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Microbiota analysis of pustules in acne vulgaris patients : special focus on *Malassezia* species

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Abstract

Acne vulgaris is a chronic, inflammatory skin disorder of pilosebaceous follicles with *Cutibacterium acnes* (formerly *Propionibacterium acnes*) as a pathogenic factor. Lipophilic yeasts of the genus *Malassezia* are the predominant fungal microbiota in normal human skin. In our analysis of the fungal microbiota of various skin diseases, the DNA of *C. acnes*, *Staphylococcus epidermidis* and *Malassezia* spp. was examined in the inflammatory pustules of patients with acne vulgaris. Pus was collected aseptically from a single pustule on the patients' face, and the DNA was extracted. The microbial DNA was detected by nested PCR using specific primers. Thirty-three patients with acne vulgaris (12 males and 21 females) were analyzed. The DNA of *C. acnes, S. epidermidis* and *Malassezia* species was detected in 39.4%, 96.9% and 69.7% of the subjects, respectively. Quantitative PCR indicated that the mean quantity of *M. globosa* DNA was relatively greater than that of *M. restricta*. In each pilosebaceous follicle, we found for the first time that either *C. acnes* or *Malassezia* spp. occupied a single pustule in the majority of the acne vulgaris patients. These results suggest that *Malassezia* spp. as well as *C. acnes* may play a role in the pathogenesis of acne vulgaris.

Introduction

Acne vulgaris is a chronic, inflammatory skin disorder of pilosebaceous follicles seen during middle to late adolescence[1)2)]. Acne vulgaris is a multifactorial disorder involving four primary pathogenic factors which interact in a complex manner to produce acne lesions : (1) sebum production by the sebaceous gland ; (2) *Cutibacterium acnes* (formerly *Propionibacterium acnes*) follicular colonization ; (3) alteration in the keratinization process ; and (4) release of inflammatory mediators into the skin[1)]. Clinical manifestations of acne vulgaris are characterized by non-inflammatory lesions consisting of closed or open comedones and inflammatory lesions consisting of erythematous macules, papules and pustules. Cumulative studies have revealed that *C. acnes* is not involved in comedogenesis or initiation of inflammation in acne lesions[1-3)]. Furthermore, *C. acnes* was not the only species of resident microorganism. Microbial analyses using culture or non-culture method indicated that the follicular contents from acne vulgaris patients contained *Staphylococcus* and *Malassezia* species as well as *C. acnes*[4-6)].

Lipophilic yeasts of the genus *Malassezia* are the predominant fungal species on normal human skin. The genus *Malassezia* currently comprises 17 species[7)], with *M. globosa* and *M. restricta* being highlighted as major elements in the skin microbiota[7-9)]. These fungi have been implicated in the pathogenesis of various skin diseases including pityriasis versicolor, *Malassezia* folliculitis, seborrheic dermatitis and atopic dermatitis[7-9)]. The mycelial form of *M. globosa* was identified as the causative organism in pityriasis versicolor [10)11)]. Meanwhile, *M. globosa*, *M. restricta* and *M. sympodialis* were detected as the dominant microbiota in *Malassezia* folliculitis[8)9)12)].

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Table 1 PCR primers and conditions for *C. acnes* and *S. epidermidis*

Species and primers used		Sequence (5′→3′)	Reaction program
1st PCR for *C. acnes*			i. Initial DNA denaturation
	F1	TGACTTTGGGATAACTTCAG	94°C for 3 min
	R1	CGCGATCAACCACCTACGAG	ii. 30 cycles of
			94°C for 30 s
			57°C for 1 min
			72°C for 50 s
			iii. Final cycle of DNA extension
			72°C for 10 min
2nd PCR for C. *acnes*			i. Initial DNA denaturation
	F2	ATAGGAGCTCCTGCTGCATG	94°C for 3 min
	R2	CAAGATTACACTTCCGACGC	ii. 30 cycles of
			94°C for 30 s
			62°C for 1 min
			72°C for 40s
			iii. Final cycle of DNA extension
			72°C for 10 min
1st PCR for *S. epidermidis*			i. Initial DNA denaturation
	F1	CTGACCCCTCTAGAGATAGAGTT	94°C for 3 min
	R1	CACCTTCGACGGCTAGCTCCAAAT	ii. 30 cycles of
			94°C for 30 s
			62°C for 1 min
			72°C for 40s
			iii. Final cycle of DNA extension
			72°C for 10 min
2nd PCR for *S. epidermidis*			i. Initial DNA denaturation
	F2		94°C for 3 min
	R2		ii. 30 cycles of
			94°C for 30 s
			62°C for 1 min
			72°C for 40s
			iii. Final cycle of DNA extension
			72°C for 10 min

In our analysis of the fungal microbiota of atopic dermatitis[13] and seborrheic dermatitis[14], we next targeted the microbiota of inflammatory lesions in acne vulgaris. We focused on the correlation between bacterial and mycological microbiota in inflammatory pilosebaceous follicles, and detected *C. acnes*, *S. epidermidis* and *Malassezia* spp. from the pustules of acne vulgaris patients.

