Research Article

Molecular Characterization of Putative Drug and Vaccine Targets for *Staphylococcus aureus* Strain Isolated from Gujrat, Pakistan

Komal Imran[1,2*], Ayesha[1], Freeha Shahbaz[1], Tahira Shaheen Butt[2], Uswa Meenal[1], Kalsoom Sughra[1], Nadia Zeeshan[1]

[1]Department of Biochemistry and Biotechnology, University of Gujrat, Pakistan; [2]Department of Biotechnology, University of Sialkot, Sialkot, Pakistan.

Abstract | *Staphylococcus aureus* a Gram-positive coccus is recognized as the most common cause of hospital and community-acquired infections in Pakistan. Due to increase in the number of multidrug-resistant bacterial strains, there is an extensive need to identify current antibiotic susceptibility patterns and novel putative candidate drug targets within multidrug-resistant (MDR) strains. In the present study, clinical MDR *S. aureus* isolates from Gujrat, Pakistan were analyzed for their antibiotic resistance patterns, providing region-specific baseline data from an underreported geographic area. Additionally, two essential proteins, Penicillin-binding protein 1 (PBP1) and UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (*MurA*), were selected for molecular and in silico characterization as potential drug targets. Antibiotic susceptibility testing was performed using standard MIC and zone of inhibition assays. *In silico* characterization of *pbpA* and *murA* candidate drug targets was performed through ExPASy-ProtParam, Pfam, Batch CD search, SOSUI server, STRING, COACH and I-TASSER. The results indicated that *pbpA* and *murA* both are essential and conserved proteins involved in peptidoglycan biosynthesis, supporting their potential as therapeutic targets and the combinatorial *murA* and *pbpA* gene knockout or inhibition would prove deleterious for antibiotic resistant strains. This study provides region-specific updated information for modern treatment guidelines and molecular insights into MDR *S. aureus* isolates from an underreported area of Pakistan and highlights pbpA and murA as promising targets for future drug discovery efforts. However, the study is limited by the small number of clinical isolates and reliance on *silico* predictions; therefore, further experimental validation and large-scale studies are required to confirm these findings.

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***Correspondence** | Komal Imran, Department of Biochemistry and Biotechnology, University of Gujrat, Pakistan; **Email:** Komalimran2000@gmail.com
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Introduction

Staphylococcus aureus is a Gram-positive cocci, has a high-risk factor of inducing infections in humans as well as in other livestock ranging from mild skin infection to life-threatening diseases (Naorem *et al.*, 2022; DeLeo *et al.*, 2010). *S. aureus* infection can be transferred from one person to another either by skin

to skin contact or by utilizing contaminated utensils. *S. aureus* has emerged as a major human nosocomial and community-acquired pathogen from the previous 30 years and a serious risk for public health (Pillai *et al*., 2012). It has been reported that high frequency of methicillin resistance *S. aureus* strains is observed in Pakistan ranging from 42 to 51% which is higher from northern Europe (Idrees *et al*., 2023; Akinkunmi and Lamikanra, 2012).

Recently, it has been discovered that newly identified *S. aureus* resistance strains are immune not only to β-lactam antibiotics (including penicillin, methicillin, cephalosporins and oxacillin) but also have potential to survive against sulfa drugs (clindamycin and tetracyclines) and Glycopeptide antibiotics (vancomycin and teicoplanin) which are declared as drugs of last resorts for cure of *S. aureus* infection (Irshad, 2025; Foster, 1996; Okuma *et al*., 2002; Huang *et al*., 2006; Moellering, 2006). Therefore, production of new antibiotics is seriously needed that have an alternative mode of action than the previous drugs against these multidrug resistant strains.

In present time researchers are working on the identification of novel drug targets and strategies to enhance the efficacy of already available antibiotics specially β-lactam antibiotics which targets peptidoglycan (PG) synthesis (Bellini *et al*., 2019). Characterization and identification of novel drug targets are verified based on two criteria. First; potentiality of a target to pathogen was determined in scenario of replication and viability and second; the target should be non-homologous to human and membrane-bounded (Khursheed, 2013).

Penicillin binding protein1 (PBP-1) in bacteria comes under class B PBPs and is a membrane bounded macromolecule. PBP-1 is responsible for the synthesis of major part of cell wall peptidoglycan (PG) (septal PG) and contributes to proper bacterial cell divison in *S.aureus* (Pereira *et al*., 2007). The *murA* gene encodes UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase, an essential enzyme that catalyzes the first committed step in peptidoglycan biosynthesis by transferring an enolpyruvate moiety from phosphoenolpyruvate to UDP-*N*-acetylglucosamine, leading to the formation of UDP-*N*-acetylmuramic acid precursor required for bacterial cell wall synthesis (Vesić and Kristich, 2012). This biochemical process of transfer is the foremost and committed step in the peptidoglycan (PG) or murein formation that is the vital structural component of *S. aureus* cell wall.

That's why, PBP-1 and *murA* can prove an important ground-breaking target for drug designing against multidrug-resistant bacteria such as methicillin resistant *S. aureus* (*MRSA)* because due to its inactivation bacteria faces structural irregularities such as the lesion, loss of selective permeability, abnormal growth, nonviable cells and elongation that can ultimately lead towards cell lysis (Sakoulas *et al*., 2014).

The identification of potential drug targets in pathogenic bacteria such as *Staphylococcus aureus* has been increasingly supported by comparative and subtractive genomics approaches combined with computational biology tools (Shahbaaz *et al*., 2016). In the present study, functional and structural characterization of two essential proteins, PBP1 and MurA, was performed to evaluate their potential as therapeutic targets against multidrug-resistant *S. aureus*. Computational analyses, including physicochemical profiling, conserved domain identification, protein interaction analysis, and three-dimensional structural modeling, were conducted to provide preliminary molecular insights into these targets.

Importantly, this study provides region-specific molecular data from clinical MDR *S. aureus* isolates obtained from Gujrat, Pakistan, an underreported geographic area with limited structural characterization studies on antimicrobial targets. The predicted structural models and active-site analyses may serve as a foundation for future molecular docking and inhibitor design studies targeting essential peptidoglycan biosynthesis pathways.

