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The activity of Selenium nanoparticles in combination with Azithromycin on Gene Expression and Genetic Variations of *ica*B and *mec*A of MDR *Staphylococcus aureus*.

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Abstract

Background. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious community well-being risk because of its amplitude for biofilm creation and antibiotic resistance. **Aims.** The current study estimated the efficiency of selenium nanoparticles (SeNPs) unaccompanied and in mixture with azithromycin (AZM) in altering the expression of biofilm-associated (*ica*B) and methicillin resistance (*mec*A) genes in *S. aureus* MRSA isolates. **Methods**. Three *Staphylococcus aureus* MRSA isolates were selected out of total seventeen isolates. Cultured in concentrations of 8 µg/mL AZM, 0.125 µg/mL SeNPs, and their mixture (SeNPs-AZM) were investigated by q-PCR, followed by DNA sequencing analysis. **Results.** Quantitative PCR analysis showed that SeNPs and SeNPs-AZM significantly down-regulated *ica*B (mean fold change: SeNPs, 0.222; SeNPs-AZM, 0.077) and *mec*A (mean fold change: SeNPs, 0.1613; SeNPs-AZM, 0.105), with $P<0.05$ used for all groups in comparison with control. on the other hand, AZM without SeNPs upregulated both genes with mean of fold change (*ica*B, 2.222; *mec*A, 2.5022). Sequence analysis revealed that SeNPs-AZM promoted the maximum number of mutations, which encompassing semi-conserved, conserved missense, and silent mutations, fulfilling in 3% nucleotide and 25% protein variations for *ica*B and 1% nucleotide and 2% protein variations for *mec*A. **Conclusions.** These results established the latent of SeNPs, particularly when mixed with AZM, to decrease biofilm creation and antibiotic resistance by damaging bacterial controlling mechanisms, supplying a talented approach antagonistic toward multidrug-resistant MRSA infections.

keywords: SeNPs, *ica*B, *mec*A, gene expression, sequence analysis

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Introduction:

Staphylococcus aureus is a gram-positive bacterium that occurs in clusters. It inhabits the human skin, conjunctiva, nasal and respiratory tracts. It can cause various illnesses, including open wounds, food poisoning, blood poisoning, and toxic shock syndrome. Some strains are antibiotic resistant, highlighting the importance of reducing antibiotic use (1). Antimicrobial resistance is a global concern. Misuse and overuse of antibiotics have led to the emergence of resistance bacteria. *S. aureus* is highly pathogenic and can cause infections. Methicillin-resistant *S. aureus* is difficult to treat due to multiple resistance genes (2). Combining antibiotics and nanoparticles can help prevent resistance. Selenium nanoparticles are studied for their low toxicity in biomedical applications. They have unique properties not found in bulk forms, including size, structure, surface area, and chemical and

biological activities. They enhance the effects of antibiotics, antifungal drugs, and anticancer drugs, and are promising for overcoming microbial resistance (4). Selenium nanoparticles synthesized by plant extracts show antimicrobial and anti-inflammatory effects. Their activity depends on size, concentration, and surface energy (5). Azithromycin is a macrolide antibiotic used mainly in pediatric medicine. Its long half-life allows for fewer doses per cycle, increasing its effectiveness (6). Amoxicillin inhibits cell wall construction, while other antimicrobials inhibit protein synthesis (7). Azithromycin specifically binds to bacterial ribosomal RNA, preventing protein synthesis and inhibiting bacterial growth. Its unique physical properties, such as an ethyl-substituted N-base, contribute to its ribosome inhibition activity (8). *Staphylococcus aureus* (*S. aureus*) is a prevalent human pathogen that remains resistance to many antimicrobial agents. Its virulence is linked to the *ica* locus which produces polysaccharide intercellular adhesin (PIA). Biofilm formation is driven by the adherence of bacteria on surfaces (9). Methicillin resistance is also common in S. aureus due to the mecA gene, leading to beta-lactam antibiotic resistance (10). Both genes are regulated by various factors and their expression levels impact biofilm formation, chronic illnesses, and drug resistance. This has been a major public health concern in recent years despite multiple antibacterial treatments (9)(11). Therefore, the current research aimed to investigate the effect of selenium nanoparticles (SeNPs) in combination with azithromycin on the expression of icaB and mecA genes of methicillin-resistance *S. aureus.*

Material and Methods

Bacterial DNA and RNA Extraction:

Three staphylococcus aureus isolates that had been previously identified using conventional methods were utilized in this study. All data related to their identification remain unpublished. Selenium nanoparticles (SeNPs) that had been synthesized and characterized in earlier experiments with unpublished data were also used. The isolates were cultured in LB broth supplemented with 1% glucose and 0.25 µg/ml of penicillin to induce the expression of icaB and mecA genes, respectively and then subjected to three treatments: 8µg/ml of azithromycin (AZM), 0.125 µg/ml of SeNPs, and (0.125/8 µg/ml) of SeNPs-AZM before extraction of nucleic acids. For DNA extraction the bacterial isolates were incubated at 37 o C for 24 hours, while for RNA extraction the bacterial isolates were incubated at 37 o C until the bacteria reached 0.8 – 1 optical density (OD600). We utilized a PrestoTM Mini gDNA Bacteria Kit and followed the instructions provided by the manufacturer (Geneaid/Taiwan). Bacterial cells were broken down using lysis buffer to fully disrupt the cell walls. After centrifugation to remove cellular debris, the DNA-containing supernatant was transferred to new tubes. The kit's wash buffers were then used to eliminate contaminants, and the purified DNA was eluted in the provided elution buffer. We utilized the easy-BLUETM Total RNA Extraction Kit and followed the manufacturer's protocol (iNtRON/ Korea) for extracting RNA. We used a specially formulated lysis solution to lyse the cells, which inhibited RNase activity and preserved RNA integrity. The lysates were then centrifuged to remove

debris, and supernatant containing RNA was transferred to new tubes. The RNA samples underwent washing steps to remove impurities, and elution of the RNA was carried out using a buffer provided by the kit.

Estimation of the Concentration and Integrity of DNA and RNA:

The purity of DNA and RNA was determined using Nanodrop (Thero Scientific NanoDrop Lite UV-Visible Spectrophotometer, USA), while the integrity of DNA was analyzed using 0.8% agarose gel electrophoresis according to (12). While for RNA using formaldehyde agarose (1.5%) gel electrophoresis described by (13).

