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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF ORAL MICROFLORA AND STUDIES ON THEIR BIOFILM PRODUCTION ABILITY AND ANTIBIOGRAM PATTERN

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ABSTRACT

Oral bacteria play an important role in body and the bacterial genus streptococcus is the dominant microflora commonly found in oral bacteria community. A biofilm is a complex structure that can be found almost everywhere associated with water. Present investigation has been carried out to study the biofilm producing organism from normal and infected oral flora and further study of their antibiotic resistance pattern. The main objective of the study was to isolate and identify the oral microflora and their biofilm production ability. In the present study total 20 mouth samples were collected from different area. During the study different samples were collected including different age group people, age group under consideration for the study was ranging between 22-16 years,We obtained 9 isolates from different samples namely S. aureus, M. luteus, Lactobacillus species, P. aeruginosa, E. coli, S. Marscence, Veillonella parvula, Streptococcus mutans, Bacillus subtilis. The isolate were screen for the biofilm formation and was evaluated qualitatively by Congo red agar medium and tube assay method. Antibiotic sensitivity testing was performed using the Kirby -Bauer disk diffusion method. Overall results revealed that Ciprofloxacin is the best antibiotic in controlling the oral infection.

INTRODUCTION

Tooth decay also known as dental caries, cavities or caries is a breakdown of teeth due to activities of bacteria (Silk, H. 2014). The cavities may be a number of different colors from yellow to black (Laudenbach J. M.; Simon, Z, 2014) complications may include inflammation of the tissues around the tooth, tooth loss, and infection or abscess formation. (F.A. Davis, 2013)

Caries is also associated with poverty, poor cleaning of the mouth and receding gums resulting in exposure of the roots of the teeth (Schwendicke *et al*., 2015) Dental caries is essentially a surface phenomenon and microorganisms behave distinctively when grown on a surface. Dental caries continues to plaque most of the world's population despite overly optimistic claims of success in the elimination of this disease it is indeed true that the prevalence of dental caries has declined in some segment of the population countries but the level of decline has been exaggerated (Sohn W., *et al.*, 2007).

Biofims are composed of primarily of microbial cells and exopolysacchride (EPS) (Flemming H. C. *et al.*, 2000). The role of biofilm is attach to abiotic surface, the epithelia of multicellular organism, and interfaces such as that between air and water (Kodali V. P. and Sen R., 2011). Biofilm may form on a wide variety of surface, including living tissues, indwelling medical device industrial or portable water system piping, or natural aquatic system (Donlan R. M., 2002)

Hence the present study was aimed to study the biofilm producing organism from oral flora and study of their antibiotic resistance pattern.

MATERIALS AND METHOD

Collection of sample

Total 20 samples were collected from normal oral and infectious mouth flora different area people and including different age group. The mouth samples swab viz dental caries, dental plaque, pyrea, tobacco chewers, gutka, ulcers and normal people were collected.

Isolation of biofilm forming microorganisms, morphological and biochemical characterization is done on the basis of Bergey's manual of determinative bacteriology (Buchanan R. E. and Gibbons N. E., 1974).

Detection of biofilm formation

The obtained isolates were further screened for presence or absence of biofilm production they were simultaneously examined by two phenotypical method.

Detection of biofilm producers by Congo red assay method

This method is based on the characteristics culture morphology of biofilm forming bacteria on Congo red medium. the medium was composed of brain heart infusion broth (BHI), 37g/l, sucrose 50 g/l, agar No. 1, 10 g/l and Congo red 0.8g/l. Congo red stain was made ready as a strong aqueous solution

and sterilized (121°C for 15 minutes) separate from the rest of the medium components and supplemented to the agar when the temperature reach 55°C. Agar plates were prepared and inoculated and kept in the incubator for 24 hours at 37°c. The production of black colonies with a dry crystalline consistency by the organism was taken to indicate biofilm production as non biofilm producing strains develop red colonies (Mathure *et al.*, 2006).

