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ORIGINAL ARTICLE

Characterization of acne patients carrying clindamycin-resistant *Cutibacterium acnes*: A Japanese multicenter study

Keisuke NAKASE,[1] Sae AOKI,[1] Sayaka SEI,[1] Sana FUKUMOTO,[1] Yuki HORIUCHI,[2] Takae YASUDA,[3] Miki TANIOKA,[4] Junichi SUGAI,[5] Wook-Kang Winnie HUH,[6] Mie KAKUTA,[7] Mayumi NOMOTO,[8] Tokihiko SHIMADA,[9] Masako WATANABE,[10] Miwa KOBAYASHI,[11] Saori MURAKAMI,[12] Chikage TAKEO,[13] Rieko TSUBOUCHI,[14] Nobukazu HAYASHI,[15] Norihisa NOGUCHI[1]

[1]*Department of Microbiology, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences,* [2]*Akihabara Skin Clinic, Tokyo,* [3]*Nakano Dermatology and Urology Clinic, Hiroshima,* [4]*Tanioka Dermatology Clinic, Kyoto,* [5]*Sugai Dermatology Parkside Clinic, Tochigi,* [6]*Hoh Dermatology Clinic, Okayama,* [7]*Kakuta Dermatology Clinic, Tokyo,* [8]*Nomoto Mayumi Dermatology Clinic, Niigata,* [9]*Shimada Dermatology Clinic, Kagoshima,* [10]*Kobayashi Dermatology Clinic, Hyogo,* [11]*Kobayashi Dermatology Clinic, Fukuoka,* [12]*Murakami Dermatology Clinic, Ehime,* [13]*Yoyogi Uehara Dermatology Clinic,* [14]*Ginza Skin Clinic,* [15]*Department of Dermatology, Toranomon Hospital, Tokyo, Japan*

ABSTRACT

Use of antimicrobials for acne treatment is correlated with an increased occurrence of antimicrobial-resistant *Cutibacterium acnes*. To clarify the role of antimicrobial use on the resistance and to investigate the characteristics of resistant strains, we conducted a multicenter study in dermatological clinics frequently visited by new patients with acne vulgaris. We collected specimens in 264 acne patients and tested 164 *C. acnes* strains isolated from 164 patients visiting 13 dermatological clinics. Antimicrobial susceptibility testing showed that the rates of resistance for tetracyclines, macrolides and clindamycin were significantly higher in *C. acnes* strains isolated from patients using antimicrobials for acne treatment than patients not using them. In particular, clindamycin-resistant strains were frequently isolated from patients with older median age (≥24 years) and severe/moderate acne. After investigating the resistance mechanism of 15 high-level clindamycin-resistant strains, the transposable clindamycin resistance genes, *erm*(X) or *erm*(50), were detected in 14 strains. Using single-locus sequence typing for *C. acnes*, the strains with *erm*(X) or multidrug resistance plasmid pTZC1 coding *erm*(50) and tetracycline resistance gene *tet*(W) were classified into clade F, which were specifically isolated from Japanese patients with acne, except for one strain. Our data showed that patients' information, such as antimicrobial use, age and acne severity, are valuable in estimating whether a patient carries antimicrobial-resistant *C. acnes*. Additionally, our results suggest that the clade F strains have a high risk of acquiring multidrug resistance.

Key words: **acne vulgaris, antimicrobial resistance, clindamycin, *Cutibacterium acnes*, patient's information.**

INTRODUCTION

Acne vulgaris is a chronic inflammatory skin disease that affects many adolescents.[1] Acne is formed on the face, breast and upper back, and though not lethal, it imposes heavy mental stress on the patients.[2,3] Overgrowth of *Cutibacterium acnes* in acne pustules is considered an exacerbation factor for acne vulgaris.[4] Antimicrobial agents, such as topical clindamycin and oral tetracyclines, are used in acne patients to decrease the *C. acnes* population. However, onset of side-effects and emergence of antimicrobial-resistant bacteria are a prime concern with antimicrobial use.[1,5,6] In Japan, topical adapalene and benzoyl peroxide were listed in the national health insurance price list in 2008 and 2015, respectively, significantly improving the choices among medicines available for acne treatment.[2]

