CHARACTERIZATION OF TYPE 6 SECRETION SYSTEM (T6SS) GENES IN EXTRA-INTESTINAL PATHOGENIC *Escherichia coli* (ExPEC)

by

ALINE LUISA DE OLIVEIRA

(Under the Direction of Catherine M. Logue)

ABSTRACT

Escherichia coli is a Gram-negative facultative anaerobe bacillus that colonizes the intestine of humans and warm-blooded animals rarely causing disease. However, some strains have become pathogenic to humans and animals via acquisition of virulence factors that make them able to colonize niches outside of the host intestines and cause extra-intestinal infections. These strains are defined as ExPEC and they include, among others, the subjects of this study: avian pathogenic *E. coli* (APEC), and neonatal meningitis *E. coli* (NMEC). APEC cause extra-intestinal infections in birds known as colibacillosis, that is one of the leading causes of economic losses worldwide in poultry production industry. NMEC is the second most common cause of neonatal sepsis and meningitis leading to mortality and morbidity rates up to 30%. To cause disease, ExPEC strains must produce virulence factors required for adherence and invasion, iron uptake, resistance to host serum and may also contain genes encoding toxins and invasins. Although many virulence factors associated with the pathogenicity of APEC and NMEC are described, their pathogenesis is not completely understood, and the role of protein secretion in their virulence remains to be defined. Type 6 Secretion System (T6SS) has

gained attention as a factor involved in pathogenicity and fitness traits of different bacterial species. In APEC and NMEC, current information on the role of T6SS in virulence is limited and warrants further investigation. The aim of this study was to perform an epidemiological analysis and characterization of APEC strains isolated from turkeys as well as the investigation of the role of T6SS genes in the virulence of APEC and NMEC strains. The epidemiological analysis of *E. coli* isolates from turkeys highlights the role of APEC in turkey cellulitis. In relation to the T6SS, we found that components of the system are involved in different virulence traits employed by APEC and NMEC to cause disease.

The main contribution of this work was to be the first one to extensively characterize a large collection of *E. coli* originated from turkeys and to demonstrate that the T6SS plays an important role in the virulence of APECO18 and NMEC 15.

INDEX WORDS: Extra-intestinal pathogenic *Escherichia coli*, APEC, NMEC, pathogenicity, protein secretion, Type 6 Secretion System.

CHARACTERIZATION OF TYPE 6 SECRETION SYSTEM (T6SS) GENES IN EXTRA-INTESTINAL PATHOGENIC *Escherichia coli* (EXPEC)

by

ALINE LUISA DE OLIVEIRA

B.S., Universidade Federal do Rio Grande do Sul, Brazil, 2011

M.S., Universidade Federal do Rio Grande do Sul, Brazil, 2015

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2021

CHARACTERIZATION OF TYPE 6 SECRETION SYSTEM (T6SS) GENES IN EXTRA-INTESTINAL PATHOGENIC *Escherichia coli* (ExPEC)

by

ALINE LUISA DE OLIVEIRA

Major Professor:	Catherine M. Logue
Committee:	Ellen Neidle
	Nicolle Barbieri
	Timothy Hoover

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
August 2021

DEDICATION

I would like to dedicate this thesis to my mother, Isabel, and my paternal grandmother, Eva (*in memoriam*), my best examples and source of inspiration.

ACKNOWLEDGEMENTS

I would first like to express my sincere gratitude to Dr. Catherine Logue. Thank you for giving me the chance to keep pursuing my dream, for helping me regain confidence in myself and for your continuous support. I would not have done it without your trust and support. I would also like to thank Dr. Nicolle Barbieri for the friendship, continuous support and contribution to my research projects. I also would like to thank my committee members: Dr. Ellen Neidle, Dr. Timothy Hoover and Dr. Nicolle Barbieri for their insight and feedback throughout the years. To the students who have helped me with my project, Darby Newman, Meaghan Young, Robert Pasko and Hillary Hsieh. I would also like to thank Brazilian Science without Borders Program for funding the first 4 years of my PhD. I would also like to express my immense gratitude to my mother, Isabel, and my sister Paula. Thank you for being my source of inspiration, for all the love and for the strength that helped me keep pursuing my goals. To my love, Gzim Pjetri, huge thank you for all the love and strength and for always reminding me that I can do anything I set my mind to and that giving up is not an option. Last but not least, I thank God for the strength and courage to keep fighting for my dreams.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AIEC	Adherent invasive *Escherichia coli*
APEC	Avian Pathogenic *Escherichia coli*
BBB	Blood-brain barrier
BMEC	Brain microvascular endothelial cells
CDEC	Cell-detaching *Escherichia coli*
CSF	Cerebro-spinal fluid
DAEC	Diffusely adherent *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extra-intestinal Pathogenic *Escherichia coli*
HBMEC	Human brain microvascular endothelial cells
InPEC	Intestinal Pathogenic *Escherichia coli*
NMEC	Neonatal meningitis *Escherichia coli*
NTEC	Necrotoxic *Escherichia coli*
T1SS	Type 1 Secretion System
T2SS	Type 2 Secretion System

T3SS	Type 3 Secretion System
T4SS	Type 4 Secretion System
T5SS	Type 5 Secretion System
T6SS	Type 6 Secretion System
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection

CHAPTER 1

INTRODUCTION

Escherichia coli is a Gram-negative facultative anaerobe bacterium that colonizes the intestines of humans and warm-blooded animals within a few hours after birth, rarely causing disease, except when the host is immunocompromised (1). However, the acquisition of virulence factors by some strains have made them able to cause infections in humans and animals (1, 2).

Pathogenic *E. coli* are classified into groups including Intestinal pathogenic *E. coli* (InPEC), which cause diarrhea-like infections, and extraintestinal pathogenic *E. coli* (ExPEC), which have acquired virulence factors that allow them to survive outside of the host intestines and cause disease in humans and animals (3). ExPEC is a diverse pathotype divided in subpathotypes that include, among others, Avian Pathogenic *E. coli* (APEC) and Neonatal Meningitis *E. coli* (NMEC) (4).

Avian pathogenic *E. coli* causes extra-intestinal infections in birds known as colibacillosis, which can manifest as localized infections known as cellulitis or systemic infections called colisepticemia. Colibacillosis is the most common infectious disease of poultry but it can also affect other species of birds (5). The disease can impact all stages of the poultry production, affecting the health of birds and quality of meat and eggs. Economic losses occur due to morbidity, carcass condemnation and increase in mortality of the birds (6). In turkey production industry, colibacillosis is one of the leading bacterial causes of economic loss worldwide (7).

Neonatal meningitis *E. coli* (NMEC) is another distinct pathotype of ExPEC that is able to survive in blood and invade the meninges of newborn infants causing meningitis (1, 8, 9). NMEC is the second most common cause of neonatal sepsis and meningitis, accounting for 30% of all early-onset infections as determined by signs of infection and isolation of the organism from the cerebrospinal fluid (CSF) cultures at ≤72 h of life (10). NMEC infections lead to high case fatality rate (15-40%) during the neonatal period (1, 11), and survivors may present with lifelong severe neurological sequelae that includes cerebral palsy, seizures, hearing loss and delayed development (12).

Several virulence factors have been described as being involved in the pathogenesis of APEC and NMEC. These factors include adhesins, invasins, serum resistance factors, iron acquisition systems and toxins (13, 14). However, the pathogenesis of APEC and NMEC strains is not completely understood. Additionally, the role of protein secretion in the pathogenesis of APEC and NMEC remains to be elucidated.

Gram-negative bacteria have evolved several secretion systems that either secrete proteins into the extracellular millieu (T1SS, T2SS, T5SS) or directly into target cells (T3SS, T4SS, T6SS) (15). The recently discovered Type 6 Secretion System (T6SS), first described in *Vibrio cholerae* (16), has gained significant attention. The system functions as a molecular syringe, secreting proteins into the external milieu or straight into the host cells. The system targets both eukaryotic and prokaryotic cells, and the effectors it delivers play various roles in impacting the host cell, including cytoskeleton rearrangement, cell invasion, cell disruption, helping the bacteria to evade host defense mechanisms, as well as other functions (17).

Several bacterial pathogens were shown to harbor a T6SS locus, including the human and animal pathogen *Burkholderia mallei,* the fish pathogen *Edwardsiella tarda,* and the human pathogen *Pseudomonas fluorescens.* In each of these pathogens, the T6SS has been associated with the infection process, as well as protecting the bacteria from host defenses (18-20).

In APEC, the role of the T6SS in pathogenicity was first investigated in the APEC septicemic strain SEPT362 , where T6SS genes *clpV* and *hcp* were found to be involved in adherence, and *hcp* was also shown to have a role in invasion (21). Another study by the same group demonstrated a T6SS *icmF* mutant presented a defective adherence and invasion, defective biofilm formation and impaired intramacrophage survival (22).

Current knowledge on the role of the T6SS in NMEC pathogenesis is limited to one study that used the prototypic NMEC strain RS218 (*E. coli* K1). RS218 harbors two T6SS clusters (T6SS1 and T6SS2). Previous work reported that T6SS2 core proteins Hcp1 and Hcp2 of RS218 play an important role in invasion, cytoskeleton rearrangement and apoptosis of human brain microvascular endothelial cells (HBMEC), which constitutes the blood-brain barrier (BBB), suggesting that the T6SS is necessary for breaching the BBB and establishment of meningitis (23).

In the first part of this study, we aimed to characterize a collection of *E. coli* isolated from turkeys with or without signs of colisepticemia regarding their genotype for virulence genes, antimicrobial and heavy metal resistance genes, phylogenetic typing, and clonal relationship among the isolates. In the second part, we focused in analyzing the role of Type 6 Secretion genes in the virulence of APEC and NMEC by generating mutants and characterizing these mutants with regards to virulence associated phenotypes.

The dissertation presented here is organized into six chapters in a journal format. Chapter 1 is an introduction with the organization of the thesis. Chapter 2 is a literature review of *E. coli* with an emphasis on the ExPEC pathotype. Chapter 3 reports on the characterization of *E. coli* isolates from turkeys regarding their virulence, antimicrobial resistance and metal resistance genotypes, phylogenetic classification and clonal relationship. Chapter 4 investigates the role of T6SS genes in the virulence of Neonatal Meningitis *E. coli* (NMEC). Chapter 5 is on the role of T6SS genes on the virulence of Avian Pathogenic *E. coli* (APEC). Chapter 6 is a conclusion of the work presented within this dissertation.

CHAPTER 2

LITERATURE REVIEW

Escherichia coli

Escherichia coli is a Gram-negative facultative anaerobe bacterium that colonizes the intestines of humans and warm-blooded animals within a few hours after birth. *E. coli* has been identified as one of the pioneer species that helps to establish the gut microbiota and is an important member of the intestinal microbiota of mammals and birds (1). *E. coli* species comprises commensal, intestinal pathogenic and extra-intestinal pathogenic subsets (24, 25). Commensal strains colonize the colon of healthy hosts and only cause disease in the presence of a large inoculum or when the host is compromised. However, the acquisition of virulence factors by some strains allows them to cause infections in humans and animals (1, 2). Pathogenic *E. coli* are classified in two groups: Intestinal pathogenic *E. coli* (InPEC), which cause diarrhea-like infections and have different strategies to invade intestinal cells (26); and extraintestinal pathogenic *E. coli* (ExPEC), which have acquired virulence factors that allow them to survive outside of the host intestines where they may cause disease in humans and animals (3).

Intestinal pathogenic *E. coli* (InPEC)

Intestinal pathogenic *E. coli* (InPEC), also known as diarrheagenic *E. coli*, cause diarrhea syndromes that can vary according to the repertoire of virulence traits harbored by the strain. The difference in the repertoire of virulence traits harbored by InPEC is the basis for the classification of the strains into sub-pathotypes that include enteropathogenic

E. coli (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent (DAEC) (3). More InPEC subpathotypes have been proposed including adherent invasive *E. coli* (AIEC), necrotoxic *E. coli* (NTEC), and cell-detaching *E. coli* (CDEC) (27, 28).

Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) is the cause of acute to persistent diarrhea in children worldwide (29). EPEC-caused disease occurs primarily in developing countries and has a mortality rate of about 30% in infants (30).

The pathogenesis of EPEC-caused diarrhea involves ingestion of EPEC through contaminated water or food, bacterial adhesion to the small intestine enterocytes, protein translocation of a receptor from bacteria to host via a type III secretion system (T3SS), and pedestal formation through actin filament rearrangement (4, 28). The intestinal microvilli are destroyed by the formation of the pedestal, which induces attachment and effacing lesions and lessens the surface area for fluid absorption contributing to diarrhea (4, 28).

The locus of enterocyte effacement pathogenicity island (LEE PAI) is one of the key virulence factors of EPEC (31). This locus encodes a Type 3 secretion system (T3SS) that includes Tir and Intimin. Tir is translocated into the host cell to serve as a receptor, while Intimin, on the surface of the bacteria, mediates attachment of EPEC to Tir (32, 33). Another virulence factor of EPEC is the small plasmid pEAF (EPEC adherence factor) (28), which encodes the bundle forming pilus (34, 35). Atypical EPEC lack the EAF plasmid, and do not make the bundle forming pilus (36).

Enterohemorraghic *E. coli* (EHEC)

Enterohemorrhagic *E. coli* (EHEC) also form a pedestal through effectors of the LEE PAI, including Tir and Intimin. However, EHEC induces attachment and effacing lesions in the large intestine, while EPEC's attachment and effacing lesions occur in the small intestine (28). The main difference between EHEC and EPEC is that EHEC strains commonly produce a Shiga toxin (Stx). However, not all Stx-producing toxin *E. coli* (37) are EHEC (38). More than 380 serotypes of *E. coli* can produce Stx, but most are not associated with disease in humans without also containing the LEE PAI (39). Therefore, EHEC may be defined as *E. coli* that produce the Stx and contain the LEE PAI (28).

Infections by EHEC occur via a fecal-oral route and a low dose, 10-100 CFUs, is sufficient for infection (39). EHEC caused disease can manifest as bloody diarrhea, non-bloody diarrhea, or hemolytic uremic syndrome (HUS). For HUS to occur, Stx is produced in the large intestine and binds to endothelial cells, being absorbed into the bloodstream and disseminated (39). HUS can result in renal failure or death (40).

Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) is the cause of approximately 15-20% of diarrheal cases in children under five years of age in developing countries and 60% of traveler's diarrhea (41). Virulence factors of ETEC include at least one enterotoxin, a heat-labile enterotoxin (LT) or a heat-stable one (ST). The LT leads to chloride secretion by secretory crypt cells, resulting in watery diarrhea (28). The ST activates kinases, which ultimately increase secretion (28). In addition to enterotoxins, ETEC harbors colonization factors that enable them to colonize the small intestine, which brings the enterotoxins produced by ETEC in close proximity to the intestinal epithelium (42).

Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* (EAEC) produce a thick biofilm and secrete toxins leading to watery diarrhea (28, 43). EAEC uses its aggregative adherence fimbriae (AAF) to colonize the mucosa of the small and large intestine (44). Several virulence factors have been described for EAEC, including the toxins ShET1, also found in *Shigella flexneri* 2a, and EAST-1, a homolog of the heat-stable enterotoxin of ETEC (45, 46). Some EAEC also contain two serine protease autotransporters (SPATEs): Pic (protease involved in intestinal colonization) and Pet (plasmid-encoded autotransporter, involved in cell rounding and detachment) (47, 48).

Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) is a major cause of dysentery in developing countries and employs pathogenesis mechanisms similar to those of *Shigella*. Diarrhea occurs following bacterial invasion of enterocytes via endocytosis, leading to host cell death (28). The plasmid pInv, which carries the invasion plasmid antigen H gene (*ipaH*), invasion-associated locus (*ial*), and invasion transcriptional regulation genes (*virF* and *virB*) is critical to EIEC virulence (49). Other potential virulence factors include the ShET1 and ShET2 enterotoxins, Pic, and autotransporters SepA and SigA (28).

Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *E. coli* (DAEC) have been implicated in diarrhea in children older than 12 months (50). DAEC have a diffuse adherence pattern on HeLa and Hep-2 cells (44). The Afa/Dr adhesins are important to the DAEC virulence as they assist in eliciting the enterocytes to develop long cellular projections, which wrap around the bacteria (28). Nevertheless, without additional virulence factors, the role of DAEC in

diarrhea is questionable as volunteers that ingested 10^{10} CFU of prototypic DAEC strains did not experience diarrhea (51, 52).

Adherent invasive *E. coli* (AIEC)

Adherent invasive *E. coli* (AIEC) colonizes the intestinal mucosa and adheres to intestinal epithelial cells. AIEC are invasive pathogens as AIEC survive and replicate intracellularly (53). Clinically, AIEC may be involved in the pathogenesis of Crohn's Disease. AIEC have been enriched from the ileal lesions of Crohn's Disease patients, and AIEC is associated with the ileal mucosa (54), where it adheres to CEACAM6, which is overexpressed in Crohn's disease patients (55).

Virulence factors of AIEC strains include type I fimbriae, long polar fimbriae, pyelonephritis-associated pili, S fimbriae, Dr antigen-specific fimbriae, K1 capsule, iron acquisition proteins, the vacuolating autotransporter toxin, alpha-hemolysin, cytotoxic necrotizing factor, and the IbeA invasin (56).

Necrotoxic *E. coli* (NTEC)

Necrotoxic *E. coli* (NTEC) are defined based on their production of cytotoxic necrotizing factor 1 (CNF1) or 2 (CNF2). NTEC strains are able to cause a variety of diseases including diarrhea, systemic infections, septicemia, and urinary tract infections (57). CNF1 is often found in other pathotypes, causing the NTEC pathotype to be a less common classification for *E. coli* isolates. As such, UPEC are occasionally subdivided by the presence of *cnf1* or *cnf2* to distinguish between those with the toxin, an NTEC UTI, and those without, an UPEC UTI (58). However, this distinction is poorly recognized among UPEC researchers.

Cell-detaching *E. coli* (CDEC)

Cell-detaching *E. coli* (CDEC) are associated with diarrhea in children (28). CDEC cause cultured epithelial cells to detach from glass or plastic. This trait is associated with cell-bound alpha hemolysin (HlyA) (59). HlyA is found in other pathotypes of *E. coli*, which makes the CDEC pathotype similar to the NTEC pathotype as other *E. coli* groupings contain their defining virulence factors. (28, 60).

Extra-intestinal pathogenic *E. coli* (ExPEC)

Extra-intestinal pathogenic *E. coli* colonize the gut of animals and humans in a similar manner to commensal strains, usually without causing infection. However, virulence factors acquired by these strains give them the unique ability to enter and survive within extra-intestinal niches and cause disease when they adapt to these niches (3). The subpathotypes of *E. coli* share a set of genes known as the *E. coli* core genome, that is common to all *E. coli*. However, they differ according to the presence of accessory traits that determine the clinical behaviors of the different strains (25).

The virulence factors that distinguish ExPEC strains from other *E. coli* include factors such as adhesins and invasins that promote attachment to and invasion of the host cell, serum resistance proteins that allow avoidance or subversion of the host defense mechanisms and toxins that lead to the injury of cells and tissues of the host resulting in disease (61).

Variation of virulence factor profiles are common even within a lineage, consistent with deletions or horizontal gene transfer of virulence factor-encoding genes. Virulence factor acquisition by horizontal gene transfer often involves blocks of DNA known as

pathogenicity-associated islands (PAIs) can occur via plasmids, phages, and other forms of recombination (62).

ExPEC is a highly diverse pathotype which comprises subpathotypes including avian pathogenic *E. coli* (APEC), neonatal meningitis *E. coli* (NMEC), uropathogenic *E. coli* (UPEC) and septicemic *E. coli* (SEPEC) (4).

Avian pathogenic *E. coli* (APEC)

Avian pathogenic *E. coli* is the causative agent of extra-intestinal infections in birds known as colibacillosis, which can manifest as localized or systemic infections. Colibacillosis is the most common infectious disease of poultry (5). The most common manifestations of APEC infections include cellulitis, a respiratory disease that frequently culminates in septicemia and lesions in organs such as liver, air sacs and heart; swollen head syndrome; omphalitis, which affects young chicks; and salpingitis, which affects breeding and laying hens (2, 63). The disease affects all stages of poultry production, resulting in quality losses. Economic losses occur due to morbidity, carcass condemnation and increased mortality of the birds (6).

Inhalation of contaminated dust is probably the primary cause of colibacillosis when infection is initiated in the upper respiratory tract. High concentrations of ammonia in the bird environment may also be a contributing factor that make birds prone to the infection as it causes damage to the respiratory tract epithelium favoring the entry of bacteria via the respiratory tract. Other factors that may favor infection are exposure of the birds to high temperatures, limited space and a deficient disinfection/hygiene control of the birds' environment (2).

In order to cause infection, APEC strains need to adhere to the upper respiratory tract cells, go through the trachea and air sacs and colonize the lungs. After colonization of the lungs and air sacs, thickening of the epithelia and the presence of exudate is observed on the surface of the respiratory tract. The first microscopic changes are the presence of edemas and the infiltration of heterophils in the respiratory epithelium. Mononuclear phagocytes are frequently found 12 h after infection (64).

Neonatal meningitis *E. coli* (NMEC)

Neonatal meningitis *E. coli* (NMEC) is a distinct pathotype of ExPEC that is able to survive in blood and invade the meninges of newborns causing neonatal sepsis and meningitis. NMEC is isolated in about 30% of all early-onset infections, characterized by signs of infection and isolation of the organism from the cerebrospinal fluid (CSF) cultures at ≤72 h of life (10). NMEC has also emerged as the most common cause of meningitis and sepsis among very low birth weight infants (<1500 g birth weight) since the 1990s (65-68). NMEC infections lead to high case fatality rate (15-40%) during the neonatal period (1, 11). Severe lifelong neurological sequelae that include cerebral palsy, seizures, hearing loss and delayed development may occur in 12% to 44% of neonatal meningitis survivors (12).

To cause meningitis in a newborn host, NMEC needs to translocate from the intestinal lumen, urinary tract, or uterus to the bloodstream; survive intravascularly and multiply and pass through the blood-brain barrier, a tight barrier formed by brain microvascular endothelial cells (BMEC); enter the central nervous system and cause inflammation of the meninges and pleocytosis of the CSF (8, 69, 70). Bacteria are usually acquired perinatally and reach the bloodstream via transcytosis through enterocytes.

Survival in blood is crucial as the progression of the disease depends on high bacteremia (>10^3 colony-forming units (CFU) per mL of blood (71). Once in the bloodstream, NMEC mimics the host immune system by producing capsular glycoproteins (K1) similar to those of humans in order to evade the host innate immune system (72). Additionally, NMEC invade and replicate in macrophages; thus, evading the immune system and further proliferating in the bloodstream (73). Such proliferation is responsible for acute bacteremia.

The virulence trait repertoire of NMEC strains was shown to be diverse, and NMEC strains were shown to have a higher average number of virulence factors when compared to human fecal *E. coli* (HFEC) (74). Some virulence factors are required for the different stages of NMEC infection. Virulence factors found to be associated with the ability of NMEC to cause disease include outer membrane proteins such as the capsular antigen K1 and OmpA, which provide protection from the host immune responses; siderophores for iron scavenging encoded by *iroN*, *fyuA* and *iutA*; adhesins P-fimbriae, S-fimbriae and type-1-fimbriae, which mediate attachment to BMEC; and invasins *ibeA*, *asl* and *cnf* (71, 75). IbeA was also proposed as a virulence factor that could be involved in the translocation of *E. coli* through the amniotic membrane. The pathogenicity island GimA, which contains the gene encoding IbeA, contributes to the invasion of the blood-brain barrier through a carbon-regulated process (76).

Uropathogenic *E. coli* (UPEC)

Uropathogenic *E. coli* (UPEC) is the cause of 80-90% of community-acquired urinary tract infections (UTIs) (77, 78). To cause disease, UPEC must: colonize the periurethral and vaginal areas, with colonization of the urethra; ascend into the lumen of

the bladder and grow in urine; adhere to the surface of bladder epithelium and interact with its defense system; form biofilm; invade and replicate within bladder cells; colonize the kidney and damage host tissue with increased risk for septicemia (79). To colonize the bladder, UPEC use a variety of virulence factors, which include lipopolysaccharide (80), capsule, flagella, outer-membrane vesicles, adhesins, outer-membrane proteins, toxins, secretion systems and iron uptake systems (81-83).

UTIs affect 150 million people annually worldwide (84, 85) and are classified as uncomplicated or complicated, and the causative agent for 65-75% of infections for both categories is UPEC (86, 87). The complication of UTIs result from the host defense or urinary tract becoming compromised via urinary obstruction, urinary retention, immunosuppression, renal failure, pregnancy, or indwelling catheters (86).

Uncomplicated UTIs are characterized by colonization of the urethra by UPEC before migration to the bladder (86). Uncomplicated UTIs affect healthy individuals who do not present structural abnormalities of the urinary tract and include cystitis and pyelonephritis (85, 86). Cystitis is the infection of the lower urinary tract (bladder), as pyelonephritis is the infection of the upper urinary tract (kidneys) (86). When left untreated, pyelonephritis can result in sepsis or renal damage or failure (88).

Complicated UTIs occur when UPEC binds to a catheter, kidney or bladder stone or is retained by an obstruction within the urinary tract (86), which can lead to urosepsis or bacteremia if UPEC crosses the tubular epithelial barrier in the kidneys (86, 89).

Virulence associated factors of ExPEC

ExPEC are distinguished from other *E. coli* strains by having specialized traits that play important roles in promoting survival in or on the host, and include factors related to adherence, acquisition of nutrients, escape from host defense mechanisms, leading to host tissue or cellular damage causing disease (61). Multiple virulence factors and even multiple representatives of a functional category of factors can be present in ExPEC strains (61).

To cause infection, ExPEC need at least one factor associated with adherence, one siderophore, and one factor associated with serum resistance. Genes that encode toxins and invasins may also be present (13). However, these strains can miss one or more of these factors and still be pathogenic. Except for genes encoding F1 fimbriae, genes that encode virulence factors are usually absent in the genomes of non-pathogenic isolates (90).

The variation in the virulence factor profiles within a given lineage is consistent with the acquisition or loss of these factors via horizontal gene transfers or deletions. Horizontal transfer of virulence genes can occur via plasmids, phages or other forms of recombination and involves blocks of DNA known as fitness island or pathogenicity islands (PAI) that may contain multiple virulence genes (62). Several virulence-associated factors have been described for APEC and NMEC strains, including adhesins, invasins, serum resistance factors, iron acquisition systems and toxins (13).

Adhesins

Adherence of bacteria to the host cell is a necessary condition for bacterial colonization and it involves specific interaction between the bacteria and host target receptors (91). The virulence factors involved in this process are called adhesins. The three main types of adhesins recognized in ExPEC are fimbriae, afimbrial adhesins (Afa), and

outer membrane proteins (Omp) (92). Adhesins initiate contact between the bacteria and the host cell, increasing the virulence of pathogenic *E. coli*. Different adhesins are adapted to colonize a specific niche. Among isolates from urinary tract infection (UTI) patients, S fimbrial adhesins (*sfa*), F1C ("pseudotype I") fimbriae (*foc*), coding P-like pili (*papC*), and Iha are the most frequently detected adhesins (93). S-fimbrial adhesins are also present in ExPEC strains that cause meningitis and sepsis and have the ability to bind extracellular matrix components on brain endothelial cells (8). Fimbrial adhesins are classified as type 1, when they have affinity for structures containing mannose residues, and type 2, when they are resistant to mannose (94).

Invasins

One of the strategies that pathogenic bacteria use to escape host defense mechanisms is cell invasion, which allows survival and multiplication of the bacteria inside the host cell. To do this, bacteria employ virulence factors knows as invasins. (70). IbeA is a well-known invasin of ExPEC. The GimA genetic island, which encodes IbeT, IbeA and IbeR, is present in *E. coli* pathogens but not present in non-pathogenic *E. coli* strains K12 (95, 96). IbeA of an NMEC K1 strain was shown to induce invasion of brain microvascular endothelial cells (BMECs), which constitute the brain-blood barrier (97).

Serum resistance

Bacterial surface structures such as capsule, lipopolysaccharide (80) and outer membrane proteins that mediate resistance to the bactericidal effect of host complement in serum have been associated with ExPEC isolates. One example is the increased serum survival *iss* gene, which has been recognized for its role in the virulence of ExPEC and was found to be significantly associated with APEC strains compared to fecal isolates of

healthy birds (98, 99). It was also found to occur in about 60% of UPEC and NMEC strains but was only present in a few human fecal commensal *E. coli* strains (99, 100).

Siderophores

Iron is essential for the growth and survival of extra-intestinal *E. coli*, which requires a higher concentration of iron than that available in physiological fluids of animals and humans. To circumvent the low iron concentration, extra-intestinal bacterial pathogens have developed high affinity systems for iron-acquisition that compete with the siderophores of the host, favoring bacterial growth (2). The siderophores produced by ExPEC are small secreted molecules with high affinity for ferric iron that can capture iron from host sources such as lactoferrin and transferrin (101). Siderophores produced by ExPEC include enterobactin, salmochelins, yersiniabactin and aerobactin (102, 103). It is not clear if enterobactin is involved in *E. coli* virulence, since its presence is common to both pathogenic and non-pathogenic *E. coli* (104). The salmochelin system receptor IroN was shown to be involved in the virulence of ExPEC strains including UPEC, NMEC and APEC (90, 105-108). Aerobactin was also found to play a role in the virulence of APEC, as strains deficient in aerobactin synthesis and uptake present with reduced virulence in a chicken systemic infection model (90).

Toxins

Another strategy used by *E. coli* to cause disease is the production of toxins. Toxins produced by *E. coli* include α-haemolysin (*hlyD*), a pore forming protein that favors the insertion of the bacteria and causes injury in the uroepithelium, cell lysis and release of intracellular micronutrients (109). Another toxin of importance is the cytotoxic necrotizing factor (*cnf1*), primarily associated with ExPEC causing urinary tract infection and

meningitis (110), contributing to invasion of HBMEC monolayer and penetration into the brain in the infant rat model of meningitis (111).

Protein secretion systems

Bacterial pathogenicity can also be influenced by protein secretion machineries known as secretions systems (112). These systems mediate the transport of effector proteins through the inner membrane, periplasm and outer membrane of the bacteria (113). Protein secretion plays a central role in modulating bacterial associations, biofilm formation, symbiosis and host-pathogen interaction (114). The understanding of the secretion mechanisms developed by bacteria is crucial to understand these processes (113).

Six secretion systems have been described for Gram-negative pathogens. Secretion Systems Type 2 and Type 5 rely on the universal secretion systems Sec and Tat to transport proteins through the inner membrane into the periplasm of bacterial cells. These proteins are then transported from the periplasm through the bacterial outer membrane via Type 2 Secretion System (T2SS) and Type 5 Secretion System (T5SS). Secretion Systems Type 1, 3, 4 and 6 translocate proteins through the inner membrane, periplasm and outer membrane of the bacterial cell in one single step (114).

In Gram-positive pathogens, proteins are usually translocated via the universal systems Sec and Tat (114), except in a few cases like *Mycobacterium* that, due to the hydrophobicity and high impermeability of its membrane, has developed a specialized system known as the Type 7 Secretion System (T7SS) (115). More recently, the extracellular nucleation-precipitation (ENP) pathway, involved in the production of the adhesive fiber curli, was proposed to be reclassified as Type 8 Secretion System (116).

Type 6 Secretion System (T6SS)

The Type 6 Secretion System, which this work focuses on, was first described in 2006 in *Vibrio cholerae* V52, which caused an outbreak of cholera in Sudan in 1968. Unlike other pandemic strains, which usually belong to serogroups O1 and O139, *V. cholerae* V52 belongs to serogroup O37. Using the amoeba *Dyctiostelium discoideum* as a host model to identify virulence mechanisms, it was found that strain V52 was resistant to predation by *D. discoideum*, an uncommon phenotype for *V. cholerae* strains. A genetic screen showed that this phenotype was due to an unknown secretion system, which the authors named Type 6 Secretion System (T6SS) (117).

T6SS operon has 15 to 20 genes, and genomic analyses has identified a set of 13 conserved proteins that constitute the core of the system (113), and a set of non-conserved proteins that play regulatory and accessory functions (118). The T6SS apparatus is described as a double membrane-spanning structure that works as a one-step mechanism by which bacterial cytoplasmic substrates are secreted into a target cell or to the extracellular space (119). A schematic representation of the T6SS is shown in Figure 2.1.

The function of some of the genes that form the system have not yet been described. It is thought that the non-secreted proteins are either structural components of the secretion apparatus or help in the secretion of effectors by providing energy for the system to work (120).

The presence of the T6SS and its importance in pathogenesis has been reported in several bacteria. In *Burkholderia mallei*, a human and animal pathogen, T6SS was shown to be important for intramacrophage proliferation, and the expression of an Hcp related protein is induced during infection *in vivo* (121). The fish pathogen *Edwardsiella tarda* has

an active T6SS (122), and, in *Pseudomonas aeruginosa*, Hcp1 is secreted by clinical isolates, and cystic fibrosis patients produce anti-Hcp1 antibody, indicating that T6SS is active during infection (123).

Besides pathogenicity, T6SS has been associated with other bacterial properties. T6SS was demonstrated to be involved in the antibacterial activity of *V. cholerae* against other Gram-negative pathogens such as *Salmonella enterica* serovar Typhimurium, *Citrobacter rodentium* and *Pseudomonas aeruginosa* (124). The system was also shown to favor *Pseudomonas fluorescens* in competition against the plant pathogen *Pectobacterium atrosepticum* (125).

The role of T6SS in the pathogenicity of APEC was first investigated in 2010 in a study that showed that an APEC septicemic strain SEPT362 expresses a T6SS and that the system contributes to the virulence of the strain. *hcp* and *clpV* deletion mutants had their adherence capability impaired; the *hcp* mutant also had its invasion capability impaired (21). Another study by the same group demonstrated that an *icmF* mutant presented a defective adherence and invasion, defective biofilm formation and had its intramacrophage viability reduced (22).

In 2013, three *loci* encoding T6SS were identified in APEC strain ED205 isolated from the brain of a duck displaying signs of colisepticemia. A screening of these loci was performed in 472 APEC isolates: 11 of them presented 2 T6SS loci. T6SS locus 1 is larger and more frequent in APEC strains and harbors 15 T6SS core genes. The difference in the number of conserved proteins in the locus and the different frequencies in which they are found suggest that the different loci may play different roles (126).

Another APEC strain, APEC TW-XM, isolated from the brain of a duck with septicemia displaying neurological signs, harbors two functional T6SS (T6SS1 and T6SS2), with T6SS1 being more associated with systemic infection, and T6SS2 associated with brain infection (127). Subsequently, it was demonstrated that the protein DotU, encoded by T6SS2 locus of *E. coli* ED719, is involved in pathogenesis of the strain, in the secretion of Hcp effector, and in the modulation of the host immune response during infection (128).

Current knowledge on the role of the T6SS in NMEC pathogenesis is limited to one study that used the prototypic NMEC strain RS218 (*E. coli* K1). RS218 harbors two T6SS clusters (T6SS1 and T6SS2). Previous work reported that T6SS2 core proteins Hcp1 and Hcp2 of RS218 play an important role in invasion, cytoskeleton rearrangement and apoptosis of HBMEC, which constitutes the blood-brain barrier (BBB), suggesting that the T6SS is necessary for breaching the BBB and establishment of meningitis (23). However, the role of T6SS in the pathogenesis of NMEC warrants further investigation. No studies have analyzed the role of T6SS1 genes in NMEC.

REFERENCES

1. Kaper JB, Nataro JP, Mobley HL. 2004. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123-40.
2. Dho-Moulin M, Fairbrother JM. 1999. Avian pathogenic *Escherichia coli* (APEC). Vet Res 30:299-316.
3. Russo TA, Johnson JR. 2000. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. J Infect Dis 181:1753-4.
4. Kaper JB, Nataro JP, Mobley HL. 2004. Pathogenic *Escherichia coli*. NatRevMicrobiol 2:123-140.
5. Nolan LK, Barnes HJ, Vaillancourt J-P, Abdul-Aziz T, Logue CM. 2013. Colibacillosis, p 751-805, Diseases of Poultry, 13th ed doi:10.1002/9781119421481.ch18. John Wiley & Sons, Ltd.
6. Dziva F, Stevens MP. 2008. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathol 37:355-66.
7. Hafez HM. Current Knowledge and Prosepctive Risk Analysis Related to Ongoing Turkey Diseases., p 138-149. *In* (ed),
8. Bonacorsi S, Bingen E. 2005. Molecular epidemiology of *Escherichia coli* causing neonatal meningitis. Int J Med Microbiol 295:373-81.
9. Glode MP, Sutton A, Moxon ER, Robbins JB. 1977. Pathogenesis of neonatal *Escherichia coli* meningitis: induction of bacteremia and meningitis in infant rats fed E. coli K1. Infect Immun 16:75-80.

10. Stoll BJ, Hansen NI, Sanchez PJ, Faix RG, Poindexter BB, Van Meurs KP, Bizzarro MJ, Goldberg RN, Frantz ID, 3rd, Hale EC, Shankaran S, Kennedy K, Carlo WA, Watterberg KL, Bell EF, Walsh MC, Schibler K, Laptook AR, Shane AL, Schrag SJ, Das A, Higgins RD. 2011. Early onset neonatal sepsis: the burden of group B Streptococcal and *E. coli* disease continues. Pediatrics 127:817-26.

11. Kim KS. 2003. Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury. Nat Rev Neurosci 4:376-85.

12. Stevens JP, Eames M, Kent A, Halket S, Holt D, Harvey D. 2003. Long term outcome of neonatal meningitis. Arch Dis Child Fetal Neonatal Ed 88:F179-84.

13. Barbieri NL, de Oliveira AL, Tejkowski TM, Pavanelo DB, Rocha DA, Matter LB, Callegari-Jacques SM, de Brito BG, Horn F. 2013. Genotypes and pathogenicity of cellulitis isolates reveal traits that modulate APEC virulence. PLoS One 8:e72322.

