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ORIGINAL RESEARCH

Characteristics of Biofilm-Forming Ability and Antibiotic Resistance of *Cutibacterium acnes* and *Staphylococcus epidermidis* from Acne Vulgaris Patients

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Introduction: Acne vulgaris (AV) is a common and chronic disorder of the pilosebaceous unit and has a multifactorial pathology, including activities of *Cutibacterium acnes* (*C. acnes*) and *Staphylococcus epidermidis* (*S. epidermidis*). Antibiotic resistance has become a major concern in dermatology daily practice, and the ability of biofilm formation by both bacteria is suggested to increase antibiotic resistance in acne.

Purpose: Our aim was to analyze the comparison of antibiotic resistance between biofilm-forming (BF) and non-biofilm-forming (NBF) strains of *C. acnes* and *S. epidermidis* towards seven antibiotics commonly used for acne.

Methods: This is a cross-sectional analytical study involving 60 patients with AV. Samples were obtained from closed comedones on the forehead using the standardized skin surface biopsy (SSSB) method at the Cosmetic Dermatology Clinic Dr. Hasan Sadikin in Bandung, Indonesia. Isolates were cultured and identified before undergoing the biofilm-forming test using the tissue culture plate method. Antibiotic susceptibility testing for each antibiotic was then performed using the disc diffusion method.

Results: The incidence of antibiotic resistance to clindamycin in BF and NBF *C. acnes* isolates was 54.5% (p=1.00), while in BF and NBF *S. epidermidis* isolates, it was 54.5% and 45.5% respectively (p=0.67). The incidence of antibiotic resistance to erythromycin and azithromycin in BF and NBF *C. acnes* isolates was 54.5% and 63.6% respectively (p=1.00), whereas for *S. epidermidis* BF and NBF isolates, it was 54.5% (p=1.00). There was no resistance observed to tetracycline, doxycycline, levofloxacin, and cotrimoxazole in all groups.

Conclusion: There were no significant differences in resistance against seven antibiotics between the *C. acnes* and *S. epidermidis* in BF and NBF groups. Furthermore, although statistically not significant, some resistances were observed against clindamycin, erythromycin, and azithromycin. Consequently, the use of these three antibiotics should be judiciously regulated.

Keywords: acne vulgaris, antibiotic resistance, biofilm, *Cutibacterium acnes*, *Staphylococcus epidermidis*

Introduction

Acne vulgaris (AV) is a common multifactorial disorder of the pilosebaceous unit.[1,2] Follicular epidermal hyperplasia, sebum production, the presence and activity of bacteria, most frequently *Cutibacterium acnes (C. acnes)*, inflammation, and immune response are the four essential components of the pathophysiology of acne vulgaris.[3] A study by Bek-

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Thomsen et al[4] showed that *C. acnes* and *Staphylococcus epidermidis (S. epidermidis)* were the predominant bacteria in AV, and they have the ability to form biofilms.[4,5] Biofilm refers to a surface-attached, structured microbial community embedded in a self-produced extracellular matrix that adheres to a biotic or abiotic surfaces.[4–6] In vitro research suggests that bacteria within a biofilm's protected microenvironment are 50–500 times more resistant to antimicrobial treatments compared to free-floating bacteria.[5] Factors contributing to this resistance include restricted penetration of antimicrobials, decreased growth rate, expression of resistance genes, and the presence of resistant "persister" cells.[1] The rise of antibiotic resistance in acne has become a major concern in dermatology practice, and the ability of *C. acnes* and *S. epidermidis* to form biofilms is believed to contribute to increased antibiotic resistance in acne.[7] Reports of antibiotic resistance in *C. acnes* and *S. epidermidis* in AV have been documented in various countries and have shown an increasing trend over the years.[7]

In this study, we examined the in vitro capacity of *C. acnes* and *S. epidermidis* to form biofilms. We then assessed the resistance of biofilm-grown bacteria to commonly used antimicrobial drugs for acne treatment. To date, no studies have compared the antibiotic resistance between biofilm-forming *C. acnes* and *S. epidermidis* species. Therefore, the aim of this study is to analyze and compare the antibiotic resistance in biofilm-forming *C. acnes* and *S. epidermidis* in AV patients at Dr. Hasan Sadikin Bandung, Indonesia.