Materials and methods

Patients and sampling

This study was conducted in the Department of Dermatology, Tokyo Medical University Hospital. The protocol was approved by the Institutional Review Board of Tokyo Medical University (No 894). Written informed consent was obtained from each subject.

Patients with acne vulgaris, diagnosed by the presence of comedones, follicular papules and pustules on the face, were enrolled in the present study. The pus used for the analysis was collected between September 2005 and February 2006, from a single pustule on the face using a sterilized disposable picker after scratching the surface of the pustule by a sterilized disposable needle. When collecting the pus, careful attention was paid to avoid contamination. The pus was placed into an Eppendorf tube directly. Only subjects with no antimicrobial or antifungal treatment during the previous two weeks were enrolled.

Table 2 Detection of C. *acnes, S.epidermidis and Malassezia* spp. in acne vulgaris patients

No.	Age	Sex	*S. epidermidis*	*C. acnes*	*Malassezia* spp.	*M. globosa*	*M. restricta*	*M. furfur*	*M. japonica*
1	14	F	+	-	+	+	-	-	-
2	31	F	-	+	-	-	-	-	-
3	24	F	+	-	+	+	-	-	-
4	31	F	+	+	-	-	-	-	-
5	20	F	+	+	-	-	-	-	-
6	26	F	+	+	-	-	-	-	-
7	-	M	+	-	+	+	-	-	-
8	38	F	+	-	+	+	+	-	-
9	32	F	+	+	-	-	-	-	-
10	21	F	+	-	+	+	-	-	-
11	20	M	+	-	+	+	+	-	-
12	20	M	+	+	-	-	-	-	-
13	20	F	+	+	-	-	-	-	-
14	21	F	+	+	-	-	-	-	-
15	20	F	+	-	+	+	-	-	-
16	20	F	+	-	+	+	+	-	-
17	34	F	+	+	-	-	-	-	-
18	21	M	+	-	+	+	-	-	-
19	20	F	+	-	+	+	-	-	-
20	22	F	+	-	+	+	-	-	-
21	65	M	+	-	+	+	+	-	-
22	46	M	+	-	+	-	+	-	-
23	47	M	+	+	+	-	+	-	+
24	30	F	+	-	+	-	-	-	+
25	53	M	+	-	-	-	-	-	-
26	15	M	+	-	+	+	+	+	-
27	29	M	+	+	+	+	+	+	-
28	17	F	+	-	+	+	+	-	+
29	19	F	+	-	+	+	+	-	-
30	27	M	+	-	+	-	+	+	-
31	31	F	+	+	+	-	+	-	-
32	19	F	+	+	+	-	-	+	-
33	40	M	+	-	+	-	+	-	+

Extraction of DNA from pus

The pus samples were placed in 100 μL of lysing solution (100 mM Tris-HCl, pH 8.0, 0.5% sodium dodecyl sulfate) and incubated for 15 min at 100°C. The suspension was transferred to another new tube, combined and extracted with phenol-chloroform-isoamyl alcohol (25 : 24 : 1, vol/vol/vol) and centrifuged at 13,700 *g*. Subsequently, the suspension was transferred to another tube, combined with chloroform- isoamyl alcohol (24 : 1, vol/ vol) and centrifuged at 13,700 *g*. The DNA was precipitated with 2.5 volume of ethanol in the presence of 3 M sodium acetate and Ethatimate (Nippon

Gene, Toyama, Japan). The DNA pellet was re-suspended in 30 μL of TE (10 mM Tris-HCl [pH8.0], 1 mM EDTA [pH8.0]) and stored at −20°C until use.

