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RESEARCH ARTICLE

Impact of Sub-Minimal Inhibitory Concentrations of Ciprofloxacin, Amikacin and Gentamicin on the Production of *Staphylococcus aureus* β- Toxin "The Biofilm Ligase"

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Abstract

Beta toxin (ß- toxin) plays an important role in *Staphylococcus aureus* infections by two mechanisms: sphingomyelinase cytotoxic activity and biofilm ligase activity. The biofilm ligase activity is considered as a promising property for biofilm matrix establishment. The present study investigated the influence of sub minimal inhibitory concentrations (sub-MICs) of ciprofloxacin, amikacin and gentamicin on the capacity of *S. aureus* (wound isolates) to produce ß-toxin and form biofilm. After determination of the MIC for each antibiotic, bacteria were grown in sub-MICs of these antibiotics then the ability of beta hemolysin production and biofilm formation were evaluated. The results indicated that 1/8 MICs of all antibiotics have induced ß- toxin and biofilms whereas the 1/2 MICs were inhibitory and a high correlation between the ß- toxin production and biofilm formation has been observed in all isolates under the influence of antibiotics, when the ß- toxin decrease, the biofilm also decrease.

Keywords: *S aureus, β- toxin, biofilm, sub-MIC, ciprofloxacin, amikacin, gentamicin.*

Introduction

Staphylococcus aureus is the most common causative agent of invasive human diseases [1, 2]. Due to the ability of this bacterium to produce number of virulence factors such as: capsule, hemolysins, leucocidin, superantigens and other enzymes, it can overcome the natural host defense mechanisms [3, 4]. Biofilm matrix consists of Exopolysaccharide (EPS), proteins and extracellular DNA (eDNA) [5, 6]. The pathogenic *S. aureus* can aggregate together and embedded in biofilm matrix, as a community of cells within an extra polymeric matrix. With the biofilms, bacteria can adhere to different surfaces and become more resistant to antibiotics [7].

Anyway, there is strong interrelate between these virulence factors, especially, "toxins" and their genes with some threatening human diseases [8]. Among these multiple exotoxins expressed by *S. aureus*, the Beta hemolysin (ß-toxin) a 35 kDa proein toxin encoded by *hlb* gene which referred to as hot-cold toxin because its hemolysis activity is enhanced after incubation at temperature below 10̊ C. The enhancing of the hemolytic activity is due to the phase separation and membrane bilayers breakdown [9, 10].

ß - toxin is also called sphingomyelinase (SMase) because it hydrolyses the plasma membrane sphingomyelin, the more plentiful sphingolipid in eukaryotic cells like erythrocytes, monocytes, lymphocytes, PMNs, keratinocytes [11, 14], leading to loss of cell integrity and subsequently death or may be not directly lysis but cells are left to be attacked by other toxins [15, 17]. On the other hand, regardless to SMase activity, ß-toxin is a member of the DNase I superfamily. It might bind or break DNA and enhance the biofilm formation by cross-linkage in the presence of e DNA. Huseby, et al., referred to this action as biofilm ligase activity [5, 18].

In the routine antibiotic susceptibility assay, when selecting an antibiotic for the treatment of *S. aureus* infections, it is important to consider the effect of certain antibiotic on bacterial toxins production. As the sub inhibitory concentration of antibiotics effect on *S. aureus* virulence factors [19, 22] Lorian [23] had observed a narrow rings of ß hemolysis surrounding the inhibition zones of *S. aureus* that grown on sheep blood agar when tested with cephalothin sensitivity disks.

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This result was attributed at that time to the interaction of the microorganism with the antibiotic. In 1998, Ohlsen and co-workers [24] found that the production of α- toxin by methicillin resistant *S. aureus* in the presence of 10 µg of methicillin was more than the case of methicillin absence. Dumitrescu and co-workers [25] had worked on *S. aureus* releasing panton valantine leucocidin (PVL), whereby they had incubated the bacteria at sub inhibitory concentrations of different antibiotics, they found that oxacillin enhanced the release of PVL, whereas, clindamycin, fusidic acid and linezolid were inhibitory.

