

Identification and antibiotic sensitivity test of isolated aerobic bacteria from acne vulgaris

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ABSTRACT: Acne vulgaris is an inflammatory skin condition caused by various factors, including bacteria in the sebaceous glands of the dermis. Inflammation in acne vulgaris can lead to the formation of purulent papules, known as acne papulopustulosa. Treatment options for acne vulgaris include topical antibiotics, such as clindamycin, and oral antibiotics, such as tetracycline, minocycline, and doxycycline. This study aimed to isolate and identify aerobic bacteria associated with acne vulgaris and assess their susceptibility to commonly used antibiotics. Aerobic bacteria from the papulopustules of six patients were isolated using the quadrant streak method on Mueller Hinton Agar (MHA) and Mannitol Salt Agar (MSA). Twelve isolates were obtained, characterized using Gram staining, catalase, and oxidase tests, and identified by 16S rRNA sequencing. The most common bacteria were *Staphylococcus* spp. (five patients), followed by *Klebsiella* sp. (two patients), *Enterobacter* sp. (one patient), and *Citrobacter* sp. (one patient). Sensitivity testing was conducted with tetracycline, minocycline, doxycycline, and clindamycin, following the Clinical Laboratory Standards Institute (CLSI) guidelines. The results showed that the effectiveness of tetracycline was 50.0%, minocycline 58.3%, and doxycycline 58.3%, while all isolates were resistant to clindamycin. Due to the small sample size, the findings should be interpreted as preliminary and descriptive, providing exploratory insight into the diversity of aerobic bacteria in acne lesions and their antibiotic susceptibility.

KEYWORDS: Acne vulgaris; aerobic bacteria; antibiotic sensitivity; identification; isolation.

INTRODUCTION

Acne vulgaris is a medical term that refers to one of the most common skin diseases, characterized by the occurrence of pimples, blackheads, whiteheads, and sometimes deeper lumps (cysts or nodules) [1]. Usually, acne primarily occurs in areas which are rich in sebaceous (oil) glands such as the face. Nevertheless, it can also develop on the neck, chest, and back [2]. The severity of acne vulgaris ranges from mild to severe and can occasionally recur, potentially leading to scarring [3]. Acne is a skin disorder that affects the aesthetic appearance of the face, the most exposed area of the body. Despite being a frequent and non-life-threatening condition, acne has a significant psychological impact and is associated with comorbidities. Acne most commonly occurs during adolescence and teenage years. Jaber et al. (2020) [4] stated that approximately 80% of teenagers worldwide were affected by this condition. It affects a patient's self-confidence, especially during adolescence, a time of personality development. Therefore, acne has a significant psychological impact, necessitating effective treatment to improve the patient's skin and self-esteem [5].

The pathogenesis of acne is multifactorial, involving hormonal changes, excessive sebum production, abnormal skin cells shedding, inflammation, and bacterial colonization. Chronic inflammation of the pilosebaceous unit can cause tissue abnormalities both non-inflammatory (open or closed comedones) and inflammatory (papules, pustules and nodules) [6]. The characterization of inflammation can vary, including non-inflammatory (open and closed comedones) and inflammatory lesions (such as papules, pustules, and nodules) [2]. Inflammation and bacterial infection trigger the formation of acne vulgaris [6]. Huang et al. (2022) [7] stated that the acne vulgaris appears when excess sebum accumulates and blocks the follicles. Bacteria proliferate and grow inside the follicles, eventually passing through the ruptured hair follicles to enter the dermal compartments.

In circumstances where the skin barrier is compromised, such as an increase in transepidermal water loss, sebum production, along with a decrease in microbes, skin diseases especially acne vulgaris, acne vulgaris. [8].

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The main bacterial species involved in acne pathogenesis is usually *Cutibacterium acnes* [9], [10]. However, several studies have reported that other bacteria can also play a role in acne pathogenesis. For example, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Micrococcus* spp. have been associated with the pathogenesis of acne vulgaris, although their roles in the pathogenesis of acne vulgaris remain controversial. Ito and Amagai (2022) [11] stated that under normal skin conditions, the skin microbiota are non-pathogenic. Nevertheless, a normal resident of the skin such as *S. epidermidis* can become opportunistic under certain conditions, such as when the skin barrier is disrupted. It may contribute to the formation of acne lesions by interacting with *C. acnes* and exacerbating inflammation. Another bacterium, *S. aureus*, can sometimes colonize acne lesions, particularly in more severe or cystic cases. Its presence may increase the risk of secondary infections, leading to worsening inflammation and the formation of pus-filled lesions.

