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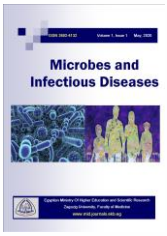

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Original article

The role of surfactants in inhibition of uropathogenic *E. coli* biofilm in catheterized patients in Assiut University Hospitals

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ABSTRACT

Background: *Escherichia coli* (*E. coli)* represent one of the major pathogens implicated in catheter associated urinary tract infection (CAUTI). Formation of biofilm by *uropathogenic E. coli* (UPEC) is a major survival and persistence mechanism of bacteria against antibiotics and host immune responses in the bladder. This study was designed aiming to evaluate the role of surfactants [non-anionic: Tween 80, anionic: sodium dodecyl sulfate (SDS), cationic: cetyl trimethyl ammonium (CTMA)] on biofilm forming CsgA gene containing strains of *E. coli*. **Methods:** A cross sectional study conducted at Assiut University Hospitals ICUs included 100 patients; 53 males and 47 females, catheterized for at least 3 days. Biofilm production by *E. coli* isolates was detected phenotypically by culture on Congo red agar plates and confirmed genotypically by detection of CsgA by conventional PCR. Overnight broth culture of each *E. coli* isolate was incubated with each surfactant at 3 different concentrations (CMC). Subculture on congo red agar plates was done. Inhibition of biofilm formation was indicted by fading or absence of black color of the colonies. **Results:** Tween 80 showed inhibition of biofilm formation by 15% of all samples at a concentration 0.1% (>CMC). SDS show biofilm inhibition by 23% at concentration 2.32mg/ml (=CMC) and at concentration 3mg/ml (>CMC). CTMA inhibited the biofilm formation by 7% at a concentration 0.4mg/ml (=CMC) and by 38% at a concentration 0.8mg/ml (>CMC). **Conclusion:** Surfactants have disruptive and inhibitory effect on biofilm formation that provides an alternative for plain and medicated catheters.

Introduction

Catheter associated urinary tract infection (CAUTI) is the most usual type of nosocomial infection globally. Catheter associated urinary tract infections are usually caused by endogenous native micro flora of the patients. However, inserted catheters serve as a conduit for bacterial entry. In addition, the imperfect catheter drainage retains some volume of urine in the bladder providing stability to bacterial residence [1].

Biofilm, that covers and secures bacteria against a catheter or mucosal surface, has been demonstrated on drainage bags, catheters and the uroepithelium. Organisms contained within the biofilm appear to be well protected from the mechanical flow of urine, host defenses and even antibiotics. The biofilm may allow the contained

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sessile organisms to establish a microenvironment from which some may move into the urine. These planktonic microbes are those that are voided and enumerated as bacteriuria by the diagnostic microbiology laboratory [2].

Uropathogenic *E. coli* (UPEC) are frequently isolated from hospital-acquired UTI [3]. Beside symptomatic infections, *Escherichia coli (E. coli)* might be found in the urine in high numbers without causing any symptoms i.e. asymptomatic bacteriuria [4].

Most isolates of *E. coli* carry a transcribable gene encoding for biofilm (curli fibers). *Escherichia coli* curli fibers are encoded on the *Csg* (curlin subunit gene) gene cluster, comprised of two differently transcribed operons, one which encodes the *CsgB, CsgA* and *CsgC* genes, and a second which encodes *CsgD, CsgE* and *CsgG* genes [5]. Curli fibers consist of polymers of a single 15-kDa protein encoded by the subunit gene *CsgA* and production of the curli fibers requires expression of both operons [6].

Basically, there are two different ways to approach the treatment of biofilms, that is, to prevent them forming or to remove already formed biofilms. In order to prevent biofilm formation, the planktonic cells attachment to surfaces or the maturation of early micro-colonies to fully structured biofilms must be prevented. Prevention of initial attachment can be approached by modifying surface to which microbes will attach, or treating microbial cells to block their attachments to surface [7,8].

Pre-conditioning of surface with some surfactants can inhibit microbial attachment [9]. Surfactants often have antimicrobial activity and interact with various cellular components, such as proteins and lipids, to reduce microbial cell growth and viability. This may provide a promising alternative for prevention of drug resistant biofilm forming bacteria implicated in CAUTIs [10].

