Malassezia furfur in Infantile Seborrheic Dermatitis

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SUMMARY Our objective was to study both incidence and various strains of *Malassezia* in infantile seborrheic dermatitis (ISD). Sixty infants between 2 weeks and 2 years old with clinical diagnosis of ISD at the Department of Pediatrics, King Chulalongkorn Memorial Hospital from May 2002 to April 2003 were recruited. *Malassezia* spp. were isolated from cultured skin samples of the patients, genomic DNA was extracted and the ITS1 rDNA region was amplified. The PCR product was examined by agarose gel electrophoresis and DNA sequences were determined. The ITS1 sequences were also subjected to phylogenetic analysis and species identification. ISD is most commonly found in infants below the age of 2 months (64%), followed by those between 2 and 4 months (28%) old. Cultures yielded yeast-like colonies in 15 specimens. PCR yielded 200-bp products (*Candida*) in 3 patients and 300-bp products *(Malassezia furfur)* in 12 patients (18%). Sugar fermentation using API 20C aux performed on the three 200-bp PCR products yielded *Candida* species. *M. furfur* was the only *Malassezia* recovered from skin scrapings of children with ISD.

Various studies have been performed on the role of *Malassezia* and seborrheic dermatitis in infants and adults.[1-6] *Malassezia* is found in many skin diseases such as psoriasis, atopic dermatitis, tinea versicolor, pityrosorum folliculitis as well as in healthy infants.[6,7] *Malassezia species* may be associated with catheter–acquired sepsis, fungemia and pulmonary infection in neonates[8-12] and also in cancer patients.[13-14]

The genus *Malassezia* can now be divided into seven species.[15] Cell-mediated immunity to *M. furfur* has been shown to be different in patients with seborrheic dermatitis and pityriasis versicolor.[16] *Malassezia* can induce cytokine production by keratinocytes[17,18] with differences in this ability among the various *Malassezia* yeasts.[19] Six different species of *Malassezia* were recovered from different dermatosis cases and different anatomical sites.[20]

The objective of this study was to investigate both incidence and species of *Malassezia* in infantile seborrheic dermatitis (ISD) based on the internal transcribed spacer 1 region (ITS1) of the ribosomal DNA (rDNA) sequence. ITS1 is located between 18S and 5.8S rDNA and has proven useful for species identification and strain typing of *Malassezia* spp.[19]

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MATERIALS AND METHODS

Study population

All infants diagnosed with ISD without prior treatment, aged between 2 weeks and 24 months were recruited into this study between May 2002 and April 2003. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Parents were informed regarding the objective of the study and their written consents were obtained before specimen collection.

Malassezia isolation and DNA extraction

We scraped ISD skin lesions for scaling and subsequently adhered slides coated with Saboraud dextrose agar (SDA) as transport medium to the skin lesions.

The transport medium was transferred to SDA overlaid with olive oil and incubated at room temperature for 4 weeks. DNA was extracted from a fraction of a colony suspended in 100 µl lysis buffer (200mM Tris-HCl, pH 8.0; SDS 0.5% w/v, 250 mM NaCl, 25 mM EDTA). After mixing by vortex for 5 seconds, the sample was incubated at 100°C for 15 minutes. Then 100 µl of 3.0 M sodium acetate were added, mixed, incubated at -20°C for 10 minutes and centrifuged at 6,060 x *g* for 5 minutes. The supernatant was extracted once with phenol:chloroform: isoamyl alcohol (25:24:1, v:v:v) and once more with chloroform. DNA was precipitated in 40 µl 3.0 M sodium acetate, 4 µl glycogen and 800 µl absolute ethanol at -70°C for 30 minutes. The resulting DNA pellet was washed in 1 ml 70% ethanol and centrifuged at 13,700 x *g* for 15 minutes. Finally, the pellet was dried and resuspended in 50 µl sterile water.

