

Mutation-induced penicillin binding protein 2A inactivation through CRISPR-dCas9 gene editing in methicillin-resistant *S. aureus*

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ABSTRACT

Background and Objectives: The global rise of antimicrobial resistance threatens effective treatment of bacterial infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen whose resistance to β -lactam antibiotics is primarily mediated by the *mecA* gene, encoding the low-affinity penicillin-binding protein PBP2a. This study aimed to investigate whether CRISPR-dCas9-mediated targeting of *mecA* could suppress resistance-associated gene expression and restore β -lactam susceptibility in MRSA.

Materials and Methods: Seven target sites within *mecA*, comprising two non-coding and five coding regions, were selected for CRISPR-dCas9 targeting. The effects of gene interference were evaluated using antibiotic disk diffusion assays, minimum inhibitory concentration (MIC) testing with gradient E-strips, RT-PCR, and Sanger sequencing.

Results: Targeting non-coding regions suppressed *mecA* transcription and reduced PBP2a production, whereas targeting coding regions generated mutations associated with dysfunctional PBP2a. CRISPR-dCas9 treatment significantly reduced *mecA* expression and increased MRSA susceptibility to β -lactam antibiotics, particularly ampicillin and cefixime. MIC values decreased by up to 12-fold and 6-fold, respectively ($p < 0.05$).

Conclusion: CRISPR-dCas9-mediated targeting of *mecA* effectively reduced β -lactam resistance in MRSA. This approach demonstrates potential for precision-based antimicrobial resistance management and warrants further investigation as a strategy for combating drug-resistant bacterial infections.

Keywords: Methicillin resistant *Staphylococcus aureus*; MRSA; Penicillin-binding proteins; CRISPR-Cas systems; Gene editing; Drug resistance; Microbial antibiotic resistance

INTRODUCTION

Introduction Antimicrobial resistance (AMR) is a huge public health issue that's really come to the forefront in this century (1). The World Health Or-

ganization (WHO) sees AMR as a significant threat that could roll back years of advancements in modern medicine, affecting things like surgeries, transplants, cancer treatments, and intensive care (2). Recent estimates show that in 2021, bacterial AMR was linked

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to around 4.71 million deaths globally, with 1.14 millions of those directly due to resistant infections. If we don't take effective action soon, these figures are expected to rise dramatically by 2050 (3). One of the most concerning resistant pathogens is Methicillin-Resistant *Staphylococcus aureus* (MRSA), which has a major impact on health outcomes, leading to increased illness, death, longer hospital stays, and higher healthcare costs worldwide. *Staphylococcus aureus* is quite the adaptable Gram-positive pathogen and can cause a range of illnesses, from basic skin infections to serious conditions like pneumonia, osteomyelitis, septic arthritis, infective endocarditis, bacteremia, and sepsis. Its ability to quickly develop resistance to various antibiotics is quite remarkable. Since the first methicillin-resistant strains were spotted in the '60s, MRSA has turned into a global concern, affecting both healthcare settings (HA-MRSA) and community environments (CA-MRSA) (4, 5). On top of that, the rise of MRSA in livestock has added to its significance, especially under the One Health approach. MRSA prevalence can vary a lot around the world, depending on factors like geography, healthcare systems, and how surveillance is done (6, 7). Recent studies suggest that MRSA makes up about 20-50% of clinical *S. aureus* cases in many parts of Asia, Africa, and Latin America. Meanwhile, several countries in Northern Europe have kept their rates below 10% thanks to strict infection-control policies. In the U.S. and parts of Southern Europe, MRSA still constitutes a large portion of serious *S. aureus* infections. The WHO's Global Antimicrobial Resistance and Use Surveillance System (GLASS) has noted that MRSA remains a common issue across over 100 countries, highlighting its ongoing importance in discussions about antimicrobial stewardship and the development of new treatments. What makes MRSA such a successful pathogen is mainly the *mecA* gene, which is found on the *Staphylococcal Cassette Chromosome mec* (SCCmec). This gene codes for penicillin-binding protein 2A (PBP2A), an alternative transpeptidase that has a significantly weaker attraction to β -lactam antibiotics (8). PBP2A is still active even when faced with high levels of methicillin, oxacillin, and other β -lactams, allowing the bacteria to keep making peptidoglycan and survive. Because of this, PBP2A is the key player in methicillin resistance and a promising target for new antimicrobial strategies (2). The rise of multidrug-resistant MRSA strains is making treatment a real challenge across the board.

