Original Research Article

Genetic analysis of Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from the population of Southern Punjab, Pakistan

ABSTRACT

The study was aimed to isolate and identify the methicillin-resistant strains and then detect the genetic variants and investigate *S. aureus* isolates that were resistant to methicillin found in the community of Southern Punjab. Collecting the isolates of *S. aureus* from the Southern Punjab region of Pakistan. Isolation and identification of these collected isolates were done by subjecting these isolates to laboratory procedures. A polymerase chain reaction was performed for the molecular and genetic analysis. 60 urine and 40 blood samples were taken from outdoor and indoor patients of the Nishtar Medical College & Hospital, Multan. Gram staining and different biochemical assays were done to confirm the presence of *S. aureus*. After the confirmation of *S. aureus*, DNA extraction was performed by a modified method of CTAB. A polymerase chain reaction was performed to analyze the size of amplicons found in the Southern Punjab community. In order to check the resistance and susceptibility pattern of *S. aureus* against beta-lactam antibiotics and fluoroquinolones, the Kirby-Bauer method was used. Out of 100 samples, 98 were cultured on blood agar and mannitol salt agar. 92 tested gram-positive and out of which only 88 gave positive results for the catalase test. When a coagulase test was performed, 85 produced coagulations with plasma in the test tubes. Upon antibiotic susceptibility testing, 50 samples were found as methicillin-resistant *S. aureus.*67% of methicillin-resistant *S. aureus* contains *mecA*[3], *femA*[3], *aac(6')/aph(2'')*, *Tet(K)*[13], *Tet(M)*[13] genes and methicillin-susceptible *S. aureus* has 33% less prevalence as compared to methicillin-resistant *S. aureus.* Southern Punjab region of Pakistan was found to possess the genes *mecA*[3], *femA*[3], *aac(6')/aph(2'')*, *Tet(K)*[13], and *Tet(M)*[13]. Southern Punjab region outnumbered in Methicillin-resistant *S. aureus*(MRSA) isolates in terms of the prevalence of *mecA*[3], *femA*[3], *aac(6')/aph(2'')*, *Tet(K)*[13], *Tet(M)*[13] genes. Non-beta lactam antibiotics can be used to treat MRSA infections.

Key words: *Staphylococcus aureus*, MRSA, Antibiotic resistance, Methicillin, Genetic characterization

1. INTRODUCTION

The *Staphylococcus* infection is normally linked with increased antimicrobial resistance in *S. aureus* [1]. *S. aureus* is a bug that generally persists in the human body and is prominent as a hazardous disease-causing agent to humans, causing considerable disease and deaths all over the world [2]. Pharmaceuticals are considered the most effective means to treat bacterial infestation, but bacteria develop the ability to resist the effectiveness of the particular drug as well as survive during drug treatment at a faster pace [3]. Noxious substances, biocatalysts, and molecular compounds having pathogenic effects are released by *S. aureus* [4]. *S. aureus* residing on the host interface does not cause any infection, but intrusive bacterial infections result from the bug affecting the first line of defense of the

host [5]. Antibacterial therapy against *S. aureus* is a major threat to healthcare professionals in treating drug tolerance [6].

The increase in resistance against antimicrobial agents is an area of concern for scientists. Infections caused by *S. aureus* increase the spread of Methicillin resistance [7]. Markedly pathogenic, resistant bacterial varieties named Methicillin-resistant *S. aureus*(superbug) are remarkably difficult to cure [8]. Superbugs augment the risk of decreasing the effectiveness of antiseptics because of their adaptation to mechanisms. Non-therapeutic use of antibiotics causes superbug infections leading to an increased death rate every year [9]. Infections caused by Methicillin-resistant *S. aureus* are responsible for more mortality as compared to non-Methicillin-resistant *S. aureus* infections [10]. Genetic modifications in Methicillin-resistant *S. aureus* allow the bacteria to develop resistance against a variety of beta-lactam antibiotics [11]. Infections cause *S. aureus* were first treated with penicillin, but extensive use of penicillin develop resistance in *S. aureus.* To fight infections of penicillin-resistant *S. aureus*, methicillin was used. Subsequent to methicillin administration, Methicillin-resistant *S. aureus* subtypes emerged [12].The transposable elements causes Methicillin-resistant *S. aureus* persistence. These transposable elements have beta-lactamase-resistant penicillin-binding protein 2a with the peptidoglycan cross linking activity. These beta-lactamase penicillin binding protein 2a establishes weak affirmation with all beta-lactam antimicrobial agents which enables the bacteria sustain cell wall synthesis in the presence of antimicrobial agents [13].

