Chapter 2

Standardization of mouse model for pyelonephritis

2.1 INTRODUCTION

Urinary tract infections by UPEC are a significant urologic condition even in women without underlying anatomic or functional abnormalities, where the bacteria ascend from the infected bladder into the kidneys to cause infection and scarring. A number of animal models have been developed until date to accurately study the host and bacterial factors that influence the pathogenesis of infection in the bladder and kidney (Chen et al., 2009). Johnson et al. (2003) proposed several models of infections generated in animals. He outlined that the essential VF’s for a strain to cause infection are the P fimbria, the S and F1C fimbria, the adhesions, the lipopolysaccharide and oligosaccharide capsule and the aerobactins and siderophores (Picard et al. 1999). The intra – vesicle inoculation of mice with uropathogenic bacteria was the widely utilized model to study unobstructed, ascending UTI (Hung et al., 2009). The major drawbacks of this method were the infection occurred due to vesicoureteral reflux after inoculation and the large inoculation volume, lead to the introduction of bacteria directly into the bladder, rather than creating an ascending mode of infection from the infected bladder. Therefore, the trans-urethral catheterization of mice as demonstrated by Hung et al. (2009), with the optimum volume of UPEC inoculum necessary to develop infection with the pathological changes similar to human infection was adapted where the inoculum was delivered in to the bladder to generate an animal model of UTI.

The widely studied strains of UPEC are E.coli UMN026, E.coli CFT073, E.coli 536 and E.coli UTI 89 were isolated in the years 1922 (University of Michigan), 1999 at the university of Maryland, 2006 (Universitat Wuzzberg, Germany) and 2009 at the University of Washington. These strains fail to depict the current scenario of antimicrobial resistance in India, where the isolated urinary pathogens are currently resistant to most of the prescribed drugs and 3rd generation cephalosporins (Ganguly et al., 2011). This issue made us to frame the objective of
this study to isolate and characterize an UPEC strain and establish a mouse model using the same to understand its virulence potential and to aid in the further studies.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and reagents

All analytical grade and extrapure chemicals, solvents and reagents used in this study were purchased from Sisco Research Laboratories (Mumbai, India) and the microbiological media from Himedia Pvt. Ltd (Mumbai, India).

2.2.2 Strains used in the study

The following strains in addition to the clinical strains under investigation were utilized as reference strains in the study: Non – pathogenic Escherichia coli strain (ATCC: 25922 / MTCC: 443) and Brevibacillus agri (MTCC: 8169) served as the standard strain for confirming its phenotypic characteristics. Escherichia coli (CFT073; MTCC: 4296) was used as the reference strain to analyze the various properties related to uropathoenic strains which helped to identify the possibility on how the strains could have undergone modification to infect the urinary bladder and kidneys.

2.2.3. Urine collection and isolation of micro-organism

A clean catch mid stream urine sample was collected in a sterile sample container from the subjects and transported in cold to the laboratory where it was processed immediately to prevent the loss of viable micro-organisms as per standard procedures for routine urine microbiological and biochemical analysis (CLSI, 2013). A 100-fold diluted urine sample was seeded on chromogenic CLED (Cysteine Lactose Electrolyte Deficient) agar medium to check for the presence of aerobic and anaerobic bacilli. The study was conducted after due approval to collect urine samples from the VIT Human Ethical Committee and the concerned hospital Institutional Review Board (SNHRC/IEC/IRB #: 11-6-8-10).

2.2.4. Phenotypic characterization

The isolated pure cultures were sub-cultured on Luria Bertani (LB) agar medium. Biochemical characterization (Logan and DeVos, 2009) and the antibiotic...
susceptibility profiling of the isolates towards Amoxicillin-Clavunualate (30 μg), Cefpodoxime (30 μg), Co-trimoxazole (25 μg), Gentamicin (10 μg), Nitrofurantoin (300 μg), Nalidixic acid (30 μg), and Tetracycline (30 μg) were carried out as per standard procedure (Lalitha, 2010). Further the structural characterization of the organism was carried out by the use of scanning electron microscopy (FEI Quanta 200F, Netherland) and transmission electron microscopy (Philips, EM201C, Netherlands). The isolates were sub-cultured on nutrient broth – 80% glycerol, and maintained as stocks at -20°C for further characterization.

2.2.5. Isolation of extra-cellular Lipo - oligosaccharide

Lipo – oligosaccharide (LOS) isolation was carried out based on the protocol by Inzana and Pichichero (1984). The bacterial suspension in LB broth was briefly centrifuged at 10,000 g for 5 min and washed with PBS (pH 7.2) containing 0.15 mM CaCl$_2$ and 0.5 mM MgCl$_2$. The cells were re-suspended in 300 μL of water and transferred to a microfuge vial containing a stir bar. Equal volume of hot (65 - 70 °C) 90% phenol was added and the mixture was stirred vigorously at 65 - 70 °C for 15 min. The suspension was cooled on ice and centrifuged at 8500 g for 15 min. The supernatant (i.e. phenol phase) was re-extracted with water and adjusted to 0.5 M with Sodium acetate. The LOS was precipitated by adding 10 volumes of 95% ethanol; the sample was stored at -20 °C overnight. The sample was centrifuged at 2000 g at 4 °C for 10 min and the obtained pellet was washed using ethanol and re-suspended in 50 μl of distilled water. The molecular weight of the isolated LOS was confirmed by silver staining a 14% Acrylamide gel (Inzana & Pichichero, 1984).