Detection of *ica*B and *mec*A Gene by PCR:

In this study, we designed four primers for the *ica* genes cluster (*ica*A, *ica*B, *ica*C, *ica*D) and primer for *mec*A as shown in Table 1. The primers were prepared by dissolving the stock solutions (100 pmol/µl) in nuclease free water according to the company's instructions (**MacroGene/ Korea**). After that, the work solutions of all primers were diluted to be (10 pmol/µL). The components (25µL) of the master mix reaction were prepared using Master Kit (GoTaq® Green) according to the company instructions as follows: 5µL template of DNA (5-50 ng), 2µL sense and antisense primer (10 pmol), 12.5 µL PCR master (GoTaq® Green) and 3.5 µL PCR water. The conditions of the PCR thermocycler were: One cycle of Initial Denaturation (95ºC, 3 min); 30 cycles of Denaturation (95ºC, 30 sec), Annealing (58ºC, 30 sec), Extension (72ºC, 1 min); One cycle of final extension (72ºC, 5 min); Hold (4ºC). Finally, the PCR product was analyzed using 1.5% of agarose gel electrophoresis according to (12).

Table 1: Primer Sequences for the Conventional PCR

PCR primer	Sequence (5`- 3`)		Size of Amplicon	NCBI Reference
*ica*A gene	**F**	TCATTGAACAAGAAGCCTGACA	972 bp	NC_017341.1
	R	GCGACTATCAATAAAGAGTGCGA		
*Ica*B gene	**F**	CCTGTAAGCACACTGGATGGT	411 bp	NC_007795.1
	R	TTCTTCCCCAACATGACCTG		
*Ica*C gene	**F**	TGTCACAGTTACTGACAACCTTGA	557 bp	NC_007795.1
	R	TGAAAAGCTGGTAACGTTCCA		
*Ica*D gene	**F**	GCTCAAGGGGGACACGAA	357 bp	KY322719.1
	R	GACAAGAACTACTGCTGCGTT		
*Mec*A gene	**F**	TCGTGTCACAATCGTTGACG	959 bp	NG_047936.1
	R	ACCACCCAATTTGTCTGCCA		

The Expression of *ica*B and *mec*A Genes by q-PCR:

The expression levels of *ica*B and *mec*A genes of *S. aureus* isolates were determined before and after being treated with AZM, SeNPs and SeNPs-AZM. To achieve that, we designed other primers for *ica*B and *mec*A genes as well as *rpo*B housekeeping gene as shown in Table 2. The primer work solutions (10 pmol/ µL) were prepared as previously described.

Table 2: Primer Sequences for the q-PCR

PCR Primer	Sequence (5`- 3`)		Size of Amplicon	NCBI Reference
*ica*B gene	**F**	TCCTTATGGCTTGATGAATGACG	95 bp	NC_007795.1
	R	TCGGAGTGACTGCTTTTTCC		
*mec*A gene	**F**	TGGCAGACAAATTGGGTGGT	108 bp	NG_047936.1
	R	GCATTGTAGCTAGCCATTCCT		
*rpo*B gene	**F**	TTCAGCAGCGACAGCATGTA	171 bp	NC_007795.1
	R	GCTGTAATAGCCGCACCAGA		

The extracted RNA was treated with DNase I enzyme kit (**Promega/ USA**) to remove any residual genomic DNA that might be present. By following the protocol provided by the company, the mixture of reaction includes 10µL total RNA 1µg, 1µL DNase I enzyme, 4 µL 10X buffer and 5µL DEPC water was incubated at 37ºC for 30 min, then 1 µL of stop solution was added. Finally, the mixture was further incubated at 65ºC to deactivate the DNase I enzyme. After that, RNA was converted to cDNA using the M-MLV Reverse Transcriptase kit (**Bioneer/ Korea**) following a two-step procedure as described by the company. The components of step 1include: 8 µL total RNA 100 ng/ µL, 1 µL random hexamer primer, and 1 µL DEPC water. These components were mixed and denatured at 65ºC for 10 min, then immediately chilled on ice. The step 1 components were added to the step 2 components (1 µL M-MLV RTase (200 µ), 4 µL 5X M-MLV RTase reaction buffer, 2 µL of 100 mM DTT, 2µ DNTP, 1µL RNase inhibitor), and then all components were vortexed and spun down for few minutes. Finally, the following thermocycler conditions (42ºC for 1 hour of synthesis of cDNA (RT step);95ºC for 5 minutes of heat inactivation) were applied to complete the conversion of RNA to cDNA. Master Mix Kit (Go Taq® qPCR) (**Promega/USA**) was used to quantify the expression level of *ica*B and *mec*A genes for MDR *S. aureus*. The components of q-PCR reaction include: 5 µL Template of cDNA (10ng); 1µL sense and antisense primer (10 pmol): 10µL master mix of qPCR; 3µL PCR-water. The conditions of q-PCR were: one cycle of Initial Denaturation (95ºC, 10 min) 39 cycles of Denaturation (95 C, 10 sec.) and Annealing\Extension\Detection (scan) (60 °C, 30 sec.); one cycle of Melting (65-95°C). The data of q-PCR were calculated and analyzed based on the Livak method.

Sequencing Analysis of *ica*B and *mec*A Genes:

The sequencing analysis of *ica*B and *mec*A genes of *S. aureus* was performed before and after treatment with AZM, SeNPs, and SeNPs-AZM to identify the genetic variations in these genes by following the sangar sequencing method (14) using sense and antisense primers. The data of sequences for both genes were read and edited by Finch TV software; the assembly of sense and antisense sequencing was done by CAP3 software; the nucleotide sequences were converted into amino acids sequences by open reading frame (ORF) program from NCBI; and sequence alignment between the control group and treated groups was achieved by blastp (protein-protein BLAST) program from NCBI.

Statistical Analysis:

The data obtained were statistically analyzed using IBM, SPSS V.23. One-way ANOVA was performed to calculate LSD of gene expression data. The values of mean ± SD were used to display the data in histograms for comparison different groups of treatments.

Results

The DNA and RNA Extraction:

The integrity of genomic DNA in the DNA gel (Figure 1A) looked to be youthful crossways all circumstances. The single separate bands detected for all groups recommended fruitful extraction of genomic DNA with high molecular weight. The control and other groups (AZM, SeNPs, SeNPs-AZM) showed a consistent band intensity, indicating optimal extraction from bacterial samples. The occurrence of separate 23S and 16S rRNA bands in the RNA gel electrophoresis (Figure 1B) referred to higher integrity of RNA. The control samples and treated samples displayed vibrant, shrill bands, confirming the extraction efficacy by easy-BLUE^{TM} Total RNA Extraction Kit.