Single Methicillin-resistant *S. aureus* subtypes do not show resistance against all antimicrobial agents. Despite advances in treatment, the issue of resistance continues to present challenges to effective treatment. The problem is the little knowledge about the resistant subtypes and its impact of resistance on development of disease [14]. The main objective of the current study was the genetic variant detection and investigation of *S. aureus* isolates resistant to methicillin and identification of the genetic variants associated with *mecA*[3], *femA*[3], *aac*(6')/*aph*(2''), *Tet(K)*[13], *Tet(M)*[13].These genetic variants study will help the molecular characterization of MRSA infection severity, minimum inhibitory concentration and infection epidemiology in Pakistan.

Gram-positive bacteria were responsible for nearly half of the cases (48.4%) with polymicrobial *S. aureus* bacteremia (p-SAB). *Enterococcus* species, *S. agalactiae*, and *viridans* group *streptococci* were the most frequent Gram-positive bacteria found. Gram-negative bacteria were discovered in 29% of cases, with the most prevalent being *E. coli* and *P. aeruginosa*. There were additional cases of mixed infections combining Gram-positive and Gram-negative bacteria (12.9%) and bacteria and fungus (9.7%). This study emphasizes the heterogeneous microbiology of p-SAB infections and the presence of both single and multiple pathogens.The study findings imply that p-SAB patients had lower incidence rates than previously reported, with Gram-positive bacteria accounting for a considerable number of cases. Furthermore, p-SAB patients had a worse survival rate than m-SAB patients. This work adds to our understanding of p-SAB in the US population [15].

S. aureus is a major cause of both community- and hospital-acquired bacteremia, with a 30% fatality rate. Methicillin-resistant Staphylococcus aureus (MRSA) bacteremia is a developing problem, with greater morbidity and mortality than MSSA bacteremia. The burden of *S. aureus* bacteremia (SAB) must be determined in order to allocate healthcare resources appropriately [16].In our study, urine and blood samples from patients were collected, and identified *S. aureus* strains, including both methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA). To determine the association between polymicrobial or monomicrobial infections and specific bacteria, a thorough analysis of the collected samples was conducted. Upon analyzing the data, it was found that *S. aureus* was frequently associated with both polymicrobial and monomicrobial infections. However, it is important to note that the prevalence and association of other bacteria in polymicrobial or monomicrobial infections varied. Certain gram-negative bacteria, such as *E. coli* and *K. pneumoniae*, were more commonly found in polymicrobial infections, while other gram-positive bacteria, such as *Streptococcus* species, were more frequently associated with monomicrobial infections.

2. MATERIALS AND METHODS

2.1 Sample collection, culturing, and preservation

To obtain a pure culture of *S. aureus*, a total of 100 clinical specimen of *Staphylococci* were collected from out and in patients hospitalized in Nishtar Medical College & Hospital Multan, in the Southern Punjab Pakistan. Sample collection lasted for 7 months from June 2022 to December 2022. *S. aureus* was obtained from urine and blood specimens of individuals aged 15 years and above without any gender discrimination. Blood and urine specimen were taken according to the standard practices. Urine samples were taken in sterile cups whereas, for blood samples Bactec Vials were used [17].

During the bacteria isolation process, the co-presence of two different strains of *S.aureus* in the same sample was observed. Urine and blood samples were inoculated onto the differential media(MSA) plates using a sterilized wire loop. Sample tagging was done and plates were incubated for a day at 37°C.MSA contains high salt content, mannitol, and sugar alcohol, this causes the *S. aureus* to ferment. Yellow colonies on MSA are termed *S. aureus* isolates, whereas red isolates are regarded as coagulase-negative Staphylococci. Some bacteria are inhibited by the media, while others are allowed to proliferate. Hemolytic pattern indicates the presence of *S. aureus*. Single yellow colony was taken to study the microscopic morphology of *S. aureus* [17].