2.2.6. Hemagglutination assay

Bacteria grown on LB agar plates were inoculated into 5 ml of phosphate buffered saline (PBS) (pH 7.4) and incubated for five days at 37 °C at static conditions, to obtain fimbriation. To 0.5 ml of fresh group B positive blood, an equal volume of Alsever’s solution was added. The washes were replicated thrice and 3% human erythrocyte suspension was prepared with PBS. The colonies were emulsified on a slide containing PBS, to form a milky white suspension. To this, an equal volume of 3% suspension of erythrocytes was added and mixed. The slide was gently rocked manually for 3-5 min, after which the presence of hemagglutination was observed. To
ascertain the mannose-resistant hemagglutination, to the emulsified bacterial suspension, a drop of 2.5% D-mannose solution was added followed by the addition of 3% erythrocyte suspension. The slides were rotated carefully and observed for hemagglutination. If agglutination of the erythrocytes was observed, it was designated as mannose-resistant hemagglutination else as sensitive hemagglutination (Forbes et al. 2002).

2.2.7. Cell surface hydrophobicity

This was determined using the ammonium sulphate by the aggregation method. A loop of bacterial suspension in phosphate buffer was mixed with an equal volume of ammonium sulphate solution in different molarity i.e. 0.3 M to 5 M, on a glass slide and observed for visible clumping of bacteria. The highest dilution of ammonium sulphate solution, giving a visible clumping of bacteria, was scored as the salt aggregation test (SAT) value as mentioned by Siegfried et al. (1994).

2.2.8. Serum resistance

The overnight culture of the bacteria was harvested and the cells were suspended in PBS (pH 7.4). The bacteria (0.05 ml) was incubated with an equal volume of serum at 37 °C for 180 min. Ten μl of the sample was withdrawn and spread on blood agar plates, which were incubated at 37 °C to determine the viable count (Raksha et al. 2003).

2.2.9. 16S rRNA gene sequencing and Phylogenetic tree construction

The genomic DNA was isolated by the method of Esteban et al. (1993). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was carried out using the primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGGTACGACTT-3’) (Lane, 1991). The amplicons were purified and sequenced using the ABI Prism big dye terminator cycle sequencing ready reaction kit v.3.1. on a ABI13730XL capillary DNA sequencer (ABI Prism 310 Genetic Analyzer, South Korea). The 16S rRNA gene sequence was aligned using ClustalW and computed using Molecular Evolutionary Genetic Analysis (MEGA) version 5 software to construct the Phylogenetic tree and evolutionary distance matrix by the neighbour joining method. The GC content of the amplified 16S rRNA gene was
determined by the GC Profile software (http://www.tubic.tju.edu.cn) (Gao & Zhang 2006).

2.2.10. Initiation of infection in mice

Female Swiss Albino mice (8 – 10 weeks) weighing 25 ± 2 g were housed in polypropylene cages (6 animals per cage) lined with paddy-husk bedding. An ambient temperature of 24 ± 1 °C, with 12 h light/dark light cycle and 65 ± 2% relative humidity was maintained. The animals were fed with food and water ad libitum. The Institutional Animal Ethical Committee (IAEC), VIT University approved the study design and protocols (Approval number: VIT/ SBST/ IAEC/III/2011/19).

The mouse were infected either with 2.5x10^8 cfu of Brevibacillus RRL - 01 or Escherichia coli RRL – 36 in 30 μl of sterile PBS under anaesthetized conditions by trans-urethral inoculation using lubricated catheters delivering at 10μl^1 of the inoculum into the bladder. After inoculation, the catheter was removed and the animal was monitored regularly to check for any discomfort, injury or inflammation due to the procedure (Hung et al., 2009). Kidneys were harvested from the control animals prior to the infection and the infected animals post inoculation. Tissues were stored in 10% neutral buffered formalin (NBF) for Haematoxylin & Eosin (H&E) staining.

2.2.11. Histopathology

The 10% NBF fixed tissues were processed for paraffin embedment and 4 μm tissues sections were stained with haematoxylin and eosin. The histological structures of the kidney and bladder were studied for evidence of UTI such as inflammation, inflammatory cell infiltrates, destruction of renal epithelium and urothelium. In addition, changes in the architecture of the glomeruli, tubules, interstitium and vessels, resolved and persistent bacteriuria and the degree of nephritis were examined using a light microscope at 400X magnification.

2.2.12. Media and culture conditions for biofilm formation

The three above mentioned E.coli strains (refer 2.2.2) were subcultured in sterile LB broth and minimal M63 medium (Schembri et al. 2003, Zhang et al. 2007)
to ascertain the medium suitable for maximal biofilm formation in a batch culture mode for a period of 5 days. The periodical changing of the media was carried out every 24 h. The minimal M63 medium was supplemented with varying concentrations of D-Mannose (w/v) as the carbon source, to ascertain the optimum concentration for biofilm formation (Lindberg et al., 2001, Martinez & Casadevall, 2007). Cells were grown at 37 °C for 24 h (stationary phase) in all experiments.

2.2.13. Cell adhesion assay

Cells grown at stationary phase were centrifuged at 4000 g for 15 min. The medium was decanted and the pellet was resuspended in LB medium. This procedure was repeated twice after which the cells were resuspended to a final concentration of $10^8$ cells ml$^{-1}$. Ten µl of the suspension was added to 290 ml of M63 medium containing 0.5% D–Mannose. The 0.5% of D-Mannose was identified to be optimum for biofilm formation in M63 medium. The suspension was mixed well and 100 µl of the suspension was transferred to a glass slide placed in a sterile petridish and viewed under an inverted microscope operated in phase contrast mode (Olympus, Japan). The suspension was viewed under 40X objective and the adhesion rate was calculated by counting the number of cells attached to the surface every 60 sec for a period of 5 min. The bacterial adhesion rate coefficient $k$, was quantified as follows: $k = \frac{J}{C_0}$, where $C_0$ is the bacterial bulk concentration and $J$ is the number of bacteria attached onto the slide surface. The experiment was carried out in triplicate, where in each run a fresh cell suspension was utilized (Rodrigues & Elimelech, 2009).

