ORIGINAL ARTICLE

Genotypic Characterization of Macrolides Resistance in Clinical Isolates of Staphylococcus Aureus from Rawalpindi, Pakistan

Iram Manzoor, Muhammad Idrees, Shumaila Naz

ABSTRACT

Objective: To determine the patterns of resistance among different macrolides and to detect macrolide resistance genes in *Staphylococcus aureus* collected from different clinical samples.

Study Design: Cross-sectional observational study

Place and Duration of Study: The clinical samples sourced from the Pathology Labs of Railway Hospital, Tehsil Head Quarter (THQ) Hospital Taxila and Wah General Hospital Wah Cantt, Pakistan was collected from 01 August 2017 to 01 February 2018.

Materials and Methods: One hundred non-repetitive clinical isolates were obtained from different clinical samples (pus, urine, sputum and blood). Each *Staphylococcus aureus* isolate was obtained from only one sample, to avoid repetition of strain. They were selected on the criteria of their growth with bright yellow color colony formation on mannitol salt agar (MSA) media along with the color change of media from red to yellow and coagulase positive results. Kirbey-Baur disk diffusion test was followed for the detection of antimicrobial sensitivity, whereas genotypic resistance drift was determined using PCR.

Results: Highest frequency of *Staphylococcus aureus* was observed in pus samples i.e. 45 (40.2%). Out of 100, 34 isolates of *Staphylococcus aureus* were resistant to macrolides group. Among 34 resistant isolates the frequency of *ermC* gene was 25 and *msrA* was 14 respectively, whereas all isolates were negative for *ermA* gene. Maximum resistance was observed against erythromycin, n=33 (29.5%) and minimum against clarithromycin, n=26 (23.3%). The highest susceptibility trend was seen against Azithromycin, n=62 (55%).

Conclusion: Resistance of *Staphylococcus aureus* may vary with different antibiotics within the same group. A high frequency of erythromycin resistance was seen in this study. The most predominant resistance was for *erm*C, among the resistance genes in isolates.

Key Words: *Drug Resistance; Genes; Macrolides; Microbial; Staphylococcus Aureus.*

Introduction

Excessive use of antibiotics for the treatment of bacterial infections has raised resistance to macrolides and lincosamides. These resistance proportions show variations with different geographical distribution and study periods. Staphylococcus aureus, facultative anaerobic bacteria that usually grows as aerobically but can survive anaerobically.[1] Most abundantly found in the nasal passage, respiratory tract and on the skin.[2] *Staphylococcus aureus causes* the *number of infections to human like scalded skin syndrome, toxic shock syndrome, septic arthritis, bacteremia and endocarditis etc.* Food-borne illness is majorly caused by enterotoxin producing strains of Staphylococcus aureus**.** The most obvious clinical manifestations are acute onset of vomiting and watery diarrhea. *Mode of infection includes two mechanisms, either by invading the tissues or by producing toxins*.[3] Staphylococcus aureus was regarded as main etiological factor for much of hospital acquired and community infections such as bacteremia, surgical attained and skin infections.[4]

Acquisition of resistance occurs either by extra chromosomal elements such as mobile DNA segments, plasmids, transposons and integrons that are attained from other bacteria in the surroundings, or by efflux pumps which releases multiple antibiotics.[5] The prevalence of these antibiotic resistance infections is more common in hospitals and in medical centers. The introduction of new antibiotics to treat the infection of Staphylococcus

Department of Bio Science
University of Wah, Wah Cantt

Correspondence:
Iram Manzoor
MS Student
Department of Bio Science
University of Wah, Wah Cantt
E-mail: iram1manzoor@gmail.com

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aureus has led to the proliferation in antibiotic resistance.[6]

Macrolides are antibiotics having 12 to 16 membered lactone rings along with some amino sugars. They are considered bacteriostatic but also act as bactericidal at very high concentrations. Resistance to macrolides in Staphylococci may arise through either target-site modification, involving genes such as erm (erythromycin ribosomal methylase) or active efflux, encoded by msrA (methionine sulfoxide reductase A) enabling the bacteria to expel the antibiotics.[7]

Literature shows many studies of macrolides resistance particularly in respiratory pathogens like Streptococcus pneumoniae[8] and Mycoplasma pneumoniae[9]. Antibiotic usage in Asia has shown increasing trend between 2000 -2015. In India, consumption surged from 3.2- 6.5 billion on defined daily doses (DDD's) marking a significant 103 % rise. Likewise, in China increase occurred from 2.3- 4.2 billion DDD's (79%), while Pakistan's antibiotic consumption has grown from 0.8 – 1.3 billion DDD's (65%).[10] Since 2010, the pattern of macrolide resistance has expanded even more, with many studies indicating a resistance rate of more than 92%.[11]