Materials and Methods

Patients

This study involved 60 patients who visited the Cosmetic Dermatology Clinic, Department of Dermatovenerology, Hasan Sadikin Bandung General Hospital, Indonesia. The inclusion criteria for our study were as follows: female patients clinically diagnosed with AV, aged between 18 to 24 years, and presenting with closed comedones on the forehead, along with positive bacterial culture results for *C. acnes* or *S. epidermidis*. Patients were excluded if they were pregnant, had received topical antibiotics in the past week and/or systemic antibiotics in the past two weeks, were using hormonal therapy or contraceptives and anti-androgenic treatment for the past three months, or had allergies to cyanoacrylate. The study protocol was approved by the Board and Ethics Committee, and written informed consent was obtained from all patients.

Bacterial Culture and Identification

The standardized skin surface biopsy (SSSB) method was used to collect microbiological samples from each patient who had closed comedones on the forehead. This method involves the use of high-bond glue (cyanoacrylate) to collect the follicular contents and the superficial portion of the horny layer, including pilosebaceous units and comedones. The collected tissue was then cultivated in fluid thioglycolate medium (FTM) and blood agar to detect *C. acnes* growth, and in tryptic soy broth (TSB), blood agar, and MacConkey agar to detect *S. epidermidis* growth. Identification of the isolates was performed using the Vitek®2 compact machine (bioMerieux, France).

Biofilm Formation Assay

In this study, the protocols for the biofilm formation assay were based on the method described by Kuehnast et al with modifications. *C. acnes* isolates were inoculated into 10 mL Brain-Heart Infusion + 1% glucose (BHI_{glu}) and incubated at 37°C for 72 hours under anaerobic conditions. The incubation product was then diluted with BHI_{glu} in a 1:100 ratio or until the opacity reached 0.5 McFarland. The diluted sample was divided into non-treated 96-well U-bottom tissue culture plates, with each well containing 0.15 mL of the sample. Each clinical isolate was cultivated in triplicates across 3 culture plates and incubated at 37°C for 72 hours under anaerobic conditions. BHI_{glu} was used as the negative control and underwent a similar process as the isolates.

Staphylococcus epidermidis isolates were inoculated into 10 mL Tryptic Soy Broth + 1% glucose (TSB_{glu}) and incubated at 37°C for 24 hours. The incubation product was then diluted with TSB_{glu} in a 1:100 ratio. The diluted sample was divided into non-treated 96-well flat-bottom tissue culture plates, with each well containing 0.2 mL of the sample.

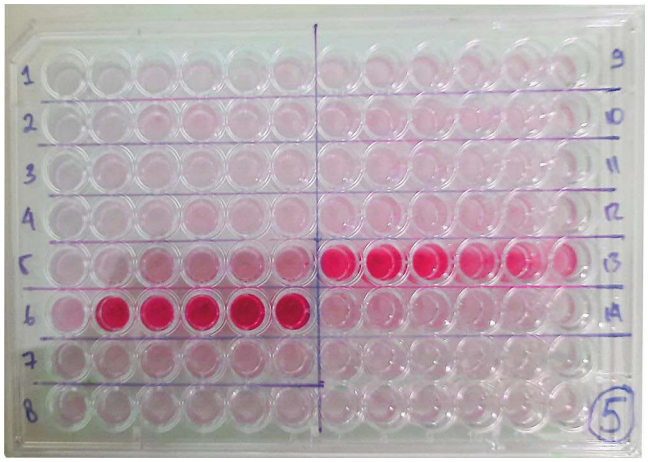

Figure 1 Biofilm production assay. Tissue culture plates show different color intensities for BF and NBF cells and measured with spectrophotometer as optical density (OD).

Each clinical isolate was cultivated in triplicates across 3 culture plates and incubated at 37°C for 24 hours. TSB_{glu} was used as the negative control and underwent a similar process as the isolates.

At the end of the incubation process, the remaining medium was discarded by gently tapping the base of the plate. The samples were washed with 0.2 mL of phosphate buffer saline (PBS) three times. The remaining biofilms at the base of the wells were dried for 50 minutes at 60°C and then fixed with 100 µL of methanol (for *C. acnes*) or with 0.2 mL of 2% CH3COONa (for *S. epidermidis*) for 10 minutes. The samples were stained with 0.15 mL of 0.1% safranin for 2 minutes (Figure 1). Afterward, the cells were washed again with 0.2 mL of PBS three times, and 0.2 mL of isopropanol was added to the plates. The biofilm-forming abilities were presented as optical density (OD), measured by a spectrophotometer (Multiskan^{TM} FC Microplate Photometer) at a wavelength (λ) of 492 nm, and further classified into biofilm-forming (BF) and non-biofilm-forming (NBF) categories (Table 1).