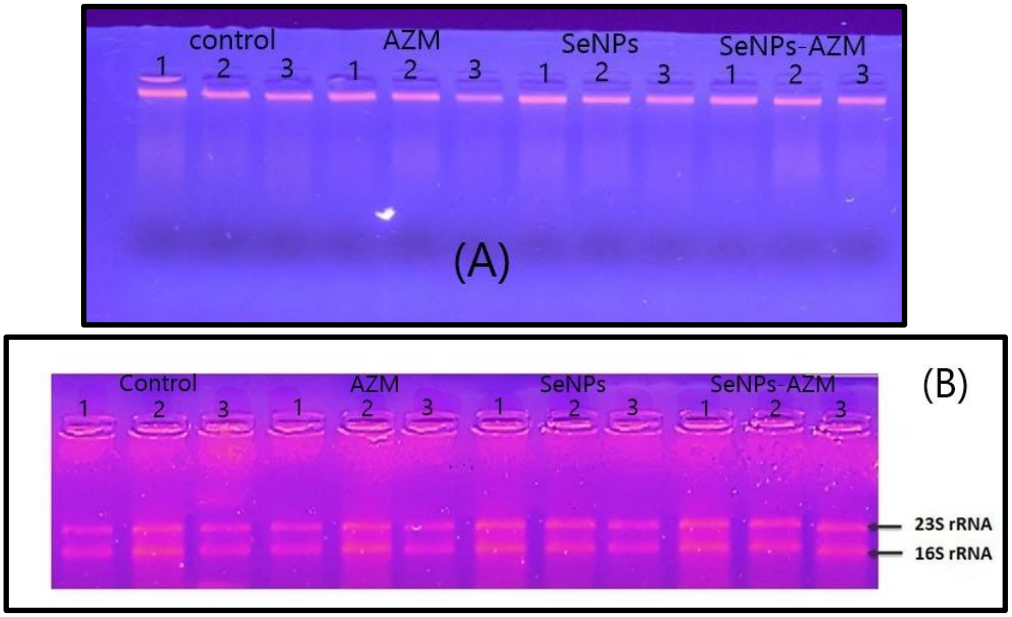

Figure 1: The gel electrophoresis of whole genomic DNA (A) and total RNA (B) of *Staphylococcus aureus* treated with AZM, SeNPs and SeNPs-AZM.

Detection of *ica*B and *mec*A Genes by PCR:

The gel electrophoresis outcomes displayed the amplification of precise *ica* cluster and *mec*A genes in s*taphylococcus aureus* isolates. For the *ica* cluster, bands matching to *ica*B (411bp) and *ica*C (557bp) were fruitfully amplified in the DNA of bacterial isolates, as demonsterated by pure, separate bands in the gel. Nevertheless, no bands were detected at 972 bp (*ica*A) and 357 bp (*ica*D), indicating the absenteeism or absence of amplification of these specific genes in the verified isolates.

On the other hand, the results of gel electrophoresis for the *mec*A gene revealed separate bands at 959 bp in wholly three isolates, ensuring the existence of this gene, which is an important indicator of methcilline resistance in *S. aureus*. The molecular weight markers in both gels authenticated the predictble band sizes and reffered to specific amplification deprived of signs of spreading or degradation as shown in (Figure 2 A and B).

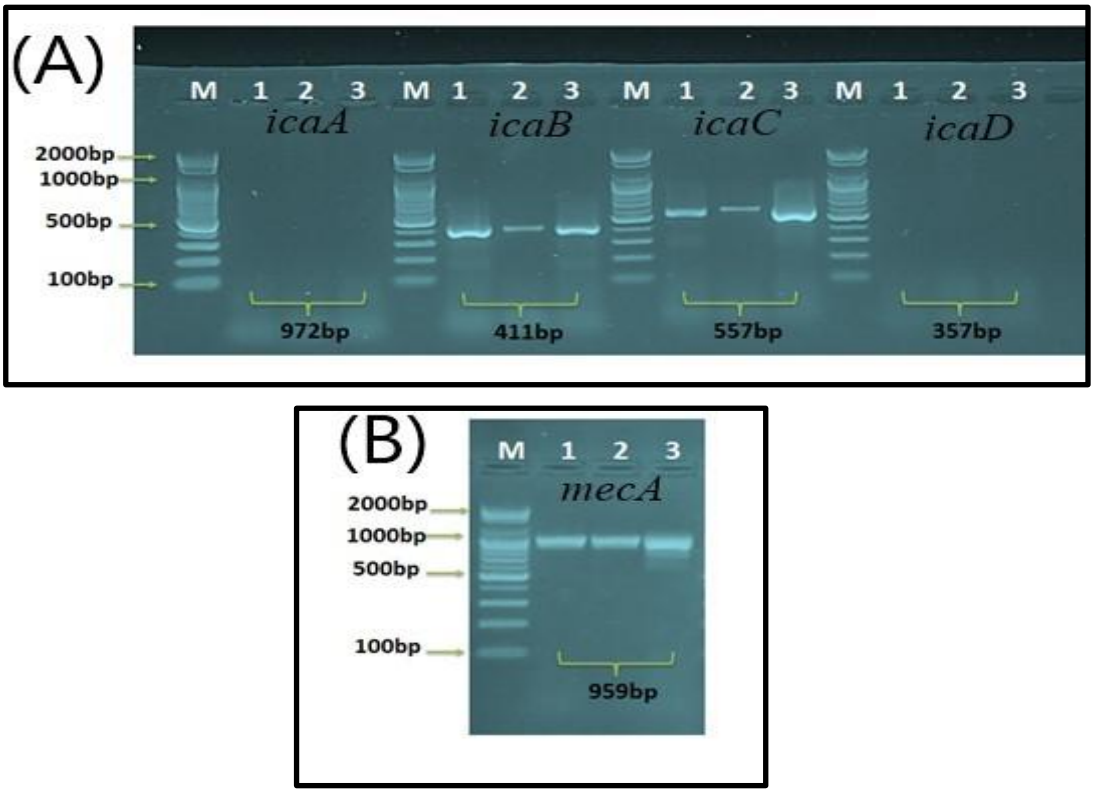

Figure 2: Agarose gel electrophoresis image that showed PCR product analysis. (A): biofilm formation (*ica*A, *ica*B, *ica*C, and *ica*D) genes in pathogenic *Staphylococcus aureus* isolates. M (Marker ladder 2000-100bp). The samples lanes showed three pathogenic *Staphylococcus aureus* isolates that show only positive for biofilm formation (*icaB* and *ica*C) genes at 411bp and 557bp PCR product size respectively; (B): methicillin resistance *(mec*A) gene in pathogenic *Staphylococcus aureus* isolates. M (Marker ladder 2000-100bp). The sample lanes showed three pathogenic *Staphylococcus aureus* isolates that show positive for methicillin resistance (*mec*A) gene at 959bp PCR product size

The Expression of *ica*B and *mec*A Genes by q-PCR:

The outcomes that existed in Figure 3 displayed the influence of AZM, SeNPs, and SeNPs-AZM on the expression of the biofilm-related gene *ica*B. The control characterized the starting point of fold chaing mean in of expression of *ica*B gene, which was 1.112. The AZM significantly P<0.05 improved the fold change into 2.222, which specified the uperegulation of *ica*B.

