Immunological and Molecular Study of Toll- Like Receptor - 4 in Patients with Urinary Tract Infections.

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Abstract

One hundred blood samples and seventy-four urine samples were collected from urinary tract patients From March to August, 2018 in five hospitals in Baghdad. The patients were divided according to gender and age, as their ages ranged from (14-65) years. E.coli bacteria were isolated and diagnosed from the urine of the patients, and all bacteria were diagnosed by biological, chemical and molecular methods, and 57 E.coli isolates were obtained.

The TLR-4 gene was detected in patients' blood by PCR technique and 100% contained the TLR-4 gene. A gene expression determination was performed by qRT PCR. The results showed an increase in gene expression compared to controls. The DNA sequence was also performed for the enlarged fragments of the PCR product. They were identical to the expected amplification size in all the genes under study which yielded positive results by PCR technology and conducted a similarity search with the basic local alignment research tool (BLAST) and confirmed that these reference strains were obtained in the bank gene and was the correct identification of all samples.

The relationship between the TLR-4 gene and the exogenous outer membrane genes in bacteria (Escherichia coli) was studied by detection of these genes (Smp A, BIC, proB, OmpA,) by PCR technology and these genes appeared in high proportions. In bacterial isolates excluding the OmpA gene appeared by a small percentage.

ELISA was analyzed for patients' serum to detect cytokines ((INT-γ and TNF – α) the results showed a marked increase in cytokines compared to controls where the relationship was negative and there were significant differences between the levels of cytokines in patients and healthy subjects and there were no significant differences in the levels of cytokines between females and males.

Conclusions: Sequencing for 20 PCR product of TLR-4 gene to detect matching of Iraqi population: The human gene expression of TLR-4 gene was elevated compared to controls result to trigger by Gram negative bacteria; The isolates E. coli bacteria were positive for the outer membrane genes (Smp A, BIC, proB, Omp). This has the effect of stimulating and increasing gene expression of TLR-4. Therefore, it was very effective by increasing the levels of INT-γ and TNF-α cytokines in infected patients. as to future work can be Study the course of signals when immune cells respond to pathogens, and determine the amount of cytokines secreted as a result of the TLR-4 immune responses.

Keywords: Immunological; Molecular; Toll- Like Receptor - 4; Urinary Tract Infections

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Introduction

Urinary Tract Infection (UTI) is an infection occur in any part of urinary system, kidney, ureters, bladder and urethra. Most infection involve the lower urinary tract, it is affect the urethra or the...
The microorganism *Escherichia coli*, known as *E. coli*, is responsible for about 80% of urinary tract infections that have not passed the urinary bladder [2]. Toll Like Receptors a family of PPRs that activated the early response to microbial components, generate signals that lead to induce adaptive immune responses, and act as a key role in the innate immune response by recognizing PAMPs [3]. Tumor necrosis factor-α is pro-inflammatory cytokines. The elevation of TNF-α levels are related to increasing of mortality in urinary tract infection [4].

**Materials and method**

**Samples Collection**

**Clinical Samples**

100 samples were collected from the patients including both sexes with (14-65) ages and urinary tract infections from five hospitals in Baghdad/Iraq, from March to August, 2018 in Baghdad city. The samples included blood and urine. They were obtained from mid stream urine from patients suffering from urinary tract infections. The urine samples were collected according to [5]. Urine was collected into sterile screw capped test tubes and cultured immediately. Urine samples were taken by sterile transport media immediately to transported laboratory then after collection (from hospital laboratory) by streaking 0.01 ml of urine (using sterile loop) on Blood agar, MacConkey agar, Mueller Hinton agar, Nutrient broth, and Eosin Methylene Blue agar in order to isolate (*Escherichia coli*) and incubated for 24 hrs at 37°C and their diagnosis was confirmed on the basis of microscopic and genetic methods, biochemical tests and final diagnosis using (BioMerieux France).

**Blood Samples Collection**

Blood samples (5ml) were collected from patients. The sample was divided into two tubes:

1- EDTA tubes for DNA isolation (Molecular genetic studies).
2- The part of blood sample was used to obtain the serum.

After collecting serum, the sample were divide into 2 appendorff tubes and stored in deep freeze at -20°C until used [6].

**Laboratory Diagnosis**

**Morphological Examination and Microscopic Examination**

The differentiations between bacterial isolates were performed by:

1- The morphology of bacterial colonies.
2- Microscopic investigation by Gram stain.
3- API 20E test system

**Identification system (API 20E)**

*E. coli*, identification was conducted using API 20E system depending on the procedure from the manufacturing company.

