CHARACTERIZATION OF AVIAN PATHOGENIC *ESCHERICHIA COLI* (APEC) AND ITS BIOFILM FORMATION

by

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(Under the Direction of Catherine M. Logue)

ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) is the etiological agent of avian colibacillosis, a leading cause of economic loss to the poultry industry. APEC has enhanced survivability due to biofilm formation. To better characterize APEC, this work evaluated *E. coli* isolated from a turkey cellulitis outbreak. Using PCR, the O serogroups of the isolates were identified, and it was found that O143, a potential emerging APEC serogroup, may be a leading cause of the outbreak. The next study aimed to better understand virulence factors that contribute to APEC biofilm formation. Signature tagged mutagenesis was used to ultimately identify nine APEC- and biofilm-associated genes. Deletion mutants were created to evaluate the role of these genes in APEC biofilm formation. In conclusion, novel genes not previously known to be APEC- or biofilm-associated were identified. This work builds the foundation for future studies to characterize these genes and better understand APEC biofilm.

INDEX WORDS: *Escherichia coli*, APEC, colibacillosis, O-type, serogroup, biofilm, virulence factors

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DEDICATION

I would like to dedicate this thesis to my parents, Deborah and William Young, Jr., for their hard work putting two first-generation students through college and for their continuous support and encouragement in my pursuit of knowledge.

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CHAPTER 1

INTRODUCTION

The purpose of this work seeks to understand the virulence factors that contribute to the biofilm formation of avian pathogenic *Escherichia coli* (APEC). APEC is the causative agent of avian colibacillosis, a disease that contributes to millions of dollars in losses in the poultry industry each year. APEC is a highly diverse pathotype with a large repertoire of virulence factors. Although research regarding biofilm formation has expanded rapidly since its discovery, little is known about the factors that contribute to the biofilm of APEC specifically. Other work has been done to identify genes that contribute to biofilm formation in APEC; however, there has not been any other study, to the author's knowledge, that evaluates which genes are most widespread and important in APEC biofilm. It is the belief of the author that this work will serve to expand the knowledge of different APEC serogroups and virulence factors that contribute to APEC biofilm formation.

The first part of the work presented in this thesis is an epidemiological study that aims to identify the O serogroups of *E. coli* that contributed to a cellulitis outbreak on a turkey farm in Iowa. APEC is commonly represented by three major serogroups (O1, O2, and O78); however, less common pathogenic serogroups have been emerging. This study utilizes a PCR-based technique to serogroup 333 *E. coli* isolates obtained from disease lesions or barn litter and to analyze their pathogenic potential through analysis of virulence factors. This information contributes to identifying emerging APEC serogroups and to better understanding the factors that contribute to avian colibacillosis.

The second part of this work aims to characterize the APEC biofilm through the identification of genes specific to APEC that are involved in the biofilm formation process. The author utilizes signature tagged mutagenesis to identify biofilm formation genes within a well-characterized and sequenced APEC strain. A PCR-based prevalence analysis was used to determine which genes were more widespread in APEC compared to avian fecal *E. coli* isolates, and then the role of the APEC genes in biofilm formation were evaluated through biofilm, growth, and gene expression assays. This work contributes to the understanding of the APEC biofilm.

The thesis presented here is organized into five chapters in a manuscript format. Chapter 1 is an introduction, which explains the purpose of this work and the organization of the thesis. Chapter 2 is a literature review of *E. coli*, with an emphasis on extraintestinal pathogenic *E. coli* and biofilm formation. Chapter 3 reports the serogroups and virulence potential of *E. coli* isolated from turkeys. Chapter 4 investigates novel genes involved in the APEC biofilm formation process. Chapter 5 is the overall conclusion of the work presented within this thesis.

CHAPTER 2

LITERATURE REVIEW

Escherichia coli

Escherichia coli is a Gram-negative facultative anaerobe that colonizes the gastrointestinal tract of most warm-blooded animals as a commensal organism (1). It was first discovered in 1884 as *Bacterium coli commune* by Theodor Escherich but was subsequently renamed *Escherichia coli* (2, 3). *E. coli* is one of the first colonizers of the gut of neonates, colonizing within a few days after birth and creating a suitable anaerobic environment for strict anaerobes to thrive (4). It has also been shown to benefit its host through colonization resistance, such that it prevents the colonization of invading pathogens (5-7). *E. coli* is one of the most well characterized organisms; it is commonly found in the environment and has a rapid growth rate, high survivability, and genetic tractability. As such, *E. coli* is widely used as a model organism for research in genetics, biochemistry, and physiology (8).

Capable of surviving in different ecological habitats, *E. coli* is a diverse and versatile species with considerable genomic plasticity. This allows for the acquisition of genetic information through horizontal gene transfer and genetic modification such as point mutations (9). Thus, commensal strains may serve as reservoirs for virulence plasmids and other factors, such as antimicrobial resistance and toxin production genes (10-13). Not only can commensal strains serve as carriers of virulence genes, but they also can gain the ability to cause disease at specific host sites through the acquisition of virulence factors (9, 14, 15). Pathogenic *E. coli* are categorized as two types: intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E.*

coli (ExPEC). InPEC are obligate intestinal pathogens that cause enteric disease and have unique features of interacting with their host (16). Conversely, ExPEC are facultative pathogens that live in the gastrointestinal tract of healthy individuals as commensal organisms but have specific virulence factors that allow them to colonize and cause disease when outside of the gut (17).

Intestinal Pathogenic *E. coli* (InPEC)

Intestinal pathogenic *E. coli* (InPEC), also known as diarrheagenic *E. coli*, causes intestinal disease commonly characterized by diarrhea. InPEC are classified based upon specific virulence traits and host interactions. There are six recognized subtypes of InPEC: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (16).

Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) was the first subtype of *E. coli* to be described. Once the most prevalent pathotype infecting children around the world, the incidence of infection has declined in developed countries, although EPEC outbreaks are still important causes of fatal infant diarrhea in developing countries (16, 18). Symptoms of EPEC infection include watery diarrhea, vomiting, and death, in severe illness. EPEC colonizes in the small intestine and is characterized by attaching and effacing (A/E) histopathology, in which the microvilli of the intestine are destroyed and pedestal-like structures on which the bacteria perch rise from the epithelial cell (19). The effacement of the microvilli leads to less surface area for absorption and results in diarrhea. The key virulence factor of EPEC is a pathogenicity island (PAI) called the locus of enterocyte effacement (LEE), which encodes the genes essential for A/E histopathology, including adhesin, intimin, a type III secretion system, and effector proteins (20, 21).

Enterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E. coli* (EHEC) is similar to EPEC in that it is characterized by A/E histopathology and the LEE PAI. However, instead of colonizing the small intestine, it colonizes the large intestine. EHEC is an important cause of diarrheal illness in developed countries, resulting in symptoms such as hemorrhagic colitis (bloody diarrhea), non-bloody diarrhea, and hemolytic uremic syndrome (HUS) (22, 23). The most important EHEC serotype in North America is O157:H7, which alone causes approximately 63,000 cases of hemorrhagic colitis annually in the United States (24). The distinguishing virulence factor of EHEC is Shiga toxin (Stx), which is structurally and antigenically similar to the toxin secreted by *Shigella dysenteriae* (25, 26). EHEC is, therefore, part of the larger branch of *E. coli* referred to as Stx-producing *E. coli* (STEC). The main reservoir for STEC is the gut of cattle, although other ruminants and wildlife can also be common carriers (27-29), and unpasteurized milk and juice and raw fruits and vegetables are additional sources of (30). EHEC in particular has a low infectious dose of 10-100 CFUs (16). As a result, EHEC can spread rapidly around countries from batches of contaminated consumables (foodstuffs), regularly causing product recalls and diarrheal outbreaks.

Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) is the leading cause of traveler's diarrhea, causing about 60% of cases each year (31), and is an important cause of illness in children in developing countries. ETEC is responsible for approximately 4-6% of diarrhea-associated deaths in children less than five years of age (32, 33) and approximately 9.5% of diarrhea-associated deaths in older children (5-14 years) (34). ETEC colonizes in the small intestine, where it secretes heat-stable enterotoxins (STs), heat-labile enterotoxins (LTs), or some combination of these. The

toxins are host specific: STa and LT-I are associated with disease in both humans and animals, whereas STb and LT-II are primarily associated with disease in animals (35). ETEC also has host-specific colonization factors, such as colonization antigen (CFA), coli surface antigen (CSA), and putative colonization factor (PCF), that allow it to bind to the intestinal epithelium and secrete its enterotoxins in close proximity (16).

Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* (EAEC) can colonize both the large and small intestines and causes watery diarrhea, sometimes with blood or mucous (36). Although a common cause of diarrhea among travelers and children in developing countries, its role as an enteric pathogen and its genomic structure are still not well defined. The current definition of EAEC is mostly phenotypic, demonstrating aggregative adhesion in a distinct "stacked-brick" formation (37), but also harboring genes for aggregative adherence fimbriae (AAF) which are encoded on the plasmid of aggregative adherence (pAA), although no one specific genotype has yet been identified (38, 39). This may be due to the genome plasticity of EAEC, as it has been found to harbor genes typical of other *E. coli* subtypes. In 2011, an O104:H4 strain of *E. coli* caused a massive diarrheal and HUS outbreak in Germany and was genotyped as a Stx-producing EAEC strain (40). There have also been reports of EAEC/UPEC hybrids (41-43), indicating how broad the definition of EAEC may be.

Diffusely Adherent *E. coli* (DAEC)

Diffusely adherent *E. coli* (DAEC) is another broadly defined subtype of InPEC that colonizes the small intestine. Phenotypic characterization involves a diffuse adherence pattern on HeLa and HEp-2 cells (44), and pathogenesis includes the development of long cellular extensions from the epithelium that wrap around the bacteria (16). However, certain virulence

factors have been associated with DAEC, including both fimbrial and afimbrial adhesins that are collectively designated as Afa-Dr adhesins (36). These adhesins bind to the decay-accelerating factor (DAF) or carcinoembryonic antigen-related cell adhesion molecule (CEACAM) on the surface of intestinal epithelial cells (45). The only other virulence factor associated with DAEC is the secreted autotransporter toxin (Sat) (46), although DAEC seem to be more associated with iron acquisition factors than EAEC or commensal *E. coli* (47). The role of DAEC in causing disease has been debated, but it appears that the highest risk of disease is associated with children one to five years of age (44).

Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) is closely related to *Shigella* spp., and they are nearly indistinguishable aside from a few minor biochemical tests (48, 49). Whereas all the previously mentioned InPEC subtypes are extracellular, EIEC is intracellular and employs pathogenic mechanisms like those of *Shigella*. The taxonomic distinction remains, however, due to the distinction in clinical significance between the two. *Shigella* causes varying degrees of dysentery and is the second leading cause of diarrhea-associated deaths (33), while EIEC causes watery diarrhea and occasionally inflammatory colitis in hosts and does not appear to be a significant cause of intestinal disease in developed or developing countries (44). EIEC invades the epithelium of the large intestine via endocytosis, leading to cell death and, consequently, diarrhea (50). The distinguishing virulence factor of EIEC is the pINV, which encodes a type III secretion system and controls the pathogen's ability to invade, survive, and diffuse within the host (50).

Extraintestinal Pathogenic *E. coli* (ExPEC)

Extraintestinal pathogenic *E. coli* (ExPEC) can colonize and cause disease outside of the gut. ExPEC strains originally exist as part of the healthy gut microbiota; however, certain strains

have unique virulence factors that allow them to survive and cause disease when exposed to extraintestinal niches (17). Therefore, ExPEC strains are classified based on site of isolation and the presence of specific virulence genes (51-53).

ExPEC strains share virulence factors that allow them to survive outside of the gut that distinguish them from InPEC. As the *E. coli* genome has high plasticity, variations in virulence profiles are common even among strains of the same subtype. In order to cause infection, ExPEC strains require at least one factor each associated with adherence, iron acquisition, and serum resistance, and they may also possess invasins, protectins, and toxins (54). They are also most often associated with phylogenetic group B2 and, to a lesser extent, group D (55, 56). However, the presence of these genes do not necessarily identify them as extraintestinal pathogens, as commensal intestinal strains may also harbor these genes, which is why site of isolation is also important in classification (9).

ExPEC is a widely diverse pathotype comprising subtypes including uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), and avian pathogenic *E. coli* (APEC).

Uropathogenic *E. coli* (UPEC)

Uropathogenic *E. coli* (UPEC) colonizes the urinary tract. It is the leading cause of community-acquired UTIs, responsible for approximately 80% of cases each year (57, 58). Pathogenesis begins with the colonization of UPEC to the periurethral area, after which it ascends through the urethra to the bladder (16). UPEC will multiply in the urine and then adhere to the host uroepithelium through fimbrial adhesin FimH (59, 60). UPEC then forms a robust biofilm, in the form of intracellular bacterial community pods, to shield itself from host defenses (61); moreover, this biofilm serves as a source of latent pathogens that can lead to persistent infections (62, 63). UPEC invades and replicates within the bladder cells, after which it may

colonize in the kidney and damage host tissue, leading to increased risk for septicemia (64). UPEC operates using a variety of virulence factors, including the capsule, lipopolysaccharide, flagella, fimbrial and afimbrial adhesins, toxins, iron-acquisition systems, and two-component signaling systems (58, 64-67).

UTIs will affect between 50% and 60% of adult women each year (68) and can be community-acquired or hospital-acquired. The intestinal tract is the primary reservoir for UPEC strains, especially those causing community-acquired UTIs (69, 70). UTIs are the most common type of hospital-acquired infection, with approximately 75% related to urinary catheters (71). The severity of UTIs varies, and they are categorized as uncomplicated or complicated.

Uncomplicated UTIs are defined as having no relevant functional or anatomical anomalies in the urinary tract, no functional renal impairment, and no comorbidities that would increase the risk of developing serious complications (72). Uncomplicated UTIs are typically found in patients with a healthy urinary tract and are differentiated as upper (kidney) and lower (bladder) infections (73). Uncomplicated UTIs can be naturally cleared by the host immune system and rarely cause serious damage, such as pyelonephritis, which can result in sepsis or renal failure (58, 74).

Complicated UTIs occur in individuals with conditions that may increase the risk of serious disease, such as comorbidities or functional or anatomic anomalies (75). Obstructions of any kind in the urinary tract, such as urinary catheters or bladder or kidney stones, provide greater surface area to which UPEC may bind, which can lead to bacteremia if UPEC crosses the tubular epithelial barrier (58).

Neonatal Meningitis *E. coli* (NMEC)

Neonatal meningitis *E. coli* (NMEC) is the primary cause of Gram-negative bacterial neonatal meningitis. Neonatal meningitis has a mortality rate of 10% to 15% in developed countries and 40% to 58% in developing countries, leaving survivors with severe neurological defects (76). Neonatal meningitis can be classified as early- or late-onset, defined by time of infection and mode of transmission (77). NMEC is the second most common cause of early-onset neonatal meningitis among all infants, isolated from about 20% to 40% of cases, and the most common cause among premature infants and those of very low birth weight (< 1500 g) (78-81). Early-onset infection occurs within the first 72 hours of life and is acquired prenatally from the mother, in which NMEC transcends from the vagina to infect the amniotic fluid that the fetus then absorbs (76, 82). Alternatively, NMEC can colonize the infant during passage through the birth canal. As with early-onset infection, NMEC is the leading Gram-negative bacterial cause of late-onset neonatal meningitis (81, 83, 84). Late-onset infection occurs after 72 hours of life and is acquired postnatally, whether from the mother or from outside sources, such as caregivers, hospital staff, and medical devices (76).

To cause meningitis in a neonate, NMEC must first translocate to the bloodstream. This is usually done via transcytosis through enterocytes. High levels of bacteremia, with $>10^4$ colony forming units per mL of blood, are required to survive in the host's blood and, therefore, progress to pathogenesis (85). The capsule provides protection for NMEC in the bloodstream through serum resistance and antiphagocytic properties (86). NMEC also invades macrophages and monocytes as an alcove for replication; it is there NMEC may reach the required bacteremia threshold to invade the blood-brain barrier (BBB) (36). The BBB is formed by brain microvascular endothelial cells (BMEC), which NMEC invades using a zipper-like mechanism

and transmigrates through the BMEC without replication, leaving the BBB intact (87). NMEC then gains access to the central nervous system, where it causes inflammation of the meninges and pleocytosis of the cerebrospinal fluid (88).

NMEC utilizes a variety of virulence factors that are specialized for different phases of pathogenesis. Notably, 80% of NMEC strains are of the K1 capsule type, which has been demonstrated to increase survival after crossing the BBB by preventing lysosomal fusion (86, 89). NMEC also uses iron-acquisition systems for survival, outer membrane proteins for survival and invasion, fimbrial adhesins for attachment to BMEC, and invasins to invade host cells and traverse the BBB (90-94).

Avian Pathogenic *E. coli* (APEC)

Avian pathogenic *E. coli* (APEC) is the etiological agent of avian colibacillosis, a leading cause of economic loss in the poultry industry worldwide (95). Colibacillosis can manifest as local or systemic infection, including cellulitis, airsacculitis, salpingitis, and colisepticemia, among others (96-99). Colibacillosis affects all species of poultry across all stages of poultry production, including but not limited to broiler and layer chickens, turkeys, and ducks, resulting in significant losses each year due to morbidity, mortality, and carcass condemnations (100).

APEC may be transferred vertically from mother to embryo or horizontally from the environment or other birds. A laying hen suffering from oophoritis or salpingitis may infect the egg during shell formation, or the egg may become contaminated during passage through the cloaca (101). Common routes of horizontal APEC infection are through the respiratory and fecal-oral routes by consumption of contaminated water or feed or inhalation of aerosols. As such, colibacillosis generally starts as respiratory disease that evolves into systemic disease (102). APEC colonizes the upper and lower respiratory tracts, where it may enter the bloodstream

through the lungs or damaged air sac interstitium (103). Dissemination into the bloodstream allows for the colonization of internal organs and bacteremia.

There are many virulence factors associated with APEC, including the ability to persist in the environment, resist antimicrobials, and acquire genetic information from other microbes (51, 104). Common virulence factors include adhesins, invasins, serum resistance mechanisms, toxins, iron-acquisition systems, two-component systems, and secretion systems (105-107). Some studies suggest that typical APEC strains will have multiple iron transport-encoding genes (107) and plasmid-associated genes (108, 109) and are more likely to have P fimbriae (56) and serum resistance genes (110) than fecal isolates. APEC has also been found to share virulence factors with human ExPEC subtypes UPEC and NMEC (111-114) and has demonstrated the ability to cause urinary tract infections and meningitis in rodent models of human disease, indicating the potential for zoonosis (115-118). Despite the multiple virulence genes associated with APEC, the mechanisms essential to APEC pathogenicity are still unknown. As APEC has such a diverse repertoire of virulence genes, there is no clearly defined genotype. Therefore, characterizing the APEC genotype warrants further investigation.

