Clinical and Experimental Health Sciences

Urine Influences Growth and Virulence Gene Expressions in Uropathogenic *E. coli:* A Comparison with Nutrient Limited Medium

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ABSTRACT

Objective: The interactions between environmental factors and microbial biological processes are well known. Urine provides host conditions probably affecting bacterial growth and gene expressions. The aim of this study was to detect the modulations of growth and gene expressions [sfa/foc, cnf1, usp and aer] of Uropathogenic *E.coli* (UPEC) strains in urine by comparing the results with serum supplemented standard American Petroleum Institute (SAPI) medium which is defined as host-like medium.

Methods: UPEC strains C7 and C149 were incubated at 37°C and growth alterations were detected by measuring the changes in the absorbance at 600 nm in four-, six – and 24 hours. Gene expression levels were analyzed by quantitative polymerase chain reaction (qPCR). Statistical analysis of fold changes in gene expression values and growths were calculated using one-way ANOVA unpaired t-test and Tukey's post hoc test, respectively.

Results: The increase of bacterial growth in urine was found to be statistically significant ($p<0.0001$). The alterations of *aer* and *sfa/foc* expression levels were statistically significant ($p<0.001$); whereas the expression levels of *cnf1* and *usp* genes were not altered ($p>0.05$).

Conclusion: According to our results, urine as an environment *in vivo* affected both the growth and gene expression in UPEC.

Keywords: Urine, Virulence, Growth, Gene Expression, UPEC

1. INTRODUCTION

It is well known that microorganisms and their hosts, including human, have co-existed for million years. The interactions between host and microorganisms during an infection were determined by microbial virulence factors, host's immune system and chemicals produced or present *in vivo* (1-4). On the other hand, the environment of microbe *in vivo* influences bacterial pathogenicity by affecting growth and virulence mechanisms. Today, behaviors of microorganisms *in vivo* dominate research and writing (5,6).

Microorganisms encounter with various host derived environmental determinants either present or produced *in vivo* such as hormones, vitamins, bile salts, sugars, antibiotics, ions, pH etc. (7-12). Urine includes such kind of various host derived determinants and, as an environment of bacteria, alters bacterial growth and gene expressions in different ways (13-22).

The purpose of the present study was to examine whether urine influences the growth and gene expressions [*sfa/foc* (encodes fimbrial adhesions SF1C), *cnf 1* (encodes cytotoxic necrotizing factor 1), *usp* (encodes a uropathogenic-specific protein) and *aer* (encodes aerobactin)] of Uropathogenic *Escherichia coli* (UPEC) which are the most common agents of urinary tract infections (UTIs). For this purpose we compared the growth and gene expression of UPEC in urine and in serum-supplemented standard American Petroleum Institute (SAPI) medium. This medium provides a nutrient limited condition and imitates *in vivo* (23-25).

2. METHODS

2.1. Strains

In the present study two different UPECs (*E. coli* C7 strain carrying *sfa/foc, cnf1* and *usp* genes and *E. coli* C149 strain carrying *aer* gene) were used which were kindly provided

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by Prof. Dr. Shingo Yamamoto (Hyogo College of Medicine, Japan). Bacteria were kept at −80 °C for all analysis.

2.2. Media

Healthy male urine and standard American Petroleum Institute (SAPI) medium supplemented with 30% (v/v) adult bovine serum (serum-SAPI) were used as growth media in this study. Healthy male urine was used after sterilized by filtration. Serum-SAPI was defined as a nutrient-limited medium which mimics *in vivo* growth conditions (23, 24). The serum-SAPI medium was prepared as previously described in studies (23,25,26). Briefly, the medium contains 6.25 mM/L ammonium nitrate, 1.84 mM/L monobasic potassium phosphate, 2.77 mM/L dextrose, 3.35 mM/L potassium chloride, and 1.01 mM/L magnesium sulphate and pH adjusted as 7.5.

2.3. Comparison of Growth in Urine and SAPI

Overnight cultures of *E. coli* C7 and C149 were prepared in SAPI medium and these were five-fold diluted and inoculated into SAPI medium and urine. Bacteria were incubated at 37 °C. Growth alterations were detected by measuring the changes in absorbance at 600 nm in four-, six – and 24-h periods. The samples were tested in duplicate and each experiment was performed twice.

