Macrolide Resistance Genes and Virulence Factors of Common Viridans Streptococci Species Colonizing Oral Cavities of Patients in Jordan

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Abstract

Aim: This study aimed to investigate the incidence of and virulence factors in oral viridans group streptococci (VGS) isolates from patients in Jordan. **Methods:** A total of 146 patients randomly attending periodontal clinic at the dental department / Jordan University Hospital in Amman, were investigated for dental diseases and presence of viridans group streptococci (VGS) using oral collected swabs for culture. A total of 81 VGS isolates were identified and investigated for antimicrobial susceptibility patterns, incidence of macrolide resistance genes and putative virulence factors using polymerase chain reaction (PCR) test. **Results:** The most frequently recovered species of VGS isolates were *S. mitis* represented by 27%, followed by 13.6% *S. mutans* and 12.3% *S. salivarius*. A total of 19/81(23%) of the VGS isolates were erythromycin-resistant, and 36/81 (44%) of VGS isolates harbored erythromycin genes. All VGS isolates were negative for the virulence factors (*ace, cylA, esp, gel E, agg*), and 32% were positive for endocarditis antigen (*efaA*). **Conclusion:** This study indicates that the common investigated species of VGS isolates carried frequently both erythromycin-resistant gene and the potential virulence factor (*efa*A) gene.

Key Words: Viridan streptococci, Erythromycin-resistant, Virulence factor

Introduction

The viridans group streptococci (VGS) are the most common pathogens isolated from human dental plaque. They are capable of causing several infections as well as invasive diseases; dental caries, purulent infections of oral and other body sites such as blood sepsis and infectiveendocarditis, despite the fact that they are generally considered to be of low pathogenic potential [1-3]. In particular, VGS are a frequent cause of native valve endocarditis in immunocompetent individuals and endocarditis or sepsis in patients with neutropenia. It is well known that streptococcalendocarditis is a life- threatening disease that requires a long period of effective treatment [3-5].

Recently, VGS have attracted attention due to their ability to act as reservoirs for antibiotic resistance genes and to transfer their resistance factors to more pathogenic organisms like *Streptococcus pneumoniae* and *Streptococcus pyogenes* [2,6]. In the past, VGS were nearly uniformly susceptible to β-lactam antimicrobial agents, aminoglycosides, tetracyclines, and macrolides. Several recently published studies from different countries reported that antimicrobial resistance is emerging in VGS isolates, mostly to penicillin and macrolides [3,6,7]. This resistance pattern has also been found frequently in species of VGS; *S.mitis, S.mutans*, and *S. salivarius* , which are important part of the normal oral flora of children and adults. In addition, these species are commonly associated with various clinical infections [2,4-5].

Therefore, it is important to follow the development of resistance patterns in these species of *streptococci* , in order to select the proper drug in chemoprophylaxis.

This study aims to investigate the common species of VGS in oral cavity of dental treated patients in Jordan, and to determine the susceptibility of isolates to erythromycin in association with certain specific potential virulence factors.

Materials and Methods

Collection of specimens

Patients were recruited from the Oral Diagnosis Clinic at the Dental Department, Jordan University Hospital(UJH) in Amman- Jordan, over the period from April to June, 2011. All participants signed an informed consent form including parents of examined children. The following demographic data were collected: name, age, sex, and a recent history of antibiotic treatment. Patients received antibiotics within the last 4 weeks were excluded from the study. All investigated patients were selected randomly according to their admission for general dental examination. Sterile swabs were used to collect plaque samples from the buccal surfaces of upper and lower first molars. The swabs were placed in brain heart infusion broth (Oxoid, England) and transferred immediately for culture at the Microbiology Research Laboratory at the Faculty of Medicine, University of Jordan. This study was approved by the ethics committee of The University of Jordan Hospital (UJH), research committee and of the Faculty of Medicine, and scientific research Deanship of The Jordan University.

Culture, isolation and identification

The collected oral swabs have been inoculated directly into brain-heart infusion broth and placed within 2-3 h in the incubator at 37°C, later 100 uL of each specimen was inoculated on trypticase soy-sucrose-bacitracin (TYS20B) agar plates [8]. After 24 hours of incubation at 37°C in Candle Jar, 5-10 catalase negative colonies were subcultured on blood agar plates 5% (v/v) human blood, and after another 24 hour of incubation, five colonies were spread on a new blood agar plate onto which optochin disk were applied . After incubation for 24-48 hrs few colonies from optochin resistant growth were cultured on bile esculin media to exclude Enterococcus species. Colonies positive or negative for alpha-hemolytic activity, catalase negative, optochin resistant, bile-esculin negative and gram positive cocci were initially identified as part of VGS and were stored in pure culture broth (15%

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glycerol-Brain Heart Infusion) at -70 ºC in Deep freezer (GFL, Germany).