**Culture**

**Urine Culture**

The urine specimens were cultured immediately on MacConkey agar plate and blood agar plate by direct streaking methods. Plates were incubated at 37°C for 18-24 hours, and then examined for bacterial growth; followed by conventional identification which includes Gram staining, microscopic characteristics, and Epi 20 test. [5].

**DNA Extraction**

**DNA Extraction from Gram Negative bacteria**

Bacterial DNA extraction was carried by using (Genomic DNA purification KitWizard® Promega. USA). Isolates inoculated on nutrient broth for 24 hr at 37 °C, then suspended in 1 ml of inoculated nutrient broth in eppendorf tube and mixed gently.
DNA Extraction from Blood  
Genomic and extraction by using Promega kit from whole blood that was collected in EDTA anticoagulant tubes. The extraction of DNA was done using genomic DNA Extraction kits (Promega) U.S.A. Based on methods.

Polymerase Chain Reaction (PCR) Amplification Technique  
A PCR technique was used with primers[7].

Primer Design  
A. Primers Selection  
The primers (IDT DNA, USA) used in PCR amplification were specific for 16S rRNA Primer of E.coli database of DNA sequences in NCBI gave the basic information to design specific primers. 

Primer sequence 
5'GGAGTTAGCCGGTGCTTCTT3'and R5'GATGACCAGCCACACTGGAA3'  

B. Primer Design TLR-4  
To amplify TLR-4 gene of human, database of DNA sequences in NCBI gave the basic information to design specific primers.  

Primer sequence 5' CATTGGTGTGTCGGTCCTCA 3' and R 5'ACTGCCAGGTCTGAGCAATC 3'  
These Primers were designed to contain 20-40 nucleotides with GC content between 55-55%, and in self complementarity 3-6 , melting temperature (Tm) between 59.96 -60°C. The primers were synthesized at Integrated and Technologies (IDT/USA). These were provided in a lyophilized form, which were re-dissolved with TE buffer (pH 8) or free DNase/RNase water and stored at -20°C

C. Sequencing and sequence alignment  
Sequencing of amplified products of TLR4 genes was done by (National Instrumentation center for Environmental management (NICEM)/Korea through using forward and reverse primers for TLR4 gene in each sequencing reaction. Homologous search was performed using Basic local alignment search tool (BLASTversion 1.1.0) program which is available at the national center biotechnology information (NCBI) online and BioEdit program. The results were compared to the references sequences of the gene that was obtained from gene bank as control.

Extraction of total RNA  
The preparation of lysates from small tissue samples (≤30mg) the SV total RNA isolation system fast and simple technique for preparing purified and intact total RNA from tissues, and used Procedure guided by the manufacturer (Promega).

Quantitative Real-Time PCR (qRT-PCR)  
Amplification of a fragment of mRNA was performed with the following master amplification reaction with the program of One-Step RT-PCR

Immunological Test  

Enzyme-Linked Immunosorbent Assay (ELISA)  
The enzyme-linked Immunosorbent Assay (ELISA) to determination of of TNF-α and INF-γ was performed as described by [8] to Determination the level of Tumor necrosis factor-α (TNF-α) and INF-γ.

1. Results and Discussion  
Isolation and Identification of bacteria from Urine Samples  

One hundred urine samples with clinically diagnosed UTI were collected aseptically from patients attending and Seventy five blood sample of the same patients with urinary tract infection were collected , and 74 healthy controls from hospitalization patient in from five hospitals in Baghdad included: AL-Kindy General Teaching Hospital, Ibn-Al Balady Hospital, Teaching Laboratories in Medical City, Education Baghdad Hospital, Al-Kadhimia teaching and Al- Harery
Hospital in Medical City for six month. From March to August, 2018 in Baghdad city. The samples included blood and urine. They were obtained from mid stream urine from patients suffering from urinary tract infections. The urine samples were collected according to [5] Urine was collected into sterile screw capped test tubes and cultured immediately. From 100 clinical isolates of gram negative bacteria primary identified as 57 clinical isolates of *Escherichia coli*.

