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Research Article

Detection of *csg* and *lux* Genes in Biofilm-Forming Uropathogenic *Escherichia coli* Associated with Urinary Tract Infections

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Abstract
Uropathogenic *Escherichia coli* (UPEC) is responsible for 80–90% of urinary tract infections (UTI) in the global population. The emergence of the increasing resistance to broad-spectrum antimicrobial agents was due to the ability to form biofilms. Cell surface factors that play a role in biofilm formation include Quorum Sensing (QS) which is encoded by the *lux*S family gene and curli by two operons, namely the *csg*BA operon. The purpose of the study is to detect the effects of 2 virulence genes (*csg*D and *lux*S) on biofilm-forming UPEC associated with UTI. As many as 76 UPEC isolates were collected from the clinical microbiology laboratories and the biofilm development was analyzed using the crystal violet method on microplate 96 wells. Using PCR assay, the two studied genes (*csg*D and *lux*S) were determined to be present in the isolates. UPEC isolates the bacteria-produced biofilms (90.80%) and nonproducers (9.20%). Most UPEC bacteria (97.36%) are known to be positive for *csg*D and *lux*S gene, while the others (92.10%) are known to be positive for the *lux*S gene. The highest proportion of the genes expressed in this study is followed by the presence of a relationship between the ability to produce biofilm and the presence of the genes under investigation, which is followed by all UPEC strains that cause UTI in humans.

Keywords: biofilm, *csg*D, *lux*S, uropathogenic *Escherichia coli*, UTI

1. INTRODUCTION

Urinary tract infection (UTI) is a term used broadly to describe infectious syndromes that can affect the urinary tract from the urethra to the kidneys [1]. UTIs can occur repeatedly, and they are frequently challenging to treat and can damage the kidney parenchyma, leading to renal insufficiency and other further complications. UTI is a serious burden on society and the health system in terms of diagnosis and management, disrupts productivity, and morbidity, and can occasionally result in death [2]. UPEC, the most common cause of UTI, can manifest as an antimicrobial resistance (AMR) and biofilm [3]. As a polymer matrix produced by the microbes themselves, biofilms are organized communities of microbes [4]. Microbes can be shielded by this EPS structure from antimicrobial substances, chemicals, drying, radiation, and other harsh conditions [5].

Biofilms are widely recognized as a major factor contributing to the high rates of recurrence and antibiotic resistance commonly associated with UTIs [6]. Biofilm formation is a significant virulence mechanism and a hallmark of multidrug-resistant (MDR) disease pathogens in hospitals [7]. The MIC for biofilms can be 100–800× greater than the MIC for planktonic cells. Additionally, single bacteria in a biofilm that have been exposed to high concentrations of antibiotics can survive and re-form a more resistant biofilm, a phenomenon known as relapse [8].

Bacterial extracellular vesicles (BEVs) are nanostructures produced by bacteria and serve an important function for molecular transport. BEV includes a variety of components, including lipids, nucleic acids (genomic DNA, plasmids, and short RNAs), proteins, and quorum sensing signaling molecules [9]. Surface factors become the determining factors in their involvement in biofilm formation, including Quorum Sensing (QS) and Curli. UPEC strains have a variety of structural (including fimbriae, pili, curli, and flagella) and QS mechanisms that contribute to their potential to cause illness, while adhesion to host epithelial cells in the urinary tract is the most significant predictor of pathogenicity [10].

Table 1. Sequences of oligonucleotide primers for PCR amplification *csg* D and *lux* S associated genes of UPEC.

Tipe Gene	Forward (F) primer	Reverse (R) primer	Annealing temperature (°C)	Amplicon size (bp)
*csg*D	CCGCTTGTGTCCGGTTTT	GAGATCGCTCGTTCGTTGTTC	56	97
*lux*S	GTTCCAGAATGTTACGCGCA	CACAGTCGATCATACCCGGA	56	425

Curli adhesive fibers influence the development of biofilms on abiotic surfaces by promoting initial interactions between cells and surfaces and later interactions between cells. Two operons, the curli export apparatus and the *csg*DEFG operon, which encode a transcription regulator *csg*D, *csg*E-G, and the *csg*BA operon, which encodes structural components, are responsible for encoding the genes involved in curli production [11]. Pathogenic bacteria in biofilms use QS mechanisms to boost their virulence and develop antibiotic resistance. QS mechanisms are also crucial for the formation and development of biofilms [12]. The QS system in *Salmonella enterica* and *Escherichia coli* is controlled by the *lux*S and *lux*R genes [13]. All components of these virulence factors can be attractive candidates for the development of new vaccines and drugs [10]. A better understanding of the pathogenesis of UTI in UPEC as a common cause is essential for the treatment and prevention of UTI [2].

