Erythromycin and penicillin resistance mechanisms among viridans group streptococci isolated from blood cultures of adult patients with underlying diseases

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SUMMARY

The aim of the study was to evaluate the species distribution, antimicrobial susceptibility and erythromycin-penicillin resistance mechanisms of viridans streptococci (VGS) isolates from blood cultures of adult patients with underlying diseases. Fifty VGS blood culture isolates were screened for their antibiotic susceptibilities against penicillin G, erythromycin and tetracycline by E-test. Clindamycin, cefotaxime, chloramphenicol, levofloxacin, linezolid and vancomycin susceptibility were performed by broth microdilution method. Erythromycin and penicillin resistance genotypes, *erm*B and *mef*A/E, *pbp*1a, *pbp*2b and *pbp*2x are amplified using PCR method. The clinical isolates included *Streptococcus mitis* (n. 19), *S.oralis* (n. 13), *S.sanguinis*, *S.parasanguinis* (n. 6, each), *S.salivarius*, *S.vestibularis* (n. 2, each), *S.constellatus*, *S.sobrinus* (n. 1, each). The percentage resistance against erythromycin and penicillin was 36% and 30%, respectively. The genotypic carriage rate of erythromycin resistance genes were: 56% *erm*B, 28% *mef*E, 8% *erm*B+*mef*E. Penicillin-resistant isolates carried *pbp*2b (33.3%) and *pbp*2x (20%) genes. Twenty-four VGS isolates were recovered from patients with cancer. *S.mitis* and *S.oralis* predominated among patients with cancer who had erythromycin and penicillin resistance isolates. The importance of classical antimicrobial agents like penicillin and erythromycin warrants the continuous surveillance of invasive VGS isolates and can guide better treatment options especially in patients with underlying diseases.

KEY WORDS: Viridans streptococci, Blood culture, *S.mitis*, *S.oralis*

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INTRODUCTION

Viridans group streptococci (VGS) are part of the oral flora and can cause severe infections such as infective endocarditis, septicaemia and neutropenia in immunocompetent and immunocompromised patients (Bruckner *et al.,* 2006). During the past decade, VGS have accounted for 25 to 30 percent of bacteremic episodes among patients with malignancies and are the most common cause of early bloodstream infections among hematopoetic stem cell transplantation recipients (Bruckner *et al.,* 2006).

Resistance to beta-lactams, macrolides and other antibiotics among blood cultures of VGS is a major concern and could compromise currently available prophylactic and therapeutic regimens (Nandhakumar *et al.,* 2008). In streptococci, there are three well-characterized macrolide resistance mechanisms. The first mechanism is the target site modification which is mediated by methylases encoded by the *erm* (erythromycin ribosome methylation) and this methylation also causes resistance to lincosamides and streptogramin B antibiotics. The second mechanism is the active efflux mechanism, encoded by the *mef* genes (macrolide efflux) causes resistance only to 14-

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and 15-membered ring macrolides and the third mechanism is the ribosomal mutations in the key antibiotic binding site (Malhotra-Kumar *et al.,* 2004). Penicillin and beta-lactam resistance among streptococci is mediated by point mutations in penicillin binding proteins (PBPs) (Chi *et al.,* 2007) In VGS there are two kinds of PBPs: PBP1 (PBP 1a, 1b) and PBP2 (PBP 2a, 2b, 2x), naming of PBPs are adapted from *Streptococcus pneumoniae* (Nakayama *et al.,* 2003). They contain some conserved amino-acid motifs (also called homology boxes) and alterations in these motifs have been reported to reduce the affinity of beta-lactams for the target enzymes and to play an important role in the development of drug resistance (Nakayama *et al.,* 2003).

The aims of the study were to evaluate the species distribution, antimicrobial susceptibility and erythromycin-penicillin resistance mechanisms of VGS isolates obtained from blood cultures of adult patients with underlying diseases in Hacettepe University Hospital, Ankara in the period between January 2005 to September 2009.

MATERIALS AND METHODS

Bacterial strains and susceptibility testing

A total of randomly selected 50 VGS blood culture isolates identified by colony morphology, Gram stain, catalase and BD Phoenix (Becton and Dickinson Diagnostic Systems, Sparks, MD) were screened for their antibiotic susceptibilities. Susceptibility to penicillin G, erythromycin and tetracycline was determined by E-test on Mueller-Hinton agar plates supplemented with 5% sheep blood. Clindamycin, cefotaxime, chloramphenicol, levofloxacin, linezolid and vancomycin susceptibility were determined by broth microdilution test according to CLSI (CLSI, 2009). One isolate per patient was used. *S. pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 were used as quality controls for all antimicrobials tested. Phenotypes of erythromycin-resistant isolates were determined using a double disc test with erythromycin (15µg) and clindamycin (2µg) discs on Mueller-Hinton agar plates containing 5% sheep blood (CLSI, 2009).

