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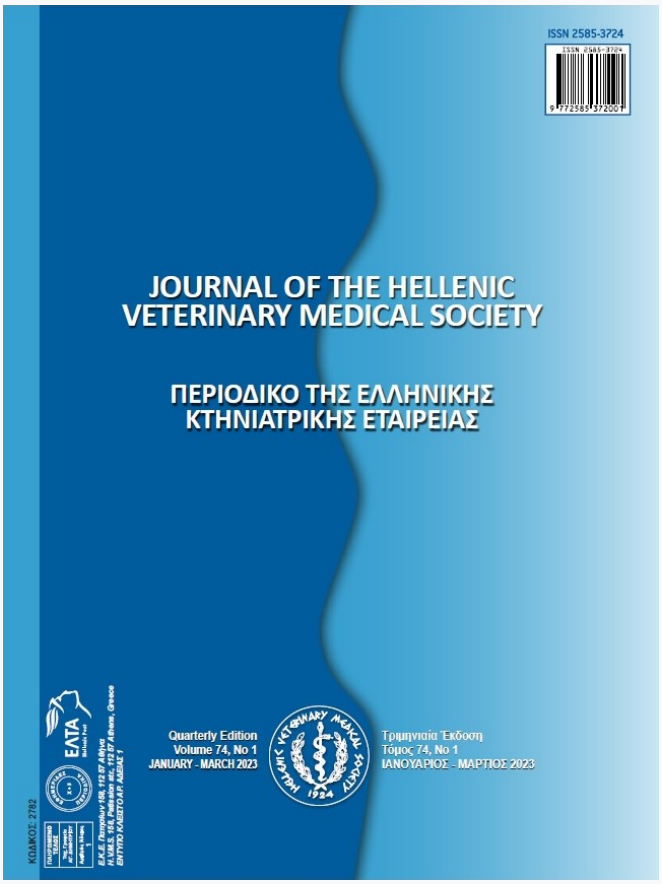

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Research article
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Effect of sub-inhibitory concentrations of antibiotic on the production and *N*-acetylglucosamine scale of methicillin- resistant *Staphylococcus aureus* biofilm

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ABSTRACT: The ability of *Staphylococcus spp.* to produce biofilm is one of the virulence factors that facilitate the adhesion and colonization on a different surface. In this study, the effects of sub minimum inhibitory concentration (sub-MIC) of some antibiotics were evaluated on induction of the biofilm producing ability and free *N*-acetylglucosamine scale in 29 isolates of methicillin resistant*staphylococcus aureus*. To this end, the antibiogram and biofilm producing functions of the studied isolates were assessed by Kirby-Bauer and tissue culture plate method, respectively. Vancomycin, trimethoprim-sulfamethoxazole and clindamycin were used in antibiogram. The free *N*-acetylglucosamine scale in the inducted biofilm after treatment with antibiotic was evaluated by thin layer chromatography (TLC) method.Based on the attained results, all the isolates were susceptible to vancomycin and were capable of producing biofilm in weak (40%), moderate (56.6%) and strong (3.33%) levels. Also, biofilm production was induced in 36.66% of isolates (11/30) from moderate to strong level by sub-MIC vancomycin. An invisible change in free *N*-acetylglucosamine scale was demonstrated in the exopolysaccharide (EPS)structure of the studied isolates biofilm.

By comparingof results and literature reviews, free *N*-acetylglucosamine scale in all studied strains was lower than 5µg in before and after inducted biofilm, or maynot exist. Certainly, for studying structural *N*-acetyl glucosamine scale, using more exact methods of extractionand measurement are needed.

Keywords: Antibiotic; Biofilm; Methicillin; *Staphylococcus aureus*.

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INTRODUCTION

Staphylococcus aureus is the etiological agent of septicemia and septic shock in 30% of central venous catheter cases (Bock et al., 1990).Methicillin Resistant *Staphylococcus aureus* (MRSA) infection,-complicated by different ranges of antibiotic resistance, could be a potential factor in biofilm formation on different surfaces (Arciola et al., 2006). Significantly, in 60% of catheter-associated infections, biofilm-producing bacteria were detected as the main agents (Cooper, 2011).Based on the studies, it is possible to reverse the biofilm structure to the planktonic state, leading to the release of the agent and the re-formation of biofilms in different parts of the body(Boles and Horswill, 2012).

