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Phenotypic And Genotypic Evaluation Of Methicillin Resistant Staphylococcus Aureus From Hospital Environment

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ABSTRACT

Methicillin-Resistant *Staphylococcus aureus* (MRSA) poses a significant public health threat in healthcare settings globally. This research aimed to conduct a thorough examination of the phenotypic and genotypic characteristics of MRSA isolated from hospital environments. The study encompassed a comprehensive analysis of 50 samples collected from various hospitals in Peshawar. Using both phenotypic and genotypic methods, MRSA strains were identified, constituting 8% of the sampled isolates. Notably, the study correlated its findings with a previous investigation, highlighting variations in MRSA prevalence attributable to sample types and hygiene conditions within Peshawar hospitals. The results underscored the persistence of MRSA strains in hospital settings, emphasizing heightened virulence and a continuous trend of antibiotic resistance. Further analyses delved into the antibiotic resistance patterns of the isolated MRSA strains, revealing comprehensive resistance to multiple antibiotics, including oxacillin, vancomycin, linezolid, ciprofloxacin, doxycycline, chloramphenicol, and clindamycin. This multidrug-resistant profile posed significant challenges for infection control practices and necessitated a re-evaluation of antibiotic usage in healthcare environments. In addition to the phenotypic assessment, a genotypic evaluation was conducted to identify specific *mecA* gene contributing to methicillin resistance. The research findings hold implications for public health, urging the development of targeted interventions, policy recommendations, and collaborative efforts with clinical teams and infection control departments. This study contributes valuable insights into the complex interplay of phenotypic and genotypic features of MRSA, guiding future strategies for infection prevention and control.

Introduction

In the ever-evolving landscape of infectious diseases, Methicillin-Resistant *Staphylococcus aureus* (MRSA) has emerged as a significant public health concern. This bacterial strain, resistant to multiple antibiotics, poses a formidable challenge to healthcare systems worldwide (Firdous *et al.*, 2023). Methicillin-Resistant *Staphylococcus aureus* (MRSA) was first identified in the early 1960s, MRSA has since evolved into a global menace, causing a wide range of infections with elevated morbidity and mortality rates. The discovery of MRSA traces back to the dawn of the

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antibiotic era when penicillin, once a panacea, was rendered ineffective against certain *Staphylococcus aureus* strains (Lee *et al*., 2018). Sir Alexander Fleming's ground breaking work paved the way for the introduction of methicillin in the 1960s. However, within a short period, resistance to methicillin was documented, giving rise to the term Methicillin-Resistant *Staphylococcus aureus* (Syiemlieh, 2020). The evolution of MRSA has since progressed, with the bacterium acquiring resistance to an array of antibiotics, including beta-lactams, making it a formidable adversary in the clinical setting (Saeed *et al*., 2023).

Staphylococcus aureus, a Gram-positive bacterium, holds a dual nature as both a benign commensal organism and a formidable pathogen capable of causing a range of infections. Its ability to switch from a harmless inhabitant of the human skin and mucous membranes to a potent pathogen is largely attributed to the acquisition of resistance mechanisms, particularly against antibiotics like methicillin(Twomey, 2023). In its commensal state, *Staphylococcus aureus* establishes a symbiotic relationship with the host, existing harmlessly on the skin and mucous membranes. This commensalism is part of the normal microbiota, contributing to the overall microbial balance on the human body. The bacterium often serves as a first line of defence against potential pathogens by occupying niches that might otherwise be exploited by harmful microorganisms(Parlet *et al*., 2019).

The transformation of *Staphylococcus aureus* into a pathogen occurs when it gains resistance to antibiotics, particularly methicillin. Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a clinical manifestation of this resistance, posing a significant challenge in healthcare settings and communities alike (Alghamdi *et al*., 2023). The acquisition of resistance is primarily facilitated by the horizontal transfer of genetic elements containing the *mec* A or *mec*C genes. The *mecA* and *mec*C genes encode for penicillin-binding proteins (PBPs) with reduced affinity for beta-lactam antibiotics. PBPs are essential components involved in bacterial cell wall synthesis. When the *mecA* or *mec*C genes are incorporated into the genome of *Staphylococcus aureus*, they result in the production of altered PBPs (Miragaia, 2018). These modified PBPs have a decreased binding affinity for beta-lactam antibiotics, rendering these antibiotics ineffective in inhibiting cell wall synthesis. The *mecA* gene, commonly associated with MRSA, encodes for a protein called PBP2a (penicillin-binding protein 2a). PBP2a has a low affinity for beta-lactam antibiotics, allowing the bacterium to maintain cell wall synthesis even in the presence of these drugs. This resistance mechanism not only confers resistance to methicillin but also extends to other beta-lactam antibiotics, including penicillin and cephalosporin (Fishovitz *et al*., 2014).

