ABSTRACT

PLUTA, DOUGLAS HERBERT. Characterization of Genetic Diversity within the FimH adhesin of Uropathogenic *Escherichia coli* and Phenotypic Outcomes in a Canine Model. (Under the direction of Dr. Megan Jacob).

Urinary tract infections (UTIs) are primary caused by uropathogenic *Escherichia coli* (UPEC), affecting more than half of all women at least once during the lifetime. Strains of UPEC usually originate from the gastrointestinal tract and share virulence factors and genetic information that allows them to survive and cause disease in the urinary environment. One of the most prevalent structures on UPEC is the type 1 pilus, the tip adhesin of which is called FimH. FimH binds to uroplakins that form hexagonal plaques on the bladder surface, allowing UPEC to adhere to the urothelium and assist in a zipper-like invasion mechanism. A variety of single nucleotide polymorphisms (SNPs) have been identified in the gene that codes for FimH although the effects of these SNPs have not been well described.

Current animal models for human UTI were reviewed and while mice are currently the most common animal model for UTI, their lack of spontaneous pathogenesis and poor urinary analogy leave a need for a more optimal model in UTI biomedical research. The canine was identified as having better genetic homology and anatomic analogy in addition to being a spontaneous model for UPEC infection. The strains that cause UPEC infection in dogs have been found to be highly similar to those that cause infection in humans and can be isolated from canine feces, identifying the dog as a potential community reservoir for human UTI. This validates the dog as a superior epidemiological model to mice as well.

One aim of this thesis was to identify SNP diversity in a large clinical sample of canine UPEC isolates as well as characterizing the phenotypic effects therein, as the association between SNP diversity and phenotype have not been well described. A PCR reaction was

designed to identify the *fim*H gene in purified DNA from UPEC collected from clinically affected dogs and the 489-bp amplicon was sequenced and subsequently genotyped. Out of 206 canine UPEC isolates collected, 72 unique *fim*H types were identified, resulting in a higher level of diversity than previously published clinical samples. The SNP diversity identified in this clinical sample was also associated with biofilm formation, multidrug resistance, and hemolysis on blood agar, although not with recurrence rates. It was found that the identified *fim*H types could be clustered by genetic similarity for more robust association analysis.

Another aim of this thesis was to characterize bacterial fitness in varying urine compositions, as this is known to change growth of UPEC *in vivo.* Dehydration, diabetes, and renal disease are all known risk factors for UTI in humans and dogs while proper hydration has a pseudo-antibacterial effect on urine. Viable cell count was assessed and growth curves were constructed after growth in treated artificial urine media for 24 hours. Biofilm formation and minimum inhibitory concentrations were assessed as measures of bacterial fitness. This study found diluted urine to grow the least bacteria as well as the least biofilms, in agreement with previously studied data. This study also blocked these isolates by three *fim*H types and found that growth, viable count, and biofilm formation in modulated artificial urine were all associated with *fim*H genotype.

It is known that *fim*H is incredibly diverse and has many possible SNPs, and our data provides the knowledge that this diversity can affect the way a given strain of UPEC will perform in a canine or human with regards to fitness and growth, virulence, and survival. Provided a way to determine the *fim*H type and interpret it, a clinician could use this information to more accurately treat or monitor a patient, reducing the disease burden of recurrent UTI and multidrug resistance.

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Characterization of Genetic Diversity within the FimH adhesin of Uropathogenic *Escherichia coli* and Phenotypic Outcomes in a Canine Model

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Comparative Biomedical Science

Raleigh, North Carolina
2021

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DEDICATION

I want to dedicate this thesis primarily to all the high school teachers who made a difference in my life. April Ellis, Dr. Tom Bauso, Dayna Mergenthaler, Lee Baldwin, Mike Evans, Chad Keister, Koko Thornton, Christine Northrup, Genise Thorsen, Megan Justice, Rafaela George, Brian Wood, Ed Constantine (Rest in Power) and probably so many more people that I interacted with along the way. So many people forget the ones that built a foundation for them along the way but each and every one of you did something for me whether you know it or not that helped me get to this point whether it was a word of encouragement, not taking life or your class too seriously, helping me believe in myself just a little bit more, or just being you. You all deserve the world and I do not think I would be doing this if I had not had the foundation each and every one of you provided me, without even half the recognition you deserve. Most of you will probably never see this, but at least this will be immortalized.

I would definitely like to dedicate this to my friends and family who may not have always understood what I was doing when I went off grid to live in the lab or stay up for days at a time to write and who may not know what “thesis” or “defense” means. Y’all kept me grounded during the pandemic and kept me from dropping out multiple times. The support means the world.

Also, I want to dedicate this to all the patients I’ve lost during my time as a first responder. Some of you, I never got to know your name, some I will never forget your name. Most have left a mark that has led me on a path that I dedicate myself to every day. This thesis will likely lead me to my next career step, but it was also a major part of a personal journey that each of you have touched as well. I thank you for letting me be part of your last moments on this Earth.

BIOGRAPHY

Doug was born in Redwood City, California to Doug and Anna Pluta in 1996. At age 4 he moved to Buffalo, NY and he finally moved to Raleigh, NC at age 10 in 2007. In 2014 he graduated with his IB Diploma, which he is very proud of to this day, from William G. Enloe High School. He then moved on to earn his B.S. in Polymer and Color Chemistry from N.C. State University in 2018. During his undergraduate career in 2015, he took a class for and became certified as an EMT and began full-time work, which is when he fell in love with the life sciences and medicine. In 2018, he earned his Paramedic certification and took a semester off of school to work in Wake County before beginning graduate school at N.C. State. He pursued dual-Master programs with a Master of Physiology and a Master of Science in Comparative Biomedical Sciences under Dr. Megan Jacob. He completed the Master of Physiology degree in May of 2021. Following graduation, he plans to work in industry while he researches PhD and MD programs before applying.

In what free time he has, he enjoys to travel, camp, hike, kayak, cook, and hang out with his dog Charlie Brown. He is lucky he enjoys what he does for work.

ACKNOWLEDGMENTS

Dr. Megan Jacob – Thank you for taking a chance on me. I came to you with no research experience, no microbiology experience, no clue what I was doing, and I had a lot of impostor syndrome, but I had a dream (tacky, I know). You gave me a chance to work with you and 2.5 years later I am a million times more confident, and I feel like a master of my craft.

Dr. Jessica Gilbertie – I would literally know 0 science without you. Every self-deprecating comment that you stopped me from making, all the encouragement, all the questions you answered, all the assays you taught me, all the advice you gave me about science and life. You simultaneously humbled me and hyped me up. I don't even know how to thank you because you are that incredible.

(Soon to be Dr.) Catherine Gensler – I now realize that whenever I ask you a question and you respond with another question, you were trying to teach me. You are the best lab mate I could ask for, best person to bounce thoughts off of, and I still need to learn more about cheese from you. Or taste your kombucha/mead/literally anything other than BEET BROWNIES.

Sam Sack and Erica Lemler – You both held this lab together in these last few months and I could not have gotten to the finish line without you both. You are both workhorses.

Dr. Gizem Levent – You are an invaluable resource. Thank you so much for being there to fill in the gaps and to even spend some time to help me learn and troubleshoot when you had no obligation to do so.

My therapist Brittney – Yeah you held me together. I'd be a wreck without you. I'm still not going to journal though.

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1. Abstract

Urinary tract infections are a highly common bacterial infection in both humans and canines, with uropathogenic *Escherichia coli* being the most common etiological agent. These strains often originate from the intestinal flora and are pathogenic when they are introduced to the conditions of the urinary tract as a result of a specific set of virulence and survival factors. Currently the mouse and pig are the two most common models for UPEC infection used in both pathophysiological and epidemiological research. Both models have been characterized to have genetic similarity and anatomic analogy with humans, but each has unique limitations that leave opportunity for an alternative model. We propose the canine as the canine has a high degree of genetic homology, anatomic and physiologic analogy, and a high degree of epidemiological relevance. Dogs also live in the same environments in humans and their feces has been shown to carry extraintestinal pathogenic *E. coli* strains with striking similarity to or, in some cases, that are the same as strains that cause UTI in humans, allowing for spatial transmission analysis. Hospitalization of canines also provides an opportunity to study the emerging issue of multidrug resistance in a setting more similar to humans than either other model. Canines have been criticized for their ethical implications, which is why more work needs to be done to support their use in experimental studies using live subjects although we believe the current literature supports the use of the canine as an optimal alternative, natural model for UPEC infection.

1.1. Introduction

Urinary tract infections (UTIs) are a financially significant and common bacterial disease affecting humans. In total, the health care costs associated with community-acquired UTI are around $1.6 billion, taking into account downstream effects such as pyelonephritis, chronic

kidney diseases, or pregnancy complications including fetal mortality (Foxman, 2003). Nearly 50% of all women will experience at least one UTI during their lifetime and around 30% will experience a UTI requiring antimicrobial treatment by age 24 (Foxman, 2003). A number of factors increase the likelihood of a female to develop a UTI, including the shortened urethra and the proximity of the urethral opening to the anus, allowing gut bacteria to be introduced to and ascend the urinary tract (Minardi et al, 2011). Enteric bacteria have been shown to be the most common cause of UTI, especially *Escherichia coli*, with one large study showing 74% of ambulatory UTI patients and 65% of hospitalized UTI patients presenting with *E. coli* as the cause of their UTI (Laupland et al, 2007). When an *E. coli* strain is able to cause a urinary tract infection, it is known as a uropathogenic *E. coli*, or UPEC.

UPEC utilizes a few key virulence factors, including a unique set of structures and proteins that allow it to survive and grow in the human urinary tract, while simultaneously causing disease in its host. One such structure is the flagellum, regulated by *flgM*, which UPEC use to ascend the urinary tract until it finds favorable conditions for growth. Once favorable conditions are found, *flgM* is no longer expressed and UPEC ceases production of flagella (Lane et al, 2007). Instead, UPEC begins to express P pili and/or type I fimbriae for adhesion to the urothelial tissue (Terlizzi et al, 2017). The PapG tip adhesin binds to receptors containing galabiose on urothelial cells and the P pilus is thought to play an important role in the development of pyelonephritis (Hultgren et al, 1989). At the tip of the type I fimbria is the FimH adhesin which binds to mannosylated uroplakin Ia receptors and has been shown to be associated with increased urothelial cell adherence as compared to PapG. FimH has also been shown to play a role in urothelial invasion, which is often not seen in PapG (Martinez et al, 2000). In the case of invasion, FimH binding with mannosylated surface receptors results in the phosphorylation of

focal adhesion kinase which forms transient complexes with phosphoinositide-3-kinase. This activates Rac1 which stimulates the formation of complexes of α-actinin and vinculin, resulting in the actin rearrangement that engulfs the adherent UPEC (Mulvey, 2002). FimH binding can also trigger autoaggregation of UPEC, allowing for the formation of surface biofilms on the urothelium (Pratt & Kolter, 1998).

In strains forming a surface biofilm, the bacteria can aggregate to those attached to the surface with the help of a few proteins. The first is autotransporter Antigen 43 which has been shown to increase interactions between bacterial cells through a self-recognition pathway. It was initially believed to be an epithelial adhesin but was then shown to play little role in adhesion to mammalian cells (De Luna et al, 2008). Curli also plays a major role in biofilm formation by interacting with the exopolysaccharides of nearby UPEC cells, creating stronger cell-to-cell interactions as well as three-dimensional structures as opposed to flat biofilms (Barnhart & Chapman, 2006). Curliated bacteria also were shown to attach and invade epithelial cells more successfully than non-curliated strains (Gophna et al, 2001). For those that do invade, UPEC often begins to replicate inside the epithelial cell vacuole and begins to form biofilm-like intracellular bacterial communities, or IBCs (Conover et al, 2016). Initially, bacteria replicate rapidly, maintaining their shape, but between 6-8 hours, replication slows and bacteria shorten and become more tightly packed, growing to fill an entire cell (Justice et al, 2004). After about 12 hours of growth, the bacteria within the IBC flux from the cell with some having returned to a standard rod size with others filamenting, growing to an average of 70μm in length (Justice et al, 2004). These filaments allow bacteria to easily reach nearby urothelial cells for further invasion and IBC formation (Justice et al, 2004). In both surface biofilms and in IBC structures, evidence exists for a quiescent state of low to absent metabolic activity and stunted growth (Wood et al,

2013, Scott et al, 2015). This reduced metabolic activity reduces the effectiveness of a variety of antibiotics that use the metabolic pathways of the pathogen to reduce replication and induce killing of the pathogen, thereby creating one mechanism for antimicrobial tolerance (Wood et al, 2013).

Currently, the mouse is the most commonly used animal model to study UPEC colonization and pathogenesis (Hung et al, 2009). The murine model has been proven over time to be a reliable model for many diseases but also comes with key anatomic and immunological differences that prevent confident comparison between murine and human UPEC pathogenesis (Hung et al, 2009). Recent studies have also explored a porcine model for specific UPEC presentations due to the similarities in urinary physiology between swine and humans although relatively few studies have been done on the molecular mechanisms of UPEC in swine (Nielsen et al, 2019). Few UTI infection studies have been done in humans and primates due to ethical concerns although epidemiological studies and urine sample analysis have been useful in UPEC characterization (Barber et al, 2016). Given the drawbacks to each of the current UPEC models, this review will further examine current literature available for the suitability of current models and will propose an alternative small-animal model, the canine, that we believe can fill many of the gaps left by existing models.

1.2. **Suitability of Current Animals for UPEC Disease Modeling**

The varying immunological responses, cellular physiology, and anatomical differences between current mammalian models for UPEC and humans creates a number of challenges in suitably replicating human disease. Current models for human urinary tract infections have been well described with regards to their anatomical, molecular, and physiological considerations relating to UPEC infection, but evaluation of a mammalian model requires continuous

reassessment of homology, analogy, as well as model fidelity (Swearengen, 2018). In order to assess analogy, one must determine whether current models, in this case swine and mice, exhibit functional or structural similarity to humans and fidelity requires an assessment of whether the model being examined can produce experimental data similar to what would be seen in a human with a UPEC infection (Swearengen, 2018, Woodward, 2016).

1.2.1. *Assessment of Validity of Current Models for UPEC Infection in Humans*

One important criterion when determining whether a model for human disease is valid is homology, or evolutionary similarity between humans and the model. A number of models beyond murine and porcine models are used to model various aspects of UPEC growth, colonization, and pathophysiology, with varying degrees of efficacy. The first of which is the nematode, specifically *Caenorhabditis elegans*, which has been shown to be an effective model for extraintestinal pathogenic *E. coli* (ExPEC) virulence given its readiness to consume pathogenic bacteria and ease of genetic analysis (Diard et al, 2007). *C. elegans* was one of the earliest invertebrates to have its genome fully characterized, offering a wealth of genomic information which has since been used extensively in bioinformatics and phylogenetics (Chervitz et al, 1998). *C. elegans* has approximately 41% homology between its genes and human genes, which is a strikingly high amount, and about 83% of *C. elegans* proteins have human orthologs, with only 11% of genes coding for nematode specific proteins, in one study (Lai et al, 2000). Given this, *C. elegans* appears to have a high degree of homology with humans, but its usefulness as a UTI model is limited to survival studies and genetics (Barber et al, 2016). Since these nematodes feed on bacteria, they can be fed strains of UPEC and death rates can be assessed rather easily (Barber et al, 2016). Additionally, they can be fed UPEC strains and further be fed bacteria that express double-stranded RNA that encode for responses similar to

certain host defenses, although their primitive immune system and lack of any developed genitourinary organs limit their translation to spontaneous disease in humans (Ewbank & Zugasti, 2011).

