*Proceeding The 1st IBSC: Towards The Extended Use Of Basic Science For Enhancing Health, Environment, Energy And Biotechnology*
*ISBN: 978-602-60569-5-5*

# Identification of Dermatophytes by Multiplex-Polymerase Chain Reaction, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism ITS1-ITS4 Primers and MvaI, and Polymerase Chain Reaction $(GACA)_4$ Primer

Rizalinda Sjahril[1], Firdaus Hamid[1] , Aan Yulianingsih[1], Novita Prastiwi[1], Awaluddin[1], Siska Nuryanti[2], Faridha Ilyas[1], Burhanuddin Bahar[3]
[1] Faculty of Medicine, Hasanuddin University, Makassar
[2] Faculty of Pharmacy, Universitas Muslim Indonesia, Makassar
[3] Faculty of Public Health, Hasanuddin University, Makassar
rizalinda@unhas.ac.id

**Abstract -** Laboratory identification of skin lesion is important for the correct diagnosis and choice of therapy. Microscopic examination of skin or nail scraping or hair fragments in 10%-KOH provides rapid result but fungal growth in culture is required for identification of species. Unfortunately, culture requires a few days to 2 weeks, and there is variable colony appearance and colour. Rapid and correct diagnosis has been enabled by Polymerase Chain Reaction (PCR), but has not yet been applied for routine diagnosis of patients. Therefore we investigated the ability of culture using Saboraud Dextrose Agar, multiplex-PCR, PCR-RFLP with ITS1-ITS4 primers and *Mva*I, and PCR with $(GACA)_4$ primer to identify of the etiology agents of 130 patients with tinea who were positive showing hyphae in 10%-KOH preparation. Skin scrapings were collected in Makassar during January-June 2016 and examinations were carried out in the Microbiology Laboratory of Hasanuddin University. Results: Dermatophytosis occurred in 73 (56,1%) males, and 57 (43,8%) females. Scraping was obtained from 78 (60%) skin and 52 (40%) nail lesions. Based on age stratification, 68 (52,3%) were 10-18 years old, 43 (33%) were 19-45 years old, and 19 (14,6%) were >45 years old. While 39 (30%) samples grew in culture, Multiplex-PCR, PCR-RFLP with ITS1-ITS4 primers and *Mva*I, and PCR with $GACA_4$ primer amplified DNA of 130 (100%), 126 (96,9%), and 106 (81,5 %) samples, respectively. Multiplex-PCR was not able to distinguish between spesies in 99 (76,2%), PCR-RFLP with ITS1-ITS4 primers and *Mva*I in 29 (22,3%) and PCR with $GACA_4$ primer in 20 (15,4%) samples.

**Keywords** - Dermatophytes, Culture, Multiplex-PCR, Restriction Fragment Length Polymorphism

## INTRODUCTION

Dermatophytosis are the most common infections of the skin, nail and hair affecting 20-25% of the world's population [7] . It is caused by dermatophytes which includes genera Epidermophyton, Microsporum, and Trichophyton. Symptoms may range from mild erythematous rashes on the skin to severe kerion-type lesions with pus formation in micro-abscesses [1].

Definitive laboratory criteria include positive microscopic evidence of septate hyphae and/or arthroconidia (10% -KOH preparation, Calcofluor white), periodic acid Schiff, and/or biopsy, and macroscopic features of colony morphology on selective media and microcopic examination of conidia [6]. Microscopic examination of skin or nail scraping or hair fragments in 10%-KOH provides rapid result but no information about the causative fungal species, meanwhile phenotypic techniques require experienced technologist and prolonged turn around time. Rapid, sensitive and specific diagnosis has been now enabled by Polymerase Chain Reaction (PCR)[9], but has not yet been applied for routine diagnostic in hospitals in Makassar, Indonesia. Therefore we investigated the ability of culture using Saboraud Dextrose Agar, multiplex-PCR, PCR-RFLP with ITS1-ITS4 primers and *Mva*I, and PCR with $GACA_4$ primer in identifying the causative fungal agents direct from scrapings of skin or nails from persons in Makassar who suffered from tinea.

