Role of Extracted Genomic DNA on Biofilm Formation by *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* *in vitro*

Sanaa R. Oleiwi
Huayda K. Abid
Dpet.of Biology/ College of sciences/ University of Baghdad

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Abstract

Bacteria form complex and highly elaborate surface adherent communities known as biofilms.Biofilm have been shown to be associated with several human diseases ,and to colonize a wide variety of medical devices . The current study focuses on contribution of extracted genomic DNA in biofilm formation by *P. aeruginosa* and *K. pneumoniae* isolates .The percentages of *Pseudomonas aeruginosa* recovery from drinking water in this study were 10%(20 positive *P. aeruginosa* samples) and *K. pneumonia*., 7%(14 positive *K. pneumonia* samples).The results showed that all *P.aeruginosa* and *K. pneumoniae* isolates (100%) were slime producer but in different degrees by forming of black colonies on congored agar: very black colonies , black colonies and red colonies .Results indicated that addition of extracted genomic DNA to microtitre-plate cultures stimulate biofilm formation(either endogenous or exogenous DNA).

Key words:biofilm ,DNA, microtitre-plates , congored agar

Introduction

Bacterial biofilms are structured communities of cells enclosed in self-produced hydrated polymatrixes that adhere to inert or living surfaces[1]. The matrix, which holds bacterial biofilms together, is a complex mixture of macromolecules including exopolysaccharides, proteins and and DNA[2,3,4].

P. aeruginosa that are widely spread in the environment and commonly occurring in soil,water and drinking water. *P. aeruginosa* is an important opportunistic human pathogen that can cause life-threatening infections, especially in patients with cystic fibrosis (CF) and individuals with a compromised immune system[5]. *K. pneumonia*e is widely distributed in environment(water and soil) ,the gastrointestinal, urinary, and respiratory tracts of healthy people and is one of the most important pathogens causing nosocomial infection [6,7]. It causes opportunistic infections, such as pneumonia , sepsis, inflammation of the urinary tract, and wound infection, in compromised patients. Biofilm-associated *K. pneumoniae* have been shown to be associated with several human diseases[8], and to colonize wide variety of medical devices[9].

Involvement of extracellular DNA in biofilm formation

The extracellular matrix, which is essential for interconnecting the bacteria in biofilms, can be composed of polysaccharides, proteins , and extracellular DNA(eDNA)[10] . Previous studies have shown that eDNA functions as a cell-to-cell interconnecting matrix compound in *P. aeruginosa* biofilms[10,11 ,12,] Subsequently, evidence was provided that eDNA functions as a matrix component in biofilms formed by many other bacterial species, e.g., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus intermedius*, , *Streptococcus mutans[13]* and *K. pneumonia*e[14].

Vilain *et al*., 2009 described DNA as an adhesin and their results indicate that biofilm formation requires DNA as part of the extracellular polymeric matrix[15], extracellular DNA(eDNA) has now emerged as one of the major components the biofilm matrix of many bacteria and has been shown to perform diverse functions in promoting the biofilm mode of existence [16].

Thus, the aim of the present study was to evaluate the role of extracted genomic DNA in the formation of biofilms formed by *P. aeruginosa* and *K. pneumoniae* *in vitro* .

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Material and Methods

Isolation and identification

1- Sample collection: The sampling was carried out during the period from the beginning of January 2014 till May, 2014; two hundred water samples were collected randomly from houses located in different parts of drinking water distribution system in Baghdad area- Iraq.

Samples were collected according to the Standard Methods [17]. Prior to collection, water was allowed to run at a uniform rate for 2–3 min., in a sterile bottles containing sodium thiosulphate to a final concentration of 0.01 % (W/V) to neutralize any free or combined residual chlorine.

One liter of water sample was collected in each bottle ,the samples were carried out to the laboratory special aspectic cool box . All the samples were analyzed within 24 h .

2- Filtration and culturing of water samples: Water was tested for the presence of *P. aeruginosa* and *K. pneumonia*e by filtering 100ml volume through 0.45 µm nitrocellulose filter (Millipore) and incubated on *Pseudomonas* agar containing cetrimide (for isolation of *P. aeruginosa*) and MacConkey agar(for isolation of *K. pneumonia*e) at 37 °C for 24h [18].

