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Research Article

Biofilm formation capability of bacteria, a threat to treat multi drug resistant diabetic foot infection isolates

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Abstract

Bacterial biofilms are complex structured communities surrounded in a matrix of extra-cellular polymeric substances growing on rough surfaces. It is involved in multiple functions like trapping of nutrients, adherence, stability of biofilm structure and the penetration of many antibiotics.250Pus samples were collected from the depth of ulcers from each patient using Amies Transport Swabs. The specimens were inoculated on appropriate media. Biochemical characteristics of pathogens were observed by performing conventional biochemical tests and Quick Test Strips. Antibiotic resistance of isolates was checked by Kirby Bauer's Disc Diffusion Method. Biofilm formation was initially detected by Congo Red Agar Method. Major biofilm forming isolates from different hospitals environment were Klebsiella spp., Corynebacterium spp., S. epidermidis while from hospital curtain's samples, Shigella dysenteriae, Candida albicans and Aspergillus flavus were having capability of biofilm formation. S. aureus showed good biofilm forming ability in presence of 0.2% glucose, sucrose and lactose, P. aeruginosa showed enhancement in biofilm formation in presence of sucrose, glucose, and maltose while E. coli indicated increased biofilm formation in presence of lactose, sucrose and glucose. Most of the biofilm forming isolates were Multi drug resistant which indicate that extracellular material released by these pathogens cover the bacteria cell and hinder the antibiotic

Keywords: Biofilm formation in isolates from diabetic Foot infection Patients

Introduction

Bacterial biofilms are complex structured communities surrounded in a matrix of extra-cellular polymeric substances growing on rough surfaces (Petrelli *et al*., 2006). *Staphylococci* biofilms are covered in a matrix that is mostly composed of exo-polysaccharides like polysaccharide intercellular adhesin (PIA) also known as slime or extra-cellular polymeric substance (EPS). It is involved in multiple functions such as trapping of nutrients, adherence, and stability of biofilm structure, pathogenesis of biofilm-associated infections. It also opposes the penetration of many antibiotics through biofilm formation resulting into reduced antibiotic efficacy (Singh *et al*., 2010). Biofilm development causes substantial problems not only in medical but also in industrial settings; biofilm-producing bacteria can tolerate the antibiotic therapy, host immune responses, and biocide actions (Harmsen *et al*., 2010). A significant feature of bacterial biofilms is extensive production of EPSs. Which joint with cell surface-associated proteins and nucleic acids, constitutes biofilm matrix. Physical nature of smooth or rough surface for colonization and bacterial adhesion has limited effect on biofilm formation (Costerton *et al*., 1995). Exo-polysaccharide matrix is secreted by sessile cells results into a biofilm structure, which is highly viscous and elastic (Hall-Stoodley and Lappin-Scott, 1998).

Surface attachment is an initial process in biofilm development, when free-floating

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bacteria attach to a surface, these bacteria grow into mature, complex biofilm progressively, and dispersal of detached bacterial cells occurs through bulk fluid. Microbial interaction with the surface is completed into various phases and requires the production of extra-cellular microbial structures that support in initial adhesion, and maintenance of biofilm structure. In non-motile bacteria like *Staphylococcus,* polysaccharide and protein adhesins have been associated to adherence, which is essential in biofilm progress. While in *P. aeruginosa*, flagella and type IV pili-mediated twitching motility both play important roles in surface aggregation (Hall-Stoodley and Stoodley, 2002).

Many environmental factors including nutritional components and intrinsic factors such as microbial diversity and their cellular processes can influence the biofilm components (Yang *et al*., 2006). Different sugars have influence on virulence and diversity of the biofilm such as extra-cellular bacterial enzymes can take part in the formation of extra-cellular polysaccharides (glucans and fructans) by utilizing sucrose. The formation of glucan is catalyzed by glucosyl transferase (GTF) and fructan by fructosyl transferase (FTF). Glucan facilitates the bacterial attachment to the tooth surface and have significance in plaque formation and biofilm development (Ismail *et al*., 2006). The major objective of this study was to investigate that biofilm formation capability of bacteria can be possible mechanism for multi drug resistance which can result into hurdle to treat complicated diabetic foot infections.

