I J R B A T, Issue (X) Vol (II) May 2022: 234-241
A Double-Blind Peer Reviewed & Refereed Journal
OPEN ACCESS
e-ISSN 2347 – 517X
Original Article

INTERNATIONAL JOURNAL OF RESEARCHES IN BIOSCIENCES, AGRICULTURE AND TECHNOLOGY
 www.ijrbat.in

TO STUDY RELATIONSHIP BETWEEN BIOFILM FORMATION AND ANTIBIOTICS RESISTANCE OF GRAM POSITIVE COCCI ISOLATED FROM DENTAL CARIES

S.N. Gawande* and V.D.Nanoty
Department of Microbiology, Shri. R. L. T. College of Science, Akola.
Email: sonalingawande@gamil.com

Communicated :18.03.2022 | Revision : 22.03.2022 Accepted :05.04.2022 | Published: 02.05.2022

ABSTRACT: Biofilm have been concerned as the main source of etiopathogenesis of dental caries, tooth decay and associated diseases. These biofilms establish a great challenge to the dental practitioner in the control and suppression of biofilm associated periodontal diseases and decaying of tooth. Out of these total 140 isolates, 98 isolates was found to be biofilm producer and 42 were biofilm non produces. These biofilm forming Gram Positive bacterial was identified by VITEK 2 instrument. Antibiotic resistance is one of the principal intimidations to worldwide health, food security and development. Streptococcus mutans along with most of the gram positive cocci was believed to be the major biofilm producer contributing in periodontal disease. In this article the relation between biofilm forming and antibiotic susceptibility of Gram positive cocci isolates was studied. Isolates from dental caries was identified and antibiotic sensitivity was also done by VITEK-2 compact system. Streptococci spp. has been showed to be significantly more resistance to antibiotic with the cause of tooth decay.

Key words: - Dental caries, Biofilm, VITEK 2, Gram positive cocci.

INTRODUCTION :

The buckle cavities have one of the maximum surface areas for microbial multiplication in human body. The dental biofilm forms initial selective adsorption of bacteria on tooth surface (Heller et al, 2015). The microflora existing in oral cavity is called oral micro flora. One of the commonly encountered problems in dentistry is loss of teeth and consequential replacement. Along with the restoration of function and aesthetic, removable prosthesis may change the oral ecology either qualitatively or quantitatively, such as increasing the total amount of oral microorganism (Azizah AL Mobereek, 2003). Oral health may lead to many oral problems such as formation of biofilm, dental caries, oral and facial pain, problems with the heart and other major organs, digestion problems and periodontal diseases. Periodontitis is frequent health difficulty caused by pathogenic biofilm forming bacteria that accelerates inflammation resulting in either reversible gingivitis or severe periodontal damage, leading to loss of healthy tooth (Gutt et al, 2018).

Biofilm have been concerned as the main source of etiopathogenesis of dental caries, tooth decay and associated diseases. However biofilm can be removed by regular oral hygiene aids or specialized dental instruments, they have the capacity to set into dental calculus making their taking away too difficult. Consequently, these biofilms establish a great challenge to the dental practitioner in the control and suppression of biofilm associated periodontal diseases and decaying of tooth.

Dental caries is the localized destruction of dental hard tissue by acidic by products from dental plaque containing acid producing bacteria (Yu O et al, 2017). Establishment of a biofilm is a multifaceted progression that follows more than a few distinctive phases, commencing with adsorption on to the tooth surface of a habituation film derived from bacterial and host molecules finally results into tooth break or

tooth cleanout. This aggregation is followed by passive transport of bacteria regulated by feeble long-range forces of attraction. Irreversible attachment occurs due to strong and weak forces created by covalent and hydrogen bonds.

Biofilms possess several properties like they are ubiquitous and form on almost all surfaces engrossed in ordinary aqueous environments. Biofilm confers assured properties to bacteria that are not usually found in the planktonic condition, this justifies credit of dental plaque as a biofilm.

