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Characterization of biofilm formed by Gram Positive Cocci in clinical samples at Damietta governorate

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ABSTRACT

Biofilm production by Gram-positive Cocci in clinical samples is widespread around the world. It is a potential threat to human health. The objective of this study was to assess the antibiotic resistance of Gram-positive Cocci in clinical samples collected from Damietta governorate both prior to and following the production of biofilm, while also examining the genetic alterations of the ClpC gene. A total of 117 clinical samples were gathered, out of which 100 were found to have pathogenic bacterial strains (i.e., urine, throat swap, vagina swap) from male and female. Fifty strains were identified as Gram-positive cocci (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Strreptococcus pneumoniae*, *Enterococcus faecalis*, and *Micrococcus varians*). All strains examined demonstrated the capacity to generate biofilm. The strains underwent sensitivity testing against a total of nineteen commercially available antibiotics before and after bacterial biofilm formation. Meropenem (MEM-10 mg) was the most commonly used drug for the treatment of bacterial strains and affected all strains, with 98% before biofilm formation and 92% after biofilm formation, while Cefazolin (CZ-30 mg) had the lowest effects (16% before biofilm formation and 6% after biofilm formation). The Multiple Antibiotic Resistance Index (MARI) was calculated for the strains under examination before and after biofilm formation. Furthermore, a quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to analyze the relative gene expression of ClpC in two samples of *Staphylococcus aureus*. ClpC is a crucial factor for stress tolerance, growth recovery, and cell death. The ClpC gene has an effect on bacterial biofilm. It was found that ClpC homologues are highly expressed in *Staphylococcus aureus* during free cells and biofilm formation cells, compared to other cells. This means that they are important in a number of different stressful and environmental situations.

Key Words:

Gram-positive, biofilm formation, clinical samples

1. INTRODUCTION

Antimicrobial drug use exacerbates the natural phenomenon of antimicrobial resistance. Due to the natural phenomenon of antimicrobial resistance and the improper use of available antimicrobial treatments, antibiotic resistance is now a worldwide problem. It is currently known that about 40–80% of bacterial cells on earth are able to form biofilms [1]. In several situations, biofilm growth was harmful. For instance, at medical facilities, biofilms have also been shown to persist on medical device surfaces and on patients' tissues, causing persistent infections [2, 3]. A biofilm is a community of bacteria that is connected to a surface and held together by a self-produced polymer matrix mostly made of polysaccharides, secreted proteins, and extracellular DNAs [4, 5]. Biofilms, so-called because they resemble a thin coating of slime under the microscope, have a characteristic architecture that consists of tower- and mushroom-shaped microcolonies wrapped in a hydrated matrix. Bacterial biofilm formation is often dependent on the interaction of bacterial cells, substrates, and the surrounding medium [6].

The formation of bacterial biofilms is a multi-step process, starting with reversible attachment to surfaces aided by intermolecular forces and hydrophobicity and progressing to extracellular polymeric substance (EPS) production, which enables the cells to permanently adhere to a surface [7, 8]. In particular, there are five main phases in the biofilm formation process: reversible attachment, irreversible attachment, EPS synthesis, biofilm maturation, and dispersal/detachment [8, 9]. However, it is also important to acknowledge that not all biofilms are detrimental to health and that some biofilms are beneficial and necessary for our survival. For example, biofilms formed by the host microbiota can act as a defense mechanism by protecting against foreign pathogens [10, 11]. An example of this is the gut microflora, which protects against food-borne pathogens that could potentially colonize the gut and cause infection [12]. The purpose of this study was to characterize the events involved in biofilm formation by Gram-positive cocci in clinical samples and identify the negative aspects associated with such formation.

2. METHODS AND MATERIALS

2.1 Collection and analysis of samples:

A total of 117 clinical samples were collected from multiple medical laboratories in Damietta Governorate. A total of fifty samples were obtained from the Al-Hayat laboratory, thirty-two samples from the doctor’s laboratory, and thirty-five samples from Tabarak’s laboratory. The collection process included adding samples into plastic bags after aseptic conditions, and the samples were transferred immediately while maintaining sterile conditions. The samples were evaluated in Tabarak’s laboratory during the period from May 2021 to December 2021.

2.2 Isolation methods:

Nutrient agar (NA), obtained before from HIMIDIA, an Indian company, was used for the isolation of the colonies. All the inoculation plates were kept at 35+2 oC for 24 to 48 hours. The colonies were then subjected to microscopic examination following the application of gram stain and tested for biochemicals [14] and morphology [13]. After that, the pure colonies were obtained, and bacterial suspensions were prepared according to the McFarland standard suspension method [15].

2.3 Identification of microorganisms:

2.3.1 Morphologically and microscopically examination:

The utilization of Gram staining is employed as the initial stage in the process of bacterial identification. The Gram-positive cocci morphological shape was determined using Gram stain, obtained from the Egyptian Diagnostic Media Company in Egypt, and observed under a microscope [13].