Methodology

Culture and sensitivity

250 Pus samples were collected from the depth of ulcers from each patient using Amies Transport Swabs (Rosa-Fraile M, 2005). The specimens were inoculated on blood agar and MacConkeys agar and chocolate agar for detection of aerobic and anaerobic isolates. Biochemical characteristics of pathogens (aerobes and anaerobes) were observed by performing conventional biochemical tests and Quick Test Strips (QTS) were used for the identification of *Enterobacteriace* (Akhter T *et al*., 2010). Antibiotic resistance of diabetic foot isolates was checked on iso sensitivity agar (Oxoid) by Kirby Bauer's Disc Diffusion Method as per Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2005).

Biofilm assay

Biofilm formation was initially detected by Congo Red Agar Method and qualitative estimation of biofilm development capability was performed as reported previously in our study (Mirani *et al*., 2012).

Effect of Different Dietary Carbohydrates on Biofilm Formation

Effect of different dietary carbohydrates on biofilm formation of multi drug resistant *S. aureus, P. aeruginosa, E. coli* was determined by modified method (Mathur *et al*., 2006 and Yang *et al*., 2006). Different dietary carbohydrates (glucose, fructose, sucrose, lactose, starch, glycogen, chitin, and cellulose) were used to check their effects on biofilm formation capability of bacteria.

Results

Major diabetic foot infection isolates (*S. aureus* and *P. aeruginosa*) were checked for antibiotic susceptibility testing in order to evaluate antimicrobial effect of commonly available antibiotics, as described in Table 1. *S. aureus* isolates were mostly resistant to

beta-lactams, Macrolides, and Aminoglycosides antibiotics while *P. aeruginosa* isolates were mainly resistant to monocyclic beta lactam, fluoroquinolones (Table1).

Major biofilm forming isolates from different hospitals environment (hospital bed samples) were *Klebsiella spp*., *Corynebacterium spp., S. epidermidis* while from hospital curtain's samples, *Shigella dysenteriae, Candida albicans* and *Aspergillus flavus* were having capability of biofilm formation as presented in Table 2. Results indicated that there was need to monitor hospital environment in order to control multi drug resistant pathogens causing nosocomial infections.

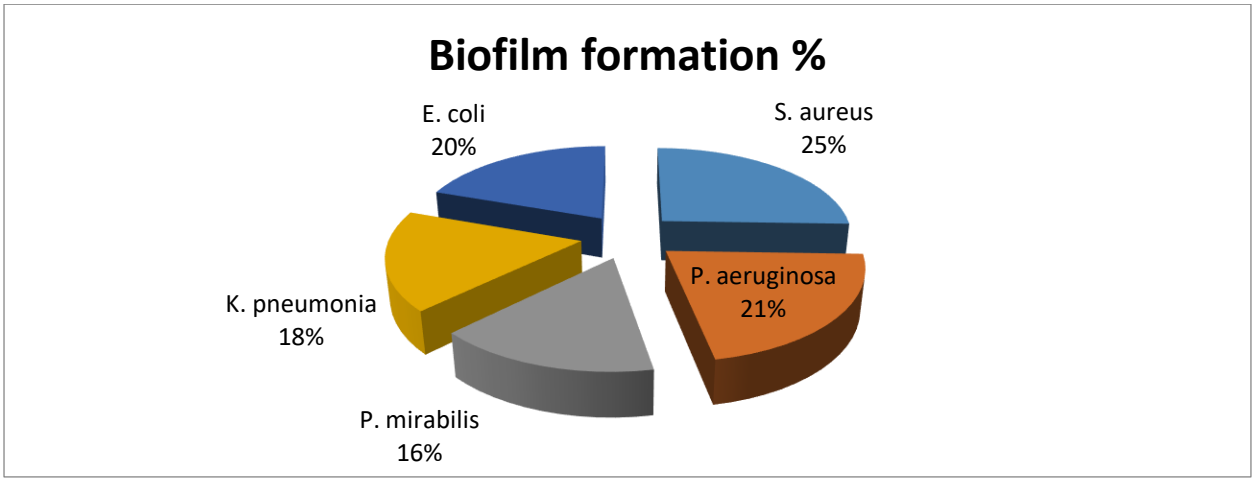
Biofilm formation capability of diabetic foot isolates was detected as *S.aureus* (25%), *P. aeruginosa* (21%), and *E. coli* (20%) were major biofilm forming isolates. Diabetic foot infection isolates were multi drug resistant (Graph 1).

