Investigating the Zoonotic Potential of Avian Pathogenic *E. coli* Isolates

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A Thesis Submitted in partial fulfilment of the requirements for the degree of

Master of Science in Industrial Biotechnology

By

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Dedicated to

My Parents and my brother

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May success follow you all, every step of the way!

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Sameen Ahmed Malik

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Abstract

Escherichia coli, a facultative anaerobe is a gram-negative bacteria which normally inhabits the gut microflora. *E. coli* is very diverse in nature and mainly act as commensal bacteria that do not cause disease, but these bacteria can become pathogenic after acquiring virulence genes by horizontal gene transfer. Pathogenic *E. coli* can be of two types that are extraintestinal pathogenic *Escherichia coli* (ExPEC) or non-extraintestinal pathogenic *Escherichia coli* (ExPEC). Extra intestinal pathogenic *E. coli* cause disease outside the intestine. ExPEC are very diverse in nature and cause different types of infection in humans, dairy, and poultry for instance UTI, Colibacillosis and Mastitis respectively. *E. coli* strains have a zoonotic risk because some of the ExPEC isolates from liver of colibacillosis infected chicken have similarities with human ExPEC, that is they have several virulence and antimicrobial resistance genes in common. Humans may acquire these genes through a variety of interactions, including as personal contact with infected birds or through the food chain. However, nothing is known about the precise rate at which handling or consumption of ExPEC-contaminated food leads to intestinal colonisation and, eventually, extraintestinal infection from a poultry source. Examining the zoonotic potential of ExPEC isolates bearing the sub pathotype Avian pathogenic *E. coli,* or APEC, is the goal of the research that has been done.

The evaluation of avian pathogenic *E. coli* isolates that cause colibacillosis in poultry, as well as the possibility that *E. coli* strains extracted from the liver of infected chickens can induce disease in chick models of avian colibacillosis and two mouse models of ExPEC-related infections, such as human sepsis and urinary tract infections, are the main foci of this research.

Through whole genome sequencing (WGS), 139 *E. coli* from liver source were categorized into ExPEC and non-ExPEC strains through screening for virulence associated genes. Based on this screening 54 out of 139 isolates were designated a ExPEC. Out of these 54, most of them belonged to APEC (45%). Among these isolates, the prevalent Sequence types were ST-131, ST-155, and ST-69. ST-69 is concerned with zoonotic infections and ST-155 is concerned with bloodstream infections. O131 and O2 were the prevalent serotypes. The bulk of the isolates from ExPEC belonged to

phylogroups B1, D, and B2. Phylogroups B2 and D are very common phylogroups where zoonotic infections are concerned. The identified strains underwent screening to determine if they carried any common VAGS (virulence associated genes) or antimicrobial resistance genes, which can spread horizontally to other bacteria and eventually to humans. Almost 99% of the ExPEC isolates contained virulence associated genes that were associated with causing zoonotic infections. ExPEC isolates from avian source resembled human ExPEC isolates causing illnesses in humans and chicken through experimental animal infection models. ExPEC isolates from liver sources were able to cause colibacillosis in chicken and showed intermediate to high pathogenicity. ExPEC isolates also caused UTI infection and lethal sepsis in mice models. 2 of the ExPEC isolates that caused colibacillosis in chick model, caused lethal sepsis in mice and enormous bacterial counts in mice. Both of these strains belonged to phylogroup D and ST-69 which were proven to have a zoonotic lineage. These findings suggest that *E. coli* isolates from liver source harbor virulence genes that can be transferred to humans and can pose a severe threat to human health.

1. Introduction

Escherichia coli (*E. coli*), which typically resides in the GI tract of healthy humans acting as a commensal organism, it is a gram-negative, facultative anaerobe with a genome size ranging from 4.5 to 5.5MB. It is a rod-shaped bacteria which is typically 2 to 6 μm long and its width ranges from 1.1 to 1.5 μm . *E. coli* possess several locomotory organs like flagella. *E. coli* is mainly commensal but they can acquire virulence genes from their environment of horizontal gene transfer and can become pathogenic, that is why they are also known as opportunistic pathogens. (Brenner, Krieg, Staley, & Garrity, 2005).

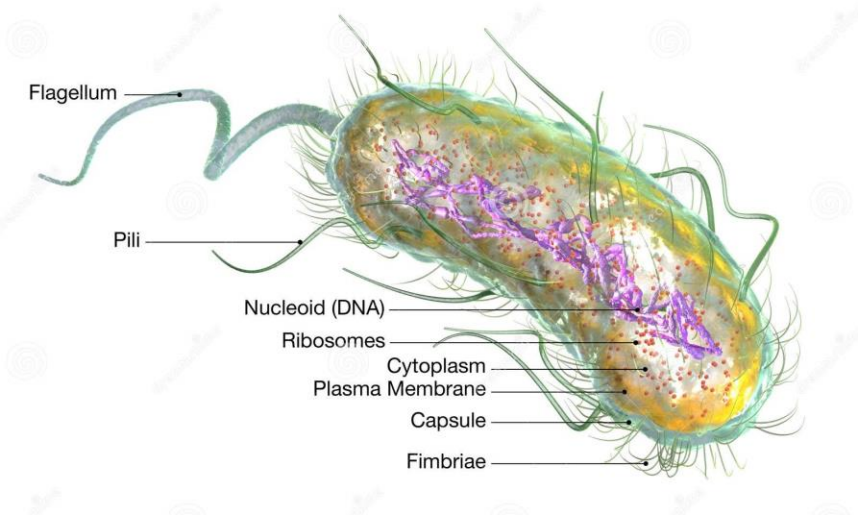

Figure 1: structure of E. coli

E. coli primarily consists of two types, one of them are commensal *E. coli*, normally they are present in the gut of human beings and other warm-blooded animals, they are harmless bacteria and do not cause disease. The other type of *E. coli* causes disease and is known as Pathogenic *E. coli*. They are associated with causing diseases to various species like mastitis in livestock industry, colibacillosis in swine industry, urinary tract infections in humans, neonatal meningitis in infants. Pathogenic *E. coli* is further categorized into Intestinal Pathogenic *E.coli* (IPEC) which is also known as Diarrheagenic *E. coli* (DEC) and Extra Intestinal Pathogenic *E. coli* (ExPEC). Diarrheagenic *E. coli* are those who can cause disease inside the intestine. But when the bacteria cross the intestinal walls and cause disease outside the intestine, then they are known as extra intestinal pathogenic *E.*

