International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 14s,2025
https://theaspd.com/index.php

Susceptibility for antibiotics and molecular studies to detect the Lipase gene in Propionibacterium acnes, isolated from a patient with acne.

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Abstract
Propionibacterium acnes (P. acnes), a usual inhabitant of the human skin, is anaerobic, Gram-positive, and rod-shaped, and it plays an important role in acne. Isolate bacteria is cultured on selective media (3-7) days anaerobically 5(9.1 %) isolates from were positive as P.acnes; since it can form a biofilm, which makes it more resistant to the antibiotic, therefor; biofilm assay by microtiter plate method, and all isolated were positive and robust; antibiotic susceptibility has been proceeded by the procedure Kirby-Bauer on the Mueller Hinton agar and chose 15 types of antibiotics the result shows that all (100%) P. acnes bacteria were resistant to ceftazidime (30µg), amoxicillin (25µg), ampicillin (10µg), ciprofloxacin (5µg), clindamycin (10µg), and pencillin (10 µg); on other hand, all 100% sensitive to doxycyclin (30 µg), azithromycin (10µg), rifampin (5µg), amikasin (30µg), azitreonam (30µg), impenine (10µg) moreover, confirm identification by polymerase chain reaction (PCR) technique by the sequence of 16 S rRNA and one of important virulence factor (lipase) play important factor in pathogensisand the first target of treatment coded by gehA. The PCR amplification products were disclosed by electrophoresis. The sizes of the amplicons 16S rRNA and Geh A size product gene were determined by comparison to the 1000 bp allelic ladder. The results showed that all isolated samples were 5(100%) positive for 16S rRNA, and the lipase gene was positive in 3 out of 5 cases (2 of them were males) and negative in the remaining 2 cases, who were from both sexes with a non-significant difference P> 0.005 (P=1.000). Considering the P-value less than 0.005, a significant

Keywords: Propionibacterium acnes,16S rRNA, lipase, biofilm, antibiotic susceptibility

INTRODUCTION

Acne or acne vulgaris is a chronic the skin inflammatory disorder of the pilosebaceous hair follicle (Vasam *et al.*, 2023). High prevalence among teenagers age group of about (80%) but this does not negate its effect on adults (Sivaramakrishnan and Jayakar, 2019); clinical features characterized by the presence of white or/and blackhead (open and closed comedones) on the non-inflammatory stage and the inflammatory features represented by the presence of papules, pustules, or nodules in some cases, (*Palaniswami et al.*, 2024). scarring is the main complication and psychosocial distress that arises from it (Sivaramakrishnan and Jayakar, 2019). The skin lesion appears on the face, chest, back, shoulder, and neck (Sadiq *et al.*, 2023). The leading bacterial cause of acne vulgaris is *P. acnes* (Lee *et al.*, 2019).

Propionibacterium acnes is a Gram-positive, anaerobic, aero-tolerant, diphtheroid, bacillus-shaped, non-spore-forming bacterium belonging to the actinobacteria phylum. Its ideal growth is at 37° C (Fournière *et al.*, 2020). It becomes a pathogenic bacteria when sebum overproduction hyperkeratinization (Achermann *et al.*, 2014). Many virulence factors participate in the pathogenesis of *P.acnes* involved, including *camp5*, *gehA*, *tly*, sialidases, neuraminidases, lipase, and endoglycocer-amidases (Al-Hasseny *et al.*, 2021). Also, *P. acnes* lipase *GehA* glycerol-ester hydrolase A has been counted as one of the most important virulence factors that played an important role in the pathogenesis of acne, which is responsible for the hydrolysis of sebum tri acylglycerols to releasing free fatty acid and glycerol (Falcocchio *et al.*, 2006).

MATERIALS AND METHODS

2.1 Bacterial Culture And Identification

In the current study, approximately 105 samples were obtained from a patient with acne vulgaris (comedon and pustule) from the face 50 only were cultured on the selective anaerobic media Wilkins Chalgren agar with 5% sheep blood and 0.02 furazolidone antibiotic to inhibit Staphylococci, after stamp incubated for 3-7days identified by colony morphology on agar and Gram stain, then at 37 °C for confirm

detection by PCR technique. Also, DNA extraction was accomplished with the genomic DNA purification kit supplemented by a manufactured company (Gene aid, UK), and assessment of the purity of DNA by Gel electrophoresis with 0.8% agarose and voltage of 70 Volts (Figure 1) shows the purity of DNA of each sample. The PCR primers are used to amplify 16S rRNA, and the lipase gene of *P.acnes* is shown in (Table 1).

