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Molecular Detection and Characterization of Efflux Pump Genes Associated with Multidrug Resistance in Clinical Isolates of *Staphylococcus aureus* in Peshawar, Pakistan

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Abstract: *Staphylococcus aureus* is an opportunistic pathogen responsible for a wide range of human diseases. It is renowned for its capacity to develop resistance to multiple antibiotics, primarily due to diverse resistance mechanisms, including efflux pumps, which significantly contribute to intrinsic resistance. This study aimed to investigate the expression of efflux pump genes, namely *Nor*A, *Mep*A, and *Mde*A, in clinical isolates of *S. aureus* obtained from Khyber Teaching Hospital and Hayatabad Medical Complex. A total of 200 clinical samples were collected. The samples were initially cultured on Mannitol salt agar and identified based on physical characteristics and biochemical tests, including catalase and coagulase assays. Molecular confirmation of *S. aureus* isolates was performed using the mecA gene, which is specific to this bacterium. Antibiotic susceptibility testing was conducted following CLSI guidelines to determine resistance patterns against a panel of antibiotics. The presence of efflux pump resistance genes (*nor*A, *mgr*A, *mep*A, *mep*R, and *mde*A) was detected using polymerase chain reaction (PCR). Among the 17 antibiotics tested, the highest resistance was observed against ciprofloxacin, penicillin, ampicillin, and cefoxitin. Out of 200 isolates, 177 were resistant, with various efflux pump genes detected. Statistical analysis using ANOVA (Single Factor) revealed significant findings ($p = 0.004$). The results highlight that efflux pumps play a crucial role in intrinsic resistance mechanisms in *S. aureus*. Consequently, this bacterium has evolved into a multidrug-resistant pathogen, posing a significant challenge to the effective treatment of infections caused by *S. aureus*.

Keywords: *Staphylococcus aureus*, antibiotic resistance, efflux pump genes, multidrug resistance, polymerase chain reaction, clinical isolates.

1. Introduction

Staphylococcus aureus is a Gram-positive, cocci-shaped, facultative anaerobe that forms clusters resembling grape-like arrangements. When grown on mannitol salt agar, it produces golden colonies. Identification of *S. aureus* typically involves biochemical tests such as catalase and coagulase assays [1]. The genome of *S. aureus* is approximately 2.8 b with a low G+C content. Humans serve as the primary reservoir for this pathogen, where it is commonly found on the skin and mucous membranes, particularly in the anterior

nares [2]. Certain populations, including healthcare workers, individuals who frequently use needles (e.g., diabetics, intravenous drug users), hospitalized patients, and immunocompromised individuals, are at higher risk of *S. aureus* infections [3]. This bacterium causes a broad spectrum of infections, ranging from superficial skin conditions such as impetigo, cellulitis, and furuncles, to more severe diseases like endocarditis, bacteremia, toxic shock syndrome, and meningitis [4]. The nature of the infection, whether toxin-mediated or invasive, depends on the infection site and host factors [5]. The treatment of *S. aureus* infections varies based on the type of infection and the presence of antibiotic resistance. Methicillin-sensitive *S. aureus* (MSSA) infections are typically treated with penicillin, while methicillin-resistant *S. aureus* (MRSA) strains require vancomycin as the first-line treatment. However, due to extensive antibiotic use, *S. aureus* has developed resistance mechanisms, rendering it a formidable multidrug-resistant pathogen [6]. Resistance mechanisms in *S. aureus* include intrinsic resistance, adaptive resistance, and acquired resistance, which are mediated by two primary pathways: (i) spontaneous mutations leading to chromosomal changes (e.g., resistance to quinolones, linezolid, and daptomycin) [7], and (ii) acquisition of mobile genetic elements (MGE) through vertical or horizontal gene transfer. Biochemical mechanisms contributing to resistance include decreased antibiotic affinity due to alterations in binding sites, enzymatic inactivation of antibiotics (e.g., β-lactam resistance via β-lactamase production), bypassing metabolic pathways targeted by antibiotics, and enhanced efflux pump activity to extrude antibiotics, such as resistance to fluoroquinolones via the *NorA* efflux pump. In *S. aureus*, efflux pumps serve as a critical first line of defense against antibiotics [8]. The chromosomally encoded *NorA* efflux pump, part of the major facilitator superfamily (MFS), is a key player in antibiotic resistance. Other efflux pumps, such as *mde*A (also belonging to MFS) and *mep*A (a member of the MATE family), further contribute to resistance by extruding macrolides and fluoroquinolones. In this study, various efflux pumps and their regulatory genes were detected in clinical isolates of *S. aureus* using polymerase chain reaction (PCR). The expression of these genes was evaluated in association with antibiotic susceptibility patterns, providing insights into the molecular mechanisms underpinning multidrug resistance in this pathogen.

