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Frequency of macrolide resistance gene *msrA* in clinically isolated erythromycin resistant *Staphylococcus aureus* strains from a tertiary care setting in Sindh, Pakistan

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Abstract: **Background**: The gene *msrA* is an efflux pump that encodes proteins causing macrolide resistance in different organisms. Macrolide resistance in *Staphylococcus aureus* is an emerging clinical challenge. The *msrA* efflux pump gene plays a significant role in mediating this resistance, making its local prevalence clinically relevant. **Objectives**: To determine the frequency of the *msrA* gene among clinically isolated erythromycin-resistant *Staphylococcus aureus* strains. **Methods**: This was a cross-sectional study carried out at the Department of Microbiology, Dow Diagnostic Research and Reference Laboratory, Karachi, Pakistan from 1st January 2018 to 31st January 2019. The frequency of *msrA* gene was determined in clinically isolated erythromycin resistant *Staphylococcus aureus* (*S. aureus*) strains. The study was reviewed and approved by the Institutional Review Board (IRB) of Dow University of Health Sciences. A total of 40 (n=40) erythromycin resistant *S. aureus* strains were isolated from the clinical samples and were obtained according to standard procedures. Antibiotic sensitivity was done as per Clinical and Laboratory Standards Institute (CLSI) guidelines. Resistant isolates were stored in Brain Heart Infusion Broth (BHI) and Polymerase Chain Reaction (PCR) were performed to detect the presence of *msrA* genes. **Results**: The frequency of *msrA* was 70% in clinically isolated erythromycin-resistant *S. aureus* strains. **Conclusion**: Based on the results, it was concluded that *msrA* is a significant contributor to the macrolide resistance in locally isolated clinical strains. A major clinical implication of this finding could be the use of efflux pump inhibitors with the macrolides may increase the sensitivity of the bacteria to the drugs, with favorable clinical outcomes.

Keywords: Drug resistance; Erythromycin; Macrolides; *msrA; Staphylococcus aureus*

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INTRODUCTION

Macrolides are a group of compounds with a wide spectrum of activity. Azithromycin, erythromycin and clarithromycin are commonly used macrolides for various infections, including chronic bronchitis, middle ear infections and acute infections of the sinuses caused by Gram-positive microbes (Dinos, 2017). They are important alternatives where penicillins and cephalosporins cannot be used or are used to treat atypical sensitive atypical bacteria (Eltayeb & Leaver, 2024).

They bind to the 50S ribosomal subunit and prevent the uncharged tRNA from being released after the shift of the amino acid to the rising nascent peptide chain, where the donor site of the 50S ribosomal subunit remains inaccessible, stopping protein synthesis (Amdan *et al.*, 2024; Naik *et al*, 2024). Since macrolides are commonly prescribed for the treatment of respiratory tract infections, especially when the first-choice drugs cannot be employed, development of resistance to them is a major issue (Davidson, 2019). A recent study at a tertiary care hospital in Rawalpindi revealed significant genetic diversity among *Staphylococcus aureus* strains, including variations in resistance genes like *msrA* (Manzoor *et al.*, 2024). This suggests a dynamic and evolving bacterial population, potentially contributing to the spread of resistance genes within healthcare facilities. In addition, *msrA* and other resistance determinants, such as *erm* genes, have become significantly more common, providing lincosamide and macrolide resistance. Treatment choices become more complex due to the possibility of a more complex resistance mechanism indicated by the co-existence of these genes.

Resistance to macrolides develops due to different types of genes *ermA, ermB, ermC, mefA and msrA* that confer resistance through different mechanisms, including structural alteration of ribosomes, removal of antibiotics and chemical inactivation of drugs (Roberts *et al*., 1999). This resistance also results in development of cross-resistance to lincosamides and streptogramin (Roberts *et al*., 1999). Data available from the Pakistan Antimicrobial Resistance Network (PARN) website shows variable macrolide resistance in *Staphylococcus* and other microbial species. These bacteria are involved in causation of various pus-producing bacterial infections, endocarditis,

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bone infections, septic arthritis, bladder and kidney infections along with septicemia, pneumonia and meningitis (Tong *et al.,* 2015).

This study was designed to investigate the frequency of *msrA* gene in clinically isolated *S. aureus* strains.

MATERIALS AND METHODS

This was a cross-sectional study, conducted at the Department of Microbiology, Dow Diagnostic Research and Reference Laboratory, Karachi, Pakistan, during the period of 1st January 2018 to 31st January 2019.

