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ISOLATION AND MOLECULAR CHARACTERIZATION OF CLINICALLY ISOLATED METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS FROM TERTIARY CARE HOSPITALS OF FAISALABAD

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ABSTRACT

B Background:

Staphylococcus aureus, a Gram-positive, round shaped bacterium is normal flora of skin and upper part of respiratory system and causative agent of food poising, folliculitis, endocarditis, osteomyelitis, arthritis, pneumonia, and skin infection. Methicillin introduced in 1959 was used against diseases caused by S. aureus which has resistant against penicillin. The resistance towards methicillin is conciliated by gene mecA that codes penicillin-binding protein 2a (PBP 2a) which leads to resistance against all beta-lactam containing antibiotics namely methicillin and oxacillin.

Materials & Methods

A total 100 specimens were collected from the patient who visited Allied hospital (n=35), General hospital Ghulam Mohammadabad (n=35) and Social security hospital Faisalabad (n=30). Samples were cultured and Gram stained. All the Gram-positive bacteria were cultured on mannitol salt agar to obtain pure colonies. Antimicrobial susceptibility testing was performed for the screening of methicillin resistant S. aureus by using Kirby Bauer disk diffusion method. After obtain required organism which was methicillin resistance S. aureus, Polymerase chain reaction was done for mecA gene that is a methicillin resistant gene. DNA was extracted for molecular studies and gel electrophoresis was performed for all positive isolates individually.

Results:

A total of 38 samples out 100 were marked positive for presence of S. aureus. Highest prevalence of S. aureus was recorded in samples collected from Allied Hospital (16/35; 45.71%) followed by General Hospital Ghulam Muhammadabad (13/35; 37.71%) while samples from Social Security Hospital (n = 09/30; 30%) showed least prevalence. All the isolates (n = 38) were tested

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for antibiotic susceptibility pattern. The readings were compared with the CLSI guidelines and highest resistance percentage was calculated for penicillin G (100%) while highest susceptibility percentage was calculated for chloramphenicol (60.53%). Prevalence of MRSA isolates was calculated by analyzing the susceptibility of the isolates against cefoxitin (30 μg). 31 out of 38 isolates (81.58%) were marked to be methicillin resistant S. aureus. All MRSA isolates were tested for the occurrence of mecA gene by PCR and gel electrophoresis. A total of 19 isolates (61.29.5%) were marked positive for mecA gene.

CONCLUSION

The on the basis of finding of current study, S. aureus is one of the most prevalent pathogen in hospital sitting equipped with the wide range of virulence factors. As S. aureus is able to cause life threatening infections, the presence of S. aureus in clinical samples followed by the prevalence of mecA gene in isolates has been a serious threat to both healthcare worker and public health.

INTRODUCTION

Staphylococci is a common category of Gram-positive, round shaped bacterium in human and veterinary medicine whose disease-causing capability ranges from innocuous commensal to fatal opportunistic. *Staphylococcus aureus* is normal flora of the body and mostly found on skin and upper part of respiratory system. It is positive for reduction of catalase and Nitrate and elective aerobes which can also grow in absence of oxygen. Methicillin was first introduced in year 1959 that was used against diseases produced by *S. aureus* which has resistant against penicillin. In year 1961, a few reports in Britain reveal that *S. aureus* had acquired resistance against methicillin and methicillin resistant *Staphylococcus aureus* (MRSA) samples were firstly collected from European states, and later from the Australia, Japan and United State. Nowadays, MRSA is most frequent issue in health care units all over the globe and its recovery rate is increasing from nursing homes and the group of population which is related to medical field. The arise of the most MRSA clones are not understood yet. Some researchers showed that all the MRSAs samples were collected from a particular ancestral *S. aureus* strain which having gene *mecA*, but current researches revealed that certain MRSAs are much variant by the fact that *mecA* has been transported in among *S. aureus* lineages (Enright et al., 2002).