Materials and Methods

Clinical isolate

Two clinically confirmed methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were obtained from the lab of Biochemistry and Biotechnology, the University of Gujrat, Gujrat Pakistan in collaboration with participating hospital, Aziz Bhatti Shaheed Hospital, Gujrat for molecular characterization and computational target analysis. These isolates were collected from the patients hospitalized in

surgical and medical wards. The targeted clinical isolates submitted for *MRSA* testing were collected from hospital laboratory at the wariness of medical personnel.

Phenotypic based identification

Preliminary morphological identification of *Staphylococcus aureus* was carried out based on the characteristic tiny, smooth, shiny golden-yellow colonies observed on culture media (Boerlin *et al*., 2003). Luria-Bertani (LB) Agar (1gm Tryptone, 1gm sodium chloride, 0.5gm Yeast Extract and 1.7gm agar in 100mL) and brain heart infusion agar (BHI), For preparation of 100 mL BHI agar, calf brain infusion solids (1.25 g), beef heart infusion solids (0.50 g), proteose peptone (1.0 g), dextrose (0.20 g), sodium chloride (0.50 g), disodium phosphate (0.25 g), and agar (1.50 g) were dissolved in distilled water and sterilized by autoclaving were used for the growth of the pathogen in the laboratory at 7 pH, 37 °C for 16-24 hrs.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out by both disk diffusion method and agar dilution minimal inhibitory concentration (MIC) method. For both above mentioned methods single colonies from 18-24 hrs old plate of *S. aureus* were used to prepare inoculum through direct colony suspension and turbidity was adjusted to 0.6 value at 600nm. Antibiotics selected for this purpose were Ampicillin (β-lactam penicillin antibiotic, semi-synthetic derivative of penicillin), Amoxicillin (the semisynthetic aminopenicillin antibiotic), Kanamycin (aminoglycoside bactericidal antibiotic), Clindamycin (semisynthetic lincosamide antibiotic) and Vancomycin (Glycopeptide Antibacterial antibiotic). The Clinical and Laboratory Standards Institute (CLSI) with US Food and Drug Administration (FDA) suggested these antibiotics for primary routine testing and reporting of *S.aureus* in laboratories (Patel *et al*., 2014). Data was interpreted according to CLSI guidelines. All antibiotic susceptibility tests (zone of inhibition and MIC assays) were performed in triplicate, and results were recorded as mean values. Performance standards for antibiotic susceptibility testing in *S. aureus* recommended by CLSI were mentioned in Table 1.

Molecular based identification of mecA, pbpA and murA genes

S.aureus genomic DNA was extracted by following the protocol described Tait *et al*. (1983). Primers were designed by utilizing a compiled Primer3 in CEMAsuite (Lane *et al*., 2015) (Table 2).

PCR reaction profiles used for amplification of *mecA*, *pbpA* and *murA* genes were as follows initial denaturation step (94°C for 3 minutes), heating step (94°C for 60 sec), extension (72°C for 60 sec) and final extension (72°C for 20 minutes). The annealing temperature for primers of genes were different in PCR reaction profile such as (50°C,49°C, 50°C for

Table 1: *Performance standards for antibiotic susceptibility testing.*

S. No	Antibiotic	Potency (µg/mL)	Zone diameters in (mm)			MIC antibiotic potency (µg/mL)		
			S	I	R	S	I	R
1	Ampicillin	10	≥ 29	-	≤ 28	≤ 4	16	≥32
2	Amoxicillin	20/10	≥ 20	-	≤ 19	≤ 2	4	≥8
3	Clindamycin	2	≤ 21	15-20	≥ 14	≤ 0.5	1-2	≥ 4
4	Kanamycin	30	≤ 18	14-17	≥ 13	≤ 16	32	≥ 64
5	Vancomycin	30	≥ 15	-	≤ 15	≤ 2	4-8	≥ 16

Table 2: *Properties of primer.*

Genes	Primers	Sequence	Length	Tm (°C)	Designed
mecA	*mecA* F	5'-ACAACAAGACCCTGGACGAC-3'	20	53.8	CEMAsuite
	mecA R	5'-TGATCTTGGCGTTGTAGCTG-3'	20	51.8	CEMAsuite
pbpA	*pbpA* F	5'- GGTGAACAGCAAGAAGAGCC-3'	20	53.8	CEMAsuite
	pbpA R	5'-TTGTCCTTCTCGGTCAGCTT -3'	20	51.8	CEMAsuite
murA	*murA* F	5'AACGGCGAGGTGAACATCAG-3'	20	53.8	CEMAsuite
	murA R	5'CCATCCTCTTCAGCTCCTCC-3'	20	55.9	CEMAsuite

60 sec), respectively. Targeted amplified products were saved T/A cloning following standard protocol of FermentasInsTAclone PCR Cloning Kit #K1214.

DNA sequencing of mecA, pbpA and murA Genes

Recombinant plasmid DNA with desired genes (pTZ *–mecA,* pTZ *–pbpA* and pTZ *–murA)* were isolated by alkaline lysis miniprep method (Birnboim and Doly, 1979). The *mecA*, *pbpA* and *murA* positive recombinant plasmids were sequenced through dideoxy chain termination method from Korea Macrogen's Express Sanger Sequencing Service.

Computational analysis

BLAST (basic local alignment search tool)

Sequences of *pbpA* and *murA* obtained after sequencing were run on BLAST against *S. aureus* genome to find out homologues sequences as our designed primers were degenerate primers. Sequences were selected on the bases of E-value (0.0) and sequence identity (>70%) (Ye *et al.*, 2006). Protein sequence of our assemble nucleotide sequences were obtained by BLASTx.

Analysis of physiochemical characteristics

Protein sequence of our assemble nucleotide sequences were further analyzed by ExPASy-ProtParam tool (www.expasy.org/tools/protparam.html) to investigate physical and chemical properties (Mohan and Venugopal, 2012).

Conserved domains prediction

Pfam and Batch CD were used for the predictions of conserved domains in PBP2a, PBP-1and MurA in order to characterize protein functions at the molecular level. The family of protein was identified by searching consensus sequence in structure coding conserved domains of both of proteins (Finn *et al.*, 2009).

Batch CD search (www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) was carried out to highlight conserved domains in PBP2a and PBP-1. This program is NCBI's web interface to explore the Conserved Domain Database within protein-coding sequences by using RPSBLAST; a variant of PSI-BLAST and Position- Specific Scoring Matrices (PSSMs) (Marchler-Bauer *et al.*, 2010).