Superficial components in bacteria such as adhesive matrix molecules of MRSA could act as an intermediator in its attachment to host molecules and subsequently, biofilm formation (Atshan et al, 2012). *Staphylococcus aureus* is found in 30% of healthy humans who are carriers (Ma et al., 2012). In cows, *Staphylococcus aureus* is reported as an etiological agent in 40.5 % of contagious mastitis cases (Workineh et al., 2002). This agent in the Australian dairy industry, known as the third largest rural industry, could be a serious cause of loss of value at wholesale ($12 billion) and export ($2.9 billion), accounting for 9 % of the world dairy trade (Dairy Australia, 2011).

The initial signal in biofilm production is the interaction between superficial factors in bacteria, such as an extracellular adhesion protein, Poly-*N*-acetylglucosamine (PNAG), autolysin, protein SasG,eDNA, fibronectin binding proteins and clumping factors, which are the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Maira-Laitren et al., 2004; Pozzi et al., 2012; Geoghegan et al., 2012; Mann et al., 2009;Szczuka et al., 2013). Furthermore, there exist different reports and comprehensive studies concerning the role of PNAG or PIA (Polysaccharide intercellular adhesion) in *Staphylococcus aureus* infections (Maira-Laitren et al., 2004; Arciola et al., 2012). Most of the clinical *S. aureus* isolates produce PNAG, with its role being unknown in in vivo infections, althoughit is associated with biofilm producing ability (Takeda and Akira, 2003;Kropec et al., 2005). However, there are some reports of in vitro settings based on biofilm production with PNAG-negative strains (Beenkeen et al., 2004).

A significant number of the human isolates of clinical *S. aureus* (70-80 %), express either CP5 or CP8;Despite this, they demonstrate varying distribution patterns in bovine mastitis strains (Fattom et al., 1996; Han et al., 2000). Recently, it has been reported that in *S. aureus* with human origin, no relationship was detected between the capsular phenotype and persistent antibiotic resistance or biofilm formation due to different (weak, moderate, or strong) producing abilities in many non-encapsulated *S. aureus* strains (Szczuka et al., 2013).

Several reports have been documented about the effects of sub-MICs of antibiotics and the naturally-occurring materials in biofilm formation (Mortensen et al., 2011; Wozniak and Keyser, 2004). Wozniak and Keyser (2004) reported the inhibitory effect of sub-MIC levels of clarithromycin on twitching motility of *P. aeruginosa* and a meaningful reduction in the matrix material of biofilm. Also, a decrease in the biomass of enteroaggregative *Escherichia coli* biofilm on a glass surface by sub-MICs of ciprofloxacin was detected by Mortensen *et al*.(Mortensen et al., 2011). Kaplan reported that the conformational structure of biofilm in the wild type (mushroom like) and the DpilHIJK mutants type (irregular and protruding structures) of treated *P. aeruginosa* with 1/4 MIC of Ceftazidime is different (Kaplan et al., 2011). In spite of numerous studies concerning the inability of sub-MIC dose in killing bacteria, their biofilm inhibitory effects have been confirmed (Otani et al., 2018). In a research conducted by Tezela et al., the maximum inhibitory effects on *Salmonella infantis* strain occurred in the presence of gentamycin sub-MIC, spectinomycin sub-MIC, nalidixic acid and neomycin, although tetracycline induced the production of biofilm ($p < 0.05$) (Uymaz Tezel et al., 2016). Based on these reports, we investigated the effects of sub-MIC of vancomycin on MRSA biofilms and the alteration of PNAG scale in the structure of the induced biofilm.

MATERIALS AND METHODS

Bacterial collection

A total of twenty-nine MRSA strains isolated from human urine samples were used in the present study. All samples were collected from patients with UTI and were identified via different biochemical tests (e.g., gram stain, catalase, oxidase, hemolysis, mannitol fermentation) (Quinn et al., 2012). The methicillin resistance specification of isolates and the investigation of *mec*A gene were specified and confirmed by Kirby-Bauer method by PCR, respectively (Sambrook and Russell, 2001). The presence and activity

of biofilm were assessed by the colorimetric method involving crystal violet (Moori-Bakhtiari et al., 2017). In all steps of the study, *S. aureus* ATCC 25923 was used as the positive control strain.

Antibiotic susceptibility test

Antibacterial susceptibility of isolates was determined according to Kirby-Bauer method. Briefly, 0.5 McFarland turbidity of each isolate was prepared in brain-heart infusion broth via incubation at 37 ºC for 2-3h. Then, the isolates were incubated with vancomycin (30µg), clindamycin (2µg) and trimethoprim-sulfamethoxazole (1.25- 23.75µg) standard discs for 24 h. The antibiotic with the most susceptibility was selected based on the inhibitory zone diameter and its comparison with the standard table.

