

Prevalence of erythromycin resistance genes among clinical isolates of viridians group *Streptococci*.

Li-qiang Zhu¹, Qin-hua Li², Gong-chang Li¹, Wu-jia Wu¹, Ning Pei¹, Yan-xia He^{1*}

¹Department of Laboratory Medicine, the Second Affiliated Hospital of Zhengzhou University, 453000, PR China

²College of Life Sciences, Zhengzhou University, Zhengzhou 450001, PR China

Abstract

Viridans Group *Streptococci* (VGS) are part of normal oral flora, causing serious infections including infective endocarditis, septicemia, and meningitis are often associated with infections in neutropenic and immunocompromised patients. Recent studies have reported that there is a concomitant increase in resistance towards erythromycin among VGS. Two hundred and twelve VGS isolates were subjected for the determination of erythromycin resistance by MIC. Isolates were tested for macrolide resistance phenotype by double disk diffusion method. Erythromycin resistance genes (*mefA*, *ermB*, *ermTR*) were detected by PCR. Fifty out of 212 isolates were found to be resistant to erythromycin by MIC respectively. Fifty out of 212 isolates were found to be resistant by double disk diffusion test, of which 44 (20.75%) isolates showed M-phenotype and amplified for *mefA* gene, 12 (2.83%) isolates showed cMLS_B phenotype and amplified for *ermB* gene. All the isolates which showed M-phenotype amplified *mefA* gene and those showed cMLS_B phenotype amplified *ermB* gene. *MefA* was the most common gene amplified.

Keywords: Viridans group *streptococci*, Macrolide resistance genes, PCR.

Accepted on August 04, 2016

Introduction

Viridans Group *Streptococci* (VGS) are part of normal oral flora, causing serious infections including infective endocarditis, septicemia, and meningitis are often associated with infections in neutropenic and immunocompromised patients [1,2]. By and large, VGS were thought to be frequently susceptible to β -lactams and macrolide however, attending increments in resistance towards these antibiotics have been accounted for among pathogenic and commensal *streptococci* [3,4]. Resistance of VGS isolated from blood towards these antibiotics is a major cause of concern and could compromise current prophylactic and therapeutic regimens [2,5]. The three major recognized mechanism of macrolides resistance are (i) target site modification which confers resistant to macrolides, lincosamides and streptogramin B components (MLS phenotype) encoded by *erm* gene, (ii) active-drug efflux pump (M phenotype) encoded by *mef* genes and (iii) ribosomal mutation in the key antibiotic binding site [6].

The conjugative transposon of Tn916-Tn1545 carries several resistant determinants and contributes to the horizontal dissemination of multidrug resistance [7]. It has been suggested that macrolide resistance genes, *erm* and *mef* of commensal VGS can be laterally transferred through transposons to pathogenic *Streptococcus pyogenes* [8]. The difference in the resistant pattern of various antibiotics drives

the necessity to increase our knowledge on antibiotics used for VGS. Thus, the present study was aimed to determine the distribution of erythromycin (*mefA*, *ermB*, *ermTR*) resistance genes among VGS isolated from blood samples of endocarditis and plaque samples of periodontitis population.

Materials and Methods

Bacterial strains

A total of 212 VGS (88-*S. mitis*, 70-*S. salivarius*, 30-*S. oralis*, 8-*S. sanguinus*, 6- *S. anginosus*, 6-*S. parasanguinis*, 4-*S. mutans*) isolated from infective endocarditis patient's blood samples (104) and plaque samples of periodontitis patients (108) were included in this study. The isolates were obtained from The Department of Laboratory, The Second Affiliated Hospital of Zhengzhou University, Henan, China. The isolates from IE patient's blood sample were designated and described as "blood group" and the isolates from the non-IE population were designated as "oral group" throughout this study.

Susceptibility testing

All the 212 VGS isolates were screened for antibiotic resistance to erythromycin (HiMedia) by Kirby Bauer disc diffusion test on Mueller-Hinton agar supplemented with 5% sheep blood (MHBA). The MIC of erythromycin, (HiMedia)

5'-GGC GTG TTT CAT TGC TTG ATG-3'

for all the isolates which showed resistance by disk diffusion method were confirmed by agar dilution method as per Clinical and Laboratory Standards Institute (CLSI) guidelines [9]. The American Type Culture Collection strains of *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 25293) were used as control strains.

