Association Between Biofilm Formation by U.P.E.C. and Serum Level of Several Cytokines

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Abstract

One hundred and eighty-nine subjects from Baghdad enrolled in this study (110 female and 79 male) and gathered into two investigated groups; the first group consisted of 149 patients, and the second group consisted of 40 healthy individuals. Results revealed after clinical laboratory diagnosis of urine samples 12 (8.1%) gave a negative bacterial culture, 137 (91.9%) were positive culture, while all urine samples of healthy control were negative. Gram staining and microscopic examination of bacterial colonies showed that 11(8.03%) out of 137 isolates were identified as Gram-positive and 126 (91.97%) as Gram-negative. After biochemical analysis and diagnosis by the Vitik system, the data demonstrated that a single infectious agent caused all U.T.I. cases. U.P.E.C. represented the most common bacterial agent because of several virulence factors responsible for its pathogenicity. The test tube method and Congo red agar medium have been used to detect biofilm formation. Results demonstrate that 129 (94.16 %) of bacterial isolates were producers, while just 8 (5.84 %) were non-producers. The results of the microtiter plate method revealed that the isolates were categorized into four groups: Strong, moderate, weak, and harmful. 22 (63.5%) were strong biofilm producers, 28 (20.44%) were moderate producers, 14 (10.22%) were weak producers, and 8 (5.84%) were unable to form biofilm. Serum levels of IL-1β, IL-6 and IL-8 were estimated by Sandwich ELISA, which were significantly higher in patients with different types of U.T.I.s than the healthy group. This study concluded that the U.P.E.C. represented the most common prevalent agent of U.T.I.s and more efficient biofilm-producer bacteria. The test tube method is the best qualitative, quick, and easy detection method of biofilm formation, while the microtiter plate is the best quantitative and sensitive method. A positive correlation was found between biofilm formation and elevated serum levels of proinflammatory cytokines, proportionally increased with advanced and severe, especially in old persons.

Keywords: UTIs; ELISA; IL-1β; IL-6; IL-8; Iraq

Introduction

Urinary tract infections (U.T.I.s) are the most common microbial infections in any part of the renal system, affecting the quality of a patient's life. These infections are characterized by frequent painful urination and are associated with significant morbidity.¹,² U.T.I.s are caused mainly by bacterial invasion into the urethra and bladder, while fungi and viruses represent rare etiologic agents of U.T.I.s. About 95% of uncomplicated U.T.I.s are caused by Gram-negative uropathogenic
bacteria from the *Enterobacteriaceae* family. These bacteria invade the opening of the urethra, travel up to the bladder, and overcome host innate immunity.\(^3\)\(^-\)\(^5\). *Escherichia coli* is the most common causative agent of U.T.I.s that usually inhabits the intestine and becomes opportunistic uropathogenic when they enter the urethra.\(^6\) *Escherichia coli* have various virulence factors such as fimbriae, capsule, biofilm, iron scavenger receptors, flagella, toxins, and lipopolysaccharides for their pathogenicity in the urinary tract.\(^7\)\(^,\)\(^8\).

Bacterial biofilm is a multifaceted structure of communities with diverse bacterial colonies of cells that can withstand harsh conditions and associate with the progression and severity of many human diseases.\(^9\) Biofilm formation is the initial stage of U.T.I. pathogenesis. It consists of extracellular lipopolysaccharides, structural proteins and cell debris, which protect bacteria from pH, nutrient deficiency and mechanical forces, tolerates antibiotics and resist phagocytosis.\(^10\)\(^,\)\(^11\).