14. Barbieri NL, de Oliveira AL, Tejkowski TM, Pavanelo DB, Matter LB, Pinheiro SR, Vaz TM, Nolan LK, Logue CM, de Brito BG, Horn F. 2015. Molecular characterization and clonal relationships among *Escherichia coli* strains isolated from broiler chickens with colisepticemia. Foodborne Pathog Dis 12:74-83.

15. Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G. 2015. Secretion systems in Gram-negative bacteria: structural and mechanistic insights. Nat Rev Microbiol 13:343-59.

16. Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, Heidelberg JF, Mekalanos JJ. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc Natl Acad Sci U S A 103:1528-33.

17. Tseng TT, Tyler BM, Setubal JC. 2009. Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. BMC Microbiol 9 Suppl 1:S2.

18. Schell MA, Ulrich RL, Ribot WJ, Brueggemann EE, Hines HB, Chen D, Lipscomb L, Kim HS, Mrazek J, Nierman WC, Deshazer D. 2007. Type VI secretion is a major virulence determinant in *Burkholderia mallei*. Mol Microbiol 64:1466-85.

19. Zheng J, Leung KY. 2007. Dissection of a type VI secretion system in *Edwardsiella tarda*. Mol Microbiol 66:1192-206.

20. Decoin V, Barbey C, Bergeau D, Latour X, Feuilloley MG, Orange N, Merieau A. 2014. A type VI secretion system is involved in *Pseudomonas fluorescens* bacterial competition. PLoS One 9:e89411.

21. de Pace F, Nakazato G, Pacheco A, de Paiva JB, Sperandio V, da Silveira WD. 2010. The type VI secretion system plays a role in type 1 fimbria expression and pathogenesis of an avian pathogenic *Escherichia coli* strain. Infection and Immunity 78:4990-8.

22. de Pace F, Boldrin de Paiva J, Nakazato G, Lancellotti M, Sircili MP, Guedes Stehling E, Dias da Silveira W, Sperandio V. 2011. Characterization of IcmF of the type VI secretion system in an avian pathogenic *Escherichia coli* (APEC) strain. Microbiology 157:2954-62.

23. Zhou Y, Tao J, Yu H, Ni J, Zeng L, Teng Q, Kim KS, Zhao GP, Guo X, Yao Y. 2012. Hcp family proteins secreted via the type VI secretion system coordinately regulate *Escherichia coli* K1 interaction with human brain microvascular endothelial cells. Infect Immun 80:1243-51.

24. Johnson JR, Oswald E, O'Bryan TT, Kuskowski MA, Spanjaard L. 2002. Phylogenetic Distribution of Virulence-Associated Genes among *Escherichia coli* Isolates Associated with Neonatal Bacterial Meningitis in The Netherlands. The Journal of Infectious Diseases 185:774-784.

25. Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P, Bingen E, Bonacorsi S, Bouchier C, Bouvet O, Calteau A, Chiapello H, Clermont O, Cruveiller S, Danchin A, Diard M, Dossat C, Karoui ME, Frapy E, Garry L, Ghigo JM, Gilles AM, Johnson J, Le Bouguénec C, Lescat M, Mangenot S, Martinez-Jéhanne V, Matic I, Nassif X, Oztas S, Petit MA, Pichon C, Rouy Z, Ruf CS, Schneider D, Tourret J, Vacherie B, Vallenet D, Médigue C, Rocha EP, Denamur E. 2009. Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. PLoS Genet 5:e1000344.

26. Reis RS, Horn F. 2010. Enteropathogenic *Escherichia coli*, *Samonella*, *Shigella* and *Yersinia*: cellular aspects of host-bacteria interactions in enteric diseases. Gut Pathog 2:8.

27. Darfeuille-Michaud A. 2002. Adherent-invasive *Escherichia coli*: a putative new *E. coli* pathotype associated with Crohn's disease. Int J Med Microbiol 292:185-93.

28. Kaper JB. 2004. Pathogenic E*scherichia coli*. Nat Rev Microbiol 2.

29. Ochoa TJ, Contreras CA. 2011. Enteropathogenic *Escherichia coli* infection in children. Curr Opin Infect Dis 24:478-83.

30. Senerwa D, Olsvik O, Mutanda LN, Lindqvist KJ, Gathuma JM, Fossum K, Wachsmuth K. 1989. Enteropathogenic *Escherichia coli* serotype O111:HNT isolated from preterm neonates in Nairobi, Kenya. J Clin Microbiol 27:1307-11.

31. McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB. 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci U S A 92:1664-8.

32. Jerse AE, Yu J, Tall BD, Kaper JB. 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc Natl Acad Sci U S A 87:7839-43.

33. Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB. 1997. Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell 91:511-20.

34. Trabulsi LR, Keller R, Tardelli Gomes TA. 2002. Typical and atypical enteropathogenic *Escherichia coli*. Emerg Infect Dis 8:508-13.

35. Girón JA, Ho AS, Schoolnik GK. 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science 254:710-3.

36. Scotland SM, Smith HR, Cheasty T, Said B, Willshaw GA, Stokes N, Rowe B. 1996. Use of gene probes and adhesion tests to characterise *Escherichia coli* belonging to enteropathogenic serogroups isolated in the United Kingdom. J Med Microbiol 44:438-43.

37. Anderson M, Bollinger D, Hagler A, Hartwell H, Rivers B, Ward K, Steck TR. 2004. Viable but nonculturable bacteria are present in mouse and human urine specimens. JClinMicrobiol 42:753-758.

38. Beutin L, Martin A. 2012. Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. J Food Prot 75:408-18.

39. Nguyen Y, Sperandio V. 2012. Enterohemorrhagic *E. coli* (EHEC) pathogenesis. Front Cell Infect Microbiol 2:90.

40. Sandvig K. 2001. Shiga toxins. Toxicon 39:1629-35.

41. Mirhoseini A, Amani J, Nazarian S. 2018. Review on pathogenicity mechanism of enterotoxigenic *Escherichia coli* and vaccines against it. Microb Pathog 117:162-169.

42. Qadri F, Svennerholm AM, Faruque AS, Sack RB. 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev 18:465-83.

43. Nataro JP, Deng Y, Cookson S, Cravioto A, Savarino SJ, Guers LD, Levine MM, Tacket CO. 1995. Heterogeneity of enteroaggregative *Escherichia coli* virulence demonstrated in volunteers. J Infect Dis 171:465-8.

44. Croxen MA, Finlay BB. 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Micro 8.

45. Savarino SJ, Fasano A, Watson J, Martin BM, Levine MM, Guandalini S, Guerry P. 1993. Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of E. coli heat-stable toxin. Proc Natl Acad Sci U S A 90:3093-7.

46. Vila J, Vargas M, Henderson IR, Gascon J, Nataro JP. 2000. Enteroaggregative *Escherichia coli* virulence factors in traveler's diarrhea strains. J Infect Dis 182:1780-3.

47. Dautin N. 2010. Serine protease autotransporters of enterobacteriaceae (SPATEs): biogenesis and function. Toxins (Basel) 2:1179-206.

48. Gomes TA, Elias WP, Scaletsky IC, Guth BE, Rodrigues JF, Piazza RM, Ferreira LC, Martinez MB. 2016. Diarrheagenic *Escherichia coli*. Braz J Microbiol 47 Suppl 1:3-30.

49. Hale TL. 1991. Genetic basis of virulence in *Shigella* species. Microbiol Rev 55:206-24.

50. Scaletsky IC, Fabbricotti SH, Carvalho RL, Nunes CR, Maranhao HS, Morais MB, Fagundes-Neto U. 2002. Diffusely adherent *Escherichia coli* as a cause of acute diarrhea in young children in Northeast Brazil: a case-control study. J Clin Microbiol 40:645-8.

51. Tacket CO, Moseley SL, Kay B, Losonsky G, Levine MM. 1990. Challenge studies in volunteers using *Escherichia coli* strains with diffuse adherence to HEp-2 cells. J Infect Dis 162:550-2.

52. Robins-Browne RM, Holt KE, Ingle DJ, Hocking DM, Yang J, Tauschek M. 2016. Are *Escherichia coli* Pathotypes Still Relevant in the Era of Whole-Genome Sequencing? Frontiers in Cellular and Infection Microbiology 6:141.

53. Boudeau J, Glasser AL, Masseret E, Joly B, Darfeuille-Michaud A. 1999. Invasive ability of an *Escherichia coli* strain isolated from the ileal mucosa of a patient with Crohn's disease. Infect Immun 67:4499-509.

54. Darfeuille-Michaud A, Boudeau J, Bulois P, Neut C, Glasser AL, Barnich N, Bringer MA, Swidsinski A, Beaugerie L, Colombel JF. 2004. High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. Gastroenterology 127:412-21.

55. Barnich N, Carvalho FA, Glasser AL, Darcha C, Jantscheff P, Allez M, Peeters H, Bommelaer G, Desreumaux P, Colombel JF, Darfeuille-Michaud A. 2007. CEACAM6 acts as a receptor for adherent-invasive *E. coli*, supporting ileal mucosa colonization in Crohn disease. J Clin Invest 117:1566-74.

56. Yang Y, Liao Y, Ma Y, Gong W, Zhu G. 2017. The role of major virulence factors of AIEC involved in inflammatory bowl disease-a mini-review. Appl Microbiol Biotechnol 101:7781-7787.

57. Mainil JG, Jacquemin E, Pohl P, Fairbrother JM, Ansuini A, Le Bouguenec C, Ball HJ, De Rycke J, Oswald E. 1999. Comparison of necrotoxigenic *Escherichia coli* isolates from farm animals and from humans. Vet Microbiol 70:123-35.

58. Rahman H, Deka M. 2014. Detection & characterization of necrotoxin producing *Escherichia coli* (NTEC) from patients with urinary tract infection (UTI). Indian J Med Res 139:632-7.

59. Sobieszczanska BM, Osek J, Gryko R, Dobrowolska M. 2006. Association between cell-bound haemolysin and cell-detaching activity of *Escherichia coli* isolated from children. J Med Microbiol 55:325-30.

60. Rodriguez-Siek KE, Giddings CW, Doetkott C, Johnson TJ, Nolan LK. 2005. Characterizing the APEC pathotype. Vet Res 36.

61. Johnson JR, Russo TA. 2005. Molecular epidemiology of extraintestinal pathogenic (uropathogenic) *Escherichia coli*. Int J Med Microbiol 295:383-404.

62. Hacker J, Carniel E. 2001. Ecological fitness, genomic islands and bacterial pathogenicity. A Darwinian view of the evolution of microbes. EMBO Rep 2:376-81.

63. Ngeleka M, Kwaga JK, White DG, Whittam TS, Riddell C, Goodhope R, Potter AA, Allan B. 1996. *Escherichia coli* cellulitis in broiler chickens: clonal relationships among strains and analysis of virulence-associated factors of isolates from diseased birds. Infect Immun 64:3118-26.

64. Horn F, Corrêa AM, Barbieri NL, Glodde S, Weyrauch KD, Kaspers B, Driemeier D, Ewers C, Wieler LH. 2012. Infections with avian pathogenic and fecal *Escherichia coli* strains display similar lung histopathology and macrophage apoptosis. PLoS One 7:e41031.

65. Stoll BJ, Hansen NI, Higgins RD, Fanaroff AA, Duara S, Goldberg R, Laptook A, Walsh M, Oh W, Hale E. 2005. Very low birth weight preterm infants with early onset neonatal sepsis: the predominance of gram-negative infections continues in the National Institute of Child Health and Human Development Neonatal Research Network, 2002-2003. Pediatr Infect Dis J 24:635-9.

66. Bizzarro MJ, Dembry LM, Baltimore RS, Gallagher PG. 2008. Changing patterns in neonatal *Escherichia coli* sepsis and ampicillin resistance in the era of intrapartum antibiotic prophylaxis. Pediatrics 121:689-96.

67. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark AR, Tyson JE, Oh W, Bauer CR, Korones SB, Shankaran S, Laptook AR, Stevenson DK, Papile LA, Poole WK. 2002. Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. N Engl J Med 347:240-7.

68. Camacho-Gonzalez A, Spearman PW, Stoll BJ. 2013. Neonatal infectious diseases: evaluation of neonatal sepsis. Pediatr Clin North Am 60:367-89.

69. Croxen MA, Finlay BB. 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Microbiol 8:26-38.

70. Kim KS. 2012. Current concepts on the pathogenesis of *Escherichia coli* meningitis: implications for therapy and prevention. Curr Opin Infect Dis 25:273-8.

71. Wooster DG, Maruvada R, Blom AM, Prasadarao NV. 2006. Logarithmic phase *Escherichia coli* K1 efficiently avoids serum killing by promoting C4bp-mediated C3b and C4b degradation. Immunology 117:482-93.

72. Kim KS, Itabashi H, Gemski P, Sadoff J, Warren RL, Cross AS. 1992. The K1 capsule is the critical determinant in the development of *Escherichia coli* meningitis in the rat. J Clin Invest 90:897-905.

73. Selvaraj SK, Prasadarao NV. 2005. *Escherichia coli* K1 inhibits proinflammatory cytokine induction in monocytes by preventing NF-kappaB activation. J Leukoc Biol 78:544-54.

74. Logue CM, Doetkott C, Mangiamele P, Wannemuehler YM, Johnson TJ, Tivendale KA, Li G, Sherwood JS, Nolan LK. 2012. Genotypic and phenotypic traits that distinguish neonatal meningitis-associated *Escherichia coli* from fecal *E. coli* isolates of healthy human hosts. Appl Environ Microbiol 78:5824-30.

75. Xie Y, Kim KJ, Kim KS. 2004. Current concepts on *Escherichia coli* K1 translocation of the blood-brain barrier. FEMS Immunol Med Microbiol 42:271-9.

76. Soto SM, Bosch J, Jimenez de Anta MT, Vila J. 2008. Comparative study of virulence traits of *Escherichia coli* clinical isolates causing early and late neonatal sepsis. J Clin Microbiol 46:1123-5.

77. Foxman B. 2014. Urinary tract infection syndromes: occurrence, recurrence, bacteriology, risk factors, and disease burden. Infect Dis Clin North Am 28:1-13.

78. Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol 13:269-84.

79. Terlizzi ME, Gribaudo G, Maffei ME. 2017. UroPathogenic *Escherichia coli* (UPEC) Infections: Virulence Factors, Bladder Responses, Antibiotic, and Non-antibiotic Antimicrobial Strategies. Frontiers in Microbiology 8.

80. Johnston PA, Liu H, O'Connell T, Phelps P, Bland M, Tyczkowski J, Kemper A, Harding T, Avakian A, Haddad E, Whitfill C, Gildersleeve R, Ricks CA. 1997. Applications in in ovo technology. Poult Sci 76:165-78.

81. Klemm P, Vejborg RM, Hancock V. 2010. Prevention of bacterial adhesion. Appl Microbiol Biotechnol 88:451-9.

82. Werneburg GT, Henderson NS, Portnoy EB, Sarowar S, Hultgren SJ, Li H, Thanassi DG. 2015. The pilus usher controls protein interactions via domain masking and is functional as an oligomer. Nat Struct Mol Biol 22:540-6.

83. O'Brien VP, Hannan TJ, Nielsen HV, Hultgren SJ. 2016. Drug and Vaccine Development for the Treatment and Prevention of Urinary Tract Infections. Microbiol Spectr 4.

84. Stamm WE, Norrby SR. 2001. Urinary tract infections: disease panorama and challenges. J Infect Dis 183 Suppl 1:S1-4.

85. Hooton TM. 2012. Clinical practice. Uncomplicated urinary tract infection. N Engl J Med 366:1028-37.

86. Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Micro 13:269-284.

87. Foxman B. 2010. The epidemiology of urinary tract infection. Nat Rev Urol 7:653-60.

88. Sivick KE, Mobley HL. 2010. Waging war against uropathogenic *Escherichia coli*: winning back the urinary tract. Infect Immun 78:568-85.

89. Ben Mkaddem S, Chassin C, Vandewalle A. 2010. Contribution of renal tubule epithelial cells in the innate immune response during renal bacterial infections and ischemia-reperfusion injury. Chang Gung Med J 33:225-40.

90. Dozois CM, Daigle F, Curtiss R, 3rd. 2003. Identification of pathogen-specific and conserved genes expressed *in vivo* by an avian pathogenic *Escherichia coli* strain. Proc Natl Acad Sci U S A 100:247-52.

91. Klemm P, Hancock V, Schembri MA. 2010. Fimbrial adhesins from extraintestinal *Escherichia coli*. Environ Microbiol Rep 2:628-40.

92. Lindberg S, Xia Y, Sondén B, Göransson M, Hacker J, Uhlin BE. 2008. Regulatory Interactions among adhesin gene systems of uropathogenic *Escherichia coli*. Infect Immun 76:771-80.

93. Hagan EC, Mobley HL. 2007. Uropathogenic *Escherichia coli* outer membrane antigens expressed during urinary tract infection. Infect Immun 75:3941-9.

94. Eto DS, Jones TA, Sundsbak JL, Mulvey MA. 2007. Integrin-mediated host cell invasion by type 1-piliated uropathogenic *Escherichia coli*. PLoS Pathog 3:e100.

95. Huang SH, Chen YH, Kong G, Chen SH, Besemer J, Borodovsky M, Jong A. 2001. A novel genetic island of meningitic *Escherichia coli* K1 containing the ibeA invasion gene (GimA): functional annotation and carbon-source-regulated invasion of human brain microvascular endothelial cells. Funct Integr Genomics 1:312-22.

96. Huang SH, Wan ZS, Chen YH, Jong AY, Kim KS. 2001. Further characterization of *Escherichia coli* brain microvascular endothelial cell invasion gene ibeA by deletion, complementation, and protein expression. J Infect Dis 183:1071-8.

97. Che X, Chi F, Wang L, Jong TD, Wu CH, Wang X, Huang SH. 2011. Involvement of IbeA in meningitic *Escherichia coli* K1-induced polymorphonuclear leukocyte transmigration across brain endothelial cells. Brain Pathol 21:389-404.

98. Pfaff-McDonough SJ, Horne SM, Giddings CW, Ebert JO, Doetkott C, Smith MH, Nolan LK. 2000. Complement resistance-related traits among *Escherichia coli* isolates from apparently healthy birds and birds with colibacillosis. Avian Dis 44:23-33.

99. Rodriguez-Siek KE, Giddings CW, Doetkott C, Johnson TJ, Fakhr MK, Nolan LK. 2005. Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology 151:2097-110.

100. Ewers C, Li G, Wilking H, Kiessling S, Alt K, Antáo EM, Laturnus C, Diehl I, Glodde S, Homeier T, Böhnke U, Steinrück H, Philipp HC, Wieler LH. 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? Int J Med Microbiol 297:163-76.

101. Skaar EP. 2010. The battle for iron between bacterial pathogens and their vertebrate hosts. PLoS Pathog 6:e1000949.

102. Johnson TJ, Kariyawasam S, Wannemuehler Y, Mangiamele P, Johnson SJ, Doetkott C, Skyberg JA, Lynne AM, Johnson JR, Nolan LK. 2007. The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. J Bacteriol 189:3228-36.

103. Miethke M, Marahiel MA. 2007. Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol Rev 71:413-51.

104. Ratledge C, Dover LG. 2000. Iron metabolism in pathogenic bacteria. Annu Rev Microbiol 54:881-941.

105. Feldmann F, Sorsa LJ, Hildinger K, Schubert S. 2007. The salmochelin siderophore receptor IroN contributes to invasion of urothelial cells by extraintestinal pathogenic *Escherichia coli* in vitro. Infection and immunity 75:3183-3187.

106. Nègre VL, Bonacorsi S, Schubert S, Bidet P, Nassif X, Bingen E. 2004. The siderophore receptor IroN, but not the high-pathogenicity island or the hemin receptor ChuA, contributes to the bacteremic step of *Escherichia coli* neonatal meningitis. Infect Immun 72:1216-20.

107. Peigne C, Bidet P, Mahjoub-Messai F, Plainvert C, Barbe V, Medigue C, Frapy E, Nassif X, Denamur E, Bingen E, Bonacorsi S. 2009. The plasmid of *Escherichia coli* strain S88 (O45:K1:H7) that causes neonatal meningitis is closely related to avian pathogenic *E. coli* plasmids and is associated with high-level bacteremia in a neonatal rat meningitis model. Infect Immun 77:2272-84.

108. Russo TA, McFadden CD, Carlino-MacDonald UB, Beanan JM, Barnard TJ, Johnson JR. 2002. IroN functions as a siderophore receptor and is a urovirulence

factor in an extraintestinal pathogenic isolate of *Escherichia coli*. Infect Immun 70:7156-60.

109. Wiles TJ, Mulvey MA. 2013. The RTX pore-forming toxin α-hemolysin of uropathogenic *Escherichia coli*: progress and perspectives. Future Microbiol 8:73-84.

110. Boquet P. 2001. The cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli*. Toxicon 39:1673-80.

111. Khan NA, Wang Y, Kim KJ, Chung JW, Wass CA, Kim KS. 2002. Cytotoxic necrotizing factor-1 contributes to *Escherichia coli* K1 invasion of the central nervous system. J Biol Chem 277:15607-12.

112. Kostakioti M, Newman CL, Thanassi DG, Stathopoulos C. 2005. Mechanisms of protein export across the bacterial outer membrane. Journal of Bacteriology 187:4306-14.

113. Shrivastava S, Mande SS. 2008. Identification and Functional Characterization of Gene Components of Type VI Secretion System in Bacterial Genomes. PLoS ONE 3:e2955.

114. Tseng T-T, Tyler BM, Setubal JC. 2009. Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. Bmc Microbiology 9.

115. Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, Appelmelk BJ, Bitter W. 2007. Type VII secretion--mycobacteria show the way. Nature Reviews in Microbiology 5:883-91.

116. Desvaux M, Hébraud M, Talon R, Henderson IR. 2009. Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. Trends in Microbiology 17:139-45.

117. Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, Heidelberg JF, Mekalanos JJ. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proceedings of the National Academy of Sciences of the United States of America 103:1528-1533.

118. Bingle LEH, Bailey CM, Pallen MJ. 2008. Type VI secretion: a beginner's guide. Current Opinion in Microbiology 11:3-8.

119. Navarro-Garcia F, Ruiz-Perez F, Cataldi Á, Larzábal M. 2019. Type VI Secretion System in Pathogenic *Escherichia coli*: Structure, Role in Virulence, and Acquisition. Front Microbiol 10:1965.

120. Filloux A, Hachani A, Bleves S. 2008. The bacterial type VI secretion machine: yet another player for protein transport across membranes. Microbiology-Sgm 154:1570-1583.

121. Schell MA, Ulrich RL, Ribot WJ, Brueggemann EE, Hines HB, Chen D, Lipscomb L, Kim HS, Mrázek J, Nierman WC, Deshazer D. 2007. Type VI secretion is a major virulence determinant in *Burkholderia mallei*. Molecular Microbiology 64 1466-85.

122. Zheng J, Leung KY. 2007. Dissection of a type VI secretion system in *Edwardsiella tarda*. Molecular Microbiology 66:1192-206. .

123. Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, Gifford CA, Goodman AL, Joachimiak G, Ordoñez CL, Lory S, Walz T, Joachimiak A, Mekalanos JJ. 2006. A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. Science 312:1526-30.

124. MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S. 2010. The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. Proc Natl Acad Sci U S A 107:19520–19524.

125. Decoin V, Barbey C, Bergeau D, Latour X, Feuilloley MGJ, Orange N, Merieau A. 2014. A Type VI Secretion System Is Involved in *Pseudomonas fluorescens* Bacterial Competition. PLoS One 9:e89411.

126. Ma J, Sun M, Bao Y, Pan Z, Zhang W, Lu C, Yao H. 2013. Genetic diversity and features analysis of type VI secretion systems loci in avian pathogenic *Escherichia coli* by wide genomic scanning. Infect Genet Evol 20:454-64.

127. Ma J, Bao Y, Sun M, Dong W, Pan Z, Zhang W, Lu C, Yao H. 2014. Two functional type VI secretion systems in avian pathogenic *Escherichia coli* are involved in different pathogenic pathways. Infect Immun 82:3867-79.

128. Wang S, Dai J, Meng Q, Han X, Han Y, Zhao Y, Yang D, Ding C, Yu S. 2014. DotU expression is highly induced during in vivo infection and responsible for virulence and Hcp1 secretion in avian pathogenic *Escherichia coli*. Front Microbiol 5:588.

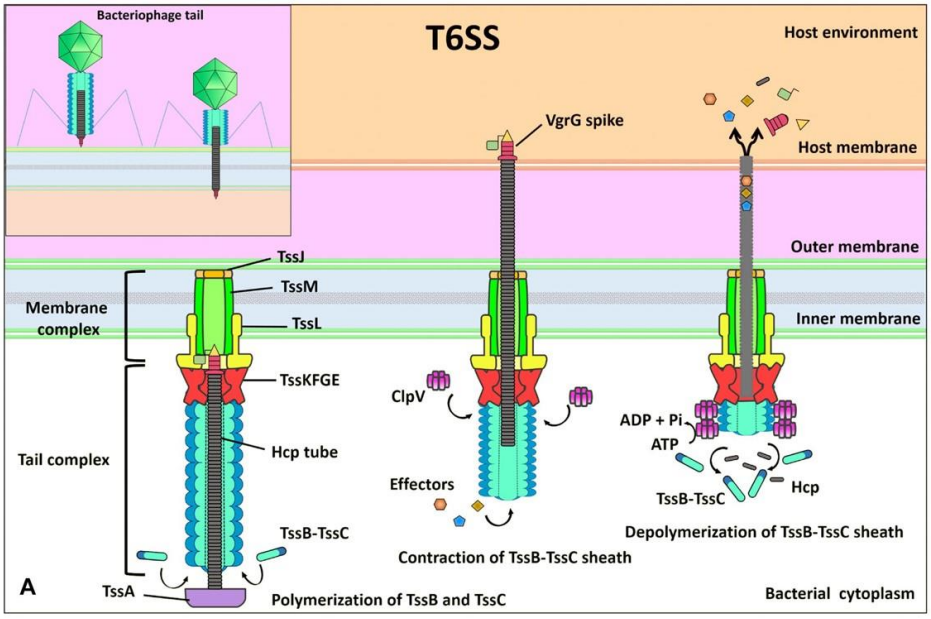

Figure 2.1. The T6SS is composed of a membrane complex, a baseplate and a tail complex. TssJ-TssL-TssM makes the membrane complex. The membrane complex is connected to the TssB-TssC tail complex and to the Hcp inner tube through the baseplate (TssK, TssE, and VgrG). Effectors are recruited to the spike–tube complex through the extension domains of VgrG and/or PAAR-repeat proteins and through incorporation into Hcp tube. An unknown extracellular signal triggers sheath contraction, which leads to the ejection of the spike–tube complex across the target membrane, thereby delivering effector proteins into the cell. The ATPase ClpV disassembles the contracted TssB–TssC sheath, which enables a new T6SS complex to be reassembled from the released subunits. Figure extracted from Navarro Garcia *et al*., 2019.

CHAPTER 3

CHARACTERIZATION OF AVIAN PATHOGENIC *Escherichia coli* (APEC) ASSOCIATED WITH TURKEY CELLULITIS IN IOWA[1]

[1] De Oliveira, A.L., D.M. Newman, Y. Sato, A. Noel, B. Rauk, L.K. Nolan, N.L. Barbieri, and C.M. Logue. 2020. Frontiers in Veterinary Science. 7:380. Reprinted here with permission of the publisher.

Abstract

Turkey cellulitis, also known as clostridial dermatitis is a significant cause of morbidity, mortality, and carcass condemnation at slaughter resulting in considerable losses for turkey producers. Here, we assessed the potential role of Avian Pathogenic *Escherichia coli* (APEC) in a cellulitis outbreak on a turkey farm in Iowa. Birds from one farm with a history of cellulitis and one farm with no history of disease (for comparison) were followed from the age of 10 weeks (before the outbreak) to 18 weeks (just prior to slaughter). *E. coli* recovered from the litter, from skin lesions of birds with cellulitis, and from systemic lesions of birds submitted for necropsy, were assessed. A total of 333 isolates were analyzed and screened for virulence-associated genes, antimicrobial resistance genes including heavy metal resistance, adhesins, invasins, and protectins, iron acquisition systems and their phylogenetic group through multiplex PCR. In addition, PCR was used to serogroup the isolates, and pulsed field gel electrophoresis (PFGE) was used to analyze a subset of strains from the farm environment (1) and birds at 17 and 18 weeks of age when the cellulitis infection appeared to peak. Overall, *E. coli* isolates recovered from cellulitis lesions and systemic infection were identified as APEC, while a lower prevalence of *E. coli* recovered from the litter met the criteria of APEC-like. Direct comparison of *E. coli* isolates from the litter, lesions, and systemic strains using PFGE failed to find identical clones across all three sources reflecting the diversity of strains present in the poultry environment causing disease. This study highlights the role of APEC in turkey cellulitis and should not be overlooked as a significant contributor to the disease in turkeys.

Keywords: *Escherichia coli*, APEC, turkey cellulitis, characterization, poultry

Introduction

The United States produced 245.2 million turkeys in 2017 and an estimated 244.75 million for 2018 (2), resulting in an industry that generates 4–4.5 billion dollars in value. The State of Iowa occupies seventh position in production ranks with 11.9 million birds produced in 2018 representing 6.8% of the total national production (2).

Colibacillosis is considered one of the leading bacterial causes of economic loss in the turkey industry worldwide (3). Turkey cellulitis (TC) is also among the top health issues in turkey producers nationally, ranking as #3 in 2019 according to the United States Animal Health Association turkey industry survey (4). Avian pathogenic *Escherichia coli* (APEC) is one of the pathogens implicated in the disease after *Clostridium* spp. Cellulitis (also known as clostridial dermatitis) is characterized by locally extensive inflammation of subcutaneous tissues of the inguinal, tail, and/or breast regions, often striking production toms at or near market age resulting in increased mortality and carcass condemnation at slaughter, leading to multi-million dollar losses for the turkey industry (5-7). Information on the role of *E. coli* in cellulitis-associated disease in turkeys is relatively limited as most often clostridia species in particular *Cl. septicum*, *Cl. perfringens*, *Cl. sordelli* have been implicated as the etiologic agents (6, 8). In addition, other microorganisms including *Streptococcus* spp. and *Staphylococcus aureus* have also been identified as potential agents of disease (6, 9, 10). Of significant interest to the current study is the view that *E. coli* is an infrequent cause of cellulitis in turkeys (10). Damage to tissues as a result of cellulitis often have their origin as a result of a trauma or injury to the bird and studies have demonstrated the importance of quality litter that is less likely to cause trauma as well as the microbiome of the litter which has the potential to affect skin health in injured or compromised birds.

Also of importance is the potential for the environment to harbor clostridia spores, which as a pathogen can contribute to the tissue destruction resulting in similar tissue damage (6). In an effort to better understand the role of *E. coli* in cellulitis-associated disease in turkeys we assessed the quality of litter, lesions from birds and systemic isolates recovered from infected birds to assess the potential role of APEC in turkey cellulitis.

Materials and methods

Sample Collection – Farms

Weekly litter samples and swabs of cellulitis lesions and birds with cellulitis were collected during the course of this study. Convenience sampling was conducted based on producer observation of infection in birds rather than sample size calculation since infection could not be predicted. Three barns were visited on a weekly basis for sample collection. Two of the barns had no history of cellulitis-associated disease (control barns) in the past 12 months (Barns A1 and B1), and the third barn had a cellulitis outbreak (case barn, Barn A2). A case farm was defined as a farm in which at least 2/3 of the flocks placed were affected with cellulitis during the previous 12 months, and an outbreak was defined when mortality due to cellulitis exceeds 0.5 per 1,000 birds for 2 consecutive days. Barn A1 was visited on eight separate occasions, starting when the turkeys were 10 weeks of age, and ending when they were 17 weeks old (preslaughter). Barn A2 was visited seven times, staring when the turkeys were 12 weeks old, and ending when they were 18 weeks (pre-slaughter). Barn B1 was visited six times, beginning at 12 weeks of age and ending at 17 weeks old (pre-slaughter). Upon arrival at each respective facility, weekly mortality and any antimicrobial treatments were noted. Each barn was divided into four quadrants, divided by fans or other markers present in the facility. A trowel was used to gather a

sample of litter ~7 cm in diameter and 4 cm deep from a random location in each quadrant. The litter was collected in a sterile whirlpak bag (Whirlpak, Nasco, Fort Atkinson, WI) and placed in an ice chest containing ice packs for transport to the lab for analysis. Four samples of litter (one per quadrant) were collected on each visit date. Common litter components included pine shavings and oat hulls mixed with sand, spread over a base of packed clay or dirt.

Tissue Sampling/Collection

To collect samples of cellulitis lesions, we relied on producer-diagnosed cases of cellulitis in birds found dead upon walking through the barn. The only barn with an outbreak of cellulitis in this study was barn A2, as defined above in sample collection. When mortality was present in cellulitis barns, up to 5 birds per week were harvested, and necropsies were performed in the field and at Iowa State University's Veterinary Diagnostic Laboratory (ISU-VDL). From a total of 11 birds, 35 lesion samples were taken using sterile cotton swabs. Swabs of the subcutis, where cellulitis lesions occur, were taken from areas of crepitus and blistering, usually on the breast, and from areas of fluid accumulation, edema, and inflammation, typically near the thigh and ventral to the inguinal region. Before any incisions were made into the affected tissue, the area was flame sterilized to avoid cross contamination. Then, using flame sterilized instruments, the skin was incised to gain access to the subcutis, a swab was inserted, and the area swabbed to include the affected tissue, the swab was then placed in a sterile Cary Blair transport media (BBL, Becton Dickinson, Franklin, NJ) tube for processing in the diagnostic microbiology lab.

Sample Processing

To process the litter sample, the procedure described by Lu et al. (11) was adapted to fit the facilities and testing procedures available for this study. Each quadrant sample was processed individually, allowing for a better profile of the barn than pooling the samples. The quadrant litter sample was mixed thoroughly, and a 2.5 g sub-sample was placed into a clean glass tube containing 15 mL of phosphate buffered saline (litter wash) (PBS, Research Products International Corp., Mt Prospect, IL). The sample was vortex mixed on the highest setting for ~5–10 s and allowed to stand briefly before pipetting 2 mL of solution into each of two, 2 mL centrifuge tubes, one for analysis and one for preservation. The tubes were centrifuged at 100 × g for 5 min to pellet large debris. The supernatant was decanted into a new tube and centrifuged at 12,000 × g for 5 min to pellet all bacteria in the solution. The supernatant from that tube was discarded, and the remaining pellet was re-suspended in 1 mL of PBS. Ten microliter of that solution was streaked onto a MacConkey agar plate using an inoculating loop and incubated overnight at 37◦C. In addition, the remaining litter wash (13 ml) was incubated for 18 h at 37◦C and struck (10 µL) to MacConkey agar as a mean to pick up additional samples that may not have been positive on initial testing from, the litter wash directly. To process the lesion sample, 2 mL of PBS was added to each tube containing a swab. The tube was closed and mixed by vortex at the highest setting. A 10 µL portion of the solution was spread on one half of a MacConkey agar plate using an inoculating loop, and the same swab was touched to the other half of the plate and spread evenly using the same inoculating loop. The plate was then incubated at 37◦C overnight.

Tissue analysis

Sections of liver, spleen, skin, and muscle tissue were submitted to ISU's VDL for microbial analysis. From those submissions, a total of 39 *E. coli* isolates were recovered, with identity confirmed by MALDI-TOF (Bruker, Billerica, MA). They were re-struck from pure cultures onto MacConkey plates and incubated overnight at 37◦C for further analysis as described below.

***E. coli* analysis**

After the plates of each sample type had grown, colonies were selected for testing. From each litter sample, three colonies were selected, totaling 12 isolates per barn per week. From each affected bird, a total of five colonies were selected from all of the swabs taken from that bird. Since the goal of this study was to identify APEC in both sample types, after overnight growth, colonies were selected for testing based on qualitative characteristics commonly found on known *E. coli* colonies. Lactose positive, dimpled, and circular colonies were chosen over lactose negative, irregularly shaped colonies. When there were not three colonies fitting that description, lactose positive colonies representative of the growth on the plate were selected. A total of 333 *E. coli* isolates (240 litter, 54 cellulitis, and 39 systemic), were isolated from all samples collected and analyzed with regard to virulence-associated genes profile (including antimicrobial resistance genes, heavy metal resistance genes, cell surface structures, iron acquisition systems), phylogenetic group, and serogroup through multiplex PCR.

DNA extraction

Bacterial DNA was obtained from whole organisms using the boil prep method. Briefly, isolates were grown at 37◦C overnight on LB agar. Next, an isolated colony was

inoculated into 1 ml of LB broth and grown overnight at 37◦C. Cultures were centrifuged at 12,000 rpm for 3 min, the supernatant was discarded and the cells were re-suspended in 200 µL of molecular-grade water and boiled 100◦C for 10 min, allowed to cool and centrifuged at 12,000 rpm for 3 min to precipitate cellular debris; 150 µL of the supernatant was transferred to a new tube and used as DNA template for gene amplification. The DNA extracts were stored at −20◦C until use.

***E. coli* confirmation**

To confirm the selected colonies were *E. coli*, isolates were screened by PCR of the 16S DNA as described previously by Lamprecht *et al*. (12). Amplification was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with the following parameters: 94◦C for 3 min; 35 cycles of 94◦C for 30 s, 60◦C for 30 s, 72◦C for 1 min; followed by a final extension of 72◦C for 10 min. PCR products were subjected to horizontal gel electrophoresis in a 2% agarose gel (LE Agarose, Lonza, Alpharetta, GA) at 100 V for 70 min. A Hi-Lo molecular weight marker (100 bp; Minnesota Molecular, Minneapolis, MN) and negative (sterile water) and positive controls from our lab collections were included on the gel for comparison and confirmation purposes. After electrophoresis, the gel was stained in 0.25% Ethidium Bromide solution (Sigma Aldrich. St. Louis, MO) for 20 min and viewed under UV light using an Omega Lum G imager (Aplegen, San Francisco, CA). Isolates were recorded as positive or negative for the 16S.