Nature and cellular location of protein

Amphiphilicity index and Hydropathy index were estimated to uncover nature of selected therapeutic drug target proteins through SOSUI server (harrier.nagahama-i-bio.ac.jp/sosui/). Nature accounts whether protein resides in the cytoplasm or spans the transmembrane (Ikeda *et al.*, 2000). SOSUI Server is an arsenal of tools such as SOSUI (Batch), SOSUIsignal, SOSUIgramN, and SOSUImp1. These Tools were also utilized during the prediction of a part of the secondary structure of selected protein from its given amino acid sequence (AAS) to estimate its location in a cell (Hirokawa *et al.*, 1998).

Protein-protein interaction network analysis

Protein in the cell environment interacts with other proteins; in silico, these interactions were studied through STRING v9.1 (Search Tool for Retrieval of Interacting Genes). STRING (http://string-db.org/) is a large repository of protein-protein interaction networks including functional interactions, regulatory interactions and stable complexes of proteins (Lewis *et al.*, 2010). The protein-protein interaction (PPIs) of selected therapeutic drug targets was searched by submitting protein query sequence in the search box of STRING was executed by molecular machines actively assembled by specific PPIs. Protein-protein interaction network would empower our current information about biochemical signaling pathways against antibiotic drug resistance (Jensen *et al.*, 2008).

Protein structure and binding site prediction

Protein structure prediction was done to estimate the location of all atoms in putative drug target molecules by using its amino acids sequence through computational methods such as I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-T ASSER/) is a hierarchical approach to protein structure and function prediction (Yang and Zhang, 2015). I-TASSER was used to understand biological functions of selected putative drug targets including its gene ontology in terms by comparing with structure and function profile of known proteins, ligand binding site prediction, solvent accessibility and enzyme commission number on the basis of sequence-to-structure-to-function paradigm (Yang and Zhang, 2015). Best models were structure with a higher C score, TM score (0.78±0.10,0.95±0.05) and RSMD 5.0±3.2Å, 3.2±2.3Å. C score is basically the confidence score related to structure prediction ranging from -5 to 2, greater the C score signifies the quality of a structure while the TM score and RSMD are the measures of accuracy of structure prediction, the higher values indicates correct topology of a protein structure that

are not due to a random chance similarity and relative to native (Naveed *et al*., 2018).

Identification of ligand binding sites

PDB validated file of best-predicted model was submitted to COACH for ligand binding site predictions by matching target models in BioLip database.

Results

Antibiotic susceptibility pattern

Staphylococcus aureus tiny, shiny gold colonies were observed on solid LB agar plates. These single colonies were further analyzed for identification of antibiotic susceptibility pattern among *S. aureus* isolates of Pakistan. The current analysis showed under examine *S. aureus* isolate was 100% resistant to ampicillin (β-lactam penicillin antibiotic, semi-synthetic derivative of penicillin), Amoxicillin (semisynthetic aminopenicillin antibiotic), Kanamycin (aminoglycoside bactericidal antibiotic), Clindamycin (semisynthetic lincosamide antibiotic) and 33% resistant to Vancomycin (Glycopeptide Antibacterial antibiotic) as the isolate was resistant to vancomycin at 4µg concentration which is a sensitive range (S) but for intermediate and resistant range (8-16 µg, ≥ 16-32µg) the zone of diameter was greater than 15mm. The zone of inhibition obtained against above-mentioned antibiotics was shown in Table 3.

Table 3: *Antibiotic susceptibility pattern of isolated S. aureus strain.*

S.	Antibiotic	Potency	Zone of inhibition (mm)	CLSI recommendations
1	Ampicillin	10µg	28±0.05	Resistant
2	Amoxicillin	20µg	15± 0.05	Resistant
3	Clindamycin	2 µg	14± 0.05	Resistant
4	Kanamycin	30 µg	12± 0.05	Resistant
5	Vancomycin	≥ 16-32 µg 8-16 µg 4 µg	29± 0.05 28± 0.05 10± 0.05	Sensitive Sensitive Resistant

Above mentioned results were reproduced by Agar dilution MIC test to differentiate between either the clinical isolate was vancomycin-intermediate isolate or resistant isolate. The range of vancomycin dilutions tested was 2-32 µg/mL. Results showed *S. aureus* clinical isolate was resistant to MIC susceptible Interpretive Criteria (≤ 4 µg/mL) while no growth pattern of *S. aureus* was observed on MIC resistant standardized (≥16 µg/mL) agar plates. For MIC intermediate interpretive criteria (8-16 µg/mL) the slight amount of growth on agar plates was observed indicating that the clinical isolate deep inside also developing resistance against this antibiotic range through unexpected and novel mechanisms of resistance showed in (Figure 1). For ampicillin (4-64 µg/mL), amoxicillin (2-16 µg/mL), clindamycin (0.5-4µg/mL) and kanamycin (16-64µg/mL) dilution ranges were tested showed the isolate was 100% resistant to all the dilutions of above-tested antibiotics. *S. aureus* percent resistance under examination of MIC resistant interpretation criteria was shown in Figure 1.

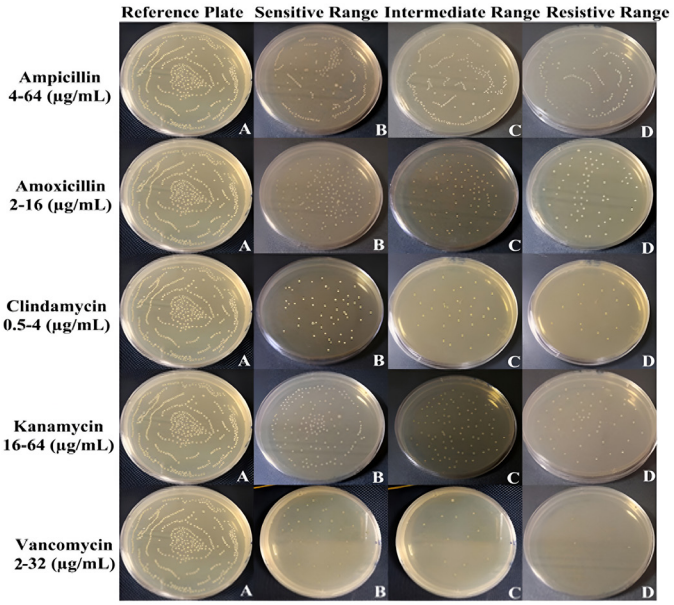

Figure 1: *MIC criteria for Staphylococcus aureus against Ampicillin, Amoxicillin, Clindamycin, Kanamycin and Vancomycin with MIC Reference Plate (0 µg/mL) (A), MIC Sensitive Interpretive Criteria (B), MIC Intermediate Interpretive Criteria (C), MIC Resistant Interpretive Criteria (D).*

