Antibiotics Resistance in Viridans Streptococci Isolated from Odontogenic Infections in Tanta University Dental Clinic, Egypt

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Abstract: In this study, a total of 166 streptococcal isolates were recovered from clinical samples. All the isolates were identified based on their colonial morphology on Mitis-Salivarius agar and API 20 Strep identification system. Six species were identified namely *S. mutans, S. mitis, S. salivarius, S. oralis, S. sanguinis, S. anginosus.* The most isolated species was *S. mutans* (22.9%) of total isolates, while the least isolated one was *S. sanguinis* (12%). The susceptibility of the recovered isolates to 10 antibiotics was determined using agar dilution method. Resistance patterns were set for the resistant isolates (58 isolate out of the recovered 166). Patterns included resistance to 2 to 9 of the tested antibiotics. The highest incidence of resistant was to macrolide antibiotics while the lowest incidence was to cefepime (4^{th} generation cephalosporin). Resistance mechanisms to tested antibiotics were investigated. Beta-lactams resistance was found to be through alterations of PBPs in resistant isolates and none of the resistant isolates showed production of β-lactamases. For macrolides and lincosamides resistance, M phenotype was predominant in the tested isolates confirmed by presence of the corresponding gene *mef(A),* followed by $cMLS_B$ then finally the $iMLS_B$, they both were confirmed by presence of the corresponding gene *erm(B).*
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1. Introduction:

Bacterial infections are common in dental practice. Reports have always confirmed that oral/dental infections are polymicrobial **(Brook *et al.*, 1991)**; including both facultative anaerobes and strict anaerobes**(Gonul *et al.*, 2013).** Of the facultative anaerobic bacteria, oral streptococci are the most frequent **(Bascones *et al.*, 2004).** Since most human oralproblems originate from odontogenic infections, prescribing antibiotics has become an important aspect of dental practice **(Dar-Odeh*et al.*, 2010)**.Although penicillins traditionally have been used for the treatment of odontogenic infections, the emergence of bacteria resistant to penicillin have caused other antibiotics to become the drugs of choice for treating those infections **(Kirkwood, 2003)**. The susceptibility of the recovered isolates to 10 commonly prescribed antibiotics in dental practice including penicillins, cephalosporins, macrolides and lincosamides was determined using agar dilution method according to CLSI (2013) breakpoints. Resistance mechanisms to tested antibiotics were investigated. Resistance to β-lactams was investigated using detection of β-lactamases enzymes production by resistant isolates and possible alteration in PBPs of resistant isolates. Resistance to macrolides in streptococci is mediated through efflux of the drug or methylation of the ribosomal target and streptococcal lincosamides resistance is mainly mediated by methylation of the ribosomal target. Resistance mechanisms to both were investigated phenotypically using disk approximation test to determine the possible mechanism of resistance and then confirmed genotypically by multiplex PCR.

2. Material and methods:

1. Collection and transport of Bacterial isolates and growth conditions:

Clinical specimens were collected from patients suffering from odontogenic infections admitted to the external clinic, faculty of Dentistry, Tanta University. Dental micro-brushes were autoclaved for sterilization and used as swabs to collect clinical specimens from infection sites in patients. After swabbing the infection site, the micro-brushes were placed in thioglycolatebroth (Oxoid, UK) which served as transport media till they were transferred to the laboratory. Micro-brushes were then streaked on surface of Mitis-Salivarius agar plates (Difco, USA). Plates were then incubated at 37ºC in anaerobic jars using gas kits (Oxoid, UK), to ensure anaerobic conditions, for 48 hours to obtain good colonial growth.

2. Identification of different oral streptococci species recovered from collected samples: -

The developed bacterial colonies on mitis salivarius agar plates were morphologically examined for their sizes, form, elevation, margins and consistency. Pure distinct colonies on Mitis-Salivarius agar plates were picked and sub cultured onto Columbia Blood agar plates containing "streptococcal

selective supplement" for enrichment of growth and for identification of their hemolytic reaction. Plates were incubated for 37ºC in anaerobic jars with gas kits for 24 hours. Hemolytic reactions of isolates were recorded. The isolates were then identified using API-20 Strep kit.