APEC minimal predictors

Screening of *E. coli* for detection of genes that define the APEC pathotype was performed by genotyping the isolates for *iroN*, *ompT*, *hlyF*, *iss*, and *iutA*, defined as minimal APEC predictors by Johnson et al. (13). Isolates with three or more of these genes

were classified as APEC if isolated from a lesion of disease (14) or APEC-like when recovered from non-disease samples (litter, feces etc.). Additional analysis included screening for 64 virulence and antimicrobial resistance-associated genes, phylogenetic analysis and PCR-based O-typing of 240 litter, 54 cellulitis and 39 systemic isolates.

Multiplex PCRs

The presence of genes encoding virulence factors was investigated through multiplex polymerase chain reaction (PCR) amplification as previously described (15, 16) Nine multiplex PCR panels were developed to detect 64 virulence genes. Primer sequences and gene definitions are shown in Table 3.1. Reactions were performed in 25 µl volume containing 2.5 µl of 10x PCR buffer, 0.4 µl 50 mM MgCl2, 1.25 µl dNTP (10µM) Pool, 2 U Taq DNA polymerase, 0.075 µl (200µM) of each primer and 2 µl of DNA sample. The conditions for the reactions were as follows, except for the annealing temperature that was adjusted according to the multiplex: 94◦C for 5 min; 30 cycles of 94◦C for 30 s, 63◦C for 30 s, 68◦C for 10 min, and a final extension step of 72◦C for 10 min. Annealing temperatures were as follows: 63◦C for multiplex 1, 2, 3, 4, 5, and 17; 60◦C for multiplex 6 and 12; and 58◦C for multiplex 8.

PCR-based phylogenetic classification

The phylogenetic group of the isolates was determined according to the *E. coli* phylogenetic typing method described by Clermont et al. (17), which assigns *E. coli* strains to the phylogenetic groups A, B1, B2, C, D, E, and F. First, a quadruplex PCR was performed for the genes *chuA*, *yjaA*, and *arpA*, and the DNA fragment TSPE4.C2. Depending on the band pattern of the isolate, it was classified as A or C, D or E, B1, C, F and B2 or E clades. If an isolate showed a band pattern that could classify it as both A or

C, or both 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). The reactions were carried out in an Eppendorf Mastercyler EP gradient 96 well block in a final volume of 25 µL containing 2.5 µl of 10x PCR buffer, 0.4 µl 50 mM MgCl2, 1.25 µl dNTP (10µM) Pool, 2 U Taq DNA polymerase, 0.075 µl (200µM) of each primer and 2 µl of DNA sample. The conditions for the reactions were as follows: 94◦C for 5 min; 30 cycles of 94◦C for 30 s, 63◦C for 30 s, 68◦C for 10 min, and a final extension step of 72◦C for 10 min.

PCR-based *E. coli* serogrouping

PCR analysis was used to screen for the most common APEC serogroups, using primers and conditions described by Iguchi *et al*. (18) with minor modifications in annealing time/temperature to accommodate the melting temperatures of the primers used.

The reactions were carried out in an Eppendorf Mastercyler EP gradient 96 well block in a final volume of 25 µL containing 2.5 µl of 10x PCR buffer, 0.4 µl 50 mM MgCl2, 1.25 µl dNTP (10µM) Pool, 2 U Taq DNA polymerase, 0.075 µl (200µM) of each primer and 2 µl of DNA sample. The conditions for the reactions were as follows: 94◦C for 5 min; 30 cycles of 94◦C for 30 s, 58◦C for 30 s, 72◦C for 2 min, and a final extension step of 72◦C for 10 min.

Pulse Field Gel Electrophoresis (PFGE)

PFGE was carried out on a select group of isolates from barn A2, the outbreak barn (weeks 17 and 18) from litter, cellulitis lesions and systemic isolates recovered from the organs of birds at necropsy. Isolates were analyzed using the method described by Ribot *et al*. (19, 20). Preparation, lysis, washing of plugs, and *XbaI* restriction were performed according to the PulseNet protocol using a CHEF mapper XA system (BioRad, Hercules,

CA). *Salmonella* Braenderup H9812 was used as the size standard. Macrorestriction patterns generated were compared using the BioNumerics Fingerprinting software (Ver 7.6, Applied Math, Austin, TX). The similarity index was calculated using the Dice coefficient, with a band position tolerance of 1% and an optimization of 0.5%. The unweighted-pair group (UPGMA) method was used to construct the dendrogram as previously described (20).

Statistical analysis

Statistical analysis was used to assess the relationship between mortality in the case and control barns compared against the number of samples collected in these barns where APEC-like strains were detected. The non-parametric Wilcoxian-matchedpair signed rank test was applied to allow comparison between data over the total samples and intervals and for individual sampling days i.e., paired data. For the analysis of virulence and resistance genes harbored by strains examined in the study the number of genes were treated as quantitative variables and the data was analyzed using nonparametric tests also due to asymmetry in the distribution of these genes. Direct comparisons (where possible) between two groups were made using the Mann-Whitney U test. All statistical analysis was performed using GraphPad Prism (Version 7.0d) for MAC OS X (GraphPad, La Jolla, CA). Statistical significance was accepted when $p<0.05$.

Results

Prevalence of APEC Genes and Mortality Rate

To assess whether the quality of the production environment (1) affected the mortality rate of the birds, we analyzed the relationship between the presence of APEC-like strains (strains harboring at least three of the 5 minimal APEC predictors) in the litter

and the mortality rate. In barn A2 flock mortality sharply increased from 1.3 to 2.7% between weeks 16 and 17 which correlated with a spike in APEC-like isolate prevalence in litter samples, which reached 88% at week 17. The prevalence of APEC-like isolates detected in the litter appeared to peak at weeks 14-16 before the mortality rate peaked (Figure 3.1A). For the two control barns assessed (Figures 3.1 B, C), mortality was relatively constant, hovering around 0.25–0.9 between weeks 12 and 17, with no significant peaks. Highest APEC-like prevalence was >80% at week 16 (Figure 3.1 B). Mortality for barn B1 hovered around 0.25% with APEC-like strain detection of 30–55% among litter samples.

When compared overall, there were no significant differences for APEC detection or mortality for all three barns; in contrast, when compared across the barns (A2 vs. A1) significant differences in APEC prevalence were noted at weeks 12 and 13 only ($p<0.05$). Similarly, when mortality rates at the case and control barns were compared (A2 vs. A1), significant differences in mortality were noted overall ($p<0.05$). The same observation was noted when the case barn was compared to the second control barn (A2 vs. B1) with mortality being significantly greater in the case barn ($p<0.05$) and across all time intervals compared ($p<0.05$).

Prevalence of Antimicrobial Resistance Genes

Genes encoding resistance to protein synthesis inhibitors tetracycline (*tetA*, *tetB*), aminoglycosides, gentamicin [*aph(3)IA*] and *aac3VI*, spectinomycin and streptomycin (*aadA*), cell wall synthesis inhibitor ampicillin (*blaTEM*), DNA synthesis inhibitor trimethoprim (*drf17*), folate synthesis inhibitor sulfonamide (*sulI*), and multidrug resistance (*qacEΔ1*) were included in the analysis (Figure 3.2 A). Genes associated with

resistance to tetracycline A (*tetA*), gentamicin [*aph(3)IA*], or ampicillin (*blaTEM*) were not detected in systemic isolates; while these isolates showed high prevalence of genes associated with sulfa (36%), spectinomycin and streptomycin (46%), *qacEΔ1* (54%). Among the cellulitis isolates, highest frequencies of resistance genes were found for *aph(3)IA* and *blaTEM* (24%), *qacEΔ1* (26%), and sulfa (31%). Among litter from control barns (A1 and B1) isolates, high frequencies were noted for *tetA* (43%), *tetB* (33%; 73%), *blaTEM* (23%), and *aph(3)IA* (22%). While a high frequency of *tetB* (44%) and *blaTEM* (41%) were found in isolates from case barn A2 (Figure 3.2 A).

Isolates were separated out for statistical analysis by source: litter in barn A1 and litter in barn B1 (control barns), and litter in barn A2 (case barn), cellulitis and systemic isolates. When compared by origin, the prevalence of *blaTEM* was significantly higher in litter from the case barn (A2) when compared to both A1 and B1 (control barns). The prevalence of *sulI* was significantly higher ($p<0.05$) in A2 (case barn) than in A1 (control). *tetA* frequency was significantly higher ($p<0.05$) in A2 than A1, but not when A2 was compared to B1 (second control barn). Surprisingly *tetB* and *aph(3)IA* were significantly higher ($p<0.05$) in either of the control barns (see Supplementary Table 3.2).

Comparing cellulitis vs. systemic isolates, *tetA*, *tetB*, *aph(3)IA*, and *blaTEM* prevalences were significantly higher in cellulitis isolates, while *aadA* and *qacEΔ1* were significantly higher in systemic isolates.

Statistical analysis was also used to compare cellulitis isolates and systemic isolates with isolates recovered from litter of barns A1, B1, and A2. The frequencies of *tetA* and *tetB* were significantly higher ($p<0.05$) in litter isolates than in both cellulitis and systemic isolates. Similarly, detection of *sulI* was higher in systemic than in control barns litters (A1

and B1) but not in case barn (A2), and in cellulitis isolates when compared to litter A1 (control). The genes *aadA* and *qacEΔ1* were significantly more prevalent in systemic isolates of *E. coli* than in litter from controls (A1 and B1) and case barn (A2).

Heavy metal Resistance Genes

Isolates were also screened for the presence of genes that encode resistance to heavy metals, including copper (*pcoA*, *pcoD*, *pcoE*), silver (*silP*), tellurite (*terD*, *terF*, *terY3*, *terX*), and arsenicals (21) (Figure 3.2 B). Genes encoding resistance to tellurite were not detected in systemic isolates; however, a high prevalence of *arsC* (72–76%), *pcoAD* (26–56%) and *silP* (28–31%) were detected in both systemic and cellulitis isolates. In Litter A2 isolates, the prevalence of *arsC* (85%), *pcoAD* (50–54%), and *silP* (62%) was higher than those in Litter A1 and Litter B1.

When compared by origin, detection of *pcoADE* was significantly higher in case barn A2 than in both control barns (A1 and B1). *arsC* frequency was significantly higher in A2 than A1 but not B1. *terY* and *terD* frequencies were unexpectedly higher in litter B1 (control) than A2 (case).

When cellulitis and systemic isolates were compared, detection of *terY* and *terD* was significantly higher in cellulitis isolates, while *pcoD* was higher in systemic isolates.

We also compared cellulitis and systemic isolates with isolates from the litter of barns A1, B1, and A2 finding *pcoD* prevalence was higher in systemic isolates when compared to control barns but not to the case barn.

Adhesins, Invasins and Protectins

The prevalence of genes encoding adhesins (*papC*, *papEF*, *papGII*, *papGIII*, *fimH*, *sfaS*, *h7*, *bmaE*), invasins (*ibeA*), and protectins, involved in the interaction of APEC with host

cells and in survival in host serum (*kpsMTI* and *kpsMT2*, *traT*, *iss*) was also analyzed (Figure 3.2 C). Among systemic isolates, the adhesion genes *papG* (II and III), *kpsMT* (I and II), and *h7* were not detected. *sfa*, *papC*, and *fimH* were detected 10, 15, and 26% of systemic isolates, respectively. The invasin *ibeA* was found in only 3% of isolates examined. A high prevalence of serum resistance *iss* (66%) and *traT* (82%) was found. With regards to fimbrial subunits, *h7* and *sfaS* were either not detected or detected at a very low prevalence (4–10%). In contrast, *fimH* (the adhesive subunit of Type 1 fimbriae) showed a high prevalence, in litter (63%) and cellulitis (44%) isolates. The overall frequency of genes of the operon *pap* (pyelonephritis-associated pilus) was low, with *papC* being the most frequent and present in only 15% of systemic isolates, 21% of litter isolates and 26% of cellulitis isolates. The invasion gene *ibeA* was found in 2% of systemic isolates, and 6–8% of cellulitis and litter isolates. P adhesin alleles were only found in litter isolates with a frequency of 3%. K1 capsule was absent in systemic isolates, and present in only 2% of litter isolates and 7% of cellulitis isolates. Heme-agglutinin *bmaE* was found in <1% of litter isolates. High frequencies were found for serum resistance genes *traT* (80–82%) and *iss* (40–66%). Genes encoding proteins involved in complement resistance (*traT*) and serum survival (*iss*) were also analyzed. *traT* was detected in 82% of systemic isolates, 80% of cellulitis isolates and 73% of litter isolates. *iss* prevalence was high in all three groups of isolates analyzed: 66% of the systemic, 55% of the cellulitis, and 40% of the litter isolates harbored the gene. When analyzing litter isolates by origin, *traT* was significantly higher ($p<0.05$) in A2 than in B1 but not in A1. Surprisingly, *papG2/3* and *fimH* were significantly ($p<0.05$) more prevalent in litter B1 (control barn) than in A2 (case barn). When cellulitis vs. systemic isolates were compared, the frequency of *papGII* was

significantly (p<0.05) higher in cellulitis isolates, while *sfaS* was significantly higher in systemic isolates. In a comparison between cellulitis and systemic isolates with isolates from litter of barns A1, B1, and A2, *fimH* was higher in litter from all three barns when compared to systemic isolates. *traT* was higher in cellulitis and systemic isolates than in litter from barn B1 (but not A1 and A2). *iss* was significantly higher in systemic than in litter isolates.

Iron Acquisition Systems

The iron-scavenge related genes analyzed in this work included *fyuA*, *iroNEC* (salmochelin), *ireA* the iron-regulated outer membrane protein, aerobactin (*aerJ*), *eitA*, and *eitB* (ABC irontransport system) (Figure 3.2 D). *fyuA* was not detected in any isolate. The most prevalent iron-related genes in systemic, cellulitis, litter A1, and litter B1 isolates were *aerJ* (27–46%), and eitA (31–41%), and *eitB* (31–41%). In litter A2 isolates, *iroN* (28%) had the higher prevalence, followed by *aerJ*, *eitA*, and *eitB* (21%) (Figure 3.2 D).

When litter isolates were analyzed by source, the prevalence of *iroN* was significantly higher in litter A2 (case) than in A1 and B1 (controls). No significant differences were observed between the frequency of the other iron acquisition genes analyzed among litter from different origins.

With regards to cellulitis vs. systemic isolates, *ireA* was found to be significantly higher in cellulitis isolates. No other significant differences were observed in iron acquisition genes between cellulitis and systemic isolates.

In a comparison between cellulitis and systemic isolates and litter isolates of A1, B1, and A2, *aerJ* was more prevalent in cellulitis isolates than in litter isolates from case

(A2) and control barn (A1). Frequencies of *eitA* and *eitB* were higher in systemic than in litter isolates from case barn (A2).

Miscellaneous

Several other virulence genes were also analyzed in this study (Figure 3.2 E). *hlyF* (hemolysin) showed a much higher prevalence (79%) in systemic isolates when compared to cellulitis (27%) and litter (41%). Genes encoding colicins were also analyzed. *cvaC* (structural gene for colicin V) was absent in systemic isolates but detected in 4% of the cellulitis isolates and 18% of the litter-associated isolates. *colB* (colicin B) was detected in 26% of the systemic and cellulitis isolates, 9% of the litter-associated isolates, and *colM* (colicin M) was present in 28% of the systemic, 24% of the cellulitis and 20% of the litter isolates. The presence of the genes encoding chaperone GroEL, integrase IntL, and the transposase IseC12 was also analyzed (Figure 3.2 E).

Additional virulence factors *malX*, *papA*, *cnf* (cytotoxic necrotizing factor), *fyuA* (siderophore), *sfa* (S fimbriae), *hlyD* (alpha-hemolysin transport), *rfc* (replication factor C), *papGI*, *gafD* (fimbrial gene cluster), *cdtB* (cytolethal distending toxin), *focG* (F1C minor fimbrial subunit), *iha* (enterobactin receptor/adhesion), and *afa* (afimbrial adhesion) were not found in any of the isolates examined. When litter isolates were compared by origin, the only gene significantly higher in case vs. control barns was *colM*. In contrast, *cvaC* and *hlyF* was significantly lower in case barn than in both controls. With regards to cellulitis vs. systemic isolates, *hlyF* and *ISEc12* were more prevalent in cellulitis than in systemic isolates. No other differences were found between frequencies of miscellaneous genes between cellulitis and systemic isolates. Comparing miscellaneous genes between systemic and cellulitis isolates and those of litter origin, we found that *hlyF*, and *intI* were

more frequent in systemic than in litter isolates from all barns; *ISEc12* was more prevalent in systemic than in litter from barns A1 and A2. *colM* was higher in systemic and cellulitis isolates when compared to litter isolates from control barns. *etsA* and *etsB* were higher in systemic than in litter from case (A2) and control (B1) barns.

Phylogenetic Groups

PCR-based phylogenetic typing was performed according to the method described by Clermont et al. (18), which assigns *E. coli* strains to groups A, B1, B2, C, D, E, and F, according to the presence of *chuA*, *yjaA* and *arpA*, and the DNA fragment *TSPE4.C2*. Most systemic isolates classified as B2 (31%), A (28%), and C (23%), with the remaining isolates classified as F (15%) and D (3%). The majority of cellulitis isolates were classified as phylogenetic groups A (41%), F (25%), and B2 (22%), with the remaining isolates belonging to phylogenetic groups B1 (8%), and C (4%). Litter isolates were primarily assigned to group A (41%), followed by B1 (33%), with the remaining isolates classified as B2 (10%), D (6%), and F (6%) (Table 3.2). Litter isolates from barn A2 were primarily classified as A (48%), B1 (18%), and B2 (17%) (Table 3.2). Litter samples were divided in two groups based on the presence of the APEC minimal predictors: “APEC-like” (3 or more of the minimal APEC predictors) or non APEC-like (2 or less of the minimal APEC predictors). Regardless of the presence of APEC predictors, most of the isolates were assigned to phylogenetic groups A and B (data not shown).

PCR-based Serogrouping

Using a PCR-based method we were able to determine the O-group of 186 isolates. In summary, the most prevalent serotype among the typed isolates was O24 (46%), followed by O25 (20%) and O8 (16%) (Figure 3.3). When examined by source, 60% (144)

of the litter isolates were typeable, 57% (22) of the cellulitis isolates and 28% (12) of the systemic isolates. O-types O24, O25 and O8 were the most frequently detected serogroup in all three groups of isolates (Table 3.3 and Figure 3.3).

PFGE Analysis

Figure 3.4 shows the PFGE profiles and dendrogram generated for 65 *E. coli* isolates: 38 were recovered from the skin and organs of 11 birds at necropsy (indicated by tissue of isolation); 17 isolates recovered from cellulitis lesions and 10 litter isolates collected from the four quadrants in barn A2 at weeks 17 and 18 of age. About 10 other isolates from litter failed to restrict using *XBaI*. At about 55% identity, the data classified PFGE patterns generated into three major clusters with overlaps in the strains found in each cluster. Cluster 1 was dominated by cellulitis strains and also included some systemic strains as indicated by organ of isolation; cluster 2 consisted primarily of litter and systemic strains while cluster 3 consisted primarily of systemic strains and some cellulitis strains. Of note, none of the exact same PFGE patterns were detected across all three sources, however there was a similar pattern detected from two individual birds (bird 6 and 7) where the same strain was recovered from a spleen in one and a liver in the second (isolate numbers 16 and 17). Similar patterns were also noted among cellulitis swabs taken from the same bird but internal cultures from tissues of these birds did not match suggesting that the infection was not exclusive to a single strain and likely was impacted by more than one disease causing strain. Among litter isolates from the same timeframe no matches with the disease strains were found but isolates in cluster 2 showed some clustering of similar patterns that were likely related and consisted of both the disease strains and litter but the PFGE restriction patterns were not identical.

Discussion

Data on modeling the prevalence of APEC in turkey barns and concurrent outbreaks and mortality associated with cellulitis is relatively limited and this study may be one of the first to use such approach in analysis (Figures 3.1 A, B). Of significant importance is the increased prevalence of APEC-like strains in the outbreak barn (Figure 3.1 A) prior to increased mortality i.e., the pathogen prevalence peaked before the disease. While multiple factors contribute to increased mortality in production birds, including the presence of other pathogens, parasites, the immune status of the birds, and other stressors both physical in the bird and the environment, this study does provide evidence of a peak in the prevalence of APEC-like isolates in the litter prior to the mortality peak suggesting that APEC may have played a role in contributing to the disease.

One of the limits of the current study however is that our analysis is based on an outbreak in one barn (A2) with a previous history of cellulitis and despite our best efforts it was not possible to identify any additional barns at the time of the study either on this farm or in the locale. This study does, however, provide novel insight into *E. coli* (APEC) associated with the outbreak and its potential role in the disease and the use of litter as an indicator of the health status of the barn. Further studies are needed to better understand APEC's role in cellulitis, however as this study is based on a naturally occurring outbreak—it is not always possible to predict where and when cases will occur, but where there is a history of outbreaks, continued monitoring is likely warranted. In this study, the occurrence of 64 virulence-associated genes in 333 *E. coli* isolates obtained from litter, cellulitis and systemic lesions from turkeys from two different farms in Iowa were analyzed (Figures 3.2 A–E). Gene screening included genes associated with antimicrobial resistance,

heavy metal resistance, iron acquisition systems, adhesion and invasion proteins and a collection of miscellaneous genes including transposase, integrase, chaperones, and toxins. This study provides a unique comparison of and likely is one on the largest reports assessing virulence genotyping of *E. coli* strains associated with turkey production with a particular focus on a barn associated with a cellulitis outbreak. The highest frequencies of antimicrobial resistance genes were found in systemic isolates and included the multidrug resistance marker (*qacEΔ1*), streptomycin (*aadA*), and sulfonamides (*sulI*). High levels of sulfonamide resistance genes found is in agreement with previous studies of pathogenic and commensal *E. coli* of turkey origin in Italy (23). A high frequency found for the streptomycin resistance (*aadA*) gene in systemic isolates also agrees with a similar study found in a collection of *E. coli* of turkey origin from Italy (24). It has been reported that resistance to streptomycin might be prevalent despite the discontinuance of its use (25). Resistance associated with ampicillin (*blaTEM*) and gentamicin (*aph(3)IA*) were not found in systemic isolates examined in the current study. Among the litter isolates examined, highest frequencies of resistance gene presence were noted for *blaTEM* (28%), tetracyclines A (30%), and B (43%). High resistance against tetracycline in pathogenic and commensal *E. coli* from turkey have been reported (24).

Our collection was also screened for the presence of *intI1*, *dfrA1* and *aadA1*. *intI1* encodes a class 1 integron that are known to play a role in acquisition and dissemination of antimicrobial resistance (26), and class 1 integrons are the most frequently detected and the best characterized, especially in Enterobacteriaceae (27). Previous studies have shown an association between the presence of class 1 integrons and the *aadA1* and *dfrA* resistance cassettes (24). Here, we found that 34 (58%) out of 58 isolates that harbored *intI1* gene

were also positive for *aadA*, which provides additional evidence for the relationship between class 1 integrons and resistance to aminoglycosides.

Previous work on virulence genotyping in *E. coli* from turkey have focused on genes that are significantly associated with highly pathogenic APEC strains, including *iutA*, *hlyF*, *iss*, *iroN* and *ompT*, *iucD*, *fyuA*, *irp-2*, *tsh*, *fimC* and *papC*, *sit A*, *cvi/cva* (23, 28, 29). Consequently, there is limited information on the frequency of most of the genes analyzed in this study in *E. coli* of turkey origin as a whole and this paper provides some new insight into virulence associated with cellulitis *E. coli* of turkeys.

The prevalence of three of the five genes determined as minimal predictors of APEC: the increased serum survival *iss*, hemolysin *hlyF* and the ferric aerobactin receptor *iutA* found in this work is comparable to the similar reports from previous studies. *iss* frequencies found were 40% in litter, 55% in cellulitis, and 66% in systemic isolates. This data agrees with previous work analyzing isolates from turkeys with colibacillosis from Brazil, where *iss* was detected in 93% and 64% of their isolates, respectively (28, 29). Another study analyzing *E. coli* from cecal swabs of healthy turkeys found *iss* in 55% of the isolates (30). *hlyF* was detected in 79% of the systemic isolates analyzed in the current study, compared to 81% found in systemic isolates in Brazil (29). *iutA* was detected in 46% of our systemic isolates compared to 64% in previous work (29).

The prevalence of the other two genes of the APEC pentaplex (13), the outer membrane protein *ompT* and the membrane siderophore receptor *iroN*, was surprisingly low in the systemic isolates analyzed in this work. *ompT* was found in only 8% compared to 81% from previous work (29). *iroN* was found in only 5% of systemic isolates in this work, but have been previously found in 69% (29) and 95% (28) of isolates examined

elsewhere. Reasons for these differences are currently unknown and will warrant further investigation.

Other genes analyzed in this and previous works include *fimH*, *K1*, *papC*, *ibeA*, *cvaC*, and *fyuA*. *fimH* was detected in 63% of litter isolates in this work. Similarly, high frequency of *fimH* (97%) was reported in a collection of *E. coli* from cecal swabs from healthy turkeys in the UK (30). The frequency of *fimH* in litter and cellulitis isolates in this study were 63 and 44%, respectively. The capsule K1 gene was detected in only 2% of litter and 7% of cellulitis isolates, which is in agreement with the low prevalence (4%) found in healthy turkey isolates elsewhere (30). The prevalence of *papC* was 21% in litter and 26% in cellulitis isolates. In systemic isolates the prevalence of *papC* was 15%, which was relatively similar to systemic isolates from turkeys with airsacculitis in Brazil (28). In contrast, the presence of *papC* in fecal isolates from healthy turkeys in UK was only 3% (30).

cvaC was not detected in any systemic isolate tested in the current study. This was unexpected, as previous studies have detected *cvaC* in 67 and 34% of systemic isolates examined (28, 29). In litter isolates, *cvaC* frequency was 18%, comparable to what was found in fecal isolates from healthy turkeys in the UK (30). *fyuA*, found in 45% of systemic isolates in a study from Brazil (29) was detected in only 1 systemic isolate in the current study. The invasin *ibeA* previously found in 31% of systemic isolates (28) in Brazil, was found in only in 3% of systemic isolates, and 7% of cellulitis and litter isolates in this study.

A surprising number of *E. coli* from all three sources examined could be serogrouped and the most common serogroups implicated included O:24, O:25 and O:8 and O:2 with smaller prevalence associated with serogroups O44, O:86, O:78, O:1, O:117,

O:18, and O23. Among APEC implicated in disease O:1, O:2, and O:78 are most often associated with disease of poultry including turkey (14, 16, 23) and often represent the majority of serogroups present. These serogroups have been reported in outbreaks dating back to the 1960s (22, 31, 32) however, in the current study they represented a small fraction of the isolates examined. More recently, however, newer serogroups including O18 and O111 have been found in *E. coli* isolated from turkeys with hemorrhagic septicemia (33) suggesting there are changes in the dominant serogroups causing disease. Of significant interest however was the high prevalence of O:24 and O:25 serogroups among isolates implicated in disease (both cellulitis lesions and on systemically infected organs) and in the litter. These would appear to be new serogroups that are not frequently implicated in disease and may therefore be emergent. Current data on *E. coli* serogroups implicated in disease of turkeys is, however, relatively limited thus curtailing our ability to perform adequate comparative analysis. Regardless, careful monitoring for changes in serogroups causing disease is warranted as there may be factors responsible for shifts in the serogroups and the selection of new types that are currently unknown.

The phylogenetic analysis of litter isolates found they primarily classified as phylogenetic group A (40%) followed by B1 (33%). Cellulitis isolates were mostly A (41%) followed by F (24%) and B2 (22%), whereas systemic isolates where mostly B2 (31%) followed by A (28%) and C (23%). In previous work on *E. coli* isolated from turkeys with airsacculitis in which the earlier Clermont phylogenetic typing scheme (34) was used that classified strains as A, B1, B2, and D, it was found that 50% of the isolates belonged to group B2, 28% to group B1, 17% to group A and 5% to group D (28).

When source of strains were compared overall it was found that there were different classification of the isolates when source types were compared. For example, almost 80% of the litter isolates were represented by phylogenetic groups A, B1, and F, in each of the barns and this prevalence was similar for cellulitis isolates (90%) and but dropped for systemic isolates to 43%. In contrast, the B2 phylogenetic group was represented in 10–20% of the litter isolates from each barn but was significantly greater among strains from cellulitis (22%) or systemic disease (31%). Assignment to phylogenetic group B2 is considered a trait of ExPEC (17, 34). Isolates of phylogenetic group C represented 23% of the systemic isolates but among all other groups was <5% prevalence. This data does however continue observations of earlier studies of this lab who noted that APEC were represented in the other phylogenetic groups aside from B2 to a greater extent (35), suggesting that APEC do not classify as easily by the Clermont method (17). This data does however also highlight the opportunistic nature of some of these APEC and APEC-like strains especially those of litter toward disease and likely is linked to additional virulence traits carried by these organisms, and litter should not be excluded as a potential source of pathogenic organisms.

PFGE Analysis of strains of APEC and APEC-like strains from the litter, lesions and tissues associated with cellulitis in the outbreak barn (A2) at its peak—i.e., weeks 17 and 18 failed to identify identical patterns across all three sources for strains implicated in disease. This observation was not however unusual as our research group has found this before and its more likely we do not find matching patterns due to the diverse nature of *E. coli* found in the environment and in disease (20) (36). In addition, the sample collection for this analysis is relatively small which may also limit our findings. Of interest however,

were identical patterns found in two different birds both isolated from organs suggesting that there may be some circulating clones causing disease.

Overall Conclusion

Turkey cellulitis continues to be a top health concern in the turkey industry, and despite *Clostridium septicum* being implicated as a major pathogen, a number of opportunistic bacteria including *E. coli* have been implicated. Isolates of *E. coli* (APEC) recovered from cellulitis lesions and systemic infection such as those examined in this study are well-developed pathogens and should not be overlooked when exploring causes of cellulitis in turkeys. Of interest would be to assess the impact and role of APEC in the disease process, which will warrant further research. As the poultry industry trends toward restricted antibiotic use, prevention and control strategies has become increasingly important. Regardless, the role of pathogenic *E. coli* in the disease process should not be overlooked.

Data availability statement

All datasets generated in this study are included in the article/Supplementary Material.

Ethics Statement

The work presented was covered under the Institutional Biosafety Committee (IBC) approval at the University of Georgia and under IBC and IACUC at Iowa State University. Written informed consent for participation was not obtained from the owners because verbal consent was part of a veterinarian visit to assess birds.

Author contributions

AO carried out the research, data analysis, and drafting of the paper. DN performed analysis for genes and co-authored the paper. YS provided assistance in sampling necropsies, analysis of farms and farm visits, and contributed to writing the paper. AN contributed to sampling at the farms and at necropsy, contributed to drafting the paper, and microbial analysis. BR contributed to sampling at the farms and at necropsy. LN provided assistance in drafting the paper and provided supplies for the study. NB provided assistance in testing strains and drafting the paper. CL helped design the study, draft the paper, and provided materials for the study. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by US Poultry and Egg Association (Project #707). The funders had no input on the study design or analysis.

Acknowledgments

Special thanks to US Poultry and Egg for financial support of this study.

Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00380/full#supplementary-material.

REFERENCES

1. Rajashekara G, Glasner JD, Glover DA, Splitter GA. 2004. Comparative Whole-Genome Hybridization Reveals Genomic Islands in *Brucella* Species. Journal of Bacteriology 186:5040-5051.
2. USDA. 2019. Poultry - Production and Value. USDA, Washington DC.
3. Hafez HM. Current Knowledge and Prosepctive Risk Analysis Related to Ongoing Turkey Diseases., p 138-149. *In* (ed),
4. Clark SR, Froebel L. Turkey industry annual report - current health and industry issues facing the US Turkey Industry, p. *In* (ed),
5. Carr D, Shaw D, Halvorson DA, Rings B, Roepke D. 1996. Excessive mortality in market-age turkeys associated with cellulitis. Avian Dis 40:736-41.
6. Clark S, Porter R, McComb B, Lipper R, Olson S, Nohner S, Shivaprasad HL. 2010. Clostridial dermatitis and cellulitis: an emerging disease of turkeys. Avian Dis 54:788-94.
7. Mandal KD, Rath PK. Clostridial Dermatitis: A New Concern among Turkey Farmers, p. *In* (ed),
8. Thachil AJ, McComb B, Andersen MM, Shaw DP, Halvorson DA, Nagaraja KV. 2010. Role of *Clostridium perfringens* and *Clostridium septicum* in causing turkey cellulitis. Avian Dis 54:795-801.
9. Lighty ME, Elvinger F, Evans RD, Sriranganathan N, LeRoith T, Pierson FW. 2016. Incidence of clostridial dermatitis (cellulitis) and factors for development of the disease in turkeys. Journal of Applied Poultry Research 25:104-112.

10. Gornatti-Churria CD, Crispo M, Shivaprasad HL, Uzal FA. 2018. Gangrenous dermatitis in chickens and turkeys. J Vet Diagn Invest 30:188-196.

11. Lu J, Sanchez S, Hofacre C, Maurer JJ, Harmon BG, Lee MD. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. Appl Environ Microbiol 69:901-8.

12. Lamprecht C, Romanis M, Huisamen N, Carinus A, Schoeman N, Sigge GO, Britz TJ. 2014. *Escherichia coli* with virulence factors and multidrug resistance in the Plankenburg River. South African Journal of Science 110:01-06.

13. Johnson TJ, Wannemuehler Y, Doetkott C, Johnson SJ, Rosenberger SC, Nolan LK. 2008. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J Clin Microbiol 46:3987-96.

14. Nolan LK, Barnes HJ, Vaillancourt J-P, Abdul-Aziz T, Logue CM. 2013. Colibacillosis, 13th ed, Ames, IA.

15. Logue CM, Doetkott C, Mangiamele P, Wannemuehler YM, Johnson TJ, Tivendale KA, Li G, Sherwood JS, Nolan LK. 2012. Genotypic and phenotypic traits that distinguish neonatal meningitis-associated *Escherichia coli* from fecal E. coli isolates of healthy human hosts. Appl Environ Microbiol 78:5824-30.

16. Rodriguez-Siek KE, Giddings CW, Doetkott C, Johnson TJ, Fakhr MK, Nolan LK. 2005. Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology 151:2097-110.

17. Clermont O, Christenson JK, Denamur E, Gordon DM. 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep 5:58-65.

18. Iguchi A, Iyoda S, Seto K, Morita-Ishihara T, Scheutz F, Ohnishi M. 2015. *Escherichia coli* O-Genotyping PCR: a Comprehensive and Practical Platform for Molecular O Serogrouping. J Clin Microbiol 53:2427-32.

19. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, Barrett TJ. 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, Salmonella, and Shigella for PulseNet. Foodborne Pathog Dis 3:59-67.

20. Hussein AH, Ghanem IA, Eid AA, Ali MA, Sherwood JS, Li G, Nolan LK, Logue CM. 2013. Molecular and phenotypic characterization of *Escherichia coli* isolated from broiler chicken flocks in Egypt. Avian Dis 57:602-11.

21. Marschall J, Agniel D, Fraser VJ, Doherty J, Warren DK. 2008. Gram-negative bacteraemia in non-ICU patients: factors associated with inadequate antibiotic therapy and impact on outcomes. JAntimicrobChemother 61:1376-1383.

22. Hemsley RV, Barnum DA, Ingram DG. 1967. Biochemical and serological studies of avian strains of *Escherichia coli*. Avian Dis 11:90-7.

23. Giovanardi D, Lupini C, Pesente P, Rossi G, Ortali G, Catelli E. 2013. Characterization and antimicrobial resistance analysis of avian pathogenic *Escherichia coli* isolated from Italian turkey flocks. Poult Sci 92:2661-7.

24. Piccirillo A, Dotto G, Salata C, Giacomelli M. 2013. Absence of class 1 and class 2 integrons among *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry in Italy. J Antimicrob Chemother 68:2683-5.

25. Bass L, Liebert CA, Lee MD, Summers AO, White DG, Thayer SG, Maurer JJ. 1999. Incidence and characterization of integrons, genetic elements mediating

multiple-drug resistance, in avian *Escherichia coli*. Antimicrob Agents Chemother 43:2925-9.

26. Mazel D. 2006. Integrons: agents of bacterial evolution. Nat Rev Microbiol 4:608-20.

27. Partridge SR, Hall RM. 2005. Correctly identifying the streptothricin resistance gene cassette. J Clin Microbiol 43:4298-300.

28. Cunha MPV, de Oliveira MGX, de Oliveira MCV, da Silva KC, Gomes CR, Moreno AM, Kn, #xf6, bl T. 2014. Virulence Profiles, Phylogenetic Background, and Antibiotic Resistance of *Escherichia coli* Isolated from Turkeys with Airsacculitis %J The Scientific World Journal. 2014:8.

29. De Carli S, Ikuta N, Lehmann FK, da Silveira VP, de Melo Predebon G, Fonseca AS, Lunge VR. 2015. Virulence gene content in *Escherichia coli* isolates from poultry flocks with clinical signs of colibacillosis in Brazil. Poult Sci 94:2635-40.

30. Al-Kandari F, Woodward MJ. 2019. Genotypic and phenotypic diversity differences of presumptive commensal and avian pathogenic *E. coli*. Br Poult Sci 60:79-86.

31. Allan BJ, van den Hurk JV, Potter AA. 1993. Characterization of *Escherichia coli* isolated from cases of avian colibacillosis. Can J Vet Res 57:146-51.

32. Altekruse SF, Elvinger F, DebRoy C, Pierson FW, Eifert JD, Sriranganathan N. 2002. Pathogenic and fecal *Escherichia coil* strains from turkeys in a commercial operation. Avian Dis 46:562-9.

33. Olsen RH, Chadfield MS, Christensen JP, Scheutz F, Christensen H, Bisgaard M. 2011. Clonality and virulence traits of *Escherichia coli* associated with haemorrhagic septicaemia in turkeys. Avian Pathol 40:587-95.

34. Clermont O, Bonacorsi S, Bingen E. 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 66:4555-8.

35. Logue CM, Wannemuehler Y, Nicholson BA, Doetkott C, Barbieri NL, Nolan LK. 2017. Comparative Analysis of Phylogenetic Assignment of Human and Avian ExPEC and Fecal Commensal *Escherichia coli* Using the (Previous and Revised) Clermont Phylogenetic Typing Methods and its Impact on Avian Pathogenic *Escherichia coli* (APEC) Classification. Front Microbiol 8:283.

