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Early detection of biofilm formation of selected bacterial isolates through a new screening method using 'image J'

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ABSTRACT

Aim: The cells growing in biofilms are physiologically distinct even from the same microorganism in the planktonic state. Although mixed-species biofilms predominate in most of the environments, single-species biofilms exist in a large variety of infections, on the surface of medical implants and parts of the machineries in the food industries. Convenient method and early detection of these biofilms are therefore, necessary to avoid or prevent the important implants from the bacterial biofilm formation. Therefore present research was undertaken with the aim to establish an *in vitro* method for early detection and screening of biofilm with easily applicable image J.
Methodology and results: Eight bacterial colonies were isolated from the poultry wastes (PW), dental plaques (DP) and hospital effluents (HE). Among them, three isolates were finally selected for the study based on their source of origin, colony characteristics and the biofilm forming ability determined by test tube assay. The isolates were provisionally identified as *Salmonella arizonae* (from PW), *Micrococcus luteus* (from DP) and *Aerococcus viridians* (from HE) and examined for their biofilm forming ability through a novel *in vitro* method, cover slip assay. The images of crystal violet stained biofilms of the isolates on the surface of the cover slips were captured and analysed by image-processing software image J. The results of the cover slip assay were then compared with those from test tube assays to conform the efficacy and reliability of the method for screening and evaluating biofilm formation. The results suggest that the novel *in vitro* method for biofilm screening by cover slip assay is effective for evaluating selected bacterial biofilms. The ability to form biofilm was not specifically correlated with the colony characteristics but the initial attachment for early development of the biofilm was significantly correlated with their motility.
Conclusion, significance and impact of study: The method of biofilm screening with the cover slip assay used in this study is novel, very simple but powerful and effective method, is expected to have significant impact and gain additional interest among the scientists for biofilm screening and study.

Keywords: Bacterial isolates, biofilm, low cost method, cover slip assay, image J.

INTRODUCTION

Biofilm is a collection of microorganisms initiating a growth process by means of proteins or polysaccharide nature peptideoglycan (Johansen *et al.*, 2009; Shumi *et. al*., 2009). On the other hand, biofilms are attached to the surfaces like plastics, stainless steel, glasses with the help of exopolysaccharides. Bacteria can live in planktonic (free living) or sessile phenotype (Mittelman, 1998; Horn and Morgenroth, 2006; Shumi *et al.*, 2013). When free living bacteria first attach to a surface, they begin to proliferate themselves, regularly aggregate and secrete exopolysaccharide (EPS) that helps to sequester nutrients and other planktonic cells. The cells-EPS complex formed and will finally mature and form water channels to pass nutrients as required, thus develop a biofilm (Criado *et al.*, 1994; Zottola *et al.*, 1994; Pompermayer and Gaylarde, 2000). The biofilm, depending on the species microcolony may be composed of 10-25% cells and 75-90% EPS matrix (Stewart and Costerton, 2001). Biofilm has an increased resistance to detergents and antibiotics. This resistance to antibiotics is due to both the stationary phase cells and the presence of persistence cells. Biofilms also provide an ideal niche for the exchange of extra chromosomal DNA (plasmid), degrading enzymes which are useful for biofilm

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development. Dispersal of cells from the biofilm colony is an essential stage of the biofilms life cycle (Bos *et al.*, 1999; Shumi *et al*., 2010).

Bacterial biofilms are also responsible for several chronic diseases that are difficult to treat. A variety of human infections such as endocarditis, caries, formation of dental plaque, gingivitis, gastrointestinal ulcers, urinary tract infections, cystic fibrosis in patients, chronic otitis, chronic lung infections are caused by several bacterial biofilm formations (Chandra *et al*., 2001; Mah and O'Tool, 2001; Hall-Stoodly *et al.*, 2004; Drago, 2009; Coenye, 2010). On the other hand, biofilms play vital roles in the ecology of the earth and the sustainability of life. They are capable of solving many problems in the cleaning of wastewater and wastewater treatment for over the century. Considering the facts, scientists are searching for new and efficient techniques for monitoring the consequences inside the biofilms. Therefore, development of new techniques and methods for early detection and monitoring of biofilm formation is necessary (Lim *et al.*, 2008). The aim of developing techniques should be simple, cost effective, efficient and harmless to biofilm so that online monitoring of biofilm development is possible to get information about their structures and functions. The type of equipment needed to investigate biofilm formation is very much dependent upon the type of questions asked (Oh *et al.*, 2009). If *in situ* or *in vivo* investigations are required, the investigator is obviously restricted in his choice since the biofilm itself proliferates in its natural system. Besides this, recently, the researcher (Jensen and Fenical, 1994; Armstrong *et al.*, 2001) stated that biofilms may be seen as a source of new bioactive agents. When bacteria are organized in biofilms, they produce some essential substances which individual bacteria are unable to produce alone. For the reason, biofilm is largely dependent on the innovation of powerful new techniques to investigate the structures and functions of biofilm development.

