RJNMSR
Review Journal of Neurological & Medical Sciences Review

PREVALENCE AND MULTIDRUG RESISTANCE PROFILING OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) ISOLATED FROM CLINICAL SPECIMENS IN TERTIARY CARE HOSPITALS OF PESHAWAR

Hidayat Ullah[1], Bilal Nasir[2], Nabeela Farman[3], Aamir Aziz[4*], Hamad Ullah[5]

[1]Sarhad Institute of Allied Health Sciences, SUIT, Peshawar
[2]Sarhad Institute of Allied Health Sciences, SUIT, Peshawar
[3]Sarhad Institute of Allied Health Sciences, SUIT, Peshawar.
*[*4]Sarhad Institute of Allied Health Sciences, SUIT, Peshawar*
[5]Sarhad Institute of Allied Health Sciences, SUIT, Peshawar
*[1]hidyatUl234@gmail.com, [2]Bilal1993@gmail.com, [3]nabeela.siahs@suit.edu.pk, [*4]aamir.biotech@suit.edu.pk,*
[5]hamadullah.siahs@suit.edu.pk

DOI: https://doi.org/

Keywords :
MRSA (Methicillin-Resistant Staphylococcus aureus)Antibiotic Susceptibility, Antibiogram, Clinical Isolates,Multidrug Resistance (MDR)

Article History
Received on 20 Nov, 2025
Accepted on 28 Dec, 2025
Published on 2 Dec, 2025

Corresponding Author: *
Aamir Aziz

Abstract

Background: Methicillin-resistant Staphylococcus aureus (MRSA) remains a critical challenge in clinical settings due to its extensive multidrug-resistance profiles. This study aimed to isolate, identify, and determine the antibiotic susceptibility patterns of MRSA strains obtained from various clinical wards at Lady Reading Hospital (LRH) and Khyber Teaching Hospital (KTH) in Peshawar, Pakistan. Methodology: Clinical samples were processed using the spread plate technique on Nutrient Agar. Suspected colonies were subjected to Gram staining and a battery of biochemical tests, including catalase, coagulase, methyl red, and urease assays. MRSA confirmation was performed using Oxacillin and Methicillin resistance tests, alongside Cefoxitin (30 µg) disc diffusion as per Clinical and Laboratory Standards Institute (CLSI) guidelines. In vitro antibiotic susceptibility against ten different therapeutic agents was evaluated using the Kirby-Bauer disc diffusion method. Results: Out of 25 initial suspected S. aureus isolates, 5 strains (20%) were confirmed as MRSA. All five confirmed strains (LRH-01, LRH-07, LRH-11, KTH-04, and KTH-09) were Gram-positive cocci and exhibited positive reactions for catalase, coagulase, methyl red, and beta-hemolysis. The antibiogram revealed significant resistance across multiple antibiotic classes. High levels of resistance were observed against Minocycline (80%), Cefotaxime (80%), Aztreonam (60%), and Azithromycin (60%). Conversely, the isolates demonstrated higher sensitivity toward Cefixime, Amikacin, and Ciprofloxacin. Notably, only one strain showed complete resistance to Cefixime and Amikacin, suggesting these may remain viable treatment options in local clinical settings. Conclusion: The study identifies a 20% prevalence of MRSA among suspected staphylococcal clinical isolates in the region. The high resistance to commonly used antibiotics like Cefotaxime and Azithromycin underscores the necessity for regular antimicrobial surveillance and the implementation of stringent infection control policies to manage the spread of resistant strains in tertiary care hospitals.

Introduction

Staphylococcus aureus is a member of natural human flora, present on skin surface and mucous membranes (particularly of nasal area), which can cause severe infections as soon as it gets a chance to penetrate the internal tissues or bloodstream (Taylor and Unakal, 2018; Weiner *et al.*, 2016). *Staphylococcus aureus* has an anomalous ability to quickly develop resistance against every other antibiotic. The mechanisms for resistance are numerous including inactivation of antibiotics by enzymes, target alteration with decreased affinity for the antibiotics, antibiotic trapping, efflux pumps etc. (Piso *et al.*, 2017).

Staphylococcus aureus is one of the most common microorganisms frequently associated with various diseases, ranging from mild infections of the skin to life-threatening endocarditis, chronic osteomyelitis, pneumonia, and bacteremia (Lowy 1998; Murray 2005). During the mid- 20th century, the introduction and use of antibiotics such as penicillin and methicillin proved successful against *S. aureus* infections. However, the bacterium quickly acquired resistance to these antibiotics posing an enormous challenge to both veterinary and human health clinicians (Brouillette & Malouin 2005).

