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Full Length Research Paper

In vitro activities of three kinds of antibiotics against Staphylococcal biofilm and planktonic cultures

T. El-Banna[1], A. Abd El-Aziz[1], A. Abo-Kamar[1], A. Ghazal[2] and R. AboZahra[3]*

[1]Microbiology Department, Faculty of Pharmacy, Tanta University, Tanta, Egypt.
[2]Microbiology Department, Medical Research Institute, Alexandria University, Alexandria, Egypt.
[3]Microbiology Department, Faculty of Pharmacy, Pharos University, Alexandria, Egypt.

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The aim of the present study is to determine the activities of three kinds of antibiotics against Staphylococcal biofilm and planktonic cultures *in vitro*, and to indicate the enhancement of biofilm formation in response to stress factors such as glucose and sub-inhibitory concentrations of antibiotics by using scanning electron microscope. Biofilm forming staphylococci were identified by using the modified microtiter plate method. And the effect of different concentrations of several antibiotics (including ciprofloxacin, gentamycin and amoxicillin-clavulanic acid) on eight isolates was determined. The result showed that out of 86 Staphylococcal isolates, eight strains were found to be strong biofilm forming. Sub-MIC of the antimicrobial agents used increased the biofilm formation in some isolates. However, the preformed biofilm was very difficult to remove with most isolates even with multiples of the MIC. The biofilm MBC reached 46 times the planktonic MBC in some isolates. Scanning electron micrographs of *staphylococcus aureus* isolate (45S) were made in order to confirm the enhanced biofilm formation in the presence of glucose and sub-MIC of ciprofloxacin and it was found that the slime layer production increases in the presence of glucose and low concentration of ciprofloxacin.

Key words: Staphylococcal biofilms, scanning electron micrograph, antimicrobial sub-MIC activity.

INTRODUCTION

Staphylococci are more frequently isolated with biomaterials and tissues in chronic bacterial infections. In tissues removed from patients with recurrent staphylococcal infections, the cells are frequently organized in confluent colonies with a biofilm-like appearance. The pathogenesis of staphylococcal infection begins with primary bacterial adhesion and colonization of the host tissues (Morikawa et al., 2005). Once a biofilm has been established, it is a major concern for clinicians in the treatment of infectious disease because of the resistance to a wide range of antibiotics (Dunne et al., 1993; Amorena et al., 1999). Therefore, a better understanding of bacterial biofilms is needed, and this may ultimately result in development of novel therapeutics for the prevention and treatment of wound infections (Davis et al., 2006). One of the factors that affect the pathogenesis of staphylococci is hypothesized to depend on the ability of the infecting organism to adhere to and form biofilm (Frank et al., 2004). The mechanism by which staphylococcus species form biofilm is being increasingly understood. Biofilm formation occurs upon initial rapid attachment of staphylococci to the surface, followed by multilayered cellular proliferation and intercellular adhesion in an extracellular polysaccharide matrix excreted by the bacteria (Gotz, 2002). Cell adhesion between staphylococci is mediated by polysaccharide intercellular adhesin (PIA), a linear homopolymer of ß-1,6-linked *N*-acetylglucosamine residues (Arciola et al., 2001). Scanning electron micrographs from early studies showed that *Staphylococcus epidermidis* forms multiple cell layers on the polymer surface; the cells in these layers are enveloped and protected by an amorphous

*Corresponding author. E-mail: rania_abozahra@yahoo.com. Tel: +2-0146969134. Fax: +2 033877149.

slimy material (Christensen et al., 1987). Several environmental factors, such as glucose, osmolarity, ethanol, temperature, and anaerobiosis, have been reported to affect biofilm formation. Some of these factors such as glucose and ethanol are shown to induce biofilm formation (Colon et al., 2002; Lim et al., 2004).

Biofilm infections are difficult to treat due to their inherent antibiotic resistance. Once staphylococcal biofilm has formed on damaged tissue, it is difficult to disrupt. Most antimicrobial therapies for biofilms have largely proven unsuccessful (Wu et al., 2003). The mechanism of biofilm-associated antibiotic resistance is uncertain and likely multifactorial. A number of factors have been postulated, including binding of antibiotic to the slime, poor penetration of antibiotic into the biofilm, slow growth rate of organisms in the biofilm, high bacterial density, and changes in gene expression in biofilm bacteria. Bacteria released from biofilms retain susceptibility to antibiotics characteristic of free-growing bacteria rather than biofilms, implying that the mechanism of resistance is not genetic change (Saginur et al., 2006). The objective of this study was to determine the effect of different antibiotics on biofilm removal and on the prevention of biofilm formation, also to compare the planktonic and biofilm MBC of staphylococcal isolates retrieved from diabetic ulcers to these antibiotics. Induction of biofilm formation in the presence of stress factors such as low concentrations of antibiotics or glucose was also done and confirmed by scanning electron micrographs.