Table1: Antibiotic resistance pattern of biofilm producer strains of *S. aureus*

Antibiotics	***S. aureus*** n= 116		**Antibiotics**	***P. aeruginosa*** n= 99	
	Sensitive (%)	**Resistant** (%)	Azetronam	**Sensitive** (%)	**Resistant** (%)
Ampicillin	20(17.2%)	94(81.03%)	Amikacin	44(42%)	55(55%)
Co-amoxiclav	64(55.1%)	52(44.8%)	Cefotaxime	60(69%)	30(39%)
Ciprofloxacin	57(49.13%)	59(50.8%)	Ceftazidime	56(56%)	43(43%)
Gentamicin	48(41.3%)	68(58.6%)	Ceftrixone	61(61%)	38(38%)
Levofloxacin	52(44.8%)	64(55.1%)	Ciprofloxacin	59 (59%)	40(40%)
Methicillin	91(78.4%)	25(21.5%)	Gentamicin	45 (45%)	54(54%)
Vancomycin	25(100%)	0(0%)	Imipenem	46 (46%)	53(53%)
Erythromycin	45(38.7%)	71(61.2%)	Levofloxacin	42 (42%)	57(57%)
Tetracycline	42(36.2%)	74(63.7%)	Meropenem	55 (55%)	44(44%)
Trimethoprim / Sulphamethoxazoe	51(43.9%)	65(56%)	Tazobactam	43 (43%)	56(56%)
Chloramphenicol	38(32.7)	78(67.2%)	Co amoxiclav	49 (49%)	50(50%)
Clindamycin	53(45.6)	63(54.3%)		51 (51%)	48(48%)

Table 2: Biofilm Production Analysis of Hospital Isolates from different Sources

Different sources of infection	**Bacterial and fungal isolates**	**Prevalence rate (%)**	**Biofilm production (%)**
Hospital bed samples n= 30			
	Staphylococcus aureus	12(40%)	(22%)
	Corynebacterium Spp.	5(16%)	(66%)
	Aspergillus niger	4(13.3%)	(20%)
	Candida albicans	4(13.3%)	(40%)
	Staphylococcus epidermidis	3(10%)	(60%)
	Klebsiella spp.	2(6%)	2(100%)
Hospital curtain samples n= 20			
	Shigella dysenteriae	7(30%)	4(57%)
	Escherichia coli	4(20%)	2(50%)
	Candida albicans	2(10%)	1(50%)
	Aspergillus flavus	2(10%)	1(50%)
	Aspergillus niger	3(15%)	1(33.3%)
	Rhizopus	2(10%)	0(0%)



Graph 1: Biofilm Production of Isolates from Diabetic Foot Infection (n=30)

Effect of different dietary carbohydrates was checked against different diabetic foot infection isolates as presented in Table 3. *S. aureus* showed good biofilm forming ability in presence of 0.2% glucose, sucrose and lactose, *P. aeruginosa* showed enhancement in biofilm formation in presence of sucrose, glucose, and maltose while *E. coli* indicated increased biofilm formation in presence of lactose, sucrose and glucose.

Table 3: Effect of Nutritional Compounds (Dietary Carbohydrates) on Biofilm Forming isolates from Diabetic Foot Infection

Dietary carbohydrates	***S. aureus*** n=30		***P. aeruginosa*** n= 30		***E. coli*** n=30	
	Biofilm formation (%)	**Non biofilm formation (%)**	**Biofilm formation (%)**	**Non biofilm formation (%)**	**Biofilm formation (%)**	**Non biofilm formation (%)**
Glucose	18(60)	12(40)	16(53.3)	14(46.6)	14(46.6)	16(53.3)
Sucrose	16(53.3)	14(46.6)	17(56.6)	13(43.3)	15(50)	15(50)
Maltose	12(40)	18(60)	14(46.6)	16(53.3)	10(33.3)	20(66.6)
Lactose	16(53.3)	14(46.6)	11(36.6)	19(63.3)	16(53.3)	14(46.6)
Cellulose	11(36.6)	19(63.3)	13(43.3)	17(56.6)	13(43.3)	17(56.6)
Sorbitol	14(46.6)	16(53.3)	12(40)	18(60)	9(30)	21(70)
Fractose	13(43.3)	17(56.6)	11(36.6)	19(63.3)	10(33.3)	20(66.6)
Manitol	11(36.6)	19(63.3)	9(30)	21(0)	8(26.6)	22(73.3)