DNA isolation

A single colony of the isolate was inoculated into 2 ml of brain-heart infusion broth and inoculated for 18 h at 37°C. After harvesting by centrifugation at 12000x*g* for 5 min, the bacterial pellet was resuspended in TE solution (10 mM Tris-HCl, pH 8, 1 mM EDTA). The cells were washed and centrifuged 3 times with 750 ml TE. The bacterial pellet was boiled for 20 min in 500 ml TE, centrifuged and the supernatant containing the DNA was stored at -20°C until use.

Detection of macrolide and penicillin resistance mechanisms

The DNA from the erythromycin-resistant isolates was amplified with primers specific for the *erm*B and *mef*A/E genes. Primers used for the amplification were as follows: ermB:5'-GAAAAGGTACTCAACCAAATA-3'(ErmB-1) and 5'AGTAACGGTACTTAAATTGTTT AC-3' (ErmB-2) and for mefA/E:5'-AGTATCATTAATCACTAGTGC-3' (MefA-1) and 5'-TTCT TCTGGTACTAAAAGTGG-3'(MefA-2) (Sutcliffe *et al.,* 1996). The PCR mixture (30 µl) contained bacterial DNA, primers (0.3 mM), PCR buffer 50 mM, 200 mM (each) deoxynucleoside triphosphates and 1.25 U Taq polymerase (New England Biolabs, USA). The mixtures were amplified in 40 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min in thermal cycler. The presence of products at 640, 348 bps indicated that the isolate carries *erm*B or *mef*A/E genes, respectively. Amplified products of *mefA/E* genes were digested with *Bam*HI (New England Biolabs, USA) restriction enzyme for the discrimination of *mef* (A) and *mef* (E) (Montanari *et al.,* 2003). PBPla gene was amplified using the following oligonucleotide primers: 5'-CGGCATTCGATTTGATT-3' and 5'-GATGTCTTCTCAGGCTTTTG-3. The PBP2x gene was amplified using the following oligonucleotide primers: 5'-CGTGGGACTATTTATGACCGAAATGG-3' and 5'-AATTCCAGCACTGATGGAAATAAACATATTA-3'. The PBP2b gene was amplified with the following oligonucleotide primers:5'-GATCCTCTAAATGATTCTCAGGTGG-3' and 5'-CAATTAGCTTAGCAATAGGTGTTGG-3' (Gillespie *et al.,* 1997). The PCR mixture (30 µl) contained bacterial DNA, primers (0.3 mM), PCR buffer 50 mM, 200 mM (each) deoxynucleoside triphosphates and 1.25 U Taq polymerase (New England Biolabs, USA). The mixtures were amplified in 40 cycles of 94°C for 1 min, 52°C for 1

min and 72°C for 1 min, with a final extension at 72°C for 10 min in thermal cycler. The presence of products at 2.3, 1.5 and 2 bps indicated that the isolate carries *pbp*1a, *pbp*2b or *pbp*2x genes, respectively. All the PCR products were run in agarose (1%) gel electrophoresis.

RESULTS

The clinical isolates included *Streptococcus mitis* (n. 19), *Streptococcus oralis* (n. 13), *Streptococcus sanguinis* and *Streptococcus parasanguinis* (n. 6, each), *Streptococcus salivarius* and *Streptococcus vestibularis* (n. 2, each), *Streptococcus constellatus* and *Streptococcus sobrinus* (n. 1, each).

The resistance rate against erythromycin was 36% (n. 18) (MIC ≥4 mg/L). Seven of the isolates (14%) were intermediately susceptible against erythromycin. Erythromycin-resistant isolates comprised of *S.mitis* (n. 7), *S.oralis* (n. 6), *S.parasanguinis* (n. 3), *S.sanguinis* (n. 1), *S.vestibularis* (n. 1).

Out of the 50 VGS tested, 11 strains (22%) were sensitive, 24 strains (48%) showed intermediate and 15 (30%) were resistant to penicillin (MIC ≥4 mg/L). Penicillin-resistant isolates comprised *S.oralis* (n. 5), *S.mitis* (n:4), *S.parasanguinis* (n:3), *S.sanguinis* (n. 2) and *S.vestibularis* (n. 1).