Table 1. Primer sequences for erythromycin resistance genes (Malhotra-Kumar et al. [10]).

Gene	Primer sequences	Amplicon size (bp)
<i>ermTR</i>	5'-GCA TGA CAT AAA CCT TCA-3'	206
	5'-AGG TTA TAA TGA AAC AGA-3'	
<i>mefA</i>	5'-AGT ATC ATT AAT CAC TAG TGC-3'	348
	5'-TTC TTC TGG TAC TAA AAG TGG-3'	
<i>ermB</i>	5'-CGA GTG AAA AAG TAC TCA ACC-3'	616

Table 2. Erythromycin resistant phenotype and genotype among blood and oral isolates of VGS.

Phenotypes	Blood group						Oral group					
	No resistant isolates	of MIC50/90 (µg/mL)	Range (µg/mL)	No positive for			No resistant isolates* of MIC50/90 (µg/mL)	Range (µg/mL)	No positive for			
				<i>mefA</i>	<i>ermB</i>	<i>ermTR</i>			<i>mefA</i>	<i>ermB</i>	<i>ermTR</i>	
M Phenotype	8	4/8	4 - 8	8	-	-	36	4/8	1 - 8	36	-	-
cMLS _B	-	-	-	-	-	-	6	>128/ >128	64 - >128	-	6	-
iMLS _B	-	-	-	-	-	-	-	-	-	-	-	-
Total	8	4/8	-	8	-	-	42	4/64	-	36	6	-

* (CHI-Square analysis: significantly higher no of isolates ($\chi^2=14.305$, DF=1, p=0.00) in blood were found to be resistant to erythromycin)

DNA extraction

DNA extraction was done by alkali lysis method. Briefly, a single colony of VGS was suspended in 100 µL of 50 mM sodium hydroxide. The suspension was incubated at 95°C for 1 min, cooled to 4°C, and then neutralized with 16 µL of 1 M Tris-HCl (pH 8.0). After centrifugation for 2 min at 14,000 rpm, supernatant was collected and stored at -20°C until further use.

Detection of erythromycin resistance genes

Detection of erythromycin resistance genes was performed by multiplex PCR [11]. PCR was done using a 50 µl master mix containing 5 µl of template DNA, 0.4 µM of each primer as described in Table 1, 200 µM of dNTPs, 1 unit of Taq polymerase enzyme and 5 µl of 10X reaction buffer. The following PCR cycling conditions were used: an initial denaturation at 95°C for 2 min followed by 30 cycles at 95°C for 1 min, 55°C for 2 min and 72°C for 10 min. After PCR, the amplicons were resolved in 1.2% agarose gel.

Determination of macrolides resistance phenotypes

All the 212 VGS isolates were screened for macrolides resistant phenotype by double disk diffusion method. Briefly, erythromycin (15 µg) and clindamycin (2 µg) disks were placed 16 mm apart on MHBA plates inoculated with the test organism. The plates were incubated at 37°C in 5% CO₂ atmosphere for 18-24 hrs and observed for three different patterns of resistant phenotypes [10]. After incubation, blunting of clindamycin zone of inhibition proximal to the erythromycin disc was interpreted as inducible type of macrolide-lincosamide-streptogramin B resistance (iMLS_B) and resistance to both clindamycin and erythromycin was interpreted as constitutive type of macrolide-lincosamide-streptogramin B resistance (cMLS_B). Susceptibility to clindamycin and resistance to erythromycin disc without blunting of the zone was indicative of M phenotype.

Statistics

A Chi-Square test for the independent of attributes between the two groups of isolates was performed using MINITAB (MINITAB, Version 13) statistical software.

Results

Antibiotic susceptibility test by disc diffusion method

Among the 212 isolates tested (104-blood group, 108-oral group) for antibiotic susceptibility by disc diffusion method, 50 (23.58%) isolates (8- blood group, 42- oral group) were resistant to erythromycin.