Antibiotic Resistance Test

The disc diffusion method was used to examine the susceptibility of each antibiotic to the bacterial isolates. A bacterial colony suspension was spread onto Mueller Hinton Agar plates. Antibiotic discs containing tetracycline, doxycycline, clindamycin, erythromycin, azithromycin, levofloxacin, and cotrimoxazole were placed on top of the agar surface using sterile techniques in triplicates (Figure 2A and B). The antibiotic resistance results were determined by measuring the zone of inhibition around the antibiotic discs after an incubation period of 24 hours for *C. acnes* and 72 hours for *S. epidermidis*. The data obtained from this procedure were analyzed according to the Clinical and Laboratory Standards Institute (CLSI) standards.

Data Analysis

All our data were analyzed using the Pearson Chi-square test, with Fisher's exact test used as an alternative when the expected value was less than 5. A p-value ≤ 0.05 was considered statistically significant. Data processing was performed

Table 1 Optical Density Measurement and Classification of Biofilm

Mean of OD	OD Classification	Biofilm Classification
OD ≤ ODc	*Non-adherent*	NBF
ODc<OD ≤ 2 × ODc	*Weakly adherent*	NBF
2×ODc <OD ≤ 4 × ODc	*Moderately adherent*	BF
4 × ODc < OD	*Strongly adherent*	BF

Notes: Optical density cut-off value (ODc) = mean of optical density (OD) from negative control + 3x standard deviation (SD) of negative control.
Abbreviations: NBF, non-biofilm forming; BF, biofilm-forming,

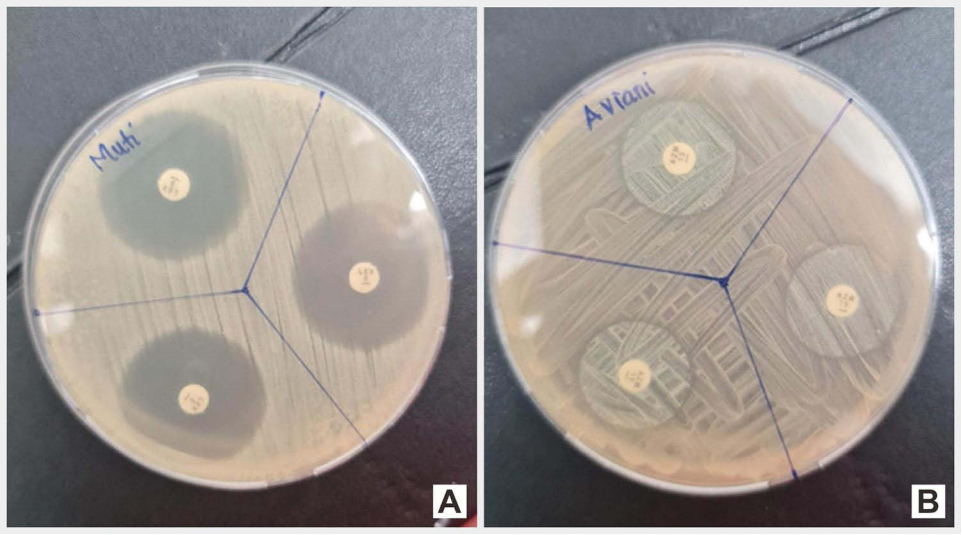

Figure 2 Antibiotic susceptibility testing with disc diffusion method. A sensitive antibiotic produce zones of inhibition (**A**), while a resistant antibiotic not produce zone of inhibition (**B**).

using the IBM® SPSS® (Statistical Package for the Social Sciences) application, version 24.0, by the International Business Machines Corporation.

Results

Subject Characteristics

Out of the 60 patients, 68 bacterial isolates were obtained, including 36 isolates of *C. acnes* and 32 isolates of *S. epidermidis*. Among the *C. acnes* isolates, 20 (55.6%) were BF and 16 (44.4%) were NBF. For *S. epidermidis* isolates, 11 (34.4%) were BF and 21 (65.6%) were NBF. Antibiotic susceptibility testing was performed on 11 isolates from each group.

The distribution of antimicrobial susceptibilities is presented in Table 2. Clindamycin, erythromycin, and azithromycin exhibited higher resistance rates among both *C. acnes* and *S. epidermidis* strains compared to other tested antibiotics.

