CORRELATING GENETIC AND PHENOTYPIC CHARACTERISTICS IN AVIAN PATHOGENIC *ESCHERICHIA COLI* AS A MODEL ENVIRONMENTAL PATHOGEN

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Food Science

Winter 2013

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ACKNOWLEDGMENTS

First and foremost, I would like to thank my wonderful advisor Dr. Kniel for all of her help and guidance throughout my time as a graduate student. Her kind and caring ways were just what I needed, and without her continued support I could not have completed this journey. I would also like to say a special thanks to Dr. Parcells for his assistance in getting me into the food science graduate program at the University of Delaware. Thinking back to my undergraduate studies, I would be remiss to not thank Dr. Gelb for being an excellent advisor and mentor.

I would like to thank Dr. Jie Wei for teaching me many of the lab skills that I would use throughout my graduate research. Without her continued encouragement I would have been lost. I would also like to thank Sarah Markland as being my "go-to" colleague, and for being a crutch when times got tough. For helping me out in the lab, I am very thankful for my lab-mates Adrienne, Kirsten, Qing, Pat, Krystal, Lindsey, Angela, and Rachel. For being my writing partner and continuously motivating me, I will always be thankful to Wenny. I am also grateful for the friendship, knowledge, and support given to me by of all the food science graduate students.

Finally, I need to take a moment to thank my family and my friends for never leaving my side for what at times was a very difficult journey. To my girlfriend Melissa; I am forever grateful for you putting up with me, and without you by my side I truly do not think I could have accomplished this. To my mom, dad, and brother; I am eternally thankful for you pushing me to be the best I can be, and more importantly, being the greatest family I could ever ask for.

This thesis is dedicated to Dr. John Dohms and his work in this field.

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ABSTRACT

Avian pathogenic *E .coli* (APEC) isolates are widespread in poultry flocks and cause colibacillosis in birds resulting in severe economic losses every year in the U.S. APEC is part of the group of extraintestinal pathogenic *E. coli* (ExPEC), a group which causes a wide variety of diseases in both animals and humans. Although APEC is not normally associated with enterohemorraghic *E. coli* (EHEC), there have been recent reports linking APEC with human disease and the finding of *stx*1 and *stx*2 genes in APEC may increase this risk. Also, APEC strains are closely related to human uropathogenic *E.coli* (UPEC), which could be a separate cause for concern.

Bacterial pathogens may pick up virulence factors that change their genotype and thus alter their phenotype. Such was the case with the novel STEC *E. coli* O104:H4 which caused a large outbreak associated with sprouted Fenugreek seeds. APEC may contain an array of virulence factors, some potentially hazardous to public health. Because APEC isolates can be isolated from a range of retail foods and have the potential to be a zoonotic risk, there is a growing concern of APEC contamination in our food supply.

The purpose of this study was to characterize APEC strains isolated from poultry flocks in Delaware and better understand their role in causing foodborne diseases and acting as a zoonotic agent. Three-hundred and twenty APEC strains had been earlier isolated from lesions in diseased poultry on the Delmarva peninsula. These APEC strains spanned a wide variety of O-types including O157 and were

isolated from a numerous different types of lesions. A total of 100 APEC isolates consisting of a variety of O-types and lesion locations were chosen for further use in this study.

The 100 APEC isolates were screened for eight different EHEC virulence genes which included *stx1*, *stx2*, *eaeA*, *espA*, *katP*, *espP*, *stcE*, and *ehxA*. A total of 28 APEC isolates possessed one gene each, with none of them having *stx1* or *stx2* and only one of them having *eaeA*. The 100 isolates were also screened for seven different ExPEC virulence genes which included *iss*, *iucD*, *papC*, *astA*, *vat*, *tsh*, and *cva/cvi*. A total of 87 APEC isolate possessed one to five of these genes. A challenge study was performed on a flock of 30 broilers to determine if the APEC infected birds would shed the bacteria in their feces. It could not be determined that the broilers were shedding APEC in their feces, because inconclusive evidence was found to support this claim.

The 28 APEC strains shown to possess an EHEC gene were chosen for further evaluation via two attachment assays. The first trial quantified the level of APEC to strongly attach to retail chicken meat. The APEC isolates attached at a wide variety of levels, but a total of five isolates all attached more strongly than both *E. coli* O157:H7 and O104:H4. The second trial tested the amount of bacteria to attach to the HCT-8 human intestinal cell-line. Again the APEC had a wide array of attachment abilities, but ten isolates were able to attach at a higher level than both of the *E. coli* outbreak strains.

The two attachment assays showed that many of the APEC isolates were able to attach at a level significantly higher than *E. coli* strains involved in large outbreaks. This shows that with the acquisition of additional EHEC virulence genes or even with

the ExPEC virulence genes they currently possess, that APEC may be a zoonotic risk. However, persistence through the farm to fork continuum must still be researched.

Chapter 1

INTRODUCTION

Although avian pathogenic *E. coli* (APEC) are a group of *E. coli* that are known to cause disease in birds, there is simply not enough known about the nature of *E. coli* as a whole to consider them as an insignificant threat for humans. In light of the recent *E. coli* O104:H4 outbreak in Germany that possessed a dual EHEC/EAEC virulence repertoire, it has become evident that the focus should not be solely on the O157:H7 serotype, the 'big six' Shiga toxigenic *E. coli* associated with beef contamination (O26, O45, O103, O111, O121, O145), or even more broadly just on the Shiga toxin-producing *E. coli* pathotype (STEC). This recent O104:H4 outbreak has demonstrated how devastating an outbreak from a pathogenic *E. coli* strain possessing dual virulence properties can be. There is an obvious and increasing need to learn more about environmental *E. coli* such as APEC to assess their potential ability to be dangerous zoonotic pathogens.

Avian pathogenic *E .coli* isolates are widespread in poultry flocks and cause colibacillosis as well as respiratory infections in birds resulting in severe economic losses every year in the United States (Barnes *et al.*, 2008). APEC strains are members of the extraintestinal pathogenic *E. coli* group along with human uropathogenic *E. coli* (UPEC), to which they are closely related. Because of this similarity, there have been recent reports linking APEC as disease-causing agents in humans (Ewers et al, 2007). Additionally, APEC have been isolated from a range of retail foods including chicken, turkey parts, ground pork, pea pods, and vegetable dip

(Johnson, 2005). The finding of particular virulence genes in APEC increases the potential risk of APEC transmission for human disease.

A goal of this project was to characterize APEC strains isolated from poultry flocks in Delaware in order to provide information to preserve public health. Information gained from these isolates could potentially be used in determining when to classify environmental isolates of *E. coli* as potential zoonotic agents. This could be achieved by analyzing whether there is a correlation between certain genes with adherence and infection.

Overall there is a need for a better understanding of environmental microbes and how they may contaminate food because microbes are ubiquitous in almost every niche of Earth. Some stunning examples of this as it applies to public health are that 51% of reusable kitchen bags are positive for coliforms, or 80% of shopping carts in Maine are positive for *E. coli* (Gerba, 2011). These two examples showcase the need to better understand the role of various types of environmental *E. coli* which could have an impact on public health. If APEC or other environmental isolates possess similar characteristics to *E. coli* pathotypes such as UPEC, EHEC, and EAEC that are known to be virulent towards humans, than they may represent a potential environmental contamination issue that must be addressed.

If APEC were to pose a serious zoonotic risk, foods such as raw poultry and fresh produce would be potential vectors. Produce could become contaminated by fecal dissemination from wild birds or through the presence of contaminated poultry litter on farms. Poultry litter is often used in composting and the final product is used as fertilizer for agricultural fields. If the composting is done improperly, then the poultry feces within the litter becomes a possible food safety hazard. It is unknown to

what degree, if any, broilers infected with APEC will shed APEC in their feces and the possible food safety hazard this could present if this pathogen is ever deemed dangerous to humans.

Classifying certain APEC or environmental *E. coli* isolates as environmental pathogens and threats to food safety requires a great deal of effort and is not simple due to the fact that hundreds and potentially thousands of diverse strains exist. A first step is to evaluate APEC for the possession of virulence factors that are known to cause disease in humans. While this may be uncommon, two fundamental evolutionary processes that could trigger this rare occurrence are sporadic mutation and horizontal transfer (Johnson, 2002). These processes could occur in broilers, poultry litter or within other areas of the poultry management environment. A second concern is to interpret whether the bacteria are transmitted through poultry and into the environment. A third and final factor for consideration is the ability of APEC to interact with food products and exert different attachment phenotypes, regardless of genotype. This question of attachment can be extended to an *in vitro* model using human cells.

Chapter 2

LITERATURE REVIEW

2.1 *Escherichia coli:* An Overview

Escherichia coli was first described in1885 by the German pediatrician Theodore Escherich (Donnenberg, 2002). It is easily one of the most studied organisms on Earth. Studies carried out on *E. coli* are responsible of our understanding of ideas such as genetic recombination, DNA replication, RNA transcription, and protein synthesis (Neidhart, 1996). *E. coli* is not only a valuable scientific tool, but it is also a multispecies pathogen of wide concern. For these reasons, *E. coli* will be studied by scientists, doctors, and epidemiologists alike for as long as it exists.

E. coli is a gram-negative, facultative anaerobic rod-shaped bacterium that is found in the lower intestine of humans and animals (Feng *et al.*, 2002). Most *E. coli* strains are part of the normal microflora of the gut and are nonpathogenic. They have a symbiotic relationship with the host by having a place to thrive in the gut while protecting the host from other pathogenic bacteria that may enter the intestine (Conway, 1995; Neill *et al.*, 1994). *E. coli* is a part of the Enterobacteriaceae family which includes other foodborne pathogens such as *Salmonella, Shigella,* and *Yersinia* (Ewing, 1986).

While most strains of *E. coli* are nonpathogenic, one of the leading pathogens responsible for causing foodborne illness is within this same genus and species of bacteria (Feng *et al.*, 2002). Birds, pigs, cattle, and humans are among susceptible

hosts, and the gastrointestinal tract, and kidneys are some of the target organs that are affected by strains of pathogenic *E. coli* (Donnenberg, 2002). There are many types of pathogenic *E. coli*, which differ in their abilities to cause disease.

The types of *E. coli* that can cause gastroenteritis can be transmitted in a number of ways including through contaminated food, drinking water, recreational water, or person-to-person contact with an infected individual. (CDC, 2012). The median incubation period for an *E. coli* infection is typically three to four days and symptoms of infections include severe stomach cramps, diarrhea, and vomiting. More serious symptoms may include septicemia, hemolytic-uremic syndrome, and thrombocytopenia. Those most at risk, especially for more severe symptoms are young children, the elderly, and people who are immunocompromised. The best ways to avoid *E. coli* infections are washing your hands thoroughly, cooking meats to the proper temperature, avoiding raw milk, being careful not to swallow the water in lakes, ponds, and swimming pools, and finally avoiding cross-contamination in the kitchen (CDC, 2012).

The fact that *E. coli* can both coexist symbiotically with its host and cause illnesses in a wide variety of species can be attributed to the broad array of pathotypes that exist. *E. coli* bacteria have been categorized into many different pathotypes based on both how they cause disease as well as the different symptoms that result from infection (Donnenberg, 2002). Some of these many pathotypes include Shiga toxin-producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and extraintestinal pathogenic *E. coli* (ExPEC). While these pathotypes serve to differentiate the multitude of *E. coli* strains, there is still some overlap that exists among them. Therefore, care should be

taken to avoid a "pigeon-holed" approach to an investigation of *E. coli* contamination in the food supply, especially since there is a risk for novel pathogenic strains to emerge.

The immensity and complexity of the *E. coli* species is due in part to the microorganism's great potential for genetic exchange. Tatum and Lederberg (1947) were the first to discover bacterial recombination in *E. coli,* and it is proven that genetic transfer is still an important mode for the species to acquire new genes by the large number of pathogenic factors found on the plasmids of the bacteria. This is not just limited to pathogenic strains as even the genome of the nonpathogenic *E. coli* K12 strain exhibits extensive transfer and elasticity (Lawrence and Ochman, 1998). When coupling this with the fact that other virulence factors in *E. coli* are encoded on portions of DNA known as pathogenicity islands (Perna *et al.,* 2001), the possibilities for novel pathogenic varieties seems endless.

2.2 Key apsects of foodborne illness estimates, outbreaks, and recalls

The Centers for Disease Control and Prevention estimate that each year roughly 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases (CDC, 2011). Pathogenic *E. coli* is one of the eight pathogens that accounts for the overwhelming majority of foodborne illnesses, hospitalizations, and deaths each year along with Norovirus, *Salmonella, Clostridium perfringens, Campylobacter* spp., *Staphylococcus aureus, Toxoplasma gondii,* and *Listeria monocytogenes*. In fact, STEC *E. coli* is number five on the list of pathogens contributing to domestically acquired foodborne illnesses resulting in hospitalization with an estimated number of 2,138 hospitalizations a year (CDC, 2011).

Pathogenic *E. coli* have been associated with outbreaks of gastroenteritis caused by consumption of various contaminated foods; including, fresh spinach, beef, frozen pizza products, prepackaged cookie dough, Romaine lettuce, cheese, in-shell hazelnuts, Lebanon bologna, and raw clover sprouts (http://www.cdc.gov/ecoli/outbreaks.html). Among the worst of these outbreaks was the fresh spinach outbreak in 2006 that resulted in 199 people from 26 different states being infected with *E. coli* O157:H7. Fifty-one percent of those who fell ill were hospitalized with 31 cases of hemolytic-uremic syndrome, and three confirmed deaths (CDC, 2006). *E. coli* O157:H7 has long been regarded as the most important strain of the species in relation to foodborne illness because of its association with large outbreaks that have severe manifestations such as HUS. After a large outbreak linked to undercooked hamburgers in 1992-1993, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) mandated that *E. coli* O157:H7 was to be considered an adulterant in ground beef (Grant *et al*., 2011). The main route for *E. coli* O157:H7 infection is fecal-oral, and thus minimizing fecal contamination in production plants has been a primary objective in fighting this pathogen.