3- Isolation and identification of colonies: After 24 h of incubation, cultures were examined for distinct colonies, the colonies were picked and recultured on to *Pseudomonas* agar and MacConkey agar plates and incubated at 37 °C for 18- 24 h in order to obtain pure well isolated colonies. All the

colonies were tested for colony morphology and colony color. All bacterial isolates were examined morphologically by Gram's stain and subjected to biochemical testes [19], and further confirmation API 20E were used in diagnosis as in (Biomerieux) France.

DNA extraction :

DNA was extracted from *P. aeruginosa* and *K. pneumonia*e isolate (isolate that had most OD value in biofilm assay)[20] as endogenous DNA and exogenous DNA , as described:

1. Spin down 5 ml of saturated cell culture(of each two species) in eppendorf tubes.
2. Add 50 µL of 10% SDS buffer,incubate at 37°C for 5-10 min until clear and viscous.
3. Put into a new tube .
4. Add 550 µL of phenol
5. Mix gently by inversion and centrifuge at 4°C at 3000 rpm for 15 min.
6. Transfer top layer to a new tube and repeat step 4.
7. Transfer top layer to eppendorf 1
8. Spin for 3 min and transfer supernatant.
9. Add 2 volumes of 100% ethanol and mix by inverting.
10. Cool sample at -20°C for 5 min.
11. Centrifuge at 3000 rpm for 15 min at 4°C.
12. Remove and discard the supernatant, resuspend the pellet by T.E buffer . [20,21] .
13. To determine the purity of the DNA and the degree of contamination with protein, spectrophotometer was used to estimate the purity ratio of DNA; and the purity ratio estimate according to the following formula,(Purity between 1.7 and 2.0 is usually accepted):

DNA purity = O.D260 / O.D280

Detection of bacterial ability to adhesion

Congo red agar method (Qualitative method)

Medium prepared according to Freeman *et al.*,1989 [22] inoculated with single colony of tested bacterial by streaking, incubated at 37º C for 24 hr.A positive result indicated by black colonies and the non-slime producers usually remained pink .

Polystyrene microtiter plates (Quantitative method)

In first step , the assay was done without addition of extracted DNA (of *P. aeruginosa* and *K. pneumonia*e) into microtitre-plate cultures.

It was done according to the method described by Pfaller *et al.*,1988 [23]as followes :

The bacterial isolates were cultured in Brain Heart Infusion (BHI) broth with 1% glucose ,incubated at 37ºC for 18 hour.After that the bacterial culture was diluted in BHI medium and adjusted in comparison to MacFarland tube No. 0. 5. Two hundred microliters of bacterial culture were used to inoculate 96-well polystyrene microtiter plates and later incubated for 24-48 hrs at 37C^{o}. After incubation periode , all wells were washed with phosphate buffer saline to eliminate the unattached cells for (2-3) times. Two hundreds µl of 1% crystal violet was added to each well , shaking the plates three times to help the colorant to get the bottom of the well. After incubation for 10 min at room temperature, each well was washed with 200 µl sterile phosphate buffer saline (PBS) to remove the planktonic cells and stain which not adhered to the surface of well. Only the adhered bacteria forming the biofilm were kept on the surface of the well. The crystal violet bound to the biofilm was extracted later with (200) µl of ethyl alcohol, and then absorbance was determined at 570 nm in an ELISA reader for determination of the degree of biofilm formation.Controls were performed with Crystal violet binding to the wells exposed only to the culture medium without bacteria. All tests were carried out in triplicate and the results were averaged.The biofilm degree was calculated as follows: Based on O.D of biofilm - producing isolates were classified into the following categories: OD ≤ ODc no biofilm producer, ODc < OD ≤ 2 × ODc weak biofilm producer, 2 × ODc < OD ≤ 4 × ODc

moderate biofilm producer and 4 × ODc < OD strong biofilm producer. (Stepanovic *et al.*, 2000) [24] .

Second step , assay was done with addition of extracted genomic DNA of *P. aeruginosa* and *K. pneumonia*e isolates into microtitre-plate cultures and biofilm assay was done in the same procedure described previously. .