Many current MRSA strains show resistance to macrolides, aminoglycosides, fluoroquinolones, tetracyclines, and lincosamides, which really limits what options doctors have. While drugs like vancomycin, linezolid, daptomycin, ceftaroline, and newer anti-MRSA treatments still work in many cases, there's been a growing number of reports about reduced effectiveness and treatment failures. Plus, infections from MRSA are associated with higher death rates, longer hospital stays, and healthcare costs that are several times greater compared to infections caused by methicillin-sensitive *S. aureus* (MSSA). These difficulties show the urgent need for innovative strategies that can directly target the resistance mechanisms instead of just focusing on finding new antibiotics (2, 9). Recent strides in molecular biology have made CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technologies crucial in genome editing and developing new antimicrobials. Initially found as a bacterial immune defense, CRISPR-associated (Cas) proteins can be guided by RNA to recognize specific DNA sequences with impressive precision. The traditional CRISPR-Cas9 system creates breaks in double-stranded DNA, but a catalytically inactive version (dCas9) still binds to DNA without cutting it (10, 11). This characteristic makes CRISPR-dCas9 systems work as programmable transcriptional repressors or gene-modulation tools without causing harmful breaks in the genome (12). There's been a lot of interest in using CRISPR technologies to tackle antimicrobial-resistant bacteria in recent times. CRISPR-based antimicrobials can specifically target resistance genes, virulence factors, plasmids, or key genomic areas, which helps restore antibiotic efficacy while minimizing disruption to helpful microbiota (13). Studies have shown promising results, like successfully targeting the *mecA* gene and other resistance factors in MRSA, which led to lower resistance levels and greater susceptibility to β -lactams (14). In a recent study, researchers used CRISPR to target multidrug-resistance genes, which significantly knocked down *mecA* expression and reduced PBP2A production, effectively reversing resistance to various antibiotics (7, 15). One emerging CRISPR strategy that's particularly appealing is the mutation-induced inactivation of PBP2A. Since PBP2A is crucial for methicillin resistance, making specific genetic changes that disrupt its structure, activity, or levels could help make MRSA responsive to standard β -lactam antibiotics again. Unlike

broad-spectrum treatments, the precise targeting of CRISPR-dCas9 might reduce the pressure that typically drives resistance development (16). Plus, the programmability of the dCas9 platform means you can target several resistance factors at once, paving the way for more precise treatments in this era of worsening AMR. Given the global impact of MRSA infections and the key role of PBP2A in β -lactam resistance, creating targeted genetic strategies to disrupt *mecA*/PBP2A function could have serious therapeutic advantages. Therefore, this study focuses on using CRISPR-dCas9 gene-editing technology to induce mutations that inactivate PBP2A in MRSA. By investigating how targeted genomic changes affect PBP2A functionality and antibiotic susceptibility, the research hopes to contribute to the growing field of CRISPR-based antimicrobial therapies and lay the groundwork for future precision strategies against multidrug-resistant *S. aureus*.

MATERIALS AND METHODS

MRSA *mecA* gene collection and alignment. Multiple *mecA* gene sequences of MRSA were retrieved from the NCBI database (multiple accession sequences). Sequence alignment was performed using ClustalW (<https://www.genome.jp/tools-bin/clustalw>) to identify conserved regions suitable for guide RNA (gRNA) editing. Conserved sequences containing Protospacer Adjacent Motifs (PAM; 5'-NGG-3'), compatibility with the Cas9 system. Corresponding CRISPR RNAs were designed as guide RNAs by Integrated DNA Technologies (IDT™) online tool (<https://sg.idtdna.com/page>) for in-silico synthesis and validation.

PBP2A protein catalytic sites identification. Penicillin Binding Protein (PBP2A) catalytic sites utilizing Protein Database (PDB) as demonstrated in Fig. 1. Guide-RNAs were designed according to the catalytic sites of PBP2a where β -lactam drugs particularly methicillin or oxacillin attaches. Five sites of PBP2A catalytic region were selected as target sites for g-RNA designing (labelled by green, yellow, pink, blue, and orange) which are given in Table 1 (17).

g-RNA designing. Seven guide-RNAs were selected from several hundreds of options. Five g-RNAs were designed against the PBP2a target sites (cata-

