ORIGINAL ARTICLE

Induction of *In Vitro* Resistance to Penicillin in Viridans Group Streptococci and Its Effect on Susceptibility Pattern of Other Antimicrobial Agents

Shree V. Dhotre[1], Basavraj S. Nagoba[2], Namdev M. Suryawanshi[2]*

[1]Department of Microbiology, Ashwini Medical College & Hospital, Kumbhari-413006, Solapur (Maharashtra) India, [2] Department of Microbiology, Maharashtra Institute of Medical Sciences & Research, Latur-413 531(Maharashtra) India

Abstract:

Background: The development of resistance to penicillin in Viridans Group Streptococci (VGS) during therapy has been reported. However, the *in vitro* development of resistance to penicillin or other antimicrobial agents in VGS is mostly overlooked and rarely reported. *Aim & Objectives:* To induce *in vitro* resistance to penicillin in VGS and to study its effect on susceptibility pattern of other structurally related (Beta–lactams) and unrelated antimicrobial agents. *Material and Methods:* Four isolates of VGS susceptible to all antimicrobial agents were manipulated *in vitro* to induce resistance to penicillin by sequential exposure to increasing concentrations of penicillin. *Results:* Increase in MIC values of penicillin from 0.06-0.12 µg/ml to 2-32 µg/ml was observed indicating development of resistance to penicillin. A significant increase in Minimum Inhibitory Concentration (MIC) values of ampicillin and slight increase in MIC values of other antimicrobial agents in some isolates was also noted. *Conclusions:* Exposure to increasing concentrations of penicillin can promote the development of resistance to penicillin and cross-resistance to other antimicrobial agents suggesting its mutagenic role.

Keywords: Cross-Resistance, *in vitro* Resistance, Penicillin, VGS

Introduction:

The development of resistance to penicillin in Viridans Group Streptococci (VGS) during therapy has been reported by earlier workers [1-4]. However, *in vitro* development of resistance to penicillin or other antimicrobial agents in VGS is mostly overlooked and rarely reported. Although the induction of drug resistance in organisms like staphylococci and *Pseudomonas aeruginosa* has been reported, there are no reports on development of drug resistance in VGS using *in vitro* methods, except for one recent report on development of high-level resistance to daptomycin *in vitro* and *in vivo* in mitis group streptococci after exposure to daptomycin [5].

To address some of these issues and present the test organism with a dynamic concentration of antimicrobial agents that more closely mimics *in vivo* human biological properties, an *in vitro* model for induction of drug resistance is proposed here which will allow maintaining desired concentration of drugs as per our needs. Using this model, it is possible to expose the study organisms to sub lethal concentration of the test drug, followed by a sequential increase in concentration of the drug so as to check the development of resistance in the study organism.

Such an *in vitro* model for induction of drug resistance would then offer a more *in vivo* like way to model physiological processes. Bacteria can be grown at densities and numbers that reflect *in vivo* infection potential. The smaller volume provides more rapid equilibration of drug concentrations and a more uniform growth environment. This

high-density culture also supports drug pathogen interactions that more accurately reflect disease states. Therapeutic modalities utilizing antibiotic agents depend not only upon a maximum tolerated dosage but the time course of administration, usually of multiple dosages. These can be easily studied using this model.

In the present study, an attempt has been made to develop *in vitro* resistance against penicillin in VGS and find out its effect on antimicrobial susceptibility pattern.

Material and Methods:

Four isolates of VGS (Three blood isolates namely, *S. oralis, S. sanguinis, S. mitis* and one isolate from subgingival plaque - *S. mutans*) with MIC in the range of 0.06 µg/ml to 0.12 µg/ml and susceptible to all the antibiotics were selected for the study of development of *in vitro* drug resistance to penicillin. All the four isolates were subjected to find out susceptibility to penicillin G, ampicillin, cefepime, cefotaxime, cefitriaxone, (beta–lactam group structurally related group) and to vancomycin, erythromycin, azithromycin, clarithromycin, tetracycline, levofloxacin, ofloxacin, clindamycin, quinupristin /dalfopristin and linezolid (structurally unrelated group of antimicrobial agents) by using automated Vitek 2 (bioMérieux) system in accordance with Clinical and Laboratory Standards Institute (CLSI) standards [6]. These isolates were manipulated *in vitro* to induce resistance to penicillin.