With the view to develop a technique for screening and evaluating the bacterial strains for biofilm formation from the poultry wastes, dental plaques and hospital effluents, we have established a novel *in vitro* method for evaluating selected bacterial biofilms, cover slip assay technique by image J software processing. Since the method is a quantitative estimation technique, the amount of microbial cells, which is responsible for biofilm development, could be measured. The exploration of the capabilities of microorganisms inside the biofilm development could be quantified.

MATERIALS AND METHODS

Bacterial strain isolation and characterization

Bacterial strains were isolated from collected raw samples of the poultry wastes, dental plaques and hospital effluents. The strains were purified through repeated plating (pour plate and streak plate) method. Several media and biochemical tests and cultural methods were used according to Shumi *et al.* (2004) for the identification of isolates. Morphological characteristics of the selected isolates were determined by cultural and microscopic methods. Cultural characterization consists of colony characteristics on agar plate, agar slants, and growth in liquid or in deep media, and microscopic features, including size, shape, arrangement and colour of the isolates were recorded. Catalase test, indole test, fermentation test, nitrate reduction test, H_2S production test, MRVP test, motility test, fermentation test, etc. and biochemical analysis were also performed for the identification of the isolates. Growth performance in different temperatures, pH and salt concentrations were examined and quantified. All the results were recorded and compared with the standard characterization of isolates.

Screening of bacterial strains for biofilm forming ability

Test tube assay

Biofilm production was carried out by the modified method of Christensen *et al*. (1982). Fresh single colony of the bacterial strain was picked up and inoculated in 5 mL LB broth medium in a test tube and incubated at 37 °C for 12 to 16 h. This culture medium was used as inoculums for the biofilm assay. Fresh inoculum at 1:20 ratio was again inoculated into 5 mL fresh LB broth and incubated at 37 °C for 24 h. For the assay of biofilm developed in medium was performed by discarding culture medium from the test tube carefully. The test tubes were rinsed (twice) with 5 mL distilled water. Five millilitre (5 mL) of crystal violet solution (1% w/v) was added to stain the biofilms and incubated at room temperature for 30 min. Excess stains were then washed with 5ml distilled water twice. The test tubes were then dried in air for 20 to 30 min. Five ml of 95% ethanol was added to the tubes and kept in room temperature for 30 min. The absorbance of the retained dye was measured by spectrophotometer at 600 nm.

Cover slip assay

First, fresh single colony was picked up and inoculated in 5 mL LB broth medium for preparing preculture. Cleaned and sterilized Petri plates and glass cover slips were prepared and poured with 15 mL fresh LB broth and fresh inoculum (1:20) were inoculated in the sterilized petri plates and 2-4 sterilized cover slips were placed into it. The plates were incubated at 37 °C for 24 h. Assay of reduced biofilm was performed by taking the cover slips from the Petri plate and rinsed with distilled water carefully. The bacterial cells attached to the cover slips were stained with ammonium oxalate crystal violet (95%)

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Table 1: Morphological and cultural characteristics of the bacterial isolate.

	Biofilm forming bacterial isolates		
	Salmonella arizonae (PW)	*Micrococcus luteus* (DP)	*Aerococcus viridians* (HE)
Agar colonies	Whitish, circular , entire, convex, smooth translucent colonies	Off-white, circular, entire, convex, smooth colonies	Lemon yellow, circular, entire, convex, smooth colonies
Vegetative cells	Short rod , 1.4 µm to 3.4 µm, occur singly occasionally occur in pair	Cocci, spherical or oval shaped, 1.7 µm in diameter, occur singly	Small cocci, spherical or oval shaped, 1.02 µm in diameter, occur singly
Spore staining	Not formed	Not formed	Not formed
Gram staining	Gram negative	Gram positive	Gram positive
Acid fast staining	Non-acid fast	Non-acid fast	Non-acid fast
NA slant	Echinulate	Filiform	Filiform
Nutrient broth	turbidity with pellicle formation	High turbidity	Moderate turbid growth.
Deep glucose agar test	Facultative anaerobic, moderate growth	Facultative anaerobic, abundant growth	Facultative anaerobic, moderate growth

Table 2: Biochemical behaviour of the biofilm forming isolates.

