Evaluating Antibiotic Resistance and Biofilm Formation of *Cutibacterium acnes* in Acne Vulgaris: Insights from Skin Sample Analysis

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Abstract:

Background: *Cutibacterium acnes* (*C. acnes*) is one of the normal microbiotas found inside the sebaceous glands that shift to an opportunistic pathogen and cause acne. Recently, the antibiotic resistance of *C. acnes* has become a major concern in dermatology clinics due to microbiome dysbiosis and the bacterial ability to form biofilms.

Aim: to evaluate the biofilm profile of different phylotypes of *C. acnes* isolated from acne patients and a healthy control, and to assess the minimum inhibitory concentration (MIC) for commonly used antibiotics in acne treatment.

Materials and Methods: From February to June 2024, a study was conducted in Basrah, Iraq, at the Basrah Teaching Hospital, involving seventy acne patients (mean ±SD: 18 ±2 years) and 70 healthy controls. We performed swab sampling from the face's surface and sent it for molecular detection and phylotyping of *C. acnes*. Subsequently, biofilm formation and MIC testing against several antibiotics were evaluated.

Results: For acne patients, *C acnes* was isolated from 37 out of 70 samples (52.8%) and IA-2 was the predominant phylotype, while healthy control samples showed more diverse bacterial clades (IA-2, IB, and II.) in 30%, 40% and 22.9% respectively. *C acnes* isolated from the patient's samples showed multiple resistance towards clindamycin, erythromycin, and levofloxacin, while sensitivity was higher for azithromycin and doxycycline. Biofilm formations were observed in both patients and control isolates; however, the healthy control samples exhibited a statistically lower biofilm concentration compared to acne patients.

Conclusion: This study highlights that specific *C. acnes* strains, namely IA2 phylotype, are key contributors to the development of acne in our locality. Furthermore, the observed rise in antimicrobial resistance among isolated strains underscores the consequences of antibiotic misuse and emphasizes the urgent need to reconsider current treatment approaches by replacing antibiotics with alternatives that would restore the *C. acnes* phylotype diversity and break down bacterial biofilms.

Keywords: *Cutibacterium acnes,* antibiotic, resistance, biofilm

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Introduction

Acne vulgaris is one of the most prevalent skin diseases, typically emerging during puberty. It affects a significant portion of the population, often leading to psychological distress, low self-esteem, and reduced productivity[1]. Pathogenesis involves a combination of factors, including increased sebum production, follicular hyperkeratinization, inflammation, immune dysfunction, and bacterial colonization by pathogens like *Cutibacterium acnes* [2] which is a Gram-positive anaerobic bacterium that acts as both a commensal and an opportunistic pathogen, it is implicated in acne pathophysiology and other conditions such as lung abscesses and prostate cancer[3]. This bacterium is genetically diverse, with six primary phylotypes: IA1, IA2, IB, IC, II, and III[4]. While IA1 and IA2 are strongly associated with acne, phylotypes II and III are predominantly found in healthy skin[5].

The growing prevalence of antimicrobial resistance in *C. acnes* has raised concerns, with strains demonstrating resistance to commonly used antibiotics such as clindamycin, erythromycin, azithromycin, and quinolones [6]. Biofilm formation is another contributing factor to increasing the resistance, as biofilms protect bacteria from antimicrobial agents and the host immune system[7]. To our knowledge, no prior published investigation has been

carried out on the Iraqi population regarding *C. acnes* phylotypes and their capacity to build biofilms that may contribute to heightened antibiotic resistance. Therefore, this work aims to evaluate the correlation between biofilm production and antimicrobial susceptibility among various *C. acnes* phylotypes, emphasizing distinctions between acne patients and healthy controls.

Materials and Methods:

Study Design

A five-month investigational comparative study was conducted in 2024 at Basrah Teaching Hospital, involving 70 acne patients and 70 healthy controls. Patients aged 10–39 (mean ±SD: 18 ±2 years) with varying degrees of acne severity were included. Exclusion criteria included pregnant women, participants with features of hormonal disorders such as hirsutism and polycystic ovarian syndrome, and underlying autoimmune diseases. This study protocol adhered to the National Research Council guidelines[8], and informed consent was obtained from all participants

Sampling;

Samples from patients were collected using sterile swabs from acne lesions and the skin surface of healthy controls. After applying pressure to extract material, samples were suspended in normal saline and transported to the lab for further processing. DNA extraction and molecular analysis were subsequently performed.

Molecular Analysis:

Genomic DNA was extracted using a commercial kit (Presto™ Mini gDNA Bacteria Kit, Geneaid, China). DNA concentration and quality were assessed before storage at -20°C.