MATERIALS AND METHODS

Bacterial strains

In this study, 140 clinical swab form foot ulcers of diabetic inpatients in the university hospital (Alexandria, Egypt) were collected. Out of 197 isolate, 86 isolates were identified as Gram positive bacteria (Staphylococi). Identification of the staphylococcal strains was done by Gram staining, catalase, coagulase tests, and cultivation on mannitol salt agar, further confirmation was carried out by using the API Staph system. Out of the 86 staphylococci 62 (70.4%) were *Staphylococcus aureus* and 25 (29.6%) were Coagulase negative staphylococci (CONS).

Quantification of biofilm formation

This was done by using the modified microtiter plate method (Stepanovic et al., 2000), where the strains were grown in TSB supplemented with 2.5% glucose (Christensen et al., 1985). All strains were categorized as, non, weakly, moderately, or strongly adherent, based on the ODs of bacterial films as described by Stepanovic et al. (2000).

Antimicrobial agents used

Gentamycin (CN) (Alexandria Co., Egypt), Ciprofloxacin (CIP) (Amriya, Egypt), and Amoxicillin-Clavulanic acid (AMC) (GlaxoWellcome) were used.

MIC and MBC determination

Susceptibility testing to each drug was performed on planktonic cultures using the two-fold dilution method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2003). MICs were performed in 96-well microplates (Greiner, Wemmel, Belgium) and results were recorded after incubation at 35°C for 18 h. For MBC determination, 10 µl aliquots were removed from the wells after incubation and spread onto Mueller–Hinton agar in Petri dishes and incubated overnight at 35°C (Tre-Hardy et al., 2008).

Effect of sub-MICs of antimicrobial agents on staphylococcal biofilm formation

Each drug was tested at one-half, one-fourth, and one-eighth the MIC to study its effect on staphylococcal biofilm formation. After overnight incubation, quantitation of biofilms was performed as described for the biofilm assay. Positive and negative-control wells were used (Bonaventura et al., 2004).

Effect of antimicrobial drugs on pre-formed biofilms

Biofilms were allowed to form as described above. The content of the wells was then aspirated and the wells were washed and fresh TSB containing two fold serial dilution of the antimicrobial agent was added to the wells as described above. The plate was then incubated at 35ºC for 24 h. The content of the wells was aspirated then washed and stained by using crystal violet as described above. The optical densities were determined after elution. Biofilm persistence in the presence of antimicrobial agents was calculated using the following formula (Tre-Hardy et al., 2008).

Percentage of biofilm persistence = $[(A_{590x} - A_{590\text{negative control}})/ (A_{590\text{ positive control}} - A_{590\text{negative control}})] \times 100$, where, ×corresponds to the antimicrobial agent used.

Scanning electron microscopy (SEM)

Overnight cultures of *S. aureus* (45S) were made in polystyrene Petri dishes to allow SEM observation. The bacteria was subcultured on TSB, TSB supplemented with 2.5% glucose and TSB supplemented with ¼ MIC of ciprofloxacin. The content of each plate was removed and the plates were washed three times with sterile physiological saline to remove the planktonic cells, the adherent cells were then fixed in 2.5% (vol/vol) glutaraldehyde in PBS (PH 7.2) for 8 h, rinsed with PBS, and then dehydrated through an ethanol series. Samples were dried and gold coated. SEM examinations were made on a JSM-6360 SEM (JEOL, Japan) (Gad et al., 2009).

RESULTS

Quantification of biofilm formation

The experiment performed was carried out to measure the degree of adherence and subsequent biofilm formation of all staphylococcal isolates. Out of the 86 staphylococcal isolates 4 (4.65%) were non-adherent, 35 (40.7%) were weakly adherent, 39 (45.35%) were moderately adherent and 8(9.3%) were strongly adherent. The eight strong biofilm forming strains (16, 17,

Table 1. MIC and MBC values for the tested antibiotics against the 8 staphylococcal isolates. The equivalent MIC breakpoints for *staphylococcus* spp. (CSLI 2005) were R: ≥ 4, S: ≤ 1 for ciprofloxacin; R: ≥8, S: ≤ 4 for gentamycin and R: ≥ 8/4, S: 4/2 for amoxicillin-clavulanic acid.