2. Methodology

A total of 200 clinical isolates were collected from pus and wound samples obtained from Hayatabad Medical Complex and Khyber Teaching Hospital. The isolates were cultured on Mannitol Salt Agar (MSA) plates and identified based on colony morphology, Gram staining, and biochemical tests such as catalase and coagulase. Molecular confirmation of the isolates was performed subsequently. Antibiotic susceptibility patterns were assessed following the Clinical and Laboratory Standards Institute (CLSI) guidelines. Tests were conducted on Mueller-Hinton Agar (MHA) using a panel of 17 antibiotics. Zones of inhibition were measured after 24 hours of incubation, adhering to CLSI standards. Genomic DNA was extracted from the isolates using the GJC® DNA Purification Kit. The DNA was analyzed through electrophoresis on a 1.5% agarose gel and visualized using a Gel Documentation System equipped with Image Lab™ software. The molecular identification of *S. aureus* isolates was confirmed by detecting the *mec*A gene as shown in Table 2, through polymerase chain reaction (PCR). The reaction mixture included 12.5 µl of Taq® Green Master Mix (2X), 1 µl of forward primer, 1 µl of reverse primer, 25 µl of PCR-grade water, and 1 µl of DNA template. The PCR was carried out under standard conditions for 35 cycles. The presence of the *mec*A gene was verified by the visualization of specific PCR bands on agarose gel. Efflux pump resistance genes (*nor*A, *mep*A, and *mde*A), along with their regulators (*mgr*A and *mep*R), were identified using PCR. The reaction mixture consisted of 12.5 µl of GoTaq® Master Mix (2X), 1 µl of forward primer, 1

µl of reverse primer, 25 µl of PCR-grade water, and 1 µl of DNA template. PCR amplification was performed under standard conditions, and the resulting bands were visualized and analyzed using a Gel Documentation System and Image Lab™ software. Of the 200 isolates, 177 harbor efflux pump resistance genes. The amplified PCR products were then subjected to Next-generation Sequencing. The obtained sequences were further analyzed against references in the NCBI database (https://www.ncbi.nlm.nih.gov/), and their mutations were identified using bioinformatics tools such as BioEdit and CLUSTALW (https://www.genome.jp/tools-bin/clustalw). The prediction of mutation stability was assessed using the online I-Mutants software (https://folding.biofold.org/i-mutant/i-mutant2.0.html).

Table 1: Specific Antibiotic used for the determination of Resistance Pattern of *S. aureus*