Discussion

Biofilm production property of isolates from diabetic foot infection was determined by qualitative tube adherence method as described by (Christensen *et al*., 1987) and tissue culture plate method (TCP) as described by (Mathur *et al*., 2006). Some virulent microorganisms produce slime which helps these pathogens in colonization on infection site, development of infections, compromising drug efficacy, antibiotic resistance, and impairing the host immune cells response (Oliveira and Cunha, 2008). According to our study, biofilm production property of isolates from diabetic foot infection was checked by tube adherence method. Biofilm was mostly observed in *S. aureus* (25%), *P. aeruginosa* (21%), *E. coli* (20%), *K. pneumoniae* (18%), and *P. mirabilis* (16%).

ELISA plate method showed slightly different results in terms of the order of predominance as *S. aureus* (43.3%), *P. aeruginosa* (36.6%), *E.coli* (33.3%), *K. pneumoniae* (30%) and *P. mirabilis* (26.6 %) were found to be strong biofilm producing isolates. Biofilm production property of isolates may be determined by different

methods, one of the most common and simple method is to detect this property by tube adherence method as described by Christensen *et al*, (1987). Tissue culture plate method can also be used, in which discrimination of strong, week and negative biofilm producers is easy as compared to the tube method due to the fact that different observations are taken in different tissue culture plates while in tube method observations are made in a single tube. Due to this reason, analysis of positive and negative biofilm producers by tube method was difficult and variability was observed. This is in agreement with an earlier report, which suggests that tube method cannot be recommended as standard method for biofilm detection (Mathur *et al*., 2006). In another study, a comparative qualitative tube method of biofilm production and quantitative micro titer assay for biofilm formation was used to observe the efficacy of these methods, biofilm formation was strongly positive in 62% of isolates in both cases and remaining were either week or non-biofilm producers and were considered as negative (Rao *et al*., 2008). Biofilm producers are more resistant to antimicrobials as compared to planktonic bacteria due to the presence of an exo-polysaccharide matrix that can cover the bacteria and slow down the diffusion of antibiotics using different resistance mechanisms (Abdi-Ali *et al*., 2006). Biofilms also have been reported for causing persistent human diseases, such as respiratory infections in cystic fibrosis patients, dental caries, gingivitis, periodontal disease, osteomyelitis, chronic prostatitis, otitis media, endocarditis, infectious kidney stones and Legionnaire's disease (Cooper, 2010). Urinary tract infection (UTI) is more complicated in diabetic patients and uropathogens have greater capability of biofilm production as was reported in our previous study (Baqai *et al*., 2008). Eradication of biofilm is possible through developing easier rapid methods for the detection of biofilm production property from different infections. Moreover, development of more new specific antimicrobial drugs and use of appropriate device surfaces in patients would also help in eradication (Baqai *et al*., 2008). In another study on biofilm production property was found in *S. aureus* (83.3%) and *S. epidermidis* (88.6%). Biofilm production genes *ica*A and *ica*D were present in all *S. aureus* biofilm producer strains. TCP method was found as an accurate and reproducible method for screening and determination of biofilm (Gad *et al*., 2009). Different molecular techniques can be utilized to visualize and genetically confirm the slime production property through PCR. As electron microscopy can be used to visualize the ultra structure and texture of biofilm, transmission electron microscope is being used to observe different biofilm production models and their characterization in different human and animal infections along with medical devices while through confocal laser scanning microscopy, the intact biofilm matrix can be visualized in situ (Donlan and Costerton, 2002).

Biofilm excrete extracellular products called polysaccharides, which are main portion of biofilm matrix, and forms a covering sheath on bacteria. In our study, effect of dietary carbohydrates (glucose, sucrose, maltose, lactose, fructose, and galactose) on biofilm development property was determined in *S. aureus* and *P. aeruginosa* isolated from diabetic foot infection. According to our

