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Genotyping of erythromycin resistant group C & G streptococci isolated in Chennai, south India

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***Background & objectives*: Increasing resistance to erythromycin has been observed worldwide in group C and group G streptococci (GCS/GGS). The information available from India is scanty. The aim of the study was to identify erythromycin resistant GCS/GGS isolates in Chennai, south India, and to compare erythromycin resistant genotypes with *emm* types.**

***Methods*: One hundred and thirty one GCS/GGS isolates were tested for erythromycin resistance by disc diffusion and agar dilution methods. Erythromycin resistance genotypes [*erm*(A), *erm*(B) and *mef*(A)] were determined by a multiplex PCR. *emm* types of erythromycin resistant GCS/GGS isolates was also assessed using *emm* gene sequencing method.**

***Results*: Sixteen of the 131 isolates (12.21%) were resistant to erythromycin. Majority of the isolates were GGS (15/16). Eight of the 16 (50%) were *S. dysgalactiae* subsps. *equisimilis.* Twelve isolates (75%) were MLS_B phenotype and four (25%) were M phenotype. Of the 12 isolates which exhibited MLS_B resistance, seven showed $cMLS_B$ phenotype and were positive for *erm*(B) gene. The remaining five were $iMLS_B$ phenotype of which three were positive for *erm*(A) gene and two for *erm*(B) gene. *erm*(A) was common among carriers whereas *erm*(B) was common among clinical isolates.**

***Interpretation & conclusions*: MLS_B was the predominant phenotype and *erm*(B) was the common genotype in the present study. The *emm* type *stC1400.0* was frequently associated with erythromycin resistant GCS/GGS in our study.**

Key words GCS/GGS - erythromycin resistance - *erm*(A) - *erm*(B) - *mef*(A)

Group C and G streptococci (GCS/GGS) are the commensal flora of the skin, genitourinary and gastrointestinal tracts. However, recent reports suggest a higher incidence of GCS/GGS causing pharyngitis, skin and soft tissue infection[1]. GCS/GGS has also been reported to cause bacteremia, cellulitis, infective endocarditis, septic arthritis, toxic shock syndrome and acute glomerular nephritis in both normal and immune compromised hosts[2]. Penicillin is the drug of choice for prophylaxis and treatment of GCS/GGS infections. Macrolides are the recommended second-line drugs in patients who are allergic to penicillin. Increasing resistance of GCS/GGS to macrolides such as erythromycin has been observed during the last decade

in several countries[3-7]. The two major mechanisms by which GCS/GGS acquire erythromycin resistance include target site modification and active drug efflux. The former is mediated by a methylase which modifies the 50s ribosomal subunit, macrolides- lincosamide - streptogramin B (MLS_B) phenotype encoded by *erm* gene and latter is M phenotype encoded by the *mef*(A) gene[6]. While there are many reports on the incidence of erythromycin resistance among GCS/GGS worldwide, there are very few reports from India[8]. Hence the present study was undertaken to determine the erythromycin resistance among the GCS/GGS isolates in Chennai, South India. In addition, erythromycin resistance genotypes of GCS/GGS were compared with *emm* types.

Material & Methods

This study was conducted in the Department of Microbiology, Dr A.L.M. Post Graduate Institute of Basic Medical Sciences, Chennai, India.

Bacterial isolates: A total of 131 GCS/GGS isolates were included in the study. Of these, 94 were throat isolates from asymptomatic school children collected during the period August 2007 to February 2009. The remaining 37 were clinical isolates from blood (2), throat swabs (25), skin swabs (5), pus (4) and pleural fluid (1), which were maintained as stock cultures in our laboratory. The swabs were processed by crystal violet filter paper method[9]. Serogrouping was performed using Histrep latex agglutination kit (Hi-media, Mumbai, India) according to the manufacturer's instructions. Institutional human ethical committee had approved the collection of throat swabs from the asymptomatic school children. Biotyping was performed for all the 131 GCS/GGS isolates by standard methods[10].