MIC Determination of selected antibiotic

First,serial dilution with ½ ratio of selected antibiotic was prepared in a final volume of 0.5 ml. Then, 0.5ml of bacterial suspension (1 McFarland turbidity) was added to each tube. A TSB medium containing 1% glucose was used as the essential medium for antibiotic and bacterial suspension. The maximum concentration of antibiotic was equalto the coated antibiotic used in the standard disc. After 24h incubation at 37 ºC, the antibiotic concentration of the last micro tube without bacterial growth was considered as the minimum inhibitory concentration (MIC).

Biofilm formation assay

The biofilm producing ability of the isolates was evaluated in the presence of sub-MIC of the selected antibiotic via two methods: 1) Incubation of bacteria with antibiotic before the biofilm producing stage for 24 h and 2) Incubation of bacteria in the presence of antibiotic during biofilm producing stage (Stepanović et al., 2000;Stepanović et al., 2007).Resistance to antibiotics could remain for 3-4 weeks after planktonic subcultures.

Evaluation of free N-acetylglucosamine in sub-MIC induced biofilm

Two isolates with a strong biofilm producing ability after antibiotic induction together with 2 negative isolates were selected and cultured in a lot (1200 ml) for biofilm production and Poly-N-acetyl glucosamine extraction. For extraction and purification of biofilm-associated EPS exopolysaccharides, the protocol designed by M. Bales et al. (2013) was used (Bales et al., 2013). In this method, after precipitation of proteins and nucleic acid by TCA in the primary solution, ethanol 95% was added to the supernatant for separating exopolysaccharide from lipids. After centrifugation, the exopolysaccharide pellet was suspended in Milli-Q water and dialyzed by means of 12kDa MWCO membrane. Finally, the collected solution was lyophilized. In every stage, the specific temperature and condition were considered based on the reference.

Detection of N-acetylglucosamine in EPSs by TLC with two visualization methods

Serial dilution (20/40/60/100%) of the prepared samples was applied on a silica gel 60 F_{254} aluminium sheet (Merck, Germany) and two different methods were used for preparing TLC: 1) the mobile phase consists of isopropanol: ethanol: H_2O 5:2:1 (v/v) and products which were detected by spraying 10% sulphuric acid in ethanol on the TLC plate followed by heating at 180°C for 3 min. The standard mixture of NAG (5-50 µg) was also run alongside it.

2) Chromatograph mobile phase comprises n-propanol/ water (7/1.5 v/v) andthe plates which were sprayed with silver nitrate solution and dried. A silver nitrate solution was prepared by adding 3 gr of silver nitrate in 12 ml of water to 500 ml of acetone and then, the spots were visualized with a 0.5 N ethanolic (95%) sodium hydroxide solution (50 ml of 10 N sodium hydroxide in 450 ml of absolute ethyl alcohol) (Gal, 1968; Itoh et al., 2005).

RESULTS

Biofilm producing ability

Biofilm formation was evaluated in TSB supplemented with or without glucose (1%) at 37 ºC for 24h. Based on the medium type, the isolates manifested different biofilm producing potentials. In TSB+glucose1%, 12 isolates (40 %) were detected as weak and 17 isolates (60 %) had moderate biofilm producing ability. Standard strain had a strong ability to produce the biofilm. In TSB without 1% glucose, 27 isolates (86.66 %) were detected as weak producers and 2 isolates (6.66%) were non-producers, with only 1 isolate (3.33%) having a moderate biofilm producing ability. Standard strain was detected to have a moderate producing ability. Having calculated the numerical difference between the positive and negative control optical densities in each method, the highest difference appeared in TSB+ glucose 1% method; therefore, this medium was selected as the most suitable for biofilm formation (Table 1).

Table 1. Comparison of biofilm producing ability results of MRSA isolates based on different medium

Biofilm producing ability	Medium	
	TSB+1% glucose (positive/total)	TSB (positive/total)
Non producer	0/30	2/30
Weak producer	12/30	27/30
Moderate producer	17/30	1/30
Strong producer	1/30	0/30

Table 2. Results of antibiotic susceptibility test by studied antibiotics

Antibiotic (µg)	Standard susceptibility range of inhibitory zone(mm)	Isolates number	Susceptibility percentage(%)
Clindamycin (2)	>21	17	55.17
Trimethoprim-sulfamethoxazole (1.25-23.75)	>16	23	75.86
Vancomycin (30)	>16	30	100