	Antibiotics (µg)	Symbol	Family	Resistant (mm)	Intermediate (mm	Susceptible (mm)
1	Ciprofloxacin (5)	CIP	Quinolone	<=15	16-20	>=21
2	Gentamicin (10)	CN	Aminoglycosides	<=12	13-14	>=15
3	Amikacin (30)	AK		<=13	14-17	>=18
4	Chloramphenicol (300)	CO	Chloramphenicol	<=12	13-17	>=18
5	Teicoplanin (30)	TEC	Glycopeptide	2	-	2
6	Penicillin (10)	P	Penicillin	<=28	-	>=29
7	Vancomycin (30)	VAN	Glycopeptide	<=16	4-8	>=2
8	Ampicillin (10)	AMP	Penicillin	<=28	-	>=29
9	Linezolid (30)	LZD	Oxazolidinones	<=20	-	>=21
10	Erythromycin (15)	ERY	Macrolides	<=13	14-22	>=23
11	Cefoxitin (30)	FOX	Cephalosporin	<=21	-	>=22
12	Moxifloxacin (5)	MXF	Quinolone	<=20	21-23	>=24
13	Cefaclor (30)	CEC	Cephalosporin	<=14	15-17	>=18
14	Tigecycline (15)	TGC	Tetracycline	<=18	-	>=18
15	Clindamycin (2)	CLI	Lincomycin	<=14	15-20	>=21
16	Fusidic acid (10)	FD	Fusidane	<=29	-	>=30
17	Azithromycin (15)	AZM	Macrolides	<=13	14-17	>=18

Table 2. List of primers designed for efflux resistance gene

	Gene	Primer sequence	GC Content (%)	Product (bp)	Annealing (°C)
1	mecA	F AGAAGATGGTATGTGGAAGTTAG R ATGTATGTGCGATTGTATTGC	39	583	55
2	norA	F CGGTTTAGTAATACCAGTCTTGCC R ACACCTGCTAATGAAACACCT	46	1053	57
3	mgrA	F AATACTTCACGTTGATCGACTTCGG RATGGGATGAATCTCCTGTAAACGTC	44	144	58
4	mepA	F CGCCAGTATTTAAAGCGATGAT R TGCTGCTAAAGCACAAAGTG	41	1256	53
5	mepR	F ATGCACATCAACAAGATGGACTG R GTCAGCCCTATATTCTTTCTTCTCG	43	160	56

6	mdeA	F ATGCACATCAACAAGATGGACTG R GTCAGCCCTATATTCTTTCTTCTCG	52	1316	57

3. Results

In the present study, a total of 200 clinical isolates were confirmed as Staphylococcus aureus through a series of identification processes. PCR analysis confirmed the high expression of efflux pump genes in the isolates that were resistant to ciprofloxacin, moxifloxacin, penicillin, cefoxitin, and ampicillin (n=177). The gender distribution of the *S. aureus* isolates revealed that 52% (n=104) were obtained from male patients, while 48% (n=96) were from female patients. The study thus showed a near-equal representation of both genders, with a slight predominance of male isolates. The age distribution of the participants showed that the highest proportion of individuals (26%, n=52) were in the 1–10 years age group, followed by 18.5% (n=37) in the 81–90 years group and 12.5% (n=25) in the 21–30 years group. The 71–80 years group represented 11.5% (n=23), while 10% (n=20) of participants were between the ages of 31 and 40. The 41–50 years age group comprised 6% (n=12), and the 61–70 years group had 6.5% (n=13). The lowest frequencies were recorded in the 51–60 years group (3.5%, n=7) and the 11–20 years group (5.5%, n=11). The resistance profile of the 200 *S. aureus* isolates tested against 17 antibiotics revealed the highest resistance against Cefoxitin, with 100% (n=200) of isolates resistant. Significant resistance was also observed against Penicillin (81.5%, n=163), Ampicillin (81%, n=162), and Moxifloxacin (75%, n=150). In contrast, Vancomycin and Teicoplanin demonstrated 100% sensitivity, with no resistance observed. Gentamicin, Amikacin, and Tigecycline exhibited high sensitivity rates of 99%, 97.5%, and 99.5%, respectively. Chloramphenicol, Linezolid, and Clindamycin also showed high sensitivity, with 96.5%, 96.5%, and 98.5% sensitivity, respectively. The overall results are shown in Table 3. Statistical analysis was performed using IBM SPSS Statistics 20 and MS Excel 2013. The antibiotic susceptibility patterns were analyzed, revealing a significant association between antibiotics with a p-value of 0.004. The molecular characterization of the efflux resistance genes revealed that the *NorA* gene was found in 133 isolates, which represents 66.5% of the samples. The *mgrA* gene was detected in 149 isolates, accounting for 74.5% of the samples. The *mepA* gene had the highest prevalence, present in 160 isolates (80%). The *mepR* gene was identified in 111 isolates, or 55.5% of the total, while the *mdeA* gene was found in 139 isolates, which is 69.5% of the samples. Sequencing and mutational analysis of efflux pump resistance genes were performed using Next Generation Sequencing (NGS) followed by mutational analysis through I-Mutant software. Mutation analysis revealed a non-synonymous mutation in the mepA gene, while no mutation was detected in the norA gene. The non-synonymous mutations in the mepA gene were as follows: at codon position 9, the reference amino acid proline (CCG) was altered to proline (CCA), leading to a change at position 3 in the amino acid sequence. Additionally, at codon position 975, the reference amino acid threonine (ACC) was altered to threonine (ACT), resulting in a change at position 325 in the amino acid sequence.