Treatment for *Staphylococcus aureu* is a concern with the emergence and spread of penicillin-resistant *S. aureus* and in turn methicillin-resistant *S. aureus* (MRSA). MRSA has become one of the most common causes of hospital-associated and community-acquired infections, and the global spread of MRSA is a matter of great concern (Grundmann *et al.* 2006). Besides, community-based MRSA has recently emerged as a potential threat, causing infections in healthy individuals with no risk factors associated with healthcare (Kluytmans-Vandenbergh & Kluytmans 2006).

Staphylococcus aureus (*S. aureus*), a major cause of infection in either hospitals or within communities across the world, has developed resistance to commonly prescribed antimicrobial agents (Goudarzi *et al.*, 2016). The most important factor contributing to the successful extensive distribution of this nosocomial pathogen is stated to be its remarkable ability to acquire resistance to new antimicrobial agents (Nezhad *et al.*, 2017).

Shortly after the introduction of penicillin as a first therapeutic option for the treatment of infections caused by penicillin-resistant *S. aureus*, methicillin-resistant *S. aureus* (MRSA) emerged in the 1960s, and since the 1980s, vancomycinhas become the drug of choice for the treatment of serious MRSA infections in many healthcare institutions (Appelbaum, 2006).

On the basis of resistance development, there are two types of *Staphylococcus aureus*. Those that are resistant to β-lactam antibiotics (a group of broad-spectrum antibiotics including some penam-penicillin derivative such as methicillin and oxacillin, and cephalosporins such as cepham are termed as are termed as MRSA-Methicillin resistant *Staphylococcus aureus*, whereas those that are susceptible to these are termed MSSA-Methicillin susceptible *Staphylococcus aureus* (Gurusami, *et al.* 2013). Antimicrobial resistance among pathogens is becoming a life threatening problem world-wide. Excessive usage of antibiotics has caused 23,000 mortality cases due to development of antibiotic resistant bacterial infections (CDC, 2013).

Antibiotic resistant infections can occur anywhere in the community, but the rate of nosocomial infections is even higher. Resistance in bacteria is a serious matter because as bacteria evolve and form ways to nullify the antibiotics, the antibiotics become ineffective, letting these deleterious organisms to survive in multiple environments (Margonis *et al.*, 2018; Wang *et al.*, 2016). Now the world is entering a 'post-antibiotic' era. In Nigeria β-lactam antibiotics don't work on 88% of *S. aureus* infections. According to Akinkunmi and Lamikanra, (2012) the prevalence of MRSA in Pakistan, is varying from 42% to 51%. In another study, β-lactam antibiotic resistant organisms are carried by 95% of adult population in India and Pakistan (Reardon, 2014). Studies show that MRSA causes around 11,000 - 18,000 deaths, and 80,000 invasive infections yearly in the US with restricted treatment choices (Morgenstern *et al.*, 2016). Multi drug resistance (MDR) is defined as resistant to at least one agent in three or more antimicrobial classes, is the most pronounced feature of MRSA isolates as they showing sensitivity only to the glycopeptides antibiotics including vancomycin (Diaz *et al.*, 2018). Conversely, vancomycin resistance has been reported in Pakistan as well (Rajaduraipandi *et al.*, 2006).

In the past few decades, long-term care facilities (LTCFs) have faced an extreme threat from multidrug resistant (MDR) infectious pathogens (Bonomo, 2001). The most abundant opportunistic pathogen is methicillin resistant *Staphylococcus aureus* (MRSA), which not only affects healthcare centers, but is also associated with community and livestock infections (Pai, 2013). The presence of *SCCmec* elements, consisting of *mecA*

The distribution of these bacteria in hospital or LTCF environments has been well reported, although there is poor documentation of the pattern of distribution in terms of the infected hosts moving between these environments (Valsesia *et al.*, 2010).

In Pakistan, which spends only a small portion of its economy on healthcare, epidemics could drive the country to a catastrophe (Hussain, 2015). For the control and prevention of such infections, knowledge about the antibiotic resistance levels of clinically isolated strains is crucial. Our present study is based on the evaluation of the sensitivity value of clinically isolated *Staphylococcus aureus* against different antimicrobial agents currently prescribed by physicians for its control.

