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Original Article

Molecular epidemiology of macrolide resistant Group A streptococci from Puducherry, India

Tintu Abraham, Sujatha Sistla

Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India

Abstract

Introduction: In penicillin allergic patients, macrolides are the most commonly used antibiotics for treating streptococcal infections, irrespective of the higher resistance rates. The objective of this study was to evaluate the comparative prevalence, phenotypes, and genetic determinants of macrolide resistance and associated *emm* types among different clinical isolates of *Streptococcus pyogenes*.

Methodology: A total of 173 *Streptococcus pyogenes* isolates were examined for macrolide resistance phenotype by double-disc test, resistance determinants by multiplex PCR and *emm* genotyping.

Results: Erythromycin resistance was found in 51.4% of isolates, with $MIC_{90} \geq 256$ µg/mL Inducible phenotype was commonly found (iMLS, 67.4%) followed by the M phenotype (32.5%). Among these isolates, 65.1% harboured *erm*B and 32.5% *mef*A as sole macrolide resistance gene, whereas presence of both, *erm*B plus *mef*A was observed in 2.2% cases. The most common types among resistant strains were *emm*63 (11.2%), *emm*44 (6.7%), *emm*42 (5.6%), and *emm*75.3, *emm*82, *emm*85, *emm*92, *emm*111.1 (4.4% each). Statistically significant association was observed between *emm*63, *emm*44 and erythromycin resistance ($p \leq 0.05$). Association of these *emm* types and macrolide resistance have not been reported earlier.

Conclusion: Higher macrolide resistance in this study can be attributed to overuse and misuse of this antibiotic. These findings indicate that macrolides should not be empirically used for treating severe streptococcal infections.

Key words: Macrolide resistance; *Streptococcus pyogenes;* phenotypes and genotypes; *emm* types

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Introduction

Streptococcus pyogenes (Group A streptococcus, GAS) is responsible for a wide array of human illnesses ranging from self-limiting pharyngitis and impetigo to flesh eating necrotizing fasciitis and other life threatening invasive infections [1]. If untreated, mild infections can lead to immune mediated post-infectious sequelae such as glomerulonephritis and acute rheumatic fever, which accounts for majority of streptococcal disease burden in the developing countries [2,3].

GAS remains universally susceptible to penicillin and this is the drug of choice for streptococcal infections. Among patients with penicillin allergy, macrolides, lincosamides and fluoroquinolones are recommended as the treatment options. Macrolides are ideal for treating streptococcal pharyngitis and other respiratory infections whereas clindamycin is preferred for treating patients with serious soft tissue infections. Although penicillin resistance has not been reported till date, resistance to other antibiotic groups has been reported worldwide [4,5].

Globally the rate of erythromycin resistance in streptococci is 20% - 40%, that is also in accordance with reports from India [4,6]. Macrolide resistance in streptococci is mediated by two major mechanisms, which include target site modification, and macrolide efflux pumps. Target site modification is affected by rRNA methylases encoded by the *erm*B gene or *erm*TR gene of the *erm*A class and is related to the MLS phenotype (resistance to macrolide, lincosamide and streptogramin B). The expression of these genes may be constitutive (cMLS) or inducible (iMLS). The second mechanism includes efflux pump encoded by the *mef*A gene and is related to the M phenotype (resistance to 14 and 15 membered ring macrolides). Other minor resistance mechanisms include mutations in the 23S rRNA gene and alterations in riboproteins L4 and L22 [7,8]. It was reported that macrolide resistance in GAS could be associated with certain *emm* types like *emm*4, *emm*12 and *emm*28 [9].

The present study was undertaken to evaluate the comparative prevalence, molecular epidemiology, phenotypes, and genetic determinants of macrolide resistant *Streptococcus pyogenes*.

Methodology

This observational study was conducted in the Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) in Puducherry, South India between November 2013 and December 2015. The study was approved by the JIPMER Scientific Advisory Committee (JSAC) and Institute Ethics Committee (IECNo. JIP/IEC/SC/2014/3/555).