Antimicrobial susceptibility tests

All VGS isolates were examined for antimicrobial susceptibility using disc diffusion method according to guidelines of Clinical and Laboratory Standards Institute (CLSI, 2011) [9]. Erythromycin resistance of VGS isolates was determined using E-test and was interpreted according to the CLSI guidelines. Erythromycin resistance was defined as having an MIC of ≥ 1, intermediate 0.5, and susceptible ≤0.25 µg/ml.

Identification of common species of VGS

DNA preparation of VGS isolates was performed using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. The DNA extracts were used for the detecting the specific genes of *S. mutans*, *S. mitis* and *S. salivarius* [10]. All PCR reactions were done at least twice using a PCR thermocycler (MJ research- INC, USA)and procedure was as follows:the initial denaturation was performed at 95ºC for 2 m, followed by 35 cycles of denaturation at 95ºC for 35 s, then annealing at 55ºC for 30s, and extension at 72ºC for 1 m , and a final extension at 72 ºC for 5 m. PCR products were analyzed by electrophoresis using 2% agarose gel (Promega, USA) containing ethidium bromide in 1× TBE buffer, and run for 1 hr with 70V, and visualized by UV Trans-illuminator (UVP,USA) and Gel Documentation System (UVP,USA). A positive control of *S. mutans* ATCC 25175, *S. mitits* ATCC 6249 and *S. salivarius* ATCC 13419 were used, and nuclease free water was used as negative control.

Detection of potential virulence genes

PCR amplification for the following genes: collagen binding protein (*ace*), haemolysin activator (*cylA*), and a surface protein (*esp*) were performed as described by Salah et al. [11], while endocarditis antigen (*efaA*),and *gel* E were detected as reported by Creti et al. [12]. All primers for detection these virulence genes were all purchased from Integrated DNA Technologies (IDT, USA). A positive control of *E. faecalis* (ATCC 29212) and (ATCC 51299) were used for the identification of the specific virulence and vancomycin resistance gene markers by PCR [13].

Detection of erythromycin-resistance genes

For the detection of resistance genes for erythromycin (ermA, ermB, ermC, mef (A/E) the same primers ad methods were carried out as reported by Sutcliffe et al. [14].

Sequencing analysis

Fifty microliter of the PCR product of *erm* B and *mef* (A/E) obtained from each positive isolates were sent to Macrogen company in South Korea for DNA purification and then DNA sequencing with (10ρ mole/µl) of each forward primers of *erm B* and *mef (A/E)*. Obtained sequences were compared to available macrolides resistant genes sequences in the GenBank database, by using the Blast server. (http:// www.ncbi.nlm.nih.gov/blast/).

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (IBM SPSS) version 19. Frequency and percentage were calculated for the categorical data. The level of significance was set at a p value of 0.05. Fisher's exact test replaces chi-squared test when the minimum expected count is less than five.

Results

This study included 146 patients aged 3 to 68 years with a mean of 26.99 (SD=15.674). Of these 66(45%) were males and 80(55%) were females. No statistically-significant difference (P 0.05) was observed between patients according to their gender, age, smoking habits, history of antibiotic treatment, oral hygiene and presence or absence of VGS. Out of 146 oral swabs, 81(56%) were positive for VGS, of these 22 (27%) were *S. mitis*, 11(14%) were *S. mutans*, 10(12 %) were *S. salivarius* and the rest 38 (47%) belonged to other unidentified species of VGS *(Table 1)*.

Table 1. Distribution of VGS species isolates from 146 oral specimens

VGS isolates	No. (%)
S.mitis	22(27)
S.mutans	11(14)
S.salivarius	10(12)
Viridans spp	38(47)
Total	81(100)

The antimicrobial resistance patterns are summarized in *(Tables 2)*.