2.3.2 Biochemical examinations and Gram-Positive microorganism ID:

Various biochemical techniques, such as the catalase test, mannitol fermentation test, glucose fermentation test, bile esculin hydrolysis test, and blood agar hemolysis test, among others, are used to determine the bacterial strain type of Gram-positive cocci. The bacterial strains were identified using Bergey's Manual of Systematic Bacteriology [13].

2.4 Antimicrobial susceptibility testing:

Transferring pure cultures from chosen strains was subjected to an antibiotic sensitivity test in both free cells and biofilm states. The procedure for determining antibiotic susceptibility (Kirby-Bauer technique) [14]. A total of 19 antibiotics were employed, as shown in Table (1). A homogeneous suspension of pure colonies of the strains was produced and gently spun into a tube containing 5 ml of sterile saline. The bacterial suspension was injected onto a Muller Hinton agar plate (from Oxford, United Kingdom) with a sterile swab, covering the whole surface of the agar (at room temperature, the plates were dried). Before being applied to the surface of the media, the antimicrobial discs were held at room temperature. The plates were then incubated at 37°C for 18–24 hours. According to CLSI recommendations, the diameters of the inhibitory zones of all surrounding discs were measured using the disk diffusion method, and the strains were classified as standards for the determination of antibiotic sensitivity (S), intermediate (I), or resistance (R) [17].

Table (1): Antimicrobial agents and the diameter of the inhibition zone, measured in millimeters

NO	Antimicrobial agents (antibiotic discs)	Symbol and concentration	The diameter of the inhibition zone, measured in millimeters		
			S	I	R
1.	Amoxicillin	AX 2 mg	≥ 18	14 -17	≤ 13
2.	Ampicillin/sulbactam	SAM 10/10 mg	≥ 15	12–14	≤ 11
3.	Azithromycin	AZM 15 mg	≥ 18	14 - 17	≤ 13
4.	Cefazolin	CZ - 30 mg	≥ 23	20–22	≤ 19
5.	Cefepime	FEP 30 mg	≥32	27 - 31	≤ 26
6.	Ceftazidime	CAZ 30 mg	≥ 21	18 - 20	≤ 17
7.	Cefuroxime	CXM 30 mg	≥ 23	15-22	≤14
8.	Choloramphenicol	C - 30 mg	≥ 18	13 - 17	≤ 12
9.	Clindamycin-2	DA-2 mcg	≥ 21	15- 20	≤ 14
10.	Doxycycline	DO 30 µg	≥ 14	11 - 13	≤ 10
11.	Erythromycin	E 15 µg	≥23	14 - 22	≤ 13
12.	Gatifloxacin	GAT 5 mg	≥ 17	13 - 16	≤ 12
13.	Linezolide	LNZ 30mg	≥ 20	19	≤18
14.	Meropenem	MEM -10mg	≥ 27	20 - 26	≤ 19
15.	Norfloxacin	NOR 10 µg	≥ 17	13 - 16	≤ 12
16.	Tetracycline	TE 30 mg	≥ 15	12 - 14	≤ 11
17.	Tobramycin	TOB 10 mg	≥ 15	13 - 14	≤ 12
18.	Trimethoprim/Sulphamethoxazole	SXT 1.25/23,75 mg	≥ 16	11- 15	≤10
19.	Vancomycin	VA 30 mg	≥ 12	10 - 11	≤9

Research on the Multiple Antibiotic Resistance Index (MARI):

Blasco's mathematical expression was used to calculate the MARI of a strain. [3].

Multiple Antibiotic Resistance Index = A/B

Identification Where (A) indicated how many antibiotics the bacterial strain was resistant to, and (B) denoted the total number of antibiotics tested against each bacterial strain.

Bacteria from a high-risk source of contamination that uses a lot of antibiotics or growth boosters have a multiple antibiotic resistance (MAR) score greater than 0.2, whereas microorganisms with a MAR index of less than 0.2 are found in environments with lower antibiotic use. A bacterial strain that is completely resistant has a MAR index of 1.0.Detection of multidrug resistance (MDR), according to Krumperman [15].

2.5 QPCR Data Analysis Using Double Delta Ct MiR-132 Gene Expression (ClpC relative gene expression levels for samples):

2.5.1 RNA purification methods:

TRIzols Reagent (15596026, Life Technologies, USA) was utilized in accordance with the manufacturer's technique for total RNA purification[16]. Invitrogen™ TRIzol™ Reagent, in a nutshell, is a ready-to-use reagent that can isolate high-quality total RNA (as well as proteins and DNA) from cell and tissue samples of human, animal, plant, yeast, or bacterial origin in under an hour. TRIzol™ reagent is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components that make it easy to isolate different RNA species with different molecular sizes. The TRIzol™ reagent enables the sequential precipitation of DNA, RNA, and proteins from a single sample [17]. The material is homogenized with TRIzol™ Reagent, chloroform is added, and the homogenate is left to separate into three layers: an interphone, a red lower organic layer, and a clear upper aqueous layer containing RNA and proteins. Isopropanol is used to precipitate RNA from the aqueous layer. Using ethanol, DNA is precipitated from the interphase/organic layer. Isopropanol precipitation is used to remove protein from the phenol-ethanol supernatant. After being cleaned of any contaminants, the precipitated RNA, DNA, or protein is resuspended [18-20] and used the colorimetric approach to determine the RNA yield.