Research on the detection and prevalence of biofilm coding genes has been carried out on various types of test pathogens, for example, the fungus *Candida albicans*, and the bacterium *Lactobacillus paraplantarum*, as well as various types of infections, for example, post-operative wound infections, nosocomial infections, recurrent urinary tract infections, skin infections, and other infections. In 2021 conducted by Bono et al. (2021) , the virulence genes *iut*A (95%), *fim*H (93%), *omp*T genes (90%), PAI (90%), and Trat (81%), were present in strong biofilm producers [14]. Research from Boroumand *et al*. indicates that out of 144 *E. coli* isolates, 22 (19.4%) developed strong biofilms, 27 (23.8%) produced moderate biofilms, and 64 (56.0%) produced weak biofilms at a rate of 3.5% [15].

This study set out to determine the frequency of the *lux*S genes, which codes for quorum sensing, and *csg*D, which codes for curli fiber in biofilm-forming UPEC isolated from UTI patients at one of the largest general hospitals in East Java. The urgency of this research is to identify indicators of the virulence factors of UPEC so that inhibition and degradation of its biofilm can be carried out. Biofilm formation is important as a form of support in the focus area of health-drug research, especially molecular, to realize independence and sovereignty in the field of medical devices.

2. MATERIALS AND METHODS

2.1. Materials and Chemicals

The UPEC utilized in this investigation was acquired from an earlier isolation [12]. The strains were isolated in pure cultures and identified in the Gastroenteritis and Salmonellosis. Laboratory of Tropical Disease Diagnostic Center Universitas Airlangga. Bacterial growth medium (nutrient agar, eosin-methylene blue agar, Luria-Bertani (LB) broth,) were purchased from Merck Company, Germany. Meanwhile, ethanol, crystal violet, saline buffer phosphate, distilled sterile water, EDTA buffer, and the other reagents were analytical grade. The 96-well plates were purchased from BD Falcon, USA.

2.2. Methods

2.2.1. Conditions for Strains, Growth, and Biofilm Detection

UPEC were grown in EMB medium (Merck Company, Germany) for 24 h at 37 °C. UPEC recognized the colonies with a green metallic shine. UPEC isolates were cultured in LB and then

incubated for 24 h at 37 °C. An overnight UPEC culture with an OD_{600} of 1 (1%) was applied to 96-well polystyrene plates, and they were then incubated for 24 h at 37 °C. As much as 150 µL of 95% (v/v) ethanol was added to each sample after it had been stained for 15 min with 0.5% crystal violet and three times cleaned with saline buffer phosphate (0.01 M, pH 7.4). The automated plate reader (Biorad) was used for quantification, and the OD_{600} value and biofilm production were interpreted in accordance with Ball et al. [16].

2.2.2. DNA Extraction, PCR and Virulence Factor Detection

Following Blanco's methodology, 75 UPEC isolates from UTI patients were examined for several genes related to UPEC adherence using polymerase chain reaction (PCR). Using the NEXprep TM Cell/Tissue genomic DNA preparation kit, genomic DNA was extracted from UPEC strains by solid phase DNA isolation methods. Bacterial pellets were made by suspending 2 mL of an overnight TSB medium (Merck) culture in 200 µL of distilled sterile water and then heating the mixture for 10 min at 80 °C. Following three freezing and thawing cycles, the samples underwent centrifugation, the suspensions were promptly cooled to -20 °C for 5 min, and the supernatants were promptly preserved as stocks of DNA templates [17].

Amplification of the virulence genes *csg*D and *lux*S was carried out by PCR using published primer pairs (macrogen Singapore), as shown in Table 1. Primer pairs were created with Primer3 using gene sequences from the National Center for Biotechnology Information (NCBI) genome databases. The amplification reactions took up a total of 25 µL and included 5 µL of DNA extract, 12,5 µL of PCR 2X Master, 1 µL of each primer, and 5,5 µL of RNAse-Free water. All genes were subjected to the same PCR conditions in a Biorad thermal cycler: 5 min of initial denaturation at 94 ° C, followed by 35 cycles of 1-min denaturation at 94 °C, 1 min of annealing at 56 °C, 1 min of extension at 72 °C, and 5 min of final extension at 72 °C. The amplified products were stained with a DNA-safe marker and then viewed using a UV transillumination imaging equipment. The amplified products were separated on a Merck 1% agarose gel using 0.5X tris borate EDTA buffer and a suitable molecular size marker (100 bp Plus DNA ladder) for 30 min at a voltage of 80 volts.

