"An Observation of the Exoproducts in Media of Uropathogenic *E. coli*: Finding a Prospective Biological Mechanism for Uroepithelial Intracellular Colonization"

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Abstract

Women are more than 50% likely to contract a urinary tract infection (UTI) in their lifetime.[1] As antibiotic resistance increases, the current methods of treatment such as ciprofloxacin and other antibiotics are becoming outdated, leaving patients at risk for recurrent urinary tract infections. Uropathogenic *Escherichia coli* (UPEC) uses many different types of virulence factors that may be directly involved with the intracellular infection of uroepithelial cells. One of these characteristics is P fimbriae which adheres to receptors on the surface of uroepithelial cells. Other proteins or factors could take a role in the biochemical mechanism of the infection. In this study, a procedure was developed to search for proteins secreted by UPEC that could be involved with urinary tract infections.

UPEC strain CFT073 was inoculated in urine and incubated at body temperature for a total of 48 hours to imitate natural conditions in the human body. The bacterial cells were removed through centrifugation, and possible secreted proteins were isolated, quantified and separated using various protein analysis kits and SDS-PAGE. The results of the CFT073 strain were compared to a laboratory strain, MG1655 (K-12) and sterile urine. The data shows that there were no recognizable secreted proteins present in the UPEC urine cultures. This could indicate that secreted proteins larger than 5 kiloDaltons (kDa) are not involved in UTIs or that uroepithelial cell tissue is required for UPEC to secrete proteins.

Background

Escherichia coli is a gram negative, facultatively anaerobic bacillus bacterium that is commonly found in the gut flora of mammals. Certain strains of *E. coli*, known as Uropathogenic *E. coli* (UPEC), are responsible "for more than 80% of uncomplicated UTIs".[2] These strains have many virulence factors such as P fimbriae, hemolysin, serum resistance, and the ability to form quiescent intracellular bacterial reservoirs (QIR)[3]. These characteristics allow the bacterial cells to intracellularly infect uroepithelial host cells, cause urinary tract infections, and provide the bacteria some antibiotic resistance.

In 2012, there were almost 600,000 people in the United States speculated to have bladder cancer.[4] A risk factor for bladder cancer is recurring UTIs. Understanding the mechanism of UTIs will lead to more efficient treatments and a lowered risk of bladder cancer cases. This study focuses on the protein output of UPEC strain CFT073 in urine compared to the laboratory strain K-12 due to their possible involvement in UTIs and the epigenetic effects on uroepithelial tissue. Our hypothesis was that the exoproteins secreted by CFT073 would be distinguishable from *E. coli* K-12 and normal urine proteins. Results would then suggest that any found proteins have the potential for being involved in the biochemical mechanism that takes place during UTIs.

Methods

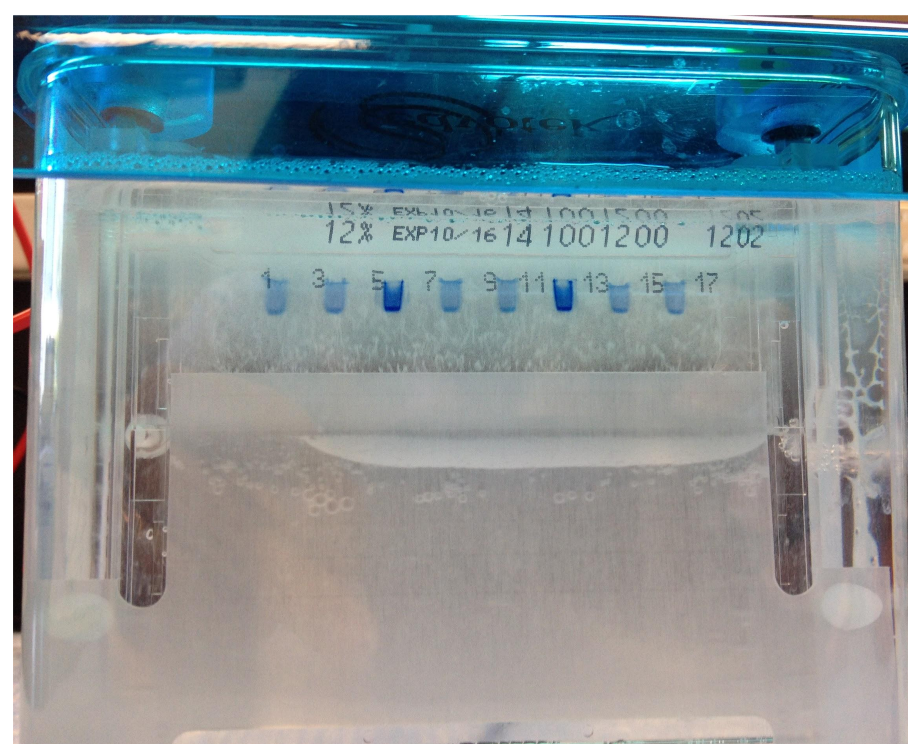
Urine was collected clean catch/mid-stream from a single donor, negative for a UTI, and filter-sterilized. The two different strains were inoculated in small samples of urine for 24 hours at body temperature. A cell counting experiment was performed on varying dilutions of the urine cultures by measuring absorbance at 600 nm (OD 600) using a spectrophotometer.[5] The number of cells/mL were determined and related to absorbance. Next the difference in growth rates between CFT073 and K-12 was established by growing the strains in nutrient broth, a 2:1 urine and nutrient broth mixture, and urine alone over a 48-hour period.