The final total volume of PCR product was 25 µl adding 12.5 of master mix Promega), five µl template DNA, two µl from forward primer, and two µl reverse primer; the final volume was completed to 25 µl by adding 3.5 µl nucleus water). Then, the protocol of pcr of denaturation at 95˚C for 30 sec., annealing at 55˚Cfor 30 sec, extension atv75˚C for 30 1mint subsequent 35 cycles. The PCR products were analyzed utilizing electrophoresis with 1.5% agarose gel mixed with safety pigment diamond (Promega) to a final concentration of 0.1 mg/mL. One hundred volts for 35 minutes. UV Trans-illuminator saw the PCR results.

Table 1: primers sequence and product size

Primers	**Sequences (5'-3')**		**Products Size bp**	**Reference**
16S *rRNA* gene *p. acnes*	F	AGTGGCGAACGGGTGAGTAA	292	This study AM902704.1
	R	ATCAGGCTTCCGCCCATTGT		
Lipase(geh A) P.acnes	F	TGCCGAACGCCTACATCAA	211	This study X99255.1
	R	TGCATCAACGCTGATCCCT		

2.2 Biofilm assay

The biofilm production is assessed by the isolate's capacity to create a biofilm using this method on flat-bottomed 96-well microtiter plates (Ruchiatan *et al.*, 2023). Incubated the isolation after being inoculated in brain heart infusion broth (BHIB) with 1% glucose for three days at 37 C; the first three wells were filled with BHIB only as a control, and then each isolated filled three wells and incubated for three days after that wash and fixation with 96% alcohol and stained with crystal violet 1% presents the biofilm development classification: strongly positive (OD450 > 0.24), mildly positive (0.12 ≤ OD450 < 0.24), or negative (OD450 <0.12)(Lin *et al.*, 2021).

2.3 Antibiotic susceptibility

Kirby-Bauer on the MullerHinton agar was the procedure used in this test. The colonies were emulsion from grown culture and diluted into the 0.5 McFarland solution. The suspension of bacteria was streaked on the Muller Hinton agar uniformly using the sterile swab and left to dry. The antibiotic discs have been proven on a plate by using sterile forceps. The cultures were for 27 hours at 37℃; anaerobically, the inhibitory zone width measurement was calculated, and the outcomes were interpreted using the CLSI, 2023 standards.

RESULTS AND DISCUSSIONS

3. 1 Culture and Identification

In this present study, 55 positive cultures growth of 105 samples were collected from a patient with acne vulgaris in AL-Karamah Teaching Hospital, cultured aerobically and anaerobically; only five positive cultures growth on Wilkins Chalgren agar with 0.02 furazolidone incubated anaerobically. The *P.acnes* colony was identified macroscopically by morphology and microscopically by Gram stain, then confirmed molecular by amplified 16 S rRNA.

These results agree with a study done by Muhammed and Dabbagh, who assume *P.acnes,* which is the most common pathogen bacteria isolated anaerobically 25 (13.02%) from a total of 160 samples were P.acnes from acne patients (Muhammed and Dabbagh, 2016). The other study by Elfekki in Egypt collected 100 samples from patients with acne and observed that the common bacteria in anaerobic culture was P. acnes, found in 44% of samples (Elfekki *et al.*, 2020). Sabah Kasim, who disagrees with the current study, found that P.acnes have an infection rate of 79 (51%) (Sabah Kasim *et al.*, 2022). This

difference may be because of the symbiosis between the *P.acnes* and S. *epidermidis* and the type of lesion *P.acnes* in closed commedon more than other forms.

3.2 Biofilm.

The results of the current study on the ability of bacteria to form a biofilm showed that all isolate *P.acnes* (100%) had strong biofilm formation according to mean OD value as shown in table 2.2 Using the Micro titer Plate Assay(MP)Amethod**.**

Table 2: Analyzing biofilm using the micro-titer plate technique

Mean OD value	Biofilm formation
OD ≤ Odc	None
ODc < OD ≤ 2× ODc	Weak
2× ODc < OD ≤ 4× ODc	Moderate
4× ODc < OD	Strong

off ;* ODc = optical density cut OD = optical density (Lin *et al.*, 2021)

These results are similar to the study results reached by Noor and Asma (2022) in Kirkuk al, Iraq.Ruchiatan *et al.*, (2023). Who displayed that among 36 *Propionibacterium acnes* as a total isolate, 20 of them were strong biofilm forming (BF) and 16 non-biofilm forming (NBF). Also, Jahns *et al.* visualized that 14 of 18 p.acnes isolates were strong (BF); thus, it is compatible with the current study (Jahns *et al.*, 2012). while Loss et al. found only 9 of 39 p.acnes isolates were strong (BF) (Loss *et al.*, 2021). The ability of bacteria to biofilm, when they become pathogenic is a prevalent virulence factor in antibiotic-resistant but may be inhibited in patients who are treated by MPA-RegulTM(medroxyprogesterone acetate)(Fournière *et al.*, 2020).