Table 2 Comparison of Antibiotic Resistance Between Biofilm-Forming and Non-Biofilm Forming of *C. Acnes* and *S. Epidermidis*

Variable	*C. acnes*					*S. epidermidis*				
	BF (n =11)		NBF (n=11)		P value	BF (n=11)		NBF (n=11)		P value
	n	%	n	%		n	%	n	%	
Tetracycline										
Resistant	0	0	0	0		0	0	0	0	
Intermediate	0	0	0	0	1.00	0	0	0	0	1.00
Sensitive	11	100	11	100		11	100	11	100	
Doxycycline										
Resistant	0	0	0	0		0	0	0	0	
Intermediate	0	0	0	0	1.00	0	0	0	0	1.00
Sensitive	11	100	11	100		11	100	11	100	
Clindamycin										
Resistant	6	54.5	6	54.5		6	54.5	5	45.5	
Intermediate	0	0	0	0	1.00	0	0	0	0	0.67
Sensitive	5	45.5	5	45.5		5	45.5	6	54.5	

(*Continued*)

Table 2 (Continued).

Variable	*C. acnes*					*S. epidermidis*				
	BF (n =11)		NBF (n=11)		P value	BF (n=11)		NBF (n=11)		P value
	n	%	n	%		n	%	n	%	
Erythromycin										
Resistant	6	54.5	7	63.6		6	54.5	6	54.5	
Intermediate	0	0	0	0	1.00	0	0	0	0	1.00
Sensitive	5	45.5	4	36.4		5	45.5	5	45.5	
Azithromycin										
Resistant	6	54.5	7	63.6		6	54.5	6	54.5	
Intermediate	0	0	0	0	1.00	0	0	0	0	1.00
Sensitive	5	45.5	4	36.4		5	45.5	5	45.5	
Levofloxacin										
Resistant	0	0	0	0		0	0	0	0	
Intermediate	0	0	0	0	1.00	0	0	0	0	1.00
Sensitive	11	100	11	100		11	100	11	100	
Cotrimoxazole										
Resistant	0	0	0	0		0	0	0	0	
Intermediate	0	0	0	0	1.00	0	0	0	0	1.00
Sensitive	11	100	11	100		11	100	11	100	

Abbreviations: BF, biofilm-forming; NBF, non-biofilm forming.

None of the isolates were resistant to tetracycline, doxycycline, levofloxacin, or cotrimoxazole. In the *C. acnes* BF group, 54.5% of the isolates were resistant to the three aforementioned antibiotics. In the NBF group, 54.5% were resistant to clindamycin, 63.6% to erythromycin, and 63.6% to azithromycin. The resistance rates in the *S. epidermidis* BF group to clindamycin, erythromycin, and azithromycin were 54.5%, while in the NBF group, the resistance rates to clindamycin, erythromycin, and azithromycin were 45.5%, 54.5%, and 54.5%, respectively.

The Chi-Square test was performed to analyze the resistance to tetracycline, doxycycline, clindamycin, levofloxacin, and cotrimoxazole, while the Fisher's exact test was used for assessing resistance to erythromycin and azithromycin. The results of these tests showed p-values greater than 0.05, indicating no significant difference in antibiotic resistance between the *C. acnes* BF and NBF groups, *S. epidermidis* BF and NBF groups, as well as among the four groups.

Discussion

Staphylococcus epidermidis and *C. acnes* are two major bacterial strains that are commonly isolated and are known to contribute as pathogenic factors in AV.[7,8] Studies have shown that *C. acnes* is the most prevalent microbe in the pilosebaceous unit, with up to 10^7 viable organisms found in a single sebaceous unit.[5] However, *S. epidermidis* counts can be equal to or higher than *C. acnes* counts in some follicles, as observed in studies that examined pooled samples of excised follicles.[8] In our previous study at this hospital, *S. epidermidis* was ranked as the second most common bacteria found in comedones of AV patients.[9] The presence of biofilms was found to be more frequent in comedone lesions compared to other inflammatory lesions, as reported in studies by Dreno et al[10] and Hindritiani et al[11] which also revealed higher rates of antibiotic resistance in closed comedones compared to skin smears or pustules. Hence, for this study, samples were obtained specifically from comedone lesions.

According to our study, out of the total number of *C. acnes* isolates, 20 (55.6%) were biofilm-forming (BF) strains, while 16 (44.4%) were non-biofilm-forming (NBF) strains. BF strains were more frequently isolated from acne sufferers compared to NBF strains. These findings support the conclusion of Jahns et al,[12] who visualized large biofilms of *C. acnes* in 14 out of 18 AV patients. However, this is in contrast to a study conducted by Loss et al,[13] which found *C. acnes* biofilms in only nine out of 39 samples (23%).

