Diagnostic value of nested-PCR for identification of *Malassezia* species in dandruff

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**Abstract.** Dandruff or pityriasis simplex is a condition of abnormal occurrence of formation of yellowish white scales from the scalp. Many factors play a role in the pathogenesis of dandruff, i.e. colonisation of *Malassezia* species. Examination of *Malassezia* species previously done by culture as the gold standard. However, there are various difficulties in doing the culture. Identification method with anested-polymerase chain reaction (nested-PCR) is expected to provide quickly and easily detected. This study aimed to determine the diagnostic value of nested-PCR in the identification of *Malassezia* species in dandruff. From 21 subjects, scales from the scalp were taken and sent to the laboratory for nested-PCR identification. Statistical analysis of diagnostic test carried out to determine sensitivity, specificity, positive predictive value, and negative predictive value. The results showed nested-PCR detected 10 sample (47.6%) positive for *Malassezia* species consist of *M. sympodialis* (23.8%); *M. slooffiae* (9.5%); *M. furfur* (4.8%); *M. globosa* and *M. furfur* (4.8%); and *M. restricta* and *M. sympodialis* (4.8%). Detection of *Malassezia* species by nested-PCR has 100% in sensitivity whereas the specificity was 55%. Nested-PCR test has high sensitivity. Therefore nested-PCR may be considered for a faster and simpler alternative examination in identification for *Malassezia* species in dandruff.

1. Introduction
Dandruff is an abnormal condition with the occurrence of scales formation or flaky scalp and usually accompanied by itching. It is the mildest form of seborrhoeic dermatitis on the scalp.[1] Until today, dandruff is still a problem in general population and can be found in almost half of post-puberty population with various gender and ethnic. This condition can caused anxiety or stress, loss of confidence, or uncomfortable for patients. The disease is often found in young adulthood but relatively rare and mild stage was found in children.[2]

The peak incidence and severity of the disease occurs at the age of about 20 years, and about 50% of the world's population has ever suffered from this disease with different degrees of severity.[3] While in Indonesia alone, according to the United States, the consensus body with the international data base in the year 2004, the estimated dandruff prevalence is approximately 18.38%.[4] There is no population in a geographical region that was not affected by dandruff in a certain stage of life. A study by Mawardi and Jusuf in 2012 showed the proportion of dandruff patient in high school student at Kualuh Hulu District, North Labuhan Batu, North Sumatera, was 58 out of 100 respondents (58%), most are 16 years old and mainly found in women (43%).[5]
Malassezia species could be the cause of dandruff besides sebum production and individual predisposition. However, Malassezia species may also be found as normal flora and colonization on the scalp.[6] Identification of Malassezia species in dandruff is performed by culture as gold standard. Eventhough, Malassezia culture giving the results not always easy to read due to the nature of its slow growth is. In addition, this fungus is difficult to grow in media culture due to dependency for long-chain fatty acids for growth in vitro, and it led requiring a special medium containing lipids that are a source of essential nutrients needed for its growth. Moreover, other disadvantages are the difficulty in distinguishing between species based on phenotype characteristics and this method also takes a long time and certain expertise.[7-10] Another option is by nested-polymerase chain reaction (nested-PCR). This method is expected to provide better and faster detection so it can be applied for the epidemiologic survey.[11] Due to the difficulties inherent in culturing Malassezia spp., this study analyzed the cutaneous Malassezia microflora directly from the skin squama of scalp patients by using a nested PCR. Hence, the study analyze the diagnostic value of nested-PCR for identification the Malassezia spp. in dandruff.[12]

2. Material and Methods

2.1. Subjects
Samples were taken from 21 subjects which are healthworkers from H. Adam Malik-General Hospital (HAM-GH) who had dandruff.

2.2. Sample collection
The sampling method was consecutive sampling which was taken from 4 different division at HAM-GH then the population which meets the inclusion and exclusion criteria will be included in the study until the required number of samples are met. The inclusion criteria for study are HAM-GH health workers with dandruff that were diagnosed by anamnesis and physical examination, the age of 18-60 years old, and agree to participate in the study by signing the informed consent. The exclusion criteria are subject to dandruff that was diagnosed with other scalp disorder, and the subject was taken antifungal therapy and/or oral steroid, topical steroid 1 month before the study. Samples were taken by scrubbing the scales from the scalp for yeast culture and nested-PCR to identify Malassezia species.