Twenty-one isolates (40%) were resistant to tetracycline. Fifteen (30%) and 10 (20%) of the isolates were resistant to clindamycin and cefotaxime, respectively. All the isolates were susceptible to vancomycin, chloramphenicol, levofloxacin and linezolid. The correlation of susceptibility patterns between penicillin and other antimicrobials that showed resistance to VGS isolates are shown in Table 1. Isolates resistant to both penicillin and erythromycin comprised 18% (n. 9) of all the isolates.

The phenotypes among the 25 erythromycin-resistant and intermediate isolates were as follows: 64% (n. 16) constitutive macrolide-lincosamide-streptogramin (cMLS) phenotype and 36% (n:9) M phenotype. The percentage genotypic carriage of each gene was: *erm*B, 56% (n. 14); *mef*A/E, 28% (n. 7); *erm*B+*mef*A/E, 8% (n. 2). Two of the isolates did not carry any of macrolide resistance genes studied here. All the *mef*A/E genes were assigned as *mef* E after *Bam*HI restriction analysis.

Five (33.3%) and three (20%) of the penicillin-resistant isolates (n:15) carried *pbp*2b and *pbp*2x genes, respectively. None of the isolates carried *pbp*1a gene.

Twenty-four (n:24) of the VGS blood culture isolates (n:50) were recovered from patients that have haematological malignancies and solid cancers, eight had heart failure (including 1 case of infective endocarditis), four had brain abscess, the rest of the isolates were recovered from patients with other types of diseases. The age range of patients was 22-95 years.

The correlation of penicillin and erythromycin-resistant (excluding intermediate) isolates in regard to species distribution, MIC, MLS and underlying diseases are shown in Table 2. Five of these patients were from intensive care units and 19 were inpatients. Twelve of them had cancer

TABLE 1 - *Comparison of susceptibility numbers(n) of antimicrobial agents that showed resistance to VGS according to penicillin susceptibility.*

	Penicillin susceptible (n:10) MICs ≤0.12 mg/L		*Penicillin intermediate (n:25) MICs 0.25-2 mg/L*		*Penicillin-resistant (n:15) MICs ≥4 mg/L*	
Antimicrobial agent	$MIC_{50/90}$	*n**	$MIC_{50/90}$	*n*	$MIC_{50/90}$	*n*
Erythromycin	0.5/1	7	1/2	19	0.5/2	6
Clindamycin	0.03/0.125	8	0.03/0.06	21	0.03/1	10
Cefotaxime	0.5/0.5	10	0.5/1	24	0.5/2	11
Tetracycline	0.064/0.25	7	0.5/4	16	0.125/2	7

*n, susceptile no

TABLE 2 - *The correlation between penicillin and erythromycin-resistant isolates according to species distribution, MIC, MLS and underlying diseases.*

S	*Age*	*Species*	*Pen MIC*	*Ery MIC*	*Mls*	*Geno type*	*Underlying diseases*	*Clinical outcome*
M	67	*S.mitis*	0.03	>256	cMLS	ermB	Squamous cell cancer	Exitus
M	39	*S.mitis*	1	>256	cMLS	ermB	Stomach cancer	Exitus
F	22	*S.mitis*	2	4	cMLS	ermB	Ewing sarcoma	Exitus
F	56	*S.mitis*	4	<0.016	-	-	Chronic myeloid leukemia	Exitus
M	78	*S.mitis*	4	32	cMLS	ermB	Brain abscess	Follow up
F	58	*S.mitis*	0.5	8	cMLS	ermB	Brain abscess	Cure
F	42	*S.mitis*	0.5	>256	cMLS	ermB	Chronic kidney failure	Follow up
M	33	*S.mitis*	4	0.5	-	-	Sinus vena thrombosis	Exitus
F	62	*S.mitis*	0.03	>256	cMLS	ermB	Chronic obstructive pulmonary disease	Cure
M	84	*S.mitis*	4	0.023	-	-	Lung cancer	Follow up
F	77	*S.oralis*	0.12	8	M	mef E	Acute myeloid leukemia	Exitus
M	46	*S.oralis*	4	>256	cMLS	ermB	Multiple myeloma	Cure
F	75	*S.oralis*	16	>256	cMLS	ermB	Sigmoid cancer	Cure
F	69	*S.oralis*	32	>256	cMLS	ermB	Burkitt lymphoma	Follow up
F	39	*S.oralis*	4	>256	cMLS	ermB	Brain abscess	Follow up
M	48	*S.oralis*	16	>256	cMLS	ermB	Chronic obstructive pulmonary disease	Cure
F	69	*S.parasanguinis*	4	>256	cMLS	ermB	Non-Hodgkin lymphoma	Follow up
F	33	*S.parasanguinis*	4	4	M	mef E	Acute myeloid leukemia	Exitus
F	71	*S.parasanguinis*	8	2	-	-	Vascular demans	Follow up
M	61	*S.parasanguinis*	1	>256	cMLS	ermB+ mef E	Chronic obstructive pulmonary disease	Cure
F	32	*S.sanguinis*	4	1	-	-	Fanconi anemia - bone marrow transplantation	Exitus
M	61	*S.sanguinis*	4	1	-	-	Intracardiak stenozis	Cure
F	65	*S.sanguinis*	1	>256	cMLS	ermB+ mef E	Acute myeloid leukemia	Exitus
F	62	*S.vestibularis*	4	>256	cMLS	ermB	Mitral valve operation	Cure