3.3Antibiotic susceptibility test

The disc diffusion method was used in an accurate study. By use of Fourteen types of antibiotics, the inhibited zone measured results compared with CSLI 2023 to give the antibiotic sensitivity as well, the result showed in Table 3, shows that all 5(100%) *P. acnes* bacteria were resistant to ceftazidime (30µg), amoxicillin (25 µg), ampicillin (10 µg), ciprofloxacin (5µg), clindamycin (10µg), penicillin (10µg) While all 5(100%) isolates were sensitive to doxycycline (30µg), azithromycin (15µg), rifampin (5µg), amikacin (30µg), aztreonam (30µg), impenine (10µg), and the incidence of antibiotic sensitivity to tetracyclin (10µg) 60%, while only 40% sensitive to erythromycin (10µg). A study agrees with this result conducted by Gozali, who assumed the highest sensitivity was to doxycycline 87.5% followed by azithromycin (67.50%) and 40% sensitive to erythromycin but disagreed with (57%) sensitivity to clindamycin (Gozali *et al.*, 2023). Moreover, results agree with Yadav *et al.*, who presume that the P.acnes antibiotic susceptibility test was 97.6% to doxycycline but disagree with susceptibility to clindamycin 88.10% and erythromycin 96.05% (Yadav et al., 2020).On the other hand, a study conducted Characteristics of Biofilm-Forming (BF)Ability and Antibiotic Resistance of *P.acnes* in Indonesia by Ruchiatan *et al.*(2022) assumed all isolates 100% were sensitive to doxycycline and tetracyclin and resistant to clindamycin in was 54.5% and resistant to azithromycin and erythromycin 63.6 and 54.5% respectively this result agrees with accurate study except azithromycin sensitive all isolate

Table 3: antibiotic susceptibility of *P.acnes*

Antibiotic	Sensitivity test		
	S no. %	I no.%	R no%
Ceftazidime (30µg)	0(0)	0(0)	5(100)
Doxycyclin (30 µg)	5(100)	0(0)	0(0)
Azithromycin (15 µg)	5(100)	0(0)	0(0)
Amoxicillin (25 µg)	0(0)	0(0)	5(100)
Ampicillin (10 µg)	0(0)	0(0)	5(100)
Ciprofloxacin (5µg)	0(0)	0(0)	5(100)
Rifampin (5 µg)	5(100)	0(0)	0(0)

Erythromycin (10µg)	2(40)	3(60)	0(0)
Tetracyclin (10µg)	3(60)	1(20)	1(20)
Clindamycin (10µg)	0(0)	0(0)	5(100)
Penicillin (10µg)	0(0)	0(0)	5(100)
Amikacin (30 µg)	5(100)	0(0)	0(0%)
Aztreonam (30 µg)	5(100)	0(0)	0(0)
Impenine (10µg)	5(100)	0(0)	0(0)

3.4 Identification of bacteria by polymerase chain reaction (PCR)

3.4.1. After DNA extraction, the purity of DNA was measured by gel electrophoresis in agarose at 0.8%, as shown in figure1.

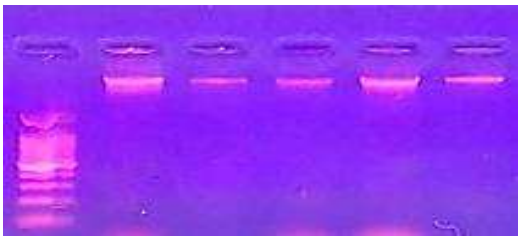

Figure 1 shows the purity of DNA in 0.8% agarose and 70-volt

3.4.2 16S rRNA gene for Identification of *P.acnes*

To more accurately identify and confirm the culture method, all five isolates are *P.acnes* by PCR, depending on the primer (16SrRNA) gene that considers the specific gene. The expected product size is 292. All 5 (100%) isolates were detected with *P.acnes*, as shown in the Figure 2.