2.2.14. Microbial – adhesion-to-hydrocarbons test

The relative hydrophilicity (i.e. the fraction of cells that partition into the aqueous phase) of the *E.coli* strains was measured by the MATH test as outlined by Walker et al., 2005. The cells were suspended in M63 medium or M63 medium supplemented with 0.5% of D-Mannose, to an optical density (OD) of 0.5 at 600 nm. The suspensions were divided into 4 ml aliquots and 1 ml of glycerol was added to each. The OD$_{600}$ nm of the aqueous phase was measured after vigourous vortexing for 30 sec. The suspensions were allowed for phase seperation for a period of 30 min, after which the OD$_{600}$ of the aqueous phase was measured. The MATH value of the
strain was calculated from the change in the optical density as follows MATH(%) = ((OD\textsubscript{600 after treatment})/ (OD\textsubscript{600 before treatment}))* 100.

2.2.15. Biofilm growth kinetics

Biofilm formation by the strains was monitored using sterile, non-treated, multi-well plates (Tarsons, India) as described by Li et al. (2001). The assay was carried out in triplicates for each of the strains. The LB broth and the M63 minimal media were supplemented with varying concentrations of D – Mannose (0% to 5%). After inoculation the plates were incubated at 37 °C for a period of 24 h, 48 h, 72 h, 96 h and 120 h. At the end of the incubation period, the plates were washed with PBS (pH 7.4) to remove the unbound cells. The cells attached to the wall of each well were stained with 300 µl of 0.1% (w/v) of crystal violet prepared in water for a period of 20 min. After the incubation period, the plate was rinsed thrice with PBS, with a time interval of 5 min between each wash, to remove the unbound crystal violet solution. The bound cells were quantified by adding 300 µl of acetone:ethanol mixture (20:80 v/v) and measuring the absorbance at 540 nm in a microplate reader (ELX800, Biotek instruments). To compare the biofilm formation among the strains in each medium, the total growth was monitored at OD\textsubscript{600}. This value was used to normalize the biofilm growth among the various strains used in the study with their total cell growth rates.

2.2.16. Exopolysaccharide quantification

The exopolysaccharides quantification in the formed biofilm was carried out by the phenol sulphuric method as outlined by Masuko et al. (2005). Briefly, the formed biofilm at the end of the incubation period was washed thrice with PBS (pH 7.4). To the washed biofilm reconstituted in 1 part of sterile water, three parts of ice-cold concentrated sulphuric acid were added. To the mixture 1 part of 5% phenol in water (w/v) was added and the contents were incubated in a static water bath maintained at 90 °C for 5 min. The plate was cooled and the absorbance of the contents were measured at 490 nm, and the concentration of the total sugars was calculated based on the D - Mannose standard curves. The protein content of the exopolysaccharide was quantified using the Bradford’s method (Bradford, 1976).
2.2.17. Structural characterization of the biofilm

In order to assess the adherence pattern of the organism to the inert surface, that facilitates the formation of micro-colonies that mature into a biofilm that are embedded along with the exopolysaccharide, the preliminary structural characterization of the formed biofilm was carried out using AFM (Nanosurf easyscan2, Switzerland) and followed by further confirmation using SEM (FEI Quanta 200F, Netherlands).

2.2.18. Statistical method

Data were represented as mean ± standard deviation of mean (SD). Mean difference between the groups were analyzed by one-way ANOVA and p value ≤ 0.05 was considered significant. Statistical analysis was performed using SPSS v16.

2.3. RESULTS

The standard UPEC strains used in the generation of mouse models of UTI were generated by genetically modifying them based on the need of the study. These strains were isolated more than a decade ago and fail to depict the current scenario of antibiotic resistance in India. In order to meet this requirement, our study focused on the isolation of pathogens from the urine of the UTI subjects. The samples under cold, sterile conditions were transported to our laboratory and processed; the isolated pathogens were characterized up to the genus level by 16S rRNA sequencing of the strains.

The gram staining of the urine deposit from subject 1 tested negative for the bacteriuria, pus cells, hematuria and nitrite. From the 1000 - fold diluted urine sample, that was seeded on CLED agar. After incubation for 24 h at 37 °C, 1.5x10⁴ cfu/ml of urine of the strain 1 was isolated. The urine deposit from subject 2 tested positive for significant bacteriuria and pus cells in the routine urine biochemical analysis and TNC colonies of the strain 2 were isolated after incubation. The two strains had similar characteristics i.e. pale yellowish, smooth, moist, slightly elevated round colonies of 2 mm diameter, when visualized after incubation at 37 °C on the CLED agar. Based on their morphological appearance the colonies were speculated to be *Escherichia coli*. 

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The gram staining of the pure isolates revealed the presence of single gram-positive rods (strain 1) and gram-negative rods in the case of strain 2. Phenotypic characterization revealed that the strain 1 was catalase positive, but negative for oxidase, nitrate, and MR-VP tests. Strain 1 also tested positive for Urease, Indole, and H₂S production and was capable of hydrolyzing casein and esculin. The growth of the microbe was enhanced in the presence of 1% D-Glucose solution (Table 2.1). The phenotypic characterization identified the strain 2 to be a gram-negative rod that was catalase positive, oxidase, nitrate and MR-VP negative. Other analyses revealed that the strain tested positive for the utilization of urease, production of indole, H₂S, hydrolysis of Caesin and Esclulin (Table 2.1). As shown in the Table 2.1, it is evident that the strain did not require the presence of glucose supplementation in the media for its growth.