Erythromycin-resistant pus samples were collected from the Microbiology section, Dow Diagnostic Research and Reference Laboratories using sterile cotton swabs or syringes and recorded with the patient's name, age and gender. In total, 40 isolates from pus samples were included in the study. The samples were transported to the Microbiology laboratory for further analysis and were refrigerated at 4°C.

After routine culture *S. aureus* was characterized using standard microbial identification techniques of Gram staining, cultural characteristics and biochemical analyses. Any isolation that did not fulfill the standard identification criteria was excluded from the study. The antibiotic susceptibility pattern of the identified strains was then determined by Kirby-Bauer disk diffusion method as per standard protocol (Bauer *et al*., 1966). Antibiotic Susceptibility Testing (AST) involved using fresh cultures of isolates on Mueller-Hinton Agar (MHA) plates, which were matched with 0.5 McFarland standards. These cultures were tested with commercially available erythromycin (15 µg) disks. According to the CLSI guidelines, the susceptibility pattern was determined as sensitive, resistant, or intermediate based on the size (mm) of the zone of inhibition (ZOI) (Humphries *et al*., 2018). The erythromycin-resistant samples were preserved in Glycerol and Brain Heart Infusion (BHI) broth at -80°C. Isolated strains were Gram-stained, and colonies were examined under a light microscope at 1000x using an oil immersion lens. A coagulase test was performed for the confirmation of *S. aureus*. A 500µL plasma sample was transferred to a small sterile test tube and mixed with a loopful of a colony of the *S. aureus.* The tubes were incubated at 37°C for a period of four hours. If the tube remained negative for coagulation for four hours, then it was further incubated at room temperature for 24 hours for confirmation (Tiwari *et al*., 2008).

For DNA extraction, the isolates were cultured in nutrient broth and incubated overnight at 37°C. Genomic DNA was extracted from *S. aureus* by using a commercially available kit (Thermo Scientific DNA extraction kit) according to the manufacturer's protocol.

PCR amplification was performed to detect *msrA* using predesigned specific primers (Forward: 5'-GCA AAT GGT GTA GGT AAG ACA ACT -3' and Reverse: 5'-TAA AAC AAA TGT AGT GTA CTA[11] -3') for *msrA* gene (Sutcliffe *et.al.,* 1996).

The final volume of 25µL using 2mM dNTP, 2µM each of forward and reverse primer, 4mM $MgCl_2$, 5units of Taq DNA polymerase, 2.5µL of KCl buffer (10X) to achieve the ultimate concentration of 1X in the total reaction volume. The final volume (25µL) was maintained by dispensing 8.5µL volume of sterile distilled water and 5µL of bacterial DNA was added. The mixture was then vortexed for five seconds and thirty-five cycles of PCR were performed using thermal cycler (AERIS ESCO AERISBLC011047-12). The first round of denaturation for the reaction mixture is conducted at 94°C for 10 minutes, while the subsequent rounds entail denaturation at 94°C for 30 seconds. Annealing temperature was set at 44°C for 1 minute, allowing primers to bind to complementary sequences. Extension at 72°C adds nucleotides, allowing amplification of the target DNA sequence. Final extension at 72°C ensures complete synthesis as well as helps to finalize the amplification process and the machine was set to hold the sample at 4°C till it was retrieved:

Amplified products from PCR were processed through electrophoresis in 1% agarose gel prepared using 1gm agarose in 100mL 1xTAE buffer. The gel solution was heated in microwave and poured into the casting tray along with 10µL of VisualaNA dye (0.01% v/v) and was left to polymerize. A total of 30µL of amplified product was placed in respective wells after the mixing of loading dye, which was 5µL in volume. 1Kbp DNA ladder was dispensed in the first well, which is located on the left side of the gel and subsequent wells were loaded with amplified PCR products. These samples were separated into 1% agarose gel in 1xTAE buffer with a running voltage of 90V. The amplified PCR products were visualized using an LED transilluminator (Cat. LED001), operating at 100–240 V AC, 50/60 Hz, 1.4 A. The gels were observed and documented under ultraviolet illumination using this system. (Lee *et al*., 2012).

RESULTS

The 40 S. aureus isolates selected for the study were confirmed based on their morphological, colonial and biochemical features. Therefore, 40 pus isolates were used in this study. The results of Gram staining of all isolates showed characteristic Gram-positive cocci in bunches, with scattered confirmation as expected from *S. aureus* isolates. Microbiological identification was carried out on blood agar, which showed β-hemolytic colonies. Isolates produced characteristic, rounded, convex colonies of creamy golden color, which were 1-4 mm in diameter, having a sharp border. Coagulase and Catalase tests came positive for *S. aureus.*

The band size of the amplified product was found to be 1 kbp on 1% agarose gel. The PCR showed 28 out of 40 (70%) isolates to be *msrA* positive.