In mild cases, acne treatment often suffices with topical medication alone. However, in moderate to severe cases, selecting the appropriate antibiotic strategy is crucial for effective healing. This is because bacterial activity contributes to the infection in acne vulgaris. Antibiotics suppress bacterial activity and have anti-inflammatory effects. However, the overuse of antibiotics can lead to bacterial resistance.

The study aims to isolate bacteria from samples directly taken from papulopustular acne lesions of six patients willing to participate in the research. Representative isolates will be molecularly identified using 16S rRNA analysis. Antibiotic sensitivity testing will be conducted using four types of antibiotics: tetracycline, minocycline, doxycycline, and clindamycin. The sensitivity testing method will be performed using paper discs, with doses administered according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [12].

▪ MATERIALS AND METHODS

Ethics statement

The study was approved by the Health Research Ethics Committee of the University of 17 August 1945 Jakarta (No. 85/KEPK-UTA45JKT/EC/EXP/02/2024, dated February 5, 2024). Written informed consent was obtained from all patients at the Dermatology and Venereology Clinic, Dr. Kun Jayanata, Jakarta, prior to sample collection. All personal and identifiable data were not disclosed to ensure patient confidentiality.

Patient and sample collection

A total of six samples were collected from male and female patients aged 20 to 35 years with moderate to severe acne. This study was designed as a primary exploratory investigation using a small size to obtain preliminary data on aerobic bacteria associated with acne lesions. Basic clinical information—including age, gender, family history of acne, sleep duration, history of consuming high-glycemic foods, previous or current vitamin consumption, and skincare treatments used—was obtained at the time of patient registration. Patients who had received antibiotic treatment in the past month or were pregnant were excluded. Participants provided informed consent and approved for obtaining a microbiological sample from their inflamed pustules. The samples were collected under aseptic conditions using a sterile comedone extractor. The pus from the pimple was inoculated into Tryptone Soy Broth (TSB) and incubated aerobically at 37°C for 24 hours.

Isolation and characterization of pustule bacteria

Bacteria from the pustules in the TSB medium were isolated using the quadrant streak method on Mueller Hinton Agar (MHA) and Mannitol Salt Phenol-Red Agar (MSA). Additionally, the TSB medium containing pustule bacteria was diluted (1:10, v/v) with fresh TSB medium and streaked onto MHA and MSA as well. The plates were then incubated at 37°C for 24 hours. Single colonies of pustule bacteria were purified and maintained on MHA medium. Characterization of the pustule bacteria involved both morphological and biochemical analyses. Morphological observations were performed using Gram staining to determine the shape and Gram type of the bacteria. Biochemical characterizations of the isolates were also done, such as catalase and oxidase tests.

Molecular identification of pustule bacteria

The isolated aerobic bacteria were identified based on 16S rRNA sequences. DNA was extracted using InstaGene Matrix (Bio-Rad, USA). The isolate was mixed with 100 µl of sterilized saline and centrifuged at

10,000 rpm for 10 minutes. After that, the supernatant was separated from the cells. The cells were then mixed with 50 μ l of InstaGene Matrix (Bio-Rad, USA), incubated at 56°C for 30 minutes, and heated at 100°C for 10 minutes. Universal primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' were used in PCR amplification. The initial denaturation of PCR was performed at 95°C for 5 minutes. The PCR cycle consisted of denaturation at 95°C for 30 seconds, annealing at 55°C for 2 minutes, and extension at 68°C for 90 seconds, with a final extension at 68°C for 10 minutes, followed by holding at 4°C. Purification of PCR products was carried out using the Montage PCR Cleanup Kit (Millipore). The PCR products were confirmed on a 1% (w/v) agarose gel stained with ethidium bromide. The purified PCR products were sequenced using primers 785F and 907R with the Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, USA). All molecular identification steps were performed by Macrogen (Seoul, Korea). The sequencing results were trimmed and assembled into contigs using Geneious Prime, and the sequences were compared using BLAST from the National Center for Biotechnology Information (NCBI). A phylogenetic tree of the isolates was constructed based on Kimura-2 parameter substitution using MEGA11.