For preservation glycerol stock technique was adopted. To make glycerol stock of pure colonies, first pure cultures were taken from MSA plates and cultured on nutrient broth. Glycerol reserves in bacteria play a crucial role in preserving plasmids for extended periods of time [18]. In case of any uncertainty in results, these preserved samples were utilized for troubleshooting.

2.2 Biochemical tests

2.2.1 Gram staining

To study the morphology of *S. aureus*, gram staining technique was applied. Staining technique used to identify the shape, size, structure and organization of *S. aureus*. Glass slides were sterilized using Bunsen burner and single colony of *S. aureus* taken from glycerol stock was placed onto the glass slide. Primary stain was applied for 60 seconds and then washed with distilled water. Fixative agent was then placed onto the glass slide and rinsed with distilled water after a minute .After a minute of applying the secondary stain and rinsing it with distilled water, glass slide was allowed to dry. Morphology was checked using a compound microscope [19].

2.2.2 Catalase test

Responses to H_2O_2 was observed at concentration of 0.5%, 1%, 2%, 3%, 4%, and 5%. Onto the sterilized glass slide, placed a single colony of *S. aureus*. Few drops of H_2O_2 were added on slide. If frequent bubble formation occurs, it confirms the presence of *S. aureus* [20].

2.2.3 Coagulase test

Onto the glass slide, place single colony of *S. aureus* and a few drops of sodium citrate anticoagulated plasma was added. Within 10 seconds of introducing bacterial cells to the plasma, look for clumping [21].

2.3 DNA extraction

To extract DNA from bacterial pallet, CTAB/Phenol-Chloroform extraction method was utilized. The microcentrifuge tubes containing bacterial pallet was combined with Tris-EDTA, 5M solution of Sodium chloride, CTAB buffer. The sample was incubated for half an hour and then treated with phenol-chloroform. DNA was precipitated with Isopropanol and was then rinsed with 70% ethanol. After that, DNA sample was preserved in PCR water [22]. The gel doc system (***BIO-RAD*** Gel Doc ™XR=with Image Lab ™Software) was used for gel visualization

2.4 Detection of genetic variants of MRSA

Methicillin resistance genes (*mecA*[3], *femA*[3],*aac(6')/aph(2")*,*Tet(K)*[13], *Tet(M)*[13]) was amplified with the help of thermal cycler PCR (Thermo Scientific) in which targeted primers for these genes were used. Table 1. enlist the primers of genes *mecA*[3],*femA*[3],*aac(6')/aph(2")*, *Tet(K)*[13],*Tet(M)*[13] to study the genetic basis of MRSA [23]. Primers used are provided in Table 1.

Table 1. Primers used for studying the genetic basis of MRSA

Gene name	Primer	Sequence 5' to 3'	Amplicons size (bp)
mecA[3]	Forward Reverse	-CCTAGTAAAGCTCCGGAA- -CTAGTCCATTCGGTCCA-	314bp
femA[3]	Forward Reverse	-AAAAAAGCACATAACAAGCG- -GATAAAGAAGAAACCAGCAG-	132bp
aac(6')/aph(2'')	Forward Reverse	-GAAGTACGCAGAAGAGA- -ACATGGCAAGCTCTAGGA-	491bp
Tet(K)[13]	Forward Reverse	-GTAGCGACAATAGGTAATAGT- -GTAGTGACAATAAACCTCCTA-	360bp
Tet(M)[13]	Forward Reverse	-AGTGGAGCGATTACAGAA- -CATATGTCCTGGCGTGTCTA-	158bp

