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DISTRIBUTION OF GENES ENCODING ERYTHROMYCIN RIBOSOMAL METHYLASES AND MACROLIDE EFFLUX PUMP IN METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATES FROM TERTIARY CARE HOSPITALS IN TAMIL NADU.

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ABSTRACT
The increasing frequency of the infections with MRSA and the changing drug susceptibility pattern have led to renewed interest in the use of Macrolide Lincosamide Streptogramin-B (MLSB) antibiotic to treat these infections. Resistance to macrolide and lincosamide is prevalent among s*taphylococci* in recent days. Hence this study was aimed to undertake the detection of resistance determinants of macrolide and methicillin resistant *S. aureus* isolates. A total of 237 *Staphylococcus aureus* isolates collected during 2013-2014, from various tertiary care hospitals were included in this study. MLSBi (inducible clindamycin resistance) was screened by using D- test. Confirmation of MRSA was done by molecular detection of *mec*A and *fem*A gene. Detection of erythromycin resistance genes (*ermA, ermC*) and macrolide efflux (*msrA*) resistance genes was done by multiplex PCR. Of the 237 *Staphylococcus aureus* isolates, 150 (63%) expressed resistance to erythromycin, 46 isolates were MSSA and 104 were MRSA. Our study showed MLSBi in 35% of MRSA and 15% of MSSA. Among MRSA, the most prevalent resistant determinant was *erm*A (22%), *erm*C in 9%, *msr*A in 16% of isolates, whereas 20% of isolates harboured multiple resistance mechanisms. Among MSSA, *erm*A gene was found in 22% of isolates, *erm*C in 9%, *msr*A in 4% of isolates, while 9% of isolates harboured multiple resistance mechanisms. In conclusion, 20% of isolates were detected in combination of *erm* genes with other resistance gene *msr*A. Though *erm*A gene was most prevalent, but isolates exhibits multiple resistance mechanism was found to be high in our study, which was less frequently reported.

KEYWORDS: (D-Test, MLSB, *msr*A gene and *erm* gene).

INTRODUCTION
The treatment of *Staphylococcus aureus* infections is becoming increasingly more complicated due to emergence of various types of antibiotic resistance. Methicillin resistant *S.aureus* (MRSA) has dramatically increased in recent years.[1] The increasing frequency of the infections with MRSA and the changing drug susceptibility patterns have led to renewed interest in the use of Macrolide Lincosamide Streptogramin B (MLSB) antibiotic to treat such infections, with clindamycin being the preferred agent due to its excellent pharmacokinetic properties.[2] However, expression of inducible resistance to clindamycin could limit the effectiveness of this drug.

The bacterial resistance to the MLSB group may be expressed through different mechanisms which include target site modification, the macrolide efflux pump and enzymatic antibiotic inactivation. The modification of the ribosomal target is encoded by a multi allele plasmid borne erythromycin ribosome methylase *(erm)* gene that causes the production of the methylase enzymes. These enzymes cause methylation of the A2058 residue which is located in the conserved domain V of the 23S ribosomal RNA component of the 50S ribosomal subunit, which leads to cross resistance and the formation of the phenotype of the resistance pattern, called as the MLSB resistance phenotype.[3, 4] The resistance to the MLSB antibiotics can be either constitutive (MLSBc) or inducible (MLSBi). If the *erm* gene are constitutively expressed, the organism may show in vitro resistance to erythromycin, clindamycin and other members of MLSB group and they are said to be of MLSBc phenotype. However if the *erm* genes require an inducing agent to express the resistance to clindamycin, then the organism are said to be of MLSBi phenotype. The organism which belongs to the MLSBi phenotype are resistant to erythromycin and sensitive to

clindamycin in vitro. The clindamycin therapy is such patient can lead to clinical failure.[5, 6] Therefore, erythromycin-resistant isolates of *S.aureus* may also be considered to be clindamycin-resistant in most geographical regions. Low levels of erythromycin is an inducer of the MLSBi phenotype and this forms the basis of the D-test.[7] The Clinical Laboratory Standards Institute (CLSI)[8] has recommended the erythromycin – clindamycin disc approximation test (D-Zone test) to detect inducible clindamycin resistance. *Staphylococci* can also develop macrolide resistance, based on the presence o the efflux pump which is encoded by the macrolide streptogramin resistance *msr*A gene, leading to resistance to macrolide and the type B streptogramins, but not to the lincosamides. These isolates are known to be of the MS phenotypes and they show in vitro resistance to erythromycin and susceptibility to clindamycin. But the clindamycin therapy can safely be given in infections which are caused by the organisms of this phenotype and there is no risk of clinical failure.[9] For *Staphylococcus aureus,* it has been shown that erythromycin resistance is caused mostly by *erm*A or *erm*C, whereas export of macrolide antibiotics by *msr*A is rare.[10,11] The present study therefore tested clinical isolates of *Staphylococcus aureus* by phenotypic and genotypic means to determine the presence and mechanisms of MLSB resistance.