Antibiotic sensitivity testing

The antibiotic sensitivity of aerobic bacteria isolates was performed using disc diffusion assay on MHA medium. The antibiotics: tetracycline (30 μ g), minocycline (30 μ g), doxycycline (30 μ g), and clindamycin (2 μ g) according to CLSI (2017) [12] guidelines. The isolates were inoculated in Mueller Hinton Broth (MHB) at 37°C for 20 hours then prepared to a 0.5 McFarland standard (absorbance of 0.08 to 0.1 at 600 nm). The suspension of isolates was swabbed onto the surface of MHA medium. Antibiotic disc containing 30 μ g tetracycline, 30 μ g doxycycline, 30 μ g minocycline, and 2 μ g clindamycin were put on the surface of agar. The plates were incubated at 37°C for 24 hours. The zone of inhibition around the disc exhibited the susceptibility of the isolates towards the antibiotics. The diameter of the zone of inhibition was measured using calipers. The results were interpreted as resistant (R), intermediate (I) or sensitive (S) according to CLSI [12].

RESULTS

Isolation and characterization of pustule bacteria

A total of 12 isolates were successfully obtained from six pustule samples, consisting of five isolates from samples coded S and seven isolates from diluted samples coded P. The number of isolates obtained should be interpreted as preliminary and descriptive, considering the small sample size evaluated in this study. The samples were diluted to reduce the number of cells for isolating pustule bacteria. This approach increased the likelihood of obtaining a single colony using the quadrant streak technique (Figure 1). Characterization of the 12 isolates revealed that eight isolates were Gram-positive cocci arranged in grape-like clusters, while four isolates were Gram-negative rods (Table 1). All isolated bacteria exhibited a positive catalase test but a negative oxidase test result.



Figure 1. Isolation bacteria pustule on MHA medium using quadrant streak method.

Molecular identification of pustule bacteria

Molecular identification revealed that five isolates were identified as *Staphylococcus epidermidis*, two isolates as *Klebsiella pneumoniae*, and the remaining isolates as *Staphylococcus caprae*, *Staphylococcus aureus*, *Enterobacter aerogenes*, and *Citrobacter koseri* (Table 1). Another isolate, P4 isolate showed a lower coverage

value due to low-quality sequencing. It was closely related to *Staphylococcus aureus*, with 79% query coverage and 99.86% identity. However, among the 12 isolates, P6.2 could not be identified at the exact species level. Sequencing data from PCR using universal 16S rRNA primers produced poor-quality results. Even after using different primers, the results were unsatisfactory. Based on Gram staining, cell morphology, and catalase-oxidase tests, the results indicate that isolate P6.2 was identified as a *Staphylococcus* sp. Therefore, only 11 isolates were included in the phylogenetic analysis. The phylogenetic reconstruction was performed using the Kimura 2-parameter method, and the results are shown in Figure 2.

Table 1. Results of molecular identification.

Sample	Isolate code	Species	Gram	Shape of cells	Sequence length	Coverage	% Identity
1	S1	<i>Klebsiella pneumoniae</i>	-	Rod	1530	96	99.52
	P1	<i>Staphylococcus epidermidis</i>	+	Coccus	1503	98	99.86
2	S2	<i>Klebsiella pneumoniae</i>	-	Rod	1530	96	99.66
3	P3.1	<i>Staphylococcus caprae</i>	+	Coccus	1492	98	99.93
	P3.2	<i>Staphylococcus epidermidis</i>	+	Coccus	1503	98	99.93
4	S4	<i>Staphylococcus epidermidis</i>	+	Coccus	1503	98	99.59
	P4	<i>Staphylococcus aureus</i>	+	Coccus	1477	79	99.86
5	P5Bs	<i>Staphylococcus epidermidis</i>	+	Coccus	1503	98	99.66
	P5Kc	<i>Staphylococcus epidermidis</i>	+	Coccus	1503	98	99.59
6	S6.2	<i>Enterobacter aerogenes</i>	-	Rod	1540	98	99.80
	S6.3	<i>Citrobacter koseri</i>	-	Rod	1494	98	98.97
	P6.2	<i>Staphylococcus</i> sp.*	+	Coccus	-	-	-