2.3. DNA extraction
Extracted DNA (2µl) were purified using Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer’s instructions.[13]

2.4. Detection of Malassezia DNA by nested PCR
To amplify rDNA from genomic DNA, a nested-PCR was carried out. Nested-PCR was conducted by using two sets of primer which are ITS-1 (5' -TCC GTA GGT GAA CCT GCG G-3'), ITS-3 (5’-ATG TCG GCA AAC AAG GCA GC-3' ) and ITS-4 (5’-TCC TCC GCT TAT TGA TAT GC-3' ).[11] For first PCR, ITS-1 and ITS-4 primers were used. The first amplification mix was carried out in 12.5µl master mix PCR consist of Taq polymerase enzyme, MgSO₄, and dNTP(Go Tag® PCR Core System, Promega); 7.5 µl nuclease-free water and 2 µl DNA template.[14] In the nested PCR step, 3 µl of the first amplification product was added to a new reaction mixture with the same composition as the first, and under aforementioned PCR conditions using primer ITS-3 and ITS-4. PCR was performed in a thermocycler (Verity 96-well Thermal Cycler,AppliedBiosystems) with an initial denaturation of 95°C for 1 min, followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min 30 s at 72°C and the final cycle comprised 1 min at 95°C, 1 min at 55°C and 5 min at 72°C.

2.5. Gel electrophoresis
All PCR products were analyzed by electrophoresis in a 2% (wt/vol) agarose gel (Promega®) by standard procedures. The result showed DNA fragment size for M. furfur 509bp, M. globosa 430bp,
3. Results

3.1. Detection of Malassezia DNA

The identification with nested-PCR found 10 samples (47.6%) of Malassezia species out of 21 samples consisting of M. sympodialis, M. slooffiae, M. furfur, M. furfur and M. globosa, M. restricta and M. sympodialis. From those results could be seen that nested-PCR may detect more than one species in 1 sample, i.e., M. furfur and M. globosa another one is M. restricta and M. sympodialis. However, there are 11 samples (52.3%) were not detected (Table 1).

| No | Malassezia species | n | %    |
|----|--------------------|---|------|
| 1  | M. sympodialis     | 5 | 23.8 |
| 2  | M. slooffiae       | 2 | 9.5  |
| 3  | M. furfur          | 1 | 4.8  |
| 4  | M. furfur and M. globosa | 1 | 4.8  |
| 5  | M. restricta and M. sympodialis | 1 | 4.8  |
| 6  | Not identified     | 11| 52.3 |
| **Total** |                     | **21** | **100.0** |

Of the 10 samples that contained Malassezia species the largest proportion of the results is M. sympodialis (n=6) followed by M. slooffiae and M. furfur (n=2) M. globosa and M. restricta (n=1) (Table 2).

| No | Malassezia species | n | %  |
|----|--------------------|---|----|
| 1  | M. sympodialis     | 6 | 50 |
| 2  | M. slooffiae       | 2 | 16.7 |
| 3  | M. furfur          | 2 | 16.7 |
| 4  | M. globosa         | 1 | 8.3 |
| 5  | M. restricta       | 1 | 8.3 |
| **Total** |                     | **12** | **100.0** |
Figure 1. NC (Negative control); *M. slooffiae* 486 bp (line 8); *M. sympodialis* 374 bp (line 10); *M. sympodialis* 374 bp (line 11); *M. sympodialis* 374 bp (line 12); *M. sympodialis* 374 bp (line 20); *M. sympodialis* 374 bp (line 21).

3.2. Sensitivity and specificity of the nested PCR assay
Statistical analysis found that sensitivity of nested-PCR was 100%, specificity was 55%, with Positive predictive value (PPV) 10.0% and Negative predictive value (NPV) was 100% (table 3).

|                  | Culture |          | Total         |
|------------------|---------|----------|---------------|
| Nested-PCR       | Positive| 1(100%)  | 9(45%)        |
|                  | Negative| 0(0%)    | 11(55%)       |
| **Total**        |         | 1(100%)  | 20(100%)      | 21(100%)      |