This model consists of a central reservoir containing the organisms, a diluent reservoir and a waste reservoir. Increasing concentrations of drug can be added to the central reservoir through the diluent reservoir which is composed of a complex media mimicking human *in vivo* nutrients designed for this model. Removal of an equal volume of drug (and organism) containing medium was achieved through the waste reservoir. This model offers a continuous culture system which mimics human *in vivo* conditions like the blood circulation.

Isolates of VGS under study were inoculated into tryptone soya broth overnight. After achieving the final bacterial concentration of $1x10^5$CFU/ml approximately (turbidity matched with McFarland tube No.1) [7], 1 ml of broth culture was inoculated into the central reservoir containing 50 ml of the complex media designed for this model and incubated at 37^0C for 72 hours in a continuous culture with inflow of media from the diluent reservoir to the central reservoir, the removal of excess media from central reservoir to waste reservoir with a steady flow rate. These strains were serially exposed to increasing concentration of penicillin starting from ½ the predetermined Minimum Inhibitory Concentration (MIC) of these isolates. The concentration of penicillin was increased after every 72 hours, serially in the following order starting from 0.05 µg/ml, 0.1 µg/ml, 0.2 µg/ml, 0.4 µg/ml, 0.8 µg/ml, 1 µg/ml, 1.2 µg/ml, 1.4 µg/ml, 1.6 µg/ml, 1.8 µg/ml and 2 µg/ml. Altogether a serial exposure of increasing concentration of penicillin was done for 10 times using the *in vitro* model [8]. MIC values of all four isolates were re-determined after serial passage to find out the changes induced in MIC values by serial exposure to increasing concentrations of penicillin in beta–lactam group (structurally related group), and in other antimicrobial agents (structurally unrelated group).

All the universal safety precautions were observed during this study to protect the workers and all the potentially harmful organisms (resistant to antimicrobial agents) produced during the study were destroyed by using appropriate sterilization methods.

Results:

Table 1 shows MIC of four selected isolates of VGS before and after development of drug resistance to penicillin and its effect on the susceptibility pattern of other beta–lactam antibiotics. Using this in vitro model, isolates of VGS were exposed to continuous culture system, to which increasing concentration of penicillin was added sequentially to induce penicillin resistance in these four isolates. MIC values of the original isolates of VGS were in the range of 0.06µg/ml to 0.12µg/ml. After exposure to increasing concentrations of penicillin, these strains developed resistance to penicillin and their MIC values against penicillin were increased up to 2µg/ml to 32µg/ml of penicillin. The antimicrobial susceptibility pattern after induction of drug resistance showed a major increase in MIC to penicillin and ampicillin, whereas no major changes in susceptibility were observed in other beta – lactam antibiotics. Table 2 shows MIC of four selected isolates of VGS to other antimicrobial agents (structurally unrelated group) before and after development of drug resistance to penicillin. The in vitro development of resistance to penicillin showed no major effect on the susceptibility pattern of other antimicrobial agents, except for slight increase in MIC values of erythromycin, azithromycin, clindamycin, cefotaxime and cefitriaxone in some of the isolates.

Table 1: MIC of VGS Strains against Structurally Related Antimicrobial Agents (Beta – lactams) before and after Induction of Resistance to Penicillin

S.N.	Source	Serotype	PEN	AMP	FEP	CTX	CRO
1	**Blood**	*S. oralis*-O	0.12	0.12	0.25	0.5	0.12
		S. oralis-M	16	08	0.25	0.5	0.12
2	**Blood**	*S. sanguinis*-O	0.12	0.25	0.12	0.25	0.25
		S. sanguinis-M	08	16	0.25	0.5	0.25
3	**Blood**	*S. mitis*-O	0.12	0.25	0.5	0.5	0.5
		S. mitis-M	32	16	0.5	0.5	0.5
4	**Sub gingival plaque**	*S. mutans*-O	0.06	0.06	0.12	0.06	0.06
		S. mutans- M	02	0.5	0.12	0.06	0.12