2.2.3. Statistical Analysis

Statistical analysis was done using SPSS software 16.0 version (IBM Corp., New York, United States). The chi-squared test and Fisher's exact test were used to compare the distribution of virulence genes among *csg*D and *lux*S genes. The p-value of less than 0.05 was considered statistically significant.

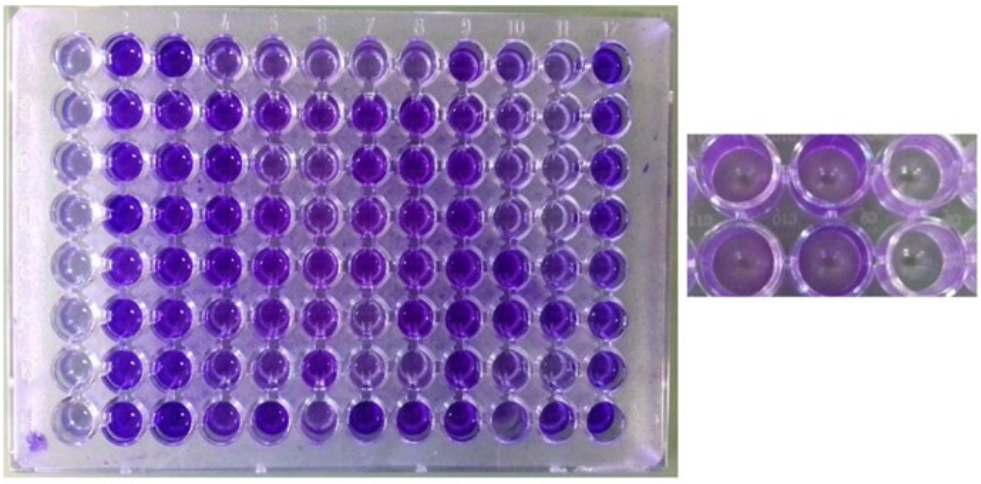

Figure 1. Crystal violet was used to stain the 96-well polystyrene microtiter plates, which were used to study the biofilm formation of UPEC strains at 37 °C.

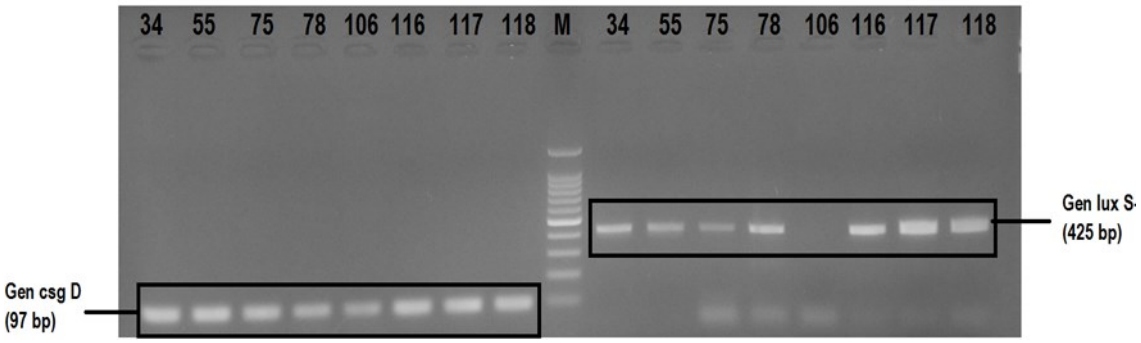

Figure 2. Genes involved in the development of biofilms can be found using PCR amplification (*csg*D and *lux*S) isolated UPEC bacteria in UTI patients.

3. RESULTS AND DISCUSSIONS

3.1. Microtiter Plate Method (MtP) Study of Biofilm Formation on UPEC

The phenotypic determination of the capability to form biofilm using the MtP method of all test isolates is shown in Table 2. The results of biofilm formation by UPEC isolates from UTI patients *in vitro* on the crystal violet method are shown in Table 2. The biofilm formation ability of UPEC was measured at OD_{495} and biofilms were classified into categories of biofilm-producing bacteria (weak biofilm, medium biofilm, and strong biofilm) and non-producers. The OD limit (ODc) of the microtiter plate assay was eight standard deviations above the mean OD of the negative controls. Strains were classified as follows: OD ≤ ODc is no biofilm, ODc < OD ≤ 2×ODc is weak biofilm, 2×ODc < OD ≤ 4×ODc is medium biofilm, and 4×ODc < OD is strong biofilm. All tests were carried out 8× and the results were averaged. The percentage of bacteria produced biofilms was 90.80% while the non producer was 9.20 %.