Detection of fungal/ bacterial DNA by nested and quantitative PCR

DNA extracted from each sample was added to 50 μL of the PCR master mix, which consisted of 5 μL of 10× PCR buffer (100 mM Tris-HCl, pH8.0, 500 mM KCl, 15 mM $MgCl_2$; Takara, Shiga, Japan), 4 μL of 200 μM deoxynucleoside triphosphates (an equimolar mixture of dATP, dCTP, dGTP, and dTTP ; Takara), 10 pmol of each primer, and 2.5 U of Ex *Taq* DNA polymerase (Takara). The first and second PCRs for *Malassezia* spp. were conducted according to the previously reported method[13]. The first primers for all *Malassezia* spp. were designed in the internal transcribed spacer (ITS) 1. Second species-specific primers were designed in the ITS 1 and intergenic spacer (IGS) 1 regions of the rRNA gene. The GenBank accession numbers are : *M. dermatis*-AB070356, *M. sympodialis*-AB070366, *M. furfur*-AB111459, *M. globosa*-AB099879, *M. japonica*-AB105064, *M. obtusa*-AB111461, M. restricta-AB178810, *M. slooffiae*-AY387151, *M. yamatoensis*-AB125266. *M. nana* and *M. pachydermatis* were excluded from the present study as they do not colonize human hosts. PCR was performed in a thermal cycler (Model 9700 ; Applied Biosystems, Foster City, CA, USA). In the second PCR step, 1 μL of the first amplification product was added to a new reaction mixture with the same composition as the first. Two major *Malassezia* spp., *M. globosa* and *M. restricta*, were then quantified using a quantitative PCR assay with the TaqMan probe according to the method of Sugita et al.[15]. Amplification and detection were performed using the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). The PCR primers and conditions for C. acnes and S. epidermidis are shown in Table 1. The primers were newly designed based on 16S rDNA sequences for *C. acnes* (AB14953) and *S. epidermidis* (AB987125). The PCR products were cloned using a TA cloning kit (Invitrogen, Carlsbad, CA), and positive clones were sequenced using an ABI PRISM cycle sequencing kit (PE Applied Biosystems). The specificity was also confirmed by comparing the sequences of several cutaneous microorganisms : *Candida parapsilosis*, *C. guilliermondii*, *Cryptococcus diffluens*, *C. liquefaciens*, *Micrococcus luteus*, *Rhodotorula mucilaginosa*, and *Staphylococcus aureus*.

Results

Thirty-three patients with acne vulgaris (12 males and 21 females : mean age ± SD, 28.0 ± 11.9 years) were analyzed in this study. DNA was extracted from a single pustule on the face of acne vulgaris patient. Table 2 shows the presence of DNA derived from *C. acnes*, *S. epidermidis* and *Malassezia* spp. The data are shown by individual case. DNA from *S. epidermidis* was detected in all the subjects except one (96.9%). Meanwhile, DNA from *C. acnes* was detected in 13 of 33 cases (39.4%).

DNA from *Malassezia* spp. was detected in 23 of 33 acne vulgaris patients (69.7%). Among these, DNA from *M. globosa* and *M. restricta* was detected in 48.4% and 39.3% of the subjects, respectively. DNA from *M. furfur* and *M. japonica* was detected less frequently (12.1% and 12.1%, respectively). DNA from *M. obtusa, M. yamatoensis, M. sympodialis, M. dermatis* and *M. sloffiae* was not detected (data not shown in the table). The detection frequency of the two major *Malassezia* spp., *M. globosa* and *M. restricta*, was high. Thereafter, quantitative analysis was carried out for these two species. As shown in Fig 1, the mean quantity of *M. globosa* DNA was relatively greater than that of *M. restricta* (1.4×10^5 and 5.4×10^4 copy/mm^3, respectively) (p=0.12).

It is well known that *C. acnes* and *Malassezia* spp. are lipophilic microorganisms. From the perspective of microbiota, Table 2 shows an interesting correlation between *C. acnes* and *Malassezia* spp. colonization. *C. acnes* DNA-positive pilosebaceous follicles were *Malassezia* DNA-negative (9/13) while *Malassezia* DNA-positive follicles were *C. acnes*-negative (19/23). Four pilosebaceous follicles were positive for both, and one follicle was negative for both. These results suggest that lipophilic *C. acnes* and *Malassezia* spp. compete for colonization in pilosebaceous follicles, resulting in colonization by only one of the species. However, unfortunately no clinical difference was observed among the follicles colonized by the different species.

Discussion

Acne vulgaris and *Malassezia* folliculitis have very similar clinical manifestations except in the distribution of lesions predominantly on the trunk and the lack of comedones in *Malassezia* folliculitis[8][9]. Therefore, the samples in this study were collected from pustules of facial lesions with comedones. Another important point in the study protocol is that a molecular-based non-culture method was used to detect microbial DNA from the pustules, allowing the identification of the microbiota within a single pustule.