Amplification of ITS1

The ITS1 region was amplified in a Mastercycler personal (Eppendorf, Hamburg, Germany) using the primer sequences previously described.[18] The forward primer was 18SF1: 5'-AGGTTTCCGT-AGGTGAACCT-3'; and the reverse primer was 58SR1: 5'-TTCGCTGCGTTCTTCATCGA-3'. Each PCR reaction mixture contained 2.5 µl 10x reaction buffer (Finnzymes, Finland), 10.0 µM each of dATP, dCTP, dGTP and dTTP (Promega, Madison, WI), 2.5 U *Taq* polymerase (Finnzymes, Finland), 0.5 µM of each primer and 1.0 µl DNA template solution. PCR was performed under the following conditions: after an initial 5-minute denaturation step at 94°C, 35 cycles of amplification were performed, each including 1 minute denaturation at 94°C, 15 seconds annealing at 60°C and 30 seconds extension at 72°C, followed by a final 10-minute extension at 72°C. Each amplified DNA sample (10 µl) was added to loading buffer (4 µl) and run on a 2.0% agarose gel with ethidium bromide on preparation (FMC Bioproducts, Rockland, ME) at 100 V for 60 minutes. The sizes of DNA products were visualized under UV transillumination.

ITS1 direct sequencing and phylogenetic analysis

For automated DNA sequencing, the PCR products of interest were excised from the gel and purified using the Gel Extraction Kit (Perfectprep Gel Cleanup, Eppendorf, Hamburg, Germany) according to the manufacturer's specifications. The purified PCR products were subjected to direct sequencing using a DNA Sequencing Kit (Applied Biosystems, CA) and primer 18SF1 in a Perkin Elmer 310 Sequencer (Perkin-Elmer, Boston) according to the manufacturer's instructions. Species identification was determined by BLAST analysis. ITS1 sequences obtained from this study were submitted to the Genbank database (www.ncbi.nlm.nih.gov) and multiple aligned with reference sequences of *Malassezia* standard strains (*M. globosa*; AB019344, *M. restricta*; AB019340, *M. slooffiae*; AB019348, *M. sympodialis*; AB019345, *M. parchydermatis*; AB019339, *M. obtusa*; AB019336 and *M. fufur*; AB019329) using the CLUSTAL X program (version 1.8). The TREEVIEW program (version 1.5) was run for phylogenetic tree construction. The sample sequences appearing on the same node as the reference sequences were classified as belonging to the same species.

RESULTS

Among the 60 infants diagnosed with ISD, 31 were male and 29 female with the majority below 2 months of age (62%), followed by 2-4 months (28%), 4-6 months (5%), 6-12 months (2%) and 12-24 months (3%). The most common site of affliction was the scalp. Most patients displayed lesions on

more than one site (Table 1). Cultures of 15 samples yielded yeast-like colonies. One of those 15 samples was from a patient with tinea versicolor. PCR was performed and yielded products of 200 bp in 3 patients and of 300 bp in 12 patients. The PCR products were stained with ethidium bromide and visualized by UV transillumination (Fig. 1).

ITS1 DNA sequences were aligned using the Clustal X computer program. The 300-bp product matched the *Malassezia furfur* sequence (Accession No. AY880912-AY880923) and the 200-bp product the *Candida* sequence. (Accession No. AY880924-AY880926). The phylogenetic tree was constructed based on the ITS1 sequence data of *Malassezia* spp. (Fig. 2). In 3 patients with a PCR product sizes of 200 bp, sugar fermentation using API 20C aux yielded *Candida* species.

DISCUSSION

Malassezia were found in many skin diseases as well as in healthy infants.[6,10] Ruiz-Maldonado *et al.*[21] reported growth of *M. furfur* in 53% of healthy infants between 1 and 24 months old and Bergbrant *et al.*[7] reported it in 87% of healthy children between 2 months and 15 years old with the largest number of colonies found among children aged between 2 and 23 months. The choice of culture media is crucial to promote growth *of Malassezia*. Tollesson *et al.*[4] demonstrated that *Malassezia* failed to grow in one particular medium (PDM; Pityrosporum Contact Plates; Biodisk AB, Solna, Sweden), whereas it yielded positive cultures with 17 out of 20 specimens obtained from healthy children using a different medium. (Max Lab Diagnostics HB, V. Frolunda, Sweden).[5]

Cultures obtained from seborrheic dermatitis in adult patients produced *M. sympodialis, M. globosa*, *M. furfur* and *M. slooffia,* with more than one *Malassezia* species recovered at different anatomical sites from both lesions and non-lesions.[20] Nakabayashi *et al.*[22] found that with seborrheic dermatitis in adults, *M. furfur* (35%) and *M. globosa* (22%) were isolated from facial skin lesions at significantly higher rates than from normal subjects, in whom *M. globosa* (22%), *M. sympodialis* (10%) and *M. furfur* (3%) prevailed.