The genus Staphylococci contains 30 species. In this genus, *S. aureus* is most common bacterium. *S. aureus* is normal Flora of skin and cause variety of skin problems like mild pimple to infection of wounds and bursting of mucosal barrier. Fatality rate of *S. aureus* has been minimized with the help of antibiotics but *S. aureus* quickly develops resistance against antibiotics. Factors like toxins, adhering proteins, enzymes, antimicrobial peptide and super-antigen make it major pathogen for human and animals (Zeconi et al., 2013).

MRSA is an important and dangerous pathogen for human equipped with various virulence factors and is the main reason of infection in community and hospitals. *S. aureus* is responsible for a variety of infection including food poising, folliculitis, endocarditis, osteomyelitis, arthritis, pneumonia, and skin infection. MRSA is one of leading cause of death in hospitals and as well as in persons with good health in the past twenty years. The worldwide spread of MRSA shielding the genes which have multi-resistance and these genes restrict the efficacy of therapeutic choices for the infections caused by staphylococci and exacerbate their medical end result (Goudarzi et al., 2016).

The resistance towards methicillin is conciliated by gene *mecA* that codes penicillin-binding protein 2a (PBP 2a) which leads to resistance against all beta-lactam containing antibiotics namely methicillin and oxacillin. The gene *mecA* exists on the moving genome island, called

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Staphylococcus cassette chromosome *mec* (SCC*mec*), and is placed in the chromosome of methicillin-resistant *S. aureus*. The size and structure of SCCmec genetic elements are different. According to its structure and size differences, there are 13 SCCmec types have been listed, called I-XIII types. According to its SCCmec type, MRSA samples have been characterized as hospital or medical institution-related MRSA (HA-MRSA) or community-related (CA-MRSA). HA-MRSA possesses type I, II, and III SCC*mec,* and on the other hand CA-MRSA contain SCC*mec* types IV, V and VI. SCC*mec* typing and other different molecular typing technique like pulsed field gel electrophoresis, multi-site sequence typing, DNA microarray and whole genome sequencing have been used to study MRSA strains in different states, distribution of clones, and has disclosed the genetic backgrounds of different geographic regions and have different genetic backgrounds (Alfouzan et al., 2019).

The MRSA, the specific bacterial strain of *S. aureus* is studied for more than half century back and have resistance towards B lactam antibiotics. According to CDC, among whole world, in Asia there is highest prevalence of MRSA (CDC 2001). The percentage of isolates of MRSA has grown from 22% to 57% in 1995-2001. In 2014 CDC issued a report that almost 60% of healthcare in USA having infection of *S. aureus* which were caused by MRSA. In 2004 MRSA has also found in animals in some countries (Grave-land et al., 2011). In Geneva, scientist found that infection rate caused by MRSA has also been more than 2000, but after 2008, reduction has been seen from 30% to 23% in 2011 (Fankhauser et al., 2011). Frequency of MRSA infection in Pakistan has been reported in urban areas with dense population (Bukhari et al., 2011).

METHODS

The study was conducted at Postgraduate Research Laboratory, Institute of Microbiology, Government College University Faisalabad, Pakistan from November 2020 to august 2021. Before starting the research work, the ethical permission was obtained from the ethical review committee, Government College University, Faisalabad and informed consent was taken from each patient before collection of samples. A total 100 specimens were collected from the patient who visited tertiary care hospitals Faisalabad; Punjab Pakistan i.e., Allied hospital, General hospital Ghulam Mohammadabad and Social security hospital Faisalabad. Samples were collected from Allied Hospital Faisalabad (n=35), General Hospital Ghulam Muhammadabad (n=35) and Social Security Hospital Faisalabad (n=30). All clinical specimens were collected under aseptic technique by using sterile needle and clean cotton swab and the sample sources were included surgical wounds, pus from skin lesions, blood and urine from the patients who suffering from urinary tract infection. All the samples were streaked on the nutrient agar plates with the help of sterile wire loop. These plates were labeled with patient I'd and sample source. Then inoculated plates were incubated for 24 hours at temperature of 37^0C in incubator. After that Gram staining was performed by using these cultures to identify Gram-positive and Gram-negative bacteria. The bacterial smear was prepared on a glass slide with the help of sterile wire loop. Firstly the smear was treated with crystal violet for the period of one minute then was rinsed with running tap water. Gram iodine was applied for the one minute and was washed off. 95% ethanol was used as declorizer for 15 seconds and rinsed off, at the end the bacterial smear was treated with counter stain safranin for one minute, and then the slide was observed under microscope at 40x and 100x lenes with the help of oil immersion.