**Cultural Characteristics of *E.coli***

The identification of presumed *E. coli* isolates was on MacConkey agar the colonies isolates appeared pink, non-viscous colonies a pinkish tint as a result to non – lactose fermenter, small in size, regular edges and no pigmentation and circular with entire edge, in appearance on MacConkey agar. Figure (3-1) on blood agar the colonies isolates appeared lactose fermentation, and grayish white, moderately opaque colonies with or without a zone of haemolysis. *E. coli* isolates was activated by its cultured on Haert t brain Agar the colonies seem as a white color and it is producing a pale color of the agar this show in Figure(3-2). On eosin methylene blue agar (EMB) (selective medium), when observed directly against light *E. coli* colonies appeared very dark, almost black. By reflecting light, a green sheen can be seen as a result to the high amount of acid produced by fermentation, and they usually appeared as ovoid or circular smooth colonies as defined by [9]. Figure (3-3).

**Microscopic examination**

Microscopic examination of Gram stained slide of *E. coli* isolates showed that the isolates were ram negative, non-spore forming rod–shaped arranged in single or aggregated in pairs and non–spore forming as described by [10].

**Biochemical Test of *E.coli***

*Escherichia coli* isolates is given in biochemical tests, a positive results for catalase test

Results of the previous biochemical tests insured what was already found by culturing on EMB agar.

It is worth to mention that the biochemical identification of *E. coli* isolates was assured by using API 20E system. The API 20E system is a fast miniaturized and standardized identification form of existing traditional procedures for the diagnosis and differentiation of members of the family Enterobacteriaceae [11]. API 20E reading table for *E.coli* isolates.

Figure (1): *Escherichia coli* growth on MaCconky Agar the colonies seem as a pink color and it is

Figure(2): *Escherichia coli* growth on Haert brain Agar the colonies seem as a white color and it is
Genotypic identification results revealed that all 57 isolates (100%) were *Escherichia coli* which showed expected amplicons size 956bp for housekeeping gene 16S rRNA. Figure (3-4) illustrated shine bands of positive isolates compared with 100bp DNA ladder. All of the isolates were collected from urine. Distribution of uropathogens may differ by type of infection or patient inhabitance.

The comparison between molecular and biochemical tests for identification of *E. coli* in the present study, it was showed that the molecular tests gave results with more accuracy, rapidly, simply and cheap. API 20E test gave 95% positive results from all isolates. This means API 20E test is less efficiency than the molecular tests in the identification of bacteria.

**Gene expression of TLR4**

The role of TLRs is very important in the pathogenesis of UTI, were detected of TLR4 have been detected in 74 patients with (14-65) ages stages and two sex of UTI.

TLR-4 Gene expressionin the blood of patients after challenge of LPS detection of TLR4 was examined in the blood isolates from patients and control. LPS in bacteria.

The *TLR4* gene were detected successfully in all samples by using PCR with used 2 specific primers as described in chapter two. The amplification accuracy of *TLR4* gene product was noticed by bands in Figure (3-5). The result of sequencing has been compared with reference sequence of TLR4 in national center biotechnology information (NCBI) Gene Bank.
Relative quantification

\[ \text{Folding} = 2^{-\Delta\Delta CT} \]

\[ \Delta\Delta CT = \Delta CT_{\text{Treated}} - \Delta CT_{\text{Control}} \]

\[ \Delta CT = CT_{\text{gene}} - CT_{\text{House Keeping gene}} \]

The results showed that the gene expression of TLR-4 compared to H.K. gene and control was higher or not significant, compared to the housekeeper that show in Figure (1), and Table (1).

The gene expression of TLR-4 was relatively high in most patients compared to healthy subjects as in Figures (2, 3, and 4).

Table (1): Gene expression of TLR-4 compare to H.K. gene and control gene by qRT PCR assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>TLR</th>
<th>H.K</th>
<th>dct</th>
<th>ddct</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>25.63</td>
<td>25.40</td>
<td>0.23</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2.</td>
<td>24.39</td>
<td>29.31</td>
<td>-4.92</td>
<td>-5.14</td>
<td>35.32</td>
</tr>
<tr>
<td>3.</td>
<td>28.14</td>
<td>31.51</td>
<td>-3.37</td>
<td>-3.59</td>
<td>12.06</td>
</tr>
<tr>
<td>4.</td>
<td>26.51</td>
<td>23.72</td>
<td>2.79</td>
<td>2.56</td>
<td>0.17</td>
</tr>
<tr>
<td>5.</td>
<td>24.22</td>
<td>27.66</td>
<td>-3.43</td>
<td>-3.66</td>
<td>12.65</td>
</tr>
<tr>
<td>6.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>28.08</td>
<td>30.26</td>
<td>-2.18</td>
<td>-2.40</td>
<td>5.30</td>
</tr>
<tr>
<td>8.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9.</td>
<td>24.86</td>
<td>25.14</td>
<td>-0.28</td>
<td>-0.50</td>
<td>1.42</td>
</tr>
<tr>
<td>10.</td>
<td>22.45</td>
<td>28.65</td>
<td>-6.20</td>
<td>-6.42</td>
<td>85.81</td>
</tr>
<tr>
<td>11.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>23.87</td>
<td>26.50</td>
<td>-2.62</td>
<td>-2.85</td>
<td>7.21</td>
</tr>
</tbody>
</table>
Figure(2): Gene expression of TLR-4 compared to H.K. gene and control gene by qRT PCR assay