		S. arizonae	*M. luteus*	*A. viridians*
H_2S production		+++	+	+++
Motility test		+++	+	++
Catalase test		+++	++	+
Starch hydrolysis		-	+++	+
Gelatin hydrolysis		+	++	-
VP test		-	-	+
MR test		-	-	+
Indole test		-	-	-
Urease test		-	-	-
Nitrate reduction		++	+	+++
Fermentation test		Alkali without gas: mannitol, raffinose, arabinose, starch, cellulose Acid without gas: lactose, fructose, glucose, sucrose	No fermentation: lactose, fructose , glucose, sucrose, arabinose, starch, cellulose Alkali without gas: mannitol, raffinose	No fermentation: lactose, fructose, glucose, sucrose, arabinose, starch, cellulose, raffinose Acid without gas: mannitol
Growth response at different pH:	4.5	+	+	+
	6.5	++++	++++	++++
	8.5	++	++	++
Growth response at different NaCl concentration:	0%	+++	++	+
	1%	++++	++++	+++
	5%	+++	++	-
	10%	-	-	-
	20%	-	-	-
Growth response at different temperature:	27 °C	+++	++	++
	37 °C	++++	++++	++++
	45 °C	+++	-	-

for 30 sec. After rinsed with distilled water, the cover slips were dried in air for 30 min. Produced biofilms on cover slips were observed under electronic microscope and images were captured.

Image processing

The biofilms formed on the surface of the glass cover slips were analysed by image processing technique 'Image J'. Image processing was performed by the

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modified method previously used by Shumi *et al*. (2009). Several images were captured and analysed to determine the quantity of attached cell. Statistical analysis was performed using computer software IBM SPSS version 21 and analysis of variance ANOVA was adopted to determine the possible treatment variation.

Evaluation of factors affecting the biofilm production

The strains, positive biofilm formers were selected for mass culture in liquid media. The production of biofilm is influenced by a number of factors such as composition of media, incubation temperature, medium pH and salt concentration. Therefore, the conditions were checked for the optimum production of biofilm by the selected strains.

Biofilm inhibition by antibiotics

Inhibition of biofilm formation by common antibiotics (Penicillin) was also studied for the isolates. To explore the effects of antibiotics on the biofilm formation of the selected isolates, the media were sterilized and mixed with antibiotics in the dose dependent manner (10 µg/L, 25 µg/L and 50 µg/L respectively) maintaining all other experimental conditions optimum for the biofilm formation. The development of biofilm was measured through the density assessment. The results were recorded and compared to the biofilm development in control culture.

RESULTS AND DISCUSSIONS

The present study was carried out with three selected bacterial strains isolated from the well-known microbial infection sites poultry wastes (PW), dental plaques (DP) and hospital effluents (HE) (Tables 1 and 2). All the three selected strains were tested for their morphological, cultural and biochemical characteristics (Tables 1 and 2). These properties were compared with the standard description of Bergey's Manual of Determinative Bacteriology, 8th edn. (Buchanon and Gibbons, 1974) and found that the isolates belonged to the genus *Salmonella*, *Micrococcus* and *Aerococcus* from PW, DP and HE respectively and provisionally identified as *Salmonella arizonae*, *Micrococcus luteus* and *Aerococcus viridians*. We investigated whether the bacterial colony morphology could be indicative of the tendency for growth on glass surfaces. Colony morphology of the three isolates was somehow different, and differed mostly in size, density and pigment of the colonies. As observed, the isolate *S. arizonae* formed relatively larger colonies with translucent density, while *M. luteus* and *A. viridians* formed small colonies with opaque density. These differences however didn't have any significant effect on the strains' ability to form biofilm. Therefore, colony morphology could be disregarded as a fundamental pathway for biofilm formation, indicating clearly that there were some other important factors such as environmental factors (diversity of bacterial community, quorum sensing, EPS production), Physicochemical characteristics (surface attachment ability, surface topography, net surface charge) involved in the formation of biofilm.

