In vitro Study of Antibiotic Susceptibility of *Propionibacterium acnes* Strains Isolated from Acne Vulgaris Patients

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Background: *Antibiotic-resistant Propionibacterium acnes has become a worldwide problem in recent years. The prevalence of antibiotic resistance to this bacterium has increased in many countries. Over the past decade, there are few previous studies regarding the incidence of antibiotic resistance and antimicrobial susceptibility of P. acnes in Thailand.*
Objective: *The aim of the present study is to determine the antibiotic susceptibility patterns of P. acnes strains from acne patients in Thailand.*
Material and Method: *A cross sectional was used to investigate in this study. Ninety-five acne patients were enrolled. Samples were collected from facial closed comedones using comedone extraction technique and isolated in an anaerobic condition. The strains were identified by phenotypic characteristics and API 20A. Antibiotic susceptibility tests and minimal inhibitory concentration (MIC) of P. acnes strains to five antibiotics that are commonly used in acne treatments in Thailand (erythromycin, clindamycin, tetracycline, doxycycline and amoxicillin), were determined by the Epsilometer test method.*
Results: *Among 95 samples, P. acnes strains were isolated from 75 patients (78.95%). MIC_{90} of doxycycline, tetracycline, amoxicillin, clindamycin and erythromycin were 1.7, 16, 0.016, 256 and 256 μg/mL, respectively. By using CLSI breakpoints for resistance, forty-eight (64%) and forty-seven (62.66%) strains resisted to erythromycin and clindamycin, while only one (1.33%) strain resisted to tetracycline. No resistance to doxycycline and amoxicillin was found in this present study. Moreover, there were statistically significant differences among age groups, history of previous antibiotic treatment and macrolide antibiotic resistance.*
Conclusion: *Among the antibiotics tested in the present study, the most common antibiotic resistance was erythromycin, followed by clindamycin and tetracycline, respectively; whereas, no resistance to doxycycline and amoxicillin was found. The antibiotic-resistant P. acnes strains have been continuously increasing in Thai acne patients from the present study.*

Keywords: *Acne vulgaris, Antibiotic resistance, Antibiotic susceptibility, Propionibacterium acnes*

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Acne is a common skin disease occurs in adolescence. It is characterized by white and black comedo, papules and pustules. Acne may precipitate psychological and social problems on individuals. It can induce stress, anxiety, depression and suicidal ideation.

The acne pathogenesis is multifactorial, which comprises of follicular hyperkeratinization, excess sebum production, inflammation and *Propionibacterium acnes* proliferation. *P. acnes* is one of the major strain isolated from acne lesions and plays an important role in acne pathogenesis. *P. acnes* stimulates inflammation and immune response via various mechanisms[1]. Recent study has been shown that *P. acnes* biofilm acts as biological glue which adheres to follicular walls leading to the reduction of responsiveness of *P. acnes* to antibiotics[2].

In the late 1970s, antibiotic resistance of *P. acnes* was first reported in Unites States[3]. From then on, antibiotics resistance of *P. acnes* has been reported in all major regions of the world[3-8]. Antibiotic resistance of *P. acnes* becomes a global problem in recent years. Most of the countries have reported that more than a half of *P. acnes* strains become resistant, predominantly to topical macrolides[4,8-15]. Generally, *P. acnes* resistant to erythromycin is the most common, followed by resistant to clindamycin and tetracycline, respectively. The clinical resistance of *P. acnes*, which manifested as a reduced response, no

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response or relapse[16] to erythromycin was reported in 2003[17].

There are various options for acne treatments including topical and systemic agents. Topical agents in acne treatment include topical antibiotics, benzoyl peroxide and retinoids. Systemic agents include oral antibiotics, oral isotretinoin, oral contraceptive pill and oral spironolactone[18]. Due to routine use of antibiotics, especially the use of topical antibiotics alone, the prevalence of antibiotic resistance has been increasing over the years[19]. Antibiotic-resistant *P. acnes* has become a global problem in recent years. Moreover, the incidence of antibiotic resistance of this bacterium has been increasing in many countries[4,19]. Over the past decades, there are few previous studies regarding the incidence of antibiotic resistance and antimicrobial susceptibility of *P. acnes* in Thailand. In addition, there was little knowledge about the antibiotic susceptibility of *P. acnes* in Thai acne patients. Hence, the antibiotic susceptibility patterns of *P. acnes* from acne patients were investigated in this study. We further investigated antibiotic susceptibility tests of *P. acnes* to amoxicillin and the difference in resistant-*P. acnes* in relation to age, disease duration, previous treatment and history of isotretinoin used.