Table 3: The result of specific antibiotic used against *S. aureus*

	Antibiotics	Resistant	Intermediate	Sensitive
1	Ciprofloxacin	176	0	24
2	Gentamicin	1	1	198
3	Amikacin	0	5	195
4	Chloramphenicol	0	7	193
5	Teicoplanin	0	0	200
6	Penicillin	163	37	0
7	Vancomycin	0	0	200
8	Ampicillin	162	38	0
9	Linezolid	0	7	193
10	Erythromycin	7	17	176
11	Cefoxitin	200	0	0
12	Moxifloxacin	150	26	24
13	Cefaclor	0	16	184
14	Tigecycline	1	0	199
15	Clindamycin	3	0	197
16	Fusidic acid	2	11	187
17	Azithromycin	10	17	173

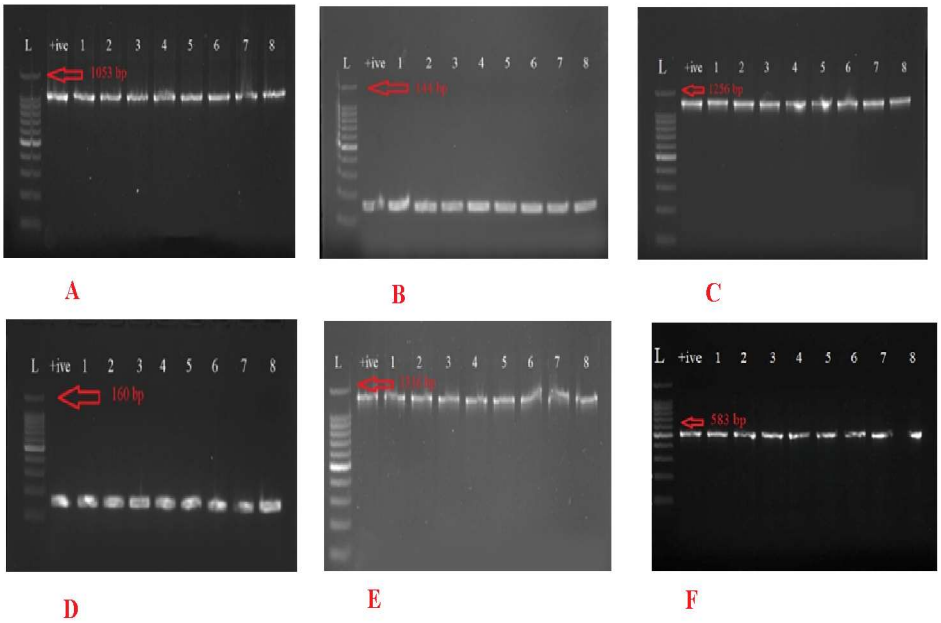

Figure 1: Electrophoretogram showing amplicons of *S. aureus* efflux resistance gene. L: 100bp molecular marker, Lane 1 to 8: positive isolates of the amplified gene **(A)** *Nor*A gene **(B)** *mgr*A gene **(C)** *mep*A gene **(D)** *mep*R gene **(E)** *mde*A gene **(F)** *mec*A gene