S:

undiluted samples; P: diluted samples; *: determined based on biochemical test

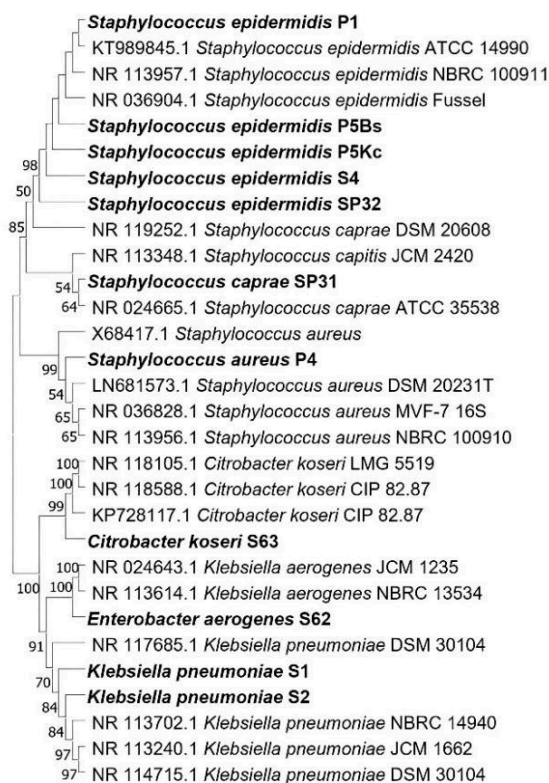


Figure 2. Phylogenetic tree of isolated aerobic bacteria from acne vulgaris based on the Kimura-2 parameter.

Antibiotic sensitivity testing

The results of antibiotic sensitivity testing using the paper disc diffusion method on MHA medium showed varying sensitivities to the tested antibiotics (Table 2). Bacterial growth inhibition was indicated by a clear zone around the paper disc (Figure 3). All isolates were resistant to clindamycin (CL). Based on these results, it can be concluded that clindamycin was the least effective antibiotic.

Table 2. The average diameter of the clear zone in the antibiotic sensitivity test.

Isolate	Antibiotic							
	Tetracycline		Minocycline		Doxycycline		Clindamycin	
	□ (mm)	Sensitivity	□ (mm)	Sensitivity	□ (mm)	Sensitivity	□ (mm)	Sensitivity
S1	-	R	6.94±0.87	R	-	R	-	R
P1	14.56±4.00	I	21.13±2.06	S	17.64±2.64	S	-	R
S2	15.76±0.97	S	12.50±1.77	R	13.90±1.00	I	-	R
P3.1	25.41±0.50	S	26.87±0.61	S	26.58±1.74	S	-	R
P3.2	14.90±2.15	I	20.64±2.75	S	17.26±1.60	S	-	R
S4	20.06±1.86	S	24.05±2.26	S	22.79±0.22	S	-	R
P4	19.33±0.70	S	20.34±2.03	S	20.64±2.33	S	-	R
P5Bs	26.64±0.57	S	26.27±1.62	S	23.87±1.41	S	-	R
P5Kc	-	R	16.60±0.58	I	-	R	-	R
S6.2	14.95±1.23	I	9.17±1.40	R	8.12±0.68	R	-	R
S6.3	12.34±2.76	I	9.98±0.80	R	10.04±1.10	R	-	R
P62	25.42±2.41	S	25.05±1.74	S	24.93±1.44	S	-	R

□ : Diameter of clear zone (mm); Sensitivity result: R : resistant; I : intermediate; S : sensitive, breakpoint determined based on CLSI guidance [12]

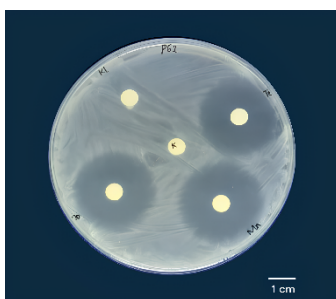


Figure 3. Results of antibiotic sensitivity testing of the P62 isolate using the disc diffusion method.

DISCUSSION

In this study, pustule samples were inoculated into TSB medium to enrich the bacteria in the samples. Tryptic Soy Broth (TSB) is a standard microbiological medium rich in proteins and glucose, commonly used for the enrichment, cultivation, and isolation of bacteria from clinical specimens, and supports bacterial growth [13]. The TSB medium contained casein, soybean peptone, dextrose, sodium chloride, and dipotassium phosphate. Casein and soybean peptone provide nitrogen and amino acids to support bacterial growth [14], whereas dextrose serves as a carbon source for bacterial growth. Sodium chloride maintains the osmotic balance of the cells, and dipotassium phosphate acts as a buffer to maintain the pH of the medium [15]. Therefore, TSB medium promotes the growth of a wide range of bacteria, including aerobic and facultative bacteria, in the gut.