## MATERIALS AND METHODS

a. Clinical Samples

From Januari to June 2016 927 participants were screened for signs of tinea by medical doctors among the authors of this study. Participants were patients attending a private skin clinic, healthy elderly residing at a government-owned elderly home care in Sungguminasa, and healthy children at 9 schools in slum districts of Makassar. All participants upon screening day admitted not having antifungal treatment in the last 2 weeks. Each participants gave a written informed consent prior to skin or nail scrapings. Each school children had a guardian or one of their parents signed the informed consent. The study was approved by the Ethical Committee of Hasanuddin University (No.195/H4.8.4.5.31/PP36-KOMETIK2016). Participants were checked for signs of tinea lesions on the skin surface, head, back, neck, abdomen, and extremities after interviewed on signs of itchiness and redness on their skin. Suspected lesions were swiped with 70% alcohol, scraped using sterile blunt knife and collected into an ultraviolet-sterilized folded black card. All skin scales or nail scrapings that showed hyphae in 10%-KOH preparation were then cultured and underwent DNA extraction.

b. Positive Control Strain

A *Trichophyton mentagrophytes* isolate that is positive by PCR and is sequencing-confirmed as a dermatophyte was used as positive control in all the molecular tests used in this study.

c. 10%-KOH Test

Using inoculation loop scales of skin or scraping of nail were placed on a slide, added a drop of 10%-KOH then a cover glass was placed on top. Microscopical observation was done to identify the presence of hyphae, microconidia and or macroconidia.

d. Culture from Skin scales and Nail Scrapings

Saboraud Dextrosa Agar (SDA) (Oxoid) was supplemented with 0.5 µg/mL cycloheximide (Oxoid) to inhibit the growth of yeasts and 0.05 µg/mL chloramphenicol (Oxoid) to avoid the growth of contaminants Scales of skin or scraping of nail were inoculated on SDA using sterile inoculation loop and allowed for incubation at 30°C[3]. Each SDA plate was observed for growth every 3 days until day 30. Strain identification was based on the combination of morphological (colour, texture) and microscopic appearance (microconidia and or macroconidia).

e. Extraction of Fungal DNA using gSYNC extraction kit (Geneaid, Taiwan)

Scales of skin or scraping of nail were placed into a 1.5 ml tube, and extraction was performed according to the guideline book included in the kit. Two hundred µL GST buffer and 20 µL Proteinase-K were added and vortexed, then allowed to incubate overnight at 60°C. Supernatant was collected and transferred to a new tube. Two hundred µl of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously for 10 seconds. All of the mixture (including

any insoluble precipitate) was transferred to the GS Column in a 2 ml collection tube. Following 1 minute centrifugation at 14-16,000 x g, the 2 ml collection tube containing the flow-through is discarded. Then the GS Column is transferred to a new 2 ml collection tube. Four hundred µl of W1 Buffer was added to the GS Column, centrifuged at 14-16,000 x g for 30 seconds and the flow-through is discarded. Then the GS Column is transferred to a new 2 ml collection tube. Six hundred µl of Wash Buffer is then is aded to the GS Column, centrifuged at 14-16,000 x g for 30 seconds then the flow-through is discarded. The GS Column is then placed back in the 2 ml collection tube. Additional centrifugation at 14-16,000 x g for 5 minutes or incubation at 60ºC for 5 minutes completely dried the GS Column. The dried Transfer GS Column was transferred to a clean 1.5 ml microcentrifuge tube and 100 Pre-heated Elution was added at the center of the column matrix. Centrifugation at 14-16,000 x g for 30 seconds eluted 200 µL of the purified DNA in the new tube. The extracted volume is kept at -20°C until ready for use.