However, unaccompanied SeNPs and accompanied SeNPs-AZM exhibited abundant minor mean fold changes, which were 0.222 and 0.077, correspondingly. These outcomes were statistically significant P< 0.05 in dipping the expression of *ica*B gene compared with together control and AZM treatments. Additionally, SeNPs-AZM was significantly higher than all treatment groups in the lowering of *ica*B gene expression, which recommended that the mixed treatment was the greatest active in the dowenregulation of biofilm gene expression.

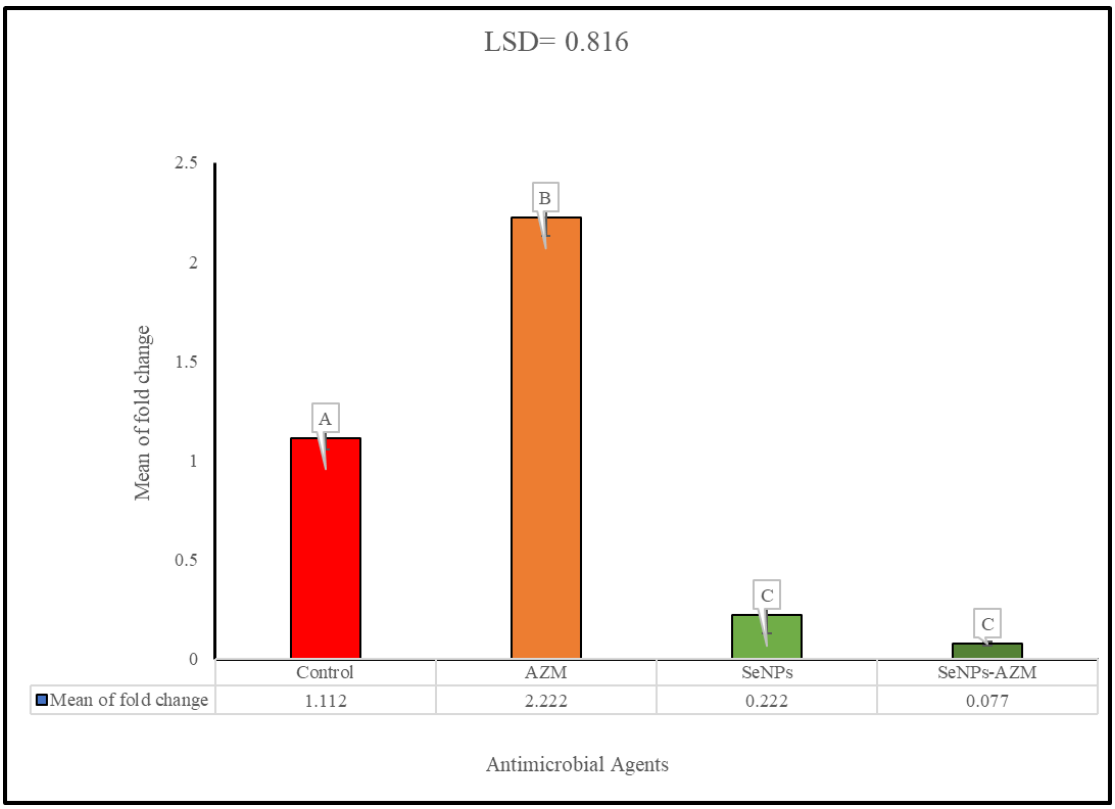

Figure 3: The mean of expression of *ica*B gene expressed by folding change in *MDR S. aureus* experimental isolate against AZM, SeNPs and SeNPs-AZM compared with untreated control isolate. Different letters indicated statistically significant at P< 0.05.

The outcomes in Figure 4 exhibited the influences of AZM, SeNPs, and SeNPs-AZM on the expression of *mec*A gene in *S. aureus.* The *mec*A gene, a serious part of methicillin resistance, is essential in the profile of bacterial resistance. The mean of fold change in *mec*A expression was 1.047 in the control group, mentioned as the baseline expression. The AZM treatment directed to a substantial upregulation of the expression of *mec*A gene, mirrored by a mean fold change of 2.502. This increase proposed that *S. aureus* might upregulate *mec*A responding to AZM, perhaps as part of it resistance mechanism to this antibiotic.Contrariwise, uncombined SeNPs and the mixture of SeNPs-AZM treatments pointedly dowenregulated the expression of *mec*A. The mean fold change for SeNPs was 0.161, wherease the SeNPs-AZM combination extra-decreased the expression, achieving a mean fold change of 0.105. These results specified that unaccompanied SeNPs, or combied with unaccompaied SeNPs, or combined with AZM, efficiently downregulated *mec*A expression, which might alleviate resistance in *S. aureus*.Statistical analysis according to the LSD value of 0.563, approved the significance of theses differences. The labelled groups with dissimilar letters referred to statistically significant differences at P< 0.05 in the expression of the *mec*A gene. Precisely, the AZM group displayed upregulation of *mec*A, which was statistically discrete from SeNPs and SeNPs-AZM groups, which established dowenregulation. This statistical difference highlighted the oppressive impact of SeNPs on the expression of the *mec*A gene in comparison with AZM, signifying that SeNPs might be a valued method in dropping the resistance of bacteria via the dowenregulation of the genes responsible for resistance.