For an invading bacterial species to interfere and gain foothold in a commensal species niche before infecting the host, the pathogen has to adhere to the biological surface (Reid 2001). Therefore, the strains were analyzed for the various virulence factors that were expressed on their surface. Both the strains were positive for mannose sensitive hemagglutination which was confirmed by the their ability to agglutinate erythrocytes in a similar fashion as that of *E.coli* CFT073. It was seen that the non-pathogenic strain *E.coli* ATCC 25922 and the *B. agri* were not capable of haemagglutination. They also had the ability to inhibit serum resistance by having the ability to opsonise the cytokines present in the serum and capable of evading the host immune response and the strain 2 tested positive for cell surface hydrophobicity assay using ammonium sulphate (Table 2.1). The strain was capable of producing lipopolysaccharide, another essential factor, which forms the hallmark of an uropathogen, which binds to the receptors on the cell surface and plays an important role in the alteration of the host immune system.

The strains were susceptible to Amoxicillin- clavunualate (30 μg) and tetracycline (30 μg). Strain 1 was also sensitive to Co-trimoxazole (25 μg) while strain 2 was susceptible to Nalidixic acid (30 μg). Both the strains were resistant to Nitrofurantoin (300 μg), Gentamicin (10 μg) and cefpodoxime (30 μg) (Table 2.2).
Table 2.1. Phenotypic characterization results of the strains Brevibacillus spp. RRL – 01 (strain 2) and E.coli RRL – 36 (strain 5)

<table>
<thead>
<tr>
<th>Phenotypic character</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
<th>Strain 4</th>
<th>Strain 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl Red</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Urease utilization</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>H₂S production</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Esculin hydrolysis</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Caesin hydrolysis</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gluc suppl growth</td>
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<td>+</td>
</tr>
<tr>
<td>Haemagglutination</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Man + Haemagglutination</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>Serum resistance</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cell surface hydrophob.</td>
<td>NA</td>
<td>0.5</td>
<td>-</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Production of LPS &amp; LOS</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The phenotypic characteristics of the strains were carried out in comparison with the following reference strains: Brevibacillus agri (MTCC #: *8169) (strain 1), non-pathogenic strain of E. coli (ATCC #: 25922) (strain 2) and E.coli CFT073 (MTCC#: 4296) (strain 3), Brevibacillus spp. RRL – 01 (strain 4) and Escherichia coli RRL - 36 (strain 5). Gluc suppl growth: Glucose supplemented growth; Man + Haemagglutination: Mannose aided haemagglutination; Cell surface hydrophob.: Cell surface hydrophobicity; LOS: Lipo oligosaccharide; LPS: Lipo polysaccharide; +: presence; -: Absence; NA: Not available.
Table 2.2. Antibiotic susceptibility pattern of Brevibacillus spp. RRL – 01 (Strain 1) and E.coli RRL – 36 (Strain 2)

<table>
<thead>
<tr>
<th>Antibiotic (µg)</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain 1</td>
</tr>
<tr>
<td>Amoxicillin- Clavulanate (30)</td>
<td>22</td>
</tr>
<tr>
<td>Cefpodoxime (30)</td>
<td>12</td>
</tr>
<tr>
<td>Co-trimoxazole (25)</td>
<td>18</td>
</tr>
<tr>
<td>Gentamicin (10)</td>
<td>13</td>
</tr>
<tr>
<td>Nitrofurantoin (300)</td>
<td>15</td>
</tr>
<tr>
<td>Nalidixic acid (30)</td>
<td>11</td>
</tr>
<tr>
<td>Tetracycline (30)</td>
<td>20</td>
</tr>
</tbody>
</table>

The TEM confirmed the rod like appearance of the strains (Fig. 2.1A & 2.1B). It was noticed that 20000X magnification, strain 1 existed as long rods (Fig. 2.1C), whereas strain 2 had two forms, a long rod and short blunt slightly rounded rod (Fig. 2.1D), indicating that it had the possibility of altering its structure based on the environment, which has been earlier identified to be a characteristic feature of uropathogens. From the EM analysis it was noticed that strain 1 were enlarged due to the presence of ellipsoidal sub - terminal spores, which gave a spindle like appearance to the rod (Fig. 2.1E) at 30000X magnification. It was interesting to note from the TEM images that the strain 2 secreted a polysaccharide matrix around each rod (Fig. 2.1F), when it encountered an abiotic surface.

The similar profile of the strains with respect to their phenotypic characters, antibiogram profile and EM characterization, instigated us to identify the strains at the genus level through 16s rRNA sequencing to establish their phylogenetic relationship with the other strains. The 16S rRNA gene sequence analysis of strain 1 (GenBank Accession number JQ398844.1) revealed that the isolate Brevibacillus spp. RRL – 01 (BRRL01) had 99% similarity with the type strain Brevibacillus agri AB112716, whereas strain 2 was identified to E.coli RRL – 36 (ECRRL36) (GenBank Accession number JQ398845.1). A phenogram reflecting the relationship among the strains and
Fig. 2.1A & C shows the presence of long rods of ECRRL36 after 48 h of incubation at 37 °C at 4500 X & 20000 X magnification respectively. Fig. 2.1B. shows the presence of polymeric capsule around the rod shaped cells at 4500 X magnification. At 20000 X magnification, the two forms of the pathogen ECRRL36 could be observed, the short blunt rods and the long rods. Fig. 2.1E. shows a sing rod shaped cell of the urinary isolate Brevibacillus RRL – 01, with a sub-terminal swollen ellipsoidal sporangia, while the Fig 2.1F, reveals the presence of dense amorphic cytoplasm at 30000 X magnification in ECRRL 36.
the candidate sequences of the related strains obtained from NCBI database are presented in Fig. 2.2A in the case of BRRL01 and Fig. 2.2B in the case of ECRRRL36.

**Figure 2.2. Phylogenetic relationships of the strains based in 16S rRNA gene sequence and related sequences**

![Phylogenetic Relationships](image)

The tree was constructed using closely related sequences based on Euclidean distance (neighbor joining algorithm with kimura parameter). The numbers of the nodes indicate the bootstrap value which indicates the number of replications per 1000 replica. Sequence accession numbers are indicated after the genus name. A: Phylogenetic relationship of BCRRRL01; B: Phylogenetic relationship of ECRRRL36.