A total volume of the PCR reaction was 25µL in which 12.5µL of Master Mix (Vazyme Biotech Co., Nanjing, China), 2.5µL of forward and reverse primers. 1µL of DNA and 6.5µL of deionized water was used. Profile for PCR amplification of *mecA*[3] and *femA*[3] gene involve following conditions: initial denaturation at 94°C for 5min followed by 35 cycles of 94 °C for 2 min, 55 °C for 2 min, 72 °C for 1 min and 72 °C for 7 min [23]. Profile for PCR amplification of *aac(6')/aph(2'')* involve following conditions: initial denaturation at 95°C for 5min followed by 30 cycles of 95 °C for 2 min, 54 °C for 1 min, 72 °C for 1 min and 72 °C for 7 min [23]. Profile for PCR amplification of *Tet(K)*[13],*Tet(M)*[13] involve following conditions: initial denaturation at 95°C for 3min followed by 30 cycles of 95 °C for 30sec, 54 °C for 30sec, 72 °C for 4 min and 72 °C for 7 min [23]. The PCR products of the anticipated size were resolved using a 1.5% agarose gel. In each well, placed 3µl-5µl of the PCR sample mixture. To determine the size of the required bands,100bp of the ladder was loaded into 1.5% agarose gel. Gel was exposed to 120 volts for 1 hour and 10 minutes. The bands were visualized when UV light pass through the gel [24].

2.5 Antimicrobial susceptibility testing

S. aureus susceptibility testing was done by Kirby-Bauer method using four different antibiotics: Methicillin (MET), Penicillin (P), Levofloxacin (LVX), and Ciprofloxacin (CIP).The choice of empirical antibiotics in our study was based on several factors, including the known spectrum of activity, clinical experience, and local resistance patterns. Penicillin, methicillin, ciprofloxacin, and levofloxacin are commonly used empirical antibiotics in clinical practice due to their broad-spectrum coverage against many common bacterial pathogens. Penicillin is effective against a wide range of gram-positive bacteria, including *streptococci*. Methicillin, a type of penicillin, is specifically used to treat infections caused by

methicillin-resistant *S. aureus* (MRSA). Ciprofloxacin and levofloxacin are broad-spectrum fluoroquinolone antibiotics that cover a wide range of gram-negative and gram-positive bacteria. These antibiotics are frequently used as empirical choices for various types of infections, including respiratory tract infections, urinary tract infections, and skin and soft tissue infections. While the specific choice of empirical antibiotics may vary depending on the type of infection and local resistance patterns, we believe that the inclusion of penicillin, methicillin, ciprofloxacin, and levofloxacin as empirical antibiotics in our study is justified based on their established efficacy and broad-spectrum coverage.

The antimicrobial susceptibility testing was done by Kirby-Bauer method. Kirby-Bauer method is the preferred method in order to determine the drug resistance. Inhibition zone near the drug indicates the resistance pattern of particular specie [25]. Because of high nutrient content for the bacterial growth, Muller Hinton agar (MHA) used for susceptibility test. Inhibitory zone was observed and measured. Those isolates having inhibitory zone less than 17mm would likely be considered as resistant to methicillin and termed as Methicillin-Resistant *S. aureus*. Whereas, isolates with inhibitory zone 17mm are sensitive and termed as Methicillin-Sensitive *S. aureus* (MSSA) [17].

3. RESULTS

3.1 Biochemical assay results

Out of total 100 isolates of *S. aureus*, 92 samples were gram positive. Upon further differentiation ,88 samples testing positive for catalase and , 85 samples produced coagulation with plasma in the test tubes. Antimicrobial susceptibility testing result confirmed 75 MRSA isolates.

3.2 Genetic factors involve in MRSA

Specific PCR primers were used to identify genes responsible for antibiotic resistance in MRSA. The *mecA*[3], *femA*[3], *aac(6')/aph(2")*, *Tet(K)*[13], *Tet(M)*[13] gene were targeted. Out of 75 samples tested, 50 were found to be positive for *mecA*[3], *femA*[3], *aac(6')/aph(2")*, *Tet(K)*[13], *Tet(M)*[13] genes. The resulting amplicon sizes were determined to be 314bp, 132bp, 491bp, 360bp, and 158bp, respectively. This suggests that 67% (n=50) of samples carried each of these genes responsible for MRSA resistance, while the others may contain analogous genes responsible for resistance. Whereas, these genes have a 33%(n=25) less prevalence rate in Methicillin Sensitive *S. aureus*. Figure 1. Shows the genes *mecA*[3] upon *S. aureus* isolation and confirmation of MRSA in 50 samples out of 75.