These results led to the speculation that the sub inhibitory concentrations or sub minimal inhibitory concentrations (sub MICs) of a given antibiotic may have a crucial role in modulation of the toxin production by certain bacteria. Results of many researchers revealed that protein synthesis inhibitors such as linezolid and clindamycin and other antibiotics at their sub-MICs have advantageous impact by weakening staphylococcal expression of virulence factors such as hemolysins, coagulase, protein A,…etc. [20,21,26,28].

With increasing of bacterial resistance to antibiotics, therapeutic options have reduced and the possibility of antibiotics in modulating the ability of bacteria to express various virulence factors may be of great importance. The purpose of this paper is to describe the impact of sub minimal inhibitory concentrations of a fluoroquinolone and two aminoglycosides on ß -hemolysin production and biofilm formation of wound isolates of *S. aureus* and to find the correlation between the two factors under variable sub inhibitory concentrations of these antibiotics.

Materials and Methods

Ethical Issues

Collection of wound swabs from the patients was approved by Al-Hussein hospital Board director at holey Karbala province and the informed consent was obtained from each patient.

Bacterial Isolates

Out of 100 clinical wound swabs, about 20 isolates of ß –hemolytic *Staphylococus aureus* were isolated from Al-Hussein hospital at holey Karbala province. Bacterial isolates were identified by biochemical and phenotypic tests according to Bergey's manual [29] and methods used by Macfaddin [30] and Collee *et al* [31]. The identification of bacteria was confirmed by API Staph. System. All of 0ur isolates were sensitive to Amikacin (Oxoid, 30 mg/disk), Ciprofloxacin (Oxoid, 5 mg/disk) and Gentamicin (Oxoid, 1mg/disk) according to antibiotic sensitivity test (CLSI, 2012). The studied isolates were able to form biofilm.

Determination of MICs

The MICs for ciprofloxacin, Amikacin and Gentamicin (Sigma) were determined by micro dilution method [32, 34]. After which the sub minimal inhibitory concentrations were selected.

Hemolysin Production

Beta hemolysin activity was measured by three protocols:

1: The classical qualitative assay: Clear zones were seen around the bacterial colonies that were grown on sheep blood agar after incubation for 24hrs at 37° C and then for 16 hrs at 4° C., 2: Semi-Quantitative assay: Bacterial filtered culture supernatant fluid was put in wells through sheep blood agar. The zones of clear hemolysis around the wells were measured by mm. and 3: Quantitative assay: This test was aided by the standard curve of RBCs breakdown by NaCl serial concentrations. According to [35, 36, 37] with little modification in wave length. The experimentally optimization of ß-hemolysin production from *S. aureus* isolates was previously described [38].

Biofilm Formation

Biofilm formation by *S. aureus* isolates was detected according to Mathur *et al* [39]. By staining bacterial growth films with crystal violet as well as the micro titer plate method which was performed according to the protocol that described by Hemati *et al* [40].As a quantitative assay.

Total Protein Concentration

Total protein concentration of bacterial culture supernatant was estimated according to Bradford *et al* [41].

Effect of Sub Minimal Inhibitory Concentrations on Hemolysin Production

The MIC, 1/2 MIC, 1/4 MIC and 1/8 MIC of ciprofloxacin, amikacin and gentamicin were

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prepared in the chemically defined medium. The flasks were inoculated with *S. aureus* isolates and then incubated in optimum conditions. The hemolysis activity of the supernatant was estimated as mentioned above.

Effect of Sub Minimal Inhibitory Concentrations on Biofilm Formation

The sub-MICs of the antibiotics were applied according to the method that described by [34, 40].For each concentration, negative control (antibiotic free medium) were applied and all experiments were performed in triplicate.

Statistical Analysis

The experiments were designed as CRD and analyzed by ANOVA test whereas the correlation was determined by Mini Tab program.

Results and Discussion

Beta hemolysin (ß-toxin) and biofilm formation are important virulence factors of pathogenic *S. aureus*.

The effect of the antimicrobial agents on these factors is an important consideration.