Antibiotic resistance is one of the principal intimidations to worldwide health, food security and development. By alterations of bacterial genes in response to use of excess medicine or antibiotic drug, antibiotic resistivity gets occurred. Finally develops resistance. These resistant bacteria possibly will infect human and causes infections which are difficult to treat than sensitive bacteria (Guidelines by WHO). Day by day antibiotic resistance emerges rapidly at very high level in all parts of world. For that reason to avoid and manage the extended use of antibiotic for avoiding resistance in diverse species of microbes, health professional can use only the local antibiotics, in specific cases; by following the guideline given by WHO. Hence the scope of present study is to exploit possible cure oral infection.

Innovation in the field of science imparts a new level isolation and identification in Life Sciences. Mechanization in Medical microbiology be present in a very primitive stage of advancement as compared to other field as in Clinical chemistry, hematology and immunology laboratories from last two decades a number of computerized systems used for the identification and antimicrobial susceptibility testing (AST) of microorganisms. Theses Computerized mechanism based on automated analysis of the outcomes of biochemical tests and microdilution trays subsequent overnight incubation and turbid metric determination of growth (Ligozzi et al 2002). Due to more clinical and financial benefits rapid bacterial identification and AST are now recognized by Advances in technology. The VITEK system originated in the 1970s as an automated system for identification and AST and has evolved today into the VITEK 2 system, which automatically performs all of the steps required for identification and AST after a primary inoculus has been prepared and standardized (Jubair, 2015). This system allows kinetic analysis by reading each test every 15 min. The optical system combines multichannel fluorimeter and photometer readings to record fluorescence, turbidity, and colorimetric signals. In This study The VITEK 2 Compact (30 card capacity) system was used , which uses a fluorogenic methodology for organism identification and a turbidometric method for susceptibility testing using a 64 well card that is barcoded with information on card type, expiration date, lot number and unique card identification number. Test kits available include ID-GN (gram negative bacillus identification), ID-GP (gram positive cocci identification), AST-GN (gram negative susceptibility) and AST-GP (gram positive susceptibility). The VITEK 2 ID-GN card identifies 154 species of *Enterobacteriaceae* and a select group of glucose non fermenting gram negative organisms within 10 hours. The VITEK 2 ID-GP card identifies 124 species of *staphylococci*, *streptococci*, *enterococci* and a select group of gram positive organisms within 8 hours or less. The VITEK 2 Antimicrobial Susceptibility Tests (AST) is for most clinically significant aerobic gram negative bacilli, *Staphylococcus spp.*, *Enterococcus spp.*, and *Streptococcus agalactiae*. Susceptibility results been available for bacteria in less than 18 hours. The databases of the VITEK 2 identification products are assembled with huge strain sets of well- differentiated microorganisms tested under

various culture conditions. These strains are derivate from a variety of clinical and industrial sources as well as from public (e.g., ATCC) and university culture collections.

Therefore present study is aim at to identify and to check antimicrobial susceptibility testing of Gram positive cocci by VITEK 2 instrument.

MATERIALS AND METHODS:

Collection of Samples:

A total 140 samples (Swab as well as extracted tooth) were collected from mouths of patients referring to dental health care centers in Akola, Maharashtra. These patients of both the genders with the mean age 28 yrs. Patients Swab sample and extracted tooth were placed in tubes contain Trypticase soy broth for 24 hrs. Then samples from broth were inoculated Congo red agar for detection of biofilm formation. Further inoculated on Mitis Salivarus agar and Blood agar aerobically at 37 °C for 24 hrs.

Identification:

After incubation, isolates were identified by colony character, gram staining. These tests were performed on all swab samples and tooth decay as per standard procedure. Gm positive cocci identified by using Vitek- 2 compact system (Biomerierx) according to the manufacturers instruction.

Biofilm Formation:

Suspension of tested strains was incubated in glass tubes containing brain heat infusion broth aerobically at 37 °C for 24- 48 hrs. After incubation Supernatant was discarded then tubes has been stained by 0.1% crystal violet solution, washed with Distilled water three times and dried naturally. Presence of a violet structure on internal wall and the bottom of test tubes confirmed biofilm formation and organism was identified as biofilm produces. And bacteria which was failed to produce such Structures were considered to be non-biofilm produces.