Figure(2): gene expression result  H.K gene

Figure(3): gene expression result  TLR-4 gene
The result of sequencing has been compared with reference sequence of TLR4 in national center biotechnology information (NCBI) Gene Bank.

The BLAST results for the first and second primer sequence show the matches between each sequence of patients with the global NCBI database, and the first hits is chosen that are more similar to query sequences. The BLAST table to examine the similarities or differences is used and also using E value to. Show that the results were obtained not by chance. Figure (5)
Infection with invasive Gram negative bacteria we observed increased response of TLR-4. These results showed that invasive Gram negative bacteria are able to stimulating LPS to speedily express TLR-4 on their surface.

**Innate immunity**

Toll-like receptors (TLRs) are a group of PAMP recognition receptors, play an significant role in innate immune signaling in response to microbial infection. The Specific TLRs illustrate functional pattern recognition of peptidoglycan and bacterial lipopeptides lipopolysaccharide (LPS; TLR4).

TLR4 functional activity was observed by measuring cytokines in serum 24-72h at patients of urinary tract infections at E. coli LPS stimulation , these results indicate that the pathway of cellular signal transduction for TLR-4 is unlike from activity that for INT-γ and TNF-α [12].

Immunity against Gram-negative bacterial component. TLR-4 can be recognized lipopolysaccharide of Gram-negative bacteria and participates in the host defense against them by evolving immune response [13].

This upregulation of TLR4 expression was synchronized with increasing in proinflammatory cytokines.

Functional TLR4 activation was assessed by subsequent stimulation with TLR agonists Escherichia coli lipopolysaccharide (LPS; TLR4 ligand) in response to LPS but when cultured with wild-type E.coli induced expression of TLR -4 on HAEC functionally reacted to E. coli LPS . Furthermore, SmpA and BIC expression elicited by E. coli LPS was high. These results illustrates that invasive E. coli stimulates expression of TLR on the surface of cells respond to defined TLR-specific ligands.

The most important target ligands for TLR4 molecules are LPS (such as UPEC and other members of Enterobacteriaceae family which are related to UTIs).

Interestingly, the number of fatty chains in lipid A relating to LPS molecule determines the level of TLR4 molecules expression and consequently the level of inflammatory responses.

By the invasion of UPEC cells into the urothelial cells of bladder and kidneys, the TLR4 molecules are expressed [14].

**Distribution of UTI patients**

In this research, female predominance is observed. There were 43 (58.11) % females and 31(41.89%) males as patients with urine positive culture of Gram negative bacteria. This preponderance is not similar to the results found by the investigation done by [15] .which females and males were 82.72% and 18.93% respectively. The Table (4-5), shows distribution of patients , according to age and sex for isolate serum that were most the patients from females in ( 14-45 ) age more high was in age (14-25) 11( 25.58) % while decrease in (46>) whereas males patients numbers were high in age ( 46-55) were10(32.26%)and age ( 66<) were 7(22.58%) but in there were low in males patients numbers in age( 14-45, Were 6 (19.35 % ).Table(1)
Table(1): Distribution of patients, according to age and sex for isolate serum

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>%</th>
<th>Mel</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14-25</td>
<td>11</td>
<td>6</td>
<td>(19.35)%</td>
</tr>
<tr>
<td></td>
<td>26-35</td>
<td>8</td>
<td>1</td>
<td>(3.22)%</td>
</tr>
<tr>
<td></td>
<td>36-45</td>
<td>9</td>
<td>3</td>
<td>(9.67)%</td>
</tr>
<tr>
<td></td>
<td>46-55</td>
<td>4</td>
<td>10</td>
<td>(32.26)%</td>
</tr>
<tr>
<td></td>
<td>56-65</td>
<td>6</td>
<td>4</td>
<td>(12.9)%</td>
</tr>
<tr>
<td></td>
<td>66&lt;</td>
<td>5</td>
<td>7</td>
<td>(22.58)%</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>31</td>
<td>43</td>
<td>(41.89)%</td>
</tr>
</tbody>
</table>