Determination of erythromycin resistance phenotypes: GCS/GGS isolates were initially screened for erythromycin resistance by Kirby-Bauer double disk diffusion assay[11] using Muller Hinton agar (Hi-media, Mumbai) supplemented with 5 per cent sheep blood. Erythromycin (15 µg) and clindamycin (2 µg) discs (Hi-media, Mumbai) were kept at a distance of 16 mm apart. The different phenotypic patterns of macrolide resistance were interpreted as $iMLS_B$ (blunting of clindamycin zone of inhibition proximal to the erythromycin disc), $cMLS_B$ (resistance to both clindamycin and erythromycin) or M phenotype (susceptibile to clindamycin and resistant to erythromycin).

Minimum inhibitory concentration (MIC) of erythromycin for all the erythromycin resistant GCS/GGS isolates was determined by agar dilution method according to CLSI (Clinical and Laboratory Standards Institute) procedures[8] using Muller Hinton agar supplemented with 5 per cent sheep blood (MHBA) and erythromycin (Hi-media, Mumbai) at a concentration ranging from 0.03 to 128 mg/l. Isolates showing an MIC of >1 mg/l were considered to be resistant.

DNA extraction: DNA was extracted from fresh subcultures of GCS/GGS by alkali lysis method[12]. In brief, the isolates were subcultured on Todd Hewitt agar (Hi-media, Mumbai) and incubated at 37°C for 18-24 h in the presence of 5-10 per cent CO_2. A single colony from the plate was suspended in 100 µl of 50 mM sodium hydroxide (SRL, India) and kept in a water bath at 95°C for 1 min. After cooling to 4°C, 16 µl of 1M Tris-HCL (SRL, India) was added and centrifuged at 12000 rpm for two minutes and the supernatant was used as template DNA.

PCR amplification of erythromycin resistance genes: Erythromycin resistant genes *erm*(A), *erm*(B) and *mef*(A) were detected in the GCS/GGS isolates by multiplex polymerase chain reaction by the method described by Bingen *et al*[13], using a 50 µl master mix containing 5 µl of template, 1 µl of 0.4 µm primers, 1 µl 200 µm of dNTPs (Medox biotech, India), 5 units of *Taq* (Bangalore Genie, India) and 5 µl of 10x PCR reaction buffer (Bangalore Genie, India). The primers for the erythromycin resistant genes are given in Table I. The PCR conditions were as follows: an initial denaturation at 95°C for 1 min followed by 30 cycles of 95°C for 1 min, 55°C for 2 min, and a final extension at 72°C for 7 min. The PCR amplicons were resolved by electrophoresis using 1.2 per cent agarose gel. The

Table I. Primer sequences of erythromycin resistant genes

Gene	Forward primer	Reverse primer
mef (A)	5'-AGT ATC ATT AAT CAC TAG TGC-3'	5'-TTC TTC TGG TAC TAA AAG TGG-3'
erm (B)	5'-CGA GTG AAA AAG TAC TCA ACC-3'	5'-GGC GTG TTT CAT TGC TTG ATG-3'
erm (A)	5'-GCA TGA CAT AAA CCT TCA-3'	5'-AGG TTA TAA TGA AACAGA-3'

expected amplicon size of *erm*(A) gene was 206 bp, *erm*(B) gene was 616bp, *mef*(A) gene was 348 bp.

Emm genotyping: Genetic relationships among the erythromycin resistant GCS/GGS isolates were determined by *emm* gene sequencing (*http://www.cdc.gov/ncidod/biotech/strep/protocols.html*). Briefly, the *emm* gene encoding the M protein was amplified with the genomic DNA described above as template and using P1 (5'-TATT(C/G)GCTTAGAAAATTAA-3') and P2 (5'-GCAAGTTCTTCAGCTTGTTT-3') primers. The cycling parameters were 94º C for 15 sec, 46º C for 30 sec, and 72º C for 1 min 15 sec for the first 10 cycles, and then 94º C for 15 sec, 46º C for 30 sec, 72º C for 1 min 15 sec (with a 10 sec increment for each of the subsequent 19 cycles) for the subsequent 20 cycles. The PCR amplicons (800 bp to 1400 bp) were submitted for sequencing (Macrogen, Seoul), and sequencing was done using primer (5'-TATTCGCTTAGAAAATTAAAAACAGG- 3') according to the standard protocol followed in Centers for Disease Control and Prevention, Atlanta, USA (*http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm*). The first 150 bp of sequence of all the erythromycin resistant isolates was compared individually with the sequences in the CDC *emm* database (*www.cdc.gov/nciod/biotech/strep/strepindex.html*) to determine *emm* types and subtypes.