Table 3. Results of biofilm production after treatment with sub-MIC of vancomycin

Isolate number	MIC(µg)	Inducible dose of vancomycin	Isolate number	MIC(µg)	Inducible dose of vancomycin
2	15	½ MIC	19	15	¼ MIC
3	7.5	⅛ MIC	21	15	¼ MIC
5	15	1/32 MIC	22	15	½ MIC
7	15	½ MIC	23	7.5	1/16 MIC
12	15	½ MIC	27	7.5	¼ MIC
18	7.5	¼ MIC	Positive control	15	Strong level

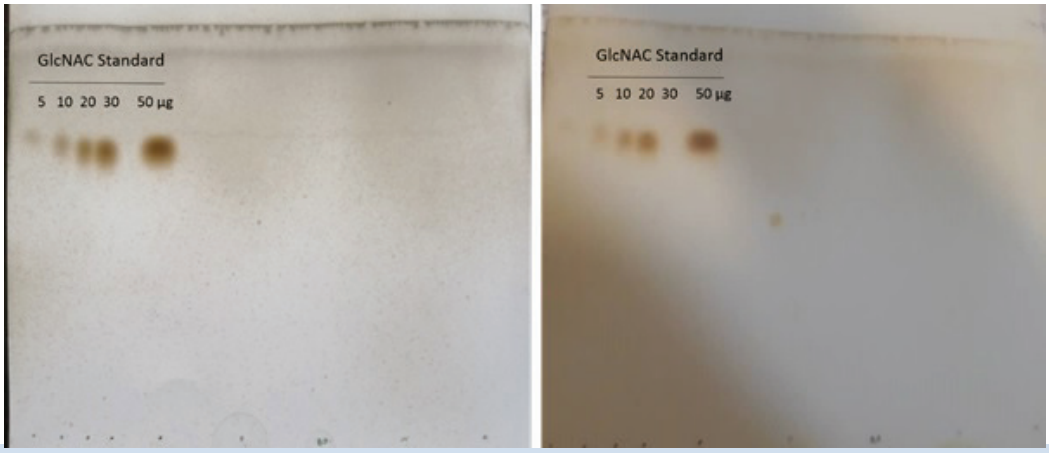

Figure 1. TLC results with two visualization methods for GlcNAC; left figure silver nitrate and Right figure: 10% sulphuric acid.

Antibiotic susceptibility test

By Kirby-Bauer method, susceptibility to clindamycin, trimethoprim-sulfamethoxazole (PadTanTeb) and vancomycin (PadTanTeb) was demonstrated in 55.17%, 75.86% and 100% of isolates, respectively. Therefore, vancomycin was selected for studying biofilm induction (Table 2).

Determining Minimum inhibitory concentration

All the isolates were evaluated by macro dilution method in the final volume of 1ml; meanwhile, the concentration spectrum of vancomycin ranged from 30 to 0.015 µg. In 17 (56.67%) isolates, MIC was calculated to be 15 µg while vancomycin accounted for7.5 µg in 13 (43.33%) isolates.

Biofilm induction by Sub- inhibitory concentration of vancomycin

Biofilm formation was evaluated in this study under the designated condition; that is, the use of TSB+

glucose 1% with 24 h incubation of bacteria in antibiotic prior to biofilm production. This method was more suitable compared to the simultaneous incubation of bacteria with antibiotic in biofilm formation process due to limited dispersion of results. Based on the results, an increase in biofilm producing ability was detected in 36.66% of isolates from moderate to strong level (11/30). The alterations in biofilm production were as follows: (36.36%) in ½ MIC (7.5 µg) and less in four isolates, (36.36%) in ¼ MIC (3.75 µg) and less in 4 isolates, (9.09%) in 1/8 MIC (1.87 µg) and less in 1 isolate, (9.09%) in 1/16 MIC (0.93 µg) and less in1 isolate, and (9.09%) in 1/32 MIC (0.01 µg) and less in1 isolate (Table 3).Meanwhile, no change was detected in biofilm producing ability of weak producer isolates.

TLC analysis of GlcNAC

TLC analysis revealed no observational level of GlcNAC monomers in the prepared EPS extracted samples at all tested concentrations (20/40/60/100%). In fact, amounts equal to or less than 5 µg of *N*-acetylglucosamine are not detectable by the TLC method.

