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Molecular epidemiology of tetracycline resistance among *viridians group streptococci* isolated from various clinical specimens.

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Abstract

Viridans Group Streptococci* (VGS) are inhabitant of normal oropharyngeal, urogenital tract and gastrointestinal tract and also considered as both commensals as well as pathogen. VGS causes serious infections which include septicemia, Infective Endocarditis (IE), meningitis and sepsis in neutropenic patients. Even though, tetracycline is occasionally used, resistance among VGS against this antibiotic has been well documented. All 106 VGS isolates were tested for tetracycline resistance by MIC. Detection of tetracycline (*tet* (M), *tet* (K), *tet* (L), *tet* (O)) resistance genes were performed by PCR. Thirty two out of 106 isolates were found to be resistant to tetracycline by MIC. Among the 32 tetracycline resistant isolates, 14 (43.75%) isolates amplified *tet* (M) gene, 3 (9.38%) isolates amplified *tet* (O) gene, 1 (3.12%) isolate amplified *tet* (M) and *tet* (O) genes, 1 (3.12%) isolate was positive for *tet* (M) and *tet* (L) genes. Among the resistance genes present, *tet* (M) was the most predominant gene reported in our isolates. The relationship of *tet* (M) gene with the conjugative transposons which are responsible for the dissemination of various resistance genes warrants a periodical surveillance of this gene, which may serve as an indicator for the dissemination of other resistance genes among VGS and other *Streptococcus sp.

Keywords: *Viridians group streptococci*, Tetracycline resistance gene, PCR.

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Introduction

Viridans Group Streptococci (VGS) are inhabitant of normal oropharyngeal, urogenital tract and gastrointestinal tract and also considered as both commensals as well as pathogen. VGS causes serious infections which include septicemia, Infective Endocarditis (IE), meningitis and sepsis in neutropenic patients [1,2]. In general, VGS were considered to be reservoir of resistance genes which often transfers resistance traits to *Streptococcus pneumonia* or *Streptococcus pyogenes* [3].

Tetracycline is utilized once in a while as a part of treatment due to their side effects [4]. Even though, tetracycline is infrequently used, resistance among VGS against this antibiotic has been well documented. It has been suggested that the reduction in the use of these antibiotics is not always followed by the reduction in the prevalence of resistant organisms [5]. Tetracycline resistance is found in varied variety of microorganisms and is encoded by an extensive range of resistance genes. Two recognized mechanisms of tetracycline are: (i) *tet* (K) and *tet* (L) genes encoding active efflux-mediated mechanism, (ii) *tet* (M) and *tet* (O) genes encoding ribosomal protection mediated mechanism [6].

The resistant determinants of tetracycline are often found on the same mobile genetic element as of erythromycin resistant determinants. Tn916 is considered as a broad host range transposon, which usually encodes tetracycline resistance and occurs naturally in both gram positive and negative microorganisms [7]. The distinction in the resistant pattern of different antibiotic drives the need to expand our insight on antibiotics used for VGS. Thus, the present study aims to determine the distribution of tetracycline resistance genes (*tet* (M), *tet* (L), *tet* (O), *tet* (T)) among clinical isolates of VGS.

Materials and Methods

Bacterial Strains

All 106 VGS (44-*Streptococcus mitis*, 35-*Streptococcus salivarius*, 15-*Streptococcus oralis*, 4-*Streptococcus sanguinus*, 3-*Streptococcus anginosus*, 3-*Streptococcus parasanguinis*, 2-*Streptococcus mutans*) isolated from blood (52), dental plaque (24), oropharyngeal (17) and nasal swabs (13) were included in this study. Isolates were previously characterized using conventional biochemical tests in our laboratory.

Susceptibility testing

All the 106 VGS isolates were tested for antibiotic resistance to tetracycline (HiMedia) by Kirby Bauer disc diffusion test on Mueller-Hinton Agar Supplemented With 5% Sheep Blood (MHBA). The MIC of tetracycline (HiMedia) 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 [8]. The American Type Culture Collection strains of *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 25293) were the control strains used in this study.

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 for further use.