Strain	Ciprofloxacin		Gentamycin		Amoxicillin-clavulanic acid	
	MIC (µg/ml)	MBC(µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC(µg/ml)
16S	0.5	1	2	4	8	32
25S	0.5	1	4	8	8	32
45S	128	512	128	256	64	256
18S	32	128	128	256	32	32
17S	8	8	64	128	8	8
78S	0.5	0.5	0.25	0.5	0.25	0.25
108S	0.125	0.5	1	2	0.25	1
90S	8	16	128	128	8	32

18, 25, 45, 78, 90 and 108S) were selected for further investigations. Three of which (16, 25 and 45S) were *S. aureus*, while 17, 18, 78, 90 and 108S were CONS.

MIC and MBC determination

MICs and MBCs recorded for the tested antimicrobial agents against the eight staphylococcal isolates are shown in Table 1. The biofilm MBCs were found to be much higher than the planktonic MBCs. These results are shown in Figure 1. In case of ciprofloxacin, the biofilm MBC was 2 to 512 times higher than the planktonic MBC. Also, the biofilm MBC for gentamycin was 2 to 256 times higher than that of the planktonic culture. Whereas, the biofilm MBC in case of amoxicillin-clavulanic acid was 4 to 64 times higher than that of the planktonic culture. Variable behavior in biofilm formation for the 8 staphylococcal isolates was observed in biofilm and planktonic cultures; no fixed increase was noticed for all strains with the different antibiotics used.

Effect of sub-MIC on biofilm formation

Relative efficacies of antibiotic treatments for prevention of biofilm formation on polystyrene plates are shown in Figure 2. In the presence of sub-MICs of the tested antibiotics, variable behavior in biofilm formation for the 8 staphylococcal isolates was observed (Figure 2). It was noticed that one-half the MIC causes marked reduction in biofilm formation in six (75%) of the tested isolates (16, 17, 78, 90, 108 and 18S), however, this reduction decreased at lower concentrations (¼, ⅛ MIC) of the antibiotics in most of the cases. Two of the tested isolates (25 and 45S) showed enhancement in biofilm formation in the presence of sub-MICs of all of the tested antibiotics, this enhancement was also noticed at lower concentrations (¼, ⅛ MIC) of the antibiotics till the biofilm reached 3.5 times the control biofilm (in absence of antibiotic) at one-fourth the MIC.

Detachment of established staphylococcal biofilms after antibiotics exposure

After finding that sub-MICs of the antibiotics enhanced the biofilm formation in some of the staphylococcal isolates in biofilm assay, the effect of MIC and its multiples on the pre-formed biofilm was tested in order to determine the biofilm removing capacity of the studied antibiotics. It was found that ciprofloxacin showed relatively the best results in biofilm detachment as it removed from 40 to 80% of an already formed biofilm in five (62.5%) of tested isolates. However, amoxicillin-clavulanic acid showed the lowest ability for biofilm detachment. On the other hand, two strains (25 and 45S) showed increase in the biofilm formation by the addition of antibiotics, these two strains were those that showed increase in biofilm formation by addition of sub-MIC of the tested antibiotics (Figure 3).

Scanning electron microscopy (SEM)

Scanning electron micrographs shows the staphylococcal biofilm in different growth conditions and they confirm the enhancement in biofilm formation at elevated glucose concentrations as in Figure 4b. Also, in the presence of sub-MIC of ciprofloxacin the polysaccharide matrix increased greatly which confirm the increase in biofilm formation in the microtiter plate assay (Figure 4).