DISCUSSION

Biofilms can protect bacterial cells from physical and chemical attacks and trap nutrients and water at the same time, which allows microorganisms to survive in harsh environments (Costerton, 1999). Comparative and genetic changes of cells in biofilm make them highly resistant to routine therapeutic doses of antimicrobial drugs which is also effective against responses of the host immune cell (Ryder, 2005). According to the literature, transfer of antibiotic resistance genes is accelerated in such structures. Also, this structure could be considered as one of the main causes of chronic infections induced by bacterial such as *S. aureus* in both human and veterinary medicine (Gad et al., 2009).

Staphylococcus aureus, among various species of *Staphylococcus*, has the highest capability due to its wide range of toxic secretions, cell surface factors, immune system escape mechanisms, and toxin production (Sakai et al., 2004).

In Hemamalini et al.'s (2015) study, *Staphylococcus aureus* infection was detected in 66.7% ofhuman septic injuries, and resistance to methicillin was reported in 35% of them (Hemamalini et al., 2015). In Libya, presence of *Staphylococcus aureus*was confirmed in 4.32% (6/139) of various food samples, all of which were reported to be resistant to clindamycin and cefotaxime, and only one isolate was methicillin-resistant (Naas et al., 2019). In Adis-Ababa (Ethiopia), 133 MRSA isolates were identified in 768 carcasses in slaughterhouses, butcheries, and equipment of these stores (Adugna et al., 2018). In the present study twenty-nine MRSA strains were isolated from 150 human urine samples and their susceptibility to vancomycin, trimethoprim-sulfamethoxazole and clindamycin was detected in 100%, 75% and 55% of isolates, respectively.Biofilm production with different levels was reported in another species of *Staphylococcus*. For example, Águila-Arcos et al. (2017), reported that, *Staphylococcus epidermidis* had a higher ability to produce biofilms compared to 25 biofilm-producing *Staphylococcus aureus* which were isolated from medical implants (Águila-Arcos et al., 2017).

The relation of biofilm production, antibiotic resistance pattern, and some adhesion factors in MRSA originating from different samples were studied in several research works (Stepanović et al., 2000). In another study, Biofilm formation was induced by MIC dose of tobramycin in *Pasteurella aerogenes* (Sohail and Latif, 2018). Kaplan et al. (2011) reported that the inhibitory concentrations of methicillin in all three studied strains of *Staphylococcus aureus* were associated with increased biofilm formation.

In gram-positive and gram-negative bacteria, treatment with sub-inhibitory concentrations (sub-MIC) of antibiotics can stimulate the production of extracellular polysaccharides. By exposing *Actinobacillus pleuropneumoniae* to Sub-MIC of penicillin G, biofilm formation was reported in 9 isolates. Penicillin G was associated with increased expression of the *pga*A gene, the protein-encoding gene participating in the structure of N-acetyl glucosamine.

Haddadin et al. (2005), after exposing *Staphylococcus aureus* to different concentrations of ciprofloxacin, was unable to detect any inhibitory or stimulating effect on biofilm production. But Roxithromycin with 45% of MIC and cephalexin with 40% of MIC, induced weak biofilm producing ability, which can be due to inhibition of the production of proteins participating in the biofilm structure (Haddadin et al., 2010).

In the Majidpour et al. study (2017), vancomycin and azithromycin had the highest inducing effect on biofilm formation of 5 studied strains of *Staphylococcus aureus* (Majidpour et al., 2017). Also, Mirani and Jamil (2011) showed that inhibitory concentrations

of vancomycin and oxacillin increased biofilm formation in *Staphylococcus aureus* on nylon and silicon surfaces (Mirani and Jamil, 2011). In the present study, the results also indicated the positive effect of vancomycin sub-MIC doses on biofilm production of studied *Staphylococcus aureus* strains.

Hsu et al. (2015) stated that in addition to improper antibiotic treatment, increasing glucose concentration in diabetic animals may facilitate the formation of vancomycin-resistant *Staphylococcus aureus* biofilm. In contrast, in the absence of glucose, vancomycin reduced the desire to form biofilms in vitro by increasing the production of proteases and DNases in vancomycin-resistant *Staphylococcus aureus* (Hsu et al., 2015).Cargill and Upton (2009), reported different responses to inhibitory concentrations of vancomycin in different strains of *Staphylococcus epidermidis.* In addition, responses ranged from a gradual reduction of density by increasing antibiotic concentration, through a stable state reaction, to increasing biofilm density in the presence of vancomycin. This increased biofilm density was repeatable and only occurred in newly formed biofilms (Cargill and Upton, 2009).

