Evaluation of the virulence potential of avian pathogenic Escherichia coli isolated from broiler breeders with colibacillosis in Mississippi

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Evaluation of the virulence potential of avian pathogenic Escherichia coli isolated from broiler breeders with colibacillosis in Mississippi

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Avian pathogenic *Escherichia coli* (APEC) is a bacterium that is responsible for colibacillosis in birds. However, information about broiler breeder APEC isolates is limited, but the data is critical due to the transfer of this bacteria down the production pyramid to progenies resulting in high mortality. Therefore, we evaluated the phenotypic virulence characteristics of 28 isolates using embryo lethality and day-old chick challenge assays. Also, the *in vitro* adhesion and invasion potential of selected nine isolates were identified. Results showed more than 1/3rd of the isolates were highly virulent and the virulence increased as the number of virulence-associated genes increased. High adhesion and invasion rates were observed among the isolates. Overall, the study helped us to evaluate the virulence characteristics of APEC from broiler breeders. However, future studies based on whole genome approach would help to identify the specific targets which can be used to develop effective interventions.
DEDICATION

I humbly dedicate this work to my family for their endless support, motivation, and encouragement throughout my career. They taught me to chase my dreams and I believe this is the best gift that I can give back to them.
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CHAPTER I
INTRODUCTION

Poultry species have the largest consumer demand among all livestock commodities around the world and is having the highest import share among livestock products globally (Matthew et al., 2022). The low cost involved in its’ production along with the high acceptance of the meat among all countries around the world, different systems of production to adjust the climatic variations across the globe, and genetic improvements throughout the past years are the main reason for this high demand and production (Brillard, 2001). Broiler breeders are the broiler parents which are critical in developing a healthy broiler flock to maintain the broiler supply chain (de Jong and Emous, 2017). Any infection affecting the broiler breeder flock would impact broiler production (Rosales, 1994). Therefore, it is essential to ensure broiler breeder flocks are free from any kind of disease (Swelum et al., 2021).

*Escherichia coli* is a gram negative, facultatively anaerobic, rod shaped bacillus present in the lower intestinal tract of most of the vertebrates. Extra-intestinal pathogenic *E. coli* is the organism which causes infections outside the intestinal tract and among them, Avian pathogenic *E. coli* (APEC) is the one causing disease in birds (Kathayat et al., 2021). APEC infections are a challenge to the poultry industry because it causes significant morbidity and mortality among birds due to respiratory and systemic infections (Dziva and Stevens, 2008). Moreover, different routes of infection such as respiratory, fecal-oral, and cloacal routes make the transmission of APEC much easy (Kathayat et al., 2021). Further, from broiler breeders, vertical transmission of
APEC has been identified and is an important factor for the increased first-week mortality among the broiler chicks which is further exacerbated by the spread within the hatchery (Yassin et al., 2009). Therefore, it is very important to control the disease in broiler breeders to ensure both broiler and broiler breeder health. For that, we need to continuously monitor the evolving bacterial populations to develop new measures that can be used to control the disease (Christensen et al., 2021).

Genotypic characterization is an excellent tool to evaluate the virulence potential of bacterial isolates (Pires-dos-Santos et al., 2013). Along with serotyping and phylogenetic classification, identification of the virulence-associated genes provides an idea about the virulence characteristics of each bacterial isolate (Nolan et al., 2019). Genes which encode for adhesion, invasion, protection from host immunity, toxin production, iron acquisition and colicin V operon were identified in the *E. coli* isolates collected from broiler breeders used in this study by Joseph et al., (2023). The presence of these genotypic virulence factors will be compared with the phenotypic findings of the current study.

Phenotypic characterization is equally important as genotypic characterization as it helps to identify the host-pathogen interaction and the specific factors responsible for bacterial virulence and pathogenesis (de Souza et al., 2016). In this study, we used embryo lethality and one-day-old chick challenge assays to evaluate the phenotypic virulence of the bacterial isolates. These phenotypic assays are considered as gold standard test to determine virulence and are regarded better than traditional method such as serogrouping (Awad et al., 2020). Further, these techniques allow us to classify the APEC isolates based on their phenotypic virulence, which will enable us to select isolates of different levels of virulence for further research, including developing an *E. coli* challenge model in broiler breeders. Additionally, *in vitro* bacterial
colonization would help us to understand the host-pathogen interaction (Le Bouguénec, 2005). Adhesion and invasion potential can be used to determine the capacity of bacteria to infect the host cells (Döpfer et al., 2000). The chicken macrophage HD11 cells can be used to mimic the host-bacterial interactions and the macrophages are the first among the cells present at the site of infections (Kogut et al., 2012). In this study, we aimed to identify the in vitro adhesion and invasion potential of E. coli isolates to chicken macrophage HD11 cell line. The main objectives of the current study are the following.

**Objective 1: Determining the virulence potential of APEC using chicken embryo lethality assay and one-day-old chick challenge study**

This study investigates the phenotypic virulence potential of broiler breeder APEC isolates using chicken embryo lethality and one-day-old chick challenge assays which helps us to classify the isolates into highly virulent, moderately virulent, and avirulent.

**Objective 2: Determining the ability of APEC to infect broiler breeders using in vitro cell culture model**

This study investigates the in vitro adherence and invasion potential of the nine broiler breeder APEC isolates that were selected based on phenotypic and genotypic virulence characteristics of isolates using the chicken macrophage HD11 cell line.
Reference


CHAPTER II
REVIEW OF LITERATURE

Abstract
Poultry meat is one of the major animal protein sources necessary to meet the global protein demand. Sustainability in broiler production is the key to achieve its’ continuous supply, and broiler breeders play a critical role in maintaining this sustainability. Colibacillosis, the disease caused by avian pathogenic Escherichia coli (APEC), causes severe economic losses to the poultry industry globally. Moreover, they cause an additional burden among broiler breeders. Besides the decrease in egg production and mortality among breeders, there is vertical transmission of APEC to the broiler chicks through eggs, resulting in increased first-week mortality and subsequent horizontal transmission at the hatchery. Of interest, the vertical transmission of antibiotic-resistance genes is another concern that needs attention. Controlling the disease in broiler breeders would possibly reduce the first-week mortality and thereby maintain the production level. For that, constant monitoring of the bacterial populations is critical. Moreover, amidst the increased antibiotic resistance pattern, more focus on alternative treatment strategies like vaccines, probiotics, and bacteriophages is necessary. Future research focusing on strategies to mitigate APEC in broiler breeders would be one of the finest solutions for sustainable broiler production.

Keywords: avian pathogenic E. coli, broiler breeders, colibacillosis, vertical transmission, first-week mortality.
Introduction

The United States is a major contributor to the world’s broiler supply. It has the highest broiler production globally and around 20 million metric tons of chicken meat production annually (Chan et al., 2022). The value of broiler production from the U.S. in 2021 was $31.5 billion, 48% higher than in 2020 (NASS. USDA, 2021). Maintaining a continuous supply of broilers in the market needs an excellent strategy for managing broilers and broiler breeders (Brillard, 2007). Broiler breeders are the parents of broilers and play a critical role in developing a healthy broiler flock (de Jong et al., 2017). Decreased fertility, hatchability, and egg production are some of the major challenges faced by the broiler breeder industry (Zuidhof, 2018). These challenges could be due to a combination of factors such as improper management, stress, inadequate nutrition, immunosuppression, and exposure to disease agents. Identifying these factors and finding solutions on time is the key strategy for producing healthy broilers for the market (Kabir, 2017).

*Escherichia coli* is a bacterium commonly found in the normal intestinal flora of humans, other mammals, animals, and birds. However, some of them can act as major pathogens causing severe disease and high death tolls (Kaper et al., 2004). Extraintestinal pathogenic *E. coli* (ExPEC) are the ones that cause the disease called colibacillosis in animals and birds. Avian Pathogenic *E. coli* (APEC) is the specific ExPEC causing disease in birds, including chickens, ducks, and turkeys (Dho-Moulin and Fairbrother, 1999). Avian colibacillosis is manifested in diverse ways, such as colisepticemia, pericarditis, peritonitis, airsacculitis, salpingitis, yolk-sac infection, coligranuloma, cellulitis, and arthritis leading to high morbidity and mortality (Dziva and Stevens, 2008). This is one of the most commonly occurring and economically devastating bacterial diseases among poultry worldwide (Nolan et al., 2019).
The primary routes of APEC entry include fecal-oral, respiratory, and vaginal origin (ascending route) through the cloaca (Dho-Moulin and Fairbrother, 1999). The fecal-oral and respiratory type-colibacillosis has received the most attention (Kathayat et al., 2021). However, the ascending route through the cloaca is also very important as it results in outbreaks characterized by salpingitis-peritonitis syndrome in broiler breeders and causes huge economic losses due to decreased egg production and quality, increased mortality, and the cost associated with treatment, culling of birds, and disposal of carcasses. This is further exacerbated by the vertical transmission of APEC from breeders to progenies leading to yolk-sac infection, omphalitis, and increased first-week mortality among broiler chicks (Welten, 2019; Swelum et al., 2021). Moreover, infected chicks, dead embryos, and eggshells act as potential sources of infection in the hatchery leading to horizontal transmission among uninfected chicks and contamination of equipment (Poulsen et al., 2017; Thøfner et al., 2019). Additionally, vertical transmission aid in the transfer of antibiotic-resistant APEC strains down the production pyramid, making their control extremely difficult (Benameur et al., 2019; Oikarainen et al., 2019).

Traditionally, prevention and control of APEC mainly focus on hygiene achieved through strict biosecurity measures and proper cleaning of facilities using disinfectants and antimicrobials, treatment using antibiotics, and vaccination strategies (Aviagen, 2018). Most of these traditional methods become ineffective over time mainly because of the evolution of resistant bacterial species (Joseph et al., 2023a). The emergence of antibiotic resistance among the bacterial population has led to restrictions on antibiotic use even for treatment purposes (Singer et al., 2019). As a result, alternatives to antibiotics, such as probiotics, prebiotics, and phage therapy, are getting more acceptance (Christensen et al., 2021). In the case of vaccines,
different types, such as inactivated, subunit, and live attenuated vaccines, are available in the market for APEC prevention such as Poulvac *E.coli* and Nobilis *E.coli*, but they provide no or limited cross-protection between strains. Hence, more potent vaccines targeting heterogenous APEC strains need to be developed (Kathayat et al., 2021). In addition, for the effective control of APEC among broilers, it should be first controlled at the highest level of the production pyramid, i.e., among broiler breeders, and subsequently should be maintained down to the lowest level (Christensen et al., 2021).

This review discusses the importance of APEC in broiler breeders as they are an understated critical point in APEC transmission and control in broiler production. We reviewed the broiler breeder industry in general, the factors contributing towards APEC infection in breeders and transmission to broilers, strategies needed to prevent and control the disease, and future research directions.

**Broiler breeder industry and its’ challenges**

The necessity for animal protein, especially poultry meat, is increasing day by day throughout the world. Per capita meat consumption in the U.S. in 2021 shows that the most consumed meat type is broiler meat, which is about 95.6 pounds. However, there is a decrease in per capita consumption in 2022, but forecasts predict it will again increase to 101.6 pounds in 2031 (Decuypere et al., 2010; Hannah et al., 2019). The high preference for broiler meat is mainly because of its availability, low-fat content, and absence of religious taboos (Leeson et al., 2009). To meet the increasing demand, there should be a continuous supply of broilers to the market. To achieve this, there is a need to effectively select and manage (health and nutrition) the broiler parent flock, the broiler breeders (Leeson et al., 2009).
In the 80s and 90s, the United States and Europe became the major markets for broiler meat. As the market matured, primary emphasis was given to breast muscle yield, and it became the main selection criterion (Leeson et al., 2009). The three major primary breeding companies in the world having access to all the genetic stock are Cobb-Vantress (Cobb 500, Cobb 700, and Cobb MV male), Aviagen (Ross, Arbor Acres, and Lohmann Indian River), and Hubbard (APHIS.USDA, 2022). These primary breeder companies have the pure line elite (pedigree), great-grandparent, and grandparent flocks, which are less in number at the top of the production pyramid. The commercial broiler companies own the parent broiler breeder and broiler flocks, which are large in numbers at the bottom of the pyramid (Thiruvenkadun et al., 2011).

From the early 90s to the 2000s, due to intense genetic selection and improvement in management practices, there was a significant improvement in production performances like live weight (2.67 kg from 1 kg) and feed efficiency (1.63 kg feed/ kg bodyweight from 4.7 kg feed/kg body weight), and a decrease in mortality (3.6% from 18%) as well as market age (6 weeks from 16 weeks) (Flock et al., 2005). However, this intense genetic selection for rapid growth has led to poor reproductive efficiency in broiler breeders causing decreased fertility, hatchability, and egg production (APHIS.USDA, 2022). Also, metabolic and physiological disorders such as ascites, tibial dyschondroplasia, and twisted leg syndrome became more common (Navarro et al., 2002; Thiruvenkadun et al., 2011; Meirhaeghe et al., 2019).

With poultry production moving towards the No Antibiotics Ever (NAE) system, another major challenge faced by the broiler breeder industry is the difficulty in controlling bacterial, viral, and parasitic diseases among birds (Singer et al., 2019). These diseases should be prevented from the highest level of the production pyramid by strict biosecurity practices (Meirhaeghe et al., 2019). The primary broiler breeder companies follow strict biosecurity
however, the integrators with broiler parents face challenges in maintaining strict biosecurity to prevent the occurrence of diseases as these birds are kept for a longer period of time compared to broilers (Hiemstra and Napel, 2013). To maintain flock fertility spike males are introduced to older flocks and this is a biosecurity breach as there is a high chance of pathogen transmission (Sabah and Yilmaz, 2023). Moreover, non-integrator farms are more difficult to control as they are not under the direct supervision of the parent company (Hiemstra and Napel, 2013). Broiler breeders are the point where the pathogens capable of vertical transmission are introduced to the production pyramid, causing further horizontal transmission among the progeny. *Mycoplasma gallisepticum, Salmonella Enteritidis, E. coli, and Avian encephalomyelitis* virus are some pathogens that are known to vertically transfer in poultry. *M. gallisepticum*, and *S. Enteritidis* are pathogens of food safety concern to humans and hence, they are closely monitored under the National poultry improvement plan program standards (NPIP) to prevent vertical transmission. However, for *E. coli* as there is no direct evidence of zoonotic potential no close monitoring is done (NPIP.USDA, 2019). Further, eggs from different breeder flocks are hatched together and if the chicks have a pathogen that is vertically transmitted, it may horizontally transmit in the hatchery, and above that, the transportation to different places may aggravate the chances of spread (Christensen et al., 2021). Chicks coming from different breeder flocks kept on a single farm are also a potential source of infection (Gelaude et al., 2014). Another challenge is the limited vaccination among broiler breeders (booster doses) and the lack of cross-protection among commercially available vaccines against different strains (Kleven et al., 2008; Fernanda et al., 2010; Kathayat et al., 2021). Identifying new vaccine targets from time to time or developing vaccines that provide cross-protection may help to control the disease in broiler breeders to a limit. More about vaccines are discussed in the following section.
**APEC in broiler breeders**

The economic impact of APEC infection in poultry worldwide is extremely high, causing severe losses due to decreased production, the cost of carcass disposal, and the increased cost of medications (Nolan et al., 2019). In the Netherlands, estimates showed a loss of 3.7 million euros due to APEC infections in poultry farms per harvest (Landman et al., 2015). While in Indonesia, the estimated loss was 1049 million and 992 million U.S. dollars per harvest for broilers and layers, respectively (Wibisono et al., 2018). In the U.S., it has been estimated that economic losses to the broiler industry can be as high as 40 million dollars annually due to carcass condemnation alone (de Brito et al., 2003). However, the data regarding overall economic impact of APEC on the U.S. poultry industry is not available. Following broiler breeder infections, the economic impact would be even higher because, if they are infected, there will be a reduction in egg production and hatchability, and increased broiler breeder mortality as well as first-week mortality in broiler chicks (Giovanardi et al., 2005; Yassin et al., 2009; Pires-dos-Santos., 2013). Hence, there is a need to estimate the overall economic impact caused by APEC on the U.S. poultry production.