2.5.2 C-DNA synthesis:

In a two-step RT-PCR procedure, 1 µg of total RNA was reverse-transcribed into single-stranded complementary DNA using a random primer hexamer and the QuantiTects Reverse Transcription Kit (Qiagen, USA) as follows [21].

2.5.3 qPCR reaction preparation:

Real-time PCR was carried out using Rotor-Gene Q (Qiagen, USA) with actin serving as the housekeeping gene to assess the mRNAs of the interleukin-1, interleukin-6, and insulin-like growth factor-1 (IGF-1) genes. Using the Maxima SYBR Green/Fluorescein qPCR Master Mix and particular primers that were constructed in accordance with the manufacturer's instructions, C-DNA amplicons were amplified.

2.5.4 Relative expression:

The relative standard curve approach (Pfaffl methodology) and double delta Ct analysis are the two primary methods for analysing qPCR data. In this study, the double delta Ct analysis assumes was used. This technique often works well for studies with a lot of DNA samples and few genes to analyse.

Values for the Double Delta Ct Analysis qPCR for:

- The gene of interest: control and experimental conditions.
- The housekeeping gene: control and experimental conditions.

Steps for Analysis of the Double Delta Ct:

1. The average of the Ct values for the housekeeping gene and the gene being tested in experimental and control conditions was calculated, yielding four values (Gene being Tested Experimental (TE), Gene being Tested Control (TC), Housekeeping Gene Experimental (HE), and Housekeeping Gene Control (HC).
2. Calculations were made to determine the difference between experimental (TE - HE) and control (TC - HC) values. Your Ct values for the experimental (CTE) and control (CTC) conditions.
3. Then the difference between the ΔCT values for the experimental and the control conditions (ΔCTE – ΔCTC) was calculated to arrive at the double delta Ct value (ΔΔCt).

4. Since all calculations were in logarithm base 2, every time there was twice as much DNA, your Ct values decrease by 1 and will not halve. Calculate the value of $2^{-\Delta\Delta Ct}$ to get the expression fold change.

Using 2-ΔΔct relative expression of target gene were estimated as follow[22]:

Control group was applied as calibrator. On the other hand, other dietaries groups were represented as tested groups for both of target and reference genes.

Threshold cycler numbers (Ct) of target gene were normalized to reference genes, for tested and control groups according to following equations:

- Δ Ct (tested) = Ct (target in the teste groups) – Ct (ref. in test group)
- Δ Ct (calibrator) = Ct (target in control) – Ct (ref. in control)

Δ Ct of tested genes were normalized to the Δ Ct of the calibrator as follow:

- ΔΔ Ct = Δ Ct (test) - Δ Ct (calibrator)

Relative gene expression fold change was estimated as follow [23]:

- Fold changes = (2-ΔΔct)

3. RESULTS

3.1 Prevalence of Gram-positive cocci from examined clinical samples:

Out of the 117 samples that underwent microbiological testing, 100 of them were discovered to have harmful bacterial strains. Among these strains, 50 were identified as Gram-positive cocci through biochemical analysis based on Bergey's Manual of Systematic Bacteriology as indicated in Table (2), 9 samples were from throat swabs, and 41 were mid-stream urine samples, as indicated in Table (2), with the *Staphylococcus aureus* strain being the most prevalent, while *Streptococcus pneumoniae* and strains were the least prevalent, as shown in figure (1).

Table (2): Strains and biochemical analysis of bacteria from clinical samples

strains	Number of strains	Gander	Sample Source	Catalase test	Manitol fermintation test	Yellow pigment colonies	Glucose Fermentation	Novobiocin sensetive test	Haemolysis	Optochin sensitivity
Micrococcus varians	3	Female	urine	(+ve)	(-ve)	(+ve)	(+ve)			
	1	Male								
Staphylococcus aureus	18	Female	urine	urine	(+ve)	(+ve)				
	9	Male								
Staphylococcus epidermidis	5	Female	urine	(+ve)	(-ve)	(-ve)		(+ve)		
	4	Male								
Streptococcus pneumoniae	2	Female	Throat swab	(-ve)					Alpha	(+ve)
	2	Male								
Enterococcus faecalis	5	Female	urine	(-ve)					Gamma	
	1	Male								