PCR Amplification and Phylotyping:

The identification of *C. acnes* was verified using target genes done by using specific primers PR-246 5'-GCAGGCAGAGTTTGACATCC-3' and PAR-2 5'-GCTTCCTCATACCACTGGTCATC-3'[9] and phylotyping was performed using primers targeting specific genes. PCR conditions included 95°C denaturation, 59°C annealing, and 72°C extension. Amplification products were visualized using 1% agarose gel electrophores

Antimicrobial Sensitivity Testing

Minimum Inhibitory Concentration (MIC): Due to the high expense of conducting the test, MIC was applied for randomly selected fifteen positive samples suffering from acne and were tested for their sensitivity against Clindamycin, Erythromycin, Levofloxacin, Azithromycin, and Doxycycline and compared with the cutoff points for each antibiotic following CLSI guidelines [11] using broth microdilution. MIC values were analyzed statistically to assess resistance distribution.

Biofilm Formation Assay: Thirty samples (15 patients and 15 control) representing different phylotypes (IA2, IB, II) were tested for biofilm formation using crystal violet staining with some modification[12].As the following: the authors prepare dilutions of anaerobically grown over night cultures in brain heart broth (obtained from individual colonies) sterile brain heart broths to achieve a final optical density reached 0.05 (A630). We then 200 µL of these diluted cultures were added to Costar® 96-well cell culture plates with a flat bottom (Corning, USA). The plates were incubated without tension before harvesting the biofilms at 37 °C for 22 hours. The culture plates washed three times with distilled water and then heat fixed at a temperature of 60°C for one hour, the plates were left to cool to room temperature before staining the biofilm mass with a 0.1% solution of crystal violet. Plates were washed three times with deionized water after staining. The biofilms were subsequently de-stained using a 33% acetic acid solution. After mixing the plates, the absorbance of the resulting solution was measured at 570 nm using BioTek 800TS microplate reader. The absorbance data were normalized to a blank acetic acid solution, and then to the harvested culture's optical densities.

Statistical Analysis: Frequency and percentage were used to display categorical or qualitative data and mean±SD and median for numerical or quantitative data. The Student T test was used to compare between the two groups using SPSS v21.0 and Microsoft Excel 2021. Statistical significance was set at $p \leq 0.05$.

Results:

Bacterial detection:

Out of 70 patients' sample, 37 (52.9%) were positive for *C. acnes* regardless the severity of acne lesions and 65 sample out of 70 (92.9%) were positive from the healthy control's samples. figure (1)

Figure (1):1% Agarose gel electrophoresis for *Cutibacterium acnes* specific primers after staining with

Red Safe dye. Lane 1-10 samples. Lane 16 ladderL100-1500; the size of product is 334 bp.

Detection of bacterial phylotypes:

In the studied population, patients' samples predominantly exhibited only one phylotype of *C. acnes* (IA-2) with no occurrence of the other phylotypes and considerable number of patients (47.1%) show negative test results. In contrast, healthy controls displayed various clades in their skin samples. The authors found that IB was the most prevalent phylotype, 28(40%), IA-2 detected in 21(30%) samples and type II in 16 (22.9%), table (2), figure 2. The difference in distribution of *C. acnes* phylotypes was statistically significant between the two groups (P<0.001).

Figure (2): Phylotypes distribution among patients and controls samples. Lane 1-5 represent IA-2 phylotypes the product size 494 bp, the lane 6-10 represent phylotypes IB product size 145 pb, the lane 11-15 represents phylotypes type II the product size351pb. LadderL100-1500.

Antibiotic sensitivity test:

Among the five antibiotics were used for Minimum Inhibitory Concentration (MIC), high resistance was found to Erythromycin (100%), Clindamycin (80%) and Levofloxacin among the 15 samples that were selected from patients with acne and identified as IA-2 phylotypes, (figure 3), while low resistance and high sensitivity for Doxycycline were observed (figure 3).

Biofilms formation:

Table 2 showed that biofilms formation was observed in both groups using crystal violet staining method and there were no statistically significant differences in biofilm

levels between patients and controls groups (p>0.05). However, low level of biofilms production was clearly identified in healthy controls compared to patients' group (0.19±0.18 µg versus 0.24±0.14 µg).

Evermore, variable levels of biofilms formation in relation to different C. *acnes* phylotypes were observed and phylotype IA2 exhibited the highest level of biofilm formation compared to II and IB phylotypes, and the difference was statistically significant, p=0.021, (figure 4).