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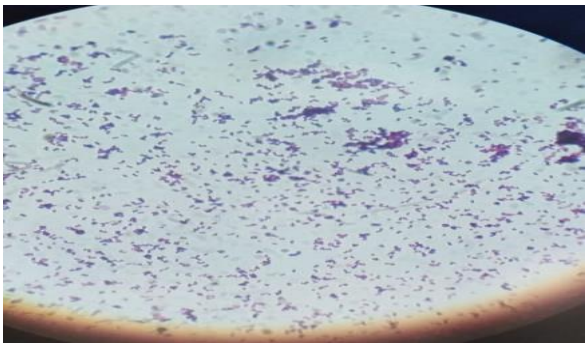

Figure 1: Gram positive bacteria after gram staining

All the Gram-positive bacteria were cultured on mannitol salt agar medium which is selective media for growth of S. *aureus* to obtain pure colonies of wanted organism. After the incubation at 37^{0}C for 24,48 hours, the positive isolates show yellow to golden color colonies on petriplates as it is characteristic feature of S. *aureus*.

Figure 2: Presenting growth of *S. aureus* on mannitol salt agar

Colonies of Gram positive bacteria on mannitol salt agar were further identified by using biochemical test.

Table 1: Biochemical tests for S. aureus

Biochemical test	Results
Catalase	Positive
Urease	Positive
Oxidase	Negative
Coagulase	Positive
Methyl red	Positive

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Citrate Utilization	Positive
Indole test	Negative
Voges Proskauer	Positive

Antimicrobial susceptibility testing was performed for the screening of methicillin resistant *S. aureus* by using Kirby Bauer disk diffusion method. *S. aureus* was grown on nutrient agar and different antimicrobial coated filter paper disks were applied on the surface of petri plates with the help of sterile forceps. After the incubation period of 24 hours at 37^{0}C, the zone of inhibition around each antimicrobial disk was measured that shows the ability of these drugs to inhibit the growth of this pathogenic organism.

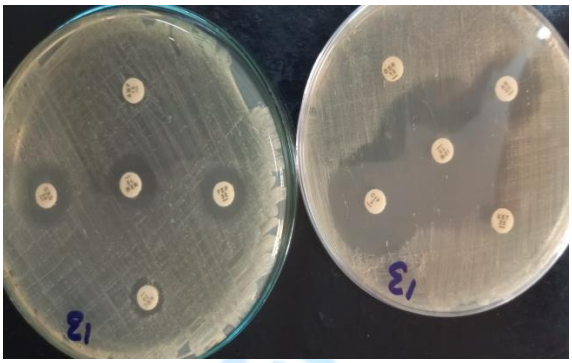

Figure 3: determination of MDR *S. aureus*

After obtain required organism which was methicillin resistance *S. aureus*, Polymerase chain reaction was done for *mec*A gene. DNA was extracted for molecular studies by picking up suitable amount of bacterial colony from mannitol salt agar plates was placed into centrifuge tubes. These tubes were centrifuged at high speed and supernatant was discarded completely. Pellet was re-suspended in 200 micro liter lysozyme mixture solutions (20mg/mL lysozyme, 20uM Tris HCL, Ph 8.0, 2Mm EDTA) and places it in incubator for 30-60 minutes. 20µL protein k and 200µL FATG2 buffer was added to the tube and after mixing thoroughly, incubation was given at 60^{0}C for 30 minutes and then at 95^{0}C for 15 minutes. 200µL of ethanol was added and mixed gently, after that transfer mixture to FATG mini Column and centrifuge for 60 seconds. 400µL concentrated buffer was putted to FATG mini column and centrifuged it. Added 750µL washing buffer to column and centrifuge again for 1 to 3 minutes to dry the column. Added 100µL of elution buffer and centrifuge at full speed for 3 minutes to elute DNA.