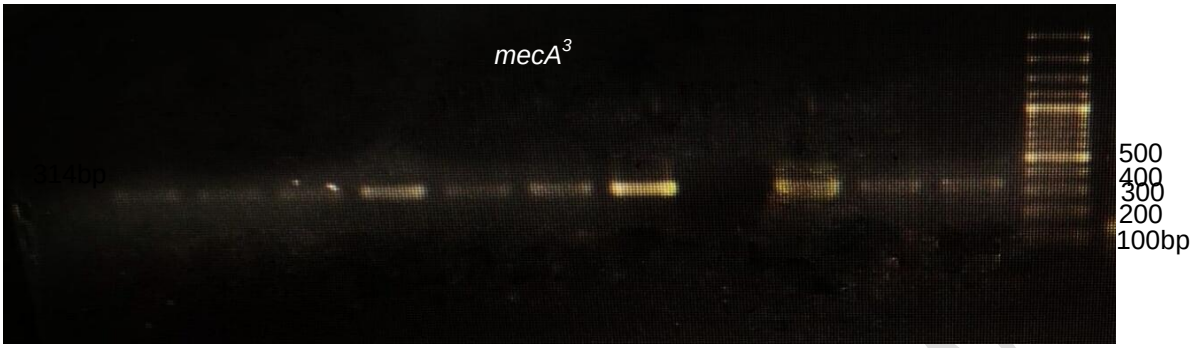

Fig 1. PCR results of *mecA*^{3}. Molecular detection and prevalence of MRSA-associated genetic factors show bands of size 314bp, which is related to *mecA*^{3} and these bands were absent in MSSA.

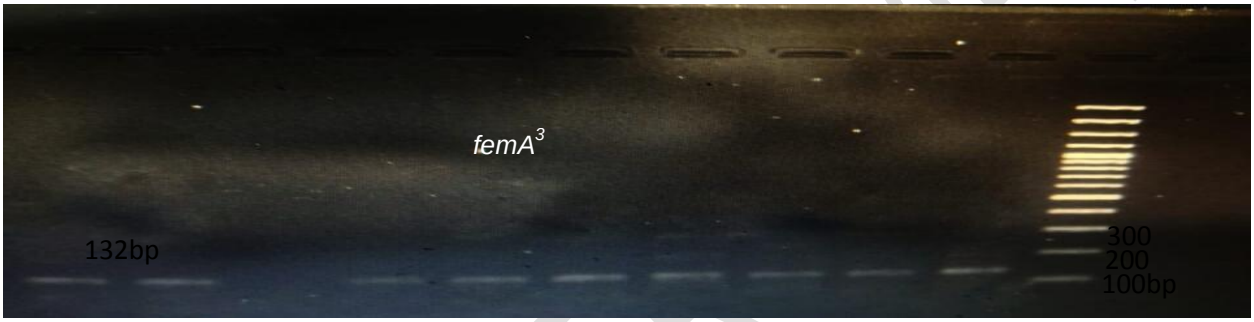

Fig 2. PCR results of *femA3.* Molecular detection and prevalence of MRSA-associated genetic factors show bands of size 132bp, which is related to *femA*^{3} and these bands were absent in MSSA.

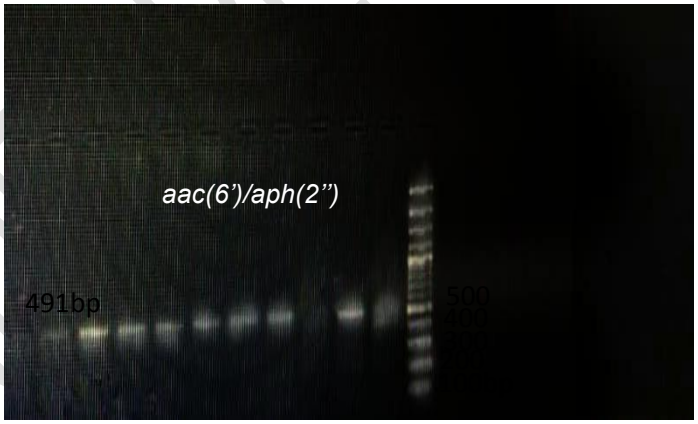

Fig 3. PCR results of *aac(6')/aph(2'').* Molecular detection and prevalence of MRSA-associated genetic factors show bands of size 491bp, which is related to ***aac(6')/aph(2'')*** and these bands were absent in MSSA.