Methods

Ethics approval

This study was approved by the Human Ethics Committee of Faculty of Medicine in Assiut University IRB no: 17100706. Informed consent was obtained before collecting samples from all patients and control subjects.

Study design: Cross sectional study.

Subjects: This study included 100 patients (47 females and 53 males, their age ranged between 23-80 years) admitted to emergency department ICU.

Inclusion criteria*:*

Eligible participants in the study fulfilled the following criteria *:*

- ICU patients.
- Period of catheterization ≥ 3 days.
- Had one of the following symptoms: fever>38℃, tenderness and turbidity of collected urine.

Exclusion criteria included*:*

- Patients with medicated catheter.
- Patients taking some drugs which affects the results, these drugs included : metronidazole, riboflavin, methocarbamol, nitrofurantoin.

Urine samples were aseptically collected from catheter tubes of these patients.

Fresh un-centrifuged urine sample was subjected to:

Naked eye examination for physical properties and viable bacterial counting by pour plate method with significant bacteriuria defined as $\geq 10^5$ colony forming units per milliliter [11].

Urine samples were centrifuged at 2000 rpm for 5 minutes and urine sediment was subjected to:

Wet mount examination, examination of Gram-stained smear and culture on blood agar, MacConkey's agar and Eosin Methylene Blue (EMB) agar for isolation *E. coli* strains. Suspected *E. coli* isolates were confirmed by subculture on Triple iron sugar (TSI) agar, Indole test Methyl Red (MR) test, Voges-Proskauer (VP) test, Simmons Citrate Agar and Christensen′s urea agar **(all culture media and biochemical tests were provided by Hi media, India)**.

Detection of uropathogenic E. coli biofilm

Phenotypic detection was performed by culture on Congo red (Sigma-Aldrich) supplemented agar plates. Black colonies with a dry crystalline consistency were considered as positive. Pink colonies and colonies with occasional darkening at the centers of colonies were considered as weak slime producers [12]. Molecular confirmation of biofilm production was performed by conventional PCR amplification of *CsgA*. DNA Extraction was performed by boiling method [13]. *CsgA* forward primer is 5'GTAGCAGCAATTGCAGCAATCG3' and *CsgA* reverse primer is 5'TTAGATGCAGTCTGGTCAACAG3' [14]. PCR reaction was performed in a 10 µl mixture containing 3 ul of template DNA (70 ng/ µl), 5 ml

of PCR master mix (COSMO PCR RED M. MIX, W1020300X, USA), and 1 ul (10 pmol) of each primer. Amplified product was detected by agarose gel electrophoresis. DNA bands were visualized by U.V. illumination at 366 nm wavelengths on U.V. Tran illuminator.

Determination of in vitro susceptibility of uropathogenic E. coli isolates

In vitro susceptibility of UPEC isolates to commercially available antibiotic discs **(provided by Titanmedia/ india**) was preformed according to modified Kirby-Bauer disk method [15]. Results were interpreted according to CLSI, 2020 guidelines. Discs of different antibiotic groups tested in the study included streptomycin, gentamycin, kanamycin, amikicin, nalidixic acid, ciprofloxacin, trimethoprim/ sulfamethooxazole (cotrimoxazole), tetracycline, chloramphenicol, imipenem, meropenem, tazobactam/ pipracillin, cefexime, ceftriaxone and ampicillin.

In vitro susceptibility of UPEC isolates to surfactants (at concentrations <CMC, =CMC & >CMC; with CMC stands for Critical Micelle Concentration) was determined by agar cup diffusion method [16]. The plates were incubated at 37℃ for 24 h and zone of inhibition of growth was measured.

Biofilm inhibition Assay by surfactant

Stock solution of each surfactant was prepared in distilled water as a solvent from which dilutions were prepared to reach concentrations =CMC, <CMC and >CMC **(Table 1)**.

For all studied surfactants, each *E. coli* isolate suspended in LB **(Hi media, India)** was categorized in a microtiter plate as four groups; one group to which 80 µL of a surfactant concentration <CMC was added, the second group to which 80 µL of a surfactant concentration =CMC was added, the third group to which 80 µL of a surfactant concentration >CMC was added and a control group (80 µL bacterial suspension in 120 µL LB without a surfactant). After 24 h of incubation at 37℃, culture from each well was done on Congo red supplemented agar plates to observe the inhibition of biofilm formation indicated by fading or absence the black colony color.