Zebrafish are another inexpensive and easily cultured model for human disease, although limited by their lack of mammalian urinary anatomy (Barber et al, 2016). In terms of homology, the zebrafish genome has been extensively characterized and studied as it relates to embryology and it has been shown that 47% of human genes are orthologous to zebrafish genes on a one-to-one basis, meaning that the human gene is orthologous to one zebrafish gene (Howe et al, 2013). Further, additional human genes were found to be orthologous on either a two-to-one, one-to-two, or other type of relationship which would further increase the degree of homology between the two species, further validating the use of the zebrafish as a model for human disease (Howe et al, 2013). One major advantage of the zebrafish model is that the zebrafish has a rather robust innate immune system that includes many components orthologous to humans including interferon production, toll-like receptors, and cytotoxic cell activity (Trede et al, 2004). Additionally, zebrafish are excellent candidates for gene manipulation, and an entire colony can be manipulated and analyzed with relative ease, thus testing of virulence and host response related to individual genes or proteins in a short time with relatively low cost (Nasevicius & Ekker, 2000). Although zebrafish are limited by the lack of a mammalian urinary system, they are inoculated through injection into the circulation post-fertilization, creating a urosepsis-like condition to assess virulence, in particular HlyA, and innate immune response (Wiles et al, 2009).

The chicken has also been identified as a potential model for UPEC disease, not because of its capacity to have natural UTIs or due to exceptional homology with humans, but because

avians are natural hosts for avian colibacillosis, caused by species of ExPEC that cause disease in avians (APEC) which have been found to have similarities to the strains of ExPEC that cause UTIs (Barber et al, 2016). In fact, homology between chickens and humans is low, with conserved sequences mostly being found within introns and orthologues representing pseudogenes as opposed to transcriptionally active genes between the two species (Hillier et al, 2004). It has been proposed that, due to these potential ExPEC strain similarities, avians could be reservoir for UPEC in humans, but one study found that significant differences were found between a sample of APEC and a sample of UPEC with regard to serogroup and virulence typing (Rodriguez-Siek et al, 2005). Still, it was proposed these strains could contribute to virulence through horizontal transmission to other bacteria (Rodriguez-Siek et al, 2005). This data, however, could be challenged by further research that showed similar upregulation of virulence genes as well as mortality and morbidity when both a UPEC and APEC strain were introduced to a murine subject and a poultry subject, suggesting that regardless of the species of host or the strain of pathogen, the bacteria will potentially behave similarly (Zhao et al, 2009). Given this result, as a large vertebrate with a low cost to maintain and high reproductive potential (Barber et al, 2016), the chicken becomes an increasingly valid model for studying molecular mechanisms of UPEC as well as identifying potential reservoirs of the bacteria.

The murine and porcine models are both commonly used today for UPEC colonization and pathogenesis characterization. The murine model has been used broadly over time in part because of its extensively homology to human genetics, with 90% of mouse genes being located on the same chromosome as in humans and with 78.5% amino acid sequence similarity among orthologous genes (Monaco et al, 2015) and this homology likely contributes to the anatomic similarities that are seen between the two species. Through years of research, a number of

genetically modified strains of mice have been developed, allowing specific experiments to be conducted targeting specific pathways or immune responses, and mice can be further genetically modified with relative ease (Barber et al, 2016). With reference to the porcine genome, although swine are less homologous to humans than mice, 112 amino acids were found to be identical in proteins between the two species where human diseases were implicated (Groenen et al, 2012). These specific similarities implicated diseases including diabetes and obesity, and swine have been further found to be susceptible to genetic manipulation and generation of transgenics, which could prove to be an important tool in their use as a model for UPEC infection (Groenen et al, 2012).

1.2.2. *Anatomical Considerations for Common UPEC Models*

Currently, the murine model is the most common animal model used to recapitulate human UPEC infection (Barber et al, 2016). In a murine model, UTI studies can be performed by inoculating with UPEC at various points in the lower urinary tract, either distal to the external urethral sphincter, between the external and internal sphincters, or directly into the bladder (Carey et al, 2016). The urethra in female mice is approximately 1cm long (Carey et al, 2016), which can be compared to the short urethral length in human women that contributes to increased incidence of UTI (McLellan & Hunstad, 2016). Additionally, given the increased length of the male urethra and the small scale on which murine experiments are performed, urethral access is complicated (McLellan & Hunstad, 2016). As a result, direct inoculation of the bladder through either suprapubic injection or through a surgical incision into the abdomen is performed, although this method is not performed in humans experimentally and more accurately represents UTI that originate from abdominal surgery (Carey et al, 2016, McLellan & Hunstad, 2016). Additionally, human urinary tract infections most commonly begin at the periurethral

boundary and ascend the urethra before attaching to the bladder epithelium, the mechanism of which is bypassed by transurethral inoculation in mice (Carey et al, 2016). In order to maintain colonization between murine subjects following inoculation, as a result of rapid urine creation and elimination, researchers often use an inoculum containing greater than 10^7 CFU/ml bacteria, which is greater than the concentration of bacteria that would be found in the bladder of a human during the beginning phases of a spontaneous UTI (Barber et al, 2016).

The other, although less common, model for human UTI is the swine. Urinary anatomy in the swine is favorable for comparison to humans in terms of relative size as well as the structure of the kidneys, ureters, and bladder, with a few exceptions (Swindle et al, 2012). Porcine bladder walls are thinner than those of humans and both proximal and distal tubules in the kidney are dilated as compared to humans (Swindle et al, 2012), although these changes are not known thus far to significantly affect UPEC colonization. Additionally, female swine have similar genitourethral anatomy as humans, with the urethral opening relatively close to the anus and a short urethra, although the male urethra is housed within a corkscrew shaped penis which diverts from human anatomy, making catheterization and urethral analysis difficult (Swindle et al, 2012). The increased size of swine as compared to mice allows for more targeted testing of UPEC upon inoculation as in a murine model, the challenge force presents a risk for the inoculum to reach the ureters in a high enough concentration to colonize and ascend to the kidneys whereas the porcine model allows for inoculation into either the urethra or bladder without much risk of inoculation of the ureter to the point of colonization (Nielsen et al, 2019). The size differences between mice and swine also can account for differences in the concentration of urine, which has been linked to UPEC phenotype, morphology, and virulence with porcine urine being much less concentrated than murine (Nielsen et al, 2019, Klein et al,

2015). Porcine models are limited by their size in that they require greater levels of anesthesia for procedures and are more expensive to maintain so as to not cause damage to the urinary tract and to prevent natural disease in their environment (Nielsen et al, 2019).

1.2.3. *Molecular and Host Response Comparisons Between Common Models*

The urinary epithelium has a highly conserved organization among mammals comprised of an apical layer of umbrella cells that are cuboidal when the bladder is empty and stretch when the bladder fills, an intermediate layer, and basal layer under which is the basement membrane separating the mucosa from the serosa (Abelson et al, 2018). Both porcine and murine models express the same four highly conserved uroplakin units on the apical surface of the urothelium (UPIa, UPIb, UPII, UPIII), similarly to humans (Wu et al, 1994). UP1a is known to be the mannose binding site for FimH on T1F, but UPIII is also known to increase UPEC-mediated apoptosis related to the exfoliation of the umbrella cell layer, exposing intermediate and basal cells (Thumbikat et al, 2009). One important molecular component that does vary between models is the toll-like receptors (TLRs). Toll-like receptors recognize pathogen-associated molecular patterns (PAMPs) which assist the host in identifying a pathogen and initiating an immune response (Dawson et al, 2017). TLR4 is known to recognize lipopolysaccharide on the surface of UPEC and has been found to be more similar in swine than in mice, both in nucleotide and amino acid sequence (Vaure & Liu, 2014). Mice also have an additional toll-like receptor, TLR11 that protects from UPEC infection in the kidneys, which is represented by a pseudogene in humans and is not found in swine (Carey et al, 2016, Clop et al, 2016). This divergence allows a unique analysis of UPEC in mice, but that analysis is not necessarily one that models spontaneous infection in humans, which could potentially be better modeled in swine.

One major advantage of the murine model is its ease of use and reliability. Due to its extensive use, it has well characterized and researchers have been able to use the model for studies on a variety of UPEC characteristics such as the growth of intracellular bacterial communities (IBCs), in which multiple studies have shown lead to the creation of neutrophil resistant bacterial filaments (Olson & Hunstad, 2016, Justice et al, 2006). It has been shown that both mice and swine, through CXCL1/2 and IL-8 respectively, are able to recruit neutrophils to the bladder in response to infection or injury, but as of yet, only mice have been used to study how neutrophils interact with UPEC in various stages of infection (Carey et al, 2016, Puyo et al, 2018). The murine model has also traditionally been the gold standard for vaccine and therapeutic pre-clinical trials as a result of its low cost, high reproducibility with regards to immune reactions, and short time frame (Barber et al, 2016). Rodents have been well studied for their pharmacological properties for a wide variety of pharmaceuticals whereas other model systems often are well-developed for specific therapeutic specialties, making the murine model versatile and reliable (Zhao et al, 2016). The amount of data collected in mice has also revealed some inconsistencies, particularly regarding macrophage antimicrobial activity where strains UTI89 and VR50 exhibited increased intracellular macrophage survival in murine macrophages but did not exhibit this increased survival in human macrophages (Bokil et al, 2011). Although similar studies have not yet been performed in other species of interest, it does appear to cast some doubt on the fidelity of the mouse as a model of human host response to UPEC infection, particularly if strains beyond those tested were to exhibit this same pattern.

1.3. **Epidemiological Considerations in Studying UPEC Infection**

Analysis of a pathogen would not be complete without an examination of the factors that contribute to its spread within and between populations as well as the population-level disease

burden that the disease carries, which is where the study of epidemiology becomes important (Krämer et al, 2009). UTIs caused by UPEC are not necessarily highly transmissible between hosts, however, UPEC strains can be readily transmitted between hosts through sexual contact or the fecal-oral route and can develop in the gastrointestinal tract of the new host prior to forming disease (Terlizzi et al, 2017). This transmission between hosts can assist in the sharing of virulence factors between discrete UPEC strains, including antimicrobial resistance plasmids, increasing the prevalence of UPEC strains that are resistant to front-line UTI treatment options (Kot, 2019). An important consideration when assessing the epidemiology of UPEC in an animal model in comparison to humans is whether the model is spontaneous or inducible, rather, whether the model can develop a UPEC infection under natural conditions or whether UPEC must be experimentally introduced into the model animal for infection to occur (Hau, 2008). Since UTIs caused by UPEC are a natural occurrence in humans, disease should ideally also develop naturally in an optimal animal model.

1.3.1. *Epidemiological Methods in the Study of UPEC Infection*

The benefit of animal models of human disease is that they can be used to collect data that would be otherwise impossible or unethical to collect in humans. From an epidemiological perspective, descriptive epidemiology, or the collection of temporal, spatial, and relational data, is rather simple to perform for humans because this data can be collected from medical records, surveys, or governmental offices (Naito, 2014). Analytic epidemiology encompasses the collection of data that analyzes the relationships between a disease, or specific components of a disease, and outcome(s) of interest, often stratified by demographic factors (Brachman, 1996). This data is often collected through case-control, cohort, or cross-sectional studies (Brachman, 1996), all of which can be performed in humans both in and out of the hospital setting. These

types of analyses can become complicated due to various forms of bias, among other limitations, chief among them is the time constraint of performing a prospective study in which subjects are identified and followed for either a defined period of time or until an outcome is reached, which could be an extended period of time (Yamaji et al, 2018). Additionally, since subjects for these observational studies must be included based on specific, selected criteria, there is the opportunity for selection bias during the enrollment process and since data is collected over a period of time during which the subject has not been or will not be controlled, external factors to the experiment could also impact the outcome being studied (Yamaji et al, 2018). It has been identified that retail poultry could be a potential reservoir for UPEC strains through assessment of grocery stores surrounding a sample of patients from whom urine samples were collected (Kapp, 2006), but performing a case-control analysis to attempt to identify the stores that were the sources of infection for a group of cases would be difficult since, for example, a case might not remember where they shopped over the time period being examined or they might have shopped at multiple places. The final form of epidemiological methodology is experimental epidemiology which would involve testing of a hypothesis, such as testing whether a gene mutation increases shedding of UPEC in urine or vaccine trials (Brachman, 1996). This type of analysis is arguably the most difficult to perform in humans from an ethical standpoint due to the ethical principle of non-maleficence, meaning preventing harm, as from most perspectives, the intentional introduction of an infectious disease would be undoubtedly harmful (Kapp, 2006).

Current animal models of UPEC are particularly useful in collecting experimental epidemiological data. Mice have been used as an optimal candidate for testing of multiple potential UPEC vaccine candidates during drug discovery trials, identifying protective immune responses when immunized with outer membrane iron receptors (Mobley & Alteri, 2015).

Survival studies in a murine model showed that one vaccine significantly increased survivability in vaccinated mice as compared to non-vaccinated mice and also increased clearance of bacteria from the kidneys in mice (Kruze et al, 1992). Results in murine models are possible to achieve on a relatively short time scale and, if the results indicate efficacy of a vaccine or drug, a study can contribute to allowing a therapeutic to move forward into clinical trials and eventually, begin to be used as a treatment option to significantly reduce healthcare costs associated with UPEC (Mobley & Alteri, 2015). Due to their shorter life span and greater reproductive success, as well as previously described urinary physiology divergences from humans, caution has been advised when comparing mice to humans, particularly when comparing murine vaccine and survival studies as the results might not necessarily correlate with the response that would be seen in a human population (Brumbaugh & Mobley, 2012). Additionally, mice are solely an inducible model of UPEC infection, in that they do not naturally develop these infections from their intestinal flora or the environment, whereas humans can spontaneously acquire the infections, so experiments based on inoculation of mice must be carefully designed to replicate the conditions found in a spontaneous human UTI to control for this limitation (Carey et al, 2016). One of the most important advantages of the porcine model is that swine are a spontaneous model for UTI, particularly sows, since the lactation and parturition processes bring the vulva much closer to the ground, often into direct contact with feces, often naturally introducing the urinary tract to the urethral opening (Glock & Bilkei, 2005), mirroring the mechanism by which UPEC and other uropathogenic bacteria from the intestinal tract are introduced to the urethral opening in humans, simply on a different scale. Vaccine and drug development is also considered an advantage of the porcine model due to their size and immune components allowing for not only a wide variety of routes of administration, but also collection of a large volume of secretions from several sites for

robust assessment of immune activity that could not necessarily be assessed in mice (Meurens et al, 2012). UPEC also presents a concern for infants and children, and swine provide an opportunity to study UPEC comprehensively through vaccination of sows and through contact tracing and observation studies between generations as sows provide passive immunity to their infants through antibodies in colostrum and milk, in contrast to mice (Robinson et al, 2014, Meurens et al, 2012).

1.3.2. *Epidemiological Assessment of Multidrug Resistance in UPEC*

As one of the most prevalent bacterial infections in humans, all forms of epidemiological studies can be important both in clearly identifying patterns of spread and for hypothesizing potential sources of outbreaks of highly resistant UPEC infections (Matsukawa et al, 2019, O'Neill et al, 1990). Extensive research has been performed on sequence typing and how it correlates to multidrug resistance (MDR) with, for, example, sequence type (ST) 131 being significantly associated with high antimicrobial resistance while resistance to ampicillin is often linked to STs 95, 127, 73, and 131 (Kot, 2019). Community sampling has further supported that MDR can be linked to O-antigen serotype and sequence typing and that, through food, water, or person-to-person contact, UPEC can easily spread throughout a community with STs with high MDR having a roughly equal distribution throughout districts within the sampling area (Matsukawa et al, 2019). As a reservoir for ExPEC, and specifically UPEC, food has been well studied with one isolate being identified in retail chicken that is indistinguishable from a strain that is known to commonly cause UTI infection, and others found in a variety of meat and ready-to-eat foods that share a high degree of genetic similarity to human UPEC strains (Vincent et al, 2010). Resistance also can have a hypothesized, rather than clear, source given circumstantial evidence similarly to when a London hospital in 1988 experienced an outbreak of highly

resistant *E. coli* and recognized that, due to the location of the Thames river, agricultural runoff was an unlikely source of the plasmids conferring the patterns of resistance that they were seeing, and that the source was more likely domestic pets like cats or dogs (O'Neill et al, 1990).

