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Impact of pyridoxin (B6) on gene expression of ica A and icaC Of Staphylococcus aureus bacteria

Muntaha R. Ibraheem[1], Riyam Alaa Mohammed[2]

[1]Biomedical department, Alkawarizmi Collage of engineering, Baghdad University, Iraq,
Email: muntaha@kecbu.uobaghdad.edu.iq
[2]Medical and molecular department, Collage ofBiotechnology, al-Nahrain University, Iraq,
Email:Riyamalaa94@gmail.com

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ABSTRACT
Pathogenic bacteria Staphylococcus aureusmostly forms biofilms by adhering themselves on both tissues and inorganic surfaces by produced polysaccharide matrix as a survival mechanism in harsh environment. Which make themunreachable to biocides, antibiotics, or host immune system. This led us to seek analternative to antibiotics that inhibitformation of biofilm, so in this studytestedthe impact of pyridoxine (vitamin B6) on the expression of icaA and icaC genes which isimportant for biofilm formation in Staphylococcus aureususing qRT-PCR. The pyridoxinewas effective against biofilm-embedded S. aureus at sub inhibitory concentrations range from 6.4 µg/mL to12.5 µg/ml.Expression levels of both icaA and icaC were reduced in 85% of S. aureus adhering strain. While increase in 15% of S. aureus adhering strain. Theresearch showed a novel mechanism of pyridoxine's action, that involved in inhibition of S. aureus adhesion by reducing the expression of both icaA and icaC genes associated with staphylococcal biofilmformation.

Keywords: pyridoxine, gene expression, icaA , icaC , staphylococcus aureus,

INTRODUCTION

From mild skin infections to more serious illnesses like pneumonia and bloodstream infections, Staphylococcus aureuscan cause a variety of infections, in which it considered a prevalent bacterial species on outer layer of skin and mucous excretion in epithelial tissue. (1)

The attachment of Staphylococcus spp. to the location of pathogen infection especially epithelial cells which represent a significant virulence factor of most Staphylococcus aureus, and the capability to form colony and adhere on the outer layer of the epithelium has been attached to the biofilms secretion, which are the basic of intricate bacterial colonies encased in a matrix of extracellular polymeric molecules.Bacteria benefit from biofilms in a number of ways, including improved immunity to the human immune system and drugs. Staphylococcus aureus forms biofilm, in which to increases its pathogenicity and makes infections more challenging to cure. On both living and non-living surfaces, including implants and catheters used in medicine, biofilm formation can take place.(2)

Staphylococcus aureus responsible for different community infections and hospital setting and that led to sever consequences, such as blood toxification with bacteria, infection of heart muscles, meningitis, liver abscess, and spinal cord epidural abscess. Some Staphylococcus aureus isolates can also develop biofilms and have multidrug resistance (MDR). This indicates that they are difficult to treat because they are resistant to numerous antibiotic types. A serious public health problem is the growth of MDR of Staphylococcus aureusstrains, such as methicillin-resistant Staphylococcus aureus (MRSA).(3)

Biofilm formation involves the production of an extracellular mucopolysaccharide, which is commonly named a “slime” and that slime comprising ofPNAG which is composed of positively charged polymer of β-1,6-N-acetylglucosamine residueswhich is a complex polysaccharide intercellular adhesin (PIA). The production of PNAG is mediated by intercellular adhesin (ica) operon and the last one is formed by the icaA, icaB, icaC and icaD genes and the regulatory gene, icaR. These genes encode protein ICAA, ICAB, ICAC and ICAD, which responsible for synthesizingthis polymer. from a clinical standpoint,biofilms have asignificant challengesince the Clustered bacteria showed antimicrobial agents’ resistances and evades the host immune defenses. Among the factors contributing to these high resistances is the physical obstacleraised by the exopolysaccharide matrix, which interfere and obstruct the activity of the antimicrobial agent. (4)

The main active form of nano pyridoxineispyridoxal 5-phosphate. Pyridoxin work as a co-factor in many enzymatic pathways that’s have a relationship with amino acid metabolism, and it is also used as an antidote for treating acute poisoning, including isoniazid overdose.(5,6)