2.3.6. Mouse model of infection

The mouse model of infection was generated by transferring \(2.5 \times 10^8\) cfu (standardized to be the optimum dose) of the respective strains in 30μl of sterile PBS by trans-urethral inoculation using lubricated catheters delivering at 10μl of inoculum into the bladder. When the *Brevibacilli* were injected into the bladder of the anesthetized mouse, it caused a pinkish colouration in the urethral region after an hour of inoculation (visual data based on comparisons drawn with control mice by the researchers). The infected mice started to die after 6 h of inoculation and within a
single day post infection all the 12 animals infected with the bacilli died. The kidneys appeared swollen and discoloured upon dissection.

When the H&E stained sections of the kidney were examined it was found that the kidney had undergone acute tubular necrosis. The entire glomerular morphology had undergone damage with complete destruction of the glomerular architecture in the cortex region of the kidney. Evidence of tubular damage with the inflammation of the tubules was also noticed in the kidney sections obtained from the animals infected with *Brevibacillus* as seen in Fig. 2.3B., when compared to the control (Fig. 2.3A). Also the inoculated pathogen was capable of causing profused hemagglutination in the tubular region (Fig. 2.3C). Brevibacilli were also found as cluster of rods (indicated by the arrows) inside the tubules that had undergone damage, with hemagglutination in the vicinity in Fig.2.3D. with the presence of intra-renal bacteria were found diffused from the renal pelvis to the medullo-cortical area.

When the ECRRRL36 were used as the urinary pathogen to infect the bladder of the mouse, it was noticed that the mice developed a low-grade fever (100 °C) on the third day. Also, it was noticed that there were elevated levels of leucocyte and nitrite in the urine of the infected mice on the 6th and 7th days post - inoculation. The infected mouse kidney sections (H&E) obtained on the 7th day post inoculation of *E.coli* in the bladder revealed that acute pyelonephritis was established in the model with neutrophil infiltration in the ureters and pelvi-ureteric junction with the sparing of the renal parenchyma with the prominent formation of foam cells (Fig. 2.3E). Dense infiltration by the lymphocytes and plasma cells, with disruption of the urothelium were also evident (Fig. 2.3F).

ECRRRL36 was capable of establishing an animal model of UTI with pyelonephritis of the kidney at the end of the 7th day. In order to survive in the host milieu and establish an infection one of the important properties of the uropathogen is to form intracellular bacterial communities within the host tissue. To access this property, the biofilm forming capability of the strain was studied *in vitro*. Identification of the surface for optimum biofilm formation *in vitro* by the culture ECRRRL36 was required. In this study the strains were allowed to form biofilm under static conditions on glass, rubber and polystyrene surfaces for a period of 120 h. The
biofilm growth was monitored in a batch culture mode. The spent medium was aseptically removed and replaced with equal volumes of fresh medium every 24 h.

**Figure 2.3.** Haematoxylin and Eosin stained sections from trans-urethrally infected mice with *Brevibacillus RRL – 01* and *Escherichia coli RRL – 36*

The sections show the presence of acute tubular necrosis in the cortex region of the kidney with the complete destruction of the glomerular architecture in section B when compared to the kidney sections obtained from the control animals (A). In addition, the destruction of tubular structures are noted in B. Figure C shows the haemagglutinating capability of the bacilli in the tubular region. The presence of intra-renal bacteria diffused in the tubular region can also be noticed in D.
At the end of the study (every 24 h) period, the biofilm formed on the surface of the material used was evaluated by the crystal violet staining method. It was noticed that on the rubber catheter, although the medium had viscous, white strand like structures formed by the bacteria at the end of 24 h, there was no adherence on the surface of the catheter, thus making it difficult to carry out further work using this as the surface for adherence. The amount of biofilm formed on the polystyrene and glass surface, were analyzed at the end of every 24 h was noticed to be greater on the glass surface and it was chosen to be the surface for further studies relating to biofilm formation.

The adhesive property of the strain was detected by two methods: the MATH test to assess the hydrophilic nature of the strain and the cell adhesion rate, to measure the number of cells adsorbed onto the surface of the glass slide. From the MATH test, it was observed that the hydrophilic nature of ECRRL36 gradually increased up to 72 h after which it started to decrease and this pattern was similar with the standard uropathogenic strain E.coli CFT073 (Figure 2.4). This high fimbriation pattern of the strain coincided with the with the results of the Mannose-sensitive haemagglutination pattern of this strain carried out by us earlier in this study. In addition, due to the high fimbriation the strain had a hydrophilicity score of 71%, which could have attributed to the high cell adhesion rate of the strain when compared to the non-pathogenic strain. The strain ECRRL36 had a adhesion rate of 360 cells per 5 min to the glass surfaces (Fig. 2.5)

Two commonly used media, LB broth and M63 minimal media were utilized for the study. The first media is utilized routinely for the maintenance of E.coli strains, while the M63 media is one where the media constitutes of ions, while the carbohydrate source is supplemented to identify the amount required by the bacteria under study for its optimal growth. In this study, up to 5% of D- Glucose (w/v) was supplemented to both the media, to aid in the formation of biofilm. The cultures were grown in batch culture mode and the media was replenished every 24 h. The assay was performed in triplicates for each culture. At the end of the incubation period, the supernatant was dispensed. The unbound cells were removed by rinsing with PBS and the biofilm was quantified using the crystal violet staining method.
Figure 2.4. Adherence capability of *Escherichia coli* RRL – 36 to glass

![Adherence capability graph](image)

Figure 2.5. Adhesion of *Escherichia coli* RRL – 36 to hydrocarbon

![Adhesion to hydrocarbon graph](image)
Figure 2.6. Biofilm formation by *Escherichia coli* RRL – 36 at different time intervals

From the assay, it was noted that at the end of 24 h, the biofilm formation in the case of M63 media the non-pathogenic strain without the supplementation of Glucose had less biofilm formation when compared to that of the pathogenic strains, ECRRL36 and *E. coli* CFT073. Of the two pathogenic strains, there was maximal biofilm production by *E. coli* CFT073, when compared to ECRRL36. In the case of the cultures grown in LB broth, there was maximal biofilm formation in the case of the non-pathogenic culture grown in the presence of 1% glucose supplementation. In all the other concentrations in both the media, appreciable growth was noticed (Fig. 2.6A).