Detection of biofilm by Standard tube method

Nutrient broth medium was prepared and 5ml of the tube medium was transferred to each of the labeled test tube. The tubes were autoclaved and they were inoculated with respective colonies. The inoculated tubes were incubated for 24 h on shaker at room temperature for growth of the organism. The tubes were decanted and washed with phosphate saline (PBS) buffer to remove the planktonic bacteria and were allowed to dry. The dried tubes were stain with 0.1% Crystal Violet solution excess stain was removed using distilled water and the tubes were then dried and observed for biofilm production (Christensen G.D. *et al.*, 1982).

Antibiotic sensitivity test

Disk diffusion method for antibiotic sensitivity test was determined following the method described by (Baron *et al.*, 1994). Firstly the nutrient broth (5 ml) was inoculated with a loopful of bacterial isolate, the culture was a incubated at 37 °C to mid log phase. A0.1ml of inoculated broth was transferred to Muller-Hinton agar plates. A sterile cotton swab used to streak that inoculum on the plate surface the inoculated plates were then place at room temperature to allow absorption of excessive moisture. With sterile forceps selected antibiotic disks(tetracycline 30 µg),(gentamycin 30 µg) (chloromphenic 30 µg) (erythromycin 15 µg) (ampicillin 10 µg) (ciprofloxacin 5 mg)were placed on the inoculated plates at 37°C for 24 hrs.after this period of incubation the diameter of inhibition zone were noted and measured by a rulers in mm,result were determined.

RESULTS AND DISCUSSIONS

Observation Table

Table No. 1 Percentage of bacterial isolates obtained from 20 samples

Sample Name	Isolates	Age group	Occurrence	Percentage
Tobacco chewers	A	22 – 56	5	25 %
Pyreya	B	40 – 53	2	10 %
Normal	C	22 – 47	5	25 %
Ulcer	D	23	1	5 %
Dental carries	E	21	1	5 %
Mouth infection	F	25	1	5 %
Gutka	G	25 – 37	3	15 %
Dental Plaque	H	76	1	5 %
Tobacco chewers	I	46	1	5 %
	Total		20	100 %

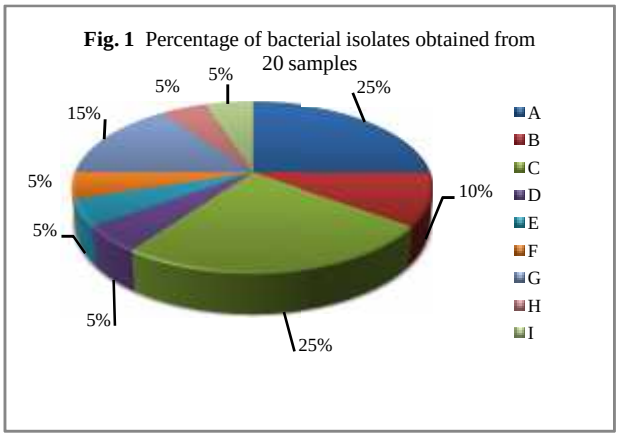

Fig. 1 Percentage of bacterial isolates obtained from 20 samples

Table 2 Biofilm producer by Tube and CRM method

Isolate	Tube method biofilm producer	Percentage	CRM of biofilm producer	Percentage
A	5	29.41%	5	33.33 %
B	2	11.76%	2	13.33 %
C	3	17.46%	2	13.33 %
D	1	5.88%	1	6.66 %
E	1	5.88%	--	0
F	1	5.88%	1	6.66 %
G	2	11.76%	2	13.33 %
H	1	5.88%	1	6.66 %
I	1	5.88%	1	6.66%
Total	17	100 %	15	100%