DISCUSSION

Biofilms, products of bacterial adherence, are structured communities of bacterial cells enclosed in a self-produced exopolysaccharide matrix and adherent to an inert or living surface. Establishment of a biofilm is the prelude to the development of various chronic, intractable infections, such as biomaterial-associated infections and pulmonary infection in patients with cystic fibrosis (Bonaventura et al., 2004). Despite various efforts, treatment of an infection after biofilm is established is

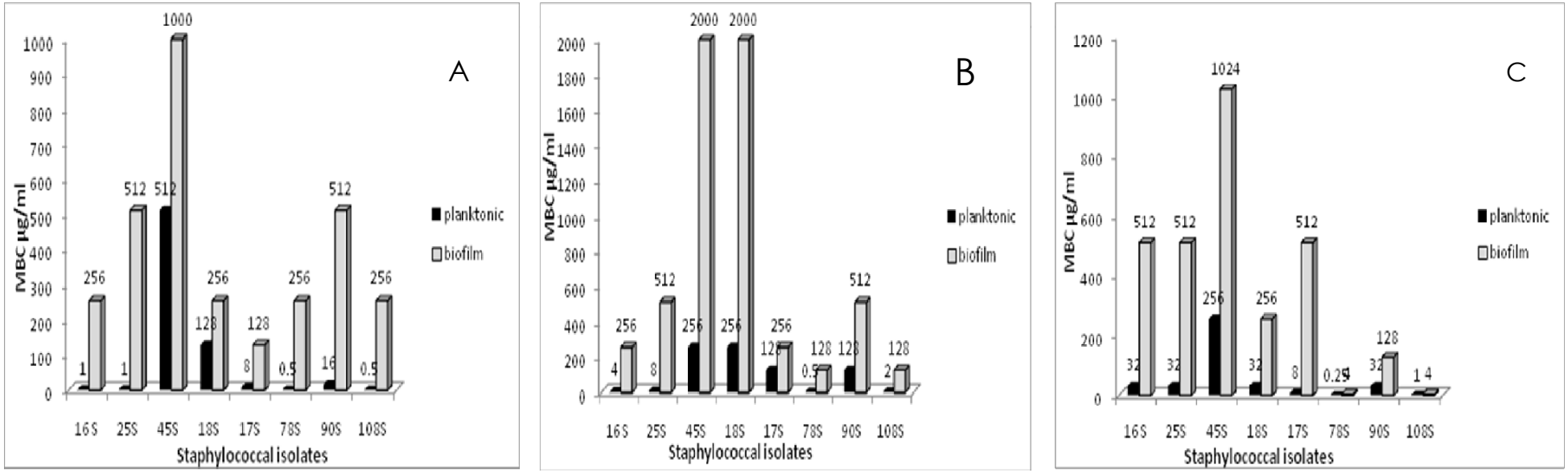

Figure 1. Comparison between planktonic and biofilm MBC of ciprofloxacin (a), gentamycin (b), and amoxicillin-clavulanic acid (c) for the 8 staphylococcal isolates.

frequently futile because of the reduced susceptibility of biofilm to antibiotics. At least three mechanisms have been proposed to account for recalcitrance of biofilms to antimicrobial agents (Mah and O'Toole, 2001): (i) failure of the antimicrobial to penetrate the biofilm, (ii) slow growth and the stress response, and (iii) induction of a biofilm phenotype (Bonaventura et al., 2004). Microtiter plate systems for quantifying adherence and biofilm formation were used in this study, these techniques have been investigated with many different organisms and stains (Christensen et al., 1985) and they have been widely used because they are simple, reproducible and quantitative methods. However, the staining measurements reflect on the total amount of biofilm (sessile cells plus exopolysaccharide matrix) but do not give any information about its viability (Griffis et al., 2009). Thus, in this study, cell viability was also measured by determination of biofilm MBC, which was found to be very high (512 × planktonic MBC) in case of the isolates 25, 78 and 108S when tested with ciprofloxacin. However, in other cases the difference between biofilm and planktonic MBC is much less (2 × planktonic MBC) in case of the isolate 18S when tested with ciprofloxacin. These results was also noticed by other investigators who found that 4 × MBC of the antibiotics used is not sufficient for killing *S. aureus* in biofilm (Amorena et al., 1999). Also *Pseudomonas aeruginosa* in biofilm showed a 4-fold greater resistance against ciprofloxacin and gentamycin compared with free-living forms (Agarwal et al., 2005).