A major concern regarding the epidemiology of MDR is within the realm of hospital-acquired infections in humans. Hospitalized inpatients, as well as older patients and those who frequently require antimicrobial therapy have been found to be more susceptible to infection by antimicrobial resistant UPEC strains (Nabti et al, 2019), which may remain from patient previously housed in a particular room, poor hand hygiene by staff, medical equipment, the environment, or visitors (Wang et al, 2019). Additionally, as a result of long term treatment with antimicrobial agents, it has been found that it is not only possible for the endogenous intestinal flora to develop resistance, it is also possible for that flora to be replaced by more resistant strains, although these resistant strains can be transient and have resistance to only certain agents dependent on the medication used in treatment of the infection at hand (Guyot et al, 1999). Patients with indwelling urinary catheters in both the acute care and long-term care settings are at increased risk for UPEC infection, particularly highly resistant strains of UPEC (Hooton et al, 2010). Systemic antibiotic use has been shown to be helpful in preventing symptomatic UTI in catheterized patients, although asymptomatic bacteremia is inevitable due to adhesion to the polymer surface of the catheter by various strains of bacteria, creating an environment favorable for colonization of other pathogenic strains and species (Stamm, 1991). Prophylactic use of antimicrobial agents in the management of catheterized patients, however, has contributed to the significant discrepancy in resistant UPEC strains found in catheterized patients as opposed to non-catheterized patients, as long term use of antibiotics create selective pressure favorable for the survival of resistant bacteria in an environment already conducive to bacterial colonization

(Nicolle, 2014). Indwelling urinary catheters also pose a significant threat of UPEC infection to the male population considering their genitourinary anatomy is unfavorable for bacterial ascension to the bladder, a catheter provides a surface for increased biofilm formation as well as reduced urine flow rate to decrease the rate of bacterial elimination (Letica-Kriegel et al, 2019). As a result of the increased burden of disease caused by catheter-associated UTI (CAUTI), hospitals have developed prevention strategies related to CAUTI including consistent surveillance of urine output and appearance as well as continued functionality of the catheter system, patient biometrics, minimizing disruption of an existing catheter and drain bad to reduce risk of contamination, and minimizing the use of indwelling catheters to only when absolutely necessary and prompt removal as soon as the catheter is no longer necessary; these prevention measures have had mixed results varying by facility, often depending on implementation and adherence to the strategies (Lo et al, 2014).

1.4. **Canines as an Alternative Model for Human UPEC Infection**

Both the porcine and murine models of UPEC infection have inherent limitations that affect their fidelity as effective models. The canine presents an opportunity to examine a spontaneous model of UPEC infection that appears to act as a reservoir for a number of the ExPEC strains that are implicated in human UTI (Valat et al, 2020). As companion animals, the canine model is one that, as it shares its environment with humans, can not only be studied on its own, but it can be used to make inferences about spatial patterns and transmission in associated human populations (Damborg et al, 2009).

1.4.1. *An Overview of the Suitability of the Canine as a Translational Model for UPEC*

Domesticated pets, canines in particular, have been well established as a potential reservoir for ExPEC strains known to cause a variety of diseases in humans including meningitis,

bacteremia, and importantly, urinary tract infections by shedding bacteria in fecal matter in areas frequented by humans (Johnson et al, 2001). The UPEC strains found in canine feces have a high degree of similarity with endogenous human UPEC strains and can invade and invade and kill human T24 cells in addition to stimulating a similar immune cascade to that of human UPEC (Nam et al, 2013). This is in part due to the highly comparable immune development between humans and canines in that, by birth, the canine has a nearly fully developed innate immune system and neonatal canines develop at a similar rate to humans (Holsapple et al, 2003). Canines do have an endotheliochorial placenta that largely prevents the transfer of maternal IgG into the fetal circulation, forcing neonates to acquire maternal IgG from colostrum although those that do consume colostrum are able to reach IgG levels similar to those of adults at a rate similar to humans (Holsapple et al, 2003). Canine TLR4 has a high degree of homology with human TLR4, the amino acid sequences of which are about 70-77% similar, although canine TLR4 is missing two exons that are present in most mammalian TLR4 genes (Vaure & Liu, 2014). Canine uroplakin plaques are also nearly identical to those found on the umbrella cell layer of the urinary epithelium, allowing for similar patterns of FimH binding on the apical surface of the bladder (Wu et al, 1994). Cytokine expression in canines has been shown to be similar in humans with TNF-α and IL-6 both having increased expression in response to UPEC infection along with IL-15 initiating bladder urothelium repair and inflammatory cell recruitment (Wood et al, 2012). Although some sources contend the mouse is more phylogenetically similar to humans than the canine, an analysis of the three genomes provided evidence that the canine genome is indeed more similar to humans than are mice, with notable implications throughout the fields of biology and medicine, including support of the hypothesis that the canine can be a suitable model for human disease, potential more so than the mouse (Cannarozzi et al, 2007). Anatomically, the

canine genitourinary systems in dogs do differ from humans in that male dogs lack seminal vesicles and Cowper's glands and there are a number of differences in the musculature of the urethra causing changes in urine pressure and flow, although these changes are not significant in the pathogenesis of UPEC (Stolzenburg et al, 2002). The female dog does compare to female humans in their shortened urethra and the anatomy of the bladder and bladder neck in both sexes had high degrees of similarity (Augsburger et al, 1993, Stolzenburg et al, 2002). Due to similarities between the urethras in both sexes in dogs and humans, catheterization procedures can be readily compared as well, however, as can catheter-associated urinary tract infections, especially among hospitalized populations (Smarick et al, 2004).

1.4.2. *Epidemiological Potential of the Canine Model*

Approximately 14% of canines will experience at least one urinary tract infection throughout their lifetime, with UPEC predominating as the causative agent, qualifying the species as a spontaneous model for UTI in humans (Thompson et al, 2011). Similarly to humans, female dogs as well as dogs of advanced age are at increased risk for urinary tract infection (KivistÖ et al, 1977). Concentration of bacteria in urine required to diagnose UTI, in CFU/ml, is also consistent between dogs and humans, allowing the two to be reliably compared based on collection of voided or manually collected urine samples (KivistÖ et al, 1977). As companion animals that develop UTIs spontaneously, a canine model can be used to study descriptive UPEC epidemiology. The presentation of a canine to a veterinary office or hospital results in the creation of a medical record and often a frozen isolate of the causative agent of infection, from which retrospective data can be collected and molecular experimentation can be done (Seguin et al, 2003). The availability of outpatient canines in a clinical setting also allows for real-time collection of urine samples from affected canines to be screened for UPEC and further analyzed

for an outcome of interest such as antibiotic resistance and sharing of data between clinical sites collecting data simultaneously allows for relatively rapid epidemiological surveillance over a large region (Cooke et al, 2002). This surveillance allows for identification of the effects of MDR as well as potential reservoirs, with feces having been identified as one of the major reservoirs for MDR canine UPEC; highly MDR UPEC strains known to cause UTI in canines have been isolated from canine feces and more significantly, some of these strains have been shown to share phylogenetics and virulence traits with human ExPEC strains, supporting the theory that the canine is a possible UPEC reservoir and supporting the canine as a model for human disease (Johnson et al, 2001, Johnson et al, 2005). Canines for sample collection can also be available as hospital inpatients, similarly to humans, with those hospitalized for greater than three days and having received prior antimicrobial therapy being at higher risk for MDR UPEC infection, which is consistent with human trends showing antimicrobial therapy and length of hospital stay as MDR UPEC risk factors (Gibson et al, 2008). For hospitalized canines, nosocomial infections also pose a similar and significant risk, as with humans. In one study, 16.5% of canine rectal swabs and 4.1% of hospital staff rectal swabs confirmed MDR ExPEC during a 12-month prospective study in Australia (Sidjabat et al, 2006). The canine model can be incredibly useful, however, in drug and vaccine development and is currently the most common non-rodent animal used in preclinical trials with a high degree of concordance with the results of clinical trials, indicating that canines can relatively accurately predict effectiveness and/or toxicology results in humans (Olson et al, 2000). This could lead to the canine being introduced into the drug development process for UPEC vaccination or for novel antimicrobials targeting ExPEC species, given the similarities between canine and human UPEC strains.

1.4.3. *Future Directions of the Canine Model in Biomedical Research*

The canine has become a major model of interest in the study of genetic and developmental diseases in humans, spurring interest in robust characterization of the genetic variation that is implicated in disease physiology (van Steenbeek et al, 2016). As such, the Dog 10K genomes project aims to collect whole exome and whole genome sequencing data as well as phenotypic data from canines internationally to better understand inter-breed variation, the relationship between phenotype and genotype, and susceptibility to disease on a genetic level while providing spatial information on genetic diversity (van Steenbeek et al, 2016). While this project is intended to identify loci implicated in diseases known or purported to be caused by genetic aberrations, mapping of the whole genome and its diversity could prove helpful in modeling UPEC pathophysiology since the use of whole genome sequencing methods have been shown to be more sensitive than SNP mapping methods (Cooke et al, 2016), which for example, could aid in identifying spatial variation or inter-breed variation in the binding site of UPIb that would theoretically alter FimH binding and adhesion, if this kind of variation were to exist. As previously described, the dog genome has previously been well described on its own and is highly homologous to humans; within breed homogeneity and analogous immune physiology have allowed for the study of canines to be used as models of heritable human diseases, particularly as a spontaneous model for cancer and autoimmune disease (Shearin & Ostrander, 2010). The comparability of the immune system does provide an opportunity for the canine to be used as a model for infectious disease in the presence of a modulated immune response given the advanced knowledge of genetic loci implicated in specific immune deviations (Shearin & Ostrander, 2010).

The principles of biomedical research ethics have long guided the use of animals in research, and particularly the canine, with the canine being one of the least commonly used animal models when compared to other species for all research purposes (Baumans, 2004). The EU and the US have both adopted regulatory requirements for the use of laboratory animals, guided by ethical guidelines including reducing the number of animals used, finding alternatives such as cell culture or modeling, and reduction of discomfort and providing appropriate housing and care (Baumans, 2004, Matthiessen et al, 2003). When searching for replacement models, guidelines have been set to seek species with the least neuro-physiological activity and pain sensitivity appropriate for the experiment being performed (Matthiessen et al, 2003). These regulations become complicated for the use of the canine experimentally because, although the model benefits from a degree of analogous physiology and anatomy as well as genomic homology, in many cases another model can be used because, in mice, for instance, pain sensitivity is much lower in mice than dogs; in fact, dogs are among a small group of species that require special ethical review in Europe due to their neurophysiology and public perception of their use as a companion animal (Matthiessen et al, 2003, Hansen, 2003). Animal rights advocacy groups in particular place pressure on the scientific community to correct perceived animal welfare issues and to reduce animal experimentation, and although some groups are extreme in their advocacy, the communication of ethical research and mitigation of animal suffering is as paramount to successful research as the data collection itself, particularly in companion animal models (Matthiessen et al, 2003).

1.5. **Conclusions**

Considering the major burden UPEC infection places on healthcare systems worldwide, research into its pathophysiological mechanisms and transmission are critical for more adequate

control and prevention. Mice have long served the biomedical community by providing a high throughput, low cost method to test molecular mechanisms of disease and the porcine model has been able to fill many gaps left by mice as it relates to anatomical analogy with both providing a high degree of genetic similarity to humans and experimental reliability. If the field of UPEC research is to advance, however, a new model that is both physiologically analogous to humans as well as epidemiologically relevant must be introduced, which is why we propose the canine. With urinary anatomy and physiology similar to that of a human in size and histology and relevant immune components comparable to humans and current models, the dog and isolates obtained from UTIs in canines can provide relevant mechanistic information about UPEC pathogenesis. Additionally, in the porcine model, isolates can be collected and stored from spontaneous UTIs, but the canine provides the unique opportunity of noninvasive collection of isolates and surveillance of clinical presentation in the clinical setting, which can be directly compared to clinical testing and treatment in humans. One major limitation of the canine model is that much of the experimental UPEC data that could be collected in a dog could be collected in another model, such as the mouse, so a proposition for use of the canine model will be met with scrutiny from the public and regulatory authorities. Since live canines have not been used widely to model infectious disease in humans experimentally, more work needs to be done to build a body of research to establish the canine as the gold standard in this field, although we believe that in terms of epidemiology and studies not involving live subjects, the current research supports use of the canine as an alternative model for human UPEC infection.

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Chapter 2 – Associations Between Genetic Diversity in *fim*H, Multidrug Resistance, and Virulence in Canine Uropathogenic *Escherichia coli*

Abstract

Urinary tract infections (UTIs) are among the most common bacterial infections, affecting nearly half of all women and about 14% of canines at least once throughout the lifetime. The primary etiologic agent of canine and human UTIs is *Escherichia coli*, strains of which originate from the intestinal tract and appear to have a specific set of specific virulence factors adapted for the urinary tract. The Type 1 fimbriae contains a tip adhesin, FimH, which is critical for attachment to the urinary epithelium and also has a role in cell invasion. Previous studies in human clinical isolates have characterized genetic diversity and found associations between *fim*H and biofilm formation. Biofilm formation in the urinary tract increases the potential for treatment failure and can increase the likelihood of recurrence of symptomatic UTI. In this study we evaluated 206 UPEC isolates collected from clinically affected canines for *fim*H genetic diversity, hemolytic activity, multidrug resistance (MDR), UTI recurrence, and the potential for isolates to form biofilm. Our study found *fim*H in 197 isolates (95.6%), representing 72 unique *fim*H types. Those types were subsequently clustered by genetic similarity. A significant association was observed between *fim*H type and hemolysis ($p<0.0001$), MDR ($p<0.002$), biofilm formation in M9 media ($p=0.01$), and biofilm formation in urine ($p=0.003$). This study has found *fim*H to be genetically diverse and have a number of potential SNP-dependent roles in UPEC phenotype. Our findings suggest that knowledge of *fim*H type in the clinical setting could aid clinicians in forming a more tailored treatment plan to accurately treat UPEC based on both genotypic and phenotypic information.

1. Introduction

Urinary tract infections (UTIs) are one of the most common types of infections encountered in the primary care setting, affecting almost half of all adult women at least once throughout the lifetime (Tan & Chlebicki, 2016). Over 80% of UTI are caused by *E. coli*, often originating from the gastrointestinal tract (Klein & Hultgren, 2020). These *E. coli* strains from the gastrointestinal tract are believed to be unique from other intestinal strains in their virulence and phylogenetic profiles and are often referred to as extraintestinal or uropathogenic *E. coli* (ExPEC or UPEC; Terlizzi et al, 2017). Uncomplicated UTI are also common in canine medicine with approximately 14% of canines presenting with UTI throughout the lifetime, even though up to 95% of UTI symptoms in dogs remain subclinical (Thompson et al, 2011). It has also been found that the UPEC or ExPEC strains implicated in UTI pathogenesis in canines and in humans have similar virulence patterns, indicating that canines could be a potential reservoir for human disease (Johnson et al, 2000).

A number of virulence factors are known to be shared between the *E. coli* associated with UTIs in both humans and canines. One such factor is α-hemolysin which is known to cause damage to the urinary epithelium, promoting inflammatory activity(Klein & Hultgren, 2020, Johnson et al, 2000). Some UPEC strains are also known to form biofilms on the urothelial surface as well as intracellular bacterial communities (IBC) within urothelial cells, increasing urinary fitness (Klein & Hultgren, 2020). Additionally, a number of genetic mechanisms in UPEC strains have contributed to an increase in multi-drug resistance, particularly to fluoroquinolones, 3rd generation cephalosporins, and β-lactams containing clavulanic acid (Thompson et al, 2011).

It is known that Type 1 fimbriae play a critical role in UPEC attachment and colonization by binding to mono-mannose oligosaccharides in the bladder (Wright & Hultgren, 2006). At the tip of the Type 1 fimbria is the FimH adhesin which contains an acidic distal mannose binding pocket that acts to perform its attachment functions (Wright & Hultgren, 2006). It is also known that biofilm formation is positively associated with the presence of the FimH adhesin in canine UPEC isolates (Gilbertie et al, 2020). Further, it has been discovered that *fim*H sequencing can be used to subtype UPEC and single nucleotide polymorphism (SNP) analysis has been effectively used to phylogenetically characterize a large clinical sample of *E. coli* isolates (Tartof et al, 2007, Dias et al, 2010).