2.4. Comparison of Gene Expressions in Urine and SAPI

Total RNA isolation and cDNA synthesis

Bacteria were grown in SAPI medium and human urine for 16–24 h at 37 °C. Total RNAs were extracted from 24-h-fresh cultures by using Tripure reagent (Roche, Switzerland) according to manufacturer's instructions. Horizontal electrophoresis gel analysis and spectrophotometer measurement used to analyze of isolated RNAs quantitatively and qualitatively. We detected the concentration and purity of total RNAs using a NanoDrop 2000 spectrophotometer Thermo Scientific (Waltham-USA). Total RNAs with a ratio (A260/A280) > 1.8 were used for quantitative PCR (qPCR) analysis. Total RNAs were also screened by 1% horizontal gel electrophoresis. High quality and amounts of RNAs were converted to cDNA for qPCR assays. Reverse transcription was carried out using commercial cDNA conversion kit (Takara, Japan) according to the manufacturer's instructions. The thermal cycle conditions were as follows: 20 min at 37°C, 5 min at 85°C and cooling at 4°C. After reverse transcription step, 1:5 diluted cDNAs were used in gene expression analysis.

Quantitative polymerase chain reaction (qPCR) analysis

Quantitative PCR reactions were carried out using SYBR Green I fluorophore dye (Takara, Japan) according to the instructions of the manufacturer. 16S rRNA gene was used as housekeeping gene. We detected the expression levels of *sfa/foc* (S and F1C fimbria), *usp* (uropathogenic-specific protein) and *cnf1* (cytotoxic necrotizing factor) for C7 strain and *aer* gene (aerobactin) for C149 strain in the presence of urine and SAPI medium. Quantitative PCRs were carried out in a reaction volume of 16 µL including 1X SYBR Green I, 0. 5 pmol of primers (Table 1) and 2 µL of cDNA equivalent to 50 ng total RNA. Quantitative PCR conditions were shown in Table 2 for all genes. Sensitivity and accuracy of the protocol were tested at different levels by performing melting curve analysis, using 16S rRNA as control. Ct values were obtained and calculated using Quant Studio 5.0 software (Applied Biosystem, USA). Relative quantification strategy was used in obtaining fold changes in gene expression using $2^{-\Delta\Delta CT}$ formula. The experiments were conducted in quadruple and the results were presented as fold change for each gene.

Table 1. *Primers used in the gene expression*

Gene	Nucleotide sequence (5'-3')	Band size (bp)	References
16S rRNA	F: CCA GGA TTT GAT YMT GGC R: GAA GGA GGT GWT CCA DCC	532	27,28
sfa/foc	F: CTC CGG AGA ACT GGG TGC ATC TTA C R: CGG AGG AGT AAT TAC AAA CCT GGC A	410	29,30
usp	F: CGG CTC TTA CAT CGG TGC GTT G R: GAC ATA TCC AGC CAG CGA GTT C	615	29
cnf1	F: AAG ATG GAG TTT CCT ATG CAG GAG R: CAT TCA GAG TCC TGC CCT CAT TAT T	498	29,30
aer	F: TAC CGG ATT GTC ATA TGC AGA CCG T R: AAT ATC TTC CTC CAG TCC GGA GAA G	602	29,30

Table 2. *Quantitative polymerase chain reaction (qPCR) conditions*

Step	Time	Temperature
Pre-denaturation	2 minute	95°C
Number of cycling: 45		
Denaturation	10 second	95°C
Annealing	15 second	58°C
Extension	20 second	72°C
Cooling	30 second	40°C

2.5. Statistical Analysis

Statistical analysis of fold changes in gene expression values were calculated using one way ANOVA unpaired t-test and growths were calculated using one-way ANOVA Tukey's post hoc test. All measurements were compared to control. All results were presented as mean±standard deviation (SD). The multiple comparisons were made at a level of $p<0.05$.

3. RESULTS

3.1. Comparison of Growth in Urine and SAPI

In order to detect the effects of human urine on growth of UPECs, the absorbance values were compared to SAPI (control medium). The growth of UPEC in urine was enhanced which was found to be statistically significant ($p < 0.001$) in the 4th, 6th and 24th hours (Figure 1, Figure 2 and Figure 3).

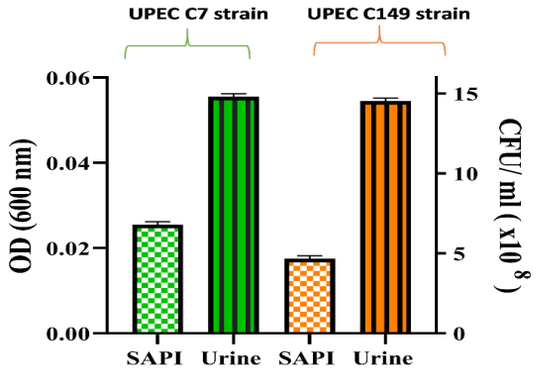

Figure 1. *Comparison of UPECs (C7 and C149 strains) growth in urine and SAPI medium in the 4th hour. The alterations were determined by comparing with control (SAPI medium). The growth alterations of bacteria in urine and SAPI were examined using one-way ANOVA followed by Tukey's post hoc test. The difference between the growth of UPECs in urine and SAPI medium were found to be statistically significant (p<0.0001).*