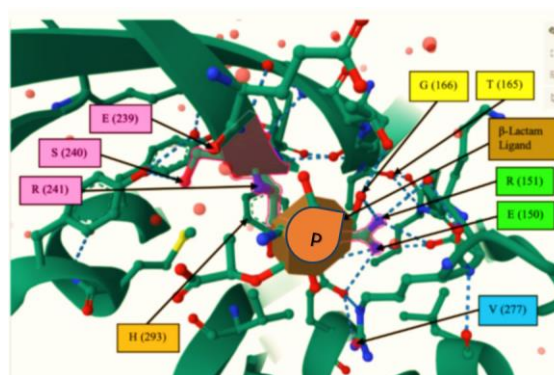


Fig. 1. PBP2A protein Catalytic activity sites (Figure adapted from PDB software). This figure shows the interactive forces, critical amino acids that stabilizes the Penicillin molecule with PBP2A protein

lytic sites), in coding region and two g-RNAs were designed in the non-coding regions (Pribnow box or promoter region). Six guide-RNA clones were requested to Synbio™ Technologies Laboratory (USA) and one g-RNA clone was made in local setting. Selected g-RNAs for cloning are given in Table 1 using referred in literature (18).

The pdCas9-sgRNA-RFP plasmid (Addgene™, USA) was selected as a delivery vector for gRNA expression in MRSA. This plasmid contains the catalytically inactive *dCas9* gene and the sgRNA scaffold, enabling simultaneous expression of both components in bacterial cells. The plasmid also carries a chloramphenicol resistance gene for selection. The plasmid was extracted from *E. coli* DH5 α stab culture following the Addgene™ plasmid extraction protocol.

pdCas9 plasmid confirmation. Plasmid confirmation was carried out by agarose gel electrophoresis and Sanger sequencing. Electrophoresis was performed using 1% agarose gel at 100 V for 45 minutes, visualized under a UV transilluminator (UVP, USA). Distinct plasmid conformations (supercoiled, linear, and open circular) were observed. Sanger sequencing (Macrogen, Korea) was conducted to verify plasmid identity and sequence integrity. Following validation, the plasmid was used for gRNA cloning.

In-silico cloning. In-silico cloning was simulated at SnapGene v8.2 (<https://www.snapgene.com/>) to model restriction digestion and ligation steps prior to laboratory execution. Restriction mapping was performed to identify suitable *BsaI* sites within both

Table 1. Selected g-RNAs for Cloning

No	g-RNAs	Protein target	Non-Coding/ Coding	Target site	Strand	PAM	g-RNA Sequence
1	IMBB-1	No Protein Site	Non-Coding Region	71	+ve	AGG	AAATGTAGTCTTATATAAGG
2	IMBB-7			133	+ve	TGG	ATAGTTGTAGTTGTCGGGTT
3	IMBB-2	E ¹⁵⁰ R ¹⁵¹	Coding Region	234	+ve	TGG	GAAAAATTAAAAATCAGAACG
4	IMBB-3	T ¹⁶⁵ G ¹⁶⁶		294	+ve	AGG	ACAGGAACAGCATATGAGAT
5	IMBB-4	E ¹⁶⁵ S ¹⁶⁶ R ²⁴¹		540	+ve	AGG	AAAGCGACTTCACATCTATT
6	IMBB-5	V ²⁷⁷		615	+ve	TGG	TATAAAGATGATGCAGTTAT
7	IMBB-6	H ²⁹³		666	+ve	TGG	AAAAAGCTCCAACATGAAGA

the *mecA* gene and pdCas9 plasmid sequence. Designed gRNA sequences were inserted in-silico into the sgRNA scaffold region by *BsaI* restriction sites to confirm proper orientation and compatibility.

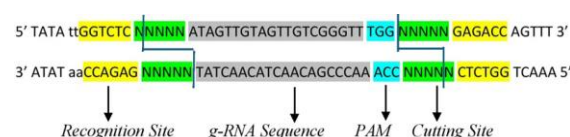
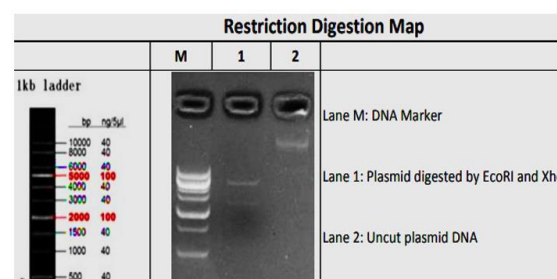
Cloning of gRNAs into pdCas9 plasmid. Cloning of gRNAs into the pdCas9 plasmid was achieved through two complementary approaches-restriction enzyme-based cloning, and Gibson Assembly-to ensure high efficiency and accuracy (19).