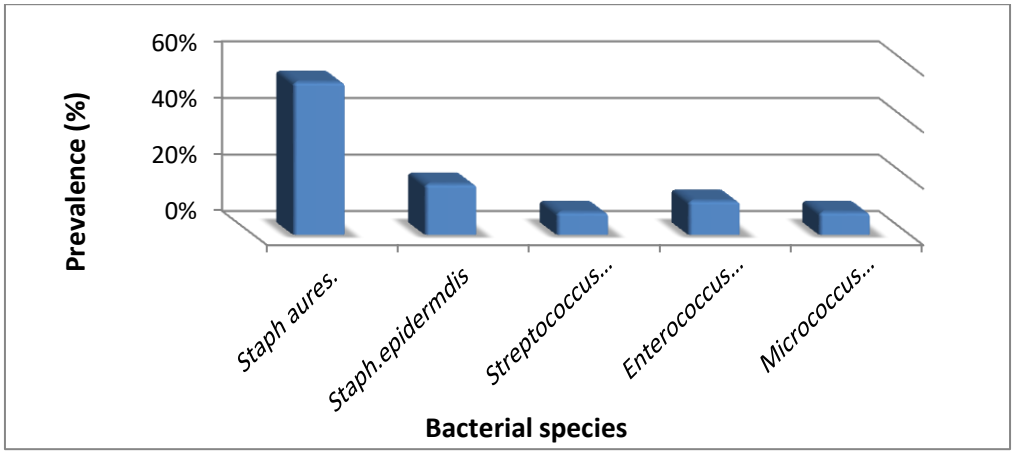

Figure (1): Prevalence of bacterial strains

3.2 Result and percentage of Antibiotics susceptibility testing:

3.2.1 Antibiotics susceptibility testing before biofilm formation: Susceptibility testing of 50 bacterial strains revealed pathogenic Gram-positive strains. Meropenem had a higher sensitivity effect (49 strains), while Cefazolin had a lower sensitivity effect (8 strains). On the other hand, Clindamycin-2 had a higher resistance effect (28 strains), while Meropenem and Doxycycline had a lower resistance effect (only one sample), as shown in figure (2).

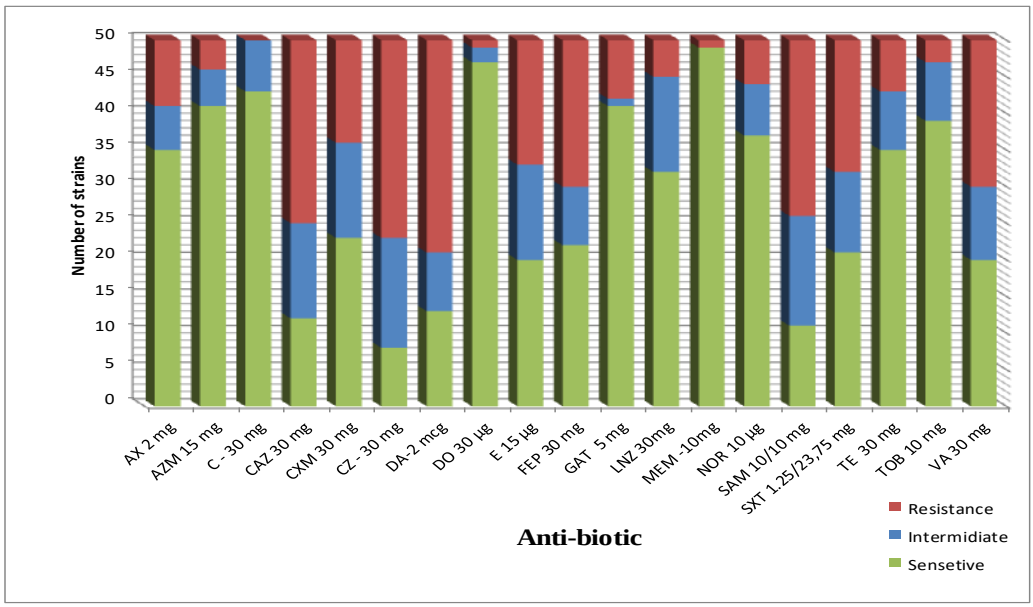

Figure (2): Antibiotic scheme for the tested strains before biofilm formation

Multiple Antibiotic Resistance index before biofilm formation: Several strains exhibited resistance to certain medicines that were tested. The calculated range of the multiple antibiotic resistance indexes was observed to be between 0.0 and 0.53. As seen in Table (3), the analysis of fifty clinical Gram-positive cocci strains indicated that one of the strains exhibited the lowest index value of 0.0, while another strain displayed a higher index value of 0.53, as depicted in figure (3).

Table (3): MAR I value with the number of strains before biofilm formation

strains	MARI= A/B	number of strains
Staphylococcus aureus	0	1
	0.05	2
	0.11	1
	0.16	5
	0.21	2
	0.26	5
	0.32	4
	0.37	6
	0.42	1
Staphylococcus epidermdis	0.11	1
	0.16	2
	0.21	2
	0.26	2
	0.32	1
	0.37	1
Streptococcus pneumoniae	0.11	1
	0.16	1
	0.26	2
Enterococcus faecalis	0.16	1
	0.26	2
	0.32	1
	0.47	1
	0.53	1
Micrococcus varians	0.16	1
	0.26	2
	0.37	1