3. Determination of minimum inhibitory concentrations (MICs) of selected antibiotics against recovered isolates:

The MICs of ampicillin, amoxicillin, cephalexin, cefuroxime, cefotaxime, ceftriaxone, cefepime, erythromycin, azithromycin and clindamycinagainst all recovered isolates were determined by agar dilution test and susceptibility groups were determined according to the procedures described by CLSI (2013).

4. Determination of beta lactams resistance mechanism in resistant isolates:

4.1. Detection of beta lactamase production by resistant isolates:

It was performed by both iodometric overlay method **(Abo-Kamar&Shohyeb, 1998)** and nitrocefin method**(Milatovic *et al.*, 1993)**. Beforedetection of the β-lactamase, bacterial isolates were first grown onto plates containing 0.1µg/ml penicillin G. for iodometric method to induce enzyme production,

4.2. Detection of penicillin binding proteins (PBPs) alterations in resistant isolates:

Alteration in PBPs were investigated in selected isolates of *S. mutans* species using sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). The PBPs were extractedaccording to the method described by **Zhao *et al.* (1991)** and labelled with fluorescent Bocillin-FL as described by **Izdebski *et al.* (2008)** through incubation with decreasing concentrations of Bocillin-FL ranging from 312.5 ng/ml to 8 µg/ml. Labeled PBPs then were separated by SDS-PAGE then detected with naked eye under UV light. The selected isolates had different resistance patterns regarding β-lactams. Isolates of *S.mutans*were selected for PBPs extraction as *S. mutans* was the most isolated species. The selected isolates for PBPs investigation had different resistance patterns regarding β-lactamsas follows; **S15**; susceptible isolate with the lowest MICs for tested beta lactam antibiotics, **S3**; isolate with resistance to tested penicillins and 1st generation cephalosporins (cephalexin), **S24**; isolate with resistance to tested penicillins, 1st and 2nd generations cephalosporins (cephalexin and cefuroxime) and **S22**; isolate with resistance to all tested penicillins and extended spectrum cephalosporins.

5. Macrolides and lincosamides resistance mechanisms:

5.1. Phenotypic detection:

The phenotypes of resistance in isolates (resistant to erythromycin but susceptible to clindamycin) were detected through disk approximation test (D-test) according to CLSI 2013.

5.2. Genotypic detection:

Macrolides resistance mechanisms among tested isolates were confirmed by detection of macrolides resistance genes; *erm(B)* and *mef(A)* was performed using multiplex PCR analysis. The DNA was extracted according to protocol of **(Moore and Dowhan, 1995).** The PCR conditions are listed in table 1.

Table 1: conditions for multiplex PCR of macrolide resistance genes.

Amplified genes	PCR conditions											
	Initial denaturation		Denaturation		Annealing		Extension		No. of cycles	Final extension		Reference
	Temp	Time (min)	Temp	Time (sec)	Temp	Time (sec)	Temp	Time (sec)		Temp	Time (min)	
Macrolide resistance genes	94°C	5	94°C	20	52°C	20	72°C	15	30	72°C	5	Ubukata *et al*., 2003

3. Results

Isolation and identification:

A total of 166 different oral streptococcal isolates were recovered from the specimens taken from 46 studied cases. All the recovered streptococcal isolates were properly identified to the species level as shown in table (2). Six streptococcal species namely *S. mutans*, *S. mitis*, *S. salivarius*, *S. anginosus*, *S. sanguinis* and *S. oralis* were identified.

As shown in table (2), It is found that *Streptococcus mutans* was the main isolated species from odontogenic infections with incidence of 82.6 % among the studied cases followed by *Streptococcus anginosus;* 71.7%. On the other hand, *Streptococcus sanguinis* is the least isolated one with incidence of 43.5% among studied cases.

Susceptibility testing:

Resistance to tested antibiotics were distributed among recovered isolates as shown in table (3). Cefepime had the least while erythromycin had the highest incidence of resistance. Patterns of antibiotics resistance among isolates are listed in table (4).