For the restriction enzyme approach, the gRNA construct IMBB-7 was cloned in-house using the *BsaI* enzyme (Thermo Fisher Scientific, USA). The pdCas9 plasmid was linearized with *BsaI* and ligated with annealed gRNA oligonucleotides to achieve directional insertion into the sgRNA scaffold region.

In Gibson Assembly method, six additional gRNAs (IMBB-1 to IMBB-6) were cloned into the same plasmid by Synbio™ Technologies (USA). The plasmid backbone was linearized with *BsaI*, and gRNA inserts were seamlessly integrated through Gibson Assembly, producing scarless constructs suitable for CRISPR editing applications (Figs. 2 and 3).

Preparation of MRSA competent cells. MRSA competent cells were prepared using the calcium chloride and rubidium chloride chemical method, as per Addgene™ bacterial transformation protocol. Out of twelve MRSA isolates, two (isolate 10 and isolate 11), exhibiting the highest MICs for β -lactam antibiotics were selected for construct transformation (20).

Transformation of MRSA competent cells. All

**Fig. 2.** Self-cloned g-RNA with recognition and cleavage sites of *BsaI* restriction enzyme.**Fig. 3.** Restriction Digestion of IMBB-5 g-RNA Plasmid clone by *EcoRI* and *XhoI* restriction enzymes.

seven recombinant pdCas9-gRNA plasmids (IMBB-1 to IMBB-7) were transformed into the two selected MRSA competent cells (Isolate 10 and Isolate 11). During Transformation cells were recovered on antibiotic-free LB medium for 1 hour at 37°C with shaking and then plated on LB agar supplemented with 10 μ g/mL chloramphenicol for selection of transformants (21).

DNA extraction and quantification. Genomic DNA from transformed MRSA colonies was extracted using the Cetyltrimethylammonium Bromide (CTAB) method (22). DNA concentration and purity were ensured by NanoDrop™ spectrophotometer reading (Thermo Fisher, USA). Samples with A260/

A280 ratios between 1.8-2.0 were considered as acceptable quality.

Conventional PCR and gel electrophoresis. Conventional PCR was performed on extracted DNA using specific primer sets targeting the *mecA* gene (Table 1). Each 25 μ L reaction contained 12.5 μ L 2 $\times$ PCR Master Mix, 0.8 μ L each of forward and reverse primers (10 μ M), 0.5 μ L template DNA, and 8.5 μ L nuclease-free water. PCR amplification was conducted using a thermal cycler (Model 2720, ABI, USA) under the following conditions: initial denaturation extracted at 95°C for 5 min; 40 cycles of 95°C for 5-10 s, 55°C for 30 s, 72°C for 25 s and a final extension at 72°C for 5 min.

For transformants from IMBB-2 to IMBB-6, (targeting coding regions) as well as IMBB-1 and IMBB-7 were amplified (targeting non-coding regions). PCR products analyzed on 1.5% agarose gels run at 100 V for 45 min and visualized under UV illumination for verification of amplicons.

Sanger sequencing, alignment, and phylogenetic analysis. Amplified *mecA* products were sent for sequencing to Macrogen (S. Korea). Sequence quality was assessed using Chromas and trimmed before alignment. Sequences were aligned with reference *mecA* sequence (Accession No. KR936061.1) using ClustalW to identify mutations or changes. Phylogenetic relationships among the transformants were inferred using the Neighbor-Joining method with 1,000 bootstrap replicates in (Phylogeny.fr France) (Figs. 4 and 5).

RNA extraction and RT-PCR of transformed MRSA cells. Total RNA was extracted from transformed MRSA cells using the TRIzol (Invitrogen, USA) reagent method and quantified by NanoDrop™ spectrophotometry. Complementary DNA (cDNA) was synthesized using 2 $\times$ RT-PCR Master Mix (Solis BioDyne, Lithuania). Secondly, real-time RT-PCR was performed to confirm *mecA* gene amplification with same primer sets as above. Expression levels were normalized against the 16S rRNA housekeeping gene. All reactions were run in triplicate under identical PCR cycling conditions.

Minimum inhibitory concentration (MIC) assay. The MICs of β -lactam antibiotics were determined for all transformants using both Kirby-Bauer disk dif-

fusion method, and E-test gradient strips, following CLSI guidelines. E-test strips for oxacillin, ceftiofur, and penicillin were used to determine quantitative MIC values. Results were recorded as the lowest antibiotic concentration inhibiting visible bacterial growth (Tables 2 a and b).