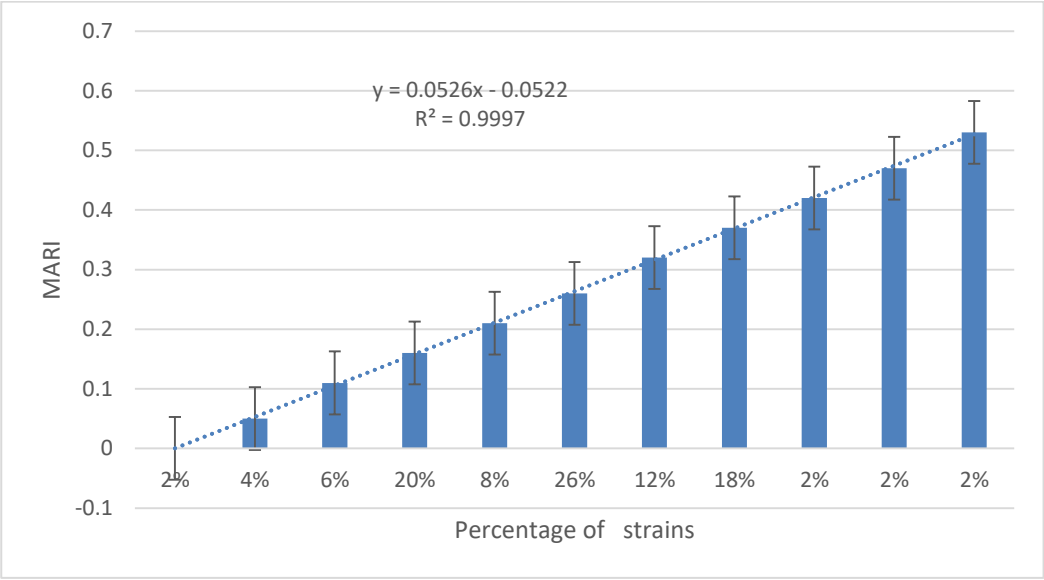

Figure (3): MARI values before biofilm formation

3.2.2 Antibiotics sensitivity test after biofilm formation: Antibiotic sensitivity of the same 50 pathogenic Gram-positive strains after cultivation on Trypticase soy broth (TSB) for 24 h to form biofilm strains revealed Meropenem had a higher sensitivity effect (46 strains), while Cefazolin and Clindamycin-2 had a lower sensitivity effect (3 strains). On the other hand, Cefazolin had a higher resistance effect (41 strains) and Meropenem had a lower resistance effect (one strain), as shown in figure (4).

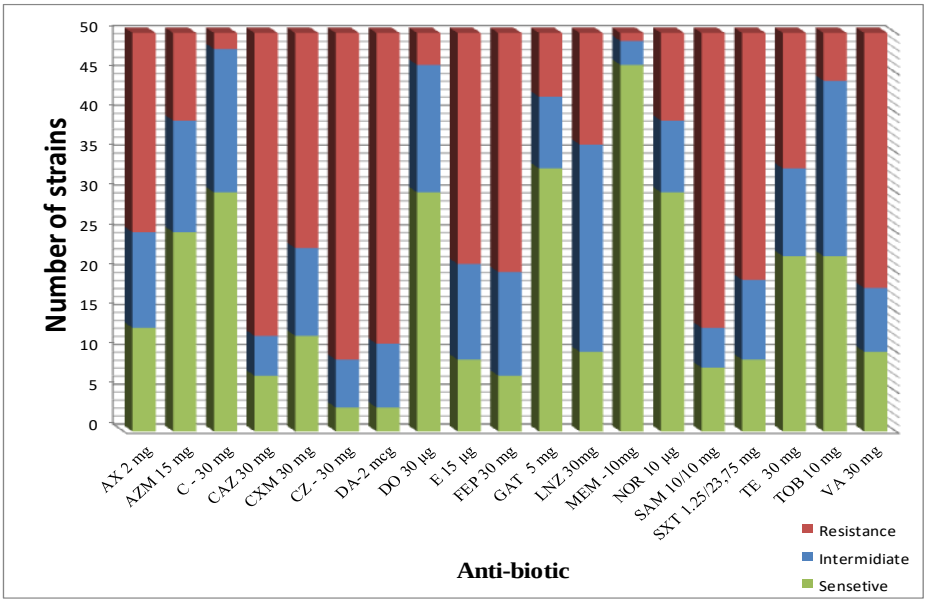

Figure (4): Antibiotic scheme for the tested strains after biofilm formation

Multiple Antibiotic Resistance index after biofilm formation: The calculated range of the multiple antibiotic resistance index after biofilm formation was observed to be between 0.05 and 0.86. As seen in Table (4), the analysis of strains after biofilm formation indicated that one of the strains exhibited the lowest index value of 0.05, while another strain displayed a higher index value of 0.86, as depicted in figure (5).