Before this step , cells of two species should be in competent state to take DNA from culture that were done as described by Sambrook,2001 [25]as follows:

Preparation of competent cell using CaCl2 method

1- Nutrient agar plates were streacked with *P. aeruginosa* and *K. pneumonia* isolates. Incubation was done at 37 ˚C overnight ,a singale colony of *P. aeruginosa* and *K. pneumonia*e were picked up ,transferred to 100 ml nutrient broth and grown overnight .

2-Two ml of suspension were transferred to each ice-cold eppendrof tubes and the culture was cooled to 0˚C by placing the tube in ice for 10 min. .

3-The cell suspension was centrifuged at 4000 rpm for 10 minutes at 4 ˚C .The tubes were then inverted for 1 min on tissue paper to allow the traces of medium to drain away .

4- The pellet was resuspended by gentle vortexing in 1 ml of ice –cold solution of 0.1 M CaCl2 .These cells can be directly used for transformation or as an aliquot of 200 µl in pre-chilled microfuge tubes kept at 4˚C.

5- Resuspend the pellet in 2ml of 100 mM CaCl2 in 15% glycerol and store it at -20 ˚C [25]. DNA of *P. aeruginosa* and *K. pneumonia* isolates was added at a final concentration of 20 µg/ml and 40 µg/ml(Spectrophotometer was used for measuring the optical density at wavelength of 260 (O.D260), then used in biofilm assay . DNA of *P.aeruginosa* supplemented to the microtiter plates culture of *P.aeruginosa (*as endogenous DNA)and of *K. pneumonia* (as exogenous DNA) .On other hand DNA of *K. pneumonia* supplemented to the microtiter plates culture of *K. pneumonia* (as endogenous DNA) and *P. aeruginosa* (as exogenous DNA) that described by Gilan, 2013[26].

Statistical Analysis

The Statistical Analysis System- SAS was used to affect of different factors in study parameters. Least significant difference –LSD test was used to significant comparison between means in this study.

Results and Discussion

Isolation and identification

Two hundred samples of drinking water were collected from different sites in Baghdad area were collected to found out the prevalence of *P. aeruginosa* and *K. pneumonia*.All the isolates were identified depending on the morphological and microscopic examinations aswell as biochemical tests; then lately conformed by API system.
The percentages of *P. aeruginosa* recovery in this study were 10%(20 positive *P. aeruginosa* samples) and *K. pneumonia*., 7%(14 positive *K. pneumonia* samples) ,these results were in agreement with other studies[27,28].

Detection of slime layer by Congo-red agar method (qualitative method)

The ability of producing slime layer by *P. aeruginosa* and *K. pneumonia* isolates was evaluated according to protocol described by Freeman *et al.*, 1989 [32]; all tested isolates were cultured on Congo-red agar plates. The results showed that all *P. aeruginosa* and *K.* .isolates (100%) were slime producer but in different degrees indicated by forming of black colonies: very black colonies(+++) , black colonies(++) and red colonies(+) as illustrated in table (1).These results were in agreement with previous studies [29,30].

Micro-titer plate assay (Quantitative method)

The ability of *P. aeruginosa* and *K. pneumoniae* isolates to produce biofilm were evaluated (quantitative) by using 96-well polystyrene microtiter plates and then absorbance was

determined at 570 nm in an ELISA reader for determination of the degree of biofilm formation for studied isolates that adhered on the surface of the microtiter wells, optical density (OD) represented the degree of the biofilm thickness tha is t formed by the studied isolates.

The results indicated that each isolate showed a different potential to form biofilm under the same condition of experiment(without addition of DNA).Biofilm producer isolates were classified using the scheme of Stepanovic ´ *et al*. (2000)[24] into groups as showed in tables 2,3.

OD values that are illustrated in table (2,3)showed that the biofilm formation of *P. aeruginosa* was higher than *K. pneumoniae* , strong group of *P. aeruginosa* had 15 isolates with mean of OD 0.813 ± 0.042 compared with strong group of *K. pneumoniae* had 8 isolates with mean of 0.776 ± 0.047.

Qualitative assays of bacterial biofilm produced by *P. aeruginosa* and *K. pneumoniae* were conducted by other researchers[31].

Effect of extracted DNA on biofilm stimulation

Results of recent study indicated that addition of extracted DNA to microtitre-plate cultures stimulate biofilm formation, in other word optical density OD values which represented the biofilm formation were higher in present of genomic extracted DNA than in absence it for both *P. aeruginosa*((P<0.05) and *K. pneumonia*e(P<0.05)(table 4) .

