Enhanced Adhesion and Cell Damage by *Escherichia coli* Harboring *hly*, *papC* and *cnf-1* genes to the Uroepithelium in Diabetic Mouse Bladder Model

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http://dx.doi.org/10.22207/JPAM.11.1.75

(Received: 24 January 2017; accepted: 04 March 2017)

The *in-vivo* mouse bladder model was sought to determine the effect of virulent uropathogenic *E. coli* (UPEC) strain harboring *hly*, *papC* and *cnf-1* genes on uroepithelium of diabetic mouse bladder infected transurethrally. The female BALB/c mice aged between 6-8 weeks were used in the study. The diabetes was induced by subcutaneous injection of alloxan hydrate (80mg / kg body weight) in mice. Two UPEC strains, one with *hly*, *papC* and *cnf-1* virulent genes and the other (hypovirulent) without *hly*, *cnf-1* and *papC* genes were selected for the study. The animals were anesthetized and 50 μl of bacterial inoculum was instilled in to bladder of DM and non-DM mice using specially devised mice catheter. The mice were sacrificed at 4 hrs, 24 hrs and 48 hrs of post infection, and the bladder was removed aseptically. One half of the bladder was homogenized and bacterial culture was performed. The other half of the bladder was used to document bacterial adhesion and invasion by histopathology and scanning electron microscopy. The exaggerated consequence of virulent UPEC strain on diabetic mouse bladder-model was documented as enhanced adhesion and extensive damage of the uroepithelium of the bladder. However, hypovirulent UPEC strain failed to produce observable pathophysiological effect. Many of the UPEC were in the filamentous form and occasionally seen looping within and between adjacent superficial cells to escape from immune mechanism like micturation and exfoliation.

**Keywords:** UPEC; *Escherichia coli*; Mouse UTI model; Diabetes mellitus.

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The *Escherichia coli* is responsible for over 80% of the community-acquired urinary infections. The ability of the uropathogenic *Escherichia coli* (UPEC) to adhere to the host bladder epithelium is considered critical to cause the disease\(^1\)-\(^2\). There is a wide array of virulence markers employed by UPEC, and the adherence is mostly caused by the presence of type 1, P, FIC and S pili. Other than pili, hemolysin, flagella, biofilm formation and iron acquisition/transport system play a crucial role in the colonization\(^3\)-\(^5\).  

Once the uropathogen adhere to the mucosal surface, a series of host defense pathways are activated. Within hours of initial adherence, uroepithelial cells exfoliate and infected cells are shed\(^6\)-\(^7\). An *in-vivo* study on mice has shown that exfoliation of uroepithelial cells prevents UPEC forming clusters and chances of biofilm formation by UPEC become high due to mild exfoliation process in the bladder\(^8\). Experimental systems have also found that UPECs have an invasive ability, following which they form intracellular
communities helping them to escape from both host innate and adaptive response and facilitate further dissemination9.

The diabetics are more prone to infection and these infections are more severe than in non-diabetics. In patients with poor glycemic control, cystitis, ascending infections leading to pyelonephritis, emphysematous complications and renal and perinephric abscesses are well recognized10. Many hypotheses have attributed to the increased UTI in patients with diabetes mellitus, such as glycosuria, impaired function of neutrophils, functional abnormalities of the urinary tract; however, these theories have inadequately understood11, 12.

In the present in-vivo mouse cystitis model study, we sought to determine the effect of virulent UPEC strain having hly, papC, and cnf-1 genes on the bladder uroepithelium of diabetic mouse infected transurethrally.

**MATERIALS AND METHODS**

**Ethical clearance**

The present study was performed in accordance with the guidelines of National Institutes of Health for the care and use of experimental animals in the research, and the protocol of animal experiment has been approved by the Institutional Animal Ethical Committee, Navodaya Medical College, Raichur, Karnataka.

**Maintenance of Mice**

The female BALB/c mice aged between 6-8 weeks were housed five in each cage, fed with pellets and tap water. They were housed under controlled environment with temperature of 23°C (± 2°C), humidity 50% (± 5%) and 10-12 hours of light and dark cycles. For anatomical reasons, only female mice were used.

**Induction of diabetes mellitus in mice**

The diabetes was induced by subcutaneous injection of Alloxan hydrate (LOBA CHEMIE PVT LTD, Mumbai) in mice, 80mg / kg body weight, after 12 hrs of fasting. The fasting blood sugar level was evaluated by Glucometer (SD Fine chemicals) after 3 days of the induction. Following the initial injection of alloxan, if the mice blood glucose level falls below 300 mg/dl, a second dose of alloxan was given to maintain blood sugar level above 300 mg/dl during the study period13.