Antimicrobial-resistant *C. acnes* have been isolated from acne patients globally.[7–9] We previously reported a significant increase in antimicrobial-resistant *C. acnes* isolated from Japanese patients seeking clinical intervention.[10,11] Additionally, we observed that antimicrobial-resistant strains were frequently isolated from patients using antimicrobials for acne treatment. Therefore, we hypothesized that characterizing the antimicrobial-resistant strains from acne patients would enable selection

Correspondence: Keisuke Nakase, Ph.D., Department of Microbiology, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. Email: nakase@toyaku.ac.jp
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of an effective antimicrobial treatment. However, in our previous study, we included patients using antimicrobials.[11] In this study, we designed our inclusion criteria to study acne patients who had not been using antimicrobials to elucidate if antimicrobials played a role in the prevalence of resistant bacteria in acne patients.

METHODS

Patients' backgrounds, bacterial strains and growth conditions

Acne pustule samples were obtained from 264 patients who visited 13 dermatological clinics in Japan between 2016 and 2017 (82 males, 170 females, 12 unknown sex) (age range, 10–58 years; mean age, 24.2 ± 8.4; median age, 23). The study was approved by the research ethics committee of the Tokyo University of Pharmacy and Life Sciences (approval no. 16-21). Patients' information was obtained using a questionnaire-based survey, and the use of antimicrobial for acne treatment in the previous 6 months was assessed. Acne severity was diagnosed based on the Japanese Acne Treatment Guideline.[2,12] Bacterial identification and culture were conducted using the methods previously reported.[10] We isolated a total of 164 strains of *C. acnes* from 164 patients (acne severity: mild, 63; moderate, 72; severe, 29) and 179 strains of *Staphylococcus epidermidis* from 179 patients (acne severity: mild, 62; moderate, 82; severe, 31; unknown, 4).

Susceptibility testing

Antimicrobial susceptibilities of *C. acnes* and *Staphylococcus epidermidis* were evaluated by measuring the minimum inhibitory concentration (MIC) using the agar dilution method prescribed by the Clinical and Laboratory Standards Institute (CLSI).[13–15] *C. acnes* JCM6425 as type strain and C. *acnes* JCM6473 were used as the susceptible controls, while methicillin-resistant *S. aureus* N315 strain was used as the resistant control and *S. aureus* JCM2874 strain was used to ensure the accuracy of the tests. We tested 17 antimicrobial agents: cefditoren sodium (Meiji Seika Pharma, Tokyo, Japan), faropenem sodium and ozenoxacin (Maruho, Osaka, Japan) were obtained from the respective manufacturers. Amoxicillin, tetracycline hydrochloride, doxycycline hyclate and ciprofloxacin hydrochloride were purchased from Sigma-Aldrich (Tokyo, Japan). Levofloxacin hydrochloride, clarithromycin, azithromycin, clindamycin hydrochloride and minocycline hydrochloride were purchased from Tokyo Chemical Industries (Tokyo, Japan). Oxacillin, nadifloxacin, erythromycin, roxithromycin and gentamicin sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). The resistance breakpoints were calculated from the MIC of the susceptible strains.[11]

Determination of antimicrobial resistance factor in *C. acnes*

Macrolide clindamycin-resistance factors in *C. acnes*, such as 23S rRNA mutation and the presence of erythromycin resistance methylase (erm)-encoding *erm*(X) and *erm*(50) genes were detected by polymerase chain reaction (PCR) and DNA sequencing.[10,16] The presence of the *mecA* gene, which encodes an altered penicillin-binding protein conferring β-lactam resistance, in *S. epidermidis* was detected by PCR, and strains carrying *mecA* were defined as methicillin-resistant *S. epidermidis* (MRSE).[17,18]

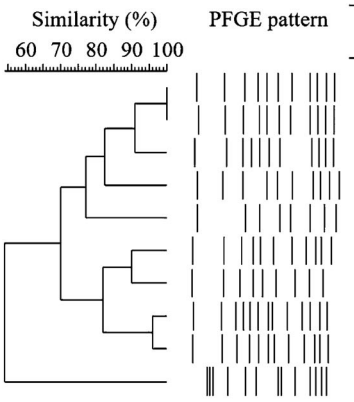
Phylogenetic analysis

The phylogenetic type of *C. acnes* was determined using single-locus sequence typing (SLST) by DNA sequence.[11,19] Pulsed-field gel electrophoresis (PFGE) for *C. acnes* DNA was carried out as previously published.[10,20] The staphylococcal