There are still various difficulties pertaining to research on *Malassezia* and ISD. Both culture me-

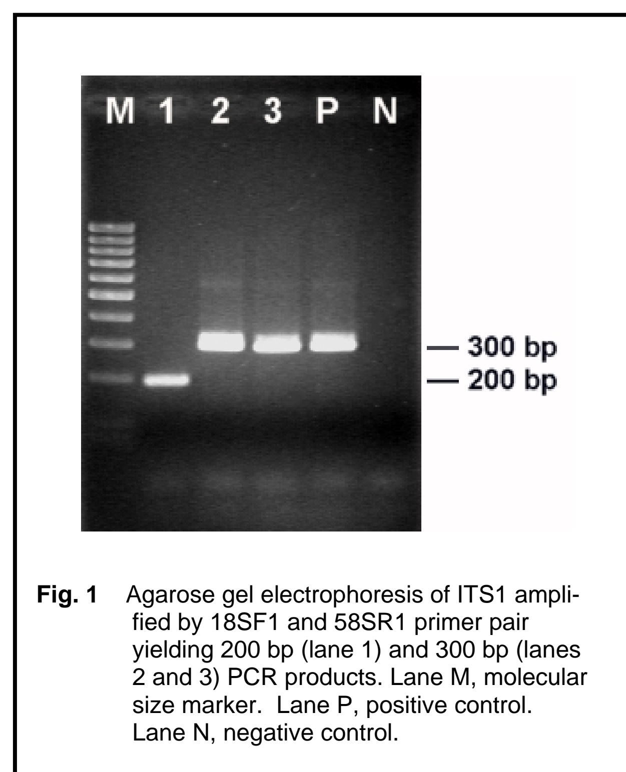

Fig. 1 Agarose gel electrophoresis of ITS1 amplified by 18SF1 and 58SR1 primer pair yielding 200 bp (lane 1) and 300 bp (lanes 2 and 3) PCR products. Lane M, molecular size marker. Lane P, positive control. Lane N, negative control.

Table 1 Sites of affliction in 60 patients presented with infantile seborrheic dermatitis

Site	1 Site n = 27 (%)	2 Sites n = 29 (%)	3 Sites n = 4 (%)
Scalp	16 (59)	20 (34)	4 (33.3)
Eyebrows	5 (19)	10 (17)	4 (33.3)
Post auricular	6 (22)	22 (37)	4 (33.3)
Cheek	-	4 (7)	-
Forehead	-	3 (5)	-