Surface Antigens

E. coli isolates are classified based upon the presence of three surface antigens: O (somatic), H (flagellar), and K (capsular). The O-antigen defines the serogroup, also referred to as the O-type, and the addition of the H-antigen and K-antigen, if present, defines the serotype of that isolate. However, since few laboratories have the capability of typing the K-antigen, the O- and H-antigens are used as the gold standard for *E. coli* serotyping (119).

The O-antigen is part of the lipopolysaccharide, and important virulence factor and structural component of Gram-negative bacteria. As the O-antigen is highly variable, it is used as

the standard identifier to classify *E. coli* isolates for taxonomical and epidemiological studies (120). There are 184 serogroups recognized by the World Health Organization Collaborating Center for Reference and Research on *Escherichia* and *Klebsiella* as of 2015 (121). These serogroups are designated O1 to O187, which include three subgroups (O18ab/O18ac, O28ab/O28ac, and O112ab/O112ac) and six excluded groups (O31, O47, O67, O72, O93, and O122). These serogroup designations are continuously changing, as demonstrated by the three added sub-serogroups and the six removed serogroups that are no longer recognized. As such, there is ongoing research to update the current understanding of *E. coli* O-antigens and their serogroup designations (122-127).

Biofilm

Microbial biofilms, which this work focuses on, were discovered in 1632 by Antonie van Leeuwenhoek at the same time he discovered bacteria. However, it was not until the 1970s that biofilms were formally characterized by J. W. Costerton after studying the bacteria inhabiting various environments. Costerton and colleagues first noticed that rumen bacteria were enveloped by a complex matrix, termed the glycocalyx, which was not present in planktonic bacteria of the same species (128, 129). After studying submerged rocks from streams, Costerton and colleagues compared the attached bacteria on the rocks to the planktonic bacteria in the stream (130, 131). A summary of their findings was published, in which Costerton coined the term "biofilm" (132).

Biofilm is an extracellular matrix composed of proteins, nucleic acids, and exopolysaccharides (EPS) secreted by microorganisms when planktonic cells attach to a surface (133). Biofilms can be formed by a single bacterial species or, as more commonly found in nature, a mixed population of bacteria (134). Biofilm formation can be divided into three main

stages: 1) attachment to a surface, 2) aggregation and formation of the mature biofilm architecture, and 3) dispersion of cells from the structure (135). A schematic representation of the biofilm formation cycle is shown in Figure 2.1. Microorganisms form biofilms as a response to stressful conditions, and it serves as a physical barrier protecting against harmful environmental factors, including antimicrobials, host defenses, and predation (136, 137). Biofilms also provide a safe environment for horizontal gene transfer and increase conjugation efficiency between bacterial cells, which can lead to increased transfer of antimicrobial resistance and other virulence genes (138, 139). The process of biofilm formation varies between species and, in the case of *E. coli*, between pathotypes; therefore, the following is a general description of *E. coli* biofilm formation.

Attachment. Bacterial attachment to a surface is further categorized as reversible attachment (first) and irreversible attachment (second). Motility has been found to play an important role in bacterial attachment, as it significantly improves the ability of the bacteria to attach to a surface and affects the overall architecture of the biofilm (140, 141). As planktonic bacteria approach a surface, there are attractive or repelling physiochemical and electrostatic forces between the bacteria and substrate, resulting in reversible attachment (133). Irreversible attachment occurs once the bacteria use their structural adhesins to attach to the surface. Flagella may be one of the first structures to initiate adhesion. In addition to their role in motility, studies have found that flagella are important for adhesion of *E. coli* to surfaces (141-143). Fimbriae also play an important role in strengthening bacteria-to-substrate attachment, namely type I fimbriae (*fim* operon), curli (*csg* operon), and conjugative pili (*tra* operon) (141, 144-149). Other outer membrane structures, such as antigen 43, outer membrane protein R, and

lipopolysaccharide, enhance adhesion for biofilm formation through cell-to-surface adhesion and cell-to-cell adhesion (150-153).

Maturation. Once the bacteria have irreversibly attached to the surface, the three-dimensional biofilm architecture will begin to form. There is a notable change in gene expression of the attached bacteria compared to their planktonic counterparts. There is a downregulation of motility genes and an upregulation favoring sessility and colanic acid (*E. coli* EPS) formation (154-156). The EPS is a major component of biofilm that is secreted by the bacterial cells to provide structure to the biofilm architecture (157). The individual bacterial cells then become a community, necessitating the need to communicate, which they do through quorum sensing. Quorum sensing utilizes small peptides and other auto-inducers to help maintain appropriate cell density, serving as the checkpoint in the biofilm cycle, or signal to cells to express certain behaviors, such as factor secretion or structure arrangement (158). Antigen 43 works in a similar way to quorum sensing, as it is a self-recognizing protein that promotes cell-to-cell adhesion (133). Many outer membrane proteins are also integral to providing structure to the biofilm, such as OmpA, Hha, and YbaJ (159, 160).

Dispersal. The final step in the biofilm formation process is the dispersal of planktonic bacteria into the environment. Instead of passive dispersal, which may happen because of shear stresses, the bacteria have evolved to perceive changes in the environment and use quorum sensing to actively disperse, given the right signals (158). Dispersal signals vary, such as changes in nutrient or oxygen availability, an increase in toxins, or another stress-inducing condition (62). The dispersal phase functions contrary to the attachment phase: genes involved in motility and EPS degradation are upregulated, whereas genes involved in attachment and EPS production are

downregulated (62). The dispersal of planktonic cells allows them to find another suitable environment to begin the biofilm formation process again.

Biofilm formation is utilized by commensal and pathogenic strains on both biotic and abiotic surfaces. Biofilms may have beneficial applications in agriculture and other industrial settings, such as plant protection, bioremediation, and wastewater treatment (161). However, the formation of biofilms by pathogenic bacteria in the environment, such as in water lines or food troughs, can lead to bacterial infection and disease persistence. For example, diarrheagenic *E. coli* can form biofilms on food processing equipment or consumables themselves, which contribute to their persistence (162). Conway and Cohen (163) hypothesize that the commensal and pathogenic *E. coli* colonize the intestinal tract as part of mixed biofilm communities, referred to as "Restaurants," where they rely on other anaerobes that live there to provide their essential nutrients. In addition, different pathotypes of *E. coli* develop different forms of biofilm. For example, EAEC, which colonizes the small or large intestine, is unique such that it forms a thick, mucus-encased biofilm on the surface of enterocytes without common factors like flagella, curli, and antigen 43 (36). EAEC instead uses aggregative adherence fimbria, which also allow EAEC to attach to and form biofilms on urinary catheters, causing catheter-associated UTIs (164). UPEC, on the other hand, invades the bladder epithelial cells and develops biofilm in the cytoplasm in the form of intracellular bacterial communities (61).

APEC Biofilm

APEC have the ability to form biofilms on biotic and abiotic surfaces. In the poultry industry, APEC biofilms are commonly found in feed and water systems, providing reservoirs for APEC contamination. Research on APEC biofilms is a growing field. Similar to other *E. coli* pathotypes, APEC require factors such as adhesins (*fimC, fimH, papC, papG, sfaS, csgA, flgE,*

aatA), invasins (*ibeA, gimB, ychO*), protectins (*waaL, sodA*), secretions systems (*hcp1, evpB, icmF*), two-component systems (*phoP/phoQ, tolC, basS/basR*), transcriptional regulators (*mcbR*), and quorum sensing (*luxS*) to mediate biofilm formation (100). Although much research has been done examining the virulence genes in APEC compared to fecal isolates (107, 117, 165-169), studies are still limited on how these factors impact APEC biofilm formation (170-174). As mentioned above, different pathotypes of *E. coli* have different mechanisms of biofilm formation, and the mechanisms of APEC biofilm formation are largely unknown. Identification of APEC-specific biofilm genes will lead to a better understanding of APEC virulence and, therefore, can lead to more efficient methods of control.

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FIGURES

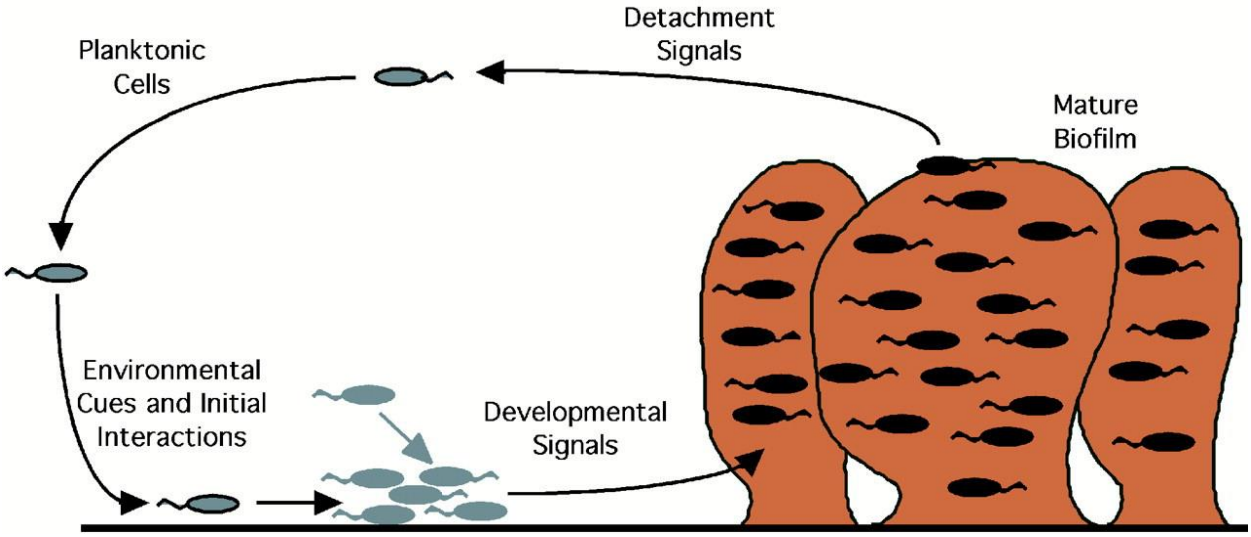

Figure 2.1: Schematic representation of the biofilm formation process. Figure extracted from O'Toole et al., 2000 (135).

CHAPTER 3

O SEROGROUP ANALYSIS OF *ESCHERICHIA COLI* ISOLATED FROM TURKEYS WITH CELLULITIS IN IOWA[1]

[1]Young, M.M., A.L. de Oliveira, N.L. Barbieri, and C.M. Logue. To be submitted to *Poultry Science*.

ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) is the etiological agent of avian colibacillosis, a leading bacterial cause of economic loss to the poultry industry worldwide. Colibacillosis can manifest locally or systemically, causing infections such as cellulitis, perihepatitis, airsacculitis, and septicemia. One of the first steps towards eliminating APEC is to identify the pathogenic serogroups (O-types) infecting birds. Therefore, the objective of this study was to analyze the distribution of *E. coli* serogroups between two turkey farms in Iowa, which included one barn with an active cellulitis outbreak (A2) and two control barns (A1, B1). Using multiplex PCR, the O serogroups for 260 isolates were identified, whereas 73 were non-typable. The most prevalent O-types in the case barn were O143 (6.3%), followed by O25, O78, and O-type Group 10 (OGp10) (4.6% each). The case barn was composed of isolates from three different origins: litter, cellulitis lesions, and tissues from diseased birds. The most prevalent O-types in the litter were OGp10 (10.0%) and O78 (7.5%); in the cellulitis lesions were O143 (13.0%), followed by O25 and O130 (7.4% each); and in the tissues were OGp14 and OGp15 (10.0% each). In contrast, the most prevalent O-types in control barn A1 were O15 (13.0%), followed by O8, O75, and O84 (4.4% each), and in control barn B1 were O111 (10.5%), O138 (9.0%), and O86 (7.5%). Although serogroups O1, O2, and O78 are the dominant O-types causing colibacillosis, they all had relatively low prevalence in the case barn compared to similar studies. Instead, O143 is the dominant serogroup in the cellulitis isolates and case barn in total and has been identified as APEC in previous studies, indicating that it may be an emergent serogroup. Vaccination programs targeting O1, O2, and O78 may be responsible for these serogroup shifts; therefore, it is important to identify any emergent APEC serogroups to ensure optimal vaccine development.

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) is the etiological agent of avian colibacillosis, an important cause of morbidity and mortality in the poultry industry worldwide. Avian colibacillosis can manifest locally or systemically, causing infections such as airsacculitis, perihepatitis, synovitis, and colisepticemia. The poultry industry is one of the cheapest sources of protein worldwide, with the value of the United States poultry production estimated to be $46.2 billion in 2018 and $40.4 billion in 2019 (1). However, condemnation ante-mortem and post-mortem can lead to heavy economic losses (2). Airsacculitis alone caused 16.1% of post-mortem condemnations in chickens and turkeys combined in 2020 (3). The 2020 Turkey Industry Annual Report scored colibacillosis as a 4.2 (out of 5) in terms of disease severity and prevalence and ranked it second (out of 36) of top current disease issues (4).

One of the first steps in controlling the spread of colibacillosis is to identify the serogroups responsible for the disease. *E. coli* is identified using surface antigens: polysaccharide O-antigens, flagellar H-antigens, and capsular K-antigens. The O-antigen identifies the serogroup and, when combined with the H-antigen and K-antigen if present, the serotype. The O-antigen is a part of the lipopolysaccharide, an important virulence factor, and is highly variable, resulting in 184 serogroups of *E. coli*. Therefore, the O-antigen is used as the standard identifier for grouping *E. coli* for taxonomical and epidemiological studies (5). The distribution of O serogroups, also known as O-types, varies by world region, but the most prominent APEC O-types causing disease include O1, O2, and O78 (6-9). Although these O-types are targeted through vaccines available in the market, emerging O-types may still increase the incidence of disease with the acquisition of new virulence and resistance factors.

This study sought to investigate the prevalence and distribution of *E. coli* O-types found across the growth phase of turkeys on two farms in Iowa. Utilizing multiplex PCR, this study aims to identify new emerging O-types of APEC that may be contributing to poultry disease in Iowa.

MATERIALS AND METHODS

Bacterial Strains and DNA Preparation

A total of 333 *Escherichia coli* isolates were obtained from a previously described study (10). These isolates were collected from three turkey barns on two farms in Iowa, of which two barns (A1 and B1, controls) had no history of cellulitis-associated disease in the past 12 months, and the third (A2, case barn) had a cellulitis outbreak. The barns were each visited once per week over the course of several weeks. Barn A1 was visited from weeks 10 to 17 of production, Barn A2 was visited weeks 12 to 18, and Barn B1 was visited weeks 12 to 17. Litter samples were collected from all three barns, and swabs from cellulitis lesions and tissue samples were collected from the case barn. As a result, isolates obtained from each barn were categorized as either litter, cellulitis, or systemic. Isolates were identified as *E. coli* by PCR of the 16S rRNA gene by de Oliveira et al. (10), and confirmed *E. coli* isolates were stored in glycerol at -80°C until further use.

Bacterial DNA was obtained from whole organisms using the boil prep method. Briefly, frozen bacterial stocks were streaked out onto Luria-Bertani agar, Miller (BD Difco™, Franklin Lakes, NJ) and grown overnight at 37°C. Using a 1-µL inoculating loop, bacteria from each plate was collected and inoculated into 200 µL of sterile Milli-Q water, boiled at 99.9°C for 10 minutes, and then centrifuged at 13,500 x g for 3 minutes to precipitate cellular debris. Afterwards, 150 µL of the supernatant containing the DNA was transferred to a fresh DNase-free

tube and used as the template for gene amplification. Bacterial DNA stocks were stored at -20°C until use.

Multiplex PCR for Serogrouping

Isolates were screened for O-types using multiplex PCR as previously described (11) with some modifications. A total of 162 PCR primer pairs were used in this study, of which 147 pairs were used to identify individual O-types and 15 pairs were used to identify each of the 15 O-type groups (OGp1 to OGp15; Table 3.1). These O-type groups comprise O-types with similar or identical O-antigen gene cluster sequences and represent 35 O-types (11). The PCR primer pairs were pooled as previously described (11) in nuclease-free water to produce 20 multiplex primer pools composed of six to nine primer pairs each.

To evaluate the *E. coli* isolates, DNA sample pools were created and composed of an equal volume of seven DNA samples each, except one pool which contained only six. PCR was conducted using an Eppendorf Mastercycler X50s (Eppendorf, Hamburg, Germany). Reactions were performed in 30 µL volumes using 5.0 µL pooled DNA, 3.0 µL PCR buffer (10x), 0.4 µL $MgCl_2$ (4 mM), 1.25 µL dNTP (10 µM) pool, 2.0 U Taq DNA polymerase, 3.52 µL multiplex primer pool (100 µM each primer), and 14.83 µL sterile Milli-Q water. The thermal cycler conditions consisted of a 5-minute activation step at 94°C, followed by 30 annealing cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 minute, and a final extension step of 72°C for 10 minutes. The amplicons were then separated by gel electrophoresis in a 1.5% agarose (SeaKem LE Agarose, Lonza, Alpharetta, GA), stained with 0.1% ethidium bromide (ThermoFisher, Waltham, MA), and visualized under UV light using a UVP GelSolo (Analytik Jena, Jena, Germany). For each sample pool that produced a band for a given multiplex primer pool, the

individual samples in that sample pool were used to perform PCR with that multiplex primer pool using the same conditions to ensure accurate assignment of the serogroup.

For each isolate that was non-typable according to the Iguchi method (11), a four-panel multiplex primer pool was used to serogroup the isolates for O1, O2, O18, and O78 (12). Even though these O-types were covered in the original Iguchi panel (11), the Wang panel (12) targeted different regions, and, therefore, could potentially serogroup the un-typed isolates. Reactions were performed in 25 µL volumes using 2.0 µL DNA, 2.5 µL PCR buffer (10x), 0.4 µL 4 $MgCl_2$ (4 mM), 1.25 µL dNTP (10 µM) pool, 2.0 U Taq DNA polymerase, 0.4 µL multiplex primer pool (100 µM each primer), and 16.5 µL sterile Milli-Q water. The thermal cycler conditions consisted of a 5-minute activation step at 94°C, followed by 30 annealing cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 minute, and a final extension step of 72°C for 10 minutes.

If an individual sample was positive for multiple O-types, then that sample was retested with each corresponding multiplex panel. If multiple O-types were still indicated, then the results were classified as a mixed reaction.