Two 24 hour 3 mL urine cultures of CFT073 and K-12 strains were made and used to find the OD 600. One milliliter of each culture was aseptically transferred to 3 mL of urine and incubated at 37°C for 24 hours. A Urine Protein Isolation and Concentration Kit by ITSI Biosciences was used to isolate and concentrate the protein from the urine. First, the samples were centrifuged to remove the bacterial cells from the protein.[6] The isolated protein was analyzed using a Total Protein Assay Kit by ITSI Biosciences to create a standard curve and determine that protein concentration of the samples were within the limits of the sensitivity of SDS-PAGE.[7]

An Edvotek vertical gel apparatus was used for SDS-PAGE. A Working Loading Buffer was prepared and added to the isolated protein samples at a ratio of 1:1. The mixture was boiled for 10 minutes to denature the proteins. Sigma-Aldrich TruePage Precast gels and SDS Running Buffer from ITSI Biosciences were added to the gel apparatus. Then samples and standards from BioRad were loaded into the gels, and electrophoresis was performed. After electrophoresis the gels were removed from the apparatus and soaked in a fixing/destaining solution. The fixed gels were placed in a Coomassie Brilliant Blue R-250 Staining Solution which binds to proteins. The stained gels were then placed in fixing/destaining solution until bands appeared by washing any unbound blue dye. The finished gels were placed in a storage solution which consisted of 0.05% acetic acid and distilled water and stored at 4°C.

Figure 1. The image to the right depicts the electrophoresis of a precast gel loaded with samples. *Lane 1* - Urine Trial 2, *Lane 3* - CFT073 Trial 2, *Lane 5* - protein standard, *Lane 7* - CFT073 Trial 1, *Lane 9* - K-12 Trial 1, *Lane 11* - protein standard, *Lane 13* - K-12 Trial 2, *Lane 15* - Urine Trial 1

The lanes depicted in the electrophoresis correspond with the finalized SDS-PAGE gel (Figure 4).