Nano Pyridoxine utilized in both as a vitamin and a medication for treating vitamin B6 deficiency and managing nausea and vomiting during pregnancy. Beside that it isused in cases of vitamin B6 dependency syndromes and it is controversially utilized in certain other disorders. This activity will emphasize the indications, mechanism of action, routed of administration, adverse effects, monitoring procedures, and contraindications associated with the use of pyridoxine on patients with vitamin B6 deficiency and other ailments.(7)

Oral pyridoxine lowering the sensitivity of peripheral chemoreflex in hypertension patients in whom baseline sensitivity (i.e., placebo condition) was the highest. The oral pyridoxine supplementation considers as a beneficial for hypertensive patients. pyridoxine had shown an efficient adjunct therapy for the linezolid toxicity prevention.(8)

Nano Pyridoxine (vitamin B_6) naturally present in foods, with a daily in take recommended dose up to 2mg. additionally, it is used widely as a dietary health supplement. While insufficiency is rare and uncommon among community with a balanced diet but it can occur in malnutrition due to starvation, alcohol adductors oreating disorder, as well as during periods of increased metabolic requirement such as pregnancy or systemic illness. Certain medications like isoniazid and levodopa/carbidopa intestinal gel can also lead to deficiency due to altered metabolism.(9).Notably, vitamin B_6demonstrated a crucial function as a possible antibacterial agent.(10)

METHODS

Sampling

This study examined the biofilm production profile of 34Staphylococcus aureus. In Which the Swap with transport media was used to isolate bacteria from different clinical sources from Al-Yarmouk Teaching Hospital (S. aureus). The isolates were enriched on Brian's heart infusion broth and then cultured on blood agar after that were tested for mannitol fermentation on MSA. The biochemical test like coagulase, hemolysis and catalase were used in detection(11).

Biofilm formation can be detected and evaluated phenotypically on microplates as follow in which all 34 isolates of Staphylococcus aureuswere cultured on blood agar for 24 h at 37°C. Then the colonies appeared were cultured on Muller Hinton agar and further incubated at 37°C for 24 h. Bacterial suspension McFarland were up to 0.5 scale and the diluted ratio was 1:10 in MH broth which supplemented with 1% glucose. After that 200 µL of this solution was transferred into individual well of one-use microplates and incubated for 24 h at 37°C without agitation. After incubation, the aquatic material was discarded and removed, the wells then were washed double time with 200µL of sterile phosphate buffer solution before being oven dried at 65°C for approximately 30 -60 min.Fallow that, 200µL of 1% crystal violate were added and stayed for 15 min in order to stain the wells. The absorbance was measured at 490 nm in ELISA reader (BIOTekreader). Uninoculated wells served as blank control containing MH broth with 1% glucose were included for comparison. The tests were repeated in triplicate. The optical density of biofilm formation was measured in order to use these optical density (OD) value that obtained to classify the strains as following: strong ≥0.3; moderate ≥0.2 and weak ≥0.1(12).

Polymerase chain reaction

DNA extraction was done for only seven isolate according to their ability to form biofilm by using ABIOPURE while Quantus Fluorometer was used to detect the concentration of extracted DNA to the Polymerase chain reaction (PCR) amplification the mixture used for the detection of the genes was carried out in 20µl volume including GoTaq® Green Master Mix (10µl), 3µl of ng/µl DNA template, 1µl of each forward and reversed primers (Table 1) (13), and 5µl of nuclease-free water to complete the amplification mixture to 20µl for 30 cycles as in All the PCR components were mixed in PCR tubes under aseptic conditions and vortexed at low speed and then placed into the thermocycler PCR machine . Amplification was performed in a thermal cycler (Veriti) programmed as followed: initial Denaturation 95°C for 5min, denaturation 95 °C for 30Sec. annealing as in(Table1) and final extension at 72°C for 30 Sec, the program was held at 4 °C, and then the products of PCR were observed by 1.5% gel electrophoresis.

