Generation of recombinant bioluminescent *Escherichia coli* for quantitative determination of bacterial adhesion

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Abstract: Bacterial adhesion to urinary catheter was evaluated by measuring the light emitted from a recombinant bioluminescent glycocalyx producer *Escherichia coli* strain. Generation of the bioluminescent strain was carried out by transforming the bacterial cells with pUCP18-GFP plasmid that contains a green fluorescence gene. Light emission measurement was closely correlated with the number of the adherent cells, giving a detectable signal from 1.2 X 10^2 cells. The efficiency of this assay was confirmed by testing the antiadherent effect of subinhibitory concentrations of ciprofloxacin with the aid of a model for *in-vitro* catheter colonization. There was no significant difference in the percentage reduction of adherent cells obtained by both light emission measurement and viable cell count techniques.

Keywords: Recombinant bioluminescent *E. coli*, pUCP18-GFP plasmid, adherence, catheter colonization, antiadherent effect.

INTRODUCTION

Bacterial adherence to surfaces and human mucosal tissues plays an important and critical early role in microbial colonization. It represents the first stage in the initiation of most infectious diseases processes (Vesterlund *et al*., 2005). As a result, there has been a growing concern regarding bacterial adherence to the surface of commonly implanted medical devices, such as intravascular catheters, urinary catheters, orthopedic implants, prosthetic heart valves and even contact lenses (Raffi *et al*., 2012). Approximately 60-70% of hospital-acquired infections, particularly in critically ill patients, are reported to be caused by medical devices (Darouiche, 2001; Bryers, 2008). Bacterial adherence has been evaluated by different techniques. Most of the conventional methods are based on plating or microscopic counting. Plating techniques usually involve surface sampling, followed by counting using viable count technique. Such techniques are tedious and the data derived from them are inherently variable depending on the efficiency of the applied methods used for sampling and for the recovery of the culturable organisms (Khardori and Yassien, 1995; Yassien *et al*., 2000). Alternatively, direct microscopic techniques such as optical, epifluorescence, phase contrast, scanning electron, transmission electron and force atomic microscopy have also been used (Thomas and McMeekin, 1981; Kim *et al*., 1996; Nostro *et al*., 2012). Nevertheless, microscopic techniques, in addition to being equally laborious as plating techniques, permit visualization of only a limited percentage of the total surface area and are dependent on the used sampling technique (Siragusa *et al*., 1999).

Microbial attachment processes are ideally observed under naturally occurring conditions with no sampling, fixation or any other treatment that may affect the biological or environmental integrity of the tested sample. Nondestructive techniques for studying the microbial adherence have been performed by ATPase and bioluminescence assays (Francis *et al*., 2000).

Bioluminescence assay, which depends on the use of bioluminescent recombinant bacteria, offers a method that is sensitive, time saving, inexpensive, innocuous and allows only the detection of viable cells.

The present study aimed to construct a recombinant plasmid (pUCP18-GFP) containing green fluorescence protein (GFP) gene. The constructed plasmid was used for the generation of bioluminescent recombinant *E. coli* pUCP18-GFP strain. This strain was used to study the primary bacterial adherence over the surface of indwelling medical devices, using a model for in-vitro urinary catheter. The efficiency of the established bioluminescent assay was evaluated by studying the antiadherent activity of sub inhibitory concentrations of ciprofloxacin.

MATERIALS AND METHODS

Organisms and plasmids

A glycocalyx producer *E. coli* clinical isolate was obtained from the Microbiology Laboratory at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. The

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plasmids and reference bacterial strains employed in this work are listed in table 1. Unless otherwise noted, cultures were maintained in Luria-Bertani (LB) broth under aerobic condition at 37°C. Media were solidified by the addition of 1.8% (W/V) agar. The cultures were stored in glycerol/DMSO mixture at -86°C.

All media and reagents were of the purest available grades, obtained from Sigma-Aldrich (MI, USA). A transformation vector carrying the green fluorescence protein gene was constructed and employed for the transformation of the positive glycocalyx producer *E. coli* strain. The transformants were designated as *E. coli* pUCP18-GFP strain.

The bacterial cultures were maintained on trypticase soy agar. The technique of Christensen *et al*. (1985) was applied for the determination of slime production. Inocula for the adhesion study were prepared by adjusting the cell suspension in saline (0.85% sodium chloride) to 10^8 CFU/ml using turbidometric method (OD_{600}) and verified by viable count technique.