MATERIALS AND METHODS

A total of 237 *Staphylococcus aureus* isolates were collected during 2013-2014, from Govt Dharmapuri Medical College Hospital, Dharmapuri, Govt kanyakumari Medical College Hospital, Nagercoil and Gopi Hospital, Salem. Most isolates were considered clinically relevant and originated from pus culture. The isolates were identified to the species level using standard biochemical techniques. The antibiotic susceptibility tests were interpreted in accordance with the CLSI guidelines.[8] Methicillin resistance was detected by taking cefoxitin disc (30µg) as a surrogate marker, Phenotypic characterization of MLSBi resistance was performed using the disk approximation test with erythromycin (E) 15 µg and clindamycin disks (CD) 2 µg. The D phenomenon was considered to be indicative of the presence of an inducible *erm* gene.[12] Molecular detection of MRSA using Triplex PCR by Nagarajan *et.al* [14] to detect femA and *mec*A which confers resistance to methicillin resistant *Staphylococcus aureus*. Detection of erythromycin resistance genes (*ermA, ermC*) was done by the method of Strommenger *et al.*, 2003. Detection of macrolide efflux (*msrA*) resistance genes was done by the method of Shittu *et al.*,2011 (Table.1).

RESULTS

Of the 237 *Staphylococcus aureus* isolates, 150 (63%) expressed resistance to erythromycin (Table.2). The constitutive phenotype was detected in 40 isolates (17%), the inducible phenotype in 43 (18%) and the MS phenotype in 67 isolates (28%). 46 isolates were MSSA (10 from Dharmapuri, 22 from Salem and 14 from Nagercoil) and 104 were MRSA (48 from Dharmapuri, 34 from Salem and 22 from Nagercoil), Percentage of macrolide resistance was higher amongst MRSA as compared to MSSA (Table.2). The result of D- test analysis and prevalence of resistance gene is shown in (Table.3).

Table.1 Primer sequence, amplicon size of target gene and method followed in this study

Target genes	Primer sequence	Amplicon size (bp)	Method followed
femA	F: AAAAAAGCACATAACAAGCG R: GATAAAGAAGAAACCAGCAG	132	Nagarajan *et.al.*, 2013
mecA	F: TGCTATCCACCCTCAAACAGG R: AACGTTGTAACCACCCCAAGA	286	
ermA	F: AAGCGGTAAACCCCTCTGA R: TTCGCAAATCCCTTCTCAAC	190	Strommenger *et al.*, 2003
ermC	F: AATCGTCAATTCCTGCATGT R: TAATCGTGGAATACGGGTTTG	299	
msrA	F: GAAGCACTTGAGCGTTCT R: CCTTGTATCGTGTGATGT	287	Shittu *et al.*,2011

Table.2 Erythromycin resistance among MRSA and MSSA in various places included in this study

Source of isolates	*Staphylococcus aureus*	MSSA (%)	MRSA(%)	Erythromycin Resistant *Staphylococcus aureus* (150) isolates(%)		
				MRSA	MSSA	Total
Govt Dharmapuri Medical College Hospital, Dharmapuri	84	20 (24)	64(76)	48(32)	10(6)	58(38)
Gopi Hospital. Salem	86	43(50)	43(50)	34(23)	22(12)	56(37)
Govt Kanyakumari Medical College Hospital Nagercoil.	67	33(49)	34(51)	22(15)	14(5)	36(25)

Table.3 Correlation between MLSB resistance phenotype with *erm* and *msr*A gene

Isolates	Methicillin Resistance	MLSB resistant Phenotype*	Genotype						
			erm A(%)	*erm*C (%)	*msr*A (%)	*erm*A/*msr*A(%)	*erm*c/*msr*A(%)	*erm*A/*erm*C /*msr*A(%)	Undetermined (%)
Erythromycin resistant *Staphylococcus aureus* isolates(150)	MRSA(104	33(cMLSB)	11(33)	5(15)	2(6)	3(9)	2(6)	1(3)	9(28)
		36(iMLSB)	6(17)	3(8)	7(19)	6(17)	1(3)	0(0)	13(36)
		35(MSB)	6 (17)	1(3)	8(23)	6(17)	1(3)	1(3)	12(34)
	MSSA(46)	7(cMLSB)	2(29)	1(14)	1(14)	0(0)	0(0)	0(0)	3(43)
		7(iMLSB)	1(14)	2(29)	0(0)	1(14)	1(14)	0(0)	2(29)
		32(MSB)	7(22)	1(3)	1(3)	0(0)	1(3)	0(0)	22(69)