Table (4): MAR I value with the number of strains after biofilm formation

strains	MARI= A/B	number of strains
Staphylococcus aureus	0.05	1
	0.26	4
	0.32	5
	0.37	3
	0.42	5
	0.47	3
	0.53	2
	0.58	2
	0.63	1
	0.68	1
Staphylococcus epidermdis	0.32	1
	0.42	5
	0.53	2
	0.58	1
Streptococcus pneumoniae	0.26	1
	0.32	1
	0.47	1
	0.58	1
Enterococcus faecalis	0.21	1
	0.32	1
	0.37	1
	0.53	1
	0.58	2
Micrococcus varians	0.37	1
	0.47	2
	0.63	1

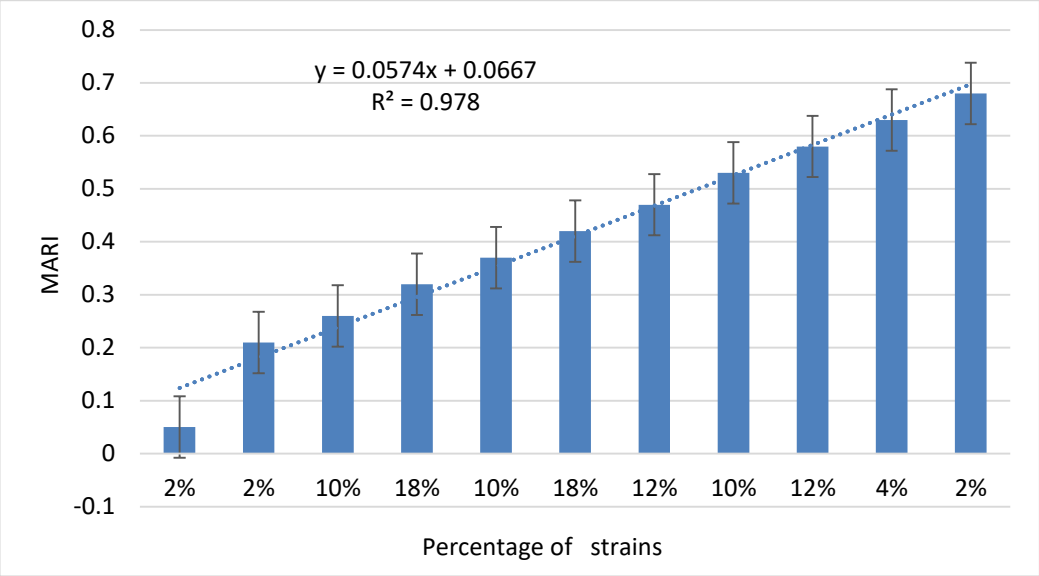

Figure (5): MARI after biofilm formation

3.2.3 Evaluating the impact of antibiotics on different strains, both in their free cell state and after biofilm formation: Upon comparing the antibiotics resistance of strains in both free cells and after biofilm formation, it was shown that the proportion of strains exhibiting resistance to the same antibiotics increased after biofilm formed, as shown in figure (6). The strains exhibited diverse responses to antibiotics, both in free cells and after biofilm formation, as seen in Table (5).

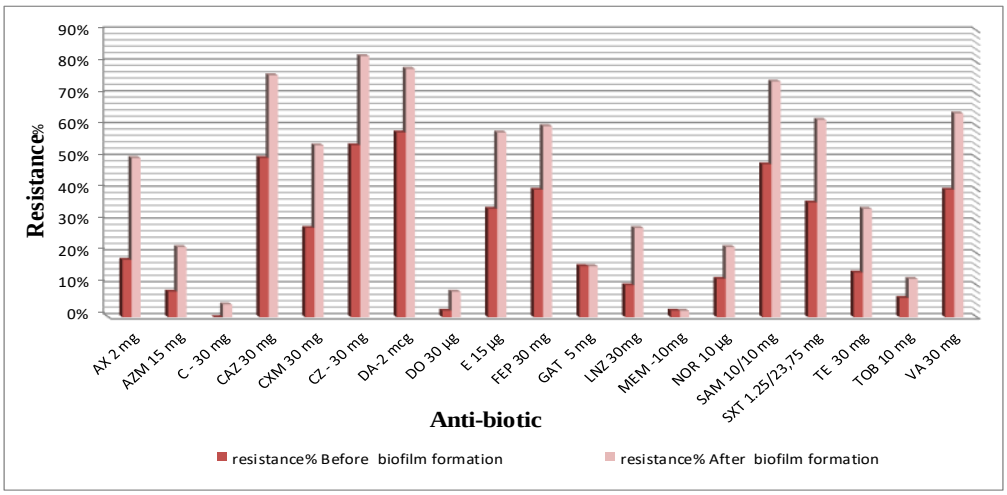

Figure (6): Antibiotic resistance of strains before and after biofilm formation

Table (5): Antibiotic efficacy against strains before and after biofilm formation

Anti-biotic	Strains sensitive%		Strains intermediate %		Strains resistance%	
	Before biofilm formation	After biofilm formation	Before biofilm formation	After biofilm formation	Before biofilm formation	After biofilm formation
AX 2 mg	70%	26%	12%	24%	18%	50%
AZM 15 mg	82%	50%	10%	28%	8%	22%
C - 30 mg	86%	60%	14%	36%	0%	4%
CAZ 30 mg	24%	14%	26%	10%	50%	76%
CXM 30 mg	46%	24%	26%	22%	28%	54%
CZ - 30 mg	16%	6%	30%	12%	54%	82%
DA-2 mcg	26%	6%	16%	16%	58%	78%
DO 30 µg	94%	60%	4%	32%	2%	8%
E 15 µg	40%	18%	26%	24%	34%	58%
FEP 30 mg	44%	14%	16%	26%	40%	60%
GAT 5 mg	82%	66%	2%	18%	16%	16%
LNZ 30mg	64%	20%	26%	52%	10%	28%
MEM -10mg	98%	92%	0%	6%	2%	2%
NOR 10 µg	74%	60%	14%	18%	12%	22%
SAM 10/10 mg	22%	16%	30%	10%	48%	74%
SXT 1.25/23,75 mg	42%	18%	22%	20%	36%	62%
TE 30 mg	70%	44%	16%	22%	14%	34%
TOB 10 mg	78%	44%	16%	44%	6%	12%
VA 30 mg	40%	20%	20%	16%	40%	64%