APEC Minimal Predictors

Isolates were screened for genes that defined the APEC pathotype by de Oliveira et al. (10) as previously described (13). Briefly, a pentaplex PCR was performed for the genes *iroN*, *ompT*, *hlyF*, *iss*, and *iutA*. Any isolate that harbored three or more of the target genes was classified as APEC if recovered from the tissue or lesion of a bird or APEC-like if recovered from the litter.

Multiplex PCR for Virulence Genes

Isolates were screened for the presence of genes encoding virulence factors by de Oliveira et al. (10) as previously described (14, 15).

PCR-Based Phylogenetic Grouping

Isolates were assigned to phylogenetic group A, B1, B2, C, D, E, or F by de Oliveira et al. (10) using a PCR-based method as previously described (16). Briefly, a quadruplex PCR was first performed for the genes *chuA*, *yjaA*, and *arpA* and for the DNA fragment *TSPE4.C2*. The isolate was then classified as "A or C," "B1," "B2," "D or E," "*E* clades," or "F" depending on the band pattern. If an isolate showed a band pattern that classified it as "A or C" or "D or E," a second reaction was performed using primers for gene C (to differentiate between A and C) or gene E (to differentiate between D and E).

Statistical Analysis

Data was analyzed using non-parametric tests due to the variability in sample size and distribution of O-types and other traits. The chi-square test was used to compare the distribution of O-types between barns. The Mann-Whitney U test was used for direct comparisons among O-types and virulence genes. All statistical analysis was performed using Microsoft Excel (Version 16.0). Statistical significance was accepted when $p < 0.05$.

RESULTS

Using multiplex PCR, 260 of the 333 isolates (78.1%) evaluated were O-typed, and 73 isolates (21.9%) could not be typed ("non-typable," NT). Of the typed isolates, 33 (12.7%) were identified as belonging to two or more O-types, thus labeled as "mixed reaction" (MR) and not included in the individual O-type counts.

Barn A2 was the case barn in this study and had a cellulitis outbreak starting from week 17. Of all the isolates tested from Barn A2, 125 (71.8%) were typed into single serogroups. The most prevalent O-type overall was O143 with 11 isolates (6.3%), which was followed by O25, O78, and OGp10 with eight isolates (4.6%) each (Figure 3.1A**)**. Isolates were collected from different sources of origin: cellulitis lesions, tissue samples (referred to as "systemic"), and litter. As such, each source provided a different distribution of O-types (Figure 3.2), with 75.9% of the cellulitis, 65.0% of the systemic, and 72.5% of the litter samples typable. The most prevalent O-type from the cellulitis isolates was O143 with seven isolates (13.0%), followed by O25 and O130 with four isolates (7.4%) each. The most prevalent O-types in the systemic samples were OGp14 and OGp15 with four isolates (10.0%) each, followed by O36 with three isolates (7.5%). The most prevalent O-type in the litter was OGp10 with eight isolates (10.0%), followed by O78 with six isolates (7.5%).

Samples were collected from Barn A2 from week 12 of production until week 18, just before slaughter. As time progressed, the O-type distribution in the barn changed, specifically related to the cellulitis outbreak during weeks 17 and 18. Serogroup O25 increased in prevalence in the litter during week 17 and again in week 18 (Figure 3.3A). There were two O25 isolates found in the cellulitis samples in weeks 17 and 18 each and one found in the systemic samples in week 17. After the appearance of serogroup O130 in week 14, it remained in the barn with relative consistency, with one isolate found in the litter during weeks 14, 15, and 16 each (Figure 3.3A) and then four isolates found in the cellulitis lesions during week 18 (Figure 3.3B). There was an increasing prevalence of OGp10 in the litter of Barn A2 until a sudden and significant drop in week 14, after which there were no detected OGp10 isolates (Figure 3.3A). There were also no OGp10 isolates detected in the cellulitis or systemic isolates (Figures 3.3B, 3.3C).

Serogroup O143 was the most prevalent O-type in the entire barn, seven of which were found in week 17 cellulitis lesions and two found in the systemic samples in week 18 (Figure 3.3B, 3.3C). There was also a significant increase in O143 in the litter from week 16 to week 17 (Figure 3.3A).

In the previous study (10), each isolate was evaluated via PCR for phylogenetic group and for the presence of five APEC-predictor genes, with the presence of three genes indicating that the isolate is APEC or APEC-like. In the case barn A2, all of the O143 isolates (n = 10) detected across all sources harbored all five APEC genes, and nine isolates (90%) were identified as phylogenetic group F and one cellulitis isolate as phylogenetic group B2. The O130 isolates (n = 7) were consistent across all sources, with all isolates identified as phylogenetic group A and harboring the same three APEC genes. The O25 isolates (n = 8) were less consistent. Although all eight isolates identified as phylogenetic group B2, only three isolates (37.5%) were positive for all five APEC genes, and the remaining five isolates did not appear to harbor any of the APEC genes. There was no apparent pattern across source or week. The OGp10 isolates (n = 8) were only present in the litter. There were six isolates (75%) that classified as phylogenetic group A and harbored none of the APEC genes. The remaining two isolates were identified as phylogenetic group B2, both of which were classified as APEC.

The control barns each had different O-type distributions both from the case barn and from each other. Of the 92 isolates recovered from Barn A1, 57 (62.0%) were typable. The most prevalent O-type from Barn A1 was O15 with 12 isolates (13.0%), followed by O8, O75, and O84 with four isolates (4.4%) each (Figure 3.1B). Isolates from Barn A1 were collected from weeks 10 to 17. The prevalence of O15 appeared to decrease with time, whereas O8 remained relatively consistent and O75 and O84 showed no apparent patterns (Figure 3.4A). Of the 67

isolates recovered from Barn B1, 45 (67.2%) were typable. The most prevalent O-type from Barn B1 was O111 with seven isolates (10.5%), followed by O138 with six isolates (9.0%), and then O86 with five isolates (7.5%) (Figure 3.1C). Isolates from Barn B1 were collected from weeks 12 to 17. The prevalence of O111 and O138 remained relatively consistent, whereas the prevalence of O86 appeared to increase with time (Figure 3.4B).

Special interest was paid to common APEC serogroups O1, O2, and O78. A summary of these O-types in this study can be found in Table 3.2. There were three O1 isolates (3.8%) found in the litter of the case barn. Although all three were APEC-like, they were only found during week 12 in the litter, which was five weeks before the cellulitis outbreak, and none were found in the cellulitis or systemic samples. Serogroup O2 in this study was categorized under the O-type group OGp7, alongside O50 (Table 3.1). In the case barn, there was one OGp7 isolate found each in the cellulitis (1.9%), systemic (2.5%), and litter (1.3%) samples, all of which were found to be APEC or APEC-like. There was one O78 isolate found in the cellulitis (1.9%) and systemic (2.5%) samples each, and six were found in the litter of the case barn (7.5%). Although there was a significantly higher prevalence of O78 in the case barn litter than the control litter, all isolates were found during weeks 12 or 13, which was four to five weeks before the cellulitis outbreak, and only two of them were considered APEC-like. In addition, the only O78 isolates found in the cellulitis or systemic samples were during week 18, a week after the outbreak began. To compare with the control barns, Barn A1 was found to have no O1 isolates, two OGp7 isolates (2.2%) during week 17, and two O78 isolates (2.2%) from weeks 11 and 15 combined. Barn B1 had neither O1 nor O78 but was found to have one OGp7 isolate (1.5%) during week 14. There was no significant difference between the case barn and either control barn for O1 or OGp7.

DISCUSSION

This study builds upon what may be one of the largest reports (10) documenting APEC in turkey cellulitis and its relationship between virulence factors, mortality, and prevalence. Here we examined the O-type distribution of *E. coli* across three barns on two turkey farms in Iowa: two control barns with no history of cellulitis in the previous 12 months and one case barn with a cellulitis outbreak. In the original study (10), a multiplex PCR panel of only 11 O-types was used to examine the O-type distribution. In this study, however, 20 multiplex PCR panels covering all 184 *E. coli* O-types that are recognized by the World Health Organization Collaborating Center for Reference and Research on *Escherichia* and *Klebsiella* (11) were used to investigate the prevalence of O-types on the farms. Screening for all *E. coli* O-types allows us to better identify emerging serogroups that are causing disease in poultry.

Certain serogroups are often associated with specific diseases and, therefore, *E. coli* pathotypes. For example, six *E. coli* O-types (O1, O6, O8, O15, O18, O25) are responsible for 75% of urinary tract infections worldwide (17, 18), and seven O-types (O26, O45, O103, O111, O121, O145, O157) are responsible for the vast majority of enterohemorrhagic *E. coli* infections in the United States (19). Likewise, serogroups O1, O2, and O78 are most often isolated from diseased birds worldwide and are responsible for 80% of avian colibacillosis cases (20-23). In this study, however, these O-types were not prominent in the case barn, comprising only 8.0% the isolates in the entire case barn. Although there was a significantly higher prevalence of O78 in the case barn litter (7.5%) compared to the control barns (2.2%, 0%), only two out of the six litter isolates in the case barn were APEC-like, and there was a low prevalence of O78 in the tissues (2.5%) and lesions (1.9%) of the diseased birds.

Instead, the most prevalent O-type in the case barn was O143, followed by O25, OGp10, and O130. The previous study (10) found O24 to be the dominant O-type and believed it to be an emerging serogroup, which was supported by another study published by this research group (22). However, the isolates were screened for only 11 O-types by de Oliveira et al. (10), which resulted in an incomplete picture as to dominant serogroups that were present and as a result identified O24 as most common. The expanded serogroup analysis used in the current study was able to classify a greater number of isolates, providing a much more accurate picture of the O-types of strains present in the control and case barns.

In the case barn, 100% of the O130 and O143 isolates and 37.5% of the O25 isolates were APEC or APEC-like and had either increasing or relatively steady prevalence in the litter before appearing in the tissues and lesions of the birds (Figure 3.3C). As reported in the previous study (10), the prevalence of APEC-like isolates in the litter increased over time and peaked just before the cellulitis outbreak began, which correlated with an increase in mortality in the case barn. Several studies have already indicated that litter quality plays an important role in the health of poultry (24-28). Although OGp10 increased in prevalence in the litter over time, there was a sudden drop in week 15, after which O25 and O143 began to be detected. In addition, the OGp10 isolates were not APEC-like except for two that also classified as a different phylogenetic group (B1) from the other six OGp10 isolates (A). When those two B1-OGp10 isolates were removed from the equation, there were significantly more virulence genes in the O25, O130, and O143 isolates compared to the A-OGp10. The lack of virulence genes in the A-OGp10 isolates paired with the sharp decrease in prevalence just before the cellulitis outbreak suggests that this O-type likely represented commensal isolates. Meanwhile, the presence of APEC-like O25, O130, and O143 isolates in the litter followed by their presence in tissues and

lesions suggests that these O-types may be, in part, responsible for the cellulitis outbreak, although further investigation is required to substantiate this claim. Instead, the source of this cellulitis outbreak may be linked to another species, such as *Clostridium septicum* or *C. perfringens*, both of which are considered the primary causes of cellulitis in turkeys (29). The investigators of the original study (10) did not discuss detection of Gram-positive bacteria, leaving room for speculation that *Clostridium* sp. or another Gram-positive bacteria was the etiological agent and *E. coli* was the opportunistic pathogen.

E. coli serogroups O130 and O143 are commonly found as the enteric pathotypes: O130 as Shiga toxin-producing *E. coli*, namely O130:H11 (30), and O143 as enteroinvasive *E. coli* (31, 32). However, there is evidence of both Shiga toxin-producing and enteroinvasive strains harboring virulence factors that are often associated with extraintestinal pathogenic *E. coli* (ExPEC), particularly uropathogenic *E. coli* (33-35). Studies have also shown that other enteric *E. coli* pathotypes may harbor ExPEC virulence factors and vice-versa, potentially creating hybrid pathotypes (36-40). However, the isolates in this study were not screened for Shiga toxin or other enteric virulence factors, and further investigation is warranted to confirm if they are indeed hybrid pathotypes.

Many ExPEC strains share virulence factors with other ExPEC (41-43), lending to the idea that APEC may be a reservoir for human ExPEC virulence factors (15, 44, 45). In a study comparing O18 APEC and neonatal meningitis *E. coli* strains (46), it was found that these subtypes were not easily differentiated based on multilocus sequence typing, phylogenetic typing, or virulence factors. Furthermore, some of the tested APEC strains were able to cause meningitis in rat models of human neonatal meningitis, supporting the hypothesis that APEC strains have zoonotic potential. As such, a serogroup of concern in this study may be O25, which

is commonly found as an uropathogenic strain (17, 47, 48). There have been numerous studies connecting poultry meat to urinary tract infections (49-53), and the presence of O25 in the case barn may be indicative of a strain with zoonotic potential. The O25 isolates in this study were previously tested (10) for the presence of some uropathogenic virulence markers (*cnf-1*, *fyuA*, *sfa-foc*, *papC*, *iha*, *sfaS*, and *chuA*) (54). Out of the eight O25 isolates in the case barn, six (75%) contained *papC* and eight (100%) contained *chuA*, but none of the isolates were found to harbor *cnf-1*, *fyuA*, *sfa-foc*, *iha*, or *sfaS*. According to this data, it seems unlikely that these isolates are uropathogenic; however, it remains important to monitor outbreaks for strains with potential hybrid pathotypes and zoonotic ability.

As previously mentioned, serogroups O1, O2, and O78 are frequently the predominating O-types causing disease in poultry and are most commonly associated with phylogenetic groups B2 and D (55). However, the most prevalent O-types in the cellulitis lesions in this study were O143 (13.0%), O25 (7.4%), and O130 (7.4%), and the majority of cellulitis isolates were classified as phylogenetic groups A (41%), F (25%), and B2 (22%). It is not unusual to see such deviations in avian colibacillosis cases, however; given the diverse nature of APEC, there may be substantial regional variation in the strains that cause disease, depending on country, state, or even farm (2). For example, in the People's Republic of China, APEC O142, a rare strain implicated in colibacillosis, was diagnosed in young broiler breeders causing black proventriculus (56). The predominant O-type in this study, O143, has not yet been identified as the leading serogroup in other colibacillosis outbreaks, but it has been found sparingly in other studies (57-60) as an APEC strain, indicating that it may be an emerging serogroup.

Vaccine programs may also elicit a shift in virulent serogroups. A study evaluating the efficacy of an autogenous vaccine against a commercial vaccine found that autogenous vaccines

shifted phylogenetic group prevalence and distribution of APEC on the tested poultry farms (61). A vaccine targeting or providing cross-protection against O1, O2, and O78 strains (62) may reduce their prevalence but also allow opportunity for other pathogenic strains to flourish and displace or change niches. It is also possible that the O1, O2, and O78 isolates identified in this study were all commensal isolates. This may have been the case with the O78 isolates, which were mostly non-APEC-like as evidenced from their lack of pathogenic genes and being isolated from non-lesion sources, i.e. litter. On the other hand, the O1 isolates identified in this study had the potential to be highly virulent, as they all had five out of five APEC predictor genes and the K1 capsule and were classified as phylogenetic group B2 (B2-O1:K1). *E. coli* B2-O1:K1 belongs to a highly pathogenic clonal group that can cause a variety of extraintestinal infections, including neonatal meningitis, urinary tract infections, and septicemia (63). Therefore, it seems likely that the turkeys may have been vaccinated against this particular serogroup. However, as the current study does not have any information on the vaccine regimen or antimicrobial treatment status of the birds examined, any reasoning for this shift in O-type dominance is purely speculation.

CONCLUSION

This study found that the usual culprits associated with avian colibacillosis (O1, O2 and O78) were not prevalent in the case barn and, instead, there were more unusual O-types implicated or associated with the cellulitis outbreak. As more vaccines targeting the dominant APEC strains are produced, the approach should be used with caution as these vaccines may be successful in protecting against the target O-types but may contribute to a serogroup shift, lending further opportunities for new serogroups to emerge. As such, it is important to continue to monitor flocks for potential APEC pathogens that are occurring. Early detection of new

emergent APEC serogroups may be key to the development of new vaccines and control of APEC.

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DISCLOSURES

The authors declare no conflicts of interest.

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TABLES AND FIGURES

Table 3.1: Groups of O-types with similar or identical O-antigen gene clusters.

Group Name	Associated O Serogroups
OGp1	O20, O137
OGp2	O28ac, O42
OGp3	O118, O151
OGp4	O90, O127
OGp5	O123, O186
OGp6	O46, O134
OGp7	O2, O50
OGp8	O107, O117
OGp9	O17, O44, O73, O77, O106
OGp10	O13, O129, O135
OGp11	O153, O178
OGp12	O18ab, O18ac
OGp13	O124, O164
OGp14	O62, O68
OGp15	O89, O101, O162

Table 3.2: Prevalence of common avian pathogenic *E. coli* (APEC) serogroups among barns. Cellulitis and systemic samples were only collected from the case barn, A2.