A recent devastating outbreak was the 2011 outbreak associated with raw sprouts in Germany. The type of *E. coli* was determined to be O104:H4 and the outbreak resulted in the staggering figure of 855 cases of HUS and 53 deaths (Robert Koch Institute, 2011). This outbreak highlighted the fact that O157:H7 is not the only strain of *E. coli* of importance to public health. The outbreak strain exhibited the typical properties of enteroaggregative *E. coli*, but it had also acquired the *stx*2 gene. It also expressed the distinct phenotypes of these two unique pathotypes, which

includes the production of Shiga toxin 2 and aggregative stacked-brick adherence to epithelial cells (Bielaszewska *et al.,* 2011). It was suggested that increased attachment to epithelial cells from the enteroaggregative phenotype may have facilitated the absorption of Shiga toxin into intestinal cells and thus the higher occurrence of HUS (Bielaszewska *et al.*, 2011). This outbreak highlights the fact that genetic recombination of *E. coli* can result in a powerful pathogen.

The *E. coli* O104:H4 outbreak in Germany further elucidated the idea that food safety and industry professionals must also focus on non-O157 *E. coli* serovars as significant foodborne pathogens. Between 1990 and 2007, 23 outbreaks associated with non-O157 STEC were reported, and the six serotypes of O26, O45, O103, O111, O121, and O145 accounted for 75% of the cases (Griffin, 2007). Because they don't have unique phenotypic characteristics, it is very difficult to readily distinguish them from other *E. coli* strains. This contributes to our limited knowledge of the full impact of non-O157 STEC on public health (Grant *et al.*, 2011). However, FoodNet has begun active surveillance of non-O157:H7 infections and has found a rate that is half that of *E. coli* O157:H7 (CDC, 2008). This is still a significant amount. The fact that the prevalence of non-O157 STEC found in up to 49% of raw beef products compared to the 1% of O157:H7 typically present in raw beef, shows these other O-types cannot be ignored (Hussein and Bollinger, 2005).

Non-O157 STEC have also become prevalent in produce and have led to a number of serious outbreaks. In 1999 there was an outbreak of O111:H8 associated with salad from a salad bar in Texas. Fifty-eight people showed the typical symptoms of an *E. coli* infection, two developed HUS, and PCR from the stool samples showed the presence of *stx1* and *stx2* (CDC, 2000). In 2010 there was a multistate outbreak of

E. coli O145 linked to shredded Romaine lettuce. Of the 26 confirmed cases from this outbreak, 12 people were hospitalized, three developed HUS, and there were no deaths. The lettuce was traced back to a single processing facility and the confirmed cases of illness came from a total of five states including Michigan, New York, Ohio, Pennsylvania, and Tennessee (CDC, 2010). Just this past year there was an outbreak of *E. coli* O26 that resulted in 29 persons infected from 11 states. The epidemiological investigation revealed that the cause of the outbreak was consumption of raw clover sprouts from Jimmy John's restaurants. The illness onset dates ranged from December 25, 2011 to March 3, 2012 (CDC, 2012).

Some of the non-O157 serotypes that have made people sick actually possess similarities to the outbreak strain from the devastating outbreak in Germany, and this should definitely cause some alarm. For example, there are other Shiga toxin-producing *E. coli* in the O104 serogroup that have a different H antigen (ECDC, 2011). In fact, an O104:H21 strain that was also *eae*-negative like the O104:H4 strain was linked to an outbreak of bloody diarrhea from the consumption of raw milk in Montana in 1994 (CDC, 1995). An outbreak of HUS in France in 1996 (Boudaillez *et al.*, 1996) also exhibited a similar pattern to the sprout outbreak in Germany, but this outbreak was not caused by the O104 serotype, but rather the O111 serotype. Like the other mixed-virulence outbreaks mentioned, this O111:H2 strain showed aggregative adhesion to HEp-2 cells and the production of Shiga toxin, but the lack of the *eaeA* gene and other EHEC plasmid markers typically used in diagnostic studies (Morabito *et al.*, 1998). This is yet another representation of why other factors besides the standard EHEC markers need to be considered when determining *E. coli* pathogenicity.

2.3 *E. coli* Virulence Factors

Pathogenic *E. coli* varieties obtain their ability to cause disease from their expression of virulence factors which include adhesins, toxins, siderophores, and secretion systems (Eisenstein and Jones, 1988). These virulence factors are not required for simple replication or host commensalism as proven by their absence from most of the nonpathogenic strains that make up the facultative intestinal flora of human and animal hosts (Johnson, 2002). Virulence factors, like proteins and toxins, give *E. coli* the unique ability to colonize host surfaces, avoid host defense systems, and to directly injure host cells and tissues (Johnson, 2002). These virulence factors include adhesive pili, the type III secretion system, hemolysin proteins, capsules, and flagella.

Virulence factors reside in a number of different "locations" within pathogenic *E. coli* strains. Pathogenicity-associated islands are genomic regions that contain multiple putative virulence genes which exhibit a codon pattern atypical for the bacterial species (Schubert *et al.*, 1998). Pathogenic strains will often contain multiple pathogenicity islands that have some overlap in terms of the virulence factor content (Swenson *et. al*, 1996). The similarities between the pathogenicity-associated islands of intestinal versus extraintestinal pathogenic *E. coli* suggest an evolutionary commonality between these two very different pathotypes (Groisman and Ochman, 1996; Hacker and Carniel, 2001). Some of these genes include the *iroN* gene that is involved with iron transport and uptake and also the *iha* gene which is an adhesin and also found in *E. coli* O104:H4 (Johnson *et al.*, 2000).

Another location of virulence factors in pathogenic *E. coli* is on plasmids. The evolutionary history of the plasmids is usually independent from the host's genome, but in certain instances they appear to have coevolved (Souza and Eguiarte, 1997).

Virulence plasmids evolve through horizontal transfer, exchange of factors with the host genome, and actual integration into the host genome (Lan *et al.,* 2001). Also of importance is that certain virulence plasmids might possess antibiotic resistance genes which supply a distinct advantage, especially if the host is receiving antibiotics (Phillips *et al.*, 1988). Some examples of plasmid-derived virulence factors include a hemolysin (*ehxA*), a catalase-peroxidase (*katP),* and a subtilase cytotoxin (*sub*AB) which are all found on the pO157 putative virulence plasmid (Bustamante *et al.,* 2011).

A third source for the horizontal transfer and acquisition of virulence genes are bacteriophages. Bacteriophages encode virulence factors that may convert their bacterial host in a process known as phage lysogenic conversion from an avirulent strain to a virulent strain or a strain with increased virulence (Boyd and Brussow, 2002). These bacteriophages are members of a range of virus families including Podoviridae, Siphoviridae, Myoviridae, and Inoviridae (Boyd and Brussow, 2002). The relationship between bacteriophages and bacteria is a very special case of co-evolution because they each provide a unique advantage to their counterpart (Desiere *et al.,* 2001). Numerous virulence factors in *E. coli* including Shiga-toxins, tellurite resistance genes, serum resistance factors, superoxide dismutase required for intracellular survival, and an intestinal colonization factor from prophage CP9330 have been found to be encoded by bacteriophage prophages and it has been postulated that the acquisition of these prophage-encoded virulence factors led to the emergence of O157 as a foodborne pathogen (Boyd and Brussow, 2002). However, this remains as one of many theories on why *E. coli* O157:H7 suddenly emerged as a foodborne pathogen after a history of being virtually unknown. One of the other popular beliefs

is that *E. coli* O157:H7 has always been prominent in certain animal populations, but that changes in the meat production industry including slaughter practices promoted the contamination of the meat with this pathogen (Armstrong *et al.,* 1996).

In addition to searching for virulence factors within the genetic makeup of *E. coli*, identifying strains based on their O serotype may also prove to be useful. The O antigen is part of the lipopolysaccharide of *E. coli* that is classically known to protect *E. coli* against host bactericidal defense factors (Russo, 2002). However, studies have been conducted which have elucidated additional findings in the association between O type and virulence. One study found that *E. coli* O157:H7 that lacked the O-antigen attached significantly less to iceberg lettuce than strains that expressed the antigen (Boyer *et al.,* 2011). Strains without the O antigen were also shown to have a greater hydrophobicity, and this was suggested as a possible reason for the greater level of attachment (Boyer *et al.,* 2011). Another study looked for the relationship between virulence genes and the O serotype in *E. coli* causing urinary tract infections. The results of this study showed a strong relationship between the O6 serotype and virulent genotypes of uropathogenic *E. coli* (Emamghorashi *et al.,* 2011). Finally, there has recently been discovered to be a link between the *E. coli* lipopolysaccharide and immune evasion in the host (Russo, 2002).

2.4 Phylogenetic Relationships in *E. coli*

Based on similar relationships discovered by techniques such as multilocus sequence typing (MLST), there are four major phylogenetic groups of *E. coli* that have been identified and they are labeled A, B1, B2, and D (Herzer *et al.,* 1990). Techniques such as MLST sample multiple selection-neutral genes throughout the genome to give an approximation of a strain's entire genome history (Selander *et al.,*

1987). By analyzing similarities and differences in the genomic histories of a wide range of *E. coli* strains, scientists and ecologists were able to distinguish the four distinct phylogenetic groups. A dendogram or phylogenetic tree can be created by multilocus enzyme electrophoresis (MLEE) of members of the *E. coli* reference (ECOR) collection (Johnson, 2002). This phylogenetic tree will then accurately depict the genotypic relationships between these four major groups.

Extraintestinal *E. coli* with the most hearty virulence factor libraries as well as those best able to infect uncompromised hosts are usually clustered in group B2 (Boyd and Hartl, 1998). Strains in phylogenetic groups A and B1 rarely cause extraintestinal infection unless they have acquired enough virulence factors by horizontal transfer (Johnson *et al.,* 2001). While the ultimate ancestral origins of virulence factors remains a blurry topic, their more recent sources can be deduced by looking at the pattern of their phylogenetic distribution (Schmidt *et al.,* 1999).

There have been many phylogenetic studies performed on a wide range of *E. coli* strains in order to try and find some organization within this very diverse species. In a study by Bert and colleagues (2010), the genetic diversity and virulence profiles of *E. coli* isolated from patients with cirrhosis were examined. Out of 34 virulence factors tested for, 18 were significantly associated with B2 isolates while none were significantly associated with non-B2 isolates. It was also hypothesized that a higher percentage of certain clonal groups present in the gut may come from a steady input from environmental reservoirs such as food (Vincent *et al.,* 2010). In a different study, 12 genomic islands from APEC O1 were compared to human ExPEC and commensal *E. coli* isolates (Johnson *et al.,* 2012). Multiple APEC pathogenicity-associated islands were found to be very prevalent among human strains that belonged

to the B2 phylogenetic group; however, despite its genetic similarity to the human strains the APEC strain was not able to cause disease in a mouse model (Johnson *et al.,* 2012).

There have been numerous similar studies to the one discussed above comparing the virulence repertoires and phylogenetic groups of APEC and human ExPEC, with the results often being unclear. The diversity seen among ExPEC virulence genes and the high degree of genetic overlap between pathogenic and nonpathogenic strains makes it nearly impossible to correlate a set of factors to a specific group (Wiles *et al.,* 2008). There is a belief among many that because there is no unique virulence profile for APEC and UPEC that this gives APEC great potential to be zoonotic agents (Rodriguez-Siek *et al.,* 2005). For a more precise comparison, Mora *et al.* (2009) focused their efforts specifically on serotype O1:K1:H7/NM which is involved in neonatal meningitis, UTI, and avian colibacillosis. They found 81% of their isolates to be positive for at least eight virulence genes which confirmed the pathogenic potential of this serogroup. Moreover, they discovered a specific clonal group (B2 ST95) within this serotype that was detected in both human ExPEC and APEC strains that were recovered from different locations at different dates (Mora *et al.,* 2009). This finding suggests that some APEC isolates could serve as human pathogens with poultry as the food vector. More comprehensive *E. coli* testing should be conducted at poultry processing plants in order to better screen for possible APEC contamination on commercial chicken breasts.

The current phylogenetic categorization of *E. coli* is not universally accepted. There is a novel idea that the adaptation of bacteria may not necessarily result in distinct species, but instead in a genetic continuum known as “fuzzy” species (Gevers

et al., 2005; Hanage *et al.*, 2005). Luo and colleagues (2011) described five *Escherichia* clades, or grouping of strains all believed to have evolved from a common ancestor, labeled C-I to C-V that were isolated from the environment and found to be indistinguishable from typical *E. coli* based on traditional testing methods. However, these environmental isolates were actually quite different from their commensal or pathogenic counterparts having genes of unknown function and many additional genes that made them more suited for survival in the environment (Luo *et al.*, 2011). The argument is that these clades should not be considered typical *E. coli* by microbiologists and therefore a more ecologic definition for species is needed.

2.5 Shiga Toxin-Producing *E. coli*

There have been over 200 types of *E. coli*, including those that fall under the enterohemorraghic pathotype, that have been reported to make Shiga toxins (Acheson and Keusch, 1996). The terminologies of pathotype and clade are not to be confused as a pathotype groups *E. coli* based on their virulence mechanisms and a clade groups strains based on their phylogenetic relationship. The most infamous of these serotypes is *E. coli* O157:H7. This serotype has been responsible for many multi-state outbreaks, including one involving fresh spinach in 2006 and one involving raw cookie dough in 2009 (CDC, 2009). This serotype was also linked to one of the largest outbreaks of foodborne illness in the world in 1996 in Japan, where over 8,000 cases were traced back to contaminated radish sprouts (Michino *et al.*, 1999).

Recently there has also been a growing awareness of the importance of non-O157 STEC in causing human disease, especially with the "big 6" O-types previously mentioned. The primary foods associated with human illness and non-O157 STEC have been salads, berries, milk, and juices (Grant *et al.*, 2011). Some recent outbreaks

involving non-O157 STEC have been an outbreak of O145 (unknown source) affecting 18 people from nine different states, and an even larger outbreak of O26 traced back to eating raw clover sprouts at Jimmy John's restaurants (CDC, 2012).

The main reservoirs of STEC in the environment are ruminants such as cattle, sheep, and goats (Beutin *et al.,* 1993). The STEC are shed in the animal's feces and can survive in the soil for a few months and in manure for up to 21 months (Fukushima *et al.,* 1999). The common route of STEC infection in humans is through fecal-oral transmission after eating contaminated food products. STEC infections will typically start with symptoms such as watery diarrhea, abdominal pain, nausea, and vomiting. The incubation period for STEC infection is typically three to four days, but may be as short as one day or as long as ten days. While most patients recover from an STEC infection without any further complications, some will develop hemolytic uremic syndrome (5-10%) which is a serious and sometimes fatal kidney disease (Thorpe *et al.,* 2002, CDC, 2012).