Inoculum preparation:

Suspensions were prepared by emulsifying bacterial isolates in 0.45% saline to the equivalent of a 0.5 McFarland turbidity standard. The same suspension was used for identification and AST for the VITEK 2 system. Suspensions for the comparative identification method were made according to the manufacturer's recommendations (VITEK 2 The Basics Manual, 2013).

Identification by VITEK 2:

The test panels (ID-GPC) contained 64 fluorimetric tests that included pH change tests and derivatives to detect aminopeptidases and -osidases. Substrates used for detection of aminopeptidases are coupled with 7-amino-methylcoumarin (7AMC); substrates for the detection of -oxidases are usually coupled with 4-methylumbelliferone (4MU). The 21 test substrates are as follows: 4M -α -L-arabinofuranoside, 4MU-α-D-galactoside, 4MU-α -D-glucoside, 4MU- α -D-N-acetylneuraminic acid, 4MU-β-D-galactoside, 4MU- β- α-D-glucoside, 4MU- β -D-glucuronide, 4MU- β -D-mannoside, 4MU-n- β acetyl-D-glucosaminide, 4MU-phosphate, alanine-7AMC, arginine-7AMC, aurease (butiloxicarbonyl-Val-Pro-Arg-AMC), histidine-7AMC, α -glutamic acid7AMC, threonine-7AMC, lysine-7AMC, phenylalanine-7AMC, proline-7AMC, pyroglutamic acid-7AMC, and tyrosine-7AMC. Additionally, the ID-GPC card includes 16 fermentation tests (for D-raffinose, amygdaline, arbutine, D-galactose, glycerol, D-glucose, L-arabinose, lactose, D-maltose, D-mannitol, N-acetylglucosamine, salicin, D-sorbitol, D-trehalose, D-melibiose, and D-xylose), two decarboxylase tests (for ornithine and arginine), and six miscellaneous tests (for urease, pyruvate, optochin, novobiocin, polymyxin B sulfate, and 6% NaCl). The card was mechanically filled by a vacuum device, sealed and inserted into the VITEK 2 reader-incubator module (incubation temperature,

35.5°C), and exposed to a kinetic fluorescence measurement every 15 min. The results were interpreted by the ID-GPC database, and final results were obtained mechanically. All cards used were mechanically discarded into a waste container (VITEK 2 The Basics Manual, 2013).

Antibiotic Sensitivity Test (AST) by Vitek-2 Compact:

One drop of the broth was routinely subculture on 5% sheep blood agar plates were incubated at 37°C for 18-20 hours to obtain isolated colonies. An isolated colony was picked and added to sterile saline solution provided by the manufacturer BioMerieux to make a suspension equivalent to a 0.5 McFarland standard, adjusted by using a DensiCHEK Plus (BioMerieux) and further processed as per the manufacturer's instruction. AST panel AST-P628 and AST-03 was used for gram positive cocci. Result was interprets as Sensitive (S), Intermediate (I) and Resistant (R) within 6 hrs.

RESULT:

Total 140 samples collected from Dental clinics for the study. Out of these 98 isolates was found to be biofilm producer and 42 were biofilm non produces which are excluded from further identification as shown in figure 1. Out of 98 isolates 90 were found to be Gram positive which was later applied for Identification by VITEK 2 as shown in figure 2. The database of VITEK 2 identification products are prepared with huge strains sets well classified microorganism tested under various culture conditions. These strains are derived from a different medical, industrial sources and public (ATCC/MTCC) and university culture collections. Test data from an unknown organism are compared to the respective database to conclude a quantitative value for vicinity to each of the database taxa. Each of the complex values is compared to the others to determine if the data are appropriately unique or close to one or more of the other database taxa. If a unique identification pattern is not recognized, a list of possible organisms is given, or the strain is determined to be outside the scope of the database. An unknown bio pattern is compared to the database of reactions for each taxon, and a numerical probability calculation is performed. Various qualitative levels of identification are allotted based on the numerical probability calculation. The different levels and associated information are shown in Table 2.