		Cellulitis		**Systemic**		**Litter**	
Barn	**O-Type**	**n**	**%**	**n**	**%**	**n**	**%**
A2	O1	0	0%	0	0%	3	3.8%
	O2	1	1.9%	1	2.5%	1	1.3%
	O78	1	1.9%	1	2.5%	6	7.5%
A1	O1	N/A	N/A	N/A	N/A	0	0%
	O2	N/A	N/A	N/A	N/A	2	2.2%
	O78	N/A	N/A	N/A	N/A	2	2.2%
B1	O1	N/A	N/A	N/A	N/A	0	0%
	O2	N/A	N/A	N/A	N/A	1	1.5%
	O78	N/A	N/A	N/A	N/A	0	0%

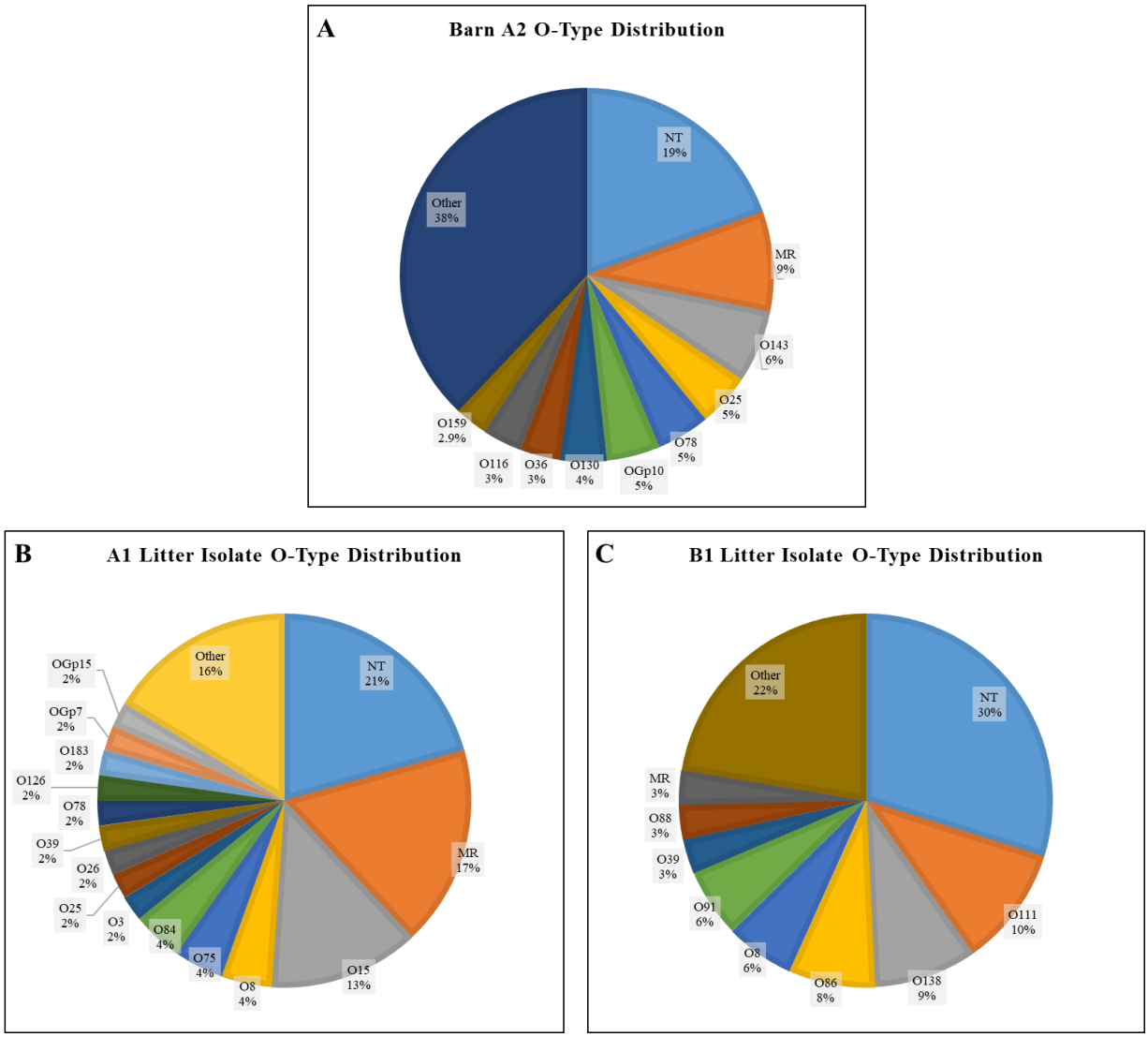

Figure 3.1: Serogroup distribution of each barn. NT = non-typable isolates. MR = mixed reaction isolates. (A) Distribution of O-types isolated from control Barn A1. (B) Distribution of O-types isolated from case Barn A2. (C) Distribution of O-types isolated from control Barn B1.

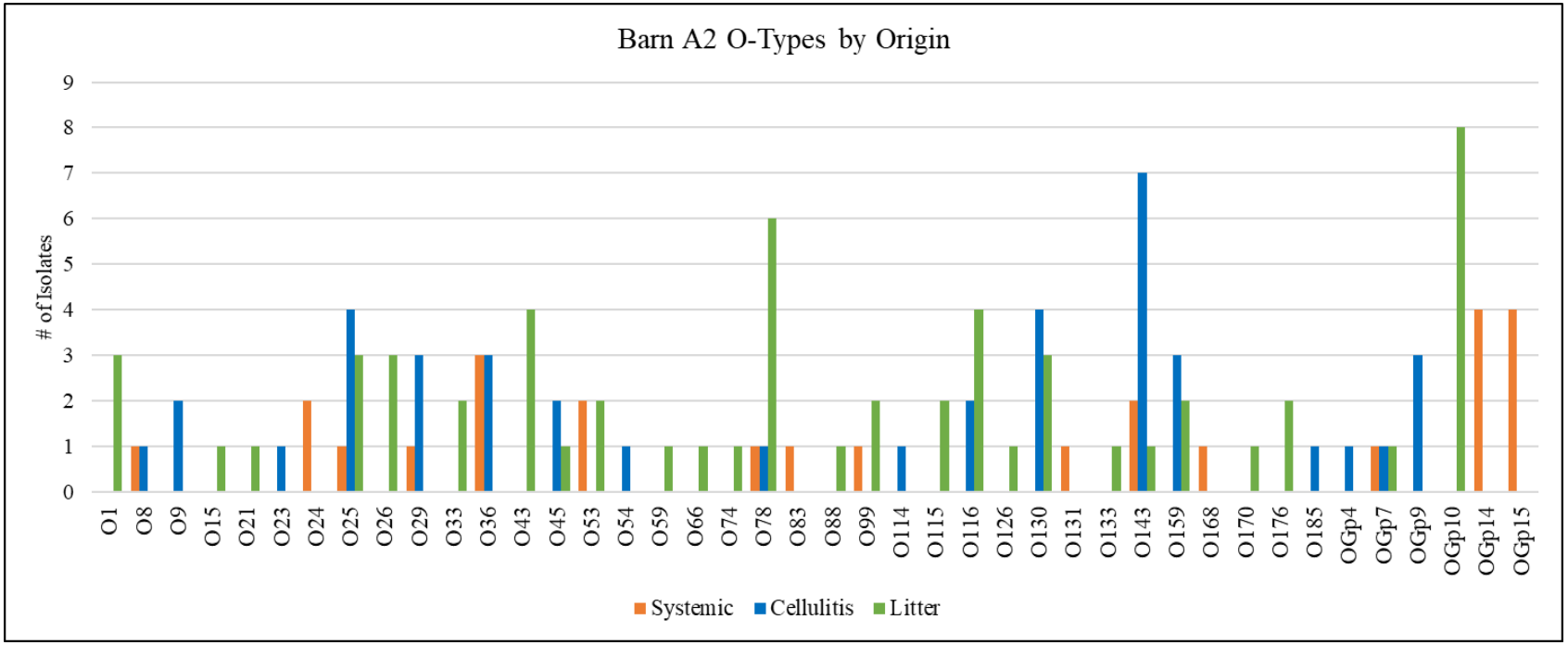

Figure 3.2: Prevalence of O-types in Barn A2 in the systemic, cellulitis, and litter isolates. The totals of non-typable (NT) and mixed reaction (MR) isolates are excluded.

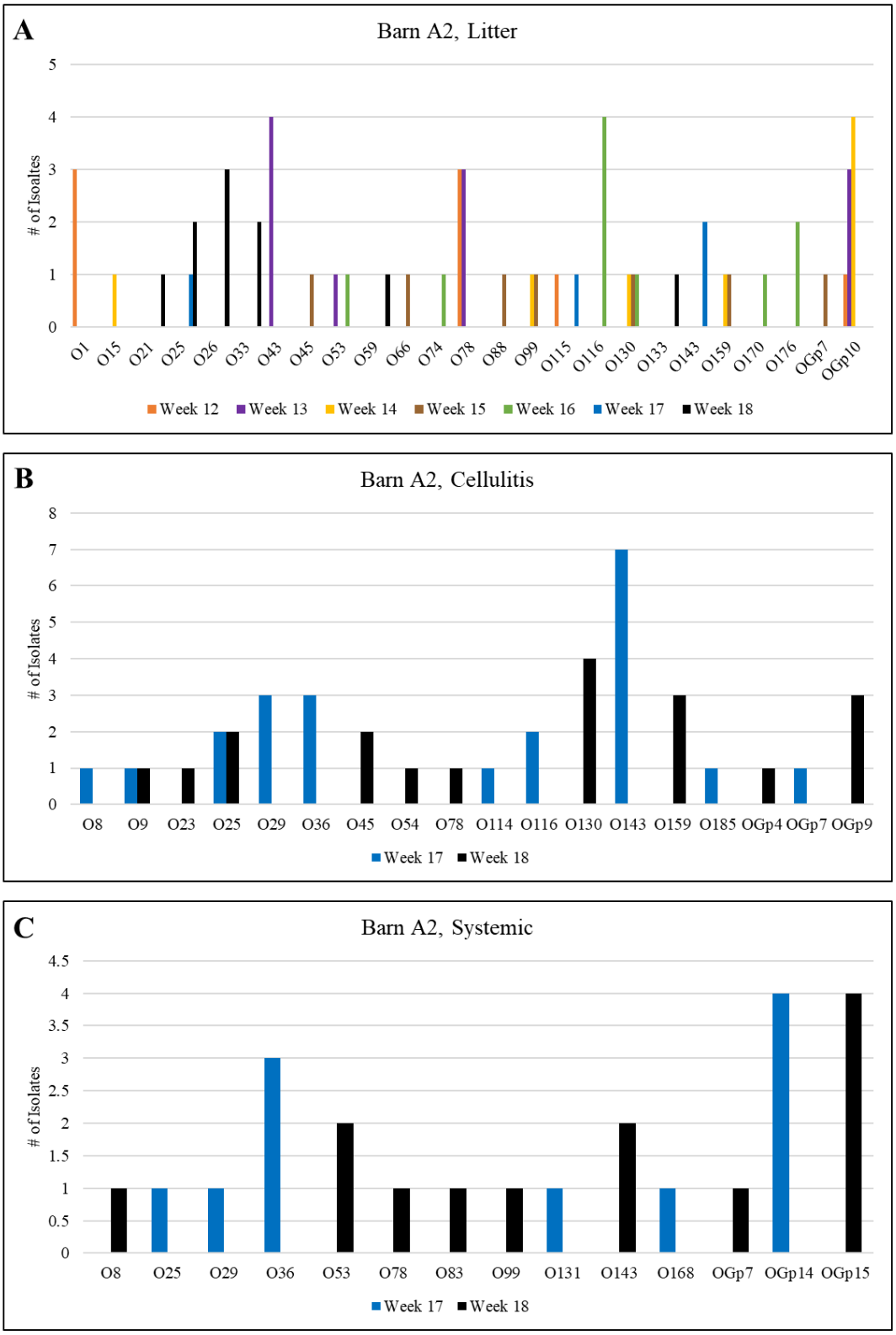

Figure 3.3: Prevalence of O-types in the case barn A2 over time. Non-typable (NT) and mixed reaction (MR) isolates are excluded. (A) Distribution of litter O-types from weeks 12 through 18. (B) Distribution of cellulitis O-types from weeks 17 and 18. (C) Distribution of systemic O-types from week 17 and 18.

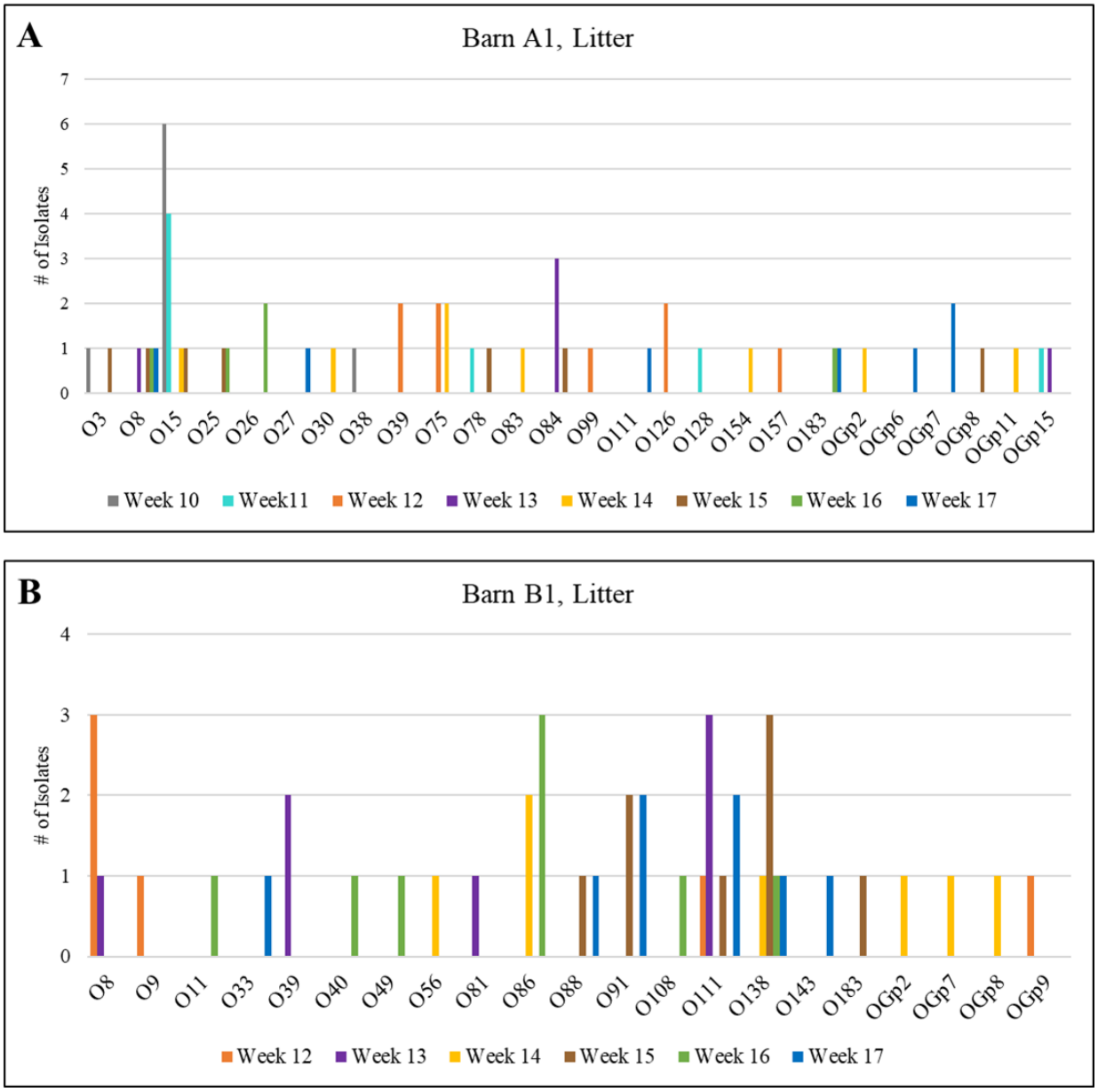

Figure 3.4: Prevalence of O-types in the control barns A1 and B1 over time. Non-typable (NT) and mixed reaction (MR) isolates are excluded. (A) Distribution of Barn A1 litter O-types from weeks 10 through 17. (B) Distribution of Barn B1 litter O-types from weeks 12 through 17.

CHAPTER 4

IDENTIFICATION OF NOVEL GENES INVOLVED IN THE BIOFILM FORMATION PROCESS OF AVIAN PATHOGENIC *ESCHERICHIA COLI*[1]

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) is the etiological agent of avian colibacillosis, a leading cause of economic loss to the poultry industry worldwide. APEC causes disease using a diverse repertoire of virulence factors, including the ability to form biofilms, which contributes to the survival and persistence of APEC. The objective of this study was to identify genes most widespread and important in APEC that contribute to APEC biofilm formation. Using APEC 380 as the template strain, a total of 15,660 mutants were randomly generated using signature tagged mutagenesis and evaluated for decreased biofilm formation ability using the crystal violet assay. Biofilm deficient mutants were sequenced, and a total of 547 putative biofilm formation genes were identified. Thirty of these genes were analyzed by PCR for prevalence among 109 APEC isolates and 104 avian fecal *E. coli* (AFEC) isolates, resulting in nine genes that were significantly more prevalent among APEC than AFEC. The expression of these genes was evaluated in the wild-type APEC 380 strain using real-time reverse-transcriptase quantitative PCR in both the exponential growth phase and the mature biofilm phase. To investigate the role of these genes in biofilm formation, isogenic mutants were constructed and evaluated for their biofilm production and planktonic growth abilities. Four of the mutants (*rfaY*, *rfaI*, and two uncharacterized genes) displayed significantly decreased biofilm formation, and of those four, one (*rfaI*) displayed significantly decreased growth compared to the wild type. Overall, this study identified novel genes that may be important in APEC and its biofilm formation. The data generated from this study will benefit further investigation into the mechanisms of APEC biofilm formation.

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) is an extraintestinal pathotype of *E. coli* that causes disease in poultry and other avian species. It the etiological agent of avian colibacillosis, a leading bacterial cause of morbidity and mortality in the poultry industry that contributes to significant economic loss worldwide each year (1). There are many virulence factors associated with APEC, including the ability to persist in the environment, resist antimicrobials, and acquire genetic information from other microbes (2, 3). Biofilm formation is a major contributing factor to the survival and persistence of APEC, and research into the genetic makeup of biofilm is a growing field.

Biofilms form when planktonic microorganisms attach to a surface and secrete exopolysaccharides, proteins, and nucleic acids to form the extracellular biofilm matrix (4). Biofilm formation can be divided into three main stages: (1) attachment to a surface, (2) aggregation and formation of mature biofilm architecture, and (3) dispersion from structure (5). Biofilm formation is a response to stressful conditions and serves as a physical barrier protecting against harmful environmental factors, including antimicrobials, host defenses, and predation (6, 7). Biofilms also provide a safe environment for horizontal gene transfer and increase conjugation efficiency between bacterial cells, which can lead to increased transfer of antimicrobial resistance and other virulence genes (8, 9).

Research into the role and function of APEC biofilm formation process is ongoing. Factors associated with biofilm include the type I fimbriae, encoded by genes on the *fim* operon, and curli proteins, encoded by genes on two *csg* operons, which are involved in the initial attachment of planktonic cells to a surface and in cell-to-cell adhesion (10, 11). Flagella have been found to play a role in the adhesion of *E. coli* to abiotic surfaces and in influencing the

biofilm architecture (12, 13). Outer membrane proteins have also been implicated in a variety of roles in biofilm formation, from cell-to-cell communication to mature biofilm development (14-16). In addition, quorum sensing has been implicated in multiple aspects of biofilm formation and development, from structural integrity to cellular dispersal (17). Despite these findings, there is still limited knowledge on which genes may be linked to biofilm formation in APEC specifically.

The purpose of this study is to uncover new genes involved in the biofilm formation process of APEC 380, a well-characterized and sequenced APEC strain, and to determine if they are most widespread and important in APEC. By discovering new genes associated with the biofilm formation of APEC, we may better characterize the virulence profile and biofilm formation ability of APEC.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Strains and plasmids are shown in Table 4.1. The wild-type template in this study was APEC 380 (denoted as A380 WT), which is a virulent O-serogroup 18, sequence type 95, and phylogenetic group B2 strain of *E. coli*. The strain was isolated from the pericardial and lung tissue of a laying hen diagnosed with colibacillosis in the United States and is a robust biofilm producer. The genome of APEC 380 was sequenced and is publicly available in GenBank (accession number CP006830) under the name "APEC O18" (18). *E. coli* DH5α was used for plasmid cloning. All strains were cultured in Luria-Bertani (LB) broth, Miller (BD Difco™, Franklin Lakes, NJ) at 37°C with agitation unless otherwise specified. The medium was supplemented with ampicillin (AMP, 100 mg/mL), kanamycin (KAN, 50 mg/mL), nalidixic acid (NAL, 30 mg/mL), or chloramphenicol (CHM, 25 mg/mL) as necessary.