Results

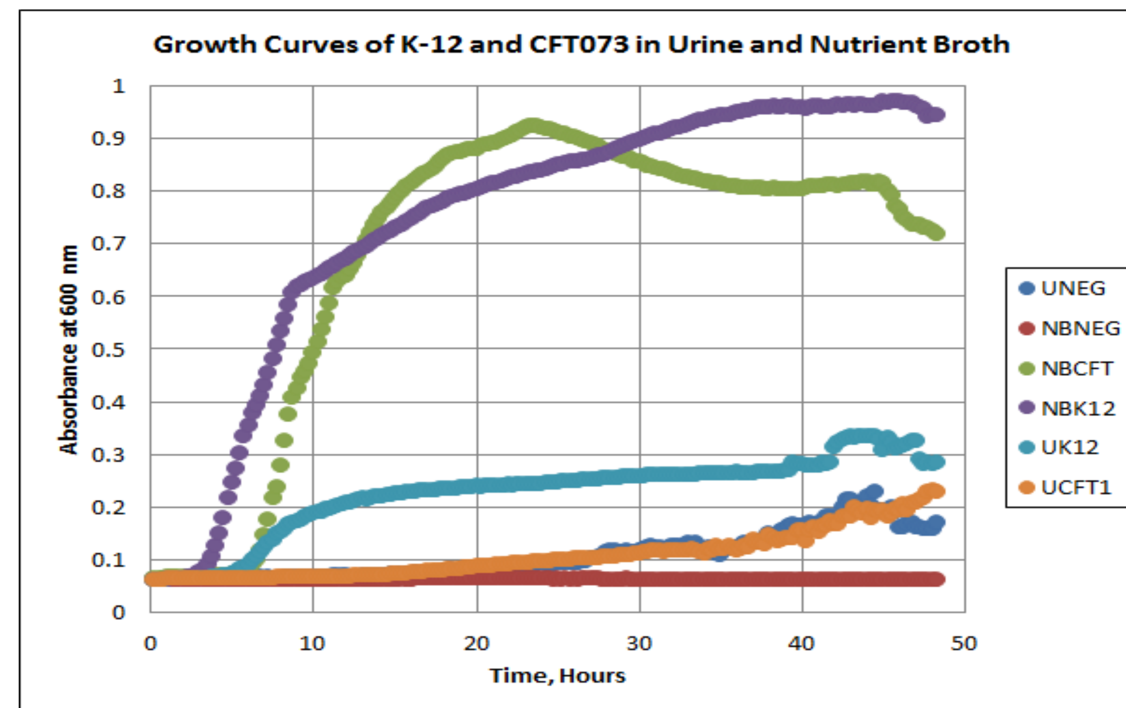


Figure 2. Growth Curve results for 12 samples of K-12 and CFT073 in nutrient broth and urine over 48 hours at 37°C with constant orbital shaking in a 96 well plate. *UNEG* - Sterile Urine, *NBNEG* - Sterile Nutrient Broth (NB), *NBCFT* - CFT073 in NB, *NBK12* - K-12 in NB, *UK12* - K-12 in Urine, *UCFT1* - CFT073 in Urine. The samples UK12 and UCFT1 resemble UNEG and NBNEG curves indicating that a larger volume of urine is necessary for growth.