Material and Method

Selection of patients

A cross sectional was used to investigate in this study. Patients with clinically diagnosed acne were enrolled. The sample size was calculated by using PS sample size program (version 3.0). The calculation was performed using a proportion (p) = 0.6, type I error (α) = 0.5, Z value at 95% confidence interval (Z) = 1.96, the degree of precision (d) = 0.05. The sample size amounted to 87 patients. From the previous study, rate of *P. acnes* isolated from closed comedone is 92 percent[20]. The final sample size is 95 patients. Age, sex, disease duration, severity of disease, treatment history and site of sampling were recorded from all patients. The study was performed at the Skin Center, Srinakharinwirot University, Bangkok, Thailand and was approved by the clinical research ethical committee of Srinakharinwirot University (Authorization No. SWUEC/E-272/2559).

Sample collection and isolation of P. acnes

Skin over the acne lesion was first decontaminated by 70% alcohol and then samples were collected from facial closed comedo using comedones extraction technique. Samples were immediately smeared onto brain heart infusion agar plates supplemented with horse serum. Inoculated plates were incubated at 37°C for 7 days under anaerobic conditions (0% O_2, 5% CO_2, 5% H_2, 90% N_2) in anaerobic jars and *P. acnes* were identified by gram stain and colony morphology. The species level of cultured organism was finally identified using an API 20A kit, a manual identification system for anaerobic bacteria (bio Meriux®, France).

Phenotypic characterization of P. acnes strains by API 20A

Carbohydrate fermentation patterns of *P. acnes* strains and biochemical reactions were determined by using API 20A kit. Species were determined by characterizing the abilities to ferment carbohydrate and comparing profiles with known carbohydrate fermentation profiles of *P. acnes* in API 20A system version 5.0. The phenotypic characterization of *P. acnes* strains by API 20A are shown in Table 1.

Antibiotic susceptibility testing

After bacterial isolation and species identification by API 20A kit, *P. acnes* isolates were selected and subcultured. Antibiotic susceptibility tests of *P. acnes* were determined by Epsilometer test (E test), a quantitative technique for determining the minimal inhibitory concentration (MIC, in microgram per milliliter). A test was performed on brain heart infusion agar using five E strips of doxycycline, tetracycline, minocycline, clindamycin and erythromycin. After 72 hours of incubation in anaerobic jar, MIC was determined and interpreted. According to the recommendation of Clinical and Laboratory Standards Institute (CLSI) 2016[21], MIC breakpoints were used to define the drug resistance.

MIC_{50} and MIC_{90} were calculated by SPSS program by using all the MIC of each strain into spreadsheet and sorting into ascending order. The MIC_{50} was the median value. Similarly, MIC_{90} was the value found in percentile 90 and represented the concentration of antibiotic that would inhibit 50% and 90% of the isolates tested, respectively.

Statistical analysis

Data were performed using IBM statistical package for the social sciences (SPSS) version 19.0. Descriptive analysis was mainly used in this present study. Frequencies were used to describe categorical data, while mean, minimum, maximum were used to

Table 1. Carbohydrate fermentation patterns of *P. acnes* strains and biochemical reactions

Test	Reactions/enzymes	*P. acnes* strain	Test	Reactions/enzymes	*P. acnes* strain
1	Indole formation	+	11	Protease hydrolysis	+
2	Urease	-	12	Beta-glucosidase	-
3	Glucose	+	13	Glycerol	+
4	Mannitol	-	14	Celobiose	-
5	Lactose	-	15	Mannose	+
6	Saccharose	-	16	Malezitose	-
7	Maltose	-	17	Raffinose	-
8	Salicin	-	18	Sorbitol	-
9	Xylose	-	19	Rhamnose	-
10	Arabinose	-	20	Trehalose	-

* The patterns were analyzed at API database indicated 99.9% identity to *P. acnes*

describe quantitative data. Pearson's Chi-square or Fisher's exact test was used to test the difference of categorical data, as appropriate.

Results

Patient demographics and treatment history

Ninety-five participants were enrolled in this study during October 2016 to February 2017. Sixty-nine (72.6%) patients were female and twenty-six (27.4%) patients were male. The average mean age was 21.74 years and ranged from 18 to 44 years. The disease duration ranged from 1 to 12 years, with a mean duration of 5.83 years. There were 69.5% presented with mild acne, 25.3% with moderate acne and 5.2% with severe acne according to Leeds revised acne grading system[22]. Concerning the history of previous acne treatment, 62.1% of patients had received acne treatment with antibiotics and 37.9% had not received any antibiotic treatment for acne. The patient demographics and treatment histories are detailed in Table 2.