At low concentrations, antibiotics are able to modulate the bacterial expression of virulence factors. In present study the antibiotics used were a quinolone (ciprofloxacin) and two aminoglycosides: (amikacin and gentamicin). Amikacin (MIC 32-64 µg/ml), ciprofloxacin (MIC 16-32 µg/ml) and gentamicin (MIC 4-8 µg/ml) showed different impacts on beta hemolysin production and biofilm formation by S. *aureus* at their sub- MICs.

Figure (1) showed that (1/8 MICs) of the three antibiotics were able to induce the production of hemolysin, the hemolytic activities (the mean hemolysis percentage) of the bacterial filtrated culture supernatants were (amikacin: 78.508%, ciprofloxacin: 69.456% gentamicin: 70.432%). At (1/4 MICs) of ciprofloxacin and amikacin the hemolysin production were induced. In contrast (1/2 MICs) of all antibiotics were significantly inhibitory ($p \leq 0.05$).

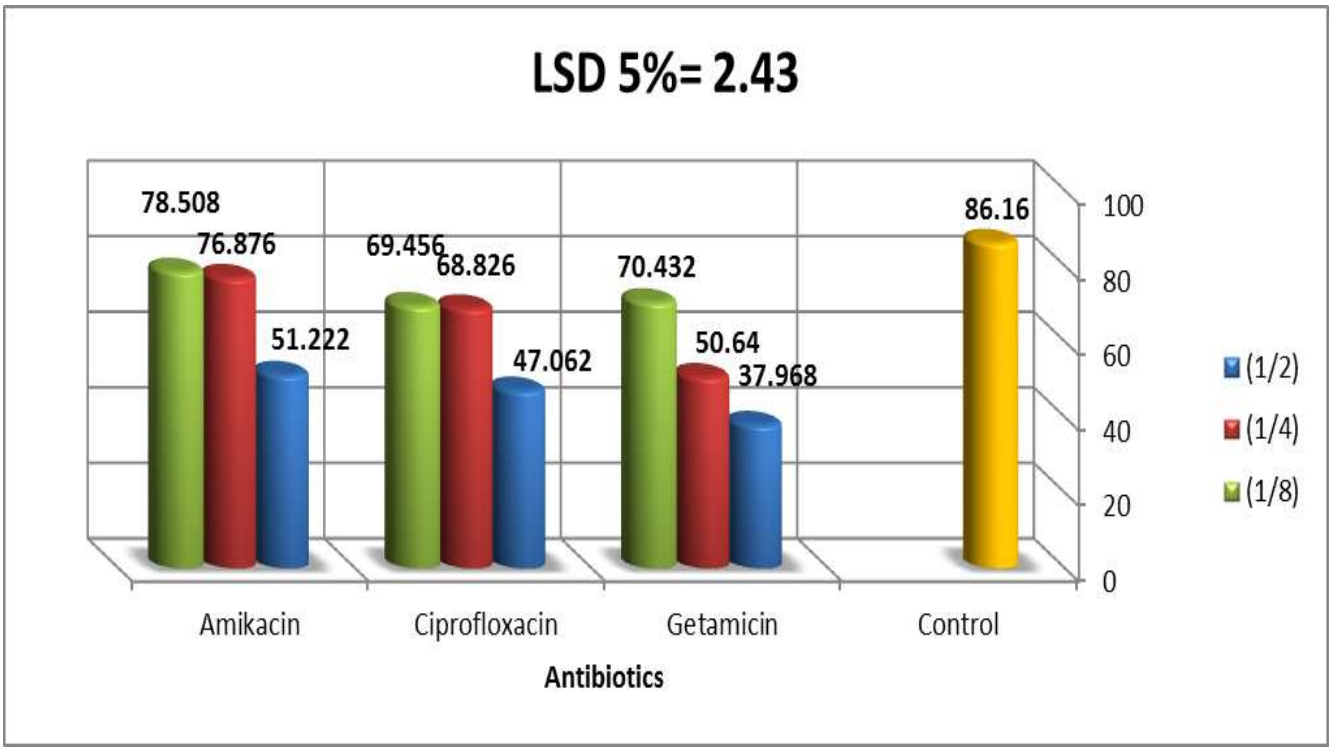

Figure 1: Mean hemolysis of *S aureus* isolates in the presence of sub-minimal inhibitory concentrations of antibiotics