f. Identification using multiplex PCR

Procedures in this multiplex PCR is based on the methods explained elsewhere [4]. The primers used were ITS1-2, 18S ribosomal RNA, and 28S ribosomal RNA. Total reaction mixture was 25 µL which included 12.5 µL of Kapa2G Fast Ready mix with dye (Kapabiosystems), 0.5µL of $MgCL_2$, 1 µL of primers, 5 µL of DNA extract, and 6 µL of nuclease free water. The reaction conditions after optimization using ITS 1 and 2 primers consist of hot step at the beginning for 5 minutes at 94°C, 34 cycles of 1 minute of denaturation at 94°C, 30 minutes of annealing at 60°C, and 1 minute of extension at 72°C, and a further 10 minutes as final extension at 72°C. The reaction conditions after optimization using 28S ribosomal RNA consist of hot step for 7 minutes at 94°C, 34 cycles of 1 minute of denaturation at 94°C, 30 minutes of annealing at 50°C, and 1 minute of extension at 72°C, and a further 10 minutes as final extension at 72°C. The reaction conditions after optimization using 18S ribosomal RNA consist of hot step for 7 minutes at 94°C, 34 cycles of 1 minute of denaturation at 94°C, 30 minutes of annealing at 57.5°C, 1 minute of extension at 72°C, and a further 10 minutes as final extension at 72°C.

All PCR in this study were performed using Icycler (Biorad) at the Microbiology Laboratory of Hasanuddin University Hospital, Makassar, South Sulawesi, Indonesia.

Five µL of the amplified DNA were observed on 2% agarose gel in TAE buffer stained with ethidium bromide (EtBr).

g. Identification using ITS-based PCR and restriction using *Mva*I (RFLP)

Identification by ITS-based was performed according to a previous publication[5], but in this study only one restriction enzyme was used. Amplification reactions were carried out with volumes of 25 uL which included 12.5 µL of Kapa2G Fast Ready mix with dye (Kapabiosystems), 0.5µL of $MgCL_2$, 0.5 µL of ITS1 primer, 0.5 µL of ITS4 primer, 5 µL of DNA extract and 6 µL of nuclease free water.The PCR conditions were as follows: 1 min of denaturation at 93° C, 1 min of annealing at 58° C, and 1 min of extension at 72° C and then a final extension for 7 min at 72°C. PCR condition was repeated for 35 cycles. Five µL of the amplified DNA were then observed on 2% agarose gel in TAE buffer stained with ethidium bromide. Five µL of the amplicons were added 1 µL of x10-buffer, 9 µL nuclease free water, and 1 µL restriction enzyme *Mva*I, incubated at 37°C for 2 hours. Twelve µL of the restricted product were then electrophoresed in a 2% agarose gel, stained with EtBr.

h. Identification using $(GACA)_4$ primer-based PCR

Amplification reactions were carried out with volumes of 25 uL which included 12.5 µL of Kapa2G Fast Ready mix with dye (Kapabiosystems), 0.5µL of $MgCL_2$, 1 µL of $(GACA_4)$ primer, 5 µL of DNA extract and 6 µL of nuclease free water. PCR was carried out for 39 cycles of denaturation at 93°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. The resulting PCR products were separated in 1% agarose gels in x0.5 TBE buffer and stained with EtBr [2,8].

Table.1. Primers used in this study

| Primer | Sequence |
|---|---|
| ITS1-2 fw | 5'-ATCATTAACGCGCAGGC-3' |
| ITS1-2 rv | 5'-TGGCCACTGCTTTTCGG-3' |
| 18S RNA fw | 5'-AAGTTGGGTCAAACTCGGT-3' |
| 18S RNA rv | 5'-TGATCCTTCCGCAGGTT-3' |
| 28S RNA fw | 5'-ACAGGGATTGCCCCAGTA-3' |
| 28S RNA rv | 5'-CTTGTTCGCTATCGGTCTC-3' |
| ITS-1 | 5'-TCCGTAGGTGAACCTGCGG-3' |
| ITS-4 | 5'-TCCTCCGCTTATTGATATGC-3' |
| (GACA)4 | 5'-GACAGACAGACAGACA-3' |

## RESULTS

a. KOH test and Culture from Skin and Nail Scrapings

Among 927 participants who were screened for tinea, 197 (21.25%) showed clinical signs of tinea. Based on age stratification, 68 (52,3%) were 10-18 years old, 43 (33%) were 19-45 years old, and 19 (14,6%) were >45 years old. Scraping consists of 78 (60%) skin scales and 52 (40%) nail scrapings.

Among 197 participants with clinical signs of tinea, 130 (65.9%) samples were positive by 10%-KOH, but only 39 (19.7%) of clinically positive samples had growing fungal colonies. The 10%-KOH positive were obtained from 73 (56,1%) males and 57 (43,8%) females The positivity rate of culture as compared to positive 10%-KOH was 39/130 (30%). *Trichophyton mentagrophytes* were the most common among all isolated fungal strains, i.e. 20 (51.28%) samples (see Table 2).