Construction of recombinant plasmid containing green fluorescence gene

The used plasmid was the shuttle vector pUCP18 (West *et al.*, 1994) that has the ability to replicate in *E.coli*. Green fluorescence protein (GFP) gene was PCR amplified, using automated thermocycler (Techne, TC-5000, UK), from pGLO plasmid (Bio-Rad Laboratories, CA, USA). Unless otherwise indicated, all the PCR reagents were obtained from Promega (WI, USA). PCR amplification of GFP gene was conducted employing two primers, namely 5'….. CCCGGGTTATTTGTAGAGCTC ATC….. 3' (*Sma*I reverse primer) and 5'….. GCATG CATGGCTAGCAAAGGAGAA……3' (*Sph*I forward primer). PCR reaction was carried out in 100µl volume containing 10 µl of buffer (10X PCR) with added *Taq* DNA polymerase, 2mM $MgCl_2$, 32pmol of every oligonucleotide primer, a 0.20mM concentration of each of all the four dNTPs, I U of *Taq* DNA polymerase and 20ng of pGLO plasmid. The amplification of GFP gene was achieved with 30 cycles at 95°C for 1 minute, 55°C for another 1 minute, and then 72°C for 1.5 minutes, and finally followed by an extension step at 72°C for 7 minutes. PCR amplified DNA was purified using gel purification kit (Qiagen Inc., CA, USA).

Ligation reaction was conducted in a total volume of 20 µl in which 5µl GFP fragment (0.25µg/µl) was mixed with 2µl of pUCP18 *Sph*I/*Sma*I treated (0.25µg/µl), 2µl of 10X ligase buffer and 2µl of DNA ligase. Reaction was incubated overnight at 15°C.

Transformation of E.coli competent cells

E. coli DH5α competent cells

E. coli DH5α competent cells were prepared by $CaCl_2$ method and transformed with the ligated hybrid plasmids (pUCP18-GFP) by heat shock method as described by Inoue *et al*. (1990). The cells were then surface inoculated on LB agar medium containing 100µg/ml ampicillin, then incubated at 37°C for 24 hr and the formed clones were picked up. Plasmids contained in the transformants were isolated using QIAprep Spin Miniprep Kit 50 (Qiagen Inc., CA, USA) and reintroduced into *E. coli* DH5α cells which were again spread on the plates. After Incubation at 37°C for 24 hr, plasmids contained in the transformants were isolated.

A glycocalyx producer E.coli competent cells

Competent cells of glycocalyx producer *E. coli* were prepared and transformed with the isolated plasmids (pUCP18-GFP) using the same procedure as described above. The treated cells were spread over LB agar medium supplemented with 100µg/ml ampicillin. The plates were kept at room temperature for 1 hr and then incubated overnight upside down at 37°C. The formed clones were picked up and streaked on LB agar plates supplemented with 100µg/ml ampicillin and 0.5mM isopropylthio-B-D-galactopyranoside (IPTG), and incubated at a temperature of 37°C for 24 hr. The cloned cells (*E.coli* pUCP18-GFP) were characterized by the production of luminescence when exposed to UV rays.

Light emission measurement as a function of the amount of cells

Two-fold serial dilutions of the bioluminescent bacterial cell suspension in saline solution were prepared, approximately from 1 x 10^5 to 1 x 10^2 CFU/ml. For each dilution, both the bioluminescence emission and the number of viable cells were determined. The emitted light was measured in a luminescence spectrometer (Perkin Elmer, LS55, UK). Bacterial free saline was used as a control.

Test for the glycocalyx production and adherence to plastic surfaces

The spectrophotometric technique described by Christensen *et al.* (1985) was used for adherence determination. In summary, 5ml of stationary 18-hr *E. coli* cultures in trypticase soybean (TS) broth were washed then diluted and standardized with fresh TS broth to contain 5 x 10^5 CFU/ml. Wells of sterile flat-bottom polystyrene tissue culture plates were filled with aliquots (0.2 ml) of the diluted cultures and incubated at 37°C for 48 hr. Using a micropipette, gentle aspiration of the contents of the tissue culture was performed followed by washing four times using sterile phosphate-buffered saline (pH 7.2). Bouin's fixative was then used to fix slime and adherent organisms overnight. Removing the fixative was carried out by washing the wells using 50% ethanol three or four times. Crystal violet was used to stain the wells and the excess stain was removed by washing the plates with running distilled water followed by air-drying. A microplate reader (Biotek, ELX808, USA) having a 490

Table 1: Reference strains and plasmids employed in this work

Plasmids or Strains	Relevant Properties	Reference or Source
Plasmids		
PUCP 18	Shuttle vector, Ampicillin^r	West *et al*., 1994
pGLO	Contains GFP gene	B*
Strains		
E coli DH5α	Sup E44 LacUI69 (80lac M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	A*

A* = Biolabs, WA, USA B* = Bio-Rad Laboratories, CA, USA ^{r} =Resistant

nm wavelength was used to read the optical density of the stained adherent films. Triplicate measurements were performed and were repeated three times.