**Broiler breeder hens**

APEC infection in broiler breeder hens characterized by salpingitis-peritonitis syndrome can affect the hens severely and is also economically devastating however, has got the least attention (Landman et al., 2016). The hens get the infection mainly through 3 routes; ascending infection through the cloaca, translocation of bacteria from the respiratory tract, and intestinal lumen. Even though the most important routes of infection are the respiratory and feco-oral routes, the ascending infection through the cloaca is also critical for this infection (Landman et al., 2016; Ewers et al., 2013). Furthermore, among multiple factors causing mortality in broiler
breeder hens, the highest mortality was due to salpingitis-peritonitis syndrome and *E. coli* was one of the major pathogens causing this systemic infection in broiler breeders (Thøfner et al., 2019). Initially, the infection in the reproductive tract will be asymptomatic and later, proceed to severe septicemia and death. Peritonitis is usually found in acute cases mostly as a complication of oviduct infection (Ozaki et al., 2018). Giovanardi et. al (2005) reported vertical transmission of APEC from broiler breeders to their progeny in an integrated poultry production chain and suggested chronic salpingitis in hens as a potential risk factor for transmitting APEC through eggs to progenies. Studies show that APEC infection and first-week mortality among chicks from aged broiler breeders (>50 weeks) are higher than from young (<30 weeks) birds, and the plausible reasons being a decrease in the eggshell quality with age and immunosuppression in birds after the peak production period (Monroy et al., 2005; Poulsen et al., 2017). However, there is only limited information on the salpingitis-peritonitis syndrome in broiler breeders and hence, further studies focused on the pathogenesis of APEC isolates causing salpingitis as well as factors promoting salpingitis in broiler breeders need to be done to develop effective control strategies in breeders.

**Broiler breeder males**

Male fertility in broiler breeders is another important aspect of production. Previous reports show that intestinal bacterial populations can invade the male reproductive system as the cloaca is the common opening for reproductive and digestive systems and thus, affecting sperm motility and fertility (Smith, 1949). Bacterial orchitis is one disease manifestation due to *E. coli* infection of the broiler breeder male reproductive system. *E. coli* mainly invade through the hematogenous route due to septicemia in this disease and affects the tubular architecture (Monleon et al., 2008). The presence of pathogenic bacteria in the semen of roosters has been
previously reported (Wilcox et al., 1958). Further, the motility of broiler breeder rooster semen samples in the presence of pathogenic bacteria such as *E. coli*, *Salmonella*, *Campylobacter*, *Clostridium*, *Bifidobacterium*, and *Lactobacillus* has been analyzed and the presence of *E. coli* in rooster semen affected its motility as well as decreased the pH of the semen sample (Haines et al., 2013). Additionally, other reports also validate that the presence of *E. coli* in the semen could affect sperm motility (Mezhoud et al., 2015). This implies that fertility could be affected by APEC infection in males due to low sperm motility and thus, lead to decreased chick production. In the broiler breeder industry even though a large number of females are fertilized by a single male (1male:10females), there is no monitoring of the semen quality of roosters. Furthermore, when the male reproductive tract gets infected, there is a high chance of transmitting *E. coli* to hens through copulation (Jacobs et al., 1978; Barnes et al., 1997; Mezhoud et al., 2015). In a study conducted by USDA’s National Animal Health Monitoring System (NAHMS), the introduction of spike males to older breeder flocks to enhance fertility was found to increase *E. coli* peritonitis as it is a breach in biosecurity (APHIS, USDA, 2012). To the best of our knowledge, there are no estimates of mortality due to APEC infections in broiler breeder males, however, periodic monitoring of roosters for *E. coli* infections will help to control the disease transmission to hens as well as throughout the broiler production chain.

**Virulence characteristics**

Virulence characterization of the bacterial populations should be done from time to time to get updated knowledge about the virulence patterns and to develop effective control measures. Multiple virulence-associated genes that enable *E. coli* to attach, invade, colonize, replicate, and damage the host cells, as well as evade the host immune response have been studied. However, there are only limited studies on the properties of APEC isolates specifically collected from
broiler breeders. The study on the virulence-associated genes of 28 clinical isolates from broiler
breeders in Mississippi, U.S. reported *iroN* (iron acquisition), *iss* and *ompT* (protectins), *hlyF*
(toxin production) as the most prevalent genes (78.6%) among the isolates (Joseph et al., 2023a).
Studies from Thailand and Australia with 200 clinical samples from broilers and broiler breeders
showed a high prevalence (100%) for *ompT* and *hlyF* (Thomrongsuwanakij et al., 2020). About
256 broiler breeder APEC non-clinical isolates from Korea also showed *ompT* as the highly
prevalent virulence gene (26%) (Kim et al., 2022). Moreover, clinical isolates from broiler
breeders in Canada also showed another set of prevalent genes pattern among the isolates (*cvaC-
*etsB-ffyuA-ireA-iroN-iss-iutA-ompT-sitA*) with *sitA* (iron acquisition) as the highest prevalent
gene (92.75%) (Varga et al., 2018). A study conducted in Brazil showed that virulence factors
such as type-1 fimbriae and pili type-1 encoded by many genes of the series *fim* aid in their
attachment to the reproductive tract of breeders (Monroy et al., 2005). Similar results were
observed in the studies conducted in different parts of the world for various isolates from
peritonitis/salpingitis lesions (Bisgaard et al., 1980; Bisgaard et al., 1981, Bandyopadhyay et al.,
(*iroN, iss, iutA, hlyF, and ompT*) and they were identified in different studies however, no
specific predictor or marker genes have been identified in broiler breeders (Dziva and Stevens,
2008; Landman et al., 2015; Joseph et al., 2023a). Therefore, studies may focus on identifying
specific targets that can reveal APEC so that, disease diagnosis becomes easier and more
reliable.

Serotyping helps to identify APEC virulence based on the determination of the presence
of various lipopolysaccharides and flagellar antigens. Three surface antigens conventionally used
to test *E. coli* includes O antigen representing Oligosaccharide units (somatic antigen), H for
flagellar antigens, and K for capsular antigens. The major O serogroups in poultry are O1, O2, and O78 while some of the major H serogroups are H1, H2, H4, and H27 (Landman et al., 2015). Regarding the serogroups among broiler breeder isolates, studies from Brazil and India identified O2, O78, and O5 as the prevalent ones (Monroy et al., 2005; Kulkarni et al., 1970). However, serogroups O6, O9, and O152 were predominant in the broiler breeder farm while O125 was identified at both breeder farm and hatchery and O3, O24, and O146 were found only at the hatchery from Mexico (Rosario et al., 2004). A study from Mississippi, U.S. identified O88, O8, and O25 as the predominant O serogroups while H9 and H21 as the predominant H serogroups in isolates from broiler breeders diagnosed with colibacillosis (Joseph et al., 2023a). These broiler breeder APEC serogroups identified in these studies are different from most predominant serogroups in poultry (Ginns et al., 1996, Nolan et al., 2019). The geographical location or the evolution of strains over time may be a reason for this difference. However, there should be more studies focusing on the identification of serogroups to get comprehensive data, especially the H serogroups. Apart from serotyping, APEC virulence can be identified based on phylogenetic classification. Phylogenetic classification mainly depends on the evolutionary similarities of isolates to categorize them. The major phylogroups observed among the APEC isolates are B2 and D according to Clermont et al. (2000) classification (Lozica et al., 2021). However according to the new classification, B2 is still a major phylogroup but other phylogroups are varying according to the characteristics of isolates (Lozica et al., 2022). Studies using broiler breeder isolates also showed prevalence of B2 phylogroup and the identified prevalence is in agreement with the general pattern in poultry which followed the old Clermont classification (Clermont et al., 2000). However, the modified classification of phylogroups has shown some shifts in some categories such as A to C and D to E or F as mentioned before, which should be considered in
future studies while classifying broiler breeder APEC isolates (Logue et al., 2017). Details regarding genotypic virulence characterization of broiler breeder APEC isolates are provided in table 2.1.

Phenotypic virulence characterization of isolates is equally important as genotypic virulence characterization. Understanding the phenotypic variation relative to genetic differences is very critical to understand bacterial characteristics and helps to identify specific factors responsible for bacterial virulence and pathogenesis (Landman et al., 2015). Day-old chick challenge and embryo lethality assays are some convenient methods to phenotypically characterize the isolates based on their virulence potential (Wooley et al., 2000; Awad et al., 2020). Traditionally, these assays use APEC clinical and non-clinical isolates and categorize them into highly virulent, intermediate virulent, or avirulent based on the percentage of embryo/chick mortality and lesions produced. Additionally, the relationship between genotypic and phenotypic virulence can also be identified (Awad et al., 2020). Oh et al., (2012) phenotypically characterized clinical APEC isolates from broiler breeder, broiler and native chicken of Korea. They found that, as the number of virulence associated genes increased per isolates, there was an increase in phenotypic virulence. Similar results were observed during phenotypic virulence characterization using clinical broiler breeder APEC isolates (Joseph et al., 2023b. But no such relationship was observed during phenotypic virulence characterization using broiler clinical APEC isolates (Awad et al., 2020). Further, in vitro cell culture studies are also helpful to characterize the isolates based on their adherence and invasive properties to eukaryotic cells. As these in vitro studies can mimic the host pathogen interaction, the adhesion and invasion potential can be used estimate its relationship with genotypic and phenotypic virulence factors of the bacterial isolates. Moreover, the above-mentioned data can be used to estimate the specific
factors responsible for virulence based on a whole genome aspect and to develop effective control methods (Ali et al., 2020). Even though the genotypic and phenotypic data provide information about the characteristics of broiler breeder APEC isolates, the vast genetic diversity is a big challenge for its control. Continuous monitoring of the APEC isolates and characterizing them both phenotypically and genotypically is critical because of the evolution of bacteria from time to time. Therefore, research in this area is inevitable.

**Antimicrobial resistance characteristics**

One of the major concerns regarding APEC colonization in broiler breeders is the vertical transmission of antibiotic-resistant genes down the poultry production pyramid (Benameur et al., 2019). Additionally, the possibility of the zoonotic potential of APEC, including the transfer of antibiotic-resistant genes to humans, has been suggested by Johnson et al, (2008). So, it is recommended to restrict the use of antibiotics for treatment alone, and that too with antibiotics less commonly used in humans. As the antimicrobial resistance pattern among poultry is continuously evolving, it is important to closely monitor the resistance pattern of isolates from breeders (Bortolaia et al., 2010). A recent study on the antimicrobial resistance pattern among the *E. coli* isolates obtained from the ovaries of broiler breeders was highly thought-provoking, as high resistance patterns toward various first-line antibiotics were observed (Benameur et al., 2018). As reports showed the vertical transmission of various antibiotic-resistant genes, identifying *E. coli* isolates in the ovary with similar resistance patterns is quite disturbing. Another major challenge faced by the poultry industry is the prevalence of multi-drug resistance among the APEC isolates. There is evidence of recovery of multidrug-resistant *E. coli* from the reproductive tract and egg contents of broiler breeders (Benameur et al., 2019). Joseph et al, (2023a) identified about 10.7% of the clinical broiler breeder APEC isolates as multi-drug
resistant which was a significant finding during the study. Even though strict biosecurity is practiced at the top level of the breeding pyramid, there are still challenges at the lower levels, especially the broiler parents. Hence, the proper use of antibiotics in broiler breeders is critical (Bortolaia et al., 2010; Benameur et al., 2018). Further, more focus on alternative treatment approaches may also help to prevent antibiotic resistance. Nowadays the poultry industry is more focused on the NAE production system. NAE programs generate more than 50% of birds produced in the U.S. (NPIP.USDA, 2019). This program is very challenging as it needs effective disease control strategies along with quality feed and strict biosecurity but would play a key role in controlling the emergence of antibiotic-resistant bacteria. Details regarding antibiotic resistance patterns among isolates from broiler breeders and first-week broiler chicks are demonstrated in table 2.2.

Another important challenge besides antibiotic resistance among APEC isolates is the resistance towards heavy metals which were used as growth promoters in the past and quaternary ammonium compounds which are part of disinfectants used for cleaning poultry facilities (Liu et al., 2016). Broiler and broiler breeder APEC isolates were reported to show a high prevalence of \textit{arsC} gene which codes for arsenic resistance. The use of arsenic-containing feed additive, Roxarsone, in poultry might be a possible reason for this increased prevalence (Li et al., 2021; Joseph et al., 2023a). Further, the presence of more than 90% silver resistance among broiler breeder APEC isolates from Mississippi, U.S. was alarming because silver compounds were used as feed additives before (Joseph et al., 2023a). Other metal resistance genes that code for tellurite and mercury are also prevalent among broiler breeder, turkey, and broiler APEC isolates (de Oliveira et al., 2020; Yang et al., 2020; Li et al., 2021). Copper resistance genes were present among the broiler breeder clinical APEC isolates tested in Mississippi. However, phenotypically
all isolates were susceptible to copper. This calls for continuous monitoring of their vertical and horizontal transmission. Furthermore, the inappropriate use of disinfectants such as the continuous use of subinhibitory concentrations in poultry facilities might be a reason for the presence of resistance against quaternary ammonium compounds among APEC isolates (Joseph et al., 2023a). New alternative strategies for disinfection or proper directive to use disinfectants at the proper concentrations for cleaning should be strictly followed to prevent bacteria from developing resistance.

**APEC transmission from broiler breeders**

**Vertical transmission**

Transmission of bacteria from broiler breeders to their progeny, possibly due to colonization in the reproductive tract or due to penetration of eggshell is described as vertical transmission. It is a major concern for APEC transmission and spread (Welten, 2019). However, only recently the high degree of genuine vertical transfer of APEC from breeders to broiler chicks have been identified. Giovanardi et al., (2005) identified the strains of *E. coli* isolated from broiler chicks were similar to that found from their parents. They identified the presence of O78 and O136 serogroups among both parent flocks and chicks along with some virulence-associated genes such as *fim/tsh/iuc* that helped to define pathotypes. Further, Multi Locus Sequence Typing (MLST) of isolates from broiler breeders, newly hatched chicks, and dead broiler chicks during the first week using Pulse Field Gel Electrophoresis (PFGE) showed that among the PFGE clusters identified, 4 of them had isolates from all 3 categories of birds and concluded the possibility of vertical transmission (Poulsen et al., 2017; Thøfner., 2019). Similarly, the same clones of APEC isolates that caused salpingitis-peritonitis syndrome were
recovered from the hatchery and day-old broiler chicks, again showing the possibility of vertical and horizontal transmission (Yassin et al., 2009; Poulsen et al., 2017).