Determination of erythromycin resistance by MIC

All the 50 VGS isolates tested for erythromycin resistance by MIC based on disk diffusion test were found to be resistant to erythromycin. The MIC₅₀ of both blood and oral group of isolates which showed M-phenotype was 4 µg/ml and the MIC range was found to be 4-8 µg/ml and 1-8 µg/ml, respectively.

The MIC₅₀ and MIC₉₀ of the 6 isolates in oral group which showed cMLS_B phenotype was >128 µg/ml and the range was between 64- >128 µg/ml (Table 2).

Determination of macrolide resistant phenotypes

Among the 212 VGS isolates tested (104-blood group, 108-oral group) for macrolide resistant phenotype by double disc diffusion test using clindamycin and erythromycin, 44 (20.75%) isolates showed M-phenotype, 6 (2.83%) isolates showed cMLS_B phenotype and the remaining 162 (76.42%) isolates were susceptible to both the antibiotics. All the 8 isolates in blood group and 36 out of 42 (85.71%) isolates in oral group showed M-phenotype. The remaining 6 (14.29%) isolates in oral group showed cMLS_B phenotype. Chi-Square test showed that significantly higher number of isolates in oral group were resistant to erythromycin ($\chi^2=14.305$, DF=1, p=0.00) (Table 2).

Detection of erythromycin resistance genes

Among the 50 macrolide resistant isolates (8-blood group, 42-oral group), all the 44 (88%) isolates (8-blood group, 36-oral group) which showed M-phenotype possesses *mefA* gene and the remaining 6 (12%) isolates in oral group, which showed cMLS_B phenotype possesses *ermB* gene. Among the various species of VGS, *mefA* was positive in *S. mitis* (20), *S. salivarius* (12), *S. sanguinus* (4), *S. parasanguinis* (4), *S. oralis* (2) and *S. anginosus* (2), whereas, *ermB* was present only in *S. mitis* (4) and *S. salivarius* (2). None of the species were positive for *ermTR* gene (Table 3).

Table 3. Erythromycin resistance genes in various species of VGS.

Species	No positive for erythromycin resistance genes			
	No of resistant Isolates	<i>mefA</i>	<i>ermB</i>	<i>ermTR</i>
<i>S.mitis</i> (n=90)	24	20	4	-
<i>S.oralis</i> (n=30)	2	2	-	-
<i>S.salivarius</i> (n=70)	14	12	2	-
<i>S.sanguinus</i> (n=8)	4	4	-	-
<i>S.parasanguinis</i> (n=6)	4	4	-	-
<i>S.anginosus</i> (n=6)	2	2	-	-
Total	50	44	6	-

Discussion

The emergence and increase in the frequency of antimicrobial resistance is of great concern as it limits the available options during the treatment of serious infections. It has been suggested that resistance rates can differ due to clinical sample, study population and country but the common point is that erythromycin resistance is increasing through the years [12]. The overall erythromycin resistance among our isolates was 23.6% which is similar to that reported from Greece, Poland and Finland [13-15]. In contrast, a higher percentage of

macrolide resistance which ranged from 25.5%-39.7% was reported from Germany, Sweden, France, Spain and Turkey [12,15-18]. Yap et al. [19] has reported that 61% of their blood isolates were resistant to erythromycin; in contrast, only 7.7% of our blood stream endocarditis isolates were resistant to erythromycin. However, 28.9% of our oral isolates were resistant to erythromycin which is less than that reported by others. Aracil et al. [20] reported that 70.6% of VGS isolated from oral cavity were resistant to erythromycin, while Zolezzi et al. [21] reported that 94% of their VGS isolates from oral, bronchial, sputum and nasal sample were resistant to erythromycin. Tazumi et al. [22] have reported that 74.7% of VGS isolated from cystic fibrosis patients were resistant to erythromycin. Among the two groups of isolates tested, we found that significantly higher number of isolates in the oral group (p=0.00) were resistant to erythromycin.