Effect of expression of GFP gene on the glycocalyx production and adherence of the bioluminescent E. coli pUCP18-GFP strain

The degree of adherence of the transformed (*E.coli* pUCP18-GFP) strain and non- transformed strains to plastic surface was determined as described earlier.

Establishment of bacterial adherence to the surface of urinary catheters

Biofilm formation and colonization of urinary catheters using the recombinant bioluminescent *E. coli* pUCP18-GFP strain was studied using an *in-vitro* model as described by Yassien and Khardori (2004).

Inoculum preparation

Harvesting of an 18 hr culture, grown in TS broth with 0.3% yeast extract, was carried out by centrifugation and washing twice using normal saline. Turbidity was adjusted using 0.5 McFarland standards. Viable count technique, using trypticase soy agar, was performed to confirm the standardization of the inoculum size. The reservoir used for colonizing the catheter segments was a 500 ml bag of sterile normal saline with added 5% dextrose (D_5NS). Inoculum size of 10^6-10^7CFU/ml was added to the infusate.

Bacterial adherence and biofilm formation

To investigate the formation of biofilms of the tested strain on urinary catheters, infusion of the solution of 5% D_5NS which contained the organism was carried out through a sterile modified Robbins device in which the segments of the catheter were placed aseptically in the inserts of the sampling device. An intravenous infusion pump (Gemini PC2, IMED, CA, USA) as well as an infusion set (Travenol Laboratory, IL, USA) were both used to infuse solution at a rate of 30 ml per hour. Following the infusion of 500ml D_5NS solution, flushing of the urinary catheters was achieved by purging the device with normal saline. An amount of 250ml of saline over 1 hr was used to remove any free-floating bacteria. Following flushing, five segments were placed in 2 ml saline, sonicated for 2 minutes at a cycle of 30% and at 3.5 output then vortexed for 1 minute. Viable count and light emission measurements were used to determine the number of cells released from the segments into normal saline.

Quantitative determination of the primary adherent cells

- *Viable count experiment.* Serial dilutions of cell suspensions were prepared, grown on TS agar plates, and incubated at 37^{o}C for 24 hr. Data were expressed as CFU/segment.
- *Light emission measurement.* A luminescence spectrometer was used to measure the emitted light, as described above. The resulting bioluminescence measurements were converted to actual cell numbers, using a calibration curve, and then compared to those obtained by direct viable count experiment.

Effect of subinhibitory concentrations of ciprofloxacin on the adherence of E. coli pUCP18-GFP using the in-vitro model of urinary catheter colonization

During colonization, ciprofloxacin was added to the inoculated infusate bags, containing 500ml of 5% glucose in saline solution, at concentrations of 1/2, 1/4 and 1/8 MICs. The control was a drug free infusate. The number of the adherent cells to the surface of urinary catheters was determined by both light emission measurement and viable count technique.

STATISTICAL ANALYSIS

Student t-test and analysis of variance were used to investigate the statistical significance between means. The statistical analysis was carried out using InStat software© (GraphPad Software Corp., CA, USA). The level of significance was set at $P<0.05$.

RESULTS

Light emission measurement as a function of the number of viable cells

GFP gene was proved to be expressed in the recombinant *E. coli* pUCP18-GFP strain, as light was emitted from the cloned cells when exposed to UV rays as shown in fig. 1. When the emitted light was measured in a luminescence

spectrometer, it was found that the minimum number of transformed bacterial cells with noticeable luminescence was approximately 1.2 x 10^2 CFU.

Further studies were carried out to measure the emitted light as a function of the number of viable cells. As shown in fig. 2, the light emission produced from the bioluminescent *E. coli* pUCP18-GFP strain closely correlates with the number of viable adherent cells over a 3-log range.