Table 1: Primers

Primer Name	Sequence 5`-3`	Annealing Temp. (°C)	source
icaA-F	GAGGTAAAGCCAACGCACTC	58	macrogen
icaA-R	CCTGTAACCGCACCAAGTTT		
icaC-F	CTTGGGTATTTGCACGCATT	56	
icaC-R	GCAATATCATGCCGACACCT		
Stau_16s-F	TGTCGTGAGATGTTGGG	52	
Stau_16s-R	CGATTCCAGCTTCATGT		

Detection of pyridoxin susceptibility by agar dilution method

Pureform of nano pyridoxin compound wasAcquired from Sigma Aldrich (Germany) company. In this study a potency of 200 mg\ ml was used in order tosetthe Minimum Inhibitory Concentration (MIC) by using the microtiter plate resazurin method according to the procedures recommended by the Nazni. (14) in which the products were serially diluted twofold from (200 mg/ml to 90µg/ml). The sterile control wells for all tasted bacteria stayed in blue color after an overnight incubation followed by a 2-4 hours incubation with resazurin(15). In compression, the growth control wells (which included both growing bacteria and medium) of all tested microorganisms was transformed to pink or pale pink. After treatment the isolates with sub inhibitory concentration for 24 hours the RNA was isolated from sample according to the protocol of TRIzol™ Reagent.

A One-Step Reverse Transcription Polymerase Chain Reaction (RT-PCR) kit was employed with three distinct primers targeting different genes. These included the housekeeping gene (stau16s) and the genes associated with biofilm intracellular adhesion (icaAC). A 10µl solution was prepared by combining 5µl of qPCR master mix, 0.25µl of MgCl2 and RT mix, 0.5µl each of forward and reverse primers, 2.5µl of nuclease-free water, and 1µl of RNA,while qPCR program the annealing temperature as in table 1.

RESULT AND DISCUSSION

Isolation and identification of Staphylococcus aureus

Out of 100 samples obtained from various clinical sources at al-Yarmouk hospital, 34 were identified as Staphylococcus aureus. The identification process involved visual examination, biochemical tests, and molecular techniques. S. aureus was recognized by its yellow appearance on agar plates, as well as through tests for coagulase, catalase, hemolysin, biofilm production, and antibiotic resistance. S. aureus is a common pathogen accountable for various infections., from minor skin issues to highly severe conditions. Identifying and characterizing S. aureus in clinical samples is crucial for determining appropriate treatment, implementing infection control measures, and understanding its prevalence and distribution in different clinical settings.

The results of biofilm show that 21% of S. aureus isolates was strong biofilm producer, this was expectedin Iraqi studies which in contrast with other studies which show that S.aureus had low percentage biofilm producing (16).All the strong biofilm producer is positive to icaA, and icaC genes, by using Polymerase chain reaction figure 1. Since the environment had huge impact on the variation of bacteria virulence factors and the Iraq environment encourage this kind of variety (17). S. aureus have the remarkable ability to produce biofilm as survival mechanisms in which this virulence Factor cause pathogenic infection. It is knowns that s. aureus hasvarious genetic and morphological factors that impact the formation of biofilmsand their clinical implications. (18)