DNA Extraction

Bacterial DNA was extracted from strains by boiling. Briefly, strains were streaked out from frozen stocks on Luria-Bertani (LB) agar, Miller (BD Difco™, Franklin Lakes, NJ, USA) and incubated overnight at 37°C. Following incubation, a 1-µL inoculating loop was used to collect bacteria from the plate and inoculate 200 µL of sterile Milli-Q water. The mixture was boiled for 10 minutes at 99°C and then centrifuged for 3 minutes at 13,000 x g to precipitate cellular debris. A volume of 150 µL of the supernatant containing the genomic material was transferred to a fresh tube and used as the DNA template for gene amplification. The bacterial DNA stocks were stored at -20°C until use.

Mutant Library Generation

A random mutant library was generated using the signature tagged mutagenesis (STM) technique using transposon pUTmini-Tn5km2, as previously described (19). Independent matings were set by growing each *E. coli* S17-1 λpir (with tag) clone and the APEC 380 nalidixic acid-resistant clone to the late log phase. To construct tagged transposon mutants of APEC 380, 400 µL of donor cells and 400 µL of recipient cells were mixed and incubated at 37°C for 8 h on plates. Cells were collected from the plates and re-suspended in phosphate buffered saline (PBS; Research Products International, Mt. Prospect, IL) and plated onto LB agar containing 50 mg/mL KAN and 30 mg/mL NAL (NAL-KAN-LB). Following selective overnight growth, single colonies were selected and inoculated into 1-mL wells of 96-well deepwell microtiter plates (Eppendorf, Hamburg, Germany). Glycerol was added to a final concentration of 80%, and the plates sealed and stored at -80°C.

Biofilm Assays for Impaired Biofilm Formation

The mutant libraries were tested for biofilm formation abilities in M63 minimal media, as previously described (20). Briefly, mutants were grown overnight in LB broth. Following incubation, the mutants were diluted 1:100 in M63 minimal media [12 g KH_2PO_4 per liter, 28 g K_2HPO_4 per liter, 8 g $(NH_4)_2SO_4$ per liter, supplemented with 1 mmol per liter of $MgSO_4$, 0.2% glucose, and 0.5% casamino acids]. Aliquots of 200 µL of each dilution were dispensed into the wells of a 96-well microtiter plate (Sarstedt AG & Co. KG, Sarstedt, Germany). Negative control wells contained uninoculated medium, and positive control wells were inoculated with A380 WT. Each mutant was tested once. Following static incubation at 37°C for 24 h, the contents of the plates were poured off and washed once with sterile Milli-Q water. Next, the wells were stained with 200 µL of 0.1% crystal violet solution (Fisher Scientific Company, Fair Lawn, NJ) for 30 minutes, washed four times with Milli-Q water to remove excess stain, and air dried for 1 h. After drying, adherent cells were re-solubilized with 200 µL of an 80:20 solution of ethanol and acetone (Fisher Scientific Company, Fair Lawn, NJ). A volume of 150 µL of this solution was transferred to a new microtiter plate, and the optical density of each well was measured at 600 nm (OD_{600}) using an automated ELx808 Ultra MicroPlate Reader (Bio-Tek Instruments, Winooski, VT). All analyses were carried out in triplicate, and the results were averaged (20).

Mutants with an OD_{600} less than 50% of the positive control were tested a second time to confirm low biofilm production. Mutants that produced less than 50% of the positive control a second time were selected for further analysis.

Mutant Sequence Analysis

Mutants were sequenced according to the primers and protocol described by (19). Briefly, selected mutants were cultured on NAL-KAN-LB agar plates and incubated overnight at 37°C.

PCR was first done to amplify the transposon sites using Arbi5 in combination with P9, a transposon-specific primer. Next, a second round of nested PCR was performed using 1 µL of each original PCR product. Arbi2 was used, as it is homologous to the 5' sequence of Arbi5, and P6 was used as a transposon I terminus-specific primer (19). The second round of PCR products were purified using ExoSAP and sent for Sanger sequencing to the Iowa State University sequencing facilities.

Data analysis of the sequenced PCR products was done using Nucleotide BLAST (N-BLAST) algorithms on public databases. Putative biofilm formation genes identified by N-BLAST analysis were selected for further analysis and consisted of 30 of most common "hits."

Prevalence Analysis

The prevalence of 30 selected putative biofilm formation genes was determined in a sample of 109 APEC and 104 avian fecal *E. coli* (AFEC) isolates collected from previous studies (21). Genes were selected if they were frequently found among the transposon mutants and if they did not have a known role in APEC-specific biofilm formation. The detection of genes was analyzed using six multiplex PCR panels. Primers used are detailed in Table 4.2. The primers for each gene were designed using A380 WT as the template with Primer3 (v0.4.0), and the multiplex panels were constructed using Geneious Prime (Version 2021.1.1). PCR was performed using 2 µL DNA, 2.5 µL PCR buffer (10x), 0.4 µL $MgCl_2$ (50 mM), 1.25 µL dNTP mixture (10µM), 2.0 U Taq DNA polymerase, 1.0 µL of multiplex primer (100 µM each primer), and 15.85 µL sterile Milli-Q water. The thermal cycle conditions consisted of a 5-minute activation step at 94°C, followed by 30 annealing cycles of 94°C for 30 s, 54-59°C for 30 s (depending on multiplex), and 72°C for 3 minutes, and a final extension step of 72°C for 10 minutes. The annealing temperate for multiplex 1, 2, and 3 was 55°C, for multiplex 4 was 59°C,

and for multiplex 5 and 6 was 54°C. The amplicons were then separated by 1.5% agarose gel electrophoresis (MSP brand Agarose LE, Atlanta, GA), stained with 0.1% ethidium bromide (Sigma Aldrich, St. Louis, MO), and visualized under UV light using a UVP GelSolo (Analytik Jena, Jena, Germany).

The prevalence of the 30 genes was also analyzed with *in silico* PCR using Geneious Prime. The genomes of 12 APEC, five human extraintestinal pathogenic (ExPEC), two intestinal pathogenic, one fecal *E. coli*, and ten laboratory (K-12 and ATCC 25922) strains of *E. coli* were imported from NCBI into Geneious Prime. Primers used were the same sequences as the those detailed in Table 4.2 for the prevalence analysis.

Construction of Mutants and Complemented Strains

Isogenic mutants were constructed using λ red mutagenesis (22). Primers for mutant construction and complementation are detailed in Table 4.2. Briefly, oligonucleotides specific to the CHM cassette flanked by 50 nucleotide extensions homologous to the 5' and 3' of the gene to be deleted were used to amplify the CHM resistance cassette from plasmid pKD3 (ATCC®, Manassas, VA). The PCR products were run on a 1.5% agarose gel, and the DNA of the resulting fragment was extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, Germantown, MD). The resulting DNA fragments were electroporated into APEC 380 containing the λ red recombinase plasmid pKD46 (ATCC®, Manassas, VA). After electroporation, the cells were grown in super optimal broth with catabolite expression (SOC) for 90 minutes at 37°C and plated on LB agar containing 25 mg/mL CHM (CHM-LB). Colonies were screened by PCR to identify deletion mutants and then confirmed using Sanger sequencing (Eurofins Genomics LLC, Louisville, KY). The CHM cassettes were cured from the strains by

transforming helper plasmid pCP20 (ATCC®, Manassas, VA) into the mutants and confirmed both phenotypically by screening for CHM-sensitive colonies and genotypically using PCR.

Complementation was performed as previously described with modifications (23). Briefly, PCR-amplified genes of interest were cloned into the BamHI and SalI restriction sites of plasmid pACYC184 (New England Biolabs, Ipswich, MA). The cloned plasmids were confirmed via Sanger sequencing, then electroporated into their mutant counterparts. After electroporation, the cells were grown in SOC for 90 minutes at 37°C and plated on CHM-LB agar. Complements were screened via PCR and confirmed via Sanger sequencing.

Growth Curve Assays

The growth rates of the A380 WT and mutant strains in M63 minimal media were analyzed. Briefly, strains were incubated statically overnight in LB broth with or without antimicrobials at 37°C. The overnight cultures were diluted 1:100 in LB broth with or without antimicrobials, then grown until the exponential growth phase (OD_{600} = ~0.5-0.6). Next, the OD_{600} of the cultures was measured using an Implen NanoPhotometer® NP80 (Implen, Munich, Germany), and cultures were diluted 1:100 in M63 minimal media. Aliquots of 200 µL were dispensed into the wells of a 96-well microtiter plate and incubated with shaking for 12 h at 37°C. Negative control wells contained uninoculated media, and the positive control wells were inoculated with A380 WT. The OD_{595} was measured every 10 minutes using a Multiskan™ FC Microplate Photometer with incubator (Thermo Scientific™, Waltham, MA). Growth curves were performed with eight technical replicates on three separate days, and the absorbance data was averaged and plotted against time to build the growth curves.

Biofilm Formation Assays

The biofilm formation of A380 WT and mutant strains was analyzed as previously described (20). Briefly, strains were incubated statically overnight in LB broth with or without antimicrobials at 37°C. The overnight cultures were diluted 1:100 in M63 minimal media. Aliquots of 200 µL were dispensed into the wells of a 96-well microtiter plate and incubated statically for 24 h at 37°C. Negative control wells contained uninoculated media, and the positive control wells were inoculated with A380 WT. Following incubation, the contents of the plates were poured off and washed once with sterile Milli-Q water to remove loosely attached cells. Next, the wells were stained with 200 µL of 0.1% crystal violet solution, washed four times with sterile Milli-Q water to remove excess stain, and air dried for 1 h. After drying, adherent cells were re-solubilized with 200 µL of an 80:20 solution of ethanol and acetone. A volume of 150 µL of this solution was transferred to a new microtiter plate, and the OD_{600} was read using an automated ELx808 Ultra MicroPlate Reader. Biofilm assays were performed with eight technical replicates on three separate days, and the absorbance data was averaged.

Expression of Biofilm Genes

Planktonic RNA extraction. RNA was extracted from planktonic A380 WT cells using the RiboPure™-Bacteria RNA purification kit from Ambion™ (Austin, TX). Briefly, A380 WT was grown overnight at 37°C in LB broth. The overnight cultures were diluted 1:100 in M63 minimal media and grown at 37°C until the exponential phase. RNA was extracted according to the manufacturer's instructions. Isolated RNA was treated with DNase I according to the manufacturer's instructions to eliminate genomic DNA. The concentration of RNA was measured using an Implen NanoPhotometer® NP80, and the samples were stored at -80°C until use.

Biofilm RNA extraction. RNA was extracted from attached A380 WT biofilm cells using the RiboPure™-Bacteria RNA purification kit from Ambion™. Briefly, A380 WT was grown overnight at 37°C in LB broth. The overnight culture was diluted 1:100 in M63 minimal media. Aliquots of 2 mL were dispensed into the wells of a 6-well non-treated multidish (Thermo Scientific Nunc™, Roskilde, Denmark) and incubated statically for 24 h at 37°C. Following incubation, the contents of the plates were poured off and washed three times with PBS. The wells were then scraped with a cell scraper and re-suspended in 1 mL of PBS. A total of five wells per plate were pooled together for the experiment. RNA was extracted, treated, measured, and stored as above.

Synthesis of first strand of cDNA. DNase-treated RNA was reverse-transcribed using the First-Strand cDNA Synthesis Kit from APExBio (Boston, MA). Briefly, 1 µg of DNase-treated RNA was mixed with 1 µL Random Primers (50 µM) and 1 µL dNTP mixture (10 mM) and adjusted to 10 µL with the provided RNase-free water. To denature the RNA, the mixture was heated at 65°C for 5 minutes and then chilled on ice for 2 minutes. The reverse transcription reaction system was prepared by adding 4 µL First-Strand Buffer (5x), 1 µL RNase Inhibitor, Murine (40 U/µL), 1 µL Reverse Transcriptase (200 U/µL), and 4 µL RNase-free water to the denatured RNA mixture, for a total volume of 20 µL. The thermocycler conditions for cDNA synthesis were as follows: 25°C for 2 minutes, 42°C for 50 minutes, and 75°C for 15 minutes. The resulting cDNA templates were stored at -20°C until use.

RT-qPCR. Gene expression was analyzed by real-time reverse-transcriptase quantitative PCR (RT-qPCR) in a qTower3 G qPCR System (Analytik Jena, Jena, Germany) and analyzed using qPCRsoft (v4.1) software, as previously described (24). Reactions were performed using SYBR® Green (GoldBio, St. Louis, MO) according to the manufacturer's instructions. Primers

(Table 4.2) were created using Geneious Prime. Each gene was analyzed with biological and technical triplicates for planktonic RNA and biofilm RNA. Each reaction mixture contained 25 ng cDNA, 1 µL forward and reverse primers (2.5 µM), and 10 µL qPCR Master Mix (2x) with SYBR® Green and was adjusted to 20 µL with nuclease-free water (Ambion™, Austin, TX). The PCR conditions consisted of a 15-minute denaturation step at 95°C, followed by 40 annealing cycles of 95°C for 5 s and 60°C for 30 s. A melting curve analysis was performed at the end to ensure amplification specificity. Threshold fluorescence was established within the geometric phase of exponential amplification. The cycle threshold (CT) was determined for each sample by qPCRsoft and averaged among replicates. The housekeeping gene 16S rRNA was used as the endogenous control to normalize expression levels. Differences in expression levels between the planktonic growth and biofilm maturation phases for each gene were calculated using the Livak method (25).

Statistical Analysis

For the prevalence analysis of genes harbored in the *E. coli* strains, the presence or absence of genes were treated as quantitative variables, and the Student's t-test was used to evaluate the statistical significance (Microsoft Excel, Version 16.0). Biofilm formation was also evaluated using Student's t-test in Excel. Gene expression Ct values were obtained using qPCRsoft and then transferred to Excel, where differences between planktonic expression and biofilm expression were analyzed using Student's t-test. Growth curves were analyzed using linear regression to compare the exponential growth rate between strains (R Studio, Version 1.4.1106). All data from growth curves was included from when the bacterial population (OD_{595}) had increased 150% from the inoculated concentration until the OD_{595} ceased to increase. Statistical significance for all tests was accepted when $p < 0.05$.

RESULTS

Transposon Library Generation and Analysis

A total of 15,660 mutants were randomly generated using the STM technique, which is three times the number of genes in the A380 WT genome. This result gave 99% confidence that there was at least one mutant per gene in the genome. Of these mutants, 2,286 (15%) had repeated decreased biofilm formation (< 50% of the positive control) and were sent for Sanger sequencing to identify where the disruption occurred.

Of the mutants sequenced, 920 mutant sequences were analyzed using N-BLAST, resulting in 547 genes of interest (Figure 4.1). Thirty genes frequently identified among the transposon mutants and not known to be involved in the APEC-specific biofilm formation process were selected for prevalence analysis. The selected genes are described in Table 4.3.

Prevalence Analysis

In this study, 213 *E. coli* (109 APEC and 104 AFEC) isolates were screened for the presence of 30 putative biofilm formation genes. Figure 4.2 shows the overall prevalence of genes among the APEC and AFEC isolates.

The APEC isolates had a high prevalence (> 90%) of the following genes: *kbaY*, *xlyF*, *msyB*, *bioCD*, *sohB*, and hypothetical proteins *hypo07*, *hypo10*, and *hypo13*. These genes were also highly prevalent in the AFEC isolates, in addition to *yjeM*, *yliE*, and hypothetical protein *hypo08*.

The APEC isolates had a low prevalence (< 10%) of the following genes: *dhaK* and hypothetical proteins *hypo02* and *hypo03*. These genes were also low in prevalence among the AFEC isolates, in addition to *nhaC*, a putative alpha-beta hydrolase (represented here as *abh*), and hypothetical proteins *hypo01* and *hypo14*.

There were nine genes that displayed statistical and biological significance in prevalence in the APEC isolates compared to the AFEC: *rfaY, nanM, nhaC, rfaI, rfaJ, abh,* and hypothetical proteins *hypo01, hypo11,* and *hypo14* ($p < 0.05$). These results are supported by the *in silico* PCR analysis from Geneious Prime (Figure 4.3), in which all nine of these genes were more prevalent among the ExPEC isolates analyzed compared to the fecal isolates and K-12 strains.

Growth Curves

In order to determine if the selected genes affected the growth of APEC, the growth curves of A380 WT, the isogenic mutants and complements, and their transposon mutant counterparts in M63 minimal media were analyzed. The growth curve of each strain is displayed in Figure 4.4. The exponential growth rate of each strain was compared to the WT using linear regression analysis. The growth rates of four of the transposon mutants were significantly lower than the WT: A380Δ*nhaC*-T, A380Δ*rfaI*-T, A380Δ*hypo01*-T, and A380Δ*abh*-T ($p < 0.0001$). Their isogenic mutant counterparts A380Δ*nhaC*, A380Δ*hypo01*, and A380Δ*abh*, however, did not have slower growth rates. Instead, the isogenic mutants with significantly decreased growth rates were A380Δ*nanM* and A380Δ*rfaI* ($p = 0.0045$ and $p < 0.0001$, respectively), although the resulting concentrations of both strains in the stationary phase were similar to that of the WT. There was a slight recovery in growth in the complement A380Δ*rfaI*-C but not A380Δ*nanM*-C. Additionally, the complemented mutants A380Δ*rfaY*-C ($p < 0.0001$), A380Δ*nhaC*-C ($p = 0.0026$), A380Δ*hypo01*-C ($p = 0.0022$), and A380Δ*hypo14*-C ($p < 0.0001$) had significantly lower exponential growth rates than the WT.

Biofilm Formation

To evaluate the importance of the selected genes in APEC biofilm formation, biofilm assays in M63 minimal media were performed for A380 WT, the isogenic mutants and

complements, and their transposon mutant counterparts. The biofilm production of each strain is displayed in Figure 4.5. In these assays, the biofilm production of all transposon mutants is significantly decreased compared to that of the WT ($p < 0.001$). The following isogenic mutants also showed decreased biofilm production: A380Δ*rfaY*, A380Δ*rfaI*, A380Δ*abh*, and A380Δ*hypo11* ($p < 0.001$, 0.001, 0.001, 0.05, respectively), although A380Δ*rfaY*, A380Δ*rfaI*, and A380Δ*abh* all had significantly increased biofilm production compared to their transposon counterparts ($p < 0.001$, 0.01, 0.001, respectively). A380Δ*abh*-C was the only complement to make a full recovery of biofilm production back to that of the WT. A380Δ*rfaY*-C, A380Δ*rfaI*-C, and A380Δ*hypo11*-C displayed no recovery. The complement A380Δ*nanM*-C also had significantly decreased biofilm production compared to its isogenic mutant counterpart ($p < 0.05$).