Methodology

1. Study Design and Sampling

The present study was conducted across two major tertiary care hospitals in Peshawar: Lady Reading Hospital (LRH) and Khyber Teaching Hospital (KTH). The research framework was developed at the Sarhad Institute of Allied Health Sciences, Sarhad University of Science and Information Technology (SUIT), Peshawar, Khyber Pakhtunkhwa. Random sampling was employed to collect clinical specimens, including pus, urine, blood, and tissue samples, from various patients. Following collection, these samples were immediately transported to the Microbiology Laboratory at SUIT for processing and analysis.

2. Initial Processing, Culture, and Isolation

Clinical specimens underwent initial macroscopic examination for color and appearance, followed by direct microscopic analysis involving centrifugation at 300 rpm for 10 minutes and the preparation of wet mount smears. For isolation, samples were serially diluted and inoculated onto Nutrient Agar and Mannitol Salt Agar (MSA) containing 7.5% NaCl using a 1 μL calibrated loop.

The plates were incubated at 37°C for 24 hours. Samples exhibiting more than 105 colony-forming units (CFU) per ml were considered positive. Suspected *Staphylococcus aureus* colonies were sub-cultured onto fresh media to ensure pure cultures for subsequent morphological and biochemical characterization.

3. Media Preparation and Sterility Quality Control

Nutrient Agar and Mueller-Hinton Agar (MHA) were prepared by measuring precise quantities of agar, peptone, yeast extract, beef extract, and starch using an electronic balance. The components were mixed with distilled water and sterilized in an autoclave at 121°C for 15 minutes. Petri plates were sterilized in a dry heat oven at 180°C. All pouring was conducted within a Laminar Flow Hood (LFH) to prevent environmental contamination. Before sample inoculation, sterility was verified by incubating the prepared plates for 24 hours at 37°C; only plates showing no growth were utilized for the study.

4. Morphological and Biochemical Characterization

Isolates were identified based on colony morphology (size, shape, color, and texture) and Gram staining. In the Gram staining procedure, 24-hour-old cultures were heat-fixed, treated with crystal violet, Gram's iodine, 90% ethanol, and safranin, then observed under oil immersion.

Biochemical identification followed Bergey's Manual of Determinative Bacteriology and included the following assays:

Catalase Test: Detection of oxygen evolution (bubbling) upon the addition of 3-4% $H2 \quad O2$.

Mannitol Fermentation: Inoculation into MSA slants, where a color change from pink to yellow indicated a positive result.

Triple Sugar Iron (TSI) Test: Stabbing and streaking TSI agar to analyze glucose fermentation through butt and slant color changes.

Motility and Urease Tests: Use of SIM medium for motility (indicated by diffuse growth) and urea agar slants to detect ammonia production via a pink color change.

Methyl Red (MR) Test: Assessment of acid production during glucose fermentation using methyl red indicator.

5. Antibiotic Susceptibility Testing (AST)

Antimicrobial susceptibility was determined using the Kirby-Bauer disc diffusion method on MHA, adhering to CLSI (2009) guidelines. Bacterial lawns were prepared by spreading 100 µL of inoculum across the agar surface. Commercially available antibiotic discs—including Amikacin, Ciprofloxacin, Chloramphenicol, and others—were applied using sterile forceps.

After incubation at 37°C for 24 hours, the diameters of the zones of inhibition were measured from three angles to calculate an average. For MRSA isolates, susceptibility was tested against a panel of 26 antibiotics representing seven different classes.

6. Statistical Analysis

All experimental data were statistically processed using SPSS version 11.0 and Graph-Pad Prism version 5. Results were interpreted through the calculation of percentages and ratios to determine the prevalence and resistance patterns of the clinical isolates.

Results

All the samples collected from clinical samples from different ward`s patients were inoculated on Nutrient ager plates using spread plate technique and then based on morphological character the suspected *MRSA* colonies were further grown and purified. The selected bacterial colonies were subjected to gram staining and biochemical tests. Oxacillin and methicillin resistant test were carry out to check MRSA. The Bacterial colonies were Isolated and identified by Morphological and biochemical Characteristics. Initially, 25 different suspected MRSA strains were isolated from the hospital clinical samples.

