Antibiotic resistance in *Escherichia coli* in the female microbiota. Phylogenetic differences within the *Escherichia coli.*

Abstract:

Urinary tract infections (UTIs) are the most common infection in women. While UTIs are most frequently caused by E. coli, it can also reside within the urinary tract as a commensal member of the urinary microbiota. Prior genomic analysis of E. coli strains associated with UTIs and commensal strains have been conducted, looking at virulence factors and antibiotic resistance distinguishing the two. UTIs are commonly treated with an antibiotic. While E. coli strains can encode for antibiotic-resistance genes naturally, they can also acquire resistance because of prior antibiotic treatment. Due to the community-acquired antibiotic resistance, there are less and less treatment options for UTIs. Recently we isolated and sequenced the genomes of 66 E. coli isolates from the bladder microbiota of women with UTIs, with urinary urgency incontinence, overactive bladder, and without LUTs. The efficacy of five commonly prescribed antibiotics on the growth of these strains was tested. Despite the presence of coding regions associated with antibiotic resistance, we found that UTI+ and UTI- strains exhibit similar sensitivities to these drugs. We also found that there is no difference within the UTI+ and UTI- strains in their placement in a phylogenetic tree based on their amino acid sequence.

Introduction:

A urinary tract infection (UTI) is an infection in any part of the urinary system, which includes the kidneys, ureters, bladder and urethra. The most widespread kind of UTI (in 80% of the cases) is found in the bladder, and UTIs are more prevalent in females than in males (1). UTIs are a significant public healthcare concern, as 1 in 3 women will develop a UTI by the age of 24. In fact, 50% of women will develop an UTI in their lifetime, and in 30% of the cases, the UTI will be reoccurring (1). There are roughly 11 milion cases reported annually in the United States, costing an estimated of $5 billion in healthcare costs each year (2). UTIs are a significant cause of morbidity in infant boys, older males, and females of all ages (3).

UTIs cause morbitidy, fever and short-term lower abdominal pain, which can result in permanent scarring of the kidney (4). *E. coli* accounts for about 90% for all UTIs. (1). In the urinary tract, *E. coli* can be beneficial, or it can assimilate genes that encode for pathogenic factors, which lead to UTIs (5). Uropathogenic *E. coli* (UPEC) was defined by having virulence factors(6). The *E. coli* which is considered UPEC is thought to be the cause of UTIs.

UPEC can colonize the bladder epithelium with the help of virulence factors (6). It is thought that *E. coli* is able to colonize the bladder and even ascend through the ureters into the kidneys, (7) functionality associated with virulence factors. In addition to virulence factors for motility (adhesions, flagella, pilli), UPECs encode for genes related to acquisition of metals, toxin production, immune evasion polysaccharide capsule, outer membrane proteins (OMPs), secretion, lipopolysaccharides (LPS), and siderophores. Furthermore, UPEC strains often form biolfims, which aid in their pathogenecity. Biofilm formation is a process whereby microorganism attach and grow on a surface. Due to the fact that UPEC strains produce LPS and other virulence factors that help in facillitating the attachement and formation of biofilms. Research has found that UPEC strains, in contrast to commensal strains, are more likely to form biofilms (8). Biofilms show decreased susceptibility to antibiotics and other antimicrobial agents (9). In order for the antibiotic to reach the *E. coli*, it has to penetrate through the biofilm layer. Even if the antibiotic penetrates the first layer, it is usally hard to reach the end (10).