VITEK 2 is compact system identifies organism on the basis of positive biochemical reactions shown by unknown organism. By which *Staphylococcus hominis* (95%), *Staphylococcus sciuri* (99%), *Staphylococcus epidermidis* (92%), *Staphylococcus aureus* (99%),*Staphylococcus gallinarum* (99%) , *Streptococcus pneumonea* (95%), *Streptococcus mutans* (97%), *Streptococcus salivaris* (98%), *Streptococcus pyogenus*(95%), *Streptococcus parasingunus* (96%) was identified(Table 4&5) Where as a study carried out by Ligozzi et al (2002), evaluation VITEK 2 system for identification and antimicrobial Susceptibility testing for medically relevant Gram positive cocci was carried out , in their study S. aureus (96.5%) , of *S.agalactiae* 96.9%, 92.7% of *Enterococcus fecalis* , 93.1% *Staphyloccos heamolyticus* and 88% of *Staphylococcus epidermidis* was identified within 3hrs. In the study of Nimer et al, (2016) identifies 74 Gram positive cocci by using VITEK 2 ID cards and AST card. Antibiotic sensitivity pattern of identified organism was carried with the same system with 16 antibiotics with their minimum inhibitory concentration (MIC). Each Antibiotic is having specific MIC pattern with respective species. As shown in Table 4, *S. heamolyticus* shows resistance against Benzyl penicillin, Oxacillin, Ciprofloxaacin, levofloxacin, Erthromycin, Cincyamicin. *S. epidermidids* shows resistance against erythromycin and Trimethoprim whereas *S. hominis* was sensitive

to all antibiotics. While coagulase positive *S. aureus* and show resistance against Benzyl penicillin, Ciprofloxacin ,levofloxacin and coagulase neagative *S. gallinarum* was found to be resistant against Clinamycin and Nitrofurontoin.

As Shown in Table 5. *Str. pneumonea* was fund to be resistant against ampicillin with intermediate sensitivity against Nitrofurantoin. Str. Mutant which is the major cause of dental carries and tooth decay shows maximum resistivity against number of antibiotic like Amoxillin, Cepoperazon, Cefipime, Gentamycin and Tiegecyclin. Where Str.salivaris was resistant to Amphicillin , Amikacin, Tigecyclines, ceftriaxone. Furthermore Str. pyogenes was found to be resistant against Amoxicillin, Meropenem, Ceftrixone while *Str. parasuinguins* was Cefuroxime and Ceftriaxone resistance. Moreover in this study it was found the Gram positive streptococci were found to be more resistant against most of the antibiotic as shown in table 5. Jubair (2015) believed that *Streptococcus mutans* were the principal etiological agent of human dental caries has become a significant species in cariogenic biofilm. In this article the correlation between biofilm forming and antibiotic susceptibility of *S. mutans* isolates was studied and identification by VITEK-2 compact system and antibiotics susceptibility by disk diffusion method. The results showed S. mutants the high percentage (71.4%) in comparison with the other streptococcus species from total dental caries samples. Out of 54 (90%) *S. mutants* have ability to forming biofilm. The high over all proportion of *S. mutans* produce biofilm. *S. mutans* biofilm have been showed to be significantly more resistance to antibiotics.

The result obatained by VITEK 2 system is fast and relaibale as compared to conventional method. The presentation of the VITEK® 2 system for direct rapid identification and antimicrobial susceptibility testing of the bacteria responsible for blood infections was determined by Nimer et al in 2016. The isolates studied included 166 Gram-negative rods and 74 Gram-positive cocci from inpatients. Compared with the standard method, 95.8% of Gram-negative rods were correctly identified by VITEK 2 and the overall level of agreement between the two methods in susceptibility testing was 92.0%. As 89.2% Gram-positive bacteria and 91.3% Gram negative bacteria were correctly identified by VITEK 2 and antimicrobial susceptibility testing discovered an overall covenant rate of these results suggest that VITEK 2 cards inoculated with fluids sampled directly from positive blood culture bottles are suitable for speedy identification and susceptibility testing of Gram-negative bacilli and Gram-positive cocci.

In Conclusion the result of study it was shown that maximum all proportion of gram positive *streptococci* produces biofilm identified by VITEK 2 and significantly more resistant to antibiotic.

