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Research Article

Macrolide resistance pattern of staphylococci collected from hospitalized patients in Egypt

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ABSTRACT

Macrolide resistance of staphylococci has risen dramatically in recent years generating a real challenge for their treatment as therapeutic options have become very limited. In this study, an antibiogram analysis of one hundred and fifty *Staphylococcus* sp. isolates collected from various clinical specimens, against erythromycin, azithromycin, spiramycin, and clindamycin was carried out. Out of the 150 collected *Staphylococcus* sp. isolates, 54 isolates (36%) showed resistance to two or more of the tested macrolides. Inducible macrolide, lincosamides and streptogramin type B resistance phenotype (iMLS) using D-test was identified in 15 of the resistant isolates (27.8%). Molecular detection of major genes coding for macrolide resistance, including erythromycin ribosomal methylase (*erm*A and *erm*C), and macrolide-streptogramin resistance gene (*msr*A) was carried out using PCR. It was found that 51.8, 37.1 and 11.1% of the resistant isolates carried one, two and three types of the resistance genes, respectively. However, *erm*C was the most frequently occurring gene (81.5%), followed by the *msr*A gene (42.6%), then *the erm*A gene (35.2%). In conclusion, the genotypic analysis revealed that the majority of the tested isolates harbored two or more macrolide resistance-coding genes where 36% displayed resistance to at least two of the most common macrolide antibiotics used in the treatment of such important pathogens particularly in patients exhibiting hypersensitivity to penicillins according to several international guidelines. Therefore, it is crucial to carry out more epidemiologic studies to clearly understand the problem of increasing macrolide resistance among Staphylococci and to implement new guidelines for the treatment of such important pathogens, particularly in Egypt.

Keywords: *Macrolides; Staphylococci; cMLS phenotype; iMLS phenotype; erm; msr*

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1. INTRODUCTION

The antimicrobial resistance among bacteria is regarded as a natural phenomenon. However, the terrifying rise in the percentages of the resistance of pathogenic bacteria to different classes of antibiotics is becoming very prominent worldwide **[1]**. Increasing rates of antibiotic resistance greatly influence the treatment outcome in seriously infected patients receiving empiric antibiotic therapy; since they result in a substantial increase in the morbidity and mortality rates in infected patients **[2]**.

Staphylococci are regarded as a normal commensal of both the skin and mucous surfaces

of humans and other animals **[3]**. There are many species of staphylococci, some of them can result in a variety of human diseases, and on the other hand, some are commensal and considered not to be pathogenic **[4]**. *S. aureus* and Coagulase-negative Staphylococci are regarded as both a commensal bacteria as well as human pathogens; since they are found as normal flora in about 30% of the total human population. At the same time, they are the major cause of a wide range of infections as bacteremia, infective endocarditis, skin infections and infections related to medical devices **[5,6]**.

Macrolide antibiotics belong to the polyketide group of natural products. Erythromycin A was the first clinically used macrolide antibiotic, first discovered in 1952 in the metabolic products of a strain of *Saccharopolyspora erythraea* **[7,8]**. The main activity of macrolide antibiotics is against Gram-positive bacteria as *Staphylococcus*, *Streptococcus*, and *Diplococcus* Gram-positive bacteria **[7]**. Macrolides are bacteriostatic **[9]**. Macrolides inhibit bacterial protein synthesis by binding to the 23S rRNA moiety of the 50S ribosomal subunit, thus interfere with protein synthesis **[10,11]**. There are three main mechanisms responsible for macrolide resistance including i) target site modification by methyltransferases encoded by *erm* genes (erythromycin ribosomal methyltransferase); ii) acquisition of efflux pumps, coded by *mef* (macrolide efflux) and *msr* (macrolide – streptogramin resistance) genes; iii) macrolide inactivation by modifying enzymes which were firstly reported in Enterobacteriaceae e.g. esterases coded by *ere* genes and phosphotransferases coded by *mph* genes **[12–14]**.