The occurrence of vertical transmission is further supported by various other studies that reported the transmission of antibiotic-resistance genes of APEC from broiler parents to chicks. In a study, the strain obtained from the chicks that died in the first week was also recovered from the broiler parents by following fluoroquinolone-resistant *E. coli* that was vertically transmitted down the integrated broiler production chain (Petersen et al., 2006). Furthermore, the Extended-Spectrum Beta-Lactamase (ESBL) and plasmid Ampicillinase C (*pAmpC*) were also found to be vertically transmitted from broiler breeders to broilers (Oikarainen et al., 2019). In fact, there are multiple reports on the vertical transmission of *pAmpC* to broilers (Nilsson et al., 2015; Benemeur et al., 2018). Another very relevant finding supporting the vertical transmission potential is the recovery of tetracycline-resistant *E. coli* from the ovary and egg contents of broiler breeders (Indrawati et al., 2021). All these findings strongly support the possibility of vertical transmission (Table 2.3), but above that, the increasing concern of the antibiotic resistance pattern in *E. coli* should be strictly monitored even though the use of antibiotics is highly controlled. In addition, developing a good strategy to control APEC infection in broilers should primarily focus on the vertical transmission potential of this pathogen as this is an underestimated factor that could cause severe loss.

One of the critical factors affecting vertical transmission is the age of the breeder flock. Monroy et al., (2005) reported that the age of broiler breeders affects the colonizing ability of APEC in the host tissue by studying the *in-vitro* adherence ability of *E. coli* in the ciliated oviduct epithelium. Interestingly, the adherence was more towards the oviduct from aged breeders (50 weeks old) than from young (12 weeks old) breeders. This difference in the
adherence pattern might be due to differences in the oviduct epithelium cell types in young and aged hens, which in turn might be the effect of hormones and egg production status. Thus, ciliated epithelium found in egg-laying hens oviducts promotes the colonization of *E. coli* (Awad et al., 2020). Moreover, the egg albumen secreted by the reproductive tract during egg production is assumed to further promote *E. coli* attachment. These host factors along with *E. coli* fimbrial proteins contribute towards oviduct adherence and colonization (Brand et al., 1956; Ge et al., 2014). Another contributing factor is the decrease in eggshell thickness with age. A study investigated this and showed increased first-week mortality due to APEC infection in chicks that hatched from older parents (>60 weeks) with thin-shelled eggs as opposed to younger parents (>30 weeks) with thick-shelled eggs. Also, floor eggs from older parents were found to have higher first-week mortality compared to that from the younger flock (Poulsen et al., 2017). Therefore, constant monitoring of the flocks with an emphasis on age is suggested to limit vertical transmission and APEC infections in broiler chicks. Figure 2.1 demonstrates the routes and mechanisms for vertical and horizontal transmissions of APEC in poultry.

**Horizontal transmission**

In horizontal transmission, the bacteria from the infected birds in a flock get transmitted to the non-infected ones through contaminated body fluids, aerosols, fluff, and feces. Broiler breeders can transmit *E. coli* among themselves and vertically to their progenies and the vertically transmitted APEC can increase the first-week mortality among the chicks because of the horizontal transmission happening at the hatchery and farm (Petersen et al., 2006; Bortolaia et al., 2010). In the hatchery, the eggs infected from the hens’ oviduct could potentially transmit APEC to other embryos during incubation as well as in the hatching basket, non-infected chicks can come in contact with infected ones via fluff and feces. All these sources can act as potent
sources of APEC infection (Lock et al., 1992; Berrang et al., 1999). APEC is a highly transmissible pathogen that can easily spread between chicks and affect the chick quality (Selby et al., 2021). Christensen et al. (2021) have previously discussed the effect of horizontal transmission in poultry. During hatch, handling, and transport of the chicks, the conditions are optimum for the spread and multiplication of APEC. To minimize this risk, it is recommended for the hatchery to be near the production facility. Also, an all-in all-out system would be an effective management strategy for preventing vertical and horizontal transmissions as there are chances of disinfection of the houses after each flock (Christensen et al., 2021).

Another important aspect that facilitates the spread of *E. coli* at farms is vectors. Studies showed that APEC isolates were able to colonize in vectors such as houseflies, wild birds, and even pigeons (Solà-Ginés et al., 2015; Borges et al., 2017; Selby et al., 2021). Of interest, beetles (*Alphitobius diaperinus*) were also found to be important in transmitting *E. coli*. Another parasite shown to be involved in the transmission is *Tetratrichomonas gallinarum* (Trichomonad spp.) (McAllister et al., 1996; Landman et al., 2017). So, focusing on controlling the vectors is essential to ensure biosecurity and prevent horizontal transmission (Landman et al., 2017). Further, the infected chicks during the first week of life may develop omphalitis, yolk sac infection, pericarditis, perihepatitis, and peritonitis. The body fluids of the infected chicks may be another important source of infection to other chicks in the flock. Moreover, one report suggests the transmission of APEC through fluff which can act as a source (Berchieri et al., 2001). Controlling horizontal transmission is essential to control the increased first-week mortality and colibacillosis in broilers and breeders.
First-week mortality among chicks

The first week of growth is crucial for a chick because it determines flock production ability, uniformity of the flock, and the age at processing (Swelum et al., 2021). APEC infections in chicks are characterized by acute and subacute septicemia. Death may occur due to acute septicemia resulting from infections in the yolk sac and respiratory system, while pericarditis, perihepatitis, and airsacculitis may develop as a result of sub-acute septicemia (Swelum et al., 2021). Chick quality can be assessed by looking into the first-week mortality pattern, which should be around 0 to 1% (Heier et al., 2002), and as per Aviagen standards, the first-week mortality should not exceed 0.7% (Aviagen, 2018). Moreover, the European Union standards suggest that the first-week mortality is indicative of the welfare of the birds (European union council, 2017). If mortality is more than 1%, it would affect the entire production system, necessitating its prevention. Even though several factors affect first-week mortality, such as breeder age, egg weight, genetics, hatchery conditions, feed quality, and house environment (Sedeik et al., 2019), the primary factor (50%) is infections due to various pathogens and *E. coli* is a major culprit in this group (Olsen et al., 2012). One study analyzed the role of *E. coli* in chick mortality 48-72 hours after hatching and found around 70% of the dead chicks had colibacillosis symptoms (Kemmett et al., 2014). Of interest, about 17% of first-week mortality among the chicks on average, *E. coli* was the major cause of death and was found to be the primary pathogen for the first-week mortality resulting from yolk sac infection (Rai et al., 2005; Abadi et al., 2013; Borges et al., 2017). As a secondary pathogen, *E. coli* along with *Enterococcus* infections was reported to be the cause of death in more than 50% of dead embryos in hatcheries. Further, as a primary pathogen, a 19.46% prevalence of *E. coli* among dead embryos was observed (Karunarathna et al., 2017).
Among chicks, the route of entry of APEC is mainly through the respiratory system, which may be through contaminated dust or fomites. Other routes of bacterial entry are vertical transmission from parents, through the contaminated floor eggs, and through the yolk sac (Nolan et al., 2019). Bacteria entering through the yolk sac, infect the chicks that retain the yolk sac for long period of time and cause the infection to peak 24-48 hrs. after hatching, and at the end of about 2- weeks the mortality may rise up to 10%-20%. Data shows that, among the surviving population of chicks, about 5% may have stunted growth and in the other birds' development will be normal (Kabir et al., 2017; Nolan et al., 2019). Another important source of infection is the feed. Reports suggest that E. coli contaminates animal feed because of the poor-quality ingredients or the poor storage facilities (Sanderson et al., 2005). Water is another important source of infection as E. coli is the indicator organism for fecal contamination (CDC, 2018). Alarmingly, scientists have identified about 13% prevalence of E. coli in the water tanks of farms. It can act as a potential source of infection and can be avoided by strict biosecurity, proper disinfection, and management (Sanderson et al., 2005). As mentioned before, Poulsen et al., (2017) identified that the age of the parent flock is an important factor affecting vertical transmission and hence it also affects first-week chick mortality. In fact, they showed that there was high first-week mortality among chicks from 60-week-old parents compared to chicks from 30-week-old parents. Moreover, floor eggs are another issue and it was reported to have high mortality among the chicks that came from floor eggs. For example, more than 60% E. coli prevalence was observed in chicks that came from floor eggs of a 60-week-old flock but from normal eggs it was only 40% (Poulsen et al., 2017). Preventing mortality in the first week of life is important, and if the mortality rates are too high, the production facilities are recommended to reduce the number of birds in the subsequent cycle or till the cause of mortality is resolved. This
is because the chicks are coming out of a conditioned environment in the hatchery to the brooder houses, where they may face many challenges such as feed, thermoregulation, and fighting infections (Yassin et al., 2009). The first-week mortality is an important challenge to the broiler industry because it determines further growth and productivity.

**Prevention and control of APEC in breeders**

**Biosecurity and management**

Biosecurity is the primary factor pertaining to the prevention and control of APEC in broiler breeders. There should be more focus on the parent stock by periodic sampling and monitoring because from the breeders the clones that cause the disease and antimicrobial resistance are transferred vertically. In addition, there are many primary bacterial and viral infections that cause a compromise in the immune barrier of birds and promotes APEC infection as a secondary pathogen. These primary disease agents should be controlled by periodic monitoring and surveillance (Meirhaeghe et al., 2019). Further, the spike males introduced to the flock must be from the same source and tested for important disease conditions including APEC infections (Sabah and Yilmaz, 2023). Another important factor is the care of birds and eggs after 40 weeks of age (Thøfner et al., 2019). When the flock ages, the bird’s immune system gets compromised, eggshell thickness decrease, and the chick quality gets affected. Proper management of the flock, including culling and disinfecting the eggs are critical in breeder flocks that are aged. Additionally, there were reports of antibiotic-resistant (ESBL/AmpC) *E. coli* which are recovered from the eggshell (Nilsson et al., 2014). Formaldehyde fumigation is widely used for disinfection of hatching eggs but is carcinogenic and has a detrimental effect on the handler (Melo et al., 2019). Alternative methods such as UV and spraying disinfectants could be practiced. For instance, a natural resin from honey bees called propolis have found to be very
effective without affecting hatchability or egg weight loss (Mousa-Balabel et al., 2016). Besides this, hydrogen peroxide and essential oils like clove oil were also found to be effective without affecting hatchability or egg weight loss (Oliveira et al., 2020; Pees et al., 2023). Disinfection procedures should be practiced regularly in the breeder house, the hatchery, and the brooder. Moreover, the floor eggs should be minimized either by discarding them or preventing their chance of getting contamination by proper cleaning or timely egg collection to reduce the contact time (Poulsen et al., 2017).

As vertical transmission results in horizontal transmission, proper disinfection of the hatcheries and practicing an all-in-all-out system might be helpful to some extent. Further, care should be taken while transporting the chicks from one farm to another to prevent the transmission of bacteria by implementing good sanitation and biosecurity programs Poulsen et al., 2017). Importantly, controlling bacterial contamination in areas such as feed mills and packaging areas and prioritizing the biosecurity measures are critical in APEC control. Above that, culling weak chicks during the first week will reduce the loss due to treatment and mortality Poulsen et al., 2017). Also, identifying the possible areas that are at risk of a breach in biosecurity is important and solutions should focus on controlling vertical transmission.

**Genetics**

Genetics is another factor that could affect APEC infection in breeders. For instance, the fast-growing lineages are found to be more susceptible to APEC infections than the slow-growing lineages (Yunis et al., 2000). Further, intense genetic selection for rapid growth and growth-related traits have increased the incidence of ascites in broilers and broiler breeders. And birds with ascites are highly vulnerable to infectious agents (Christensen et al., 2021). As a result, Denmark is slowly trying to remove the fast-growing lineages from their production.
system and grow more slow-growing lines; and this could help to make the birds healthier (Anon, 2017). Furthermore, scientists are working on identifying the possibility of developing breeds that are resistant to *E. coli* infections. They tried to identify the effect of maternal inheritance on disease resistance since the heritability of gamma globulins are 2.5 times higher in female parents than in male parents. Immunized parents are crossed in different ways and the effects were monitored. A significant maternal effect has been identified, but the reciprocal crosses had an intermediate effect for both parent lines. The reason for this need to be investigated, and studies in this area would be highly impactful as there is evidence of genetic improvement that results in disease resistance (Leitner et al., 1994).

**Antibiotics**

Various antibiotics are used to treat APEC infections in birds. Antibiotics such as tetracyclines, aminoglycosides, macrolides, sulphonamides, penicillins, cephalosporins, trimethoprim, quinolones, polymyxins, chloramphenicol, lincosamides are routinely used for the treatment of colibacillosis (Agunos et al., 2012). However, recent studies have reported resistance of APEC towards most of these antibiotics mainly because of their previous improper and unrestricted use in animal agriculture. For example, Colistin is an important drug in human therapeutics however, Colistin resistant *E. coli* species were recently found in swine and poultry (Welten, 2019). Moreover, isolates collected from broiler breeders were resistant to many of the above-mentioned antibiotics (Table 2.2) (Petersen et al., 2006; Chafik et al., 2015). Several countries, including the U.S., have restricted the use of antibiotics for non-therapeutic purposes (growth promoters) in animal production and there is a high acceptance of the NAE production system in the market (Singer et al., 2019). Limiting the use of certain antibiotics for animal treatment and/or developing specific antibiotics against animal/bird pathogens would be
beneficial. The increased occurrence of this resistance pattern among bacteria in broiler breeders needs much attention (Welten, 2019). Also, constant monitoring of the antibiotic resistance pattern among disease-causing APEC isolates will be beneficial from a treatment standpoint.

**Vaccination**

Vaccination is one of the important strategies to limit APEC infections. Birds are vaccinated at day 1 and then around 12-14 weeks of age against *E. coli*. Developing efficient vaccines to prevent APEC infections is an important solution for limiting the use of antibiotics for treatment (Ewers et al., 2004; Ghunaim et al., 2014). Moreover, preventing first-week mortality can be achieved through proper vaccination of the parent flocks (Heller et al., 1990; Kariyawasam et al., 2004). Initially, only bacterins or inactivated vaccines were used against APEC, but after years of research, scientists developed live and subunit vaccines that became more popular (Bolin et al., 1987). Inactivated vaccines were an important category of vaccines concerning broiler breeders because initially, there were studies regarding the possibility of vaccinating the broiler parents to get protection in the broilers to reduce the cost of vaccination in broilers (Rosenberger et al., 1985). The study also showed that antibodies derived from broiler parents could protect broiler chicks for up to two weeks and then, the level of antibodies got reduced (Chaffer et al., 1997). Nobilis *E. coli* is a commercially available inactivated vaccine by MSD Animal Health for active immunization of broiler breeders to provide passive immunization to broiler chickens. However, a study has shown a reduction in overall broiler breeder mortality but no effect on first-week chick mortality after Nobilis *E. coli* vaccination (Gregersen et al., 2010). Also, there are limitations for inactivated vaccines as they are effective only against homologous challenges. Also, other factors such as frequency and route of administration and the age of the bird at vaccination also affect the efficacy (Ghunaim et al.,
Subunit vaccines that have been studied include iron-regulated outer membrane protein-based, fimbriae-based, and increased serum survival proteins-based vaccines. Overall, the subunit vaccines are more effective than inactivated vaccines as they provide protection against heterologous challenges in addition to homologous challenges but continuous follow-up studies in a commercial setting are necessary to confirm the field success of these vaccines (Ghunaim et al., 2014). Live attenuated vaccines are getting more acceptance as they are available for mass immunizations along with evidence of cell-mediated immunity rather than circulating antibodies alone (Filho et al., 2013). Poulvac *E. coli* is a commercially available modified live vaccine by Zoetis. In addition to providing protection in layers and broilers, a trial conducted by a broiler breeder company in South Africa using this vaccine showed a decrease in mortality by curbing peritonitis (International hatchery practice, 2019; Christensen et al., 2021). Another trial was conducted in Denmark to evaluate the efficacy of the autogenous vaccine in the broiler breeder flock already vaccinated with Poulvac. However, there was no protection against homologous or heterologous infection even though there was an increased antibody response (Li et al., 2017). *In-ovo* administration of Poulvac demonstrated that the air cell route is better than the amniotic route in terms of embryo mortality and hatchability. However, further studies are needed to confirm the long-term efficiency or protection to birds following *in-ovo* administration of the vaccine (Lindsey et al., 2022).