Management of the growth and understanding the virulence of UPEC in a clinically affected human or canine patient is critical for reduction of healthcare costs and prevention of recurrent UPEC infection. Although previous studies have shown that FimH is genetically diverse, these studies have used small samples and clinical studies have used both UPEC as well as other ExPEC to assess this diversity (Tartof et al, 2007, Dias et al, 2010). Additionally, certain phenotypic characteristics have been shown to be associated with the presence of FimH (Gilbertie et al, 2020), but the extent of these associations has yet to be sufficiently explored in the context of the genotypic diversity of FimH. Thus, the aim of this study is to assess, using a large random sample, the extent of genetic *fim*H variation in uropathogenic *E. coli* collected from clinically affected canines. Further, we aim to identify associations between *fim*H type and phenotypic outcomes including virulence and biofilm formation.

2. Materials and Methods

2.1. Sample Collection and Isolate Preparation

Escherichia coli isolates (n = 206) were obtained from the Microbiology and Molecular Diagnostic Laboratory at NC State University Veterinary Hospital (Raleigh, NC) from canine patients clinically presenting with UTIs between 2012 and 2020. Specimens were aseptically cultured onto Trypticase Soy Agar with 5% Sheep blood (BAP; Thermo Scientific™ Remel™, Waltham, MA) and MacConkey Agar (Thermo Fisher Scientific, Waltham, MA) and enriched in thiogylocate broth (Thermo Scientific) and incubated for up to 72 hours at 37°C according to laboratory standard operating procedures. Isolates were confirmed by biochemical (Sensititre, Thermo Fisher) or MALDI-TOF (Vitek MS, Biomerieux; Marcy-l'Étoile, France) to be *E. coli*. Isolates were preserved in Tryptic Soy Broth with 15% Glycerol at -80°C for further analyses. The clinical presentation, collection method, recovered *E. coli* concentration, and antimicrobial susceptibility results were all stored in the patient's electronic medical record. Recurrence was determined through assessment of medical records to determine whether the canine from which the isolate was collected has had more than two infections in six months or three infections in a year. Additionally, we assessed recurrence based on the likeness of the etiologic agent, regardless of frequency. If the etiologic agent of all infections over the time period was UPEC, the dog was considered to have recurrent UTI, but if one or more bacteria was not UPEC, for the purposes of this study, it was not classified as recurrent. Cases involving pyelonephritis were also classified as non-recurrent as well given the differing mechanisms between upper and lower urinary tract infections.

For this study, isolates were removed from the freezer and streaked for isolation onto a BAP and incubated at 37°C overnight in aerobic conditions. Inclusion criteria included

specimens obtained from the urine, urethra, bladder, and urinary catheter. Samples were excluded from analysis in this study if the canine patient had more than a single organism recovered from the culture, if the CFU/ml was inadequately described, missing, or <1,000 CFU/ml, and if the source of collection was one other than the acceptable sites listed above, regardless of the patient's UTI status. Samples were also excluded if, during data collection, a change in phenotype was observed and an alternative stock of the isolate was not available. After restreaking, hemolysis was observed by visual assessment on BAP and recorded. For the purposes of this study, hemolysis was determined on a binary scale (present/absent) and hemolysis type was not a factor.

2.2. Antimicrobial Susceptibility

Antimicrobial susceptibility data was obtained directly from the Microbiology and Molecular Diagnostic Laboratory for all isolates; the assay was conducted during clinical assessment using abbreviated microbroth dilution (Sensititre), and interpretations were based on Clinical and Laboratory Standards (CLSI) guidelines. Multidrug resistance (MDR) was considered resistance to >3 unique antimicrobial classes.

2.3. Biofilm Biomass

Biofilm biomass was determined for all isolates using a previously described protocol (Gilbertie et al; 2020) with some modifications. A 10µl suspension of a 0.5 McFarland solution in phosphate buffered saline (PBS) was added to each well of a 96-well tissue culture treated U-shaped bottom plate (Corning, Glendale, AZ) containing 90µl of either M9 minimal media (Sigma-Aldrich, St. Louis, MO) or filter-sterilized canine urine in three separate, randomized pools with a minimum of three unique dogs represented in each pool. Free-catch canine urine was collected from randomly selected dogs and biochemical testing was performed by urine

dipstick after pooling to ensure the pooled urine represented that of a healthy canine. Three replicates of every isolate were added to each plate and three independent researchers performed the protocol for each isolate. Biofilms were incubated at 37°C for 24 hours under microaerophilic conditions. Additionally, control strain K12 (American Type Culture Collection 29425, Manassas, VA) was prepared and included on each plate. Wells containing uninoculated media were included as a negative control. After incubation, plates were washed with 150µl deionized water, stained with 200µl 2% crystal violet (Becton Dickinson, Franklin Lakes, NJ) in deionized water, eluted with 200µl 100% ethanol, and absorbance was measured at 570nm with the optical density unit (ODU) being recorded and replicate values being averaged. The average ODU reading for each isolate was normalized by subtracting the average ODU value of the wells containing blank media. The normalized values for each researcher were then averaged for the final biomass value for each isolate.

2.4. DNA Extraction and *fim*H PCR

DNA was extracted from all isolates grown on BAP using the DNeasy Blood & Tissue kit (QIAgen, Valencia, CA) according to manufacturer instructions. DNA purity and yield was determined following extraction by placing 1µl of extracted DNA onto a NanoDrop 2000 Spectrophotometer (Thermo Fisher) and analyzing. DNA extraction was performed again if the A260/280 value for an isolate was less than 1.75 or greater than 1.93 or if the extraction procedure yielded less than 18ng/µl of DNA. All samples were aliquoted to a concentration of 18ng/µl and stored at -80°C.

A protocol for PCR detection of *fim*H was optimized using GoTaq Colorless MasterMix (Promega, Madison, WI, USA) and previously described *fim*H primers (Dias et al, 2010). Primers were optimized at a concentration of 7.5X and loading DNA was optimized to a

concentration of 18ng/µl. The PCR reaction consisted of denaturation for 2 minutes at 95°C, followed by 30 cycles of 95°C for 1 min for denaturation, 56.5°C for 1 minute for annealing as optimized using a gradient PCR, and 72°C for 50 seconds for extension, followed by 72°C for final extension. PCR product was visualized for the presence of the *fim*H gene with a product size between 800 and 848 base pairs using the Qiaxcel Advanced (QIAgen, Valencia, CA) capillary electrophoresis system. Sequenced PCR product was stored at 4°C for further analysis.

2.5. *fim*H Sequencing and Analysis

Forward and reverse Sanger sequencing of the *fim*H gene was performed by the NC State University Genomic Sciences Laboratory (Raleigh, NC, USA). An aliquot of 6µl of PCR product was placed into a semi-skirted 96-well plate (Genesee Scientific, San Diego, CA) with 12 wells for each *E. coli* isolate. Six replicates for each isolate were sequenced using the forward primer and six were sequenced using the reverse primer (Primers: Dias, et al, 2010). Sequencing results were analyzed using Geneious Prime 2020.2.2 (https://www.geneious.com). The six forward reads for a given isolate were aligned using the built in Geneious alignment program and the same was performed for the six reverse reads. A consensus sequence was created using these two alignments. DNA sequences were checked against provided chromatograms and manual base calls were performed as necessary. The resulting 489bp sequence was entered into the Center for Genomic Epidemiology FimTyper Database to provide a *fim*H type based on previously described genotyping methods (Roer et al, 2017). Nucleotide sequences were compared against the publicly available sequence of strain K12 for analysis. Nucleotide sequences were also translated using Geneious Prime into protein sequences of 163 peptides in length for additional analysis.

2.6. Statistical and Bioinformatic Analysis

Statistical data analysis was performed using JMP Pro 15.2 software (SAS Institute Inc., Cary, NC). Summary statistics were collected for continuous variables including sample means and standard deviations as well as interquartile ranges when appropriate. For analysis of significance, 95% confidence intervals (CI) were also calculated and in all cases $p<0.05$ was determined to be statistically significant. The Shapiro-Wilk test was used to assess the normality of continuous data and as none of the data proved to be normal, all data was further analyzed using the 1-way nonparametric Kruskall-Wallis test. To assess for individual differences between *fim*H types or groups, a paired Wilcoxon signed-rank test was performed for each pair of observations. Multivariate correlation analysis was performed to assess relationships between outcomes within individual groups or samples. When appropriate, least squares regression was performed to assess for goodness of fit when comparing data sets.

GrapeTree v1.5 (Zhou et al, 2018) was used to produce a minimum spanning tree using MSTreeV2 to easily visualize clustering and scaling of prevalence within the distribution. Multiple sequence alignment of 197 fimH sequences was performed using MUSCLE v.3.8.31 (Edgar, 2004) and a phylogenetic tree was inferred with the best-fit nucleotide substitution model using ModelFinder (Kalyaanamoorthy et al, 2017) and IQ-TREE v.1.6.12 (Nguyen et al, 2015). Finally, the constructed maximum-likelihood tree was annotated and the phylogram was visualized using ITOL v.6 (Netunic et al, 2021).

3. **Results**

A total of 206 isolates were included in the complete analysis of phenotypic and genotypic characteristics of UPEC from canine sources. Retrospective analysis of medical records identified 80 (38.8%) as recurrent isolates. This number did not include any isolates with

a history of causing pyelonephritis or which were isolated from dogs with a history of previous infections with etiologic agents other than UPEC. On restreak from frozen stock, 88 of 206 (42.7%) hemolyzed BAP media. Although β-zone hemolysis is expected in hemolytic *E. coli*, the degree and type of hemolysis from the UPEC samples analyzed differed so hemolysis was recorded as a positive/negative result only. A total of 79 (38.3%) isolates were positive for MDR. Resistance was most seen in β-lactam antibiotics, with >90% of the analyzed isolates showing resistance to ampicillin and/or amoxicillin-clavulanic acid. The next most frequent resistance was seen in fluoroquinolones, specifically ciprofloxacin, and 3rd generation cephalosporins. Specific analysis was complicated by variation in antimicrobial susceptibility testing, leading to certain antibiotics being tested against some isolates but not others, likely due to these isolates having been collected over the course of nine years. Amikacin was a commonly tested aminoglycoside and zero isolates showed full resistance to it with only two isolates showing intermediate resistance. Biofilm formation in M9 media resulted in a mean OD_{570} value of 0.301 and a median of 0.1445 with an interquartile range (IQR) of 0.301. Biofilm formation in canine urine had a mean result of 0.429, median of 0.257, and IQR of 0.393, indicating an overall increase in biofilm formation in canine urine compared to M9 media.

Of the isolates included, 197 were found to contain the *fim*H gene (95.6%); *fim*H was absent in 9 (4.4%) subsequently referred to as *fim*H-. Of those isolates positive for *fim*H, 8 isolates (4.1%) were identified as newly discovered *fim*H types not previously recognized by the typing database. One additional newly discovered *fim*H type was excluded from analysis due to contamination during analysis without a pure stock. Within the population analyzed, 72 unique *fim*H types were identified. Of these 72 types, 45 *fim*H types were represented by only one isolate (62.5%). Although only 16 *fim*H types out of 72 unique types (22.2%) had three or more

isolates identified during the sequencing process, those 16 types make up 63.1% of the biobank (130/206). The most prevalent *fim*H type was *fim*H9 with 33 isolates out of 206 (16%). The distribution of *fim*H types is visualized in Fig. 2.1, in which larger, colored nodes indicate more prevalent types with smaller, white nodes indicate less prevalent nodes. Distance between nodes represents relative genetic difference. The full distribution of these types is presented in Appendix A.

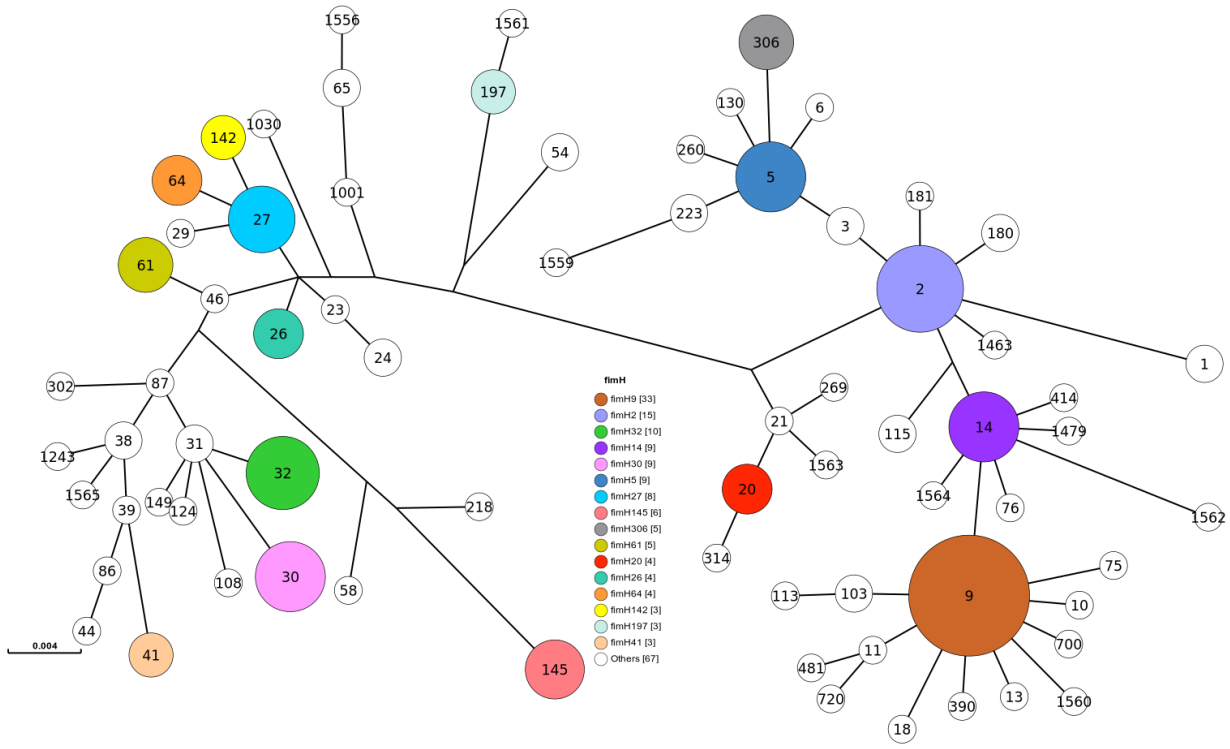

Figure 2.1. Distribution of *fim*H types recovered from canine UPEC isolates as visualized by GrapeTree Size of each node is relative to the prevalence of the corresponding *fim*H type and the colored nodes correspond to the 16 most prevalent types. Length of branches and distance between nodes is relative to genetic distance between two nodes.

When *fim*H type was assessed against a phenotype for hemolysis, biofilm, and MDR we observed several significant associations. A significant association was found between *fim*H type and MDR (p=0.002). Describe what that association is. Additionally, A strong association

between hemolysis and *fim*H type was seen ($p<0.0001$). A significant association was also seen between biofilm formation in M9 minimal media and *fim*H type ($p=0.01$). Paired analysis showed that specific *fim*H types frequently exhibited significantly ($p<0.05$) increased or decreased activity for each phenotype when compared to other types and the results of the paired analysis are summarized in Table 2.1.

Table 2.1. Paired Wilcoxon signed rank test results per phenotype compared against *fim*H type

Phenotype	Increased Activity	Decreased Activity
MDR	*fim*H-, *fim*H30	*fim*H14
Hemolysis	*fim*H2, *fim*H9	*fim*H-, *fim*H30, *fim*H32
Biofilm formation in M9	*fim*H2, *fim*H9	*fim*H-
Biofilm formation in Urine	*fim*H9	*fim*H-, *fim*H2

No significant associations were found between recurrence and *fim*H type or between recurrence and other phenotypic findings. When MDR was assessed against biofilm formation in M9, it was found that negative MDR is associated with higher biofilm formation ($p<0.0007$). Interestingly, when the analysis was reversed and biofilm formation in M9 was assessed against MDR, no significant association was found. Additionally, it was found that negative MDR is also significantly associated with positive hemolysis ($p<0.0007$). A summary of the overall findings of sequencing, phenotypic assessments, as well as phylogeny is visualized in Fig. 2.2 with colored sections corresponding to the most prevalent *fim*H types, similarly to Fig. 2.1. According to Bayesian information criteria, TIM3e+R2 was chosen as the best fit model with cross-validation for development of the cladogram. Among a total number of 489 nucleotide sites, 64 were single-nucleotide polymorphism (SNP) sites.