36. Maluta RP, Logue CM, Casas MR, Meng T, Guastalli EA, Rojas TC, Montelli AC, Sadatsune T, de Carvalho Ramos M, Nolan LK, da Silveira WD. 2014. Overlapped sequence types (STs) and serogroups of avian pathogenic (APEC) and human extra-intestinal pathogenic (ExPEC) *Escherichia coli* isolated in Brazil. PLoS One 9:e105016.

37. Johnson JR, Stell AL. 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis 181:261-72.

38. Rodriguez-Siek KE, Giddings CW, Doetkott C, Johnson TJ, Fakhr MK, Nolan LK. 2005. Comparison of *Escherichia coli* Isolates Implicated in Human Urinary Tract Infection and Avian Colibacillosis. Microbiology 151:2097-2110.

39. Johnson TJ, Wannemuehler YM, Nolan LK. 2008. Evolution of the iss gene in *Escherichia coli*. Appl Environ Microbiol 74:2360-9.

40. Clermont O, Bonacorsi S, Bingen E. 2004. Characterization of an anonymous molecular marker strongly linked to *Escherichia coli* strains causing neonatal meningitis. J Clin Microbiol 42:1770-2.

41. Lescat M, Clermont O, Woerther PL, Glodt J, Dion S, Skurnik D, Djossou F, Dupont C, Perroz G, Picard B, Catzeflis F, Andremont A, Denamur E. 2013. Commensal *Escherichia coli* strains in Guiana reveal a high genetic diversity with host-dependant population structure. Environ Microbiol Rep 5:49-57.

Table 3.1. Primer sets and multiplex PCRs used in analysis of APEC.

Gene	Amplicon size (bp)	Primer Sequence (5'-3')	Description	Ref
Multiplex 1				
malX	925	ggacatcctgttacagcgcgca tcgccaccaatcacagccgaac		
papA	717	atggcagtggtgttttggtg cgtcccaccatacgtgctcttc	*pap* operon	(37)
fimH	508	tcgagaacggataagccgtgg gcagtcacctgccctccggta	Type 1 fimbriae	(37)
kpsIII	392	tcctcttgctactattccccct aggcgtatccatccctcctaac	Capsule	(37)
papEF	326	gcaacagcaacgctggttgcatcat agagagagccactcttatacggaca	*pap* operon	(37)
ireA	254	gatgactcagccacgggtaa ccaggactcacctcacgaat	iron acquisition	(38)
ibeA	171	aggcaggtgtgcgccgcgtac tggtgctccggcaaaccatgc	invasin	(37)
Multiplex 2				
cnf-1	1105	atcttatactggatgggatcatcttgg gcagaacgacgttcttcataagtatc	CNF	(37)
fyuA	787	tgattaaccccgcgacgggaa cgcagtaggcacgatgttgta	iron acquisition	(37)
ironEC	667	aagtcaaagcaggggttgcccg gacgccgacattaagacgcag	salmochelin	(38)
bmaE	507	atggcgctaacttgccatgctg agggggacatatagcccccttc	heme-agglutinin	(37)
sfa-foc	410	ctccggagaactgggtgcatcttac cggaggagtaattacaaacctggca	fimbriae	(37)
aerJ	302	ggctggacatcatgggaactgg cgtcgggaacgggtagaatcg	aerobactin	(37)
papGIII	258	ggcctgcaatggatttacctgg ccaccaaatgaccatgccagac	*pap* operon	(37)
Multiplex 3				
hlyD	904	ctccggtacgtgaaaaggac gccctgattactgaagcctg	hemolysin D	(38)
rfc	788	atccatcaggaggggactgga aaccataccaaccaatgcgag	O antigen pol.	
ompT	559	atctagccgaagaaggaggc cccgggtcatagtgttcatc	OM protein	(38)
papGI'	479	ctactatagttcatgctcaggtc cctgcatcctccaccattatcga	*Pap* operon	(37)

Gene	Amplicon size (bp)	Primer Sequence (5'-3')	Description	Ref
papGI	461	tcgtgctcaggtccggaattt tggcatcccccaacattatcg	*Pap* operon	(37)
kpsII	272	gcgcatttgctgatactgttg catccagacgataagcatgagca	capsule	(37)
papC	205	gtggcagtatgagtaatgaccgtta atatcctttctgcagggatgcaata	*pap* operon	(37)
Multiplex 4				
gafD	952	tgttggaccgtctcagggctc tcccggaactcgctgttact	fimbria	(37)
cvaC	679	cacacacaaacgggagctgtt cttcccgcagcatagttccat	*colIV* operon	(37)
fliC	547	acgatgcaggcaacttgacg gggttggtcgttgcagaacc	flagellar gene	(38)
cdtS	430	gaaaataaatggaacacacatgtccg gaaagtaaatggaatataaatgtccg	toxin	(37)
focG	364	cagcacaggcagtggatacga gaatgtcgcctgcccattgct	fimbrial subunit	(37)
traT	290	ggtgtggtgcgatgagcacag cacggttcagccatccctgag	complement^{R}	(37)
papGII	190	gggatgagcgggcctttgat cgggccccaagtaactcg	*pap* operon	(37)
Multiplex 5				
papGI	1140	ctgtaattacggaagtgatttctg ttccagaaatagctcatgtaacccg	*pap* operon	
papGII&III	1070	ctgtaattacggaagtgatttctg actatccggctccggataaaccat	*pap* operon	
iha	829	ctggcggaggctctgagatca tccttaagctcccgcggctga	UPEC island	
afa	594	ggcagagggccggcaacaggc cccgtaacgcgccagcatctc	adhesin Afa	
iss	323	cagcaacccgaaccacttgatg agcattgccagagcggcagaa	serum survival	(39)
sfaS	244	gtggatacgacgattactgtg ccgccagcattccctgtattc	fimbria subunit	(37)
K1	153	tagcaaacgttctattggtgc catccagacgataagcatgagca	capsule	(37)
Multiplex 6				
hlyF	599	ggcgatttaggcattccgatactc acggggtcgctagttaaggag	hemolysin F	
etsB	537	cagcagcgcttcggacaaaatctcct	transport system	

Gene	Amplicon size (bp)	Primer Sequence (5'-3')	Description	Ref
		ttccccaccactctccgttctcaaac		
colM	498	cagcgccattaccccataaatagtga ggttcgttcgccggtgtaagcgttag	colicin M	
etsA	450	caactgggcgggaacgaaatcagga tcagttccgcgctggcaacaacctac	transport system	
colB	430	acaagacagcaccagttatgggtatt gttgttggttttgttggcgtagttat	colicin B	
eitB	380	tgatgccccgccaaactcaaga atgcgccggcctgacataagtgctaa	iron transport	
eitA	284	acgccgggttaatagttgggagatag atcgatagcgtcagcccggaagttag	iron transport	
Multiplex 8				
blaTEM	558	atgtgcgcggaacccctatttgttta aaaaagcggttagctccttcggtcct	ampicillin^{R}	
aac3VI	502	ggcacccgcgacgccctggtccaaaag gggcccggcgccgatcgacaggattt	gentamicin^{R}	
tetB	446	aacgcgtgaagtggttcggttggt ttcgccccatttagtggctattcttc	tetracycline^{R}	
tetA	372	cggggcgactggggcggtagc caaagcgcggccggcacctgt	tetracycline^{R}	
groEL	318	cgccggcatgaacccgatggacctca tcggcctgcatcgactgcgggttgttg	chaperone	
aph(3)IA	278	tcgggcaatcaggtgcgacaatcta tgccagcgcatcaacaatattttcacc	gentamicin^{R}	
drf17	243	atatcccgtggtcagtaaaaggtg gacccccgccagagacata	trimethoprim^{R}	
Multiplex 12				
terX	576	atgcgccgcctgcctgtttaccttgtta cgcgcttgtgctgccggaagaca	tellurite^{R}	
pcoA	507	atccggaaggtcagcaccgtccatagac gacctcgcggatgtcagtggctacacct	copper^{R}	
terF	428	ccgacaaacttccagaagatggggtagt gaggcagcggttgcatttgtacttgacg	tellurite^{R}	
pcoE	385	gtggggcagcttttgctcagtccagtga cgaagctttcttgcctgcgtctgatgtg	copper^{R}	
terY3	302	cctggggccgtcagcggacctg tccttgctggtggccgttcatacttcat	tellurite^{R}	
terD	231	ccactgcgcggaatttccactcaccat acgccgtcccgtctgatgttgacaag	tellurite^{R}	
arsC	153	ccagcctgcggcacctcgcgtaatac	arsenic^{R}	

Gene	Amplicon size (bp)	Primer Sequence (5'-3')	Description	Ref
		acgcagcagcgctcgtactgaaataccc		
Multiplex 17				
silP	603	acaccccggcctgggctcctt	silver^{R}	
		tgcgggcacgggaacaaacctc		
intl1	545	cactccggcaccgccaactttc	integrase	
		gaacgggcatgcggatcagtgag		
pcoD	502	ggcgcccagaatgataatcgcaaca	copper^{R}	
		gggcgtggcgctggctacactt		
sulI	462	cgccgctcttagacgccctgtcc	sulfa^{R}	
		caacggtggcgcccaagaaggat		
iseC12	404	cgcggccacgtaaaccgaaagataaa	transposase	
		gcgcgggtgcacagcaacctc		
aadA	365	taacggcgcagtggcggttttca	aminoglycoside^{R}	
		aagctcgccgcgttgtttcatcaag		
aac3VI	302	gggcaagcgccgcgtcacttatt	gentamicin^{R}	
		cgcggcgttgtttcggcttca		
qacEΔ1	246	tcggcctccgcagcgacttcc	ammonium^{R}	
		cttgccccttccgccgttgtctaat		
Phylogenetic typing				
chuA	288	atggtaccggacgaaccaac		(17)
		tgccgccagtaccaaagaca		(34)
yjaA	211	caaacgtgaagtgtcaggag		(17)
		aatgcgttcctcaacctgtg	Quadruplex PCR	(17)
TspE4.C2	152	cactattcgtaaggtcatcc		(17)
		agtttatcgctgcgggtcgc		(17)
arpA	400	aacgctattcgccagcttgc		(17)
		tctccccataccgtacgcta		(40)
*arpA**	301	gattccatcttgtcaaaatatgcc	Group E	(41)
		gaaaagaaaaagaattcccaagag		(41)
trpA	219	agttttatgcccagtgcgag	Group C	(41)
		tctgcgccggtcacgccc		(41)

Table 3.2. Phylogenetic classification of isolates examined.

	Litter A1		Litter A2		Litter B1		Cellulitis (n=54)		Systemic (n=39)	
Group	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
A	39	42	37	48	19	28	22	41	11	28
B1	27	29	14	18	37	55	4	7	0	0
B2	6	7	13	17	4	6	12	22	12	31
C	4	4	3	4	2	3	2	4	9	23
D	10	11	4	5	1	1	0	0	1	3
E	0	0	1	1	0	0	0	0	0	0
F	6	7	5	6	4	6	13	24	6	15

Table 3.3. O-type distribution according to origin of isolates.

	Litter A1		**Litter A2**		**Litter B1**		**Cellulitis**		**Systemic**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
O1	0	0	2	4	0	0	0	0	1	9
O2	3	5	2	4	3	7	2	6	1	9
O8	12	22	1	2	14	34	2	6	2	18
O17	0	0	0	0	0	0	0	0	0	0
O18	0	0	2	4	0	0	0	0	0	0
O23	0	0	0	0	0	0	0	0	1	9
O24	33	60	18	38	15	37	17	55	3	27
O25	5	9	21	44	3	7	5	16	3	27
O44	0	0	0	0	2	5	4	13	0	0
O78	1	2	2	4	0	0	0	0	0	0
O86	0	0	0	0	4	10	0	0	0	0
O117	1	2	0	0	0	0	1	3	0	0

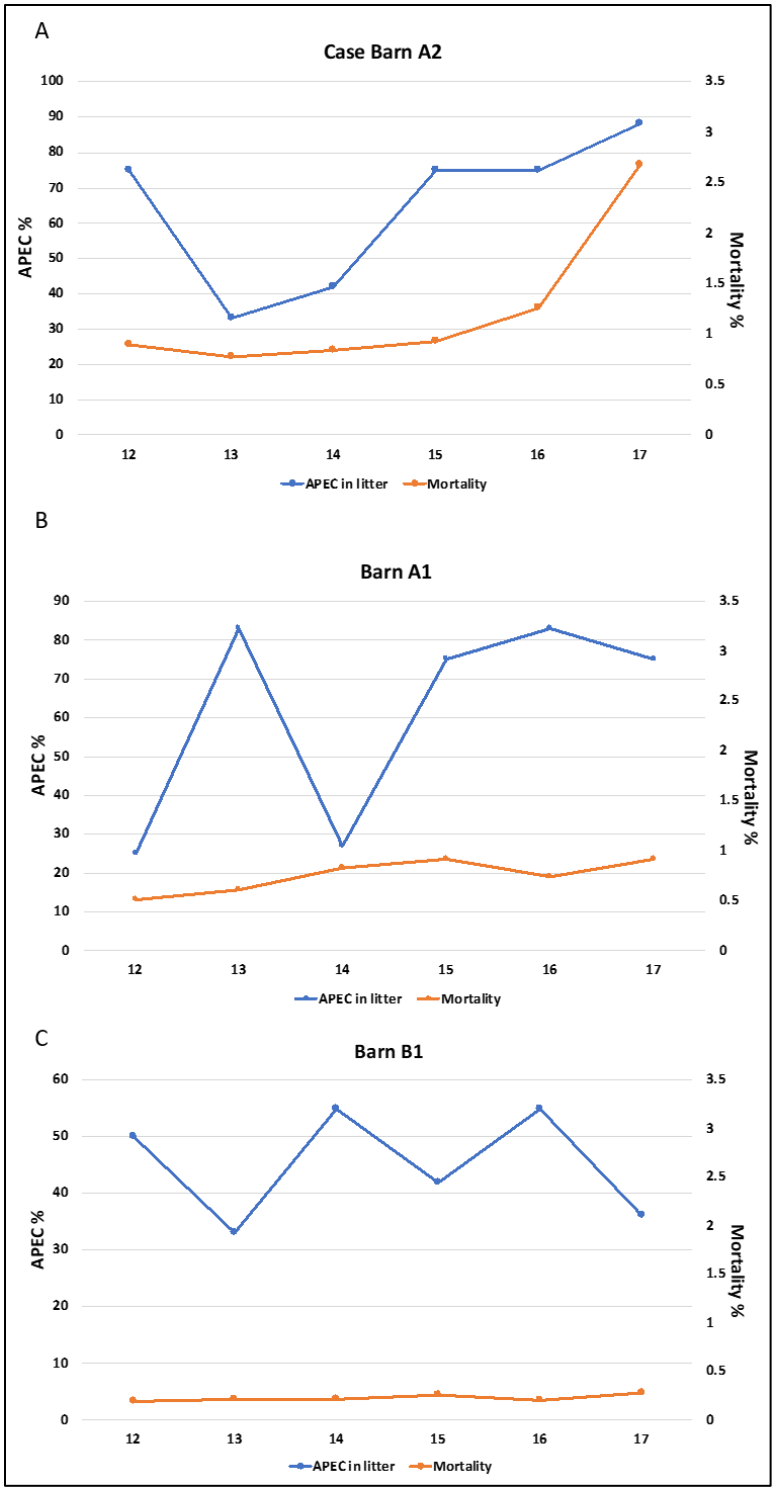

Figure 3.1. (A) Prevalence of APEC-like strains in the litter compared with mortality for the case barn (A2). (B) Prevalence of APEC-like strains in the litter and mortality in control barn A1. (C) Prevalence of APEC-like strains in the litter and mortality in control barn B1.

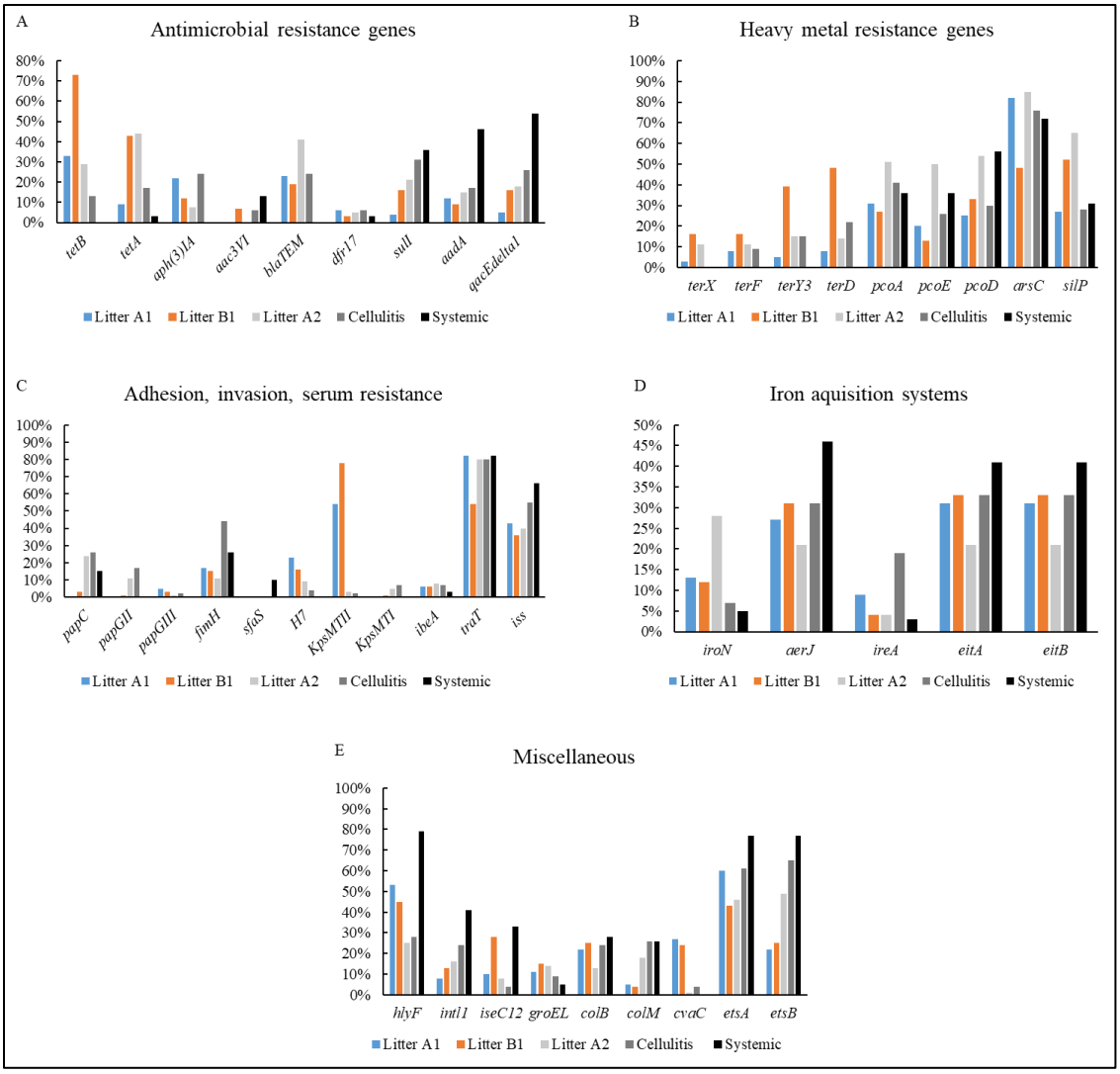

Figure 3.2. (A) Prevalence of APEC-like strains in the litter compared with mortality for the case barn (A2). (B) Prevalence of APEC-like strains in the litter and mortality in control barn A1. (C) Prevalence of APEC-like strains in the litter and mortality in control barn B1.

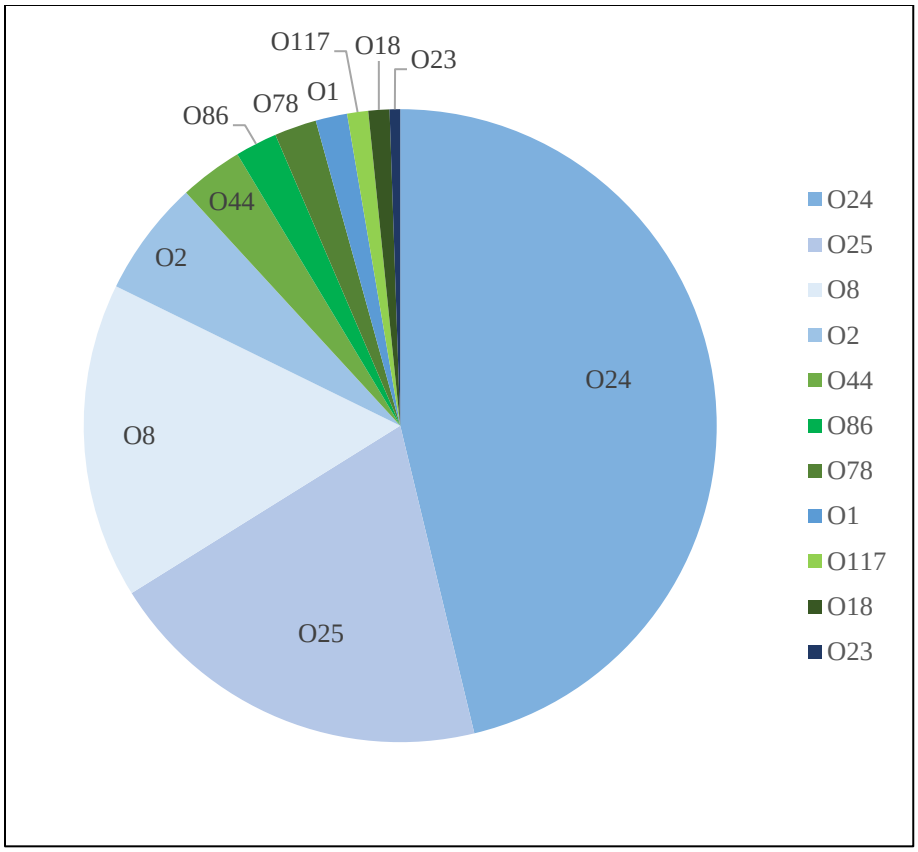

Figure 3.3. PCR based O-typing. Distribution of APEC most common O-types among litter, cellulitis and systemic isolates includes only isolates that were assigned to one of the O-types screened.

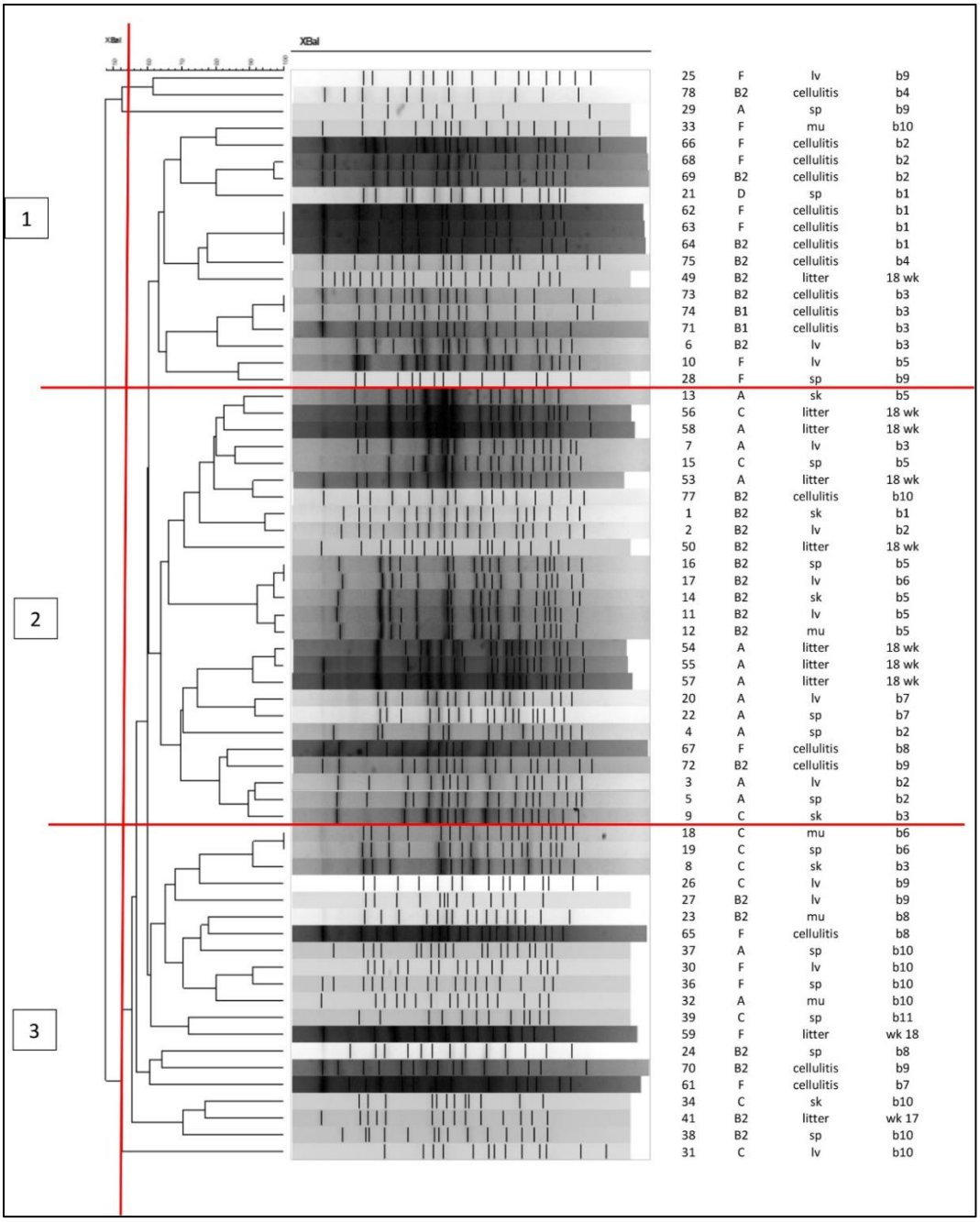

Figure 3.4. PFGE analysis of APEC and APEC-like strains analyzed at 17 and 18 weeks in litter, cellulitis and systemic strains from barn A2. First column shows the isolate ID, column 2 shows the phylogenetic group, column 3 the source of isolate (litter, cellulitis, lv, liver; sp, spleen; sk, skin; mu, muscle), column 4 is the bird identification numbered 1–11 and week litter was collected (wks 17 or 18).

CHAPTER 4

TYPE 6 SECRETION SYSTEM (T6SS) PLAYS A ROLE IN THE VIRULENCE OF NEONATAL MENINGITIS *Escherichia coli* NMEC15[1]

[1]De Oliveira, A.L., N.L. Barbieri, D.M. Newman, M.M Young, and C.M. Logue. To be submitted to Infection and Immunity.

Abstract

Neonatal meningitis *Escherichia coli* (NMEC) is the second most common cause of neonatal sepsis and meningitis with fatality rates of 15-40%. Survivors may present with lifelong neurological sequelae that include cerebral palsy, seizures, hearing loss and delayed development. NMEC strains have a diverse virulence trait repertoire, and a higher average number of virulence factors when compared to human-fecal *E. coli* (HFEC). Despite the identification of virulence factors that contribute to meningitis, the pathogenesis of meningitis remains to be elucidated. The Type 6 secretion system (T6SS), recently identified in *Vibrio cholerae*, has recently gained attention due to its reported role in the infection process and protection of bacteria from host defenses in a number of pathogens. In NMEC strain RS218, T6SS Hcp proteins were shown to play a role in invasion, cytoskeleton rearrangement and apoptosis of human brain microvascular endothelial cells (HBMEC), which constitute the blood-brain barrier (BBB), suggesting that the T6SS is involved in the establishment of meningitis. Here, we analyzed the frequency of T6SS genes *hcp*, *impK*, *evpB*, *vasK* and *icmF* in a collection of NMEC strains and their potential role in virulence associated phenotypes of NMEC 15. We demonstrated that ImpK and IcmF are involved in the adherence of NMEC15 to HBMEC, and Hcp, ImpK and IcmF were involved in invasion of HBMEC. Additionally, deletion of *hcp* affected resistance of NMEC15 to predation by *D. discoideum* and the formation of biofilm. These studies confirm that the T6SS plays a significant role in the virulence of NMEC.

Keywords: T6SS, NMEC, protein secretion, pathogenesis, neonatal meningitis

Introduction

Neonatal meningitis *Escherichia coli* (NMEC) is a distinct pathotype of Extra-intestinal pathogenic *E. coli* (ExPEC) that is able to survive in blood and invade the meninges of newborn infants causing meningitis (1-3). NMEC is the second most common cause of neonatal sepsis and meningitis and is isolated in about 30% of all early-onset infections, characterized by signs of infection and isolation of the organism from the cerebrospinal fluid (CSF) cultures at ≤72 h of life (4). NMEC has also emerged as the most common cause of meningitis and sepsis among very low birth weight infants (<1500 g birth weight) since the 1990s (5-8). Neonatal meningitis caused by NMEC results in high mortality with a case fatality rate of 15-40% (1, 9). Severe, lifelong neurological sequelae that includes cerebral palsy, seizures, hearing loss and delayed development may occur in 12% to 44% of neonatal meningitis survivors (10).

To cause meningitis, NMEC must enter the bloodstream and cross the blood-brain barrier, a tight barrier formed by brain microvascular endothelial cells, enter the central nervous system, and cause inflammation of the meninges and pleocytosis of the CSF (11). Bacteria are acquired perinatally and reach the bloodstream via transcytosis through enterocytes. Survival in blood is crucial as the progression of the disease depends on high bacteremia ($>10^3$ colony-forming units (CFU) per mL of blood) (12).

The virulence trait repertoire of NMEC strains was shown to be diverse, and NMEC strains were shown to have a higher average number of virulence factors when compared to human fecal *E. coli* (HFEC) (13). Recognized virulence factors involved in NMEC pathogenesis include capsule, which provides protection from the host immune responses, and the outer membrane protein A (OmpA), which provides resistance (12). Attachment to

BMEC is mediated by the FimH subunit of Type 1 pili and OmpA (14, 15), and invasion depends on Ibe proteins, FimH, OmpA and cytotoxic necrotizing factor 1 (CNF1) (16). Despite the identification of several *E. coli* determinants that contribute to meningitis, including adhesins, invasins, serum resistance factors, iron acquisition systems and toxins (17, 18), the pathogenesis of meningitis caused by NMEC is not completely understood. Additionally, the role of protein secretion in the pathogenesis of ExPEC, especially NMEC, remains to be elucidated.

Protein secretion is a primary virulence property of bacteria, and six secretion systems have evolved in Gram-negative bacteria, designated Type 1 secretion system through Type 6 secretion system (T1SS-T6SS). These systems either secrete proteins into the extracellular milieu (T1SS, T2SS, T5SS) or directly into target cells (T3SS, T4SS, T6SS) (19). The recently discovered T6SS, first described in *Vibrio cholerae* (20), has gained attention due to its role in a number of bacteria including the human and animal pathogen *Burkholderia mallei*, the fish pathogen *Edwardsiella tarda*, and the human pathogen *Pseudomonas fluorescens.* In each of these pathogens, the T6SS has been associated with the infection process as well as protecting the bacteria from its host defenses (21-23).

The T6SS is composed of 13 conserved proteins that constitute the core of the system and a set of non-conserved proteins with regulatory and accessory functions (24). These components assemble into a membrane complex, a baseplate and a tail-like structure (contractile sheath, inner tube and puncturing spike) (25-27).

The T6SS is described as a membrane spanning machine that functions as a molecular syringe, secreting proteins into the external milieu or straight into the host cells.

The effectors delivered by this system target both eukaryotic and prokaryotic cells, and the effectors it delivers play different roles on the host cell, including cytoskeleton rearrangement, cell invasion, cell disruption, as well as helping the bacteria to evade host defense mechanisms among others (28).

The T6SS is an atypical secretion system in which one protein may play a role as a structural component and secreted effector. This is the case of the better characterized protein in this system, Hcp (Hemolysin-coregulated protein) (29). Hcp is the hallmark of T6SS, and it forms a transportation channel between the inner and outer bacterial membranes. Hcp is also a secreted protein with various roles in different bacteria, being involved in cytotoxicity in *D. discoideum* amoebae and J774 murine macrophages during *V. cholerae* infection (20). Hcp may also play a role in facilitating efficient tumorigenesis in *Agrobacterium tumefasciens* (30), and Hcp1 was detected in the sera of cystic fibrosis patients, and is produced *in vivo* during *Burkholderia mallei* infection in humans and horses (21).

Current knowledge on the role of the T6SS in NMEC pathogenesis is limited to one study that used the prototypic NMEC strain RS218 (*E. coli* K1). RS218 harbors two T6SS clusters (T6SS1 and T6SS2). Previous work reported that T6SS2 core proteins Hcp1 and Hcp2 of RS218 play an important role in invasion, cytoskeleton rearrangement and apoptosis of HBMEC, which constitutes the BBB, suggesting that the T6SS is necessary for breaching the BBB and establishment of meningitis (31). However, the role of T6SS in the pathogenesis of NMEC warrants further investigation. No studies to date have analyzed the role of T6SS1 genes in NMEC.

In this study, we investigated the prevalence of T6SS1 genes in a collection of 86 NMEC and 179 HFEC (fecal *E. coli* isolated from healthy humans) isolates. We also investigated the role of T6SS1 genes *hcp*, *evpB*, *impK*, *vasK*, and *icmF* in the pathogenesis of NMEC15. We determined the role of these genes in the ability of NMEC15 to resist predation by *Dictyostelium discoideum*, form biofilm on abiotic surface, adhere to and invade HBMEC, and secrete T6SS effector protein Hcp with the goal of better understanding the role of T6SS in NMEC pathogenesis.

Materials and methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids are shown in Table 4.1. The WT NMEC15 strain was isolated from the cerebrospinal fluid of a newborn infant (< 28 days old) with meningitis from the Netherlands (32). The O serogroup was identified at the *E. coli* reference center at Pennsylvania State University.

E. coli DH5α was used for cloning. All *E. coli* strains were grown in Luria-Bertani (LB) broth, Miller (BD Difco™, Franklin Lakes, NJ) at 37°C with shaking, unless otherwise specified. The medium was supplemented with ampicillin (Amp 100 µg/mL), chloramphenicol (Cm 10-25 µg/mL) or L-arabinose (6.5 mM) as necessary.

DNA extraction

Bacterial DNA was obtained from whole organisms using the boil prep method. Briefly, isolates were grown at 37°C overnight on LB agar (BD Difco™, Franklin Lakes, NJ). Next, an isolated colony was inoculated into 1 ml of LB broth and grown overnight at 37°C. Cultures were centrifuged at 16,700 x *g* for 3 minutes. The supernatant was discarded, and the cells were re-suspended in 200 µL of nuclease-free water, boiled for 10

minutes, cooled and centrifuged at 16,700 x *g* for 3 minutes to precipitate cellular debris; 150 µL of the supernatant was transferred to a new tube and used as DNA template for gene amplification. The DNA extracts were stored at -20°C until use.

Detection of T6SS genes by PCR

The presence of genes from T6SS *hcp*, *evpB*, *impK*, *vasK*, and *icmF* was analyzed in a collection of 86 NMEC and 179 HFEC by Polymerase chain reaction (PCR) amplification. NMEC strain RS218 was used as template for primer design. Primers were purchased from Sigma (St. Louis, MO). Reactions were performed in 25 µl volume containing 2.5 µl of 10x PCR buffer, 0.4 µl 50 mM $MgCl_2$, 1.25 µl dNTP (10 µM) Pool, 2 U Taq DNA polymerase, 0.075 µl (200 µM) of each primer and 2 µl of DNA sample. The conditions for amplification were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 68°C for 3 min, and a final extension step of 72°C for 10 min. Primers used for screening are shown in Table 4.2.

PCR product analysis

PCR products were subjected to horizontal gel electrophoresis in a 1.5% agarose gel (LE Agarose, Lonza, Alpharetta, GA) at 200 V for 70 min. A Hi-Lo molecular weight marker (100 bp; Minnesota Molecular, Minneapolis, MN) and negative (sterile water) and positive controls from our lab collections were included in the gel for comparison and confirmation purposes. After electrophoresis, the gel was stained in 0.25% ethidium bromide solution (Sigma Aldrich. St. Louis, MO) for 20 min and viewed under UV light using an Omega Lum G imager (Aplegen, San Francisco, CA).

Construction of mutants and complemented strains

Isogenic mutants were constructed using the Lambda-Red recombination system (33). Briefly, oligonucleotides specific to the chloramphenicol cassette flanked by 50 nt extensions homologous to 5'- and 3'-ends of the gene to be deleted were used to amplify the chloramphenicol resistance cassette from plasmid pKD3 (ATCC®, Manassas, VA). The PCR products were run on a 1.5% agarose gel, and gel extraction of the specific fragment was performed using QIAquick Gel Extraction Kit (Qiagen, Germantown, MD). The extracted fragments were electroporated into NMEC 15 containing the lambda-red expression plasmid pKD46 (ATCC®, Manassas, VA). After electroporation, the cells were grown in super optimal broth with catabolite repression (SOC) for 90 min and plated on LB agar containing 25 µg/mL chloramphenicol. Colonies were screened by PCR to identify deletion mutants. The chloramphenicol resistance cassette was cured by transforming helper plasmid pCP20 (ATCC®, Manassas, VA) into the mutants and screening for chloramphenicol sensitive colonies. *In trans* complementation was performed by cloning PCR-amplified genes into the *XbaI* and *HindIII* restriction sites of plasmid pBAD24 (ATCC®, Manassas, VA), and transforming the construct into their mutant counterparts. Primers used are listed in Table 4.2.