Understanding the genetic and molecular underpinnings of MRSA is crucial for developing effective treatment strategies and preventing further spread. The *mecA* and *mec*C genes are typically carried on mobile genetic elements, such as staphylococcal chromosomal cassettes (SCC), which can be horizontally transferred between *Staphylococcus aureus* strains. This transferability enhances the adaptability and persistence of MRSA in diverse environments. The genetic diversity of MRSA strains contributes to the complexity of the resistance problem. Various clonal complexes and sequence types have been identified, each with distinct genetic backgrounds and resistance profiles Molecular techniques, including polymerase chain reaction (PCR)

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and whole-genome sequencing, play a pivotal role in characterizing the genetic diversity of MRSA isolates, aiding in epidemiological studies and the development of targeted interventions (Preeja *et al*., 2021).

Methodology

Study Design and sample collection

The study project was conducted in the Microbiology Lab at the Sarhad Institute of Allied Health Sciences, Sarhad University of Science and Information Technology (SUIT), Peshawar, Khyber Pakhtunkhwa (KPK). The *Staphylococcus aureus* samples were collected from three different hospitals in Peshawar, i.e., Lady Reading Hospital (LRH), Khyber Teaching Hospital (KTH), and Hayatabad Medical Complex (HMC). The research study lasted 6 months, from May 2023 to November 2023.

Inclusion Criteria and exclusion criteria

Samples were exclusively collected from hospitals within the designated geographical area. Any isolates not identified as *Staphylococcus aureus* were excluded from the study. Isolates lacking resistance to methicillin were excluded to maintain a focus on methicillin-resistant strains. Samples not originating from hospital environments were excluded to align with the study's specific focus on healthcare settings. Isolates with incomplete or insufficient phenotypic and genotypic data were excluded to maintain the integrity of the analysis.

Sample Collection

Randomly samples of *Staphylococcus aureus* were collected from Hospitals environments i.e. surgical tools, Dialyser Machine, Operation theatre instruments, Patients wounds and clinical specimens like Pus, urine and blood in Leady Reading Hospital, Hayatabad Medical Complex and Khyber Teaching Hospital. The samples were immediately transported to Microbiology Lab at Sarhad Institute of Allied Health Sciences, Sarhad University of Science and information technology (SUIT), Peshawar, Khyber PukhtoonKhwa (KPK).

Sample processing

All randomly collected *S. aureus* samples were cultured on nutrient agar. The medium was prepared by weighing the required components, dissolving them in distilled water, and autoclaving at 121°C for 15 minutes. Sterilized media were poured into petri plates under laminar airflow and allowed to solidify. The samples were then streaked onto the plates and incubated at 37°C for 24 hours. After incubation, plates were examined for growth and contamination. Pure colonies were selected and subjected to microscopic, biochemical, and molecular identification.

Confirmation of the Methicillin-Resistant *Staphylococcus aureus* (MRSA)

The cefoxitin disc diffusion method is routinely used to detect Methicillin-Resistant *Staphylococcus aureus* (MRSA). In this technique, a standardized bacterial suspension of *S. aureus* is inoculated onto Mueller–Hinton agar plates, and a disc containing a defined concentration of cefoxitin, a cephalosporin antibiotic, is placed on the surface. After incubation at 37°C for 24 hours, the zone of inhibition around the disc is

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measured. A reduced or absent inhibition zone indicates resistance and confirms the presence of MRSA, while a clear zone suggests methicillin sensitivity.

Phenotypic identification of MRSA

The isolated MRSA from the processed samples underwent phenotypic characterization (Gram straining) and Biochemical characterization according to burgay`s manual of Bacteriology (Holt *et al*., 1994).