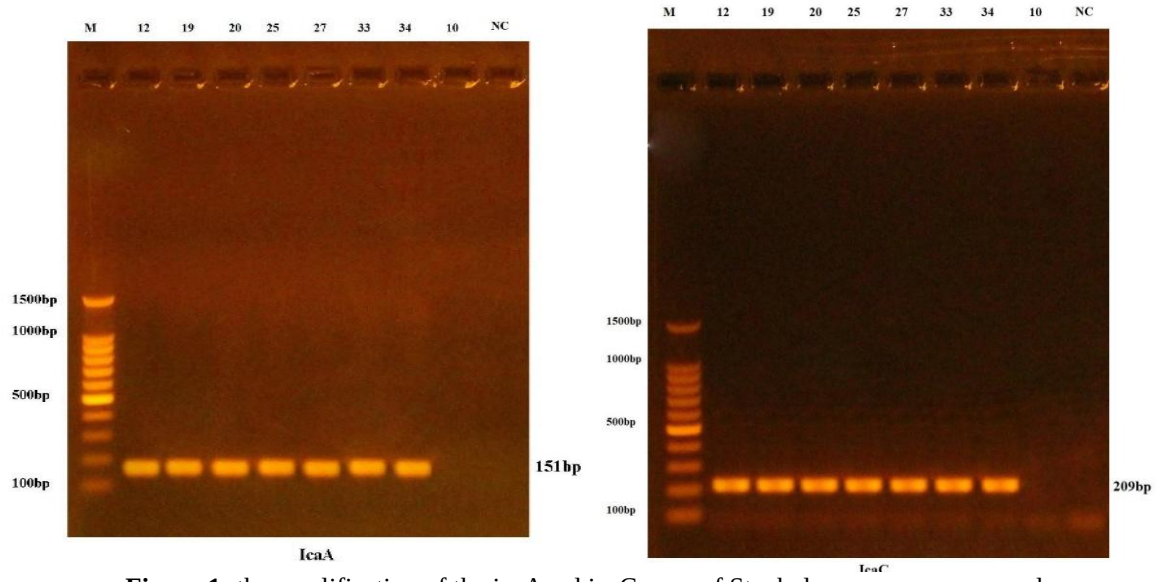

Figure 1: the amplification of the icaAand icaC gene of Staphylococcus aureus samples.

The study to investigate how antibiofilm agents work to inhibit or disrupt these biofilmswere very little known.So the minimum inhibitory concentration (MICs) of the products pyridoxin was measured using a Resazurin -based turbidimetric (TB) assay. The results show that the pyridoxin has an effect on the strong biofilm Staphylococcus aureus the figure 2 showes that at 200 mg\ml inhibited the growth of bacteria and the minimum inhibitary concentration was 12.5-6.4 mg\ml.From our results the nano pyridoxin has very excellent effects on pathogenic bacteria in our opinion it is effect on bacterial survival in which one of the survival

mechanisms is the production of biofilm.Nanoparticle effect on proteins in bacteria cell wall, that's another reason for inhibiting bacterial growthand consider as bactericidal(6,19).

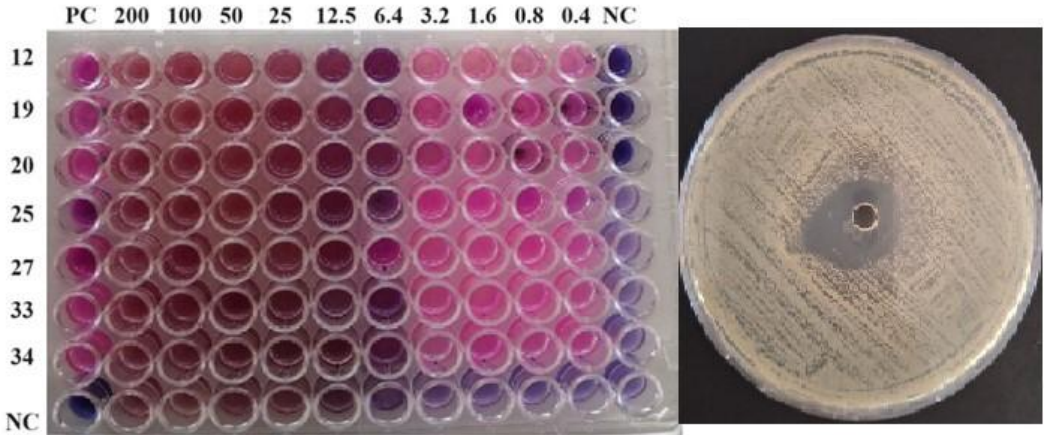

Figure 2: shows the well diffusion assay and microtiter plate.