At the end of 48 h (Fig. 2.6B), we found substantial biofilm formation by the non-pathogenic strain *E. coli* ATCC 25922 in all the concentrations of M63 media and
maximal growth in the media which lacked glucose supplementation was observed. A similar fashion was noticed in the biofilm formation by the strains ECRRRL36 and E.coli CFT073. However, the overall biofilm was considerably less in ECRRRL36 when compared to that of E.coli ATCC 25922 and E.coli CFT073. When the biofilm formation in the different strains grown in LB media supplemented with glucose concentration was assessed, it was noticed that although there was substantial growth in all the concentrations, this growth was less when compared to that in M63 media. In the LB media, the pathogenic strains expressed a marginally higher growth when compared to the non-pathogenic strain (Fig. 2. 6B).

However, a striking change in the biofilm formation of the strains was noticed when the growth pattern was analyzed at the end of 72 h. The strains had a reduced biofilm formation pattern in the M63 media except at 0.5% of glucose. In the LB media, the pathogenic strains also exhibited a reduced biofilm formation in all the concentrations of glucose supplementation. When the growth pattern of ECRRRL36 was noticed in LB media without any supplementation of glucose and in that of M63 media with 0.5% of glucose supplementation, it was interesting to note that they had similar biofilm formation (Fig. 2. 6C). Therefore, we decided to continue to use the routine bacteriological media LB broth for the biofilm formation without the supplementation of glucose for further studies.

At the end of incubation i.e., 24 h, 48 h, 72 h, 96 h and 120 h the supernatant from each tube was discarded and the formed biofilm was harvested. It was rinsed thrice with PBS. The biofilm was scraped from the glass and the exopolysaccharide (Fig. 2.7) and protein content (Fig. 2.8) of the biofilm were analyzed and it was identified to be the maximum at the end of 72 h.

The biofilm formed at the end of 24 h and 72 h by ECRRRL36 were rinsed in PBS and analyzed by AFM. It was noticed that in the deflection scanning forward mode at the end of 24 h, there was adsorption of rod like structures on the surface of the glass, with irregulat deposition of material across the surface of the glass. These rod like structures were also found to be in close association as seen in the Fig. 2.9. It was noticed that the end of 72 h there was the formation of a mature biofilm, in which a number of rod like to circular cells were adhered close to each other. It could also be
Figure 2.7. Exopolysaccharide content in the biofilm formed by *Escherichia coli* RRL – 36

![Exopolysaccharide content graph](image1)

Figure 2.8. Protein content in the biofilms formed by *Escherichia coli* RRL – 36

![Protein content graph](image2)
inferred that in this association there was a close contact with the neighbouring biofilms present through detached cells. Also the covering on the surface of the glassslide had also been increased by many folds.

The SEM of the glass slides obtained at the 72 h revealed the presence of bacteria entrapped in the exopolysaccharide (Fig. 2.10C). From the images it was also evident that the amount of exopolysaccharide formed by CFT073 was considerably higher than the strain under the study. It was noticed that although the amount of exopolysaccharide formed by ECRRRL36 was less in comparison to *E.coli* CFT073, the number of bacteria bound to the matrix was considerably equal. It was also interesting to note, that upon further magnification to 50,000X and 100,000X, a number of crevices were noted on the surface of the exopolysaccharide (Fig. 9F). Most of these crevices were small and they did not contain bacteria. However, it was noticed that when these were large enough, bacteria were entrapped within the matrix (Fig. 2.10G).

### 2.4. DISCUSSION

Infectious diseases rank the highest cause of significant morbidity and mortality in human beings. Although considerable progress in understanding the bacterial pathogenesis mechanisms and the underlying molecular events evoked by the host pathogen interactions is underway, emerging pathogens among humans, animals and plant populations have always been the redefining criteria for the diagnostic and treatment strategies involved in the disease management (Larios et al., 2010). The dynamics of this biological invasion by the pathogen is a field that remains poorly understood. Pathogenesis of a new species can be attributed to any of the following reasons: changes in the host environment, or host behaviour and movements, or host phenotype due to a recent illness, or host genetics, or pathogen genetics (Woolhouse et al., 2005). The changes occurring in the pathogen genetics seem to rather play a greater role, when compared to the other changes mentioned, as these determine its ability to infect a compatible host (Woolhouse et al., 2001) by breaking free the ‘species barrier’ (Blancou and Aubert, 1997).
Figure 2.9. Morphological analysis of biofilm formed by *Escherichia coli* RRL – 36 using AFM

Figure 2.10. Morphological analysis of biofilm formed by *Escherichia coli* RRL – 36 using SEM

A, B, C were taken at 20000X magnification at 20kV. A: the biofilm formed on the glass surface by the non-pathogenic strain *E.coli* ATCC 25922. B: biofilm formed on the glass surface by the pathogenic strain *E.coli* CFT073. C: the biofilm formed on the glass surface by the clinical isolate *E.coli* RRL - 36. D: the isolate ECRRL36 bound to the exopolysaccharides matrix of a mature biofilm at 72 h and was captured at 30000X magnification at 20kV. E: represents the minor crevices (yellow arrow) and major crevices observed in the biofilm formed by *E.coli* RRL - 36. F: bacteria entrapped in the major crevices of the biofilm formed *E.coli* RRL - 36.
Brevibacillus, a soil aerobic microbe has been reported as a uropathogen through our study. Earlier studies have indicated that this strain could be a possible clinical pathogen, residing in the bronchi-alveolar lavage, and breast abscess (Logan et al., 2002). Members belonging to this genus have been isolated from estuarine sea grass rhizosphere, mushroom compost, public water supply, pharmaceutical fermenter plant and its antibiotic raw product, gelatin processing plant and contaminated foodstuffs such as sterilized milk, spinach and cheese (Singh and Kumar, 1998).