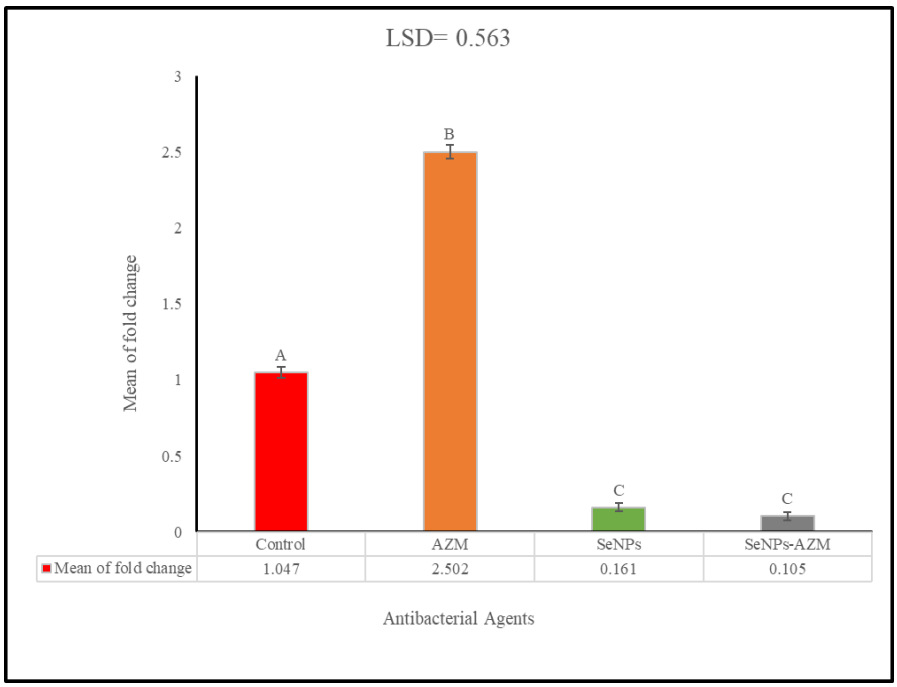

Figure 4: The mean of expression of *mec*A gene expressed by folding change in *MDR S. aureus* experimental isolate against AZM, SeNPs and SeNPs-AZM compared with untreated control isolate. Different letters indicated statistically significant at P< 0.05.

Sequencing Analysis of *ica*B and *mec*A Genes

The sequence examination of the *ica*B gene in *Staphylococcus aureus* revealed various nucleotide (N) and protein (P) sequence polymorphisms across treatments together with variations in sequence identity (Figure 5, 6 and Table 3). Implementing the AZM treated group, a single nucleotide replacement occurred which makes a semi-conservative missense transition (325 A>G) (T>A, Thr/Ala), and 99% N identity, 96% P identity, 1% nucleotide variation, and 4% P variation. In the SeNPs-treated group, 322 G>A, 331 T>C, 338 A>G, 354 A>G, 358 A>G, 383 T>G, and 395 T>G mutations resulted in 98% N and 82% P sequence identity and 2% N and 16% P sequence variation. These mutations included semi-conserved missense mutations (3 P>C, 6 Y>H, 15 K>E, 25 E>C) and a conserved mutation (8 N>S), along with two silent mutations: Two SNPs are identified to be 354 A>G (Lysine, K) and 395 T>G (Asparagine, N). SeNPs-AZM treated group yielded nucleotide changes 330 T>A, 338 A>G, 354 A>G, 358 A>G, 382 T>G, 387 T>G, 395 T>G, out of which, 97% N and 75% P sequence identity with the reference sequence and 3% N and 25% P sequence variation. This group exhibited semi-conserved missense mutations (7 E>G, 8 S>Q, 10 Y>H, 16 Y>H, 23 P>V, 24 P>M) and a conserved mutation (19 P>Q), alongside four silent mutations: Substitutions were identified at positions 330 T>A (serine, S), 354 A>G (lysine, K), 382 T>G (tyrosine, Y), and 387 T>G (tyrosine, Y). Specifically, these findings indicated that both SeNPs and SeNPs-AZM treatments encouraged additional nucleotide and protein polymorphisms than AZM treatment, where silent mutations sustain amino acid sequences while missense semi-conserved, less conservation) and conserved mutations. may alter the structural or functional characteristics of the *ica*B protein.

(A)

Score	Expect	Identities	Gaps	Strand
734 bits(397)	0.0	399/400(99%)	0/400(0%)	Plus/Plus

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Sbjct  1    ............................................................  60

Query  61   CTGAAATATAAAGAAAATAGTGCTCTGGCATTAAATTATCACCGTGTAAGAAAAGCGAAT  120
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(B)

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(C)

Score	Expect	Identities	Gaps	Strand
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Figure 6: Pairwise sequence alignment of *ica*B gene of MDR *S. aureus*. (A) AZM against control;(B) SeNPs against control and (C) SeNPs-AZM against control. Query referred to control isolate, while sbjct referred to treated isolates

In this study, polymorphism in nucleotide (N) and protein (P) *mec*A gene sequences of *Staphylococcus aureus* was conducted based on three treatments (Figure 7, 8 and Table 4). In the AZM-treated group, functional mutations of a nucleotide polymorphism (415 A>G, 504 T>A) caused conserved missense mutation (97 K>E, 126 I>N) resulting in 99% identity and 1% variation for both N and P. For the SeNPs-treated group, nucleotide changes (575 A>C, 585 A>G, 677 T>C, 678 A>G, 755 A>G, 766 C>G, 772 A>G, 799 T>A, 841 A>G) resulted in missense mutations, including semi-conserved (92 L>H, 146 K>E, 207 A>P) and conserved (124 K>R), along with three silent mutations 678 (A>G), 772 (A>G) and 861 (A>G) where Alanine, Lysine and Glutamic Acid remained unchanged. This group maintained 99 % N identify 1 % variation and 98% P identify 2% variation.

In the SeNPs-AZM-treated group, nucleotide polymorphisms (505 T>A, 529 A>T, 576 T>C, 663 G>A, 679 C>T, 764 T>A, 769 A>G, 804 C>G, 824 A>C, 847 A>C, 861 A>G, 871 T>A, 873 A>G) resulted in semi-conserved missense mutations (150 G>A, 179 G>D, 190 P>S, 233 N>D, 245 R>R, 250 P>G) and seven silent mutations 692 (C>T), 706 (T>A), 790 (A>G), 808 (A>G), 824 (A>G), 847 (A>G) and 861 (A>G) where Threonine, Phenylalanine, Glutamic acid, Glutamic acid, Lysine, Glycine and Glutamic acid remained unchanged. This group also had 99% N identity (1% variation) and 98% P identity (2%). Silent mutations over the cell maintained the protein sequence, while semiconservative and coarse conservative missense mutations caused structural and functional alterations in the protein encoded by *mec*A. These results show that both SeNPs and SeNPs-AZM as a treatment place more selective evolutionary pressure on the *mec*A gene than that of AZM treatment alone.