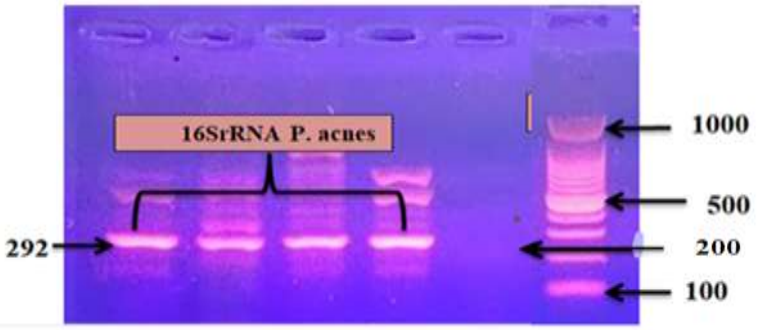

Figure 2: 1.5 % Agarose gel electrophoresis at 100 volts for 35 min for par gene PCR products visualized under UV light at 280 nm after staining with diamond safety pigment. L100:1000bp ladder; lane were positive for this gene, and the product size is 292 bp.

A study conducted in Babylon province by Al-Hasseny *et al.*(2021) demonstrated that only 9 (64.3%) isolates from 200 samples were *P.acnes* using a specific primer to confirm the bacterial diagnosis; on the other hand; these results agree with another study conducted in Iran by Naghdi and Ghane (2017), who mentioned that 82.85% of isolates were *p.acnes* by PCR methods (Naghdi and Ghane, 2017). in contrast, Published *et al.*(2020) showed that PCR results using the 16 SrRNA gene of *P. acnes* were 68 (36.5%) out of 186 positive isolates for amplifying the 16S rRNA gene.

3.4.3. identification of lipase gene

Although *P.acnes* is considered normal flora in the skin, it is regarded as the leading cause of acne vulgaris and maintains its role in acne vulgaris; the most important virulence factor is lipase extracellular excretion, which encoded by gene *geh A* gene lipase functions to metabolic the lipid in the pilosebaceous gland to produce free fatty acids that important nutrition element of *P. acnes*. The lipase gene was screened by PCR using a specific lipase primer; the trial results were only three positive isolates (2 male) and one female. The other two were negative (male and female), as shown in Figure 2.

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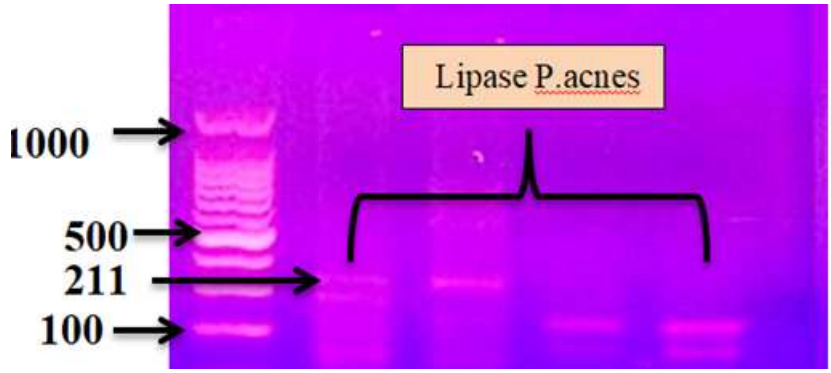

Figure 2: 1.5 % Agarose gel electrophoresis at 100 volts for 35 min for par gene PCR products visualized under UV light at 280 nm after staining with diamond safety pigment. L100:1000bp ladder; lane were positive for this gene, and the product size is 211 bp.

Furthermore, Mahmood *et al.*(2020) assume that lipase produced by *P.acnes* isolated from acne lesions in all clinical isolates without treatment. On the other hand, it inhibits when patients are treated with oral isotretinoin. Since the positive isolates were males, the reason may be that most females use a skincare routine that includes lotion and tocotrienol. In addition, some cosmetics contain substances that inhibit bacteria or their virulence factors (Mahmood *et al.*, 2020); moreover, Nakamura *et al.*(2003) demonstrate that lipase gene visualization is not on all strains of *P.acnes* (Nakamura *et al.*, 2003)

4. CONCLUSION

P.acnes is one of the bacteria that cause acne vulgaris; current identification by 16 sRNA, lipase is the most critical extracellular excretion enzyme, and it is considered an important virulence factor that plays a role in the pathogenesis of acne. This enzyme is significantly affected by changes in skin conditions (PH and excessive sebaceous); however, it can be the main target in treating acne vulgaris. *P.acnes* can form biofilm and resist antibiotics; therefore, the treatment must not last long (more than six months). Moreover, a combination of topical(topical retinoids, topical antibiotics) and oral antibiotic treatment gives the best and fastest treatment with limited use of systemic antibiotics to reduce bacterial resistance to antibiotics.

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