The rational use of antimicrobials is the cornerstone of good clinical practice; to increase the therapeutic efficacy, and minimize the risk of treatment failure and emergence of resistant microorganisms **[15]**. Due to the abuse and overuse of antimicrobials in Egyptian hospitals, there is progressive development of bacterial resistance to antibiotics **[16]**. Therefore, it is of crucial importance to identify the mechanism of resistance to macrolide antibiotics in Egyptian hospitals to be able to identify new approaches for the treatment of bacterial infections and try to avoid the transfer of the resistant genes and dissemination of antimicrobial resistance among bacteria. Therefore, this study aimed at detection and analysis of the different macrolide resistance phenotypes, and correlates these phenotypes with the previously mentioned macrolide resistance genes among pathogenic Staphylococci collected from hospitalized patients from a certain clinical setting in Egypt.

2. MATERIALS AND METHODS

2.1. Microorganisms and culture media

A total number of 150 *Staphylococcus* isolates including *S. aureus* (97 isolates; 64.7%) and coagulase-negative *Staphylococcus* sp, (53 isolates; 35.3%) were collected from different clinical specimens including pus (87; 58%), blood (32; 21.3%), sputum (24; 16%) and bronchoalveolar lavage (7; 4.7%) from the Microbiology diagnostic laboratories of Al-Demerdash Hospital during the period from October 2015 to March 2016. The whole study was approved by the Faculty of Pharmacy, Ain Shams University Research Ethics Committee (ENREC-ASU-Nr. 94). Mueller Hinton, blood, and mannitol salt agar were used to culture and purify the recovered isolates which were identified microscopically and biochemically thereafter **[17]**. *S. aureus* isolates were distinguished from other Staphylococci by giving yellow colonies after culture on mannitol salt agar **[18]**. *S. aureus* ATCC® 25923 strain was used for the quality control of antimicrobial susceptibility tests.

2.2. Antimicrobial susceptibility test

The susceptibility of the collected clinical isolates was evaluated against four antibiotics: erythromycin (15 μg), azithromycin (15 μg), spiramycin (10 μg) and clindamycin (2 μg) by Kirby-Bauer disc diffusion method using Mueller-Hinton agar, and the results were explained according to CLSI breakpoints 2016. The antibiotic disks were obtained from Oxoid®, UK.

2.3. Detection of inducible resistance phenotype

The inducible resistance phenotype was detected by the double-disc diffusion test (D test) as described previously by Coutinho and co-workers **[19]**.

2.4. Detection of macrolide resistance genes

The chromosomal DNA of the resistant isolates was extracted using the Zyppy™ Genomic DNA purification Kit purchased from Sigma Scientific Services Company (Cairo, Egypt). The extracted DNA of each isolate was tested as a PCR template for the detection of erythromycin ribosomal methylase gene (*erm*A and *erm*C) and macrolide-streptogramin resistance gene (*msr*A) using the appropriate primers **(Table 1)**.

Table 1: The primer sequences used in this study and the expected PCR product sizes

Primer	Target gene	Primer sequence (5'→3')	Ta(°C)	PCR product (Kb)	Reference
ErmA-f	*erm*A	TATCTTATCGTTGAGAAGGGATT	50	139	**[21]**
ErmA-r		CTACACTTGGCTTAGGATGAAA			
ErmC-f	*erm*C	AATCGTCAATTCCTGCATGT	51	299	**[22]**
ErmC-r		TAATCGTGGAATACGGGTTTG			
Msr-f	*msr*A	TCCAATCATAGCACAAAATC	47	163	**[21]**
Msr-r		AATTCCCTCTATTTGGTGGT			