4. Discussion

4.1. Detection of Malassezia DNA
This study found that *M. sympodial* was the most number of *Malassezia* species that identified from dandruff followed by *M. slooffiae* and *M. furfur*. In 2002, a study by Gaitanis et al. in Greece reported that there were several *Malassezia* species that can be identified from the pathologic skin, such as seborrheic dermatitis, using the nested-PCR method. The identified species were *M. furfur, M. globosa, M. restricted, M. sympodialis, M. pachydermatis, M. obtuse,* and *M. slooffiae*, although *M. pachydermatis* was rarely found in human skin. The method that was used in this study can directly identify several *Malassezia* species at the same time from a specimen, whereas two or more amplified products from PCR can be obtained using DNA that was extracted from pathologic skin. This condition can be expected because human skin contains more than one species of *Malassezia*. The
result shows that non-culture diagnostic method from the dermatologic specimen can be used regularly for detection and identification of Malassezia species.[11]

Other research by Lian et al. in 2014 in China reported that Malassezia species that can be isolated from seborrheic dermatitis patient are M. furfur (76.5%) followed by M. sympodialis (12.5%).[15] A study by Gonzales et al. in 2015 in Mexico, used PCR to detect Malassezia species in seborrheic dermatitis patient and the species that often found are M. furfur (20%), M. globosa (16.7%), M. sympodialis (6.7%), M. restricta (3.3%), and M. slooffiae (1.7%).[16]

Mahmoudabadi et al. in 2014 conducted a research in Iran about the detection of Malassezia species from seborrheic dermatitis patient using nested-PCR assay. The study found that the most frequent isolate that is detected in seborrheic dermatitis patient is M. restricta. It also concluded that nested-PCR using 2 sets of the primer could be done quickly, repeated to identify Malassezia species and recommended to use in many patients.7] Lee et al. in 2012 in Korea also found that the most identified Malassezia species by PCR analysis from seborrheic dermatitis patients was M. restricta.[17]

4.2. Comparison between Nested-PCR assay and Culture
This study found only 1 sample (4.8%) from 21 samples that positive with a culture which is M. furfur (4.8%) while nested-PCR detected 10 samples positive Malassezia species (47.6%) with M. sympodialis was found as the large number. The nested-PCR also identified the presence of 2 Malassezia species simultaneously in a single specimen. Comparison of identification of Malassezia species between nested-PCR and culture from dandruff showed that nested-PCR detected Malassezia species higher, including type and number of Malassezia species compare to identification by culture.

4.3. Sensitivity and specificity of the nested PCR assay
Fungal culture assay is a conventional method that can be found in the most laboratory, but this essay has disadvantages such as the inability to differentiate Malassezia species by their phenotype characteristics, it takes a lot of time and certain expertise. On the other hand, molecular biology assay such as nested-PCR is thought as the most reliable tool for Malassezia species identification. It can identify one or more Malassezia species on the same specimen and discovered the potential interaction between Malassezia species or other yeast species in human skin at same time. The nested-PCR had the advantages of simplicity, more sensitive, and rapid turn around for results, but may be less accurate due to low specificity. A major disadvantage of using ITS primer for PCR concerns the frequent contamination of reagents and materials with traces of fungal DNA.[18] As per the ubiquitous nature of fungi, virtually every reagent may show low-level contaminations, e.g., primers, Taq polymerase, etc., or contamination becomes introduced during sampling. Strict precautions are necessary, including careful quality control of the reagents, but environmental contamination remains a problem.[19]

5. Conclusion
Detection of Malassezia species by nested-PCR has high sensitivity. Therefore nested-PCR may be considered for a faster and simple alternative examination in identification for Malassezia species in dandruff. This study identified 5 Malassezia species; M. sympodialis, M. slooffiae, M. furfur, M. globosa, and M. restricta, with the most number, was M. sympodialis. The sensitivity of nested-PCR in this study was 100%, and the specificity was 55%.