Growth curve analysis

The growth of WT NMEC15 and mutant strains in minimal medium M9 was analyzed. Briefly, strains were incubated overnight in LB broth containing chloramphenicol (10 µg/mL) with incubation at 37°C. Next, OD_{600} of the cultures was measured and cultures were diluted to an OD600 of 0.05 in M9 supplemented or not with L-arabinose (Sigma Aldrich, St. Louis, MO) at a final concentration of 0.2%. Cultures were

incubated at 37°C with shaking at 220 rpm, and OD_{600} measurements were obtained every 30 minutes. To measure OD_{600}, 300 µL of the growing culture was dispensed in a well of a 96 well plate, and the absorbance was read using an ELX 808 Ultra microplate reader (Bio-Tek Instruments, Winooski, VT). The experiment was performed with biological replicates and the absorbance data was averaged and plotted against time to build the growth curves. Growth curves were carried out for a total of 7-8 hours.

Analysis of Expression of T6SS genes

RNA extraction

To assess the expression of *hcp*, *evpB*, *impK*, *icmF* and *vasK* genes, NMEC15 was grown overnight at 37°C statically. Next day, the cultures were diluted 1:100 in either LB broth or DMEM:F-12 (ATCC®, Manassas, VA) and incubated at either 37°C or 42°C until exponential phase (OD600 ~0.6). RNA from two biological replicates was extracted using the RiboPure™ RNA purification kit (Ambion, Austin, TX) according to manufacturer's instructions. RNA of NMEC15 was also extracted after 1h and 3h of contact with HBMEC cells to test whether cellular contact induces the expression of T6SS genes. Isolated RNA was treated with DNase 1 according to the manufacturer's instructions to eliminate DNA trace amounts from the eluted RNA. The concentration of RNA samples was determined using a NanoPhotometer® NP80 (Implen, Munchen, Germany), and the samples were stored at -80°C until use.

Synthesis of the 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 DNAase-treated RNA was mixed with

1µL of Random primers (50 µM), 1 µL of 10 mM dNTP mixture, and adjusted to 10 µL with RNase-free water. The mixture was heated at 65 °C for 5 min and chilled on ice for 2 minutes for denaturation. The mixture was then centrifuged, and the cDNA synthesis mix was prepared by adding 4 µL of 5x first-strand buffer, 1 µL of RNase inhibitor, 1 µL of Reverse transcriptase and RNase-free water up to 20 µL. cDNA synthesis reaction was set as follows: 2 min at 25 °C, 50 min at 42 °C, and 15 min at 75 °C. cDNA was diluted 1:20 before use as template for qRT-PCR and stored at -20 °C until use.

qRT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed in a qTower^3 G qPCR System (Analytik Jena, Jena, Germany). Primers for qRT-PCR were purchased from Sigma Aldrich (St. Louis, MO). Reactions were performed using either qPCR Master Mix with Sybr® Green (Goldbio, St. Louis, MO) or Platinum™ SYBR™ Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA). Each reaction was performed in a final volume of 20 µl containing 25 ng cDNA (1 µL diluted cDNA), 1 µL each primer (10 µM), 10 µL 2x qPCR Master Mix Sybr® Green (Goldbio, St. Louis, MO) or 10 µL Platinum™ SYBR™ Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA) and nuclease-free water up to 20 µL. When using Platinum™ SYBR™ Green qPCR SuperMix-UDG. 1 µL of (1:10) ROX Reference Dye was added to each reaction. PCR conditions were as follows: 50 °C for 2 min hold, 95 °C for 2 min hold, and 40 cycles of 95 °C for 15 sec and 60 °C for 30 sec.

Threshold fluorescence was established within the geometric phase of exponential amplification, and the cycle threshold (CT) (34) was determined for each sample RNA. The CT from duplicates was averaged. Expression levels were normalized using the

housekeeping gene 16S rRNA as endogenous control. Melting curve analyses were performed after each reaction to ensure amplification specificity. Differences (nfold) in transcripts were calculated using the relative comparison method (35).

Adherence and invasion assays

Cell adherence and invasion assays were performed as previously described (36). Human brain microvascular endothelial cells (HBMEC) were used in this study and the cell line was cultured in 75 cm^2 cell culture flasks (Corning®, NY) in DMEM-F12 (ATCC®, Manassas, VA) containing 10% fetal bovine serum (FBS; Sigma, MO) and 0.1% endothelial growth supplement (ECGS, Cell Applications, Inc., San Diego, CA) with incubation at 37°C and 5% CO_2. Cells were transferred to sterile 24-well-plates with $1x10^5$ cells/well 48h prior each experiment. Wild-type NMEC15, mutants and complemented strains were grown statically in LB broth overnight. Next, cultures were diluted 1:50 in fresh LB broth and grown at 37°C with shaking at 220 rpm for 2h. Cultures were then induced with 0.2% L-arabinose for 2h. After induction, cultures were washed with PBS and adjusted to $1x10^8$ cells/mL. 10 µL of bacterial suspension was used for infection. HBMEC cells were washed once with PBS and then exposed to bacteria at a multiplicity of infection (MOI) of 10. The 24-well-plates were centrifuged at 500g for 5 min and incubated for 1h for adherence and 4h for invasion.

For the adherence assays, after the 1 h infection, cells were washed four times with 1 mL of PBS and then lysed with 1 mL of 0.1% Triton-X100 for 10 min at room temperature. Serial dilutions of cell suspension were spread onto MacConkey agar plates and incubated at 37°C overnight, and CFU were counted.

For the invasion assays, after 1 h infection, cells were washed four times with sterile PBS to remove unattached cells, and then re-incubated with fresh DMEM:F-12 containing 100 µg/mL gentamicin for 3h. At 4h post infection, cells were washed four times with 1 mL of PBS and then lysed with 1 mL of 0.1% Triton-X100 for 10 min at room temperature. Serial dilutions of cell suspensions were plated onto MacConkey agar and CFU were determined following overnight incubation of plates at 37°C. The input dilution of bacteria was also plated and counted to determine the CFU for each inoculum used in the assay.

To visualize the association between bacteria and HBMEC, 1 x 10^5 cells per well were plated on glass coverslips in 24-well-plates and infected with a MOI of 10 CFU/cell as described above. After 1 or 4 h post-infection, cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and stained with Giemsa for 20 min at room temperature. Samples were then washed with water and observed under a light microscope at 1,000x and photographed.

Plaque assay

Plaque assay was performed as previously described (37) with slight modifications. Overnight cultures of wild type NMEC 15 and mutant strains were diluted 1:50 and grown to exponential phase at 37°C with shaking. Then 0.2% arabinose was added to the cultures of the complemented strains for induction of gene expression, and then incubated for 2 more hours. Bacteria were then pelleted by centrifugation at 5,700 x *g* for 7 min, washed with SorC (16.7 mM Na_2H/KH_2PO_4/50µM $CaCl_2$, pH 6.0) and diluted in SorC to 5x10^7 CFU/mL.

D. discoideum cells were cultured in HL5 axenic medium and collected by centrifugation at 500 x *g* for 5 min, washed once with SorC, resuspended in SorC to 5 x 10^6 cells/mL, and serially diluted in 10-fold increments in SorC. *D. discoideum* dilutions were mixed 1:1 with bacteria to generate bacterium-to-amoeba ratios ranging from 10:1 to 10^6:1. Aliquots of 10 µL of the suspensions were spotted onto SM/5 agar plates and allowed to dry in a laminar hood under a sterile airflow. Plates were incubated at 22°C and examined after 3 and 5 days of incubation for plaque formation by *D. discoideum*, and photographed. Strains were then classified as sensitive or resistant to predation by *D. discoideum* based on the presence or absence of "predation plaques" formed by the amoeba.

Analysis of biofilm formation

To assess the involvement of the T6SS genes in the formation of biofilm by NMEC, mutants and complemented strains were screened for biofilm formation in M63 minimal media as previously described (38). Briefly, strains were incubated overnight in LB broth supplemented or not with arabinose 0.2% (Sigma Aldrich, St. Louis, MO). Next, cultures were diluted in minimal medium M63 with or without arabinose at a final concentration of 0.2% to an OD_{600} of 0.05 in 8 wells of a 96-well plate and incubated at 37°C for 24 hours. Blank wells were filled with M63 only. Cultures were poured out and plates were gently rinsed with de-ionized (DI) water. 200 µL of 0.1% crystal violet was added to each well and plates were incubated at room temperature for 30 min. The plates were then rinsed 4 times with DI water and allowed to dry at room temperature for 1 h. Biofilm was solubilized by adding 200 µL of a 80:20 ethanol:acetone mixture. 150 µL of the

resolubilized biofilm was transferred to a new 96-well plate, and OD_{600} was read using an ELX 808 Ultra microplate reader (Bio-Tek Instruments, Winooski, VT).

Statistical analysis

For the analysis of the prevalence of T6SS genes harbored by strains from different collections examined in the study, the number of genes were treated as quantitative variables and the data was analyzed using non-parametric tests due to asymmetry in the distribution of the genes. Non-parametric analysis was also applied to compare the phenotypes presented by the mutants in comparison to the WT strains. Direct comparisons (where possible) between two groups were made using the Mann-Whitney U test. All statistical analysis was performed using GraphPad Prism (Version 7.0d) for Windows (GraphPad, La Jolla, CA). Statistical significance was accepted when $p \leq 0.05$. For adherence and invasion assays, bacterial groups were compared using Student's *t*-test and differences were considered significant when $p<0.05$.

Results

Prevalence of T6SS genes is significantly higher in NMEC than in HFEC isolates.

To gain insight into the relationship between the presence of T6SS genes and the pathogenicity of NMEC, we used PCR to screen a collection 86 NMEC isolates and 179 HFEC isolates. Prototypic strain NMEC RS218 was used as template for designing the primers. Genome analysis shows that this strain harbors at least two T6SS clusters. Most genes analyzed in this study (*hcp, evpB, impK and vasK*) belong to cluster 1, which is approximately 30-kb in size and contains 24 genes, including T6SS components and hypothetical proteins. *icmF* belongs to cluster 2.

The collections were analyzed for the presence of T6SS genes *evpB* (contractile sheath major subunit), *impK* (membrane complex), *hcp* (secretion tube and effector), *vasK* (membrane complex) and *icmF* (membrane complex; T6SS loci 2, not shown).

The prevalence of all 5 genes was significantly higher ($p<0.05$) in NMEC than in HFEC isolates. Among the NMEC isolates *evpB* was detected in 76%, *impK* in 65%, *hcp* in 78%, *vasK* in 38% and *icmF* in 49% of the isolates. Among HFEC isolates, *evpB* was detected in 28%, *impK* in 41%, *hcp* in 9%, *vasK* in 8% and *icmF* in 25% (Figure 4.1). These findings support our hypothesis that T6SS contributes to the virulence of NMEC.

Mutants in T6SS genes were constructed in NMEC15.

To analyze the role of T6SS genes in the pathogenesis of NMEC strains, isogenic deletion mutants for *evpB, impK, hcp, icmF* and *vasK* were generated using NMEC15 as the parental strain. NMEC15 belongs to O18 serotype and B2 phylogenetic group. Prototypic strain NMEC RS218 was used as template for designing the primers (Figure 4.2).

Deletion of *hcp, evpB* and *impK, vasK* and *icmF* did not affect the growth of NMEC15 in minimal medium.

To assess whether T6SS1 mutations affect the growth of NMEC15, wild-type, mutants and complemented strains were assessed for their ability to grow in minimal medium M9. As shown in Figure 4.3, deletion of *hcp, evpB, impK, vasK* or *icmF* did not affect growth of NMEC15 in minimal media.

NMEC15 expresses a T6SS.

The expression of T6SS genes by NMEC15 was analyzed using semi-quantitative real time PCR. The expression of T6SS genes was analyzed in NMEC15 grown in LB and

DMEM at 37°C or 42°C, as T6SS secretion has been shown to be temperature-dependent in other bacterial species (39). Experiments were performed in biological and technical duplicates. Genes encoding 16S rRNA were used as endogenous control for data normalization. As seen in Figure 4.4, NMEC15 expressed T6SS genes in both LB and DMEM with some significant differences ($p<0.05$) according to the media or temperature used. For instance, *icmF* expression was enhanced at 42 °C and in DMEM; *vasK* expression was higher at 37 °C; *hcp* expression was enhanced at 42 °C.

T6SS gene expression has been shown to be upregulated in the presence of the host (40). To assess whether the expression of T6SS genes by NMEC15 is upregulated under contact with host cells, we analyzed the expression of T6SS genes after 1 h and 3 h post infection of HBMEC cells. Figure 4.5 shows that the expression of *evpB* is significantly ($p<0.05$) upregulated at 3 h post-infection.

ImpK and IcmF are involved in adherence of NMEC15 to HBMEC.

Because T6SS *hcp* has been previously described to be involved in binding and invasion of HBMEC by NMEC strain RS218 (31), we assessed whether *hcp* and other T6SS components (*evpB*, *impK*, *icmF* and *vasK*) are involved in the adherence to and invasion of HBMEC by NMEC15. The adherence rate of NMEC15*ΔicmF* was only 28% (or 3.5 times smaller) of that observed in the WT strain, and the adherence rate of NMEC15*ΔimpK* strain to HBMEC was only 48% of the adherence rate of the WT. Statistical analysis demonstrated the decrease in adherence was statistically significant ($p<0.05$). The phenotype was restored upon complementation with *icmF* and *impK* on the arabinose inducible vector pBAD24. These results suggest that IcmF and ImpK contribute

to adherence of NMEC15 to HBMEC. In contrast, deletion of *hcp* and *evpB* did not significantly affect adherence of NMEC15 to HBMEC ($p \geq 0.05$) (Figure 4.5).

ImpK, IcmF and Hcp are involved in invasion of HBMEC by NMEC15.

Deletion of T6SS *hcp* has been shown to affect the invasion of HBMEC by NMEC strain 218 (31). Because of this, we assessed the invasion capability of NMEC15 WT, mutant and complemented strains. We found that deletion of *impK*, *icmF* and *hcp* significantly impaired the strains' ability to invade HBMEC. The invasion by NMEC15*ΔicmF* was only 53% of the rate observed in the WT. Invasion by NMEC15*ΔimpK* and NMEC15*Δhcp* to HBMEC was only 44% (2.2x smaller) and 63% (1.6 x smaller) compared to the rate observed in the WT strain. Statistical analysis demonstrated the decrease in adherence was statistically significant ($p<0.05$). The phenotype was fully restored upon complementation with *icmF* or *impK* or *hcp* on the arabinose inducible vector pBAD24. These results suggest that IcmF, ImpK and Hcp contribute to invasion of HBMEC by NMEC15 (Figure 4.6).

Deletion of *hcp* increases sensitivity of NMEC15 to predation by *D. discoideum*.

The amoeba *Dictyostelium discoideum* feeds on bacteria via phagosomal and endolysosomal mechanisms similar to those employed by mammalian phagocytes (41-45). Due to the fact that some genes required for resistance to predation by *D. discoideum* are also involved in replication or survival of bacteria in mammalian macrophages (46-50), and in some cases in causing disease in animals (51-57) we explored the use of *D. discoideum* as a model for pathogenic interaction between NMEC15 and mammalian macrophages.

We assessed the ability of NMEC15 WT, mutant and complemented strains to resist predation by *D. discoideum.* We did not observe a difference in phenotype between the wild-type and Δ*impK*, Δ*evpB*, Δ*icmF* and Δ*vasK* strains (data not shown). However, deletion of *hcp* affected the strain's sensitivity to predation by *D. discoideum*. At the bacteria amoeba ratio of 10^1:1, the bacterial lawn was completely consumed and replaced by *D. discoideum* fruiting bodies in the WT, mutant, and complemented strain. At the ratios 10^2:1 and 10^3:1 as expected, the amoeba consumed most of the bacteria, but some bacterial lawn was still observed at the edges of the spot in the WT, mutant, and complemented strain. The difference between the WT strain and Δ*hcp* was observed at bacteria-amoeba ratios of 10^4:1 and 10^5:1. At these ratios, a difference was observed in the size and number of predation plaques between the WT and Δ*hcp* strain. This result indicates that Hcp is involved in the ability of NMEC15 to resist to predation by *D. discoideum* (Figure 4.7).

Role of T6SS in biofilm formation by NMEC15

Given that T6SS have been previously associated with biofilm formation by APEC (40), EAEC (58) and other bacteria (59, 60), we investigated whether mutations in T6SS genes would affect the ability of NMEC15 to form biofilm. As shown by the growth curves, the mutants do not show growth defects compared to the wild-type strain. We used the crystal violet biofilm assay to assess biofilm formation by NMEC15 wild-type, mutants, and complemented strains. All mutants formed significantly less biofilm than the wild-type ($p<0.05$). Δ*hcp*, Δ*evpB*, Δ*impK*, Δ*vasK* and Δ*icmF* formed 71%, 79%, 83% 80% and 75% of the biofilm formed by the wild-type, respectively. The complemented strains, however, did not restore the phenotype.

Discussion

NMEC is the second most common cause of neonatal sepsis and meningitis, and it has emerged as the most common cause of meningitis and sepsis among very low birth weight infants (VLBW, <1500 g birth weight) since the 1990s (5, 8, 61). NMEC presents with a diverse virulence trait repertoire, with recognized virulence factors involved in its pathogenesis (13). Among its virulence genes, the T6SS gene *hcp* was shown to play an important role in the virulence of the meningitis-causing *E. coli* K1 strain RS218 (31). Here, we analyzed the role of *hcp* and other T6SS components, *evpB*, *impK*, *vasK* and *icmF* in virulence related traits of NMEC15.

This is the first report to characterize an entire collection of NMEC and HFEC isolates regarding the presence of T6SS genes. The screening of these collections showed that T6SS genes are more prevalent in NMEC than in HFEC isolates, which led us to further characterize the system in NMEC15. We found that T6SS genes are involved in virulence related traits of NMEC, including adherence to and invasion of HBMEC, resistance of NMEC15 to predation by *D. discoideum*, and biofilm formation.

Adherence of bacteria to host cells is important during initial stages of colonization and is usually the first step in infection caused by *E. coli* (62). T6SS has been previously associated with the adherence of ExPEC, including NMEC, to their host cells. In *E. coli* K1 strain RS218 the T6SS core component gene cluster (from *evfB* to *hcp1*) deletion mutant was shown to be defective in HBMEC binding (63).

Here, we found that Δ*impK* and Δ*icmF* were defective in NMEC15 binding to HBMEC. IcmF is an inner membrane protein of the T6SS, and it was shown to be involved in intracellular multiplication by other pathogens (64, 65). IcmF is required for survival

and replication in macrophages, intracellular growth in amoeba, intracellular replication in eukaryotic cells and immediate cytotoxicity and exit from the phagosome (66-69). In avian pathogenic *E. coli* (APEC), which are closely related to ExPEC that cause disease in humans, including NMEC, IcmF was shown to be important for adherence of APEC SEPT362 to HeLa cells (40).

ImpK is an essential component of the T6SS *Agrobacterium tumefaciens.* It is an inner membrane protein that interacts with ImpL via its N-terminal domain in the cytoplasm and is essential for secretion of Hcp by the plant pathogen (70). In another plant pathogen, *Acidovorax citrulli,* that causes bacterial fruit blotch (BFB) deletion of *impK* led to a decrease on seed to seedling transmission of disease, as well as in the colonization of melon seeds during germination and in biofilm formation (71). To date, there is no information on the role of *impK* homologs in ExPEC strains and its association with virulence.

Deletion of *impK* and *icmF* also affected invasion of HBMEC by NMEC15. Decreased invasion by the Δ*icmF* mutant agrees with a previous report that showed that deletion of *icmF* is important for invasion of HeLa cells by APEC SEPT362 (40). Another mutant that presented with decreased invasion was Δ*hcp*. Hcp is both a structural component and a secreted effector of T6SS that forms a transportation channel between the inner and outer membranes of the bacteria (29). Deletion of *hcp* has also been shown to lead to defective invasion of host cells by *E. coli* K1 RS218 (63).

We have also analyzed the resistance of WT and mutants to predation by *Dictyostelium discoideum,* that feeds on bacteria through well-characterized phagosomal and endolysosomal mechanisms similar to those of mammalian phagocytes (41-45).

Studies of bacterial pathogens have showed that genes required for resistance to predation by *D. discoideum* are also involved in replication or survival of bacteria in mammalian macrophages (46-50), and in some cases in causing disease in animals (51-57). Additionally, T6SS was implicated as a virulence determinant of *V. cholerae* using the *D. discoideum* model system (20). Thus, we explored *D. discoideum* as a model for pathogenic interactions between NMEC15 and macrophages. Results found in this work show that deletion of *hcp* led to an increased sensitivity of the strain to the predation by *D. discoideum*. Previous studies have shown that deletion of T6SS genes caused the strain to be avirulent towards *D. discoideum.* In *V. cholerae*, deletion of T6SS genes VCA0109 through VCA0114 and VCA0119 made the strain avirulent towards the amoeba (72).

In addition to being involved in adherence to host cells, T6SS has also been associated with biofilm formation by APEC (40). To check whether this is the case in NMEC, we assessed biofilm formation by NMEC15 wild-type and mutants. All mutants analyzed in this study had their ability to form biofilm impaired in comparison to the wild-type strain. However, no restoration of phenotype was observed in the complemented strains. Further investigation of the role of these genes in the formation of biofilm by NMEC is warranted to rule out polar effects of the mutations in this phenotype.

Conclusion

The T6SS plays an important role in NMEC pathogenesis as evidenced by its prevalence in NMEC compared with HFEC isolates, its involvement in NMEC15 ability to adhere and invade HBMEC, the role in resistance of NMEC to predation by *D. discoideum*, and the impact in biofilm formation. Further work including *in vivo* assays to

determine the role of T6SS on the virulence of NMEC and its effects on the host is warranted.

REFERENCES

1. Kaper JB, Nataro JP, Mobley HL. 2004. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123-40.
2. Bonacorsi S, Bingen E. 2005. Molecular epidemiology of *Escherichia coli* causing neonatal meningitis. Int J Med Microbiol 295:373-81.
3. Glode MP, Sutton A, Moxon ER, Robbins JB. 1977. Pathogenesis of neonatal *Escherichia coli* meningitis: induction of bacteremia and meningitis in infant rats fed E. coli K1. Infect Immun 16:75-80.
4. Stoll BJ, Hansen NI, Sanchez PJ, Faix RG, Poindexter BB, Van Meurs KP, Bizzarro MJ, Goldberg RN, Frantz ID, 3rd, Hale EC, Shankaran S, Kennedy K, Carlo WA, Watterberg KL, Bell EF, Walsh MC, Schibler K, Laptook AR, Shane AL, Schrag SJ, Das A, Higgins RD. 2011. Early onset neonatal sepsis: the burden of group B Streptococcal and *E. coli* disease continues. Pediatrics 127:817-26.
5. Stoll BJ, Hansen NI, Higgins RD, Fanaroff AA, Duara S, Goldberg R, Laptook A, Walsh M, Oh W, Hale E. 2005. Very low birth weight preterm infants with early onset neonatal sepsis: the predominance of gram-negative infections continues in the National Institute of Child Health and Human Development Neonatal Research Network, 2002-2003. Pediatr Infect Dis J 24:635-9.
6. Bizzarro MJ, Dembry LM, Baltimore RS, Gallagher PG. 2008. Changing patterns in neonatal *Escherichia coli* sepsis and ampicillin resistance in the era of intrapartum antibiotic prophylaxis. Pediatrics 121:689-96.
7. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark AR, Tyson JE, Oh W, Bauer CR, Korones SB, Shankaran

S, Laptook AR, Stevenson DK, Papile LA, Poole WK. 2002. Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. N Engl J Med 347:240-7.

8. Camacho-Gonzalez A, Spearman PW, Stoll BJ. 2013. Neonatal infectious diseases: evaluation of neonatal sepsis. Pediatr Clin North Am 60:367-89.

9. Kim KS. 2003. Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury. Nat Rev Neurosci 4:376-85.

10. Stevens JP, Eames M, Kent A, Halket S, Holt D, Harvey D. 2003. Long term outcome of neonatal meningitis. Arch Dis Child Fetal Neonatal Ed 88:F179-84.

11. Croxen MA, Finlay BB. 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Microbiol 8:26-38.

12. Wooster DG, Maruvada R, Blom AM, Prasadarao NV. 2006. Logarithmic phase *Escherichia coli K1* efficiently avoids serum killing by promoting C4bp-mediated C3b and C4b degradation. Immunology 117:482-93.

13. Logue CM, Doetkott C, Mangiamele P, Wannemuehler YM, Johnson TJ, Tivendale KA, Li G, Sherwood JS, Nolan LK. 2012. Genotypic and phenotypic traits that distinguish neonatal meningitis-associated *Escherichia coli* from fecal *E. coli* isolates of healthy human hosts. Appl Environ Microbiol 78:5824-30.

14. Khan NA, Kim Y, Shin S, Kim KS. 2007. FimH-mediated *Escherichia coli* K1 invasion of human brain microvascular endothelial cells. Cell Microbiol 9:169-78.

15. Prasadarao NV. 2002. Identification of *Escherichia coli* outer membrane protein A receptor on human brain microvascular endothelial cells. Infect Immun 70:4556-63.

16. Kim KS. 2008. Mechanisms of microbial traversal of the blood-brain barrier. Nat Rev Microbiol 6:625-34.

17. Barbieri NL, de Oliveira AL, Tejkowski TM, Pavanelo DB, Rocha DA, Matter LB, Callegari-Jacques SM, de Brito BG, Horn F. 2013. Genotypes and pathogenicity of cellulitis isolates reveal traits that modulate APEC virulence. PLoS One 8:e72322.

18. Barbieri NL, de Oliveira AL, Tejkowski TM, Pavanelo DB, Matter LB, Pinheiro SR, Vaz TM, Nolan LK, Logue CM, de Brito BG, Horn F. 2015. Molecular characterization and clonal relationships among *Escherichia coli* strains isolated from broiler chickens with colisepticemia. Foodborne Pathog Dis 12:74-83.

19. Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G. 2015. Secretion systems in Gram-negative bacteria: structural and mechanistic insights. Nat Rev Microbiol 13:343-59.

20. Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, Heidelberg JF, Mekalanos JJ. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc Natl Acad Sci U S A 103:1528-33.

21. Schell MA, Ulrich RL, Ribot WJ, Brueggemann EE, Hines HB, Chen D, Lipscomb L, Kim HS, Mrazek J, Nierman WC, Deshazer D. 2007. Type VI secretion is a major virulence determinant in *Burkholderia mallei*. Mol Microbiol 64:1466-85.

22. Zheng J, Leung KY. 2007. Dissection of a type VI secretion system in *Edwardsiella tarda*. Mol Microbiol 66:1192-206.

23. Decoin V, Barbey C, Bergeau D, Latour X, Feuilloley MG, Orange N, Merieau A. 2014. A type VI secretion system is involved in *Pseudomonas fluorescens* bacterial competition. PLoS One 9:e89411.

24. Boyer F, Fichant G, Berthod J, Vandenbrouck Y, Attree I. 2009. Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? BMC Genomics 10:104.

25. Cascales E, Cambillau C. 2012. Structural biology of type VI secretion systems. Philos Trans R Soc Lond B Biol Sci 367:1102-11.

26. Cascales E. 2008. The type VI secretion toolkit. EMBO Rep 9:735-41.

27. Planamente S, Salih O, Manoli E, Albesa-Jove D, Freemont PS, Filloux A. 2016. TssA forms a gp6-like ring attached to the type VI secretion sheath. Embo j 35:1613-27.

28. Tseng TT, Tyler BM, Setubal JC. 2009. Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. BMC Microbiol 9 Suppl 1:S2.

29. Pukatzki S, McAuley SB, Miyata ST. 2009. The type VI secretion system: translocation of effectors and effector-domains. Curr Opin Microbiol 12:11-7.

30. Wu HY, Chung PC, Shih HW, Wen SR, Lai EM. 2008. Secretome analysis uncovers an Hcp-family protein secreted via a type VI secretion system in *Agrobacterium tumefaciens*. J Bacteriol 190:2841-50.

31. Zhou Y, Tao J, Yu H, Ni J, Zeng L, Teng Q, Kim KS, Zhao GP, Guo X, Yao Y. 2012. Hcp family proteins secreted via the type VI secretion system coordinately

regulate *Escherichia coli* K1 interaction with human brain microvascular endothelial cells. Infect Immun 80:1243-51.

32. Johnson JR, Oswald E, O'Bryan TT, Kuskowski MA, Spanjaard L. 2002. Phylogenetic Distribution of Virulence-Associated Genes among *Escherichia coli* Isolates Associated with Neonatal Bacterial Meningitis in The Netherlands. The Journal of Infectious Diseases 185:774-784.

33. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-5.

34. Decoin V, Barbey C, Bergeau D, Latour X, Feuilloley MGJ, Orange N, Merieau A. 2014. A Type VI Secretion System Is Involved in *Pseudomonas fluorescens* Bacterial Competition. PLoS ONE 9:e89411.

35. Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW. 2000. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. Anal Biochem 285:194-204.

36. Barbieri NL, Vande Vorde JA, Baker AR, Horn F, Li G, Logue CM, Nolan LK. 2017. FNR Regulates the Expression of Important Virulence Factors Contributing to the Pathogenicity of Avian Pathogenic *Escherichia coli*. Front Cell Infect Microbiol 7:265.

37. Hasselbring BM, Patel MK, Schell MA. 2011. *Dictyostelium discoideum* as a Model System for Identification of *Burkholderia pseudomallei* Virulence Factors. Infection and Immunity 79:2079-2088.

38. Skyberg JA, Siek KE, Doetkott C, Nolan LK. 2007. Biofilm formation by avian *Escherichia coli* in relation to media, source and phylogeny. J Appl Microbiol 102:548-54.

39. Bladergroen MR, Badelt K, Spaink HP. 2003. Infection-blocking genes of a symbiotic *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein secretion. Mol Plant Microbe Interact 16:53-64.

40. de Pace F, Boldrin de Paiva J, Nakazato G, Lancellotti M, Sircili MP, Guedes Stehling E, Dias da Silveira W, Sperandio V. 2011. Characterization of IcmF of the type VI secretion system in an avian pathogenic *Escherichia coli* (APEC) strain. Microbiology (Reading) 157:2954-2962.

41. Buczynski G, Grove B, Nomura A, Kleve M, Bush J, Firtel RA, Cardelli J. 1997. Inactivation of two *Dictyostelium discoideum* genes, DdPIK1 and DdPIK2, encoding proteins related to mammalian phosphatidylinositide 3-kinases, results in defects in endocytosis, lysosome to postlysosome transport, and actin cytoskeleton organization. J Cell Biol 136:1271-86.

42. DeShazer D, Brett PJ, Woods DE. 1998. The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. Mol Microbiol 30:1081-100.

43. Maniak M, Rauchenberger R, Albrecht R, Murphy J, Gerisch G. 1995. Coronin involved in phagocytosis: dynamics of particle-induced relocalization visualized by a green fluorescent protein Tag. Cell 83:915-24.

44. Rezabek BL, Rodriguez-Paris JM, Cardelli JA, Chia CP. 1997. Phagosomal proteins of *Dictyostelium discoideum*. J Eukaryot Microbiol 44:284-92.

45. Rupper A, Cardelli J. 2001. Regulation of phagocytosis and endo-phagosomal trafficking pathways in *Dictyostelium discoideum*. Biochim Biophys Acta 1525:205-16.

46. Andrews HL, Vogel JP, Isberg RR. 1998. Identification of linked *Legionella pneumophila* genes essential for intracellular growth and evasion of the endocytic pathway. Infect Immun 66:950-8.

47. Hägele S, Köhler R, Merkert H, Schleicher M, Hacker J, Steinert M. 2000. *Dictyostelium discoideum*: a new host model system for intracellular pathogens of the genus Legionella. Cell Microbiol 2:165-71.

48. Solomon JM, Leung GS, Isberg RR. 2003. Intracellular replication of *Mycobacterium marinum* within *Dictyostelium discoideum*: efficient replication in the absence of host coronin. Infect Immun 71:3578-86.

49. Solomon JM, Rupper A, Cardelli JA, Isberg RR. 2000. Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. Infect Immun 68:2939-47.

50. Steinert M, Heuner K. 2005. *Dictyostelium* as host model for pathogenesis. Cell Microbiol 7:307-14.

51. Alibaud L, Köhler T, Coudray A, Prigent-Combaret C, Bergeret E, Perrin J, Benghezal M, Reimmann C, Gauthier Y, van Delden C, Attree I, Fauvarque MO, Cosson P. 2008. *Pseudomonas aeruginosa* virulence genes identified in a *Dictyostelium* host model. Cell Microbiol 10:729-40.

52. Cosson P, Zulianello L, Join-Lambert O, Faurisson F, Gebbie L, Benghezal M, Van Delden C, Curty LK, Köhler T. 2002. *Pseudomonas aeruginosa* virulence analyzed in a *Dictyostelium discoideum* host system. J Bacteriol 184:3027-33.

53. Finck-Barbançon V, Goranson J, Zhu L, Sawa T, Wiener-Kronish JP, Fleiszig SM, Wu C, Mende-Mueller L, Frank DW. 1997. ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Mol Microbiol 25:547-57.

54. Hauser AR, Kang PJ, Engel JN. 1998. PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. Mol Microbiol 27:807-18.

55. Pearson JP, Feldman M, Iglewski BH, Prince A. 2000. *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. Infect Immun 68:4331-4.

56. Pukatzki S, Kessin RH, Mekalanos JJ. 2002. The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba Dictyostelium discoideum. Proc Natl Acad Sci U S A 99:3159-64.

57. Vance RE, Rietsch A, Mekalanos JJ. 2005. Role of the type III secreted exoenzymes S, T, and Y in systemic spread of *Pseudomonas aeruginosa* PAO1 in vivo. Infect Immun 73:1706-13.

58. Aschtgen MS, Bernard CS, De Bentzmann S, Lloubès R, Cascales E. 2008. SciN is an outer membrane lipoprotein required for type VI secretion in enteroaggregative *Escherichia coli*. J Bacteriol 190:7523-31.

59. Stewart BJ, Enos-Berlage JL, McCarter LL. 1997. The lonS gene regulates swarmer cell differentiation of *Vibrio parahaemolyticus*. J Bacteriol 179:107-14.

60. Waite RD, Paccanaro A, Papakonstantinopoulou A, Hurst JM, Saqi M, Littler E, Curtis MA. 2006. Clustering of *Pseudomonas aeruginosa* transcriptomes from planktonic cultures, developing and mature biofilms reveals distinct expression profiles. BMC Genomics 7:162.

61. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark AR, Tyson JE, Oh W, Bauer CR, Korones SB, Shankaran S, Laptook AR, Stevenson DK, Papile LA, Poole WK. 2002. Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. NEnglJMed 347:240-247.

62. Antão EM, Wieler LH, Ewers C. 2009. Adhesive threads of extraintestinal pathogenic *Escherichia coli*. Gut Pathog 1:22.

63. Zhou Y, Tao J, Yu H, Ni J, Zeng L, Teng Q, Kim KS, Zhao G-P, Guo X, Yao Y. 2012. Hcp Family Proteins Secreted via the Type VI Secretion System Coordinately Regulate *Escherichia coli* K1 Interaction with Human Brain Microvascular Endothelial Cells. Infection and Immunity 80:1243-1251.

64. Hilbi H, Segal G, Shuman HA. 2001. Icm/dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. Mol Microbiol 42:603-17.

65. Watarai M, Derre I, Kirby J, Growney JD, Dietrich WF, Isberg RR. 2001. *Legionella pneumophila* is internalized by a macropinocytotic uptake pathway controlled by the Dot/Icm system and the mouse Lgn1 locus. J Exp Med 194:1081-96.

66. Kirby JE, Vogel JP, Andrews HL, Isberg RR. 1998. Evidence for pore-forming ability by *Legionella pneumophila*. Mol Microbiol 27:323-36.

67. Pratt LA, Kolter R. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol 30:285-93.

68. Purcell M, Shuman HA. 1998. The Legionella pneumophila icmGCDJBF genes are required for killing of human macrophages. Infect Immun 66:2245-55.

69. VanRheenen SM, Duménil G, Isberg RR. 2004. IcmF and DotU are required for optimal effector translocation and trafficking of the *Legionella pneumophila* vacuole. Infect Immun 72:5972-82.

70. Ma LS, Lin JS, Lai EM. 2009. An IcmF family protein, ImpLM, is an integral inner membrane protein interacting with ImpKL, and its walker a motif is required for type VI secretion system-mediated Hcp secretion in *Agrobacterium tumefaciens*. J Bacteriol 191:4316-29.

71. Tian Y, Zhao Y, Wu X, Liu F, Hu B, Walcott RR. 2015. The type VI protein secretion system contributes to biofilm formation and seed-to-seedling transmission of *Acidovorax citrulli* on melon. Mol Plant Pathol 16:38-47.

72. Zheng J, Ho B, Mekalanos JJ. 2011. Genetic analysis of anti-amoebae and anti-bacterial activities of the type VI secretion system in *Vibrio cholerae*. PLoS One 6:e23876.

73. Guzman LM, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol 177:4121-30.

Table 4.1. Plasmid and strains used in this study.

Plasmids	**Genotype/description**	**Ref.**
pKD46	Lambda Red recombinase expression plasmid	(33)
pKD3 Amp^r	template plasmid for FRT-flanked Amp cassette	(33)
pKD3 Cm^r	template plasmid for FRT-flanked Cm cassette	(33)
pCP20	FLP recombinase expression plasmid	(33)
pBAD24	Cloning vector	(73)
pBAD24-*hcp*	pBAD24 with *hcp*	This work
pBAD24-*evpB*	pBAD24 with *evpB*	This work
pBAD24-*impK*	pBAD24 with *impK*	This work
pBAD24-*vasK*	pBAD24 with *vasK*	This work
pBAD24-*icmF*	pBAD24 with *icmF*	This work
pBAD24-FLAG:*hcp*	pBAD24 with FLAG-*hcp*	This work
Strains	**Genotype/description**	**Ref.**
E. coli DH5α	F−Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ– thi-1 gyrA96 relA1	Lab stock
NMEC15	NMEC15 WT	Lab stock
NMEC*Δhcp*	NMEC15 with *hcp* deleted by λ red rec.	This work
NMEC*ΔevpB*	NMEC15 with *evpB* deleted by λ red rec.	This work
NMEC*ΔimpK*	NMEC15 with *impK* deleted by λ red rec.	This work
NMEC*ΔvasK*	NMEC15 with *vasK* deleted by λ red rec.	This work
NMEC*ΔicmF*	NMEC15 with *icmF* deleted by λ red rec.	This work
NMEC*Δhcp*-p*hcp*	NMEC*Δhcp* with *hcp* cloned into pBAD24	This work
NMEC*ΔevpB*-p*evpB*	NMEC*ΔevpB* with *evpB* cloned into pBAD24	This work
NMEC*ΔimpK*-p*impK*	NMEC*ΔimpK* with *impK* cloned into pBAD24	This work
NMEC*ΔvasK*-p*vasK*	NMECΔ*vasK* with *vasK* cloned into pBAD24	This work

Strain	Genotype/description	Ref.
NMEC*ΔicmF*-p*icmF*	NMEC*ΔicmF* with *icmF* cloned into pBAD24	This work

Table 4.2. Primers used for screening, deletion and complementation of T6SS genes.