O- original, M-mutant, PEN- penicillin, AMP- ampicillin, FEP- cefepime, CTX- cefotaxime, CRO- ceftriaxone

Table 2: MIC of VGS Strains against Structurally Unrelated Antimicrobial Agents (Agents other than Beta – lactams) before and after Induction of Resistance to Penicillin

S.N.	Source	Serotype	VAN	ERY	AZM	CLR	TCY	LVX	OFX	CLI	LNZ	QDA
1	**Blood**	*S. oralis*- O	0.12	0.25	0.25	0.12	1	0.5	2	0.25	0.5	1
		S. oralis- M	0.12	0.5	0.5	0.12	1	0.5	2	0.5	0.5	1
2	**Blood**	*S. sanguinis*- O	0.25	0.12	0.5	0.25	0.5	1	1	0.25	1	0.5
		S. sanguinis- M	0.25	0.25	0.5	0.25	0.5	1	1	0.25	1	0.5
3	**Blood**	*S. mitis*- O	0.25	0.25	0.5	0.25	2	1	2	0.25	2	1
		S. mitis- M	0.25	0.5	1	0.25	2	1	2	0.5	2	1
4	**Sub gingival plaque**	*S. mutans*-O	0.06	0.12	0.06	0.06	0.25	0.5	0.5	0.06	0.25	0.06
		S. mutans- M	0.06	0.25	0.12	0.12	0.25	0.5	0.5	0.06	0.25	0.06

O-original, M-mutant, VAN-vancomycin, ERY-erythromycin, AZM-azithromycin, CLR- clarithromycin, TCY-tetracycline, LVX- levofloxacin , OFX-ofloxacin, CLI- clindamycin, QDA- Quinupristin/Dalfopristin, LNZ-Linezolid.

Discussion:

In the present study, selected blood and subgingival plaque isolates of VGS were exposed to sequential increasing concentration of penicillin in the *in vitro* drug resistance model containing a special medium to develop an environment mimicking human *in vivo* conditions, to induce resistance to penicillin in all the four isolates of VGS, as evidenced by their increase in MIC (Table 1).

The *in vitro* model used in this study to induce drug resistance in the study organisms has demonstrated distinct advantages when compared to previously used methods to study drug resistance. This model not only offers an advantage to determine optimum dosing schedules, but also helps in revealing the mechanisms of resistance development. Historical *in vitro* methods for evaluating efficacy of antibiotics suffer from two fundamental shortcomings. The first is that antibiotic concentrations remain static; they are not varied in a dynamic fashion as they would be when administered *in vivo*. Flux in concentration of antibiotic should reflect the adsorption rate, bioavailability, volume of distribution and excretion rate. None of these parameters can be controlled using current methods. The second shortcoming is the number of organisms exposed to the drug is necessarily limited so mechanisms of resistance cannot be studied effectively.

A major change in the MIC of VGS to penicillin and ampicillin after exposure to penicillin in the *in vitro* model indicates development of resistance to penicillin and cross resistance to ampicillin– a structurally related antimicrobial agent to penicillin. Also changes in MIC of erythromycin and azithromycin have been observed, indicating development of low level of cross resistance following exposure to penicillin in these agents which are not structurally related to penicillin. No major changes in the MIC of tetracycline,

cephems, fluroquinolones, streptogramins, glycopeptides, oxazolidinones indicates that no cross resistance has been developed to these antimicrobial agents (Table 2).

In one study, norfloxacin resistance was induced in nine clinical isolates of coagulase negative staphylococci by means of serial passage in brain heart infusion broth containing increasing concentrations of norfloxacin [9]. In another study, resistance to ciprofloxacin was induced in *P. aeruginosa* by exposing the organism in separate culture media containing increasing concentrations of ciprofloxacin, a total of 14 serial passages were carried out to induce drug resistance in the microorganism [8]. Similarly, in the present study, it was possible to induce drug resistance in VGS strains to penicillins using the *in vitro* model. We induced drug resistance in all the selected VGS strains capable of causing infective endocarditis and dental caries in humans, which included *S. sanguinis, S. oralis, S. mitis* and *S. mutans.*