References
[1] Cardin C W 1998 Textbook of cosmetic dermatology 2nd edition ed Baran R and Maibach H I (London: Martin Dunitz) pp 193-200
[2] Jusuf N K 2011 Proc. conf. on social dermatovenerology 13th perdoski national congress (Manado: Konas XIII Perdoski) p 43
[3] Ashadi L N and Pendit B U 2014 *Everything about hair* (Surabaya: Fakultas Kedokteran Universitas Indonesia Press) pp 112-22

[4] Mawardi A and Jusuf N K 2011 Gambaran pengetahuan dan sikap siswa-siswi SMA terhadap ketombe / dandruff di kecamatan kualuhulu, kabupaten labuhan batu utara (Medan: Karya Tulis Ilmiah Fakultas Kedokteran Sumatera Utara) pp 25-39 (unpublished paper)

[5] Statistics by Country for Dandruff [Internet]. [updated 2003 May, cited 2017 September 4] Available from: http://www.cureresearch.com/d/dandruff/stats-country_printer.htm

[6] Hay R J 2011 Malassezia, dandruff and seborrhoeic dermatitis: an overview *Brit. J. Dermatol.* **165** 2–8

[7] El-Hadidy G S, Gomaa N I M, AboBakar R A E and Metwally L A 2007 Direct molecular identification of *Malassezia* species from skin scales of patients with seborrhoeic dermatitis by nested terminal fragment length polymorphism analysis *Egypt. J. Med. Microbiol.* **16** 437-44

[8] Mahmoudabadi A Z, Zarrin M and Azish M 2014 Detection of *Malassezia* species isolated from patients with *Pityriasis versicolor* and seborrhoeic dermatitis using nested-PCR *Jentashapir J. Health Res.* **5** e26683

[9] Chua K B, Chua I L, Chua I E, Chong K A and Chua K H 2005 A modified mycological medium for isolation and culture of *Malassezia furfur* Malay. *J. Pathol.* **27** 99–105

[10] Gueho-Kellermann E, Boekhout T and Begerow D 2010 Biodiversity, phylogeny, and ultrastructure *Malassezia and the skin* ed Boekhout T, Gueho-Kellermann E, et al. (Berlin: Springer-Verlag) pp 17-63

[11] Gaitanis G, Velegkri A, Frangoulis E, Mitroussia A, Tsigonia A, Tzimiogianni A, et al. 2002 Identification of *Malassezia* species from patient skin scales by PCR-RFLP *Clin. Microbiol. Infect.* **8** 162–73

[12] Sugita T, Suto H, Unno T, Tsuboi R, Ogawa H, Shinoda T and Nishikawa A 2001 Molecular analysis of *Malassezia* microflora on the skin of atopic dermatitis patients and healthy subjects *J. Clin. Microbiol.* **0095-1137** 3486–90

[13] Technical Manual Wizard® Genomic DNA Purification Kit [Internet] (Wisconsin: Promega Corporation) [cited 2017 September 4] Available from: https://ita.promega.com/-/media/files/resources/protocols/technical-manuals/0/wizard-genomic-dna-purification-kit-protocol.pdf

[14] Technical Bulletin GoTaq® PCR Core Systems[Internet] (Wisconsin: Promega Corporation) [cited 2017 September 4] Available from: https://worldwide.promega.com/-/media/files/resources/protocols/technical-bulletins/0/gotaq-pcr-core-systems-protocol.pdf

[15] Lian C, Shen L, Gao Q, Jiang M, Zhao Z and Zhao J 2014 Identification of *Malassezia* species in the facial lesions of Chinese seborrhoeic dermatitis patients based on DNA sequencing *Mycoses* **57** 759-64

[16] Gonzalez F K S, Lozano O W, Lopez R O, Cabrera L V, Candiani J O, Avila D E Z, et al. 2016 Distribution of *Malassezia* species in Mexican seborrhoeic dermatitis patients *Afr. J. Microbiol. Res.* **10** 185-90

[17] Lim S H, Kim Y R, Jung J W, Hahn H J, Lee Y W, Choe Y B, et al. 2012 A comparison study between culture based technique and op-site non-culture based technique for identifying *Malassezia* yeasts on normal skin *Korean J. Med. Mycol.* **17** 217-29

[18] Loeffler J, Hebart H, Bialek R, Hagmeyer L, Schmidt D, Serey F P, et al. 1999 Contaminations occurring in fungal PCR assays *J. Clin. Microbiol.* **37** 1200–2

[19] Silvana K R, Andrea Z, Roberto F S and Guido V B 2016 Similar efficacy of broad-range ITS PCR and conventional fungal culture for diagnosing fungal infections in non-immunocompromised patients *BMC Microbiol.* **16** 132