Due to poor cross-protection of commercially available vaccines, efforts to develop autogenous vaccines against APEC in broiler breeders have been found to be more effective (Landman et al., 2014). A study on autogenous vaccines reported that it influenced the selection of phylogroups after vaccination. They repressed most of the phylogroups but resulted in some strain shifts. For example, in one farm, the prevalence of phylogroup A decreased after
vaccination even though it was present in the vaccine whereas the prevalence of phylogroup F increased after vaccination which was not present in the vaccine (Lozica et al., 2021). Research on autogenous vaccines focusing on the target virulence factors which promote cross-protection would provide more efficient vaccines (Lozica et al., 2022). Further, there are certain criteria to be followed to develop effective vaccines. Firstly, it should be able to provide cross-protection against multiple APEC serogroups. Secondly, the vaccine needs to be stable for administering in a way that could be suitable for bulk vaccination in birds. Lastly, vaccines should be able to be administered at a suitable age to get maximum protection (Dho-Moulin and Fairbrother, 1999).

**Probiotics and prebiotics**

Following restrictions on antibiotics, the use of alternatives to control APEC has been investigated for a long time. Probiotics are microbial feed supplements that are live but inactivated and provide health benefits such as protection from infectious agents while prebiotics are non-digestible supplements that promote the growth of beneficial bacteria in the gut and thereby provide immunity (Hajati et al., 2010; Wang et al., 2017). Many scientists investigated the effect of *Lactobacillus plantarum* B1 probiotic in the feed of broilers and observed improvement in growth parameters along with a reduction in cecal *E. coli* output (Hajati et al., 2010; Wang et al., 2017; Ding et al., 2019; Redweik et al., 2020). Additionally, the use of Fructose Oligosaccharide (FOS) along with *Lactobacillus plantarum* B1 was also tested, and improved performance was noted (Redweik et al., 2020). A challenge study using the O78 APEC strain to identify the effect of *Enterococcus faecalis-I* showed improvement in the performance and reduction in mortality among the challenged broiler birds (Tarabees et al., 2019). Prebiotics such as FOS is also shown to be very effective in preventing bacterial infections, especially *E. coli*. FOS can act as a substrate for *Bifidobacteria* which can synthesize bacteriocin that can
suppress various pathogens (Tarabees et al., 2019). Another important prebiotic is the Mannose Oligosaccharide (MOS) which can interfere with the bacterial adhesion mechanism. The most commonly used polysaccharide prebiotic in poultry is the partially hydrolyzed guar gum which is basically a MOS (Hajati et al., 2010). Several probiotic and prebiotic mixes in the field are known to enhance immunity in the intestine as it is one of the most compromised areas that allow the spread of infection. Moreover, research should also focus on the reproductive system health because in broiler breeders’ bacteria entering through the ascending route through the cloaca can infect the reproductive tract.

**Bacteriophages**

Bacteriophages are another possible source of treatment that could effectively prevent colibacillosis in chickens. They are viruses that target infectious bacteria without affecting the normal microflora Huff et al., 2005; Oliveira et al., 2010; Kaikabo et al., 2017; Naghizadeh., 2019). A phage mixture using SPR02 and DAF6 was administered through the air sac route against experimental infection of APEC O2 and showed a significant reduction in mortality in broilers (Huff et al., 2005). Studies using phage cocktails supplemented through intra-tracheal and intra-venous routes also significantly reduced mortality and APEC load in the liver, lungs, and heart in broilers (Kaikabo et al., 2017; Naghizadeh., 2019). Phage-loaded chitosan particles administered orally have also shown a significant reduction in mortalities following experimental APEC infection in chickens. However, there are challenges in the practical application of these phage therapies because of the constraints in large-scale production and use in the poultry industry (Zbikowska et al., 2020). Studies on the effect of phage therapy in broiler breeders infected with APEC, including its effect on preventing vertical transmission, need to be done to develop phage as an alternative to antibiotic treatment in breeders.
Miscellaneous

Advanced techniques to control APEC infections are also promising. Innate immune stimulants are one among them which can stimulate the immune responses against pathogens. Cytosine-phosphodiester-guanine motifs are one of the important innate immune stimulants which activate pathogen-associated molecular patterns (PAMPs) (Goonewardene et al., 2020). Additionally, antimicrobial peptides are short positively charged peptides that act against most bacteria including antibiotic-resistant ones. D-analog of chicken cathelicidin-2 is an important antimicrobial peptide tested against APEC in an in-vivo challenge study in broilers (Cuperus et al., 2016; Mahlapuu et al., 2016). Moreover, virulence inhibitors mainly cause the virulence mechanisms in bacteria unable to make virulence factors thus, disarming the bacteria and not producing bacterial resistance (Totsika et al., 2017). In the future, in vivo and in vitro challenge studies in broiler breeders might reveal the efficiency of this alternative control method against broiler breeder APEC infections.

Conclusion

Broiler meat is one of the major sources of animal protein that is preferred globally and broiler breeders play an inevitable role in sustainable broiler production thus, the challenges in broiler breeder rearing should be minimized. APEC in broiler breeders is critical because it causes disease in breeders as well as is vertically transmitted from the hens’ reproductive tract through eggs to chicks and further horizontally transmitted between chicks. Moreover, evidence showing the transfer of antibiotic resistance genes vertically points out the importance of preventing and controlling APEC in broiler breeders. Using alternatives to antibiotics such as efficient vaccines, probiotics and prebiotics, and bacteriophages along with strict biosecurity and management practices could help limit the infection. Constant research focusing on the
phenotypic and genotypic characterization of APEC isolates from broiler breeders is necessary because of the vast genetic diversity of this bacteria and its evolution as time passes.
Table 2.1  Genotypic virulence characteristics of *E. coli* strains isolated from broiler breeders from different geographical locations.

<table>
<thead>
<tr>
<th>Location</th>
<th>E. coli Isolated From</th>
<th>Virulence-Associated Genes</th>
<th>Serogroups Identified</th>
<th>Phylogroups Identified</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>United States</td>
<td>Broiler breeder</td>
<td><em>iroN</em>, <em>iutA</em>, <em>iss</em>, <em>hlyF</em>, <em>ompT</em>, <em>papC</em>, <em>tsh</em>, <em>ibeA</em>, <em>astA</em> and <em>cva/cvi</em></td>
<td>O88, O8, O25, O115, O166, O161, O1, O55, H9, H21, H4, H7</td>
<td>B1, B2, D</td>
<td>(Joseph et al., 2023a)</td>
</tr>
<tr>
<td>Thailand</td>
<td>Broiler breeder</td>
<td><em>iroN</em>, <em>iutA</em>, <em>iss</em>, <em>hlyF</em> and <em>ompT</em></td>
<td>N/A</td>
<td>N/A</td>
<td>(Thomrongsuwannakij et al., 2020)</td>
</tr>
<tr>
<td>Australia</td>
<td>Broiler breeder</td>
<td><em>iroN</em>, <em>iutA</em>, <em>iss</em>, <em>hlyF</em> and <em>ompT</em></td>
<td>N/A</td>
<td>N/A</td>
<td>(Thomrongsuwannakij et al., 2020)</td>
</tr>
<tr>
<td>Country</td>
<td>Strains</td>
<td>Antigens</td>
<td>Serotypes</td>
<td>References</td>
<td></td>
</tr>
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<td>---------</td>
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<td></td>
</tr>
<tr>
<td>Korea</td>
<td>Broiler breeder</td>
<td><em>iroN</em>, <em>iutA</em>, <em>iss</em>, <em>hlyF</em> and <em>ompT</em></td>
<td>N/A</td>
<td>N/A</td>
<td>(Kim et al., 2022)</td>
</tr>
<tr>
<td>Canada</td>
<td>Broiler breeder, Broiler</td>
<td><em>sitA</em>, <em>iroN</em>, and <em>iutA</em>, <em>iss</em>, <em>ompT</em>, <em>etsB</em>, <em>cvaC</em>, <em>ireA</em></td>
<td>N/A</td>
<td>N/A</td>
<td>(Varga et al., 2018)</td>
</tr>
<tr>
<td>Korea</td>
<td>Broiler breeder, Layer, Broiler</td>
<td><em>fimC</em>, <em>tsh</em>, <em>fyuA</em>, <em>irp2</em>, <em>iucD</em>, <em>cav/cvi</em>, <em>iss</em>, <em>astA</em>, <em>vat</em></td>
<td>O1, O6, O8, O15, O18, O25, O26, O28, O78, O111, O112, O115, O125, O119, O126, O167</td>
<td>N/A</td>
<td>(Oh et al., 2012)</td>
</tr>
<tr>
<td>Denmark</td>
<td>Broiler Breeder</td>
<td><em>papAH</em>, <em>papEF</em>, <em>papGII</em>, <em>ireA</em>, <em>iroN</em>, <em>iucD</em>, <em>yuA</em>, <em>kpsMTK1</em>, <em>kpsMTII</em>, <em>malX</em> [PAI], <em>fimH</em>, <em>iss</em>, <em>traT</em>, <em>sitA</em>, <em>vat</em>, <em>astA</em>, <em>cvi/cva</em>, <em>ibeA</em>, <em>tsh</em>, <em>ompT</em></td>
<td>N/A</td>
<td>A, B1, B2, D</td>
<td>(Pires-dos-Santos et al., 2013)</td>
</tr>
<tr>
<td>Country</td>
<td>Description</td>
<td>Strain</td>
<td>Other Strains</td>
<td>Source</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>---------------------</td>
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<td>--------------</td>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td>Colombia</td>
<td>Broiler breeder</td>
<td>N/A</td>
<td>O1, O2, O5, O36, O45, O53, O78</td>
<td>(Monroy et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>Broiler breeder, Hatchery</td>
<td>N/A</td>
<td>O19, O18, O8, O78, O6, O9, O52, O125</td>
<td>(Rosario et al., 2004)</td>
<td></td>
</tr>
<tr>
<td>Croatia</td>
<td>Broiler breeders</td>
<td>N/A</td>
<td>N/A</td>
<td>A, B1, B2, C, D, E, F</td>
<td>(Lozica et al., 2021)</td>
</tr>
</tbody>
</table>
Table 2.2  Antibiotic resistance pattern observed among *E. coli* isolates from broiler breeders/ broiler chicks reported worldwide.

<table>
<thead>
<tr>
<th>Location</th>
<th><em>E. coli</em> Isolated From</th>
<th>Antibiotic Resistance Genes Identified</th>
<th>Resistant Antibiotics Identified by Susceptibility Testing</th>
<th>Multidrug Resistance Identified</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algeria</td>
<td>Broiler Breeder</td>
<td>-</td>
<td>TET, NAL, AUG, AMP, SXT, ENR, NIT, NEO, CHL, GEN</td>
<td>Yes</td>
<td>(Chafik et al., 2015)</td>
</tr>
<tr>
<td>Egypt</td>
<td>Broiler Chicks- First week</td>
<td>-</td>
<td>AMP, AUG, CTX, CST, TET, DOX, GEN, NEO, OFX, ENR, CIP, FLO</td>
<td>Yes</td>
<td>(Awad et al., 2020)</td>
</tr>
<tr>
<td>Thailand</td>
<td>Broiler Breeder</td>
<td>-</td>
<td>AMX, CEF, CHL, CIP, ENR, FLO, GEN, NAL, SXT, TET</td>
<td>Yes</td>
<td>(Thomrongsuwannakij et al., 2020)</td>
</tr>
<tr>
<td>Australia</td>
<td>Broiler Breeder</td>
<td>-</td>
<td>AMX, CEF, CHL, CIP, ENR, FLO, GEN, NAL, SXT, TET</td>
<td>Yes</td>
<td>(Thomrongsuwannakij et al., 2020)</td>
</tr>
<tr>
<td>Algeria</td>
<td>Broiler Breeder</td>
<td><em>blaCTX-M-1, qnrS1</em></td>
<td>AMP, TET, PRL, CZN, CXM, NAL</td>
<td>Yes</td>
<td>(Beameur et al., 2018)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Broiler Breeder</td>
<td><em>GyrA, ParC</em></td>
<td>ENR</td>
<td>-</td>
<td>(Petersen et al., 2006)</td>
</tr>
</tbody>
</table>
Table 2.2 (continued)

<table>
<thead>
<tr>
<th>Country</th>
<th>Breeder Type</th>
<th>Antibiotic Resistance</th>
<th>Resistance to Infection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Korea</td>
<td>Broiler Breeder</td>
<td>blaTEM-1, blaTEM-135, blaTEM-176, blaCTX-M-1, blaCTX-M-55, qnrS, qnrB, qnrA, cmlA, catA1, aac(6')-Ib, aac(3)-II, tetA, tetB, tetC</td>
<td>TET, NAL, AMP, CEP, SXT, CHL, CZN, GEN, LVX</td>
<td>Yes</td>
</tr>
<tr>
<td>Italy</td>
<td>Broiler Breeder</td>
<td></td>
<td>AMX, ENR, TET, SXT</td>
<td>Yes</td>
</tr>
<tr>
<td>Denmark</td>
<td>Broiler Breeder/Broiler</td>
<td></td>
<td>AMP, NAL, CIP</td>
<td>Yes</td>
</tr>
<tr>
<td>Sweden</td>
<td>Broiler Breeder</td>
<td>blaCMY-2</td>
<td>AMP, NAL, TET, OTC, GEN, CHL</td>
<td>Yes</td>
</tr>
<tr>
<td>Indonesia</td>
<td>Broiler Breeder/Layer Breeder</td>
<td>tetA, tetB</td>
<td>CHL</td>
<td>Yes</td>
</tr>
<tr>
<td>France</td>
<td>Broiler chicks- First week</td>
<td></td>
<td>TET, AMX, SXT, NAL, GEN</td>
<td>-</td>
</tr>
<tr>
<td>Algeria</td>
<td>Broiler Breeders</td>
<td>blaCTX-M-1, blaSHV-12, blaTEM-1</td>
<td>NAL, UB, CIP, LVX, AMP, AMC, XNL, TET, TPM, SXT, NEO, CHL</td>
<td>Yes</td>
</tr>
<tr>
<td>Country</td>
<td>Broiler Breeder (Roosters)</td>
<td>Antibiotics</td>
<td>Source</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------</td>
<td>-------------</td>
<td>---------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Belgium</td>
<td>Broiler Breeder</td>
<td>CTX, XNL, AMX, CFM, TMP, NAL, CZM</td>
<td>(Mezhoud et al., 2015)</td>
<td></td>
</tr>
<tr>
<td>United States</td>
<td>Broiler Breeders</td>
<td>blaTEM, blaCTX, aph3IA, TET, STM, KAN, GEN, SXT, Yes</td>
<td>(Joseph et al., 2023a)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3 Comprehensive data from different geographical locations showing vertical transmission of *E. coli* from broiler breeders to chicks.