The standard treatment for UTIs are antibiotics, which overtime can create antibiotic resistant UTIs. The treatment includes several antibiotics such as ampicilin, cephalotin, ciproofloxacin annd nitrofurantonin, β-lactams, trimethoprim, nitrofurantoin (11, 12). Through the repetitive treatment of antibiotics, *E. coli* can develop resistance. The mechanisms that is thought to give antibiotic resistance varies widely. *E. coli.*In *E. coli* antibiotic resistance is given through mobile genetic elements, jumping genes (13). Resistance is often plasmidic in *E. coli* (10). Antibiotic resistance correlates with the increase in community acquired antibiotic resistance, which can lead to creating super-bugs (10). Multiple studies of UTI *E. coli* have explored the correlation of antibiotic resistance of *E. coli* that comes from an UTI patients and the standard antibiotic treatment given to the patient (4, 11, 14, 15). These studies have showed that resistance in UPEC strains different from drug to drug, but there is an overall resistance to antibiotics. In a study that looked at the antibiotic resistance in *E. coli* that come from a UTI patient , the authors found that antibiotic resistance varies widely in UTIs. The study found that antibitotic resistance is as high as 39.6 % in ampicillin, 23.8% trimethoprim, 22.4%, trimethoprim/sulfamethoxazole, 16.7% amoxillin/clavulanic acid and 15.1% ciprofloxacin (15). This shows that antibiotic resistance is found often within UPEC strains.

Another study tried to evaluate the phenotypic virulence factors in UPEC isolates and correlate them with their antibiotic resistance pattern. The virulence factors such as hemolysin, Types 1 fimbrae, and biofilm formation are related to UPEC strains (16). It is suggested that for

the best practices against developing a super-bug that would be resistant to all types of antibiotics, antibiotics in the case of UTIs should be admistered only if there is a clear sign of bacterial infection and to use appropriate antibiotics (14). Antibiotic resistance is increasing rapidly in comparision with older UPEC strains (17). UPEC strains that form biofilms have a higher antibiotic resistance rate than UPEC strains that do not form biofilms(18). Antibiotic resistance is not only in eldery patients that could have acquire antibiotic resistance over time, but it is prevalent in the younger population (19).However, none of these studies looked at antibiotic resistance in non-UTI *E. coli* strains.It is thought that there is a diffrence between the evolutionary trait of virulence factors in pathogenic versus non-pathogenic *E. coli. E. coli* has 4 main phylogenetic groups A, B1, B2, and D (20). Studies show that group B2 is predominant in UPEC strains (21). However, this study did not look at the *E. coli* from patients with the symptoms of a UTI (UTI+) versus patients without the symptoms of UTI (UTI-).

According to the current research, UPEC strains have an increase in antibiotic resistance and they are part of its own clade genetically. This has motivated this current study which looks at UTI+ and UTI- samples and compares their antibiotic resistance. A phylogenetic tree was created as described in methods. The phylogenetic tree looked at UTI+ and UTI-

Methods

Patient recruitment

The patient recruitment received the approval from the Loyola University Medical Center (LUMC) Institutional Review Board. The patient gave written and verbal approval for chart abstraction and urine collection with analysis for research purposes. The admission and urine collection of the patients was done by the members from Loyola Urinary Education and Research Collaborative. The members are part of the clinical practice of the Female Pelvic Medicine and Reconstructive Surgery Center at LUMC. Patients were recruited as part of separate studies [CITATION TBD].

Urine collection and EQUC bacterial culturing. The ascetically collection of urine was done with a transurethral catheter and was placed in a BD Vacutainer Plus C&S preservative tube for culturing. The urethra, vagina and vulva are bypassed by aspiration; therefore, the niche must be the bladder. Standard urine culture (SUC) was performed on all the samples, as well as expanded quantitative urine culture (EQUC). 1 µl of urine was inoculated onto 5% sheep blood agar plate (BAP) and MacConkey agar plate (BD BBL prepared plated media), incubated aerobically at 35 °C for 24 h, for the SUC. The detection level for the colony growth on either plate was set to 1000 CFU/ml. EQUC was performed as described previously (22). The 100 µl of urine was

Grown under 5 different conditions in BD BBL prepared plated media: (1) BAP in CO_2 for 48 h, (2) chocolate agar (CHOC) in CO_2 for 48 h, (3) colistin and nalidixic acid (CNA) agar in CO_2 for 48 h, (4) CDC anaerobe BAP in an anaerobic jar for 48 h, and (5) BAP in aerobic conditions (BD GasPak Anaerobe Sachets) for 48 h. The threshold level for detection was CFU/ml, which was signified by 1 colony of growth on any of the plates. On different plate of the same medium, each morphologically distinct colony type was isolated. This was done to prepare a pure culture which would be used for the identification. In order to identify the bacterial strains which were described (22), Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrophotometry with the MALDI Biotyper 3.0 software program

(Bruker Daltonics, Billerica, MA) was used . The strains which were identified as *E. coli* via MALDI-TOF were selected for sequencing.