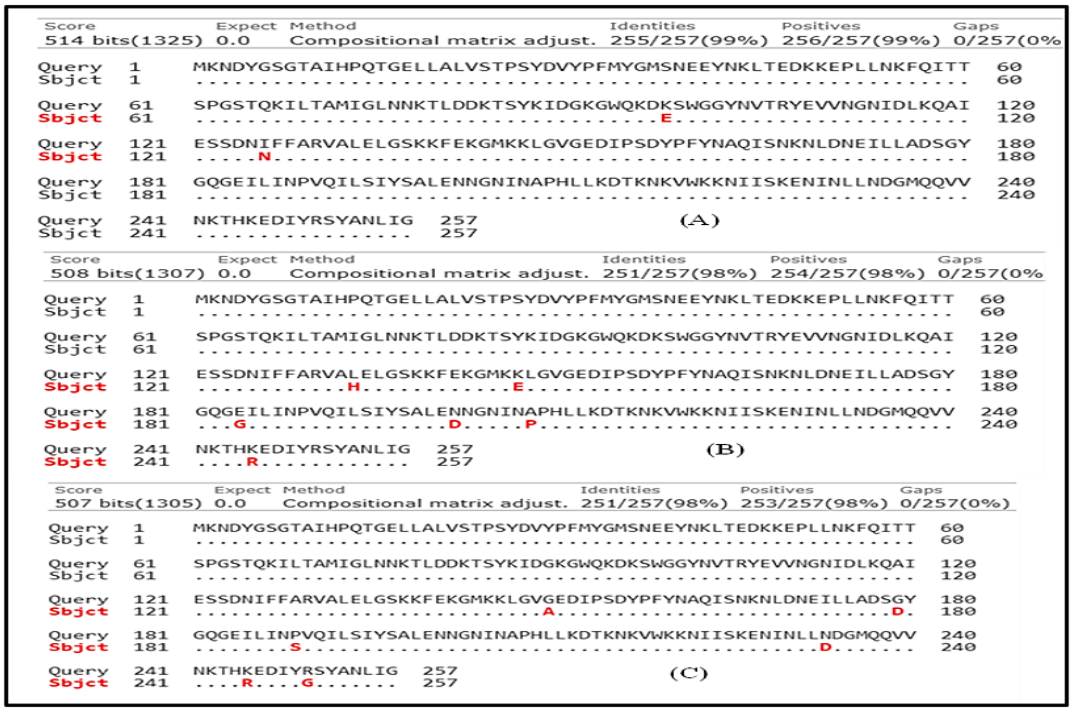

Figure 7: Pairwise sequence alignment of PBP 2a of MDR *S. aureus*. (A) AZM against control; (B) SeNPs against control and (C) SeNPs-AZM against control. Query referred to control isolate, while sbjct referred to treated isolates.

(A)

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Query  661  AGGTTACGGACAAGGTGAAATACTGATTAACCCAGTACAGATCCTTTCAATCTATAGCGC  720
Sbjct  661  ..............................................................  720

Query  721  ATTAGAAAATAATGGCAATATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGT  780
Sbjct  721  ..............................................................  780

Query  781  TTGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAAATGATGGTATGCAACA  840
Sbjct  781  ..............................................................  840

Query  841  AGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTTATGCAAACTTAATTGGCAA  900
Sbjct  841  ..............................................................  900
```

(B)

Score	Expect	Identities	Gaps	Strand
1613 bits(873)	0.0	891/900(99%)	0/900(0%)	Plus/Plus

```
Query  1    TCGTGTCACAATCGTTGACGATAATAGCAATACAATCGCACATACATTAATAGAGaaaaa  60
Sbjct  1    ..............................................................  60

Query  61   gaaaaaaGATGGCAAAGATATTCAACTAACTATTGATGCTAAAGTTCAAAAGAGTATTTA  120
Sbjct  61   ..............................................................  120

Query  121  TAACAACATGAAAAATGATTATGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATT  180
Sbjct  121  ..............................................................  180

Query  181  ATTAGCACTTGTAAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGTAA  240
Sbjct  181  ..............................................................  240

Query  241  CGAAGAATATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCCAGAT  300
Sbjct  241  ..............................................................  300

Query  301  TACAACTTCACCAGGTTCAACTCAAAAAATATTAACAGCAATGATTGGGTTAAATAACAA  360
Sbjct  301  ..............................................................  360

Query  361  AACATTAGACGATAAAACAAGTTATAAAATCGATGGTAAAGGTTGGCAAAAAGATAAATC  420
Sbjct  361  ..............................................................  420

Query  421  TTGGGGTGGTTACAACGTTACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACA  480
Sbjct  421  ..............................................................  480

Query  481  AGCAATAGAATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGTAA  540
Sbjct  481  ......................A.......................................  540

Query  541  GAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATATACCAAGTGATTATCC  600
Sbjct  541  ......................G.......................................  600

Query  601  ATTTTATAATGCTCAAATTTCAAACAAAAATTTAGATAATGAAATATTATTAGCTGATTC  660
Sbjct  601  ..........................................C...................  660

Query  661  AGGTTACGGACAAGGTGAAATACTGATTAACCCAGTACAGATCCTTTCAATCTATAGCGC  720
Sbjct  661  ..................G...........................................  720

Query  721  ATTAGAAAATAATGGCAATATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGT  780
Sbjct  721  .......G..................C.........................G........  780

Query  781  TTGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAAATGATGGTATGCAACA  840
Sbjct  781  ..................A...........................................  840

Query  841  AGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTTATGCAAACTTAATTGGCAA  900
Sbjct  841  ..................G...........................................  900
```

(C)

Score	Expect	Identities	Gaps	Strand
1591 bits(861)	0.0	887/900(99%)	0/900(0%)	Plus/Plus

```
Query  1    TCGTGTCACAATCGTTGACGATAATAGCAATACAATCGCACATACATTAATAGAGaaaaa  60
Sbjct  1    ..............................................................  60

Query  61   gaaaaaaGATGGCAAAGATATTCAACTAACTATTGATGCTAAAGTTCAAAAGAGTATTTA  120
Sbjct  61   ..............................................................  120

Query  121  TAACAACATGAAAAATGATTATGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATT  180
Sbjct  121  ..............................................................  180

Query  181  ATTAGCACTTGTAAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGTAA  240
Sbjct  181  ..............................................................  240

Query  241  CGAAGAATATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCCAGAT  300
Sbjct  241  ..............................................................  300