Effect of surfactant on microbial growth

Optical densities of the bacterial growth in the wells of microtiter plates were read at the absorbance wavelength of 630 nm before incubation and after overnight incubation of the plates at 37℃ to assess the inhibitory effect of different concentrations of surfactants added to the wells on bacterial growth.

Inhibition (%) = $[(A_C - A_T)/A_C] \times 100$.

A_C is the absorbance of control well and A_T is the absorbance of treated well with particular surfactant concentration [16].

Table 1. Surfactant structural formula and concentrations.

Types of surfactants	Structural formula	Different surfactants concentrations		
		<CMC	=CMC	>CMC
Tween 80 (ml/ml)	$C_{64}H_{124}O_{27}$	0.001	0.01	0.1
Sodium lauryl sulfate (SDS) (mg/ml)	$C_{12}H_{25}SO_4Na$	1.14	2.36	4.72
Cetyl Trimethyl Ammonium Bromide (CTMA/ CTAB) (mg/ml)	$C_{19}H_{42}Br$	0.2	0.4	0.8

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences version 16.0 (SPSS, Chicago, IL, USA). The statistical significance of differences between groups was expressed as *P* value with *p* value <0.05 was regarded as statistically significant.

Results

Out of 100 urine samples examined by viable count, revealed 82% significant bacteriuria. The most common isolated microorganism was *Candida* species (32%) and *Staphylococci* species (17%). The most common isolated Gram-negative bacilli from urine samples included *E. coli* (13%) followed by *Klebsiella* (8%), *Pseudomonas* (4%), *Proteus* (4%) and other lactose non-fermenters (4%).

The 13 *E. coli* isolates identified on culture media and by biochemical reactions were biofilm producers as detected by colony color on Congo red agar plates and confirmed by conventional PCR amplification of *CsgA* gene **(Figures 1 and 2)**.

The biofilm forming *E. coli* isolates were 100% resistant to nalidixic acid, ceftriaxone,

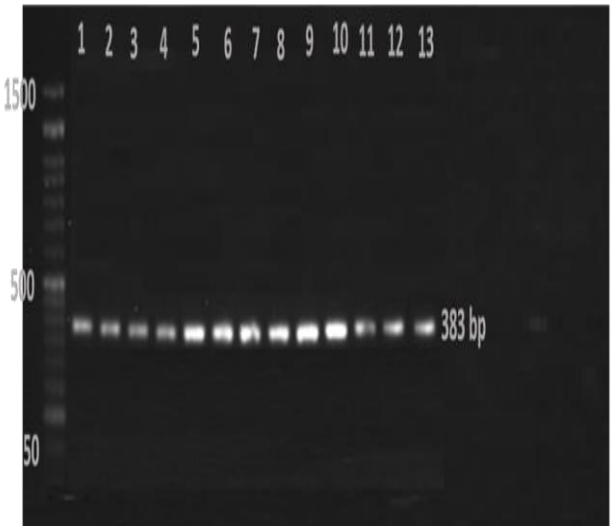
tetracycline, imipenem, ciprofloxacin, kanamycin, ampicillin, cefixime and tazobactam/ piperacillin. These isolates were 92.4% resistant to trimethoprim/ sulfamethooxazole (Cotrimoxazole) and 84.6% resistant to streptomycin, gentamycin, amikacin and chloramphenicol. on the other hand, lowest resistance was to meropenem (53.9%). The in vitro susceptibility of *E. coli* isolates to the surfactants proved marked zone of inhibition to SDS and CTMA both at concentration =CMC and >CMC **(Table 2)**.

The inhibitory effect of surfactants on biofilm formation among *E. coli* isolates is shown in **figure (3)**.