**Selection of strains**

The molecular characterization of UPEC strains were done by PCR amplification using the primers previously described by Johnson and Stell16.

Two UPEC strains selected for the study were from symptomatic UTI patients, and strains having following characteristics were selected for the study and inoculated in DM and non-DM mouse model transurethrally:

1. **Uropathogenic E. coli with hly, cnf-1 and papC genes**, and belonging to (extraintestinal) phylogenetic group B2 (Virulent strain).
2. **Uropathogenic E. coli without hly, cnf-1 and papC genes**, and belonging to (intestinal) phylogenetic group A (Hypovirulent strain).

**Inoculum Preparation:**

These Bacteria were inoculated from nutrient agar deeps in to BHI broth and incubated at 37°C.

Fresh bacterial cultures were pelleted by centrifugation (2000 x g for 20 minutes), and resuspending in PBS and diluted appropriately to yield 50 µl inocula of 1-2 x 10⁷ Colony Forming Units (CFU)/ml.

**Mouse model of UTI**

The preparation of urethral catheter and inoculation procedures to induce UTI in mice model was followed as per Johnson DE et al protocol17. Briefly, the mouse urinary bladder was voided by gentle massage over bladder (lower abdomen) before infection. A drop of urine was collected directly at urethral orifice with a calibrated loop, and by spreading on the surface of Mac Conkey agar plate sterility was tested. The mice urine showing bacterial count >10⁷ per ml were excluded from the study.

The animals were anesthetized by injecting 0.05 ml of Ketamine intraperitoneally (50mg/ml). The 50 µl of inoculum prepared was instilled in to urinary tract through a soft polyethylene catheter (Outer diameter 0.61mm) adapted to a 0.4 x 20 mm needle on a tuberculin syringe. After injection, the catheter was immediately withdrawn, and no further manipulation was performed17. Control animals were injected with 50 µl of sterile PBS transurethrally.

Out of 26 female mice selected for the study, a group of 13 were injected with alloxan
hydrate to induce diabetes (DM) and the other group of 13 were used as non-diabetic (non-DM) controls. The mice were tested for the presence of bacteriuria by collecting urine directly on calibrated loop by gentle abdominal massage, and the mice showing bacterial count d” 10^2 UFU/ml were included in the study.

**Detection of adhesion and Invasion of UPEC to uroepithelium**

Once the animals are sacrificed, the bladder was taken out aseptically. One half of the bladder was homogenized, and bacterial cultures were performed. The other half of the bladder is used to register bacterial adhesion by histopathology and scanning electron microscopy.

**Histopathology**

A bit of bladder tissue was placed in 10% formalin for 24 hrs. The resulting formalin-fixed tissue was embedded in paraffin and cut in to 3 µm thick sections. The slides were stained with haematoxylin and eosin stain. The histopathology slides were graded by a qualified pathologist in a blinded manner. For each slide, histopathological parameters recorded were – interstitial edema, hemorrhage, leucocyte infiltration, and uroepithelial cell damage. A semi quantitative severity scale (0-4 of each parameter) was used for the evaluation. For each of the four categories assessed, average score of less than one was considered mild effect, score of 2-3 as moderate effect and greater than 3 as severe effect of the strain on the histopathological feature.

**Bacterial Culture**

The viable count was performed by serial dilutions of homogenized bladder in PBS and plated on Mac Conkey’s agar plates. The number of bacteria was expressed as the number of CFU per gram tissue.

**Tissue fixation for scanning electron microscopy (SEM)**

The bladder tissue in its intact form was fixed overnight at 4°C in 0.1M cacodylate buffer (pH 7.2) containing 0.5% gluteraldehyde. The next day, these fixed tissues, were washed three times with cacodylate buffer and transported to Indian Institute of Chemical Technology (IICT), Hyderabad for SEM.

**RESULTS**

From each group of 13 mice, six were inoculated with virulent UPEC strains having hly, pap C and cnf-1 gene, another six with hypovirulent UPEC strains devoid of hly, papC and cnf-1 gene and one with sterile PBS transurethrally in to the bladder. Each set of four mice in combination of DM+ virulent strain, DM+ hypovirulent strain, non-DM + virulent strain and non-DM+ hypovirulent strain were sacrificed at 4 hrs, 24 hrs and 48hrs of post infection. Before sacrificing mice, urine was collected directly on to the loop and cultured for estimation of bacterial count.

**Histological score of mice bladder at different time interval**

To measure the in-vivo effect of UPEC strains on mouse bladder epithelial cells in different host conditions histological scoring was done (Table -1).