Table 2. Patients demographics and treatment histories

	No. patients (%)/ descriptive statistics (n = 95)
Age (years)	
Mean (min/max)	21.74 (18 to 44)
Sex	
Female	69 (72.6)
Male	26 (27.4)
Durations of acne (years)	
Mean (min/max)	5.38 (1 to 12)
Severity of acne at enrolled	
Mild	66 (69.5)
Moderate	24 (25.3)
Severe	5 (5.2)
Acne treatment history	
Yes	68 (71.6)
No	27 (28.4)
Treatment with antibiotics	
Yes	59 (62.1)
No	36 (37.9)
Treatment with topical antibiotics	51 (53.7)
Topical clindamycin	51 (53.7)
Topical erythromycin	0 (0)
Treatment with systemic antibiotics	29 (30.5)
Doxycycline	17 (17.9)
Amoxicillin	12 (12.6)

Antibiotic susceptibility of P. acnes isolates and minimal inhibitory concentration (MIC)

Not all of *P. acnes* strains were susceptible to all antibiotics. Among 95 samples, *P. acnes* strains were found and isolated in 75 patients (78.95%). The mean MIC of doxycycline, tetracycline, amoxicillin, clindamycin and erythromycin were 0.45, 1.54, 0.017, 154.11 and 163.84 µg/mL, respectively. By using CLSI breakpoints for resistance, there were forty-eight (64%) and forty-seven (62.66%) strains resisted to erythromycin and clindamycin, while only one (1.33%) strain resisted to tetracycline. No resistance to doxycycline and amoxicillin were found in this present study. The MIC and *P. acnes* resistance are shown in Table 3.

The clinical factors associated with *P. acnes* resistance were analyzed and shown in Table 4. There was statistically significant differences between age groups and macrolide antibiotic resistance (clindamycin; $p = 0.011$, erythromycin; $p = 0.009$).

Table 3. Antibiotic susceptibility of *P. acnes* strains

Antibiotics	MIC (µg/mL) mean (min-max)	MIC_{50}* (µg/mL)	MIC_{90}** (µg/mL)	MIC breakpoints (µg/mL)	*P. acnes* resistance n (%)
Doxycycline	0.45 (0.016 to 3)	0.047	1.7	4	0 (0)
Tetracycline	1.54 (0.016 to 48)	0.047	2	16	1 (1.33)
Amoxicillin	0.017 (0.016 to 0.125)	0.016	0.016	2	0 (0)
Clindamycin	154.11 (0.016 to 256)	256	256	6	47 (62.66)
Erythromycin	163.84 (0.016 to 256)	256	256	2	48 (64)

* MIC_{50} = minimal concentration required to inhibit the growth of 50% of organisms
** MIC_{90} = minimal concentration required to inhibit the growth of 90% of organisms

Table 4. Association between clinical characteristics of patients and resistance in *P. acnes* isolates

Characteristic	Number of resistant *P. acnes*				
	Doxycycline	Tetracycline	Amoxicillin	Clindamycin	Erythromycin
Age range					
<25 years (n = 85)	0	1	0	45	46
≥25 years (n = 10)	0	0	0	2	2
p-value	N/A	1.000	N/A	0.011*	0.009*
Duration of disease					
<2 years (n = 12)	0	0	0	5	5
≥2 years (n = 83)	0	1	0	42	43
p-value	N/A	1.000	N/A	0.486	0.480
Previous antibiotic treatment					
Yes (n = 46)	0	1	0	36	37
No (n = 29)	0	0	0	11	11
p-value	N/A	1.000	N/A	0.001*	0.001*
History of isotretinoin intake					
Yes (n = 13)	0	1	0	6	6
No (n = 82)	0	0	0	41	42
p-value	N/A	1.000	N/A	1.000	1.000

* Significant at 0.05 level, χ^2 or Fisher's exact test, as appropriate, N/A = Not available

Table 5. Change in MIC_{90} of *P. acnes* to various antibiotics in Thailand and antibiotic resistance of *P. acnes* strains

	MIC_{90} (µg/mL) / percentage of resistance *P. acnes*				
	Doxycycline	Tetracycline	Amoxicillin	Clindamycin	Erythromycin
Poomsuwan P and Noppakhun N	0.094/0	0.125/0	N/A	0.38/6.15	0.032/6.15
This study	1.7/0	2/1.33	0.016/0	256/62.66	256/64

N/A = not available

Furthermore, the greatest number of antibiotic-treated acne patients was resistance to clindamycin and erythromycin. A statistically significant difference was also found between history of previous antibiotic

treatment and macrolide antibiotic resistance (p = 0.001), whereas there was no statistically significant difference between duration of disease and history of isotretinoin intake with *P. acnes* resistance.