Tween 80 has insignificant inhibitory effect on bacterial growth which was clear when growth of bacterial isolates (measured at the absorbance wavelength of 630 nm) in the wells containing tween 80 at different concentrations was compared to bacterial growth in control wells (containing no tween 80) after overnight incubation. On the other hand, SDS has inhibitory effect on growth of *E. coli* isolates because there was a statistically significant difference in the absorbance between the wells containing SDS at a concentration <CMC and the control wells (*p* value 0.008), the wells containing SDS at a concentration =CMC and the control wells (*p* value 0.001) and the wells containing SDS at a concentration >CMC and the control wells (*P* value 0.000) after overnight incubation. Also, CTMA has inhibitory effect on growth of *E. coli* isolates when used at a concentration higher than the CMC because there was a statistically significant difference in the absorbance between the wells containing CTMA at a concentration >CMC and the control wells after overnight incubation (*p* value 0.000).

Figure 1. Black colonies of biofilm producing *E. coli* isolates Congo red supplemented agar plate.

Figure 2. Gel electrophoresis of the PCR –amplified products for detection of *CsgA* gene at 383 bp.



Lane M is a DNA marker (50-1500bp). Lanes 1-13 are positive for *CsgA* gene.

Figure 3. Percent of inhibition of biofilm formation by the 13 *E. coli* isolates by using different concentrations of the surfactants**.**

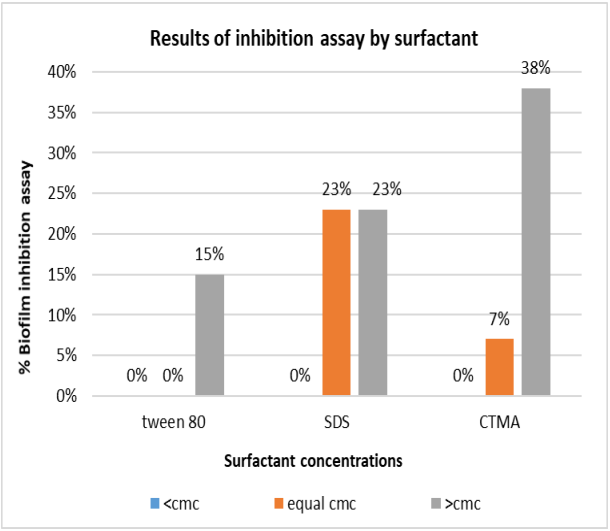


Table 2. In vitro susceptibility results of *E. coli* isolates to both commercially available antibiotic discs and surfactants.

Antibiotics	**Antibiotic (zone of inhibition in mm)**
Antibiotics to which *E. coli* isolates were 100% resistant	Nalidixic acid (≤13)
	Ceftriaxone (≤19)
	Tetracycline (≤11)
	Imipenem (≤19)
	Ciprofloxacin (≤21)
	Kanamycin (≤13)
	Ampicillin (≤13)
	Cefixime (≤15)
	Tazobactam/Piperacillin (≤17)
Antibiotic to which *E. coli* isolates were 92.4% resistant	Trimethoprim/ Sulfamethoxazole (Cotrimoxazole) (≤10)
Antibiotics to which *E. coli* isolates were 84.6% resistant	Streptomycin (≤11)
	Gentamycin (≤12)
	Amikacin (≤14)
	Chloramphenicol (≤12)
Antibiotic to which *E. coli* isolates were 53.9% resistant	Meropenem (≤19)
Surfactants	**Surfactant concentration (zone of inhibition in mm)**
Tween 80	<CMC (0)
	=CMC (0)
	>CMC (0)
SDS	<CMC (0)
	=CMC (13-33)
	>CMC (30-35)
CTMA	<CMC (0)
	=CMC (19.4)
	>CMC (21-26)

Discussion

Among the 100 urine samples included in the study, 82% of these samples were reported as positive for nosocomial UTIs.

As concluded in **Talaat et al.** [17], the most common isolated organisms were *candida* species (39%) and *Staphylococci* species (20%), *E .coli* (13%), *Klebsiella* (9%), *pseudomonas* (4%), *proteus* (4%) and other Lactose non- fermenters (4%).

In our study, all isolated *E. coli* were biofilm producers that was detected by congo red agar supplemented plates and confirmed by detection of *CsgA* gene by conventional PCR. According to **Wojnicz et al.** [14], the PCR products gave positive bands at 383 bp.

The antibiotic susceptibility pattern of isolated *E. coli* results were matched with **Ponnusamy et al.** [18] and **Ramadan et al.** [19] studies that demonstrated a significant correlation between biofilm production and antibiotic resistance.