INF-γ levels in study groups enrolled in the study

Levels of INF-γ with UTI patients distributes according age and sex compare with control were. Mean of serum concentration INF-γ ± S.E was 68.50±4.352 pg/ml in the females patients and 0.18±0.007 pg/ml in the control at age stage (46-55) was higher than other age but lower level was 18.267 ±9.57pg/ml at age stage 66< in females, while in the males patients at age stage (56-65), mean of INF-γ concentration was 73.32 ±20.1 pg/ml and the control 0.298 ±0.138pg/ml that was higher than other age but lower level was 50.14 ±20.36pg/ml at age stage 66< in males. Mean of concentration was significantly high in the patients than in the controls (P≤0.000), The results also showed that there were no significant differences between males and females in a levels INF-γ as shown in (Figar1&2).

Figure(1): Levels of INF-γ with UTI patients (female) according age compare with control

Figure(2): Levels of INF-γ with UTI patients (male) according age compare with control

TNF-α levels in study groups enrolled in the study

In an females patients, mean of TNF-α concentration at higher age stage pg/ml and 10.48±5.72pg/ml=(46-55) was 10.38±7.24 and(14-25)respectively but for their control0.21±0.026 pg/ml while in the males patients, Mean of TNF-α concentration was lower level was 3.583±4.06 at age (14-25)pg/ml, the control 0.155±0.005pg/ml and higher other age was 12.53±2.12 pg/ml, 11.11±3.91pg/ml at age stage (36-45), (46-55), (56-65) respectively. The results showed that there are no significant differences between males and females in a levels TNF-α but
the mean of TNF-α concentration in patients was high significantly compared with control group \((P ≤0.001)\). As shown in Figar (3&4).

To demonstrate TLR-4 function and study its regulatory role in induced inflammation ELISA analysis of some anti-inflammatory and anti-inflammatory cytokines was performed. Inflammatory cytokines have been studied such as TNF-α, IFN-γ, and the very important anti-inflammatory cytokines that have been extensively studied up to now have been measured.

The serum level of IFN-γ increased at the patients UTI and bacteria in its urine of patients which at (14-25) age and then gradually started decreased until (56-65) age and more, this likely men of late age in certain chronic is infected or immunological diseases or their easy exposure Viral infections as a result of lower immunity in these ages ( Table 3-4 )

General Mean of serum concentration of INF-γ and TNF-α concentrations compare with control. That INF-γ was52.371±18.138 pg/ml in the patients group and 0.2237±0.0608pg/ml in control group.

Mean serum TNF-α of patients was 8.006±7.050pg/ml but in control group 0.184±0.0267.

Mean serum TNF-α was significantly higher in the patients group than in the control group (p-value ≤ 0.000000), as shown in Table ( 2)

Table (2): Mean of INF-γ and TNF-α concentrations patient compare with control

<table>
<thead>
<tr>
<th></th>
<th>TNF-α</th>
<th>INF-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>patients</td>
</tr>
<tr>
<td>Mean</td>
<td>0.2237</td>
<td>52.371</td>
</tr>
<tr>
<td>SD</td>
<td>0.0608</td>
<td>18.138</td>
</tr>
</tbody>
</table>

P.value ≤ 0.000000

This result agrees with [15], who pointed to increasing of production of tumor necrosis factor-alpha (TNF-α) during human aging, who founded that, levels of TNF-α has been increased in the circulation after progression of age-related inflammatory diseases.

The results are similar to [16], who indicated elevation of (TNF-α) in the elderly population. This signifies a state of continuous stimulation of the immune system, thus, the condition of continuous
subclinical inflammation, which will explain the development and development of many pathological processes in the elderly.

Stimulation with *E. coli* LPS induced cytokines production by presence TLR-4 but not in uninfected. *E. coli* LPS did not the production of INF-γ and TNF-α in serum of patients the increase in INF-γ and TNF-α levels was as a result to the interaction between TLR4 expression and *E. coli* LPS induced by invasive Gram negative bacterial infection.