Antibiotic Susceptibility Testing

The Kirby–Bauer disc diffusion method was used to evaluate antibiotic susceptibility on Mueller–Hinton agar. The antibiotics tested included chloramphenicol, clindamycin, vancomycin, linezolid, ciprofloxacin, and doxycycline. Standardized bacterial suspensions were evenly spread on the agar surface, and antibiotic-impregnated discs were placed onto the inoculated plates. After incubation at 37°C for 24 hours, zones of inhibition were measured. Results were interpreted according to established guidelines to classify isolates as susceptible, intermediate, or resistant. This method provides a clear profile of antimicrobial resistance and supports appropriate therapeutic decision-making (Yin *et al*., 2021).

Molecular Identification of MRSA

The sample was forwarded to the Macrogen Company in Korea for 16S rRNA sequencing for the molecular identification of MRSA strains. The sample was forwarded to the Macrogen Company in Korea for 16S rRNA sequencing for the molecular identification of MRSA strains.

Identification of the *mecA* Gene

The *mecA* gene encodes PBP2a, a modified penicillin-binding protein that gives resistance to beta-lactam antibiotics, including methicillin. It is commonly detected by polymerase chain reaction (PCR) (Lade & Kim, 2023).

Steps involved:

DNA Extraction: Bacterial DNA is isolated from the suspected MRSA sample.

PCR Amplification: Specific primers are used to amplify the *mecA* gene if present.

Gel Electrophoresis: PCR products are separated by size on an agarose gel.

Visualization: DNA bands are observed under UV light. The presence of the expected band confirms the *mecA* gene (Lade & Kim, 2023).

Primer used for identification of mecA gene

For the identification of the mecA gene, specific primers were designed to target regions within the gene during the polymerase chain reaction (PCR) amplification process.

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Forward Primer 5'-GTAGAAATGACTGAACGTCCGATAA-3'
Reverse Primer 5'-CCAATTCCACATTGTTTCGGTCTAA-3'

Data analysis

All the data collected from the current study was analysed statistically using SPSS-11.0 for windows 10 along with Graph-pad Prism version 5. Percentages and ratios were calculated or different variables.

Results

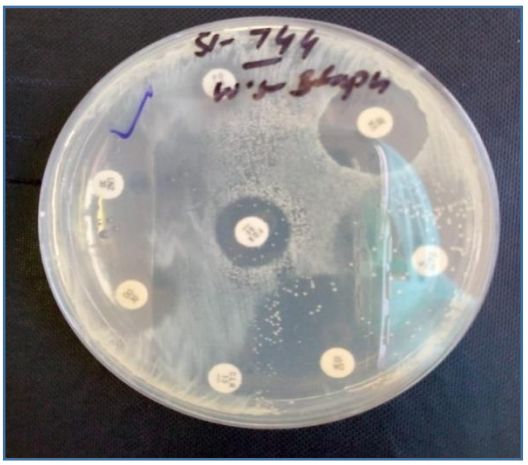
Isolation of Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Samples were collected from surgical instruments, patient wounds, and operation theatres of major hospitals in Peshawar, including Lady Reading Hospital, Hayatabad Medical Complex, and Khyber Teaching Hospital. All specimens were cultured on mannitol salt agar. Based on colony morphology, four methicillin-resistant Staphylococcus strains were isolated. These isolates were purified and confirmed through Gram staining, biochemical testing, and 16S rRNA gene sequencing.

Screening of MRSA

The purified isolates were screened using the cefoxitin disc diffusion method on Mueller–Hinton agar. Four isolates showed zones of inhibition less than 23 mm, indicating methicillin resistance. *Staphylococcus aureus* ATCC 25923 was used as the control strain (Fig.1)