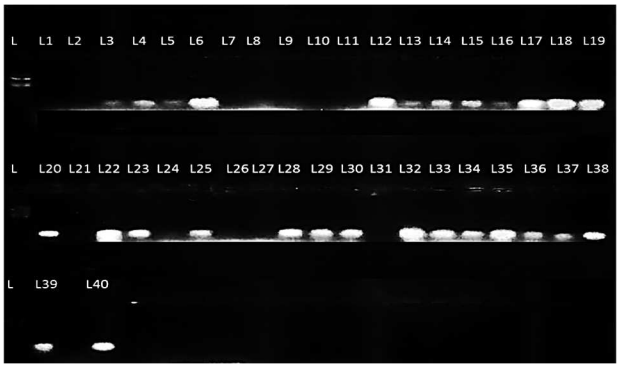

Fig. 1: PCR Amplification of *msr* (*A*) Gene: PCR amplification of *msr* (*A*) gene showed single band of 1 Kbp in 28 isolates. DNA molecular weight marker (ladder) is shown on left hand land.

DISCUSSION

This study showed that about two-thirds of the erythromycin-resistant isolates were positive for the presence of the *msrA* gene. This gene has been reported to be at a considerably low frequency in earlier European studies. In a study from 24 European hospitals, the most common determinant was *ermA* in Methicillin-Resistant *S. aureus* (MRSA) (88%), while *ermC* was found more frequently in Methicillin Sensitive *S. aureus* (MSSA) strains (47%). On the other hand, *msrA* was found in only MSSA strains with a frequency of only 13% (Schmitz *et al*., 2000). Another study from French hospitals from 1999 reports the frequency of *msrA* to be only 2.1% (Lina *et al*., 1999). More recent reports from our own region show a different picture. Though resistance to Erythromycin was reported from 70% to 99% (Sarwar *et al.,* 2020; Ullah *et al.*, 2016). The *ermC* gene was reported to be present in 41-42% in macrolide resistance isolates (Manzoor *et al.,* 2024, Madzgalla *et al.,* 2016). In an Iranian study, the frequency of *msrA* gene was reported to be 28% to as high as 68% in Coagulase-negative *Staphylococci* (Khashei *et al.,* 2018) while another study from Nepal showed the frequency to be only 10% (Nagarkoti *et al.,* 2021). This could be because of various reasons, including stricter regulations regarding antibiotic prescription, differences in the genetic structure of the bacterial population or environmental factors. It is important to mention that *msrA-carrying* strains develop resistance through an efflux mechanism and have been reported to stay negative for inducible clindamycin resistance (Steward *et al.,* 2005), which is among the drugs used for MRSA strains (Adhikari *et al.,* 2017). Those resistant through other molecular mechanisms, such as those mediated by *erm* genes, may develop induced resistance to clindamycin during therapy (Adhikari *et al.,* 2017). Although a phenotypic D test is available, which differentiates inducible strains for clindamycin resistance from non-inducible strains (Adhikari *et al.,* 2017). A PCR-based method for MRSA detection could be implemented to genotype strains for inducible clindamycin resistance in erythromycin-resistant strains.

The major limitation of our study was that we did not consider the simultaneous presence of other resistance genes; however, to the best of our knowledge, there is little data available on the frequency of different genetic determinants of macrolide resistance from Pakistan (Madzgalla *et al.,* 2016).

From the probabilistic point of view, it will be rare to find two or more genetic determinants simultaneously in a single strain and the frequency of single gene conferring resistance would be higher than that of resistance due to multiple genes. A future investigation in this direction may provide useful insights into the molecular epidemiology of macrolide resistance in Pakistan.

CONCLUSION

Our study showed that the *msr* (*A*) gene was present in approximately two-thirds of the erythromycin-resistant isolates.

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Author's contributions
MA, MYN, MH, and ShSh conceived and designed the study. MA and SaSh performed laboratory work and data collection. MYN, ShSh and MH contributed to study supervision and data interpretation. MA and MYN wrote and revised the manuscript for important intellectual content. All authors reviewed, edited, and approved the final version of the manuscript.

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Data availability statement
Data are already available in the form of published M. Phil thesis of Muhammad Asif. Raw data can be shared on request.

Ethical approval
The study was ethically reviewed and approved by the Institutional Review Board (IRB) of Dow University of Health Sciences (IRB-951/DUHS/Approval/2017/3).

Conflict of interest
None

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