*3.2. Detection of csg*D *and lux*S *Genes*

In this research, genotypic characterization of genes responsible for biofilm formation was carried out, and PCR assay was used to detect *csg*D and *lux*S genes that contribute to the formation of UPEC biofilm from UTI patients. The *csg*D gene was found in 74 UPEC isolates (97.36%) and 82.40% of UPEC biofilm formers. The *lux*S gene was found in 70 UPEC isolates (92.10%) and 95.70% of UPECs that formed biofilms. The relationship between the *csg*D and *lux*S genes, and biofilm forming capacity in MtP method was 76 different UPEC clinics associated with UTI are shown in Table 2. Based on Table 2 and Figure 1, it is known that most UPEC bacteria (97.36%) are known to be positive for *csg*D and *lux*S gene, while 92.10% are known to be positive for the *lux*S gene (Figure 2).

3.3. Discussion

UTIs, which commonly affect the urethra, bladder, or kidneys (pyelonephritis), are among the most prevalent infectious diseases in the world. They cause significant morbidity and mortality as well as high treatment costs [18]. Between the ages of 16 and 35, UTIs are at least four times more prevalent in women than in men, At least 40% to 60% of women will get an infection at some point in their lives, and 10% of women will get an infection annually [14].

This research shows that most UPEC isolates can form biofilms using the crystal violet method. One mechanism that helps the pathogenesis of *E. coli*, and to help keep it in the urinary tract, and hinders its eradication is the biofilm formation [19]. UPEC strains possess several virulence factors, which contribute to the adherence and colonization of bacteria in the uroepithelium. The most crucial factor for the establishment, persistence, and recurrence of UTIs is the biofilm formation ability of UPEC [20]. UPEC has many virulence factors with a role in the urinary tracts, therefore, persistence and biofilm formation cause pyelonephritis and even chronic and recurrent UTI. It leads to an increase in antimicrobial resistance and the severity of infection [21].

After the invasion, UPEC can form intracellular

bacterial communities (IBC) in the cytoplasm of bladder epithelial cells (BEC) to elude the host immune response. This is a primary cause of UTIs [22]. Once a biofilm has formed, pathogenic bacteria may be able to evade immune defense mechanisms, and infect the urinary tract by colonizing there. Additionally, biofilms have high levels of drug resistance, which makes it challenging for drugs to pass through the biofilm and cause recurrent and chronic UTIs [23]. Bacteria living in biofilms have a 10 to 1000-fold increase in drug resistance, notably antibiotic resistance, as compared to bacteria living in planktonic environments which is caused by the structural characteristics of biofilms and the bacteria that make them up [24]. Bacterial adhesion, which starts with the attachment of cells to the supporting surface and continues with the attachment of cells and the formation of microcolonies, initiates biofilm development after the formation of the conditioning layer. Cell proliferation is the third stage, which is followed by the production of mucus, cell proliferation, and finally the maturation of the biofilm in four stages. Bacterial dispersal, which is the fifth and last stage of the biofilm life cycle, is made possible by the mature biofilm [25].

Conjugative pili, curli fibers, and type 1 fimbriae are three different types of organelles that *E. coli* uses to perform this irreversible attachment. Numerous proteins and regulatory mechanisms are involved in the intricate process by which *E. coli* transitions from the planktonic to the biofilm form. The most essential ones for regulating *E. coli* biofilms include c-di-GMP, two-component signaling systems (TCS), the *Rcs*CDB regulator, and QS [26].

The curli synthesis gene (*csg*), specially *csg*A gene is partially transcribed by the *crl* gene, which encodes the primary gene for the structural subunit of curli fimbriae. Curli fimbriae are one of the significant biofilm components, implicated in UPEC attachment to uroepithelium and abiotic surfaces [20]. Gram-negative and Gram-positive bacteria both contain small signal molecules known as autoinducers (AI), which regulate gene expression based on cell density. The most extensively researched AI in *E. coli* is AI-2., which is produced by the *lux*S enzyme associated with biofilm for QS, a cell density-dependent chemical signaling system. Once optimal bacterial density is reached, *lux*S regulation is downregulated, inhibiting AI-2 production. This AI production is controlled and quickly secreted out via the LSR transporter [26].

Enteric bacteria exhibit curly expression when exposed to demanding environmental circumstances that promote biofilm formation over planktonic cell proliferation. This encases individual bacterial cells and produces interwoven strands that support the ECM. Curly proteins form thin amyloid fibers on the surface of enteric bacteria that range in width from 4 to 10 nm and have a -sheet-rich structure with the sheet strands aligned perpendicular to the fiber axis. Curly-deficient bacteria only grow in a single layer of cells and do not form full three-dimensional biofilms [27].