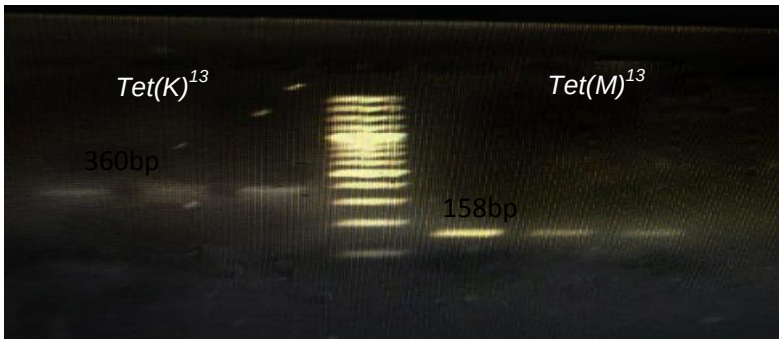

Fig 4. PCR results of *Tet(K)*[13], *Tet(M)*[13]. Molecular detection and prevalence of MRSA-associated genetic factors show bands of size 360bp and 158bp, which are related to *Tet(K)*[13], *Tet(M)*[13] respectively, and these bands were absent in MSSA.

Graphical representation in Figure 5. shows the prevalence of genes in MRSA and MSSA isolates

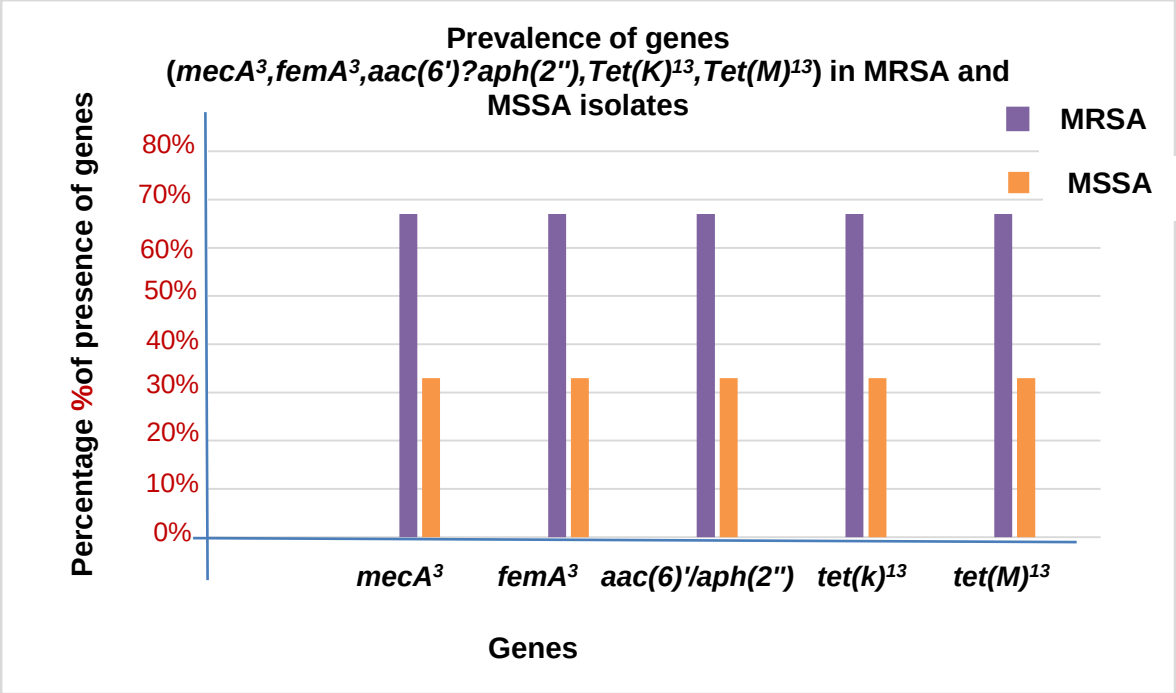

Fig 5. Prevalence of *mecA*[3], *femA*[3] ,*aac(6')/aph(2'')*, *Tet(K)*[13],*Tet(M)*[13]

3.3 Antibiotic resistance and susceptibility pattern in *S. aureus*

The resistance pattern of MRSA and susceptibility pattern of MSSA for methicillin(MET), penicillin(P), levofloxacin (LVX), ciprofloxacin (CIP) determined by Kirby-Bauer method. Figure 6 represents the presence of resistance and susceptibility pattern in *S. aureus*.