Gene Expression

To better understand the behavior of APEC during planktonic growth and biofilm maturation, the expression of the target genes of interest was analyzed and compared in both phases. The 16S rRNA housekeeping gene was used as the endogenous control to normalize expression levels. The expression levels of *rfaY* and *rfaI* were unable to be analyzed due to a lack of primer binding to the sequence. As shown in Figure 4.6, there was a significant increase in expression of *nanM*, *nhaC*, *hypo11*, and *hypo14* during planktonic growth compared to the mature biofilm phase (fold changes: 16.61, 10.76, 11.88, and 115.74, respectively). Although *hypo01* and *abh* displayed numerically higher gene expression in the planktonic growth phase, there was no significant difference between planktonic growth and biofilm maturation expression levels.

DISCUSSION

APEC is a major problem for the poultry industry worldwide. It is a leading cause of morbidity and mortality, leading to substantial economic losses annually (1). APEC also has the potential to cause disease in humans, including urinary tract infections and meningitis (26-28). Biofilms, therefore, are an important virulence factor that contribute to the survival and persistence of APEC in the environment and the host. In order to better characterize the APEC biofilm, we used STM to identify APEC genes that contribute to biofilm formation in a well-characterized APEC strain.

From the 547 genes identified in this study, some previously described as being associated with biofilm formation were also found. Putative biofilm formation genes identified in this study included type I fimbriae genes *fimA, fimC, fimD, fimF, fimG, fimH,* and *fimI* and the transcriptional regulator *csgD*, which is a major biofilm regulator in both *E. coli* and *Salmonella* spp. (29-31). Multiple outer membrane proteins were also found, including *pgaA, pgaB, ompA,* and both *envZ* and *ompR* of the EnvZ-OmpR two-component regulatory system, all of which have been shown to be involved in or regulate biofilm formation (32-34). Other virulence genes were found also, such as hemolysin co-regulated protein (Hcp) subunit *hcp1*. Hcp is a major component of the type VI secretion system, which is a virulence factor of APEC involved in adherence and pathogenicity and has been demonstrated to contribute to biofilm formation in APEC (24, 35). The finding of these biofilm-related genes validates the credibility of this study and allows us to move forward with our hypothesis. Since the disruption of these biofilm-related genes caused a decreased biofilm-formation ability in the APEC 380 mutants, it is reasonable to assume that the other genes found in our analysis may also be related to biofilm formation.

To test our hypothesis, isogenic mutants and complements were created for eight of the putative APEC biofilm genes (*rfaY, nanM, nhaC, rfaI, hypo01, abh, hypo11,* and *hypo14*). An isogenic mutant was not created for *rfaJ* due to its close proximity to *rfaY* and *rfaI* on the genome. These mutants were evaluated for their role not only in the biofilm formation of APEC but also in its planktonic growth. Biofilm formation begins with the attachment of planktonic bacteria to a surface; therefore, the first step of biofilm formation relies on the fitness of planktonic bacteria. Slow-growing and otherwise-hindered bacteria will form biofilms more slowly and incompletely, resulting in poor biofilm structure, as seen in previous studies (36, 37). From this study, the deletion of three genes (*rfaY, abh,* and *hypo11*) were found to decrease biofilm production without decreasing the growth rate of APEC 380, while the deletion of *rfaI* induced both decreased planktonic growth and biofilm production.

A limitation of this study included the decrease in the growth rate and subsequent biofilm production of the complemented strains. This, however, may have been because the genes were re-inserted back on a plasmid, which may hinder full function. Previous studies have shown that plasmids increase the metabolic burden of the host bacteria, which may result in slower growth rates (38-41). This observation may also explain the lack of recovery of the complemented strains in growth and biofilm formation, as the complemented strains themselves were impaired by the burden of the plasmid and displayed decreased growth when their deletion mutant counterparts did not.

In addition to the plasmid-bearing complemented strains, a limitation of this study was the inability to obtain results for expression of *rfaY* and *rfaI* in the planktonic growth and mature biofilm maturation stages as a result of inhibitors present. It is well-known that reverse transcriptase (RT) can have inhibitory effects on PCR (42-44). RT inhibition may also be

mediated through specific primer-template interactions (45, 46), which would explain why the other primers could bind to the cDNA and exhibit gene expression. Although two-step RT-qPCR should reduce the risk of RT interference, untreated cDNA, as was used in this study, may still harbor RT that will interfere with the RT-qPCR reaction (42). Therefore, the presence of RT from the untreated cDNA interacting with the *rfaY* and *rfaI* primers resulted in the inhibition of primer binding to the template and the inability to analyze the transcripts.

Of the eight putative APEC biofilm formation genes analyzed in this study, four have been characterized (*rfaY, nanM, nhaC, rfaI*) and four are uncharacterized or identified as hypothetical proteins (*abh*, *hypo01*, *hypo11*, *hypo14*). *rfaY* and *rfaI*, also referred to as *waaY* and *waaI* or *waaO*, respectively, are both involved in the synthesis of the lipopolysaccharide (LPS), a major virulence determinant in *E. coli*. LPS constitutes a large portion of the outer membrane of Gram-negative bacteria and is composed of three structural domains: the lipid A endotoxin, the core oligosaccharide, and the O-antigen polysaccharide (47). Additionally, LPS contributes to biofilm formation through bacterial adhesion and biosynthesis of colanic acid, the main exopolysaccharide of *E. coli* (4, 48). Both *rfaY* and *rfaI* are involved in the synthesis of the core oligosaccharide, with *rfaY* contributing to the inner core and *rfaI* to the outer core (49, 50). A previous study showed that the deletion of *rfaY* and *rfaI* in *E. coli* W3110 significantly decreased biofilm formation without decreasing the growth rate (51). However, that previous study used LB broth to culture the strains for the growth curve compared to minimal media in the current study, which may account for the difference in growth, as nutrient availability in media impacts bacterial growth rates (52).

Interestingly, when *in silico* PCR was performed, the primers used for the *rfaY* or *rfaI* of APEC 380 ($rfaY_{A380}$ and $rfaI_{A380}$) did not bind with *E. coli* W3110. A subsequent alignment of

rfaY$_{A380}$ and *rfaI*$_{A380}$ to *E. coli* W3110 using Geneious Prime displayed significant variation between the *rfaY*$_{A380}$ and *rfaI*$_{A380}$ and those of *E. coli* W3110. Further alignments between *rfaY*$_{A380}$ and *rfaI*$_{A380}$ and other *E. coli* strains indicated that, although *rfaY* and *rfaI* genes are found in the *E. coli* K-12 strains examined, they also have significant variations from *rfaY*$_{A380}$ and *rfaI*$_{A380}$ (approximately 60% pairwise identity). However, 16 out of 17 of the APEC and other ExPEC strains analyzed harbored genes that fully align with *rfaY*$_{A380}$ and *rfaI*$_{A380}$, indicating that these genes may be specific to APEC or ExPEC**.** This may be due to the LPS core structure of each strain. Thus far, there are five characterized LPS core structures, denoted K-12, R1, R2, R3, and R4 (47). APEC 380 and *E. coli* W3110 have different LPS core structures, classified as R1 and K12, respectively. Research regarding the relationship between LPS core type and *E. coli* pathogenicity is limited; however, there may be a correlation between ExPEC and LPS core type based upon phylogenetic group and virulence genes (53-55). According to this data and considering the significant difference in prevalence of *rfaY*$_{A380}$ and *rfaI*$_{A380}$ among the APEC (65% and 78%, respectively) and AFEC (14% and 31%, respectively) isolates analyzed by conventional PCR, the variations of *rfaY* and *rfaI* found in APEC 380 may be specialized for ExPEC.

Another characterized gene in this study was *nanM*, previously referred to as *yjhT*, which encodes a mutarotase of N-acetylneuraminic acid (Neu5Ac), a member of the sialic acid family (56). *nanM* is a part of the *nan* operon and accelerates the spontaneous conversation of α-Neu5Ac to β-Neu5Ac, which is more stable and accessible for bacteria that obtain sialic acid from their environment (56). Sialic acid is an energy source for *E. coli*, and it contributes to pathogen survival in the host and its ability to interact with host-cell surfaces (57, 58). Sialic acid regulation is also linked to the formation of biofilm-like intracellular bacterial communities in

uropathogenic *E. coli* (59). In this study, *nanM* was upregulated while growing in minimal media compared to when the biofilm was mature. Since sialylation contributes to biofilm formation (60-62), *nanM* may have been upregulated as a result of the stressful environment in the minimal media, converting α-Neu5Ac to β-Neu5Ac to prepare for biofilm formation. The nutrient-deficient environment in the minimal media also may have upregulated *nanM* as a means to acquire more energy through sialic acid. In the phenotypic biofilm assay, there was no significant decrease in biofilm formation when *nanM* was deleted from APEC 380. This may be because of compensatory mechanisms in *E. coli* for sialic acid uptake, such as *nanS*, which is a putative sialate esterase that allows *E. coli* to grow on alternative sialic acids (63). Although research on the role of sialic acid in APEC pathogenesis is limited, since APEC and uropathogenic *E. coli* (UPEC) are known to share virulence factors (27, 28), it is reasonable to assume that sialic acid is important for APEC virulence also. This observation is supported by the prevalence of *nanM* in the APEC isolates (64%) compared to the AFEC isolates (15%) (Figure 4.2). Therefore, *nanM* may be a potential marker of APEC strains.

The final characterized gene was *nhaC*, which encodes the sodium:proton (Na^+/H^+) antiporter NhaC. *E. coli* is usually known to only carry Na^+/H^+ antiporters NhaA and NhaB: NhaA is the primary Na^+/H^+ antiporter responsible for conferring resistance to high levels of sodium and lithium, and NhaB is an alternate Na^+/H^+ antiporter when NhaA is not activated (64, 65). *nhaC* has been characterized in other species such as *Bacillus* spp. (66, 67), and its insertion been found to functionally complement a *nhaA* deletion in *E. coli* (68). *nhaC* is homologous to the *E. coli* gene *ibeT*, which encodes the putative transporter IbeT belonging to the Na+/H+ antiporter family (69). *ibeT* resides on the *ibeRAT* operon on the GimA genomic island. It has been implicated in the adhesion of neonatal meningitis *E. coli* (NMEC) to brain microvascular

endothelial cells (BMEC) both *in vitro* and *in vivo* and may also coordinately contribute to invasion with *ibeA* (70, 71). The upregulation of this gene during the growth phase in minimal media may have been a mechanism of energy acquisition or preparation for attachment. However, neither *nhaC* nor *ibeT* have been directly implicated in biofilm formation to date, and the gene deletion did not significantly impair biofilm formation in APEC 380, although there was a numerical decrease observed. *ibeA*, on the other hand, has been associated with biofilm formation in APEC (72). Therefore, similar to the role in BMEC invasion, *ibeT* may coordinately contribute to APEC biofilm formation with *ibeA*, although further research is required for confirmation.

The remaining four genes identified in this study were all uncharacterized. Full gene sequences were analyzed using N-BLAST, but no characterized identical proteins could be found for the hypothetical proteins *hypo01*, *hypo11*, or *hypo14*. Interestingly, the expression of *hypo11* and *hypo14* in the planktonic phase of growth were both significantly higher than that observed during biofilm maturation. Even more noteworthy was that *hypo14* was expressed 115.74 times higher in the planktonic growth phase than in the biofilm maturation phase (Figure 4.6). Considering attachment factors are upregulated prior to bacterial attachment (4), *hypo11* and *hypo14* may potentially have involvement in adhesion. However, further characterization assays are required to elucidate the function of these genes.

The putative alpha-beta hydrolase (*abh*) returned one gene out of 696 identical protein accession numbers, resulting in the identification of carboxylesterase B *caeB*. Lun and Bishai (73) characterized the *caeB* paralog *caeA* in *Mycobacterium tuberculosis* and found that it encodes a cell wall-associated carboxylesterase that is required for *M. tuberculosis* virulence. However, *M. tuberculosis* is a Gram-positive species, and *E. coli* is Gram-negative. Goullet et al.

(74) characterized some properties of *caeB* in *E. coli*, but the exact role of *caeB* in *E. coli* has yet to be identified. In the present study, the deletion of this putative *caeB* exhibited decreased biofilm formation in APEC 380 but did not decrease planktonic growth. In addition, its location on the APEC 380 genome is near genes encoding other hypothetical proteins and tail fiber assembly proteins. Therefore, *caeB* may play a role in adhesion or motility, but further studies are required to prove that the putative hydrolase in this study was *caeB* and what exact role it may play in APEC biofilm formation.

Transposon insertions can lead to incomplete disruptions in the genes or may block expression of downstream genes if inserted into an operon (75). Polar effects of the transposon on the expression of adjacent genes may explain why the deletions of *nanM*, *nhaC* (*ibeT*), *hypo01*, and *hypo14* did not result in reduced biofilm formation in APEC 380 like that of the transposon mutants. For example, *ibeT* is on the same operon as and downstream of *ibeA*. The transposon may have been inserted near the beginning of *ibeT* or between the two genes, therefore disturbing the function of *ibeA*, which is known to contribute to biofilm formation (72). Similarly, the *nanM* is upstream of *nanS*, an alternate sialic acid enzyme. The transposon insertion in *nanM* could have also disrupted the function of *nanS*, severely inhibiting APEC 380's ability to acquire sialic acid. The same logic applies to any other gene whose isogenic mutant does not match the properties of its transposon mutant counterpart.

In conclusion, eight genes have been found to be widespread in APEC and may contribute to APEC biofilm formation. This work lays the groundwork for further research, including how prevalent these genes are in APEC and how they contribute to APEC biofilm formation. In addition, four novel hypothetical proteins have been identified that appear to be

widespread in APEC and may contribute to APEC biofilm formation. Further characterization assays are required to elucidate their functions and what exact roles they may play in *E. coli*.

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DISCLOSURES

The authors declare no conflicts of interest.

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TABLES AND FIGURES

Table 4.1: Strains and plasmids in this study.

Plasmid	**Genotype/Description**	**Reference**
pUTmini-Tn5*km2*	Insertion mutagenesis transposon	Li et al., 2005
pKD46	λ Red recombinase expression plasmid	Datsenko and Wanner, 2000
pKD3	Template plasmid for FRT-flanked CHM resistance cassette	Datsenko and Wanner, 2000
pCP20	Temperature-sensitive FLP recombinase expression plasmid	Datsenko and Wanner, 2000
pACYC184	Cloning vector	de Pace et al., 2011
pACYC184-*rfaY*	pACYC184 with *rfaY*	This study
pACYC184-*nanM*	pACYC184 with *nanM*	This study
pACYC184-*nhaC*	pACYC184 with *nhaC*	This study
pACYC184-*rfaI*	pACYC184 with *rfaI*	This study
pACYC184-*hypo01*	pACYC184 with hypo01	This study
pACYC184-*abh*	pACYC184 with *abh*	This study
pACYC184-*hypo11*	pACYC184 with *hypo11*	This study
pACYC184-*hypo14*	pACYC184 with *hypo14*	This study
Strain	**Genotype/Description**	**Reference**
A380 WT	Wild-type template	Lab stock
E. coli S17-1 λpir	Donor strain for conjugation	Li et. al., 2005
DH5α	Plasmid cloning vessel	Lab stock
A380Δ*rfaY*-T	A380 with *rfaY* disruption by pUTmini-Tn5*km2* transposon insertion	This study
A380Δ*rfaY*	A380 with *rfaY* deletion by λ red recombinase	This study
A380Δ*rfaY*-C	A380Δ*rfaY* with pACYC184-*rfaY*	This study
A380Δ*nanM*-T	A380 with *nanM* disruption by pUTmini-Tn5*km2* transposon insertion	This study
A380Δ*nanM*	A380 with *nanM* deletion by λ red recombinase	This study
A380Δ*nanM*-C	A380Δ*nanM* with pACYC184-*nanM*	This study
A380Δ*nhaC*-T	A380 with *nhaC* disruption by pUTmini-Tn5*km2* transposon insertion	This study
A380Δ*nhaC*	A380 with *nhaC* deletion by λ red recombinase	This study
A380Δ*nhaC*-C	A380Δ*nhaC* with pACYC184-*nhaC*	This study
A380Δ*rfaI*-T	A380 with *rfaI* disruption by pUTmini-Tn5*km*2 transposon insertion	This study
A380Δ*rfaI*	A380 with *rfaI* deletion by λ red recombinase	This study

Strain	Genotype/Description	Reference
A380Δ*rfaI*-C	A380Δ*rfaI* with pACYC184-*rfaI*	This study
A380Δ*hypo01*-T	A380 with *hypo01* disruption by pUTmini-Tn5*km2* transposon insertion	This study
A380Δ*hypo01*	A380 with *hypo01* deletion by λ red recombinase	This study
A380Δ*hypo01*-C	A380Δ*hypo01* with pACYC184-*hypo01*	This study
A380Δ*abh*-T	A380 with *abh* disruption by pUTmini-Tn5*km2* transposon insertion	This study
A380Δ*abh*	A380 with *abh* deletion by λ red recombinase	This study
A380Δ*abh*-C	A380Δ*abh* with pACYC184-*abh*	This study
A380Δ*hypo11*-T	A380 with *hypo11* disruption by pUTmini-Tn5*km2* transposon insertion	This study
A380Δ*hypo11*	A380 with *hypo11* deletion by λ red recombinase	This study
A380Δ*hypo11*-C	A380Δ*hypo11* with pACYC184-*hypo11*	This study
A380Δ*hypo14*-T	A380 with *hypo14* disruption by pUTmini-Tn5*km2* transposon insertion	This study
A380Δ*hypo14*	A380 with *hypo14* deletion by λ red recombinase	This study
A380Δ*hypo14*-C	A380Δ*hypo14* with pACYC184-*hypo14*	This study

Table 4.2: Primers used in this study.