The effects of sub minimal inhibitory concentrations of several antibiotics on bacterial toxins were exhibited by [20, 42, 45]. These were illustrated by their impacts on protein synthesis, for example ciprofloxacin inhibits the DNA gyrase while amikacin and gentamicin as aminoglycosides interfere with ribosomal subunits [46, 50]. In this study, the effect of antibiotics on protein synthesis was indicated by the estimation of total protein concentration in bacterial filtered supernatant for each treatment. Additionally, results of quantitative assay of hemolysin were supported in Figure (2) that illustrated the diameters of hemolytic zones on sheep blood agar (with or without antibiotics) after

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hot-cold incubation. According to Hemati *et al* [40].The biofilm formation of *S. aureus* isolates was classified to (no biofilm, weak, moderate and strong). About (85%) of them were strong biofilm formers. When the bacterial isolates were exposed to the (sub-MICs) of antibiotics, their biofilms were clearly affected (Table 1). There was cut clear decrease in biofilm formation by increasing the antibiotic concentration

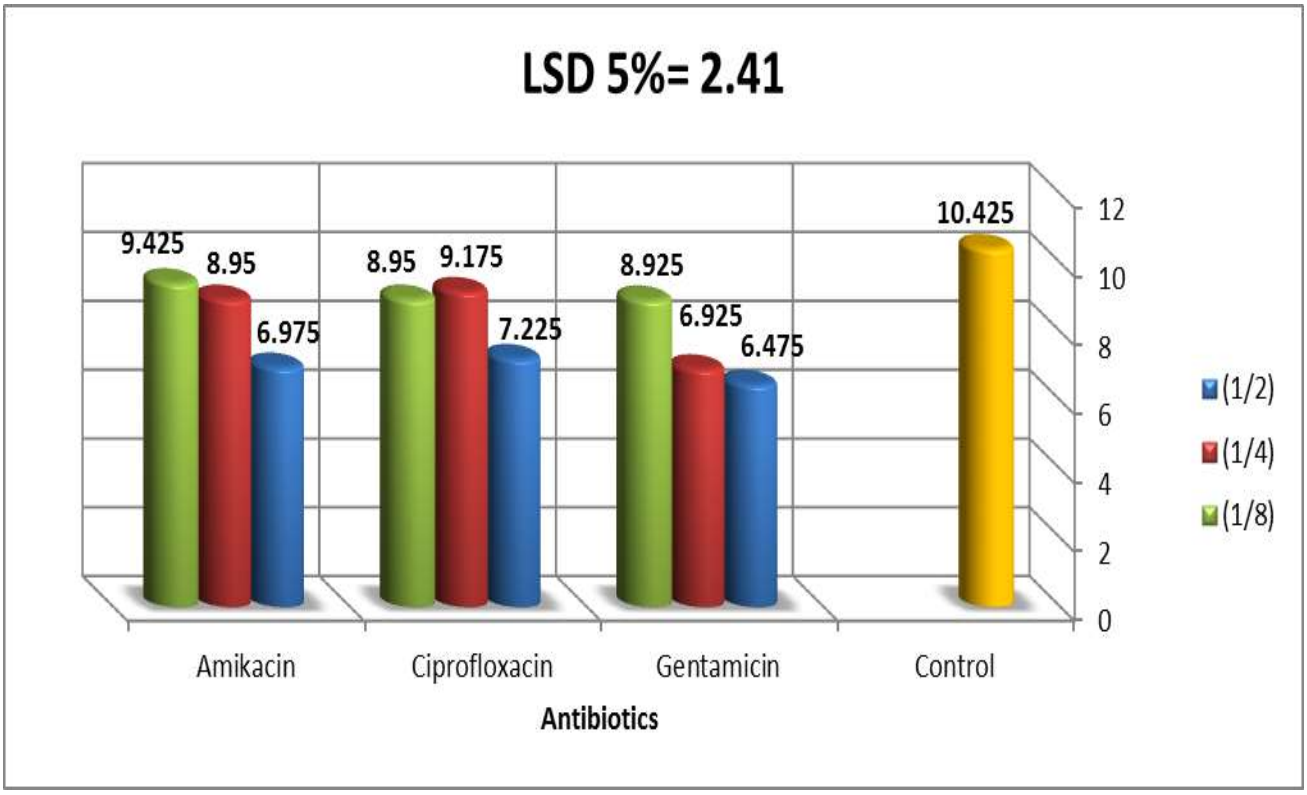

Figure 2: Mean hemolysis diameters of *S aureus* isolates in the presence of sub-minimal inhibitory concentrations of antibiotics