**Virulent UPEC strain**: Moderate leucocyte infiltration (Grade 1.5) and sub mucous edema (Grade 2.0) of mouse bladder was seen DM

<table>
<thead>
<tr>
<th>Combination of E. coli strain &amp; mice DM status</th>
<th>Adherence 4h</th>
<th>Adherence 24h</th>
<th>Adherence 48h</th>
<th>Histological Score Edema 4h</th>
<th>Histological Score Edema 24h</th>
<th>Histological Score Edema 48h</th>
<th>Histological Score Hemorrhage 4h</th>
<th>Histological Score Hemorrhage 24h</th>
<th>Histological Score Hemorrhage 48h</th>
<th>Histological Score Leucocyte infiltration 4h</th>
<th>Histological Score Leucocyte infiltration 24h</th>
<th>Histological Score Leucocyte infiltration 48h</th>
<th>Histological Score Epi. Damage 4h</th>
<th>Histological Score Epi. Damage 24h</th>
<th>Histological Score Epi. Damage 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM + Virulent strain</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>0.5</td>
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<td>0.5</td>
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<tr>
<td>DM + hypovirulent strain</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>1</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>non DM + Virulent strain</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
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<td>0.5</td>
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<tr>
<td>non DM + hypovirulent strain</td>
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</tbody>
</table>

Average score of < 1: mild histological effect
Average score of 1-3: moderate histological effect
Average score of >3: severe histological effect
mice after 24 hrs of challenge. In non-DM mice leucocyte infiltration and edema was shown a similar pattern with comparatively low histological grade. Hemorrhagic response (grade 1) was seen after 48 hrs of challenge in both DM and non-DM mice. Enhanced bacterial adherence (grade 1.5-2.0) and extensive epithelial cell damage was noticed in DM mice, after 48 hrs of post infection (Figure-1).

**Hypovirulent UPEC strain:** Mild degree (Grade 0.5-1.0) of leucocytic infiltration and edema was seen after 24 hrs of post infection in DM mice and after 48 hrs of post infection in non-DM mice. No hemorrhage was noticed in both the study groups. Minimum amount of bacterial adherence to uroepithelial cells at 24 hrs and sparse epithelial cell damage at 48 hrs of post infection was noticed in DM mice. Neither bacterial adherence to epithelial cells nor epithelial damage was recorded at any time of post infection in non-DM mice.

**Bacterial count from DM mouse bladder and urine**

**Virulent UPEC strain:** Decreased colony count of $10^5$ CFU/gm compared to initial bacterial inoculation of $10^7$ CFU/gm was observed at 4 hrs of post infection and was gradually increased up to $10^{12}$ CFU after 48 hrs of post infection. UPEC colony count showed an increasing trend in the bladder culture compared to the urine culture.

**Hypovirulent UPEC strain:** The Colony count from urine was dropped from $10^4$ to $10^3$ CFU/gm after 48 hrs. However, the colony count from bladder was remained at $10^6$CFU/gm at 4 hrs as well as at 48 hrs. of post infection. Similar to virulent strain, hypovirulent A strain has also showed higher colony count of UPEC in the bladder compared to that in the urine (Graph -1).

**Bacterial count from non-DM mouse bladder and urine**

**Virulent UPEC strain:** Colony count from bladder was increased gradually from $10^6$ to $10^8$
CFU/gm at 48 hrs of post infection. However, colony count from urine dropped from $10^6$ to $10^5$ CFU/ml.

**Hypovirulent UPEC strain:** Colony count from bladder dropped down from $10^6$ to $10^5$ CFU/gm at 24 hrs. and maintained the same after 48 hrs of post infection. Bacterial count from urine gradually decreased from $10^5$ CFU/ml at 4 hrs to $10^6$ CFU/ml at 48 hrs of post infection (Graph - 2).

**Scanning electron microscopic study of bacterial attachment**

**Non-DM mouse bladder challenged with the virulent strain**

SEM of the bladder mucosa showed bacterial adherence after 4 hrs and 24 hrs of challenge. Moderate amount of discrete bacterial population was recorded after 48 hrs post infection (Fig. 2).

**DM mouse bladder challenged with the virulent strain**

SEM of the bladder mucosa showed bacterial adherence in single and clusters after 4 hrs, Patchy epithelial damage and large population of bacteria adhering all over the surface after 24 hrs, and epithelial damage with heavy population of bacteria in large clusters was seen after 48 hrs of post infection. Many bacteria appeared in elongated filamentous form. These filamentous bacteria were occasionally seen looping within and between adjacent superficial cells (Fig. 3).

**DISCUSSION**

In the present study, mouse model was chosen, as the other experimental animals like rabbits, rats, and guinea pigs do not parallel urinary tract of humans in receptors for attaching bacteria. Also, it has been shown that the mouse diabetic UTI model is consistent with the features of diabetic human’s urinary tract with infection, and can be an important tool for understanding the UPEC pathogenicity in such subjects.