Table 2. Identified Colonies on SDA Culture

| **Strain** | **N (% among 130 cultured samples)** | **N (% among 39 positive culture)** |
|---|---|---|
| *Microsporum audouinii* | 5 (3.8) | 5 (12.8) |
| *Microsporum canis* | 2 (1.5) | 2 (5.12) |
| *Microsporum ferruginosa* | 1 (0.8) | 1 (2.56) |
| *Microsporum gypseum* | 1 (0.8) | 1 (2.56) |
| *Trichophyton mentagrophytes* | 20 (65.4) | 20 (51.28) |
| *Trichophyton rubrum* | 4 (3.1) | 4 (10.25) |
| *Trichophyton soudanense* | 2 (1.5) | 2 (5.12) |
| *Trichophyton schoenleinii* | 2 (1.5) | 2 (5.12) |
| *Trichophyton tonsurans* | 1 (0.8) | 1 (2.56) |
| *Trichophyton verrucosum* | 1 (0.8) | 1 (2.56) |
| No growth | 91 (70) | - |
| Total | 130 (100) | 39 (30) |

ISBN: 978-602-60569-5-5

b. PCR tests

In contrast to 39 (30%) samples which grew in SDA culture, Multiplex-PCR, PCR-RFLP with ITS1-ITS4 primers and restriction with *Mva*I, and PCR with $(GACA)_4$ primer were able to amplify DNA of 130 (100%), 126 (96,9%), and 106 (81,5 %) samples, respectively.

Multiplex-PCR with ITS1-2 amplified fragments of 400 bp, 502 bp, 509 bp, or 560 bp on ITS1-2. By 18S RNA primer, no fragments, 50 bp, 60 bp, 70 bp or 80 bp may be amplified. By 28S RNA primer, no fragments, or 300 bp may be amplified. A pattern formed by a combination of the 3 pairs of primers will determine the dermatophyte strain. The size of fragments amplified by ITS1-2 and 18S RNA are depicted in figure 1, 2 and 3.

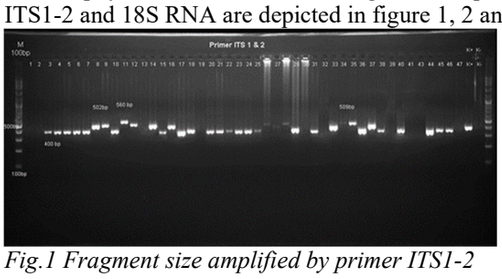


*Fig.1 Fragment size amplified by primer ITS1-2*

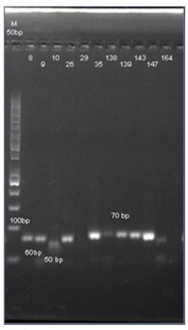


*Fig. 2 Fragment size amplified by primer 18S RNA*

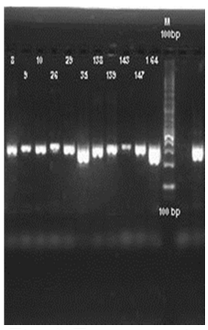


*Fig. 3 Fragment size amplified by primer 28S RNA*

Although Multiplex-PCR showed 100% detection rate as compared to 10%-KOH result (See Table 3) , the limitation by Multiplex-PCR based on the ITS1-2, 18S and 28S RNA specific for dermatophytes is that we were not able to distinguish the strains in 99 (76,2%) samples because the band patterns were different from the patterns of the 11 known standard strains amplified by the ITS1-2, 18S and 28S RNA primers[4]. The 11 known pattern were from standard strains *E. floccosum*, *M. canis*, *M. audouinii*, *M. gypseum*, *M. fulvum, T. rubrum*, *T. violaceum*, *T. mentagrophytes var mentagrophytes*, *T. mentagrophytes var interdigitale*, *T. tonsurans*, and *T. verrucosum*.

By PCR-RFLP with ITS1-ITS4 primers and restriction by *Mva*I we were able to identify 74.6% dermatophytes consisting 6 strains (See Table 4), but there were undetermined or unclassified strains in 29 (22,3%) samples and there were 4 (3.1%) samples not amplified. This method showed lower sensitivity as compared to multiplex PCR.