Using nested PCR, we analyzed for the presence of DNA derived from *C. acnes*, *S. epidermidis* and *Malassezia* spp. Although quantitative PCR was not performed due to the limited quantity of DNA from a single pustule, we were able to determine the presence or absence of *C. acnes*, *S. epidermidis* and *Malassezia* spp.. Surprisingly, DNA from *S. epidermidis* was detected in almost

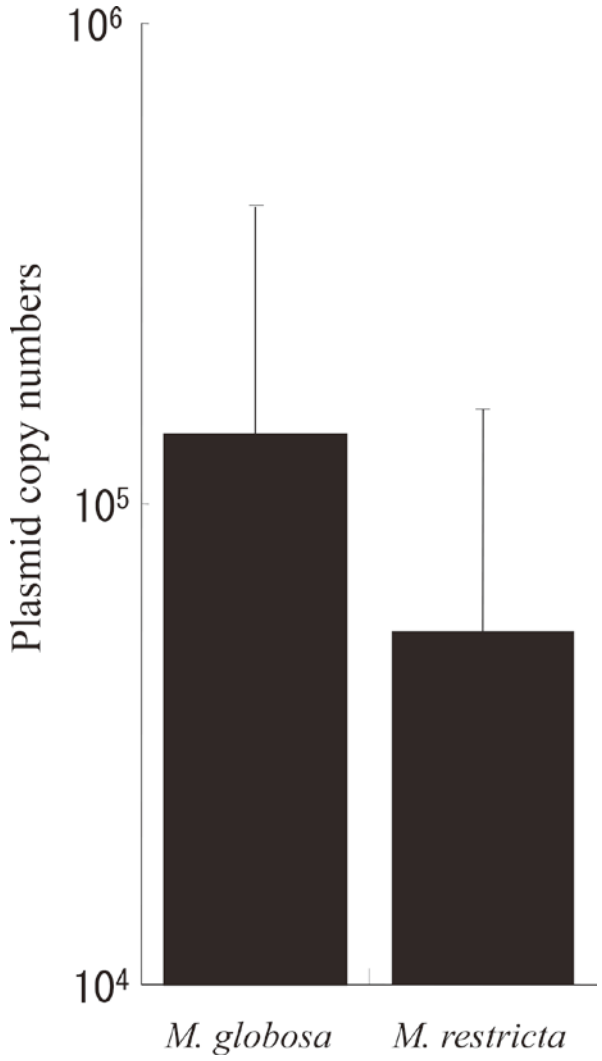

Fig. 1 Quantitative analysis of DNA of *M. globosa* and *M. restricta*
DNA samples collected in Table 1 were quantified using a quantitative PCR assay for *M. globosa* and *M. restricta*. Error bars show SD. *M.globosa*-positive patients : n= 16, *M.restricta*-positive patients : n=13

all subjects (96.9%) while DNA from *C. acnes* was detected only in 13 of 33 subjects (39.4%). In previous reports examining the microbiota of multiple follicular contents together by culture or non-culture DNA detection methods, *Cutibacteirum* spp. predominated, followed by *Staphylococcus* spp. *and Malassezia* spp.[4-6]. Since we did not use a quantitative method, we were unable to determine whether *S. epidermidis* was saprophytic flora or a pathogenic factor. As shown in Table 2, DNA from *C. acnes* was detected in 13 of 33 subjects (39.4%) while that from *Malassezia* spp. was detected in 23 of 33 subjects (69.7%). Interestingly, we found for the first time that either *C. acnes* or *Malassezia* spp. occupied one pustule in most of the acne pustules. *C. acnes* and *Malassezia* spp. compete for colonization in a pilosebaceous follicle, resulting in the exclusive presence of one of the species. This phenomenon may be due to their similar biological characteristics ; *C. acnes* and *Malassezia* spp. are lipophilic microorganisms, and *C. acnes* is an anaerobic bacterium and *Malassezi*a spp. is a microaerobic fungus. To confirm our hypothesis, studies of more cases of acne vulgaris using a next generation sequencer and those comparing before and after the specific anti-microbial treatments are required.