Detection of beta lactamase enzymes:

Isolates resistant to tested beta-lactams were examined for beta lactamase production using iodometric as well as nitrocefin methods and none of the isolates showed production of beta lactamase.

Detection of PBPs alteration among resistant isolates:

From the results indicated in figure (1). It was noted that tested resistant isolates (b, c and d) hadPBPs pattern distinct from the susceptible one (a) and also from each other. In figure (1-a), it was observed that all separated PBPs didn't show any decrease in intensities with decreasing Bocillin concentrations till lane 7 where the corresponding concentration (0.125 µg/ml) didn't saturate the PBPs while in figure (1-b), both PBPs 1a and 2x showed decreased intensities at 0.25 µg/ml and PBP 2a showed decreased intensities at 0.25, 0.5 and 1 µg/ml. However, in figure (1-c), PBPs 1a, 2x and 2b showed decreased intensities at 0.25 and 0.5 /ml, but PBP 2b showed decreased intensities at 0.25/ml whereas figure (1-d) showed PBPs 1a, 2x and 2b with decreased intensities at 0.25, 0.5 and 1 /ml and PBP 2b with decreased intensities at 0.25 and 0.5 /ml. Intensity of PBP3 band didn't change in any of the tested resistant isolates relative to the susceptible one.

Table (2): **Phenotypic characters of recovered oral streptococci species.**

Colonial morphology on Mitis-Salivarius agar	Corresponding API 7-digit No.	Isolated Streptococcus species	No. of isolates (%)	Incidence among cases (46 cases)
Rough colonies that look like granular frosted glass in appearance,undule-shaped colonies (making dextran from sugar).	5240770 5240550	*Streptococcus mutans*	38 (22.9%)	**82.6%**
Medium sized round blue colonies	5061411 5061415	*Streptococcus anginosus*	33 (19.9%)	**71.7%**
Small, flat, light blue colonies.	0040401 0061401	*Streptococcus mitis*	29 (17.5%)	**63%**
Small round blue colonies.	0260441	*Streptococcus oralis*	24 (14.5%)	**52.2%**
Mucoid, smooth colonies resembling gum drops (using the sugars to produce a gummy-like levan, thus producing sticky, mucoid, gum-drop colonies.)	5050451 5040441 5050461	*Streptococcus salivarius*	22 (13.2%)	**47.8%**
Hard, rubbery, adherent colonies called"zoogela".	0241451	*Streptococcus sanguinis*	20 (12%)	**43.5%**

Table (3): Incidence of tested antimicrobials resistance among isolated streptococcal species.

AMA*	Number (%) of resistant isolates					
	S. mutans (n=38)	*S. anginosus* (n=33)	*S. mitis* (n=29)	*S. oralis* (n=24)	*S. salivarius* (n=22)	*S. sanguinis* (n=20)
	R*	R	R	R	R	R
AMP	11 (29%)	7 (21%)	2 (7%)	2 (8%)	2 (9%)	1 (5%)
AML	10 (26%)	7 (21%)	1 (3%)	1 (4%)	2 (9%)	--
CL	10 (26%)	6 (18%)	1 (3%)	1 (4%)	2 (9%)	--
CXM	10 (26%)	7 (21%)	--	--	1 (5%)	--
CRO	5 (13%)	4 (12%)	--	--	1 (5%)	--
CTX	6 (16%)	4 (12%)	--	--	1 (5%)	--
FEP	2 (5%)	1 (3%)	--	--	1 (5%)	--
E	15 (39%)	10 (30%)	4(14%)	7 (29%)	4 (18%)	5 (25%)
AZM	14 (37%)	8 (24%)	4 (14%)	6 (25%)	3 (14%)	3 (15%)
DA	2 (5%)	3 (9%)	1(3%)	1(4%)	--	--

Table (4): patterns of resistance to tested antibiotics:

Pattern code	Resistance markers*	Distribution among recovered species					
		S. mutans	*S. anginosus*	*S. mitis*	*S. oralis*	*S. salivarius*	*S. sanguinis*
I	E	---	---	---	1	---	1
II a	E+AZM	11	6	3	5	3	3
IIb	AMP+E	1	---	---	---	---	1
III a	AMP+AML+CL	---	---	1	1	1	---
IV a	AMP+AML+CL+CXM	2	2	---	---	---	---
IV b	AMP+E+AZM+DA	---	---	1	1	---	---
V a	AMP+AML+CXM+E+DA	---	1	---	---	--	---
VI a	AMP+AML+CL+CXM+CTX+CRO	3	1	---	---	---	---
VII a	AMP+AML+CL+CXM+E+AZM+ DA	2	---	---	---	---	---
VII b	AMP+AML+CL+CXM+CRO+CTX+FEP	2	---	---	---	---	--
VII c	AMP+AML+CL+CXM+CTX+E+ AZM	1	---	---	---	---	---
VIII	AMP+AML+CL+CXM+CRO+CTX+FEP+E	---	1	---	---	1	---
IX	AMP+AML+CL+CXM+CRO+CTX+E+AZM+DA	---	2	---	---	---	---
	Total no.of resistant isolates	22	13	5	8	5	5