In the study, diabetic mouse cystitis model showed enhanced inflammatory reaction, bacterial adhesion and destruction of uroepithelium compared to non-diabetic mouse cystitis model. Bacterial adherence and cell destruction was more pronounced by virulent UPEC strain ($hly^+$, $papC^+$ and $cnf1^+$ gene) in DM mice. The hypovirulent strain showed no appreciable inflammatory reaction and adherence after 48 hrs of post infection in non-DM mice.

The increased leucocyte infiltration and submucous edema of mouse bladder tissue was recorded with virulent strain compared to hypovirulent strain in both DM and non-DM mice at 24 hrs of post infection. Further, the virulent strain showed enhanced bacterial adherence and epithelial damage in DM mice. Hagberg et al selected bacterial growth after 24 h and found it as better measure of the early bacterial establishment in the urinary tract. The activation of host defense mechanism in the tissue was indicated by the accumulation of inflammatory cells in the bladder tissue.

Previously, Smith and co-workers evaluated the relative impact of expression of CNF1 and Hly on bacterial colonization and
histopathology in female mice, and reported that
the hemolysin of UPEC evokes widespread flaking
of the uroepithelium from the bladder and
hemorrhage in bladder tissue within the first 24 hrs
after intraurethral inoculation of mice. They have
also observed that the combination of CNF1 and
Hly contribute to leucocyte infiltration into the
bladder at day 1 after infection.

Mo et al hypothesized that the (Tamm-
Horsefall protein) THP abnormalities have been
associated with diabetes, and such subjects had
shown profound reduction in urinary THP. Though
granulocyte dysfunction in diabetic subjects could
also render more susceptibility to UTI, THP
dysfunction can also contribute significantly to
the propensity to develop UTI in diabetics
independent of the neutrophil status.

When DM mice bladder model was
challenged with virulent UPEC strain, decreased
colony count of \( 10^5 \) CFU/gm compared to initial
bacterial inoculation of \( 10^7 \) CFU/gm was observed
in the early stage of infection and was gradually
increased up to \( 10^{12} \) CFU/gm at 48 hrs of post
infection. The kinetics of urinary tract colonization
by \( E. coli \) was compared by Rosen et al between
diabetic and healthy control mice. They observed
that \( E. coli \) efficiently colonized mouse bladder as
early as 6h post-infection in both healthy and
diabetic mice. The bacterial count in infected
bladders of healthy mice decreased to a geometric
mean of less than \( 10^3 \) CFU per bladder by 72 hrs
post infection. Diabetic mouse bladder, on the other
hand, retained a high level of bacterial colonization
at 72 h post infection.

Our study was in agreement with the
report of Mulvey et al that the number of bacteria
within the bladder decreased substantially (an
average of 3 log units) during the first 12 hrs of
inoculation via transurethral catheterization in to
female mice. The reduction of bacteria correlates
with considerable exfoliation of the superficial
epithelial cells in mice bladder, and the incursion
of neutrophils into the bladder tissue in response
to infection.

The scanning electron microscopy of
diabetic mouse bladder showed bacterial
adherence in single and in clusters at 4 hrs, patchy
epithelial damage with a large population of bacteria
adhering to all over the surface at 24 hrs of post
infection and extensive epithelial damage and
heavy population of bacteria in large clusters was
observed at 48 hrs of challenge. Many of the
bacteria were in the filamentous form after 48 hrs
of challenge. Mulvey et al have also observed that
the bacteria associated with the dying superficial
cells at 6 hrs after inoculation were frequently
elongated, some times reaching the length of above
50 µm. These filamentous bacteria were
occasionally seen looping within and between
adjacent superficial cells. These observations
suggest that UPEC has the capacity to multiply
within the superficial bladder cells. Consequently,
the UPEC escape from micturation before the host
cell absolutely gets exfoliated.

To conclude, our findings suggest that
the combined effect of virulent UPEC strain and
diabetic status of mouse showed enhanced
adhesion and extensive damage of the
uroepithelium in the bladder. It was evident from
the study that the majority of bacteria persisted
within the mouse bladder at 48 hrs of post infection
was protected from the host immunity. Based on
these observations, it is possible that many
recurrent UTIs may also occur due to revival of
UPEC from dormant reservoirs status established
within the bladder mucosa following an initial acute
infection. Other pathogenic bacteria that are
traditionally considered non-invasive may acquire
similar strategies to that of UPEC to establish long-
term bacterial reservoirs in other tissues, and this
in-vivo experiment may help us to explain the
recurrent nature of many infectious diseases.

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