Isolation of pustule bacteria was performed on MHA and MSA media. MHA is a nutrient-rich, non-selective medium that supports the growth of most bacterial species. Its composition makes it versatile for a wide range of Gram-positive and Gram-negative bacteria. The main components of MHA are beef

extract, acid hydrolysate of casein, and starch. Beef extract and acid hydrolysate of casein provide amino acids, peptides, vitamins, and nitrogenous compounds that support protein synthesis and bacterial growth, respectively. Starch in the medium absorbs toxic by-products that may be produced by bacteria during growth and contributes to the ability of the medium to neutralize bacterial toxins. The composition of MHA ensures that most bacterial strains can grow without inhibiting specific types of bacteria [16]. MSA is a selective and differential medium used to isolate and differentiate *between Staphylococcus* species. The selectivity of MSA is due to its mannitol and high salt concentrations. Only certain bacteria can metabolize mannitol as a carbon source, while the high salt concentration (7.5%, v/v) inhibits most bacteria except staphylococci. If *S. aureus* is present, it ferments mannitol, causing the pH of the medium to become acidic. This change alters the color of phenol red in the medium from red to yellow [17]. Based on the isolation results, 12 isolates were obtained from the pustule samples, consisting of Gram-positive cocci (eight isolates) and Gram-negative rods (four isolates). Davis (1996) [18] reported that both Gram-positive and Gram-negative bacteria can be isolated from pustule samples.

Molecular identification revealed six species: *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Staphylococcus caprae*, *Staphylococcus aureus*, *Enterobacter aerogenes*, and *Citrobacter koseri*. One isolate could only be identified as *Staphylococcus* sp. based on its phenotypic characteristics. The diversity of skin conditions can affect the normal microflora of the skin. However, the skin's bacteria are predominantly Gram-positive, such as the coccus group (Staphylococci, Micrococci, and Diphtheroids) [18]-[19]. Other studies conducted by Sitohang et al. (2019) [20], Zhao et al. (2021) [21] and Legiawati et al. (2023) [22] found that *Staphylococcus* was the dominant bacterium. This bacterium is a normal component of the skin's microflora [18]. *S. epidermidis* contributes to healthy skin homeostasis [23]. Abdulatif (2023) [24] reported that *S. epidermidis* was the dominant bacterium isolated from acne samples. Huang et al. (2022) [7] reported that both *S. epidermidis* and *S. aureus* can be isolated on tryptic soy agar medium.

The lack of *C. acnes* detection in this study likely reflects that the isolation was performed only under aerobic conditions, which do not support the growth of anaerobic bacteria. Additionally, the abundant growth of *S. epidermidis* in acne lesions could suppress the growth of *C. acnes*. Dessinioti & Katsambas (2024) [23] also reported that the *C. acnes* can be hindered by *S. epidermidis*. This is due to the staphylococcal strains have anti-*C. acnes* activities. Therefore, in this study we did not obtain the *C. acnes* from the samples.

In addition to Gram-positive bacteria, Gram-negative bacteria have also been found in acne. Gram-negative bacteria constitute a small portion of the skin flora [18]. The study by Legiawati et al. (2023) [22] showed that *Citrobacter*, *Enterobacter*, and *Klebsiella* can be isolated from acne. The presence of these Gram-negative bacteria may be due to their introduction to the skin, for example, during face washing if the skin is not dried immediately. According to Davis (1996) [18], drying is a key factor in preventing the growth of Gram-negative bacteria on intact skin.

The results of the antibiotic sensitivity tests for tetracycline, minocycline, doxycycline, and clindamycin are presented in Table 2. The isolated bacteria, *S. epidermidis*, *S. aureus*, and Gram-negative bacteria (*Klebsiella* sp., *Enterobacter* sp., and *Citrobacter* sp.) all showed resistance to clindamycin. Rahmani et al. (2021) [25] stated that *S. epidermidis* strains were resistant to many antibiotics, including clindamycin. Long-term systemic antibiotic therapy has been associated with an increase in resistant strains of *S. epidermidis*. Sitohang et al. (2019) also found that *S. epidermidis* was resistant to clindamycin. Research by Hassanzadeh et al. (2008) [26] revealed that clindamycin is the least effective antibiotic against acne-causing bacteria, particularly *Propionibacterium* sp. Asditya et al. (2019) [27] found that clindamycin was the least sensitive antibiotic against *S. aureus* and *P. acnes* compared to doxycycline.