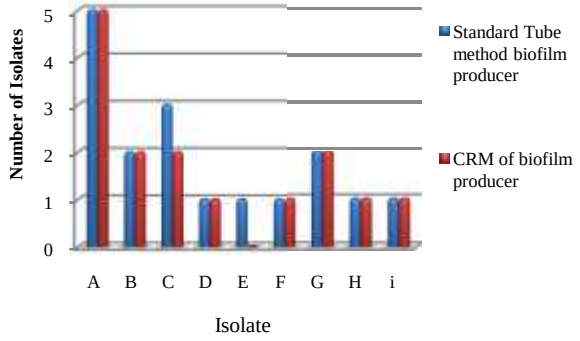

Fig. 2 Biofilm producer by Standard Tube and CRM method

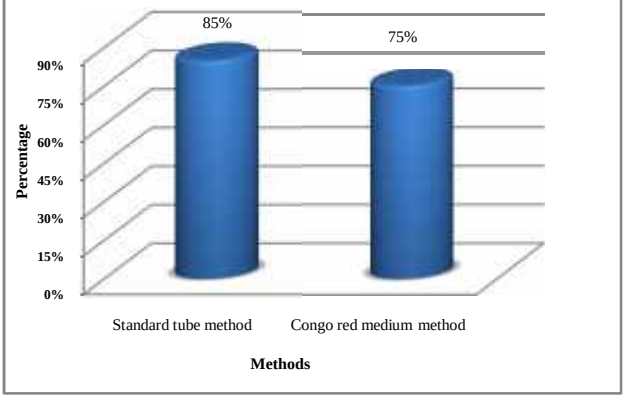

Fig. 3 Detection of biofilm producing isolated by ST and CRM

Table 3 Antibiotic sensitivity test of isolate

Isolate	Ciprofloxacin		Chloromphenicol		Ampicilin		Tetracycline		Gentamycin	
	Zone in mm	S/R	Zone in mm	S/R	Zone in mm	S/R	Zone in mm	S/R	Zone in mm	S/R
A	24	S	12	R	-	R	18	I	20	S
B	27	S	11	R	24	S	16	I	18	S
C	20	I	12	R	-	R	17	I	8	R
D	32	S	10	R	-	R	-	R	12	R

Fig. 4 Antibiotic sensitivity test of isolate

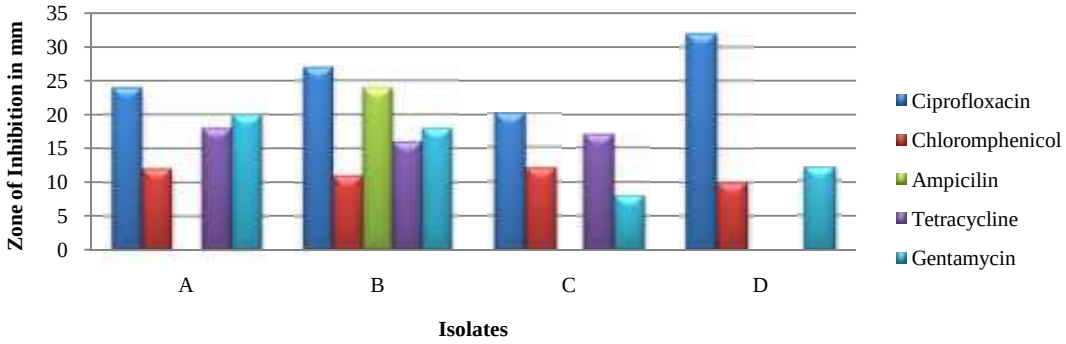

Table 4 Antibiotic sensitivity test of isolate

Isolate	Erythromycine		Chloromphenicol		Ampicilin		Tetracycline		Gentamycin	
	Zone in mm	S/R	Zone in mm	S/R	Zone in mm	S/R	Zone in mm	S/R	Zone in mm	S/R
E	22	I	20	I	-	R	15	I	13	I
F	-	R	-	R	-	R	-	R	23	S
G	12	R	20	I	-	R	-	R	15	S
H	12	R	22	I	-	R	-	R	18	S
I	10	R	15	S	-	R	10	R	13	I