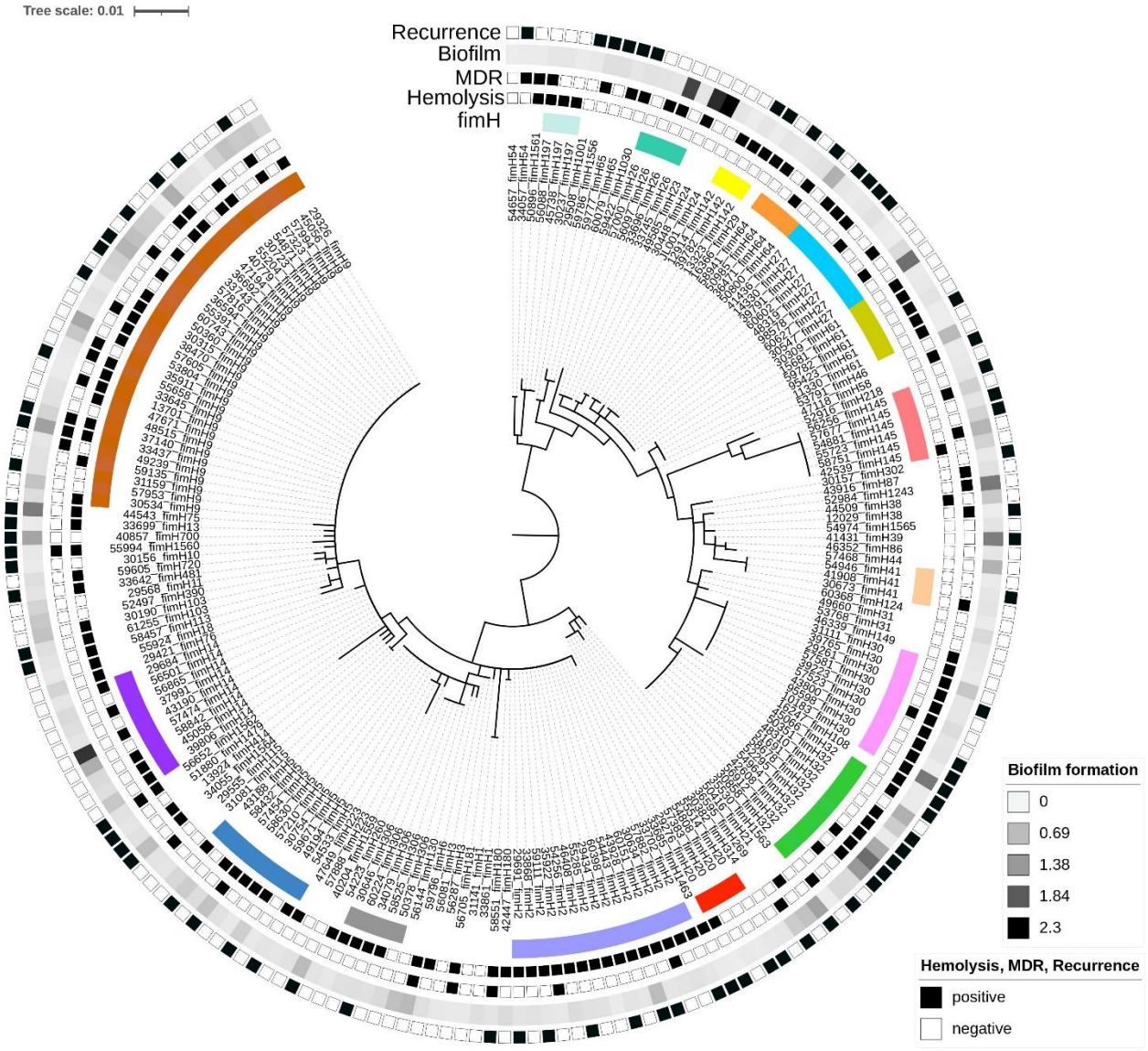

Figure 2.2. Phylogenetic profile of 197 isolates with phenotypic characteristics Cladogram representing phylogeny of the 197 isolates containing *fim*H with profile of phenotypic outcomes assessed in this study. Black squares represent presence of hemolysis, MDR, or recurrence and white squares represent absence. Biofilm formation is represented as a grayscale heatmap. Colored sections indicate the 16 most prevalent *fim*H types. Tree scale of 0.01 is equal to 3.3 SNPs.

Following genotypic assessment of sequencing results and visualization, it was found that *fim*H types could be sorted into 13 discrete clusters based on genetic similarity. It was also found that when nucleotide sequences were translated, *fim*H types could be further grouped based on matching 163-residue amino acid sequences. Many *fim*H types that were clustered based on

genetic similarity were grouped differently when translated or were not grouped at all due to matching with one or more genetically distant types or due to having a single, unique amino acid sequence when translated.

The largest group of matching amino acid sequences, ProteinA, contained 129 isolates (62.6% of isolates) representing 30 unique *fim*H types (41.6% of types). Within this amino acid sequence group, 11 out of the 13 nucleotide sequence clusters were represented (84.6%). Conversely, the largest nucleotide sequence cluster, *fim*H(A), contained 47 isolates and was split into 11 discrete amino acid sequence groups including six isolates that had unique amino acid sequences and could not be placed into a matching cluster. A full breakdown of clustering for nucleotide-based clusters and protein sequence-based groups can be found in Appendix A.

All of these clusters were also assessed against the phenotypic outcomes of interest and significant associations were found between nucleotide sequence clustering and biofilm formation in M9 ($p=0.01$), hemolysis ($p<0.0001$), and MDR ($p<0.0001$) although no significant association was found with recurrence. Additionally, no significant association was found when phenotypic outcomes were assessed against amino acid sequence clustering. The results of pairwise analysis are summarized in Table 2.2.

Table 2.2. Paired Wilcoxon signed rank test results per phenotype compared against nucleotide sequence cluster

Phenotype	Increased Activity	Decreased Activity
MDR	H, M, N	A, B, C, D, E
Hemolysis	A, B, D, E, F	C, G, H, J, K, L, M, N
Biofilm formation in M9	A	M, N
Biofilm formation in Urine	A	D, F, M

On multivariate analysis, *fim*H- as well as nucleotide sequence clusters M and N all showed a significant negative association between MDR and biofilm formation in M9 media (p=0.0003, p=0.003, p=0.02, respectively), indicating that positive MDR could lead to a decrease in biofilm formation in isolates in these groups. Nucleotide sequence cluster E showed a significant positive association between MDR and biofilm formation in M9 (p=0.0016), as well as a significant positive association between MDR and hemolysis (p=0.03) and a significant negative association between hemolysis and biofilm formation in M9 (p=0.02). Additionally, although protein sequence clustering had not shown any previous significant associations, protein cluster A showed a significantly negative association between MDR and hemolysis (p=0.013).

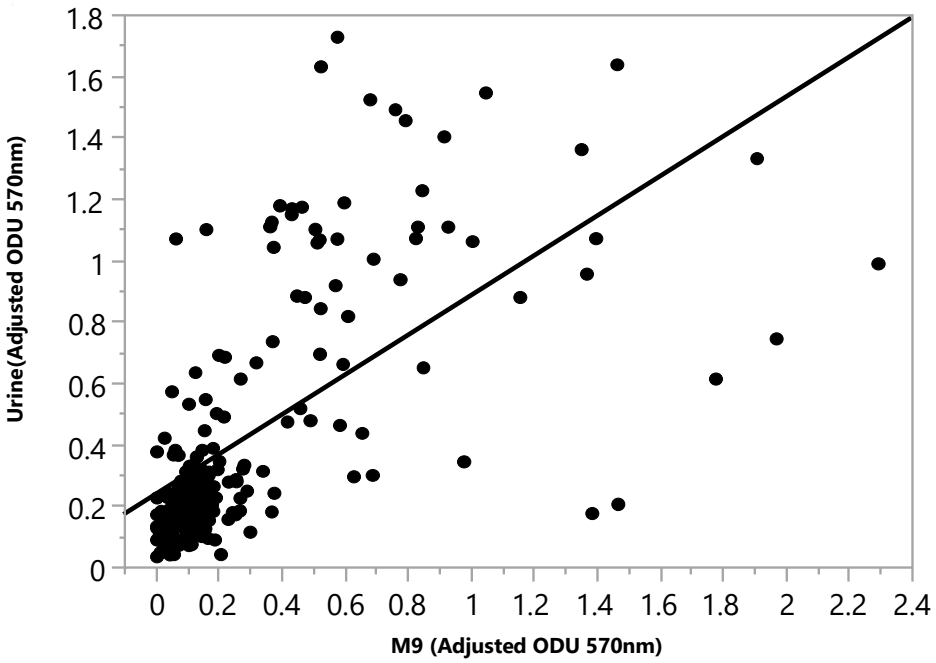

Figure 2.3. Linear regression model of UPEC biofilm formation data in M9 and urine
Biofilms were grown in both M9 minimal media and in pooled canine urine, with three replicate pools with at least three canines per pool. Biofilms grew for 24 hours at 36°C then were stained with crystal violet and washed. Biofilm data points were paired and plotted then linear regression was performed with regression line shown in plot (R^2=0.41).

Biofilm formation ability was also assessed in pooled canine urine. No significant correlation was found when biofilm formation in M9 was compared to biofilm formation in urine (R^2=0.41, Lack of Fit $p < 0.0001$); this effect can be seen in Fig. 2.3. A t-test for paired samples was performed to compare the two media types and the mean difference of 0.1288 between the two media types was found to be significant ($p<0.0001$), which can be seen in Fig. 2.4. Additionally, biofilm formation in pooled urine showed strong significant associations with both *fim*H type as well as nucleotide sequence clustering (p=0.003, p=0.0006, respectively). Least squares regression was performed using both *fim*H type and nucleotide sequence cluster to analyze suitability of the two media types. In both cases, both grouping methods showed statistically significant effects on biofilm formation in M9 and urine. Interestingly, the p-values for urine for both analyses were smaller than those for M9 (p=0.0046 vs p=0.025 for *fim*H type; p=0.0061 vs p=0.01 for nucleotide sequence clustering).

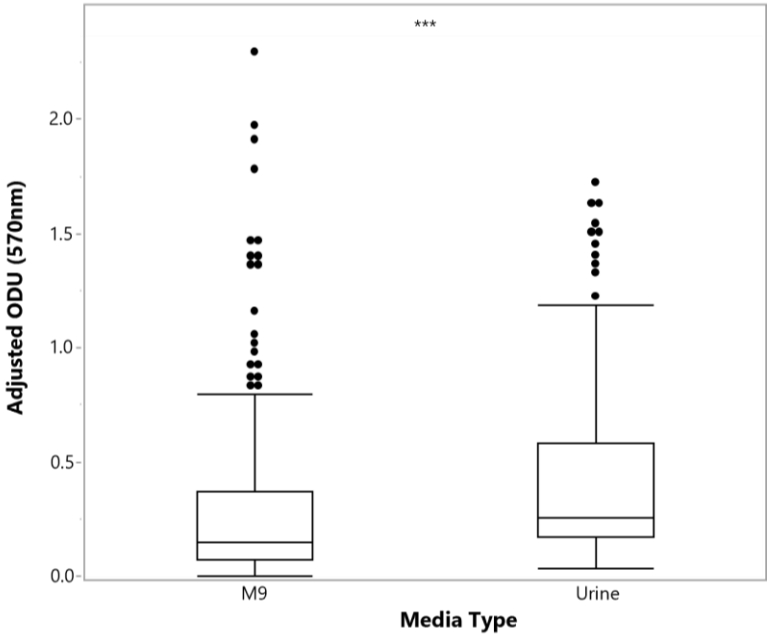

Figure 2.4. Boxplot of adjusted ODU(570nm) values for canine UPEC biofilm formation results in M9 and urine Biofilms were grown as previously described and were analyzed. Data sets were compared against each other rather than being paired and means were assessed via Wilcoxon signed rank test to determine whether the two sets were significantly different ($p<0.0001$).

4. Discussion

Urinary tract infections, both uncomplicated and catheter-associated, are most frequently caused by *E. coli* infection (Foxman, 2010). Both humans and canines are highly likely to experience at least one UTI throughout their lifetime, with up to 4.5% of canines experience recurrent UTI (Tan & Chlebicki, 2016, Thompson et al, 2011). It is known that the Type 1 fimbria, particularly the FimH adhesin, is critical for attachment to the urothelial surface to prevent bacterial elimination as a result of urine flow and can aid in invasion through a zipper-like mechanism into individual cells (Stærk et al, 2016, Wright & Hultgren, 2006). Within the population sequencing performed herein revealed 72 unique *fim*H types in a sample of 206 canine urine isolates (34.9%) which surpasses previously published results that found 27 unique *fim*H types in a sample of 165 isolates from multiple clinical sample types (Dias et al, 2010). Additionally, *fim*H was absent in only 4.4% of our isolates whereas it was absent in 8% of those in the previous study (Dias et al, 2010) which could be due in part to the fact that samples in the current study were only collected from urine from canines with uncomplicated UTI compared to a variety of sources including blood, respiratory samples, and catheter tips. The current study found nine isolates that had not been previously identified by the FimTyper database, which is consistently updated (Roer et al, 2017), although it is not clear whether these isolates are unique to canines or whether further studies with large sample sizes in either canines or humans would continue to identify new *fim*H types.

Previous studies have supported the hypothesis that FimH plays a role not only in attachment and invasion but also in virulence and multidrug resistance, and our study aimed to elucidate those associations further. Our results further support previous findings that FimH is significantly associated with biofilm formation in M9 media and that isolates lacking FimH are

poor biofilm formers (Gilbertie et al, 2020). Our study further shows that *fim*H2 and *fim*H9 are particularly severe biofilm formers when compared to other *fim*H genotypes in M9. These same types were shown to be significantly associated with positive hemolysis activity but were not associated with MDR, while isolates lacking FimH were significantly lacking in hemolysis activity and were significantly associated with the presence of MDR, supporting the theory that UPEC strains select for either virulence or survival (Gilbertie et al, 2020). Analysis based on *fim*H type was complicated by the fact that, with many unique types, many types were represented by a small number of isolates for analysis. Further studies could increase the total sample size to potentially increase the number of samples per *fim*H type, although it is not possible to select for *fim*H type when collecting isolates. This could potentially lead to an even larger distribution of types with few isolates per type.

Given the limitation of comparative sample size, samples were clustered based on genetic similarity, which increased the number of isolates per comparison group. In general, Cluster A exhibited patterns consistent with virulence-associated strains whereas clusters M and N exhibited patterns consistent with strains that prioritize antimicrobial resistance. On multivariate analysis, cluster E did show a positive association between MDR and biofilm formation in M9 as well as between MDR and hemolysis, which is not explained by the theory of virulence versus survival. Interestingly, when clustered, all clusters were represented in either the increased or decreased activity sections of the pairwise analysis of hemolytic activity. Additionally, eight out of 13 clusters were represented in the pairwise analysis for MDR. This could indicate that these genetically similar clusters share one or more SNPs that heavily affect these phenotypic traits more than the full *fim*H sequence does. Additional SNP analysis and mutant studies could be done to determine which exact SNPs are shared between these clusters that could cause these

associations and a larger sample size could further refine the clustering process to identify the most genetically similar clusters using the most *fim*H types available.