PCR amplification and visualization of mecA, pbpA and murA genes

Methicillin resistance in *S. aureus* clinical isolate was determined by molecular level identification of *mecA* gene (Penicillin-binding protein PBP2a) through PCR. The amplified part corresponds to 750bp and visualized on an agarose gel in gel documentation system in front of the 3rd band of 1Kb DNA marker. Penicillin-binding protein 1 (*pbpA*) gene and UDP-N-acetyl glucosamine 1-carboxyvinyltransferase enzyme (*murA*) was identified by PCR utilizing primers designed by CEMAsuite. Following PCR amplification, the products were resolved on agarose

gel electrophoresis and visualized under UV light using a gel documentation system. The amplified fragments corresponding to approximately 1000 bp and 1246 bp confirmed successful amplification of the target genes shown in Figure 2.

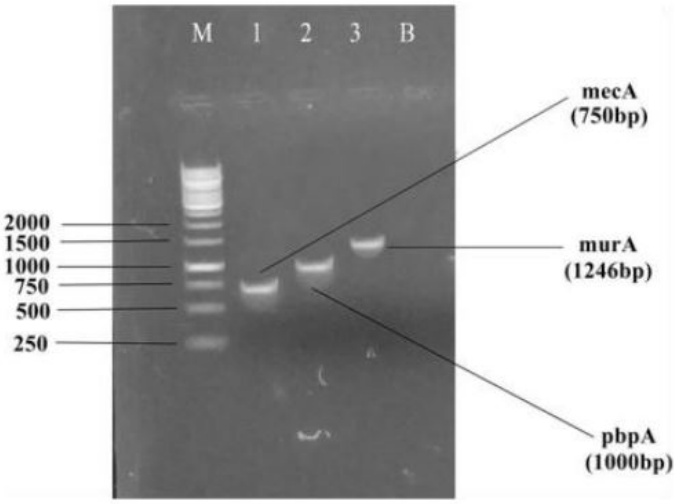

Figure 2: *Molecular based identification of mecA, pbpA and murA Genes by PCR.*

Sequences of mecA, pbpA and murA genes

The nucleotide sequence of *mecA*, *pbpA* and *murA* genes were determined through dideoxy chain termination method. The text files of both genes in FASTA format quality were checked and analyzed by using the software BioEdit. The assembled coding sequence of *mecA* comprised of 737 bp, *pbpA* of 870 bp and *murA* of 1246bp. The nucleotide sequences obtained in this study have been successfully deposited in the NCBI GenBank database to ensure public accessibility and data transparency, with the following accession numbers: *mecA* (MH106551), *pbpA* (MH106552), and *murA* (MH106553).

Bioinformatics analysis results

Physiochemical properties of proteins

The finalized assembled sequences of *pbpA* and *murA* proteins physiochemical properties such as the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY) estimated by ProtParam tool showed in Table 4.

The physiochemical properties computed by ProtParam such as MW and pI would assist in proteins separation by 2-D gel electrophoresis and Extinction Coefficient (EC) would assist in pure protein quantitative estimation in the sample. Instability index (II) refersPBP-1 and MurA proteins are stable and the grand average of hydropathy (GRAVY) accounts hydrophilic nature of proteins.

Table 4: *Physiochemical properties of PBP-1 and MurA by ProtParam tool.*

Physiochemical properties		
Protein	**PBP-1**	**MurA**
Molecular weight	79645.10	40467.58
Theoretical pI	9.12	5.65
Ext. coefficient	82740	10430
Instability index	26.57	29.27
Aliphatic index	68.35	103.51
GRAVY	-0.713	-0.013

Conserved domains of proteins

The Pfam and Batch CD search Tool showed the PBP-1 protein exhibited PBP_dimer, Transpeptidase, and PASTA and FstI domains. Transpeptidase domain is involved in cell wall synthesis, PASTA provides binding sites for β-lactam antibiotics and FstI involved in cell division by regulating peptidoglycan synthesis at division septum. While MurA protein contained ESPS synthase family (3-phosphoshikimate phosphate synthase) domain. All these domains functionally characterized PBP-1 and MurA protein as an important putative drug target by enrapturing signaling pathways from cell wall synthesis to availability of binding sites for antibiotics to a division of a bacterium.

Nature and cellular location of protein

SOUSI server predicted PBP-1 and MurA both are soluble proteins with an average hydrophobicity score -0.73 and -0.013, respectively. The other in build software of SOSUI server such asSOSUImp1 revealed PBP-1 is secretory type of protein while MurA is cytoplasmic in nature. SOSUIsignal predicted both have no signal peptide and SOSUIgramN predicted Cytoplasm is subcellular localization site of MurA while PBP-1 exhibited in Extracellular Matrix (EC) showed in Table 5.

PBP-1 protein drug targets

PBP-1 and MurA are human non-homologous essential proteins that were assorted as a potent drug target due to its involvement in bacterial survival

pathways. The 15 drugs interacting with PBP-1 were found in Drug Bank database that can be used as inhibitors of this protein are showed in Table 6.

Protein-protein network analysis

STRING analysis showed PBP-1 and MurA are making interactions with the following neighboring proteins in the result of biochemical events and/or electrostatic forces to perform various molecular processes for the survival of bacteria in (Figure 3A, B). By literature viewing, a comprehensive understanding of the functioning of these proteins in cell wall synthesis and antibiotic resistance development was made.