All the 25 strains were subjected to Oxacillin and methicillin resistant test. Among the 25 isolated strains only 20% (05 strains) showed resistant to Oxacillin and methicillin. Thus confirmed as MRSA. Among the selected 25 bacterial strains tested, 20% (05) samples showed growth of *MRSA.* Different Morphological and biochemical tests like gram straining, catalase test, coagulase test, urease test, Indol test, motility and Methyle red) were performed to confirm *MRSA* (the results of Morphological and biochemical identifications are shown in table 4.1)

Table 4.1: ***Morphological and biochemical identification of the selected bacterial strains***

Strain	Gram Reaction	Oxacillin/ methicillin resistance	Biochemical tests Performed				Identified strain
			Catalase	Coagulase	Methyl Red	Hemolysi s	
LRH- 01	+ve	Resistant	Positive	Positive	Positive	Positive (Beta)	*MRSA*
LRH- 07	+ve	Resistant	Positive	Positive	Positive	Positive (Beta)	*MRSA*

LRH- 11	+ve	**Resistant**	**Positive**	**Positive**	**Positive**	**Positive (Beta)**	***MRSA***
KTH- 04	+ve	**Resistant**	**Positive**	**Positive**	**Positive**	**Positive (Beta)**	***MRSA***
KTH- 09	+ve	**Resistant**	**Positive**	**Positive**	**Positive**	**Positive (Beta)**	***MRSA***

All identified *S. aureus* isolates from various clinical specimens were subjected to *in vitro* antibiotic susceptibility test by the Kirby–Bauer disc diffusion method following Clinical and Laboratory Standards Institutes (CLSI) guidelines (CLSI, 2015). Ten different antibiotics (Chloramphenicol, Streptomycin, Ciprofloxacin, Aztreonam, Amikacin, Cefixime, Minocyclin, Sulfamethoxazole, Cefotaxime and Azithromycin) were used to determine the antibiotic susceptibility profile of selected *MRSA* bacterial strains. For identification of MRSA, cefoxitin disc (30 µ) was used. A zone of inhibition less than 22 mm or any discernible growth within zone of inhibition by *S. aureus* against cefoxitin in Muller hinton agar (MHA) plate was indicative of methicillin resistance. *S. aureus* ATCC 25923 was used as a standard control strain. Methicillin resistance was tested for all the *S. aureus* isolates by the agar screening method using MHA.

The results of antibiotic sensitivity assay are shown in table 4.2 to 4.7, figure 4.5, figure 4.6, figure 4.7 and figure 4.8. The National Committee for Clinical Laboratory Standards (NCCLS) interpretive criteria was used regarding the effectiveness of an antibiotic (Table 4.2).

Table 4.2: ***Antibiotics Standard Zone Diameters***

No	**Antibiotics**	**Disk content**	**Zone diameter (mm) Interpretive criteria**		
1	Chloramphenicol	5ug	≥ 18	13-17	≤ 12
2	Streptomycin	10ug	≥ 15	12-14	≤ 11
3	Ciprofloxacin	5ug	≥ 21	16-20	≤ 15
4	Aztreonam	30ug	≥ 21	18-20	≤ 17
5	Amikacin	30ug	≥ 17	15-16	≤ 14
6	Cefixime	5ug	≥ 19	16-18	≤ 15
7	Minocyclin	30ug	≥ 16	13-15	≤ 12
8	Sulfamethoxazole	25ug	≥ 16	11-15	≤ 10
9	Cefotaxime	30ug	≥ 26	23-25	≤ 22
10	Azithromycin	15ug	≥ 18	15-17	≤14

The isolates showed high level of resistance to Streptomycin, Aztreonam, Sulfamethoxazole, Cefotaxime, Azithromycin and Minocycline were sensitive to Chloramphenicol, Ciprofloxacin, amikacin, Cifixime.

Table 4. 1: Antibiotic susceptibility pattern

S.NO	Antibiotic	Disk content	No. of Susceptible *MRSA* Strains	No. of Intermediate *MRSA* Strains	No. of Resistant *MRSA* Strains
1	Chloramphenicol	5ug	01	03	01
2	Streptomycin	10ug	01	02	02
3	Ciprofloxacin	5ug	02	02	01
4	Aztreonam	30ug	02	00	03
5	Amikacin	30ug	03	01	01
6	Cefixime	5ug	04	06	01
7	Minocycline	30ug	00	01	04
8	Sulfamethoxazole	25ug	01	02	02
9	Cefotaxime	30ug	01	00	04
10	Azithromycin	15ug	02	00	03