findings, biofilm production was enhanced in the presence of 0.2% sucrose and glucose as compared to other sugars. Other reports indicated the importance of biofilm production and effect of sugars on biofilm development properties as in dental caries. It is difficult to treat dental diseases due to presence of sugars in daily diet and its influence on dental plaque biofilm. In another study on *P. aeruginosa*, effect of different sugars (glucose, lactose, sucrose, galactose and fructose, at a concentration of 0.2%, was checked on the biofilm exopolysacchrides. It was noticed that in the presence of 0.2% sucrose, lection binding was enhanced to a considerable level than in presence of other sugars (Yang *et al*., 2006). Oral bacteria metabolize sugars leading to the production of organic acids in sufficient concentration to lower the pH of dental plaque and increase the concentration of glucan and fructan polysaccharides as reported previously. Biofilm production capability was enhanced in the presence of different dietary sugars and sucrose, lesser growth time was required for biofilm formation. (Tam et *al*., 2007). In another study increased concentration of sucrose up to 1 % can enhance biofilm production in *Strep. mutans* while separation process can start in higher concentration of sucrose. (Tahmourespour *et al*., 2010).

Conclusion

Most of the biofilm forming isolates were Multi drug resistant which indicate that extracellular material released by these pathogens cover the bacteria cell and hinder the antibiotic action on cell leading to higher prevalence of multi drug resistant strains in diabetic foot infections.

References

ABDI-ALI, A., MOHAMMADI-MEHR, M. & AGHA ALAEI, Y. 2006. Bactericidal activity of various antibiotics against biofilm-producing Pseudomonas aeruginosa. *Internatioal Journal of Antimicrobial Agents,* **27,** 196-200.

Akhter T, Baqai R, Aziz M (2010) Antibacterial effect of NSAIDS on clinical isolates of urinary tract infection and diabetic foot infection. Pak j of pharmaceutical scien; **23**(1): 108-113

BAQAI, R., AZIZ, M. & RASOOL, G. 2008. Urinary tract infection in diabetic patients and biofilm formation of uropathogens. *Infectious Disease Journal of Pakistan,* **17,** 7-9.

BROWN, M. L., ALDRICH, H. C. & GAUTHIER, J. J. 1995. Relationship between glycocalyx and povidone-iodine resistance in Pseudomonas aeruginosa (ATCC 27853) biofilms. *Applied Environmental Microbiology,* **61,** 187-93.

BJARNSHOLT, T., KIRKETERP-MOLLER, K., JENSEN, P. O., MADSEN, K. G., PHIPPS, R., KROGFELT, K., HOIBY, N. & GIVSKOV, M. 2008. Why chronic wounds will not heal: a novel hypothesis. *Wound Repair and Regeneration,* **16,** 2-10.

COOPER, R. 2010. Biofilms and wounds: much ado about nothing. *Wounds UK,* **6,** 84-90.

COSTERTON, J. W., LEWANDOWSKI, Z., CALDWELL, D. E., KORBER, D. R. & LAPPIN-SCOTT, H. M. 1995. Microbial biofilms. *Annual Review of Microbiolgy,* **49,** 711-45.

CHRISTENSEN, G. D., BADDOUR, L. M. & SIMPSON, W. A. 1987. Phenotypic variation of Staphylococcus epidermidis slime production in vitro and in vivo. *Infection and Immunity,* **55,** 2870-7.

DONLAN, R. M. & COSTERTON, J. W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiolgy Review,* **15,** 167-93.

GAD, G. F. M., EL-FEKY, M. A., EL-REHEWY, M. S., HASSAN, M. A., ABOLELLA, H. & EL-BAKY, R. M. A. 2009. Detection of icaA, icaD genes and biofilm production by Staphylococcus aureus and Staphylococcus epidermidis isolated from urinary tract catheterized patients. *Journal of infection in developing countries,* **3,** 342-351.

HARMSEN, M., YANG, L., PAMP, S. J. & TOLKER-NIELSEN, T. 2010. An update on Pseudomonas aeruginosa biofilm formation, tolerance, and dispersal. *FEMS Immunol Med Microbiol,* **59,** 253-68.

HALL-STOODLEY, L. & LAPPIN-SCOTT, H. 1998. Biofilm formation by the rapidly growing mycobacterial species Mycobacterium fortuitum. *FEMS microbiology letters,* **168,** 77-84.

HALL-STOODLEY, L. & STOODLEY, P. 2002. Developmental regulation of microbial biofilms. *Curr Opin Biotechnol,* **13,** 228-33.