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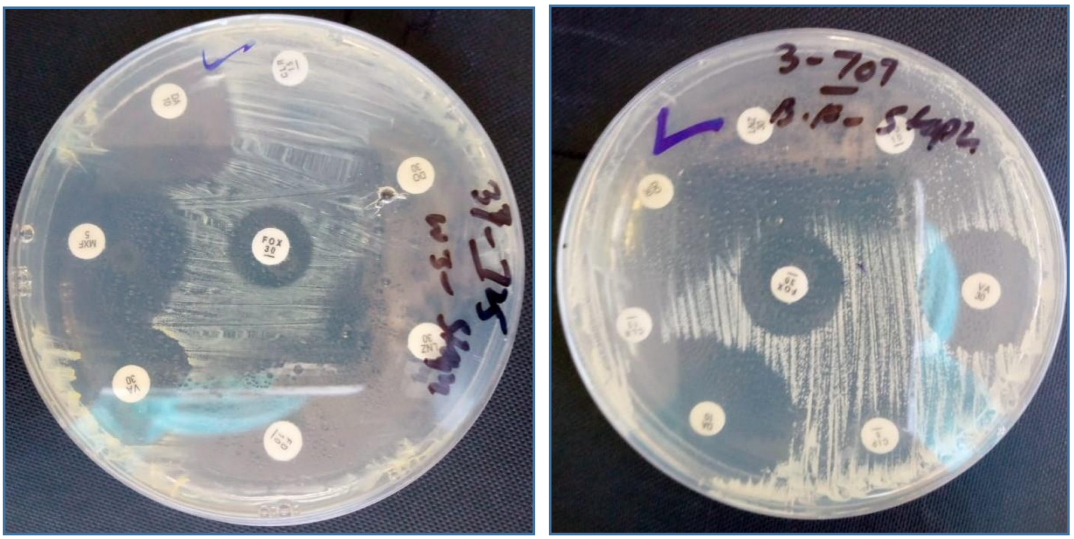


Fig.1. Diagrammatic illustration of *MRSA* screening

Biochemical characterization

Table.1. Morphological and biochemical Characterization of *MRSA*

Isolated Strains	**Oxacillin Disc Diffusion Test**	**Gram Staining**	**Biochemical Test**					**Identified Strains**
			Coagulase	**Oxidase**	**Catalase**	**Methyl Red Test**	**Hemolysis Test**	
LRH-1	Resistant	Positive	Positive	Positive	Positive	Positive	Positive (Beta)	*MRSA*
LRH-2	Resistant	Positive	Positive	Positive	Positive	Positive	Positive (Beta)	*MRSA*
KTH-1	Resistant	Positive	Positive	Positive	Positive	Positive	Positive (Beta)	*MRSA*
HMC-1	Resistant	Positive	Positive	Positive	Positive	Positive	Positive (Beta)	*MRSA*

Molecular Identification (16s rRna) of the MRSA

To confirm the MRSA isolated from the hospital enivornments, 16S rRNA sequencing was conducted by Macrogen Company in South Korea. Following sequencing, the isolated strains exhibited 99% homology with *Staphylococcus aureus*, as depicted in

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Figure 4.9. The Phylogenetic tree of these strains were constructed using Mega 11 software based on the 16S rRNA sequencing data obtained from Macrogen Company, South Korea by likelihood method with 500 bootstrap.

Fig.2. Phylogenetic tree showing 99 % similarity with *Staphylococcus aureus*

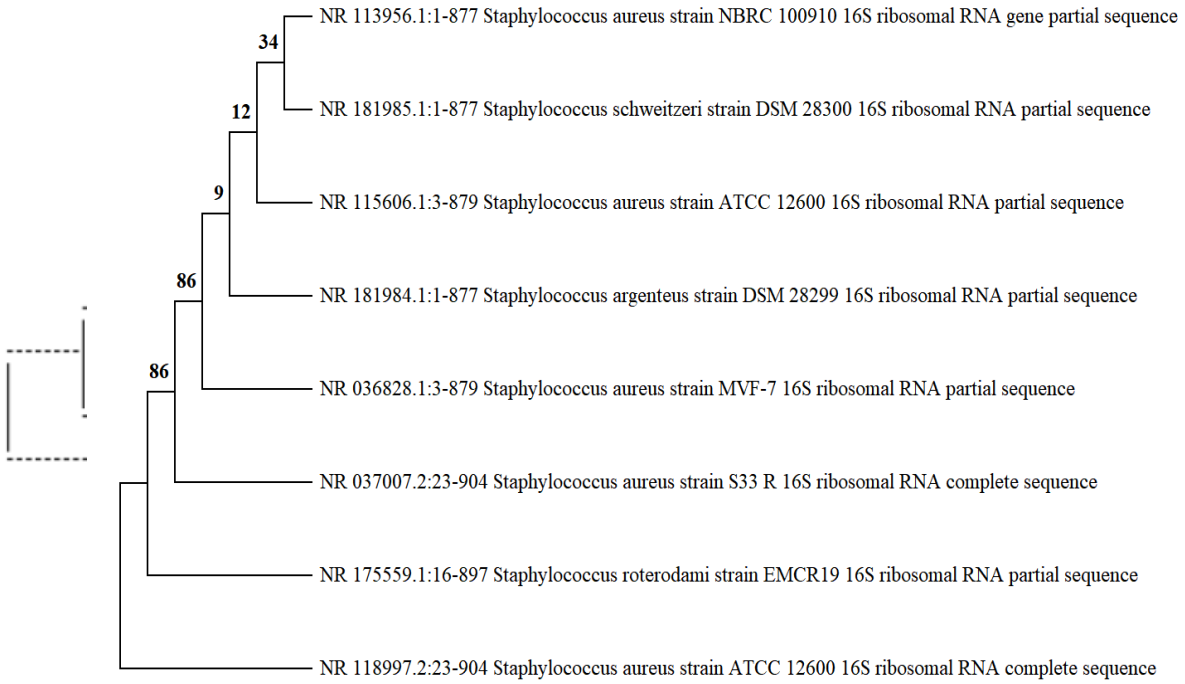
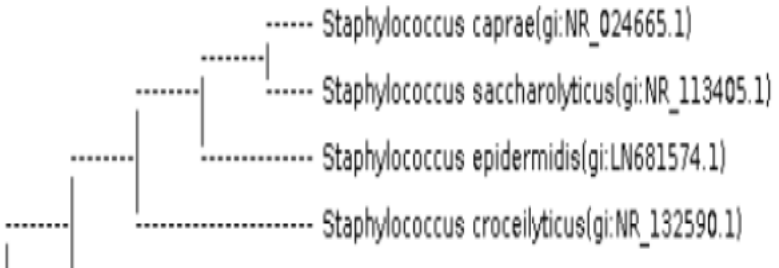