REFERENCES:

Al-Mobeireek AZ. Qualitative changes in oral flora before and after the insertion of removable prosthesis. Pak Oral Dent J. 2003;23:51-6.

bioMerieux Vitek, IncGNI technical bulletin.1996bioMerieux Vitek, Inc.Hazelwood, Mo

Gutt B, Ren Q, Hauser-Gerspach I, Kardas P, Stübinger S, Astasov-Frauenhoffer M, Waltimo T. Beneficial oral biofilms as smart bioactive interfaces. Frontiers in Microbiology. 2018 Jan 31;9:107.

Heller D, Helmerhorst EJ, Gower AC, Siqueira WL, Paster BJ, Oppenheim FG. Microbial diversity in the early in vivo-formed dental biofilm. Applied and environmental microbiology. 2016 Mar 15;82(6):1881-8.

Heller D, Helmerhorst EJ, Gower AC, Siqueira WL, Paster BJ, Oppenheim FG. Microbial diversity in the early in vivo-formed dental biofilm. Applied and environmental microbiology. 2016 Mar 15;82(6):1881-8.

Jubair HH. The relationship between biofilm forming and antibiotics resistance of streptococcus mutans isolated from dental caries. Int. J. Curr. Microbiol. App. Sci. 2015;4(5):568-74.

Ligozzi M, Bernini C, Bonora MG, De Fatima M, Zuliani J, Fontana R. Evaluation of the VITEK 2 system for identification and antimicrobial susceptibility testing of medically relevant gram-positive cocci. Journal of clinical microbiology. 2002 May;40(5):1681-6.

Monteiro AC, Fortaleza CM, Ferreira AM, Cavalcante RD, Mondelli AL, Bagagli E, da Cunha MD. Comparison of methods for the identification of microorganisms isolated from blood cultures. Annals of Clinical Microbiology and Antimicrobials. 2016 Dec;15(1):1-1.

Nimer NA, Al-Saa'da RJ, Abuelaish O. Accuracy of the VITEK 2 system for a rapid and direct identification and susceptibility testing of gram-negative rods and gram-positive cocci in blood samples. EMHJ-Eastern Mediterranean Health Journal. 2016;22(3):193-200.

Pincus DH. Microbial identification using the bioMérieux Vitek® 2 system. Encyclopedia of Rapid Microbiological Methods. Bethesda, MD: Parenteral Drug Association. 2006:1-32.

Table 2. Identification level probability

Table 2. Identification Levels		
Sr. No.	**ID Message Confidence level**	**% Probability**
1	Excellent	96-99%
2	Very Good	93-95%
3	Good	89-92%
4	Acceptable	85-88%
5	Low Discrimination	N/A
6	Unidentified organism	Very atypical bio pattern. Does not correspond to any taxon in database.