The finding of this study that development of resistance to penicillin has been associated with development of low level of cross resistance to erythromycin and azithromycin is similar to earlier report in which development of low level of resistance to antimicrobial agents such as cefepime, amikacin and imipenem (structurally unrelated antibiotics) following exposure to ciprofloxacin *in vitro* has been reported which is evidenced by MIC determination before and after exposure to ciprofloxacin [10]. In another study, exposure of coagulase negative staphylococci to norfloxacin has resulted in 18 to 20 times increase in MIC of norfloxacin and change in *in vitro* susceptibility to ciprofloxacin, pefloxacin, ofloxacin, kanamycin, neomycin and tobramycin, indicating development of cross resistance to fluoroquinolones and aminoglycosides. Their results show that exposure to increasing concentrations of norfloxacin can induce the development of resistance to various antimicrobial agents [9]. In the present study also, on exposure to increasing concentration of penicillin, the VGS strains have developed low level of resistance to erythromycin, azithromycin, clindamycin, cefotaxime in some of the isolates along with development of resistance to penicillins.

Conclusion:

Exposure of susceptible VGS to sublethal concentration of penicillin results not only into development of resistance to penicillin as evidenced by increased MIC values, but also results into development of resistance to other antibiotics. These results show that administration of antibiotics in adequate doses for appropriate time period is most critical part in avoidance of development of resistance during therapy.

This *in vitro* model not only served to induce drug resistance in the study organisms, but also proved to be an effective model that could mimic human *in vivo* conditions. This model could be a helpful tool for the future researchers in understanding the mechanisms of development of the drug resistance by the microorganisms in a more realistic way.

Further studies on molecular basis of development of resistance in VGS and its clinical significance are necessary to achieve more useful conclusions.

References

1. Doern GV, Ferraro MJ, Brueggemann AB, Ruoff KL. Emergence of high rates of antimicrobial resistance among viridans group streptococci in the United States. *Antimicrob Agents Chemother* 1996; 40:891-894.
2. Farber BF, Eliopoulos GM, Ward JI, Ruoff KL, Syriopoulou V, Moellering RC Jr. Multiply resistant viridans streptococci: susceptibility to beta-lactam antibiotics and comparison of penicillin-binding protein patterns. *Antimicrob Agents Chemother* 1983; 24:702–705.
3. Pottumarthy S, Morris AJ. Detection of decreased penicillin susceptibility in viridans group streptococci. *Pathology* 1998; 30:188–191.
4. Jones RN, Wilson WR. Epidemiology, laboratory detection, and therapy of penicillin-resistant streptococcal infections. *Diagn Microbiol Infect Dis* 1998; 31:453–459.
5. Garcia-de-la-Maria C, Pericas JM, Del Rio A, Castaneda X, Vila-Farres X, Armero Y, Paula A. Espinal, Carlos Cervera, Dolors Soy, *et al*. Early *in vitro* and in vivo development of high-level daptomycin resistance is common in Mitis group streptococci after exposure to daptomycin. *Antimicrob Agents Chemother 2013*; 57:2319–2325.
6. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; Twenty-third informational supplement. Wayne, PA, USA: CLSI document M100-S23; 2013.
7. McFarland J. Nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *J Am Med Asso* 1907; 14:1176-1178.
8. Nagoba BS, Deshmukh SR, Wadher BJ, Gude UG, Gomashe AV, Tumane PM. Induction of resistance to ciprofloxacin and other fluoroquinolones in clinical and environmental isolates of *Pseudomonas aeruginosa*. *Ind J Med Microbiol* 1998; 16:29-30.
9. Deshmukh SR, Nagoba BS, Wadher BJ, Tumane PM. Norfloxacin induced resistance to fluoroquinolones & structurally unrelated antimicrobial agents in coagulase negative staphylococci. *Ind J Med Res* 1997; 106:461-464.
10. Joan Fong- Tomc, Benjamin K, Bonner DB. Ciprofloxacin induced low level resistance to structurally unrelated antibiotics in *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1993; 37:1289-1296.

**** Author for Correspondence:*** *Dr. B. S. Nagoba, Assistant Dean (Research & Development), Maharashtra Institute of Medical Sciences & Research, Latur-413 531,M.S., India Email: dr_bsnagoba@yahoo.com, bsnagoba@gmail.com Cell: 9423075786/7588237531*