Using PCR with $(GACA)_4$ primers we were able to identify 66.15% consisting 6 strains (See Table 5), and can distinguish *Trichophyton mentagrophytes variant mentagrophytes* and *variant interdigitale*. Unfortunately there were undetermined or unclassified strains in 20 (15.4%) and there were 24 (18.4%) samples that were not amplified.

Table 3. Identification using Multiplex PCR

| Strain | N | % |
|---|---|---|
| *Microsporum audouinii* | 16 | 12.3 |
| *Microsporum canis* | 1 | 8 |
| *Microsporum vulvum* | 2 | 15 |
| *Microsporum gypseum* | 4 | 3.1 |
| *Trichophyton mentagrophytes* | 6 | 4.6 |
| *Trichophyton tonsurans* | 1 | 0.8 |
| *Trichophyton violaceum* | 1 | 0.8 |
| Unclassified* | 99 | 76.2 |
| No amplification | 0 | 0 |
| Total | 130 | 100 |

*Unclassified refers to those band patterns different to the 11 known patterns of dermatophytes.

Table 4. ITS 1-4 and restriction by *Mva*I enzyme

| Strain | N | % |
|---|---|---|
| *Microsporum canis*# | 29 | 22.3 |
| *Epidermophyton floccosum* | 1 | 0.8 |
| *Trichophyton rubrum* | 13 | 10 |
| *Trichophyton tonsurans* | 5 | 3.8 |
| *Trichophyton verrucosum* | 3 | 2.3 |
| *Trichophyton mentagrophytes* | 46 | 35.4 |
| Unclassified* | 29 | 22.3 |
| No amplification | 4 | 3.1 |
| Total | 130 | 100 |

# based on known amplicon size prior to restriction, but no known cut band size, if any such dermatophyte is present.

*Unclassified refers to those band patterns different to the 5 known patterns of cut band size of dermatophytes restricted by *Mva*I enzyme.

Table 5. PCR by $GACA_4$ primer

| Strain | N | % |
|---|---|---|
| *Microsporum canis* | 14 | 10.7 |
| *Epidermophyton floccosum* | 1 | 0.8 |
| *Trichophyton rubrum* | 14 | 10.7 |
| *Trichophyton violaceum* | 10 | 7.7 |
| *Trichophyton mentagrophytes variant mentagrophytes* | 30 | 23.1 |
| *Trichophyton mentagrophytes variant interdigitale* | 17 | 13.1 |
| Unclassified* | 20 | 15.4 |
| No amplification | 24 | 18.4 |
| Total | 130 | 100 |

* Unclassified refers to those band patterns different to the 6 known patterns of cut band size of 5 dermatophytes(Shehata, 2008, Faggi, 2001)

## DISCUSSION

In this study we compared the results obtained from culture and 3 molecular detection methods which were based on ITS1-2, 18S RNA, 28SRNA, ITS1 and ITS1-ITS4 followed by enzyme restriction with *Mva*I and by using a single $(GACA)_4$ primer. It is clear that the detection rate of molecular tests are much higher and much faster than culture. Molecular detection requires turn-around time of 24-48 hours, while culture requires average of 10-14 days.

Multiplex-PCR, PCR-RFLP with ITS1-ITS4 primers and *Mva*I, and PCR with $(GACA)_4$ primer were able to amplify the dermatophytes' DNA in 130 (100%), 126

(96,9%), and 106 (81,5 %) samples, respectively, more sensitive than 39 (30%) samples positive by SDA culture. Multiplex PCR showed consistent result with the 10%-KOH test. The inability of Multiplex-PCR in differentiating strains in 99 (76,2%), PCR-RFLP with ITS1-ITS4 primers and *Mva*I in 29 (22,3%) and PCR with $(GACA)_4$ primer in 20 (15,4%) samples is explained through understanding that each molecular methods used in this study had limited known reference patterns to compare with. Therefore there is a proportion of Makassar strains that was catagorized into "unclassified" found in all three molecular methods. This requires further study to know the DNA patterns using other reference dermatophytes strains.