In our study, 88% of erythromycin resistant isolates exhibited M-phenotype which was higher than that reported from Finland, Greece, Spain and Turkey [12-14,21]. Moreover, 12% of our erythromycin resistant isolates showed cMLS_B phenotype which is lower than that reported from Belgium [6] and none of our isolates showed iMLS_B phenotype. The isolates, which showed M-phenotype were amplified for *mefA* gene and those which showed cMLS_B were amplified for *ermB* gene which is similar to that reported earlier [6,21]. As reported by several authors [18,22] *mefA* gene was predominantly (88%) present among our isolates. Zolezzi et al. [21] have also reported that 59.6% of VGS isolates had *mefA* gene. We report that 12% of our resistant isolates were amplified for *ermB* gene which is comparable to that reported by Kouidhi et al. [4] and lower than that reported earlier [12,22]. Hence this study shows that M-phenotype is the predominant phenotype and *mefA* is the predominant gene present among VGS. It has been spotlighted in several studies that the use of various antibiotics may lead to antibiotic resistance among oral streptococci [23]. We also recorded higher percentage of erythromycin resistance in VGS isolated from the oral cavity, suggesting that these strains accomplish a pool of erythromycin resistance within the oral cavity. Since, the resistance rates vary due to geographical limits and source of the samples, continual monitoring of antimicrobial resistance is needed for effective therapeutic strategy.

References

1. Bruckner L, Gigliotti F. Viridans group streptococcal infections among children with cancer and the importance of emerging antibiotic resistance. Semin Pediatr Infect Dis 2006; 17: 153-160.
2. Balletto E, Mikulska M. Bacterial infections in hematopoietic stem cell transplant recipients. Mediterr J Hematol Infect Dis 2015; 7: e2015045.
3. Rodriguez-Avial I, Rodriguez-Avial C, Culebras E, Picazo JJ. Distribution of tetracycline resistance genes tet(M), tet(O), tet(L) and tet(K) in blood isolates of viridans group streptococci harbouring erm(B) and mef(A) genes.