It is necessary to have a clear understanding of the pathogenesis of *Staphylococcus spp.* biofilm in order to evaluate therapeutic strategies. Significant progress has been made in this area in recent years, leading to a clearer understanding of the stages related to the development of Staphylococcal biofilm. The role of different factors in biofilm formation, especially in the initial contact of bacteria to the surface, which is a key factor in the continuation of the formation of this structure, has been identified by various studies;- for example, Bap in *Staphylococcus aureus* strains caused mastitis in cattle (Tormo et al., 2007).Moreover, regarding the Poly n-acetyl glucosamine, which has an established role in the pathogenesis and biofilm production in *Staphylococcus aureus,*has been reportedto be absent in strains having the ability to produce biofilms (Beenkeen et al., 2004).It is not clear whether the presence of Poly *N*-acetylglucosamine causes infection or not, but the presence of this compound in most strains isolated from clinical cases can be considered as a sign of its importance (Kropecet al., 2005). This polysaccharide has a disordered structure and protects the bacterial cells against the host immune system (Takeda and Akira, 2003).

As mentioned earlier, the exopolysaccharide PNAG is a major matrix component in the biofilms of many gram-positive and -negative bacterial species. It consists of a chain of GlcNAc units, produced by a membrane associated enzyme complex and secreted into the extracellular space (Roux et al., 2015). The PNAG of exopolysaccharide has a key role in biofilm that has made it one of the main subjects in biofilm research. Labeling the GlcNAc monomer by chemical methods is complicated by its presence in many bacterial surface structures, and there have been no reports of metabolic incorporation of unnatural sugars into PNAG. Also, enzymatic removal of PNAG leads to near total disruption of PNAG-dependent biofilms. Therefore, different PNAG enzymatic digestion and chromatography methods have been described previously (Itohet al., 2005; Gökçen et al., 2013).

TLC methods have been used to separate low molecular weight of oligosaccharides and in the present study, TLC evaluation of exopolysaccharides extracted from bacterial biofilms, before and after exposure with sub-MIC concentration of vancomycin, revealed no observational level of GlcNAC residues at the tested concentration, which can be representative of substrates being out of detection range. Studies on pathogen bacteria such *as Staphylococcus epidermidis* showed that in normal conditions, the amount of PNAG in antibiotic induced exopolysaccharide production was only 3.5% mole, and in the case of non-exposure to antibiotics, its level is expected to be lower. Although we should remember that the dilute solution of EPS which has been used in research is not representative of biofilm environments, and PNAG is not distributed homogenously on the cells but concentrates in discrete areas which can be lost in different stages of preparation methods (Eddendenet al., 2020). Thereby, in these times, a highly sensitive and selective HPLC-MS-MS or GS/Ms as precise methods are required for quantitative evaluation. On the other hand, as mentioned earlier, in some strains of *Staphylococcus aureus* PNAG has not been observed in biofilm structure. Hence, it can be argued that, in studied strains in the present research, there was no such combinationfrom the beginning. Future studies are needed to extend our knowledge.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

REFERENCES

Adugna F, Pal M, Girmay G. (2018) Prevalence and Antibiogram assessment of *Staphylococcus aureus* in beef at municipal abattoir and butcher shops in Addis Ababa, Ethiopia. BioMed Res Int 2: 237-241.

Águila-Arcos S, Álvarez-Rodríguez I, Garaiyurrebaso O, et al. (2017) Biofilm-forming clinical *Staphylococcus* isolates harbor horizontal transfer and antibiotic resistance genes. Front Microbiol 8: 201-9.

Arciola CR, Campoccia D, Baldassarri L, et al. (2006) Detection of biofilm formation in Staphylococcus epidermidis from implant infections. Comparison of a PCR method that recognizes the presence of *ica* genes with two classic phenotypic methods. J Biomed Mater Res 76: 425-30.

Arciola CR, Campoccia D, Speziale P, et al. (2012) Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm resistant materials. Biomaterials 33: 5967-5982.

Atshan SS, Nor Shamsudin MN, Sekawi Z, et al. (2012) Prevalence of adhesion and regulation of biofilm-related genes in different clones of *Staphylococcus aureus*. J BioMed Biotech 1-10. Article ID 976972.

Bales PM, Renke EM, May SL, et al. (2013) Purification and characterization of biofilm-associated EPS exopolysaccharides from ESKAPE organisms and other pathogens. Plos One 8 : 67950.

Beenkeen KE, Dunman PM, McAleese F, et al. (2004) Global gene expression in *Staphylococcus aureus* biofilms. J Bacteriol 186: 4665-4684.