4. Discussion

In *S. aureus*, resistance is primarily attributed to intrinsic resistance mechanisms, particularly due to the presence of efflux pumps. A key player in the intrinsic resistance of *S.*

aureus to fluoroquinolones is the *norA* gene. A case study from Tehran reported the presence of the *norA* gene in clinical isolates, with 24% of isolates resistant to ciprofloxacin, 68% resistant to methicillin, and 32% susceptible to methicillin [13]. Another study demonstrated resistance rates to Gentamicin (12.5%), ciprofloxacin (42.5%), Cotrimoxazole (12.5%), Amoxicillin (87.5%), and Erythromycin (50%), and found that the *norA* gene and its regulator were present in ciprofloxacin-resistant isolates. Real-time PCR further revealed that these ciprofloxacin-resistant isolates expressed efflux pump genes, confirming a link between ciprofloxacin resistance and efflux pump expression in *S. aureus* [14]. In line with these findings, a study confirmed the presence of efflux pump genes in ciprofloxacin-resistant isolates of *S. aureus*. Of 50 isolates, 68% were methicillin-resistant *S. aureus* (MRSA), and 24% of these were resistant to ciprofloxacin. All these isolates were positive for the *norA* gene along with its regulator [15]. Another study corroborated the presence of the *mecA* gene in MRSA isolates, revealing that the efflux pump genes *norA*, *mepA*, and *mdeA* were present in these isolates, with *mdeA* showing the highest prevalence, followed by *mepA* and then *norA* [16]. The regulator *mgrA* was identified as an activator for the *norA* gene, while it acted as a repressor for other efflux pumps. These findings support the idea that *mgrA* regulates efflux pump genes differently depending on the specific pump involved [17]. Additionally, it was confirmed that *mepR* functions as an autoregulatory repressor for its gene as well as for *mepA*. The repressive activity of *mepR* allows the release of *mepA* by binding to its substrate, thereby modulating the expression of the *mepA* gene [18]. Efflux pump genes were also found to be present in ciprofloxacin-resistant isolates of MRSA, with most of these isolates exhibiting resistance to ciprofloxacin [19]. Furthermore, a study published in 2020 confirmed the presence of the *MpeA*, *NorA*, and *MdeA* genes in ciprofloxacin-resistant MRSA isolates, with prevalence rates of 10%, 60%, and 6%, respectively [20]. Another study in 2020 reported that 70% of isolates showed strong efflux activity, while 30% exhibited intermediate activity. The *norA* and *mdeA* genes were identified as major contributors to the strong efflux activity, while other efflux pumps played a minor role [21]. The expression of these genes, along with their regulators, highlights the complexity of resistance mechanisms and suggests that targeting efflux pumps could be a promising strategy to combat resistant strains. Overall, the identification of these efflux genes in clinical isolates reinforces the need for ongoing surveillance and the development of novel therapeutic approaches to overcome antibiotic resistance in *S. aureus*.

5. Conclusions

The findings of this study conclude that Staphylococcus aureus exhibits a significant capacity for resistance to a wide range of antibiotics, affecting individuals across all age groups. This highlights that infections caused by *S. aureus* are not confined to any specific demographic. Fluoroquinolones were found to be the most resistant antibiotic in clinical isolates, with a high prevalence in pus and wound swab samples. The primary mechanism of intrinsic resistance observed in this study was the activity of efflux pumps, which provides critical insight for selecting more effective treatment strategies for *S. aureus*-associated infections.

Author Contributions

Aqsa Zafar conducted experiments and contributed to data analysis and manuscript drafting. Hoor Hamid supervised the study and finalized the manuscript. Hadiba Saadat, Talha Imtiaz, and Khushal Akbar assisted with genetic analysis, data validation, and technical support.

Conflicts of Interest

The authors declare no conflicts of interest.

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