Frequent use of macrolides plays a crucial role in shaping the resistance pattern of this group among different countries. Hsia research highlights the commonly used antibiotic for hospitalized children in China, Japan, and South Korea is azithromycin, with a prescription rate of 11.8%[12] while inappropriate prescription rate of macrolide group in Pakistan is 74.6 %[13]. To the best of our knowledge the resistance rate of macrolide particularly in Staphylococcus aureus is not studied in Rawalpindi region. The present research mainly focuses on the frequency of macrolides resistance in Staphylococcus aureus in Rawalpindi and molecular characterization of macrolide resistance genes in the strains as these antibiotics were chosen because of their common choice of selection by physicians.

Materials and Methods

A cross-sectional observational study was conducted between 01st August 2017 and 01st February 2018, and the data was collected from various hospitals (Railway Hospital, Tehsil Head Quarter (THQ) Hospital Taxila and Wah General Hospital Wah Cantt in Rawalpindi, Pakistan) by using non-probability (purposive sampling technique). Ethical approval for this study was obtained from the University of Wah, Pakistan under Ref# UW/BIS/18/222. Post-operative wound pus sample, urine samples of UTI and sputum samples of individuals with cough and pulmonary disease were collected. Samples were obtained from individuals of both genders aged 10 years and older, individuals with post-operative wound pus samples, with urine samples diagnosed with urinary tract infections and with sputum samples exhibiting symptoms of cough and pulmonary disease were included. Repetitive isolates of Staphylococcus aureus along with those samples which were contaminated or showed signs of improper handling and those isolates that were not purified were excluded from the study.

Hundred non-repetitive isolates of Staphylococcus aureus were purified from different clinical samples (pus, urine, sputum and blood) in Pathology lab of Railway Hospital, Rawalpindi. Purified isolates were then taken to the Lab of Department of Biosciences, University of Wah. Mannitol Salt Agar media was used for growth of Staphylococcus aureus. The purified isolates were Gram's stained and checked for color and morphology. Catalase, oxidase, and coagulase tests were also performed.

Disk diffusion method was used to check the susceptibility of bacterial isolates against commercially available macrolide antibiotics. Disk Diffusion method devised by Kirby-Bauer was used for antimicrobial drug susceptibility. Zone of inhibition were measured according to Clinical and Laboratory Standard Institute (CLSI) guidelines.[14]

Extraction of genomic DNA of resistant strains was done by Geneaid (Cat.# GEE150). Macrolides resistant genes erm(A), erm(B), erm(C), and mrs(A/B) were detected by the PCR amplifications using particular oligonucleotide primers (Table I).

Multiplex PCR assays were performed in 25 µl PCR mixture. Mixture contained a DNA template (20 µg), 2 µl of dNTP's, 0.5 µl of Go Taq DNA polymerase, 2.5 µl green Go Taq buffer (10X), 0.5 µL of each forward and reverse primers were used in concentrations of 25 pM. Primers of ermA, ermC and msrA were selected from different literatures[15,16] (Table I). The PCR was performed with some modifications, initial denaturation at 95 °C for 5 minutes, followed by 30

cycles of 30 seconds at 95°C for denaturation, 58 °C for 30 seconds (annealing of erm), 60°C for 30 seconds (annealing of msr) extension at 72 °C for 1 minute then final extension 72 °C for 7 minutes.[17] Amplicons were analysed on 1% agarose gel and visualized in Syngene InGenius3: gel documentation system. Amplicons size was checked by using 1Kb ladder as a marker.

Data analysis was performed by IBM SPSS version 26.0. Descriptives including frequencies and percentages were reported in a tabular and bar chart.