In the present study, the effects of several antibiotics on Staphylococcal adherence were tested. The studied antibiotics were chosen for several reasons. Amoxicillin-clavulanic acid was tested because it is frequently used in the therapy of Staphylococcal infections. Quinolone (ciprofloxacin) was chosen because of their interesting activity against gram-negative (Baskin et al., 2002) and gram-positive (Wilcox et al., 1991) bacterial biofilms. Currently, there are no antibiotics on the market specifically indicated for the prevention of diabetic foot infections. Furthermore, diabetic ulcers are often associated with vascular disease and restricted peripheral blood flow, which may render systemically acting antibiotics less effective. By achieving very high localized concentrations of antibiotic, gentamycin may be used to overcome these concerns if used locally. Here, the effect of the antibiotics on biofilm formation was studied by adding sub-MIC of

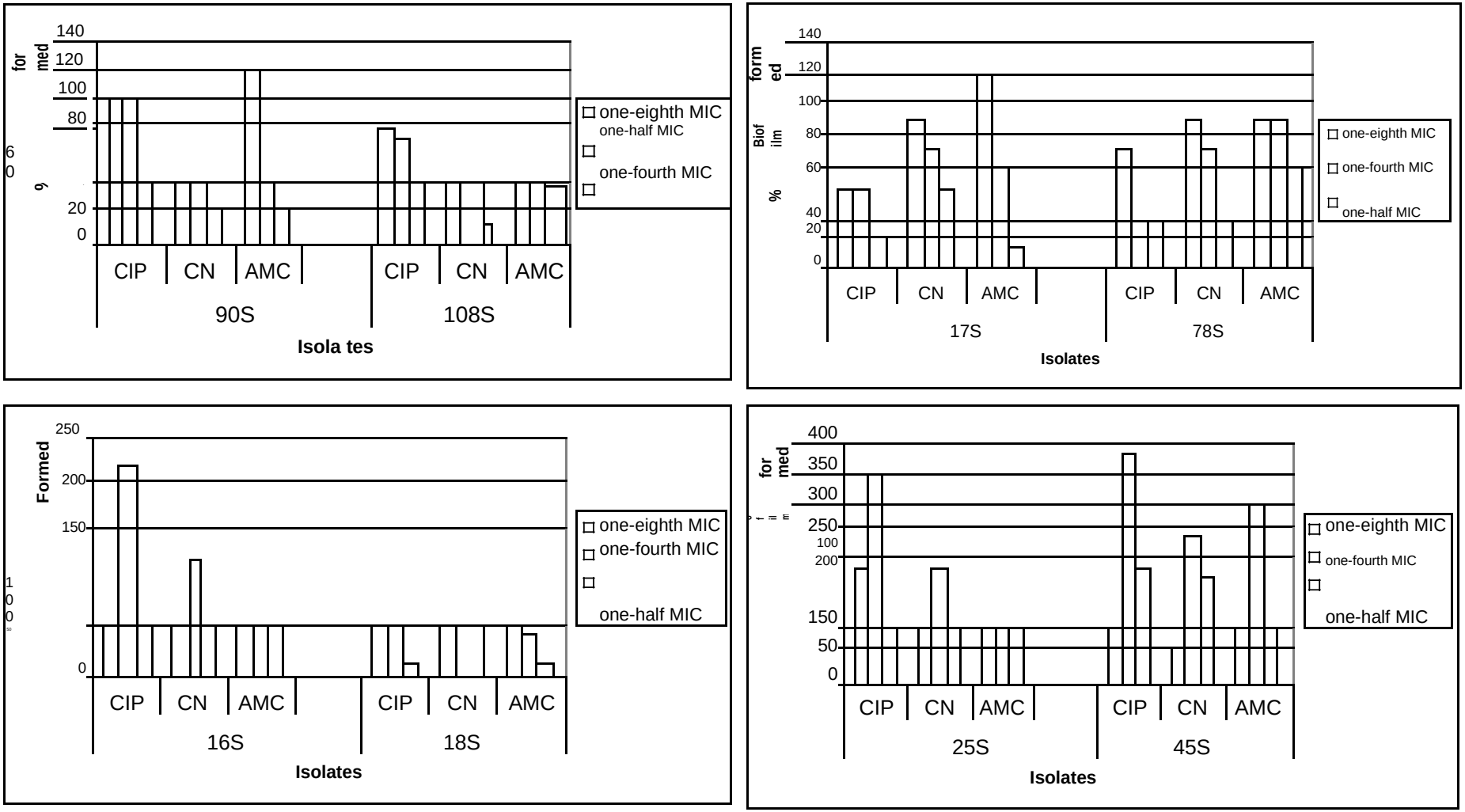

Figure 2. Effect of sub-MIC of ciprofloxacin, gentamycin and amoxicillin-clavulanic acid on biofilm formation.

several antibiotics with the growing biofilms to see how it affects the formation, and in another experiment the biofilms were allowed to be formed then the antibiotics were added to study the effect of the antibiotics on the detachment of already formed biofilms.