<table>
<thead>
<tr>
<th>Location</th>
<th>Source</th>
<th>Factors/techniques used to identify vertical transmission</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td>Broiler breeders, Eggs,</td>
<td>Bacterial count and lesions</td>
<td>(Thøfner et al., 2019)</td>
</tr>
<tr>
<td></td>
<td>Broiler breeders, Live chicks</td>
<td>PGFE and MLST*</td>
<td>(Poulsen et al., 2017)</td>
</tr>
<tr>
<td>Finland</td>
<td>Broiler breeders, Eggs,</td>
<td>Antibiotic resistance genes</td>
<td>(Oikarainen et al., 2019)</td>
</tr>
<tr>
<td></td>
<td>Poultry house</td>
<td>(ESBL/pAmpC)</td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>Broiler breeders, Dead chicks</td>
<td>Serogrouping (O78 and O139)</td>
<td>(Giovanardi et al., 2005)</td>
</tr>
<tr>
<td>Denmark</td>
<td>Broiler breeders, Broilers</td>
<td>Antibiotic resistance (AMP &amp; NAL) *</td>
<td>(Bortolaia et al., 2010)</td>
</tr>
<tr>
<td>Sweden</td>
<td>Broiler breeders, Broilers,</td>
<td>Antibiotic resistance gene</td>
<td>(Nilsson et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Hatchery</td>
<td>(pAmpC)</td>
<td></td>
</tr>
<tr>
<td>United States</td>
<td>Broiler breeders</td>
<td>Ec (NAL)*</td>
<td>(Montgomery et al., 1999)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Broiler breeders, Live chicks</td>
<td>Antibiotic resistance gene</td>
<td>(Zurfluh et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Environmental sample</td>
<td>(blaCTX-M-1)</td>
<td></td>
</tr>
<tr>
<td>Netherlands</td>
<td>Broiler breeders, Environmental sample</td>
<td>Antibiotic resistance genes</td>
<td>(Dierikx et al., 2013)</td>
</tr>
</tbody>
</table>

* PGFE- Pulsed-field gel electrophoresis, MLST- Multilocus sequence typing, AMP- Ampicillin, NAL- Nalidixic Acid, Ec (NAL)- *Escherichia coli* in Nalidixic Acid medium.
Figure 2.1  Image showing the APEC route of entry, vertical transmission to the chicks, horizontal transmission in the hatchery and among broiler breeders
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CHAPTER III
PHENOTYPIC VIRULENCE CHARACTERIZATION OF AVIAN PATHOGENIC
ESCHERICHIA COLI (APEC) FROM BROILER BREEDERS WITH
COLIBACILLOSIS IN MISSISSIPPI

Abstract
Avian pathogenic *Escherichia coli* (APEC) causes extraintestinal infections called
colibacillosis in various poultry species and are responsible for huge economic losses worldwide.
The disease in broiler breeders needs to be controlled because APEC can be vertically
transmitted from the breeders to the offspring through contaminated eggs. Moreover, bacterial
populations are continuously evolving from time to time creating high genetic diversity.
Therefore, in this study, we evaluated the phenotypic virulence characteristics of 28 APEC
isolates from broiler breeders with colibacillosis in Mississippi using Embryo Lethality Assay
(ELA) and Chick challenge study. Additionally, the relationship between genotypic and
phenotypic virulence patterns was determined. A total of 558 broiler breeder-hatching eggs were
used for ELA. On day 12 of incubation, 0.1mL of an overnight culture of bacteria at a final
concentration of 100-500 CFU/mL was injected into the allantoic sac of embryonated eggs. The
eggs were candled daily for 7d post-inoculation and embryo mortality was recorded. For the
chick challenge study, 256-one-day-old female chicks were used and 0.1mL of an overnight
culture of bacteria at a final concentration of $10^8$ CFU/mL was subcutaneously injected into the
neck region of each chick. Then, the chicks were monitored for 7d post challenge and mortality
along with lesions following necropsy was recorded. A pathogenicity score (PS) was assigned to each isolate based on the day of chick death and lesions during necropsy and then, isolates were classified based on pathogenicity. Pearson correlation analysis using SAS 9.4 was performed to determine the relationship between ELA, chick challenge, and the presence of virulence-associated genes. Overall, 39.3% of the isolates were highly virulent and 3.5% were avirulent during both assays. The mortality following ELA ranged between 6% to 78% and for the chick challenge, it was from 0% to 100%. There existed a strong positive correlation between embryo mortality and the chick challenge study ($R= 0.73$, $P<0.01$). Based on virulence, among the 28 isolates, 11 (39%) were classified as highly virulent, 6 (21%) as intermediate, and 11 (39%) as avirulent. Moreover, positive correlations were observed for embryo mortality and pathogenicity score with the presence of virulence genes that predict APEC, $iroN$, $iss$, $ompT$, $hlyF$, and $iutA$. In conclusion, this study helped us to evaluate the virulence characteristics of APEC isolates obtained from diseased broiler breeders in Mississippi based on their phenotypic virulence and its relationship with genotypic virulence. Future research to determine their vertical transmission potential need to be done along with in-vitro adhesion and invasion assays to identify the infection potential of the phenotypically categorized isolates and to compare them with genotypic virulence.

**Keywords:** Avian pathogenic *Escherichia coli*, broiler breeder, embryo lethality assay, chick virulence assay, virulence characterization.

**Introduction**

Avian pathogenic *Escherichia coli* (APEC) causes colibacillosis in various poultry species such as chickens, ducks, and turkeys and it leads to severe economic loss to the poultry industry worldwide. In general, the disease is manifested as peritonitis, oophoritis, and salpingitis.
in adult egg-laying birds and omphalitis in chicks (Dho-Moulin and Fairbrother, 1999; Barnes et al., 2003; Ewers et al., 2004). The reproductive tract infections in broiler breeder hens lead to severe egg production loss as well as vertical transmission of APEC to chicks followed by horizontal transmission in the hatchery that finally causes increased first-week chick mortality at the farm (Yassin et al., 2009; Nilsson et al., 2014). The high genetic diversity among the bacterial species is the primary challenge associated with controlling APEC infections in broiler breeders (Nolan et al., 2019). In addition, there is only limited information on the APEC isolates that specifically cause disease in broiler breeders. Hence, continuous monitoring of disease-causing APEC isolates from broiler breeders is required.

In general, the genotypic virulence characteristics of APEC isolates from poultry have been widely studied (Thomrongsuwannakij et al., 2020; Newman et al., 2021). However, its’ diverse nature due to the presence of plasmid-mediated pathogenicity islands makes its virulence prediction based on genes, serogroups, and phylogroups very difficult (Guabiraba & Schouler, 2015; Kathayat et al., 2021). Additionally, another challenge is differentiating primary pathogens from secondary or non-pathogenic isolates (Wooley et al., 2000). As a result, phenotypic virulence characterization methods like embryo lethality and day-old chick challenge assays have been widely used for the virulence assessment of E. coli isolates. In these phenotypic assays, the time of death following the challenge as well as lesions in the organs are considered to determine virulence making it more robust than the traditional genotypic methods (de Souza et al., 2016; Awad et al., 2020). These assays are regarded as gold standard tests and are routinely used as diagnostic tests to determine E. coli virulence (Wooley et al., 2000; Filho et al., 2019).

Recently, our research team collected APEC isolates from broiler breeders diagnosed with colibacillosis, and their genotypic virulence and antimicrobial resistance properties were
studied (Joseph et al., 2023). About 96% of the isolates were found to carry at least one virulence-associated gene. Specifically, the genes encoding iron acquisition (iroN and iutA), protection (iss and ompT), and toxin production (hlyF) showed the highest prevalence among the multiple virulence genes tested. This was followed by genes for adhesion (papC and tsh), colicin V plasmid operon (cva / cvi), and invasion (ibeA). Interestingly, a high prevalence of newly evolving serogroups such as O88 and H21 were identified along with a significant prevalence for phylogroup B2 among the isolates. As a matter of concern, 93% of the isolates were found to carry at least one antimicrobial resistance gene, and a high level of multidrug resistance was also observed (Joseph et al., 2023). As multiple factors such as geographical location and poultry species are known to impact the genetic diversity of APEC (Nolan et al., 2019), these findings provide insight into APEC specifically from broiler breeders in Mississippi. However, to develop effective treatment and control measures a thorough knowledge on the pathogenesis of APEC including the relationship between genotypic and phenotypic virulence are required. Hence, the current study aims to identify the phenotypic virulence pattern of these APEC isolates collected from broiler breeders with colibacillosis in Mississippi using embryo-lethality assay and one-day-old chick challenge assay. Further, the relationship between phenotypic virulence as observed from this study and genotypic virulence factors identified by Joseph et al. (2023) will be determined.

Materials and methods

Source of APEC isolates

Twenty-eight APEC isolates were obtained from Mississippi Veterinary Research and Diagnostic Laboratory (MVRDL)/ Poultry Research and Diagnostic Laboratory (PRDL). They were isolated from lesions of diseased broiler breeders of various age groups diagnosed with E.
coli infection (Table 3.1). Isolates were confirmed as E. coli using real-time PCR for the presence of ybbw gene during the previous study from our lab (Joseph et al., 2023).

**Bacterial culture preparation for challenge**

E. coli were retrieved from frozen stock by streaking onto MacConkey agar plates (Becton, Dickinson and Company, Sparks, MD) and were incubated at 37°C for 18 to 24 hrs. For each isolate, a single, lactose-positive colony was picked from the plates, inoculated into LB broth (Becton, Dickinson and Company, Sparks, MD), and were grown at 37°C for 18 h with shaking. Post incubation, bacterial cells were pelleted, washed two times, resuspended in PBS (Thermo Fisher Scientific, Vilnius, Lithuania), and then, the cells were held overnight at 4°C. To determine the stock concentration for the challenge, E. coli CFU enumeration was done by serial dilution and plating onto MacConkey agar plates. Next day, the day of challenge, the desired concentration was obtained by serially diluting the stock culture.

**Embryo lethality assay**

A total of 558 broiler breeder-hatching eggs from a 38-week-old Ross x YPM breeder flock were received from a commercial hatchery. The eggs were randomly divided into 31 groups (28 APEC isolates and 3 control groups) with 18 eggs per group (n=18). The control groups were PBS-injected, dry punch, and non-injected. On day zero, the eggs were labeled and arranged in two GQF incubators (GQF Manufacturing Inc., 1502 Digital Sportsman incubator; Savannah, GA) in such a manner that all groups were equally distributed at all three levels of the incubator (93 eggs per level). Eggs were incubated at standard conditions (Temperature 37.5 ºC, humidity 50-55%, and turning at a 45º angle every hour). On day 10 of incubation, all the eggs were candled, and the infertile eggs and eggs with dead embryos were discarded. On day 12 of
incubation, eggs with live embryos were manually injected with APEC strains as previously described by Wooley et al, (2000) and Nolan et al, (1992). Briefly, 0.1 mL of the bacterial culture at a concentration of 100-500 CFU/mL was injected into the allantoic sac of embryonated eggs using a 1 mL syringe fitted with 22G x 1-inch needle (Becton, Dickinson and Company, Sparks, MD) directed through a punctured hole in the egg shell perpendicular to the air cell. All embryonated eggs were candled daily for 7 days post-challenge and daily mortality was recorded. Based on the embryo mortality the APEC isolates were classified into highly virulent (mortality >29%), moderately virulent (mortality between 10% -29%), and avirulent (mortality <10%) (Wooley et al., 2000).

Five dead embryos per group were aseptically collected in Whirl Pak bags (M.G Scientific, Pleasant Prairie, WI) for bacteriological analysis. After adding PBS to each bag, it was processed using a Seward stomacher (Cole-Palmer, Vernon Hills, IL) and then, a loop full of sample was streaked onto MacConkey agar plates and incubated overnight at 37ºC to identify the presence of pink-lactose positive colonies.

**One day-old chick challenge study**

A total of 500 one-day-old Ross x YPM chicks, not vaccinated against *E. coli*, were received from a commercial hatchery. All chicks were feather-sexed immediately after arrival. In addition, five chicks were randomly euthanized and tested negative for *E. coli* as described below. Only female chicks were used in this study. A total of 256 female chicks were housed in wire cages (8 chicks/cage) and placed in two rooms of the ABSL-2 experimental facility at the Mississippi State University Poultry Research Unit. Chicks had access to *ad libitum* feed and water. Chicks were randomly assigned to 32 groups; 28 isolates and 4 controls (n=8 chicks/group). Controls were 2 PBS-injected (sham) groups and 2 non-injected groups (negative
control). After placement, the chicks were provided two days to acclimatize to the environmental conditions in the animal research facility before the challenge. For the challenge, each chick was injected with 0.1mL of 1x 10^8 CFU/ml of APEC isolate subcutaneously into the neck region (Wang et al., 2015). Then, the chicks were monitored 4 times a day for 7 days post-challenge and daily mortality was recorded. Necropsy was performed on all dead chicks and lesions were collected from 4 chicks per group in Whirl Pak bags. After adding PBS to each bag, it was processed using a Seward stomacher and then, a loop full of sample was streaked onto MacConkey agar plates and incubated overnight at 37°C to identify the presence of pink-lactose positive colonies.

To evaluate and classify the isolates based on virulence, a pathogenicity score (PS) was assigned to each isolate according to the time of death and lesions observed during the necropsy (de Souza et al., 2016). For the time of death, the highest value given was “one” on the first day and with each day, the value reduced by a factor of 0.1428. For the presence of a particular lesion, a score of “one” was assigned and when the lesion is absent, a score of “zero” was given. An isolate was considered highly virulent if its' pathogenicity score was between 7 and 10, moderately virulent if between 4 and 6.99, and avirulent if between 1 and 3.99.

PS= (TD*5) + Pc+ Ph+ A+ C+ O

TD- Time of death, Pc- Pericarditis, Ph- Perihepatitis, A- Airsacculitis, C- Cellulitis O- Omphalitis

**Ethics statement**

All experiments concerning the handling of chicken embryos and chicks were approved by the Institutional Animal Care and Use Committee of Mississippi State University (IACUC#22-399).
PCR analysis of virulence-associated genes

Previously, all 28 isolates were tested for the presence of 10 virulence-associated genes, \( papC \) (P-fimbriae), \( tsh \) (temperature-sensitive hemagglutinin), \( ibeA \) (invasion of the brain endothelium protein A), \( iutA \) (aerobactin siderophore receptor), \( iroN \) (salmochelin siderophore receptor), \( iss \) (increased serum survival), \( ompT \) (outer membrane protease), \( astA \) (heat-stable enterotoxin), \( hlyF \) (putative avian hemolysin), and \( cva/cvi \) (Colicin V plasmid genes) (Joseph et al., 2023).

Statistical analysis

Pearson correlation analysis using SAS 9.4 was used to determine the relationship between percentage embryo mortality following embryo lethality assay, pathogenicity score following one-day-old chick challenge study, and the presence of virulence-associated genes among the 28 broiler breeder APEC isolates.

Results

Embryo lethality assay

Results showed that embryo mortality varied between 6 to 78% among the isolates (Table 3.1). Based on embryo mortality, 23 (82.14%) isolates were categorized as highly virulent, 4 (14.28%) isolates as moderately virulent, and 1 (3.58%) isolate as avirulent. All isolates showed some level of mortality within the first two days of the challenge.