S, Sex; Pen, Penicillin; Ery, Erythromycin; MLS, Macrolide-Lincosamide-Streptogramin phenotype.

and nine died due to their underlying diseases. The age range of patients with penicillin and erythromycin-resistant isolates was 22-84 years.

DISCUSSION

The incidence of invasive viridans group streptococci infections and erythromycin and penicillin resistance have been increasing over the years (Reilly *et al.,* 2007). Viridans group streptococci can cause significant morbidity and mortality in patients with cancer since these patients frequently receive antibiotic therapy subsequent to chemotherapy, that may select for antibiotic resistance especially penicillin-resistant strains of VGS (Reilly *et al.,* 2007, Westling *et al.,* 2004). In the present study, reduced susceptibility against erythromycin and penicillin reached a high rise trend among our VGS blood culture isolates from patients with underlying diseases between the years 2005 and 2009.

Macrolide-resistant VGS isolates have been introduced in various studies around the world.

Resistance rates can differ due to clinical sample, study population and country but the common point is that macrolide resistance increases through the years. Uh *et al.* (2004) from South Korea indicated 33.9% erythromycin resistance among VGS from blood cultures while in the same year, Achour *et al.* (2004) from Tunisia found 70% resistance against erythromycin in *S.mitis* isolates recovered from neutropenic patients. Brown *et al.* (2008) reported their susceptibility trends between years 2001-2006 in the UK in VGS strains isolated from blood cultures and indicated that the resistance rate increased over the years reaching 58.5% in 2004 and declining to 31.3% in 2006. Lindgren *et al.* (2007) from Finland reported erythromycin resistance as 17.5% among invasive VGS isolates. Several studies have focused on macrolide resistance in VGS relating to MLS_B and M phenotypes, namely *erm*B and *mef*A/E genes, respectively. Rodriguez-Avial *et al.* (2003) from Spain indicated as the most prevalent type as MLS_B phenotype (57%) with *erm*B gene alone or with both *erm*B and *mef*A genes among their macrolide-resistant blood isolates. In contrast to the above study, Zolezzi *et al.* (2004) from Spain reported that the M phenotype was predominant (59.38%) among their erythromycin-resistant VGS isolates from oropharynx samples, and they found the *mef*A/E gene in all the strains with M phenotype and *mef*E was the predominant subclass (95.36%). Tazumi *et al.* (2009) from Ireland indicated the carriage of genes, *erm*B (25.3%), *mef*A (1%), *mef*E (75.8%) and *erm*B+*mef*E (11.1%) from patients with cystic fibrosis. In our study, erythromycin-resistant and intermediate isolates found to carry genes; *erm*B (56%), *mef*E (28%) and *erm*B+*mef*E (8%) in rank of order. This finding is in contrast with Tazumi *et al.*'s study. This is probably due to the study population and the clinical sample obtained from patients. Usually sputum samples tend to have more efflux type (*mef*E) macrolide resistance because of the long-term usage of macrolides that may affect VGS in this anatomical region. In most studies, it is usually assumed that the most common encountered gene among erythromycin-resistant VGS isolated from bloodstream is *erm*B gene (Zolezzi *et al.,* 2004). We found a similar result on the dominancy of *erm*B gene as the erythromycin-resistant mechanism among our VGS blood isolates.