In case of prevention of biofilm formation, all antibiotics tested at Sub-MIC markedly reduced the adherence of most (6/8) 75% of Staphylococcal isolates to polystyrene in a dose-dependent manner. However, biofilm formation was influenced by the presence of sub-MIC of the

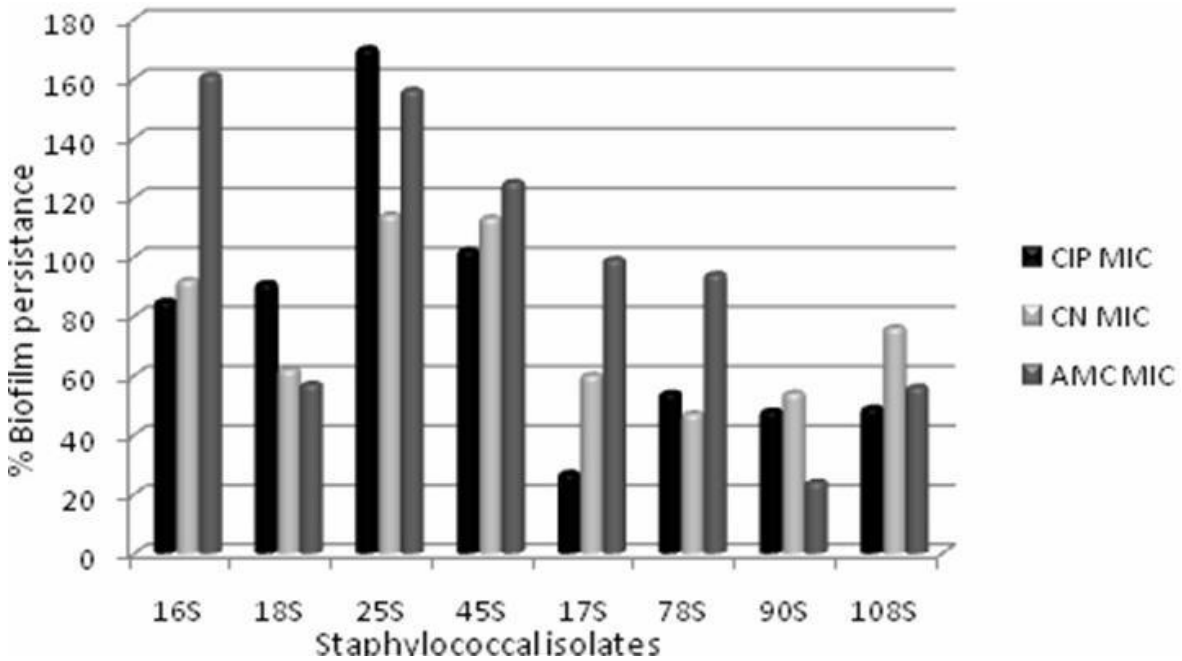

Figure 3. Effect of the MIC of each antibiotic on the removal of a pre-formed biofilm.