RESULTS

Transformation of MRSA competent cells. MRSA transformants were isolated on LB agar with Chloramphenicol drug as a selection marker. An uncountable, thousands of colonies, were isolated that showed very high transformation efficiency. Randomly number of transformants were purified into single colony isolation protocol.

Sanger sequencing of transformants *mecA* gene alignment. In the experiment a few bases were deleted with the help of designed gRNA-4 PAM sequence (AGG) such as CTT bases deleted and T base near the PAM region also deleted showing that the mutations occurred in the region of gRNA-4 in the Transformant 10⁴ sequence.

In the experiment with 11⁶, specific nucleotide deletions were noticed with the assistance of designed gRNA-6 PAM (TGG) sequence such as TCCA nucleotides were deleted and C base nearby the PAM sequence also got deleted representing the mutations in the gRNA-6 region in the Transformant 11⁶ sequence.

MIC determination of the MRSA transformants. After the confirmation from the sequencing results, g-RNA transformants, MIC of the MRSA transformants were done by disk diffusion as well as gradient E-strip method. After the validation of the disk diffusion assay, MIC were determined by gradient strip method (six transformants) are given in Table 2.

The Minimal Inhibitory Concentrations were determined by gradient strip method for Ampicillin resistance which was decreased by 12-fold after CRISPR-dCas modifications. There were no major changes in MICs of Mecillinam, Vancomycin, and Ofloxacin as these antibiotics are from the different antibiotic groups (Fig. 6).

Statistical analysis. The initial MRSA and later after CRISPR-dCas9 edited MIC was determined for Ampicillin and Cefixime along with other E-test

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MRSA10      CGTAACTATCCTCTAGAAAAGCGACTTCACATCTATTAGGTTATGTTGGTCCCATTAAAC
104          --TACGTATTCTAA---TAAATCGA---AATACCTAGGAGGT-AC TTCGTC CCGGTCA--
              **  **  **  **  **  **  **  **  **  **  **  **  **  **  **  **

MRSA10      GTAAGCACACCTTCATATGACGTCTATCCATTATGTATGGCATGAGTAACGAAGAATAT
104          GTG-GCGCGCGCTCCTCTG-CGGCGAGGCGCCTGCGAA---CTTTGTTGAGAAGGGGTGG
              **  **  **  **  **  **  **  **  **  **  **  **  **  **  **  **

MRSA10      AATAAATTAACCGAAGATAAAAAA---GAACCTCTGCTCAACAAGTTCAGATTACAAC
104          CACAAAGTGACCGAGGAACGCGCTCAAAGTCCTCTTGAAGAATTGAAGAAGACAGGG
              *  **  *  **  **  *  *  *  **  **  *  *  *  **  **  **  **

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Fig. 4. Sanger Sequence of *mecA* gene of MRSA Transformant 10⁴ Aligned with original MRSA-10 isolate Sanger Sequence. gRNA-4 shown by pink color and PAM region by yellow color as gRNA-4 clone was transformed in MRSA Competent cells 10. Several base deletions and mutations had occurred in the region of gRNA-4 in the Transformants 10⁴ sequence.

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MRSA11      AAGGGACTCGAAAACTTTACGATAAAAAGCTCCAACATGAAGATGGCTATCGTGTACACA
116          AGCGCTCTGGTA----CTTCGGAACAAAC----GGAGGGGGCTGG-TATGACCTCCA-
              *  *  *  *  *  *  **  **  *  **  *  *  *  *  **  **

MRSA11      ATCGTTGACGATAATAGCAATACAATCGCACATACATTAATAGAGAAAAAGAAAAAGAT
116          ACC-TCGAAGGGGG-GGTTTTACGGACGGACGC-CACTGGGAACGAGAAGTGAAAAAA
              *  *  *  *  *  *  **  **  **  **  **  **  *  *  **  **

MRSA11      GGCAAAGATATTCAACTAA-CTATTGATGCTAAAGTTCAAAGAGTATTTATAACAACAT
116          GGGAAATGGGAGCCGACGAACTTTTCGGTTGGAA--CAAGGGTGAACCTATCATAAG-
              **  **  *  *  **  **  **  **  *  *  *  **  *  *  **  **

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Fig. 5. Sanger Sequence of *mecA* gene of MRSA Transformant 11⁶ Aligned with original MRSA-11 isolate Sanger Sequence. gRNA-6 shown by red color and PAM region by yellow color as gRNA-6 clone was transformed in MRSA Competent cells 11. Several base deletions and mutations had occurred in the region of gRNA-6 in the Transformant 11⁶ sequence.