Results

Of the 131 isolates, 15 (11.4%) were GCS and 116 (88.5%) were GGS. Sixteen (12.2%) of the 131 GCS/GGS isolates were resistant to erythromycin by double disc test. Of these, 15 were GGS and one was GCS. Of the 16 resistant isolates, eight (50%) were *Streptococcus dysgalactiae* subsps. *equisimilis*, six (37.5%) were *S. dysgalactiae* subsps. *dysgalactiae* and two (12.5%) were *S. equi* subsps. *zooepidemicus* (Table II)

Of the 16 erythromycin resistant GCS/GGS isolates, five were from patients with tonsillopharyngitis, three from pyoderma, one from wound infection and seven were from carriers. One of the 16 isolates showed intermediate resistance. Both the MLS_B and M phenotypic patterns of erythromycin resistance were observed. Twelve out of 16 (75%) erythromycin resistant GCS/GGS isolates showed MLS_B resistance and four isolates (25%) were M phenotype. Of the 12 isolates which exhibited MLS_B resistance, seven were of $cMLS_B$ phenotype and five showed $iMLS_B$ phenotype. Among the 16 erythromycin resistant GCS/GGS isolates, nine (56.25%) were positive for *erm*(B) gene, three (18.75%) for *erm*(A) gene and four (25%) were positive for *mef*(A) gene (Table III). The seven $cMLS_B$ phenotype isolates were positive for *erm*(B) gene. Of the five $iMLS_B$ isolates, three were positive

Table II. Genotype and phenotype characteristics of the erythromycin resistant groups C and G streptococci

S. no.	Source	Serogroup	Biotype	*emm* type	Phenotype	Genotype
1	Pharyngitis	G	*S. dysgalactiae* subsps. *dysgalactiae*	*stG6792.3*	cMLS	erm B
2	Pharyngitis	G	*S. dysgalactiae* subsps. *dysgalactiae*	*stC1400.0*	cMLS	erm B
3	Pharyngitis	G	*S. dysgalactiae* subsps. *dysgalactiae*	*stG6792.3*	cMLS	erm B
4	Pharyngitis	G	*S. dysgalactiae* subsps. *equisimilis*	*stC1400.0*	cMLS	erm B
5	Pharyngitis	G	*S. dysgalactiae* subsps. *equisimilis*	*stC1400.0*	M	mefA
6	Pyoderma	G	*S. equi* subsps. *zooepidemicus*	*stG2574.0*	cMLS	erm B
7	Pyoderma	G	*S. dysgalactiae* subsps. *dysgalactiae*	*stC1400.0*	M	mefA
8	Pyoderma	G	*S. dysgalactiae* subsps. *equisimilis*	*stC2sk.0*	cMLS	erm B
9	Pus	G	S. dysgalactiae subsps. *dysgalactiae*	*stC1400.0*	cMLS	erm B
10	Carrier	G	*S. dysgalactiae* subsps. *equisimilis*	*stC5345.1*	iMLS	erm A
11	Carrier	G	*S. dysgalactiae* subsps. *equisimilis*	*stG10.0*	iMLS	erm A
12	Carrier	G	*S. dysgalactiae* subsps. *equisimilis*	*stGLp1.0*	iMLS	erm A
13	Carrier	G	*S. dysgalactiae* subsps. *equisimilis*	*stG2574.0*	M	mefA
14	Carrier	G	*S. equi* subsps. *zooepidemicus*	*stG866.0*	M	mefA
15	Carrier	C	*S. dysgalactiae* subsps. *equisimilis*	*stGLp1.0*	iMLS	erm A
16	Carrier	G	*S. dysgalactiae* subsps. *dysgalactiae*	*stG643.0*	iMLS	erm A

Table III. Erythromycin resistant phenotypes and genotypes in relation to MIC

Erythromycin resistance phenotype	Total no. isolates (N=16)	Minimum inhibitory concentration (mg/l)			Erythromycin resistance genotypes		
		MIC_{50}	MIC_{90}	Range	*erm*(A)	*erm*(B)	*mef*(A)
$cMLS_B$	7	>128	>128	4 - >128	-	7	-
$iMLS_B$	5	4	>128	0.5 - >128	3	2	-
M	4	8	>128	4 - >128	-	-	4

for *erm*(A) gene and two were positive for *erm*(B) gene. Four isolates with M phenotype were positive for *mef*(A) gene (Table IV). *erm*(B) was the most common genotype in clinical isolates whereas *mef*(A) was frequent among carriers (Table II).