Following bacteriological analysis of embryos, \( E. coli \) colonies were recovered from all 28 isolates, including the four isolates in which mortality occurred in less than four embryos and live embryos were used for \( E. coli \) recovery. \( E. coli \) were not recovered from any of the control groups.
One day-old chick challenge study

Overall, mortality among the isolates ranged from 0 to 100%. Four of the isolates (14.30%) showed 100% mortality within 7 days post-challenge and 8 (28.60%) of them showed zero mortality. Following necropsy, all isolates (100%) were found to cause cellulitis, 89% caused pericarditis, 75% caused airsacculitis, 71% caused omphalitis, and 57% caused perihepatitis. In some chicks’ minor lesions were also observed in the femur, hock, proventriculus, and kidney. Furthermore, at least one lesion associated with colibacilosis was observed in all the chicks challenged with the APEC isolates. The pathogenicity score varied between 1 and 9.5 (Table. 3. 2). Based on pathogenicity score, 11 isolates (39.30%) were classified as highly virulent, 6 isolates (21.42%) as moderately virulent, and 11 isolates (39.30%) as avirulent. However, no isolates were non-pathogenic with a pathogenicity score of zero.

Following bacteriological analysis of the organs collected, E. coli colonies were recovered from all 28 groups. E. coli were not recovered from any of the control groups.

Relationship between embryo lethality assay, one-day-old chick challenge study, and virulence-associated genes

There exists a positive correlation between embryo mortality and chick pathogenicity score (r = 0.73, P < 0.01; Figure 3.1). A positive relationship was also seen between the mortality estimates of embryos and chicks (r = 0.70, P < 0.01). Further, based on both embryo lethality and chick challenge assays, among the 28 isolates, 11 (39.3%) isolates were classified as highly virulent while only one isolate (3.5%) was found to be avirulent in both studies. However, there was no isolate common for the moderately virulent group following the two assays (Table 3.2).
Additionally, 74% of the isolates that were found to be highly virulent following embryo lethality assay were found to be high or moderately virulent following the chick challenge study.

Of the ten virulence-associated genes tested, positive correlations were observed for both % embryo mortality and pathogenicity score with the presence of five virulence genes, *iroN, iss, ompT, hlyF*, and *iutA* (Table 3.3). These genes have been previously described as the APEC minimal predictors by Johnson et al. (2008). In fact, as the frequency of these five genes increased, there was an increase in % embryo mortality and pathogenicity score (Figures 3.2a &b). However, no significant relationship was observed with the rest of the genes tested (Table 3).

**Discussion**

The current study focused on the phenotypic virulence characterization as well as the relationship between phenotypic and genotypic virulence characteristics of broiler breeder APEC isolates from Mississippi. Overall, based on the embryo lethality and one-day-old chick challenge assays, 39.3% of the isolates were found to be highly virulent, 3.5% were avirulent while the isolates in the moderately virulent group varied according to the type of challenge performed. Further, we observed that phenotypic virulence was complemented by the presence of certain virulence-associated genes.

A high mortality pattern was observed among the APEC isolates following the embryo lethality assay. Around 82% of the isolates showed a mortality pattern greater than 29% and were classified as highly virulent. On the other hand, during the chick challenge study, the pathogenicity score among our isolates varied between 1 and 9.5. More than 60% of the isolates were belonging to the highly and moderately virulent categories. *E. coli* recovery was 100% for embryo lethality assay and for the chick challenge study. At least one organ per bird tested
positive for *E. coli* in all our samples except for the controls in the chick challenge. Similar high *E. coli* recovery was observed from APEC isolates from laying hens with *E. coli* peritonitis syndrome (Landman et al., 2021). The embryo mortality, pathogenicity score, and *E. coli* recovery show the virulence potential of the APEC clinical isolates collected from broiler breeders that were used in this study.

The high embryo mortality rate following embryo lethality assay was in agreement with many of the previous studies. For instance, Oh et al. (2012) reported that majority of the clinical APEC isolates from broilers, broiler breeders, layers, and native birds in Korea were classified as highly virulent following the embryo lethality assay. Further, Ozaki et al, (2017) found that APEC isolated from layer chickens with lesions such as pericarditis and perihepatitis were highly virulent whereas the majority of the isolates from birds with salpingitis were avirulent or intermediate virulent and none of the isolates from fecal samples of healthy birds caused any embryo mortality. The virulence pattern of isolates following the chick challenge study showed that 39.2% of the isolates were belonging to both highly virulent as well as avirulent categories while 21.4 % of the isolates were moderately virulent. On the other hand, in a study conducted in China on the characterization of *E. coli* strains obtained from liver samples of dead chickens, a majority of the isolates were exclusively belonging to the highly virulent category (Wang et al., 2015). Additionally, Awad et al, (2020) used clinical broiler APEC isolates and inoculated subcutaneously in chicks and showed that, the majority of their isolates as intermediate or low pathogenic. The above-mentioned studies (Wang et al., 2015 and Awad et al., 2020) used the same classification criteria for the isolates which considered lesions and mortality percentage as described by Rosenberger et al., (1985); however, it was different from the criteria we used which focused on the pathogenicity score as per de Souza et al, (2016). Hence, this difference in
virulence prevalence observed between studies might be an effect of the classification criteria used, but at the same time the effect of pathogen characteristics should be explored further. Interestingly, all chicks showed at least one lesion during the necropsy and most of the lesions were found in the heart, liver, and skin. The skin lesion (cellulitis) was present in all birds except the control groups which validates that the subcutaneous route of challenge was successful. Similar results were found in other studies also (de Souza et al., 2016; Awad et al., 2020). Therefore, the spread or severity of lesions along with the mortality percentage would point towards the virulence potential of the isolates.

There exists a positive correlation between the pathogenicity score following the chick challenge and the percentage embryo mortality. Most of the isolates categorized in the highly virulent group (39 %) were consistent with both challenge studies. However, these isolates were collected from different organs and no pattern was observed between virulence and the source of the isolates. Gibbs et al. (2004) compared several challenge models to study colibacillosis in poultry and found a high association between the results of the embryo lethality assay and chick challenge via the subcutaneous and intravenous routes. However, Awad et al, (2020) found no correlation between embryo lethality and chick challenge models with all the isolates causing high embryo mortality for the broiler APEC isolates tested. They concluded that the developed immune system of the chicks compared to embryos might be the reason for this difference among both assays. Even though there was a high correlation between the two assays in our study, the presence of 36% of isolates in the intermediate pathogenic group varied between the assays. In addition to the difference in immune response between chicks and eggs, a possible reason for this variation may be because these isolates were secondary pathogens or
opportunistic pathogens which cause the disease when the birds are immunocompromised or stressed.

The vertical transmission potential of broiler breeder APEC isolates has been previously reported (Nilsson et al., 2014; Poulsen et al., 2017). Therefore, identifying the possible virulence pattern of isolates that were related to reproductive tract origin is critical. Among the isolates in our study, the isolate P7 caused clinical lesions solely in the oviduct but was found to be highly virulent and avirulent following embryo lethality assay and one-day-old chick challenge study, respectively. Among the 5 isolates which caused lesions in the ovary, 3 of them were highly virulent during both assays however, the actual site of their collection is unknown (Tables 3.1 & 3.2). The reason for the variation of the phenotypic virulence among these isolates which caused lesions in the reproductive tract might be the geographical location of collection of these isolates, the health status of birds, and co-infections with other pathogens. Above that, the assay parameters such as % embryo mortality and pathogenicity score could also have effects on these results (Fujimoto et al., 2021). Moreover, there were reports of ascending infections of *E. coli* through the cloaca to reproductive tracts and it is an important site for the dissemination of bacteria from broiler breeder hens vertically to their progeny. So, identifying the characteristics of the isolates from the reproductive tract would help to control the possibility of vertical transmission by suitable interventions (Yassin et al., 2009; Olsen et al., 2012; Olsen et al., 2016).

The presence or absence of virulence-associated genes could provide an idea about the genotypic virulence characteristics of the isolates which can be correlated with the phenotypic virulence to provide the overall virulence potential of isolates (Pires-dos-Santos et al., 2003; Oh et al., 2012; Kathayat et al., 2021). Results showed that, as the number of virulence-associated genes increased per isolate there was an increase in phenotypic virulence. Oh et al, (2012) found
a similar pattern of genotypic and phenotypic virulence for another set of genes in which some of the genes tested were different. Even though the genes were different, the overall pattern was significant. We tested 10 virulence-associated genes in this study and among them, papC, tsh, ibeA, cva/cvi, and astA were not significantly correlated with phenotypic virulence in both assays and their prevalence was less than 45% among the isolates. However, the other five genes, iroN, iutA, iss, hlyF, and ompT, showed strong positive correlations with embryo mortality and chick pathogenicity score. Moreover, these five genes were highly prevalent among our isolates which were about 78.6% for iroN, iss, hlyF,ompT, and 68% for iutA (Joseph et al., 2023). Interestingly, Johnson et al., (2008) identified these five genes as predictors of APEC virulence. Scientists had tried to characterize APEC isolates from broiler breeders based on their genotypic characteristics including these genes and they found a high virulence potential for the isolates which had these genes (Varga et al., 2018; Thomrongsuwannakij et al., 2020; Kim et al., 2022). However, few studies found prevalence of different set of genes as responsible for bacterial virulence. For instance, the gene sitA was prevalent among the broiler breeder clinical isolates from Canada in addition to the five APEC predictor genes mentioned above (Varga et al., 2018). The gene iucD was the prevalent virulent gene among broiler breeder, broiler, and layer isolates from Korea (Oh et al., 2012). The emergence of new virulence patterns among bacterial populations is inevitable and constant monitoring is required (Dho-Moulin and Fairbrother, 1999; Kathayat et al., 2021). Even though the virulence pattern among our broiler breeder isolates was related to the five APEC-predicting genes, further characterization focusing on the whole genome sequence of these isolates would help to make sure the prevalence is even and identify any new virulence factors that would promote APEC control in broiler breeders.
Conclusion

We classified 28 APEC isolates obtained from diseased broiler breeders based on their phenotypic virulence pattern by performing an embryo lethality assay and a one-day-old chick challenge study. Based on these two assays, 39.3% of the isolates were found to be highly virulent, 3.5% were avirulent while the isolates in the moderately virulent group varied according to the type of challenge performed. Moreover, we identified a positive relationship between phenotypic virulence and the frequency of virulence-associated genes. This will allow us to explore more about the virulence patterns in APEC to develop effective control strategies. However, as a next step, we need to inspect how these isolates respond to live cells based on their adhesion and invasion capabilities which are important in bacterial infections and also what are the specific genes which are causing this virulence pattern on a whole genome approach to achieve this goal.
Table 3.1 Information regarding the 28-broiler breeder *E. coli* isolates used in this study. The isolates were obtained from the lesions of diseased birds diagnosed with *E. coli* infection.

<table>
<thead>
<tr>
<th>Isolate*</th>
<th>Hen age</th>
<th>Organs/Sites were lesions observed</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>24w</td>
<td>Ovary, peritoneum</td>
<td><em>E. coli</em> infection</td>
</tr>
<tr>
<td>P2</td>
<td>36w</td>
<td>Bone marrow, ovary, air sac</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P3</td>
<td>28w</td>
<td>Heart, ovary, lung</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P4</td>
<td>56w</td>
<td>Ovary, heart</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P5</td>
<td>41w</td>
<td>Ovary, liver, abdomen</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P6</td>
<td>49w</td>
<td>Bone marrow, abdomen</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P7</td>
<td>55w</td>
<td>Oviduct</td>
<td><em>E. coli</em> infection</td>
</tr>
<tr>
<td>P8</td>
<td>57w</td>
<td>Liver</td>
<td><em>E. coli</em> infection</td>
</tr>
<tr>
<td>P9</td>
<td>23w</td>
<td>Bone marrow, liver</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P10</td>
<td>53w</td>
<td>Bone marrow</td>
<td><em>E. coli</em> infection</td>
</tr>
<tr>
<td>P11</td>
<td>42w</td>
<td>Bone marrow, heart</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>E. coli</em> related bacterial synovitis and hock joint arthritis</td>
</tr>
<tr>
<td>P12</td>
<td>14w</td>
<td>Pericardium</td>
<td><em>E. coli</em> related bacterial arthritis</td>
</tr>
<tr>
<td>P13</td>
<td>18w</td>
<td>Hock/joint</td>
<td><em>E. coli</em> related bacterial arthritis</td>
</tr>
<tr>
<td>P14</td>
<td>23w</td>
<td>Liver</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P15</td>
<td>17w</td>
<td>Hock joint, heart</td>
<td>Colisepticemia and arthritis</td>
</tr>
<tr>
<td>P16</td>
<td>23w</td>
<td>Liver, bone marrow</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P17</td>
<td>19w</td>
<td>Heart, air sac, eyes, hock joints</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P18</td>
<td>27w</td>
<td>Abdomen, sternum, tendon</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P19</td>
<td>36w</td>
<td>Heart, bone marrow, liver</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P20</td>
<td>3d</td>
<td>Yolk sac, pericardium, liver</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P21</td>
<td>4d</td>
<td>Liver, yolk sac</td>
<td><em>E. coli</em> related yolk sac infection</td>
</tr>
<tr>
<td>P22</td>
<td>10d</td>
<td>Hips</td>
<td><em>E. coli</em> related bacterial arthritis</td>
</tr>
<tr>
<td>P23</td>
<td>7d</td>
<td>Heart swab</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P24</td>
<td>21d</td>
<td>Heart and abdomen swabs</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P25</td>
<td>6d</td>
<td>Pericardium, abdomen, yolk sac</td>
<td>Colibacillosis</td>
</tr>
<tr>
<td>P26</td>
<td>15d</td>
<td>Hock joint and femur</td>
<td><em>E. coli</em> related bacterial Polyarthritis</td>
</tr>
<tr>
<td>P27</td>
<td>1d</td>
<td>Liver, yolk sacs, peritoneum</td>
<td>Colisepticemia</td>
</tr>
<tr>
<td>P28</td>
<td>1d</td>
<td>Liver, yolk sacs, peritoneum</td>
<td>Colisepticemia</td>
</tr>
</tbody>
</table>