Primer	Sequence (5' - 3')
	Prevalence Analysis
rfaY_F1[1]	TTAAAGTCTTTGCCCCGAAA
rfaY_R1	AGGCATGCAATTTTTCCATT
kbaY_F1	CCTCGAAGTGTGCAGTGAAA
kbaY_R1	CTTGTGGATCGGTCAGGAAT
nanM_F1	CCGAGAGATCAAGCAACCTC
nanM_R1	CCCCTCCGGCAAATATAAGT
nhaC_F1	CATGCCGATCTTCCAATTCT
nhaC_R1	ATGGTGCCAAAATCTTCCAG
yjeM_F1	CTCCACCTGGGTTTCTACCA
yjeM_R1	TGTATTTCCACCACGCAAAG
xylF_F2	CGATGCGGATATCGATTTTT
xylF_R2	GGTTAGCGCGTTTTCCATAA
waaV_F2	AGGGGATGCGATTTATTGTG
waaV_R2	AGATTCTTTCGTGCGCAAAT
rfaI_F2	GCTTAAGGCATTACCGACGA
rfaI_R2	ATAAAAGGTTGCGCACTTGG
gatC_F2	TTAACGTGGCGATGCTACTG
gatC_R2	TCCACAGGGTGATGCTCATA
yliE_F2	AGGCGATGCGAAAGAGATTA
yliE_R2	CCCCTTGTTTACGCAGTTGT
hypo02_F3	GGCATGAAAGAACTTGCTGA
hypo02_R3	GGTCAAGCGATGACCATTAAA
msyB_F3	GATGCGCAACGAACTGTTTA
msyB_R3	CAAATGCGACACACCTTTTG
rfaJ_F3	TTTTGGTCGTACGCGACATA
rfaJ_R3	TGTTCACGCGCGATATTAAA
hypo01_F3	CGTATCCCTGTGCACCTTCT
hypo01_R3	TGTGGTCATCAGGCTTTCTG
hypo03_F3	GCAGGTGAAAACGGAGATGT
hypo03_R3	CATTCATAACGCCAAGTTCG
bioD_F4	CCTTACACCTTCGCAGAACC
bioD_R4	AGTCCGGCGTGTAGTATTGC
sohB_F4	GCGTCTGCGTGATAAAAACA
sohB_R4	TTTCATCAACCAGGCCTTTC
dhaK_F4	CACACCAGACCAAATGATCG
dhaK_R4	GTGCTGCCAAGTCCGTTAAT

Primer	Sequence (5' – 3')
	Prevalence Analysis
yicL_F4	GCTGTGGCAGTGGGATAAAT
yicL_R4	AAAGATCACCGAACCGTCAG
abh_F4	TGGTGAGGAAAAACCGTCTC
abh_R4	ATTAATCCAGGGCGTTGTTG
hypo06_F5	GGGAAAACCAAATCCCTGAT
hypo06_R5	CGTCGCGTATGAACCTGTAA
hypo11_F5	AAACTGGCGTGAGGATGAAC
hypo11_R5	CATCGCGCATAAACACTCAT
hypo13_F5	TGGCCTACTTTCAGGCGTAT
hypo13_R5	GCGTATGGCGAATCATTTCT
hypo12_F5	TACTTCCTTTGTCCCGGTTG
hypo12_R5	GCGCTCTGATTTGTTCCTTC
hypo05_F5	TGCTTTCCGAGTCTGTTCCT
hypo05_R5	GCGTAATCCAGTTTCCGTGT
hypo09_F6	TCAGCCAGAAAAATGTGGTG
hypo09_R6	CGCTGACTGTCTGACCAAAA
hypo14_F6	CGAAAATGCGCTCAATGTTA
hypo14_R6	TTTCCCCCAACTTTTTACCC
hypo08_F6	TAAAAACGTTCCCCAGCAAC
hypo08_R6	TCATAATTCACAGGCGACCA
hypo07_F6	TGCACCCGATCTCAATATCA
hypo07_R6	GCTTCTGCATCGCAATGTTA
hypo10_F6	AAAGCCGCACTTGACCTTTA
hypo10_R6	CGACCAGCGATAATCACCTT
	Gene Deletion
rfaY_MUT_F	ATGATTACAAGTATACGCTATCGCGGCTTCTCATTTTATTACAAAGATAAtgtaggctggagctgcttcg[2]
rfaY_MUT_R	TTACGCTTTGCCTTTTAATTTTTTAATAAATTTGCGTAGTTTGGTTCTGTatgggaattagccatggtcc[3]
nanM_MUT_F	ATGAATAAAACAATAACGGCGCTTACTATCATAATGGCTTCATTTGCCACtgtaggctggagctgcttcg
nanM_MUT_R	TTAGTTTTGTACTGTGACTTTATTATCCTTCACAGAAATCAAAACTGAATatgggaattagccatggtcc
rfaI_MUT_F	ATGAGTGCCCACTATTTTAATCCACAAGAGATGATCAATAAGACAATCATtgtaggctggagctgcttcg
rfaI_MUT_R	TCATTTTATTATCTTTAAATAAAAATAATAAATATAATTCATTATCCCGTatgggaattagccatggtcc
nhaC_MUT_F	ATGAGAGAAAAACCCAGTTTTTATGTCGCGCTTACACCGATCATTTTTATtgtaggctggagctgcttcg
nhaC_MUT_R	TCAGGCCTTTGCTTCGTTGAAGCGCAGTAAACGGAAACCTGTAGAAGCATatgggaattagccatggtcc
hypo01_MUT_F	ATGAAAACGAATAATGCCGGTTATATTATCGGCGCGTATCCCTGTGCACCtgtaggctggagctgcttcg
hypo01_MUT_R	TTAACGAGATTCATTCAGAGAGTGAATGCCGTTGCGTAATATTTCGATGCatgggaattagccatggtcc
abh_MUT_F	ATGCGAAAAATAATTACTCATTTCAAAGTTGTTTTAACGTTACTTCTACCtgtaggctggagctgcttcg
abh_MUT_R	TTATTGTATTTCTGCTTCAGAATTTCCTGGGCAGTATATATTTTCTGGTTatgggaattagccatggtcc

Primer	Sequence (5' – 3')
	Gene Deletion
hypo11_MUT_F	ATGCCCCCCACTCCTGCCATGCAGGCATTAATTGAGCAGATATATCATATtgtaggctggagctgcttcg
hypo11_MUT_R	TTATTTTCCTGGTGATTCGGATGATGCGTCATACATCGCGCATAAAACACTatgggaattagccatggtcc
hypo14_MUT_F	ATGAAATCCGAGACGCTAACTGTCCAACAACTTTTTCAAGACCGCCGACAtgtaggctggagctgcttcg
hypo14_MUT_R	TTAATCGTGTTTTGGCCATACTTTCAATGCAATTTCCCCCAACTTTTTACatgggaattagccatggtcc
	Check Deletion
rfaY_check1_F	TTTAATATCGCGCGTGAACA
rfaY_check1_R	TGCCGCACCAAATAAAAAGT
nanM_check1_F	AGGGTGTTTACAACGGCAGA
nanM_check1_R	GCATCTTCCTTGTCCGGTAA
rfaI_check1_F	AGGAACAAGGGCTGCTGTC
rfaI_check1_R	CTCTGGGGCGTTTTCTTTTT
nhaC_check1_F	TGTCACTGGAGAAGGTGCAG
nhaC_check1_R	CGAGCTGGAGATTGTGCTTA
hypo01_check1_F	CACTACGGCAATACGCAAGA
hypo01_check1_R	GCCTTCGAGTATCCGTTTCA
abh_check1_F	GATTTTCTCCCCGGTGGTAT
abh_check1_R	TGGCACGTCAACTTTTGATT
hypo11_check1_F	GAGGAAAGCTGTTGGGACTG
hypo11_check1_R	CAGGAGCGGAAAGGAGAATA
hypo14_check1_F	CCGATGGGATGATGAAGAGA
hypo14_check1_R	ACGTAATGCGCTGAACTGTG
	Gene Complementation
rfaY_comp_F	TACGGATCCACATGATTACAAGTATACGCTA[4]
rfaY_comp_R	TAAAGTCGACACTTACGCTTTGCCTTTTAATT[5]
nanM_comp_F	TACGGATCCACATGAATAAAACAATAACGGC
nanM_comp_R	TAAAGTCGACACTTAGTTTTGTACTGTGACTT
rfaI_comp_F	TACGGATCCACATGAGTGCCCACTATTTTAA
rfaI_comp_R	TAAAGTCGACACTCATTTTATTATCTTTAAATAAAAATAA
nhaC_comp_F	TACGGATCCACATGAGAGAAAAACCCAGTTT
nhaC_comp_R	TAAAGTCGACACTCAGGCCTTTGCTTCGTTGA
hypo01_comp_F	TACGGATCCACATGAAAACGAATAATGCCGG
hypo01_comp_R	TAAAGTCGACACTTAACGAGATTCATTCAGAG
abh_comp_F	TACGGATCCACATGCGAAAAATAATTACTCA
abh_comp_R	TAAAGTCGACACTTATTGTATTTCTGCTTCAG
hypo11_comp_F	TACGGATCCACATGCCCCCCACTCCTG
hypo11_comp_R	TAAAGTCGACACTTATTTTCCTGGTGATTCGGATGATGCG
hypo14_comp_F	TACGGATCCACATGAAATCCGAGACGCTAACTGT

Primer	Sequence (5 – 3')
	Gene Complementation
hypo14_comp_R	TAAAGTCGACACTTAATCGTGTTTTGGCCATACTTTCAATGC
	Check Complementation
pACYC_F	CGACCACACCCGTCCTGT
pACYC_R	AAGGCTCTCAAGGGCATCG
	RT-qPCR
rfaY_RT_F	CCGAAAACGAAAAGGAATGA
rfaY_RT_R	AGCTCTACGCCCTCGACATA
nanM_RT_F	GGCGGTTGTGAATAAAGGTG
nanM_RT_R	CCCCTCCGGCAAATATAAGT
rfaI_RT_F	TGCCATGTATTTCCGTTTTG
rfaI_RT_R	TCGCGTTCAGTAACAACAGC
nhaC_RT_F	ATGCCGATCTTCCAATTCTG
nhaC_RT_R	CATTCCCAGCCAGGTAAAAA
hypo01_RT_F	CGTATCCCTGTGCACCTTCT
hypo01_RT_R	TTTCCAGCTCTCTGGCGTAT
abh_RT_F	GACCTTCTGCTATGGCGTTC
abh_RT_R	TGCTCATCCCCCTGATCTAC
hypo11_RT_F	TTGCGAGCGATTCCTTATTC
hypo11_RT_R	TCCTCACGCCAGTTTTCTTT
hypo14_RT_F	CATTTTAATCACCCGCCATC
hypo14_RT_R	ATTTGGGCATCATCTTCAGC

[1]Number indicates which multiplex panel primer is in (1, 2, 3, 4, 5, or 6).
[2]Forward primer extension to amplify chloramphenicol resistance cassette (tgtaggctggagctgcttcg).
[3]Reverse primer extension to amplify chloramphenicol resistance cassette (atgggaattagccatggtcc).
[4]Forward primer extension to amplify BamHI restriction site (GGATCC).
[5]Reverse primer extension to amplify SalI restriction site (GTCGAC).

Table 4.3: Description of the genes used for the PCR prevalence analysis. Protein IDs were obtained from the GenBank database.

Gene Code	Gene Name	Protein ID
rfaY/waaY	Lipopolysaccharide core heptose(II) kinase	AKK35220.1
kbaY	Tagatose-bisphosphate aldolase	AKK34749.1
nanM/yjhT	N-acetylneuraminic acid mutarotase	AKK35882.1
nhaC/ibeT	Sodium:proton antiporter	AKK35912.1
yjeM	Putative inner membrane transporter YjeM	AKK34702.1
xylF	Xylose ABC transporter substrate-binding protein	AKK35168.1
waaV	Beta1,3-glucosyltransferase	AKK35218.1
rfaI/waaI/waaO	Lipopolysaccharide 1,3-galactosyltransferase	AKK35222.1
gatC	PTS galactitol transporter subunit IIC	AKK33632.1
yliE	Cyclic di-GMP phosphodiesterase	AKK36780.1
msyB	Putative acidic protein MsyB	AKK35996.1
rfaJ/waaJ	Lipopolysaccharide 1,2-glucosyltransferase	AKK35221.1
bioD	Dithiobiotin synthetase BioD	AKK36724.1
sohB	Inner membrane peptidase	AKK32900.1
dhaK	Dihydroxyacetone kinase subunit K	AKK35900.1
yicI	Alpha-glucosidase	AKK35256.1
abh[1]	Putative alpha-beta hydrolase	AKK36538.1
hypo01[2]	Hypothetical protein	AKK35178.1
hypo02	Hypothetical protein	AKK32566.1
hypo03	Hypothetical protein	AKK32562.1
hypo05	Hypothetical protein	AKK32896.1
hypo06	Hypothetical protein	AKK34206.1
hypo07	Hypothetical protein	AKK35454.1
hypo08	Hypothetical protein	AKK36190.1
hypo09	Hypothetical protein	AKK37046.1
hypo10	Hypothetical protein	AKK35691.1
hypo11	Hypothetical protein	AKK35017.1
hypo12	Hypothetical protein	AKK32843.1
hypo13	Hypothetical protein	AKK34817.1
hypo14	Hypothetical protein	AKK34205.1

[1]The putative alpha-beta hydrolase is uncharacterized and thus referred to as "*abh*" for the purpose of this study.
[2]Hypothetical proteins are referred to as "*hypo#*" for the purpose of this study.

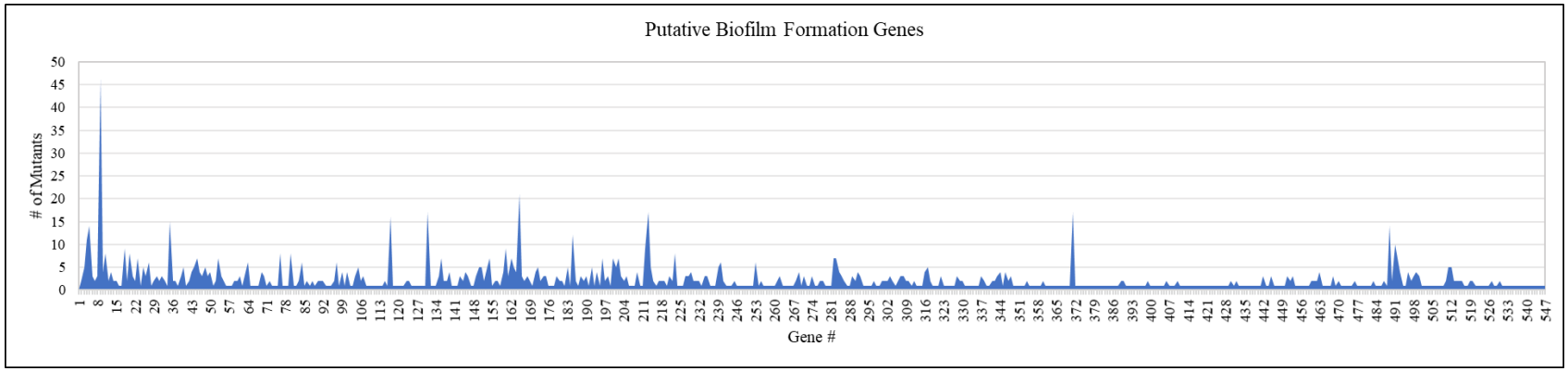


Figure 4.1: The frequency in which all the 547 discovered putative biofilm formation genes were found among the transposon mutants.

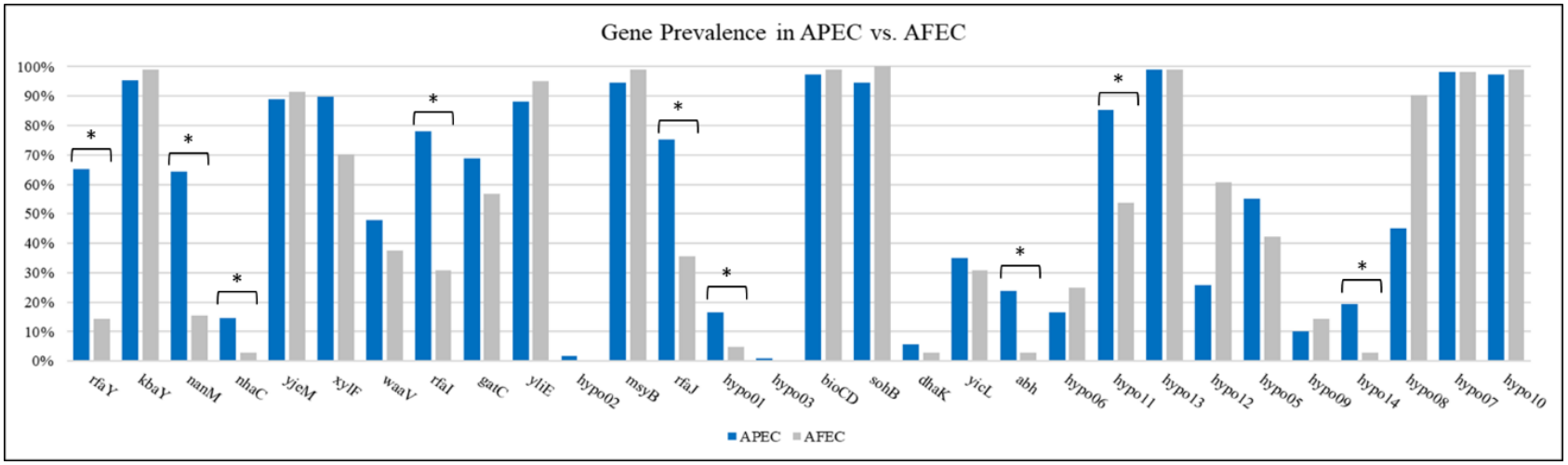
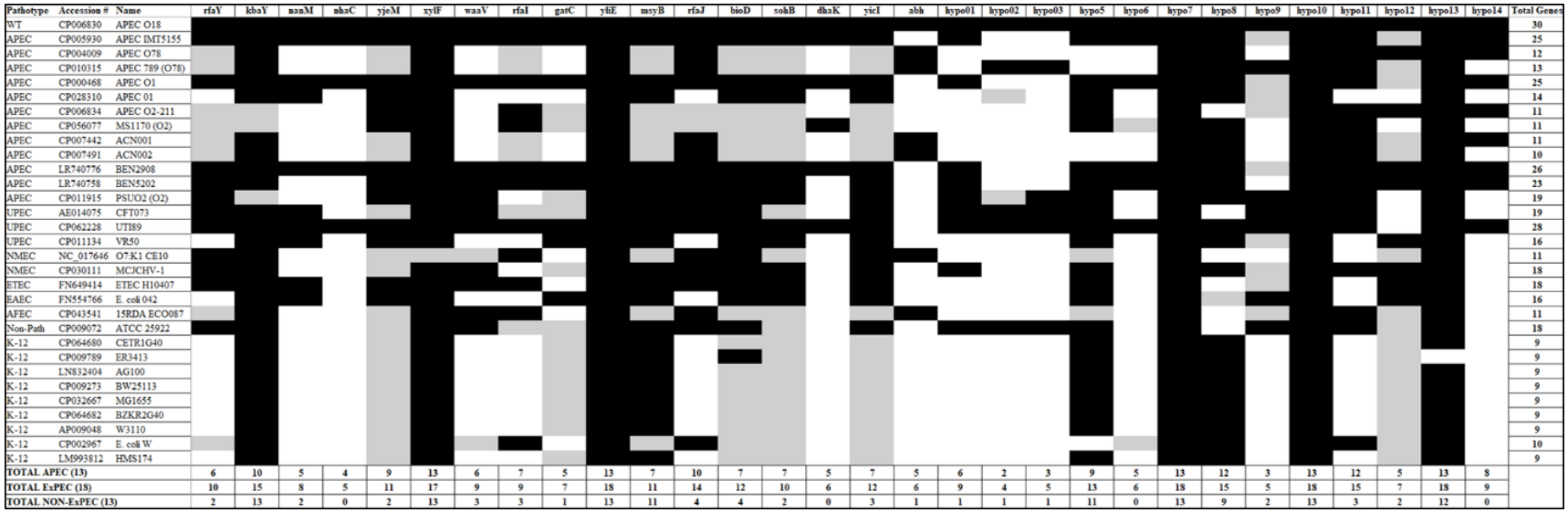


Figure 4.2: Prevalence analysis results. Asterisks above brackets indicate significant difference between those strains. *p < 0.05.