Hemati *et al* [34]. Found that among several antibiotics, ciprofloxacin induced the biofilm formation of *Pseudomonas aeruginosa* and biofilm formation decreased by increasing antibiotics concentration. The results might be attributed to the antimicrobials in use, the bacterial strain and the matrix composition. All of these factors are associated with the response of bacteria to the sub minimal inhibitory concentrations of an antibiotic; inter microbial signaling and microenvironment system [34, 51]. Haddadin *et al* [22].Examined the sub minimal inhibitory concentrations of several antibiotics on some virulence factors of *S aureus* biofilms, they attributed the inhibitory effects of some antibiotics to their ability to inhibit the synthesis of some proteins important for growth and biofilm formation.

Table 1: Percentage of isolates according to biofilm formation subjected to sub- MICs of antibiotics measured by micro titer plate method

Antibiotics / Concentration	Ciprofloxacin	Amikacin	Gentamicin
Zero	++ (85%)	++ (85%)	++ (85%)
1/2 MIC	+^{w} (75%)	+ (70%)	+^{w} (80%)
1/4 MIC	+ (80%)	++ (60%)	+ (75%)*
1/8 MIC	+ (80%)	++ (60%)	+ (80%)**

+^{w}, +, ++ were weak, moderate and strong biofilm formation respectively
*10% of isolates were strong biofilm formers
** 15 of isolates were strong biofilm formers

Significantly, our data showed a high correlation between the two factors: hemolysin production and biofilm formation (r = 0.84-0.86), i.e., indicated that when hemolysin increases, biofilm also tends to increase (Figure 3).

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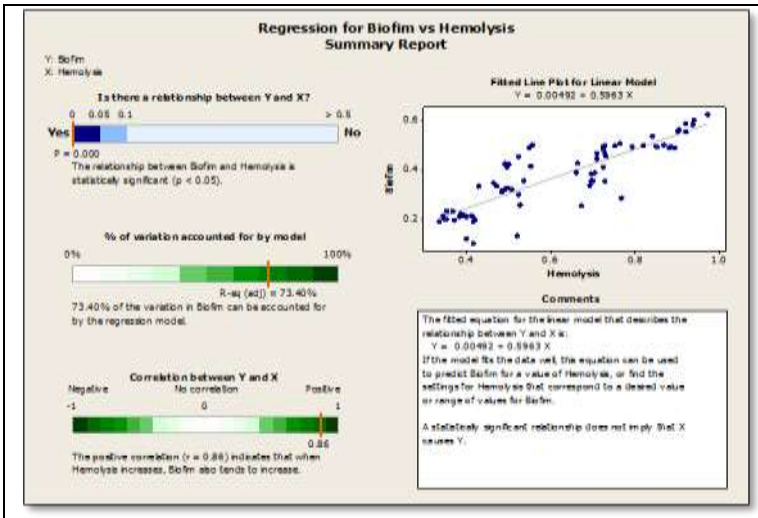
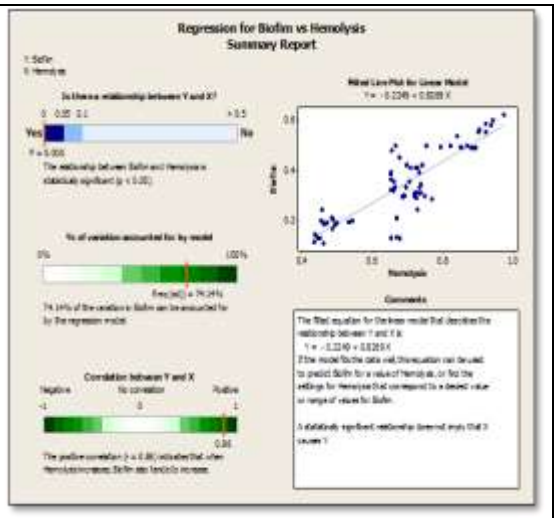
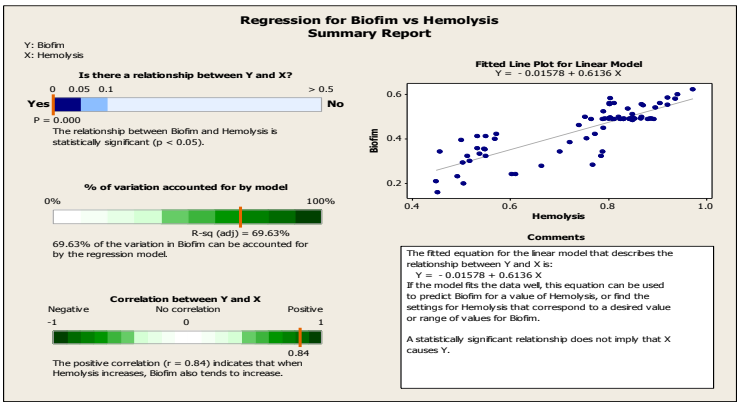