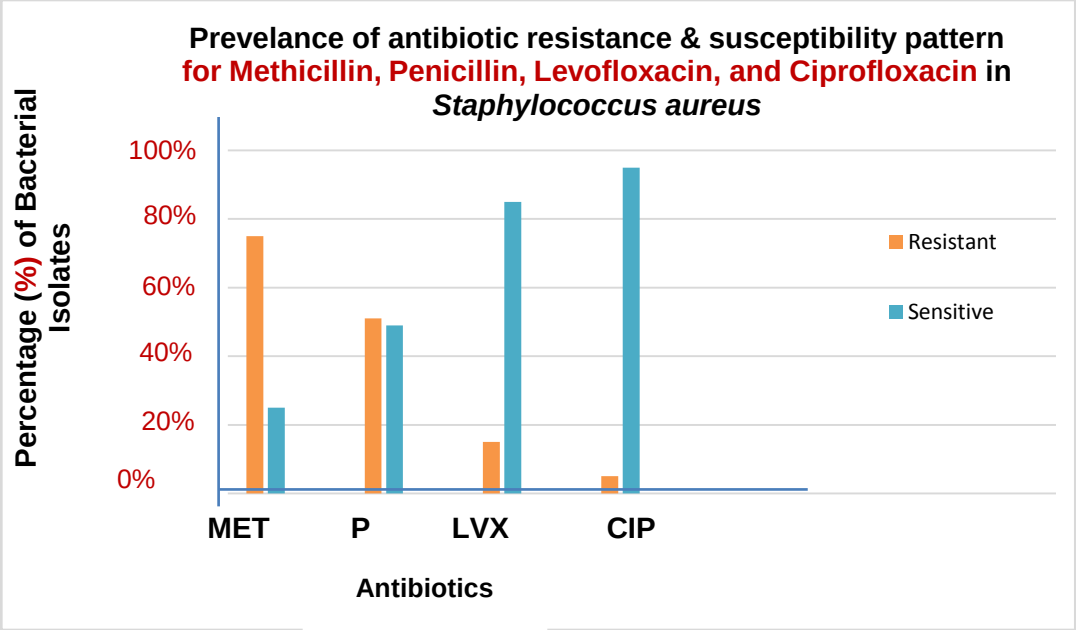

Fig 6. Prevalence of antibiotic resistance & susceptibility pattern in *S. aureus*

4. DISCUSSION

The study investigated the genetic variants and antibiotic resistance & susceptibility patterns in *S. aureus* isolates using beta-lactam as well as fluoroquinolones antibiotics. This data can assist healthcare professionals in identifying local resistance patterns and building local treatment methods, and non-beta-lactam medicines may be beneficial in treating MRSA infections in the Southern Punjab region. *S. aureus* were isolated from patients with different diseases by taking their blood and urine samples. If *S. aureus* enters the blood stream it can leads to high fever, chills, and even pneumonia and the condition is termed as “Bacteremia”. The possibility of this condition often occurs in Type 2 Diabetic patients whose immune system already weakened. *Staphylococcal* infections affect skin, lungs, kidneys, throat and leads to pus formation. Urinary tract infection (UTI’s) patients were instructed for providing their blood and urine samples for the detection of *Staphylococcal* infection.

After the isolation and detection by different biochemical assays, 75 out 100 isolates were confirmed as *S. aureus*. Polymerase chain reaction was performed for the molecular identification. As *S. aureus* gave positive gram staining results, it confirmed the presence of thicker cell wall so a modified approach of CTAB was applied for the extraction of DNA. And all gram- negative isolates were not found to produce coagulation in the plasma. Upon testing by using beta-lactam antibiotics and fluoroquinolones, 75% (N=50) were methicillin-resistant *S. aureus* and 33%(n=25) were methicillin susceptible *S. aureus*.66.7% isolates were recognized as MRSA and 33.3% as MSSA in the territory of REHIM YAR KHAN [26]. And according to the reports from the territory of ISLAMABAD around 65% isolates of *S. aureus* that shows resistance to Methicillin were also found resistant to antibiotic termed cefoxitin [26].It is

crucial to note that resistance patterns might change among areas and even across different healthcare institutions within the same location. Local prescription practices, antibiotic use, and infection control methods can all contribute to these disparities. As a result, it would be beneficial to compare the findings of this study with those of other studies done in other countries in order to acquire a better knowledge of the variances in antibiotic resistance patterns and genetic profiles of *S. aureus* strains.About 85% isolates were found susceptible to fluoroquinolone (Ciprofloxacin) according to reports from territory of REHIM YAR KHAN [27].