Bacterial strains

All the consecutive, non-repetitive isolates of GAS from various clinical cases (tonsillitis, necrotizing fasciitis, pyoderma, folliculitis, wound infection, abscess and sepsis) were included in the study. Identification was done by routine laboratory techniques involving bacitracin sensitivity, PYR test, latex agglutination (STREP Test kit, Plasmatec, Dorset, UK) and confirmed by *spy*1258 PCR [10].

Antibiotic susceptibility and MIC determination

Isolates were tested for susceptibility to erythromycin and clindamycin by disc diffusion, minimum inhibitory concentration (MIC) was determined by use of the standard protocol [11], Erythromycin resistant isolates were checked by E-test (Biomerieux, La Balme Les Grottes, France).

Detection of macrolide resistance phenotypes

Macrolide resistance phenotype was detected based on their susceptibility by double disc tests involving erythromycin (15µg) and clindamycin (2µg) discs, as previously described [12].

DNA extraction

Genomic DNA was extracted using mericon DNA Bacteria plus kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Detection of macrolide resistance genes

All erythromycin resistant isolates were screened by a multiplex PCR for the presence of genes *erm*B, *erm*A and *mef*A as described previously [13], using 25µl reagent that contains 2×Taq PCR Smart Mix (Origin, Karunagappalli, Kerala, India), 10 pmol of each primers and 10 ng of sample DNA. The PCR cycling conditions were as follows: initial heating at 93°C for 3 minutes, followed by 30 cycles of denaturation at 93°C for 1 minute, annealing at 61.8°C for 1 minute, extension at 65°C for 1 minute and final extension for 3 minutes after the last cycle. The 16SrRNA gene specific for genus *Streptococci* was included as an internal control (Table 1).

Emm typing

Emm sequencing was performed according to the protocol of the Center of Disease Control (CDC) International Streptococcal Reference Laboratory with slight modifications (http://www.cdc.gov/ncidod/biotech/strep/protocols.htlm).

Statistical analysis

Analysis were carried out using OpenEpi software (Version3.03a). The prevalence of macrolide resistance was expressed in percentages. The Chi- Square test was used to calculate the association of *emm* types with macrolide resistance. p value of ≤ 0.05 was considered as statistically significant.

Results

A total of 173 *Streptococcus pyogenes* were recovered from various clinical samples like pus, wound swab, throat swab, blood, tissue during the study period. Out of 173, 130 (75.14%) were isolated from patients with non-invasive superficial and respiratory

Table 1. Primer sequences for macrolide resistance genes and internal control.

Primers	Amplicon size
ermA forward - 5' CCC GAA AAA TAC GCA AAA TTT CAT 3' *ermA* reverse – 5' CCC TGT TTA CCC ATT TAT AAA CG 3'	590 bp
*erm*B forward – 5' TGG TAT TCC AAA TGC GTA ATG 3' *erm* B reverse – 5' CTG TGG TAT GGC GGG TAA GT 3'	745 bp
mef A forward- 5' CAA TAT GGG CAG GGC AAG 3' *mef* A reverse - 5' AAG CTG TTC CAA TGC TAC GG 3'	317 bp
16S rRNA forward – 5' GAG TAC GAC CGC AAG GTT GA 3' 16S rRNA reverse - 5' CTG GTA AGG TTC TTC GCG TTG 3'	100 bp

infections, while 43 (24.86%) were isolated from patients with invasive infections like necrotizing fasciitis, sepsis, osteomyelitis, septic arthritis etc. Out of all, 89 (51.4%) were resistant to erythromycin. Majority of the isolates (60/173; 67.4%) presented with inducible phenotype (iMLS) followed by the M phenotype (29/173; 32.5%). There was a complete absence of cMLS phenotype. iMLS phenotype was predominant among non-invasive isolates in comparison with invasive isolates. These isolates showed high-level resistance to erythromycin ($MIC_{90} \geq$ 256 µg/mL), whereas those with the M phenotypes had lower erythromycin MICs (1-12 µg/mL) (Table 2).