HOIBY, N., KROGH JOHANSEN, H., MOSER, C., SONG, Z., CIOFU, O. & KHARAZMI, A. 2001. Pseudomonas aeruginosa and the in vitro and in vivo biofilm mode of growth. *Microbes and Infection,* **3,** 23-35.

ISMAIL, W. N. H. W., RAZAK, F. A. & RAHIM, Z. H. A. 2006. The Role of Sucrose in the Development of Oral Biofilm in a Simulated Mouth System. *Online Journal of Biological Sciences,* **6,** 62-66.

MAH, T. F. C. & O'TOOLE, G. A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in microbiology,* **9,** 34-39.

MATHUR, T., SINGHAL, S., KHAN, S., UPADHYAY, D. J., FATMA, T. & RATTAN, A. 2006. Detection of biofilm formation among the clinical isolates of Staphylococci: an evaluation of three different screening methods. *Indian Journal of Medical Microbiology,* **24,** 25-9.

Mirani, Z. A., Khan, M. N., Aziz, M., Naz, S., & Khan, S. I. (2012). *Effect of stress on biofilm formation by icaA positive and negative strains of methicillin resistant Staphylococcus aureus. Journal of the College of Physicians and Surgeons--Pakistan: JCPSP, **22**(1), 10-14.*

Moghadam, S. O., Pourmand, M. R., & Aminharati, F. (2014). Biofilm formation and antimicrobial resistance in methicillin-resistant Staphylococcus aureus isolated from burn patients, Iran. *The Journal of Infection in Developing Countries, **8***(12), 1511-1517.

NGO Q, V. K., DEVA AK (2007) (SUPPL 1): A66 2007. Role of bacterial biofilms in chronic wounds. *ANZ J Surg,* **77,** A66-67.

OLIVEIRA, A. & CUNHA, M. 2008. Bacterial biofilms with emphasis on coagulase-negative staphylococci. *Journal of Venomous Animals and Toxins including Tropical Diseases,* 572-96.

PETRELLI, D., ZAMPALONI, C., D'ERCOLE, S., PRENNA, M., BALLARINI, P., RIPA, S. & VITALI, L. A. 2006. Analysis of different genetic traits and their association with biofilm formation in Staphylococcus epidermidis isolates from central venous catheter infections. *Eurpian Journal of Clinical Microbiology and Infectious Disease,* **25,** 773-81.

RAO, R. S., KARTHIKA, R. U., SINGH, S. P., SHASHIKALA, P., KANUNGO, R., JAYACHANDRAN, S. & PRASHANTH, K. 2008. Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of Acinetobacter baumannii. *Indian Journal of Medical Microbiology,* **26,** 333-37.

Rosa-Fraile M, Camacho-Muñoz E, Rodríguez-Granger J ,Liébana-Martos C (2005) Specimen storage in transport medium and detection of group B streptococci by culture. J cl microbiol;**43**(2):928-930

SINGH, R., RAY, P., DAS, A. & SHARMA, M. 2010. Penetration of antibiotics through Staphylococcus aureus and Staphylococcus epidermidis biofilms. *Journal of Antimicrobial Chemotherapy,* **65,** 1955-58.

SUCI, P. A., MITTELMAN, M. W., YU, F. P. & GEESEY, G. G. 1994. Investigation of ciprofloxacin penetration into Pseudomonas aeruginosa biofilms. *Antimicrobial Agents and Chemotherapy,* **38,** 2125-33.

TAM, K., KINSINGER, N., AYALA, P., QI, F., SHI, W. & MYUNG, N. V. 2007. Real-time monitoring of Streptococcus mutans biofilm formation using a quartz crystal microbalance. *Caries Research,* **41,** 474-83.

TAHMOURESPOUR, A., KERMANSHAHI, R. K., SALEHI, R. & PERO, N. G. 2010. Biofilm formation potential of oral streptococci in related to some carbohydrate substrates. *African Journal of Microbiology Research,* **4,** 1051-1056.

WOLCOTT, R. D. & EHRLICH, G. D. 2008. Biofilms and chronic infections. *The Journal of the American Medical Association,* **299,** *2682-84*

YANG, Y., SREENIVASAN, P. K., SUBRAMANYAM, R. & CUMMINS, D. 2006. Multiparameter assessments to determine the effects of sugars and antimicrobials on a polymicrobial oral biofilm. *Applied and Environmental Microbiology, **72,** 6734-42.*