*The letter P is used to numerically arrange the isolates for identification purpose.*
Table 3.2  Results for embryo lethality assay and one-day-old chick challenge study. A total of 28 *E. coli* isolated from broiler breeders diagnosed with colibacillosis were used for challenge. For embryo lethality assay, n=18 eggs and one-day-old chick challenge study, n= 8 chicks were used.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>No. of Dead embryos</th>
<th>Total no. of live embryonated eggs incubated</th>
<th>Embryo Lethality Assay</th>
<th>One Day-Old Chick Challenge Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>13</td>
<td>18</td>
<td>72.2</td>
<td>Highly virulent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pathogenicity Score (PS)**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chick Mortality (%) ***</td>
</tr>
<tr>
<td>P2</td>
<td>9</td>
<td>17</td>
<td>53</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P3</td>
<td>3</td>
<td>17</td>
<td>17.64</td>
<td>Moderately virulent</td>
</tr>
<tr>
<td>P4</td>
<td>8</td>
<td>18</td>
<td>44.5</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P5</td>
<td>1</td>
<td>17</td>
<td>5.9</td>
<td>Avirulent</td>
</tr>
<tr>
<td>P6</td>
<td>8</td>
<td>18</td>
<td>44.5</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P7</td>
<td>8</td>
<td>18</td>
<td>44.5</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P8</td>
<td>6</td>
<td>18</td>
<td>33.4</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P9</td>
<td>5</td>
<td>18</td>
<td>27.8</td>
<td>Moderately virulent</td>
</tr>
<tr>
<td>P10</td>
<td>3</td>
<td>17</td>
<td>17.6</td>
<td>Moderately virulent</td>
</tr>
<tr>
<td>P11</td>
<td>5</td>
<td>16</td>
<td>31.2</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P12</td>
<td>13</td>
<td>17</td>
<td>76.5</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P13</td>
<td>8</td>
<td>18</td>
<td>44.5</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P14</td>
<td>9</td>
<td>18</td>
<td>50</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P15</td>
<td>12</td>
<td>18</td>
<td>66.7</td>
<td>Highly virulent</td>
</tr>
<tr>
<td></td>
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<tr>
<td>---</td>
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</tr>
<tr>
<td>P16</td>
<td>12</td>
<td>18</td>
<td>66.7</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P17</td>
<td>14</td>
<td>18</td>
<td>77.8</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P18</td>
<td>12</td>
<td>18</td>
<td>66.7</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P19</td>
<td>8</td>
<td>16</td>
<td>50</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P20</td>
<td>6</td>
<td>18</td>
<td>33.4</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P21</td>
<td>10</td>
<td>18</td>
<td>55.6</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P22</td>
<td>6</td>
<td>18</td>
<td>33.4</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P23</td>
<td>10</td>
<td>18</td>
<td>55.6</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P24</td>
<td>10</td>
<td>17</td>
<td>59</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>P25</td>
<td>10</td>
<td>18</td>
<td>55.6</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>p26</td>
<td>13</td>
<td>18</td>
<td>72.2</td>
<td>Highly virulent</td>
</tr>
<tr>
<td>p27</td>
<td>3</td>
<td>18</td>
<td>16.7</td>
<td>Moderately virulent</td>
</tr>
<tr>
<td>p28</td>
<td>6</td>
<td>18</td>
<td>33.4</td>
<td>Highly virulent</td>
</tr>
</tbody>
</table>

* Embryo Mortality(%) = (No of dead embryos /Total number of live embryonated eggs incubated per group) x 100; **Pathogenicity Score= (TD*5) + Pc+ Ph+ A+ C+ O, where TD- Time of death, Pc- Pericarditis, Ph- Perihepatitis, A- Airsacculitis, C- Cellulitis, and O- Omphalitis; ***Chick Mortality (%)= (No of dead chicks /Total number of chicks per group) x 100.
Table 3.3  Relationship of percentage embryo mortality following embryo lethality assay and pathogenicity score following one-day-old chick challenge study with the presence of virulence-associated genes.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Embryo Mortality (%)</th>
<th>Pathogenicity Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r-value</td>
<td>P-value</td>
</tr>
<tr>
<td>iroN</td>
<td>0.59</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>iss</td>
<td>0.59</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>ompT</td>
<td>0.41</td>
<td>0.03</td>
</tr>
<tr>
<td>hlyF</td>
<td>0.41</td>
<td>0.03</td>
</tr>
<tr>
<td>iutA</td>
<td>0.43</td>
<td>0.02</td>
</tr>
<tr>
<td>papC</td>
<td>-0.19</td>
<td>0.32</td>
</tr>
<tr>
<td>tsh</td>
<td>-0.04</td>
<td>0.83</td>
</tr>
<tr>
<td>ibeA</td>
<td>-0.10</td>
<td>0.59</td>
</tr>
<tr>
<td>astA</td>
<td>0.15</td>
<td>0.43</td>
</tr>
<tr>
<td>cva/cvi</td>
<td>-0.21</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Figure 3.1  Relationship between pathogenicity score and % embryo mortality.

Correlation between pathogenicity score following one-day-old chick challenge and mortality percentage following embryo lethality assay. Line equation: $y = 0.1239x - 0.7367$. $r = 0.73$, $P < 0.01$. 
Figure 3.2  Correlation between genotypic and phenotypic virulence.

(a) Correlation between the number of virulence-associated genes and embryo mortality following embryo lethality assay having line equation: $y = 5.2245x + 26.679$ and $r=0.53$, $P<0.01$.

(b) Correlation between the number of virulence-associated genes and pathogenicity score following one-day-old chick challenge study having line equation: $y = 0.7125x + 2.3182$ and $r=0.42$, $P=0.02$. APEC minimal predictor genes: $iroN$, $iss$, $ompT$, $hlyF$, and $iutA$. 
Reference


Abstract

Avian pathogenic *Escherichia coli* is a gram-negative bacterium that causes colibacillosis infections in various poultry species. Controlling these bacteria in broiler breeders is critical as it may cause vertical and horizontal transmission and there is only limited information about the isolates collected from breeders. Therefore, characterizing the isolates *in vitro* and comparing them with phenotypic and genotypic virulence characteristics would provide a better idea about the virulence potential of broiler breeder APEC isolates. In this study, we evaluated the adhesion and invasion potential of nine broiler breeder APEC isolates towards chicken macrophage HD11 cells. Each bacterial isolate was grown to a concentration of 8 logs CFU/mL and challenged the HD11 cells having a final concentration of $10^6$ cells/mL giving a Multiplicity of Infection (MOI) 1:100 in 24 well plates. After 3 hrs. of incubation with the bacteria, cells were collected to determine adhesion potential and a gentamycin protection assay was done to determine the invasion potential. All the samples were plated in MacConkey agar plates to determine the concentration of bacteria adhered and invaded. From the results, there were about 8 logs CFU/mL for adhesion for all isolates except one while, invasion concentration varied between 3 logs to 7 logs CFU/mL except for one isolate which had no invasion at all. Also, most of the
isolates were significantly different from each other in terms of adhesion and invasion. However, the adhesion and invasion potential of the isolates were not correlated to the genotypic and phenotypic virulence factors. More studies based on a whole genome aspect would provide factors responsible for this adhesion and invasion and can be used to identify the characteristics of these isolates for further used studies to identify vaccine targets and to develop an *E. coli* challenge model in broiler breeders.

**Keywords:** APEC, broiler breeder, HD11 chicken macrophage cells, adhesion, invasion

**Introduction**

The extra-intestinal *Escherichia coli* that causes systemic infections in poultry species is termed Avian Pathogenic *E. coli* (APEC). They are gram-negative coliforms with multiple virulence factors such as, adhesins, invasins, toxins, iron aquisition factors, lipopolysaccharides and this makes their control difficult (Dziva and Stevens, 2008; Kathayat et al., 2021). Exploring these virulence factors continuously will help to understand the changes occurring among APEC isolates (Dho-Moulin and Fairbrother, 1999; Sarowska et al., 2019). Further, the APEC isolates from broiler breeders are much less studied compared to other species of birds, but broiler breeders are critical in spreading APEC due to the involvement of vertical transmission of the bacteria from parents to the offspring (Nilsson et al., 2014, Poulsen et al., 2017). This vertical transmission causes huge economic loss to the poultry industry as it affects all levels of the production pyramid (Giovanardi et al., 2005, Monroy et al., 2005, Yassin et al., 2009). Thus, to develop effective diagnosis, treatment, and control measures, the virulence and pathogenesis properties of APEC from broiler breeders should be studied (Poulsen et al., 2017; Thøfner et al., 2019; Christensen et al., 2021).
Adhesion to the host cell surface is the first step towards bacterial colonization in host tissue and it is a requirement for bacterial pathogenesis as it helps to overcome the host defense mechanisms (Döpfer et al., 2000). Adhesins are proteins that help APEC to attach to the host cell surface. Adhesins include fimbrial (chaperone- usher fimbriae, curli and type IV fimbriae), non-fimbrial (afimbrial adhesins and autotransporters), and atypical (Type VI secretion system, flagella, and lipopolysaccharide) proteins (Aleksandrowicz et al., 2021). Moreover, the process of adhesion enables the bacteria to activate other mechanisms of virulence which include secretion systems and other effector proteins, and also helps the bacterial appendages like fimbriae to specifically attach to receptors (Rendón et al., 2007; Aleksandrowicz et al., 2021). Following adhesion, invasins help the bacteria to enter inside the host cells, and they are readily activated in the initial phases of infection. There are several invasins present in E. coli but some of them specific to APEC include invasion proteins, toxigenic invasion locus, and genetic island associated with neonatal meningitis (Dziva and Stevens, 2008; Sarowska et al., 2019). Following invasion, mucociliary colonization and systemic translocation happen depending on the physical and immunological barriers as well as virulence of the APEC strains. Depending on the above-mentioned factors, bacteria may either be phagocytized or result in localized or systemic infections (Pourbakhsh et al., 1997; Alber et al., 2020). Hence, identifying the adhesion and invasion characteristics is important to evaluate the virulence potential of APEC.

APEC colonization in poultry occurs through different routes of entry however, when infection happens, the bacteria enter the bloodstream and affect most of the internal organs which leads to septicemia eventually (Pourbakhsh et al., 1997). Innate immune responses get activated whenever the microbes encounter the host immune cells and among them, macrophages are the first ones to get activated following an infection. As the first line of defense,
macrophages phagocytize the bacteria and then produce multifunctional compounds such as reactive oxygen species, nitric oxide, and cytokines to kill them as well as send signals to other immune cells (Kogut et al., 2012). Hence, identifying the host-pathogen interaction is a major step to evaluate bacterial virulence. HD11 cells are chicken macrophage-like cell lines that mimic the host cell reactions in vitro and enable to study the effects of the virulence potential of bacterial isolates (Lee et al., 2018).

Additionally, the genes responsible for virulence are another important tool to characterize APEC (Nolan et al., 2017). Joseph et al, 2023b identified the prevalence of ten virulence-associated genes among broiler breeder clinical APEC isolates. It includes papC (P-fimbriae), tsh (temperature-sensitive hemagglutinin), ibeA (invasion of the brain endothelium protein A), iutA (aerobactin siderophore receptor), iroN (salmochelin siderophore receptor), iss (increased serum survival), ompT (outer membrane protease), astA (heat-stable enterotoxin), hlyF (putative avian hemolysin), and cva/cvi (Colicin V plasmid genes). In this study, we evaluated the in vitro adhesion and invasion potential of nine of these clinical APEC isolates from broiler breeders and also evaluated its relationship with presence/absence of virulence-associated genes and also with embryo mortality and pathogenicity score following embryo lethality assay and one-day-old chick challenge study, respectively.

Materials and methods

Selection of APEC isolates

For this experiment, nine isolates were selected from a total of 28 broiler breeder APEC isolates which were collected from the Mississippi Veterinary Research and Diagnostic Laboratory (MVRDL)/ Poultry Research and Diagnostic Laboratory (PRDL). They were selected based on the phenotypic virulence pattern observed following embryo lethality and one-
day-old chick challenge assays along with the genotypic virulence patterns (Joseph et al., 2023a; Joseph et al., 2023b). The information on the isolates used in this study are detailed in Table 4.1.

**Bacterial culture preparation**

The nine isolates were already confirmed as *E. coli* by the presence of *ybbw* gene using real-time PCR during the previous study by Joseph et al., 2023b. The isolates were retrieved from -80°C and streaked individually onto MacConkey agar (Becton, Dickinson and Company, Sparks, MD) plates and grown for 18-24hrs. at 37 °C. Following that, a single pink lactose-positive colony was selected from each isolate and cultured in Luria-Bertani (Becton, Dickinson and Company, Sparks, MD) broth for 16 hrs. at 37 °C in a shaker incubator at 200 rpm to get the bacterial stock culture for challenge.

**Standardization of bacteria**

From the overnight bacterial culture, 1 mL was transferred to a 1.5 ml Eppendorf tube (Waltham, MA) and centrifuged at 1,2000 rpm for 10 mins in a benchtop centrifuge (Eppendorf North America, Enfield, CT). The bacterial pellet was then washed two times and resuspended in PBS (Thermo Fisher Scientific, Vilnius, Lithuania). The resuspended culture was then serially diluted ten-fold and dilutions 10⁻⁵ to 10⁻⁷ were plated in duplicate MacConkey agar plates and incubated at 37 °C overnight. Plates with a countable number of colonies were used to determine the Colony Forming Units per mL (CFU/mL). Also, the bacterial pellet after centrifugation as mentioned above was resuspended in cell culture media and optical density (OD₆₀₀) was measured using a microplate reader (Citation1 Image reader, BioTek, USA). The remaining bacterial culture was pelleted down and stored at 4 °C. Following that, the pellet was
resuspended in cell culture media and standardized to $10^8$ CFU/mL based on the plate counts and OD$_{600}$ which is then used for the challenge.

**Preparation of HD11 (chicken macrophage) cell lines**

The thawed chicken macrophage cells were mixed with prewarmed fresh cell culture media containing Advance Dulbecco’s Modified Eagle’s medium (ADMEM) (Gibco, Billings, MT) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 1% GlutaMAX (Gibco, Billings, MT), and 1% penicillin and streptomycin (10000U/ 10000 μg/mL) (Gibco, Billings, MT). It was then suspended in cell culture media and grown up to 70-90% confluency at 5% CO2 and 37 °C in T-25 flasks (Corning, Glendale, AZ) followed by two passages in 100mm petri dishes (Corning, Glendale, AZ). Finally, the cells that reached 90% confluency were seeded in 24 well-tissue culture-treated plates (Corning, Glendale, AZ) for adhesion and invasion assays.

**Adhesion and invasion assay**

Adhesion and invasion properties of these *E.coli* isolates were examined according to Almeida et al, (1996) with slight modifications. Briefly, each well in the 24-well plate was seeded with chicken macrophage cells at a concentration of ~ $10^5$ cells/mL on the day before the challenge and incubated at 5% CO2, 37 °C until a monolayer was formed the next day. Each plate had 3 replicate wells each for adhesion and invasion assays for each isolate and 6 wells as controls with media containing 1% FBS and 1% GlutaMAX in ADMEM having HD11 cells. The cell concentration was determined by counting the cells with a hemocytometer (EMS, Hatfield, PA) using trypan blue diluent and confirmed $10^6$ cells/mL with 70-90% confluency in the monolayer. Each of the 3 replicate wells for an isolate was challenged with corresponding
standardized bacteria in cell culture media (ADMEM supplemented with 1% FBS and 1% GlutaMAX) at a concentration of $10^8$ CFU/mL and incubated for 3 hrs. at 37 °C with 5% CO2 giving a Multiplicity of Infection (MOI) 1:100. (Lee and Falkow, 1990; Schierack et al., 2011; Peng et al., 2018). After incubation, the media was removed from the culture plate and each well was washed thrice with PBS to remove the unattached bacteria and 300 μL of 0.1% Triton X-100 (Saint Louis, MO) was added to harvest all the cells. The harvested cells were then ten-fold serially diluted to $10^{-7}$ dilution and dilutions $10^{-4}$ to $10^{-6}$ were plated and incubated at 37 °C for 24h.

After the adhesion assay, the remaining 3 wells/isolate was used for the invasion assay. All isolates were confirmed for their resistance towards Gentamycin. For which, each well was added with 500 μL Gentamycin (Gibco, Grand Island, NY) at a concentration of 10 mg/mL to kill the extracellular bacteria and the plates were incubated for 3 hrs. (Döpfer et al., 2000). After that, each well was washed thrice with PBS and 300 μL of 0.1% Triton-X (Saint Louis, MO) was added to harvest all the cells. The harvested cells were then ten-fold serially diluted to $10^{-4}$ and dilutions $10^{-2}$ to $10^{-4}$ were plated and incubated at 37 °C for 24h to determine the invasion (log CFU/mL) by counting the colonies from the above-mentioned incubated plates. The adhesion (log CFU/mL) was calculated by deducting the invasion (log CFU/mL) from the total (log CFU/mL) that was obtained by counting the colonies from the incubated plates. Adhesion and invasion assays were repeated two independent times with 3 replicates each time.