Following proteins in PBP-1 string network were found; pbp2 (Penicillin-binding protein 2), SACOL1122 (Cell cycle protein FtsW), ftsW (Cell cycle protein FtsW), mgt (Monofunctional Glycosyltransferase), pbp4 (Penicillin binding protein 4), SACOL1779 (Trans glycosylase domain-containing protein), murG (UDP-diphospho-muramoyl-pentapeptide beta-N-acetylglucosaminyltransferase), murD (UDP-N-acetylmuramyl-L-alanyl-D-glutamate synthetase, mraY (phospho-N-acetylmuramoyl-pentapeptide-transferase), ftsL (Cell division protein; Essential cell division protein) are playing eager roles in the regulation of cell wall biosynthesis and cell division by establishing interactions within PBP-1 signaling pathway.

We found following proteins in string network; *murB*, *murC*, *murD*, *murE*, *murF*, *murG*, *pbpA*, *ddl*, *mnaA* and *glum* are making interaction with MurA showed its involvement in biosynthesis of cell wall and also in several metabolic pathways such as the formation of nucleotide precursors in the bacterial cell. The synergetic combinations of PBP-1 and MurA inhibitors with β-lactam antibiotics can increase overall efficacy of antibiotic combinations to fight against MDR organisms causing infections in humans.

PBP-1 structure prediction

PBP-1 proteins structure was predicted by I-TASSER for their comprehensive structural and functional understanding accounting their ligand binding sites. I-TASSER predicted the secondary structure of PBP-1 and MurA being enriched with α Helixes and

Table 5: *Nature and cellular location of PBP-1 and MurA.*

Protein	SOSUI (server)		SOSUIsignal		SOSUIgramN	SOSUImp1
	Nature	Percentage	Signal peptide	Nature	Subcellular localization site	Type
PBP-1	Soluble Protein	100%	No	Soluble	Extracellular matrix	Secretory
MurA	Soluble protein	100%	No	Soluble	Cytoplasm	Cytoplasmic

Table 6: *PBP-1 and MurA protein drug targets.*

PBP-1			MurA		
Drug name	Drug group	Action	Drug Name	Drug Group	Action
Imipenem	Approved	Inhibitor	Uridine-Diphosphate-N-Acetylglucosamine	experimental	Inhibitor
Cefoperazone	Approved	Inhibitor	Fosfomycin	Approved	Inhibitor
Ceftizoxime	Approved	Inhibitor	(S)-2-{Methyl-[2-(Naphthalene-2-Sulfonylamino)-5-(Naphthalene-2-Sulfonyloxy)-Benzoyl]-Amino}-Succinicacid	experimental	Inhibitor
Cefradine	Approved	Inhibitor	Aminomethylcyclohexane	experimental	Inhibitor
Cefazolin	Approved	Inhibitor	CyclohexylammoniumIon	experimental	Inhibitor
Cefoxitin	Approved	Inhibitor	3'-1-Carboxy-1-Phosphonooxy-Ethoxy-Uridine-Diphosphate-N-Acetylglucosamine	experimental	inhibitor
Cefonicid	Approved	Inhibitor	1-Anilino-8-Naphthalene Sulfonate	experimental	inhibitor
Ceftibuten	Approved	Inhibitor			
Cefpiramide	Approved	Inhibitor			
Ceftazidime	Approved	Inhibitor			