Discussion:

The microflora on human skin mostly belong to one of these genera; *corynebacteria*, *Propionibacterium* and *staphylococci* that contribute in skin homeostasis and maintain healthy skin and prevent other pathogenic bacteria for colonization [13].In contrary, *Propionibacterium acnes* (recently names as *Cutibacterium acnes*) can turn to opportunistic pathogens and cause acne vulgaris[14].Bacterial growth previously was believed is the trigger of the developing the disease, new finding approved that loss of microbial equilibrium between bacteria skin population and *C.acnes* phylotypes can truly lead to chronic skin infection[15].Studies using different DNA-based techniques evaluated the inordinate variety of *C.acnes* strains each method have advantage and drawbacks. Lomholt and Kilian suggest that IA strain was frequently found in acne skin than phylotypes IB, II and III [16] . In the current study, we found that phylotype IA2 was the predominant strain among acne isolates, in contrast, healthy skin demonstrates more diversity in phylotypes distribution especially types IB (40%), IA2(30%), II (22.9%). The variety of strains in control groups and the predominancy of one strain in patient's isolates can be explained by the microbial dysbiosis that observed in acne lesions with loss of microbiome diversity and the predominance of pathogenic strains that prefer to proliferate inside inflammatory lesion[17].In contrast, some researchers reported that phylotypes IA-1 are more seen in patients with acne while type IA-2,IB,II were associated with healthy control[18]. Furthermore, the distribution of phylotypes in correlation to the severity of acne, many studies reported that there was no significant different in mild and severe acne in relation to a specific phylotypes [19]. In the current study, because of the small sample size, we were unable to stratify the degree of biofilm formation according to disease severity.

A lot of different antibiotics have been used to treat acne. However, recently, the rise of antimicrobial resistance has become a major global concern. This is on top of the fact that C. acnes naturally is insensitive to some antibiotics, like 5-nitroimidazole agents (metronidazole tinidazole, and ornidazole), aminoglycosides, and sulfonamides. Furthermore, studies have reported high antibiotic resistance against macrolides and clindamycin, and less resistance to tetracycline [20]. In line with the above observations, our findings showed that different isolates, particularly those from severe cases, had increased

resistance to clindamycin, erythromycin, and levofloxacin, while their sensitivity to azithromycin and doxycycline was high. The mechanism of antibiotic resistance in C. acnes primarily stems from chromosomal point mutations, particularly in the 23S rRNA gene for macrolides and the 16S rRNA gene for tetracycline [21].

Along with the development of resistance, bacterial biofilm formation may also play a part in making C. acnes less susceptible to antibiotics and increasing resistance. Researchers thought that this, along with the presence of virulence factors, explains antimicrobial resistance [22]

Recent data suggest that the pathogenesis of acne involves the formation of biofilms by *C. acnes*, a complex protective shell that acts as a wall to enable the bacteria to survive in harsh environments, as first described in 2007 [23]. In 2012, a study revealed that IA and II phylotypes, capable of forming large colonies, producing biofilms, and containing secreted bacterial proteins, were involved in acne cases. [24]. Even though patients in the current study tended to form biofilms more than healthy controls, there were no significant differences ($p = 0.059$) in the levels of biofilm between patients and controls. However, when we measured biofilm formation by IA2, II, and IB phylotypes, we found statistically significant differences ($p=0.021$), with biofilm levels significantly higher in the IA2 phylotype than in the II and IB phylotypes. These findings support the hypotheses that in acne patients, the imbalance in *C. acnes* phylotype with the predominance of pro-pathogen IA2 phylotype that exhibits the highest biofilm formation plays key roles in the pathogenesis of acne [25].

Conclusion:

This study indicates that C. acnes phylotype AI2 was predominantly linked to acne in our patients relative to healthy controls. The research supports the idea of microbial dysbiosis, with the dominance of pathogenic strains significantly contributing to acne development. The ability of *C. acnes* to create biofilms within sebaceous units may enhance the antibiotic resistance of various isolated clades. This may also demonstrate the abuse of antibiotics, underscoring the necessity to reevaluate existing treatment practices. Further multicenter studies with a substantial patient cohort are essential to elucidate our findings.

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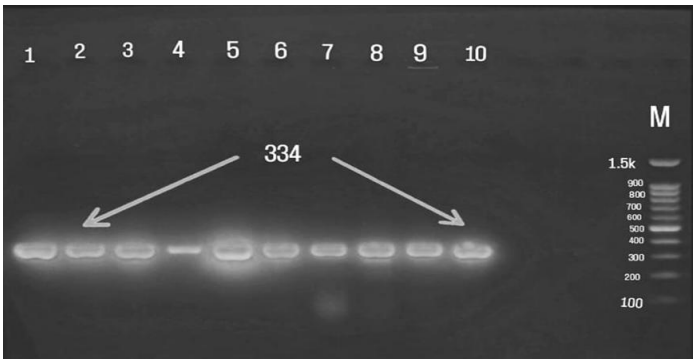

Figure (1): 1% Agarose gel electrophoresis for *Cutibacterium acnes* detection lane 1-10, the band 334bp.