Table 1. Antimicrobial susceptibilities for *Cutibacterium acnes* isolated from acne patients who previously used or did not use any antimicrobials

	Antimicrobial used (n = 100)			No antimicrobial used (n = 64)			Total (n = 164)		
Antimicrobial agent	MIC range	MIC_{90}	R (%)	MIC range	MIC_{90}	R (%)	MIC range	MIC_{90}	R (%)
Amoxicillin	≤0.06–0.25	0.13	0	≤0.06–0.25	0.13	0	≤0.06–0.25	0.13	0
Cefditoren	≤0.06–0.25	0.13	0	≤0.06–0.25	0.13	0	≤0.06–0.25	0.13	0
Faropenem	≤0.06	≤0.06	0	≤0.06–0.25	≤0.06	0	≤0.06–0.25	≤0.06	0
Levofloxacin	0.25–16	8	11.0	≤0.06–16	0.5	3.1	≤0.06–16	1	7.9
Nadifloxacin	0.13–16	4	ND	≤0.06–8	0.5	ND	≤0.06–16	1	ND
Ozenoxacin	≤0.06–0.5	0.13	ND	≤0.06–0.25	≤0.06	ND	≤0.06–0.5	≤0.06	ND
Erythromycin	≤0.06–≥256	≥256	40.0*	≤0.06–≥256	≤0.06	7.8	≤0.06–≥256	≥256	27.4
Clarithromycin	≤0.06–≥256	≥256	40.0*	≤0.06–≥256	≤0.06	7.8	≤0.06–≥256	≥256	27.4
Roxithromycin	≤0.06–≥256	≥256	40.0*	≤0.06–≥256	≤0.06	7.8	≤0.06–≥256	≥256	27.4
Azithromycin	≤0.06–≥256	≥256	40.0*	≤0.06–≥256	≤0.06	7.8	≤0.06–≥256	≥256	27.4
Clindamycin	≤0.06–≥256	≥256	29.0*	≤0.06–≥256	0.25	7.8	≤0.06–≥256	128	20.7
Gentamicin	≤0.5–32	8	ND	≤0.5–32	8	ND	≤0.5–32	8	ND
Doxycycline	≤0.06–16	4	6.0	≤0.06–1	0.5	0	≤0.06–16	2	3.7
Minocycline	≤0.06–8	2	0	≤0.06–1	0.5	0	≤0.06–8	1	0

MIC_{90} values indicates the minimum inhibitory concentration (MIC) (μg/mL) that inhibits the growth of 90% of the strains. R (%), the rate of resistant strains. ND, nadifloxacin, ozenoxacin and gentamicin, topical agents, were not defined resistance breakpoints.
*Asterisks indicate significant difference versus "no antibiotics used" by Fisher's exact test ($P < 0.05$).



Strain	Clinic	SLST	Acne severity
AS31a	Tokyo A	F19	Mild
AS56a	Tokyo A	F4	Moderate
AS21a	Tokyo A	F1	Moderate
AS3a	Tokyo A	F4	Moderate
AS60a	Tokyo A	A5	Moderate
YU4a	Tokyo D	F1	Moderate
YU10c	Tokyo D	F1	Moderate
KA5a	Tokyo B	F1	Mild
NA31a	Hiroshima	F4	Moderate
JCM6473	-	K9	-

Figure 1. Pulsed-field gel electrophoresis (PFGE) analysis of *Cutibacterium acnes* carrying *erm*(50). PFGE data could not be obtained for one strain.

cassette chromosome (SCC) *mec* type of *S. epidermidis* was determined as previously published.[17,18]

Statistical analysis

The comparison between the two groups was analyzed using Student's *t*-test and Fisher's exact test (JMP Pro; SAS Institute, Tokyo, Japan).