Discussion

Antibiotic resistance of *P. acnes* occurs naturally overtime, usually through genetic changes; nevertheless, misuse and overuse of antibiotics are accelerating this process. Over several decades, antibiotics have been used in the treatment of acne vulgaris, sometimes with topical antibiotics against *P. acnes* acne monotherapy[23]. Resistance of *P. acnes* to antibiotics mostly occurs from genetic mutations. Recent research showed the association between point mutations in gene encoding 23S rRNA and clindamycin, erythromycin resistance. In addition, the research has identified four phenotypes with cross sensitivity to macrolide, lincosamide and streptogramin B (MLS) antibiotics. On the other hand, tetracycline resistance is associated with a point mutations in 16S rRNA[24].

This present study is the second study of antibiotic susceptibility patterns of *P. acnes* isolated from acne patients in Thailand. In 2001, Poomsuwan P and Noppakun N[25] firstly reported the antibiotic susceptibility of *P. acnes* in 70 Thai acne patients using the E strip of doxycycline, tetracycline, minocycline, clindamycin and erythromycin. Accordingly, this present study was aimed to determine the antibiotic susceptibility patterns of *P. acnes* to commonly prescribed antibiotics for acne: erythromycin, clindamycin, tetracycline, doxycycline and amoxicillin. Amoxicillin is one of the commonly prescribed antibiotics for acne in our country so we included amoxicillin in this present study. Although minocycline was reported to be very effective in acne treatment it was not included to this present study because it is not available in Thailand.

The previous study collected samples from 70 patients and *P. acnes* strains were isolated from 65 participants[21]. It revealed that the prevalence of antibiotic-resistant *P. acnes* was 6.15% to erythromycin and clindamycin and there was no strain resistant to doxycycline and tetracycline. The MIC_{90} of *P. acnes* to antibiotics and prevalence of resistance were detailed in table 5. In this present study, *P. acnes* isolates were frequently resistant to erythromycin (64%) and clindamycin (62.66%) but rarely to tetracycline (1.33%). To compare with the previous study, there was a tenfold increase in the prevalent rate of antibiotic-resistant *P. acnes* to clindamycin and erythromycin. In addition, in this study, some *P. acnes* strains were resistant to tetracycline (1.33%), whereas all *P. acnes* strains were sensitive to tetracycline in the previous study. In Thailand, the pattern of drug resistance is similar to other countries such as Europe, Egypt, Hong Kong, Singapore, Korea and Japan[4,6,8,9,13,15]. In this study, the MIC of antibiotics has increased over several years. This could be due to misuse and overuse of antibiotics as treatment for Thai acne patients. Moreover, a variety of topical and systemic antibiotics are available in Thailand and sometimes patients can also buy as an over-the-counter drug.

This study found that there was statistically significant difference between age group and macrolide antibiotic resistance. A higher rate in *P. acnes*-resistant antibiotics in young patients could be due to the young patients often concern about how their face looks so the young patients usually go to the dermatologist for acne problems. Consequently, the younger patients may be exposed more frequently to antibiotics. Furthermore, the result of this study also proposes an association between history of previous antibiotic treatment and macrolide antibiotic resistance. Most of the resistant isolates were collected from patients with reported history of antibiotic treatment because their misuse and overuse of antibiotics. However, the resistant strains were found in patients with no history of previous antibiotic treatment. The de novo colonization of resistant strains and carriage of resistant strains by the contacts of acne patients may explain this finding.

Although there was not statistically significance, this study found that there was much lower resistant strains in isotretinoin-treatment group. It could be from the indirect suppression of numbers of *P. acnes* by oral isotretinoin which can suppress the sebum excretion. The insignificant results can reflect an inadequate sample size so the larger sample size would be required in the next study.

In the present situation, the prevalence of antibiotic-resistant *P. acnes* has been increasing. Therefore, it is crucial to choose antibiotics cautiously in treating acne. To defeat the problem of antibiotic resistance, The Global Alliance to Improve Outcomes in Acne Group has recommend of that antibiotics should be co-prescribed with benzoyl peroxide, which is effective in the prevention of bacterial resistance. Physicians should keep antibiotic courses short, ideally less than 3 months, and do not use for maintenance treatment. In addition, topical antibiotics should never be used as monotherapy for the

treatment of acne vulgaris.

Conclusion

This present study showed that *P. acnes* strains were frequently resistant to erythromycin and clindamycin but rarely to tetracycline in Thailand. Moreover, the prevalence of resistance is associated with increased age and also history of antibiotic treating acne. Physicians should carefully prescribe antibiotics to acne patients. Formulation of guidelines should be adjusted accordingly to reflect changes in *P. acnes* resistance, because of the rapid development of antibiotic-resistant *P. acnes* in many parts of the world.