Lipopolysaccharide is an integral component of the cell wall, and biofilm layers activate the host immune response and induce cytokine production, enhancing the inflammatory response and synthesizing specific antibodies to the somatic antigen\(^12\)\(^,\)\(^13\)\(^,\)\(^14\). TLR4 in the epithelial cells of the urinary tracts can recognize L.P.S. and initiate a downstream signaling cascade that produces different cytokines such as IL-1β, IL-6, and IL-8\(^15\)\(^,\)\(^16\). It is a crucial mediator of the inflammatory response, essential for the host response and resistance to pathogens, produced and secreted by various cell types of the innate immune system.\(^17\) High IL-1β levels in serum promote the differentiation of monocytes into conventional dendritic cells and support the proliferation of activated lymphocytes and their differentiation into plasma cells. Its combination with IL-2 promoted the expansion of N.K. cells and CD4+, CD8+ and T-lymphocytes.\(^18\)\(^,\)\(^19\). IL-1β is expressed by bladder epithelial cells and is essential for the clearance of uropathogenic bacteria and stimulates secretion of IL-6 and IL-8.\(^20\)\(^-\)\(^22\). IL-6 is an interleukin that acts as a pleiotropic cytokine; the IL6 gene encodes it. It is responsible for stimulating acute phase protein synthesis and the production of neutrophils in the bone marrow and supports the growth of B cells. L-6 interacts with its receptor IL-6R (CD26) proteins to form a complex to initiate a signal transduction cascade.\(^23\)\(^,\)\(^24\). It is a chemoattractant cytokine produced by various tissue and blood cells. Bacterial adherence to epithelial cells is a virulence trait of pathogenic bacteria for establishing U.T.I. and induction of IL-8 expression in different uroepithelial cells.\(^25\) Macrophages produce the chemokine IL-8 in response to TNF-α, IL-1β and IL-2, essential for recruiting neutrophils during U.T.I.s and stimulation of phagocytosis.\(^26\)\(^,\)\(^27\). The urothelium secretes and responds to chemokines and cytokines as an essential component of its response to U.T.I. For example, urothelial production of IL-8 and receptor expression following *Escherichia coli* exposure serve critical roles in neutrophil recruitment with stimulation of IL-1β and IL-6 rapid secretion by urothelial cells.\(^28\).

**Materials and Methods**

This study included 189 people of both sexes, 113 females and 76 males, who attended the Urology Unit at Al-Yarmook Teaching Hospital in Baghdad from the 1st of October (2020) to the 1st of July (2021). The individuals were divided into two groups primarily based on their clinical diagnosis, which was determined by symptoms and laboratory tests.

Five mL plastic disposable syringes, which were used to collect blood from all individuals through venipuncture, were allowed to clot at room temperature for 15 minutes before being centrifuged for 15 minutes at about 5000 rpm to separate serum for estimation of the levels of cytokines. Midstream urine samples were collected from patients and healthy people using dry, clean, tightly sealed disposable containers to reduce contamination until transported to the lab for diag-
nosis by the Vitik system, an automated bacterial identification and susceptibility testing system. Biofilm formation qualitative assessment was carried out by test tube method and Congo red agar medium. At the same time, quantitative assessment was carried out by microtiter plate method. According to manufacturer instructions, sandwich ELISA was used to estimate the serum level of IL-1β, IL-6 and IL-8. All data were documented and statistically analyzed using the ANOVA test in the S.P.S.S. Software. Differences were considered significant at P<0.05.