Our study assessed recurrence by assessing canine medical records to evaluate whether the isolate in question was potentially the result of a failure of treatment for a previous UTI or a resistant organism. Previous data would indicate that MDR plays a role in recurrence of UPEC, particularly in UPEC (Thompson et al, 2011); however, our data showed no significant association between MDR or recurrence. In fact, our data showed no significant association between recurrence and any other phenotypic outcome or between *fim*H type or nucleotide sequence cluster. Additionally, we translated nucleotide sequences into 163-residue amino acid sequences and grouped them based on identical sequences. The amino acid sequence that was created encoded for the mannose binding pocket of the FimH binding pocket and we hypothesized that alterations in this sequence would alter UPEC phenotype, particularly biofilm formation. This grouping method, however, showed no significant association with any phenotypic outcomes on Wilcoxon signed rank analysis. Further studies could assess whether variation in this amino acid sequence would alter affinity for mannose which could have implications for cellular attachment or invasion, but that is beyond the scope of this study. What was found when amino acid sequence clustering was assessed was within protein group A, positive hemolysis activity was significantly associated with negative MDR. This could be seen as protein group A contained more than half of the analyzed isolates and could be used to describe an overall effect for the population. As nucleotide sequence clustering did result in significant changes to phenotype, we can further hypothesize that *fim*H SNPs play a role in *fim*H replication or transcription, although more work would have to be done to determine what that role is.

Previous research has shown that biofilm formation for *E. coli* varies based on method and media used (Naves et al, 2008). While M9 minimal media has been proven to be an effective media choice for UPEC isolates to distinguish between minimal biofilm formers and extreme biofilm formers (Gilbertie et al, 2020), it was also decided to assess formation in pooled canine urine to simulate the clinical environment of interest. When we compared biofilm outcomes when isolates were grown in M9 minimal media or pooled canine urine, the substrate type was significantly different. Additionally, no significant correlation or covariance was found between the two sets. Further, it is notable that when tested in M9, *fim*H2 was frequently seen as a high biofilm former as compared to other *fim*H types whereas in urine, *fim*H2 was seen as a lower biofilm former compared to other types. A lack of relationship between the two suggests M9 media may poorly model for biofilm formation *in vivo*. As such, least squares regression was run with biofilm formation against both *fim*H type and nucleotide sequence clusters. For both media types, the comparator group showed a significant effect, although the associations seen in urine appeared stronger for both *fim*H type ($p=0.0046$ vs $p=0.025$) and for sequence clusters ($p=0.0061$ vs $p=0.01$). Since all results were statistically significant, we can infer that both substrates are valid measures of biofilm formation but given the knowledge that the data sets are statistically different from each other, we can use these differences to infer that urine potentially produces results closer to what would be seen *in vivo*. One limitation that might have impacted this biofilm formation study is that some of the isolates in the biobank were collected as far back 2012, meaning that several isolates could have undergone a number of freeze-thaw cycles since collection which potentially has altered true biofilm formation although this effect would likely be seen in both M9 and urine.

5. Conclusion

In conclusion, the FimH tip adhesin is a genetically diverse protein that is significantly associated with biofilm formation in both M9 media and urine, hemolysis activity, and multidrug resistance. These associations have been found to be affected by the genetic character of *fim*H or by groups of genetically related *fim*H genes. These *fim*H dependent associations appear to further differentiate UPEC strains into virulent strains or resistant strains which can be an important piece of clinical information. Future typing of *fim*H in the clinical setting could further aid both canine and human clinicians in tailoring treatment plans for UTI patients so as to provide more appropriate care and prevent recurrent infection.

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Chapter 3 – Assessing the Role of *fim*H on Uropathogenic *Escherichia coli* Fitness in Modulated Urine Environments: An *in vitro* Study Using Canine Isolates

Abstract

A number of physiological states and pathologies can change the composition of urine, all of which have been shown, in some way, to alter susceptibility to urinary tract infection (UTI). The most common etiological agent of human and canine UTIs is uropathogenic *Escherichia coli* (UPEC). This study assessed the effects of varying urine compositions, using a base artificial urine (AU) formulation, on the growth dynamics of UPEC. Additionally, we evaluated impact of the diversity of the *fim*H tip adhesin on growth dynamics in these various urine compositions as *fim*H known to play a role in urothelial attachment and invasion and has been shown to be associated with biofilm formation in canines. The base AU was diluted, concentrated, and supplemented with glucose or protein and growth was tested by measuring optical density (OD) at 600nm over 24 hours with shaking in aerobic conditions. Viable cell counts, biofilm formation, and minimum inhibitory concentrations were also assessed as measures of the effect of AU on the fitness of UPEC strains. Significant associations were found between urine composition and UPEC concentration over time ($p<0.0001$), as well as the potential to produce biofilm ($p=0.01$). Significant associations were also found between *fim*H genotype and biofilm formation ($p<0.0001$), yeast agglutination for *fim*H expression ($p=0.0003$). Our results suggest a possible role in the growth dynamics and susceptibility profile of UPEC in different physiological conditions and provide preliminary data to support a role of *fim*H diversity in UPEC fitness in urine.

1. Introduction

Uropathogenic *Escherichia coli* (UPEC) has been consistently shown to be the primary cause of both complicated and uncomplicated urinary tract infection (UTIs), significantly increasing risk of 6-month recurrence of UTI in women (Medina & Castillo-Pino, 2019). UPEC is also associated with development of asymptomatic bacteriuria defined by the colonization of bacteria within the urinary tract in the absence of clinical signs of UTI (Nicolle et al, 2005). Regardless of treatment of bacteriuria, presence of asymptomatic bacteriuria can predispose patients to development of symptomatic infection within one year of diagnosis (Asscher et al, 1969). Canines are also susceptible to subclinical bacteriuria through colonization of multidrug susceptible or resistant organisms, challenging clinicians to decide whether to treat or monitor the dog's condition (Johnstone, 2020). Canines with kidney disease and diabetes have increased incidence of subclinical bacteriuria although incidence of symptomatic lower UTI has been inconsistent (Lamoureux et al, 2019, McGuire et al, 2002). In populations with comorbidities such as diabetes or impaired renal function, progression to symptomatic infection is considered a complicated UTI and is associated with higher risk of treatment failure as well as higher mortality (Sabih & Leslie, 2021).

Diseases affecting the kidney often cause metabolic changes that affect host response to infection as well as composition of urine that increase risk of UTI, as seen in acute kidney injury and chronic kidney disease (Dimitrijevic et al, 2021). Proteinuria has been established as one of the standard markers of renal disease for its reliability in determining glomerular filtration status; the presence of urine protein indicates that renal injury has increased glomerular capillary pressure, leading to dysfunctional podocytes and the inability of the glomerulus to filter large molecules (Cravedi & Remuzzi, 2013). Urine concentration has also been shown to affect UPEC

growth. Increased urine osmolality has been shown to reduce UPEC growth *in vivo* in canines while reduced osmolality increased growth (Thornton et al, 2018). Hydration status is usually the primary cause of urine osmolality changes in humans, although dysfunctional metabolic, endocrine, cardiovascular, and renal processes can all cause significant changes to urine concentration, with increased osmolality also causing reduced UPEC growth in humans (Beetz, 2003). Diabetes is also a known risk factor for UTI in canines and humans, with peripheral neuropathy reducing the ability to fully void urine which is usually a host defense mechanism against bacterial colonization (Nitzan et al, 2015, Morgan et al, 2008). Changes in blood glucose can also create oxidative stress and alter the immune response to pathogens by stimulating chronic inflammation and elevating circulating leukocytes (Daryabor et at, 2020). In human diabetic patients, UPEC remains the primary pathogen and women remain the primary patient population, but interestingly, risk for UTI in diabetic patients increases as a function of time from diabetes diagnosis, likely because of the systemic effects of the disease that affect the urinary system (Nitzan et al, 2015).

One way that UPEC is potentially able to evade host defenses is to use urine as a growth medium in which to replicate rapidly (Reitzer & Zimmern, 2020). Growth in urine is dependent on a number of conserved bacterial metabolism pathways and appears to be relatively independent of the UPEC virulence factors (Mobley, 2016). One virulence factor that appears to have a positive association with UPEC fitness is FimH, which is the tip adhesin of the Type 1 pilus, and is critical for adherence to the bladder urothelium and has been implicated in cell invasion and formation of intracellular bacterial communities (IBCs) (Chen et al, 2009). The formation of these IBCs and other biofilm structures allow UPEC to enter a quiescent state that is practically immune from host defenses, increasing the host's risk of developing a recurrent

infection (Mulvey et al, 2001). One indication of the formation of IBCs is the presence of bacteria with filamentous morphology, which is associated with more highly concentrated urine in mice and humans (Murray et al, 2021), indicating a potential impact of urine composition on FimH. FimH is a genetically diverse protein as well, although the phenotypic role of this diversity has not been well described (Dias et al, 2010).

Given the increased severity of infections that patients with comorbidities face, it is important to understand the way that UPEC behaves in aberrant urine conditions so as to provide more accurate treatment to these populations. Previous studies have described dilute urine as protective against UTI, as well as showing concentrated urine as a risk factor for UTI (Beetz, 2003). Studies have also suggested glucosuria increases risk for UTI, although the studies on renal failure and chronic kidney disease have been mixed with the consensus being that kidney disease is invariably a risk factor for UTI but that proteinuria as the result on a test strip may be the result of renal disease or may be the result of a UTI (Geerlings et al, 2014, Carter et al, 2006). Our objective was to assess the growth dynamics, biofilm potential, and antibiotic susceptibility of canine UPEC in five different artificial urine formulations to determine associations based on urine composition. We also aimed to determine if these associations varied by fimH type and expression.

2. Materials and Methods

2.1. UPEC isolates

E. coli isolates were obtained from urine diagnostic submissions to the Microbiology and Molecular Diagnostics Laboratory at the College of Veterinary Medicine, North Carolina State University (Raleigh, NC), all of which originated from canines presenting to the veterinary hospital for evaluation. Isolates were included in the biobank if they were identified as the sole

uropathogen of canine UTI (defined as > 1,000 CFU/ml) and if the source of collection was urine, urinary catheter, urethra, or bladder. Further, isolates were included in this study if they represented one of three *fim*H genotypes, *fim*H9, *fim*H2, or *fim*H32, based on previous characterization (Pluta et al., unpublished data). These genotypes were selected because they had >10 representative isolates in the biobank; 10 isolates per genotype were chosen randomly to create three equal groups. Isolates were stored at -80°C in Tryptic Soy Broth with 15% Glycerol (Thermo Scientific™, Waltham, MA) until needed for phenotypic analysis. Isolates were cultured for isolation at 37°C overnight on Trypticase Soy Agar with 5% Sheep blood (BAP; Thermo Scientific™ Remel™) in aerobic conditions unless otherwise noted.

2.2. Media Preparation

A standard artificial urine (AU-S) media was prepared as previously described (Sarigul et al, 2019) with 0.005g/L yeast extract and 1g/L casamino acids (all chemicals for AU: Sigma-Aldrich, St. Louis, MO) added to aid in bacterial growth. This standard AU media was standardized to a specific gravity (SG) of 1.012 ± 0.002 and was diluted and standardized to a SG of 1.001 ± 0.002 (AU-D). The protocol was also scaled to make a concentrated AU (AU-C) with a standardized SG of 1.044 ± 0.002. These cutoffs were based on a euhydration value of approximately 500mOsm/kg which can be estimated by a specific gravity of 1.013 (Perrier et al, 2017). Bovine serum albumin protein (BSA, Sigma-Aldrich) was used in a concentration of 2.5g/L to simulate proteinuria (AU-P) which, when compared to the concentration of creatinine in the standard AU formulation, resulted in a protein:creatinine ratio of 320.5mg/mmol; this value meets the proteinuria criteria for nephrotic syndrome in adults (Hull & Goldsmith, 2008). An additional formulation was created with 1g/L D-glucose (AU-P, Sigma-Aldrich) added to simulate a urine glucose clearance of 100mg/dL, well above pathologic limits of 25mg/dL (Kim

et al, 2017). All media was sterile filtered using a 0.2μm cellulose acetate filter (Corning, Glendale, AZ) and standardized to 6.0pH before storage at room temperature prior to use, with the exception of AU+BSA which was stored at 4°C.

2.3. *E. coli* Growth Dynamics

Each well on the outside edge of a 96-well tissue culture treated U-bottom microtiter plate (Corning) was filled with 200μL of sterile deionized water to prevent evaporation of experimental inoculum. The remaining wells were filled with 180μL of AU and 20μL of a 0.3 McFarland solution of one *E. coli* isolate in phosphate buffered saline (PBS). Each isolate was evaluated in triplicate. The plates were incubated at 37°C with slow shaking in a microplate reader (BioTek, Winooski, Vermont, US) for 24 hours with the optical density at a wavelength of 600nm (OD_{600}) being recorded every 15 minutes. This process was repeated for all isolates in each of the five AU formulations. Growth rate was determined for each *fim*H genotype in each urine formulation by averaging the OD_{600} values for all triplicates, and across samples within a genotype; the $\ln(OD_{600})$ values were plotted against time. A random sample of three isolates of each *fim*H genotype was selected for serial dilution and plating to determine the viable concentration. At T=0, the 0.3 McFarland for each selected isolate was serially diluted 1:10 in PBS in a 96-well plate. In triplicate, 20μL of each dilution was plated on BAP for each isolate and incubated overnight at 37°C. In a 96-well plate, each isolate was plated, in triplicate, in each of the five AU formulations and incubated with shaking for 24 hours at 37°C. At T=24, each sample was serially diluted 1:10 and 20μL each dilution was plated in triplicate on BAP and incubated overnight at 37°C. Following each incubation, the number of colonies in the most countable spot was determined, the counts averaged and the concentration calculated.

2.4. Crystal Violet Biofilm Assay

Ability to form biofilms in five AU formulations was performed using a previously described biofilm assay (Kern et al, 2018). In contrast to the previous protocol, the isolates in our study were not enriched in lysogeny broth and were taken directly from BAP, and inoculated into PBS to make a 0.5McF solution. *E. coli* laboratory strain K12 (American Type Culture Collection 29425, Manassas, VA) and triplicate uninoculated media wells were included for each media type as controls. A 10µL aliquot of each McFarland solution was added to 90µL of each of the five AU media types, in triplicate. Further, in our study, after overnight incubation with shaking, excess media was shaken and plates were inverted to drain. Plates were then stained with 200µL of 0.1% crystal violet for 15 minutes, washed twice with 150µL of deionized water for 1 minute each, and treated with 100µL of 100% ethanol to solubilize the stain. Plates were analyzed using the microtiter plate reader (BioTek) and OD_{570} values were recorded. The OD_{570} values for each isolate in each media were averaged and the average OD_{570} measurement of the blank wells were subtracted from the sample measurement to normalize values across plates.

2.5. Minimum Inhibitory Concentration Measurement

The minimum inhibitory concentration for the *E. coli* isolates was determined by microdilution method as described by established protocols (CLSI, 2012). Isolates were standardized to a 0.5McF in PBS and were diluted 1:20. The antibiotics chosen for analysis were ciprofloxacin, amoxicillin-clavulanic acid (4:1), and cephalexin (all: Sigma Aldrich) on the advice of an expert in canine UTI treatment. Stock solutions of each antibiotic were created at 10mg/ml in PBS and were adjusted to appropriate beginning concentrations and stored based on previous studies (Andrews, 2001). Each well of a 96-well non-tissue culture treated U-bottom plate (Thermo Fisher) was filled with 100µL of either untreated AU media or cation-adjusted

Mueller-Hinton broth (Thermo Fisher) with each well in the first column receiving 100μL antibiotic solution. The solution in the first column was serially diluted 1:2 across the plate, leaving the final column without antibiotic as a negative control. A 10μL aliquot of each *E. coli* isolate suspension was added to each well except the final column and the plate was incubated for 21-24 hours at 37°C. After incubation, plates were visually assessed to determine at which concentration of antibiotic bacteria were completely inhibited from growing in media.

2.6. *fim*H Expression Assay

Expression of *fim*H was assessed by yeast agglutination, as previously described (Stærk et al, 2016). *Saccharomyces cerevisiae* (American Type Culture Collection 9763) was cultured for 24-48 hours at 30°C with shaking in yeast peptone dextrose broth (Thermo Fisher) to a concentration of approximately 1.27 x 10^8 CFU/ml. Single colonies of each UPEC sample were taken from BAP and were enriched in lysogeny broth (Sigma Aldrich) for 3 hours with shaking at 37°C. Following incubation, enrichments were centrifuged and washed twice with PBS and standardized to a McFarland of 3.0. A 160μL aliquot of each suspension was added in triplicate to the first row of a 96-well flat bottom non-tissue culture treated plate (Thermo Fisher) and 80μL of a 1:10 dilution of the yeast suspension was added to each well in the plate. The contents of the first row were diluted 2:3 down the plate to maximize discretion between each row. The plate was plate on an orbital shaker for 5 minutes before being visualized under light microscopy to visualize the lowest concentration of bacteria necessary to agglutinate yeast.