**Statistical analysis**

Statistical analysis was done using the PROC GLM procedure of SAS 9.4 to identify the statistical significance of each isolate during adhesion and invasion and Fisher’s protected LSD
was used for mean separation with significance set at $P<0.05$. Also, Pearson’s correlation analysis was performed to identify relationship of adhesion and invasion with virulence factors (Joseph et al., 2023b) as well as the phenotypic virulence during embryo lethality assay and chick challenge study (Joseph et al., 2023a).

**Results**

**Adhesion and invasion assay**

Following the adhesion assay, all isolates showed at least 8 logs CFU/mL on average of bacterial adhesion to the HD11 cells. The highest adhesion was shown by P15 with 9.08 logs CFU/ mL and the lowest was P8 with 8.08 logs CFU/ mL of bacterial adhesion (Figure 4.1). The different APEC isolates were found to have a significant impact on chicken macrophage adherence ($P < 0.0001$). Moreover, all isolates except P12, P19, and P27 had a significant effect on the mean response.

During the invasion assay, all isolates except P27 showed some level of invasion into the host cells. The APEC isolates were found to have a significant impact on chicken macrophage invasion ($P$ value < 0.0001). The highest invasion was shown by isolate P18 which was 7.29 logs CFU/ mL and the lowest was P10 with 3.98 logs CFU/ mL. However, the level of invasion was not statistically different between P9, P10, P12, and P15. For the highest invasion, P18 was followed by P19, P5, and P8 ($P <0.0001$; Figure 4.2).

**Relationship between adhesion and invasion assays**

There was a reduction in bacterial concentration following invasion compared to the adhesion among all isolates with P27 showing no invasion and had the highest difference between the two assays. There was around 4 logs CFU/ mL reduction between adhesion and
invasion for P10 and P12 while around 1 log CFU/mL reduction was shown by P8, P18, and P19. Isolate P5 showed 2 logs CFU/mL reduction while P9 and P15 showed a reduction of 2 logs CFU/mL.

**Relationship of adhesion and invasion assays with genotypic and phenotypic virulence factors**

There was no correlation between adhesion and invasion with virulence-associated genes that were previously tested by Joseph et al., 2023b and phenotypic virulence factors during the day-old chick challenge study and embryo lethality assay for these 9 isolates (Joseph et al., 2023a). Details for the genes tested and P values and R values are provided in Table 4.2.

**Discussion**

This study aimed to evaluate the *in vitro* virulence potential of broiler breeder APEC isolates and identify their relationship with genotypic and phenotypic virulence factors. We observed a high adhesion and invasion potential for all isolates towards the chicken macrophage cell line except P27 which did not show any invasion.

Macrophages are the first among various cell types to reach the site of infection. So, *in vitro* bacterial challenge studies using chicken macrophage-like cell line HD-11 would mimic the host-pathogen interaction (Lee and Falkow, 1990). Hence, the adhesion and invasion potential of multiple broiler breeder APEC isolates were evaluated using the HD11 cells. Results showed around 8 logs CFU/mL of bacterial adhesion to the HD11 cells for all the isolates tested except for P15 which showed 9 logs CFU/mL. However, for the invasion, the intracellular bacterial concentration varied from 3 logs to 7 logs CFU/mL. The isolates which showed the highest adhesion potential were P15, and P18, and for invasion, it was P18 and P19. Interestingly, P15 and P18 were highly virulent during our previous embryo lethality and chick challenge assays.
and P19 was moderately or highly virulent. Additionally, all of them had 7 among the 10 virulence-associated genes tested. (Joseph et al., 2023a; Joseph et al., 2023b). However, there was no statistical relationship between presence of virulence genes and *in vitro* adhesion and invasion potential during our study. Previously, APEC clinical isolates collected from pheasants and wild birds were used to estimate the adhesion and invasion potential to Hep2 cells and also to estimate the *in vivo* virulence. In that study, scientists found significant adhesion and invasion of the bacteria towards the cells, but the MOI was higher (1:1000) and there were prominent lesions and recovery of isolates following the day-old chick challenge study (La Ragione et al., 2002).

To identify the effect of flagellar proteins (*fliG*) in adhesion, APEC isolates from diseased birds showing clinical symptoms of colibacillosis with a MOI (1:100) using chicken embryo fibroblast cells showed high adhesion (7 logs CFU/mL) and invasion (5 logs CFU/mL) potential. Moreover, they found a 100% mortality following the chick challenge with the same wild-type strain used for adhesion and invasion (Yin et al., 2021). Further, they did not analyze the statistical relationship between the assays and phenotypic virulence. However, the *in vitro* and *in vivo* pathogenicity of the isolates was high (>60%). Silveira et al. (2002) examined the relationship between the adhesion potential with virulence factors like pathogenicity during chick challenge. Similar to our study no correlation was detected. But some human isolates showed a significant correlation between these factors (Germani et al., 1996). The nine APEC isolates used might be a possible reason for the absence of a statistical relationship during our study. Future studies with a greater number of broiler breeder APEC isolates is required to identify the relationship between *in vitro* and *in vivo* challenge studies.

Isolate P5 was classified as avirulent following the phenotypic studies however, it showed a significant adhesion (8 logs CFU/mL) and invasion (6 logs CFU/mL) to
macrophages. Moreover, only 2 among the 10 virulence-associated genes were positive for P5. Another interesting finding of the study was the absence of invasion for isolate, P27. It was also an avirulent / moderately virulent isolate with only one virulence-associated gene. Sometimes, the genes responsible for virulence may not be expressed due to some environmental factors including *in vivo* conditions (Peng et al., 2018; Mol et al., 2019). Additionally, there may be other genes that are responsible for virulence besides the genes tested in this study. Also, co-infections with other diseases may affect gene expression which results in variations in virulence and immune responses (Ariaans et al., 2008). Scientists have identified the possible virulence-associated genes that result in the pathogenesis of bacteria helping adhesion and invasion. Various experiments including deletion of specific genes were done previously. Wang et al., 2017 identified the importance of *EtrA* gene which is a part of the type 3 secretion system in *E. coli* by estimating the *in vitro* adhesion and invasion potential of isolates with chicken fibroblast cells. Yin et al, (2021) identified the effect of *fliG* which codes for flagella in *E. coli* using HD11 cells. In that study, they found the wild strain which was non-mutated had a high adhesion and invasion potential. On the other hand, there was a significant reduction in both adhesion and invasion for the bacteria which was subjected to *fliG* deletion. Such genes which help in the virulence of bacteria should be studied in detail and evaluated among broiler breeder APEC isolates.

Following adhesion, a reduction in bacterial concentration was noticeable for invasion for all the isolates used in this study. As mentioned before, P27 did not have any invasion. All other isolates, the reduction varied between 1 log CFU/mL and 4 logs CFU/mL. A similar reduction in bacterial concentration was noticed in previous studies (Wang et al., 2017; Peng et al., 2018). In addition to P27, a high reduction was also shown by the isolates P10 and P12 which are
avirulent/moderately virulent and highly virulent with 1 and 7 virulence-associated genes, respectively. But, none of the genes related to invasion except for P27 which had 1 virulence associated gene present which is *ibeA* (invasion of the brain endothelium protein A). Therefore, the reason for this variation of phenotypic/ genotypic virulence and *in vitro* virulence, especially the absence of invasion should be explored further based on a whole genome sequencing analysis. Moreover, identifying the immune responses developed during host-pathogen interaction is important because it will provide information on how the host responded to the incoming pathogen and might be the reason for the reduced invasion of these isolates. Therefore, identifying the immune-related genes expression in the host cells and virulence-related genes expression in the bacterial cells is critical (Pourbakhsh et al., 1997; Stehling et al., 2003; Alber et al., 2020).

**Conclusion**

This study helped us to identify the adhesion and invasion potential of nine broiler breeder APEC isolates which were selected based on phenotypic and genotypic virulence characteristics that were analyzed previously. Bacterial concentration for adhesion varied between 9.08 logs CFU/ mL and 8.08 logs CFU/ mL while the invasion concentration varied between 3.98 logs CFU/ mL and 7.29 logs CFU/ mL with P27 having no invasion potential. Moreover, there was no correlation between adhesion and invasion with genotypic and phenotypic virulence. More research focusing on other factors which resulted in the adhesion and invasion pattern and the gene expression studies of the host and bacterial cells would possibly help to understand these isolates better in terms of virulence and pathogenicity.
**Table 4.1** Details regarding the APEC isolates used for adhesion and invasion assays. The isolates were selected based on the phenotypic virulence classification and presence of virulence associated genes.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Phenotypic classification following chick challenge</th>
<th>Phenotypic classification following embryo lethality</th>
<th>Number of virulence-associated genes present out of the ten genes tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5</td>
<td>Avirulent</td>
<td>Avirulent</td>
<td>4</td>
</tr>
<tr>
<td>P8</td>
<td>Avirulent</td>
<td>Highly virulent</td>
<td>1</td>
</tr>
<tr>
<td>P9</td>
<td>Avirulent</td>
<td>Moderately virulent</td>
<td>1</td>
</tr>
<tr>
<td>P10</td>
<td>Avirulent</td>
<td>Moderately virulent</td>
<td>1</td>
</tr>
<tr>
<td>P12</td>
<td>Highly virulent</td>
<td>Highly virulent</td>
<td>7</td>
</tr>
<tr>
<td>P15</td>
<td>Highly virulent</td>
<td>Highly virulent</td>
<td>7</td>
</tr>
<tr>
<td>P18</td>
<td>Highly virulent</td>
<td>Highly virulent</td>
<td>7</td>
</tr>
<tr>
<td>P19</td>
<td>Moderately Virulent</td>
<td>Highly virulent</td>
<td>7</td>
</tr>
<tr>
<td>P27</td>
<td>Avirulent</td>
<td>Moderately virulent</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4.2  Results of Pearson’s correlation analysis for adhesion and invasion with phenotypic and genotypic virulence factors.

<table>
<thead>
<tr>
<th>Virulence Factors</th>
<th>Adhesion</th>
<th>Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r-value</td>
<td>P value</td>
</tr>
<tr>
<td>% Embryo mortality during embryo lethality assay</td>
<td>-0.12</td>
<td>0.75</td>
</tr>
<tr>
<td>Pathogenicity Score during chick challenge</td>
<td>0.15</td>
<td>0.68</td>
</tr>
<tr>
<td>Ten virulence-associated genes*</td>
<td>0.38</td>
<td>0.31</td>
</tr>
<tr>
<td>APEC minimal predictor genes**</td>
<td>0.34</td>
<td>0.36</td>
</tr>
<tr>
<td>Antibiotic resistance genes***</td>
<td>0.29</td>
<td>0.43</td>
</tr>
<tr>
<td>Metal resistance genes$</td>
<td>-0.54</td>
<td>0.12</td>
</tr>
<tr>
<td>Antimicrobial resistance genes$</td>
<td>-0.19</td>
<td>0.36</td>
</tr>
</tbody>
</table>


**APEC minimal predictors- \textit{iroN}, \textit{ompT}, \textit{hlyF}, \textit{iss}, and \textit{iutA}.


$$Antimicrobial resistance genes include both antibiotic and metal resistance genes.
Figure 4.1  Results for adhesion potential of APEC isolates.

The mean CFU/mL for adhesion of each isolate is represented as a bar graph. The error bars represent the Standard Error of Mean (SEM) for the two independent experiments.
Figure 4.2  Results for invasion potential of APEC isolates.

The mean CFU/mL for invasion of each isolate is represented as a bar graph. The error bars represent the Standard Error of Mean (SEM) for the two independent experiments. P27- Isolate had low invasion.
Reference


CHAPTER V
SUMMARY

Current findings

Avian pathogenic *Escherichia coli* (APEC) is a gram-negative bacterium that is responsible for the disease, colibacillosis in birds which causes a severe economic burden to the poultry industry worldwide. This necessitates the development of effective control strategies to prevent the disease. There is a large amount of data regarding APEC isolates from all species and categories of birds however, there is only limited information about the characterization of clinical APEC isolates from broiler breeders. Additionally, there are reports of vertical transmission of the broiler breeder isolates to the progenies and horizontal transmission in the hatchery which increases the first-week broiler chick mortality. Therefore, the 2 chapters of this thesis mainly focus to characterize broiler breeder APEC isolates phenotypically, to evaluate the *in vitro* virulence potential, and to compare them with genotypic virulence factors for the isolates described previously by Joseph et al, 2023.

As mentioned above, the first objective of the study was to evaluate the phenotypic virulence potential of 28 broiler breeder APEC isolates based on an embryo lethality assay and a one-day-old chick challenge study. Overall, we were able to classify the isolates based on their virulence into highly virulent, moderately virulent, and avirulent. Based on the two assays, 39.3% of the isolates were highly virulent and 3.5% of isolates were avirulent while the isolates in the moderately virulent group varied according to the type of challenge performed. Moreover,
as the number of virulence-associated genes increased per isolate there was an increase in pathogenicity score following the day-old chick challenge study and % embryo mortality following embryo lethality assay. Moreover, five virulence associated genes (\textit{iroN, iss, hlyF, ompT, iutA}) that can predict APEC isolates which is described by Johnson et al, 2008 was significantly correlated with pathogenicity score ($P=0.02$) and % embryo mortality ($P<0.01$).

The second objective was to identify the \textit{in vitro} adhesion and invasion potential of nine isolates which are selected from the first study based on their phenotypic and genotypic virulence patterns. We observed a high adhesion pattern for all isolates which was above 8 logs CFU/ mL and invasion from 3 to 7 logs CFU/ mL except for one isolate that had zero invasion. However, we couldn’t find a statistically significant correlation between adhesion and invasion potential with genotypic and phenotypic virulence factors that are studied previously. The reason for this might be due to the environmental effects on the pathogen or it might be a secondary pathogen. But, the relationship between genes and virulence was evident during phenotypic virulence characterization. Specific factors which led to adhesion and invasion should be identified.

\textbf{Recommendation}

The findings of this study would help the poultry industry to visualize better the broiler breeder APEC clinical isolates since the data about isolates characteristics is much limited. The phenotypic virulence pattern observed in this study is important because more than 1/3rd of the isolates were consistently virulent during both assays and the virulence increased as the number of virulence-associated genes increased. Also, five virulence associated genes (\textit{iroN, iss, hlyF, ompT, iutA}) that can predict APEC isolates in broilers showed strong correlation with the phenotypic virulence and they have the potential to be developed as predictors for broiler breeder APEC isolates.
This helps to explore further to develop specific target-based control measures or either update/ modify them. Additionally, the high adhesion and invasion potential of the isolates are also significant because these are two important steps in bacterial colonization therefore, identifying specific factors responsible for the adhesion and invasion would help to find isolate characteristics and control measures in the future.

**Implications and future research**

The study is very relevant as this is among the first study in Mississippi to characterize clinical APEC isolates collected from broiler breeders. Therefore, it will be a good reference for upcoming studies. The changing bacterial populations from time to time is a serious concern and therefore, updating the information about their characteristics and developing more effective control strategies is critical. Future research should focus on the identification of more virulence-associated factors based on a whole genome approach. Therefore, we would be able to identify the factors responsible for high adhesion and invasion potential and the ones which resulted in the phenotypic virulence. Exploring the gene expressions and proteins developed during *in vitro* assays is also critical to identify the host-pathogen response. Developing a challenge model for broiler breeders would help to select targets that can be used to develop a vaccine or effective drug against APEC infection.