DNA Extraction and Genome Sequencing

DNA was extracted from liquid culture using the Qiagen DNeasy UltraClean Microbial Kit following the manufacturer's protocol. DNA concentration was quantified using the Quibit fluorometer. DNA libraries were constructed using the Nextera XT DNA Library preparation kit and sequenced on the Illumina MiSeq platform using the MiSeq Reagent Kit v2 (500-cycles) at Loyola University Chicago's Genomics Facility (Maywood, IL United States). Raw sequencing reads were deposited in NCBI's SRA database. The SRAs numbers are as follow SRA Accession Numbers are provided in the additional data section.

Assembly and Annotation

Raw reads were first trimmed for quality using the tool sickle (https://github.com/najoshi/sickle) and then assembled by SPAdes (v3.10.1) using the assembly-only option (23). Contigs less than 500 nucleotides in length were removed from further consideration. Assembled contigs were then manually inspected and individually queried against the nr/nt database via megaBLAST. The genome coverage of each assembly was calculated using BBMap's bbwrap and pileup scripts (https://sourceforge.net/projects/bbmap/). Each assembly was annotated the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (24).

Core Gene Tree

Amino acid sequences for each genomes' RefSeq annotation derived by PGAP were concatenated into a single fasta format file and sorted by length and clustered using usearch (25) via the clusterfast method. Sequence identity thresholds of 70, 80, and 90% were used and inspected; the clusters generated for 80% were selected for further analysis. These clusters were parsed using Python to generate a gene presence/absence matrix for the genomes. With this matrix hierarchical clustering was performed using SciPy (www.scipy.org) and the Ward variance minimization algorithm. This tree was converted to Newick format and visualized using iTOL (26).

Antibiotic susceptibility testing

Using an inoculation loop, the *E. coli* was cultured in 3 ml of Luria Broth (LB) and incubated at 37°C for 24 h. After, 2 ml of the overnight culture was added to 2 ml of LB and incubated for 8h at 37°C. 1 ml of the culture was spread on a 1.7% LB agar plate and left to dry for 10 m. The antimicrobial susceptibility BD BBL™ Sensi-Disc™ were pressed into the bacterial lawn and performed in triplicate. The following antibiotics were tested in triplicate: ciprofloxacin 5 mcg, amoxicillin with clavulanic acid 20/10 µg (amoxicillin w/clav) , fosfomycin 200mcg, sulfamethoxazole-trimethoprim 75/1.25 mcg (sulfmeth trimth), cefpodxm 10mcg.They were incubated for 24 h at 37°C. Each plate was examined for the zone of inhibition, and the diameter was measured. The antibiotic diameter specified by the manufacturer for the strain to be resistant (R) are as follows: fosfomycin range of 0 to 1.2 cm, ciprofloxacin range of 0 to 1.5, amoxicillin w/clav range of 0 to 1.3 cm, sulfmeth trimth range of 0 to 1.7 and cefpodxm 0 to 1.7 cm. For the strain to be intermediate resistant (I) the ranges are fosfomycin range of 1.2 to 1.6 cm, ciprofloxacin range of 1.5 to 2.1, amoxicillin w/clav range of 1.3 to 1.8 cm, sulfmeth trimth range of 1.7 to 1.2 and cefpodxm 1.7 to 2.1 cm. If the ring had a diameter

bigger than the following, the strain is susceptible to the respective antibiotic: fosfomycin 1.6 cm, ciprofloxacin 2.1, amoxicillin w/clav 1.8 cm, sulfmeth trimth 1.7 and cefpodxm 10mcg 2.1 cm.