A total of *S. epidermidis* isolates, 11 (34.4%), were biofilm-forming (BF), including 2 (18.1%) isolates characterized as strong biofilm producers, while 21 (65.6%) were non-biofilm-forming (NBF). The proportion of NBF strains was higher than BF strains isolated from acne patients. This result contrasts with a study conducted by Farran et al[14] which found biofilm-forming *S. epidermidis* in 91.4% of samples. In their study, they used PCR to screen for the presence of the intracellular adhesion (ica) operon in *S. epidermidis* isolates, which is correlated with the prevalence of biofilm formation. However, this was not assessed in our study.

Our study showed that the BF group of *S. epidermidis* was more frequently resistant to antibiotics compared to the NBF group. On the other hand, in *C. acnes* strains, the NBF group showed a tendency to be more resistant to antibiotics, although this difference was not statistically significant. A comparative study on antibiotic resistance and biofilm formation ability between *C. acnes* and *S. epidermidis* isolates, as used in this study, has not been previously assessed.

The topic remains controversial, as other authors have reached radically different conclusions using various approaches and different species of therapeutically relevant bacteria. A study by Donadu et al[15] in Italy found that 51.7% of S. aureus and 62.8% of non-aureus staphylococcal strains were strong biofilm producers, but they found no variations in biofilm formation based on methicillin resistance. Similar findings were reported in a study by Gajdacs et al[16] which found no significant connections between the rate of biofilm formation and antibiotic resistance in 302 isolates of Pseudomonas aeruginosa.

Cutibacterium acnes and *S. epidermidis* are known to be biofilm producers.[1,5,17] Biofilms are aggregates of mono- or multispecies bacterial communities, consisting of diverse exopolysaccharides (EPS), environmental DNA, and other biomolecules such as lipids, proteins, and carbohydrates.[4] Biofilms provide protection against shear forces, maintain an inflammatory environment in vivo, and promote the transformation of *C. acnes* and *S. epidermidis* into metabolically dormant persister cells.[5,14]

Cutibacterium acnes and *S. epidermidis* possess an extensive repertoire of virulence factors. AV is caused by *C. acnes* virulence factors including camp5, gehA, tly, sialidases, neuraminidase, and endoglycoceramidases. The lipoglycan-based cell envelope and the extracellular secreted lipase, particularly triacylglycerol lipase encoded by the gehA gene, aid in the adherence and colonization of the bacterium to the sebaceous follicle. Additionally, the gehA gene product contributes to acne formation by damaging host tissue.[18] One of the key virulence factors produced by *S. epidermidis* is the fatty acid modifying enzyme, which converts bactericidal fatty acids in the skin into cholesterol. *S. epidermidis* also secretes the exopolysaccharide intercellular adhesin (PIA), responsible for adhesion and biofilm formation on the skin surface, providing protection against components of the human innate host defense. These biofilms create favorable anaerobic conditions necessary for the growth of *C. acnes*.[14]

Apart from their propensity to form biofilms, the ability of *C. acnes* and *S. epidermidis* to establish chronic infections and persist in vivo enhances their survival in adverse environmental conditions.[14,18] In chronic infections, where *C. acnes* and *S. epidermidis* establish long-term persistence within biofilms, the expression of virulence factors is downregulated to accommodate the lower metabolic activity within the EPS. This can result in therapeutic failure and a decreased quality of life for affected patients.[4] Furthermore, the chemical composition of biofilms inhibits the diffusion of antimicrobials, acting as a pharmacokinetic barrier to these drugs. Consequently, the minimum inhibitory concentrations (MICs) required to target biofilm-embedded bacteria may be 10^1–10^4 times higher than those needed for planktonic bacteria.[14]

The environment has a significant impact on biofilm formation, and researchers are intrigued by the mechanisms through which gene expression in individual cells influences biofilm formation. Environmental factors determine whether a cell forms a biofilm or not.[18] Additionally, the structure of biofilms is highly dependent on the surrounding environment, indicating that biofilms adapt to local conditions. Second messengers, such as cAMP and c-di-GMP, play a crucial role in the interplay between environmental factors and gene regulation. Cell-to-cell communication, known as quorum sensing (QS), is a vital component in biofilm formation.[19] QS is one of the primary mechanisms responsible for regulating the expression of virulence factors and biofilm formation.[6,19] However, several antibiotics can also affect these QS systems in *C. acnes* and *S. epidermidis* by directly influencing gene expression or degrading the signal molecules involved.[19]