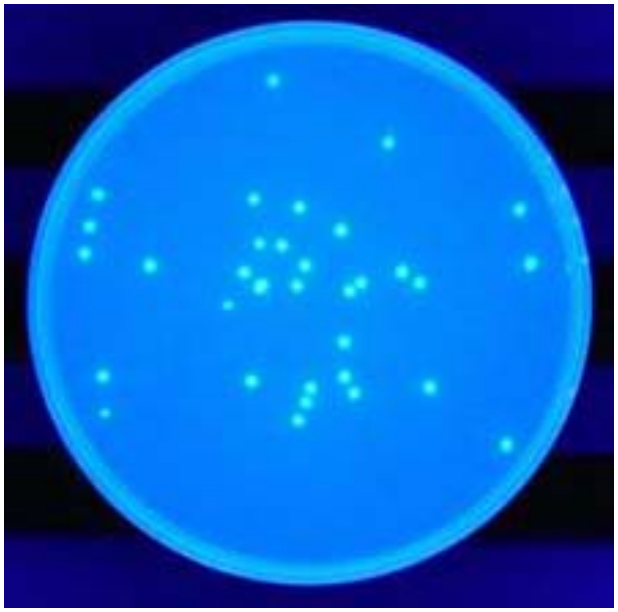

Fig. 1: Colonies of the cloned *E coli* pUCP18-GFP on LB agar emitted light when exposed to UV rays.

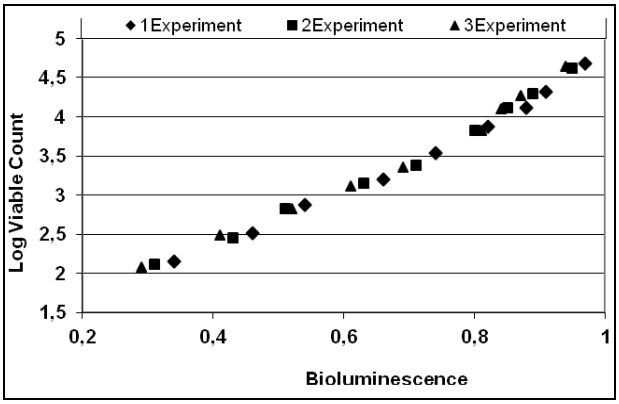

Fig. 2: Correlation of light emission and viable plate count of bioluminescent *E coli* pUCP18-GFP.

Effect of expression of GFP gene on the glycocalyx production and adherence of bioluminescent E. coli strain pUCP18-GFP

The optical density of the adherent biofilms was measured by spectrophotometric method. The results showed that the optical density of the formed adherent biofilms of the transformed and non-transformed *E. coli* were 0.46±0.08 and 0.47± 0.06, respectively.

Effect of subinhibitory concentrations of ciprofloxacin on the adherence of E.coli pUCP18-GPF to the surface of urinary catheter segments

The presence of sub-MICs of ciprofloxacin inhibited the adherence of the bacterial cells to urinary catheters segments and its effect was concentration dependent. In the presence of 1/2, 1/4 and 1/8 MIC of the tested ciprofloxacin, the range of the percentage of the adherent cells, determined by light emission measurement, was reduced to 28.6-34.8%, 44.1-51.4% and 66.8-72.6% of the control, respectively. On the other hand, using the viable count technique, the percentage of the adherent cells was reduced to 29.1-35.4%, 42.2-50.8% and 68.2-74.3% of the control, respectively (fig. 3). According to the obtained results, there is no significant difference between the results obtained by the two techniques.

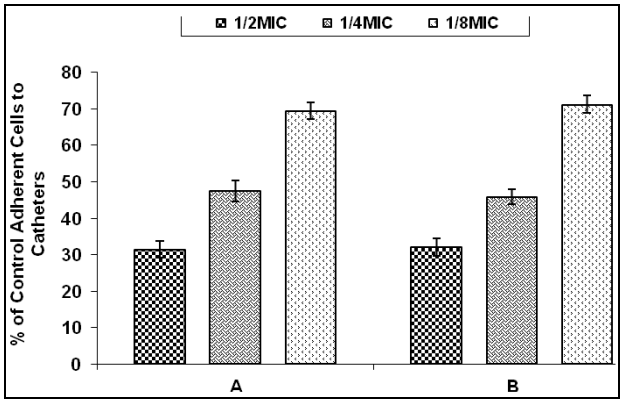

Fig. 3: Relative inhibition of adherence of bioluminescent *E coli* pUCP18-GFP to the urinary Catheter segments by different sub inhibitory concentrations of ciprofloxacin as measured by (A) Light emission measurement (B) Viable count technique.