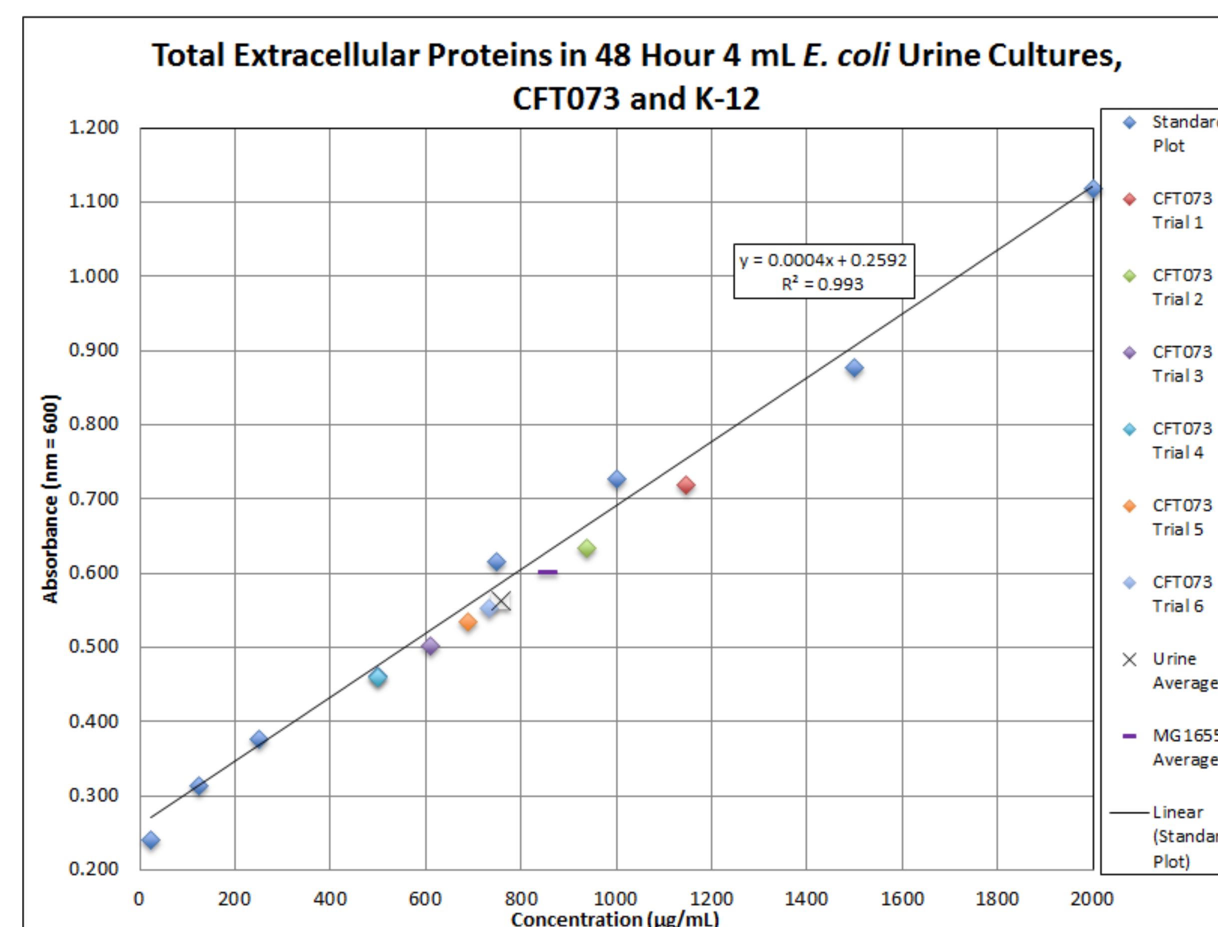


Figure 3. Average Total Extracellular Proteins in 48 hour cultures of CFT073 and K-12. The Standard Plot was determined with the Total Protein Assay Kit. The "X" on the plot designates the average total protein found in sterile urine. This figure demonstrates that CFT073 does not always secrete proteins in urine. This does not include proteins under 5 kDa.