According to 1990s surveillance studies, intermediate penicillin susceptibility among VGS blood isolates ranged between 14 and 64% and in 2000s surveillances, penicillin resistance ranged between 2 and 45% (Jalava *et al.,* 2009). Highly resistant VGS strains can be found and the resistance rates can be on a large scale. Wisplinghoff *et al.* (1999) from Germany reported a 2% high penicillin resistance and 19% intermediate penicillin susceptibility among their neutropenic cancer patients caused by *S.mitis* and *S.oralis*. Potgieter *et al.* (1992) found 38% penicillin resistance among 211 VGS strains isolated from blood cultures. The strains were susceptible to cephalosporins, imipenem and vancomycin. Kennedy *et al.* (2001) reported 45% and 38% penicillin resistance in their two-period study among paediatric patients with malignancy. Lyytikainen *et al.* (2004) found penicillin resistance as 7% and in another study the same year, Westling *et al.* (2004) reported penicillin resistance as 25% among their patients with haematological diseases.

Paulus *et al.* (2009) from Canada reported 13% penicillin resistance among VGS-related bacteremia in pediatric oncology patients. In our study, we found that 30% of the isolates were resistant to penicillin. None of the studies above dealt with the *pbp* profiles of their VGS isolates, we found that 33.3% and 20% of the isolates carry *pbp*2b and *pbp*2x genes and none carry *pbp*1a. Beta-lactam-resistant strains of VGS are prevalent in countries with a high incidence of penicillin-resistant pneumococci. According to the 2008 EARSS (European resistance surveillance system) report, reduced susceptibility against penicillin for pneumococci was assigned as 34% in our country which is marked as high (EARSS, 2008). In our isolates, due to low level isolation of *pbp*2b and *pbp*2x genes, penicillin resistance mechanisms can partially be explained so the reason of having penicillin-resistant VGS isolates might be related to the penicillin resistance in pneumococci isolates. Based on the data obtained from *S.pneumoniae*, it is assumed that highly resistant strains of VGS have accumulated several mutations in PBPs and these highly resistant strains may also need mutations other than PBP and by transformation and recombination between closely linked microorganisms *S.pneumoniae* and VGS might produce beta-lactam resist-

ant VGS with mosaic PBP genes (Dowson *et al.,* 1990).

VGS bacteremia has become a common problem in neutropenic adults and children. Among immunocompetent persons VGS can occasionally cause subacute endocarditis and invasive pyogenic infections, but in contrast these organisms occur more frequently and are more pathogenic when they reside in neutropenic patients with cancer (Huang *et al.,* 2007). Syrjala *et al.* (2010) from Finland indicated that viridans group streptococci were most commonly observed (in 20.4% of positive blood cultures) after high-dose cytarabine and idarubicin treatments. The high prevalence of penicillin and macrolide resistance among special groups of patients were expressed in various studies.

In general, it is assumed that children are colonized with penicillin-resistant strains of VGS more often than are adults. Recent studies among children with cancer or receiving an stem cell transplantation have found 21 to 37% of VGS blood isolates to be highly penicillin-resistant (MIC ≥4 µg/ml) (Reilly *et al.,* 2007, Westling *et al.,* 2004). This phenomenon can be seen in adult patients as well. For example, Achour *et al.* (2004) reported 83% penicillin G and 70% macrolide resistance in *S.mitis* among isolates recovered from patients hospitalized at bone marrow transplant center. Mrazova *et al.* (2005) indicated that penicillin resistance is an important risk factor in the mortality of patients with VGS bacteremia and erythromycin resistance is also a mortality factor when concomitant with penicillin resistance. In our study, the age range of patients was 22-95 years.

As the study was planned retrospectively, we have limited clinical data about the patients but twenty-four of the VGS (n. 50) strains were isolated from patients with cancer. *S.mitis* and *S.oralis* predominated among patients with cancer (n. 9) that had erythromycin and penicillin resistance isolates. *S.oralis* isolates tend to have higher MIC values than *S.mitis* (Table 2). It is noteworthy that all the isolates are susceptible to vancomycin, levofloxacin and linezolid.

As a result, our study found that there is an elevated rise in non-susceptibility against erythromycin and penicillin in our VGS blood isolates from patients with underlying diseases. Predominance of *erm*B and *mef*E genes are the cause of erythromycin resistance. Existence of *pbp*2b and *pbp*2x can partially explain the penicillin resistance. The importance of classical antimicrobial agents like penicillin and erythromycin warrants the continuous surveillance of invasive VGS isolates and can guide better treatment options especially in patients with underlying diseases.

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