Discussion

S. aureus infection causes a health burden, particularly in low- and middle-income countries. MRSA is emerging as a serious threat to public health worldwide. MRSA has significantly increased morbidity and mortality rate of patients along with the increased length of hospital stay. *S. aureus* infections are a significant clinical problem in medical practice as the organism shows resistance to the commonly used first-line drugs. The prevalence of MRSA, however, varies markedly by country. All the samples with clinically detected *S. aureus* may serve as a reservoir of MRSA, which may transmit the infection in a community. Thus, there is a chance of a rapid increase in the development of community- acquired MRSA infection. Though infected persons with *S. aureus* are a direct vital source, it is also reported to be isolated from fomites (Neely and Maley, 2000). Fomites can be considered as an indirect transmission route for *S. aureus* as well as other infectious diseases in hospital (Dudhagara *et al.*, 2011). So, environmental sources and fomites cannot be ignored. Antimicrobial resistance (AMR) remains a global setback in managing common bacterial infectious diseases in both the resource-limited and developed countries (Hart and Kariuki, 1998). The World Health Organization has indicated that the current drift in the progression in AMR and drug development could lead to a “post-antibiotic era” (World Health Organization, 2018) with more than 4 million deaths projected per year by 2050, in Asia alone (O’Neill, 2014). To the best of our knowledge, this is the first attempt to systematically unify the individual studies that address MRSA isolation from the hospital environment.

In the present study, 05 (20.3%) *S. aureus* isolates were found out of 25 environmental samples. The results are similar to those reported by Mukhiya *et al.*, with growth positivity of 17.4% (Mukhiya *et al.*, 2012). The differences in MRSA prevalence in hospital environments may be due to the sample type and local hygiene conditions. . Our results implied that MRSA strains were present with high occurrence in moist environments, which agreed with other investigations, as moist samples act as prominent hotspots of MRSA distribution (Kaito *et al.*, 2011). Over the course of time, *S. aureus* has developed resistance to different, conventionally used antibiotics. All the MRSA isolates were resistant to more antibiotics than MSSA isolates. All identified *S. aureus* isolates from various clinical specimens were subjected to *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 high level of resistance to Streptomycin, Aztreonam, Sulfamethoxazole, Cefotaxime, Azithromycin and Minocycline were sensitive to Chloramphenicol, Ciprofloxacin, amikacin, Cifixime. The increasing rate of the Staphylococcal infections among the patients and the changing patterns of AMR has led to a renewed interest in the use of the clindamycin therapy in treating such infections (Aleksandra *et al.*, 2014). The overall findings suggest an alarming situation of AMR accompanied by *S. aureus*. The development of antibiotic-resistant properties is troublesome and has been described as a serious public health concern, particularly in developing countries (Vandepitte *et al.*, 2003).

All MRSA isolates encountered in this study were completely resistant to antibiotics, such as cotrimoxazole and erythromycin. A similar result was noted for erythromycin among MRSA strains from Trinidad and New York (Akpaka *et al.*, 2006). Similarly a comparable result was reported for cotrimoxazole in Islamabad (Hussain *et al.*, 2005).

The resistance rate of MRSA isolates to vancomycin was found to be 5.6% (see Table 4). This result was higher when compared to previous findings from different parts of the world, including Ethiopia. These reports revealed that there is no MRSA isolate resistance to vancomycin (Hussain *et al.*, 2005). This variation may be due to timely emergence of resistance strains. However, our result is lower in contrast to report from New York (10%) (Barie *et al.*, 2006). This lower finding may be explained by method variation, since assessment of the previous study was in a controlled clinical trial. In this study, high prevalence of multidrug resistant MRSA was observed. This may predispose patients to infection with intractable isolates and emphasizing the need for improved infection control practices and guidelines for 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, amoxicillin). Arora *et al.* revealed that, 73% of MRSA strains were multidrug resistant (Arora *et al.*, 2010). This indicated that resistant

strains were 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 threaten the success of an MRSA control program. Molecular confirmation of MRSA strains was not conducted due to absence of molecular techniques in the study set up.

Conclusion

The Hospital Environments including Wards, Waiting rooms, Washrooms, Corridors and Doctor/ Nurses Staff rooms were found to be contaminated with MRSA. The prevalence of *S. aureus* and MRSA in different wards of both hospitals (Lady reading Hospital Peshawar and Khyber teaching Hospitals Peshawer) were found to be high. The rate of MRSA strains among clinical isolates was also high. Vancomycin was relatively effective drug for *S. aureus* and MRSA infections. High level of resistance was observed to erythromycin, cotrimoxazole and gentamycin among *S. aureus* isolates from surgical ward. In addition, high level of resistance was observed to erythromycin, cotrimoxazole, gentamycin, ampicillin, amoxicillin and penicillin G among isolates from gynaecology and obstetrics ward. Moreover, all MRSA isolates were multidrug resistant (≥6). As a recommendation, the rationale of some antibiotic combinations requires evaluation and the establishment of antibiotic policy and treatment guidelines

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