RESULTS

Antimicrobial resistance for *C. acnes*

We measured the antimicrobial susceptibility of 164 *C. acnes* strains (Table 1). Our results showed that the resistance rates against roxithromycin and clindamycin were 27.4% (45/164) and 20.7% (34/164), respectively. Moreover, all clindamycin-resistant strains also exhibited cross-resistance for macrolides. Resistance rates against levofloxacin and doxycycline were 7.9% (13/164) and 3.7% (6/164), respectively. In patients who previously used antimicrobials, the resistance rates of macrolides and clindamycin were significantly higher than in patients who did not use antimicrobials ($P < 0.05$). Interestingly, a significant difference in the clindamycin resistance rate was found among the clinics (0–62.5%, Table S1). We then analyzed the 45 macrolide clindamycin-resistant *C. acnes* strains to investigate the resistance mechanism. Our results showed that 68.9% (31/45) of the strains had 23S rRNA mutation (Table S2). These 23S rRNA mutants were isolated from all of the dermatological clinics except two. By contrast, the exogenous resistance gene *erm*(X) and the pTZC1 plasmid coding for the *erm*(50) and *tet*(W)genes were found in 8.8% (4/45) and 22.2% (10/45) of resistant strains, respectively. The strains carrying *erm*(X) and pTZC1 were isolated from two and four clinics, respectively. Furthermore, the strains carrying pTZC1 might have spread between acne patients, because they were all isolated from clinics located in Tokyo. However, the results of PFGE analysis showed that the strains carrying pTZC1 with different band patterns were not the same strains (Fig. 1). Moreover, the antimicrobial resistance rate in *S. epidermidis* strains were higher in the strains isolated from patients who previously used antimicrobials for acne treatment (Table S3). Additionally, the prevalence of MRSE in patients who used antimicrobials (57.7%) was significantly higher than in patients

Table 2. Relationship between the history of use of antimicrobials and isolation of antimicrobial-resistant *Cutibacterium acnes*

	Resistance rate (%)*		
Antimicrobial agent	Antimicrobial used	Not used	*P*
Oral macrolides	59.3	21.2	**<0.01**
Topical clindamycin	31.1	15.5	**0.02**
Quinolones	13.0	5.9	0.12
Oral tetracyclines	11.8	1.5	**0.02**

The patients of "antibiotics used" had used antibiotics for treatment acne vulgaris within the past 6 months.
*Oral macrolides, topical clindamycin, quinolones and oral tetracyclines showed the resistance rates of roxithromycin, clindamycin, levofloxacin and doxycycline, respectively.

Table 3. Characteristics of acne patients in whom clindamycin-resistant *Cutibacterium acnes* were isolated

Feature	Resistant ($n = 34$)	Susceptible ($n = 130$)	*P*
Age, years			
Range	15–45	11–58	-
Mean	27.1	24.2	**<0.05**
Female, *n* (%)	25 (73.5)	84 (64.6)	0.22
Family history of acne, *n* (%)	14 (41.2)	49 (37.7)	0.43
Acne severity, *n* (%)			
Mild	7 (20.6)	56 (43.1)	**<0.05**
Moderate	21 (61.8)	51 (39.2)	**<0.05**
Severe	6 (17.6)	23 (17.7)	0.59
Disease duration of acne, years	9.9	7.6	0.07

Table 4. Relationship between acne patients' age and resistant rate of clindamycin

Acne severity	Resistance rate (%) Younger (11–23 years)	Older (24–58 years)	*P*
Mild	11.1	11.1	0.65
Moderate	13.9	44.4	**0.02**
Severe	10.5	40.0	**0.04**

who did not (20.0%) ($P < 0.05$). No significant difference was found between these groups in the SCC*mec* MRSE type, with the most common strain being SCC*mec* type IV (previously used, 58.3%; not used, 46.7%).

Patients' backgrounds and characterization of antibiotic-resistant determinants

To further understand the effect of antimicrobial use for acne treatment on the appearance of *C. acnes*-resistant strains, we divided our patients based on the type of antimicrobial used (Table 2). The resistance rate of *C. acnes* strains isolated from patients who used topical clindamycin (31.1%, 19/61) was significantly higher than the patients who did not use it (15.5%, 16/103) ($P < 0.05$). Similarly, resistance rates for roxithromycin and doxycycline of *C. acnes* strains isolated from patients who used oral macrolides and tetracyclines were significantly higher than strains from patients who did not use these treatments ($P < 0.05$).