The ability of this microbe to grow on nutrient agar, CLED agar and LB broth reflects that it is a heterotrophic consumer of nutrients and its growth is enhanced in by 1% glucose supplementation. This suggests that the bacteria are capable of utilizing the available glucose concentration in the urine of the person initially to rapidly multiply and form a bacterial community in the urethral region before subsequent colonization of the superficial facet cells of the urinary bladder (Geerlings et al., 1999). This enhanced growth in the presence of glucose coincides with the existing reports that mention glucose is essential for the fimbriation of the uropathogens (Muller et al., 2009).

This enhanced growth of the Brevibacillus in the presence of glucose is supported by the source of isolation, urine, which could have contained glucose, as the patient was a diabetic individual. This bacteria was isolated from the urine of a diabetic subject, where the glucose concentration in the urinary milieu, would have been many fold, when compared to that of normal subjects, thereby justifying the fact that this could have been one of the reasons, why this pathogen have caused UTI in the subject due to rapid multiplication (Fünfstück et al, 2012). The capability of the isolates belonging to this genus to produce extra-cellular lipo-oligosaccharides, suggests that these carbohydrate moieties could also play a possible role, similar to that of lipo-polysaccharides of other species, in the initiation of infection and in triggering the immune response of the host.

Escherichia coli, among the bacterial pathogens, being a facultative, gram-negative rod still holds many facets, commonly referred to as virulence factors (VF’s), which enable it to be an etiologic agent of various intestinal and extra-intestinal infections (Johnson and Russo 2005). Uropathogenic E.coli have been reported to
belong to the phylogroup B2, along with the other *E.coli* responsible for neonatal meningitis, bacteraemia, septicaemia, and avian pathogenic *E.coli* (Johnson & Russo 2002).

The virulence potential of *E.coli* are attributed to the VF’s that are dispersed as pathogenic islands across the bacterial chromosome or plasmids (Ewers et al. 2007). The roles of these VF’s, although mostly putative have been widely accepted to play specific roles during pathogenesis by aiding in mucosal colonization, subverting systemic host defences, acquisition of nutritional requirements, invasion of host tissues and also for the stimulation of inflammatory host response beneficial to the invading pathogen (Johnson and Russo 2005). The expression of these VF’s depends on the immune competence of the host, geographical location of isolation, pathotype and virulence potential of strain causing the infection (Tenaillon et al. 2010).

The bacteria invade and rapidly replicate to form intracellular bacterial communities and filamentous form of the pathogen (Hunstad and Justice 2010). This morphological difference as seen in the pathogen ECRRL36, aids the bacteria in further colonization, where the long filamentous structures are effective in further colonization of the urinary system with special reference to the underlying tissues. The small blunt rods help in the formation of micro-colonies and in the rapid multiplication of the bacteria in the colony and the subsequent biofilm formed by asexual reproduction, which was also visible from the TEM images of the strain.

The changes *in vivo* in the induced animal model using BRR01, such as the ability of the pathogen to destruct the glomerular and tubular architecture, cause hemagglutination in the tubules, to be present within the renal tubules and intra-renally in within the renal lumen and cause anaemia in the clinical subject, suggest that the bacteria could have possesed a varied virulence mechanism with which it was capable of establishing an infection. Also, the possibility of septisemia by the strain BRRL01, was considered. To rule out this possibility, the blood from the infected animals was cultured asectically on the LB agar plates and observed for growth after 24 h incubation at 37 °C. The absence of colonies on the agar plates, indicated that the strain, upon inoclation into the bladder, had ascended up the urinary tract to cause
pyelonephritis of the kidneys. This was also confirmed by the presence of large clusters of the bacillus in intact tubular pockets.

The uropathogen with the help of the adhesions such as the T1F, P fimbriae, S fimbriae and F1C fimbriae bind to the superficial cells of the urothelium lining the urinary system. Initially the fimbriation of the strain would have just been limited to the adhesive pili which were expressed as the strain started to adhere to surface; but after the strain was able to adapt to the environment it would have started to express the genes for the other fimbriae which were essential for other functions such as nutrient scavenging, entry into the host cell etc. The presence of the various renal proteins, commonly referred as antimicrobial proteins (AMP), on the superficial epithelial cells of the urinary system aids in the adhesion of the uropathogen to it. AMP’s are elevated during infection and the removal subsequent to their binding to the pathogen precludes infection. They can also regulate the innate immunity to recognize and opsonise the pathogen before it propagates in the urinary milieu (Zasloff, 2007).

The adhesions at the surface of the invading pathogens bind to the receptors of the extracellular matrix (ECM) and activate a series of complex signal transduction cascades in the host cell, which are both beneficial and inhibitive to the bacterial invasion (Soto & Hultgren, 1999). The integrin receptors at the ECM surface help in the binding of the adhesive pili to the molecules such as fibronectin, collagens, laminin, vitronectin, thrombospondin, elastin, sialoproteins and GAG’s such as heparin, heparan and chondroitin sulphate to the MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules) (Ljungh et al. 1996, McDevitt et al. 1997, O’Connell et al. 1998, Patti et al. 1994).