Based on our experimental data, we did not find any significant differences or notable relationship between biofilm formation and antibiotic resistance. This could be explained by the fact that microorganisms adapt their virulence factor expression only in survival-critical situations, or it may suggest that our in vitro methodologies were not sophisticated enough to detect their association. Additionally, while most laboratory biofilm studies are conducted under static conditions, natural conditions exhibit fluctuations.[16]

The emergence of *C. acnes* and *S. epidermidis* isolates with varying capacities to form biofilms may provide insights into the genetic heterogeneity within these species, which is a crucial factor in the effectiveness of AV infection.[19] However, it is not yet fully understood how this heterogeneous gene expression contributes to the adaptability and flexibility of biofilms in different environments.[16] Additionally, discrepancies in phenotypes and susceptibility patterns may also be attributed to the geographical origins of these isolates.[6,19]

Cutibacterium acnes phylogenetic groups exhibit distinct genetic and phenotypic characteristics.[20] To date, six recognized phylotypes of *C. acnes* exist. Kuehnast et al[17] conducted a study to compare the dynamics of biofilm formation, biofilm morphology, and adherence ability among these six phylotypes of *C. acnes*. The results revealed a correlation between biofilm formation and the phylotypes of *C. acnes*. The IA1 phylotype was found to have thicker biofilms, and IA1, IA2, and IC phylotypes exhibited higher adhesion ability to abiotic surfaces. The study concluded that the *C. acnes* phylotype determines the quality of biofilm formation. Rachmawati et al[21] conducted a study that demonstrated a significant correlation between the expression of the icaA and icaD genes, encoding intracellular adhesion proteins, and *S. epidermidis* biofilm formation in vitro. However, our study did not assess the phylotype of *C. acnes* or the expression of the icaA and icaD genes in *S. epidermidis*, thus limiting our ability to evaluate their biofilm-forming capacity.

The emergence of *C. acnes* and *S. epidermidis* isolates with varying capacities to form biofilms may provide insights into the genetic heterogeneity within these species, which is a crucial factor in the effectiveness of AV infection.[19] However, it is not yet fully understood how this heterogeneous gene expression contributes to the adaptability and flexibility of biofilms in different environments.[16] Additionally, discrepancies in phenotypes and susceptibility patterns may also be attributed to the geographical origins of these isolates.[6,19]

Treatment with oral antibiotics should be avoided due to the high rates of antimicrobial resistance reported in AV worldwide.[7] A study conducted by Platsidaki et al[20] in the UK, Spain, Italy, Greece, Sweden, and Hungary reported that out of 664 patients, the prevalence of *C. acnes* resistance rates ranged from 50.8% to 93.6% to various antibiotics (tetracycline, macrolide, lincosamide, and streptogramin B). A previous study conducted in Bandung in 2019 revealed that *C. acnes* and *S. epidermidis* were the most commonly found bacteria in AV, and they exhibited high resistance rates towards clindamycin (62.5%), azithromycin (60.7%), and erythromycin (57.1%), respectively.[9] These results are similar to our findings. Macrolide-resistant *C. acnes* is frequently isolated from AV patients, with the majority of resistant isolates having the 23S rRNA mutation.[7,22] Clindamycin has become the most commonly used antibiotic for acne treatment.[22] However, the uncontrolled use of clindamycin can lead to a high frequency of antimicrobial resistance among AV patients.[7] The rate of tetracycline resistance was lower compared to clindamycin and erythromycin.[20] Additionally, some antibiotics require specific conditions to work effectively. For example, clindamycin and erythromycin need to be in a basic environment (pH > 7) to be effective. In infected tissues, this condition may not always occur, resulting in a poor therapeutic outcome.[23]

Conclusion

There were no significant differences in antibiotic resistance against tetracycline, doxycycline, clindamycin, erythromycin, azithromycin, levofloxacin, and cotrimoxazole between the *C. acnes* and *S. epidermidis* in BF and NBF groups ($p > 0.05$). The use of clindamycin, erythromycin, and azithromycin should be judiciously regulated, while tetracycline, doxycycline, levofloxacin, and cotrimoxazole remain sensitive antibiotic treatments for AV.

Ethical Statement

This study complies with the Declaration of Helsinki and was performed according to the approval from the Research Ethics Committee of Universitas Padjadjaran (No. 164/UN6.KEP/EC/2021).

Consent Statement

The authors certify that they have obtained all appropriate patient consent forms.

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Disclosure

The authors report no conflicts of interest in this work.

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