Although we lump all *E. coli* that produce Shiga toxins into one broad category, there is actually quite a bit of diversity among STEC, even among the toxins themselves. Shiga-toxin 1 (*stx1*) is closely related in amino acid sequence to the Shiga toxin of *Shigella dysenteriae* whereas Shiga toxin 2 (*stx2*) is much less related and will not be neutralized by the antibodies for *stx1* (O'Brien and Holmes, 1987). It has been found that *stx2* is often associated with the *eae* gene involved with attachment and is most frequently found in human cases, especially those more severe in nature and leading to HUS. The *stx1* gene is the opposite; found more often in non-human cases and in less serious cases of uncomplicated diarrhea (Boerlin *et al.,* 1999).

Colonization can begin once STEC have reached the lower gastrointestinal tract. The locus for enterocyte effacement (LEE) pathogenicity island encodes for genes that are responsible for bacterial adherence to intestinal epithelial cells through attaching and effacing (A/E) lesions. Genes on the pathogenicity island encode a type III secretion system. This type III secretion system injects the translocated intimin receptor (Tir) into the host cell where it acts as a receptor for intimin which is an outer membrane protein present on the bacterium. The association between Tir and intimin is an integral part in the formation of the A/E lesion. Once the bacteria have adhered to the intestinal epithelial cells they produce Shiga toxin which is absorbed into the systemic circulation of the body and causes disease (Thorpe *et al.*, 2002).

As with most types of *E. coli*, enterohemorrhagic *E. coli* has a number of important genes that play a role in the expression of key virulence factors. The most commonly sought after genes are the *stx1* and *stx2* genes which will confirm the presence of STEC in a sample (Paton and Paton, 2001). The genes responsible for the pathogenicity of the bacteria including those that contribute to the attaching and effacing lesions such as the *eaeA* and *espA* genes are found on a chromosomal pathogenicity island known as the locus of enterocyte effacement (McDaniel *et al.*, 1995).

In addition to this, all clinical isolates of *E. coli* O157:H7 possess a virulence plasmid called pO157. Several of the genes in this plasmid have been characterized including *ehxA, espP, katP, stcE, and subA.* The *ehxA* gene codes for a hemolysin, *katP* assists in reducing oxidative stress for the pathogen, *espP* cleaves pepsinA and human coagulation factor V, and *stcE* is a metalloprotease (Bustamante *et al.*, 2011).

Bustamante and colleagues (2011) developed a multiplex PCR assay which could detect the five putative virulence plasmid genes. This assay was proposed to provide additional characterization of STEC strains past the genes that are typically screened for on the LEE. This additional information could be of great epidemiological value (Paton and Paton, 2002). Being able to detect pathogenic strains of *E. coli* based on various different virulence genes would greatly enhance the current monitoring and surveillance programs. Also, a more precise characterization of outbreak strains will perpetuate an easier and faster trace-back process. This pentavalent multiplex PCR assay showed high sensitivity and specificity and can be applied to the characterization of *E. coli* strains from the environment, food, or patients (Bustamante, *et al.,* 2011). Since the full array of virulence factors necessary to cause severe disease in humans is not known, being able to identify an increasing amount of these factors can have important public health implications (Wickham *et al.,* 2006). Comprehensive data on the virulence properties of STEC strains could allow for a correlation between genes present and the severity of the disease (Wickham *et al.,* 2006).

2.6 Enteroaggregative *E. coli*

One of the defining characteristics of enteroaggregative *E. coli* is the "stacked brick" adherence of the bacteria on the surface of cells. There have not been any outbreaks associated only with EAEC that have had the same severity as the worst EHEC outbreaks. Patients with an EAEC infection will typically develop a watery diarrhea that lasts for several weeks (Cobeljic *et al.,* 1996). An EAEC infection can be diagnosed by isolating *E. coli* that displays the aggregative adherence pattern in the HEp-2 assay (Nataro and Steiner, 2002). There are also genetic markers you can look

for when diagnosing an EAEC infection. Some of these include the EAEC virulence plasmid (*aatA*), the pilin subunit of aggregative adherence fimbriae (*aggA*), and the protein involved in intestinal colonization (*pic*) (Bielaszewska *et al.*, 2011).

In terms of food safety, enteroaggregative *E.coli* long existed in the same realm as pathotypes such as enteropathogenic, enterotoxigenic, and enteroinvasive *E. coli*: it was known to be important, but it was not a real concern and was thus kept out of the public eye. It was always *E. coli* O157:H7 that got all of the attention, and deservedly so due to the numerous large outbreaks it was associated with. However, enteroaggregative *E. coli* vaulted to the forefront of the food safety world after one of the worst food outbreaks in recent memory was attributed to an *E. coli* strain with a blended EAEC/EHEC virulence repertoire (CDC, 2011).

This outbreak of *E. coli* O104:H4 in Germany saw an astonishing 855 cases of hemolytic uremic syndrome with 53 deaths (Robert Koch Institute, 2011). The Institute for Hygiene and the National Consulting Laboratory for Hemolytic Uremic Syndrome in Germany characterized the outbreak strain and found that it showed a stacked-brick adherence to intestinal epithelial cells which is representative of EAEC. However, they tested for genes of EHEC, EAEC, EPEC, ETEC, and EIEC, and genes from both EHEC (*stx2, iha*) and EAEC (*aggA, aggR*) were present (Bielaszewska *et al.*, 2011). This outbreak demonstrates that blended virulence profiles in enteric pathogens, introduced into susceptible populations, can have extreme consequences for infected individuals (Bielaszewska *et. al*, 2011). In another study, an O111:H2 strain of *E. coli* from an outbreak of hemolytic uremic syndrome possessed this same unique combination of virulence factors of Shiga toxin production and enteroaggregative adhesion ability (Morabito *et al.*, 1997).

One of the things that was so unique about this outbreak was that out of 588 clinical EHEC strains present in the National Consulting Laboratory for HUS and the Reference Laboratory for Enterobacteriaceae, only one belonged to serotype O104:H4 (Mellman *et al.,* 2008). This proves that the potential for exchange of virulence factors between strains may allow once seemingly harmless serotypes to evolve into lethal pathogens, possibly even more dangerous than some strains of O157:H7. Thirteen member states in the European Union were not able to easily identify the strain, showing the many gaps in the detection systems across Europe (Rosseneu, 2011). This outbreak in the EU was first associated with Spanish cucumbers and later correctly associated with Fenugreek seeds from Egypt (Rosseneu, 2011). Global conversations focused on the constant evolution of nature and the notion that humans only look for what they know because of the innate way in which we categorize things (Rosseneu, 2011). Several scientists focusing on *E. coli* are beginning to ask questions on whether we should be testing for different strains or virulence factors of *E. coli*.

2.7 Broadening Our Vision of *E. coli*

Among other things, the *E. coli* O104:H4 outbreak in Germany reinforced the need to expand the food safety outlook of disease-causing *E. coli* serotypes and pathotypes. Testing and monitoring efforts should not solely focus on the O157:H7 serotype, rather research should also focus on ways to elucidate the potential of other pathotypes and serotypes of *E. coli* to cause foodborne illness. In searching for answers, it is critical to remember that the emergence of pathogenic strains, both new and old, result from the evolutionary processes of mutation and horizontal transfer (Guttman and Dykhuizen, 1994; Hartl and Dykhuizen, 1984). DNA fragments or

plasmids can be horizontally acquired through bacterial conjugation, phage-mediated transduction, or passive means and have a profound effect on the recipient's genomic backbone and overall genetic content (Milkman and Bridges, 1990; Boerlin *et al.*, 1998).

Testing for other O-serotypes of *E. coli* in our food has already begun. On June 4, 2012 ground beef testing as directed by the USDA-FSIS began to focus more attention on non-O157 STECs and the "big 6" serotypes of O26, O45, O103, O111, O121, and O145 (Grant *et al.*, 2011, USDA-FSIS, 2012). Other than O157, these are the six STEC O-serotypes associated with the most human disease outbreaks. Also, detection methods for non-O157 STECs are being created, and this is a huge step in the right direction. The current procedure for STEC detection, in short, starts with selective enrichment, followed by screening for *stx* and *eae* genes based on a real-time PCR assay. For positive samples from this assay, a second real-time PCR assay investigates the presence of O-serotypes. Based on an immunomagnetic assay developed for each of the top-6 non-O157 STECs, each pathogen from positive samples will be isolated for further biochemical confirmation and presence of *stx* and *eae* genes (Stockbine, 2011).

Several studies testing for the presence of non-O157 STEC in food and water sources have been conducted. A study in the Washington D.C. area looked for the presence of non-O157 STEC in retail ground beef and pork through the use of PCR and colony hybridization (Ju *et al.*, 2012). This study found 32 STEC isolates in the 480 samples with 31.3% of the isolates harboring *stx1*, 68.7% with *stx2*, 21.9% with *hlyA*, and none of the isolates carrying the *eae* gene (Ju *et al.*, 2012). Nine of the isolates were identified as O91, and none were in serogroup O157. Also, a different

study found 4% of post-intervention beef carcasses to possess non-O157 STEC (Barkocy-Gallagher *et al.,* 2003).

In addition, the ill-effects of novel outbreaks can also impact the thirst for knowledge about little-studied *E. coli* pathogens. For example, Rasko *et al.* (2011) used third-generation DNA sequencing to identify the entire genome sequence of the *E. coli* O104 German outbreak strain along with other strains of enteroaggregative *E. coli*. The German outbreak strain was found to be unique from the other strains because of a distinguishable set of virulence factors acquired through lateral genetic transfer including a prophage encoding the *stx2* gene (Rasko *et al.,* 2011).

2.8 Extraintestinal Pathogenic *E. coli*

Strains of *E. coli* that are able to manifest themselves in bodily sites outside of the gastrointestinal tract are known as extraintestinal pathogenic *E. coli* or ExPEC (Smith *et al.,* 2007). In humans, ExPEC can cause diseases such as urinary tract infections and neonatal meningitis (Smith *et al.,* 2007). Subtypes of the ExPEC pathotype include meningitis-associated *E. coli* (MAEC), uropathogenic *E. coli,* and avian pathogenic *E. coli* (Russo and Johnson, 2000). Similar serogroups, virulence genotypes, and phylogenetic groups are shared among human and animal ExPEC. This has introduced the idea of APEC as zoonotic pathogens, and the isolation of ExPEC strains from food products such as poultry has initiated the idea that food could be the transmission vehicle for these organisms (Smith *et al.,* 2007).

2.8.1 Uropathogenic *E. coli*

One of the more common sites of bacterial infection in our bodies is the urinary tract, and *E. coli* is easily the most abundant pathogen at this location (Kunin,

1987). In humans, UPEC is responsible for 80% of urinary tract infections (Allsopp *et al.,* 2010). The individuals at highest risk for urinary tract infections are neonates, sexually active women, and the elderly (Bahrani-Mougeot *et al.,* 2002). Uropathogenic *E. coli* is the leading cause of cystitis, bladder infection, and acute pyelonephritis, an infection of one or both kidneys (Burman *et al.,* 1988; Warren and Mobley, 1996).

UPEC possesses a number of genetic factors that contribute to its pathogenicity and these include hemolysins, fimbriae, protein toxins, and iron-acquisition systems (Bahrani-Mougeot *et al.,* 2002). The genes that encode these virulence factors are typically found on pathogenicity islands. When certain phenotypes are expressed more often from *E. coli* strains isolated from UTI patients than from strains isolated from their healthy counterparts, this implies that these factors play a part in causing the disease (Bahrani-Mougeot *et al.,* 2002). Several models have been used to study the pathogenesis of UPEC with the most significant being the use of adult volunteers suffering from recurring UTIs (Andersson *et al.,* 1991).

There have been numerous studies that have shown the possibility for humans to potentially be infected with ExPEC through food or water sources. One study was carried out in Southeast Queensland, Australia in order to investigate the presence of 20 virulence genes in rainwater tanks (Ahmed *et al.,* 2011). Twenty-two of 30 tanks surveyed were positive for *E. coli,* and virulence genes belonging to the ExPEC pathotype were found in 68% of these positive samples (Ahmed *et al.,* 2011). These genes included adhesins, toxins, and invasins among others. Also of interest was that 15% of the *E. coli* isolates were positive for the *eaeA* gene (Ahmed *et al.,* 2011). This study supports the idea that with this diverse mix of virulence genes, the acquisition of

additional genes by particular isolates could reequip them to become dangerous pathogens. This study can be looked at as a microcosm of our environment as a whole. If this amount of diversity among *E. coli* virulence factors exists in rainwater barrels, it can be inferred that there are other environments with a similarly complex ecosystem of *E. coli*. It is possible that environments tied to food production such as chicken houses and agricultural fields may contain this diverse grouping of *E. coli*, and it is for reasons like this why we must become more aware of potential novel environmental pathogens.

2.8.2 Avian Pathogenic *E. coli*

APEC strains are members of the extraintestinal pathogenic *E. coli* pathotype. In birds, APEC begins as a respiratory infection and then progresses to a systemic infection causing diseases such as colibacillosis and septicemia (Barnes *et al.*, 2008; Dozois *et al.*, 2003). The most common route of infection is through inhalation of fecally-contaminated dust, and the mortality and reduced feed efficiency from the resulting disease results in economic losses of millions of dollars (Tivendale *et al.*, 2000). Because of similarities between the virulence factors of APEC and UPEC strains such as the *iss* gene for serum survival, it is hypothesized that APEC may be a foodborne risk for human acquired ExPEC (Johnson *et al.*, 2005). A study found startling similarities between APEC and UPEC with only 4.5% of a sequenced APEC genome not found among three sequenced UPEC genomes (Johnson, 2007). Therefore, there is an increasing need to learn more about environmental *E. coli* such as APEC to discover whether they are harmless to humans or have the ability to be dangerous zoonotic agents. In this same regard, it is extremely important to remember that unspecified agents account for 38.4 million out of 48 million (80%) of foodborne

illnesses each year in the United States (CDC, 2011). APEC may play a critical role in this gap.

If APEC were to pose a serious zoonotic risk, foods such as raw poultry and fresh produce could be vectors. Poultry could become contaminated during processing, especially during evisceration and by cross-contamination in the chiller. Produce could become contaminated by fecal dissemination from wild birds or through the presence of contaminated poultry litter on farms (Hoover *et al.,* 2010). In a study from Japan, *E. coli* strains were isolated from wild birds and screened for intimin (*eae*) and Shiga toxins (*stx1, stx2*) ; two virulence properties that are known to be important in causing human illness. Intimin was found in 25% of the strains, Shiga toxin was found in 5% of the strains, and of real interest is that very few of the strains these genes were associated with were found to be enterohemorrhagic *E. coli* (Kobayashi *et al.,* 2009). The near absence of enterohemorrhagic *E. coli* coupled with the presence of these significant virulence genes indicates that these atypical enteropathogenic *E. coli* strains present in wild and/or domesticated birds could be a source of contamination in our food supply.