The PCR was carried in the thermocycler (Nyx-Technik Inc. Personal, Cycler ATC401, USA) using the following conditions: initial denaturation at 95 °C for 4 min, followed by 30 cycles of: denaturation at 95 °C for 30s, annealing for 30s (the annealing temperature was adjusted according to the melting temperature of the primers used) and extension at 72 °C for 1 min, this was followed by one cycle of final extension at 72 °C for 5 min, then the reaction was held at 4 °C for 10 min. The PCR products of the tested genes were analyzed via 0.8% agarose gel electrophoresis containing 0.5 μg/mL ethidium bromide using a 1 kb DNA ladder (Thermo Scientific, USA) **[20]**. Purification of the PCR products was carried out using the GeneJET™ PCR Purification kit (Fermentas, Waltham, Massachusetts, USA) at Sigma Scientific Services Company, Egypt. Finally, sequencing of the PCR products was done at GATC Biotech Company (Konstanz, Germany), through Sigma Scientific Services Company (Egypt) by the use of ABI 3730xl DNA Sequencer.

3. RESULTS

The Antibiogram analysis showed that, out of the 150 collected *Staphylococcus* sp. isolates, 54

isolates (36%) were resistant to 2 or more of tested antibiotics. Out of the resistant isolates, thirty-five isolates (64.8%) were found to be *S. aureus*; while the other 19 isolates were coagulase-negative Staphylococci (35.2%). It was found that 34 (63%, n= 54) isolates are resistant to the four tested antibiotics (cMLS phenotype); sixteen isolates (30%, n= 54) showed resistance to all macrolides and inducible resistance to lincosamides (iMLS phenotype), which was demonstrated by formation of D-shaped inhibition zone after erythromycin-clindamycin disk approximation test (D-test); while only 4 isolates (7%, n= 54) exhibited resistance to macrolides only (MS phenotype). The percentages of macrolide resistance phenotypes are shown in **Fig (1)**, and the D-test showing inducible resistance is shown in **Fig (2)**.

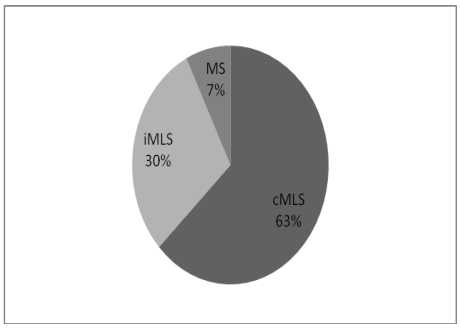

Fig. 1. Distribution of different macrolide resistance phenotypes among the resistant *Staphylococcus* sp. isolates. Percentages were calculated as compared to the total number of resistant *Staphylococcus* spp. isolates (n= 54). cMLS, cMLS phenotype, isolates exhibited resistant to the four tested antibiotics; MS, MS phenotype (isolates exhibited resistance to macrolides only; iMLS, iMLS phenotype (isolates resistance to all macrolides and inducible resistance to lincosamides).

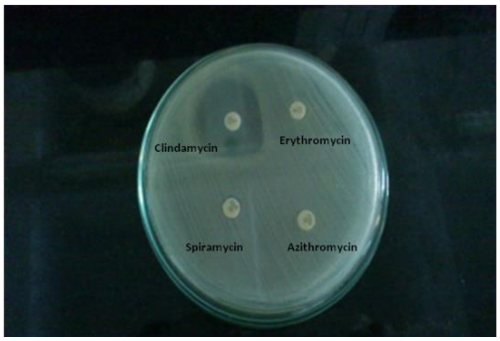

Fig. 2. D-test showing D-shaped inhibition zone

The macrolide resistance genes were detected by polymerase chain reaction (PCR) using chromosomal DNA of the resistant isolates as templates, and primers previously mentioned in **Table 1**. PCR results revealed that 57.4% of the resistant isolates carried only one type of resistance genes, while 33.3% carried two types of genes, and finally three types were detected in 9.3% of the resistant isolates. *erm*C was the most frequently occurring gene (79.6%), followed by *msr*Agene (48.9%), then *the erm*A *gene* (31.5%). The data showing the distribution of the tested genes among the resistant isolates were illustrated in **Fig. 3**. The agarose gel electrophoresis revealing PCR products of the resistant genes is shown in **Fig. 4&5**. The distribution of the macrolide resistance phenotypes and genotypes among the tested isolates, both *S.aureus* and coagulase-negative Staphylococci, is shown in **Table 2)**.