This results came in accordance with Whitchurch,*etal*.,2002[10] who found that DNA is required for the initial establishment of biofilms .,and reported that the majority of the extracellular material in biofilm was not exopolysaccharide but DNA and therefore hypothesized that this DNA may play a functional role in biofilms, but until now there have been no local studies on the role of extracted genomic DNA on biofilm formation by *P.aeruginosa* and *Klebsiella pneumonia in vitro.*

The results of recent study are supported other studies which were conducted on other bacteria suggest that DNA important for biofilm formation, of Streptococcus [,32], *K. pneumonia* [33],*Bordetella*[34]andStaphylococcus[13].

Current results showed that addition of genomic extracted DNA of *P. aeruginosa* in conc. 20 µg/ml into culture of *P. aeruginosa*(endogenous) increased OD value from 0.729 to 1.014 but when addition of genomic extracted DNA of *P. aeruginosa* in conc. 20 µg/m into *K. pneumonia* culture(exogenous DNA) increased OD value from 0.594 to 0.957 .(table 4) .

In other hand addition of genomic extracted DNA of *K. pneumonia* in conc. 20 µg/ml into *K. pneumonia* culture(endogenousDNA) increased OD value from 0.594 to 0.937 but when addition of of genomic extracted DNA of *K. pneumonia* in same conc. into *P. aeruginosa* culture (exogenous DNA) increased OD value from 0.729 to 1.022 . Addition of excess DNA(DNA in final concentration 40 µg/ml)

had approximately the same effect of DNA in final concentration 20 µg/ml on biofilm formation ,in other hand the DNA sources (from *P. aeruginosa* ,or *K. pneumonia*) also had approximately the same effect on biofilm formation (two type of DNA had effect on biofilm formation in same degee , these results agreed with Liu *etal*., 2008 [35] which they found that DNA promoted bacterial aggregation in a type-nonspecific way . These results suggest that DNA functions as a molecular bridge to promote aggregation.

The structural role of biofilm is further reinforced by the effect obtained when the bacteria were supplemented with DNA (either endogenous or exogenous), which enhanced biofilm formation. [22]. Because DNA can function as a cell-to-cell connector[10,6] , it is likely that DNA also has a role in the accumulative phase of biofilm formation.

Qin *etal.*, [13] suggest that extracellular DNA is important for the initial phase of biofilm development by S.epidermidis on polystyrene or glass surfaces.

Bacteria in natural environments predominantly live in biofilms, i.e. surface-attached microbial communities embedded in a self-produced extracellular matrix[3].

DNA has been implicated as a major structural component for the initial establishment of biofilms in bacteria [10,36]. Moreover, it has been observed that free DNA plays an important role in accumulation phase in biofilm formation [37].

DNA has an essential role in efficient attachment of cells to a surface, Vilain *etal* demonstrate DNA as adhesins that contribute to bacterial biofilm. This property of extracellular DNA(eDNA) based mainly on the observation that addition of DNase I significantly reduced cellular attachment, resulting in reduced biofilm formation. Vilain, *etal* .,2009[15].

Comparisonof extracellular DNA and(genomic) chromosomal DNA by the use of polymerase chain reaction and Southern analysis suggested that the extracellular DNA is similar to whole-genome DNA.[11] and had the same role in biofilm formation (in current study chromosomal DNA was used.

There are several proposals concerning the role of extracellular DNA in biofilm function.The possible roles of extracellular DNA as a polymeric substancea structural componen [38,39] , and an energy and nutrition source [40], and gene pool for horizontal gene transfer (HGT) in naturally competent bacteria have been considered [22].

It was previously shown that the the extracellular DNA of *P. aeruginosa* , *Klebsiella pneumonia* and *Streptococcus pneumonia* could change the properties of the biofilms formed by these bacteria [14,10] .

Others explain this property of DNA as DNA Facilitates Twitching Motility–Mediated Biofim Expansion. As biofilms of *P. aeruginosa* contain large quantities of eDNA[10,11] and the type IV pili (tfp) of *P. aeruginosa* have been shown to bind DNA[41] ,we explored the possibility that eDNA may also contribute to the twitching motility–mediated biofilm expansion, and this indicates that DNA is important in coordinating bacterial movements during biofilm expansion and self-organization of bacterial biofilms is facilitated by extracellular DNA[37].