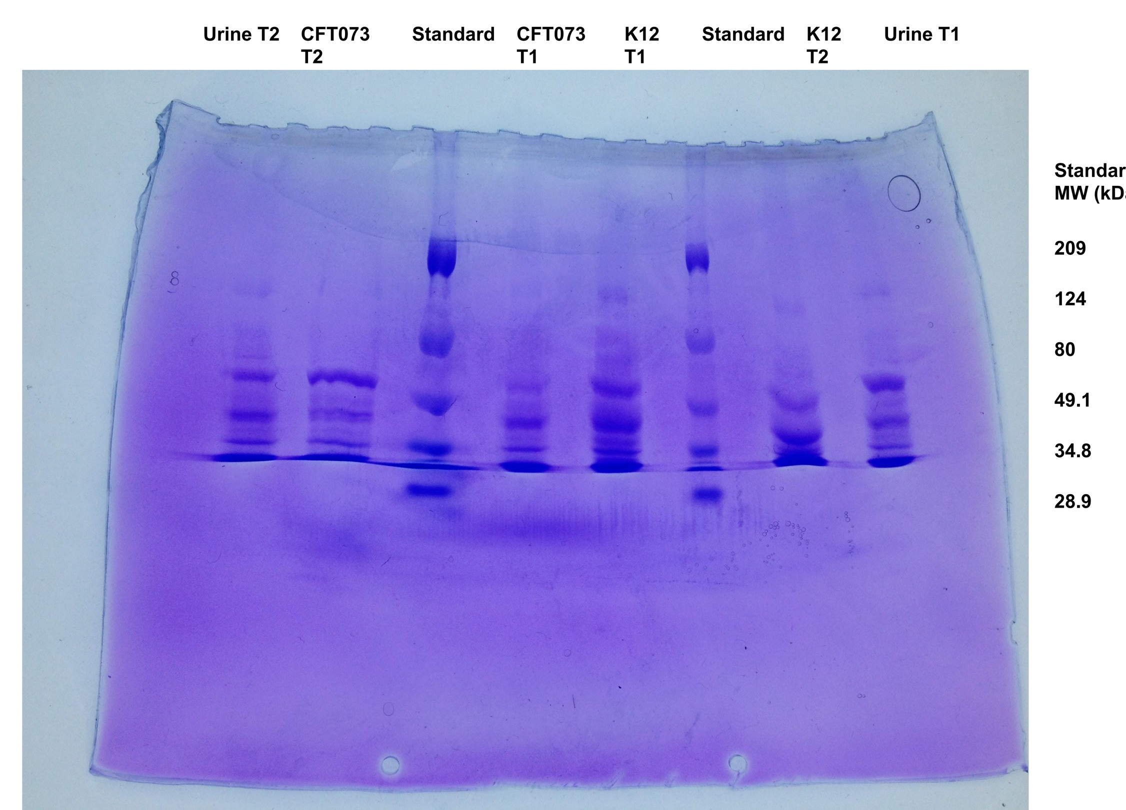


Figure 4. Finished SDS-PAGE gel. The well contents are labeled just above the figure. T1 and T2 represent Trial 1 and Trial 2. The molecular weights in kDa of the Prestained SDS-PAGE Standards are to the right of the figure lined up to the corresponding band. In this gel and all other gels during the experiment, there was no significant pattern of banding that would suggest that CFT073 or K-12 secretes proteins in urine in the 48 hour incubation period.

Discussion

The cell counting experiment revealed that there were about 3.70E+08 cells/mL in the CFT073 urine culture and that there were about 1.23E+06 cells/mL in the K-12 urine culture. According to these results, there are about 300 times more cells per mL in a CFT073 culture than a K-12. Since both strains were inoculated in a similar fashion, this result can suggest that CFT073 has a higher growth rate than K-12 in urine. Optical density (OD) was used to represent the measurement of the bacterial cell concentration at 600 nm. The average OD 600 at 24 hours for the K-12 strain was 0.12 and the average OD 600 for the CFT073 strain was 0.23. However, the results in **Figure 2** were obtained using the Epoch Microplate Spectrophotometer. At 24 hours, the microplate results showed that K-12 had an OD 600 of nearly 0.25, and the CFT073 culture had an OD 600 that was close to the negative controls. Many factors could have contributed to this inaccuracy: a difference in inoculation technique, the size of the wells or contamination in the blank urine samples. Throughout the rest of the experiment, the bacteria were grown in 3-4 mL of urine and and gave a consistent reading at OD 600. Urine has significantly fewer nutrients than nutrient broth, so the smaller volume of 350 µL in the 96 well plate may not have had enough nutrients for the bacteria to grow as in the larger volumes of urine.

The total amount of Extracellular Proteins in 48 hour 4 mL cultures of both strains are shown in **Figure 3.** Significant data for K-12 was not obtained, therefore a statistical comparison between K-12 and CFT073 could not be performed. However, since the averages between CFT073 and the sterile urine samples were similar it can not be concluded that there were extracellular protein secretions. The results demonstrated by the gel in **Figure 4** corroborate the findings from the protein quantification analysis. This gel and all of the other gels showed no significant evidence of extracellular proteins in excess of normal urine protein. It was expected that there would be background banding from the urine, because a random urine sample has a protein concentration ranging from 0 to 20 mg/dL.[8] There are many different types of trace amounts of protein found in urine ranging from uroepithelial tissue and glands, blood, and seminal proteins from the reproductive tract in males.[9]

Conclusion

In this experiment, there was no evidence to suggest that there are exoproteins larger than 5 kDa secreted by the UPEC strain CFT073 in urine. It is possible that these proteins were transient and exist only long enough to assist in the infection. Since there was no uroepithelial tissue to infect, transient proteins would have dissociated. The limits of the experiment could have affected the results. Proteins under 5 kDa were not analyzed by the Total Protein Assay Kit and were separated from the samples that were loaded into the gels. The SDS-PAGE protein standard did not go below 5 kDa, and the 20.6 and 7.1 kDa standards did not show up on the gels. This suggests that there could have been inconsistencies with the electrophoresis procedure. The sensitivity of the materials used for SDS-PAGE could have prevented small quantities of protein from being noticed. Many bands on the gel are too light to be able to compare the blank samples to the test samples. The presence of uroepithelial cells may be necessary for UPEC to secrete proteins. Extracellular proteins may not be involved in the infection. Other factors such as microRNA could be used in triggering the UPEC to infect the host. There is also a possibility that the P fimbriae is the only mechanism in which UPEC adheres to and infects uroepithelial cells. Our research suggests that future investigations should focus on immediately produced small peptides or factors other than secreted proteins.

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