Results

Frequency and Percentages of Staphylococcus aureus in samples were n=23 (20.5%) in blood, n=18 (16.1%) in urine, n=45 (40.2%) in pus, n=26 (23.5%) in sputum. The highest frequency of Staphylococcus aureus strains was in pus samples. (Figure 1)

Among all the macrolides maximum resistance in bacterial strains was against erythromycin with the percentage of n=33 (29.5 %), and minimum resistance was against clarithromycin with the percentage of n=26 (23.3 %), whereas azithromycin susceptibility was highest n=62 (55.4 %) among the group. (Figure 2)

Thirty-Four strains were found to be resistant, and they were subjected to PCR amplification for macrolides resistance genes detection (msrA, ermC, ermA). Twenty-five (73.5 %) strains were positive for ermC gene, n=15 (44.1 %) for msrA whereas n=11 (32.4 %) strains were positive for both ermC and msrA. While 05 strains were negative for all the three primers. In contrast, ermA gene was not detected in 34 resistant strains. (Figure 3)

Lane M; marker of 10Kb, Lane 1; as Positive control for ermC and msrA , Lane 2 (IM 40), Lane 3; Negative control, 4 (IM 45), 5 (IM 70), 6 (IM 36) , 7 (IM 23), 8 (IM 108), 9 (IM 106), 11(IM 112), 13 (IM 05) and 14 (IM 33). Samples in lane 2, 4, 5, 9, 11, 12 and 14 showed positive result for msrA with product size of 939 bp. Whereas samples in lane 6, 7, 10 and 13 showed positive result for ermC with product size of 321 bp. (Figure 4)

Discussion

The highest frequency of Staphylococcus aureus was recorded in pus sample, n=45 (40.2%) from wound infections. This high incidence was also found in Nepal in 2018.[18]This increase existence in pus sample

Table I : Primers of Macrolides Resistance Gene along with their Sequences

Gene	Primer	Oligonucleotide seq(5'->3')	Product size(bp)	Tm Ċ	GC%
msrA1	msrA1-F	GGC ACA ATA AGA GTG TTT AAA GG	939	55.79	39
	msrA1-R	AAG TTA TAT CAT GAA TAG ATT GTC CTG TT		57.02	28
ermA	ermA-F	AGCGGTAAACCCCTCTGAG	457	57.78	58
	ermA-R	TAGTGACATTTGCATGCTTCAA		55.66	38
ermC	ermC-F	ACTTGTTGATCACGATAATTTCCA	321	58.41	33
	ermC-R	TCTACTTAATCTGATAAGTGAGCTATTCAC		57.21	33

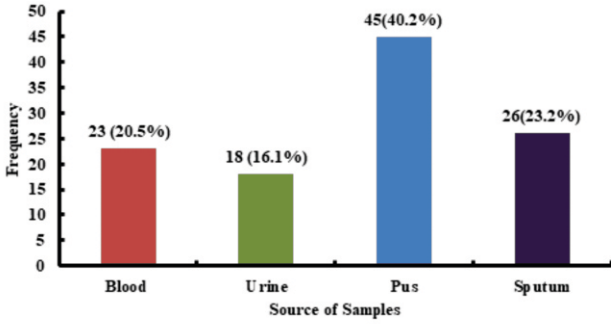

Figure 1: Frequency and Percentages of Staphylococcus Aureus in different Samples

Table II: Susceptibility Patterns of Staphylococcus Aureus to Different Macrolides

Antibiotics	Resistant	Intermediate	Susceptible
Azithromycin	29	9	62
Clarithromycin	26	14	60
Erythromycin	33	32	35
Telithromycin	29	15	56

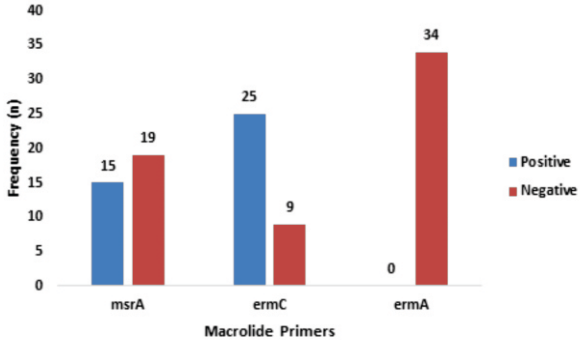

Figure 2: PCR of Different Macrolide Resistance Genes

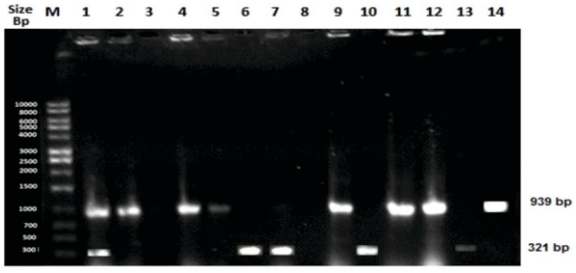

Figure 3: ermC and msrA typing of Gel with Control and Selected Samples

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may occur as Staphylococcus aureus is a habitant of skin when it gets inside the wounds or cut becomes pathogenic in nature. The pattern of resistance of macrolides in Staphylococcus aureus was recorded as erythromycin > azithromycin = telithromycin > clarithromycin and their percentages were 29.5% > 25.9% = 25.9% > 23.2%. The highest percentage of resistance, at 29.5%, was observed for erythromycin. It is notably a lower resistance fraction as compared to the findings of other studies from Pakistan. For instance, previous research in Peshawar[19] recorded a peak resistance rate of 99%, while 72% in Lahore.[20]

