was 79.1%(3) 62.8%14 [16]

By using dermatophyte media with chloramphenicol and cycloheximide the contamination is reduced to minimum. The growth is soon identified by colour change in the medium because of production of alkaline biproducts by growth of dermatophyte and non-dermatophyte produces no colour change.SDA medium there is greater chances for contamination and by the time the appears the medium is subjected to dehydration [23.]

Recently DNA BASED techniques like species-specific PCR, PCR-RFLP, arbitrarily primed PCR [AP-PCR], sequencing of different regions of the DNA,real-time PCR, nested PCR, and various targets such as ribosomal-DNA (rDNA),beta tubulin gene, and mitochondrial DNA (mtDNA) are found to be useful for identification of dermatophytes [24,25]

Different studies show that PCR – RFLP is easy to perform,rapid,inexpensive and reliable method to differentiate dermatophytes to species level.Thus the present study is done to evaluate the accuracy of species identification by PCR – RFLP.The most interesting finding in our study was comparison of conventional culture methods and molecular methods.(DNA based).T.interdigitale is often confused with T.rubrum ,colony morphology of both are similar.That can be distinguished by PCR.[26] T.interdigitale produces scattered microconidia with no macroconidia,so it may resemble T.rubrum.[27]Microsporum canis which was misdiagnosed by conventional culture methods were identified by PCR – RFLP in present study.

Thus in conventional culture methods the species identification is often mistaken.For epidemiological purposes,out break investigation the conventional methods are almost of no use and can be done by genotype based methods.However its cost effectiveness precludes primary diagnosis of various infections apart from dermatophytosis.

Conclusion

The dermatophytic infections are common in males with age group 30-40years and are common among superficial infections.For laboratory identification& diagnosis even though microscopy is Gold standard , isolation by using DTM for morphological identification& speciation or by rapid modern molecular techniques are useful for their prevention and cure.

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