3.3 The relative gene expression of ClpC in two samples (*Staphylococcus aureus*):

Gene expression in two different types of *Staphylococcus aureus* was assessed. They found that the genetic representation of the gene doubled when biofilms formed compared to when the same strain's cells were free. This finding is presented in Table (6).

Table (6): QPCR Data Analysis Using Double Delta Ct MiR-132 ClpC gene expression

samples	Gene being Tested Experimental C_t Value (TE)	Gene being Tested Control Housekeeping Gene Experimental C_t Value (HE)	Gene being Tested Control C_t Value **(TC)**	Housekeeping Gene Experimental	ΔC_t Experimental Value **($\Delta\Delta$CTE)**	ΔCt values for the control **($\Delta\Delta$CTC)**	**$\Delta\Delta$Ct Value**	expression fold change fold Expression level in the experimental condition the expression as in the control condition **(2^-$\Delta\Delta$Ct)**
Before biofilm production	25.2	24.3	21.2	20.3	0.9	0.9	0	1
After biofilm production	24.5	26.3	21.2	20.3	-1.8	0.9	-2.7	6.5

4. DISCUSSION

Gram-positive cocci are the most frequent pathogenic bacteria. In the past, most research has concentrated on growing free cells in vitro. However, recent studies have revealed that bacterial cells are frequently found in close proximity to surfaces and interfaces, in the form of aggregates known as biofilms [24]. Furthermore, once established, biofilm-originating diseases can be extremely difficult to battle due to the resilience of this growth phase against removal by human immune defense mechanisms and antimicrobial medications. It has been proposed that adhering and biofilm bacteria behave differently and express a different set of genes than their free cells cousins [25]. Importantly, all biofilms can act as reservoirs of infection and lead to human disease [26, 27].

The current investigation conducted a comparative analysis of the impact of antibiotics on Gram-positive cocci both prior to and subsequent to biofilm development. This finding is presented in Table (5). Additionally, the study examined the ClpC gene expression patterns of Gram-positive cocci cultivated in biofilm and free-cell environments. The significance of this study lies in its ability to demonstrate the impact of regular biofilms on bacterial sensitivity to antimicrobial medicines, even in an in vitro setting.

This investigation involved the examination of a total of 50 urine and throat swab samples obtained from both male and female individuals, as depicted in Table (2). The objective of this analysis was to ascertain the prevalence of pathogenic Gram-positive cocci. The sample analysis revealed that the bacterial species were *Staphylococcus aureus* (54%), *Staphylococcus epidermidis* (18%), *Streptococcus pneumoniae* (12%), and Enterococcus faecalis (8%). Based on the findings, it was observed that *Staphylococcus aureus* bacteria exhibited the highest prevalence among the clinical samples obtained, whereas *Enterococcus faecalis* demonstrated the lowest prevalence, as indicated in figure (5).

All bacterial strains in this investigation showed the ability to generate biofilms after growth on TSB culture media, hence validating the dissemination of biofilms and their inherent capability to develop within pathogenic bacterial cells. This finding aligns with the results obtained in prior studies [28, 29]. Gram-positive biofilm development was identified using the Tube method (TM). This was consistent with much earlier research [30, 31].

The results of the antibiotic susceptibility analysis revealed that the free-cell strains obtained in this study demonstrated varying levels of resistance to different antibiotics. Among the strains, 58% were found to be resistant to Clindamycin-2, followed by 54% were resistant to Cefazolin, 50% were resistant to Ceftazidime, 48% were resistant to Ampicillin/sulbactam, 40% were resistant to Cefepime, 40% were resistant to Vancomycin, 36% were resistant to Trimethoprim/Sulphamethoxazole, 34% were resistant to Erythromycin, 28% were resistant to Cefuroxime, 18% were resistant to Amoxicillin, 16% were resistant to Gatifloxacin, 14% were resistant to Tetracycline, 12% were resistant to Norfloxacin, 10% were resistant to Linezolide, 8% were resistant to Azithromycin, 6% were resistant to Tobramycin, 4% were resistant to Doxycycline, 2% were resistant to Meropenem, and 0% were resistant to chloramphenicol, as shown in figure (6). These results are in line with previous data [32], which recorded that Gram-positive cocci were resistant to clindamycin.