M phenotype GAS isolates with (n = 29) macrolide resistance carried only *mef*A as sole macrolide resistance determinant. Fifty-eight out of sixty with iMLS phenotype carried only the *erm*B gene as the only resistant determinant whereas one throat and one skin isolate harbored *mef*A gene along with *erm*B gene. In the present work, the *erm*B gene was the most prevalent macrolide resistance determinant followed by *mef*A gene (Figure 1).

Thirty four *emm* types were observed in the erythromycin resistant population and the most prevalent were *emm*63 (11.2%), *emm*44 (6.7%), *emm*42 (5.6%), and *emm*75.3, *emm*82, *emm*85, *emm*92, *emm*111.1 (4.4% each). The majority of the resistant isolates were recovered from superficial infections. The *erm*B genotype was most commonly found in *emm*44 (6/6), *emm*42 (5/5), *emm*111.1 (3/4), *emm*82.1 (4/4), *emm*92 (4/4) and *emm*85 (4/4). The *mef*A genotype was most common in *emm*63 (9/10), *emm*75.3 (4/4) and *emm*81.11 (2/2). Statistically significant association was observed between *emm* 63, *emm* 44 and erythromycin resistance ($p \leq 0.05$).

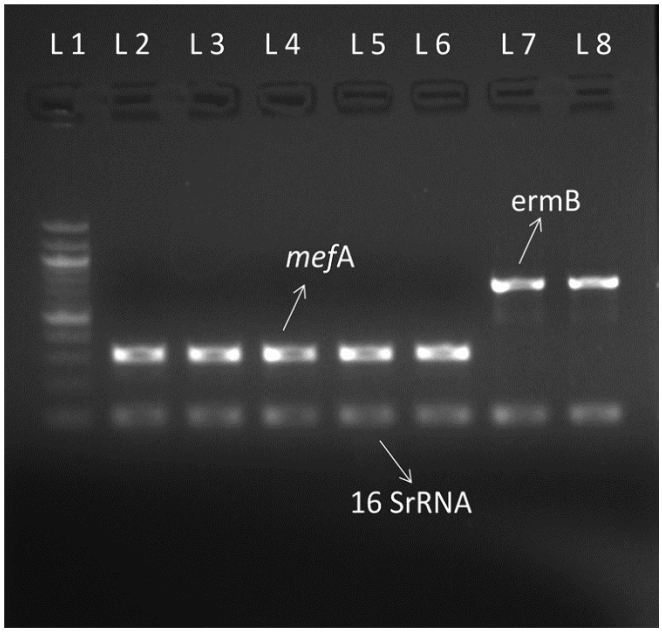
Discussion

GAS erythromycin resistance was first reported in 1955 and since then the resistance has been progressively increasing worldwide. The rates vary between 5% and 40%, with the highest prevalence in Asia and lowest prevalence in Europe and USA. This is a major concern, as macrolides are the main treatment option for streptococcal infections in penicillin allergic patients for treating streptococcal infections. Higher resistance could be attributed to the over prescription and misuse of these antibiotics.

Recent reports show that there is a fluctuating trend in the macrolide resistance pattern across the globe with unexpected reductions in the resistance rates especially from Spain, Portugal and China, where the resistance used to be high [14,15]. Lower macrolide resistance (10-15%) continued to be reported from countries like Finland, Greece, Italy, Germany, Mexico, USA and Canada [8].

Previous studies from India reported that there is a steady increase of GAS erythromycin resistance ranging from 2% to 38.13%. In the present study we report an overall erythromycin resistance of 51.4% which is very high comparing to reports from other parts of India [16,17]. In this study we observed that the erythromycin resistance was 48.8% among invasive

Figure 1. Results of multiplex PCR for erythromycin resistance genes.



Lane 1- 100bp ladder, lanes 2-8: 100bp product positive for PCR internal control (16SrRNA), lanes 2- 6: 317bp product positive for *mef*A gene and lanes7,8: 745bp product positive for *erm*B gene.

Table 2. Distribution of phenotypes, genotypes and MIC values among macrolide-resistant *S. pyogenes* isolates.