Query  301  TACAACTTCACCAGGTTCAACTCAAAAAATATTAACAGCAATGATTGGGTTAAATAACAA  360
Sbjct  301  ..............................................................  360

Query  361  AACATTAGACGATAAAACAAGTTATAAAATCGATGGTAAAGGTTGGCAAAAAGATAAATC  420
Sbjct  361  ..............................................................  420

Query  421  TTGGGGTGGTTACAACGTTACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACA  480
Sbjct  421  ..............................................................  480

Query  481  AGCAATAGAATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGTAA  540
Sbjct  481  ......................A.......................G...............  540

Query  541  GAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATATACCAAGTGATTATCC  600
Sbjct  541  ..........................................C...................  600

Query  601  ATTTTATAATGCTCAAATTTCAAACAAAAATTTAGATAATGAAATATTATTAGCTGATTC  660
Sbjct  601  ..............................................................  660

Query  661  AGGTTACGGACAAGGTGAAATACTGATTAACCCAGTACAGATCCTTTCAATCTATAGCGC  720
Sbjct  661  ..A.........................T.............A.....................  720

Query  721  ATTAGAAAATAATGGCAATATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGT  780
Sbjct  721  ..............................................................  780

Query  781  TTGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAAATGATGGTATGCAACA  840
Sbjct  781  .........G..................G.................G.............  840