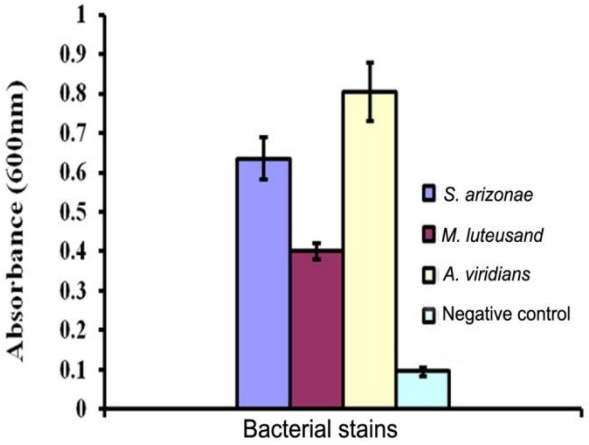

Figure 1: Absorbance (600 nm) for the isolates *S. arizonae*, *M. luteusand* and *A. viridians* after 34 h of growth. Negative control of LB medium incubated in the same conditions as the other samples. Background signal from negative control was not deducted.

Since this research work has been undertaken to establish an *in vitro* screening method for biofilm formation and quantification, we also used a well-accepted standard test tube method and proposed a new image processing system (image J) for early and advanced detection of biofilm on the glass cover slip. After comparing the results of the test tube assay method (Figure 1) with the cover slip assay (Figure 2), we found our new image processing method was more efficient and much advanced for the biofilm study than the test tube method. Our results suggest that this image processing method was effective and provided reliable result and displayed strong correlation with the biofilm-positive isolates (Figure 2).

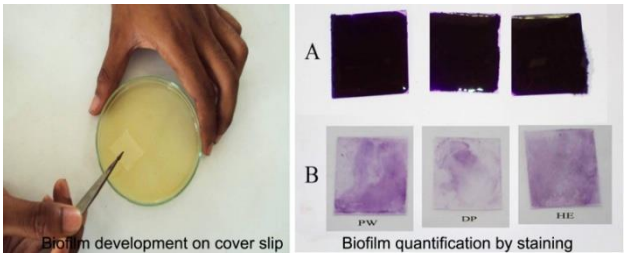

Figure 2: Glass cover slip was taken from the LB medium after developing the biofilm. Crystal violet staining of biofilm produced by the stains isolates *S. arizonae* (PW), *M. luteusand* (DP) and *A. viridians* (HE), on glass cover slip during the crystal violet staining (A) and Biofilm stained with crystal violet on the surface of the glass cover slip (B).

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Table 3: Optimum conditions for the biofilm development by the selected isolates. The results represent as mean value of triplicates.

Required conditions for biofilm formation		Biofilm absorbance (600 nm)		
		Biofilm forming isolates		
		S. arizonae	*M. luteus*	*A. viridians*
Medium	LB	0.54*	0.30*	0.70*
	NB	0.51	0.10	0.23
Incubation temperature	27 °C	0.445	0.12	0.13
	37 °C	0.79*	0.80*	0.80*
	45 °C	0.31	0.29	0.075
Medium pH	4.5	0.43	0.01	0.005
	6.5	0.82*	0.25*	0.32*
	8.5	0.505	0.17	0.13
Required salt concentration (%)	0	0.05	0.00	0.02
	1	0.79*	0.27*	0.74*
	5	0.13	0.01	0.19

*marks indicted the highest value in that condition.

The use of crystal violet allowed us for direct visualization of biofilm formation in the test tube walls. This result clearly showed that the crystal violet bounded ring formation around the air-liquid interface of the tubes in all positive strains, somewhat with different thickness. The cover slip assay also showed the similar pattern of attachment on the surface of glass cover slip (Figure 2). The images captured from the cover slip assay (Figure 3) showed the positive correlation with the standard test tube assay. These experiments showed us that there were sharp differences in the degree of attachment of bacterial cells, the retention of crystal violet by the cells and the absorbance of crystal violet at 600 nm among the biofilm forming strains. The method (cover slip assay measured by image J) was also verified in other laboratories, indicating the acceptance of the method for the purpose.

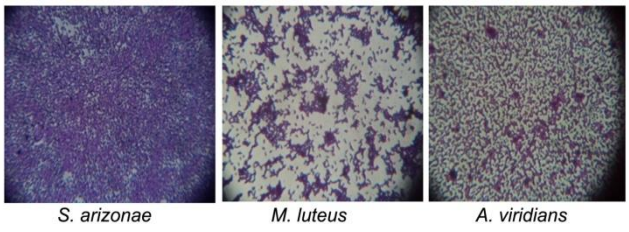

Figure 3: Captured images of *S. arizonae*, *M. luteus* and *A. viridians* biofilms observed under microscope after staining the surface of glass cover slip.