*Table 2. Antimicrobial resistance pattern of 81 VGS isolates. *All isolates showed erythromycin MIC of 3-256µg/ml. **Co-resistance with erythromycin*

Antibiotics	Resistant isolates No. (%)
Erythromycin*	19 (23)
Ampicillin	9 (11)
Clindamycin**	7 (9)
Levofloxacin**	7 (9)
Vancomycin	3 (4)
Chloramphenicol	Null

The rates of VGS isolates resistant to erythromycin in vitro susceptibility test were (23%) followed by ampicillin (11%), levofloxacin and clindamycin (9%), vancomycin (4%). All VGS isolates were susceptible to chloramphenicol . The distribution of 19 erythromycin-resistant isolates included each were 4 *S. mitis* and *S. mutans*, 1 *S. salivarius* and 10

belonged to others VGS isolates *(Table 3)*, whereas 36/81 (44%) VGS isolates harbored erythromycin genes as shown in *Table 3*.

*Table 3. Distribution of erythromycin resistance genes among 81 VGS isolates. *Erythromycin-resistant isolates (19) detected in vitro susceptibility test and included each 4 S.mitis and S.mutans, 1 S.salivarius and 10 belonged to others Viridans species isolates*

VGS Resistant Genes	No. Erm B	No. Mef (A/E)	No. Erm C	No. Erm B, mef (A/E)	No. Erm B, ermC	No. ermC, mef (A/E)	No. ermB,ermC,mef (A/E)	Total no. isolates (%)
S. mitis	11	1	2	1	3	0	0	18/22(82)
S. mutans	2	1	0	3	2	1	1	10/11(91)
S. salivarius	2	0	1	3	0	0	2	8/10 (80)
Viridans spp.	0	0	0	0	0	0	0	0/38 (0)
Total no. (%)	15(42)	2(6)	3(8)	7(19)	5(14)	1(3)	3(8)	

There were no erythromycin -resistant genes detected in other VGS isolates. All VGS isolates were negative for presence of the virulence factors genes (*ace, cylA ,esp, gel E*). Endocarditis antigen A (*efaA*) gene was detected in 26/81 (32%) of the isolates, of these 9/22 (41%) were *S. mitis*, 7/11(67) were *S. mutans* and 8/10(80) were *S. salivarius*, whereas only 2/38 (5%) of viridans spp carried (*efaA*) gene as shown in *Table 4* and *Figure 1*.

*Table 4. Distribution of potential virulence factor (efa A) among 81 VGS isolates. * Significant compared to other VGS isolates.*

VGS isolates	No. (%) isolates carried (efaA) gene	P value
S.mitis	9/22(41)	0.001*
S.mutans	7/11(67)	0.001*
S.salivarius	8/10(80)	0.001*
Viridans spp.	2/38(5)	0.48
Total	26/81(32)	-

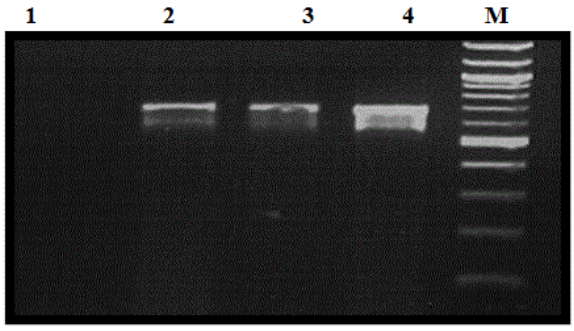

Figure 1. Agarose gel electrophoresis of amplified efaA genes by the uniplex PCR assay. Lanes: M, 1000-bp DNA ladder; lane 1 to 4, the Streptococcus viridans isolates,3,4 which were positive to efaA (735 bp) ; lane 1, negative control; lane 2, positive control (E. faecalis ATCC 29212).

Forward primers and PCR products for both *erm* (B), *mef* (A/F) genes were sent for capillary sanger sequencing company, and the resultant nucleotide sequences were analyzed using method of sequence alignment online tool. (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were confirmed to hit against *erm* B database registered gene sequence with an identity homology of 98-99%; and to hit against *mef* (A/F) database registered gene sequence with an identity homology of 97-99%.

Discussion

This study has shown that the most frequently isolated species of VGS (53%) from oral cavity of patients in Jordan were *S. mitis* , *S. mutans* and *S. salivarius* , and the rest 47% were other species of viridans streptococci . Most recent studies from different parts of world have reported that *S. mitis* and *S. salivarius* represent the most frequently isolated species among viridans streptococci recovered from neutropenic patients and healthy populations [5,7,15-17]. **Bruckner** and **Gigliotti** [4] reported that the incidence and severity of VGS infections have increased during the past 15 years and account for about one third of all bacteremic episodes. Risk factors of developing viridians streptococci infection include severe neutropenia, mucositis, gastrointestinal toxicity, pneumonia, and high-intensity chemotherapy. In addition, treatment of infections caused by viridans streptococci will becoming more difficult due to the emerging antimicrobial resistance to frequently used drugs in many developed and developing countries[3-4,6,11, 16-17].