Results and Discussion

The 66 *E. coli* samples come from catharized urine samples from women. These women were diagnosed based on their symptoms with one of the following, UTI, urge urinary incontinence (UUI), or overactive bladder (OAB), or were from a control population (no lower urinary tract symptoms (no LUTS)). The CFU was counted for each strain, and it has variation of 10 CFU per strain to 100,000 CFU per strain. From the samples, 42 are classified as UTI, 13 UUI, 6 No LUTS, 5 OAB. Each of the samples were sequence and assembled using the method described above.

Their genome size ranges from 4.6 mbp to 5.6 mbp with an average length of 5.1 mbp. The assemblies include between 33 to 161 contigs per assembly; on average, each genome was assembled into 72 contigs. The N50 ranged from 72,489 to 508,087 with an average of 250,943. The core genome and pangenome was calculated for the 66 genomes generated in this study and a tree was generated based upon their gene content. As Figure 1 shows, there is no significant correlation between gene content and symptom state. This tree clearly shows that UTI+ strains are found within two distinct clades. Prior studies have found that the UTI+ *E. coli* or UPEC and UTI- *E. coli* are not always found in the same clade(27). This study has looked at the phylogenetic closeness within recurrent UTIs in 2 stages, when the patient had UTI symptoms and when the patient did not have UTI symptoms (27). The findings show that 67% of the samples were part of the clade B2, however it does not look at samples from patients that did not have a UTI. Other studies are consistent with the fact that UTI+ *E. coli* are found in clade B, but they do not look at the phylogenetic clade of UTI- *E. coli* (28, 29). In the 2 major clades showed in Figure 1, there are all 4 types of *E. coli*, neither of them being too close together. There are 2 UTIs that are more closely related (UMB 6712 and UMB 6653), but their actual distance is of 6.32 branches away, and connecting to those 2 is UMB 7413 a distance of 6.9 branches away, which is UTI as well. The 3 strains are most closely together related to a non-UTI strain, an UUI strain UMB 1727 which is only 17. 24 branches away from the 3 strains. A closer look at Figure 1, indicates that the overall seen trend in this phylogenetic tree is that there is no correlation in which clade the *E. coli* falls under. There is no evolutionary trait that an UTI *E. coli* has acquired over time, versus a non-UTI *E. coli.*

A growing body of literature has found that there is a difference between the antibiotic resistance of UTI versus non UTI *E. coli* found in the female microbiota (17). Five commonly prescribed antibiotics for UTI were tested, the results of which are shown in Figure 2. In the antibiotic resistance testing, it was noticed that 0% of 65 *E. coli* samples are resistant to fosfomycin, while 73.8 % are resistant to sulfamethoxazole-trimethoprim. The antibiotic resistance shows no significant difference between the UTI+ and UTI- samples (UUI, no LUTS, OAB). There are 0 strains resistant to fosfomycin. For the ciprofloxacin, 8 UTI samples are resistant and 7 UTI- samples are resistant. These 7 UTI- samples include 2 OAB, 3 UUI, and 3 No LUTS. The amoxicillin w/clav had a very small resistance overall; 0 UTI- and 1 UTI+ strain showed resistance. Sulfmeth trimth showed the highest resistance among the strains tested. 19 UTI- strains were resistant to sulfamethoxazole-trimethoprim, while 29 UTI+ strains showed resistance. The 19 UTI- strains include 4 OAB, 5 no LUTS, and 10 UUI. In the 66 *E. coli* strains tested, only 5 UTI- strains and 13 UTI+ strains are cefpodxm .All of the 24 UTI- strains were

susceptible to Fosfomycin (5 OAB, 6 no LUTS, 12 UUI), while 38 UTI+ strains were susceptible. 12 out of the 25 UTI- strains were susceptible to ciprofloxacin, and 27 UTI+ strains were also susceptible. The UTI- susceptible strains include 2 OAB, 2 no LUTS, and 8 UUI.