Table 2. a) Zone of Inhibition Measurements (MIC) are given in µg (Isolate 10) Resistant and Sensitive isolates were labelled as R and S respectively. b) Zone of Inhibition Measurements (MIC) are given in µg (Isolate 11) Resistant and Sensitive isolates were labelled as R and S respectively.

MRSA Transformants (specific gRNA)	AMP		MEC		CFM		VA		OFX	
	Before	After	Before	After	Before	After	Before	After	Before	After
a										
10-4	12 µg (S)	1.0 µg (S)	0 µg (R)	0 µg (R)	0 µg (R)	6 µg (S)	3 µg (S)	1.0 µg (S)	6 µg (S)	16 µg (S)
10-5	12 µg (S)	1.5 µg (S)	0 µg (R)	0 µg (R)	0 µg (R)	12 µg (S)	3 µg (S)	0.38 µg (S)	6 µg (S)	8 µg (S)
10-6	12 µg (S)	1.5 µg (S)	0 µg (R)	0 µg (R)	0 µg (R)	16 µg (S)	3 µg (S)	0.38 µg (S)	6 µg (S)	6 µg (S)
b										
11-2	16 µg (S)	2 µg (S)	0 µg (R)	0 µg (R)	0 µg (R)	12 µg (S)	0.5 µg (S)	1.5 µg (S)	6 µg (S)	8 µg (S)
11-3	16 µg (S)	1.5 µg (S)	0 µg (R)	0 µg (R)	0 µg (R)	16 µg (S)	0.5 µg (S)	0.5 µg (S)	6 µg (S)	6 µg (S)
11-7	16 µg (S)	4 µg (S)	0 µg (R)	0 µg (R)	0 µg (R)	24 µg (S)	0.5 µg (S)	0.5 µg (S)	6 µg (S)	8 µg (S)

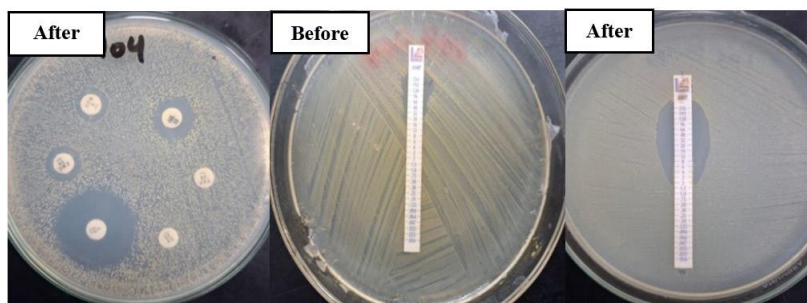


Fig. 6. MIC Determination of MRSA isolate 10 (Before) and MRSA Transformant 10^4 (After) on MH agar plates by Disk Diffusion Test and Gradient E-Strip Test

strips with all parameters for two-way ANOVA and Pearson's and Spearman's correlations. The Gradient MIC determination was highly significant for Ampicillin and Cefixime.

DISCUSSION

The emergence of MRSA is one of the severest issues in the clinical microbiology and the general population health worldwide. The resistance of this pathogen is mainly due to the acquisition of the *mecA* gene, which is present in the Staphylococcal Cassette Chromosome location *mec* (SCC*mec*), and codes the modified penicillin-binding protein PBP2a. PBP2a, in contrast to native PBPs, has a much lower affinity to β -lactam antibiotics and, therefore, PBP2a permits cells to continue biosynthesizing their cell walls despite the administration of such antibiotics (23). The lack of cure of the MRSA infection, especially in hospitals, is complicated by its capacity to avoid the immune system and the formation of biofilms as well as the horizontal transfer of resistance determinants, making eradication processes more difficult (24). A decade of innovations in antibiotics has not yet produced sufficient treatment options to counter the dissemination of MRSA and instead, new gene-based therapies are required to be developed with the capacity to selectively interfere with their resistance mechanisms at their genetic origin.