Primer	Sequence (5'-3')	Ref.
	Screening of T6SS genes	
*hcp*_Fw	cgaaggtagcatcgaagtgg	This work
*hcp*_Rv	ttaaactccgccgttttcag	This work
*evpB*_Fw	agcgattcacgttctgcttt	This work
*evpB*_Rv	tgcacacaccagccattatt	This work
*impK*_Fw	ccactccagtcgcatttctt	This work
*impK*_Rv	attcagccagtggtgtagcc	This work
*vasK*_Fw	tcagcctgcaaacgtatctg	This work
*vasK*_Rv	cggaagtttttcagctccag	This work
*icmF*_Fw	acaacgaggcggtaaaacag	This work
*icmF*_Rv	atgtaacgaacggcttccac	This work
	Gene deletion	
*hcp*Del_F:	atggctattcctgcttatctctggctgaaagatgacggcggcgcggatat[1]	This work
*hcp*Del_R:	tcaggcggaaggacgctcattccacgagtcggaatgaatgatgttgccgt[2]	This work
*evpB*Del_F	atgctgatgtctgtacaacaagaacattccacctctgaaactgcaacact[1]	This work
*evpB*Del_R	tcaggctttcgctttcggcatctgggaaaccagagaaaggttgatatcca[2]	This work
*impK*Del_F:	atgaaaaaagatatggatatcaatatcgatgcgctgctgcgcgacacgtt[1]	This work
*impK*Del_R:	ttaacgcaggctttgcggcagcagttcatccaccagtacattcagccagt[2]	This work
*vasK*Del_F:	atgcatactcagcgttacggtttctggggatatttaattctgattattgg[1]	This work
*vasK*Del_R:	tcactcatcggcgtcttccccatactcttccgcatccttcatactcctgc[2]	This work
*icmF*Del_F:	gtgttcaaatttcccacatcccgactgttcagcacgttgaaatctgcgct[1]	This work
*icmF*Del_R:	ttaatacaacgtatccggtaaacggaacaggctgaacagaccgccggtga[2]	This work
	Check deletion	
*hcp*Check_F:	atcagtcttgttccgcgttc	This work
*hcp*Check_R:	tcaccagattgtgggtatgc	This work
*evpB*Check_F	tcagaactgcgtgatgaactg	This work
*evpB*Check_R	ctgctgctgaaactgctgag	This work
*impK*Check_F:	gctggatatgcacagtgacg	This work
*impK*Check_R:	aaacactgaccacagcacca	This work
*vasK*Check_F:	ggccctacaggagagtttat	This work
*vasK*Check_R:	cgcgtatacgcaggaaatcg	This work
*icmF*Check_F:	cagcagtaccggatgctctt	This work
*icmF*Check_R:	cagtttccagttcagctccg	This work
	Gene complementation	
*hcp*_Fw_*XbaI*	gcgtctagagatggctattcctgcttatct	This work
*hcp*_Rv_*HindIII*	gcgttcgaagctcaggcggaaggacgctcat	This work

Primer	Sequence (5'-3')	Ref.
*evpB*_Fw_*XbaI*	gcgtctagagatgctgatgtctgtacaaca	This work
*evpB*_Rv_*HindIII*	gcgttcgaagctcaggctttcgctttcggca	This work
*impK*_Fw_*XbaI*	gcgtctagagatgaaaaaagatatggatat	This work
*impK*_Rv_*HindIII*	gcgttcgaagcttaacgcaggctttgcggca	This work
*vasK*_Fw_*XbaI*	gcgtctagagatgcatactcagcgttacgg	This work
*vasK*_Rv_*HindIII*	gcgttcgaagctcactcatcggcgtcttccc	This work
*icmF*_Fw_*XbaI*	gcgtctagaggtgttcaaatttcccacatc	This work
*icmF*_Rv_*HindIII*	gcgttcgaagcttaatacaacgtatccggta	This work
Check insertion in pBAD24		
pBAD24_Fw	atgccatagcatttttatcc	(73)
pBAD24_Rv:	gatttaatctgtatcagg	(73)
Hcp-FLAG fusion		
*hcp*_N-Flag_Fw	gatgatgataaaatggctattcctgcttatctc	
*hcp*_N-Flag_Rv	atctttataatcctctagaggatccccggg	

[1] Forward primer extension to amplify chloramphenicol resistance cassette (tgtaggctggagctgcttcg).
[2] Reverse primer extension to amplify chloramphenicol resistance cassette (atgggaattagccatggtcc).

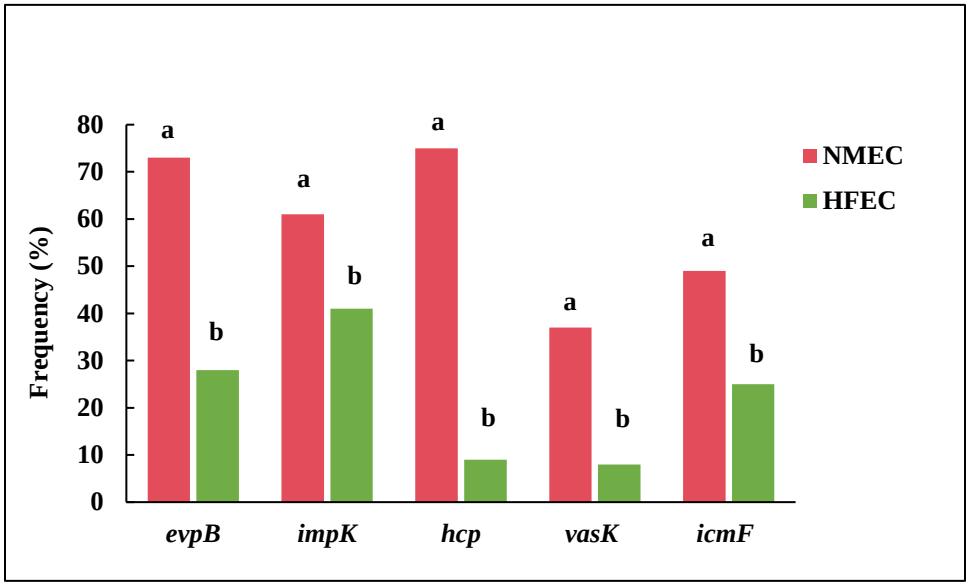

Figure 4.1. Histogram comparing the frequencies of genes *evpB*, *impK*, *hcp*, *vasK* and *icmF* between NMEC and HFEC strains. Different letters above bars indicate that the prevalence of the gene was significantly different between NMEC and HFEC ($p<0.05$).

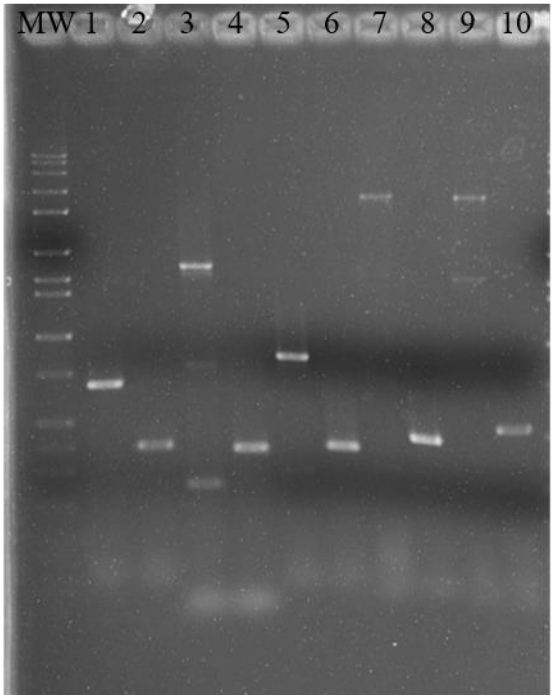

Figure 4.2. Construction of T6SS mutants in NMEC 15. MW - Molecular weight marker. Lane 1: WT *hcp*; Lane 2: Δ*hcp*; Lane 3: WT *evpB*; Lane 4: Δ*evpB*; Lane 5: WT *impK*; Lane 6: Δ*impK*; Lane 7: WT *icmF*; Lane 8: Δ*icmF*; Lane 9: WT *vasK*; Lane 10: Δ*vasK*.

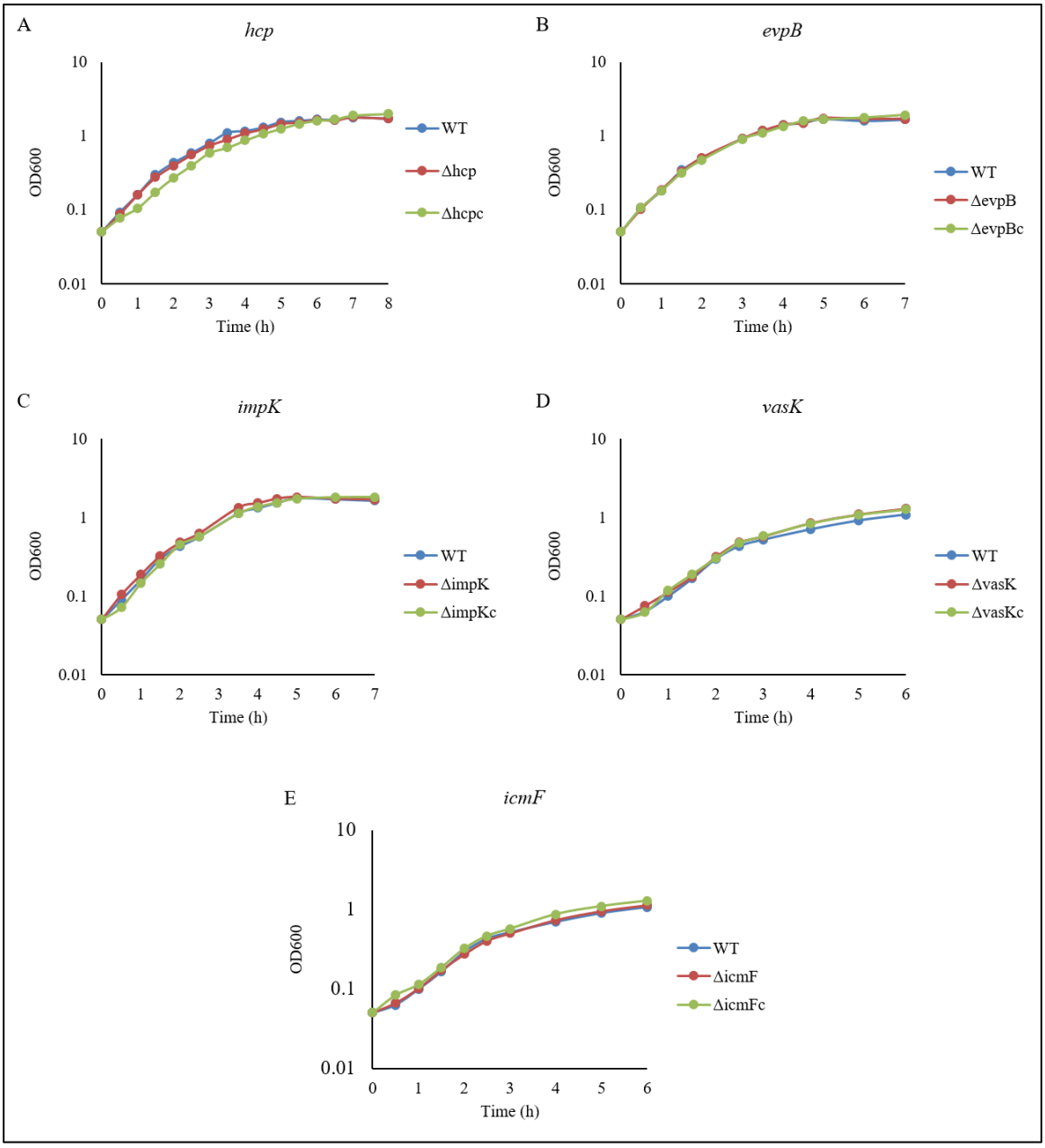

Figure 4.3. Deletion of T6SS genes does not affect the growth of NMEC15 in minimal media. Growth curves of (A) NMEC15 WT, Δ*hcp*, Δ*hcp*c, (B) NMEC15 WT, Δ*evpB*, Δ*evpB*c, (C) NMEC15 WT, Δ*impK*, Δ*impK*c, (D) NMEC15 WT, Δ*vasK*, Δ*vasK*c, (E) NMEC15 WT, Δ*icmF*, Δ*icmF*c at 37°C. The expression of the genes in the complemented strains was induced with 6.5 mM L-arabinose.

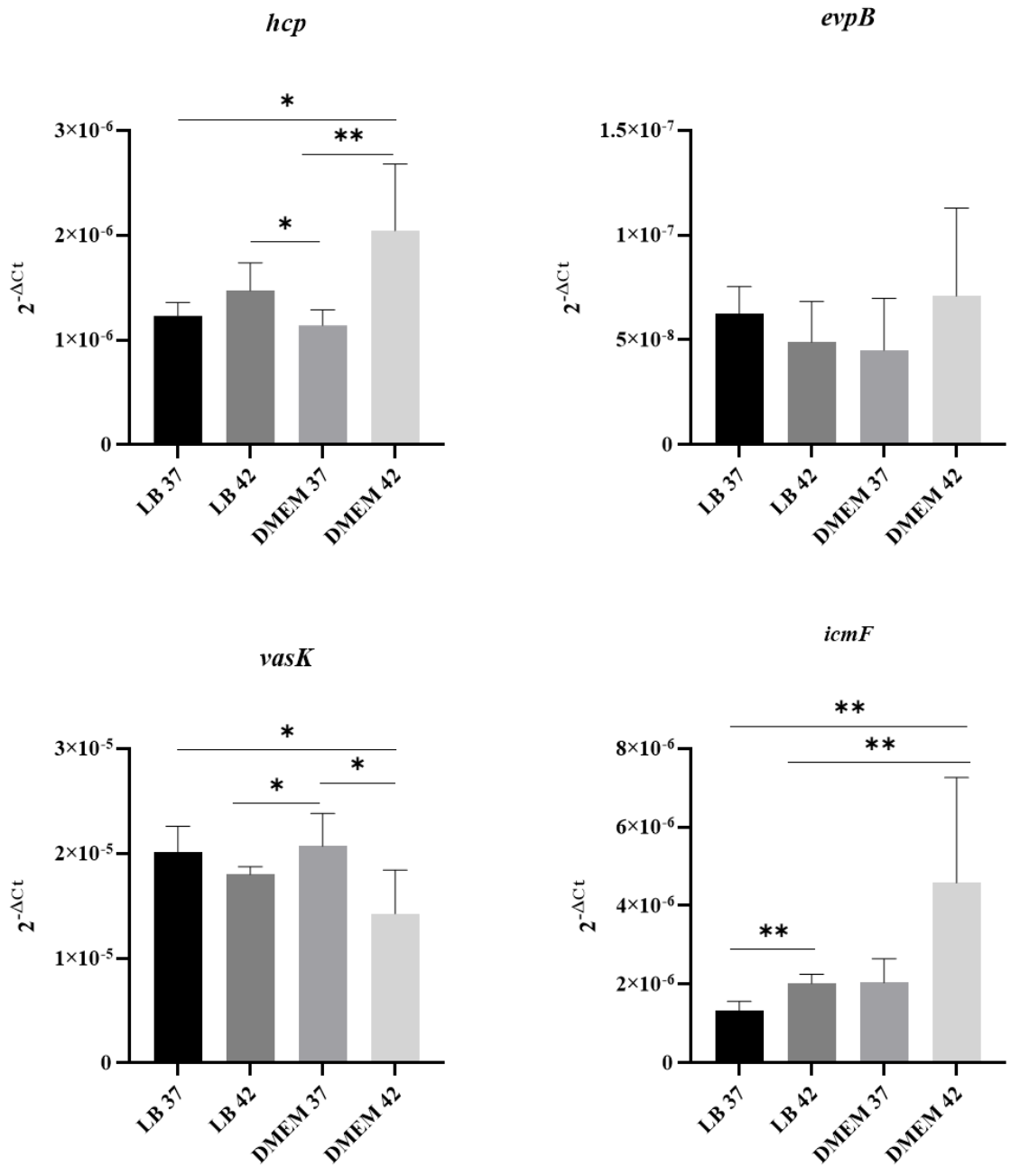

Figure 4.4. NMEC15 expresses genes encoding a T6SS. qRT-PCR of *hcp*, *evpB*, *vasK* and *icmF* genes in the NMEC15 strain in LB and DMEM at 37°C and 42°C. *, $p<0.05$.

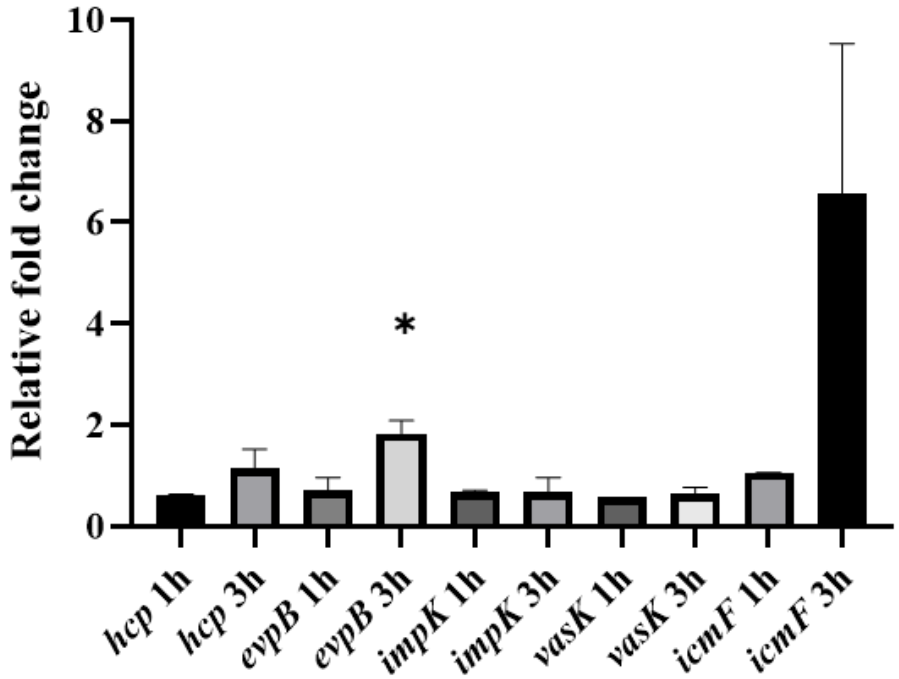

Figure 4.5. Expression of *evpB* by NMEC15 is enhanced at 3h post infection of HBMEC cells. Fold change is relative to NMEC15 grown in DMEM at 37°C. *, $p<0.05$.

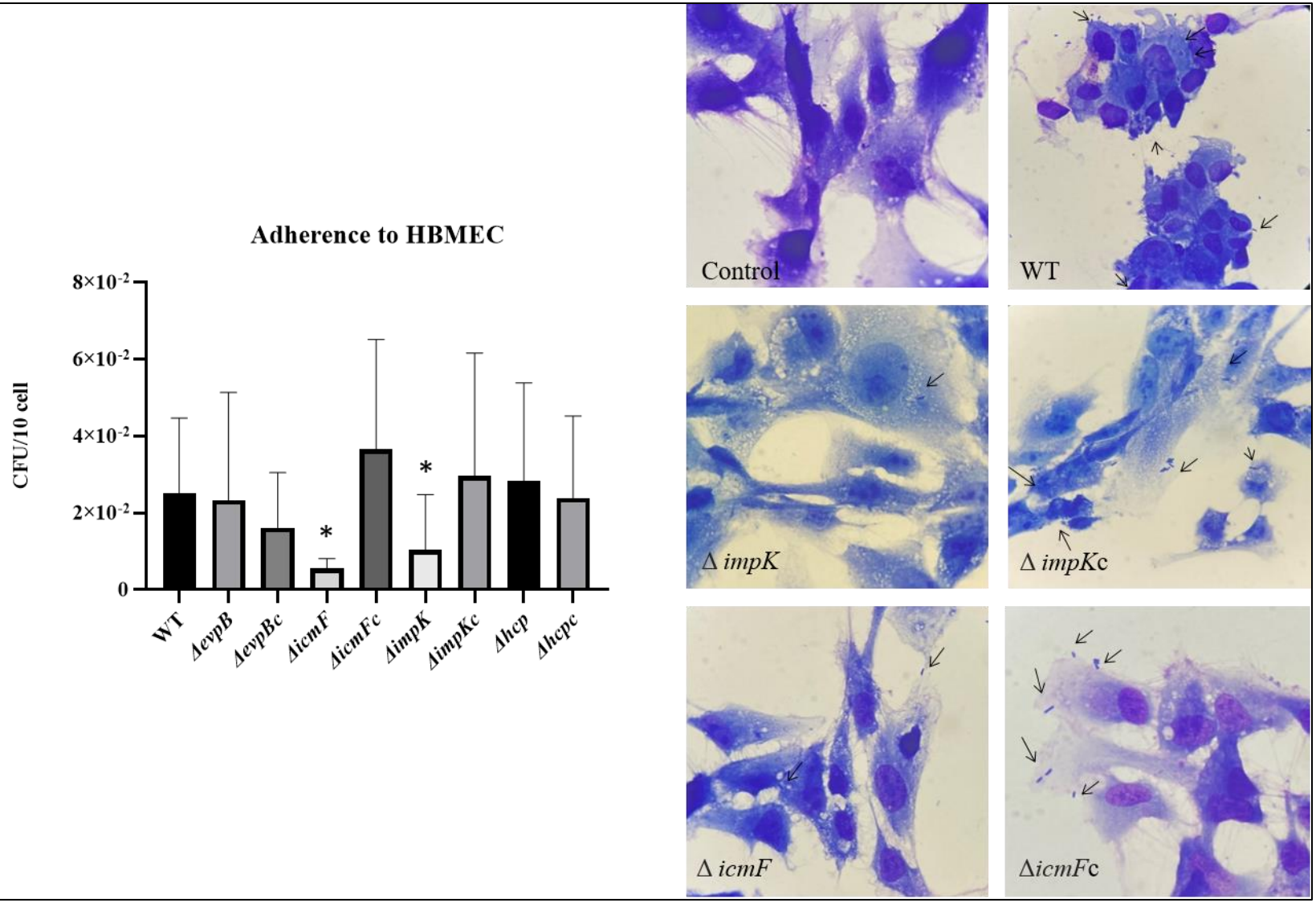
Adherence to HBMEC
CFU/10 cell
8×10-2
6×10-2
4×10-2
2×10-2
0
*
*
WT
ΔevpB
ΔevpBc
ΔicmF
ΔicmFc
ΔimpK
ΔimpKc
Δhcp
Δhcpc
Control
WT
Δ impK
Δ impKc
Δ icmF
ΔicmFc

Figure 4.6. T6SS is involved in adherence of NMEC15 to HBMEC cells. (A) Quantification of adherence of strain NMEC15 WT, mutants and complemented strains to HBMEC (*, $p<0.05$). (B) Giemsa stain of NMEC15 WT, mutants and complemented strains adhering to HBMEC. Arrows indicate adherent bacteria.

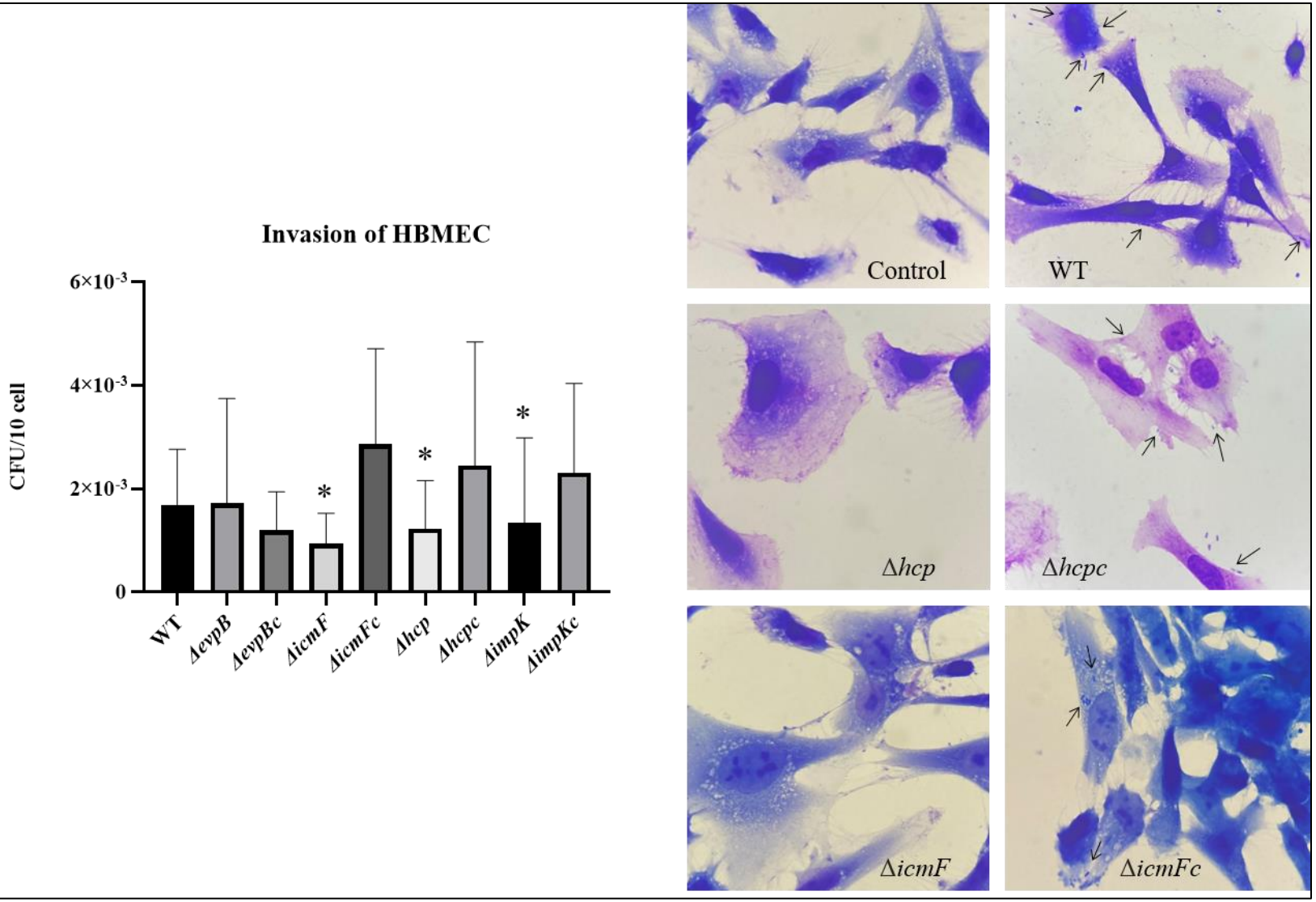
Invasion of HBMEC
CFU/10 cell
6×10-3
4×10-3
2×10-3
0
WT
ΔevpB
ΔevpBc
ΔicmF
ΔicmFc
Δhcp
Δhcpc
ΔimpK
ΔimpKc
*
Control
WT
Δhcp
Δhcpc
ΔicmF
ΔicmFc

Figure 4.7. T6SS is involved in invasion of HBMEC by NMEC15. (A) Quantification of invasion of HBMEC by NMEC15 WT, mutants and complemented strains (*, $p<0.05$). (B) Giemsa stain of NMEC15 WT, mutants and complemented strains invading HBMEC. Arrows indicate invading bacteria.

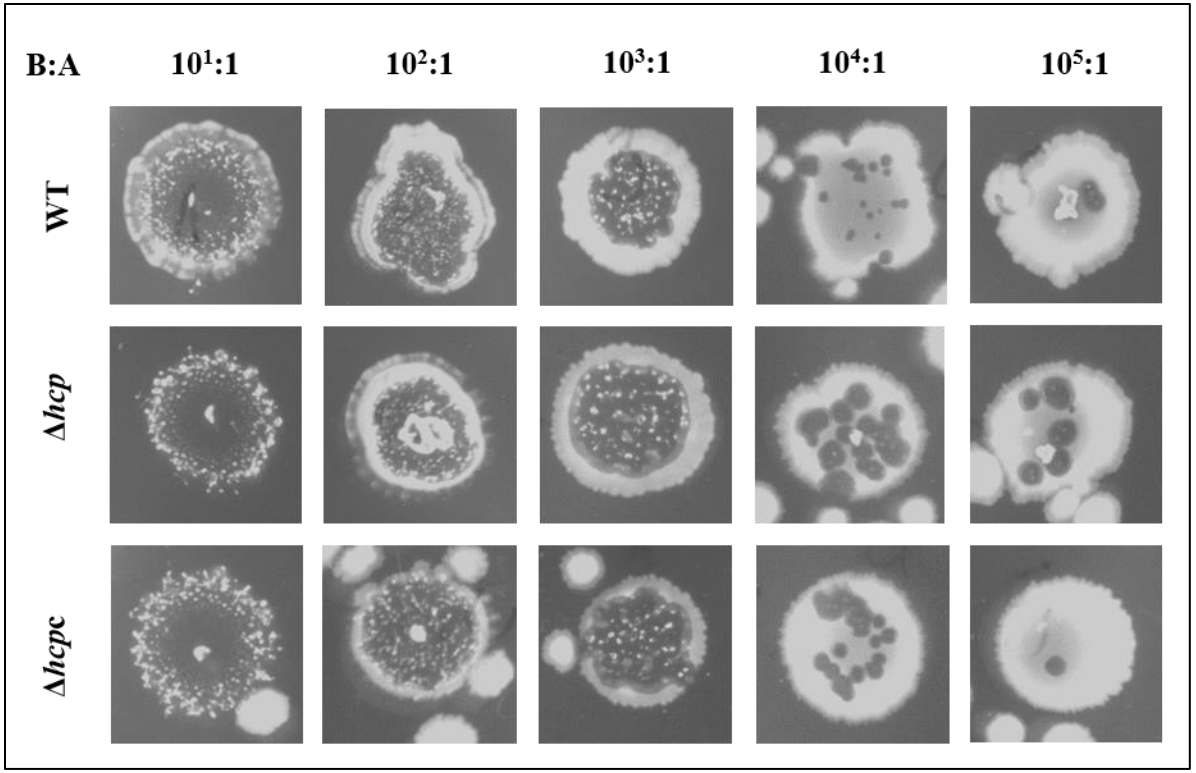

Figure 4.8. Deletion of *hcp* increases sensitivity of NMEC15 to predation by *D. discoideum*. Figure shows predation plaque assay for wild-type, mutant (Δ*hcp*) and complemented strain (Δ*hcpc*).

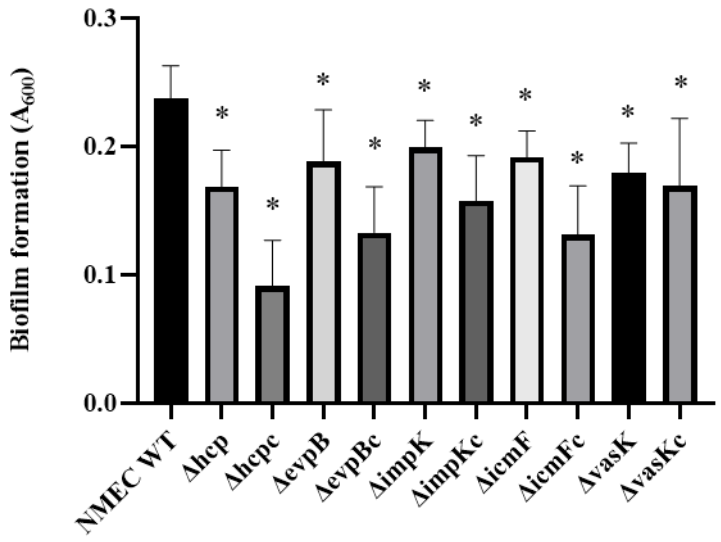

Figure 4.9. Biofilm formation by NMEC15 wild-type, mutants and complemented strains. Cells were grown for 24 h in a 96-well plate containing M63. *, $p<0.05$, indicates statistically significant difference between the strain and the wild-type.

CHAPTER 5

TYPE 6 SECRETION SYSTEM (T6SS) PLAYS A ROLE IN THE VIRULENCE OF AVIAN PATHOGENIC *Escherichia coli* APECO18[1]

[1]De Oliveira, A.L., N.L. Barbieri, D.M. Newman, M.M Young, and C.M. Logue. To be submitted to PLOS One.

Abstract

Avian pathogenic *E. coli* is the causative agent of extra-intestinal infections in birds known as colibacillosis, which can manifest as localized or systemic infections. The disease affects all stages of poultry production, resulting in economic losses that occur due to morbidity, carcass condemnation and increased mortality of the birds. APEC strains have a diverse virulence trait repertoire, which includes virulence factors involved in adherence to and invasion of the host cells, serum resistance factors, and toxins. However, the pathogenesis of APEC infections remains to be fully elucidated. The Type 6 secretion (T6SS) system has recently gained attention due to its role in the infection process and protection of bacteria from host defenses in human and animal pathogens. Previous work has shown that T6SS components are involved in the adherence and invasion of host cells, as well as in the formation of biofilm, and intramacrophage bacterial replication. Here, we analyzed the frequency of T6SS genes *hcp*, *impK*, *evpB*, *vasK* and *icmF* in a collection of APEC strains and their potential role in virulence-associated phenotypes of APECO18. We demonstrated that T6SS genes are significantly more frequent in APEC than in fecal *E. coli* isolates from healthy birds. We also generated mutants for *hcp*, *impK*, *evpB*, and *icmF* and characterized these mutants regarding virulence-associated phenotypes, including adherence and invasion to host model cells, biofilm formation and the resistance to predation by *Dictyostelium discoideum*. Deletion of *hcp* reduced resistance of APECO18 to predation by *D. discoideum*, while deletion of *evpB* or *icmF* impaired biofilm formation on a polystyrene surface. These data suggest the T6SS plays a significant role in the virulence of APEC.

Introduction

Avian pathogenic *Escherichia coli* cause extra-intestinal infections in birds known as colibacillosis, which can manifest as localized or systemic infections. Colibacillosis is most common in poultry but can also occur in other species of domestic and wild birds. The severity of the infection depends on the virulence traits of the strain, host status and predisposing factors (1). The most common manifestations of APEC infections include cellulitis, a respiratory disease that frequently culminates in septicemia and lesions in organs such as liver, air sacs and heart; swollen head syndrome; omphalitis (inflammation of the yolk sac) and salpingitis (inflammation of the oviduct) (2, 3). Colibacillosis affects all stages of the poultry production, resulting in economic losses occur due to morbidity, carcass condemnation and mortality of the birds (4).

Although the route of infection by APEC is not clearly defined, the oral and respiratory tracts seem to be the primary mode of entry (5). When the infection initiates in the upper respiratory tract it is probably due to the inhalation of contaminated dust. High concentration of ammonia in the birds' environment may be a factor that make the birds prone to the infection as it causes damage to the respiratory tract epithelium and favors the entry of bacteria via the respiratory tract. Other factors that may favor infection are the exposure of the birds to high temperatures, the limited space and insufficient disinfection of the environment (2). Even though several virulence factors that are involved in different stages of the infection process have been described for APEC, the pathogenesis of APEC strains remains unclear.

Secretion of proteins via protein machineries is recognized as a primary virulence property of bacteria, and Gram-negative bacteria have developed several secretion systems

(T1SS-T6SS) that transport proteins either into the extracellular milieu (T1SS, T2SS, T5SS) or directly into target cells (T3SS, T4SS, T6SS) (6). The Type 6 Secretion System (T6SS), (7) has recently gained attention with regards to its role in bacterial pathogenesis. The T6SS is composed of 13 conserved proteins that constitute the core of the system and a set of non-conserved proteins with regulatory and accessory functions (8). These proteins assemble into a membrane complex, a baseplate, and a tail-like structure (comprised of the contractile sheath, inner tube and puncturing spike) (9-11). Acting as a molecular syringe, the system targets both eukaryotic and prokaryotic cells, and its effectors play various roles on the host cell, including cytoskeleton rearrangement, cell invasion, and bacterial escape from the host defense mechanisms (12).

The type 6 secretion system is an atypical secretion system in which one protein may play a role as a structural component and secreted effector. This is the case of the best characterized protein in the system, Hcp (Hemolysin-coregulated protein) (13).

The presence of the T6SS has been reported in a number of bacteria including the human and animal pathogen *Burkholderia mallei*, the fish pathogen *Edwardsiella tarda*, and the human pathogen *Pseudomonas fluorescens.* In each of these pathogens, the T6SS has been associated with the infection process, as well as protecting the bacteria from host defenses (14-16).

The role of T6SS in the pathogenesis of APEC was demonstrated recently (17, 18). The septicemia causing strain SEPT362 expresses a T6SS, and core components of the system Hcp, ClpV and IcmF are involved in biofilm formation, intramacrophage replication, *in vivo* virulence and in interaction with model host cells (19, 20). A subsequent genome analysis showed that APEC may harbor up to three *loci* encoding T6SS and these

vary in size and gene content (21). Another APEC strain, TW-XM, harbors 2 functional T6SS involved in different pathogenic pathways (22).