Phenotype	GENES	No. of positives (n = 89)	MIC value (µg/mL)
	erm B	58	
	erm B + *mef* A	2	
M 29 (32.6%)	*mef* A	29	1-12
cMLS	*erm* A	0	

strains (n = 21/43) and 52.3% among non-invasive strains (n = 68/130).

Earlier studies from India have reported the prevalence of M type [18], but given our data the iMLS phenotype was predominant (67.4%), followed by M phenotype (32.5%) which is in agreement with a recent study from Chennai [16]. Isolates with the iMLS phenotype showed high-level resistance to erythromycin ($MIC_{90} \geq 256$ μg/mL) and inducible resistance to clindamycin, whereas those with M phenotypes showed lower erythromycin resistance values and good susceptibility to clindamycin. Another notable feature is that M phenotype was predominant in countries with lower resistance. Clindamycin is recommended in treating severe skin and soft tissue infections due to the higher tissue permeability, inhibition of toxin production, and promotion of phagocytic activity. The higher rate of inducible clindamycin resistance (67.4%) in our study is a serious issue since it can result in unexpected treatment failures. The cMLS phenotype is commonly reported from European countries [19]. The complete absence of cMLS phenotype among our study population is in contrast with previous reports, which document the presence of cMLS as one of the minor phenotype in India [18]. The genetic determinants of macrolide resistance in Indian GAS population have not been extensively studied. The only Indian study discussing the genetic basis of macrolide resistance showed the predominance of *erm*B gene [16]. Our findings reveal a predominance of *erm*B gene (65.1%) followed by *mef*A gene (32.5%). A similar result was also previously reported from Belgium, France and Italy [20, 21]. Results of the present study also show that the *mef*A gene is always associated with the M phenotype and is consistent with other reports [22] from the literature. In addition, our data demonstrated that the iMLS phenotype was associated with *erm*B gene which is in contrast to other studies [7] where iMLS was reported to be associated with *erm*A gene.

Among erythromycin resistant isolates in the present study, the most common emm types were *emm*63 (11.2%), *emm*44 (6.7%), *emm*42 (5.6%), and *emm*75.3, *emm*82, *emm*85, *emm*92, *emm*111.1(4.4% each), which was entirely different from those reported from other geographical areas. In the only other published report from India where such association was studied, detected that *emm*49 and *emm*56 were associated with macrolide resistance [23]. The *emm*28 and *emm*4 types were reported to be associated with macrolide resistance in Europe, Spain, Finland and Quebec whereas *emm*12 was found to be the main resistant *emm* type in Germany, Greece, Italy, Portugal and Israel [13]. In the United States, *emm*75 and *emm*12 accounted for majority of erythromycin resistant *S. pyogenes* [24]. The lack of association between any particular *emm* type and macrolide resistance may attest to the fact that these two genetic properties are totally unrelated. This could also be due to the relatively large number of *emm* types (n = 34) encountered in the present study while in the Western studies published in the literature, such associations have been more regularly found. The only importance for such a perceived association would be the possible success of a particular clone in dissemination and/or causation of a particularly severe type of infection, as, an added feature of antibiotic resistance would further complicate the situation. The observation that macrolide resistance in *S. pyogenes* is associated with particular *emm* types could be just a suspicion without any clinical or epidemiological conformational relevance.

Conclusions

In the present study, the rate of macrolide resistance is higher compared to reports from other regions of the world, with iMLS as the predominant phenotype and *erm*B as the main genetic determinant. This may be due to the over the counter availability and misuse of these antibiotics in this area without regular surveillance of resistant GAS genotypes and phenotypes. Findings also indicate that macrolides should not be empirically used for treatment of severe streptococcal infections. In order to control the spread of resistant clones, it is advisable to use erythromycin only after conformational laboratory tests that indicate susceptibility. The prevalent *emm* types associated with macrolide resistance in this geographical area is entirely different from those of Western countries; the reason requires further investigations.

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Corresponding author

Sujatha Sistla, MD
Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER)
Gorimedu, 605006, Puducherry, India
Phone: +91 9894058062
Fax: +91 413 2296251
Email: sujathasistla@gmail.com

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