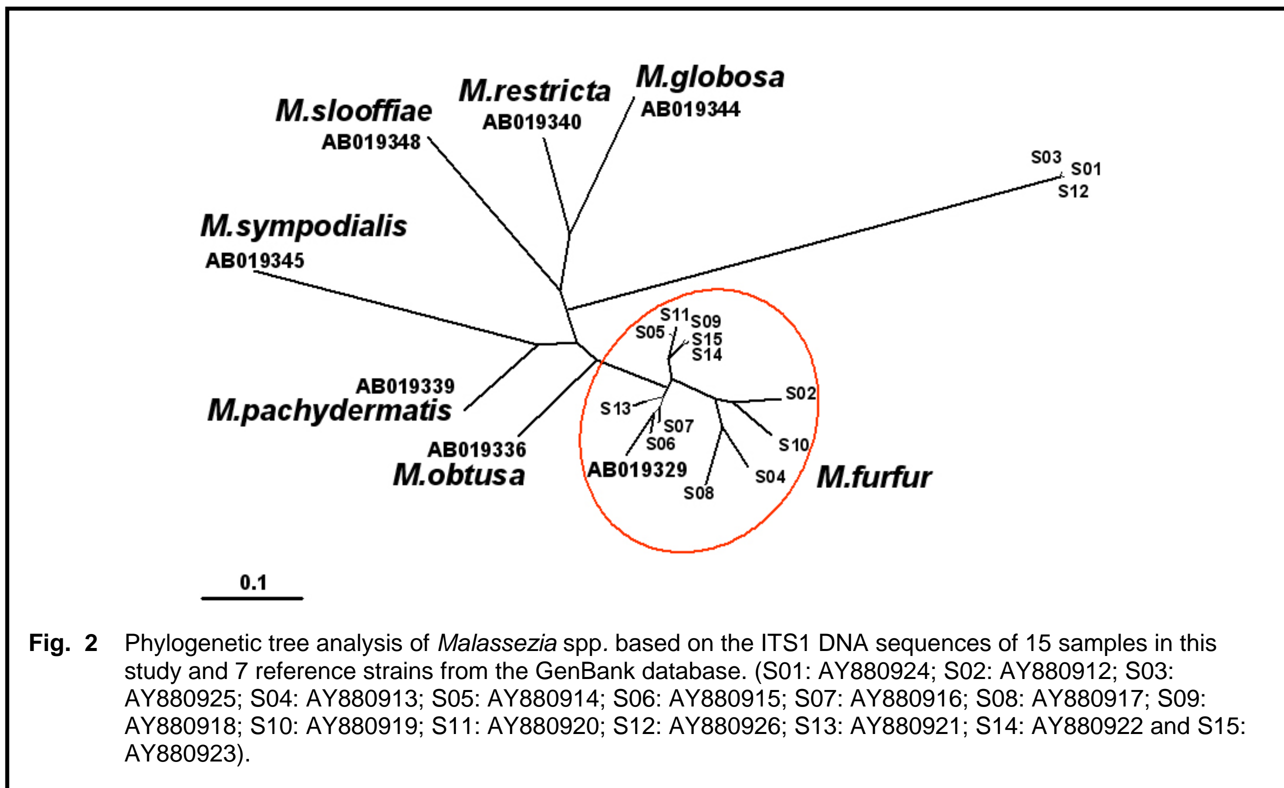

Fig. 2 Phylogenetic tree analysis of *Malassezia* spp. based on the ITS1 DNA sequences of 15 samples in this study and 7 reference strains from the GenBank database. (S01: AY880924; S02: AY880912; S03: AY880925; S04: AY880913; S05: AY880914; S06: AY880915; S07: AY880916; S08: AY880917; S09: AY880918; S10: AY880919; S11: AY880920; S12: AY880926; S13: AY880921; S14: AY880922 and S15: AY880923).

dia and the method of specimen collection certainly affect the growth of *Malassezia.* The pathogenesis of *Malassezia* is still controversial as it can induce cytokine production by keratinocytes,[17,18] but can also be found on non-lesion skin and even in healthy infants. There are conflicting results relating to its growth subsequent to treatment in that there has been some evidence of reduction[23] which has been contradicted by other authors.[4] Faergemann[23] demonstrated a significantly reduced number of cultured *Pityrosporum orbeculare* colonies in all groups treated with miconazole-hydrocortisone, miconazole and hydrocortisone, yet in the hydrocortisone group it was still significantly higher than in other groups. On the other hand, Tollesson *et al.*[5] showed that the number of *M. furfur* colonies did not significantly change during the treatment and follow-up periods.

In our study, we found *M. furfur* in only 18% ISD lesions which is very low in comparison with previous studies (70-90%).[5,6] The low prevalence of *Malassezia* in this study may be due to different culture media, the specimen collection technique applying a contact plate, as well as the environment and methods of child rearing which in turn can affect the growth of microorganisms. Therefore, further studies especially targeting prevalence are required. Novel and effective methods pertaining to both culture and culture media are imperative to study the prevalence of *Malassezia* species and their role in skin diseases.

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REFERENCES

1. Faergemann J, Jones TC, Hettler O, Loria Y. *Pityrosporum ovale* (*Malassezia furfur*) as the causative agents of seborrhoeic dermatitis: new treatment options. Br J Dermatol 1996; 134: 12–5.
2. Janniger CK. Infantile seborrheic dermatitis: an approach to cradle cap. Cutis 1993; 51: 233–5.