2.7. Statistical Analysis

Statistical analysis was performed using JMP Pro 15.2 software (SAS Institute Inc., Cary, NC). Summary statistics including mean, standard deviation, 95% confidence intervals, and median were calculated when appropriate. Data was analyzed for normality using the Shapiro-

Wilk test and data with a normal distribution was tested for significance with a one-way analysis of variance test. Nonparametric data was analyzed using the Wilcoxon signed rank test, or Kruskal-Wallis test by rank when more than two groups were being analyzed. Additionally, to test for significance between individual groups within the same experiment, the Wilcoxon signed rank test for matched pairs was used. A dependent t-test for paired samples was used to test for significant differences between paired samples within experimental groups. For all tests for significance, $p<0.05$ was considered significant.

3. Results

Over the course of the study, media was tested regularly for stability and was noticed to be pH stable under chosen storage conditions for approximately one month, at which point the pH began to reduce. Further, AU with protein was noted to be pH stable in the refrigerator indefinitely although protein molecules would begin to fall out of solution over time. Media preparations were noted to be susceptible to contamination to both bacterial and fungal growth. Solid precipitation or contamination were cause for immediate waste and synthesis of new media. The growth curves of the 30 isolates, averaged across all samples to create a representative composite growth curve for each AU formulation are in Fig. 3.1. A Kruskal-Willis test by rank was performed and the results were found to be significant ($p<0.0001$). Further, when each formulation was paired with another, each pairing was found to be significantly different ($p<0.0001$ for all pairs). The growth curves for each of the three *fim*H genotypes were evaluated independently for each AU formulation, and results are visualized in Fig. 3.2. In all urine formulations with the exception of AU-P, the same pattern held true; *fim*H32 had a significantly higher mean OD than both *fim*H2 ($p<0.0001$ for all AU) and *fim*H9 ($p<0.0001$ for all AU) and *fim*H9 had a statistically lower mean than *fim*H2 ($p<0.0001$ for all AU). In the case

of AU-P, *fim*H32 was significantly lower than *fim*H2 ($p<0.0001$) and *fim*H9 was significantly lower than *fim*H2 ($p<0.0001$) with no significant association between *fim*H32 and *fim*H9.

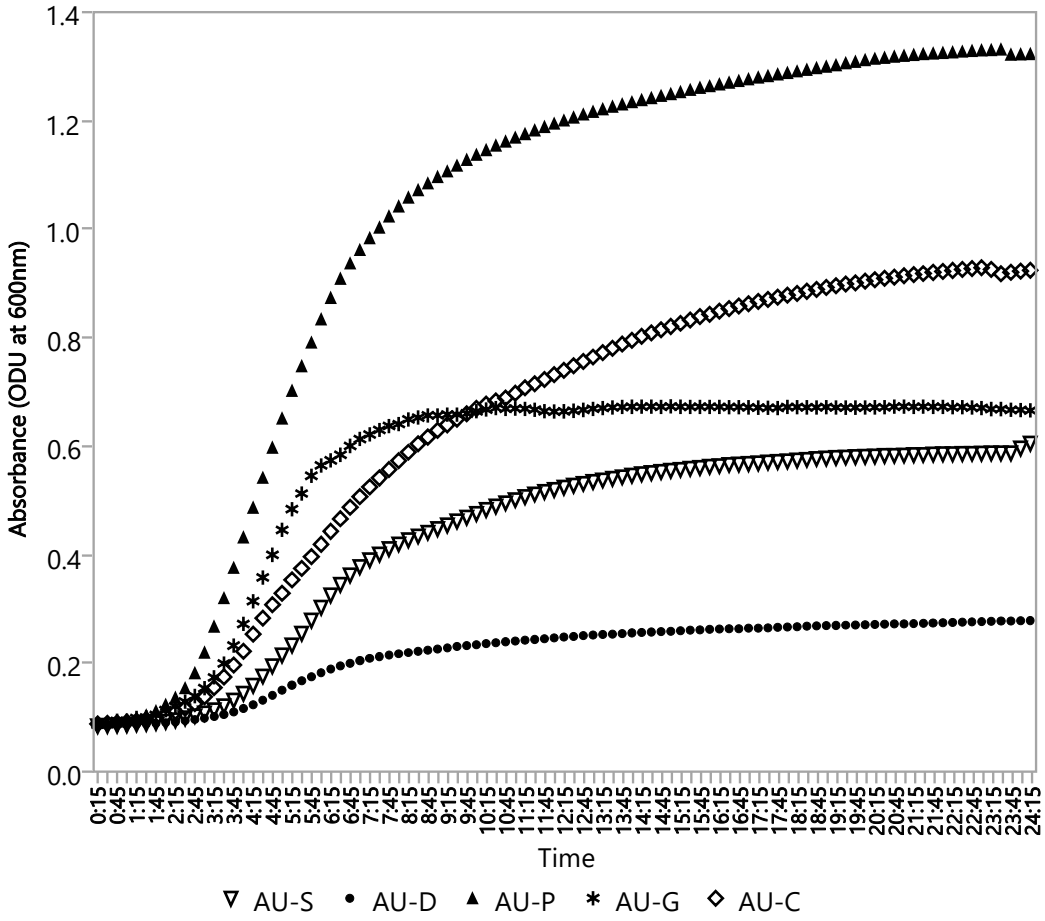

Figure 3.1. Comparison of absorbance (OD_{600}) over time for all uropathogenic *Escherichia coli* isolates in five different artificial urine (AU) formulations
Isolates were measured using an absorbance spectrophotometer at 15-minute intervals for 24 hours and OD_{600} values were plotted against time to visualize the bacterial growth curve for each media type (S = standard, D = dilute, P = protein, G = glucose, C = concentrated)

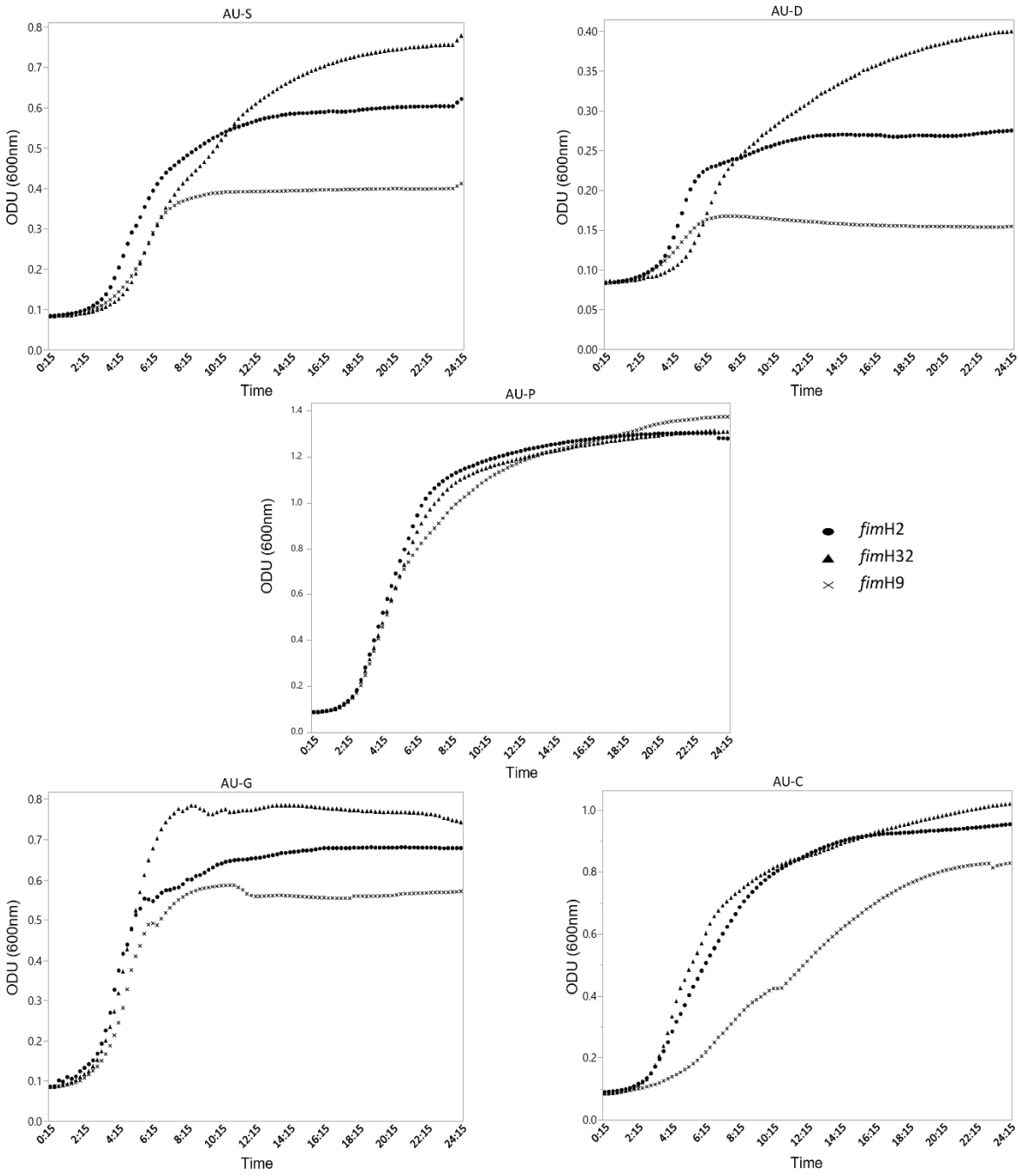

Figure 3.2. Comparison of growth curves between three *fim*H genotypes blocked by artificial urine (AU) formulation After growth curve assays were run, the composite growth curves for each *fim*H genotype were compared to each other blocked by AU formulation to assess for associations between genotype and growth in each AU formulation. Genotypes were statistically compared using a student's t-test for paired samples. (AU abbreviations – S = standard, D = dilute, P = protein, G = glucose, C = concentrated)

Total time to stationary phase, which was determined as the time point on the ln(OD_{600}) vs time plot at which growth began to level off from the period of exponential growth and which includes both lag and log phase, was noted for each genotype in each AU. A one sample t-test was performed to determine significance using 360 minutes as the hypothetical mean, estimating time to stationary phase of *E. coli* in lysogeny broth. The only significant association that was found was AU-P was found to have a significantly longer time to stationary than the hypothetical mean (p=0.039). No significant associations were found in the other AU types or between the *fim*H types. The time to stationary phase results can be found in table 3.1.

Table 3.1. Total time to stationary phase of three UPEC *fim*H genotypes in various AU formulations, in minutes. The final column reports p-values for the data in each AU formulation.

	*fim*H2	*fim*H32	*fim*H9	p-value
AU-S	405	570	420	0.1844
AU-C	525	375	615	0.1741
AU-D	345	435	375	0.4444
AU-G	345	405	390	0.3828
AU-P	420	405	450	0.0390

Abbreviations: AU = artificial urine (S = standard, C = concentrated, D = dilute, G = glucose, P = protein), UPEC = Uropathogenic *Escherichia coli*

In addition to time to stationary phase, viable counts were assessed for a random sample of each genotype to determine whether the AU formulations were resulting in cell death or an increase in viable cells. When the viable counts of all AU types were blocked by *fim*H genotype, it was found that *fim*H32 resulted in significantly greater viable cell counts after 24 hours than *fim*H2 (p=0.012), and *fim*H9 resulted in significantly greater viable counts than either *fim*H2 or *fim*H32 (p<0.0001, p=0.0252, respectively). This is in contrast to the previous finding that *fim*H9

consistently had significantly lower OD_{600} values during the 24-hour growth curve experiment. No significant association was found regarding the viable counts between the AU types. A comparison of the viable counts for each AU formulation blocked by *fim*H genotype is visualized in Fig. 3.3.

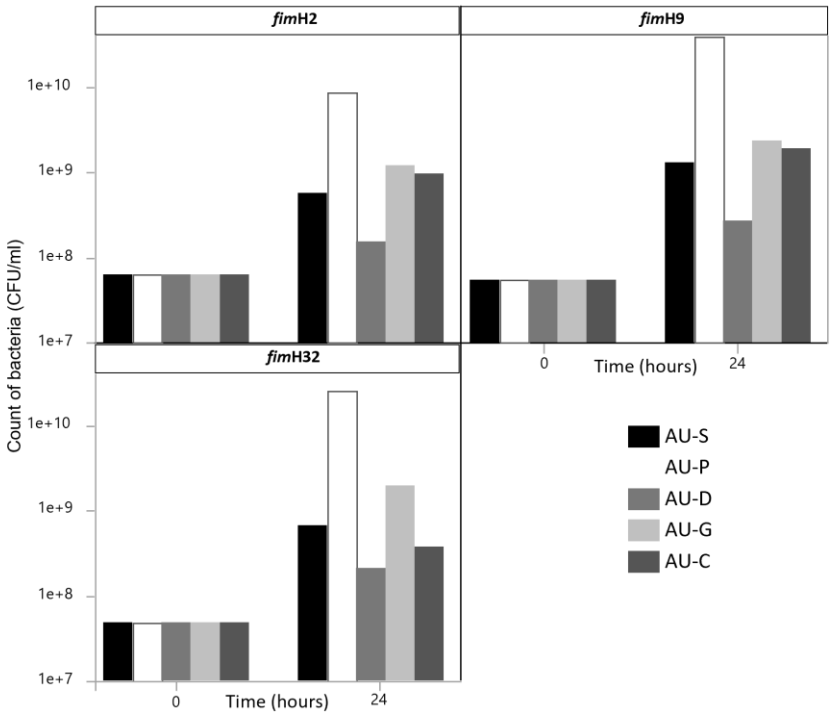

Figure 3.3. Viable count in CFU/ml of uropathogenic *Escherichia coli* isolates blocked by *fim*H genotype in five artificial urine (AU) formulations A random sample from each genotype was selected to be have 24-hour viable counts assessed. Viable counts were assessed by serial dilution followed by spot plating on BAP. The same inoculum was used for each urine type, hence T=0 having the same viable count for every AU type. (AU abbreviations – S = standard, D = dilute, P = protein, G = glucose, C = concentrated)

Biofilm formation was assessed in each AU formulation and a significant association was found between AU treatment and biofilm formation ability ($p=0.0119$). When individual AU types were paired, AU-P was significantly associated with higher biofilm formation than both AU-D and AU-G ($p=0.001$, $p<0.05$, respectively), and AU-D was significantly lower than both AU-C and AU-S ($p<0.05$ in both cases). Additionally, a significant association was found

between *fim*H genotype and biofilm formation regardless of AU type ($p<0.0001$). For paired tests, *fim*H9 and *fim*H32 were both found to be significantly higher than *fim*H2 ($p<0.0001$ in both cases). When AU type was blocked by *fim*H genotype, the only significant differences that were found were within AU-C and AU-D, and both showed that *fim*H9 formed significantly greater biofilms than *fim*H2 ($p<0.0001$ in both cases). The average biofilm formation for each AU type is visualized in Fig. 3.4.