Fig. 5 Antibiotic sensitivity test of isolate

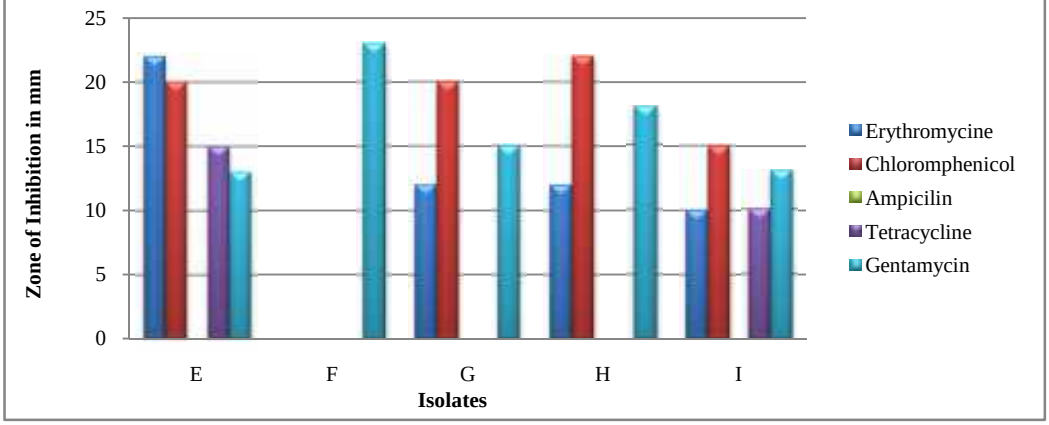

Photo : Biofilm Producer positive test on Congo Red Agar

Photo : Strong and Weak Biofilm Producer by Tube Method

DISCUSSION

Present investigation has been carried out to study the biofilm producing organism from normal and infected oral flora and further study of their antibiotic resistance pattern. The main objective of the study was to isolate and identify the oral microflora and their ability to form biofilm production. In the present study total 20 mouth samples were collected from different area including different age group people. The samples were collected from college campus of Shivaji College, Dental hospital and Villages nearby to Akola. During the study different samples were collected including different age group people, age group under consideration for the study was ranging between 22 - 76 years. we got 5 isolate (25%) from tobacco chewers, 2 isolate (10%) from pyrea infection, 5 isolate (25%) from normal oral flora ,only 01 isolate (5%) was obtained from infection of mouth ulcers, dental caries, dental plaque, 3 isolate (15%) were obtained from gutka chewers. Tabulated in table No 1 and graphically represented in Fig -1.

Isolation and Morphological characterization of biofilm forming bacteria

Nine different colonies were grow on selective and differential media and were selected for characterization out of nine isolate most of the isolate shows circular, entire and convex elevation appearance where as remaining isolate shows roughly circular, oval and raised colony appearance with different colony colour as per the medium used. The microscopic observation revise that out of nine isolate three shows Gram -ve rod's, while one isolate shows Gram-ve cocci where as the other five shows Gram +ve cocci arranged in bunches or in tetrads similarly isolate (I) shows Gram -ve rod. Our results are in accordance with the result of Rahman Monzilur *et al*, 2015 They isolate oral bacterial species of the genus. *Streptococcus, Lactobacillus, Enterococcus, Staphylococcus, Cornybacterium Veillonella*, prominent bacteria commonly found in the oral cavity. Reports from Loo.C.Y *et al.*, 1999 showed that the Viridance *Streptococci*, which include *Streptococcus gordonii*, are pioneer oral bacteria that initiate dental plaque formation Sessile bacteria in a biofilm exhibit a mode of growth that is distinct from that of planktonic bacteria biofilm formation of *S. gordonii* Challis was characterized using an in vitro biofilm formation assay on polystyrene surfaces. Our results and not in accordance with the results stated by Loo C. Y. *et al.,* (1999).

Biochemical Characterization

Various biochemical test have been performed for biofilm yielding strain. Result of biochemical characterization are summarized.Sugar fermentation test was performed by using 3 sugar ie glucose, lactose, sucrose and checked for acid and gas production. Most of the isolate were positive for different sugars for acid and gas production ,except isolate (I) and (G) shows negative acid production for glucose whereas (D), (F) and (I) shows gas production positive. Rest of other isolates show negative gas production. Somewhat similar results were obtained in case of sucrose and lactose sugar. All the isolates were further processed for IMViC test and Enzyme study. Indol test was positive for (E) and (H) isolates only in case of methyl red (B),(C) and (H) isolates shows negative test .50% of the isolate shows VP and Citrate test positive. Enzyme test was carried out with catalase, gelatinas, urease and oxidase.