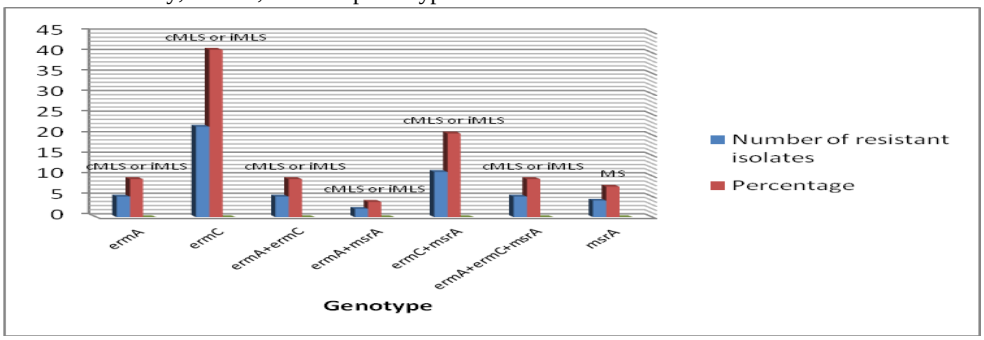

Fig. 3. Correlation between different MAC resistance genotypes and phenotypes among *Staphylococcus* sp. resistant isolates. Percentages were calculated as compared to the total number of resistant *Staphylococcus* sp. isolates (n= 54). *erm*A, erythromycin ribosomal methylase A, *erm*C, *erm*C), *msr*A, macrolide-streptogramin resistance gene. cMLS phenotype (constitutive macrolides, lincosamides, and streptogramin type B), iMLS phenotype (inducible macrolides, lincosamides and streptogramin type B), MS phenotype (macrolides and streptogramin type B)

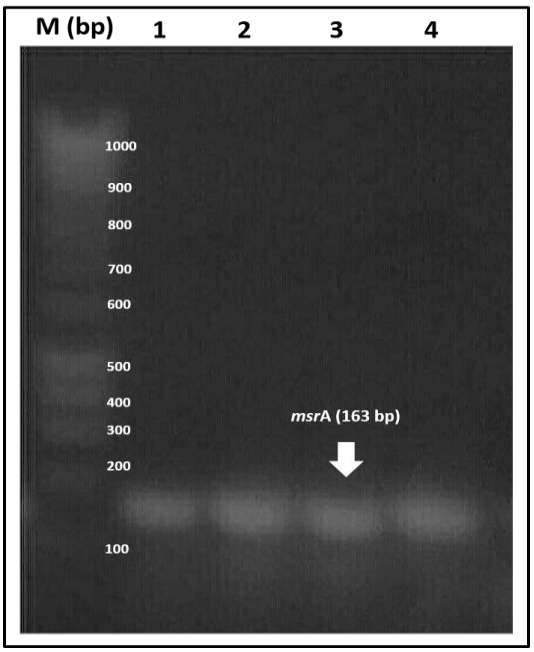

Fig.4. Agarose gel electrophoresis of the PCR product for *the msr*A gene in some tested resistant *Staphylococcus* isolates. Lane: 1 (isolate code S57); Lane2 (isolate code S64); Lane3 (isolate code S88); Lane 4(isolate code S89); and M, 1kb size marker (Thermo Scientific, USA).