We also investigated the initial attachment capability of the selected isolates on the non-living surface especially on the glass surfaces either on the test tube walls or on the surface of the cover slip. Generally, motile bacteria utilize their flagella for spreading across the surface, and then anchor onto the surface with pili and possibly outer membrane proteins (O'Toole *et al.*, 2000). In the present study, the isolate *S. arizonae* was found much motile than the *A. viridians* and *M. luteus* which were the weaker motile (Table 2). The results of the biofilm formation with different environmental conditions showed that the weakly motile isolate *M. luteus* formed less biofilm than the isolates *S. arizonae* and *A. viridians* (Table 3). These results pointed out that the initial attachment capability is correlated with their motility for early development of their biofilm.

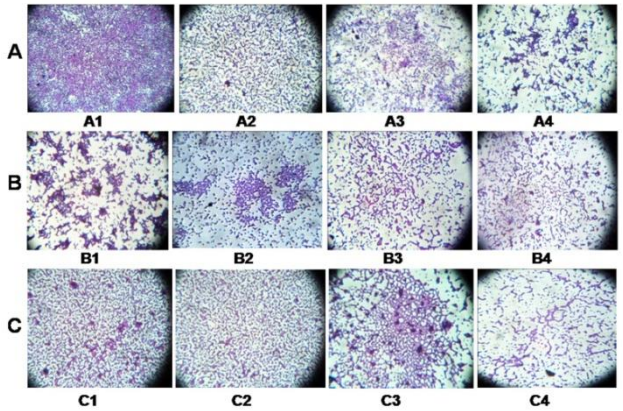

Figure 4: Microscopic images show the inhibitory effects of penicillin against the bioflm formation on the glass cover slip by the (A) *S. arizonae*; (B) *M. luteus*; (C) *A. viridians*. The biofilms were stained with the crystal violet on the surface of glass cover slip prior to capturing the images. A1, A2, A3, and A4 represent the biofilm formed by *S. arizonae* in 0 µg/L, 10 µg/L, 25 µg/L and 50 µg/L penicillin respectively. B1, B2, B3, B4 represent the biofilm developed by *M. luteus* in 0 µg/L, 10 µg/L, 25 µg/L and 50 µg/L penicillin respectively and C1, C2, C3, C4 represent the biofilm formation by the *A. viridians* in 0 µg/L, 10 µg/L, 25 µg/L and 50 µg/L penicillin respectively.

Table 3 showed the required condition for the maximum biofilm development by the three isolates. The LB medium (with 1% NaCl) at pH 6.5 and incubation temperature 37 °C enhanced the biofilm formation. Whereas, in nutrient medium all the bacterial strains produced less amount of biofilm compared to LB medium in the optimum conditions. All the bacterial strains were inhibited by the use of antibiotics penicillin (Figure 4). The images showed that increased concentration (10 µg/L, 25 µg/L and 50 µg/L) of penicillin decreased the development of biofilm. That means the presence of antibiotics has an inhibitory effect on the formation of biofilm and the increased concentrations of antibiotics inversely affect the amount of biofilm formation (Figure 4).

Our result showed that in control condition (without antibiotic), cells of bacterial strains attached on the solid support, whereas, presence of antibiotic reduces the cell attachments on the surface. Similar results were exhibited by all the three bacterial strains (Figure 4). The result was correlated with those obtained by the test tube assay (Ryu and Beuchat, 2005; Parinia and Pitt, 2006). Besides, this image processing provided us some more information about the attachment ability of bacterial stains, i.e. how the cells were attached and the arrangements of binding

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on the solid support could be obtained. In Figure 4, control *S. arizonae,* showed uniformly distributed or homogeneously attached on the glass cover slip. However, with the increased level of antibiotic, the numbers of cells on the surfaces of the cover slips decreased, and their binding capacity showed different mode of attachment ability. Mah and O'Toole (2001) explained how microorganisms changed their cell surface for the attachment on the solid supports in presence of antibiotics. Since several organisms have different cell surface and binding capability, their mode of biofilm formation varied, however in presence of antibiotics they have changed their surface protein for attachment on the solid support, resulting, changing the mode of binding. Similarly our selected bacterial strains showed changing mode of cell attachment on the glass cover slip which was a very important phenomenon for the biofilm study could not be provided by the test tube assay method.

Here we introduced a new method i.e. image processing software image J for the estimation of biofilm development. The images captured from the biofilm (which developed on the solid support) could be analysed by the image processing software (image j). Recent research has begun a shed light on a simple method to know how the surface-attached microbial communities developed. Finally, this method is very simple, easy to operate, require less space to set the experiment, cost and time effective. Considering the above facts, our proposed method for the estimation and evaluation of the biofilm can be effective and useful for the selected bacterial isolates.

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