The possibility that the isolated strain could be an UPEC probed us to analyze the adhesive potential of this strain when exposed to the negatively charged glass surface (Striker et al. 1995). When the media (LB broth or M63 minimal media) and the pathogen are exposed to the abiotic surface, a series of small molecules such as water and salt ions adsorb on the surface to create a thin film (Gristina, 1987; Schneider and Marshall 1994). Subsequently a layer of organic molecules add the thin film to form a conditioning film onto which the first microorganisms, brought to the
surface by the Brownian motion, gravitation, intrinsic motility or by the diffusion of the pathogen to adhere. As this adherence of the microbes is a reversible adsorption and its integrity and strength depends on the structure of the conditioning film (Busscher and van der Mei 1995, Busscher et al. 1992). The secretions of the exopolysaccharide substances by the adsorbed pathogens results in the conversion of the initial reversible to an irreversible adsorption and strengthen its cohesiveness (Dufrene et al. 1996, Neu and Marshall 1990).

The formation of thin film and the conditioning film in the case of this strain ECRRL36 were noticed by the end of 4 h in the AFM images as the orange-white patches on the orange background (represents the surface of the glass slide as seen in Fig. 2.9). When the ECRRL36 started to adhere to the conditioning film from the deflection scan forward mode, the presence of a few bacteria were noticed at the 24 h. It was also visible that this adherence was aided by the presence of a matrix, which formed a coat over the irregular glass surface. By the end of the 72 h it was noticed from the AFM images that the bacteria had started to form a spherical colony, that consisted of numerous bacteria, that were embedded in the exopolymeric matrix secreted by the ECRRL36 as result of adsorption onto the glass surface, was confirmed by SEM (Fig. 2.10).

In the in vivo system, when the pathogen enters the urinary system, the urine in the bladder acts like a conditioning film, onto which the bacteria adsorb and rapidly grow by utilizing the nutrients at their disposal from the urine. Once the pathogen has established a microcolony at the surface, they rapidly evoke the host immune system by the help of the conserved N and C terminal regions of the protein flagellin, the subunit of flagellum (Hayashi et al, 2001). The flagellum not only aids in the motility of the pathogen to the cell surface, but also in the secretion of extracellular non – flagellar virulence associated effector proteins and polypeptides (Duan et al. 2013, Young et al. 1999, Majander et al. 2005). The UPEC exhibit the presence of the FimH adhesions, a 30 kDa protein composed of 300 residues (Hultgren et al, 1996), that bind to the mannosylated integral membrane proteins such as uroplakins, located at the luminal surface of the bladder urothelial cells (Neu 1996, Mulvey et al. 1998). In addition, it has been identified these fimbriae are generally shorter in length (0.12 µm).
in contrast to the non-contacting pili, thereby providing way for the internalization of the invading UPEC.

The previous reports have also pointed out that the initial attached by UPEC to silicon surfaces or patterned surfaces, resulting in the formation of bumps and sub-micrometer crevices, the role of the adhesins was essential. These crevices are generally too narrow for the bacteria to bind to, and therefore after the initial attachment, there was an alteration in the expression pattern of the virulence genes, resulting in over-expression of various flagellar genes, that proved to be essential in the attachment of the pathogen to the patterned surfaces (Giron et al. 2002, Mahajan et al. 2009).

The other aspect of virulence that still remains a gray area in the case of UPEC are the protein and polypeptides that are secreted by the flagellin upon binding to the toll like receptors (TLR). This binding in the case of S. typhi and S. typhimurium, have been indicated to result in the formation of lysophospholipids, which help in the penetration of the host cells, and also aid in the modulation of the host cell immune response (Subramanian and Qadri 2006). Other that TLRs, gangliosides have also been reported to be effective receptors for flagellin (Feldman et al. 1998, McNamara et al. 2001).

Once the bacteria are bound to the surface, they initiate the mechanism of secretion of the exopolysaccharides. This polysaccharide matrix envelopes the bacterial colony and effectively inhibits phagocytosis, makes the biofilm immune to antibiotics and helps in the persistence of infection (Erdem et al. 2007, Eaves-Piles et al. 2008). Once a mature biofilm is formed, the number of microorganisms present in the biofilm is determined by the in situ bacterial growth. Also, the surface of the biofilm is rapidly altered depending on the active compounds secreted by the microorganisms in the biofilm, the nutrients available in the niche, the attraction and adhesion to other microbes present in the environment, thus leading to further colonization.

This biofilm formation of the pathogen could have been the reason for the establishment of infection in the mice model after a period of seven days with all the
pathological conditions similar to humans such as bacteriuria, pyuria, neutrophil infiltration in the kidneys with damage to the glomerular and tubular region and inflammation in the tubular region (Anderson et al., 2003).

2.5. Conclusion

The results from this study shed light on the expanding spectrum of various strains becoming possible pathogens of the urinary tract and also calls for both, the extensive and exhaustive analysis of the genome of the isolated strain, to discriminate its pathogenetic mechanisms, which forms the crux of its virulence factor study, and the need for developing a surveillance database to include the emerging uropathogens for efficient diagnosis. This study provides an overview on how the genotypic changes, occurring in the soil microbe Brevibacillus alter its pathogenicity and make it an uropathogen (reported for the first time), which were confirmed in vitro by the Mannose sensitive heme-agglutination, serum resistance and cell surface hydrophobicity assays due to the horizontal gene transfer mechanisms, through which the genetic material could have been transferred from one microbe to the other in the same niche. Also this study succeeded in establishing and standardising a mouse model of UTI with the uropathogen Escherichia coli RRL – 36 and its virulence factor characterization that make it a representative of the uropathogenic strains isolated from this region of the country. In addition, standardization of the various conditions for the growth of biofilm and characterization of the formed biofilm, provide light on how this pathogen behaves in vivo.