3. Rajka G. Infantile seborrheic dermatitis. In: Harper J, Orange A, Prose N, eds. Textbook of Pediatric Dermatology. Oxford, Blackwell Scientific 2000; pp. 255-9.
4. Ljubojevic S, Skerlev M, Lipozencic J, Basta-Juzbasic A. The role of *Malassezia furfur* in dermatology. Clin Dermatol 2002; 20: 179-82.
5. Tollesson A, Frithz A, Stenlund K. *Malassezia furfur* in infantile seborrheic dermatitis. Pediatr Dermatol 1997; 14: 423-5.
6. Broberg A, Faergemann J. Infantile seborrheic dermatitis and *Pityrosporum ovale*. J Dermatol 1994; 21: 334-40.
7. Bergbrant IM, Brobug A. *Pityrosporum ovale* culture from the forehead of healthy children. Acta Derm Venereol 1994; 74: 260-1.
8. Danker WM, Spector SA, Furer J, *et al. Malassezia* fungemia in neonates and adults: complication of hyperalimentation. Rev Infect Dis 1987; 9: 743-53.
9. Larocco MA, Dorenbaum A, Robinson A, Pickering LK. Recovery of *Malassezia pachydermatis* from eight infants in a neonatal intensive care nursery: clinical and laboratory features. Pediatr Infect Dis J 1988; 7: 398-401.
10. Mickelsen PA, Viano-Paulson MC, Stevens DA, *et al.* Clinical and microbiological features of infection with *Malassezia pachydermatis* in high-risk infants. J Infect Dis 1988; 157: 1163-8.
11. Richet HM, McNeil MM, Edwards MC, *et al.* Cluster of *Malassezia furfur* pulmonary infections in a neonatal intensive care unit. J Clin Microbiol 1989; 27: 1197-200.
12. Welbel SF, McNeil MM, Pramanik A, *et al*. Nosocomial *Malassezia pachydermatis* bloodstream infections in a neonatal intensive care unit. Pediatr Infect Dis J 1994; 13: 104-8.
13. Francis P, Walsh TJ. Approaches to management of fungal infections in cancer patients. Oncol 1992; 6: 133-48.
14. Samonis G, Bafaloukas D. Fungal infections in cancer patients: an escalating problem. In Vivo 1992; 6: 133-48.
15. Gupta AK, Kohli Y, Li A, Faergemann J, Summerbell RC. In vitro susceptibility of the seven *Malassezia* species to ketoconazole, voriconazole, itraconazole and terbinafine. Br J Dermatol 2000; 142: 758-65.
16. Bergbrant IM, Andersson B, Faergemann J. Cell-mediated immunity to *Malassezia furfur* in patients with seborrhoeic dermatitis and pityriasis versicolor. Clin Expl Dermatol 1999; 24: 402-06.
17. Faergemann J, Bergbrant IM, Dohse M, Scott A, Westgate G. Seborrhoeic dermatitis and *Pityrosporum (Malassezia)* folliculitis: characterization of inflammatory cells and mediators in the skin by immunohistochemistry. Br J Dermatol 2001; 144: 549-56.
18. Watanabe S, Kano R, Sato H, Nakammura Y, Hasegawa A. The effects of Malassezia yeasts on cytokine production by human keratinocytes. J Invest Dermatol 2001;116: 769-73.
19. Makimura K, Tamira Y, Kudo M, Uchida K, Saito H. Species identification and strain typing of Malalassezia species stock strains and clinical isolates base on the DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. Mycol Med 2000; 49: 29-35.
20. Gupta AK, Kohli Y, Summerbell C, Faergemann J. Quantitative culture of Malassezia species from different body sits of individuals with or without dermatoses. Med Mycology 2001; 39: 243-51.
21. Ruiz–Maldonado R, Lopez–Matinez R, Chavarria EP, Castanon LR, Tamayo L. *Pityrosporum ovale* in infantile seborrheic dermatitis. Pediatr Dermatol 1989; 6: 16–20.
22. Nakabayashi A, Sei Y, Guiuol J. Identification of *Malassezia* species isolated from patients with seborrheic dermatitis; atopic dermatitis, pityriasis versicolor and normal subjects. J Mycol Med 2000; 38: 337-41.
23. Faergemann J. Seborrheic dermatitis and *Pityrosporum orbiculare*: treatment of the seborrheic dermatitis of the scalp with miconazone-hyerocortisone (Daktacort), miconazole and hydrocortisone. Br J Dermatol 1986; 114: 695-700.