Polymerase chain reaction was performed to check out the presence of *mec*A gene that is a methicillin resistant gene. PCR was performed for all positive isolates individually. Different ingredients like master mix, distilled water, extracted DNA and forward and reverse primer for *mec*A gene were used to complete the reaction.

RESULTS

A total of 38 samples out 100 were marked positive for presence of *S. aureus*. Highest prevalence of *S. aureus* was recorded in samples collected from Allied Hospital (16/35; 45.71%) followed by General Hospital Ghulam Muhammadabad (13/35; 37.71%) while samples

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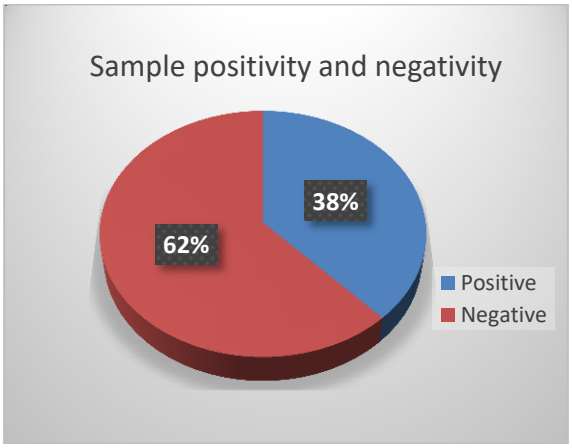

Figure 4: Prevalence of *S.aureus*

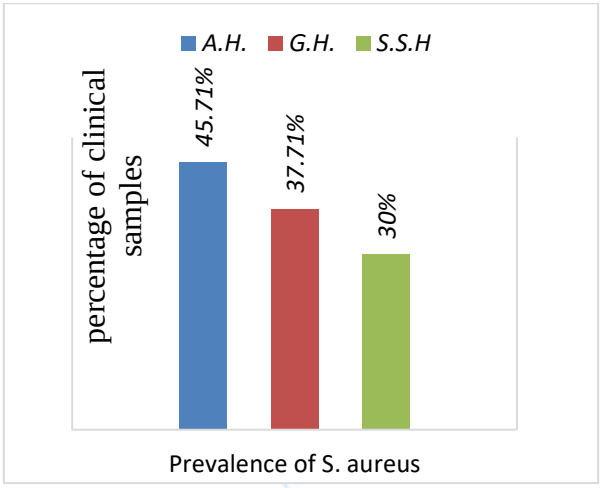

Figure 5: Graph presenting hospital wise prevalence of *S. aureus* in clinical samples.

All the isolates (n = 38) were tested for analysis of antibiotic susceptibility pattern of the *S. aureus* isolates. The readings were noted after 24 hours and compared with the CLSI guidelines for interpretation of resistance, intermediate and sensitive pattern of the isolates against the tested antibiotics.

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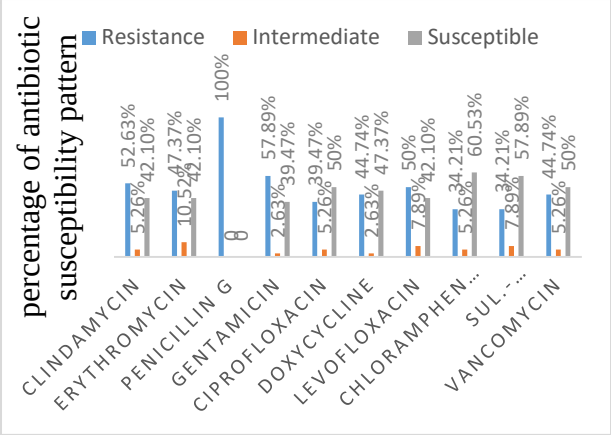

Figure6: Presenting antibiotic susceptibility pattern of *S. aureus* isolates

Prevalence of MRSA isolates was calculated by analyzing the susceptibility of the isolates against cefoxitin (30 µg). 31 out of 38 isolates (81.58%) were marked to be methicillin resistant *S. aureus* isolates on basis of this testing and results are presented in Table 4.5 and Figure 4.16 and 4.17