Detection of tetracycline resistance genes

Detection of tetracycline resistance gene was performed as described by Malhotra-Kumar et al. [9]. 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, 300 µM of dNTP's, 2 units of Taq polymerase enzyme and 5 µl of 10X reaction buffer. PCR cycling conditions were as follows: an initial denaturation for 3 min at 93°C followed by 30 cycles at 93°C for 1 min, 62°C for 1 min, 65°C for 4 min and final extension for 3 min at 65°C. After PCR, the amplicons were resolved in 1% agarose gel.

Table 1. *Primer sequences used for the detection of tetracycline resistance genes.*

Gene	Primer sequences	Amplicon size (bp)
tet (K)	5'-GATCAATTGTAGCTTTAGGTGAAGG-3' 5'-TTTTGTTGATTTACCAGGTACCATT-3'	155
tet (L)	5'-TGGTGGAATGATAGCCCATT-3' 5'-CAGGAATGACAGCACGCTAA-3'	229
tet (M)	5'-GTGGACAAAGGTACAACGAG-3' 5'-CGGTAAAGTTCGTCACACAC-3'	406
tet (O)	5'-AACTTAGGCATTCTGGCTCAC-3' 5'-TCCCACTGTTCCATATCGTCA-3'	515

Statistics

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

Results

Antibiotic susceptibility test by disc diffusion method

Among the 106 isolates screened for antibiotic susceptibility by disc diffusion method, 32 (30.19%) isolates (16-blood isolates, 16-non-blood isolates) were resistant to tetracycline. The distribution of tetracycline resistance by various species was given in Table 2.

Table 2. *Tetracycline resistance by disk diffusion among various species of VGS.*

Species	Tetracycline resistance
Staphylococcus mitis (n=44)	15 (33.33%)
Staphylococcus salivarius (n=35)	8 (22.86%)
Staphylococcus oralis (n=15)	5 (33.33%)
Staphylococcus sanguinus (n=4)	3 (75.00%)
Staphylococcus parasanguinis (n=3)	1 (33.33%)
Staphylococcus anginosus (n=3)	0
Staphylococcus mutans (n=2)	0
Total (n=106)	32 (30.19%)

Determination of tetracycline resistance by MIC

All the 32 VGS isolates tested for tetracycline resistance by MIC based on disk diffusion test were found to be resistant to tetracycline. The MIC breakpoints of VGS for tetracycline were as follows: susceptible ≤ 2 µg/ml, intermediate 4 µg/ml and resistance ≥ 8µg/ml. An overall MIC50/90 was 32/64 µg/ml with the range of 8-128 µg/ml. The MIC50 of both blood and non-blood isolates was 32 µg/ml and the MIC range was found to be 16-64 µg/ml and 8-128 µg/ml, respectively. The MIC90 of blood and non-blood isolates was 64 µg/ml and 128 µg/ml, respectively as shown in Table 3.

Table 3. *Distribution of tetracycline resistance genes and MIC values among VGS.*

Isolates	No. of resistant isolates	Minimum inhibitory concentration		Tetracycline resistance genes				Not amplified
		MIC50/90 (µg/mL)	Range (µg/mL)	*tet* (M)*	*tet* (O)	*tet* (M) and *tet* (O)	*Tet* (M) and *tet* (L)	
Blood	16	32/64	16-64	3	3	1	-	9
Non-blood	16	32/128	8-128	11	-	-	1	4
Total	32	32/64	8-128	14	3	1	1	13

*(Chi-Square analysis: significantly higher no of non-blood isolates (χ^2=8.127, DF=1, p=0.004) were positive for *tet* (M) gene).

Detection of tetracycline resistance genes

Among the 32 tetracycline resistant isolates, 14 (43.75%) isolates amplified *tet* (M) gene, 3 (9.38%) isolates amplified *tet* (O) gene, 1 (3.12%) isolate amplified *tet* (M) and *tet* (O) genes, 1 (3.12%) isolate amplified *tet* (M) and *tet* (L) genes and the remaining 13 (40.63%) isolates did not amplified any of the tested genes. Of the 16 resistant blood isolates, 3 (18.75%) isolates amplified *tet* (M) gene, 3 (18.75%) isolates amplified *tet* (O) gene and 1 (6.25%) isolate possessed both *tet* (M) and *tet* (O) genes. Of the 16 resistant non-blood isolates, 11 (68.75%) isolates amplified *tet* (M) gene and 1 (6.25%) isolate had both *tet* (M) and *tet* (L) genes. Chi-Square test showed that the occurrence of *tet* (M) gene was significantly higher in non-blood isolates (χ^2=8.127, DF=1, p=0.004) as shown in Table 3.