Numerous recent studies have been done to try and evaluate the possibility of avian pathogenic *E. coli* as a zoonotic pathogen. A study by Vincent and colleagues (2010) explored food as a reservoir for ExPEC by gathering isolates from women with UTIs, retail meat, and ready-to-eat foods from the same time period and geographic area. Two *E. coli* isolates from retail chicken and one isolate from honeydew were genotypically closely related to certain isolates from human UTIs; which provides strong evidence for food being a potential vehicle for ExPEC infections (Vincent *et al.,* 2010). In another study, from 737 samples of retail beef, pork, and chicken, 41

ExPEC isolates were found with an overwhelming majority (71%) coming from retail chicken (Bergeron *et al.,* 2012). These results suggest that ExPEC involved in UTIs may be transmitted from food-animal sources, and that APEC may be of particular concern because of the strong possibility of chicken as a reservoir (Bergeron *et al.,* 2012). Of particular food safety concern would be handling raw poultry, cross-contamination with raw poultry in the kitchen, or eating undercooked chicken.

An additional concern with APEC is its proven resistance to antibiotics. Eighty-three APEC strains that were isolated between 2001 to 2006 were found to be resistant to ampicillin (77%), oxytetracycline (76%), and kanamycin (36%) among numerous other antimicrobials (Ozawa *et al.,* 2008). Another study looked at 101 APEC isolates that were recovered between 1985 and 2005. This group of APEC were found to be highly resistant to tetracycline (84%), streptomycin (84%), enrofloxacin (71%), and ampicillin (67%) (Kim *et al.,* 2007).

While antibiotic resistance may be an important mechanism for surviving poultry production practices, acid tolerance would be key for survival in the human digestive system. In one experiment, tested strains of *E. coli* were shown to be resistant to deoxycholate which is one of the most abundant bile salts present in humans (Mellata *et al.,* 2012). Further support for acid tolerance is evident from a study that showed 59% of APEC isolates tested to be resistant to nalidixic acid (Zhao *et al.,* 2005). This proven resistance to antibiotics and demonstrated acid tolerance is important in the overall persistence and versatility of APEC as a potential human pathogen.

2.9 Genomic Evaluation of Avian Pathogenic *E. coli*

Bacterial pathogens may pick up virulence factors that may alter their phenotype (Donnenberg, 2002). Such was the case with the novel *E. coli* O104:H4 strain which caused a large outbreak associated with sprouted seeds (CDC, 2011). It is known that avian pathogenic E. coli contains an array of virulence factors for pathogenesis in poultry (Tivendale *et al.*, 2011). However, it remains to be determined whether APEC has a virulence repertoire similar enough to those *E. coli* that causes human illness where a simple gene acquisition could transform APEC into a human pathogen. Therefore, continued research needs to be done in order to better understand the genome of avian pathogenic *E. coli*.

APEC is not normally associated with the crucial virulence genes from enterohemorraghic *E. coli* such as the Shiga toxin and intimin genes (Mora *et al.*, 2009). However, assessing APEC as a potential foodborne pathogen cannot be complete without comparison to the EHEC pathotype that causes the most serious foodborne illnesses. A group from Canada conducted an experiment in order to determine the presence of significant EHEC genes in a set of APEC isolates through PCR and colony blots. They examined a total of 97 APEC isolates which originated from avian cellulitis, septicemia, swollen head syndrome, and diseased turkeys, as well as five *E. coli* isolates from the feces of healthy birds (Parreira and Gyles, 2002). Fifty-three percent of the APEC isolates carried *stx* sequences with the large majority possessing *stx1*, one isolate with *stx2* and one possessing both *stx1* and *stx2* (Parreira and Gyles, 2002). All of the isolates were negative for the *eae* and E-*hlyA* genes that encode intimin and hemolysin, respectively. The five isolates from healthy birds possessed no virulence genes (Parreira and Gyles, 2002). Lastly, the O1, O2, and O78 serogroups had very high percentages of being *stx*-positive (Parreira and Gyles, 2002),

and these same serogroups have been labeled as STEC of bovine or human origin, showing a possible zoonotic link (Bettelheim, 2001).

Along with testing for the presence of EHEC genes, it is also of critical importance to evaluate APEC for different ExPEC virulence genes in order to achieve a better understanding of its pathogenicity as well as to look for similarities with human UPEC. The diversity of APEC strains is due to multiple virulence factors such as adhesins, toxins, the colicin V plasmid, F hemolysin, temperature-sensitive hemagglutinin (Tsh), and lipopolysaccharide complexes (Dho-Moulin and Fairbrother, 1999). Many of these same virulence factors are also found in human *E. coli* strains, like UPEC, which indicates that they are not host-specific (Mokady *et al.*, 2005). There is concern about the possibility of transference of ExPEC from birds to humans, and the fact that APEC can routinely be isolated from retail poultry meat compounds this fear (Johnson *et al.,* 2005).

In a study by Vidotto and colleagues (2011) in Brazil, 185 APEC isolates and 80 retail poultry *E. coli* (RPEC) isolates were tested for the presence of the five plasmid-based virulence genes, *hlyA, iroN, iss, iutA,* and *ompT*. It was found that 10% of RPEC possessed all five of the virulence genes of APEC, and with RPEC harboring APEC traits that are known to contribute to ExPEC infection in human beings, the threat of a zoonotic infection becomes quite alarming (Vidotto *et al.,* 2011). In a different study, a multiplex polymerase chain reaction was developed to detect eight different virulence genes in APEC isolates. It was found that the genes, *papC, irp2, iss,* and *iucD* were sporadically found throughout both the 14 APEC strains and five UPEC strains analyzed in this study (Ewers *et al.,* 2004). This great genetic similarity

reveals the concern of the very close relation between these two pathotypes which fall under the same ExPEC distinction.

2.10 Attachment of *E. coli* to Food Surfaces and Human Cells

The attachment abilities of *E. coli* are a very important part of its pathogenicity. Without the ability to attach to food matrices or cells within our bodies, *E. coli* would not be a primary threat to public health. There are many virulence factors that help *E. coli* bind to food surfaces; these include type IV pili (Donnenberg, 2002) and curli fibers (Macarisin *et al.*, 2012). A first step in causing infection once EHEC enters the body through contaminated food is adherence to the large intestinal mucosa by means of an attaching and effacing lesion (Torres *et al.*, 2005). Depending on the pathotype of the *E. coli*, the way in which the bacteria adhere to intestinal cells varies greatly.

Attachment to food products is one of the first necessary steps for *E. coli* to cause foodborne illness; therefore, there have been many studies done to learn more about this process. One study looked at the role that cellular appendages such as curli fibers might play in attachment to spinach leaves. The authors found that curli-producing strains of *E. coli* O157:H7 were able to form a very strong attachment to the leaf surface while curli-deficient mutants had a much weaker association (Macarisin *et al.*, 2011). They also differentiated between loosely and strongly attached bacteria and found a direct correlation between attachment strength and the ability to produce curli fibers (Macarisin *et al.*, 2011). Other studies suggest the role of other virulence factors becoming involved in adherence such as flagella (Xicohtencatl-Cortes *et al.*, 2009) and the type III secretion system (Saldana *et al.*, 2009). Finally, a study found

the presence of a capsule to be significant in attachment of *E. coli* O157:H7 to the surface of lettuce and apples (Hassan and Frank, 2004).

For *E. coli* to be able to effectively cause disease it must be able to attach to host cells and then proceed to colonize host tissues. An excellent way to test the attachment capabilities of various *E. coli* strains to human cells is through the use of cell culture lines. One such cell line is Caco-2 cells which are human colonic adenocarcinoma cells (ATCC, 2012). An experiment by Halpin and colleagues (2009) out of Dublin Ireland tested the effects that bovine whey products have on the association of *E. coli* O157:H7 to Caco-2 cells. They were able to devise and perform two assays that helped quantify the association and the invasion of the pathogenic *E. coli* to Caco-2 cells (Halpin *et al.,* 2009). In a separate study, the researchers were able to use a very similar Caco-2 adherence assay to determine that deletion of the *minD* gene (cell division) from *E. coli* O157:H7 resulted in decreased adherence to human intestinal cells (Parti *et al.,* 2011).

While much work has been performed to better understand the attachment of EHEC to both food surfaces as well as human cell lines; the same does not apply for APEC. A thorough evaluation of APEC as a possible zoonotic pathogen must include learning more about its attachment to food products such as raw produce and its ability to adhere to human intestinal cell lines, as has been done with other *E. coli* pathotypes.

Chapter 3

MATERIALS & METHODS

3.1 Isolation of APEC Isolates from Commercial Broilers

APEC isolates were collected from lesions of diseased chickens from commercial broiler houses throughout the Delmarva Peninsula (Dohms and Boettger, 2008, Characterization of APEC strains isolated from Delmarva, Animal Health Project No. DEL00643, unpublished results). The collected isolates were from multiple lesions of individual broilers that included the liver, hock joint, pericardium, yolk, crop, air sac, ceca, intestine, and cellulitis infection. A total of 320 isolates were collected, and each one was given a unique identification number. APEC strains were sent to University of Pennsylvania New Bolton Center (Kennett Square, PA) for O typing. More simplified numbers were given to strains used in further characterization.

3.2 Additional Reference Strains Used

While the APEC isolates were the main focus of this study, a host of other *E. coli* strains were also used for comparison, control, and reproducibility purposes. APEC strains were obtained from broilers in Delmarva. The EHEC strain used was *E. coli* O157:H7 strain 4407 (clinical isolate from 2006 spinach outbreak). Also, three nonpathogenic commensal strains of *E. coli* originally isolated from cabbage in Maryland and labeled as MW 416, MW 423, and MW 425 (Patel *et al.*, 2009) were used. A mutant strain of curli-deficient *E. coli* was used for comparison purposes

(received as a gift from Jitu Patel, USDA-ARS, Beltsville, MD). Additionally, two clinical strains of uropathogenic *E. coli* were provided by Don Lehman of the Department of Medical Laboratory Science at the University of Delaware (Newark, DE). One EAEC strain was used: a clinical isolate of *E. coli* O104:H4 from the German sprout outbreak (ATCC # BAA-2326), and a third UPEC strain was used: a typical uropathogenic *E. coli* (ATCC 700928). Both were purchased from the American Type Culture Collection (ATCC) in Manassas, Virginia. Bacterial strains were grown on selective media such as MacConkey Agar (MAC) or Sorbitol MacConkey Agar (SMAC) (Fisher Scientific, Fair Lawn, NJ).

3.3 Multiplex PCR Screening for *E. coli* Genes of Interest

Two multiplex PCR assays were developed to characterize the APEC isolates based on enterohemorrhagic *E. coli* virulence genes of interest. A multiplex PCR to test for genes on the putative virulence plasmid pO157 was developed based on a previous study (Bustamante *et al.*, 2011). The primers are described in Table 1.

A second multiplex PCR was set up to test for virulence genes from various sources including the LEE pathogenicity island. Three of the primer sets for this reaction were based on those in a previous study (Paton & Paton, 1998). Information regarding these primers is listed in Table 2.

A third multiplex PCR was designed in order to characterize the APEC isolates based on extraintestinal pathogenic *E. coli* virulence genes. The primers for this reaction were chosen based on previous work and are listed in Table 3 (Ewers *et al*., 2005).

3.4 Polymerase Chain Reaction

To prepare for PCR, Sorbitol MacConkey agar (SMAC) plates were quadrant-streaked with cultures of the APEC isolates and incubated at 37°C overnight in order to obtain single colonies. A single APEC colony was picked from each SMAC plate and dissolved in 50 µl of nuclease-free water to serve as the template DNA. Then the Master Mix and the primers were vortexed and centrifuged at 1,073 x g for one minute in order to achieve homogeneous solutions. The following 25 µl reaction volume was prepared on ice for each APEC isolate: 12.5 µl of GoTaq Green Master Mix (Fisher Scientific, Fair Lawn, NJ), 2X, 0.6 µl of 10uM upstream primer (Sigma-Genosys, Woodlands, TX), 0.6 µl of 10uM downstream primer, 1 µl of the DNA template, and 6.7 µl of nuclease-free water (Fisher Scientific, Fair Lawn, NJ). The samples are dispensed into 0.2 ml PCR tube strips and loaded into an Eppendorf thermocycler (Hamburg, Germany). To begin amplification by PCR a 5-minute initial denaturation step at 95°C is performed. Then, 35 cycles of a 95°C denaturation step for 45 seconds, a 58°C annealing step for 45 seconds, and a 72°C extension step for 1 minute is performed. A final extension step at 72°C for 5 minutes was carried out.

PCR-banding patterns were identified by gel electrophoresis on a 2% agarose gel and visualized by ethidium bromide using an AlphaImager UV light cabinet for observation and data collection.

3.5 MUG Test for *E. coli*

The MUG test is a procedure useful to detect the presence of *E. coli* from coliforms present in food or water samples. The premise of the test is detection of *E. coli* based on the beta-glucuronidase enzyme that most strains of *E. coli* possess. If the *E. coli* strain possesses this enzyme it will be able to hydrolyze 4-

methylumbelliferyl-beta-D-glucuronide (MUG), and this hydrolysis will result in a fluorescent end product (ATCC, 2012). This test was used here to determine whether or not the APEC isolates possessed beta-glucuronidase like most *E. coli* strains and could therefore be detected by the MUG test.

MacConkey agar plates supplemented with MUG were purchased from Fisher Scientific (Fair Lawn, NJ). Overnight cultures of the APEC isolates were grown in 9 ml of LB broth (Fisher Scientific, Fair Lawn, NJ) at 37°C. The following day each plate was partitioned into three different sections and subsequently streaked with three distinct APEC isolates. After incubation at 37°C overnight, the plates were observed in a dark room for fluorescence with a handheld UV lamp. The "Colilert" test kit (IDEXX, Westbrook, ME) was also performed on all of the isolates as additional confirmation for the presence or absence of beta-glucuronidase.