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TableNo. (1) :The results of slim layer production of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* isolates

Isolates	Degrees of biofilm		
	+	++	+++
P. aeruginosa	5 isolates (25%)	7 isolates (35%)	8 isolates (40%)
K. pneumoniae	3 isolates (21.43%)	4 isolates (28.57%)	7 isolates (50%)

Table No.(2): The groups of biofilm producer *Pseudomonas aeruginosa* isolates according to optical density(OD) values by using the scheme of Stepanovic´ *et al.* (2000)[OD value of control was 0.15

OD Groups	No.(%) of isolates	Mean of OD ± S.E
Weak	0(0)%	0.00 ± 0.00
Moderate	5(25%)	0.496 ± 0.021
Strong	15(75%)	0.813 ± 0.042
LSD value	---	0.191
P-value	---	0.0001

Table No.(3) : Thr groups of biofilm producer *Klebsiella pneumoniae pneumoniae* isolates according to Optical density(OD) values by using the scheme of Stepanovic´ *et al.* (2000)[OD value of control was 0.15] .

OD Groups	No.(%) of isolates	Mean of OD ± S.E
Weak	2(14.28%)	0.235 ± 0.010
Moderate	4(28.57%)	0.409 ± 0.026
Strong	8(57.14%)	0.776 ± 0.047
LSD value	---	0.184
P-value	---	0.001

Table No. (4): OD values represent the biofilm formation of *Pseudomonas aeruginosa* and *Klebsiella pneumonia* under experimental different condition (in present and absence of genomic DNA of *P. aeruginosa* and *K. pneumonia*)

Experimental conditions	Mean of OD ± SE	
	P. aeruginosa	***K. pneumonia*e**
Without extracted DNA	**0.729 ± 0.042 b**	**0.594 ± 0.066 b**
With extracted DNA of *P. aeruginosa* in concentration 20 µg/ml	**1.014 ± 0.059 a**	**0.957 ± 0.048 a**
With extracted DNA of *P. aeruginosa* in concentration 40 µg/ml	**1.112 ± 0.048 a**	**1.031 ± 0.052 a**
With extracted DNA of *K. pneumonia*e in concentration 20 µg/ml	**1.022 ± 0.034 a**	**0.937 ± 0.047** a
With extracted DNA of *K. pneumonia*e in concentration 40 µg/ml	**1.029 ± 0.037 a**	**1.058 ± 0.054 a**
LSD- value	**0.209 ***	**0.273 ***
P-value	**0.0467**	**0.0361**
*** (P<0.05)**		

a:in present DNA **,b** :in absence of DNA

دور DNA المستخلص في تكوين الغشاء الحيوي لبكتريا *Pseudomonas aeruginosa , Klebsiell pneumonia* خارج الجسم الحي

سناء رحمن عليوي
هويدا كريم عبد
قسم علوم الحياة / كلية العلوم / جامعة بغداد

استلم البحث في : 22/ حزيران / 2014 , قبل البحث في : 20/ تشرين الاول /2014

الخلاصة

تكون البكتريا معقدا ومجتمعات ملتصقة بالسطوح بصورة متقنة جدا يسمى الغشاء الحيوي. يرتبط الغشاء الحيوي ببعض امراض الانسان وكذلك يستوطن شتى الادوات الطبية , ركزت الدراسة الحالية على دور DNA المستخلص على تشكيل الغشاء الحيوي بواسطة P. aeruginosa و K. pneumonia .كانت نسبة عزل هذه البكتريا من مياه الشرب في هذه الدراسة هي 10%(20 عزلة P. aeruginosa) و7%(14 عزلة K. pneumonia).اظهرت النتائج ان جميع عزلات P.aeruginosa و K. pneumoniaeكانت منتجة للطبقة اللزجة لكن بدرجات مختلفة بتكوين المستعمرات السود على وسط اكار احمر الكونغو :مستعمرات سود غامقة وسود و حمر .اشارت النتوائج الى ان اضافة DNA الى الوسط يحفز تشكيل الغشاء الحيوي (سواء كان DNA ذاتي او خارجي).

الكلمات المفتاحية :الغشاء الحيوي ,دنا .اطباق المعايرة ,اكار احمر الكونغو