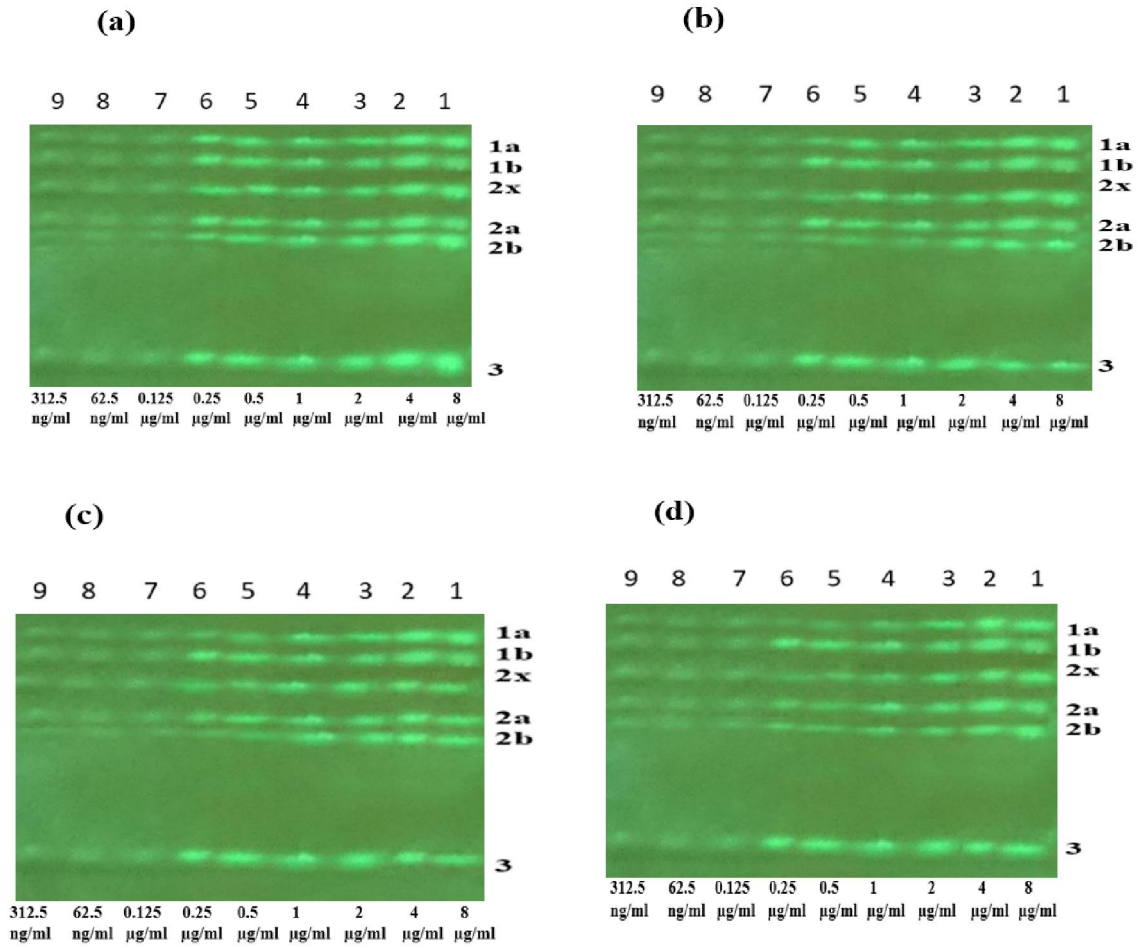

Figure (1) PBP profiles of selected resistant *S. mutans* isolates: (a) S15, (b) S3, (c) S24 and (d) S22.

Phenotypic detection of macrolides and lincosamides resistance:

It was found that 6 out of 40 (15%) selected isolates showed positive D-test indicating incidence of inducible resistance to clindamycin through methylation of the ribosomal target in presence of inducers such as erythromycin ($iMLS_B$), while the remaining 34 isolates were negative which confirm their resistance to only macrolides without lincosamides through efflux of the drug out of the bacterial cell (M phenotype).

Detection of macrolide resistance genes among selected macrolide resistant isolates:

As shown in figure (2), the results revealed that *mef(A)* gene was detected in all isolates that showed M phenotype in the D-zone test (34 isolates) whereas *erm(B)* gene was detected in all the isolates with $iMLS_B$ (6 isolates). Two of the isolates with $iMLS_B$ phenotype had the *mef(A)* gene in addition to the *erm(B)* gene, represented by lane 2 and lane 5 in electrophoregram (a).

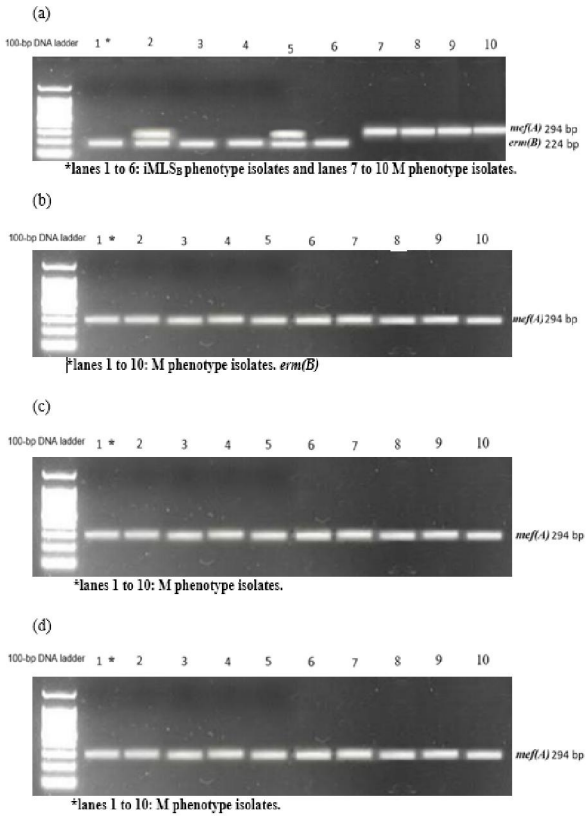

Figure (2) Electrophoregrams of amplified products of tested isolates.

4. Disscusion

In our study, 166 different oral streptococci isolates were recovered from the 46 studied cases with odontogenic infections. The recovered isolates were as follows: *S. mutans* (82.6%), *S. anginosus* (71.7%), *S. mitis* (63%), *S. oralis* (52.2%), *S. salivarius* (47.8%) and *S. sanguinis* (43.5%).The same species were recorded by **Diaz *et al.* (2006).** *Streptococcus mutans* was the most commonly isolated species and this finding might confirm its involvement in disease production **(Thurnheer *et al.*, 2001; e Franco *et al.*, 2007** and **Maripandi *et al.*, 2011).**