Fig.3 . Phylogenetic tree showing 99 % similarity with *Staphylococcus aureus*



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Fig.4. Phylogenetic tree showing 99 % similarity with *Staphylococcus aureus*

Antibiotic Sensitivity Testing

The isolated S. aureus were checked to antibiotic susceptibility test by the Kirby Bauer disc diffusion method following Clinical and Laboratory Standards Institutes (CLSI) guidelines (CLSI, 2015). The seven different antibiotics (chloramphenicol, clindamycin, vancomycin, linezolid, ciprofloxacin, and doxycycline) were used to check the antibiotic susceptibility of the identified MRSA bacterial strains. The results of antibiotic sensitivity assay as shown in table 2. The National Committee for Clinical Laboratory Standards (NCCLS) interpretive criteria was used regarding the effectiveness of an antibiotic. The isolates *S. aureus* showed high level of resistance to oxacillin, vancomycin, linezolid, Ciprofloxacin, chloramphenicol and clindamycin respectively and showed more sensitive to doxycycline as shown in table 2.

Table 2. Antibiotic susceptibility pattern of MRSA Isolated from Hospital Environments

S.NO	**Antibiotic**	**Disk content**	**No. of Susceptible *MRSA* Strains**	**No. of Intermediate *MRSA* Strains**	**No. of Resistant *MRSA* Strains**
1	Chloramphenicol	5ug	02	02	01
2	Clindamycin	10ug	01	02	01
3	Linezolid	5ug	02	02	03
4	Vancomycin	30ug	02	00	04
5	Ciprofloxacin	30ug	03	01	01
6	Doxycycline	5ug	04	06	00
7	Oxacillin	1ug	02	01	05

Polymerase chain reaction (PCR) and Molecular Method

The *mec* A gene, responsible for methicillin resistant it was identified using specific primers designed with the NCBI Primer-BLAST tool. PCR was employed to amplify the target gene from bacterial genomic DNA. Gel electrophoresis confirmed the

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presence of the *mec*A gene by detecting bands of the expected size, providing valuable insights into the genetic potential of the isolated *S. aereus*.

Locus	Primer	Oligonucleotide sequence (5'-3' specificity	Location	Amplicon size (bp)
mec A	*MECA* P4	5'-TCCAGATTACAACTTCACCAGG-3'	1190-1211	
	MECA P7	5'-CCACTTCATATCTTGTAACG-3'	1351-1332	162

Discussion

Methicillin-resistant *Staphylococcus aureus* (MRSA) was a formidable bacterium that had garnered considerable attention in healthcare settings worldwide. This strain of *Staphylococcus aureus*, a common bacterium that normally resides on the skin and in the nasal passages, had developed resistance to multiple antibiotics, including methicillin and other beta-lactam antibiotics (Algammal *et al*., 2020). MRSA posed a significant public health concern due to its ability to cause severe and potentially life-threatening infections, especially in hospitals and other healthcare facilities. Its resistance to conventional antibiotics complicated treatment, making MRSA infections challenging to manage. Understanding the phenotypic and genotypic aspects of MRSA was crucial for devising effective control measures and improving patient outcomes in healthcare environments (Boswihi *et al*., 2018).