A study from Finland has shown that 81% of streptococci isolates from oral cavity belonged to *S. mitis*, followed by the other species of VGS [2], while a study from France has reported that out of 236 VGS isolates, 125 isolates were *S. mitis* and 17 were *S. salivarius* [18]. A study from England has also reported that *S. mitis* accounted for the majority of viridans streptococcus bacteremia (55%) in children on chemotherapy for cancer [19], whereas a study from Kuwait found that *S. salivarius* (21.5%) and *S. sanguis* (16.3%) were the most prevalent species in the oral specimens of 102 examined healthy children [17].

This study shows that majority of the VGS isolates were susceptible to ampicillin, clindamycin, vancomycin, levofloxacin and chloramphenicol in the range between 89 and 100%, respectively, while only (23%) of VGS isolates were erythromycin resistant and carried erythromycin resistance genes. Most previous studies published in the 21th century have reported wide range of resistance rates to erythromycin (5.0% to 51%) among various VGS species isolates from healthy person or clinical specimens of patients [5,16,19-21]. A higher resistance rates to erythromycin has been also reported from France, where 53% of *S. mitis* and 41% *S. salivarius* isolates were resistant to erythromycin [18].

This study has also found that (44%) of VGS isolates were either resistant or susceptible for erythromycin in vitro test in association with presence of erythromycin gene *erm B*, whereas *mef* (A/E) was detected in few isolates (6%), and the *erm* C was present only in (8%) of the isolates. In addition, VGS isolates that expressed three erythromycin-resistant genes (*ermB, ermC, mef* (A/E)) were limited only to few isolates (8%). The absence of erythromycin-resistant in certain isolates in vitro test may be due to low level or down regulation of gene expression or by the presence of a silent gene as reported in other bacteria species [22].

A recent study has reported that *S. mitis* and *S. oralis* were frequently recovered from patients with cancer who had erythromycin and penicillin resistance isolates [3]. A study carried out by Malhotra-Kumar et al. [23] has found that high oropharyngeal carriage of macrolide-resistant VGS is associated with co-resistance to tetracycline and fluoroquinolones among healthy Belgian adults. In this study, 9% of macrolide-resistant VGS isolates demonstrated co-resistance with clindamycin/ levofloaxcin or both. In addition, it has been reported VGS play a significant role as a reservoir of antimicrobial resistance genes, transferring different resistance genes to more pathogenic organisms like *S. pneumoniae* and *S. pyogenes* [2,19].

The present study has found that all VGS isolates were negative for the presence of virulence factors which are commonly found in *Enterococcus fecalis* (*ace, cylA ,esp, gel E*) [11,12]. This study has shown that *S. mitis*, *S. salivarius* and *S. mutans* isolates carried high percentage of only (*efaA*) virulence factor which was in the range of 41% to 80% , whereas only 5% of other VGS isolates harbored this virulence factor *(Table 4, Figure 1)*. Despite the fact that VGS and *E. fecalis* are quite different genus, the presence of (*efaA*) gene in certain species of VGS is interesting and further investigations are necessary to document its presence and how acquired this virulence gene. Most previous studies have investigated the pathogenesis of oral viridians streptococci in relation to their biofilm formation, enzymic action of the glucosyltransferase enzymes, pneumolysin and other potential factors, except the virulence factor (*efaA*) which was not included [5,24,25,26].

Conclusion

This study shows that erythromycin-resistant is moderately found among VGS isolate in oral cavity of patients in Jordan, and about one-third of these frequently detected streptococci species; *S. salivarius*, *S. mutans* and *S. mitis* carried the potential virulence factor (*efaA*) which might contribute for their pathogenicity in endocarditis.

Competing Interest

The authors declared that they have no competing interests regarding publication of this paper.

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Contributions of Each Author

RfAZ conducted the lab work, generated and analyzed the data as well as has prepared the first draft of manuscript. NDO has investigated the patients and supervised collection of clinical specimens. AAS has supervised all laboratory works. All of the authors read and approved the final manuscript.

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