While we originally hypothesized that antibiotic resistance would differ between UTI+ and UTI- strains, no significant difference was observed. The average percentage of UTI resistant to a specific antibiotic was calculated, as well as the average percentage of non-UTI resistant to a specific antibiotic (Table 1). A two tailed t test was performed. The statistical analysis for the resistant strains gives us a value of 0.812, which indicates that there is no statistical difference between the observed antibiotic resistance of UTI+ and UTI- strains. The statistical analysis for the intermediate strains gives a value of 0.54. The statistical analysis for the susceptible strains gives a value of 0.816, again indicating no statistical difference between the observed antibiotic resistance of UTI+ and UTI- strains. The analysis of the antibiotic resistance shows that my first hypothesis was wrong, and that there is no difference between the two types of *E. coli.*

Our results for the antibiotic testing were consistent with the literature in the UTI+ case (15, 17). Our resistance rates for the UTI+ are very similar with other studies, even with studies with a larger sample pool (30, 31).

	Fosfomycin	Ciprofloxacin	Amoxicillin w/clav	Sulfmeth trimth	Cefpodxm
RESISTANT					
UTI -	0.00%	29.17%	0.00%	79.17%	20.83%
UTI +	0.00%	19.51%	2.44%	70.73%	31.71%
SUSCEPTIBLE					
UTI -	100.00%	50.00%	66.67%	20.83%	54.17%
UTI +	92.68%	65.85%	56.10%	29.27%	34.15%
INTERMEDIATE					
UTI -	0.00%	20.83%	33.33%	0.00%	25.00%
UTI +	7.32%	14.63%	41.46%	0.00%	34.15%

Table 1. The results of the antibiotic testing in percentage. For 2 UTI types were calculated their resistance (resistant, susceptible and intermediate) to the 5 antibiotics. The 5 antibiotics were Fosfomycin, ciprofloxacin, amoxicillin w/clav, sulfmeth trimth, cefpodxm.