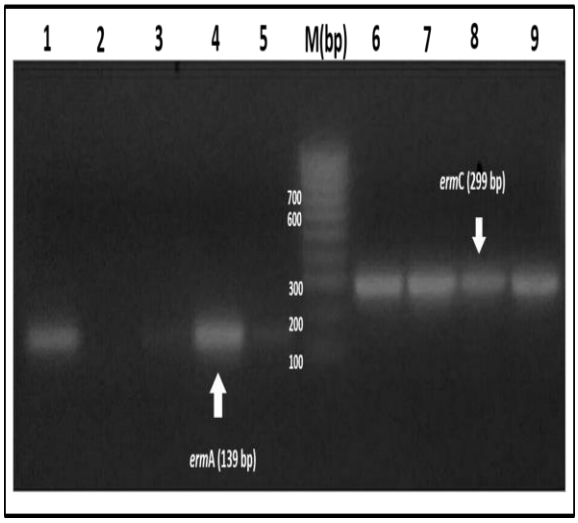

Fig. 5. Agarose gel electrophoresis of the monoplex PCR products for *erm*A and *erm*C genes in some *Staphylococcus* resistant isolates. For *erm*A: lane 1 (isolate code S55); lane 2 (isolate code S48); lane 3 (isolate code S10);lane 4 (isolate code S5);lane 5 (isolate code S3). For *erm*C: lane 6(isolate code S11); lane 7 (isolate code S7); lane 8 (isolate code S5); Lane9 (isolate code S1). Lane M, 1kb size marker (Thermoscientific, USA).

Table. 2. distribution of the macrolide resistance phenotypes and genotypes among the tested isolates

Isolate	Number	Resistance phenotype			Resistance genotype		
		cMLS	iMLS	MS	*erm*A	*erm*C	*msr*A
Staphylococcus aureus	35	22 (62.9%)	10 (28.5%)	3 (8.6%)	11	28	11
Coagulase-negative Staphylococci	19	12 (63.1%)	6 (31.6%)	1 (5.3%)	6	15	11

cMLS (constitutive macrolides, lincosamides, and streptogramin type B), iMLS (inducible macrolides, lincosamides, and streptogramin type B), MS (macrolides and streptogramin type B).

4. DISCUSSION

Macrolide resistance among pathogenic bacteria has been increased in recent years worldwide particularly among Gram-positive cocci including Staphylococci and streptococci and therefore difficulty in their treatment **[23,24]**. The major goal of our study was to screen and evaluate the macrolide resistance among pathogenic Staphylococci recovered from different clinical specimens from the Microbiology diagnostic laboratories of Al-Demerdash Hospital Egypt. In this study, one hundred and fifty *Staphylococcus* isolates, collected from various clinical specimens, were subjected to antibiotic susceptibility test against, erythromycin, azithromycin, spiramycin, and clindamycin. Out of the 150 collected *Staphylococcus* sp. isolates, 54 isolates (36%) showed resistance to two or more of the tested antibiotics. Inducible macrolide, lincosamides and streptogramin type B resistance phenotype (iMLS) was identified in 15 of the resistant isolates (27.8%) by showing D-shaped inhibition zone after approximation of erythromycin and

clindamycin discs (D-test).

The MLS resistance among Staphylococci can be attributed to either one of two mechanisms which are: i) target site modification due to *erm* genes, and ii) active-efflux due to *the msr* gene **[25-27]**. The most prevalent subclasses of *the erm* gene among Staphylococci are *erm*A, *erm*B, and *erm*C **[28]**. There are four different phenotypes of macrolide resistance; two of them are coded by *erm* genes including both cMLS and iMLS phenotypes, where, the other two phenotypes are coded by *msr* genes which can affect only either MACs (M phenotype) or MACs and streptogramin type B (MS phenotype) **[29]**. Accordingly, identifying the type of MLS resistance is crucial, and important from the clinical point of view. Owing to excessive use of macrolide antibiotics in the healthcare settings, iMLS phenotype can be converted into cMLS phenotype and this conversion can result in the treatment failure in patients suffering from serious staphylococcal infections **[30]**. In our study, the cMLS resistance phenotype has the upper hand since it was present in 34 resistant *Staphylococcus* isolates (63%, n= 54), while the iMLS phenotype was detected in only 16 isolates (30%, n= 54). Moreover, the MS phenotype was detected in only 4 resistant isolates (7%, n= 54). The findings of our study agree, to some extent, with the results of another study conducted in Texas, where the cMLS phenotype was the major resistance phenotype (41.7%), while both iMLS and MS phenotypes have only 3.3% each **[31]**. In another study carried out in Serbia, the iMLS phenotype was detected in the majority of the collected isolates (33.4%) followed by cMLS phenotype (8.9%) **[4]**. We can explain the high prevalence of the iMLS phenotype to the increased misuse of macrolides and lincosamides in healthcare settings **[29]**.