Similar finding were obtained from Sonkusale K. D and Tale V.S (2015) they were observed that biochemical test use in isolate was 3 sugars .ie ribose, lactose, manitol they reported that *Streptococcus species*, *Staphylococcus species* and *Enterobactor species* shows acid and gas production positive for all the 3 isolates while *Psudomonas species* shows acid and gas production test positive for ribose sugar. Where as lactose ,manitol shows negative test. According to them, during biochemical characterization they noticed that Indol and MR test was negative for *Enterobactor species* and *Psudomonas species* while VP and Citrate test was positive. Similarly they observed catalase test positive for Psudomonas species, *Staphylococcus species* and for all the isolates of *Enterococcus species*

Biofilm formation assay

The isolate were screen for the biofilm formation and were confirmed by Congo red agar method. Total 20 samples were collected and nine isolates was obtained on Congo red agar medium the isolate A, B, C, D, F, G, H, I shows positive results and were indicated by the black colonies with a dry crystalline consistency indicated positive test for biofilm formation overall result indicates that 75% of isolates shows positive Congo red test.The biofilm formations was evaluated and were qualitatively by Congo Red Agar Medium and tube assay method. The tubes were stain with crystal violet and for Standard tube method 85% of the isolate shows, adherence of the stain of tube to the wall and bottom of the test tube. In present study shows it was observed that strong biofilm formation for the tube method was found in isolate A,B,H,D,I where as isolate C and G shows weakly biofilm formation. Intermediate or moderate biofilm formation activity was shown by isolate E, F.According to Sonkusale D. K. (2015), on the chrecterization of the biofilm forming bacteria from oral microflora the qualitative and quantitative estimation revels that oral microflora contain all four types of biofilm former i.e. weak, strong, moderate, intermediate. The sucrose concentration is mainly responsible for the biofilm formation thus the effect of 2%, 5%, 10% and 15% sucrose concentration was checked on biofilm formation by the isolates. The strong biofilm formation was observed in three isolates A, C and E in all the sucrose concentration where as maximum biofilm formation was in 15% sucrose concentration by these isolate .The five isolates (B, D, 3A, V, and IV) exhibited weak to moderate biofilm formation.

Antibiotic sensitivity test

Antibiotic sensitivity testing was performed using the Kirby-Bauer disk diffusion method determining and sensitivity resistance of bacteria to antibiotic by measuring the diameter of inhibition zone in mm and then compaired with standerd diameter installed in the standard scale.The most useful antibiotic in this study were Ciprofloxacin, Chloromphenicol, Erethromycin, tetracycline, gentamycin although the spectrum of agent causing oral infection is relatively constant but their antibiotic susceptibility pattern are different for different organism. In the Present study it was noticed that Chloromphenicol, Ampicilin, Erethromycin and tetracycline was no longer found to be effective controlling oral infections as 80% of oral pathogen show high degree of resistance to this antibiotic. The broad spectrum activity was shown by Ciprofloxacin and gentamycin, showing zone of inhibition ranging from 20 mm to 32 mm where as gentamycin shows zone of inhibition in between 18 to 20 mm. overall results revealed that Ciprofloxacin is the best antibiotic in controlling the oral infection presented in Table 3.

The results reported from Essam F.A Al-Jumaily (2014) revealed that during their study it was noticed that all the obtained isolate shows the resistance to bacitracin where as some isolate shows sensitivity towards optochin and vancomycin.

CONCLUSION

In the current study we had isolated *S. aureus, Micrococcus luteus, Lactobacillus species, Pseudomonas aeruginosa, Streptococcus mutans, E. coli, Bacillus subtilis, Veillonella parvula , Serratia Marscence* from oral flora. *S. aureus, Lactobacillus* species and *Veillonella parvula* is predominant in all the isolated species and play a important role in dental caries progression. *S. aureus, Micrococcus luteus, Streptococcus mutans, P. aeruginosa, B. subtilis* had shown strong activity in biofilm production. The emergence of antibiotic resistance suggesting excessive uses of antibiotic in human resulting in increasing risk for human health. Controlling the use of antibiotic and wisely usages of it is needed to reduced to spread of antibiotic resistance among oral pathogens.

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