Next, we analyzed the characteristics of patients from whom clindamycin-resistant strains were isolated. We found that their mean age was significantly higher, and the frequency of mild acne was significantly lower than patients from whom clindamycin-susceptible strains were isolated ($P < 0.05$) (Table 3). Then, we analyzed the relationship between age and acne severity in patients with clindamycin-resistant strains (Table 4). Clindamycin resistance was significantly higher in older patients with severe or moderate acne (≥24 years) than in younger patients (≤23 years) ($P < 0.05$). A similar trend was observed in the patients from whom roxithromycin-resistant strains were isolated. However, the trend could not be analyzed in patients from whom doxycycline- and levofloxacin-resistant strains were isolated due to the small sample size (data not shown).

Characterization by phylogenetic analysis of *C. acnes*

We determined the phylogenetic type of *C. acnes* strains by using SLST. The *C. acnes* strains were classified into 28 different types belonging to eight clades (A–K) (Fig. 2). Clade A

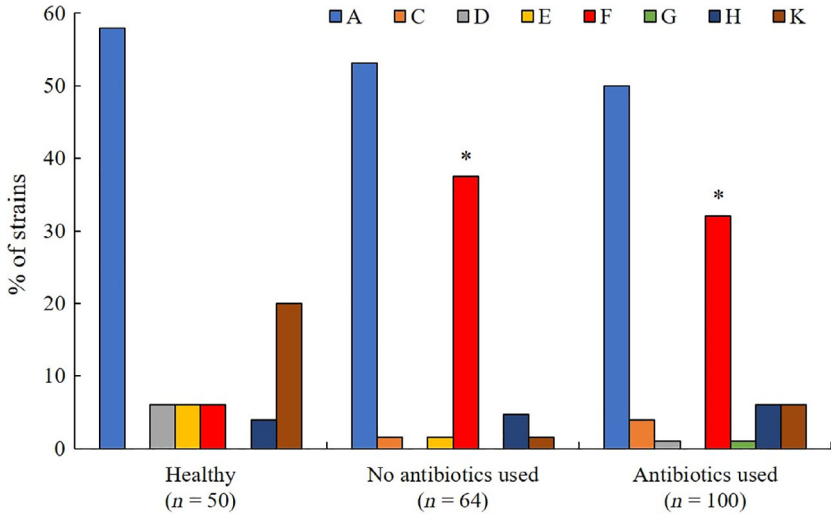
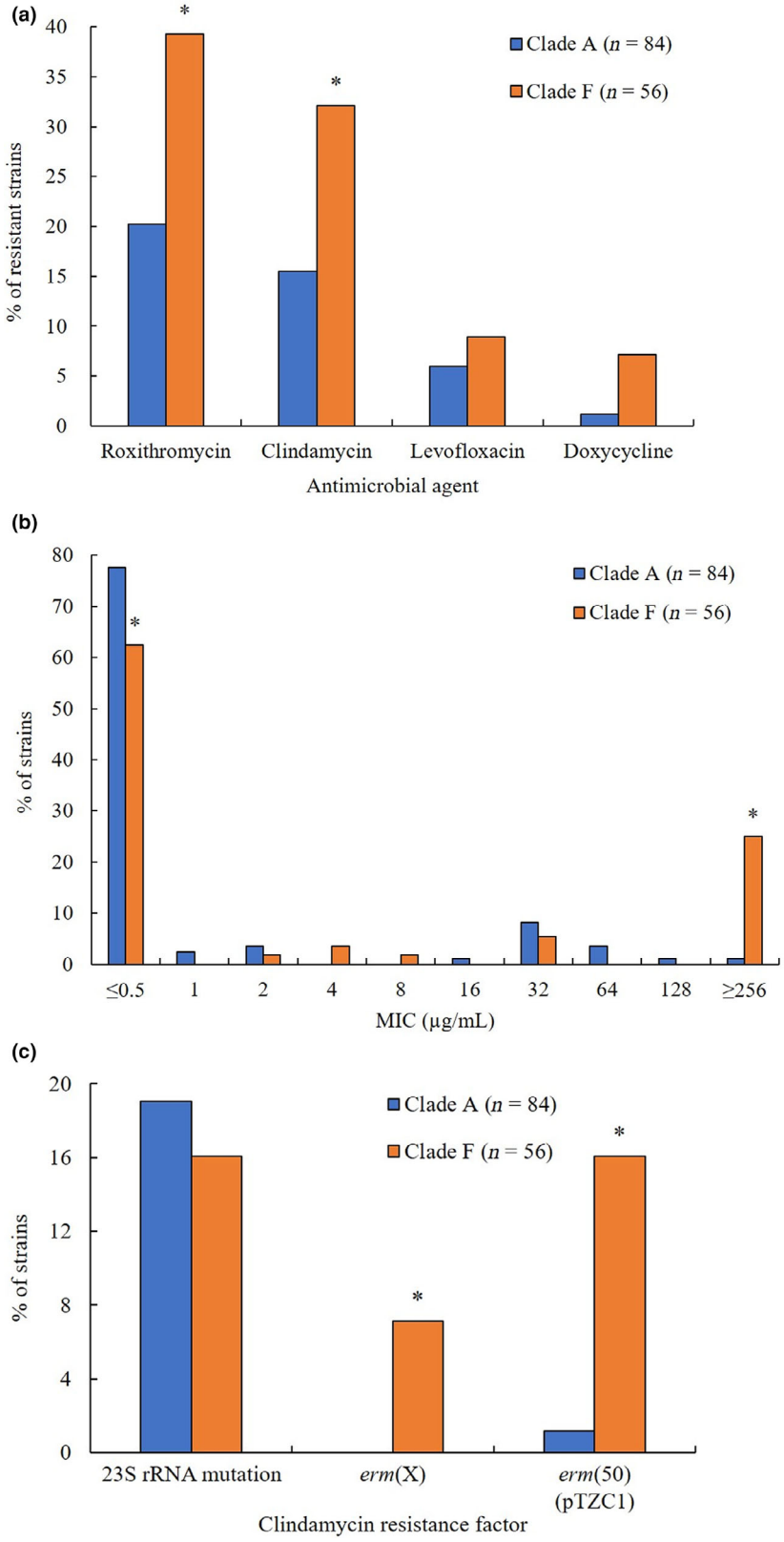