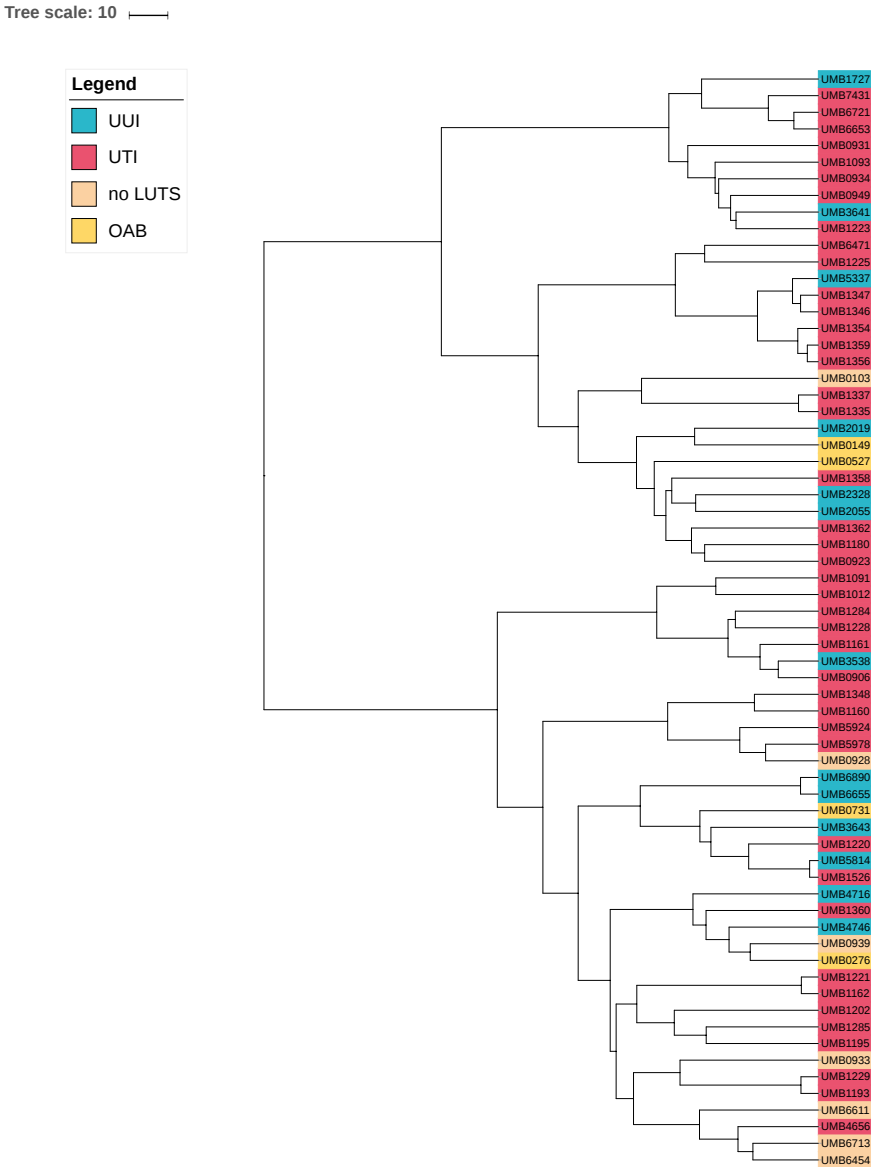

Figure 1. Phylogenetic tree of *E. coli* found in the female urine. The tree was created as discussed in methods. The samples were color coded according to the legend.