Figure 1 Showing Number of Biofilm producer and Non Biofilm producers

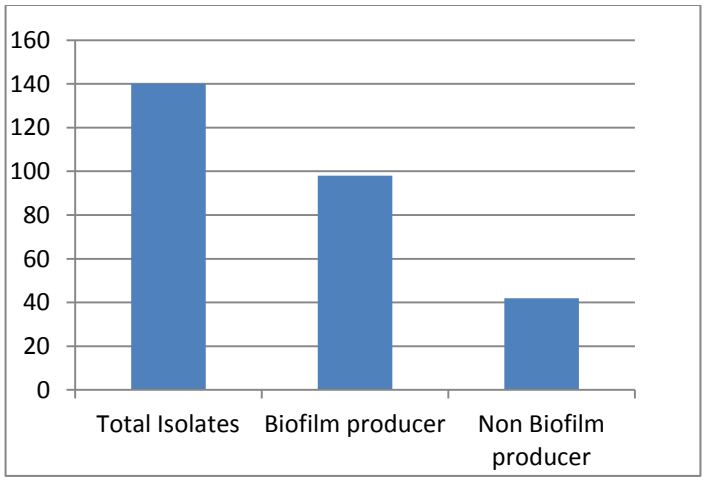

Figure 2 Showing percent distribution of Gm + ve and Gm -ve isolates

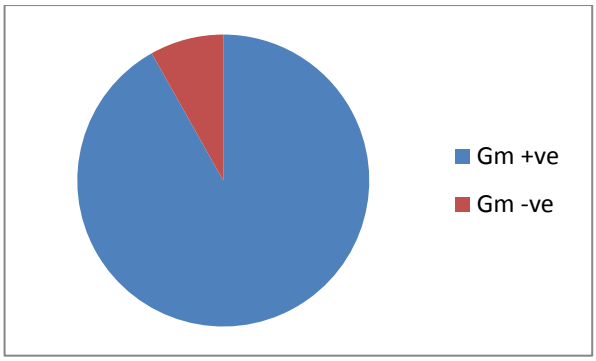

Figure. Showing number of isolates

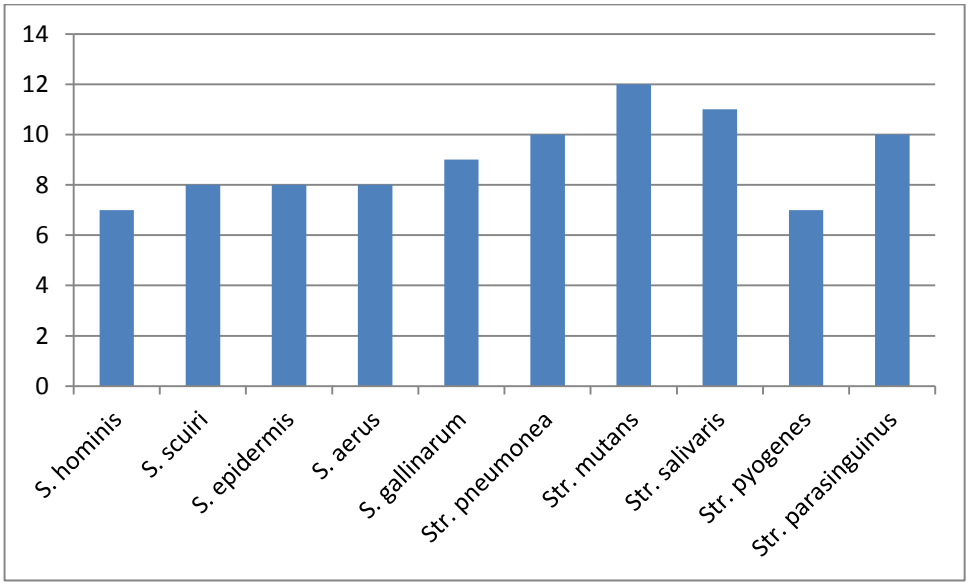

Table 3. Showing sample distribution percentage with probability

Sr. No.	Isolates	No. of Isolates	Percentage%	Probability % by VITEK 2
1.	*S. hominis*	7	7.7	93%
2.	*S. scuiri*	8	8.8	99%
3.	*S. epidermidis*	8	8.8	92%
4.	*S. aerus*	8	8.8	99%
5.	*S. gallinarum*	9	10	99%
6.	*Str. pneumonea*	10	11.1	95%
7.	*Str. mutans*	12	13	97%
8.	*Str. salivaris*	11	12	98%
9.	*Str. pyogenes*	7	7.7	95%
10.	*Str. parasinguinus*	10	11.1	96%
	Total	90	100%	

Table 4. Antibiotic Susceptibility pattern shown by identified organism (*Staphylococci spp)*