Antibiotics were used to test how resistant the same 50 pathogenic Gram-positive strains were after they had formed biofilms. Strains revealed that (82%) were able to resist Cefazolin, followed by (78%) were able to resist Clindamycin-2, (76%) were able to resist Ceftazidime, (74%) were able to resist Ampicillin/sulbactam, (64%) were able to resist Vancomycin, (62%) were able to resist Trimethoprim/Sulphamethoxazole, (60%) were able to resist Cefepime, (58%) were able to resist Erythromycin, (54%) were able to resist Cefuroxime, (50%) were able to resist Amoxicillin, (34%) were able to resist Tetracycline, (28%) were able to resist Linezolide (22%) were able to resist Norfloxacin, (22%) were able to resist Azithromycin, (16%) were able to resist Gatifloxacin, (12%) were able to resist Tobramycin, (8%) were able to resist Doxycycline, (4%) were able to resist Chloramphenicol, and (2%) were able to resist Meropenem. Multidrug resistance refers to the occurrence of resistance to two or more

antibiotics across all drugs that have been evaluated. In order to ascertain the antibiotic resistance pattern of the strains, an analysis was conducted on the characteristics of their Multi-Drug Resistance (MDR).

Previous research has also shown that biofilm-bacterial communities display higher levels of resistance in comparison to immune defense mechanisms and antimicrobial medications. It has been proposed that adhering and biofilm bacteria behave differently and express a different set of genes than their free cell cousins. This resistance is primarily related to the existence of a strong polymeric matrix that obstructs the penetration of antibiotics [33]. The results of this study suggest that bacterial biofilms display significant resistance to commonly used antimicrobial agents. Specifically,it was noted that Clindamycin-2 had the highest degree of resistance among strains prior to the formation of biofilms, with around 58% of strains demonstrating resistance. Nevertheless, subsequent to the establishment of biofilm, the aforementioned proportion experienced a notable escalation, reaching 78%. In the context of biofilms, it was seen that the bacterial cells within the films exhibited significant resistance to routinely employed antibiotics. Notably, Cefazolin has shown the lowest efficacy, with 82% of the strains displaying resistance to this antibiotic. This resistance percentage represents an increase from the pre-biofilm formation stage, where only 54% of the strains exhibited resistance to Cefazolin.Based on the results acquired, it can be inferred that Meropenem exhibits the most pronounced efficacy against strains both prior to and after biofilm development. Before the formation of biofilm, it was shown that a significant proportion of strains, specifically 98%, exhibited susceptibility to Meropenem. Nevertheless, subsequent to the establishment of biofilm, the susceptibility of the strains to Meropenem exhibited a modest reduction to 92%. The findings of our investigation are consistent with previous research that has demonstrated a positive association between biofilms and increased antibiotic resistance. This effect was also seen and recorded in a study conducted by Najar-Peerayeh et al. [34, 35]. Furthermore, it was noted that microorganisms capable of forming biofilms displayed a notably elevated Multiple Antibiotic Resistance Index in relation to microorganisms that do not form biofilms. The findings of our investigation are consistent with prior studies that have demonstrated a positive association between biofilms and increased levels of antibiotic resistance [34, 35].

The present study demonstrates the change in the genomic and transcriptome structure of the ClpC gene before and after biofilm formation and provides valuable insights into its physiological importance. It is widely accepted that protein degradation is a critical mechanism that plays a pivotal role in maintaining optimal cellular function. This mechanism works to prevent the accumulation of misfolded proteins and regulate the stability of key regulatory proteins, thus contributing to overall cellular functioning [36]. However, research into the biological functions of ClpC in *Staphylococcus aureus* is still limited in the scientific literature. However, new research suggests that ClpC plays a critical function in maintaining cellular vitality during the aging process. A study conducted by Chatterjee et al [37]. The results of the current study demonstrated the prevalence of the ClpC gene in two strains of *Staphylococcus aureus* obtained from clinical samples during the free cell state as well as after being induced to form biofilms in a laboratory environment. Given the absence of disparity between the experimental group and the control group, a fold change value of 1 signifies that the gene expression in the free cells from the test condition is equivalent to 100% of the gene expression in the control condition. A fold-change value exceeding 1 signifies that the gene of interest exhibited higher expression levels compared to the control (6.5=650%) following the formation of biofilm by the strains. The estimation of the relative expression of the target gene was conducted using the 2-ΔΔct method [38].

5. CONCLUSION

Biofilm is made up of intricate bacterial colonies living in an exopolysaccharide matrix that clings to external surfaces. In therapeutic settings, biofilm frequently results in nosocomial, long-term infections.

Treating infections caused by biofilm with antibiotics alone is futile because the bacteria in the biofilm have become resistant to them. The high rate of biofilm-related infections brought on by medical devices calls for the use of cutting-edge solutions to handle the challenges that biofilm presents. These findings emphasize the importance of implementing regulations on antibiotic usage and establishing antibiotic stewardship programs in hospitals. To effectively prevent the transmission of drug-resistant bacteria and diseases, it is imperative to acknowledge the importance of biofilms and establish specific diagnostic criteria for biofilm infections. Furthermore, it is imperative to develop drugs that efficiently address these disorders.

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