M. globosa and *M. restricta* are known to be major microbiota in healthy skin and various skin diseases though the relative detection rate of the two major species differs among skin disorders[7-9]. This observation was enabled by the molecular-based, culture-independent method described by Sugita et al. in 2001[13]. In the culture method, the detection rate of *M. restricta* was very low due to the lack of an appropriate isolation medium or isolation technique[8]. In our study, *M. globosa* was detected in the pilosebaceous follicles of 16 of 23 subjects (69.6%), *M. restricta* in 13 of 23 subjects (56.5%), and both species in 8 of 23 subjects (34.8%). Quantitative analysis of these two species also indicated a relatively greater colonization rate for *M. globosa* though the difference was not statistically significant (Fig. 1). Therefore, we were unable to confirm that acne vulgaris is a disease with *M. globosa* as the major fungal microbiota. Furthermore, phylogenetic analysis of the isolated strains of two species, using the IGS region in the rRNA gene, failed to show any acne-specific strain of either *M. globosa* or *M. restricta* when compared with atopic dermatitis patients and healthy subjects (unpublished data).

The role of *C. acnes* in the pathogenesis of acne vulgaris has been intensively studied[1]. *C. acnes* contributes to the inflammatory stage of acne by inducing monocytes to secrete inflammatory cytokines and releasing lipases, proteases and hyaluronidases, which contribute to tissue injury[16)17)]. *C. acnes* was found to bind to toll-like receptor-2 on monocytes and keratinocytes[16)18)]. Meanwhile, the role of *Malassezia* spp. in the pathogenesis of acne vulgaris and *Malassezia* folliculitis has not been intensively studied. *Malassezia* spp. can live as commensal flora in normal skin and therefore has less inflammation-inducing activity than other microorganisms. Yamasaki et al reported that *Malassezia* spp. binds to Mincle, a C-type lectin receptor, on macrophages, which produce inflammatory cytokines and chemokines[19].

The reason for relatively low detection rate of *C. acnes* compared with the findings of previous reports[3] is obscure. One possibility is the change in microbiota due to extended use of antibiotics against *C. acnes* though antibiotic-treated patients were excluded from the present study. For refractory cases of acne vulgaris,

administration of an antifungal agent is an alternative treatment option. Before-and-after clinical studies of specific antimicrobial treatments may elucidate the role of *Malassezia* spp. in the pathogenesis of acne vulgaris.

Conclusion

We examined the detection rate of *C. acnes*, *Staphylococcus epidermidis* and *Malassezia* spp. DNA in inflammatory pustules from 33 acne vulgaris patients. The DNA was extracted from a single pustule on the face of each patient, and the microbial DNA was detected by nested PCR. The DNA of *C. acnes, S. epidermidis* and *Malassezia* spp. was detected in 39.4%, 96.9% and 69.7% of the subjects, respectively. Quantitative PCR indicated that the mean quantity of *M. globosa* DNA was relatively greater than that of *M. restricta*. In each pilosebaceous follicle, we found for the first time that either *C. acnes* or *Malassezia* spp. occupied a single pustule in the majority of the acne vulgaris patients. These results suggested that *Malassezia* spp. as well as *C. acnes* may play a role in the pathogenesis of acne vulgaris.

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ざ瘡患者膿疱の菌叢解析：特にマラセチア属に注目して

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【要旨】 ざ瘡は脂腺性毛包の慢性炎症性皮膚疾患であり、*Cutibacterium acnes*（従来名 *Propionibacterium acnes*）が病原因子として働いている。脂質要求性酵母である *Malassezia* 属は正常皮膚菌叢において優勢な真菌である。我々は種々の皮膚疾患の真菌叢を調べる過程で、本試験ではざ瘡患者の炎症性膿疱から *C. acnes*、*Staphylococcus epidermidis* と *Malassezia* 属の DNA の検出を試みた。膿は顔面の単一の膿疱から無菌的に採取し、DNA を抽出した。菌由来 DNA は菌種特異的プライマーを用いて nested PCR により検出した。その結果 33 人の被験者（男性 12 名、女性 21 名）が解析された。*C. acnes*、*S. epidermidis*、*Malassezia* 属の DNA はそれぞれ 39.4%、96.9%、69.7% の被験者から検出された。定量的 PCR では *M. globosa* の DNA 量は *M. restricta* の量よりも多く検出された。大多数のざ瘡患者でひとつの脂腺性毛包には *C. acnes* か *Malassezia* 属のいずれかの菌種が占拠していることを初めて見出した。これらの結果からざ瘡の病態において *C. acnes* だけでなく *Malassezia* 属も重要な役割を果たしていることが示唆された。

〈キーワード〉 *Malassezia*, *Cutibacterium acnes*, *Staphylococcus epidermidis*, ざ瘡, PCR