The total number of resistant oral isolates to any of the tested antimicrobials was 58 out of the 166 (34.9%), distributed between different species as follows; *S. mitis* isolates had the lowest resistance incidence (17%) among isolated specieswhile *S. mutans* isolates showed the highest one (58%) and each of the other isolated species showed resistance percent as follows: *S. anginosus* 39%, *S. oralis*33%,*S. salivarius* 23%, *S. sanguinis* 25%.These high incidences of resistance among the oral microorganisms especially among the viridans group streptococci (VGS) was comparable with the data of antibiotic resistance rates which was documented by **Teng *et al.*(1998), Rosser *et al.* (1999), Rotimi *et al.* (2005), Mokaddas *et al.* (2007)** and **Dar-Odeh *et al.*(2010).**

According tothe current study, the obtained resistance rates among recovered isolates to tested antibiotics were lower than that reported by **Rotimi *et al.* (2005);** who reported that resistance rates to amoxicillin, erythromycin, cefuroxime, cephalothin and clindamycin were 40.8, 32.6, 27.5, 25.3 and 15.4%, respectively. However, both the data of the present study and the data reportedby **Rotimi & Salako *et al.* (2005)** show species-related difference in the susceptibility pattern which then underscores the importance of accurate identification.

Considering β-lactamases, they weren't known to occur in streptococcus species**.** However, in 1986, streptococci producing β-lactamase were isolated from the subgingival plaque of adults with periodontitis **(Kinder *et al.*, 1986; Doern *et al.*, 1996; Hakenbeck, 1998; Chambers, 1999 andSweeney *et al.*, 2004).** Therefore, our recovered isolates that were resistant to β-lactams were tested for possible production of β-lactamase. None of the tested isolates showed production of β-lactamase enzyme. Hence, β-Lactam resistance in isolated viridans streptococci is likely to be mediated by altered PBPs which have decreased affinity to antibiotics and then higher concentrations of drugs are thus both required to inhibit the altered PBPs **(Dowson *et al.*, 1990; Hakenbeck *et al.*, 1998; Amoroso *et al.*, 2001; Sauvage*et al.*, 2008; Chardin *et al.*, 2009 and Haenni *et al.*, 2010).** The SDS-PAGE of the susceptible isolateshowed decrease in bands intensity (which is the indicator of decreased affinity of binding to Bocillin FL and hence decreased affinity for β-lactams) started from the concentration 0.125

µg/ml. On the other hand, in the resistant isolates, decreased affinity for β-lactamsstarted from higher concentrations than 0.125 µg/ml.In **Nakayama and Takao** study **(2003)** on *S. mitis*, it was found that the MIC of penicillin G for strains with PBP 2x alterations remained low, while the MICs of cephalosporins, including CTX, were slightly higher than those for strains without any alterations. This finding was also reported by **Ubukata *et al.* (1997) and Asahi *et al.* (1999)**. Ourstudy similarly demonstrated that decreased affinity of PBP 2b was associated with resistance to penicillins while resistance to cephalosporins was associated with decreased affinity of PBP 2x and high resistance to both antibiotics was associated with decreased affinity of PBP 1a beside PBP 2x and PBP 2b alterations.

The phenotype of isolates resistant to MLS_B antibiotics tells which mechanism of resistance to them is present in this isolate. The phenotype may be either M phenotype, $cMLS_B$ phenotype or $iMLS_B$ phenotype. M phenotype indicates efflux resistance mechanism while both $cMLS_B$ and $iMLS_B$ indicates resistance through methylation of ribosomes **(Leclercq, 2002).** The current study showed predominance of M phenotype among isolated erythromycin resistant oral streptococci as it was detected in 34 out of the 56 tested isolates (60.7%) while MLS_B phenotype was represented by 39.3% of the isolates divided between $iMLS_B$ (6 isolates)and $cMLS_B$ (16 isolates) phenotypes. All isolates with $iMLS_B$ phenotype showed incidence of *erm(B)* gene with two of them had additional *mef(A)* genes together with the *erm(B)* gene while all the M phenotype isolates showed presence of *mef(A)* gene. Predominance of M phenotype was similarly reported by **Aracil *et al.* (2001), Zolezzi *et al.* (2004), Ioannidou *et al*. (2001) and Seppälä *et al.*, 2003).**

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