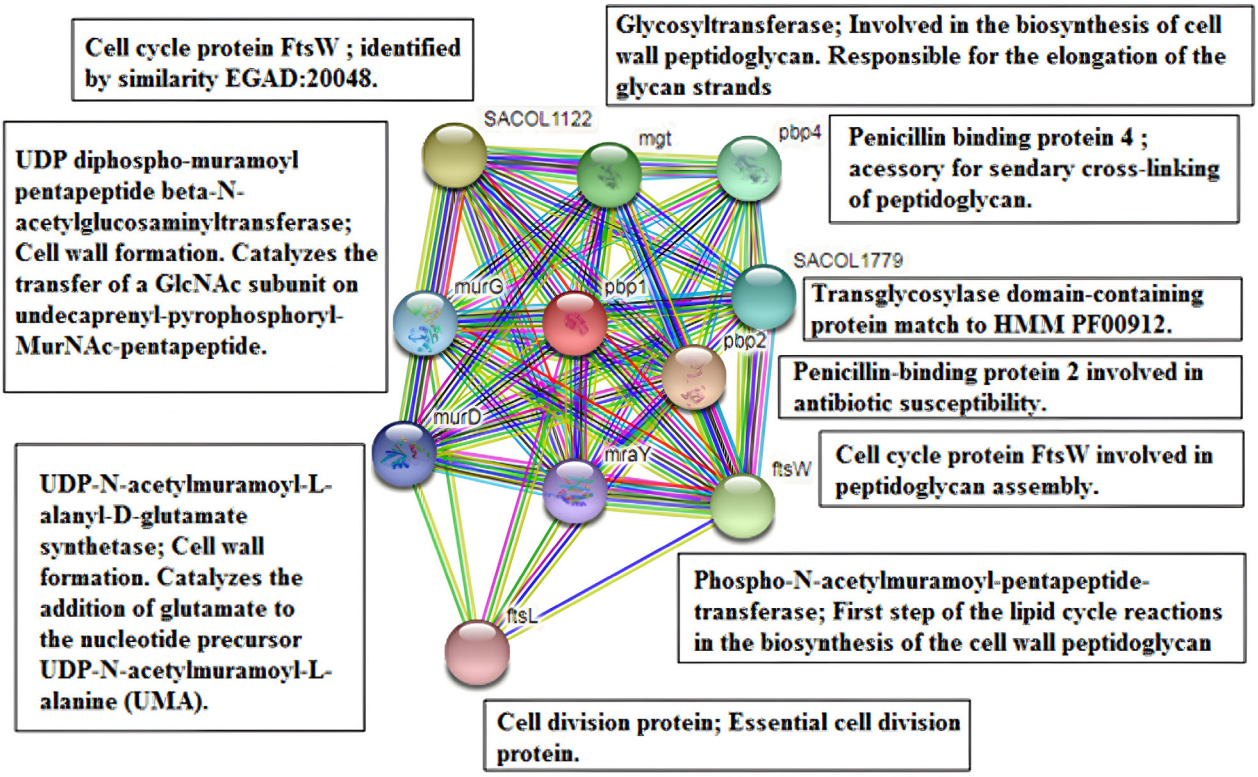

Figure 3A

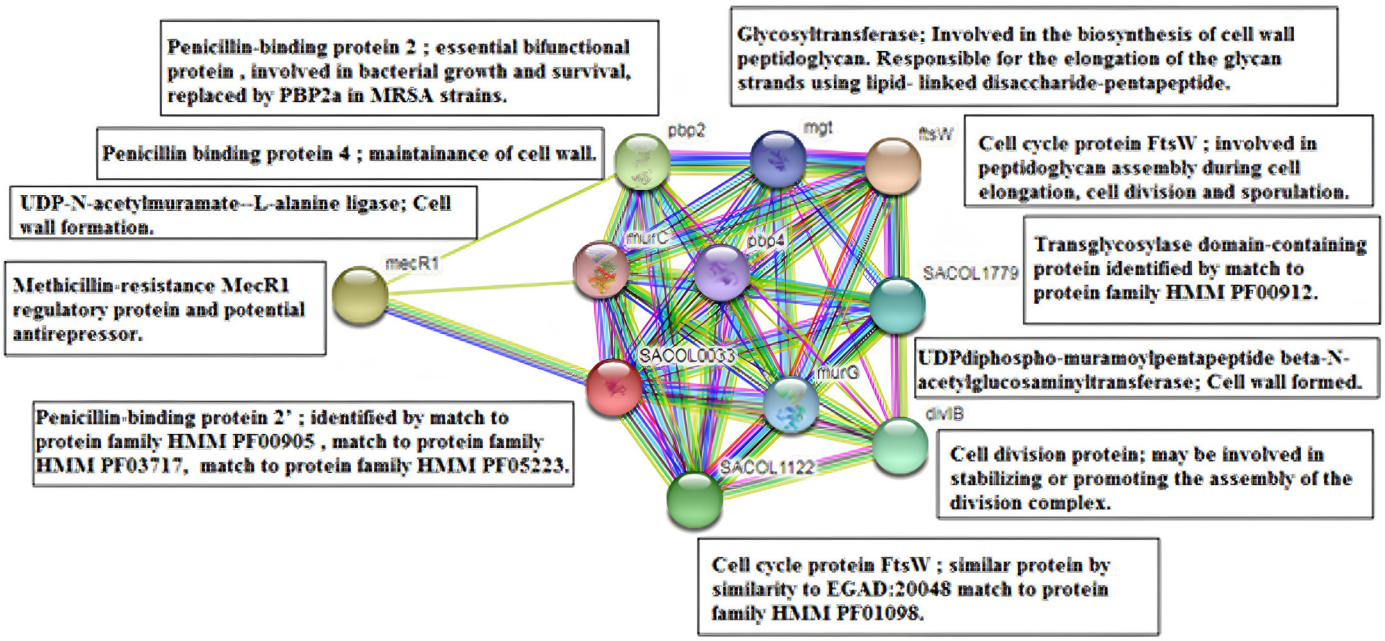

Figure 3B

Figure 3: *A: STRING Analysis of PBP-1. B: STRING Analysis of murA.*

long stretches of the coil with a confident score reaching to 9 estimated the core region of PBP-1 contains folding elements indicate presence of domains in the query sequence of protein. I-TASSER predicted 5 models for query protein among these the one best model structure of PBP-1 and MurA was selected with a higher C score (0.51, 0.71), TM score (0.78±0.10,0.95 ±0.05) and RSMD 5.0±3.2Å, 3.2±2.3Å showed in (Figure 4A, B). C score is basically the confidence score related to structure prediction ranging from -5 to 2, greater the C score signifies the quality of a structure while the TM score and RSMD

are the measures of accuracy of structure prediction, the higher values indicates correct topology of a protein structure that are not due to a random chance similarity and relative to native. The above-obtained values showed the predicted models were of good quality.

Among the five models predicted by I-TASSER model 3eqvA was selected for PBP-1 with a C score of 0.304 and TM 0.686 illustrating it as serine-type D-ala D-ala carboxypeptidase with an Enzyme Commission (EC) number 3.4.16.4 while for MurA 2rl2A model was selected with a C score of 0.496 and TM 0.99 illustrating it as UDP-N-acetylglucosamineenol pyruvyl transferase with an Enzyme Commission (EC) number 2.5.1.7. The predicted active site residues were at 72, 75, 167, 169 positions for PBP-1 and at 167, 193, 196, 212, 302, 304, 306, 308 for MurA. At the end gene ontology (GO) section illustrated function of PBP-1 and MurA comprised of two parts; first showed 10 homologous GO templates while second part goes for consensus prediction of GO terms including molecular function, biological process, and cellular component.

The best template 2z2lB was selected on the basis of homology score (Fh score) 0.85 with the target protein and a C score of 0.50. GO predicted the cellular compartment of PBP-1 is plasma membrane or intrinsic component of the membrane having some covalently attached portion. The Consensus prediction of GO illustrated PBP-1 is involved in peptidoglycan biosynthesis process, cell wall organization regulating the cellular shape and cell division. The best template 2rl2A was selected illustrated MurA is involved peptidoglycan (PG) biosynthesis in *S. aureus* and cell wall development.

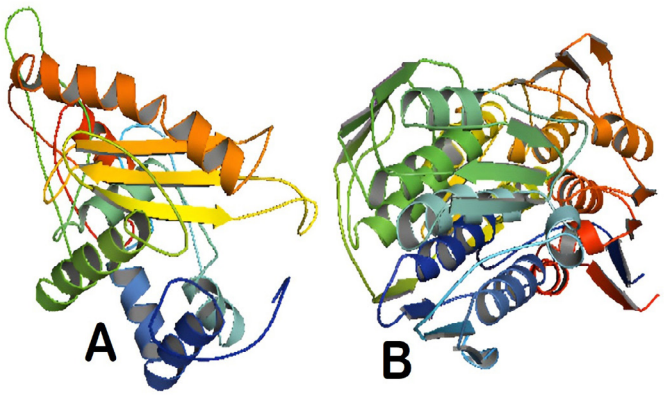

Figure 4: A*: predicted 3D structure of PBP-1, B: predicted 3D structure of MurA.*

PBP-1 ligand binding interactions

PDB file of best predicted PBP-1 and MurA model were submitted to COACH for ligand binding site predictions by matching target models in BioLip database. The first PDB hit 4r23A is selected for PBP-1 with a C score 0.61 and cluster size 144 binds to the DXU (dicloxacillin) ligand. Except for DXU, it can also bind with other ligands available in "mult" link. There are 11 ligand binding sites in first top selected model and residues are as following 22, 23, 96, 99, 123, 124, 125, 126, 127, 128, 164, 166, 167, 168, 307, 308, 330, 331, 334, 373. The first PDB hit 3su9A is selected for MurA with a C score 0.76 and cluster size 72 binds to the EPZ ligand. Except for EPZ, it can also bind with other ligands available in "mult" link. There are 20 ligand binding sites in first top selected model and residues are as following 22, 23, 96, 99, 123, 124, 125, 126, 127, 128, 164, 166, 167, 168, 307, 308,330, 331, 334, 373. COACH also combines the results of five algorithms including COFACTOR, TM-SITE, S-SITE, FindSite and ConCavity to show ligand binding interactions in best-predicted modelain (Figure 5A, B).