In the present study, we investigated the prevalence of T6SS1 genes in a collection of 454 APEC isolates and 102 litter isolates, as well as 106 avian fecal *E. coli* (AFEC) isolates from healthy birds. We also investigated the role of T6SS1 genes *hcp, evpB, impK,* and *icmF* in the pathogenesis of APECO18. We determined the role of these genes in the ability of APECO18 to resist predation by *Dictyostelium discoideum*, form biofilms on abiotic surface, adhere to and invade DF-1 chicken fibroblasts, and secrete T6SS effector protein Hcp with the goal of better understanding the role of T6SS in APEC pathogenesis.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids are shown in Table 5.1. The WT APEC strain was isolated from the pericardium of a chicken with signs of colisepticemia (23). The O serogroup was identified at the *E. coli* reference center at Pennsylvania State University.

E. coli DH5α was used for cloning. All *E. coli* strains were grown in Luria-Bertani broth (BD Difco™, Franklin Lakes, NJ) at 37°C with shaking, unless otherwise specified. The medium was supplemented with ampicillin (Amp 100 µg/mL), chloramphenicol (Cm 10-25 µg/mL) or L-arabinose (6.5 mM) as necessary.

DNA extraction

Bacterial DNA was obtained from whole organisms using the boil prep method. Briefly, isolates were grown at 37°C overnight on Luria-Bertani agar (BD Difco™, Franklin Lakes, NJ). Next, an isolated colony was inoculated into 1 ml of LB broth and grown overnight at 37°C. Cultures were centrifuged at 16,700 x *g* for 3 minutes. The

supernatant was discarded, and the cells were re-suspended in 200 µL of molecular-grade water, boiled for 10 minutes, and centrifuged at 5,700 x *g* for 3 minutes to precipitate cellular debris; 150 µL of the supernatant was transferred to a new tube and used as DNA template for gene amplification. The DNA extracts were stored at -20°C until use.

Detection of T6SS genes by PCR

The presence of T6SS genes *hcp*, *evpB*, *impK*, *vasK* and *icmF* was analyzed in a collection of 179 avian fecal *E. coli* (AFEC), 102 litter *E. coli* and 454 APEC isolates by polymerase chain reaction (PCR) amplification. APECO18 was used as template for primer design. Primers were purchased from Sigma (St. Louis, MO). Reactions were performed in a 25 µl volume containing 2.5 µl of 10x PCR buffer, 0.4 µl 50 mM $MgCl_2$, 1.25 µl dNTP (10uM) Pool, 2U Taq DNA polymerase, 0.075 µl (200 µM) of each primer and 2 µl of DNA sample. The conditions for amplification were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 68°C for 3 min, and a final extension step of 72°C for 10 min. Primers used are listed in Table 5.2.

PCR product analysis

PCR products were subjected to horizontal gel electrophoresis in a 1.5% agarose gel (LE Agarose, Lonza, Alpharetta, GA) at 200 V for 70 min. A Hi-Lo molecular weight marker (100 bp; Minnesota Molecular, Minneapolis, MN) and negative (sterile water) and positive controls from our lab collections were included in the gel for comparison and confirmation purposes. After electrophoresis, the gel was stained in 0.25% ethidium bromide solution (Sigma Aldrich. St. Louis, MO) for 20 min and viewed under UV light using an Omega Lum G imager (Aplegen, San Francisco, CA).

Construction of mutants and complemented strains

Isogenic mutants were constructed using the Lambda-Red recombination system (24) using the APECO18 as parental strain. Briefly, oligonucleotides specific to the chloramphenicol cassette flanked by 50 nt extensions homologous to 5'- and 3'-ends of the gene to be deleted were used to amplify the chloramphenicol resistance cassette from plasmid pKD3 (ATCC®, Manassas, VA). The PCR products were run on a 1.5% agarose gel, and gel extraction of the specific fragment was performed using QIAquick Gel Extraction Kit (Qiagen, Germantown, MD). The extracted fragments were electroporated into APECO18 containing the lambda-red expression plasmid pKD46 (ATCC®, Manassas, VA). After electroporation, the cells were grown in SOC (super optimal broth with catabolite repression) for 90 min and plated on LB agar containing 25 µg/mL chloramphenicol. Colonies were screened by PCR to identify deletion mutants. The chloramphenicol resistance cassette was cured by transforming the helper plasmid pCP20 (ATCC®, Manassas, VA) into the mutants and screening for chloramphenicol sensitive colonies. *In trans* complementation was performed by cloning PCR-amplified genes into the *XbaI* and *HindIII* restriction sites of plasmid pBAD24 (ATCC®, Manassas, VA), and transforming the construct into their mutant counterparts. Primers used are listed in Table 5.2.

Growth curve analysis

The growth of WT APECO18 and mutant strains in minimal medium M9 was analyzed. Briefly, strains were incubated overnight in LB broth containing chloramphenicol (10 µg/mL) with incubation at 37°C. Next, OD_{600} of the cultures was measured and cultures were diluted to an OD600 of 0.05 in M9 supplemented or not with arabinose at a final concentration of 0.2%. Cultures were incubated at 37°C with shaking

at 220 rpm, and OD_{600} measurements were obtained every 30 minutes. To measure OD_{600}, 300 µL of the growing culture was dispensed in a well of a 96 well plate, and the absorbance was read using an ELX 808 Ultra microplate reader (Bio-Tek Instruments, Winooski, VT). Absorbance of duplicates was averaged, and data was plotted against time to build the growth curves. Growth curves were carried out for a total of 7-8 hours.

Analysis of Expression of T6SS genes

RNA extraction

To assess the expression of *hcp*, *evpB, impK*, *icmF* and *vasK* genes, APECO18 was grown overnight at 37°C statically. Next day, the cultures were diluted 1:100 in either LB broth or DMEM (ATCC®, Manassas, VA) and incubated at either 37°C or 42°C with shaking and grown to exponential phase (OD600 ~0.6). RNA from biological duplicates was extracted using the RiboPure™ RNA purification kit (Ambion, Austin, TX) according to manufacturer's instructions. RNA of APECO18 was also extracted after two hours of contact with DF-1 chicken fibroblasts to test whether cellular contact induces the expression of T6SS genes. Isolated RNA was treated with DNase 1 according to the manufacturer's instructions to eliminate DNA trace amounts from the eluted RNA. The concentration of RNA samples was determined using a NanoPhotometer® NP80 (Implen, Munchen, Germany), and the samples were stored at -80°C until use.

Synthesis of the 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 DNAase-treated RNA was mixed with 1 µL of Random primers (50 µM), 1 µL of 10mM dNTP mixture, and adjusted to 10 µL with RNase-free water. The mixture was heated at 65 ℃ for 5 min and chilled on ice for 2 minutes for denaturation. The mixture was then centrifuged, and the cDNA synthesis mix was prepared by adding 4 µL of 5x first-strand buffer, 1 µL of RNase inhibitor, 1 µL of Reverse transcriptase and RNase-free water up to 20 µL. cDNA synthesis reaction was set as follows: 2 min at 25 °C, 50 min at 42 °C, and 15 min at 75 °C. cDNA was diluted 1:20 before use as template for qRT-PCR and stored at -20 °C until use.

qRT-PCR

Quantitative real-time RT-PCR (qRTPCR) was performed in a qTower^3 G qPCR System (Analytik Jena, Jena, Germany). Primers for qRT-PCR were purchased from Sigma Aldrich (St. Louis, MO). Reactions were performed using either qPCR Master Mix with Sybr® Green (Goldbio, St. Louis, MO) or Platinum™ SYBR™ Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA). Each reaction was performed in a final volume of 20 µl containing 25 ng cDNA (1µL diluted cDNA), 1 µL each primer (10µM), 10 µL 2x qPCR Master Mix Sybr® Green (Goldbio, St. Louis, MO) or 10 µL Platinum™ SYBR™ Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA) and nuclease-free water up to 20 µL. When using Platinum™ SYBR™ Green qPCR SuperMix-UDG. 1 µL of (1:10) ROX Reference Dye was added to each reaction. PCR conditions were as follows: 50 °C for 2 min hold, 95 °C for 2 min hold, and 40 cycles of 95 °C for 15 sec and 60 °C for 30 sec.

Threshold fluorescence was established within the geometric phase of exponential amplification, and the cycle threshold (CT) (25) was determined for each RNA sample. The CT from each replicate was averaged. Expression levels were normalized using the housekeeping gene 16S rRNA as endogenous control. Melting curve analyses were performed after each reaction to ensure amplification specificity. Differences (nfold) in transcripts were calculated using the relative comparison method (26).

Adherence and invasion assays

Cell adherence and invasion assays were performed as previously described (27) using chicken fibroblasts DF-1 cells. The cells were cultured in 75 cm^2 cell culture flasks (Corning®, NY) in DMEM-F12 (ATCC®, Manassas, VA) containing 10% fetal bovine serum (FBS; Sigma, MO) with incubation at 37°C and 5% CO_2. Cells were transferred to sterile 24-well-plates at a concentration of $1x10^5$ cells/well 48 h prior each experiment. Wild-type APECO18, mutants and complemented strains were grown statically in LB broth overnight. Next, cultures were diluted 1:50 in fresh LB broth and grown at 37°C with shaking at 220 rpm for 2h. Cultures were then induced with 0.2% L-arabinose for 2h. After induction, cultures were washed with PBS and adjusted to $1x10^8$ cells/mL. 10 µL of bacterial suspension was used for infection. DF-1 cells were washed once with PBS and then exposed to bacteria at a multiplicity of infection (MOI) of 10. The 24-well-plates were centrifuged at 500 x g for 5 min and incubated for 1 h for adherence and 4 h for invasion.

For the adherence assays, after the 1h infection, cells were washed four times with 1 mL PBS and then lysed with 1 mL 0.1% Triton-X100 for 10 min at room temperature. Serial dilutions of cell suspension were spread onto MacConkey agar plates and incubated at 37°C overnight, and CFU were counted.

For the invasion assays, after 1 h infection, cells were washed four times with sterile PBS to remove unattached cells, and then re-incubated with fresh media containing 100 µg/mL gentamicin for 3 h. At 4 h post infection, cells were washed four times with 1 mL PBS and then lysed with 1 mL 0.1% Triton-X100 for 10 min at room temperature. Serial dilutions of cell suspensions were plated onto MacConkey agar and CFU were determined following overnight incubation at 37°C. The input dilution of bacteria was also plated and counted to determine the CFU for each inoculum used in the assay.

To visualize the association between bacteria and DF-1, $1x10^5$ cells per well were plated on glass coverslips in 24-well-plates and infected with an MOI of 10 CFU/cell as described above. After 1 h or 4 h post-infection, cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and stained with Giemsa for 20 min at room temperature. Samples were then washed with water and observed under a light microscope at 1,000x and photographed.

Plaque assay

Plaque assay was performed as previously described (28) with slight modifications. Overnight cultures of wild type APECO18, mutant and complemented strains were diluted 1:50 and grown to exponential phase at 37°C with shaking. Then 0.2% arabinose was added to the cultures of the complemented strains for induction of gene expression, and then incubated for 2 additional h. Bacteria were then pelleted by centrifugation at 5,700 x *g* for 7 min, washed with SorC (16.7 mM Na_2H/KH_2PO_4/50µM $CaCl_2$, pH 6.0) and resuspended in SorC to $5x10^7$ CFU/mL.

D. discoideum cells were cultured in HL5 broth and collected by centrifugation at 500 x *g* for 5 min, washed once with SorC, resuspended in SorC to $1x10^6$ cells/mL, and

serially diluted in 10-fold increments in SorC. *D. discoideum* dilutions were mixed 1:1 with bacteria to generate bacterium-to-amoeba ratios ranging from $5x10^2$:1 to $5x10^6$:1. Aliquots of 10 µL of the suspensions were spotted onto SM/5 agar plates and allowed to dry in a laminar hood under a sterile airflow. Plates were incubated at 22°C, examined at 3 and 5 d after incubation for plaque formation by *D. discoideum*, and photographed. Strains were then classified as sensitive or resistant to predation by *D. discoideum* based on the presence or absence of "predation plaques" formed by the amoeba.

Analysis of biofilm formation

To assess the involvement of the T6SS genes in the formation of biofilm by APECO18, wild-type, mutants and complemented strains were screened for biofilm formation in M63 minimal media as previously described (29). Briefly, strains were incubated overnight in LB broth that was supplemented with arabinose 0.2% (Sigma Aldrich, St. Louis, MO) where indicated. Next, cultures were diluted in minimal medium M63 supplemented with 0.2% arabinose when indicated to an OD_{600} of 0.05 in 8 wells of a 96-well plate and incubated at 37°C for 24 h. Blank wells were filled with M63 only. Cultures were poured out and plates were gently rinsed with de-ionized (DI) water. 200 µL of 0.1% crystal violet was added to each well and plates were incubated at room temperature for 30 min. The plates were then rinsed 4 times with DI water and allowed to dry at room temperature for 1 hour. Biofilm was solubilized by adding 200 µL of a 80:20 ethanol:acetone mixture. 150 µL of the resolubilized biofilm was transferred to a new 96-well plate, and OD_{600} was read using an ELX 808 Ultra microplate reader (Bio-Tek Instruments, Winooski, VT).

Statistical analysis

For the analysis of the prevalence of T6SS genes harbored by strains from different collections examined in the study, the number of genes were treated as quantitative variables and the data was analyzed using non-parametric tests due to asymmetry in the distribution of the genes. Non-parametric analysis was also applied to compare the phenotypes presented by the mutants in comparison to the WT strains. Direct comparisons (where possible) between two groups were made using the Mann-Whitney U test. All statistical analysis was performed using GraphPad Prism (Version 7.0d) for Windows (GraphPad, La Jolla, CA). Statistical significance was accepted when $p<0.05$. For adherence and invasion assays, bacterial groups were compared using Student's *t*-test and differences were considered significant when $p<0.05$.

Results

Two T6SS gene clusters are present in the genome of APECO18.

T6SS clusters are common in the genomes of *E. coli* pathotypes and are divided into three groups based on the homology and structural analysis of clusters (21). Additionally, APEC strain TW-XM was shown to harbor two clusters encoding T6SS (22). Here, genomic analysis found that APECO18 harbors two putative T6SS clusters, T6SS1 and T6SS2. The T6SS 1 cluster is 30.2 kb in length, with a GC content of 52.2%, and is flanked by tRNA genes. The T6SS2 cluster is 27.9 kb in length, with a 52% GC content and also flanked by tRNA genes. Most of the genes analyzed in this study, namely *evpB, impK* and *hcp* belong to T6SS1 cluster. Additionally, we analyzed the *icmF* gene from cluster 2. Schematic representation of these clusters is shown in Figure 5.1.

Prevalence of T6SS genes is significantly higher in APEC than in litter and AFEC isolates.

To gain insight into the relationship between the presence of T6SS genes and the pathogenesis of APEC, we used PCR to screen a collection 454 APEC, 102 litter and 106 AFEC isolates. Prototypic strain APECO18 was used as template for designing the primers. Genome analysis shows that this strain harbors at least two T6SS clusters.

The collections were analyzed for the presence of T6SS1 genes *evpB* (contractile sheath major subunit), *impK* (membrane complex), *hcp* (secretion tube and effector), *vasK* (membrane complex) and *icmF* (membrane complex). Most genes analyzed in this study (*hcp*, *impK*, *evpB* and *vasK*) belong to cluster 1, that is approximately 30-kb in size and contains 24 genes, including T6SS components and hypothetical proteins. *icmF* belongs to cluster 2.

The prevalence *evpB*, *impK* and *hcp* was significantly higher ($p<0.05$) in APEC than in litter and AFEC isolates; prevalence of *impK* and *hcp* was also significantly higher in AFEC than in litter isolates. The prevalence of *vasK* was significantly higher ($p<0.05$) in APEC than in litter isolates. *icmF* was not detected in AFEC isolates, and the prevalence did not significantly differ between litter and APEC isolates (Figure 5.1). These findings support our hypothesis that T6SS contributes to the virulence of APEC.

Mutants of T6SS genes were constructed in APECO18.

To further investigate the role of T6SS1 genes in the pathogenesis of APEC strains, isogenic deletion mutants for *evpB*, *impK*, *hcp* and *icmF* were generated using APECO18 as the parental strain (Figure 5.2). Prototypic strain APECO18, which belongs to O18 serotype and B2 phylogenetic group, was used as template for designing the primers.

Deletion of T6SS genes did not affect the growth of APECO18 in minimal medium.

To exclude that the mutations have an influence on the growth rate of the mutants, APECO18 wild-type, mutants and complemented strains were assessed for their ability to grow in minimal medium M9. No differences in the growth rate of *hcp*, *evpB*, *impK* or *icmF* mutants compared to the wild-type were detected in minimal medium (Figure 5.3).

APECO18 expresses genes encoding a T6SS.

The expression of T6SS genes by APECO18 was analyzed using semi-quantitative real time PCR. Expression analysis for T6SS genes was analyzed in *E. coli* grown in LB and DMEM media at 37°C or 42°C, as T6SS secretion has been shown to be temperature dependent in other organisms (30). All experiments were performed in biological and technical duplicates. Genes encoding 16S rRNA was used as endogenous control for data normalization. As seen in Figure 5.5, APECO18 expressed T6SS genes in both LB and DMEM with significant differences ($p<0.05$) observed according to the media or temperature used. For instance, *evpB* expression was higher in LB regardless of the temperature; *vasK* expression was higher in DMEM at 42°C.

Because expression of T6SS has been shown to be upregulated in the presence of the host (18), we assessed whether the expression of T6SS genes by APEC O18 was upregulated in the presence of chicken fibroblasts DF-1. Figure 5.6 shows that the expression of *evpB* is significantly ($p< 0.05$) enhanced at 3 h post-infection, and expression of *icmF* is significantly enhanced at 1 h post-infection and enhanced further at 3 h post-infection (Figure 5.6).

Role of T6SS in the adherence of APECO18 to chicken fibroblasts DF-1.

Because T6SS genes *icmF*, *hcp*, and *clpV* has been previously recognized as being involved in the binding of APEC Sept362 to HeLa cells (17, 20), we assessed whether *hcp* and other T6SS components (*evpB, impK*, and *icmF*) are involved in the adherence of APECO18 to chicken fibroblasts DF-1.

Surprisingly, we did not observe a negative effect of the mutation of T6SS genes *hcp*, *evpB*, *impK*, and *icmF* in the adherence capability of APECO18. An unexpected significant increase ($p<0.05$) of 90% in the adherence APECO18*ΔimpK* in relation to the WT strain was observed. Complemented strains APECO18*ΔimpK*c, APECO18*ΔevpB*c and APECO18*ΔicmF*c showed an adherence that was 2.27, 16.30 and 6.47 times higher than the wild-type respectively ($p<0.05$). These results suggest that the T6SS genes analyzed in this study are not involved in the adhesion of APECO18 to chicken fibroblasts DF-1.

Role of T6SS in the invasion of chicken fibroblasts DF-1 by APECO18.

Deletion of *hcp* and *icmF* has been shown to affect the invasion of HeLa cells by APEC Sept362 (17, 20). To investigate whether *hcp*, *evpB, impK* and *icmF* are involved in invasion of chicken fibroblasts DF-1 by APECO18, we assessed the invasion capability of APECO18 WT, mutants and complemented strains.

Surprisingly, we did not observe a negative effect of the mutation of T6SS genes in the invasion capability of the mutants compared to the wild-type strain. The decrease of invasion of 61%, 78% and 78% presented by APECO18*Δhcp*, APECO18Δ*evpB*, and APECO18*ΔvasK* respectively in relation to the wild-type strain was not statistically significant ($p>0.05$). An unexpected increase of 20% in the adherence of APECO18*ΔimpK* compared to the WT strain was observed, but it was not significant ($p>0.05$).

Complemented strain APECO18*ΔevpB*c showed an adherence that was 3.13 times the adherence presented by the wild-type APECO18 ($p<0.05$).

Deletion of *hcp* increases sensitivity of APECO18 to predation by *D. discoideum.*

The amoeba *Dictyostelium discoideum* feeds on bacteria via phagosomal and endolysosomal mechanisms similar to those employed by mammalian phagocytes (31-35). Because some genes required for resistance to predation by *D. discoideum* are also involved in replication or survival of bacteria in mammalian macrophages (36-40), and in some cases in causing disease in animals (41-47) we explored *D. discoideum* as a model for pathogenic interaction between APECO18 and macrophages.

We assessed the ability of APECO18 WT, mutants and complemented strains to resist to predation by *D. discoideum.* We did not observe a difference in phenotype between the wild-type and Δ*impK*, Δ*evpB*, and Δ*icmF* strains (data not shown). However, deletion of *hcp* affected the strain's sensitivity to predation by *D. discoideum*. At the bacteria to amoeba ratio of $5x10^2$:1, the bacterial lawn was completely consumed and replaced by *D. discoideum* fruiting bodies in the WT, mutant, and complemented strain. At the ratio $5x10^3$:1 the amoeba consumed most of the bacteria, but some of the bacterial lawn was still observed at the edges of the spot in the WT, mutant, and complemented strains. The difference between the WT strain and Δ*hcp* was observed at the bacteria-amoeba ratio of $5x10^4$:1. At this ratio, the WT strain was resistant to predation by the amoeba, with no plaques observed. However, the mutant strain became sensitive to predation with a large predation plaque observed on the bacterial lawn. The phenotype was restored in the complemented strain. This result indicates that Hcp is involved in the ability of APECO18 to resist predation by *D. discoideum* (Figure 5.6).

Effect of T6SS genes in biofilm formation by APECO18

Given that T6SS have been previously associated with biofilm formation by APEC (17), EAEC (48) and other bacteria (49, 50), we assessed whether mutations in T6SS genes would affect biofilm formation by APECO18. As shown by the growth curves, the mutants do not show growth defects compared to the wild-type strain. Using the crystal violet biofilm assay, we observed that the deletion of *hcp* and *impK* did not affect the formation of biofilm by APECO18. However, deletion of *evpB* and *icmF* significantly decreased biofilm formation on polystyrene compared to the wild-type strain ($p \leq 0.05$). Δ*evpB* formed only 58% of the biofilm formed by the wild-type APECO18, and Δ*icmF* formed 73% of the biofilm of the wild-type strain. Unexpectedly, the complemented strains APECO18Δ*evpB*c and APECO18Δ*icmF*c formed only 70% and 68% of the biofilm formed by the wild-type strain, respectively, not restoring the phenotype. Indeed, levels of biofilm formation in these complemented strains was lower than the level of the *icmF* mutant itself.

Discussion

APEC cause extra-intestinal infections in production birds known as colibacillosis, which can manifest as localized or systemic infections. The severity of the infection depends on the virulence traits of the strain, host status and predisposing factors (1). APEC presents with a diverse virulence trait repertoire, with recognized virulence factors involved in its pathogenesis (51). Although several virulence factors have been described so far, the pathogenesis of APEC strains remains unclear. Among its virulence genes, T6SS genes, *hcp*, *clpV* and *icmF* were shown to be involved in virulence-associated phenotypes displayed by APEC strain Sept 362 (17, 18). Here we analyzed the role of *hcp* and other T6SS components, *evpB*, *impK*, and *icmF* in virulence-associated traits of APECO18.

This study is also the first report the characterization of a large collection of APEC, litter-associated *E. coli,* and AFEC isolates regarding the presence of T6SS genes. The screening of these collections showed that T6SS genes are more prevalent in APEC than in AFEC and litter-associated *E. coli* isolates, which led us to further characterize the system in APECO18. We generated mutants of *hcp, evpB, impK,* and *icmF* in APECO18 and characterized these mutants regarding virulence-associated traits of APEC, including adherence to and invasion of DF-1 chicken fibroblasts, resistance to predation by *D. discoideum*, and biofilm formation.

Adherence of bacteria to host cells is important during initial stages of colonization and is usually the first step in infection caused by *E. coli* (52). T6SS has been previously associated with the adherence of APEC to their host cells. In Sept362, mutation of the T6SS genes *hcp*, *clpV* and *icmF* led to a significant reduction of the adherence of the strain to HeLa cells (17, 18). Surprisingly, we did not observe a significant negative effect of the mutation of T6SS genes in the adherence capability of APECO18. In contrast, the expression of the genes by the expression vector significantly increased the adherence of the strains compared to the wild-type.

T6SS genes have also been found to play a role in invasion of host cell model by APEC. In Sept362, *hcp* and *icmF* deletion significantly decreased the ability of the strain to invade HeLa cells (17). In the present work, we did not observe any effect from the deletion of *hcp, evpB, impK* or *icmF* in the ability of APECO18 to invade chicken fibroblasts DF-1.

We have also analyzed the resistance of wild-type and mutants to predation by the amoeba *D. discoideum*, that feeds on bacteria through well-characterized phagosomal and

endolysosomal mechanisms similar to those of mammalian phagocytes (31-35). Studies of bacterial pathogens have showed that genes required for resistance to predation by *D. discoideum* are also involved in the replication or survival of bacteria in mammalian macrophages (36-40), and in some cases in causing disease in animals (41-47). Additionally, T6SS was implicated as a virulence determinant of *V. cholerae* using the *D. discoideum* model system (7). Thus, we explored *D. discoideum* as a model for pathogenic interactions between APECO18 and macrophages. Results from ourf work show that deletion of *hcp* led to an increased sensitivity of the strain to the predation by *D. discoideum*. Hcp is both a structural component and a secreted effector of T6SS that forms a transportation channel between the inner and outer membranes of the bacteria (13). Previous studies have shown that deletion of T6SS genes caused the strain to be avirulent towards *D. discoideum.* In *V. cholerae*, deletion of T6SS genes VCA0109 through VCA0114 and VCA0119 made the strain avirulent towards the amoeba (53).

In addition to being involved in adherence to host cells, T6SS has also been associated with biofilm formation by APEC (54), EAEC (48) and other bacteria (49, 50). To check whether T6SS plays a role in the ability of APECO18 to form biofilm, we assessed biofilm formation by APECO18 wild-type, mutants and complemented strains. We did not observe an effect of deletion of *impK* and *hcp* in the formation of biofilm by APECO18. However, APECO18Δ*icmF* presented with a decrease in its biofilm formation, which agrees with previous study that showed deletion of *icmF* in Sept362 impaired its ability to form biofilms (17). IcmF is an inner membrane protein of the T6SS, and it was shown to be involved in intracellular multiplication by other pathogens (55, 56). IcmF is required for survival and replication in macrophages, intracellular growth in amoeba,

intracellular replication in eukaryotic cells and immediate cytotoxicity and exit from the phagosome (57-60).

APECO18Δ*evpB* also had its ability to form biofilm impaired. *evpB*, a structural gene of T6SS, is the large subunit that forms the contractile sheath. Previous work has shown that *evpB* mutant of the fish pathogen *E. tarda* showed lower replication ratios in phagocytes and displayed an autoaggregation phenotype when grown in DMEM (61). However, there is no information of association between *evpB* and biofilm formation by other bacteria. The complementation of the mutants with the respective genes did not restore the wild-type phenotype. This could result from the enhanced dosage of *evpB* in the complemented strain affecting expression of additional genes needed for biofilm formation. Further investigation of the role of these genes in the formation of biofilm by APEC is warranted to rule out polar effects of the mutations in the formation of biofilm.

Conclusion

T6SS plays a role in APEC pathogenesis as evidenced by its prevalence in APEC compared with AFEC isolates, its involvement in the ability of APECO18 to resist predation by *D. discoideum*, and impact in biofilm formation. Further work including *in vivo* assays to determine the role of T6SS on the virulence of APEC and its effects on the host is warranted.

REFERENCES

1. Barnes HJ, Gross WB. 1997. Colibacillosis, P 131-141, Disease Of Poultry 10th Ed.
2. Dho-Moulin M, Fairbrother JM. 1999. Avian Pathogenic *Escherichia coli* (APEC). Vet Res 30:299-316.
3. Ngeleka M, Kwaga JK, White DG, Whittam TS, Riddell C, Goodhope R, Potter AA, Allan B. 1996. *Escherichia coli* cellulitis in broiler chickens: clonal relationships among strains and analysis of virulence-associated factors of isolates from diseased birds. Infect Immun 64:3118-26.
4. Dziva F, Stevens MP. 2008. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathol 37:355-66.
5. Harry EG, Hemsley LA. 1965. The association between the presence of septicaemia strains of *Escherichia coli* in the respiratory and intestinal tracts of chickens and the occurrence of coli septicaemia. Vet Rec 77:35-40.
6. Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G. 2015. Secretion systems in Gram-Negative bacteria: structural and mechanistic insights. Nat Rev Microbiol 13:343-59.
7. Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, Heidelberg JF, Mekalanos JJ. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc Natl Acad Sci U S A 103:1528-33.

8. Boyer F, Fichant G, Berthod J, Vandenbrouck Y, Attree I. 2009. Dissecting the bacterial Type VI Secretion System by a genome wide *in silico* analysis: what can be learned from available microbial genomic resources? BMC Genomics 10:104.

9. Cascales E, Cambillau C. 2012. Structural biology of Type VI Secretion Systems. Philos Trans R Soc Lond B Biol Sci 367:1102-11.

10. Cascales E. 2008. The Type VI Secretion Toolkit. EMBO Rep 9:735-41.

11. Planamente S, Salih O, Manoli E, Albesa-Jove D, Freemont PS, Filloux A. 2016. Tssa forms a Gp6-Like ring attached to the Type VI Secretion sheath. Embo J 35:1613-27.

12. Tseng TT, Tyler BM, Setubal JC. 2009. Protein secretion systems in bacterial-host associations, and their description in the gene ontology. BMC Microbiol 9 Suppl 1:S2.

13. Pukatzki S, Mcauley SB, Miyata ST. 2009. The Type VI Secretion System: translocation of effectors and effector-domains. Curr Opin Microbiol 12:11-7.

14. Schell MA, Ulrich RL, Ribot WJ, Brueggemann EE, Hines HB, Chen D, Lipscomb L, Kim HS, Mrazek J, Nierman WC, Deshazer D. 2007. Type VI Secretion is a major virulence determinant in *Burkholderia mallei*. Mol Microbiol 64:1466-85.

15. Zheng J, Leung KY. 2007. Dissection of a Type VI Secretion System in *Edwardsiella tarda*. Mol Microbiol 66:1192-206.

16. Decoin V, Barbey C, Bergeau D, Latour X, Feuilloley MG, Orange N, Merieau A. 2014. A Type VI Secretion System is involved in *Pseudomonas fluorescens* bacterial competition. Plos One 9:E89411.

17. De Pace F, Boldrin De Paiva J, Nakazato G, Lancellotti M, Sircili MP, Guedes Stehling E, Dias Da Silveira W, Sperandio V. 2011. Characterization of Icmf of the Type VI Secretion System in an Avian Pathogenic *Escherichia coli* (APEC) strain. Microbiology (Reading) 157:2954-2962.

18. De Pace F, Nakazato G, Pacheco A, De Paiva JB, Sperandio V, Da Silveira WD. 2010. The Type VI Secretion System plays a role in type 1 fimbria expression and pathogenesis of an Avian Pathogenic *Escherichia coli* strain. Infect Immun 78:4990-8.

19. De Pace F, Boldrin De Paiva J, Nakazato G, Lancellotti M, Sircili MP, Guedes Stehling E, Dias Da Silveira W, Sperandio V. 2011. Characterization of Icmf of the Type VI Secretion System in an Avian Pathogenic *Escherichia coli* (APEC) strain. Microbiology 157:2954-62.

20. De Pace F, Nakazato G, Pacheco A, De Paiva JB, Sperandio V, Da Silveira WD. 2010. The Type VI Secretion System plays a role in type 1 fimbria expression and pathogenesis of an Avian Pathogenic *Escherichia coli* strain. Infection And Immunity 78:4990-8.

21. Ma J, Sun M, Bao Y, Pan Z, Zhang W, Lu C, Yao H. 2013. Genetic diversity and features analysis of Type VI Secretion Systems loci in Avian Pathogenic *Escherichia coli* by wide genomic scanning. Infect Genet Evol 20:454-64.

22. Ma J, Bao Y, Sun M, Dong W, Pan Z, Zhang W, Lu C, Yao H. 2014. Two functional Type VI Secretion Systems in Avian Pathogenic *Escherichia coli* are involved in different pathogenic pathways. Infect Immun 82:3867-79.

23. Mangiamele P. Toolbox for exploring Avian Pathogenic *Escherichia coli* (APEC) pathogenesis and control, P. *In* (Ed),

24. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-5.

25. Decoin V, Barbey C, Bergeau D, Latour X, Feuilloley MGJ, Orange N, Merieau A. 2014. A Type VI Secretion System is involved in *Pseudomonas fluorescens* bacterial competition. Plos ONE 9:E89411.

26. Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW. 2000. Quantitative Reverse Transcription-Polymerase Chain Reaction to study mRNA decay: comparison of endpoint and real-time methods. Anal Biochem 285:194-204.

27. Barbieri NL, Vande Vorde JA, Baker AR, Horn F, Li G, Logue CM, Nolan LK. 2017. FNR Regulates the expression of important virulence factors contributing to the pathogenicity of Avian Pathogenic *Escherichia coli*. Front Cell Infect Microbiol 7:265.

28. Hasselbring BM, Patel MK, Schell MA. 2011. *Dictyostelium discoideum* as a model system for identification of *Burkholderia pseudomallei* virulence factors. Infection And Immunity 79:2079-2088.

29. Skyberg JA, Siek KE, Doetkott C, Nolan LK. 2007. Biofilm formation by avian *Escherichia coli* in relation to media, source and phylogeny. J Appl Microbiol 102:548-54.

30. Bladergroen MR, Badelt K, Spaink HP. 2003. Infection-blocking genes of a symbiotic *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein secretion. Mol Plant Microbe Interact 16:53-64.

31. Buczynski G, Grove B, Nomura A, Kleve M, Bush J, Firtel RA, Cardelli J. 1997. Inactivation of two *Dictyostelium discoideum* genes, *ddpik1* and *ddpik2*, encoding proteins related to mammalian phosphatidylinositide 3-kinases, results in defects in endocytosis, lysosome to postlysosome transport, and actin cytoskeleton organization. J Cell Biol 136:1271-86.

32. Deshazer D, Brett PJ, Woods DE. 1998. The type II o-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. Mol Microbiol 30:1081-100.

33. Maniak M, Rauchenberger R, Albrecht R, Murphy J, Gerisch G. 1995. Coronin involved in phagocytosis: dynamics of particle-induced relocalization visualized by a green fluorescent protein tag. Cell 83:915-24.

34. Rezabek BL, Rodriguez-Paris JM, Cardelli JA, Chia CP. 1997. Phagosomal proteins of *Dictyostelium discoideum*. J Eukaryot Microbiol 44:284-92.

35. Rupper A, Cardelli J. 2001. Regulation of phagocytosis and endo-phagosomal trafficking pathways in *Dictyostelium discoideum*. Biochim Biophys Acta 1525:205-16.

36. Andrews HL, Vogel JP, Isberg RR. 1998. Identification of linked *Legionella pneumophila* genes essential for intracellular growth and evasion of the endocytic pathway. Infect Immun 66:950-8.

37. Hägele S, Köhler R, Merkert H, Schleicher M, Hacker J, Steinert M. 2000. *Dictyostelium discoideum*: a new host model system for intracellular pathogens of the Genus *Legionella*. Cell Microbiol 2:165-71.

38. Solomon JM, Leung GS, Isberg RR. 2003. Intracellular replication of *Mycobacterium marinum* within *Dictyostelium discoideum*: efficient replication in the absence of host coronin. Infect Immun 71:3578-86.

39. Solomon JM, Rupper A, Cardelli JA, Isberg RR. 2000. Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. Infect Immun 68:2939-47.

40. Steinert M, Heuner K. 2005. *Dictyostelium* as host model for pathogenesis. Cell Microbiol 7:307-14.

41. Alibaud L, Köhler T, Coudray A, Prigent-Combaret C, Bergeret E, Perrin J, Benghezal M, Reimmann C, Gauthier Y, Van Delden C, Attree I, Fauvarque MO, Cosson P. 2008. *Pseudomonas aeruginosa* virulence genes identified in a *Dictyostelium* host model. Cell Microbiol 10:729-40.

42. Cosson P, Zulianello L, Join-Lambert O, Faurisson F, Gebbie L, Benghezal M, Van Delden C, Curty LK, Köhler T. 2002. *Pseudomonas aeruginosa* virulence analyzed in a *Dictyostelium discoideum* host system. J Bacteriol 184:3027-33.

43. Finck-Barbançon V, Goranson J, Zhu L, Sawa T, Wiener-Kronish JP, Fleiszig SM, Wu C, Mende-Mueller L, Frank DW. 1997. ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Mol Microbiol 25:547-57.

44. Hauser AR, Kang PJ, Engel JN. 1998. Pepa, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. Mol Microbiol 27:807-18.

45. Pearson JP, Feldman M, Iglewski BH, Prince A. 2000. *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. Infect Immun 68:4331-4.

46. Pukatzki S, Kessin RH, Mekalanos JJ. 2002. The human pathogen P*seudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. Proc Natl Acad Sci U S A 99:3159-64.

47. Vance RE, Rietsch A, Mekalanos JJ. 2005. Role of the type III secreted exoenzymes S, T, and Y in systemic spread of *Pseudomonas aeruginosa* PAO1 *in vivo*. Infect Immun 73:1706-13.

48. Aschtgen MS, Bernard CS, De Bentzmann S, Lloubès R, Cascales E. 2008. SciN is an outer membrane lipoprotein required for Type VI Secretion in enteroaggregative *Escherichia coli*. J Bacteriol 190:7523-31.

49. Stewart BJ, Enos-Berlage JL, Mccarter LL. 1997. The *lonS* gene regulates swarmer cell differentiation of *Vibrio parahaemolyticus.* J Bacteriol 179:107-14.

50. Waite RD, Paccanaro A, Papakonstantinopoulou A, Hurst JM, Saqi M, Littler E, Curtis MA. 2006. Clustering of *Pseudomonas aeruginosa* transcriptomes from planktonic cultures, developing and mature biofilms reveals distinct expression profiles. BMC Genomics 7:162.

51. Logue CM, Doetkott C, Mangiamele P, Wannemuehler YM, Johnson TJ, Tivendale KA, Li G, Sherwood JS, Nolan LK. 2012. Genotypic and phenotypic traits that distinguish Neonatal Meningitis-Associated *Escherichia coli* From Fecal *E. Coli* isolates of healthy human hosts. Appl Environ Microbiol 78:5824-30.

52. Antão EM, Wieler LH, Ewers C. 2009. Adhesive threads of Extraintestinal Pathogenic *Escherichia coli*. Gut Pathog 1:22.