Query  841  AGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTTATGCAAACTTAATTGGCAA  900
Sbjct  841  ......G.............G.........A...G.............................  900
```

Figure 8: Pairwise sequence alignment of *mec*A gene of MDR *S. aureus*. (A) AZM against control; (B) SeNPs against control and (C) SeNPs-AZM against control. Query referred to control isolate, while sbjct referred to treated isolates.

Table 3: NCBI Homology Sequence Identity of *ica*B Gene and its PIA de-acetylase Protein of MDR *S. aureus* Isolate before and after Treatment With AZM, SeNPs, and SeNPs-AZM

Isolate before treatment	Isolates after treatment	NCBI homology sequence identity						
		Nucleotide polymorphism	Amino acid Polymorphism	Type of polymorphism	Percentage of identity		Variations of Percentage	
					N	P	N	P
Control	AZM	325 A>G	4 T>A	Missense	99 %	96 %	1 %	4 %
Control	SeNPs	322 G>A, 331 T>C, 338 A>G, 354 A>G, 358 A>G, 383 T>G, 395 T>G	3 E>K, 6 Y>H, 8 N>S, 15 K>E, 23 F>C	Missense	98 %	82 %	2 %	16 %
Control	SeNPs-AZM	325 A>G, 330 T>A, 335 A>G, 338 A>T, 343 T>C, 354 A>G, 361 T>C, 369 A>T, 371 C>A, 381 T>G, 386 T>G	7 E>G, 8 N>I, 10 Y>H, 16 Y>H, 19 P>Q, 23 F>V, 24 I>M	Missense	97 %	75 %	3 %	25 %

Table 4: NCBI Homology Sequence Identity of *mec*A Gene and its PBP 2a Protein of MDR *S. aureus* Isolate before and after Treatment with AZM, SeNPs, and SeNPs-AZM

Isolate before treatment	Isolates after treatment	NCBI homology sequence identity						
		Nucleotide polymorphism	Amino acid Polymorphism	Type of polymorphism	Percentage of identity		Variations of Percentage	
					N	P	N	P
Control	AZM	416 A>G, 504 T>A	97 K>E, 126 I>N	Missense	99 %	99 %	1 %	1 %
Control	SeNPs	525 T>A, 563 A>G, 637 T>C, 678 A>G, 728 A>G, 746 G>C, 772 A>G, 799 T>A, 861 A>G	132 L>H, 146 K>E, 207 A>P, 244 K>R	Missense	99 %	98 %	1 %	2 %
Control	SeNPs-AZM	505 T>A, 529 A>G, 576 G>C, 663 G>A, 692 C>T, 706 T>A, 790 A>G, 808 A>G, 824 A>G, 847 A>G, 861 A>G, 871 T>A, 875 A>G	150 G>A, 179 G>D, 190 P>S, 233 N>D, 245 K>R, 250 R>G	Missense	99 %	98 %	1 %	2 %

Discussion

The DNA and RNA Extraction:

These results recommend that both DNA and RNA were fruitfully isolated with maximum value from *S. aureus* below diverse investigational circumstances. The outcomes established the vigorous procedures used to extract DNA and RNA, guaranteeing the conservation of integrity although bacterial isolates were treated with different antibacterial agents. The uniformity of DNA and RNA quality in SeNPs-treated samples recommend that selenium nanoparticles, utilized uncombined or combined with AZM, apply negligible hostile impacts on stability through the growth of bacteria. These observations offer a basis for additional genetic analyses to discover the responses of *S. aureus* to these treatments at genomics and transcriptomics levels.

Detection of *ica*B and *mec*A Genes by PCR

The absenteeism of *icaA* and *icaD* gene intensification in the isolates, herein, referred to numerous likelihoods. Firstly, it is conceivable that these genes do not exist in the verified *S. aureus* isolates, signifying that these isolates could absence precise components of the *ica* operon in charge of biofilm formation. This might reverberate genetic changeability among diverse *S. aureus* isolates, as not wholly medical isolates accommodate a whole *ica* operon [(15)]. Otherwise, mutations in the primer binding regions of these genes might block amplification, causing the absenteeism of bands. Additional possible elucidation might be the difference in the expression or regulation of *icaA* and *icaD* genes (16)(17). These genes might exist but are quiet or expressed at low-slung levels below the verified circumstances, requisition their amplification stimulating deprived of precise environmental incentives or stress issues that upregulate the *ica* operon (18). These results recommended that the verified *S. aureus* isolates might not depend on the whole *ica* operon for biofilm formation, possibly representing substitute biofilm formation pathways. The existence of *icaB* and *icaC* proposed fractional operon functionality, which could be adequate for certain features of biofilm production (19). The simultaneous recognition of the *mec*A gene established the nature of methicillin-resistant isolates, focusing on their medical importance. Generally, these outcomes highlighted the genetic variety of *S. aureus* and the necessity for additional investigations to know the controlling pathways and genetic factors affecting the expression and functionality of the *ica* operon in diverse isolates.

The Expression of *ica*B and *mec*A Genes by q-PCR

The downregulation effect of SeNPs and the improved influence when syndicated with AZM could be indicated via numerous mechanisms. Nanoparticles like SeNPs might damage the expression of biofilm genes concluded direct communication with the cell wall of bacteria, subsequent in oxidative stress and damaging cell signaling pathways essential for biofilm establishment (20). In mixture treatment, SeNPs might perform synergistically with AZM to improve the penetrability of bacterial cells to AZM, which means increasing the destruction of biofilm gene expression (21). This cooperation might also be attributed to SeNPs possibly dropping antibiotic resistance mechanisms, permitting AZM to be more active in minor doses (22). The current outcomes were in line with a study conducted by Haddadian and his coauthors who exhibited that the expression level of *ica*D of *S. aureus* was significantly downregulated when

preserved with SeNPs-niosome compared with SeNPs and noisome alone (23). An additional study offered that the use of SeNPs as a co-adjuvant in vaccine preparation for MRSA improved the response of immunity and emphasized their probable role in fighting antibiotic-resistant isolates (24). These results recommend that SeNPs unaccompanied or in combination with antibiotics might present a brilliant strategy toward *S. aureus* infections, encompassing those produced by resistance isolates. The current results were paralleled with some results that used SeNPs against antibiotic resistance genes such as the results found that SeNPs (1500 µg/mL) alone and SeNPs with ampicillin (375 µg/mL) significantly downregulated the *oqx*B gene of *Klebsiella pneumoniae* (25). An Iraqi local study revealed that the biogenic SeNPs (250 µg/ml) downregulated the expression of *mex*B of *Pseudomonas aeruginosa* (26). A study conducted by Zhang and his colleagues showed that the biological SeNPs downregulated the expression of antibiotic-resistance genes and virulence factors in broiler manure by damaging seleno-compound metabolism and chemotaxis pathways in several bacterial genera (*Butyricimonas*, *Odoribacter*, *Paraprevotella*,*Rikenella*,*Lactobacillus*, *Candidatus,Borkfalkia*, *Merdimonas*, *Oscillibacter*, *Intestinimonas*, *Megamonas*, and *Desulfovibrio*) (27). One possible method through which SeNPs downregulate *mec*A is the activation of oxidative stress in bacterial cells. SeNPs are recognized to produce reactive oxygen species (ROS) when introduced to bacterial cells. Raised levels of ROS able to damage cellular parts, such as DNA, proteins, and lipids, which perhaps cause a decrease in the expression of the *mec*A gene as an essential element of a comprehensive bacterial pressure response. Extreme oxidative stress can damage the resistance mechanisms of bacteria and, as a result, improve sensitivity to antibacterial drugs (28).

The penicillin-binding protein 2a (PBP2a) is encoded by the *mec*A gene, and PBP2a plays a serious function in the synthesis of bacterial cell walls and offers resistance against β-lactam antibiotics (29). Investigations have revealed that SeNPs are able to interact with the safety and function of the cell wall, possibly suppressing the activity and expression of PBP2a (30). The biosynthesis pathways of the cell wall are directly targeted via SeNPs and influence the monitoring networks' regulatory expression of the *mec*A gene, thus downregulating it (31). Some studies recommend that metal nanoparticles, such as SeNPs, be able to impact the expression and epigenetic mechanisms of bacterial genomics. SeNPs can modify the governing pathways of the gene required in resistance, whether via direct contact with DNA or via affecting transcriptional regulators (32). This could damage the transcription of *mec*A, subsequently decreasing the levels of PBP2a and reducing resistance. The mixture of SeNPs-AZM probably applies to a synergistic impact, where SeNPs damage the safety of the cell wall, raising the permeability of the membrane, and therefore enabling the access of AZM to bacterial cells. This mixture is able to improve the activity of antibacterial toward *S. aureus*, downregulating the expression of *mec*A as essential components of bacterial defense response. The efflux pumps are vital for bacteria to be resistant, as they vigorously convey antibiotics outside of bacterial cells (33). SeNPs, mainly combined with AZM, have been shown to decrease the activity of efflux pumps in some bacterial types. By damaging these agents, SeNPs-AZM is able to hold sophisticated intracellular concentrations of AZM, raising its efficiency and donating

the downregulation of *mec*A as the cell's resistance mechanisms are overcome (25). Azithromycin is one of the macrolide antibiotics, the primary is inhibiting the protein synthesis of bacteria. Once mixed with SeNPs, which are able to damage cellular structures and oxidative equilibrium, these double pathways can provide a robust repressive impact on PBP2a protein synthesis. This suppression caused a reduction in the expression of *mec*A as the bacterial cell reallocates references to respond to the double pressures forced by SeNPs and AZM.

Sequencing Analysis of *ica*B and *mec*A Genes

The relative expression levels of the *ica*B and *mec*A genes in *Staphylococcus aureus* are varied depending on the type of mutation; semi-conserved, conserved missense and silent type of mutations. Conserved and semi-conserved missense mutations saturate the divergent amino acids. They, therefore, may change the physicochemical properties of the amino acids, and as a result, compromise the transcriptional regulatory active sites or domains (34). For example, the observed higher fold change in the AZM-treated group may be due to occasional changes, so while the gene cannot produce its original product in full, it may remain somewhat active. On the other hand, fold changes in *ica*B and *mec*A expression were obtained from *S. aureus* after treatment with SeNPs and SeNPs-AZM are appreciably lower. Multiple simultaneous missense mutations may weaken the proteins encoded by *ica*B and *mec*A or affect transducers which regulate the promoter binding of both genes (35). Also, silent mutations which do not change the amino acid sequence may still affect the efficiency of mRNA stability or codon usage, or alter other secondary structures which also decrease gene expression (36) Observations on downregulated of both genes and cumulative mutation signify that the particular set of mutations overwhelms the normal hypothetical gene regulatory network to bring about a state of attenuation in the RNA transcription or translation. This illuminates the opportunity for SeNPs-based treatment in down-regulating gene expression that leads to biofilm and antibiotic resistance development and bacterial persistence.

Conclusions:

Depending on the outcomes obtainable, the study established that selenium nanoparticles (SeNPs), alone or in a mixture with azithromycin (AZM), successfully downregulated the expression of ***ica*B** and ***mec*A** genes in methicillin-resistant *Staphylococcus aureus* (MRSA). This proposes a considerable decrease in biofilm development and antibiotic resistance. The SeNPs, mainly when mixed with AZM, promote oxidative stress and damaged bacterial regulatory mechanisms, improving the antibacterial efficiency of AZM while aiming for genetic pathways vital for resistance. These results indicate that SeNPs are a talented adjunctive therapy policy to fight multidrug-resistant *S. aureus* infections and inhibit or block antibiotic resistance.

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