The effect of pyridoxin was confirmed by the experiment ofgene expression on the seven isolates MDR and strong biofilm-producing of S. aureus after determining their minimum inhibitory concentration (MIC). The analysis involved exposing the isolates to sub-inhibitory concentrations for 24 hours. Then the gene expression of icaA and icaC genes, along with the housekeeping gene 16s rRNA, was measured using Real-time Polymerase Chain Reaction (RT-qPCR). The levels of gene expression were normalized to the housekeeping gene and quantified using the fold change (2^-ΔΔCt) and ΔCt values. melt curve analysis of the samples using RT-qPCR revealed a single peak for the amplicons of icaA, icaC, and 16s rRNA genes as in figures 3,4 and 5.
This observation indicates that the melt curve represents a pure single amplicon in the samples. It also suggests that the amplification specificity of the assay using the intercalating dye is high. the results show that's 85% of the isolates for these two genes (icaA and icaC)was downregulated about half proportional except 14% of the isolates was upregulated two-fold
It was found that co-expression of some genes such as icaA and icaC are necessary for phenotypic expression of biofilm in many isolates(19).Biofilm formation can be raised due to circumstances thatharmful and poisoned to colonies of bacteria.One of these circumstances is the sub-minimum inhibitory concentrations (MICs) of certain antimicrobial and some vitamin, this effect is specific to each strain and is linked to the prompting of stress pathways in S. aureus, which subsequently activate the expression of genes associated with biofilm formation(20).In our investigation we tested the influence of pyridoxin on the expression of icaA and icaC genes within the ica operon. Our findings showed thatthe presence of pyridoxin at a concentration ranging from12.5 to 6.4 mg\ml,will decrease the expression of icaA and icaC genes in biofilm formed by highly adherent strains after 24 hours. Pyridoxine mainly downregulated the expression of both genes in 85% of strongly adherent strains, while increasing expression levels in 15% of the remaining strains. This suggestion may illustrate that pyridoxinat sub-inhibitory concentration like a lot ofother potentialhazardous substances,might activate stress pathways within the bacterial population., leading to that preferred form involves increased the expression of genes linked to biofilm formation.and favored mode of bacterialsurvival.

CONCLUSION

One of the crucial defense mechanisms of bacteria is biofilm formationthat enhance and increase the onset and persistence of contagion. The capacity of MRSA to generate biofilm corelated to challenging -to-cure contagion makesS.aureus a serious and considerablerisks to health of individual,these cases is linked with tolerance of staphylococcal biofilms against antimicrobial and this is leading to ineffectiveness of antibiotic treatments. Therefore,it is essential to develop new therapiesaimed to hinder the formation of biofilms or disrupt them activelyand disrupt its existing. The finding of this study showed a new aspectof pyridoxinsaction involved in the halting of biofilm formation. At concentrations of12.5-6.4 mg\ml of pyridoxin cell viability of S. aureus within the biofilm and the expression levels of genes associated with the production of binding factors and polysaccharide intercellular adhesin (PIA) were notably decreased.. This downregulation of icaA and icaC genes, which encode PIA proteinsmay Impede the colonization of host tissues, including damaged tissues in wounds. Our study showed that pyridoxine inhibits S. aureus adhesion to host extracellular matrix factors through a previously unidentified mechanism involving the reduced expression of crucial genes associated with biofilm formation by S. aureus.

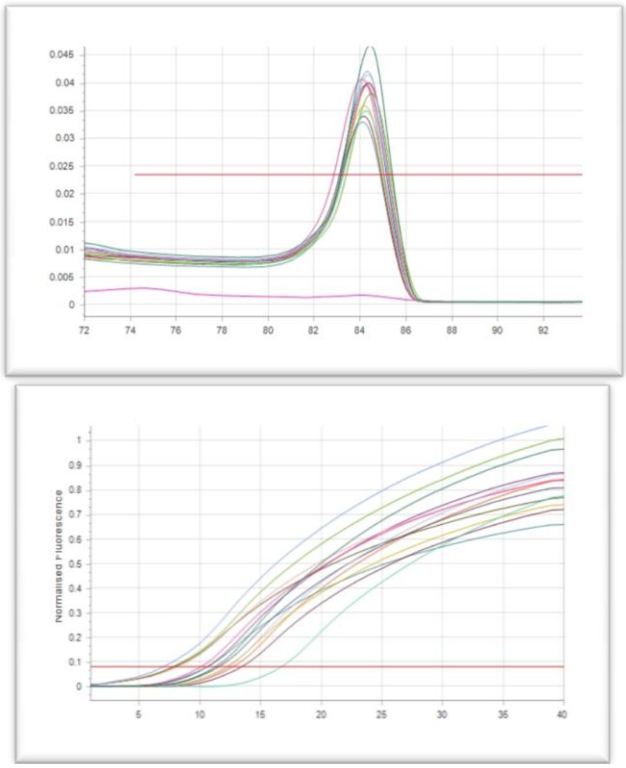

Figure 3: Melt curve and Amplification plots of 16s rRNA gene amplicons after RT-qPCR.