53. Zheng J, Ho B, Mekalanos JJ. 2011. Genetic analysis of anti-amoebae and anti-bacterial activities of the Type VI Secretion System in *Vibrio cholerae*. Plos One 6:E23876.

54. Sarachu M, Colet M. 2005. Wemboss: A web interface for EMBOSS. Bioinformatics 21:540-541.

55. Hilbi H, Segal G, Shuman HA. 2001. Icm/Dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. Mol Microbiol 42:603-17.

56. Watarai M, Derre I, Kirby J, Growney JD, Dietrich WF, Isberg RR. 2001. *Legionella pneumophila* is internalized by a macropinocytotic uptake pathway controlled by the Dot/Icm system and the mouse *lgn1* locus. J Exp Med 194:1081-96.

57. Kirby JE, Vogel JP, Andrews HL, Isberg RR. 1998. Evidence For Pore-Forming Ability By *Legionella pneumophila*. Mol Microbiol 27:323-36.

58. Pratt LA, Kolter R. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type i pili. Mol Microbiol 30:285-93.

59. Purcell M, Shuman HA. 1998. The *Legionella pneumophila* IcmGCDJBF genes are required for killing of human macrophages. Infect Immun 66:2245-55.

60. Vanrheenen SM, Duménil G, Isberg RR. 2004. Icmf and Dotu are required for optimal effector translocation and trafficking of the *Legionella pneumophila* vacuole. Infect Immun 72:5972-82.

61. Rao PS, Yamada Y, Tan YP, Leung KY. 2004. Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. Mol Microbiol 53:573-86.

62. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-6645.

63. Guzman LM, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. J Bacteriol 177:4121-4130.

Table 5.1. Plasmids and strains used in this study.

Plasmids	**Genotype/description**	**Ref.**
pKD46	Lambda Red recombinase expression plasmid	(62)
pKD3 Amp^r	template plasmid for FRT-flanked Amp cassette	(62)
pKD3 Cm^r	template plasmid for FRT-flanked Cm cassette	(62)
pCP20	FLP recombinase expression plasmid	(62)
pBAD24	Cloning vector	(63)
pBAD24-*hcp*	pBAD24 with *hcp*	This work
pBAD24-*evpB*	pBAD24 with *evpB*	This work
pBAD24-*impK*	pBAD24 with *impK*	This work
pBAD24-*vasK*	pBAD24 with *vasK*	This work
pBAD24-*icmF*	pBAD24with *impG*	This work
pBAD24-FLAG:*hcp*	pBAD24 with FLAG*hcp*	This work
Strains	**Genotype/description**	**Ref.**
E. coli DH5α	F−Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ– thi-1 gyrA96 relA1	Lab stock
APECO18 (380)	APECO18 WT	Lab stock
APEC*Δhcp*	APECO18 with *hcp* deleted by λ red rec.	This work
APEC*ΔevpB*	APECO18 with *evpB* deleted by λ red rec.	This work
APEC*ΔimpK*	APECO18 with *impK* deleted by λ red rec.	This work
APEC*ΔicmF*	APECO18 with *icmF* deleted by λ red rec.	This work
APEC*Δhcp*-p*hcp*	APEC*Δhcp* with *hcp* cloned into pBAD24	This work
APEC*ΔevpB*-p*evpB*	APEC*ΔevpB* with *evpB* cloned into pBAD24	This work
APEC*ΔimpK*-p*impK*	APEC*ΔimpK* with *impK* cloned into pBAD24	This work
APEC*ΔicmF*-p*icmF*	APEC*ΔicmF* with *icmF* cloned into pBAD24	This work

Table 5.2. Primers used in this study.

Primer	Sequence (5'-3')	Ref.
	Screening of T6SS genes	
*hcp*_Fw	cgaaggtagcatcgaagtgg	This work
*hcp*_Rv	ttaaactccgccgttttcag	This work
*evpB*_Fw	agcgattcacgttctgcttt	This work
*evpB*_Rv	tgcacacaccagccattatt	This work
*impK*_Fw	ccactccagtcgcatttctt	This work
*impK*_Rv	attcagccagtggtgtagcc	This work
*vasK*_Fw	tcagcctgcaaacgtatctg	This work
*vasK*_Rv	cggaagtttttcagctccag	This work
*icmF*_Fw	acaacgaggcggtaaaacag	This work
*icmF*_Rv	atgtaacgaacggcttccac	This work
	Gene deletion	
*hcp*Del_F:	atggctattcctgcttatctctggctgaaagatgacggcggcgcggatat[1]	This work
*hcp*Del_R:	tcaggcggaaggacgctcattccacgagtcggaatgaatgatgttgccgt[2]	This work
*evpB*Del_F	atgctgatgtctgtacaacaagaacattccacctctgaaactgcaacact[1]	This work
*evpB*Del_R	tcaggctttcgctttcggcatctgggaaaccagagaaaggttgatatcca[2]	This work
*impK*Del_F:	atgaaaaaagatatggatatcaatatcgatgcgctgctgcgcgacacgtt[1]	This work
*impK*Del_R:	ttaacgcaggctttgcggcagcagttcatccaccagtacattcagccagt[2]	This work
*vasK*Del_F:	atgcatactcagcgttacggtttctggggatatttaattctgattattgg[1]	This work
*vasK*Del_R:	tcactcatcggcgtcttccccatactcttccgcatccttcatactcctgc[2]	This work
*icmF*Del_F:	gtgttcaaatttcccacatcccgactgttcagcacgttgaaatctgcgct[1]	This work
*icmF*Del_R:	ttaatacaacgtatccggtaaacggaacaggctgaacagaccgccggtga[2]	This work
	Check deletion	
*hcp*Check_F:	atcagtcttgttccgcgttc	This work
*hcp*Check_R:	tcaccagattgtgggtatgc	This work
*evpB*Check_F	tcagaactgcgtgatgaactg	This work
*evpB*Check_R	ctgctgctgaaactgctgag	This work
*impK*Check_F:	gctggatatgcacagtgacg	This work
*impK*Check_R:	aaacactgaccacagcacca	This work
*vasK*Check_F:	ggccctacaggagagtttat	This work
*vasK*Check_R:	cgcgtatacgcaggaaatcg	This work
*icmF*Check_F:	cagcagtaccggatgctctt	This work
*icmF*Check_R:	cagtttccagttcagctccg	This work
	Gene complementation	
*hcp*_Fw_*XbaI*	gcgtctagagatggctattcctgcttatct	This work
*hcp*_Rv_*HindIII*	gcgttcgaagctcaggcggaaggacgctcat	This work

Primer	Sequence (5'-3')	Ref.
*evpB*_Fw_*XbaI*	gcgtctagagatgctgatgtctgtacaaca	This work
*evpB*_Rv_*HindIII*	gcgttcgaagctcaggctttcgctttcggca	This work
*impK*_Fw_*XbaI*	gcgtctagagatgaaaaaagatatggatat	This work
*impK*_Rv_*HindIII*	gcgttcgaagcttaacgcaggctttgcggca	This work
*vasK*_Fw_*XbaI*	gcgtctagagatgcatactcagcgttacgg	This work
*vasK*_Rv_*HindIII*	gcgttcgaagctcactcatcggcgtcttccc	This work
*icmF*_Fw_*XbaI*	gcgtctagaggtgttcaaatttcccacatc	This work
*icmF*_Rv_*HindIII*	gcgttcgaagcttaatacaacgtatccggta	This work
Check insertion in pBAD24		
pBAD24_Fw	atgccatagcatttttatcc	
pBAD24_Rv:	gatttaatctgtatcagg	

[1] Forward primer extension to amplify chloramphenicol resistance cassette (tgtaggctggagctgcttcg).
[2] Reverse primer extension to amplify chloramphenicol resistance cassette (atgggaattagccatggtcc).

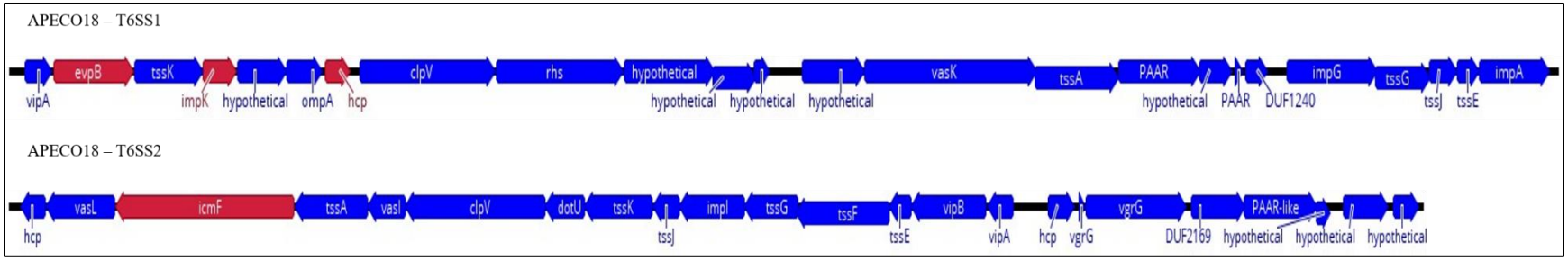

Figure 5.1. Schematic diagram of the genetic organization of APECO18 T6SS gene clusters. The direction of the arrows indicates the direction of transcription. Arrows colored in red indicate the genes that were individually deleted from the APECO18 genome.

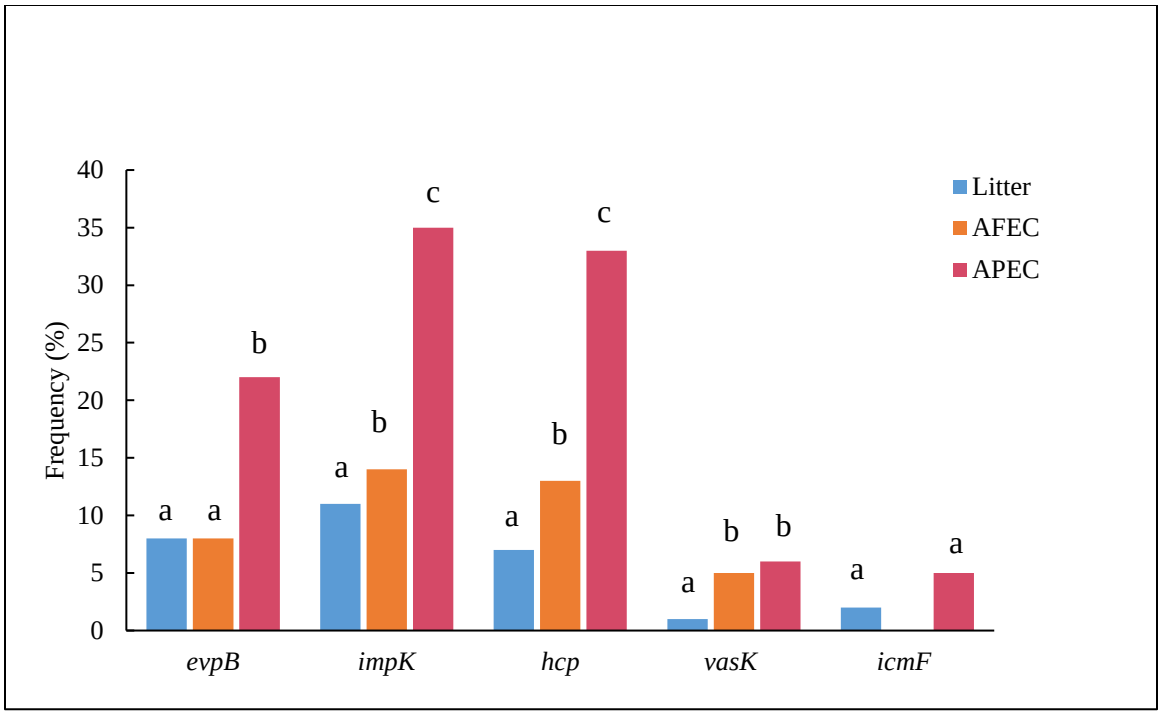

Figure 5.2. Histogram comparing the frequencies of genes *evpB*, *impK*, *hcp*, *vasK* and *icmF* in litter, AFEC and APEC isolates. Different letters above bars indicate that the prevalence of the gene was significantly different between groups ($p<0.05$).

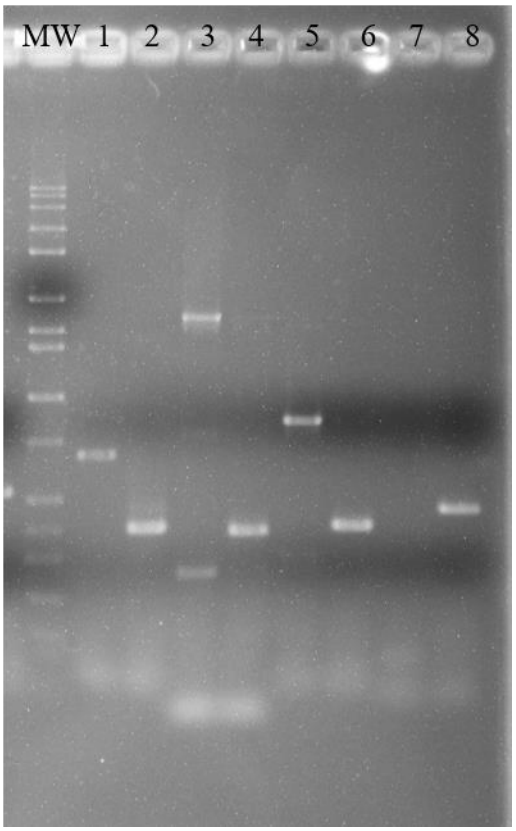

Figure 5.3. Construction of T6SS mutants in APECO18. MW - Molecular weight marker. Lane 1: WT *hcp*; Lane 2: Δ*hcp*; Lane 3: WT *evpB*; Lane 4: Δ*evpB*; Lane 5: WT *impK*; Lane 6: Δ*impK*; Lane 7: WT *icmF*; Lane 8: Δ*icmF*.

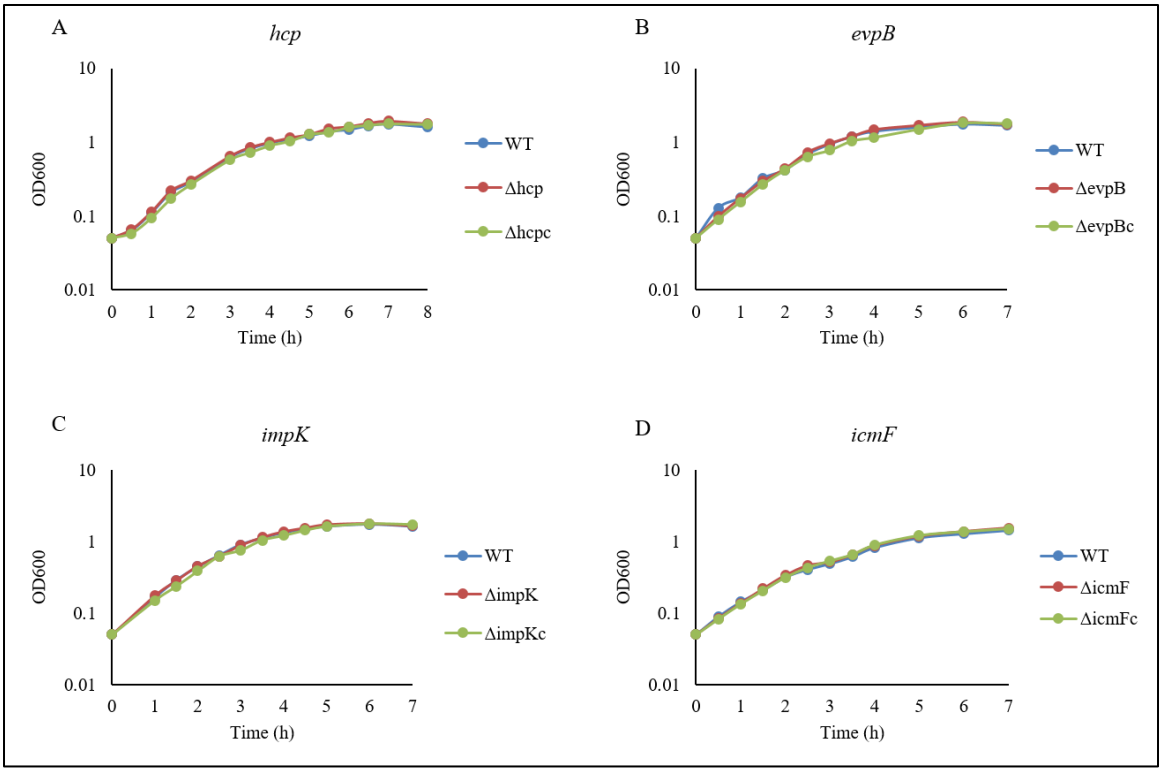

Figure 5.4. Deletion of T6SS genes does not affect the growth of APECO18 in minimal media. Growth curves of (A) APECO18 WT, Δ*hcp*, Δ*hcp*c, (B) APECO18 WT, Δ*evpB*, Δ*evpB*c, (C) APECO18 WT, Δ*impK*, Δ*impK*c, (D) APECO18 WT, Δ*icmF*, Δ*icmF*c at 37°C. The expression of the genes in the complemented strains was induced with 1.5 mM L-arabinose.

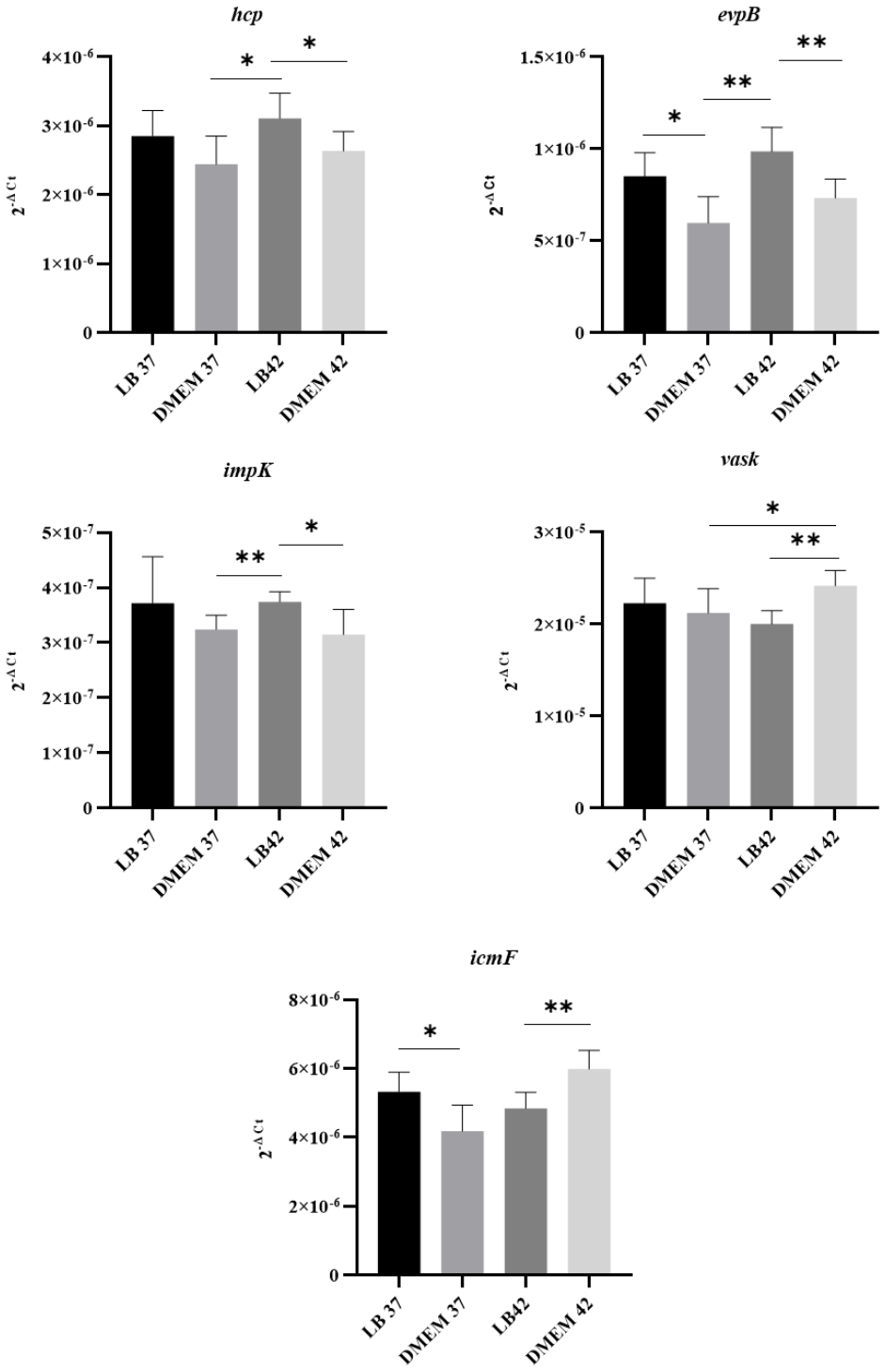

Figure 5.5. APECO18 expresses genes encoding a T6SS. qRT-PCR of *hcp*, *evpB*, *impK*, *vasK* and *icmF* genes by APECO18 in LB and DMEM at 37°C and 42°C. *, $p<0.05$.

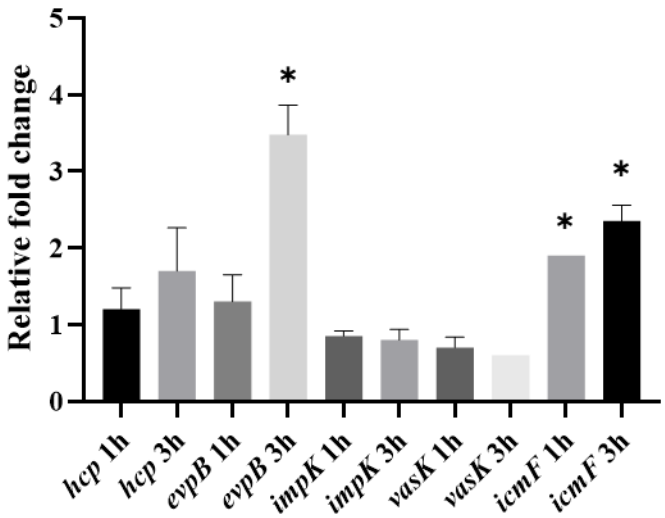

Figure 5.6. Expression of T6SS genes by APECO18 is enhanced post-infection of DF-1 cells. Expression of *evpB* enhanced at 3h post infection, and expression of *icmF* is enhanced at 1h and 3h post infection. Fold change is relative to APECO18 grown in DMEM at 37°C. *, $p<0.05$.

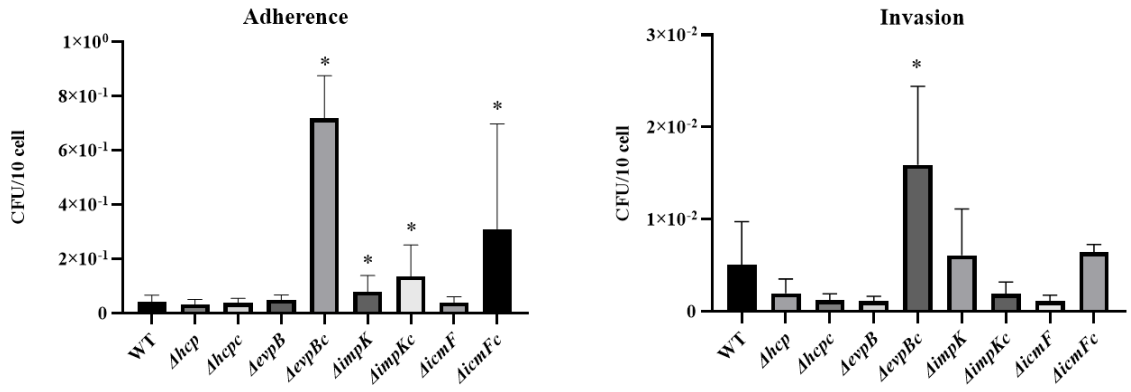

Figure 5.7. Role of T6SS in adherence to and invasion of DF-1 chicken fibroblasts by APECO18. (A) Quantification of adherence of APECO18 wild-type, mutant and complemented strains to DF-1. (B) Quantification of invasion of DF-1 by APECO18 wild-type, mutant and complemented strains (*, $p<0.05$).

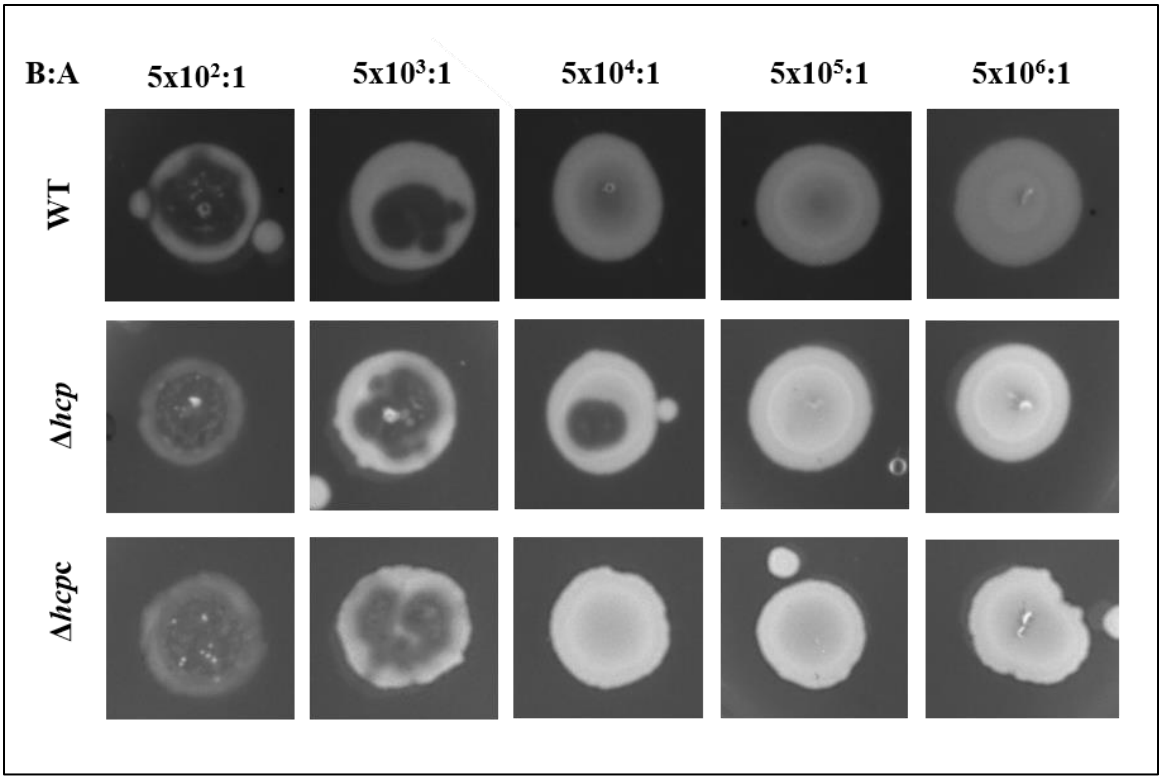

Figure 5.8. Deletion of *hcp* increases sensitivity of APECO18 to predation by *D. discoideum*. Figure shows predation plaque assay for wild-type, mutant (Δ*hcp*) and complemented strain (Δ*hcpc*) at different bacteria-to amoeba rations (B:A).

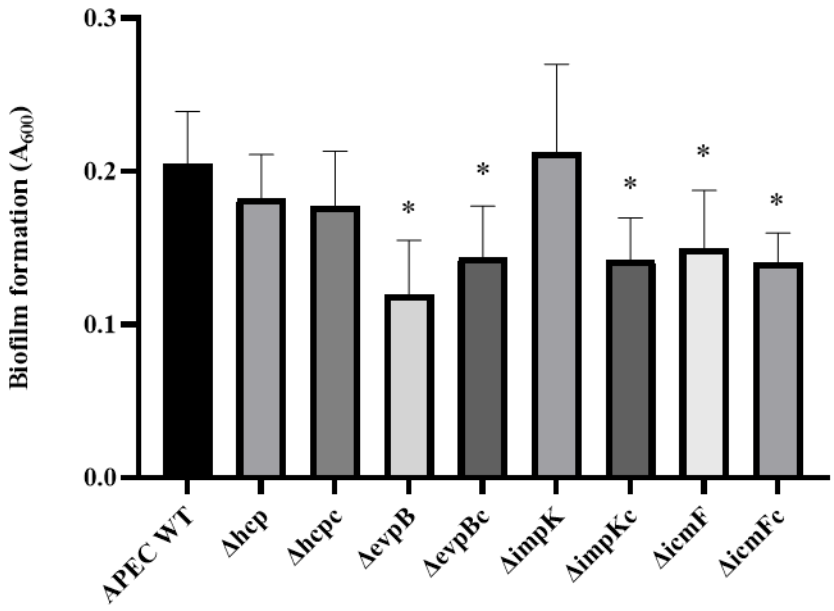

Figure 5.9. Biofilm formation by APECO18 wild-type, mutants, and complemented strains. Cells were grown for 24 h in a 96-well plate containing M63. *, $p<0.05$, indicates statistically significant difference between the strain and the wild-type.

CHAPTER 6

COMPLETE GENOME SEQUENCE OF THE NEONATAL MENINGITIS *Escherichia coli* SEROTYPE O18:K1 NMEC15[1]

[1]De Oliveira, A.L., T. J. Johnson, N.L. Barbieri, and C.M. Logue. To be submitted to Microbiology Resource Announcements (MRA) - ASM.

Abstract

Neonatal meningitis *E. coli* (NMEC) is the second leading cause of sepsis and meningitis in neonates worldwide. Here we report the genome sequence of NMEC, belonging to serotype O18:K1, isolated from the cerebrospinal fluid (CSF) of an infant with neonatal bacterial meningitis (NBM) from the Netherlands.

Announcement

Neonatal meningitis *E. coli* (NMEC) is the second leading cause of sepsis and meningitis in neonates worldwide (1). However, these strains have emerged as the most common cause of meningitis and sepsis among very low birth weight infants (<1500 g birth weight) since the 1990s (2-5).

Strain NMEC15 (serotype O18:K1) was isolated from the CSF of a newborn infant (< 28 days old) with meningitis from the Netherlands (6). O serogroup was identified at the *E. coli* reference center at Pennsylvania State University. NMEC15 belongs to serotype O18:K1, similarly to prototypic NMEC strain RS218 (7). Here we present the genome sequence of NMEC15.

NMEC15 was grown on LB agar and subsequently in Luria-Bertani broth at 37˚C. Genomic DNA (gDNA) was extracted using the ChargeSwitch gDNA mini bacteria kit (Life Technologies, Carlsbad, CA) for Illumina sequencing. DNA yields were quantified using a Qubit fluorimeter double-stranded DNA (dsDNA) HS kit (Life Technologies). The QIAseq FX kit (Qiagen, Germantown, MD) was used to prepare the genomic library for Illumina 2X300bp MiSeq sequencing. Raw reads were subjected to quality processing using Trimmomatic to remove low quality reads/regions and remove Illumina adapters. Reads were then assembled using Shovill with the SPaDES assembler. Following

trimming, a total of 124,468,910 base reads were used for assembly, corresponding to approximately 23.5x average genome coverage. The assembled genome size was 5,291,404 bp organized into 268 contigs. The strain harbors plasmids IncFIB and IncFII.

Two clusters for T6SS were found in NMEC15 genome: T6SS1 and T6SS2. The T6SS 1 cluster is 30.2 kb in length, with a GC content of 52.2%. T6SS2 cluster is 27.9 kb in length, with a 52% GC content.

Resistance genes included *sul1*, for resistance to sulfonamide, and *aadA1*, for resistance to aminoglycoside. *E. coli* virulence factors include increased serum survival gene (*iss*), the S-fimbriae minor subunit (*sfaS*), and the vacuolating autotransporter toxin (*vat*).

Data availability

The genome has been deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) under BioProject PRJNA732675, BioSample Accession SAMN19334708.

REFERENCES

1. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark AR, Tyson JE, Oh W, Bauer CR, Korones SB, Shankaran S, Laptook AR, Stevenson DK, Papile LA, Poole WK. 2004. To tap or not to tap: high likelihood of meningitis without sepsis among very low birth weight infants. Pediatrics 113:1181-1186.
2. Stoll BJ, Hansen NI, Higgins RD, Fanaroff AA, Duara S, Goldberg R, Laptook A, Walsh M, Oh W, Hale E. 2005. Very low birth weight preterm infants with early onset neonatal sepsis: the predominance of gram-negative infections continues in the National Institute of Child Health and Human Development Neonatal Research Network, 2002-2003. Pediatr Infect Dis J 24:635-9.
3. Bizzarro MJ, Dembry LM, Baltimore RS, Gallagher PG. 2008. Changing patterns in neonatal *Escherichia coli* sepsis and ampicillin resistance in the era of intrapartum antibiotic prophylaxis. Pediatrics 121:689-96.
4. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark AR, Tyson JE, Oh W, Bauer CR, Korones SB, Shankaran S, Laptook AR, Stevenson DK, Papile LA, Poole WK. 2002. Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. N Engl J Med 347:240-7.
5. Camacho-Gonzalez A, Spearman PW, Stoll BJ. 2013. Neonatal infectious diseases: evaluation of neonatal sepsis. Pediatr Clin North Am 60:367-89.
6. Johnson JR, Oswald E, O'Bryan TT, Kuskowski MA, Spanjaard L. 2002. Phylogenetic Distribution of Virulence-Associated Genes among *Escherichia coli*

Isolates Associated with Neonatal Bacterial Meningitis in The Netherlands. The Journal of Infectious Diseases 185:774-784.

7. Wijetunge DS, Karunathilake KH, Chaudhari A, Katani R, Dudley EG, Kapur V, DebRoy C, Kariyawasam S. 2014. Complete nucleotide sequence of pRS218, a large virulence plasmid, that augments pathogenic potential of meningitis-associated *Escherichia coli* strain RS218. BMC Microbiol 14:203.

CHAPTER 7

CONCLUSION AND FUTURE WORK

Neonatal meningitis *E. coli* and avian pathogenic *E. coli* are both a concern for public health. NMEC is one of the most common causative agents of neonatal meningitis in newborns and low birth weight infants. It is estimated that about 20% of cases of neonatal meningitis are caused by *E. coli*. APEC, the etiological agent of extra-intestinal infection in production birds, leads to a reduced quality of poultry and turkey production causing significant economic impact for the industry. Additionally, APEC strains present a potential for zoonotic transmission as poultry serves as the main host for APEC, and the poor handling or consumption of undercooked meat can lead to potential infection of humans, which can serve as a reservoir for this pathotype. Besides poultry, APEC can also affect other bird species, including turkey.

This work is the first to provide an extensive characterization of a large collection of *E. coli* isolated from turkey lesions and litter, presented in chapter 3 of this dissertation. *E. coli* isolates were analyzed regarding their virulence-associated, antimicrobial resistance and heavy metal resistance genotypes. Additionally, phylogenetic grouping and PCR-based serogrouping, and PFGE analysis were performed. Results found in this analysis highlight the importance of not overlooking this organism as an impactful pathogen for turkey production also.

Understanding the traits involved in the virulence of ExPEC is crucial for understanding the infection process and for the development of means to control it. The

mechanisms of pathogenesis for APEC and NMEC still warrant investigation as there are many aspects that are still poorly understood. Secretion systems have been characterized as important tools involved in bacterial pathogenesis and fitness and have recently gained interest as a possible virulence factors of APEC and NMEC strains.

Chapters 4 and 5 of this dissertation consist in the characterization of T6SS genes in APECO18 and NMEC15. A large collection of APEC, AFEC, NMEC, and HFEC isolates were characterized regarding the presence of several T6SS genes. The frequency of these genes was found to be significantly higher in the pathogenic *E. coli* isolates than in isolates from healthy hosts, providing further evidence of the involvement of T6SS in the pathogenesis of ExPEC. The creation of deletion mutants for the T6SS genes aimed to characterize these genes and analyze their role in virulence-related phenotypes in both APEC and NMEC to help build the knowledge of T6SS in the pathogenesis of APEC and NMEC.

Deletion of T6SS genes in APECO18 and NMEC15 affected different virulence-associated phenotypes of the strains, including adherence and invasion to host cell models, resistance to predation by the amoeba *D. dictyostelium* and the ability of the strain to form biofilm. These findings corroborate with previous findings and contribute to the knowledge on the role of T6SS in ExPEC strains, which is still a growing field with only a few reports in the literature. Finally, this work provides some new evidence of the role of the T6SS in APEC and NMEC which to date has not been well characterized, and the contribution of this research will significantly enhance our knowledge of how this pathogen behaves and its impact on the hosts. This work builds for future work to continue to dissect the role of secretion systems in ExPEC and their role in pathogenesis and disease.

Future directions of this work include the analysis of the expression of a recombinant FLAG-tagged Hcp protein by APECO18 and NMEC15 as well as the analysis of secretion of this protein by wild-type and mutant strains. Hcp secretion is the hallmark of T6SS and it has been previously shown that deletion of core structural genes of T6SS prevent the secretion of this effector. It will be of interest to analyze if any of the structural T6SS genes, namely *evpB*, *impK*, *vasK* and *icmF*, analyzed in this work, would have a direct effect on the secretion of the recombinant protein, FLAG-Hcp, by APECO18 and NMEC15. The FLAG-Hcp recombinant protein is the approach chosen used as the availability of commercial Hcp antibodies is scarce and the variability of the Hcp proteins, even within the same cluster is high, making it hard to select a commercially synthetized antibody for this purpose.

The FLAG-Hcp expressing the FLAG tag on its N-terminus was already constructed, and the cloning vector containing the recombinant protein was transformed into APECO18 and NMEC15 wild-type and mutants. Expression and secretion of FLAG-Hcp will be analyzed by Western blot using an anti-FLAG antibody. We expect that deletion of at least one of the structural genes *evpB*, *impK*, *vasK* or *icmF* should impair the strains' ability to secrete the effector Hcp.