*interpretation based on D –test

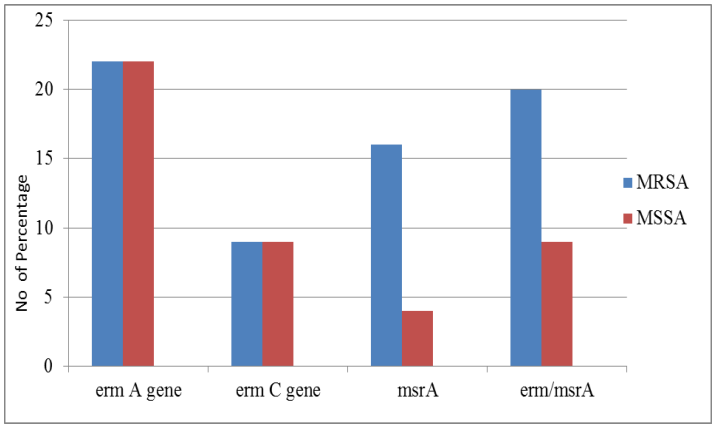

Figure:1 Distribution of *erm* and *msr*A genes among MSSA and MRSA

DISCUSSION

The multiplicity of mechanisms that confers resistance to macrolides is reflected by the complexity of the resistance phenotypes; however, the most clinically important and widespread determinants in *Staphylococus aureus* are the methylase and efflux genes. Identification of the resistance mechanisms is important with regard to the use of clindamycin and 16-membered ring macrolides. The double-disk diffusion technique with erythromycin and Lincomycin (or Clindamycin) is useful to guide interpretation of the susceptibility test.[19] The incidence of resistance is highly variable with regard to the country and, most importantly, the patterns of infections observed among patients. For this reason, local statistics are of crucial value for empiric therapy. Surveillance of both the incidence of macrolide resistance and the respective prevalence of the various resistance mechanisms is justified by the rapid variations in macrolide resistance observed in several countries.[20] There have been various reports on the pattern of the MLSBi resistance among the *Staphylococcus aureus*; a study from Tamil Nadu demonstrated MLSBi in 28% of the MRSA and in 11% MSSA.[13] In North India, it was reported to be 30% in the MRSA and 10% in MSSA [14], our study showed MLSBi in 35% of MRSA and 15% of MSSA, whereas in two studies from Karnataka, the MLSBi phenotype was seen in 63%[15] and 55.26%[16] among *S.aureus* isolates, the reason for this lower incidence may be the geographical and environmental factors, which were entirely different in different clinical set ups. In our study MLSBc phenotype was identified among 32% of MRSA and 15% of MSSA isolates respectively. Difference in incidence of MLSBi and MLSBc phenotype were less in our study while rest exhibiting the MS phenotype.

MLSB Resistance genotypes

The distribution of resistance genes among 150 isolates resistant to erythromycin (104 MRSA and 46 MSSA) as determined by PCR amplification of *erm*A, *erm*C and *msr*A, is displayed in Table 1. Among MRSA (104) the most prevalent resistant determinant was erm A, which was detected in 22% of the isolates. The *erm*C determinant was found in 9% of the isolates, *msr*A was found in 16% of isolates, whereas 20% of isolates harboured two different resistance mechanisms , of which 14% of isolates contained both *erm*A and *msr*A gene, 4% of isolates contained *erm*C and *msr*A and 2% of isolates contained *erm*A, *erm*C and *msr*A gene. Among MSSA (46), *erm*A gene was found in 22% of isolates, *erm*C in 9% and *msr*A alone in 4% of the isolates, while 9% of isolates harboured multiple resistance mechanisms, of which 3% contained *erm*A and *msr*A, 6% of isolates contained *erm*C and *msr*A determinants. Though *erm*A gene was most prevalent in our study , we found no difference in distribution of *erm* A gene among MRSA and MSSA (Figure.1). The multiplicity of resistance mechanisms which confers macrolide resistance is less frequently identified in *Staphylococcus aureus* with reference to the previous reports.[17, 18] In our study we found 20 % of MRSA

isolates exhibits multiple resistance mechanism carrying both *erm* and *msr*A determinants.

CONCLUSION

Staphylococcus aureus isolates from three different region used in this study showed (63%) of resistance to MLS antibiotics and inducible clindamycin resistance was higher in MRSA than MSSA. The most dominant resistance determinant was *erm*A gene among the MLS resistant isolates and also exhibits in association with different *erm* genes were detected in our study. About 20% of isolates were detected in combination of *erm* genes with other resistance gene *msr*A, which was less frequently reported. Although *erm*A gene was highest in the study, isolates showing multiple resistance mechanism was high in our study.

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CONFLICT OF INTEREST: None.

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