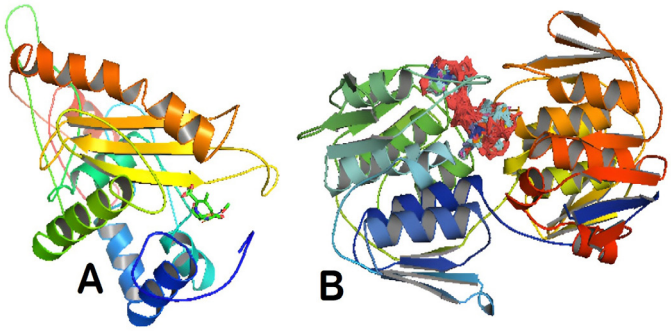

Figure 5: *Predicted ligand binding sites of PBP-1 (A) and MurA (B).*

Biochemical pathway of PBP-1 and MurA

The antibiotic-resistant strains provoked the need of newer antibiotics in the arsenal for fighting against such resistant strains. One of the main targets of the bacterial antibiotics is different enzymatic steps of the cell wall synthesis or cell envelope. The cell wall of Gram-positive bacteria comprises a multi-layer heteropolymer which is up to 70% enriched with peptidoglycan (Typas *et al.*, 2012). Bacterial peptidoglycan facilitates bacteria to stand against intracellular pressure and gives a profound shape to bacteria so that it can be produced generation after generation. The peptidoglycan is composed of linear glycan chains (alternating units of β-1,4 linked N-acetyl-glucosamine (GlcNAc) and

N-acetylmuramic acid (MurNAc) that are interlinked by short peptides (Barreteau *et al.*, 2008). Bacterial cell wall is synthesized into three stages illustrated in (Figure 6), The first stage is the generation of the soluble UDP-MurNAc-pentapeptide precursor (Park`s nucleotide) mediated by MurA and MurF (Lupoli *et al.*, 2011). The second stage is the generation of lipid I mediated by MraY. Then disaccharide precursor lipid II is produced under the action of MurG and FemXAB (Heijenoort, 2001; Navarre and Schneewind, 1999). Afterward lipid II is translocated to the outside surface through unknown mechanism. In the third stage, lipid II is being incorporated into the nascent peptidoglycan mediated by penicillin-binding proteins (PBPs).

Penicillin binding protein1 (PBP-1) comes under class B PBPs and is a membrane bounded macromolecule. PBP-1 is responsible for the synthesis of major part of cell wall peptidoglycan (PG) (septal PG), cell division β-lactam resistance (Pereira *et al.*, 2007). Proper bacterial cell wall synthesis is a key of bacterial growth, cell integrity and cell division. Many studies have reported the inactivation PBP1 can cause bacterial structural irregularities such as lesion, loss of selective permeability and elongation ultimately leading towards cell lysis and death of bacteria (Foulquier *et al.*, 2014; Wada and Watanabe, 1998). That's why PBP-1 is an important ground-breaking target for drug designing against multidrug resistant bacteria, due to its efficient role in bacteria survival.

The *murA* gene encodes UDP-N-acetyl glucosamine 1-carboxyvinyltransferase enzyme catalysis the replacement of enolpyruvyl group from phosphoenolpyruvate (PEP) to a tetrahedral intermediate of substrates as UDP-N-acetyl glucosamine (UDPAG) and form UDP-N-acetyl glucosamine enol pyruvate and also inorganic phosphate (Pi) (Green, 2002; Yadav *et al.*, 2012). UDP-N-acetyl glucosamine enol pyruvate (EP-UNAG) is an initiator of N-acetylmuramic acid that substitutes with N-acetyl glucosamine results in the formation of glycan's chains leading to peptidoglycan layers in cell wall synthesis (Vesić and Kristich, 2012). This biochemical process of transfer is the foremost and committed step in the peptidoglycan (PG) or murein formation that is the vital structural component of *S.aureus* cell wall (An *et al.*, 2003). As the cell wall structure and composition is considered as the essential components that are necessary for the bacterial survival in host cell body (McDevitt *et al.*, 2002). The combinatorial *murA* and *pbpA* gene knockout would prove deleterious for antibiotic resistant strains in future.