Difference may be raised because of advancements in medical facilities in Rawalpindi, a neighbor city of capital of Pakistan, infection control awareness among the medical professionals is high as compared to the peripheral cities. Erythromycin resistance in Pakistan, particularly in the Rawalpindi region is less as compared to other Asian countries like India, Nepal[21] and Iran[22]. The variation in resistance rate may be due to geographical factors and small sample size. This shows Rawalpindi has a better situation, with significantly lower resistance to erythromycin.

This research has highlighted pattern resistance within the macrolide group in which erythromycin has shown its peak as compared to its other group members (azithromycin, clarithromycin and telithromycin). High resistance to the erythromycin or any drug of this class which are commonly used in medical practices for respiratory infections can limit the treatment options and complicate the infection control measures. This can lead to prolonged illness, increased severity of symptoms, and a higher risk of complications as multi-drug resistance in microbes has emerged as concerning global issue within medical community.[23] This increasing resistance of bacteria to various drug poses a considerable threat to the efficacy of antibiotics.

Maximum rate of erythromycin resistance may co-relate with the high fraction of ermC gene (73%) in S. aureus isolates. msrA and erm (A and C) genes were selected for detection of macrolides resistance in S. aureus in recent study. Out of 100 S. aureus isolates 34 were resistant to different macrolides; further on these resistant isolates were subjected to PCR amplification for msrA and erm (A&C) gene finding.

Our study findings indicate that ermC (73%) is the conspicuous factor for erythromycin resistance in contrast to the msrA and ermA. These results are in accordance with the reports from Iran[22] and Japan where ermC gene was attributed for major erythromycin resistance. However, these values conflict with other investigations from several countries for instance a study from Korea, Turkey and Egypt[24], reported ermA gene as a major cause of resistance against macrolides. This high prevalence of ermC gene poses a risk of gene elements transformation to other species as it is transferable. Frequency of msrA in our study was also high 44% which seems to be very similar (43.6%) with study from Iran[25]. Notable results of present research were zero frequency of ermA gene in Staphylocoocus aureus strains. Probably the zero frequency of ermA was responsible for the low percentage of erythromycin resistance as compared to other studies. Another interesting finding of our study is high percentage of co-existence of ermC and msrA 11 out of n=34 (32%) which depicts mechanism of combined resistance is increasing in Rawalpindi. ermC and msrA both genes are linked with resistance to macrolides and lincosamides, and their co-existence may confer the higher level of resistance to both classes of drugs making it more challenging to treat the infections for which they are used. The co-occurrence of resistance genes may suggest their presence on the same genetic elements, can contribute to spread of multi drug resistance through horizontal gene transfer between bacteria.

Limitations

- Antimicrobial resistance (AMR) evolves over the time that's why a short period of 6 months may not highlight the long-term trends of resistance.
- Bacterial resistance is multifactorial so the study of just one factor (resistance genes) may affect the results. More factor like virulence gene study can add effective contribution to the solution.

Conclusion

Staphylococcus aureus is currently exhibiting maximum resistance against erythromycin and minimum against clarithromycin. Azithromycin exhibits the highest susceptibility, emphasizing its effectiveness as a choice of drug for the treatment of infections previously treated with erythromycin. To avoid further rise in erythromycin resistance in

Staphylococcus aureus and its potential transmission to other bacterial strains, both clarithromycin and azithromycin are recommended as alternatives.

Among three genes (msrA, ermA and ermC), ermC has high percentage in resistant isolates. This ermC gene is a key contributor in erythromycin resistance. Concurrently co-existence of ermC and msrA gene is also responsible for increasing rate of macrolides resistance. In future long-term studies to monitor the patterns of resistance against macrolides and other classes of antibiotics are recommended to control multi-drug resistance in Staphylococcus aureus.

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CONFLICT OF INTEREST

Authors declared no conflicts of Interest.

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DATA SHARING STATMENT

The data that support the findings of this study are available from the corresponding author upon request.