MRSA infection was first treated by antibiotic, vancomycin. But after the resistance pattern against vancomycin arose, linezolid was suggested for bacterial infections. Linezolid works by inhibiting the protein synthesis at the 23S ribosomal site of bacterial ribosome [15]. *mecA*[3], *Tet(K)*[13], *Tet(M)*[13], and *aac(6')/aph(2")* genes were found in ISLAMABAD territory and the prevalence rate reported was 54%, 87%, 80% and, 75% respectively [26].

This genomic research sheds light on the processes driving antibiotic resistance and aids in understanding the genetic variety of *S. aureus* strains in the region. It is important to note that resistance patterns can vary between regions and even within different healthcare facilities within the same region. Factors such as local prescribing practices, antibiotic usage, and infection control measures can contribute to these differences. Therefore, it would be valuable to compare the findings of this study with other studies conducted in different regions to gain a broader understanding of the variations in antibiotic resistance patterns and genetic profiles of *S. aureus* strains.

The study's findings on MRSA isolates in the Southern Punjab region are relevant and applicable in a variety of ways. To begin, the researchers identified certain genes found in MRSA isolates, such as *mecA*[3], *femA*[3], *aac(6')/aph(2"), Tet(K)*[13], and *Tet(M)*[13]. This knowledge is critical for making informed treatment decisions since MRSA infections frequently require different medications than MSSA to infections. The study also found that MRSA isolates outnumbered MSSA isolates in terms of the prevalence of the detected genes. This difference emphasizes the significance of precise MRSA diagnosis and tailored therapy.

Furthermore, the study found that non-beta lactam antibiotic resistance was low in the Southern Punjab region. This shows that non-beta lactam antibiotics may be useful in the treatment of MRSA.In terms of comparisons with other areas and localities, it is worth noting that the study was limited to the Southern Punjab region. As a result, without more investigation, the findings may not be immediately relevant to other areas or locales. Variations in MRSA frequency and antibiotic resistance profiles have been identified in different geographical locations, emphasizing the importance of region-specific research.To completely comprehend the variations in findings among areas, this study should be compared to other studies done in other regions. Such comparisons would give a more comprehensive

picture of MRSA frequency, antibiotic resistance patterns, and the practical consequences of treatment decisions in different places.

5. CONCLUSION

MRSA isolated from the Southern Punjab region were found to possess the genes *mecA*[3], *femA*[3], *aac(6')/aph(2'')*, *Tet(K)*[13], and *Tet(M)*[13]. Methicillin-resistant *S. aureus*(MRSA) isolates outnumber Methicillin-sensitive *S. aureus* (MSSA) isolates in terms of the prevalence of *mecA*[3], *femA*[3], *aac(6')/aph(2'')*, *Tet(K)*[13], *Tet(M)*[13] genes.This distinction is critical for making appropriate treatment decisions, as MRSA infections frequently require different medicines than MSSA infections. Antibiotic resistance was minimal for non-beta lactam drugs. It's possible that the genetic elements responsible for those drugs were missing or were not expressed. As a result, non-beta lactam antibiotics may be used to treat MRSA infections in certain areas.The future work could include investigating the mechanism of antibiotic resistance in MRSA isolates that are resistant to non-beta lactam antibiotics.A larger study could also include other bacterial pathogens, such as *K. pneumonia*, which is often associated with hospital-acquired infections and has been reported to exhibit high levels of antibiotic resistance.Association of these genes with other antibiotics. Similar diagnostics methods can be used for treatment.

Ethical Approval:

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

Consent

As per international standard or university standard, patient(s) written consent has been collected and preserved by the author(s).

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