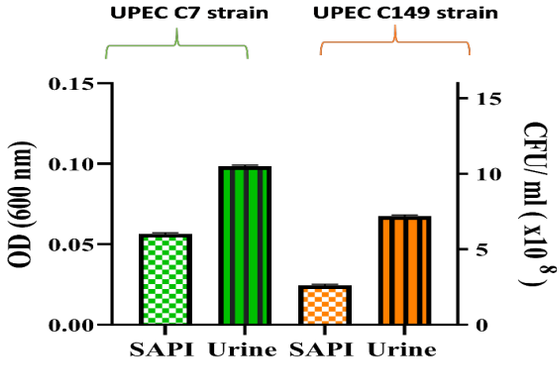

Figure 2. *Comparison of UPECs (C7 and C149 strains) growth in urine and SAPI medium in the 6th hour. The alterations were determined by comparing with control (SAPI medium). The growth alterations of bacteria in urine and SAPI were examined using one-way ANOVA followed by Tukey's post hoc test. The difference between the growth of UPECs in urine and SAPI medium were found to be statistically significant (p<0.0001).*

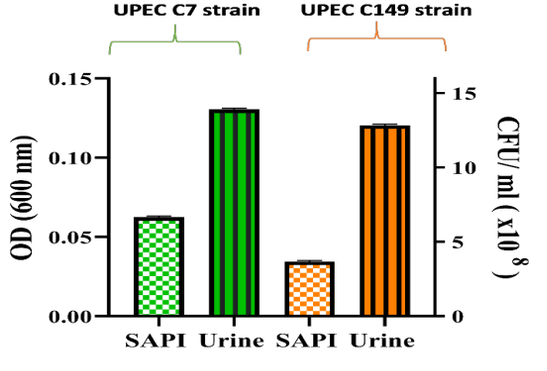

Figure 3. *Comparison of UPECs (C7 and C149 strains) growth in urine and SAPI medium in the 24th hour. The alterations were determined by comparing with control (SAPI medium). The growth alterations of bacteria in urine and SAPI were examined using one-way ANOVA followed by Tukey's post hoc test. The difference between the growth of UPECs in urine and SAPI medium were found to be statistically significant (p<0.0001).*

3.2. Comparison of Gene Expressions in Urine and SAPI

The down regulation of *aer* gene was found to be statistically significant in urine (p<0.01). The expression of *sfa/foc* gene was increased in urine (p<0.01). However, no statistically significant difference was found in the expression levels of *cnf 1* and *usp* (p>0.05) (Figure 4). Ct values in all genes ranged from 6.60 to 26.64.

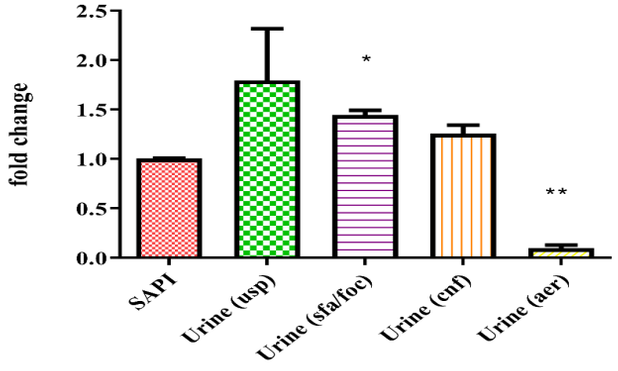

Figure 4. *Comparison of gene expressions in urine and SAPI. The alterations were determined by comparing with control (SAPI medium). Statistical analysis was calculated using one-way ANOVA unpaired t test. *, **: Significant at p<0.01 level and p < 0.001 level values, respectively.*

4. DISCUSSION

Globally a large number of people suffer from UTIs which are mostly caused by UPEC strains. UPECs have different virulence factors as compared to other *E.coli* strains. Some of these virulence factors are fimbrial adhesins encoded by *sfa/foc* genes, a siderophore for iron uptake encoded by *aer* gene, cytotoxic necrotizing factor 1 encoded by *cnf1* gene, and uropathogenic-specific protein which has endonuclease activity encoded by *usp* gene (31,32). The expression of UPEC virulence genes may be affected by environmental conditions determined by host factors. It is well known that during infectious process, bacteria sense *in vivo* environmental changes and adapt to them.