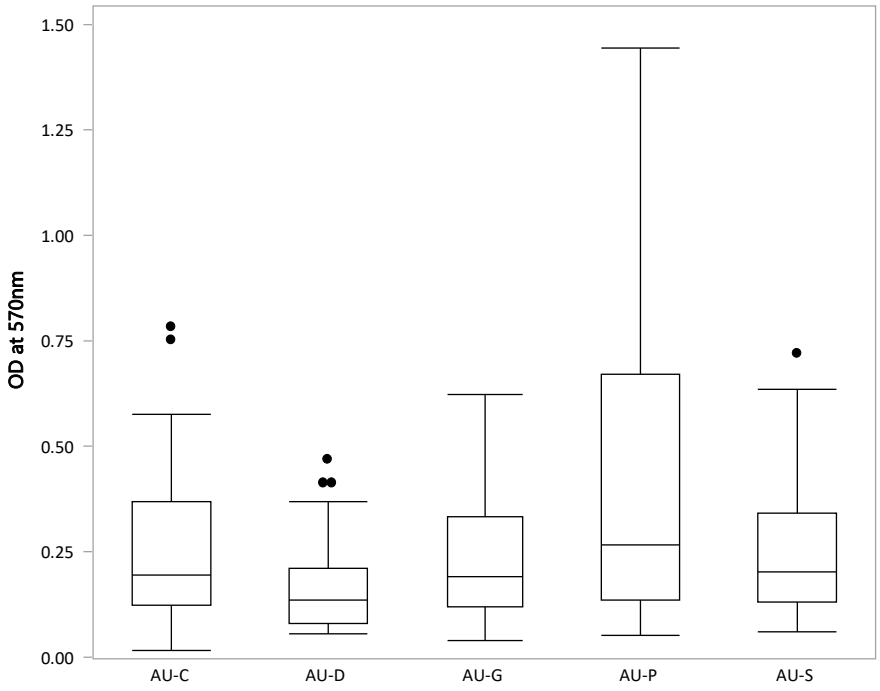

Figure 3.4. Comparison of mean biofilm formation among five artificial urine (AU) formulations for 30 uropathogenic *Escherichia coli* isolates Isolates were assessed for 24-hour biofilm formation, stained using crystal violet, washed, and assessed at OD_{570} for biofilm formation. Triplicate values for each isolate were averaged and the average for a media blank control was subtracted, after which the average of all 30 isolates was calculated for each media type and visualized. (AU abbreviations – S = standard, D = dilute, P = protein, G = glucose, C = concentrated)

Minimum inhibitory concentrations (MIC) of amoxicillin-clavulanic acid (AMC), cephalexin (LEX), and ciprofloxacin (CIP) were assessed in AU-S and cation adjusted Mueller-Hinton broth (CAMHB) for all 30 isolates in this study. No significant differences in MIC were

observed between media types for CIP or LEX, but a significant association was found between media type and MIC in AMC (p=0.0022). Further significant associations were found when MIC data was blocked by *fim*H genotype; these associations are summarized in table 3.2.

Table 3.2. Mean MIC values for AMC, LEX, and CIP in AU-S and CAMHB blocked by *fim*H genotype Genotypes within blocks with the same letter are not significantly different (p>0.05)

Media	Antibiotic	Genotype	Mean MIC (µg/ml)	Significance
CAMHB	AMC	*fim*H2	22.5	b
		*fim*H9	33.4	a,b
		*fim*H32	74.8	a
	LEX	*fim*H2	109.2	b
		*fim*H9	112	b
		*fim*H32	336.8	a
	CIP	*fim*H2	0.0156	c
		*fim*H9	0.0687	b
		*fim*H32	21.604	a
AU-S	AMC	*fim*H2	5.05	b
		*fim*H9	22	a,b
		*fim*H32	52.5	a
	LEX	*fim*H2	92	a
		*fim*H9	169.6	a
		*fim*H32	300.8	a
	CIP	*fim*H2	0.125	b
		*fim*H9	0.284	b
		*fim*H32	20.843	a

Abbreviations: MIC – Minimum Inhibitory Concentration, AMC – Amoxicillin/Clavulanic Acid, LEX – Cephalexin, CIP – Ciprofloxacin, AU-S – Standard artificial urine, CAMHB – Cation-adjusted Mueller-Hinton broth

Yeast agglutination was used as a method to test type 1 fimbriae (T1F) expression and as a result, *fim*H expression, in lysogeny broth. A strong significant association was found between yeast agglutination and *fim*H genotype (p=0.0003). A paired Wilcoxon signed rank test showed that *fim*H32 required a significantly higher concentration of bacteria to agglutinate yeast than either *fim*H2 or *fim*H9 (p=0.0003, p=0.0225, respectively) and that *fim*H9 required a significantly lower concentration of bacteria to agglutinate yeast than *fim*H2 (p=0.0149). A lower concentration of bacteria required for agglutination corresponded to a higher expression of T1F and *fim*H (Stærk et al, 2016). A comparison of mean bacterial concentration is visualized in Fig. 3.5.

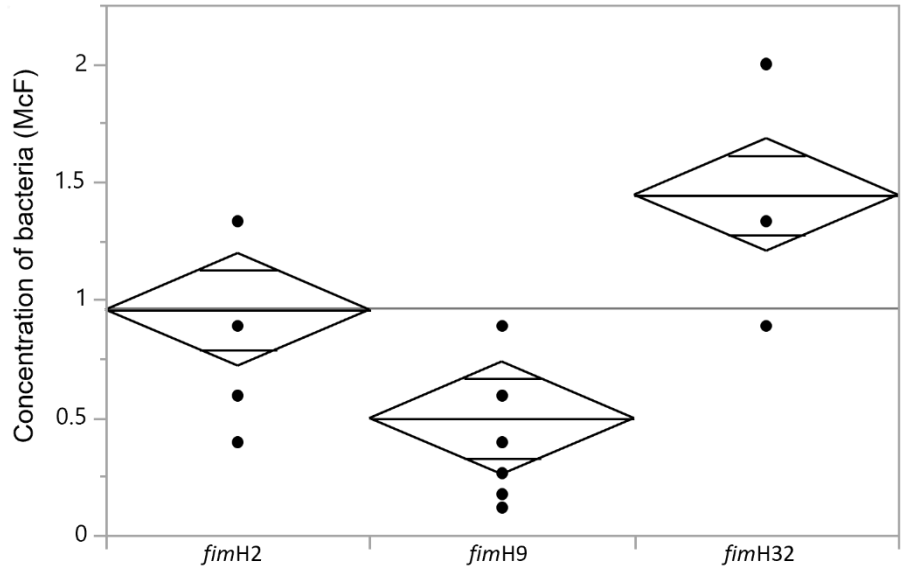

Figure 3.5. Mean bacterial concentration required to agglutinate yeast by *fim*H genotype After growing isolates to approximate mid-exponential phase in lysogeny broth, they were standardized to a McFarland of 3.0 and introduced to yeast then diluted 2:3. The concentration at which yeast no longer was agglutinated was noted, in terms of approximate McFarland, based on the dilution factor in the well being visualized.

4. Discussion

Uropathogenic *E. coli* is the primary pathogen responsible for the vast majority of urinary tract infection cases in both humans and canines (Foxman, 2010, Johnstone, 2020). Understanding the way that UPEC grows in urine as a media is important for not only developing more accurate treatment options, but also for choosing more appropriate animal models for human UPEC infection. It is known that UPEC grows rapidly in urine, utilizing a variety of metabolites and electrolytes naturally present in urine to fuel different energy-producing mechanisms (Reitzer & Zimmern, 2019). Protein has been shown to be a potential source of energy for bacteria although it has been argued that UTIs might be a source of proteinuria (Reitzer & Zimmern, 2019, Carter et al, 2006), although our results support the theory that proteinuria preceding a UTI would act as an energy source for bacteria as it relates to growth. Additionally, dilute urine has been shown to reduce UPEC growth in both humans and canines, while more concentrated urine has been shown to increase UPEC growth (Beetz, 2003, Thornton et al, 2018); these findings were supported by our data. Currently the mouse is the primary model used in UPEC animal studies, but mice have naturally highly concentrated urine (Parfentjev & Perlzweig, 1933). This potentially provides an indication to choose alternate models for human UPEC infection, as the primary method to alter urine concentration in mice is to create genetic knockouts which might become time-consuming or costly (Yang & Bankir, 2005). When growth measured by OD_{600} was evaluated by *fim*H genotype, *fim*H32 was noted to have consistently and significantly higher measurements than the other two genotypes, with only the exception of AU-P. Interestingly, when viable cell counts were measured, *fim*H9, which consistently had significantly lower OD measurements than the other two genotypes, had the highest viable cell count. This is likely due to the fact that it also had high biofilm formation

ability, or that other *fim*H types had already started to reach death phase. It is likely that the majority of *fim*H2 remained planktonic and therefore metabolically active so turnover was likely high, leading to an accumulation of dead cells that contributed to the OD readings during the growth curve assay. In the case of *fim*H9, by forming biofilms and entering a sessile state that is metabolically stunted, there is a reduction in the accumulation of dead bacteria that would contribute to the OD readings (Beloin et al, 2008). We can theorize that *fim*H32 formed some biofilms and also grew rapidly planktonically, leading to high OD readings that recognized viable and dead planktonic bacteria as well as the biofilm structure. Regardless of genotype, it should be noted that AU-D was a poor medium for biofilm growth, further supporting previous data stating that dilute urine had a protective effect against UTI. Specifically, AU-C grew significantly more biofilm than AU-D ($p<0.05$), offering a potential mechanism by which concentrated urine predisposes someone to UTI as compared to dilute urine. AU-P also grew significantly more biofilm than both AU-G or AU-D, indicating that the protein in the urine could provide either energy for the bacteria prior to or entering or during its sessile state or UPEC could use the protein in the urine as a building block for the biofilm matrix. Further work will need to be done to confirm these mechanisms and their potential molecular pathways.

In addition to understanding the growth dynamics of UPEC, we aimed to determine whether media had an effect on antimicrobial susceptibility of the UPEC strains we tested. Antimicrobial susceptibility testing (AST) data acquired on initial presentation of the patient to the clinic was reviewed although this data could not be used in final analysis. Isolates used in this study were collected between 2016-2020 and were tested using various AST protocols based on availability of materials. This variability resulted in differences in reporting of data with some isolates not being tested against certain antibiotics and with MIC data being reported simply as

greater than or less than an established breakpoint, making interpretation difficult. Across all antimicrobials and both media types tested with the exception of cephalexin in AU-S, *fim*H32 had a higher mean MIC than *fim*H2. In both media with ciprofloxacin and in CAMHB with cephalexin, *fim*H32 was also found to have statistically significant difference with *fim*H9. Only in CAMHB with ciprofloxacin was *fim*H9 significantly higher than *fim*H2. The phenotypic effects of the genotypic diversity of *fim*H have not yet been well studied, but these results offer interesting preliminary data for further research into the effects of *fim*H diversity in various physiological media. This study did assess expression of these *fim*H genotypes in lysogeny broth and found that *fim*H9 expresses T1F significantly more than either of the other two genotypes while *fim*H32 expresses T1F significantly less than either of the other two. Our expression data is limited in that the experiment was only performed in lysogeny broth because of the predictability of the media on both UPEC and the yeast that was being used. Additionally, the previous study used this agglutination assay in both lysogeny broth and urine as well as in flow chamber conditions to assess for adhesion and invasion (Stærk et al, 2016) whereas the assay was used in our study as a simple screening tool to assess potential differences in expression as a function of genotype. With the knowledge that the three selected genotypes express *fim*H differently, at least in lysogeny broth, further research can be done to assess for patterns among a larger sample of genotypes or to determine whether these differences persist in human or canine urine. Our results could indicate that higher expression of T1F in *fim*H9 could be associated with its increased biofilm formation and that reduced T1F expression in *fim*H32 could be associated with its increased mean MIC across the spectrum of antimicrobials we tested, although more research would need to be done to confirm these hypotheses.

5. Conclusion

The composition of urine has an undeniable impact on the growth dynamics of the most significant cause of urinary tract infections, UPEC. Understanding this impact can help human and veterinary clinicians make more informed decisions on the best course of treatment for their patients. As subclinical bacteriuria is common in canines with and without comorbidities, this data is particularly applicable to the veterinary field in providing clinicians some context since our samples were sourced from canines and it can help guide the decision of how and whether to treat subclinical bacteriuria cases. Additionally these results can provide information to the research community regarding the use of appropriate animal models for human UPEC infection. Murine models have long been at the forefront of UPEC research, but the results of our study suggest that, given the increased concentration of their urine, using the mouse as a model might produce results that do not mimic the pathogenesis of UPEC in humans. We also believe our results regarding the genetic diversity of *fim*H as it relates to growth dynamics and antimicrobial susceptibility provide a strong foundation for future research in this field.

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APPENDIX

Appendix A

Complete Distribution of *fim*H Types and Clustering

Table A.1. **Full distribution of *fim*H types and clustering based on genetic similarity and matching amino acid sequence** The quantity column shows how many isolates in the biobank were identified during sequencing as being of a given *fim*H type. Types with matching nucleotide cluster identifiers were identified as being highly genetically similar and those having matching amino acid cluster identifiers had identical amino acid sequences. Amino acid identifiers labeled "Protein#" did not match any other *fim*H amino acid sequence and were uniquely identified using the *fim*H type number.

*fim*H Type	Qty	Nucleotide Sequence Cluster	AminoAcid Cluster
fimH-	9	N/A	N/A
fimH1	2	fimH(D)	ProteinA
fimH10	1	fimH(A)	ProteinJ
fimH1001	1	fimH(J)	ProteinA
fimH103	2	fimH(A)	ProteinE
fimH1030	1	fimH(K)	ProteinA
fimH108	1	fimH(H)	Protein108
fimH11	1	fimH(A)	Protein11
fimH113	1	fimH(A)	Protein113
fimH115	2	fimH(B)	ProteinA
fimH124	1	fimH(H)	ProteinA
fimH1243	1	fimH(G)	ProteinI
fimH13	1	fimH(A)	Protein13
fimH130	1	fimH(E)	ProteinG
fimH14	9	fimH(B)	ProteinA
fimH142	3	fimH(N)	Protein142
fimH145	7	fimH(L)	ProteinA
fimH1463	1	fimH(D)	ProteinB
fimH1479	1	fimH(B)	ProteinF
fimH149	1	fimH(H)	ProteinI
fimH1556	1	fimH(J)	Protein1556
fimH1559	1	fimH(E)	ProteinE
fimH1560	1	fimH(A)	Protein1560
fimH1561	1	fimH(F)	Protein1561
fimH1562	1	fimH(B)	Protein1562
fimH1563	1	fimH(C)	ProteinF
fimH1564	1	fimH(B)	ProteinD
fimH1565	1	fimH(G)	Protein1565
fimH18	1	fimH(A)	ProteinB
fimH180	2	fimH(D)	ProteinJ
fimH181	1	fimH(D)	ProteinC
fimH197	3	fimH(F)	Protein197
fimH2	15	fimH(D)	ProteinA
fimH20	4	fimH(C)	ProteinA
fimH21	1	fimH(C)	ProteinA
fimH218	1	fimH(L)	ProteinA
fimH223	2	fimH(E)	ProteinE
fimH23	1	fimH(K)	ProteinA
fimH24	2	fimH(K)	Protein24
fimH26	4	fimH(K)	Protein26
fimH260	1	fimH(E)	ProteinC
fimH269	1	fimH(C)	ProteinE
fimH27	8	fimH(N)	ProteinI
fimH29	1	fimH(N)	Protein29
fimH3	2	fimH(D)	ProteinA
fimH30	9	fimH(H)	ProteinA
fimH302	1	fimH(G)	ProteinA
fimH306	5	fimH(E)	Protein306
fimH31	2	fimH(H)	ProteinA
fimH314	1	fimH(C)	Protein314
fimH32	10	fimH(H)	ProteinA
fimH38	2	fimH(G)	ProteinA
fimH39	1	fimH(G)	ProteinA
fimH390	1	fimH(A)	ProteinA
fimH41	3	fimH(G)	ProteinA
fimH414	1	fimH(B)	ProteinG
fimH44	1	fimH(G)	Protein44
fimH46	1	fimH(M)	ProteinA
fimH481	1	fimH(A)	Protein481
fimH5	9	fimH(E)	ProteinA
fimH54	2	fimH(F)	Protein54
fimH58	1	fimH(L)	ProteinA
fimH6	1	fimH(E)	Protein6
fimH61	5	fimH(M)	ProteinA
fimH64	4	fimH(N)	Protein64
fimH65	2	fimH(J)	ProteinA
fimH700	1	fimH(A)	ProteinD
fimH720	1	fimH(A)	Protein720
fimH75	1	fimH(A)	ProteinA
fimH76	1	fimH(A)	ProteinE
fimH86	1	fimH(G)	ProteinA
fimH87	1	fimH(G)	ProteinA
fimH9	33	fimH(A)	ProteinA