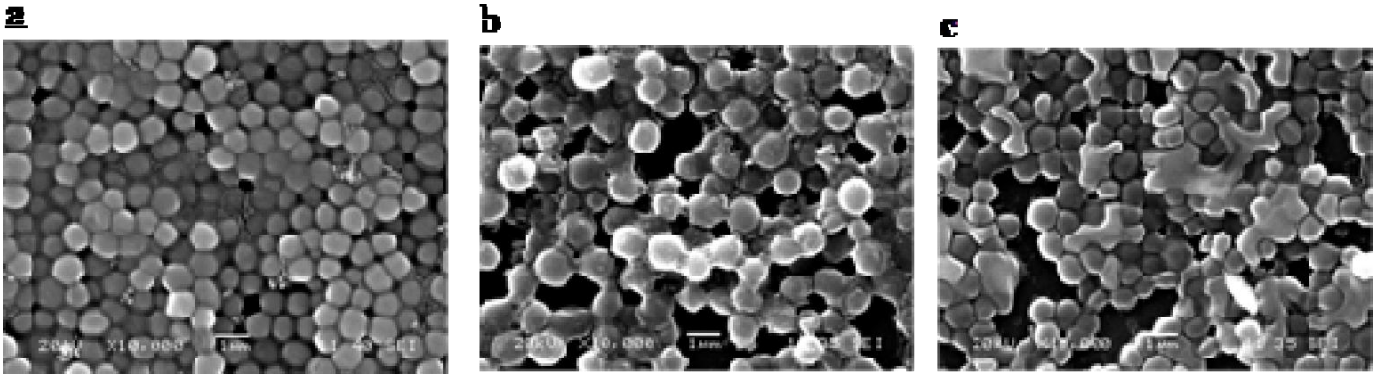

Figure 4. Scanning electron micrograph showing the adherent biofilm of *S. aureus* 45S on polystyrene plate in (a): TSB, (b): TSB with 2.5% glucose and (c): TSB with ¼ MIC of ciprofloxacin.

antibiotics in two (25%) of the tested isolates. Kadry et al. (1999) reached a similar conclusion about the reduction of biofilm formation at low concentrations of ciprofloxacin and other antimicrobial agents in case of mucoid *S. epidermidis*, while Rachid et al. (2000) reported that induction of biofilm gene expression was observed by applying sub-MICs of protein synthesis inhibitors such as tetracycline, quinupristin and dalfopristin to growing cells of biofilm forming *S. epidermidis*. These results are also supported by Knobloch et al. (2001) who stated that PIA synthesis and biofilm formation by *S. epidermidis* are significantly increased by environmental stresses like osmolarity and the presence of ethanol. Also, the presence of glucose significantly enhances the staphylococcal adherence (Stepanovic et al., 2000).

Whereas, results obtained by Perez-Giraldo et al. (2004) with 6 strains of *S. epidermides* showed that subinhibitotry concentrations of moxifloxacin did not significantly modify biofilm formation, whereas, 2, 10, 50 and 100*MIC produced a log decrease in the viable count included in the biofilm. In this study, it is assumed that the staphylococcal isolates respond to the presence of very low antibiotic concentrations as external stresses, which lead to increase in the biofilm formation.

As to the preformed biofilm, ciprofloxacin showed the best results in biofilm removal. Concentrations ranging from 0.5 to 2 MIC caused removal of 50% of an already formed biofilm in five (62.5%) of the tested isolates, followed by gentamycin where the MIC caused the removal of 50% of an already formed biofilm in only four

(50%) of the tested isolates. However, amoxicillin-clavulanic acid showed the least results in biofilm removal (Figure 3). In addition, the effect of MIC of the tested antibiotics on a preformed biofilm showed that there is no fixed pattern for the effect of antibiotics on this preformed biofilm but it was noticed that the bacterial behavior in response to the different antibiotics applied is strain dependant, which requires further investigations. These results suggest that ciprofloxacin is able to penetrate staphylococcal biofilm with a strain-specific efficiency, as suggested by the high strain-to-strain variation, probably due to chemical and physical heterogeneity of staphylococcal biofilms. This difficulty in biofilms removal with antibiotics alone led Krespi et al. (2010) to use ciprofloxacin in combination with different types of lasers to remove more than 80% of Staphylococcal biofilm cells. Other investigators used

other approaches to treat biofilm-associated staphylococcal infections such as cell-wall degrading enzymes (Son et al., 2009).

Scanning electron microscope (SEM) of the isolate 45S was used in this study to confirm the enhancement of biofilm formation in the presence of glucose where it was found that there was increase in the polysaccharide material produced by the staphylococcal cells, also there is an observed increase in the cell size which may explain the increase in the absorbance reading in the microtiter plate assay as was found in previous studies (Stepanovic et al., 2000; Christensen et al., 1985). In the presence of ¼ MIC of ciprofloxacin the biofilm increased by 350% (Figure 2), this was confirmed by the scanning electron micrograph where it was found that there is a great increase in the polysaccharide material that seems to completely cover most of the cells in that case. From this study, we can conclude that once biofilms are formed they are very difficult to be removed even by applying multiples of the MIC of the tested antibiotics. And it was found that there is a high strain-to-strain variation in the behavior towards the tested antibiotics, so it is advised to study each strain separately. It was also noticed that the use of sub-MIC of the tested antibiotics enhanced the biofilm formation of some isolates; this enhancement was confirmed by scanning electron micrographs. So, adjustment of the given dose is very important.

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