Results
One-handed and eight nine Iraqi subjects have been included, 110 (58.20%) females and 79 (41.80%) males. The patient’s group comprised 97 females and 52 males, while the healthy individuals comprised 13 females and 27 males. Females had a higher prevalence and incidence of urinary tract infections than males. The patient’s urine sample culture results showed that 137 (91.9%) gave positive bacterial cultures. Only 12 (8.1%) were negative bacterial cultures, and all 40 urine samples (100%) of healthy individuals were negative bacterial cultures. AlChalabi (2016) found that 71% of samples had positive bacterial cultures, and 29% had negative cultures. The differences in the percentages could be related to differences in the sample size, the seasons, medications taken before sampling, and the study’s geographic location. Gram staining and microscopic analysis of bacterial colonies isolated from urine samples revealed that 11 (8.03%) out of 137 isolates were Gram-positive bacteria, and 126 (91.97%) were Gram-negative. The G-ve bacteria was discovered to be the most common bacterial causative agent of U.T.I.s. After diagnosis by the vitik system. The data demonstrated that a single infectious agent caused all U.T.I. cases: Citrobacter Freund [5 (3.65 %)], Streptococcus agalactiae [4 (2.92%)], Pseudomonas aeruginosa [6 (4.37%)], Staphylococcus aureus [7 (5.10%)], Klebsiella pneumoniae [17 (12.41%), Proteus mirabilis [22 (16.1 %)], and E. coli [76 (55.47%)]. Kaduma et al. recorded that the positive culture of urine resulted from Klebsiella pneumoniae (37%), E. coli (28%), P. aeruginosa (7%) and P. mirabilis (5%), while Citrobacter freundii (1%) [46]. The single Gram-positive isolate was S. aureus (23%). In Iraq, Abbass and Al-Mathkhury (2020) used the V.I.T.E.K. 2 compact system to confirm the identification of uropathogenic isolates after morphological and biochemical testing in all growing colonies. Escherichia coli was the most common isolate (67.5%), followed by S. aureus (11.5 %), P. mirabilis (10%), P. aeruginosa (4%), and K. pneumoniae (5 %). The findings are consistent with those of Assafi et al. (2022), who isolated E. coli from midstream pee specimens of symptomatic U.T.I. patients admitted to Zakho Hospital and revealed that females had much higher U.T.I. prevalence and incidence rates than males, as well as multidrug-resistant E. coli, which was responsible for 85.3 % of all cases due to antibiotic misuse and overuse. The test tube method and Congo red agar medium are the easiest and cheapest methods used for qualitative detection of biofilm production. Only 8 (5.84%) bacterial isolates were found to be non-biofilm producers, while 129 (94.16%) isolates were found to be producers. Environmental factors and changes in the culture media influence adhesion and biofilm formation. A microtiter plate method is a valuable tool for quantitative assessment. The results revealed that isolates were categorized into four groups: strong, moderate, weak, and negative and the the findings were 22 (63.5%) for strong biofilm producers, 28 (20.44%) for moderate producers, 14 (10.22%) for weak producers, and 8 (5.84%) for unable to form biofilm. Bacterial infection has become a considerable hazard to public health and the economy due to the advent of multidrug resistance. One of the leading causes of bacterial resistance is the production of bacterial biofilms. The
complexity of the chemical composition and physical structure of mature biofilms makes their removal a challenging task \(^4^9\). The importance of cytokines such as IL-1\(\beta\), IL-6, IL-8 in the progression and severity of U.T.I.s is considerable. The Sandwich ELISA technique estimates serum levels of determined immunological parameters because of its high specificity and sensitivity. Serum levels of IL-1\(\beta\) significantly high in patients when compared to the controls, with a mean level of 47.15 and 10.13 pg/ml, respectively. IL-6 is a multifunctional cytokine found to be at a higher level in the serum of the patients than in the healthy control, which is associated with a number of biological functions. The mean serum levels of IL-6 in sera of patients were elevated to reach 69.27 pg/ml when compared with healthy, 8.61 pg/ml. IL-8 is a unique mediator that participates in all of the proinflammatory processes, which is recorded significantly higher levels in sera of patients (58.48 pg/ml) than in healthy (17.53 pg/ml). The urothelium secretes and responds to chemokines and cytokines as a critical component of its response to U.T.I. The IL-8 production and its receptor expression serve critical roles in neutrophil recruitment. Likewise, IL-6 is rapidly secreted by urothelial cells following Escherichia coli exposure \(^2^8, 5^0\).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum level (Mean ± S.E. pg/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1(\beta)</td>
<td>IL-6</td>
<td>IL-8</td>
</tr>
<tr>
<td>Patients</td>
<td>47.15±2.25</td>
<td>70.36±5.12</td>
<td>58.84±2.97</td>
</tr>
<tr>
<td>Healthy</td>
<td>10.13±0.55</td>
<td>8.61±0.45</td>
<td>17.53±2.26</td>
</tr>
<tr>
<td>P Value</td>
<td>0.00041</td>
<td>0.0001</td>
<td>0.001</td>
</tr>
</tbody>
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Table 1. Serum level of cytokines (IL-1\(\beta\), IL-6 and IL-8).

<table>
<thead>
<tr>
<th>Biofilm Production</th>
<th>IL-1(\beta) (Mean ± SE) pg/ml</th>
<th>IL-6 (Mean ± SE) pg/ml</th>
<th>IL-8 (Mean ± SE) pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>75.64 ± 1.83</td>
<td>94.48 ± 7.04</td>
<td>81.48 ± 5.19</td>
</tr>
<tr>
<td>Moderate</td>
<td>68.55 ± 1.17</td>
<td>86.40 ± 7.82</td>
<td>76.62 ± 6.84</td>
</tr>
<tr>
<td>Weak</td>
<td>27.15 ± 1.47</td>
<td>56.95 ± 1.17</td>
<td>64.21 ± 3.66</td>
</tr>
<tr>
<td>Non-producer</td>
<td>17.26 ± 0.87 c</td>
<td>39.26 ± 0.87</td>
<td>13.06 ± 4.04</td>
</tr>
</tbody>
</table>

Table 2. Association between serum levels of cytokines and biofilm production.