Figure 2. Distribution of single-locus sequence typing (SLST) clades in *Cutibacterium acnes* isolated from acne patients depending on the use of antibiotics for acne treatment. The patients of "antibiotics used" had used antibiotics as acne treatment within last 6 months. The "healthy" group showed the skin sample of healthy individuals in our previous report.[11] Asterisks indicates significant difference by Fisher's exact test (vs healthy, $P < 0.05$).

Figure 3. Comparison of antimicrobial resistance in *Cutibacterium acnes* belonging to single-locus sequence typing clade A and F. (a) Proportion of antimicrobial resistance. (b) Minimum inhibitory concentration (MIC) distribution of clindamycin MIC in *C. acnes*. Asterisks indicate significant difference calculated by Fisher's exact test between the rates of clade A and clade F between the same MIC ($P < 0.05$). (c) Prevalence of the resistant factors of clindamycin in *C. acnes* strains. *erm*(X) was found only in clade F strains (4/56, 7.1%). The *erm*(50) gene coded in multidrug resistance plasmid pTZC1 was detected in nine clade F strains (16.1%) but only in one clade A strain (1.2%).



(traditional type IA_1) is the major clade found on Japanese healthy individuals and was also the most isolated clade in our patients (50.0% in the group using antimicrobials and 53.8% in the group not using them).[11] We also isolated the clade F strains (traditional type IA_2), which are rarely isolated from acne patients,[21] in 32.0% of the patients using antimicrobials and 36.9% of the patients who did not. Therefore, our data suggested that antimicrobial use has no effect on the type of *C. acnes* strain present in acne pustules.