Antibactrial Agaent	***S. heamolyticus***		***S. epidermidis***		***S. hominis***		***S. scuiri***		***S.aurreus***		***S. gallinarum***	
	MIC	**INTR**	**MIC**	**INTR**	**MIC**	**INTR**	**MIC**	**INTR**	**MIC**	**INTR**	**MIC**	**INTR**
Benzyl penicillin	>=0.5	**R**	<=0.03	S	<=0.3	S	0.06	S	>=0.5	**R**	<=0.3	S
Oxacillin	>=4	**R**	>=0.25	S	<=0.25	S	2	S	0.5	S	<=0.25	S
Gentamycin	<=0.5	S	<=0.5	S	<=0.5	S	<=0.5	S	<=0.5	S	<=0.5	S
Ciprofloaxacin	>=8	**R**	<=0.5	S	<=0.5	S	<=0.5	S	>=8	**R**	<=0.5	S
levofloxacin	>=8	**R**	1	S	<=0.12	S	0.5	S	4	**R**	<=0.12	S
Erythromycin	>=8	**R**	>=8	**R**	>=8	S	<=0.25	**R**	<=0.25	S	>=8	S
Clindamycin	>=4	**R**	0.25	S	<=0.12	S	0.5	S	<=0.12	S	<=0.12	**R**
Linezolide	2	S	1	S	2	S	2	S	2	S	2	S
Daptomycin	1	S	0.5	S	1		2	S	0.5	S	1	
Teicoplanin	4	S	16	I	>=32	S	2	**R**	<=0.5	S	>=32	S
Vancomycin	1	S	4	S	1	S	<=0.5	S	<=0.5	S	1	S
Tetracyclin	2	S	<=1	S	<=1	S	<=1	S	<=1	S	<=1	S
Tigecycline	0.5	S	<=0.12	S	<=0.12	S	<=0.12	S	<=0.12	S	<=0.12	S
Nitrofurantoin	<=16	S	<=16	S	<=16	S	<=16	S	<=16	S	<=16	**R**
Rifampicin	<+0.0	S	0.03	S	<=0.03	S	<=0.03	S	<=0.03	S	<=0.03	S
Trimethoprim/ Sulphamethoxazole	<=10	S	>=320	**R**	<=10	S	<=10		<=10	S	<=10	S
Where, R= Resistant, I= Intermediate, S= Sensitive												

Table 5. Antibiotic Susceptibility pattern shown by identified organism (*Streptococci spp.)*

Antibacterial Agent	***Str. pneumonea***		***Str. mutans***		***Str. salivarius***		***Str. pyogenes***		***Str. parasanguinis***	
	MIC	**INTR**	**MIC**	**INTR**	**MIC**	**INTR**	**MIC**	**INTR**	**MIC**	**INTR**
Ampicillin	>=32	**R**	<=0.3	S	>=32	**R**	0.06	S	>=32	S
Amoxillin	<=2	S	<=0.25	**R**	<=2	S	2	**R**	<=2	S
Piperacillin	<=4	S	<=0.5	S	<=4	S	<=0.5	S	<=4	S
Cefuroxime	<=1	S	<=0.5	S	<=1	S	<=0.5	S	<=1	**R**
Cefuroxime	<=1	S	<=0.12	S	<=1	S	0.5	S	<=1	S
Ceftriaxone	<=1	S	>=8	S	<=1	**R**	<=0.25	**R**	<=1	**R**
Cefoperazon	<=8	S	<=0.12	**R**	<=8	S	0.5	S	<=8	S
Cefepime	<=1	S	2	**R**	<=1	S	2	S	<=1	S
Ertapenem	<=0.5	S	1	S	<=0.5	S	2	S	<=0.5	S
Imipenem	<=0.25	S	>=32	S	<=0.25	S	2	S	<=0.25	S
Meropenem	<=0.25	S	1	S	<=0.25	S	<=0.5	**R**	<=0.25	S
Amikacin	<=2	S	<=1	S	<=2	**R**	<=1	S	<=2	S
Gentamicin	<=1	S	<=0.12	**R**	<=1	S	<=0.12	S	<=1	S
Nalidixic acid	4	S	<=16	S	4	S	<=16	S	4	S
Ciprofloxacin	<=0.25	S	<=0.03	S	<=0.25	S	<=0.03	S	<=0.25	S
Tigecyclines	<=0.5	S	<=10	**R**	<=0.5	**R**	<=10	S	<=0.5	S
Nitrofurantoin	64	I	<=8	S	64	S	<=1	S	64	S
Colistin	<=0.5	S	<=1	S	<=0.5	S	<=1	S	<=0.5	S
Trimethoprim	<=20	S	<=0.5	S	<=20	S	<=1	S	<=20	S
Where, R= Resistant, I= Intermediate, S= Sensitive										