Gentamicin



Ciprofloxacin



Amikacin

Figure 3: The correlation between hemolysin production and biofilm formation of S. aureus isolates in the presence of sub-minimal inhibitory concentrations of antibiotics

The results of Caiazza and O′ Toole [52] showed that an antibiotic such as cephalexin has an indirect effect on biofilm formation via induction of staphylococcal α- toxin (an important compound at second phase of biofilm formation). To our knowledges, the contribution of hemolysin in the biofilm formation is rather elucidated. Huseby *et al* [18]. Worked on the role of ß- toxin encoding gene (*hlb*) in the biofilm formation *in vitro*, they found that *S. aureus* COL (*hlb*$^{+}$) strain formed thicker and faster biofilm than *S. aureus* COL (*hlb*$^{-}$) strain. They submitted a suggestion that when produced, ß- toxin may be important in endocarditis, due to its contribution in biofilm formation by biofilm ligase activity. The descriptive study of Herrera *et al* [53].

Was carried on the active site of DNA biofilm ligase and they submit a suggestion that according to this activity ß- toxin does not have any impact on lethality but induce the increase of vegetative size. ß- Toxin is of multiple roles, SMase activity (erythrocyte lysing and killing the proliferating lymphocytes) and biofilm matrix establishment. It can bind to carbohydrates or proteins and its large homology to DNase I family makes it participate in biofilm formation (54). By its DNA biofilm ligase activity, ß- toxin able to cross-link itself in the presence of eDNA and the formed covalent oligomers precipitate like a biofilm [18, 53]. Additionally, *S. aureus* may use the biofilm ligase activity to protect eDNA from enzymatic degradation [53].

But this does not mean that *S. aureus* cannot form biofilms except with the presence of hemolysin, many strains lacking ß- toxin can form complex biofilm in the presence of extracellular DNA, EPS and proteins. Thus, due to this correlation, our finding confirmed that antibiotics that affect the ß- toxin production also have impact on biofilm formation. Lastly, ß- hemolysin and its role in the establishment of bacterial biofilm opened many doors to new targeting therapeutic

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treatments for bacterial infections, especially with their significant roles in the pathogenesis of bacteria.

Conclusion

Overall, the results showed a clear impact of the sub-MICs of antibiotics on the production of beta toxin and biofilm formation in all *S. aureus* isolates, ciprofloxacin, amikacin and gentamicin were able to modulate bacterial expression of these important virulence factors. The data collectively indicated the high correlation between the beta toxin production and biofilm formation in all isolates under the influence of antibiotics.

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