Bock SN, Lee RE, Fisher B, et al. (1990) A prospective randomized trial evaluating prophylactic antibiotics to prevent triple-lumen catheter-related sepsis in patients treated with immunotherapy. J ClinOncol 8: 161-9.

Boles BR, Horswill AR (2012) Swimming cells promote a dynamic environment within biofilms. National Academy of Sciences 109: 12848-12849.

Cargill JS, Upton M. (2009) Low concentrations of vancomycin stimulate biofilm formation in some clinical isolates of staphylococcus epidermidis. J ClinPathol62: 1112-6. doi: 10.1136/jcp.2009.069021.

Cooper IR (2011) Microbial biofilms: case reviews of bacterial and fungal pathogens persisting on biomaterials and environmental substrata. In: Mendez-Vilas A, editor. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. Badajoz, Spain: Formatex Research Centre: pp 807-17.

Costerton JW. (1999) Introduction to biofilm. Int J Antimicrob Agent 11: 217-221.

Dairy Australia (2011) Dairy Australia Strategic Plan 2011-2015. http://www.dairyaustralia.com.au/. Accessed December 2010.

Eddenden A, Kitova EN, Klassen JS, et al. (2020) An Inactive Dispersin B Probe for Monitoring PNAG Production in Biofilm Formation. ACS ChemBiol 15(5): 1204-1211. doi: 10.1021/acschembio.9b00907.

Fattom AI, Sarwar J, Ortiz A, et al. (1996) *Staphylococcus aureus* capsular polysaccharide (CP) vaccine and CP specific antibodies protect mice against bacterial challenge. Infect Immun 64: 1659- 1665.

Gad GFM, El-Feky MA, El-Rehewy MS, et al. (2009) Detection of *icaA*, *icaD* genes and biofilm production by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from urinary tract catheterized patients. J Infect Dev Ctries 3: 342-351.

Gal AE. (1968) Separation and identification of monosaccharides from biological materials by thin-layer choromatography. Anal Biochem 24: 452-461.

Geoghegan JA, Corrigan RM, Gruszka DT, et al. (2010) Role of surface protein SasG in biofilm formation by *Staphylococcus aureus*. J Bacteriol 197: 5663-5673.

Gökçen A, Vilcinskas A, Wiesner J. (2013) Methods to identify enzymes that degrade the main extracellular polysaccharide component of Staphylococcus epidermidis biofilms. Virulence 4(3): 260-70. doi: 10.4161/viru.23560.

Haddadin R, Saleh S, Al-Adham I, et al. (2010) The effect of subminimal inhibitory concentrations of antibiotics on virulence factors expressed by *Staphylococcus aureus* biofilms. J ApplMicrobiol 108: 1281-1291.

Han HR, Pak SI, Guidry A. (2000) Prevalence of capsular polysaccharide (CP) types of *Staphylococcus aureus* isolated from bovine mastitic milk and protection of *S. aureus* infection in mice with CP vaccine. J Vet Med Sci 62: 1331-1333.

Hemamalini V, Kavitha V, Ramachandran S. (2015) In vitro antibiogram pattern of *Staphylococcus aureus* isolated from wound infection and molecular analysis of *mec*A gene and restriction sites in methicillin resistant *Staphylococcus aureus*. J Adv Pharm Technol Res 6: 170.

Hsu CY, Shu JC, Lin MH, et al. (2015) High glucose concentration promotes vancomycin-enhanced biofilm formation of vancomycin-non-susceptible *Staphylococcus aureus* in diabetic mice. PloS One 10: 0134852.

Itoh Y, Wang X, Hinnebusch BJ, et al. (2005) Depolymerization of beta-1,6-N-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. J Bacteriol 187(1): 382-7. doi: 10.1128/JB.187.1.382-387.2005. PMID: 15601723.

Kaplan JB. (2011) Antibiotic-induced biofilm formation. Int J Artif Organs 34: 737-751.

Kropec A, Maria-Litran T, Jefferson KK, et al. (2005) Poly-N-acetylglucosamine production in *Staphylococcus aureus* is essential for virulence in murine models of systemic infection. Infect Immun 73: 6868-6876

Ma Y, Xu Y, Yestrepsky BD, et al. (2012)Novel inhibitors of *Staphylococcus aureus* virulence gene expression and biofilm formation. PLoS One 7: e47255.

Maira-Laitren T, Kropec A, Goldmann D, et al. (2004) Biologic properties and vaccine potential of the staphylococcal Poly-N-acetyl glucosamine surface polysaccharide. Vaccine 22: 872-879.