3.6 Fecal Shedding Challenge Study

Thirty one-day-old broiler chicks were received from a local grower in Delmarva (Millsboro, DE). The birds were grown up to two weeks of age in a small colony house located on the College of Agriculture and Natural Resources Poultry Farm (Newark, DE). The feed, water, bedding and temperature conditions of the house were set-up to mimic commercial broiler production. At two weeks of age, the broilers were moved to a larger block house with 12 separate cages where they were split up into ten groups of three birds each. Groups one through eight served as the experimental groups which would each be challenged with unique isolates of APEC and human pathogenic *E. coli*. Group nine was a negative-control group that was sham-inoculated with tryptic soy broth (TSB). Group ten was a secondary negative-control group that was left completely untreated. The six APEC isolates that were

used in this experiment had all been isolated from lesions of diseased chickens from commercial broilers throughout the Delmarva Peninsula. The APEC isolates that were selected were chosen either because of their O-type (O157) or because they possessed an EHEC virulence gene as determined in the multiplex PCR experiments described previously.

Broilers were challenged at 17 days of age with 0.01ml of culture containing an inoculum level of 10^6 cfu/ml. Before inoculation, cloacae swabs of each bird were collected to determine fecal contents and commensal gut microflora before being challenged with *E. coli*. Cloacae swabs were also collected on days 0, 3, 5, 7, 14, 19, and 26-days post-inoculation. Swabs from each group were pooled together and bacteria enumerated on SMAC after incubation for 24 hours at 37º C.

Because of the challenge of detecting our particular *E. coli* isolates among the plethora of bacteria present in broiler feces, additional advanced detection techniques were also used. Rainbow Agar (Fisher Scientific, Fair Lawn, NJ) was used in which different pathotypes of *E. coli* all grow with different distinct colors. For any of the strains that were presumptive positive for the O157 serotype, an O157 spot test kit (Oxoid, UK) was used. Finally, suspect colonies as well as DNA from the broilers' ceca were evaluated by multiplex PCR.

Throughout the course of the six and a half week experiment, the broilers were observed for any obvious outward signs of illness. Any birds that appeared to be suffering as a result of possible APEC infection or other factors such as leg problems were culled from the experiment. The birds were euthanized by the AVMA-approved method of cervical dislocation. Any birds that were culled for severe illness or died during the course of the experiment were necropsied and observed for clinical signs of

illness such as gross lesions. The experiment was terminated on day 45 and all remaining birds were euthanized by cervical dislocation. Necropsies were performed on at least one bird from each group, and a piece of their ceca was harvested for further analysis. Due to the challenging nature of this experiment, some strains were tested in duplicate in a second trial.

3.7 Evaluation of APEC Attachment to Retail Chicken Breast

Whole chicken breast tenderloins were purchased from a local grocery store (Newark, DE). Overnight cultures were prepared as described previously. A total of 28 APEC isolates were selected for this particular experiment based on the presence of EHEC virulence genes in the multiplex PCR study discussed previously. The breast tenderloins were aseptically cut into 2.5 cm. X 2.5 cm. squares in a biosafety cabinet. The 9 ml overnight cultures were centrifuged at 581 x g for 10 minutes and resuspended in 1ml of LB broth to achieve an inoculum level of 10^8 cfu/ml. Each square of chicken was spot inoculated with 10 µl of a unique APEC isolate, and each isolate was tested in duplicate. The chicken pieces were then left to dry for 30 minutes in the biosafety cabinet.

Each sample was aseptically transferred to 99 ml buffered peptone water (BPW) and inverted 25 times as a light wash step. One milliliter was drawn from each bottle and serially diluted in 9 ml BPW. Each serial dilution was spread plated onto SMAC and the colonies representing the typical morphology of smooth and circular *E. coli* colonies were counted and referred to as loosely attached bacteria. The chicken samples were then aseptically transferred to 99 ml Whirlpack® bags of BPW and hand massaged for 1 minute. Serial dilutions and spread-plating were performed again, and these recovered *E. coli* colonies were referred to as strongly attached bacteria. Based

on a previously cited formula we were able to determine the percentage of the total bacterial population that was strongly attached or the S_R value (Dickson and Koohmaraie, 1989). The S_R values were calculated as (strongly attached bacteria)/(loosely + strongly attached bacteria).

In addition to the chicken breast attachment experiment, a brief antibiotic resistance trial was conducted with gentamycin. Overnight cultures of the 28 APEC isolates were grown as previously described. In a 96-well plate, 100 µl of each isolate was dispensed into its own well. Each isolate was challenged with 50 µl of 50 µg/ml of gentamycin. From each well 10 µl was spot inoculated onto SMAC and grown at 37° C overnight.

3.8 HCT-8 Cell Line

The final phase of this comprehensive APEC characterization study was an experiment to examine the adherence capabilities of APEC to human cells. HCT-8 human ileocecal colorectal adenocarcinoma cells (ATCC # CCL-244) were grown in Roswell Park Memorial Institute medium (RPMI 1640) (Mediatech Inc., Manassas, VA) with L-gluatamine and 25 mM HEPES. The medium was either supplemented with 2, 5, or 10% fetal bovine serum (FBS).

3.9 HCT-8 Cell Attachment Assay

The following cell association assay was based on a similar experiment conducted with mammalian cell culture (Halpin *et al.*, 2009). The HCT-8 cells were grown to confluency in large cell culture flasks in RPMI 1640 media with 10% FBS. Once confluent, the media was removed and the cells were washed with 5ml of Hank's Balance Salt Solution (HBSS) (Mediatech Inc., Manassas, VA). The cells

were then split by adding 2 ml of trypsin (Mediatech Inc., Manassas, VA) to each flask and incubating at 37°C and 5% CO_2 for 15 minutes. After the cells were lifted from the surface of the flask they were resuspended in 15 ml of 10% RPMI medium. The collected cells were then centrifuged at 581 x g for 10 minutes to remove medium, serum, and debris. The cells were then resuspended in 12 ml of 10% RPMI and two 6-well plates seeded at ~10^6 cells per well and grown to confluency at 37°C and 5% CO_2.

The cell monolayers were challenged with a multiplicity of infection of ~100:1 (bacteria:cells). Overnight cultures of APEC were centrifuged at 581 x g and resuspended in RPMI so that 500 µl contains a total of ~10^8 cfu/ml. The medium was removed from the cell plates and each well washed twice with 1 ml of HBSS. Each challenge well then received 500 µl of bacterial cells in RPMI and each control well contained 500 µl RPMI. Samples were analyzed in duplicate. The plates were incubated at 37°C and 5% CO_2 for 1 hour.

After incubation the medium was removed and the cells washed twice with 1 ml of HBSS. Each well was overlaid with 500 µl of RPMI and incubated for an additional 30 minutes at 37°C and 5% CO_2. Lastly, the medium was removed and the monolayers lysed by addition of one ml of 1% Triton-X-100 prepared in HBSS. From this final 1 ml volume, serial dilutions were prepared in BPW. Subsequently, SMAC spread plates were prepared and represent the amount of bacteria that attached to the HTC-8 cells. As in the chicken breast attachment study, the 28 APEC isolates chosen for this study were those that were found to possess at least one EHEC virulence gene.

3.10 Statistical Analysis

For the chicken breast attachment work, data was recorded as the S_R mean ± the standard deviation. A student's t-test was used to determine the significance of this data (p-value <0.5). For the cell line attachment study, data was analyzed as the mean of the replicate log values ± the standard deviation. Significance was determined by the student's t-test (p-value <0.5). Correlation analysis was used to compare the chicken breast attachment and cell line experiments. Statistical analysis was performed with JMP 10 software (SAS Cary, NC).

Chapter 4

RESULTS AND DISCUSSION

4.1 Preliminary Characterization of the APEC Isolates

A total of 320 isolates were collected from lesions of diseased birds across the Delmarva Peninsula. O serotyping revealed that there were a total of 24 different O types present in the 320 APEC isolates; however, many of the isolates proved to be untypable. The O serotypes that appeared the most frequently were O8, O35, O78, and O157. Of the "Big 6" non-O157 STEC, the only O-type that was present was O45. From the common O types among the APEC isolates, O6, O45, and O157 have previously been associated with human disease (Mathusa *et al.*, 2010).

PCR was performed in order to screen for five genes that are typically associated with pathogenicity in APEC. These five genes were *intl1, tsh, traT, iss,* and *iucC.* Out of the 320 APEC isolates, 23 were found to be positive for all five genes, 38 were found to be negative for all five pathogenic traits, and the remaining isolates revealed varying combinations of genes.

4.2 Multiplex PCR for EHEC Genes

Two multiplex PCR assays were designed to each screen for four different genes commonly associated with the EHEC pathotype. The first reaction was set-up to screen for the *stx1, stx2, eae,* and *espA* genes. The *stx* genes are of utmost importance because these encode for the production of Shiga toxin which is the reason why STEC and EHEC can cause such potent disease (Paton and Paton, 2001). The

eae gene encodes for an outer membrane protein known as intimin which is key for attachment to epithelial cells (Yu and Kaper, 1992). Lastly, the *espA* gene has been shown to be an integral part of the type III secretion system and the formation of attaching and effacing lesions (Kenny *et al.*, 1996). The second multiplex PCR screened for accessory virulence genes found on the pO157 virulence plasmid that also play a host of key roles in pathogenesis. While these accessory genes are often not considered as hallmarks of disease like those discussed above, they play crucial roles such as pathogen survival in the host (Bustamante *et al.*, 2011). The presence of any combination of these eight genes along with viable attachment properties could mean a strong indication of a potentially pathogenic *E. coli* strain.

A total of 100 different APEC isolates were selected to be screened for these eight different genes using these two multiplex PCRs. While the 100 isolates were essentially chosen at random, there was an emphasis on selecting strains with a variety of different O types as well as a variety of different isolation locations. A strong emphasis was placed on choosing O157 isolates because of the large number of outbreaks associated with this O type. The *stx1*, *stx2*, and *espA* genes were not found to be present in any of the 100 isolates that were tested. One isolate out of the 100 possessed the *eaeA* gene (Figure 1). This was an O13 APEC isolate with an identification number of 07-5668, which was originally obtained from a diseased broiler chicken.

There have been numerous studies looking for the presence of cytotoxins in APEC. In 1992, an experiment reported 18 of 82 (22%) chicken isolates produced a cytotoxin that was active on Vero cells (Emery *et al.*, 1992). A study in 1994 found production of verotoxin from 11% of *E. coli* isolated derived from septic chickens

(Fatinatti *et al.*, 1994). More recently in 2001, colony hybridization was used to detect the *stx1* gene in two *E. coli* isolates from chickens with diarrhea (Mellata *et al.*, 2001).

While these studies all showed a degree of cytotoxicity, an experiment out of Canada by Parreira and Gyles (2002) produced much more alarming results. They examined 97 APEC isolates from lesions of septicemia, cellulitis, and swollen head syndrome in chickens. Through PCR and colony hybridization, they found 53% of the isolates contained *stx* gene sequences: one *stx2* only, 2 with both *stx* sequences, and 49 with *stx1* only. All of the *stx*-positive isolates were also screened for *eae* and *E-hlyA* genes, but none were found to possess either of these genes (Parreira and Gyles, 2002). These results are vastly different from our current study where no *stx* genes were found in 100 screened APEC isolates. This may indicate that geographic location alone plays a very key role in the presence of *stx* in APEC.

Studies have indicated that *stx* has been found in a wide multitude of environments. Cattle have been shown to be a major reservoir for *stx*-producing bacteria. With the PCR approach of testing bovine fecal samples, the general range for those that contained *stx* was between 18-25% (Rogerie *et al.*, 2001; Jenkins *et al.*, 2002). Several other farm animals have also been shown to contain large populations of *stx*-positive bacteria in their feces including sheep and goats (Mauro and Koudelka, 2011). *Stx* is also commonly found in many wild animals. In a study looking at fecal samples from 50 wild deer in Pennsylvania, nearly 50% of the samples were shown to possess *stx* (Kistler *et al.*, 2011). In another study, 12 wild bird species tested positive for *stx1* in their feces and another 30 species tested positive for *stx2* (Hughes *et al.*, 2009). In addition to all of these animal sources, *stx* has also been found in varying levels in air samples, drinking water, recreation water, and at high levels in wastewater

(Mauro & Koudelka, 2011). With this seemingly omnipresence of *stx* in the environment, it can be seen why it was necessary to test our APEC isolates for the presence of both *stx1* and *stx2*.

The multiplex PCR targeted towards virulence plasmid genes revealed several positive results. Out of the 100 APEC isolates that were screened, 15 were positive for *katP*, 8 for *espP*, 2 for *ehxA*, and 2 for *stcE*. The screening results for both sets of EHEC virulence genes can be seen in Table 4. In addition to the screening of the 100 APEC isolates some additional strains of *E. coli* were also tested for reference purposes. *E. coli* O157:H7 strain 4407 was used as a positive control and shown to contain all 8 of the virulence genes screened for. In comparison, *E. coli* O157:H12 was used as a negative control and tested negative for all 8 genes. For testing the accuracy of the reactions, the *E. coli* strain O104:H4 from the outbreak in Germany was also screened and was shown to possess *stx2* and *espP* genes which is consistent with the literature (Bielaszewska *et al.*, 2011). Additionally, three clinical isolates of UPEC were screened and one of these isolates was found to contain *stcE* and one was found to contain *espP*. Finally, the three nonpathogenic environmental isolates (Patel *et al.*, 2009) were found to be clear of any of these virulence genes. A gel image from a PCR screening for these four virulence plasmid genes can be seen in Figure 2.

4.3 Multiplex PCR for ExPEC Genes

The 100 APEC isolates were screened for ExPEC virulence genes of interest. The seven genes encode for a number of different virulence elements including: P-fimbriae, an adhesion factor (*papC*), a temperature-sensitive hemagglutinin (*tsh*), an iron-acquisition system (*iucD*), a protein for increased serum survival (*iss*), a colicin V plasmid (*cva/cvi*), an enteroaggregative heat-stable toxin (*astA*), and a vacuolating

autotransporter toxin (*vat*) (Ewers *et al.*, 2004). Unlike the previous PCR results revealing a maximum of one virulence gene per isolate, many of the isolates possessed 2, 3, 4, and even 5 of the ExPEC genes. In total, 18% of the APEC isolates had one ExPEC gene, 39% had two, 26% had three, 3% had four, and 1% had five. This was highly expected since APEC is a subset of the ExPEC pathotype. A gel after a multiplex PCR screening for these ExPEC genes is pictured in Figure 3.