Curli are extracellular amyloid fibers that aggregate on the surface of many Gram-negative bacteria, including *E. coli*, and are encoded by two differentially transcribed operons, *csg*BAC and *csg*DEFG, respectively. Up to 85% of the extracellular matrix is made up of the primary protein curli, which is also in charge of forming the overall structure of the biofilm. Environmental

Table 2. Analisis variasi gen *csg*D dan *lux*S pada UPEC.

	Number of isolates (N= 76)	Biofilm formation (n= 76)	Biofilm formation (%)
*csg*D gene			
Positive	74 (97.36%)	61	82.40
Negative	2 (2.64%)	2	100
*lux*S gene			
Positive	70 (92.10%)	67	95.70
Negative	6 (7.89%)	5	83.30

elements such as temperature, growth phase, and concentrations of the second messenger cyclic-di-GMP (c-di-GMP) regulate the expression of *csg*D. The *csg*D activates *csg*BAC expression, resulting in the production of the mammalian cell adhesion molecule, and upstream genes involved in biofilm formation are activated as a result of increased intracellular c-di-GMP levels [28].

The *csg*BAC and *csg*DEFG operons are a group of differentially transcribed operons that encode *csg*. The *csg*A and *csg*B are released outside the cell, and *csg*B works as a nucleator protein to help in the creation of amyloid fibers, which are normally located around cells but are not covalently connected to cell surface components. The *csg*E and *csg*F help *csg*B and csgA transport and secretion, as well as the appropriate assembly of amyloid fibers at the cell surface. The *csg*G is an outer membrane lipoprotein that oligomerizes to produce a secretory channel that allows *csg*A, *csg*B, and *csg*F to be exported. Curls are a crucial regulator of curli, in addition to boosting biofilm development, enhancing adherence to mammalian and plant cells, and contributing to pathogenicity [29].

The periplasmic protein *csg*F interacts directly with *csg*G at the outer membrane to promote the secretion of *csg*A, type VIII secretion (or precipitation-nucleation) is the mechanism by which *csg*B is localized at the cell surface and then polymerized to form curli fimbriae. A member of the FixJ/UhpA family of transcriptional regulators, *csg*D positively controls the expression of curli by the *csg*BA operon. It is thought to be a key biofilm regulator in *E. coli* [30].

A bacterial communication system called QS uses chemical molecules as communication signals to allow bacteria to coordinate their behavior. It is important to recognize that the QS communication system aims to control the behavior of clustered bacteria in order to maximize benefits for the population within the biofilm, including optimal nutrient utilization, increased pathogenicity, and increased survival rates. However, it is reasonable to expect a certitude that the QS communication system will not be completely successful [31].

Five main QS systems are present in pathogenic *E. coli* bacteria, and they are as follows: (i) the *lux*S enzyme produces AI-2 signaling; (ii) SdiA signaling (cell division inhibitor/suppressor); (iii) a host-bacterium communication pathway involving AI-3, epinephrine, and norepinephrine; (iv) self-produced peptides that deliver extracellular death factor (EDF) signaling, which activates the toxin-antitoxin system, and (v) indole signaling, which is mediated by self-produced effector indole, are the other two examples of signaling that is carried out within cells [32].

Key phenotypes in *E. coli*, such as biofilm development, motility, and drug resistance are all influenced when quorum sensing is activated by the detection of molecular signals from the environment during stressful situations, such as the presence of antibiotics [33]. The AI-2-based signaling in UPEC correlates with biofilm development in *E. coli* via up-regulation of the RNA interference *mqs*R and subsequent activation of the two-component system *qse*BC to increase motility [34]. Through a process known as quorum sensing, which is carried out with the help of autoinducers like acyl-homoserine lactone (ahl-1), which is released by the enzyme acyl synthase (*lux*I), thus Gram-negative and Gram-positive bacteria can sense one another [35].

4. CONCLUSIONS

The degree of biofilm formation differs between different Uropathogenic *Escherichia coli*. The *csg*D and *lux*S genes have a significant role in biofilm formation of Uropathogenic *Escherichia coli*. The highest proportion of the gene expressed in this study is also consistent with the absence of a relationship between the ability to produce biofilm and the presence of the genes under investigation and all of the UPEC strains that cause UTIs in human studies that produce both positive and negative biofilm. The high recurrence of capacity to create biofilms, may be a potential danger to successful treatment and may increment horribleness and mortality in tainted patients.

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Conflicts of Interest

The authors declare no conflict of interest.

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