Pathotype	Accession #	Name	rfaY	kbaY	nanM	nhaC	yjeM	xylF	waaV	rfaI	gatC	yhiE	nuyB	rfaJ	bioD	sohB	dhaK	yicI	abh	hypo01	hypo02	hypo03	hypo5	hypo6	hypo7	hypo8	hypo9	hypo10	hypo11	hypo12	hypo13	hypo14	Total Genes
WT	CP006830	APEC O18																														30
APEC	CP005930	APEC IMT5155																														25
APEC	CP004009	APEC O78																														12
APEC	CP010315	APEC 789 (O78)																														13
APEC	CP000468	APEC O1																														25
APEC	CP028310	APEC 01																														14
APEC	CP006834	APEC O2-211																														11
APEC	CP056077	MS1170 (O2)																														11
APEC	CP007442	ACN001																														11
APEC	CP007491	ACN002																														10
APEC	LR740776	BEN2908																														26
APEC	LR740758	BEN5202																														23
APEC	CP011915	PSUO2 (O2)																														19
UPEC	AE014075	CFT073																														19
UPEC	CP062228	UTI89																														28
UPEC	CP011134	VR50																														16
NMEC	NC_017646	O7:K1 CE10																														11
NMEC	CP030111	MCJCHV-1																														18
ETEC	FN649414	ETEC H10407																														18
EAEC	FN554766	E. coli 042																														16
AFEC	CP043541	15RDA ECO087																														11
Non-Path	CP009072	ATCC 25922																														18
K-12	CP064680	CETR1G40																														9
K-12	CP009789	ER3413																														9
K-12	LN832404	AG100																														9
K-12	CP009273	BW25113																														9
K-12	CP032667	MG1655																														9
K-12	CP064682	BZKR2G40																														9
K-12	AP009048	W3110																														9
K-12	CP002967	E. coli W																														10
K-12	LM993812	HMS174																														9
TOTAL APEC (13)			6	10	5	4	9	13	6	7	5	13	7	10	7	7	5	7	5	6	2	3	9	5	13	12	3	13	12	5	13	8	
TOTAL ExPEC (18)			10	15	8	5	11	17	9	9	7	18	11	14	12	10	6	12	6	9	4	5	13	6	18	15	5	18	15	7	18	9	
TOTAL NON-ExPEC (13)			2	13	2	0	2	13	3	3	1	13	11	4	4	2	0	3	1	1	1	1	11	0	13	9	2	13	3	2	12	0	

Figure 4.3: Results of *in silico* PCR on Geneious Prime. Black boxes represent both forward and reverse sequences binding to genome, gray represents only one sequence, and white represents no sequences. The "Total" column is the sum of all primers that have both the forward and reverse sequences bound.

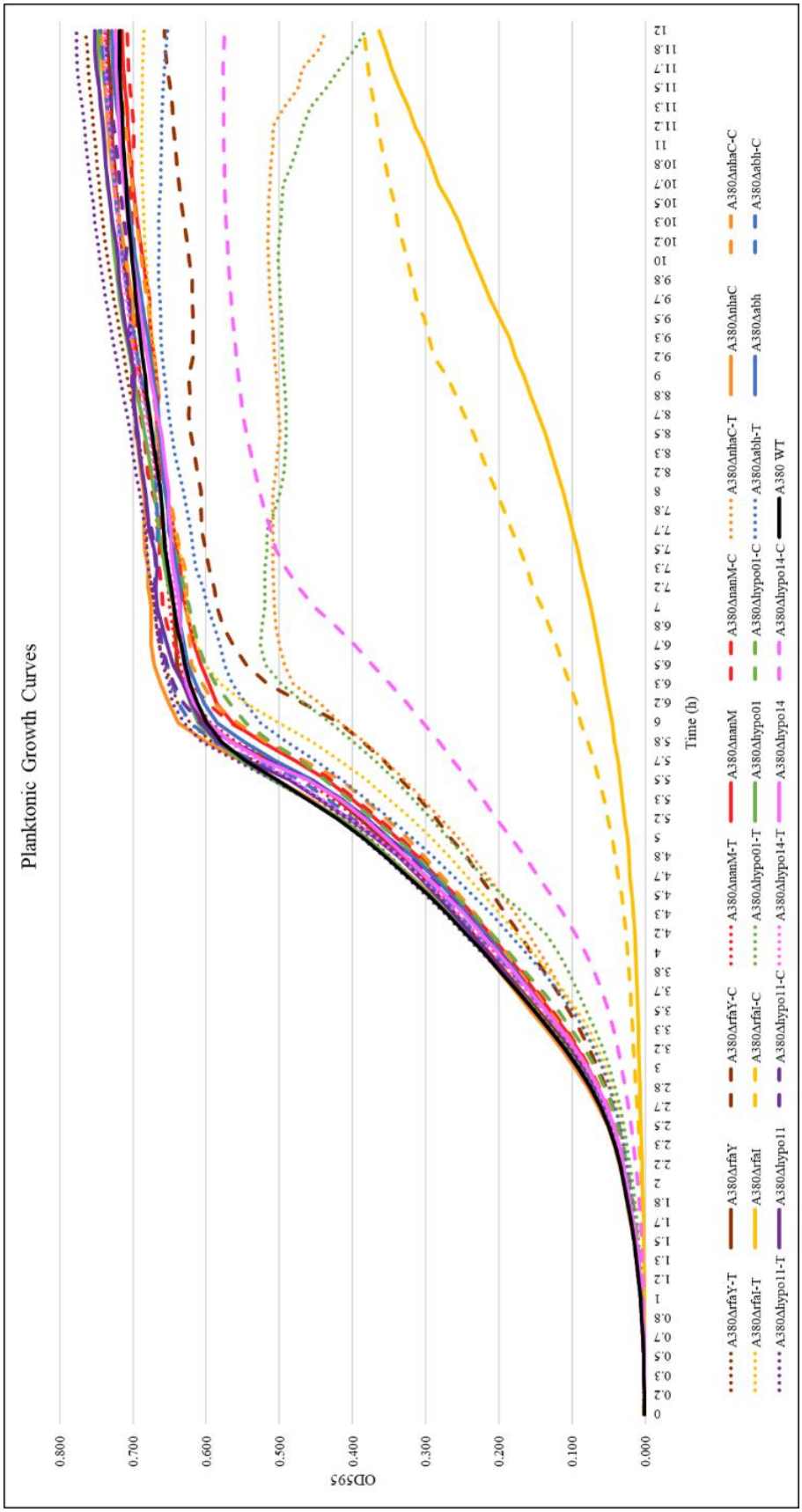


Figure 4.4: Growth curves of all the strains tested.

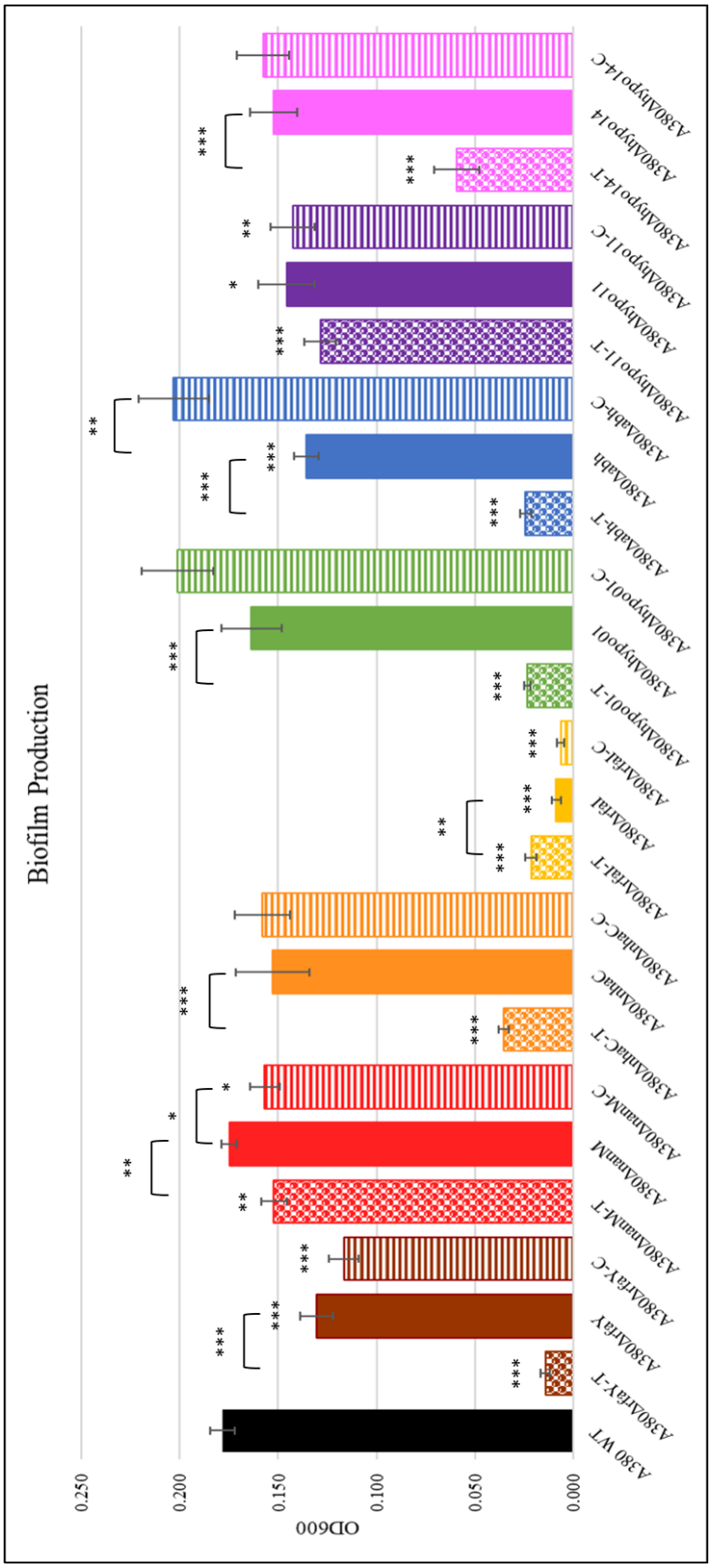


Figure 4.5: Biofilm production of all the strains tested. Asterisks (*) above individual bars indicate a significant difference between that strain and APEC 380 WT. Asterisks above brackets joining two bars indicates significant difference between those two strains. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

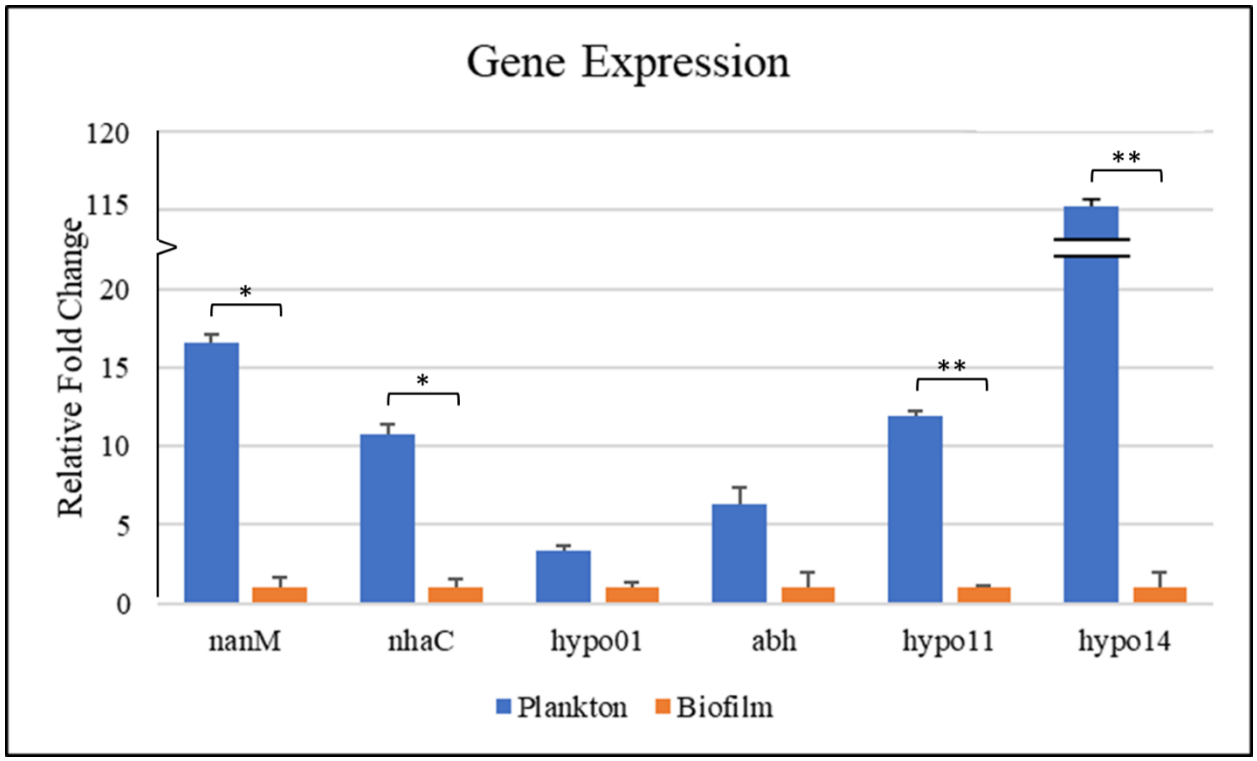


Figure 4.6: Comparison of expression of *rfaY*, *nanM*, *nhaC*, *rfaI*, *hypo01*, *abh*, *hypo11*, and *hypo14* during exponential growth and mature biofilm phases. Expression is measured in fold change. *$p < 0.01$, **$p < 0.001$.

CHAPTER 5

CONCLUSION AND FUTURE WORK

Avian pathogenic *Escherichia coli* (APEC) is a concern for both animal and public health. It is the etiological agent of avian colibacillosis, an extraintestinal disease of birds that can cause illness in chickens, turkeys, ducks, and quail, among others. Avian colibacillosis in the poultry industry leads to reduced quality of poultry production, causing significant economic impact worldwide. Additionally, APEC presents the potential for zoonosis, as it shares virulence traits with human extraintestinal pathogenic *E. coli* that cause diseases such as meningitis and urinary tract infections.

This work builds upon what may be the largest report characterizing *E. coli* isolates collected from turkey cellulitis lesions and litter, presented in Chapter 3 of this thesis. *E. coli* isolates were analyzed using a PCR-based method for their O-type distribution to determine the potential *E. coli* serogroup responsible for the cellulitis outbreak. Utilizing data collected from the original report, the serogrouped isolates were then evaluated for their pathogenic potential by analyzing their virulence factors. Although the usual APEC O-types causing disease in birds are O1, O2, and O78, this case had a relatively low prevalence of such serogroups. Therefore, this study highlights the importance of monitoring flocks for atypical APEC serogroups causing disease in birds as new pathogenic serogroups may be emergent.

An important means of predicting emerging APEC serogroups and devising methods of control is understanding the mechanisms of virulence associated with APEC. A common mechanism for survival and persistence in pathogenic *E. coli* is the ability to form biofilms. Each

pathotype has unique features that contribute to biofilm formation. However, since APEC is a highly diverse pathotype with a wide array of virulence factors, the APEC biofilm is still yet poorly characterized.

Chapter 4 of this thesis aimed to better understand the virulence factors that contribute to APEC biofilm formation. A well-characterized and sequenced strain of APEC was used as the template to generate a random transposon mutant library, from which all mutants were then evaluated for their abilities to form biofilm. After the biofilm-deficient mutants were identified and sequenced and their gene disruptions identified, several putative biofilm formation genes were selected and analyzed amongst avian pathogenic and fecal *E. coli* isolates, resulting in the identification of a selection of genes that were more prevalent in the pathogenic *E. coli* strains than those isolated from healthy birds. This finding supports the hypothesis that there are specialized APEC virulence genes that contribute to the biofilm formation process. The creation of the deletion mutants and analysis of gene expression then characterized these genes to determine their contribution to the APEC biofilm formation process. Overall, this study identified novel genes that were more widespread in APEC and appeared to contribute to biofilm formation.

A variety of putative biofilm formation genes identified in this work were found to have various functions including roles associated with lipopolysaccharide biosynthesis, energy acquisition, nutrient transport, and unknown functions of hypothetical proteins. This work identified biofilm genes that have not yet been associated with APEC and found most were widespread in APEC isolates compared to commensal avian fecal isolates. As such, these genes may serve as potential identifiers of APEC in future studies. This work also identified genes that have not yet been associated with biofilm that contribute to the biofilm formation of the APEC

strain analyzed. These findings together help to increase our understanding of APEC and its biofilm formation and set the groundwork for future studies regarding APEC characterization and virulence factors that contribute to APEC biofilm formation.

Future directions of this work will aim to characterize the genes identified in this study and determine their roles in biofilm formation. With four uncharacterized genes identified in this work, characterization assays evaluating their roles in motility, adhesion, invasion, and survivability will be necessary to elucidate their functions in APEC. In addition, considering the high prevalence of the lipopolysaccharide-associated genes in the pathogenic APEC isolates compared to the commensal isolates, future directions may also explore the relationship between the lipopolysaccharide core structure and *E. coli* pathogenicity.

In conclusion, the work described in this thesis contributes to the overall understanding of APEC, which is a highly diverse and evolving pathotype that has a significant impact on animal and public health. In order to control the spread of APEC, we must identify virulence factors associated with APEC that can be leveraged as control targets. This work and the work that builds from it will increase our understanding of APEC and its pathogenic mechanisms.