The 7 ExPEC genes that were screened for were present in a variety of different levels in the APEC isolates. Genes were present in the 100 APEC isolates at varying proportions as outlined in Table 5. The reference strains were also screened for typical UPEC genes to serve as comparisons. The outbreak isolates of *E. coli* O157:H7 strain 4407 and *E. coli* O104:H4 did not possess any of these UPEC genes. More interestingly, two of the three clinical UPEC isolates tested positive, with one containing the *iss* and *iucD* genes and the other containing the *papC* gene. Finally, the *cva* gene was found to be present in one of the three nonpathogenic environmental isolates that were tested. These results were consistent with a number of studies including a similar study by Ewers and colleagues (2004). They also found a much higher prevalence of these ExPEC virulence genes among APEC and UPEC strains than they did in other pathotypes or nonpathogenic strains (Ewers *et al.*, 2004).

4.4 MUG Analysis

Testing for the presence of beta-glucuronidase through the MUG test is critical because this is one of the means of *E. coli* detection in water samples. Any of the *E. coli* isolates that have a negative result in the MUG test will not be recognized as *E. coli* when testing a water sample. The results of the MUG assessment are shown in Table 6. This table highlights the 28 APEC isolates that were chosen for further

analysis based on EHEC gene presence and the five reference strains used for comparison and reproducibility purposes.

4.5 Assessment of APEC Fecal Shedding by Commercial Broilers

A large population of bacterial species makes up the natural microflora present in the avian digestive system; therefore, conducting an experiment to determine whether or not broilers were shedding one particular microorganism was a difficult task. In order to achieve as accurate of an evaluation as possible, a number of different testing methods were conducted.

Fecal materials were enumerated on selective media. Before being challenged with the APEC isolates, cloacae swabs were conducted to determine the appearance of their natural microflora on selective agars. Bacterial enumeration by typical microbiological methods indicated that the bacteria already present in the guts of these broilers had the same magenta color and coccoid morphology on MAC and SMAC agars as our APEC culture stocks. This prevented detection of the APEC inoculum from the cloacae swambs. Microbial detection of APEC colonies on Rainbow agar was also not possible. However, the use of SMAC agar did allow verification that the group inoculated with *E. coli* O157:H7 strain 4407 did not shed these bacteria in their feces since this strain grows colorless on SMAC, but the swabs from this group of chickens did not show any colorless colonies on SMAC, only the typical magenta. Furthermore, after inoculation the plates from the negative control groups showed no differences in color, morphology, or bacterial load than plates from the experimental groups. Thus, no evidence was provided that the experimental groups were shedding any differently than the control groups.

Bacterial colonies were tested at random using an O157 agglutination spot-test from broilers inoculated with O157 APEC isolates. While stock colonies of both the O157:H7 reference strain and the five O157 APEC isolates were positive by this test, all of the randomly selected colonies from the fecal samples within these groups were negative by this test.

PCR was used in a last attempt to determine APEC-shedding by the broilers. Bacterial colonies from each group's swabs were evaluated alongside the corresponding inoculum isolate for similarities in genes and banding patterns. It was clear that the colonies from the swabs did not possess the same genes as their "parent stocks." Total DNA from the ceca was also isolated and assessed by multiplex PCR. DNA profiles did not match their group's corresponding DNA profile of the APEC isolate.

While APEC-shedding could not be determined, levels of illness associated with APEC inoculation were observed. At 4 days post-infection (dpi), a total of 3 birds were found dead. The necropsies of these birds revealed bloated intestines and hemorrhaging in the breast muscle. These birds had been inoculated with APEC 7 (x2) and APEC 25. At 19 dpi, a bird inoculated with APEC 3 was culled due to illness. From observation alone it could be seen that this bird had ruffled feathers, labored breathing, and outwardly appeared ill. A bird from the negative control group was also culled for comparison at necropsy. The ill bird showed signs of pericarditis, perihepatitis, and pleuropneumonia, all typical of an APEC infection; while the appearance of the negative control bird was what you would expect to see in a healthy broiler. Figure 4 is a picture illustrating some of the lesions caused by APEC. At 20 dpi another bird inoculated with APEC 3 died spontaneously, with the total APEC

related mortality of the flock to 5 broilers. At the conclusion of the experiment only two other broilers showed signs of APEC illness at necropsy, neither from a group that experienced mortality. These necropsies revealed dark and pronounced veins on their bloated intestines as well as ascites or water belly. All groups inoculated with APEC were observed to have diarrhea throughout the experiment which is another indication of colibacillosis.

4.6 Attachment of APEC to Retail Chicken Breast

Attachment to chicken breast muscle was used to gain a better appreciation for the potential of APEC transfer to consumers while handling contaminated raw poultry, or during the consumption of undercooked poultry. The concern for zoonotic risk is a main focus of this research. Attachment is determined through an S_R value which is a calculated number that represents the percentage of the total bacterial population that is strongly attached to the chicken breast (Dickson and Koohmaraie, 1989). As previously discussed, a total of 28 APEC isolates where chosen for this portion of the study based on the presence of an EHEC virulence gene in an earlier performed multiplex PCR.

Table 7 shows the S_R mean ± the standard deviation for each isolate. A student's t-test was also performed on the output from the 33 isolates in order to compare which isolates were significantly different from each other in terms of attachment capability to chicken breast tissue. This can be seen in Table 8. After examination of the various levels of significance, it is clear that the APEC exhibit a "flowing spectrum" of their attachment strength. For comparison purposes, the attachment capabilities of the outbreak and clinical UPEC strains were tested. Since attachment is a hallmark of pathogenesis, and these strains have already been proven

to cause human disease, it is crucial to compare the attachment capabilities of APEC to these strains. Additionally, the results from the gentamycin resistance trial revealed that all 28 APEC strains used were resistant to this antimicrobial. This is an important piece of evidence showing APEC as a hearty and versatile pathotype of *E. coli*.

From the levels of significant difference presented in the student's t-test in Table 8, some important conclusions can be drawn. First, attachment strength to chicken breast varies greatly among the APEC isolates. However, of more importance is that 10 APEC isolates had a significantly stronger S_R value than *E. coli* O104, 5 APEC isolates had a significantly stronger S_R value than *E. coli* O157:H7 strain 4407, and 3 APEC isolates had a significantly stronger S_R value than the strongest attaching UPEC. These results are interesting because they illustrate that certain strains of APEC have a very strong affinity for attachment to chicken breast, and even outperform strains involved in clinical human disease. This is important because with such strong attachment properties certain APEC isolates could become a real zoonotic threat if they were to acquire the proper genetic material.

4.7 HCT-8 Cell Attachment Assay

To further understand attachment, the 28 APEC isolates and references strains were assessed for their ability to attach to a human cell line. An initial inoculum of ~1.0 X 10^8 cfu/ml was used. This experiment was run in duplicate and Table 9 shows the mean log attachment ± the standard deviation for each isolate. A student's t-test was performed on the data and similar to the chicken breast experiment, the APEC again exhibited a "flowing spectrum" of how well they attached.

From the levels of significant difference presented in the student's t-test in Table 10, some important conclusions can be drawn. First, attachment strength to the HCT-8 mammalian cell line widely varies among the APEC isolates. However, of more importance is that all 28 APEC isolates attached significantly stronger than *E. coli* O157:H7 strain 4407, 12 APEC isolates attached significantly stronger than the highest performing UPEC, and 10 APEC isolates attached significantly stronger than *E. coli* O104:H4. Also of interest is that APEC 22 attached at a significantly higher level than all 32 of the other isolates tested. Something of note is that although strain 4407 did not attach to HCT-8 cells, it did show some attachment to Caco-2 cells that were tested in a side project. Similar to the chicken breast attachment work, certain APEC were shown to have a strong affinity for attachment to the HCT-8 human cell line. The ability to effectively attach to cells is a key factor in pathogenicity, and this furthers the argument as to why APEC can be considered a potential zoonotic concern.

This attachment to mammalian cells is important because it is the first step of bacterial colonization. After *E. coli* is ingested it must survive the acidic conditions of the stomach, pass into the intestine, and adhere to the epithelial cells of the colon in order to colonize the host (Torres *et al.*, 2005). In EHEC, this intimate attachment is aided by virulence factors such as intimin encoded by the *eae* gene and a host cell-bacteria bridge encoded by *espA*, *espB*, and *espD* (Frankel *et al.*, 1998). For APEC, a number of other attachment factors may be involved such as the *E. coli* pilus, curli fibers, and bundle-forming pili (Parti *et al.*, 2011).

4.8 Conclusions

In summary, a number of different techniques were used in order to better characterize APEC as an *E. coli* pathotype and to continue the discussion of whether

or not this group of bacteria should be viewed as a zoonotic risk. The experiments that were performed were chosen in such a way as to evaluate multiple different aspects of the bacteria's disease-causing capabilities. Virulence genes of both EHEC and ExPEC nature were screened for in order to determine a virulence repertoire of APEC as well as compare this repertoire to know human pathogenic varieties of *E. coli*. A challenge study was used to assess whether or not commercial broilers infected with APEC would shed the bacteria in their feces and thus lead to its spread into food environments. Lastly, APEC attachment to a food matrix as well as a human cell-line was evaluated since these types of attachment are crucial components in disease-causing *E. coli*. In general, the results from each experiment were quite variable among all of the APEC isolates tested, and this speaks to the diverse nature of the *E. coli* species as a whole.

The genetic profiling of the APEC strains for the EHEC genes was similar to what was hypothesized. We didn't expect many of the APEC to possess the EHEC genes which they were screened for because these are defined as two distinct *E. coli* pathotypes (Donnenberg, 2002). In total, 27 of the 28 APEC genes found to possess an EHEC gene contained one of the accessory virulence genes including *katP* (reduces oxidative stress), *espP* (protease), *stcE* (contributes to attachment), and *ehxA* (enterohemolysin). However, while none of the APEC isolates were positive for either *stx1* or *stx2*, there was one isolate that contained the *eaeA* gene which is widely considered a very important virulence factor in the pathogenicity of EHEC. Identifying this one APEC isolate with the *eaeA* gene is definitely an important finding since this gene is a known EHEC marker. Yet the other 27 isolates should not

be cast aside as these genes too are crucial in the causation of disease as seen by *E. coli* O157:H7 strain 4407 possessing all of them.

The results from the screening of the ExPEC genes was also similar to what was hypothesized. The APEC isolates possessed a much higher percentage of these genes in comparison to the EHEC genes. Since APEC is a subset of the ExPEC pathotype, this followed the trend that we expected to observe. Additionally, the UPEC reference strains also possessed a number of these ExPEC genes while the EHEC reference strains were shown to not contain any of them. This furthers the notion that APEC and UPEC are at least somewhat related and are thus grouped under the same ExPEC umbrella. This genetic similarity between APEC and UPEC and their genetic dissimilarity to EHEC agrees with results shown in other studies (Ewers *et al.,* 2004; Delicato *et al.,* 2003).

Besides the comparison of APEC to both the EHEC and ExPEC pathotypes, the characterization work also revealed a number of noteworthy APEC isolates. APEC 2 was a unique isolate because this was the only strain out of all 28 that was beta-glucuronidase negative and therefore undetectable by the MUG test. Also, APEC 23 was interesting because its O-type was O157, its isolation location was from the intestines, and it was not shown to possess any of the ExPEC genes. Therefore, this isolate shares many similarities with typical EHEC. Finally, APEC 24 was significant because this was the only isolate out of 100 screened that contained a gene from the LEE pathogenicity island which is directly associated with EHEC virulence.

Assessing whether APEC-infected birds would shed the bacteria in their feces was a crucial question for the spread of this microorganism. From the results of our challenge study, we were not able to conclude that the APEC bacteria were shed in the

feces of infected broiler chickens. However, we faced many difficulties due to the large amount of background microbiota present in the chickens; therefore, additional research with more precise experimental designs must be conducted in this area. Possible solutions to reduce the background microbiota would include incubating the plates at 44°C or supplementing the selective agar with nalidixic acid since many APEC isolates are known to be resistant. The threat of *E. coli* from avian sources is still a real issue. Studies have isolated *E. coli* containing either intimin or Shiga toxin from the feces of wild birds (Kobayashi *et al.*, 2009) as well as identified the presence of APEC on retail chicken meat (Vidotto *et al.*, 2010).

One of the more significant findings from this study was how well certain isolates of APEC were able to attach to both a food matrix such as chicken breast and human intestinal cells. In each assay, a significant number of APEC isolates outperformed both of the outbreak strains of *E. coli* and the clinical UPEC strains as discussed earlier. The biggest issue present with the attachment work was how variable the APEC strains performed between the two tests. When each data set was broken up into three equal tiers of eleven, there was a total of eight isolates that were in the top tier for one assay and the bottom tier for the other assay. A correlation was run on JMP to check for a relationship between the two tests, but the test only revealed an extremely weak negative correlation (-0.11) that was not significant. However, of great interest was that 3 APEC isolates (1, 4, & 27) performed in the top tiers for both of the attachment tests, suggesting the potential for certain strains to attach strongly to both food matrices and human cells. This is an important finding in regards to the possible transmission of APEC to humans.

The main goal of this work was to achieve a better understanding of APEC and better understand the threat it could pose as an emerging foodborne pathogen. Our results showed the ability of certain isolates of APEC to attach at very high levels to both chicken breast meat as well as human intestinal cells. Therefore, if these APEC isolates possessed the right combination of virulence factors, they could pose a similar threat to public health as current human pathogenic strains of *E. coli* such as O157:H7. While our study did not show APEC to be a threat in terms of genetic virulence factors, other studies have shown more alarming results (Parreira and Gyles, 2002; Mellata *et al.*, 2001). Further research needs to be conducted on the genetic characteristics of APEC, especially on the presence of the *stx1*, *stx2*, and *eaeA* genes. Additionally, studies should focus on whether or not APEC can act as a source of food-acquired extraintestinal infection. Finally, a comprehensive study following the persistence of APEC through the farm to fork continuum would be very helpful in solving many of the unknown questions regarding this pathotype.