Among the various species of VGS, *tet* (M) gene was positive in *Staphylococcus mitis* (8), *Staphylococcus salivarius* (4), *Staphylococcus sanguinus* (1) and *Staphylococcus oralis* (1); *tet* (O) gene was present in *Staphylococcus sanguinus* (2) and *Staphylococcus oralis* (1) whereas, one *Staphylococcus oralis* had both *tet* (M) and *tet* (O) genes and one *Staphylococcus salivarius* had both *tet* (M) and *tet* (T) genes as shown in Table 4.

Table 4. *Distribution of tetracycline resistance genes among various species of VGS.*

Species	No of isolates	Tetracycline resistance genes				Not amplified
		tet (M)	*tet* (O)	*tet* (M) and *tet* (O)	*tet* (M) and *tet* (L)	
Staphylococcus mitis	15	8	0	0	0	7
Staphylococcus oralis	5	1	1	1	0	2
Staphylococcus salivarius	8	4	0	0	1	3
Staphylococcus sanguinus	3	1	2	0	0	0
Staphylococcus parasanguinis	1	0	0	0	0	1
Total	32	14	3	1	1	13

Discussion

Although, tetracycline is less frequently used, the spread of its resistance among *streptococci* may be particularly relevant form an ecological point of view. In this study, 30.19% of our isolates were resistant to tetracycline which is slightly higher than that reported from USA (16.8%) and lower than that reported from Spain (35%) and Canada (34%) [10-12]. We found that 29.63% of our blood stream isolates were resistant to tetracycline which is comparable to that reported by Horaud and Delbos [13]. In contrast, Wisplinghoff et al. [14] reported a higher percentage (39%) of blood stream VGS isolates from neutropenic cancer patients were resistant to tetracycline, while Yap et al. [15] reported a lower percentage (18%) of tetracycline resistance among their community acquired blood stream isolates.

We found that, 30.8% of the non-blood isolates were resistant to tetracycline which was comparable to that reported by Seppala et al. [16] from Finland, who found that 27.3% of VGS isolated from oral cavity were resistant to tetracycline. In Poland, Rozkiewicz et al. [17] reported that 52% of VGS isolated from plaque samples of healthy school children were resistant to tetracycline which is higher than that reported in our study. A relatively lower percentage of tetracycline resistance among oral isolates of VGS was reported from Greece (23%), United Kingdom (11%) and Tunisia (15.6%) [18-20].

It has been reported that *tet* (M) and *tet* (O) were the most common tetracycline resistance genes present in *streptococci* [9] which forms the basis for the selection of detected genes in this study. As reported earlier, [20] *tet* (M) gene was predominantly (43.8%) present in our isolates. Our result was comparable to that reported from Belgium (46.4%) [9]. However, studies from Spain and UK reported a higher percentage (78%-79%) of *tet* (M) gene among the VGS isolates [10,20]. We also found that significantly higher (p=0.004) number of our non-blood isolates possessed *tet* (M) gene. Moreover, 9.37% of tetracycline resistant isolates possessed *tet* (O) gene which is comparable to that reported earlier [9,10,20]. In contrast, a study from Tunisia reported that *tet* (O) was the predominant gene present among the VGS [18]. Only 3.1% of our VGS isolates possessed both *tet* (M) and *tet* (O) genes which is similar to that reported earlier [9] and 40.62% of tetracycline resistant isolates did not amplified for any of the tested genes. A possible reason for this could be that other tetracycline resistance genes which were not tested in our study may be responsible for their resistance.

It has been assumed that, resistance to tetracycline in *Staphylococcus pneumoniae* was primarily because of the existence of conjugative transposons that encoded *tet* (M) gene [21]. Tn916, is considered as a broad host range transposon

that occurs naturally in both gram positive and negative microorganisms and acts as a powerful vehicle for the spread of antibiotic resistance genes [7]. These elements can carry resistant determinants, which lead to the dissemination of multidrug resistance among VGS. The high presence of *tet* (M) gene in our study which can be associated with conjugative transposons may act as a pool for the spread of resistance genes among VGS. Thus surveillance of tetracycline resistance gene particularly *tet* (M) is highly warranted which may act as an indicator for the dissemination of other resistance genes among VGS and other *Streptococci sp.*

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