Gram negative (*Escherichia coli*) as a result of the big majority of UTI in both of hospitalized patients and other. These infections can differ from a simple cystitis to a serious parenchymatous attack. The degree of severity according to the virulence of the responsible strains and on the susceptibility of the host, particularly if there is a urological illness concomitant. A best knowledge of the virulence factor characteristics of the microorganism causing the infection will allow expecting to a point, the progress of infection in the host organism.

Table (3): Number and percentage of positive isolates for presence of screened LPS genes and Outer membrane adhesions of 57 *E.coli* isolates

<table>
<thead>
<tr>
<th>Genes</th>
<th>Number and Percentage of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane Integrity</td>
<td><em>E.coli</em></td>
</tr>
<tr>
<td><em>Mot/OmpA</em></td>
<td>45</td>
</tr>
<tr>
<td><em>BIC</em></td>
<td>82</td>
</tr>
<tr>
<td><em>SmpA</em></td>
<td>95</td>
</tr>
<tr>
<td><em>proB</em></td>
<td>90</td>
</tr>
</tbody>
</table>

**LPS (outer membrane) genes**

To investigate the role of bacteria to adhere to the living surface and non-living surfaces, the role of (*SmpA, proB, BIC, OmpA*), has been studied in stimulating and increasing gene expression for TLR-4.

Diagnostics of *E. coli* bacteria were carried out by morphology, chemical and molecular methods using PCR technology by the housekeeping gene and the electrophoresis gel was transported, after the diagnosis the LPS genes were detected (*Bic, Smp A, pro B, Omp A*) by PCR technology, where the results showed the emergence of a gene Bic in 82% of isolates the Smp A gene in 95% of isolates as in Figure (5;6;7) and Table (3).

Also, and the *OmpA* and *pro B* genes of *E. coli* bacteria were detected and appeared to possess the *pro B* gene at 90%. the *OmpA* gene it did low appear in the isolates of *E. coli* at 45%, respectively as in Figure (6) and Table (3).

These results show and confirm that the possession of Gram-negative bacteria for these genes has an important role in stimulating innate immunity, which increases the expression of the TLR-4 gene, which has an important role in identifying these bacteria and thus by means of signals stimulates the immune cells to secrete cytokines and INF and TNF.

Figure (5): Gel electrophoresis detect, Smpa (186bp), using DNA Ladder (1500bp) promega.

Evidence for Bic and SmpA genotypes with high House Keeping in vivo and in vitro is an increase in TLR-4 gene expression and its effect on effective secretion of cytokines (INF-γ and TNF-α) when compared to healthy controls.

It should be noted that to clarify the effect of antibiotics used in the present study, we note that there is no effect of antibiotics or the indication on gene inhibition.

TLRs as Sensors, it identify and classify opportunistic pathogens that breach epithelial barriers and enter tissue sites. This pathogen sensory function is carried out on TLR expressing host cells including macrophages, mast cells, immature dendritic cells (DCs), and both endothelial and epithelial cells. These TLR expressing cells continually screen both the external (skin, mucosal surfaces) and internal environments for the presence of pathogens [17].

Though its accurate role in virulence was unidentified, presence of SmpA in infective clinical isolates and within the outer membrane vesicles of infesting clinical strains makes it as an essential substance [18].

The suggestions (SmpA) play an important role in antibiotic resistance for bacteria under study because; in a way that deficiency of them may be incapable to assemble OMPs in the outer membrane. Another study showed the SmpA deficiency may present the high tended sensitivity to antibiotics, [19].The Blc gene is poorly transcribed, suggesting that the normal concentration of Blc in the outer membrane is low. Other findings indicate host pathogenesis [20].

The greatest factors for the occurrence of E. coli in UTI are gene coding of fimbrial adhesive systems.

In this study, explain that live invasive Gram negative bacteria induce TLR-4 expression . studies mention that E.coli LPS and fimbriae , as a purified components, induce expression of TLR
on the surface, indicate that stimulation of TLR surface expression demands interaction of live, fimbriated of *E. coli*. Important, proved that the increased TLR-4 result from induce with infection of invasive Gram negative bacteria was able of functionally reactive with *E. coli* LPS as a TLR4-LPS ligand. These results indicate that the levels of TLRs expressed on the surface immune cell are modified by Gram-negative bacteria. Recent reports was showed, TLRs are selectively up-regulated during infection and inflammation next stimulation with microbial products [21].

### References