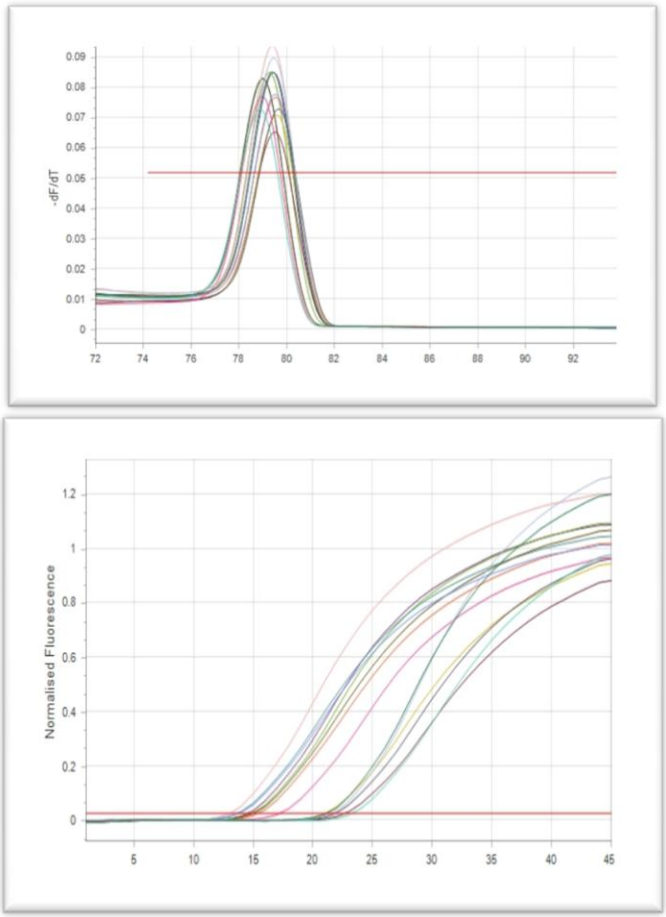

Figure 4: Melt curve and Amplification plots of icaA gene amplicons after RT-qPCR.

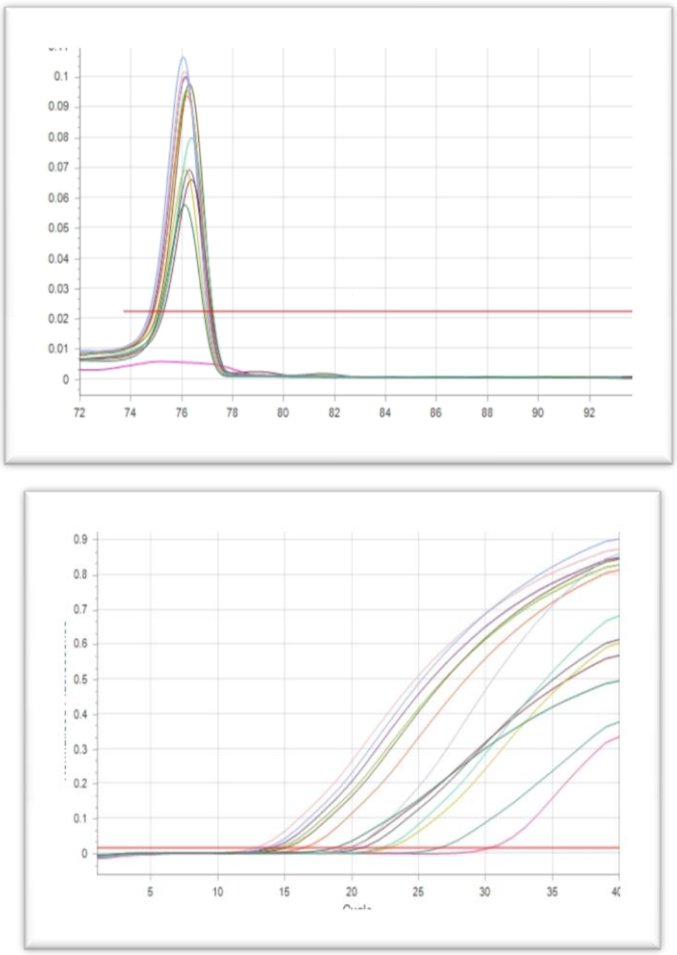

Figure 5: Melt curve and Amplification plots of icaC gene amplicons after RT-qPCR.

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