A total of eight different *emm* types were identified among the erythromycin resistant GCS/GGS isolates, of which *stC1400.0* was most common and $cMLS_B$/ *erm*(B) and M/ *mef*(A) were the most common associations (Table IV). Other *emm* types identified in this study were *stG6792.3*, *stG2574.0, stGLp1.0*, *stG10*, *stG643.0*, *stG866.0*, *stC2k.0* and *stG5345.1*. Four different *emm* types were seen in clinical isolates and six different *emm* types were seen in carrier isolates. *stC1400.0* was commonly seen in clinical isolates and *stGLp1.0* in carrier isolates. *stG2574.0* was seen in both carrier and clinical isolates. Of the five *stC1400.0* isolates, three were $cMLS_B$ phenotype and expressed *erm*(B) gene. The remaining two isolates were M phenotype and expressed the *mef*(A) gene (Table IV).

Table IV. Association of *emm* types with erythromycin resistant phenotypes and genotypes

emm types	No. of isolates showing erythromycin resistance			
	$iMLS_B$/ *erm*(A)	$iMLS_B$/ *erm*(B)	$cMLS_B$/ *erm*(B)	M/ *mef*(A)
stG1400.0	-	-	3	2
stG6792.3	-	-	2	-
stG2574.0	-	-	1	1
stGLp1.0	1	1	-	-
stG10	-	1	-	-
stG643.0	1	-	-	-
stG866.0	-	-	-	1
stC2k.0	-	-	1	-
stG5345.1	1	-	-	-

Discussion

Macrolides are important in the treatment of streptococcal infections. *erm* genes which target site modification are predominant in group G streptococci from Europe[6] while erythromycin-resistant group G streptococci from Asia and the United States have shown a higher prevalence of *mef* genes[14]. The new *mef* sequence variant which has been identified in group G streptococci suggests that macrolide resistance continues to evolve[3].

Occurrence of erythromycin resistance among GCS/GGS in different countries shows a high degree of variability. In the present study erythromycin resistance was detected in 12.2 per cent of GCS/GGS which is high when compared to reports from other regions of the world (6.7% from Turkey[4], 3.5-3.6% from Finland[5], 10.7-12.5% from Spain[6], 3-8-6.2% from UK[2]). High prevalence of erythromycin resistance (21%) in GCS/GGS has been reported in Hong Kong[5]. Lateral gene transfer from group A Streptococcus is said to be the contributing factor for the emerging resistance among GCS/GGS[6]. In the present study, three distinct genotypes of erythromycin resistance *erm*(A), *erm(B)* and *mef*(A) were documented, suggesting a possible polyclonal origin of resistance in the same geographical area which is in agreement with previous studies reported from Hong Kong, Spain, Turkey and Finland[4-7]. Both $cMLS_B$ and M phenotype were seen in the clinical isolates included in our study. This is in contrary to the previous reports, where $iMLS_B$ and M phenotype were common in clinical isolates of GCS/GGS[4,6]. In agreement with previous studies, isolates with $iMLS_B$ phenotype frequently expressed the *erm*(A) gene[3]. The correlation between erythromycin resistance and *emm* types is well documented in *Streptococcus pyogenes*[15]. Previous studies have shown that the *emm* types *emm4*, *st1815*, *emm12*, and *emm75* are commonly

erythromycin resistant[15]. In our study it was found that the *emm* types were generally heterogeneous and only one genotype (*stC1400.0*) appeared to be more common among our isolates. The carrier isolates appeared to be more heterogeneous than the clinical isolates suggesting that these would have originated from diverse resistant clones.

In conclusion, it is clear that there are geographical differences in the mechanisms of erythromycin resistance. Macrolides are widely used in India yet regular surveillance of resistant genotypes of beta haemolytic streptococci, is not frequently done. Increase in erthromycin resistance among the GCS/GGS isolates is a matter of concern and it may be advisable to use erythromycin only after laboratory tests indicate susceptibility in order to check the multiplication and spread of resistant clones.

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