Among the resistant *S. aureus* isolates in our study, it was found that cMLS phenotype was the most predominant resistance phenotype, since it was detected in 22 resistant isolates (62.9%); followed by iMLS phenotype which was found in 10 isolates (28.5%); then MS phenotype in only 3 isolates (8.6%). The results of our study agree with the results of another study conducted in Iran; where the ranking of the resistance phenotypes among resistant *S. aureus* isolates is the same compared to our study. Also, the *erm*C gene was found to be the most prevalent macrolide resistance gene in both studies **[32]**. However, the results of our study are completely different from another study carried out by Zachariah and co-workers; wherein the latter study, the MS phenotype was the most prevalent resistance phenotype; followed by iMLS phenotype; then cMLS phenotype **[30]**. Among the resistant coagulase-negative Staphylococci isolates in our study, it was found that cMLS phenotype was the most predominant resistance phenotype, since it was detected in 12 resistant isolate (63.1%); followed by iMLS phenotype which was found in 6 isolates (31.6%); then MS phenotype in only 1 isolates (5.3%). The results of our study agree with the results of another study conducted in Poland; where the cMLS phenotype was the most predominant resistance phenotype **[23]**. The MLS resistance phenotype, either constitutive or inducible, may vary significantly based on different factors as a geographical region and population variations **[4]**.

Another important aim of our study was to investigate the correlation between the genotypes and phenotypes of the recovered resistant *Staphylococcus* sp. isolates collected from patients suffering from serious infections at one of the major clinical settings in Egypt. Our findings revealed that all isolates showing resistance to both macrolides and lincosamides; MLS phenotype either constitutive or inducible; were found to harbor at least one type of *erm* genes (*erm*A or *erm*C). The difference between

both phenotypes is in the type of mRNA of each methylase which is active and produced in absence of inducer in bacteria showing cMLS phenotype, while it is inactive in bacteria displaying iMLS phenotype and become active and being translated only in the presence of inducer **[4]**. The genotype of the four isolates exhibiting MS resistance phenotype showed that only *msr*A was present, which explains the resistance of these isolates only to macrolides, but not to clindamycin.

Conclusion

PCR analysis, used to detect the macrolide genes revealed that the majority of the tested isolates harbored two or more macrolide resistance-coding genes where 36% displayed resistance to at least two of the most common macrolide antibiotics. This finding is of important value for the clinicians to select the appropriate macrolide antibiotic for the treatment of resistant Staphylococci particularly in patients exhibiting hypersensitivity to penicillins. Therefore, it is crucial to carry out more epidemiologic studies to clearly understand the problem of increasing macrolide resistance among Staphylococci and to implement new guidelines for the treatment of such important pathogens, particularly in Egypt.

Declarations

Ethics approval and consent to participate

The whole study was approved by the Faculty of Pharmacy, Ain Shams University Research Ethics Committee (ENREC-ASU-Nr. 94).

Consent to publish

Both informed and written consents were obtained from patients or parents of patients after explaining the study purpose

Availability of data and materials

All data generated or analyzed during this study are included in this published article in the main manuscript.

Competing interests

The authors declare that have no competing interests

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