Table 1: Virulence Plasmid (pO157) Genes Multiplex PCR Parameters

Primer Name	Primer Sequence (5'→3')	Amplicon Size
katp (F)	GCGCCAGTGGTGGTCAGCAA	914 bp
katp (R)	ATATCGGGCTGCCGGTCCCA	
espP (F)	GCTGGCAACCAGCAACAGCG	774 bp
espP (R)	CGGTAGCCCGCTTCTGCACC	
stcE (F)	GGCTCCGGAGGTGGGGGAAT	399 bp
stcE (R)	GAAGCCGGTGGAGGAACGGC	
ehxA(F)	ACAGCTGCAAGTGCGGGTCTG	262 bp
ehxA (R)	GGGATGCACTGGAGGCTGCAC	

Table 2: Shiga toxins & Pathogenicity Island Genes Multiplex PCR Parameters

Primer Name	Primer Sequence (5'→3')	Amplicon Size
eaeA (F)	GACCCGGCACAAGCATAAGC	384 bp
eaeA (R)	CCACCTGCAGCAACAAGAGG	
stx1 (F)	ATAAATCGCCATTCGTTGACTAC	180 bp
stx1(R)	AGAACGCCCACTGAGATCATC	
stx2 (F)	GGCACTGTCTGAAACTGCTCC	255 bp
stx2 (R)	TCGCCAGTTATCTGACATTCTG	
espA (F)	GCCAAACTTCCTCAAGACGTG	100 bp
espA (R)	CACCAGCGCTTAAATCACCAC	

Table 3: ExPEC Virulence Genes Multiplex PCR Parameters

Primer Name	Primer Sequence (5'→3')	Amplicon Size
iucD (F)	ACAAAAAGTTCTATCGCTTCC	714 bp
iucD (R)	CCTGATCCAGATGATGCTC	
vat (F)	TCCTGGGACATAATGGTCAG	981 bp
vat (R)	GTGTCAGAACGGAATTGT	
astA (F)	TGCCATCAACACAGTATATCC	116 bp
astA (R)	TCAGGTCGCGAGTGACGGC	
iss (F)	ATCACATAGGATTCTGCCG	309 bp
iss (R)	CAGCGGAGTATAGATGCCA	
papC (F)	TGATATCACGCAGTCAGTAGC	501 bp
papC (R)	CCGGCCATATTCACATAA	
tsh (F)	ACTATTCTCTGCAGGAAGTC	824 bp
tsh (R)	CTTCCGATGTTCTGAACGT	
cva A/B	TGGTAGAATGTGCCAGAGCAAG	1181 bp
cvi cvaC	GAGCTGTTTGTAGCGAAGCC	

Table 4: Presence of EHEC Virulence Genes in 100 Screened APEC Isolates

stx1	0/100	**katP**	15/100
stx2	0/100	**espP**	8/100
eaeA	1/100	**stcE**	2/100
espA	0/100	**ehxA**	2/100

Table 5: Percentage of ExPEC Virulence Genes in 100 Screened APEC Isolates

tsh	5%	**cva/cvi**	30%
papC	12%	**iss**	39%
astA	17%	**iucD**	60%
vat	23%		

Table 6: Summary of Significant Isolates Chosen for Further Evaluation

Isolate	**O-type**	**Location**	**MUG**	**EHEC Genes**	**ExPEC Genes**
APEC 1 *(03-2096)*	O20	Unknown	+	katP	iss, iucD
APEC 2 *(05-2561)*	Untypable	Unknown	-	katP	papC, iucD
APEC 3 *(05-2565)*	O157	Unknown	+	espP	iss, iucD, vat
APEC 4 *(05-2884)*	Untypable	Unknown	+	katP	iss, iucD, tsh
APEC 5 *(05-3800)*	O35	Unknown	+	katP	iss, iucD
APEC 6 *(06-3320)*	O6	Unknown	+	katP	iss, iucD
APEC 7 *(06-5822)*	O157	Unknown	+	espP	none
APEC 8	O157	Unknown	+	katP	iss

(07-0550)					
APEC 9 *(07-0551)*	O157	Unknown	+	espP	iss, vat, cva
APEC 10 *(07-0624)*	Untypable	Unknown	+	katP	iss, iucD, tsh
APEC 11 *(07-0717)*	O157	Pericardium	+	espP	iss, iucD, vat
APEC 12 *(07-0764)*	O1	Liver	+	stcE	iss, tsh
APEC 13 *(07-0969)*	O102	Pericardium	+	katP	astA, iss, iucD
APEC 14 *(07-1078)*	O8	Hock Joint	+	espP	iss, iucD
APEC 15 *(07-1204)*	O157	Yolk	+	ehxA	astA, iss, iucD
APEC 16 *(07-1232)*	O5	Pericardium	+	katP	astA, iucD
APEC 17 *(07-1307)*	O8	Hock Joint	+	ehxA	iss, iucD, vat
APEC 18 *(07-1312)*	O78	Hock Joint	+	espP	iss, iucD
APEC 19 *(07-1316)*	O12	Hock Joint	+	espP	iss, iucD, vat
APEC 20	O78	Pericardium	+	katP	iss, iucD,

(07-1351)					tsh
APEC 21 *(07-1352)*	O78	Pericardium	+	katP	iss, iucD, vat
APEC 22 *(07-1520)*	O9	Yolk	+	katP	iss, iucD
APEC 23 *(07-2520)*	O157	Intestine	+	espP	none
APEC 24 *(07-5668)*	O13	Unknown	+	eaeA	iss, iucD
APEC 25 *(07-6849)*	O157	Unknown	+	katP	iucD, vat
APEC 26 *(97-1009)*	Untypable	Unknown	+	katP	astA, iss, iucD
APEC 27 *(99-872)*	O5	Liver	+	katP	astA
APEC 28 *(99-144)*	O2	Hock Joint	+	stcE	iss, iucD, vat
UPEC 1	X	Clinical	+	stcE	none
UPEC 2	X	Clinical	+	espP	iss, iucD
UPEC 3	X	Clinical	+	none	papC
4407	O157	Outbreak	+	All 8	none
O104:H4	O104	Outbreak	+	stx2, espP	none

Cells with an "X" indicate that this particular data was not provided for this isolate.

Table 7: S_R Attachment Values on Retail Chicken Breast

Isolate	**S_R Mean ± SD**	**Isolate**	**S_R Mean ± SD**	**Isolate**	**S_R Mean ± SD**
APEC 1	62.3±9.1	APEC 12	8.8±1.8	APEC 23	13.9±0.6
APEC 2	28.9±1.9	APEC 13	21±0.2	APEC 24	31.6±0.7
APEC 3	35.2±1	APEC 14	17±1.9	APEC 25	7.2±3.8
APEC 4	70.5±6	APEC 15	36.1±12.8	APEC 26	5.3±0.3
APEC 5	12.4±2.3	APEC 16	13.2±5	APEC 27	44.4±24
APEC 6	16.9±1.7	APEC 17	24.8±12	APEC 28	14.2±2.3
APEC 7	44.4±12.7	APEC 18	12.7±0	UPEC 1	31.8±5.8
APEC 8	27.2±6.5	APEC 19	34.8±3.1	UPEC 2	23.8±5.2
APEC 9	50±1	APEC 20	6±0.5	UPEC 3	32.1±6
APEC 10	12.3±0.3	APEC 21	15.8±12.6	4407	27.7±2.4
APEC 11	16.6±7	APEC 22	0±0	O104:H4	13.4±1.8

*S_R value = (strongly attached bacteria)/(loosely + strongly attached bacteria)

Table 8: Student's t-test for S_R Attachment Value to Chicken Breast

Level												Mean
APEC 4	A											70.508705
APEC 1	A	B										62.321278
APEC 9		B	C									50.009132
APEC 7			C	D								44.407190
APEC 27			C	D								44.361063
APEC 15			C	D	E							36.128057
APEC 3				D	E							35.246583
APEC 19				D	E	F						34.840682
ATCC				D	E	F						32.062947
UPEC 1				D	E	F						31.831336
APEC 24				D	E	F						31.558823
APEC 2					E	F	G					28.909383
4407					E	F	G	H				27.699013
APEC 8					E	F	G	H	I			27.204449
APEC 17					E	F	G	H	I	J		24.833997
UPEC 6					E	F	G	H	I	J		23.846562
APEC 13						F	G	H	I	J	K	20.963630
APEC 14							G	H	I	J	K	L 17.025459
APEC 6							G	H	I	J	K	L 16.850479
APEC 11							G	H	I	J	K	L 16.615532
APEC 21							G	H	I	J	K	L 15.764572
APEC 28								H	I	J	K	L 14.248907
APEC 23								H	I	J	K	L M 13.902084
O104:H4									I	J	K	L M 13.416210
APEC 16									I	J	K	L M 13.227735
APEC 18										J	K	L M 12.732575
APEC 5										J	K	L M 12.417614
APEC 10										J	K	L M 12.266053
APEC 12											K	L M 8.832936
APEC 25											K	L M 7.216347
APEC 20												L M 5.953412
APEC 26												L M 5.265593
APEC 22												M 0.000000

Levels not connected by same letter are significantly different.

Table 9: Log Attachment to HCT-8 Cell Line

Isolate	**Log Mean ± SD**	**Isolate**	**Log Mean ± SD**	**Isolate**	**Log Mean ± SD**
APEC 1	6.93±0.32	APEC 12	2.7±0	APEC 23	6.88±0.06
APEC 2	6.4±0.11	APEC 13	6.92±0.02	APEC 24	6.08±0.09
APEC 3	6.27±0.1	APEC 14	5.54±0.1	APEC 25	6.79±0
APEC 4	6.79±0.05	APEC 15	3.78±0.2	APEC 26	6.83±0.17
APEC 5	6.78±0.12	APEC 16	7±0.13	APEC 27	7.16±0.01
APEC 6	6.83±0.13	APEC 17	5.97±0.07	APEC 28	6.19±0
APEC 7	6.46±0.24	APEC 18	5.44±0.03	UPEC 1	6.43±0.32
APEC 8	6.51±0.03	APEC 19	6.12±0.13	UPEC 2	6.13±0.03
APEC 9	2.98±0.28	APEC 20	6.95±0	UPEC 3	0±0
APEC 10	3.52±0.06	APEC 21	6.62±0.09	4407	0±0
APEC 11	6.04±0.03	APEC 22	8.39±0.12	O104:H4	6.53±0.14

Table 10: Student's t-test for Log Attachment to HCT-8 Cell Line

Level															Mean
APEC 22	A														8.3944376
APEC 27		B													7.1552936
APEC 16		B	C												6.9964975
APEC 20		B	C												6.9542425
APEC 1		B	C												6.9344691
APEC 13		B	C												6.9162545
APEC 23			C	D											6.8783941
APEC 26			C	D											6.8309063
APEC 6			C	D											6.8297204
CD			C	D											6.8116246
APEC 4			C	D											6.7941919
APEC 25			C	D	E										6.7888608
APEC 5			C	D	E										6.7811464
APEC 21				D	E	F									6.6187719
O104:H4					E	F	G								6.5273065
APEC 8						F	G								6.5114203
APEC 7						F	G								6.4566420
UPEC 1						F	G	H							6.4334389
APEC 2						F	G	H							6.4007019
APEC 3							G	H	I						6.2689284
APEC 28								H	I	J					6.1861061
UPEC 6									I	J					6.1344761
APEC 19									I	J					6.1207857
APEC 24									I	J					6.0831262
APEC 11									I	J					6.0350559
APEC 17										J					5.9659577
APEC 14											K				5.5449526
APEC 18											K				5.4386857
APEC 15												L			3.7762733
APEC 10												L			3.5226615
APEC 9													M		2.9771213
APEC 12														N	2.6989700
ATCC														O	0.0000000
4407														O	0.0000000

Levels not connected by same letter are significantly different.

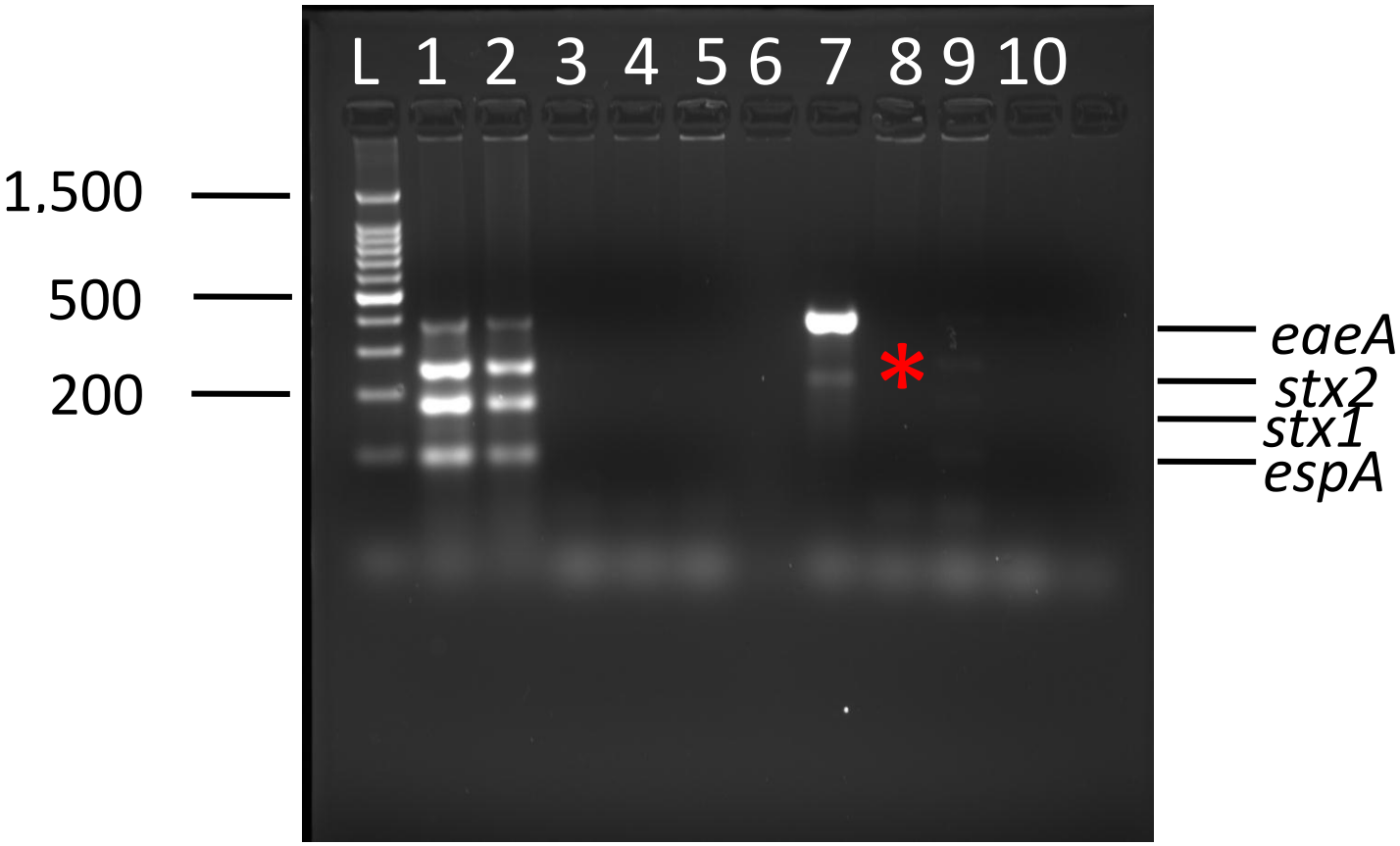

Figure 1. Agarose gel image of multiplex PCR. L) molecular weight ladder; 1 & 2) O157:H7 positive controls; 3) O157:H12 negative control; 4-11) sample APEC isolates showing presence of *eaeA* in lane 7.