As it was stated above, not only physiological conditions such as pH, temperature, but also many host determinants either present or produced *in vivo* regulate the growth and gene expression of microorganism during infection (3 – 6,14,18,19,22,33-35). It was suggested by some authors that, urine contains high concentrations of various metabolites which induce the growth of bacteria, whereas the others define the urine as a nutrient limited medium (with regard to amounts of iron, amino acids and nucleotides) (14,16,17,20,36-38).

There are many studies aimed on the effect of human urine on bacterial growth and gene expression in different bacteria. It was shown that pH, the amount of glucose, iron, osmolality, ammonium, organic acids, creatinine and urea of urine are important factors affecting growth of bacteria (16-22, 39-41).

Our study was designed in a different way to examine the influence of human urine on growth and virulence gene expression in UPEC (urine compared to serum SAPI medium).

The bacterial growth is the first step in the infection process which is necessary for avoidance of immune response and infection. Roos et al. have shown that the *in vitro* growth ability of *E.coli* in urine was strain depended. *E. coli* 83972 strain was grown well in urine depending on the individual batch of urine used, but *E. coli* K-12 reference strain MG1655 could not grow in urine. They also reported that *E.coli* 83972 strain was grown better than the K12 strain both in Luria Bertani (LB) broth, a nutritionally rich medium and human urine. They also showed that strain 83272 was grown better than some clinical isolated *E.coli* strains (536, CFT073, NU14, and 1177) in urine which were isolated from patients with UTIs (39). Alteri and Mobley have indicated that the *in vitro* growth rates in urine of enteropathogenic and commensal *E. coli* strains was generally similar to that of UPEC's (40). In contrast to these results, Aubron et al. suggested that the *in vitro* growth of different *E. coli* strains (8 UPEC, 1 Enterohemorrhagic *E. coli*, 9 asymptomatic bacteriuria causing strains and 3 commensal strains) was significantly less than in LB medium compared to growth levels in urine. They also found that the growth ability of asymptomatic bacteriuria strains in the urine was not better than of UPEC and commensal *E.coli* strains (41).

In the present study we found that urine induced the growth of UPEC significantly ($p < 0.001$) when compared to serum-supplemented SAPI medium. Our results are consistent with previous studies; serum-SAPI medium has reduced the growth of UPEC when compared to Tryptic Soy Broth, Dulbecco's Modified Eagle's Medium etc. in the 4^{th}, 6^{th} or 24^{th} hours (27,28,42). Urine seems a good growth medium for *E. coli* due to contain several inorganic and organic compounds and the growth of UPEC was provided effectively by fresh urine for this study.

The effects of urine on microbial virulence related gene expressions were shown in many *in-vivo* and *in-vitro* studies (18, 19, 22, 33, 34, 40, 43, 44). Greene et al. concluded that when *E. coli* grown in urine, *fim* expression was found to be prevented. They also suggested that urine has inhibitory effects on FimH function (18). In a study of Hancock and Klemm the expression levels of 815 genes were examined in urine by comparing planktonic and biofilm forming strains and they reported that the expression levels of *pap* and *foc* genes were found to be not altered but *fyuA* gene (encoding siderophore receptor) expression was upregulated (19). Russo et al. found that expression level of $iroN_{E.coli}$ (encoding siderophore receptor) was increased in urine (22). King et al. found K1 capsule genes of a UPEC strain were down-regulated in urine (34). Roos et al. showed that expression levels of iron-uptake, transport system and adhesion-associated genes, *pap, sfa/foc* genes, were upregulated when *E.coli* strain grown in urine (39). Snyder et al. reported the expression levels of *E.coli* CFT073 genes both of *in vivo* and *in vitro* (urine compared to LB) conditions. They reported that many of the most highly expressed genes *in vivo* conditions were also among the most highly expressed in urine. Some of the upregulated genes were reported as related with iron acquisition systems, capsular compositions, microcin secretion genes (43). In consistent with Snyder et al., Hagan et al. suggested that *E. coli* gene expressions were generally similar in urine provided from women with UTIs and infected mouse. The most significantly differences in expression levels were reported for genes encoding adhesins (44).

In our study, expression of *aer* gene which is responsible for iron uptake in UPEC was found to be down-regulated; this result is inconsistent with other studies. The upregulation of *sfa/foc* gene related to adhesion of the pathogen to the urinary tract; this result was consistent with the previous studies (39,43). In line with all these results, we may suggest that these discrepancies related to be depend on strain, compared to medium/conditions.

5. CONCLUSION

In many studies mentioned above, the comparison of growth, expression levels of genes encoding virulence factors were investigated by using urine and standard bacteriological culture media such as Luria-Bertani, Tryptic soy broth etc. However, in our study nutrient limited SAPI medium was used which mimics *in vivo* environment. For detection of behaviors of pathogens *in vitro,* studies are needed to prepare proper alternatives for culture media to provide mimicking *in vivo* conditions.

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