Results showed that the strength of biofilm production by uropathogenic bacteria correlated with the progression of U.T.I.s, significantly higher in patients with a more severe form of the disease and inducing a concentration-dependent increase of cytokine levels. IL-1\(\beta\), IL-2 and IL-8 gene expression increased in response to stimulation by L.P.S. in the composition of biofilm and bacteria cell wall, which causes recruitment of neutrophils with induction of other cytokine production levels such IL-6, I.F.N. \(\gamma\) and TNF-\(\alpha\). Serum levels of IL-1\(\beta\), L-6 and IL-8 are essential markers for diagnosing U.T.I.s severity and differentiating between acute and chronic recurrent U.T.I.s.

**Discussion**
The results showed that U.T.I.s affect women up to ten times more than males for various reasons, including urethral length, sensitive skin, sexual contact, pregnancy, menopause, specific methods of contraception and urethral posture \(^3^4, 3^5\). Although women are more likely than men to get UTIs, urinary infections affect men and women of all ages. Specific populations, such as pregnant women, the elderly, or patients with spinal cord injuries, catheters, or diabetes, are also at higher risk of U.T.I.s becoming more common as people age \(^3^6\) to \(^3^7\). Older adults are
more susceptible to U.T.I.s than younger people due to more excellent rates of urine retention, incontinence, long-term hospitalizations, comorbidities, urinary catheterizations and declining immune responses. Urinary tract abnormalities, especially in those with urinary retention or incontinence (e.g., prostatic hyperplasia), diabetes mellitus, urinary catheterization and sexual intercourse, which are leading to U.T.I.s in both men and women as they age, are all modifiable risk factors for U.T.I.s in the elderly. According to symptoms and signs, diagnosis by ultrasound, and all laboratory diagnostic tests requested by the consultant urologist, cystitis is the most common uncomplicated kind, especially in ladies. In contrast, prostatitis is the most common type during the years of peak sexual activity, which is usually between the ages of 18 and 39 years old. Young women are equally prone to uncomplicated recurring U.T.I.s. Following an initial episode of a U.T.I., 27% of women experience a verified recurrence within 6 months, and 2.7% experience a second recurrence within the same time frame. U.T.I.s are among pregnant women’s most common clinical bacterial infections, accounting for around 25% of all infections. Women are more prone to U.T.I.s due to anatomical, physiological, and hormonal changes during pregnancy. Untreated U.T.I. in pregnancy is associated with complications like pyelonephritis, sepsis, severe sepsis and septic shock, hypertensive disease of pregnancy, anemia, acute and chronic renal failure, intrauterine growth restriction, premature delivery, fetal mortality and increased cesarean delivery. Each year, around 250,000 cases of pyelonephritis are detected in the United States, with females having a higher incidence. Pyelonephritis affects 28 out of every 10,000 women between the ages of 18 and 49 years, with 7% of cases necessitating hospitalization. Cultural and genetic factors may impact the prevalence and recurrence of U.I.T.s. Infection with members of the Enterobacteriaceae family is the most prevalent cause of bacterial prostatitis, represented as the primary type of U.T.I.s in 50-90% of older men.

Conclusions
Urinary Tract Infections (U.T.I.s) are the most common infectious illness in Iraqi society, with an increasing incidence rate each year. Because of its efficient pathogenicity due to multiple virulence factors, E. coli is the most common uropathogenic bacterial species. Biofilm formation and thickness are essential virulence parameters that influence bacterial pathogenicity, severity, and progression of U.T.I.s. IL-6 and IL-8 might be produced by responding to bacterial infections, representing important potential bioindicators of U.T. severity.


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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Department of Molecular and Medical Biotechnology in the College of Biotechnology (Al-Nahrain University, Baghdad, Iraq).

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Conflicts of Interest: The authors declare no conflict of interest.

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