What is already known on this topic?

In Thailand, there were few reports on resistance of *P. acnes* to antibiotics. The previous study showed that *P. acnes* resisted to erythromycin and clindamycin.

What this study adds?

This study adds the test of antibiotic susceptibility of *P. acnes* with amoxicillin. It was found that *P. acnes* resisted erythromycin, clindamycin and tetracycline with a higher prevalence.

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Potential conflicts of interest

None.

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การศึกษารูปแบบความไวของสายพันธุ์โปรปิโอนิแบคทีเรียมแอคเน่จากผู้ป่วยสิวต่อยาปฏิชีวนะ

อาภากร เหล่าชุนสุวรรณ, มาลัย ทวีโชติภัทร์, มนตรี อุดมเพทายกุล

ภูมิหลัง: *ปัจจุบันการดื้อต่อยาปฏิชีวนะของสายพันธุ์ Propionibacterium acnes นั้นเป็นปัญหาใหญ่ทั่วโลกและพบว่ามีอุบัติการณ์การดื้อต่อยาที่สูงขึ้นในหลายประเทศสำหรับประเทศไทยในช่วง 10 ปีที่ผ่านมายังไม่พบมีรายงานถึงอุบัติการณ์การดื้อยาและรูปแบบความไวของสายพันธุ์ P. acnes ต่อยาปฏิชีวนะ*

วัตถุประสงค์: *เพื่อศึกษารูปแบบความไวของสายพันธุ์ P. acnes ต่อยาปฏิชีวนะชนิดต่างๆ ที่นิยมใช้รักษาสิวในประเทศไทย*

วัสดุและวิธีการ: *การศึกษานี้เป็นการศึกษาเชิงพรรณนา ณ จุดเวลาใดเวลาหนึ่งแบบตัดขวาง ทำการคัดเลือกอาสาสมัครที่มีสิวจำนวน 95 คน เก็บตัวอย่างจากสิวอุดตันหัวปิดที่บริเวณใบหน้าโดยการกดสิว จากนั้นนำตัวอย่างที่ได้ไปเพาะเลี้ยงในสภาวะไร้ออกซิเจนเพื่อคัดแยกและพิสูจน์เอกลักษณ์สายพันธุ์ P. acnes โดยอาศัยลักษณะทางฟีโนไทป์และ API 20A นำมาทดสอบความไวของสายพันธุ์ต่อยาปฏิชีวนะที่นิยมใช้รักษาสิวในประเทศไทย คือ doxycycline, tetracycline, amoxicillin, clindamycin และ erythromycin โดยการหาค่าความเข้มข้นที่ต่ำที่สุดของยาปฏิชีวนะที่ยับยั้งการเจริญของสายพันธุ์ได้ minimal inhibitory concentration (MIC) ด้วยวิธี epsilometer test*

ผลการศึกษา: *จากการศึกษานี้คัดแยกสายพันธุ์ P. acnes ได้จากอาสาสมัคร 75 คนจากทั้งหมด 95 คน พบค่าความเข้มข้นที่ต่ำที่สุดของยาปฏิชีวนะที่ยับยั้งการเจริญของสายพันธุ์ได้ 90 คนจาก 100 คน (MIC_{90}) ของยา doxycycline, tetracycline, amoxicillin, clindamycin และ erythromycin มีค่า 1.7, 16, 0.016, 256 และ 256 ตามลำดับ พบสายพันธุ์ P. acnes ดื้อต่อยา erythromycin จำนวน 48 คน (64%), clindamycin จำนวน 47 คน (62.66%) และมีเพียง 1 คน (1.33%) ดื้อต่อ tetracycline การทดลองไม่พบสายพันธุ์ที่ดื้อต่อยา doxycycline และ amoxicillin นอกจากนี้ จากการทดลองพบว่ามีความสัมพันธ์ระหว่างสายพันธุ์ P. acnes ที่ดื้อต่อยากลุ่ม macrolide กับ อายุ และประวัติการรักษาด้วยยาปฏิชีวนะมาก่อนอย่างมีนัยสำคัญทางสถิติ*

สรุป: *อุบัติการณ์การดื้อยาปฏิชีวนะของสายพันธุ์ P. acnes นั้นสูงขึ้นเมื่อเทียบกับในอดีต ยาปฏิชีวนะที่พบว่า เชื้อดื้อมากที่สุดคือ erythromycin ตามด้วย clindamycin และ tetracycline ตามลำดับ*