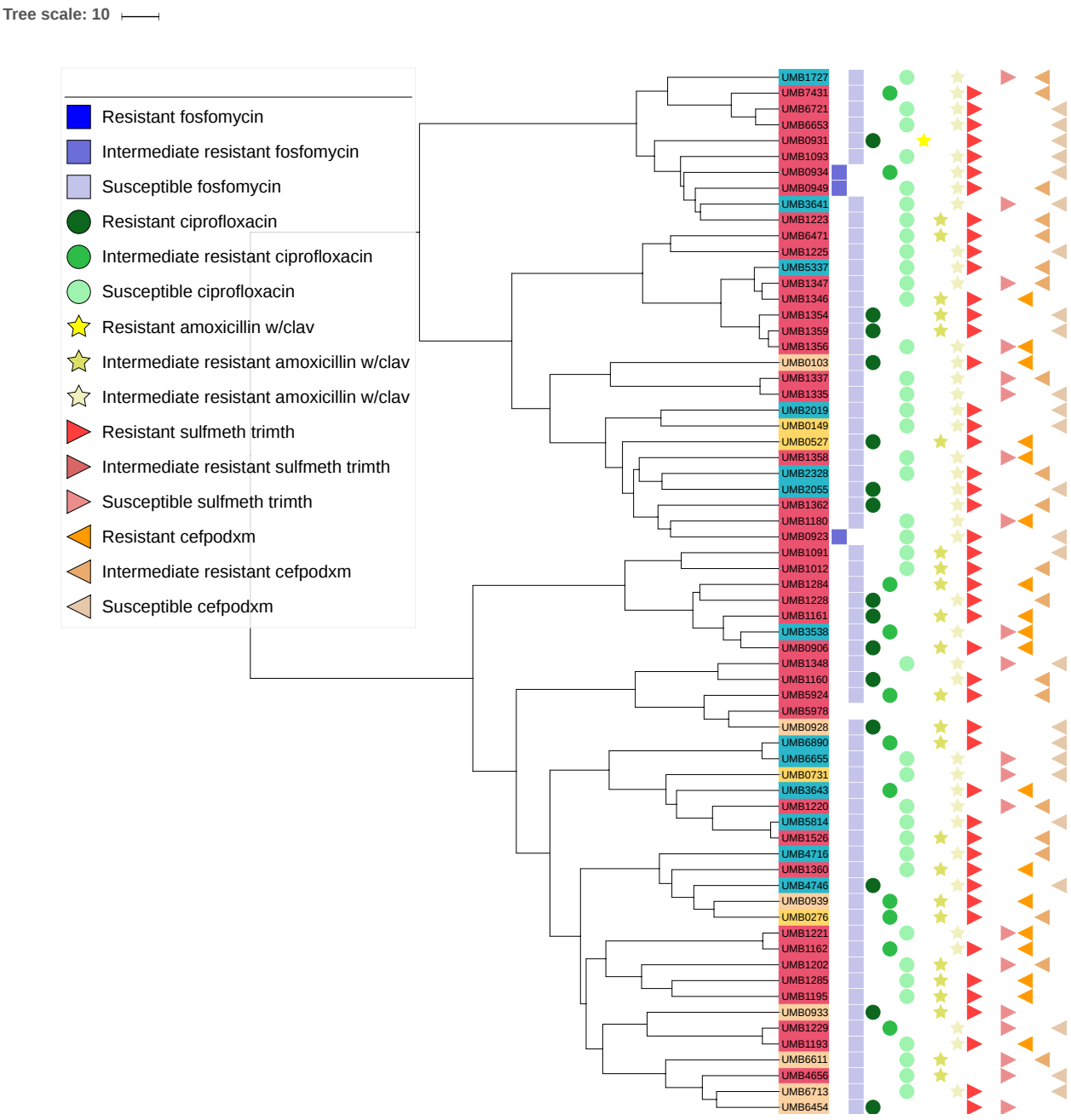

Figure 2. Phylogenetic tree of *E. coli* found in the female urine. The tree was created as discussed in methods. The antibiotic resistance was calculated as described previously in methods. The symbols are used to show the type of resistance to each antibiotic.

Conclusions

Even though it was believed that there is an evolutionary drive between pathogenic and non-pathogenic *E. coli* (32), there is not enough evidence to conclude it. Until now, studies have primarily focused on antibiotic resistance within UTI+ strains. Here we have compared both UTI+ and UTI- *E. coli* strains from the bladder. As my results have showed, there is no difference between UTI+ and UTI- strains. In conclusion, I think more research is needed to investigate the difference between UTI+ and UTI- *E. coli* strains. One of the next questions that we could propose is if there is another microbe that lets the *E. coli* become pathogenic, and if this commensal relationship is the cause of UTI. This commensal relationship can be more investigated in future studies.

ADDITIONAL DATA:

SRE NUMBERSs:

SRA Accession Number
SRR7534319, SRR7534320, SRR7534321,
SRR7534322, SRR7534279, SRR7534316, SRR7534282, SRR7534318, SRR7534313,
SRR7534314, SRR7534305, SRR7534289, SRR7534306, SRR7534307, SRR7534308,
SRR7534290 SRR7534309, SRR7534291, SRR7534310, SRR7534311, SRR7534312,
SRR7534297,SRR7534298, SRR7534271, SRR8185535, SRR7534270, SRR7534273,
SRR7534272,SRR7534267, SRR7534266, SRR7534269, SRR7534268, SRR7534277,
SRR7534276, SRR7534303, SRR7534304, SRR7534301, SRR7534302, SRR7534299,
SRR7534300, SRR7534285, SRR7534274, SRR7534315, SRR7534325, SRR7534280,
SRR7534278, SRR7534293, SRR7534286, SRR7534292, SRR8185536, SRR7534284,
SRR7534287, SRR7534283, SRR7534281, SRR7534275, SRR7534324, SRR7534323,
SRR7534294, SRR7534327, SRR7534295, SRR7534296 (SRR8182356), SRR7534326,
SRR7534329, SRR7534328 (SRR8182355), SRR7534331, SRR7534330 (SRR8182357),

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