Over the past few years, genome editing using dCas has become a revolutionary technology in bacterial genetics, which allows control of gene expression accurately and programmably. CRISPR-dCas9 (dead Cas9), a catalytically inactive system that does not possess a nuclease, is a system that works by sterically blocking transcription or inducing targeted mutagenesis by introducing base-pair mismatches

during DNA repair (25). In contrast to the traditional CRISPR-Cas9, which introduces two strands' breaks resulting in the lethality of cells in bacteria, CRISPR-dCas9 provides a reversible and non-lethal gene interference platform that can be applied to the essential or resistance genes¹⁶. In the current experiment, CRISPR-dCas9 was used to target the *mecA* gene, namely, to silence its expression by binding promoter and non-coding sequences or to cause inactivation by mutagenesis by targeting the active site of PBP2a with the use of conserved coding domains. This dual targeting approach was aimed to inhibit protein expression in addition to interfering with structural integrity which would effectively reduce functional resistance phenotype.

The CRISPR-dCas9 system consisting of a single-guide RNA (sgRNA) that is complementary to a set of *mecA* loci assembles mechanically into a ribonucleoprotein complex, which complexes with the intended DNA sequence without cleaving it. The steric hindrance caused by dCas9 does not allow the binding or elongation of RNA polymerase and thus silences the expression of *mecA* (26). The system can also disrupt the process of DNA replication or cause the mismatch of base pairs during repair, resulting in the occurrence of point mutations or frameshifts, which produce nonfunctional versions of PBP2a (27). In the paper, seven target sites were chosen of which two were in regulatory noncoding regions and five were in the coding sequence which enabled a systematic comparison between transcriptional repression and mutational disruption. Targeting non-coding regions had high downregulation of *mecA* transcripts, which was confirmed by RT-PCR quantification and targeting of the coding region had sequence-level changes confirmed by Sanger sequencing. Such mutations, particularly those that involved the transpeptidase domain, probably affect-

ed cross-linking of peptidoglycan layer in response to β -lactams like ampicillin and cefixime, reinstating its sensitivity (28).

The phenotypic validation in terms of antibiotic susceptibility tests showed that there was considerable re-sensitization of CRISPR-dCas treated isolates of MRSA. The disk diffusion and gradient E-strip MIC tests indicated that MICs of ampicillin and cefixime were decreased to 1 μ g and 6 μ g, respectively, which is 12-fold and 6-fold lower than the untreated controls ($P < 0.05$). These results correlate with all the previous experiments in which silencing of *mecA* or related controllers (*mecI*, *blaZ*) using CRISPR restored β -lactam sensitivity in resistant isolates (29). The transcriptional silencing observed is consistent with the currently described process of gene knockdown by dCas9, whereas the presence of base replacements in coding regions confirms the possibility of loss of function through mutation. The two mechanisms come together to reduce the production and activity of PBP2a which reduces the ability of the bacterial defense against the β -lactam antibiotics.

This transcriptional silencing observed is consistent with the currently described process of gene knockdown by dCas9, whereas the presence of base replacements in coding regions confirms the possibility of loss of function through mutation. The two mechanisms come together to reduce the production and activity of PBP2a which reduces the ability of the bacterial defense against the β -lactam antibiotics. The therapeutic consequences of this kind of genetic manipulation are immense. In classical antibiotic treatment, the spectrum of influence is usually broad-spectrum, killing any variants resistant to antibiotics, but non-resistant microbiota is not targeted. CRISPR-dCas9 allows focusing on specific resistance determinants, without exerting any effect on non-resistant microbiota (30). Furthermore, the sgRNA design can be rapidly adapted to novel resistance alleles or new resistance genes in bacterial populations because of the modularity of the sgRNA design. The challenge of getting CRISPR constructs into MRSA cells, albeit the technical difficulty of their entry into cells because of the thick peptidoglycan layer and restriction-modification blocks of the bacteria, can be overcome with bacteriophage vectors, electroporation, or conjugative plasmids (31). In vivo applications, and phage-mediated delivery, in particular, hold promise as a method of specific-

ly targeting the delivery of CRISPR-dCas cargo to pathogenic *S. aureus* strains with reduced off target effects because of the specificity of bacteriophage actions.