In this research study, MRSA strains were detected by phenotypic and genotypic methods. 08% of MRSA were identified out of 50 samples from hospital environments in Peshawar, in which two samples were isolated from Leady Reading Hospital (LRH), one from Khyber Teaching Hospital (KTH), and one from Hayatabad Medical Complex (HMC). This result was correlated with a previous study reported by Majeed *et al*., 2012. The differences in MRSA prevalence in Peshawar hospital environments may have been due to the sample type and low hygiene conditions. From this study, it was concluded that MRSA strains were present with a high occurrence in hospital environments due to increased virulence and continuous growing antibiotic resistance (Rudkin, 2012).

Over the course of time, *S. aureus* had developed resistance to different conventionally used antibiotics. All the MRSA isolates were resistant to more antibiotics than the MSSA isolates. All identified *S. aureus* isolates from various clinical specimens were subjected to an in vitro antibiotic susceptibility test by the Kirby-Bauer disc diffusion method following Clinical and Laboratory Standards Institutes (CLSI) guidelines (CLSI, 2015). The isolates showed a high level of resistance to oxacillin, vancomycin, linezolid, ciprofloxacin, doxycycline, chloramphenicol, and clindamycin, respectively. The increasing rate of staphylococcal infections among patients and the changing patterns of AMR had led to a renewed interest in the use of clindamycin therapy in treating such infections (Pandey *et al*., 2012) . The overall findings suggested an alarming situation of AMR accompanied by *S. aureus*. The development of antibiotic-resistant properties was troublesome and had been described as a serious public health

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concern, particularly in developing countries(Kimang 2012). All MRSA isolates encountered in this study were completely resistant to antibiotics, such as oxacillin and vancomycin. A similar result was noted for vancomycin among MRSA strains from Trinidad and New York (Zarifian *et al.*, 2012). The resistance rate of MRSA isolates to oxacillin and vancomycin was found to be 5.6%. This result was higher when compared to previous findings from different parts of the world, including Ethiopia. These reports revealed that there was no MRSA isolate resistance to oxacillin and vancomycin (Memon *et al*., 2012). This variation may be due to the timely emergence of resistant strains. However, our result was lower in contrast to a report from New York (10%)(Barie, 2012). This lower finding may be explained by method variation, since the assessment of the previous study was in a controlled clinical trial. In this study, a high prevalence of multidrug-resistant MRSA was observed. This may have predisposed patients to infection with intractable isolates and emphasized the need for improved infection control practices and guidelines for the use of antibiotics in this setting. Moreover, all MRSA strains isolated in our investigation were resistant to ≥3 antibiotics tested, excluding β-lactams (penicillin G, ampicillin, and amoxicillin). Basak *et al*. 2012) revealed that 73% of MRSA strains were multidrug-resistant. This indicated that resistant strains had emerged, and the emergence of those resistant strains, especially for the most bactericidal anti-MRSA agents, may have further aggravated the emergence of multidrug-resistant MRSA, and it may have threatened the success of an MRSA control program. The MRSA strains were confirmed by molecular techniques.

Conclusion

The study underscored the critical public health challenge posed by Methicillin-resistant *Staphylococcus aureus* (MRSA) in healthcare settings globally. With a focus on hospital environments in Peshawar, the research identified an 8% prevalence of MRSA among 50 samples. The study reaffirmed the alarming presence of MRSA, indicating a high occurrence in hospital settings due to increased virulence and persistent antibiotic resistance. The isolated MRSA strains exhibited comprehensive resistance to multiple antibiotics, including oxacillin, vancomycin, linezolid, ciprofloxacin, doxycycline, chloramphenicol, and clindamycin. The findings revealed a high prevalence of multidrug-resistant MRSA, posing significant challenges for effective infection control practices and the use of antibiotics in healthcare settings.

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