DISCUSSION

Different techniques, such as direct viable count techniques and radiolabelled cell procedures have been used for quantitative evaluation of primary bacterial adherence to the surface of indwelling devices such as catheters and even contact lenses (Fleiszig *et al*., 1996; Ahearn *et al*., 2000; Yassien and Khardori, 2004). ATP analysis, a rapid method that was originally used for the determination of bacterial densities in water and industrial raw materials, has also been used for the determination of cell densities on inert surfaces (Brolin and Wettermark, 1991; Costanzo *et al*., 1998; Gracia *et al*., 1999).

In this work, generation of bioluminescent *E. coli* pUCP18-GFP strain allowed a quantitative determination of bacterial adhesion by bioluminescence assay. The glycocalyx producer *E. coli* strain was transformed with pUCP18-GFP plasmid that contains GFP gene (under the control of *Lac* promoter) and B-lactam resistance gene (West *et al*., 1994). The recombinant *E. coli* pUCP18-GFP clone cells were selected on media containing 100

µg/ml ampicillin. IPTG was added to the growth medium to regulate the activity of *Lac P* promoter and consequently activate the expression of GFP gene and the production of GFP protein resulting in appearance of luminescence when the cells were exposed to UV. The minimum number of cells detected by their bioluminescent phenotype was 1.2×10^2 cells indicating a relatively strong expression of GFP gene in the transformed *E. coli* strain. Therefore, the obtained bioluminescent *E. coli* pUCP18-GFP strain could be used to establish a method for quantitative determination of the bacterial adhesion to the surface of catheters depending on the light emission measurement.

The bioluminescence assay is an accurate and relatively inexpensive method. It allows sensitive and rapid determination of the number of viable cells without the need for overnight incubation on agar plates. In addition, the bioluminescence assay is more accurate than other techniques such as ATPase assay. The latter technique depends on the luminescence developed from the interaction between the exogenous factor luciferase enzyme and the substrates Luciferin, Oxygen, and bacterial ATP. Whereas, the bioluminescence assay eliminates any variability caused by substrate availability as well as streamlines, as it does not require an exogenous substrate.

The study of bacterial adhesion using bioluminescent bacteria strains has been formerly reviewed. A bioluminescent *E. coli* O157:H7, transformed with pCGLS1 plasmid that carries the entire luciferase (*lux*) operon, was used for studying bacterial adherence to beef carcass surface tissue (Siragusa *et al*., 1999). Different recombinant bioluminescence *E. coli* strains were used for accurate assessment of bacterial adherence in the presence of HeLa human epithelial cells (Brovko *et al*., 2011).

Comparative study of the primary bacterial adhesion between the transformed *E coli* pUCP18-GFP and non-transformed *E. coli* strains showed no significant difference in the bacterial adherence to the surface of the catheters, indicating that insertion of pUCP18-GFP plasmid, and expression of GFP gene has no effect on the production of glycocalyx and consequently on the degree of bacterial adhesion.

When light emission was measured as a function of the number of viable cells, it was found that the light emitted from the bioluminescent *E. coli* pUCP18-GFP cells was directly proportional to the number of the CFU/ml, and hence, a linear relationship could be obtained. The linear relationship between the level of light emission of bioluminescent bacteria and their viable counts was reported previously (Siragusa *et al*., 1999; Rocchetta *et al.*, 2001).

In the present study, antiadherent activity of sub inhibitory concentrations of ciprofloxacin on the bacterial adherence to the surface of urinary catheters was carried out using an *in-vitro* model of catheter colonization. Quantitative determination of bacterial adherence to the surface of the catheters was carried out by direct viable count and light emission measurement. The results revealed that no significant difference in the values of bacterial counts was observed by the two techniques indicating the efficiency of using the bioluminescence assay as a rapid method for quantitative determination of bacterial counts.

Accordingly, the bioluminescent *E. coli* pUCP18-GFP was found to be a versatile tool for applying a simple, rapid and sensitive method (bioluminescence assay) in the bacterial adhesion studies. In addition, the constructed pUCP18-GFP plasmid can be used for transformation and generation of other interesting bioluminescence strains such as *Serratia marcescens* or *Staphylococcus aureus* to be used for further studies on the bacterial adherence to the surface of inanimate objects or even living tissues.

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