- Susceptibility to quinupristin/dalfopristin and linezolid. *Int J Antimicrob Agents* 2003; 21: 536-541.
4. Kouidhi B, Zmantar T, Hentati H, Najjari F, Mahdouni K, Bakhrouf A. Molecular investigation of macrolide and Tetracycline resistances in oral bacteria isolated from Tunisian children. *Arch Oral Biol* 2011; 56: 127-135.
 5. Nandhakumar B, Senthilkumar S, Menon T, Shanmugasundaram S. Penicillin-resistant viridans group streptococci from blood cultures of infective endocarditis patients in South India. *Int J Antimicrob Agents* 2008; 32: 543-544.
 6. Malhotra-Kumar S, Lammens C, Martel A, Mallentjer C, Chapelle S, Verhoeven J, Wijdooghe M, Haesebrouck F, Goossens H. Oropharyngeal carriage of macrolide-resistant viridans group streptococci: a prevalence study among healthy adults in Belgium. *J Antimicrob Chemother* 2004; 53: 271-276.
 7. Ljiljana P, Edita S, Sandra Z, Jadranka Z, Jelena A, Pavlovic N. Determination of presence of resistance to erythromycin and tetracycline antibiotics in isolated *Streptococcus* species from group C and group G. *Acta Veterinaria (Beograd)* 2010; 60: 487-495.
 8. Jönsson M, Swedberg G. Macrolide resistance can be transferred by conjugation from viridans streptococci to *Streptococcus pyogenes*. *Int J Antimicrob Agents* 2006; 28: 101-103.
 9. Clinical and Laboratory Standards Institute. Performance Standards for antimicrobial susceptibility testing. 2013; M100-S23, Wayne PA.
 10. Seppala H, Nissinen A, Yu Q, Huovinen P. 1993. Three different phenotypes of erythromycin-resistant *Streptococcus pyogenes* in Finland. *J Antimicrob Chemother* 1993; 32:885-891.
 11. Malhotra-Kumar S, Lammens C, Piessens J, Goossens H. Multiplex PCR for simultaneous detection of macrolide and tetracycline resistance determinants in streptococci. *Antimicrob Agents Chemother* 2005; 49: 4798-4800.
 12. Ergin A, Eser ÖK, Hasçelik G. Erythromycin and penicillin resistance mechanisms among viridans group streptococci isolated from blood cultures of adult patients with underlying diseases. *New Microbiol* 2011; 34: 187-193.
 13. Ioannidou S, Tassios PT, Kotsovoli-Tseleni A, Foustoukou M, Legakis NJ, Vatopoulos A. Antibiotic resistance rates and macrolide resistance phenotypes of viridans group streptococci from the oropharynx of healthy Greek children. *Int J Antimicrob Agents* 2001; 17: 195-201.
 14. Seppälä H, Haanperä M, Al-Juhaish M, Järvinen H, Jalava J, Huovinen P. Antimicrobial susceptibility patterns and macrolide resistance genes of viridans group streptococci from normal flora. *J Antimicrob Chemother* 2003; 52: 636-644.
 15. Rozkiewicz D, Daniluk T, Sciepek M, Zaremba ML, Cylwik-Rokicka D, Luczaj-Cepowicz E, Milewska R, Marczuk-Kolada G, Stokowska W. Prevalence rate and antibiotic susceptibility of oral viridans group streptococci (VGS) in healthy children population. *Adv Med Sci* 2006; 51: 191-195.
 16. Wisplinghoff H, Reinert RR, Cornely O, Seifert H. Molecular relationships and antimicrobial susceptibilities of viridans group streptococci isolated from blood of neutropenic cancer patients. *J Clin Microbiol* 1999; 37: 1876-1880.
 17. Ardanuy C, Tubau F, Liñares J, Dominguez MA, Pallares R, Martín R. Spanish Pneumococcal Infection Study Network (G03/103). Distribution of subclasses *mefA* and *mefE* of the *mefA* gene among clinical isolates of macrolide-resistant (M-phenotype) *Streptococcus pneumoniae*, viridans group streptococci, and *Streptococcus pyogenes*. *Antimicrob Agents Chemother* 2005; 49: 827-829.
 18. Westling K, Julander I, Ljungman P, Jalal S, Nord CE, Wretling B. Viridans group streptococci in blood culture isolates in a Swedish university hospital: antibiotic susceptibility and identification of erythromycin resistance genes. *Int J Antimicrob Agents* 2006; 28: 292-296.
 19. Yap RL, Mermel LA, Maglio J. Antimicrobial resistance of community-acquired bloodstream isolates of viridans group streptococci. *Infection* 2006; 34: 339-341.
 20. Aracil B, Miñambres M, Oteo J, Torres C, Gómez-Garcés JL, Alós JI. High prevalence of erythromycin-resistant and clindamycin-susceptible (M phenotype) viridans group streptococci from pharyngeal samples: a reservoir of *mef* genes in commensal bacteria. *J Antimicrob Chemother* 2001; 48: 592-594.
 21. Zolezzi CP, Rubio Calvo MC, Millán L, Goñi P, Canales M, Capilla S, Durán E, Gómez-Lus R. Macrolide resistance phenotypes of commensal viridans group streptococci and *Gemella* spp. and PCR detection of resistance genes. *Int J Antimicrob Agents* 2004; 23: 582-589.
 22. Tazumi A, Maeda Y, Goldsmith CE, Coulter WA, Mason C, Millar BC, McCalmont M, Rendall J, Elborn JS, Matsuda M, Moore JE. Molecular characterization of macrolide resistance determinants [*erm(B)* and *mef(A)*] in *Streptococcus pneumoniae* and viridans group streptococci (VGS) isolated from adult patients with cystic fibrosis (CF). *J Antimicrob Chemother* 2009; 64: 501-506.
 23. Bryskier A. Viridans group streptococci: a reservoir of resistant bacteria in oral cavities. *Clin Microbiol Infect* 2002; 8: 65-69.

*Correspondence to

Yan-xia He

Department of Laboratory Medicine

The Second Affiliated Hospital of Zhengzhou University

Henan-453000

P.R China