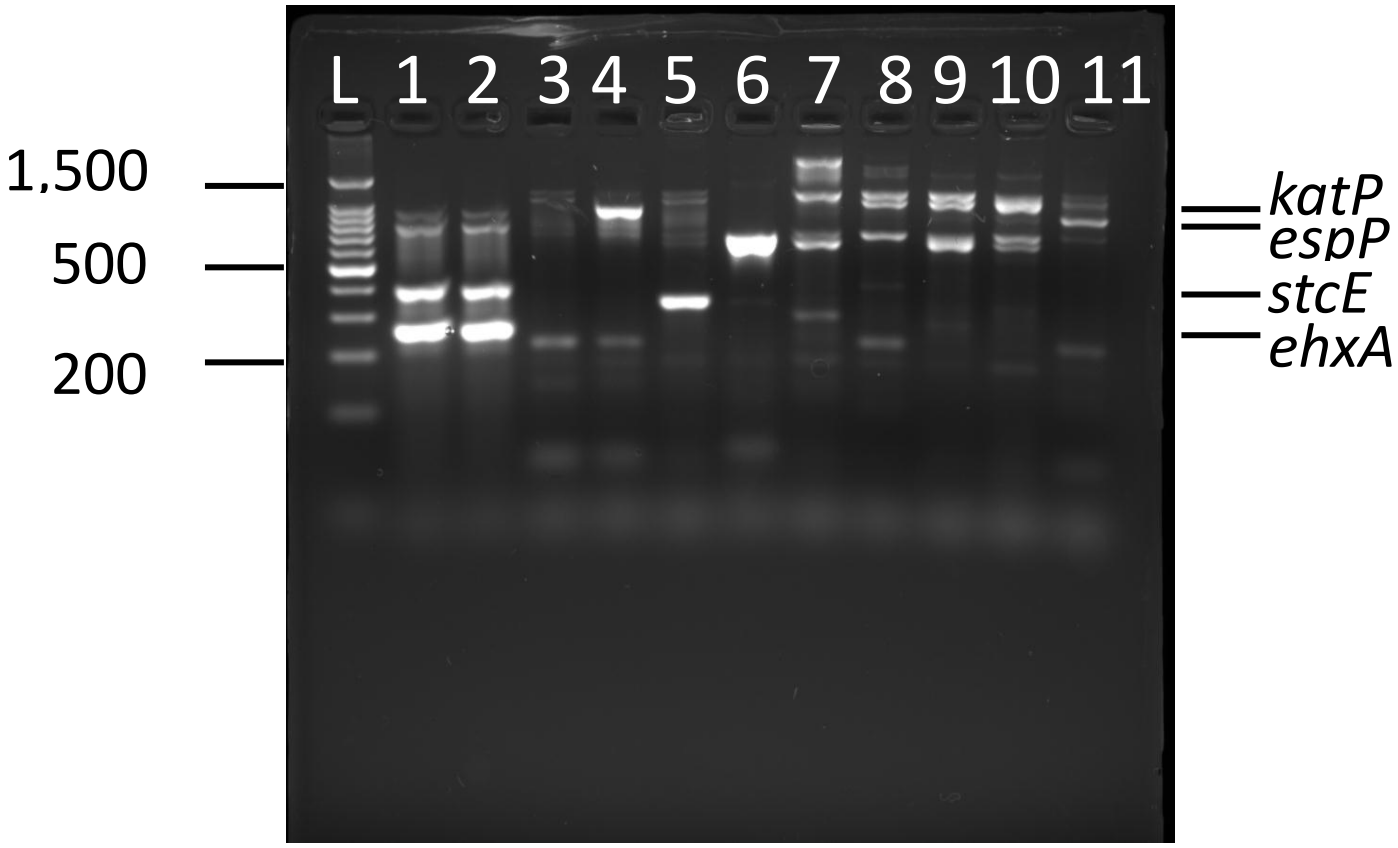

Figure 2: Agarose gel image of multiplex PCR for virulence plasmid. L) molecular weight ladder; 1 & 2) O157:H7 positive controls; 3) O157:H12 negative control; 4-11) sample APEC isolates showing presence of *katP* in lanes 4, 8-10, *espP* in lane 11, and *stcE* in lane 5.

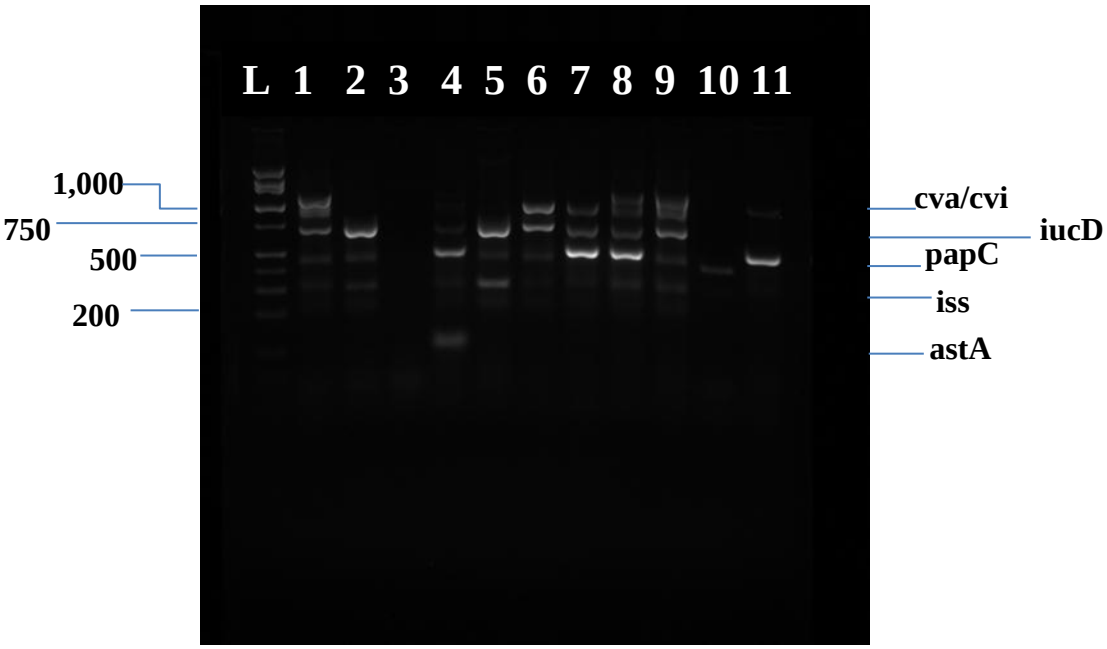

Figure 3: Agarose gel image of multiplex PCR for ExPEC genes. L) molecular weight ladder; 1-11) sample APEC isolates showing presence of astA in lane 4, iss in lane 5, papC in lanes 4, 7, 8, & 11, iucD in lanes 1, 2, 5, 7, 8, & 9, and cva/cvi in lanes 1 & 6.

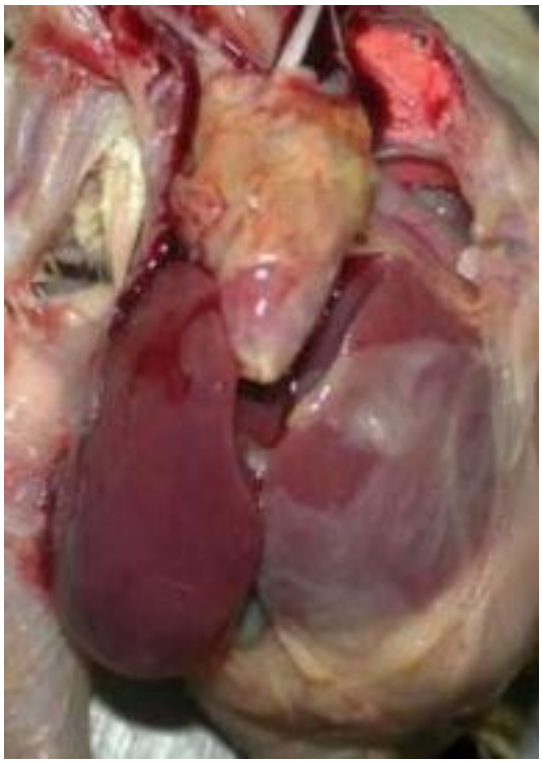

Figure 4: Picture of pericarditis & perihepatitis in a broiler with APEC infection.

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Appendix

AACUC SUBMISSION FORM

UNIVERSITY OF DELAWARE

COLLEGE OF AGRICULTURE AND NATURAL RESOURCES

AGRICULTURAL ANIMAL CARE AND USE COMMITTEE

Application for Use of Agricultural Animals

In Teaching or Research

AACUC Protocol Number: (1) 09-27-11T

TITLE OF PROJECT: Avian Pathogenic *E. coli* Challenge Study

INSTRUCTOR/PRINCIPAL INVESTIGATOR

Kalmia Kniel
Printed Name **Signature**
Date

(This section for Committee use only)

Application Approved (date) _____

Application Rejected (date) _____

Reason for Rejection _____

_____ _____

Signature, Animal Care and Use Committee **Date**

1. Proposals, grant applications, and research contracts designating the use of agricultural animals for research and/or teaching at the University of Delaware, including Agricultural Experiment Station Farm (Newark), Research and Education Center (Georgetown), or Lasher Lab (Georgetown) must receive approval of the Agricultural Animal Care and Use Committee prior to initiation of the project. Approval by the Committee will be obtained as part of the grant proposal approval process of the College of Agriculture and Natural Resources. Documentation of approval will be retained in the Office of the Animal and Food Sciences Department, College of Agriculture and Natural Resources and will not be processed further with the proposal unless required by the granting agency.

2. The Secretary of the Committee has the authority to approve applications that follow approved farm management standard operating procedures and procedures that have been pre-approved by the Committee. Applications that include procedures not pre-approved by the Committee require committee review and approval.

3. Questions concerning the approval process should be directed to the Committee Secretary or Chairperson.

4. The investigator should know, when possible, the source of the animal subjects and the animal's genetic make-up, environmental background and health history.

5. Please read this form and fill out all sections. Failure to do so may delay review of the application.

6. Applications requiring Committee review and approval should be submitted electronically to: Secretary
   Agricultural Animal Care and Use Committee
   044 Townsend Hall
   University of Delaware

7. If University facilities are used, the Principal Investigator must be a University faculty or professional staff member.

APPLICATION INFORMATION:

Title: **Avian Pathogenic *E. coli* Challenge Study**

Instructor/Principal Investigator: **Kalmia Kniel**

Address: **044 Townsend Hall**

Telephone: **302-831-6513** Email: **kniel@udel.edu**

People involved in animal care (not listed above) for this protocol:

Name	Email	Office Phone #	Home/Cell Phone #	Received Animal Care Training	
				Yes	No
Co-investigator(s)					
Kyle LeStrange	klestra@udel.edu	N/A	631-678-6603	X	
Sarah Markland	sarmark@udel.edu	N/A	302-299-8236	X	
Krystal Shortlidge	kshort@udel.edu	N/A	302-250-8043	X	

Name the person(s) responsible for conducting the training.

Robert Alphin trained Kyle LeStrange, and Kyle trained the other co-investigators.

If after hours participation is required by students, please describe how this is being handled. (e.g. supervisors, assistants, etc.) Please include the times and days that students may be on site.

Checking of the birds on the weekend conducted by Kyle LeStrange, Kali Kniel, and co-investigators.

Has everyone listed above read the application and is familiar with the proposed work?

YES **X** NO □

If no, identify those needing to read application.

New or Three Year Review (mark one)

NEW □ THREE YEAR **X**

If this is a 3 year renewal, what is the assigned existing protocol number?

(1) 09-27-11T

Teaching or Research Application (mark one)

TEACHING □ RESEARCH **X**

If TEACHING box was checked, select from the following:

Demonstration □ Laboratory □ Student Project □

Proposed start date: 2/1/12 End date: 3/2/12

Are all proposed animal care management procedures 1) defined as "pre-approved" by the Animal Care and Use Committee, or 2) part of the Standard Operating Procedures developed by the Animal Care and Use Committee for that particular species?

YES **X** NO □ to be determined by AACUC □

Has everyone been trained in all procedures that are listed in this protocol? Yes **X** No □

Who has not been trained?

_____ _____ _____

ANIMAL INFORMATION:

Common Name of the Animal Requested: **Broiler Chicks**

Amount Being Requested: **30**

Source of Animals: **Local grower**

Where are the animals being housed: **Block House #9**

Briefly Describe the Goals or Objectives of this Application (use additional space as needed).

The goal of this application is to determine whether the feces from broilers infected with APEC can be a viable transmission route to spreading the pathogen onto crops.

Please state or attach your animal protocol.

How did you determine the number of experimental animals you are requesting? If you have a table showing treatment groups and animal numbers please insert here or include as an attachment.

There were a total of 8 *E. coli* isolates we wanted to initially test to see if they were shed by the birds. Because we wanted each group to be performed in triplicate for statistical accuracy, we needed a total of about 30 birds.

Please verify that the research involved in this protocol is new and is not a duplication of work already performed.

Yes, this is new work.

Does this procedure involve surgery? YES □ NO **X**

If yes, explain in detail the surgery.

Will the animals experience pain? YES □ NO **X**

If so, what is your pain management protocol? Please insert here or include as an attachment (euthanasia is an acceptable means of pain management):

_____
_____

Are drugs, vaccines and/or medications being used? YES □ NO **X**

If yes, describe what is being used. Include dosages and sites.

How often are animals monitored and how are sick or injured animals being handled?

Birds will be checked at least once every day. Any birds that are sick or injured and cannot reach food or water will be euthanized.

What is the method of euthanasia?

Cervical dislocation

List the veterinarian who is on-call.

Dr. Miguel Ruano **302-831-1539**
Name Telephone

Does this application need approval from EHS? YES □ NO **X**

If yes, what form(s) are attached?_____

NOTE: EHS approval is required for experiments involving the administration of hazardous or biological materials such as pathogens, carcinogens, highly toxic, or radioactive materials.