In this study, Tween 80 showed inhibition of biofilm formation by 15% of all samples at a concentration 0.1% (>CMC) which is consistent with **Sloup et al.** [20] that found that Tween 80 treatment was able to disperse biofilms resulting in a strong inhibition of biofilm formation.

Wu et al. [21] found that Tween 80 has a higher propensity persist as micelle near and above CMC and accumulates at air-liquid interface competing with cell adsorption and prevent cells from mechanically coupling at the interface. This inhibition of pellicle formation appeared as disappearance or fading of black color on CRA.

In this study SDS show biofilm inhibition by 23% at concentration =2.36mg/ml and at concentration=4.72mg/ml. **Taş et al.** [22] reported that SDS significantly affects a membrane protein's functions, without a gross destruction of the membrane. At higher concentrations, closer to the surfactant CMC, equilibrium is established between the cell membrane components associated with the lipid bilayer phase and a co-existing micellar pseudo phase in the aqueous medium that results in dissolution of several components of the lipid bilayer into micelles, destruction of cell membrane integrity, and cell lysis.

Santos et al. [23] who supported our findings by demonstrating that low concentrations of SDS did not alter protein conformation. At a concentration equal to CMC SDS began to induce protein unfolding, at the concentrations above CMC SDS decreased surface tension and formed free micelles after saturation.

In this study we found that CTMA inhibited the biofilm formation at CMC =0.4mg/ml by 7% and above CMC =0.8mg/ml by 38%.

The ability of Cetyl Trimethyl Ammonium Bromide (CTMA/ CTAB) surfactant to inhibit *E. coli* biofilm observed in this study was due to the fact that *E. coli* cells were subjected to purely oxidative stress as a result of CTAB treatment. Since superoxide and hydrogen peroxide were made. CTAB, when used at the appropriate concentrations, caused cell lysis and bacterial growth in the short term [24].

In this study, Tween 80 has insignificant inhibitory effect on bacterial growth. On the other hand, SDS has inhibitory effect on growth of *E. coli* isolates when used at a concentration <CMC and =CMC and CTMA have inhibitory effect on growth of *E. coli* isolates when used at a concentration >CMC.

Odahara [25] and **Abboud et al.** [26] said that certain concentrations of SDS could be toxic to microorganism because SDS adsorption produces depolarization of cell membrane and consequently decreases the absorption of nutrient and modifies the substances from cell metabolism. The bacteria may also be killed by possible detergents effect that strips the Lipopolysaccride outer layer of gram-negative bacteria when the SDS concentration is high. **Salton et al.** [27] said when *E. coli* cells suspensions treatment as 1.5 mg dry weight bacteria with 45 μg. CTAB/ml. for 5 min. at 20° results in the death of *c*. 30 % of the cells and initiates the leakage of cellular constituents.

Conclusion

This study concluded that the biofilm producing UPEC strains were resistant to most tested antimicrobial agents that calls for an urgent need to control the overuse of antibiotics. Therefore, surfactants that have inhibitory and disruptive effect on biofilm formation and inhibitory effect on growth of UPEC were evaluated in this study. The inhibition Assay of the *E. coli* biofilm by surfactant agents resulted in ability of inhibition of Tween 80 by 15%, SDS by 23% and CTMA by 38.4% above CMC, however at CMC Tween 80 by 0%, SDS by 23% and CTMA by 7%. The three types of surfactants used in the study gave inhibition of biofilm formation when tested by laboratory trials. Therefore, our results should be confirmed in clinical trials to be able to recommend tested surfactant in prevention of and treatment of UTIs.

Limitations of the study

To be more informative, this study has to be compared with the *in vivo* testing of antimicrobial activity of the studied surfactants, as it is a well-known fact that many *in vivo* factors can alter the pattern of results (mainly the host immune response). However, *in vivo* studies require a prior large-scale evaluation of the safety of the studied surfactants.

Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Authors' contributions

Shereen M. Mohammed conceived and designed the study and wrote the original draft of the manuscript. Omnia A. Ahmed administered the project, collected and analyzed the data. Mostafa Samy Abbas provided clinical evaluation of study groups and helped in collecting participants' data. While Ismail S. Mohamed and Wegdan A. Mohamed participated in revising the article

critically and approved its intellectual content. All authors have reviewed and approved the final version of the manuscript.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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