We then compared the rate of antimicrobial resistance between A and F clades. We found that the clade F strains showed higher resistance to roxithromycin, clindamycin, levofloxacin and doxycycline (Fig. 3a). In particular, the resistance rates of roxithromycin and clindamycin in the clade F strains were remarkably higher. Additionally, the high-level clindamycin-resistant strains (MIC $\geq$ 256 μg/mL) were only detected from clade A and F strains, and their proportion was significantly higher in the clade F strains ($P < 0.05$) (Fig. 3b). When we investigated the resistance mechanism for 15 high-level clindamycin-resistant strains, one, four and 10 strains had 23S rRNA mutation, acquisition of *erm*(X) and pTZC1, respectively. No difference was found in the isolation frequency of 23S rRNA mutants between the strains of clade A (18.8%) and F (16.1%). In contrast, the strains having *erm*(X) or pTZC1 were classified into clade F except for one strain (Fig. 3c). The acquisition ratio of exogenous antimicrobial resistance genes in clade F strains was 19.5-fold higher than in the clade A strains. Furthermore, the strains carrying the multidrug resistance pTZC1 plasmid encoding the *tet*(W) gene showed resistance or low susceptibility to tetracycline (MIC range, 8–32 μg/mL). Based on our results, we strongly suggest that the clade F strains are more susceptible to acquiring multidrug resistance.

DISCUSSION

Our previous study of hospital outpatients showed that the resistance of *C. acnes* strains to roxithromycin and clindamycin in acne patients was 44.3% and 38.6%, respectively.[11] The present study for dermatological clinic patients showed a lower resistance rate (roxithromycin, 27.4%; clindamycin, 21.1%). Further, the number of patients with a history of antimicrobial use was lower for dermatological clinic patients (60.6%) than hospital outpatients (81.4%).[11] Therefore, it appears that the proportion of patients who used antimicrobials is directly correlated with the antimicrobial resistance rate. In the present study, 32.1% of the patients who used clindamycin topically presented clindamycin-resistant strains; in contrast, only 16.5% of patients who did not use clindamycin presented with clindamycin-resistant strains. Moreover, a similar trend was observed in patients who used oral macrolide, quinolones and oral tetracyclines. Therefore, our data suggest that antimicrobial use for acne treatment strongly relates to the appearance of antimicrobial-resistant *C. acnes*.

Antimicrobial-resistant strains were isolated more frequently from older patients with severe or moderate acne. Usually, the duration of the antimicrobial treatment is longer for acne than for other infections,[2] and therefore patients affected by severe acne have longer treatment time. Therefore, our data suggest that the patients' information could be useful to estimate whether a patient carried antimicrobial-resistant *C. acnes*.

We previously reported that the clade F strains were more abundant in acne patients than healthy individuals.[11] In the present study, our results showed that the rate of clade F in dermatological clinic patients was similar to that previously reported in hospital outpatients.[11] In contrast, in France, the isolation frequency of clade F strains was reportedly low (<10%), and there was no difference in the isolation rate between acne patients and healthy individuals.[21] Therefore, we suggest that the clade F strains specifically correlate to acne pathology in Japanese patients. The resistance rates for roxithromycin, clindamycin and doxycycline observed in clade F strains were higher than in clade A strains. Additionally, the acquisition ratio of exogenous antimicrobial resistance genes in clade F strains was remarkably higher (19.5-fold) than in clade A strains. Previously, it was reported that the clade F strains acquired *erm*(X) with high frequency.[22] Likewise, the clade F strains could possibly become multidrug resistant by acquiring the pTZC1 plasmid, coding for the *erm*(50) and *tet*(W) genes, with high frequency.

In conclusion, our data showed that the presence of antimicrobial-resistant *C. acnes* in acne patients could be estimated from the patients' information. Furthermore, our results revealed that the clade F strains could be a risk factor for the increase of antimicrobial resistance in Japanese patients.

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CONFLICT OF INTEREST: None declared.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Antimicrobial susceptibilities for *Cutibacterium acnes* isolates at each dermatological clinic
Table S2. Detection rate of macrolide clindamycin resistance factors at each clinic
Table S3. Antimicrobial susceptibilities for *Staphylococcus epidermidis* isolates