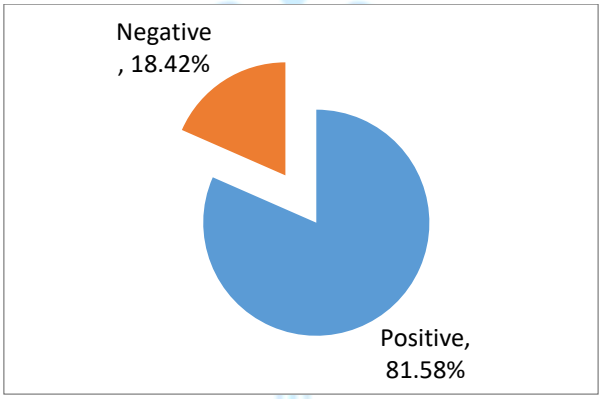

Figure 7: Pie presenting the prevalence of MRSA isolates

Agarose powder was used for the preparation of gel with TAE buffer for the procedure of gel electrophoresis for the detection of Methicillin resistant *mec*A genes from the MRSA isolates.

A total of 31 *S. aureus* isolates were tested for the occurrence of *mec*A gene. A total of 19 isolates (61.29.5%) were marked positive for occurrence of *mec*A gene and results are presented in table 4.6 and figure 4.18 and 4.19

Table 2: Presenting the prevalence of mecA gene in S. aureus isolates

Total isolates tested	Frequency of *mec*A gene	Prevalence of *mec*A gene
31	19	61.29%

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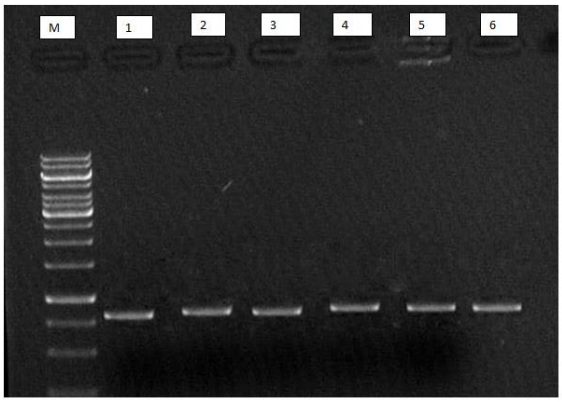

Figure 8: Gel electrophoresis of mecA gene in MRSA isolates (Lane M; DNA marker, lane 1-6; mecA gene)

DISCUSSION

The aim of this study was to isolate and identify the MRSA from clinical specimens, confirmation and phenotypic detection of MRSA by performing antimicrobials susceptibility testing by Kirby Bauer disk diffusion assay and cefoxitin disk method and determination of *mec*A gene by polymerase chain reaction. The current study has proved that S. aureus is one of the leading causes of nosocomial infection and shows strong resistance towards the most antibiotics including methicillin. Molecular assay confirmed that mecA gene is responsible for the resistance towards methicillin. The results of our study showed a resemblance to the study reported by Fatima et al. (2019) in Lahore, Pakistan. They concluded in their study that the isolates which were resistant to cefoxitin and oxicilin, most of them (67.27%) detected positive for *mec*A gene. Our results were also similar to a previous study conducted by Sadiq et al. (2020). Out of 150 isolates, 96 (63%) showed resistance to cefoxitin which is potential marker for identification of MRSA. A study conducted by fazal et al. (2020) showed comparatively similar result to our study. They concluded that 51% isolates of *S. aureus* were found positive for mecA gene. A comparative study was also conducted by Albarrag et al. (2020) on the clinical samples in Saudi Arabia and our results were found to be quite relevant and similar with the results presented by him. They showed in the result that 85% (17/20) isolates of *Staphylococcus aureus* were detected positive for *mecA*. The results of most of the studies on methicillin resistance *S. aureus* that had been conducted on samples from the hospital patients were quiet similar to our research.

CONCLUSION

The on the basis of finding of current study, *S. aureus* is one of the most prevalent pathogen in hospital sitting equipped with the wide range of virulence factors. As *S. aureus* is able to cause life threatening infections, the presence of *S. aureus* in clinical samples followed by the prevalence of *mec*A gene in isolates has been a serious threat to both healthcare worker and public health.

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