Clindamycin is an antibiotic classified under the lincosamide group. It is predominantly used topically for acne treatment but can also be administered orally. The drug has a relatively narrow to moderate spectrum, primarily targeting Gram-positive bacteria and anaerobes [28]. In this study, all Gram-negative bacteria showed resistance to clindamycin. Previously, antibiotics like erythromycin and clindamycin were commonly used, but they are now seldom prescribed due to high resistance levels [29]. Clindamycin works by inhibiting peptide bond formation during protein synthesis at the 50S ribosomal subunit, which prevents bacterial multiplication. It functions as a bacteriostatic agent at lower doses and can become bactericidal (killing bacteria) at higher concentrations [30]. Due to the risk of serious side effects, oral clindamycin is less frequently used for acne. One significant risk associated with clindamycin is the development of *Clostridioides difficile*-associated colitis, which can lead to severe diarrhea and colon inflammation [30]-[31].

Data in Table 2 show that *S. epidermidis* from sample P5Kc was resistant to doxycycline and minocycline. Meanwhile, *S. epidermidis* isolated from P1 and P3.2 showed intermediate reactions, while the isolate from P5Kc was resistant to tetracycline. It appears that doxycycline and minocycline were more effective in inhibiting the growth of acne-causing bacteria from the *Staphylococcus* group. Hassanzadeh et al. (2008) [26] revealed that tetracycline was the least effective antibiotic for *Staphylococcus aureus* in an in vitro assay. Doxycycline and minocycline are both part of the tetracycline class and share a similar structure consisting of four fused hydrocarbon rings, commonly known as a naphthalene ring system. They are classified as second-generation tetracyclines, while tetracycline itself represents the first generation and the prototype of this class [32], [33]. Tetracycline is a broad-spectrum antibiotic effective against a wide range of Gram-positive and Gram-negative bacteria by inhibiting protein synthesis within bacterial cells [34]. Nevertheless, these antibiotics have limited efficacy against Enterobacteriaceae, and resistance to them is prevalent among these bacteria. Species within the Enterobacteriaceae family, such as *Escherichia coli* and *Klebsiella*, are showing increasing resistance to tetracycline [35].

In this study, the Gram-negative isolates *Klebsiella pneumoniae* (S1 and S2), *Enterobacter aerogenes* (S6.2), and *Citrobacter koseri* (S6.3) mostly showed resistance to doxycycline, minocycline, and tetracycline. Several factors may contribute to the antibiotic resistance observed in these species. One possible reason is the presence of resistance genes that enable bacteria to withstand antibiotics. For example, resistance to clindamycin is frequently linked to *erm* genes (erythromycin ribosomal methylase genes), which methylate the bacterial ribosome. This modification prevents clindamycin from binding to its target, thereby neutralizing its effect [36]. Another resistance mechanism involves efflux pumps, which actively expel antibiotics from bacterial cells, thereby reducing the drug's intracellular concentration and allowing the bacteria to survive. Efflux pumps are particularly common in tetracycline resistance [37]. Resistance may also arise from mutations in ribosomal target sites. Since clindamycin inhibits protein synthesis by binding to the 50S ribosomal subunit, mutations in this target site can diminish the antibiotic's binding affinity, leading to resistance [30]. Additionally, some bacteria produce proteins that protect ribosomes from tetracycline. Ribosomal protection proteins (RPPs) dislodge tetracycline from its binding site on the 30S ribosomal subunit, allowing protein synthesis. Common RPP genes such as *Tet(M)*, *Tet(O)*, and *Tet(Q)* can also be transferred between bacteria [38]. Certain bacteria may also produce enzymes such as *Tet(X)* that chemically modify tetracycline, rendering it ineffective by preventing it from binding to the ribosome [39]. Although less common, this form of resistance is significant. Additionally, some bacteria develop resistance by reducing the permeability of their cell membranes to tetracycline, preventing the antibiotic from entering the cell. Alterations in membrane porins or other surface structures can limit tetracycline uptake, reducing its effectiveness, a mechanism often seen in Gram-negative bacteria with complex outer membranes [37].

CONCLUSION

Isolation of aerobic bacteria from acne vulgaris revealed the presence of *S. aureus*, *S. caprae*, *S. epidermidis*, *K. pneumoniae*, *E. aerogenes*, and *C. koseri*. Among these, *S. epidermidis* was predominantly found in papulopustular samples. Clindamycin was ineffective against all the bacteria tested. The highest antibiotic effectiveness against the bacterial isolates was demonstrated by minocycline and doxycycline. Since it was a preliminary study describing a small population, further research with larger sample sizes is required to confirm these findings and provide more representative data on antibiotic resistance patterns in acne associated bacteria.

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