However, there are still several biological and regulatory factors. The possibility of horizontal transfer of CRISPR-Cas complex or unwanted off-target binding is one of the primary factors of concern and can impact non-target genes that play a vital role in bacterial physiology (32). SgRNA sequence computational prediction and optimization, as well as whole-genome sequencing validation, are, therefore, crucial to specific editing. Also, the presence of un-edited *mecA* alleles in subpopulations that are currently not eradicated may result in a repetition of resistance to a selective antibiotic pressure, and that is why combinatorial therapy with CRISPR-dCas editing / interference with regular antibiotics are crucial. The other issue is that CRISPR constructs are not so stable in bacterial cells and can be overcome by relying on the plasmid backbones with stable replication origins or strategies of chromosomal integration (33). The results of the present research highlight the possibility to revolutionize the management of antimicrobial resistance with the help of the CRISPR-dCas9 technology (16). CRISPR editing directs specific antibody to the *mecA* gene, directly disrupting the main mechanism of resistance offered by MRSA and returning β -lactam susceptibility and thereby leading to less frequent use of last line drugs like vancomycin or linezolid. Notably, the MRSA is not the only pathogen that can be targeted using such gene-editing techniques but can be applied to other clinically relevant pathogens with resistance determinants like *bla*_{NDM-1} *vanA*, or *tetM* (34). CRISPR-dCas9 has shown precision, reversibility, and adaptability, and it can be used to develop the next generation of antimicrobial therapeutics that can target the genetic aspect and not just employ chemical inhibition (3).

We used CRISPR-dCas9 technology to suppress the *mecA* gene expression of MRSA which produces PBP2A protein to reduce the antibiotic resistance of MRSA especially β -lactam drugs resistance. Seven target sites were selected, five against the coding region and two against the non-coding region of the *mecA* gene. The plasmid clones were transformed in MRSA cells, and the *mecA* gene expression and MIC testing of MRSA were analyzed to determine the percentage of antibiotic resistance reduction. MIC

showed that gRNA-4 (T¹⁶⁵ and G¹⁶⁶), gRNA-5 (E²³⁹, S²⁴⁰, and R²⁴¹), and gRNA-6 (V²⁷⁷) clones had more inhibitory activity than other clones and gRNA-7 had lowest inhibitory activity. Transformants of competent cells 10 showed greater sensitivity as compared to transformants of competent cells 11. *mecA* gene sequence of MRSA transformants (mutants) was analyzed to identify the mutations. Sequence Alignment revealed that numerous mutations and base deletions had occurred in the g-RNA and PAM region and near that region. RT-PCR was also performed to analyze the *mecA* gene expression of MRSA to evaluate the percentage of gene expression suppression. RT-PCR results exhibited that gRNA-4, gRNA-5, and gRNA-6 had highest gene expression reduction than other gRNAs. Transformants of competent cells isolate 10 indicated more reduction of *mecA* gene as compared to transformants of competent cells 11. The plasmid clones against the non-coding region of the gene whether completely inhibit the transcription of *mecA* gene leading to no production of PBP2A protein or partial gene transcription inhibitions producing immature protein. The plasmid clones against the coding region of the gene mutated the *mecA* gene of MRSA which produced mutated PBP2A protein with low affinity to β -lactam drugs whether it does not bind the drug anymore or attaches loosely leading to reduced antibiotic resistance of β -lactam drugs.

CONCLUSION

Conventional antibiotics could be utilized for the treatment of antibiotic-resistant bacterial infection. Such as beta-lactam drug oxacillin or methicillin could be reused to treat MRSA infections and the deadly bacteria could be treated. The antibiotic resistance has become a global threat as there is no treatment option for multidrug-resistant (MDR) bacterial strains. This system is very useful as it provides further opportunity to develop new antibiotics and resensitize available antibiotics to treat drug resistant bacteria.

This study has several limitations that should be considered when interpreting the findings.

Conducted under controlled laboratory conditions, which may not fully reflect the complexity of MRSA infections in vivo, including host immune responses, tissue penetration, and biofilm-associated resistance.

Efficiency of CRISPR-dCas9 delivery into MRSA may vary across strains dependent, and the results obtained from a limited number of isolates may not be although dCas9-based editing is designed to avoid double-strand DNA breaks, off-target binding or unintended transcriptional effects may still occur and could influence bacterial fitness or gene expression beyond *mecA*/PBP2A inactivation.

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Study may not fully assess the long-term stability of the induced mutations or the possibility of compensatory mutations that restore resistance over time.

Phenotypic susceptibility testing alone may not capture subtle changes in virulence, persistence, or tolerance that could remain after gene editing.

All in all the delivery system, scalability, and safety of CRISPR-based approaches remain major barriers to clinical translation.

Study focuses on a single resistance mechanism; it may not address other coexisting resistance determinants that contribute to multidrug resistance in MRSA.

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