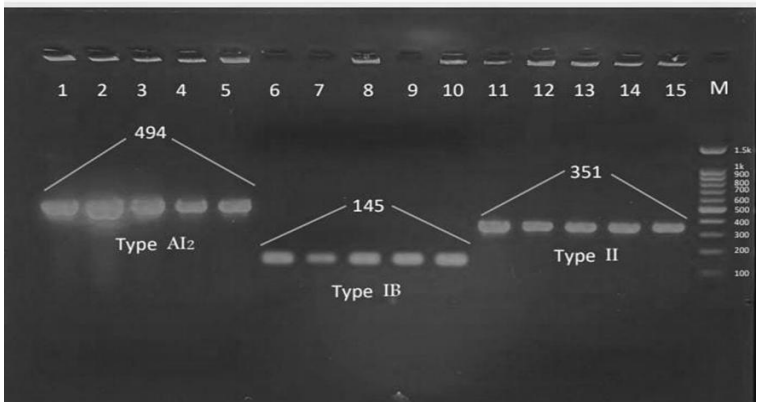

Figure (2): phylotypes distribution among patients and controls lane 1-5 show 1A-2 phylotypes 494bp ,6-10 represent IB phylotypes 145bp, lane 11-15 represent 351bp

Table (1): Distribution of patients and controls samples according to the *C. acnes* phylotypes

Group (No)	IA-2	II	IB	Negative
Patients (70)	37(52.9%)	0(0%)	0(0%)	33(47.1%)
Controls (70)	21(30%)	16(22.9%)	28(40%)	5(7.1 %)
p-value	<0.001	<0.001	<0.001	

Table 2: the biofilms level formation among studied groups

Variables	Patients	Control	P-value
No of samples	15	15	
Mean±SD (µg)	0.24±0.14	0.19±0.18	0.125
Median (Min.-Max.)	0.16 (0.11-0.51)	0.14 (0.07-0.77)	

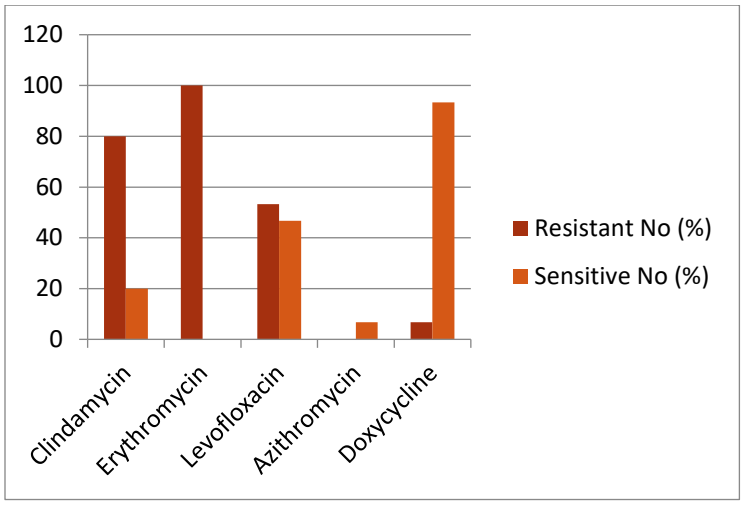

Figure (3): Distribution of resistance and sensitivity to the studied antibiotics

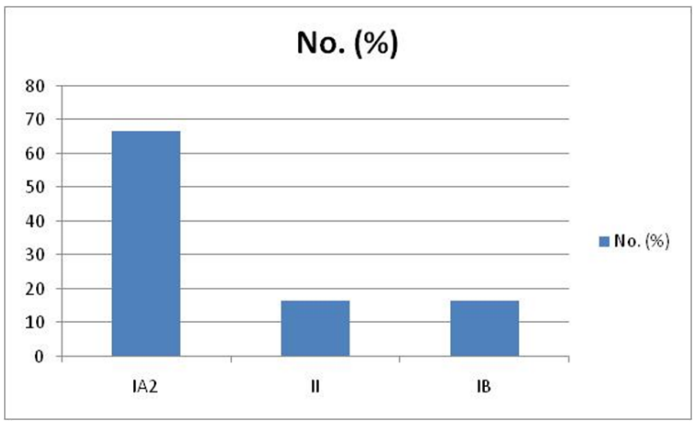

Figure (4): Biofilms level formation in different *C. acnes* phylotypes