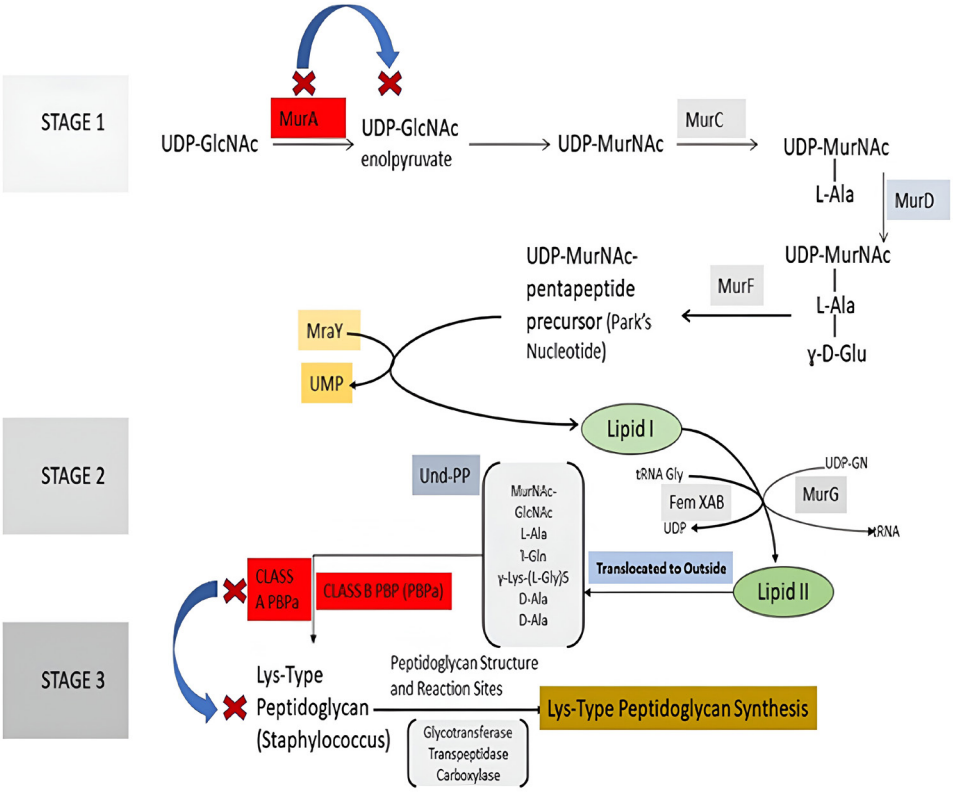

Figure 6: *Biochemical pathway of PBP-1 and MurA.*

Discussion

Multidrug-resistant (MDR) bacterial strains have become a serious public health problem nowadays. *Staphylococcus aureus* resistant strains have caught the eyes of researchers due to their alternative pattern of resistance against all so far discovered front-line antibiotics (Min *et al*., 2025). Therefore, it is the need of hour to support new management strategies and purpose novel essential therapeutic targets against these resistant strains for better surveillance of infections in future (Douglas *et al*., 2023). Similar regional studies have reported variable resistance patterns in *S. aureus*, emphasizing the importance of continuous local surveillance to guide empirical antibiotic therapy and update treatment guidelines (Bellini *et al*., 2019).

In the present study, clinical MDR isolates from Gujrat, Pakistan demonstrated resistance to multiple antibiotics, supporting earlier findings that regional isolates often exhibit broader resistance profiles, likely due to selective antibiotic pressure and uncontrolled usage in clinical settings. These observations reinforce the necessity of region-specific antimicrobial monitoring programs, particularly in underreported areas (Idrees *et al*., 2023).

The results reported clinical isolate with methicillin resistance was also resistance to other second-generation antibiotics (Ampicillin, Amoxicillin), Clindamycin, Kanamycin and Vancomycin. The increase in the emergence of antibiotic resistance specifically Vancomycin among Pakistan isolated clinical strain compromised previously using treatment guidelines, have increased the risk of mortality, morbidity and the greater period of hospital stay with long hospital costs (Muzammil *et al*., 2023). That's why routine monitoring and surveillance of antibiotic susceptibility pattern of local regional *S. aureus* isolates is essential to update information for modern treatment guidelines in order to the selection of appropriate antimicrobial therapy (Hu *et al*., 2024).

The increased antimicrobial resistance provoked the identification of novel putative candidate drug targets within local *S. aureus* resistant isolates for better efficient treatment of infections (Lakhundi and Zhang, 2018). Previous studies have established that MurA catalyzes the first committed step in peptidoglycan biosynthesis, while PBP1 plays a critical role in cell wall assembly and bacterial cell division, making both enzymes highly conserved and attractive targets for antibacterial drug development (Sangshetti *et al*., 2017). The *silico* structural and functional analysis performed in this study further supports their essentiality by identifying conserved domains, predicted active sites, and functional interaction networks. These findings are consistent with earlier computational and experimental reports that highlight the central role of peptidoglycan biosynthesis enzymes in bacterial survival (Ambade *et al*., 2023). The PBP-1 and MurA3D structure was predicted by I-TASSER, COACH, and tracked the active ligand binding sites in the protein.

A key novelty of this work lies in the integration of phenotypic antibiotic susceptibility profiling with *in silico* structural characterization of PBP1 and MurA from clinically isolated MDR strains of *S. aureus* obtained from Gujrat, Pakistan. This provides region-specific molecular insights that are currently underrepresented in the literature and contributes baseline data for future drug discovery efforts targeting local resistant strains. Furthermore, the study suggests that simultaneous targeting of PBP1 and MurA may represent a potential combinatorial strategy to disrupt multiple steps of peptidoglycan biosynthesis, although this remains a theoretical approach requiring experimental validation.

However, the present study has certain limitations, including the small number of clinical isolates analyzed and its reliance on computational predictions without in vitro or in vivo validation. Therefore, the findings should be interpreted as preliminary, and future studies involving larger sample sizes, molecular docking, and functional assays are required to validate these targets and their therapeutic potential.

Conclusion

This study provides region-specific insights into the antibiotic resistance patterns of multidrug-resistant *Staphylococcus aureus* isolates obtained from Gujrat, Pakistan, emphasizing the growing challenge of antimicrobial resistance in local clinical settings. The observed resistance of the clinical isolates to methicillin and multiple additional antibiotics highlights the urgent need for continuous surveillance and the development of alternative therapeutic strategies against MDR strains. In silico characterization

revealed that PBP1 and MurA are conserved proteins involved in essential peptidoglycan biosynthesis pathways, supporting their potential as candidate drug targets against MDR *S. aureus*. The study further suggests that simultaneous targeting of these proteins may offer a promising strategy for future antimicrobial development. However, the findings are limited by the small number of clinical isolates and reliance on computational analyses; therefore, further experimental validation and large-scale studies are necessary to confirm their therapeutic potential.

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Novelty Statement

This study provides preliminary region-specific molecular characterization of PBP1 and MurA from multidrug-resistant Staphylococcus aureus isolates obtained from Gujrat, Pakistan. The integration of local antibiotic resistance profiling with in silico structural and functional analysis highlights these conserved proteins as potential targets for future antimicrobial drug development.

Author's Contribution

KI, Ay conducted Research and wrote Manuscript.
FS and TSB do Bioinformatics Analysis and Data Curation.
UM, Dr. KS and Dr. NZ reviewed, edited and provided expert opinions on Manuscript
All authors read and approved the Final Version of the Manuscript.

Generative AI and AI assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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