Majidpour A, (2017) Dose-dependent effects of common antibiotics used to treat *Staphylococcus aureus* on biofilm formation. Iran J Pathol 12: 362.

Mann EE, Rice KC, Boles BR, et al. (2009) Modulation of eDNA release and degradation affects Staphylococcus aureus biofilm maturation. PLoS One 4: e5822.

Mirani ZA, Jamil N. (2011) Effect of sub-lethal doses of vancomycin and oxacillin on biofilm formation by vancomycin intermediate resistant *Staphylococcus aureus*. J Basic Microbiol 51:191-195.

Moori-Bakhtiari N, Moslemi M. (2017) Phenotypic evaluation of biofilm producing ability in Methicillin Resistant *Staphylococcus aureus*. Feyz 20: 525-31 [Article in Persian].

Mortensen NP, Fowlkes JD, Maggart M, et al. (2011) Effects of sub-minimum inhibitory concentrations of ciprofloxacin on enteroaggregative*Escherichia coli* and the role of the surface protein dispersin. Int J Antimicrob Agents 38: 27e34.

Naas HT, Edarhoby RA, Garbaj AM, et al. (2019) Occurrence, characterization, and antibiogram of *Staphylococcus aureus* in meat, meat products and some seafood from Libyan retail markets. Vet World 12: 925.

Otani S, Hiramatsu K, Hashinaga K, et al. (2018) Sub-minimum inhibitory concentrations of ceftazidime inhibit *Pseudomonas aeruginosa* biofilm formation. J Infect Chemother 24: 428e433. https://doi.org/10.1016/j.jiac.2018.01.007

Pozzi C, Waters EM, Rudkin JK, et al. (2012) Methicillin resistance alters the biofilm phenotype and attenuates virulence in *Staphylococcus aureus* device-associated infections. PLoSPathog 8: e1002626.

Quinn PJ, Carter ME, Markey B, et al. Clinical veterinary Microbiology. Lynton House, 7-12 Tavistock Square, London WC1H9LB, England: Mosby, 2012.

Roux D, Cywes-Bentley C, Zhang YF, et al. (2015) Identification of Poly-N-acetylglucosamine as a major polysaccharide component of the bacillus subtilis biofilm matrix. Int J BiolChem 290: 19261−19272.

Ryder MA. (2005) Catheter-related infections: it's all about biofilm. Top AdvPractNurs J 5: 1-6.

Sakai H, Procop G, Kobayashi N, et al. (2004) Simultaneous detection of *Staphylococcus aureus* and coagulase-negative staphylococci in positive blood cultures by real-time PCR with two fluorescence resonance energy transfer probe sets. J ClinMicrobiol 42: 5739-5744.

Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 2001.

Sohail M, Latif Z. (2018) Molecular analysis, biofilm formation, and susceptibility of methicillin-resistant *Staphylococcus aureus* strains causing community-and health care-associated infections in central venous catheters. Rev Soc Bras Med Trop 51: 603-609.

Stepanović S, Vuković D, Dakić I, et al. (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 40: 175-179.

Stepanović S, Vuković D, Hola V, et al. (2007) Quantification of biofilm in microtiter platesoverview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 115: 891-899.

Szczuka E, Urbańska K, Pietryka M, et al. (2013) Biofilm density and detection of biofilm-producing genes in methicillin-resistant *Staphylococcus aureus* strains. Folia Microbiologica 58: 47-52.doi: 10.1007/s12223-012-0175-9.

Takeda K, Akira S. (2003) Toll receptors and pathogen resistance. Cell Microbiol 5: 143-153.

Tormo MA, Ubeda C, Martí M, et al. (2007) Phase-variable expression of the biofilm-associated protein (Bap) in *Staphylococcus aureus*. Microbiology 153: 1702-1710.

Uymaz Tezel B, Ak¸celik N, NeslihanYuksel F, et al. (2016) Effects of sub-MIC antibiotic concentrations on biofilm production of *Salmonella Infantis*. BiotechnolBiotechnol Equip 30: 1184-1191. http://dx.doi.org/10.1080/13102818.2016.1224981

Workineh S, Bayleyegn H, Mekonnen H, et al. (2002) Prevalence and aetiology of mastitis in cows from two major Ethiopian dairies. Trop Anim Health Prod 34: 19-25.

Wozniak DJ, Keyser R. (2004) Effects of sub-inhibitory concentrations of macrolide antibiotics on *Pseudomonas aeruginosa*. Chest 125: 62Se9S.