That $(GACA)_4$ primer could not amplify any DNA in 18.4% samples does not indicate a limited ability of one molecular method compared to another, because the three molecular tests used known specific primers for dermatophytes, same volume and same batch of DNA extract. We consider this as reflection on the differences in detection limit of each molecular methods.

We address several limitations of this study. First, among 130 samples, although by multiplex PCR all were positive reflecting that at least one certain strain of dermatophyte is present, occurrance of mixed infections with other dermatophytes is possible. To proof this requires further test by ITS1-ITS4 primer followed by other restriction enzymes such as *Hinf*I and *Hae*III. Second, molecular detection is very specific thus rare strains of dermatophytes may not be identified, and causes false negatives. Third, the concentration of the DNA in the extract was not determined prior to PCR testings, therefore the detection limit of each molecular method cannot be determined. Fourth, these three molecular tests can not identify the causative fungal agents in cases of non-dermatophyte origin tinea.

## CONCLUSION

Considering the specificity, sensitivity and short turn-around time for a definitive diagnosis of tinea, in order to start a correct and prompt antimycotic treatment, we recommend PCR test be applied for laboratory diagnosis if hospitals are prepared with basic molecular equipments.

## ACKNOWLEDGEMENTS

Sincere thanks to Dianawati Amiruddin, MD from a private clinic in Makassar who gave access to authors in this study to involve the participation of patients with tinea, and to the Management officers of the Sungguminasa elderly home care, and the headmasters of 9 primary schools within slum districts in Makassar, South Sulawesi for their assistance during screening of study participants. Many thanks also to Syafri for his assistance in the laboratory.

## REFERENCES

[1] BORMAN, A. M., CAMPBELL, C. K., FRASER, M. & JOHNSON, E. M. 2007. Analysis of the dermatophyte species isolated in the British isles between 1980 and 2005 and review of the world wide dermatophytes trend over the last three decade. Medical Mycology, 45, 131-141.

[2] ELAVARASHI, E., KINDO, A., KALYANI, J. & SUDHA, R. 2014. APplication of PCR finger printing usin (GACA)4 primer in the rapid discrimaination of dermatophytosis. Indian J of Medical Microbiology, 32, 236-239.

[3] KAUFMANN, R., BLUM, S. E., ELAD, D. & ZUR, G. 2016. Comparison between point of care dermatophye medium and mycology laboratory culture for diagnosis of dermatophytosis in dogs and cats. Vet Dermatol, 1-6.

[4] KIM, J. Y., CHOE, Y. B., AHN, K. J. & LEE, Y. W. 2011. Identification of dermatophytes using multiplex Polymerase Chain Reaction. Ann Dermatol, 23, 304-312.

[5] MIRZAHOSEINI, H., OMIDINIA, E., SHAMS-GHAHFAROKHI, M., SADEGHI, G. & RAZZAGHI-ABYANEH, M. 2009. Application of PCR-RFLP to rapid identification of the main pathogenic dermatophytes from clinical specimen. Iranian J Public Health, 38, 18-24.

[6] SCHER, R. K., TAVAKKOL, A., SIGURGEIRSSON, B., HAY, R. J., JOSEPH, W. S., TOSTI, A., FLECKMAN, P., GHANNOUM, M. A., ARMSTRONG, D. G., MACKINSON, B. C. & ELEWSKI, B. E. 2007. Onichomycosis: Diagnosis and definition of cure. The American Academy of Dermatology, 939-944.

[7] SEEBACHER, C., BOUCHANA, J.-P. & MIGNON, B. 2008. Updates on the epidemiology of dermatophytes infections. Mycopathologia, 166, 335-352.

[8] SHEHATA, A. S., MUKHERJEE, P. K., ABOULATTA, H. N., EL AKHRAS, A. I., ABBADI, S. H. & GHANNOUM, M. A. 2008. Single step PCR Using (GACA)4 primer: Utility for rapid Identification of dermatophyte species and strains. Journal of Clinical Microbiology, 46, 2641-2645.

[9] WIEGAND, C., BAUER, A., BRASCH, J., NENOFF, P., SCHALLER, M., MAYSEN, P., HIPLER, U.-C. & ELSNER, P. 2016. Are the classic diagnostic methods in mycology still the state of the art. Deutsch Dermatologische Gesellschact, 490-494.