coli or ExPEC. ExPEC exhibits the potential to cross the intestinal barrier and the sub pathotypes associated with ExPEC are Avian Pathogenic *E. coli*, Neonatal Meningitis *E. coli*, Urinary Pathogenic *E. coli* (UPEC), Sepsis-associated Pathogenic *E. coli* (SePEC), and Mammary Pathogenic *E. coli* (MPEC) (Johnson & Russo, 2002).

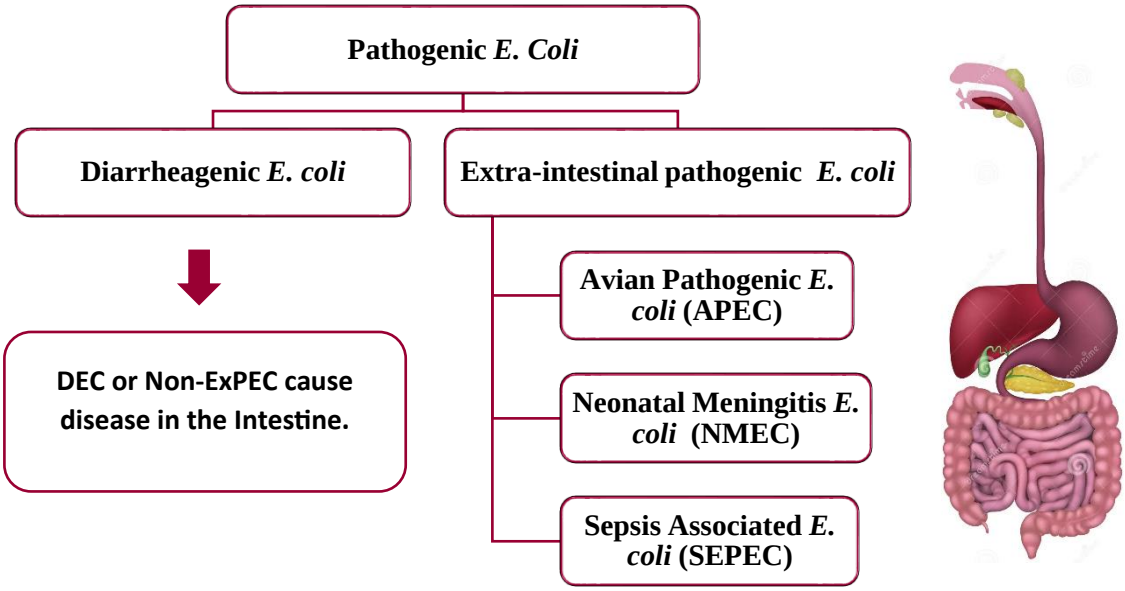

Figure 2: classification of *E. coli*

Amongst the sub pathotypes of ExPEC, APEC is the major causative agent of colibacillosis in chicken and other swine species, these swine species include birds like turkey. Colibacillosis is a syndrome and many risk factors are associated with it. Some of them are pericarditis, perihepatitis, swollen head syndrome, cellulitis etc. Colibacillosis is associated with 8-10% mortality rates in young chicks. APEC infections are associated with major economic losses. Annually 13.06% loss to poultry industry is associated with APEC infections. It is a major cause of decreased hatching rates, live weight reduction and decreased egg production in poultry.

According to the One Health Concept, the health of animals and humans is interconnected with their shared environment. Various studies have suggested that ExPEC might have zoonotic potential based on several reasons. Avian ExPEC strains harbor a variety of virulence associated genes, that can be transferred to humans via horizontal gene transfer if humans consume the infected poultry, some of the human and avian ExPEC also have

similar virulence associated genes. According to various studies, some of the ExPEC strains isolated from liver source and some of the human ExPEC strains share somewhat similar phylogenetic background. If Avian species encounter any bacterial infection, they are treated heavily with antibiotics. So, avian ExPEC strains harbor a variety of Antibiotic resistance genes (ARGS) due to the over and misuse of antibiotics for the treatment of infections, as growth promoters etc. The antibiotic resistance genes of the avian ExPEC has the ability to be transferred to humans, if they consume the infected poultry, they can catch the disease. These genes can also be transferred to humans via direct or indirect contact. Indirect contact can be made through contaminated feces, posing a severe threat to human health. In the swine industry, large amounts of antibiotics, such as aminoglycosides, beta-lactams, quinolones and are used to prevent infection and increase the growth rate of chickens. This may give rise to one of the greatest health threats: antibiotic resistance in bacteria such as *E. coli.* All these factors contribute to the zoonotic potential of ExPEC strains. According to various studies ExPEC strains with ST95 and ST23 can cause disease both in chicken and humans and can by potentially zoonotic (Asai, 2008).

However, there is no sufficient data present in Pakistan, that can give insight regarding several VAGS and the antibiotic susceptibility patterns among ExPEC strains. On the other hand, zoonotic potential of ExPEC strains isolated from poultry has also not been established yet. Therefore, this research was done to cater all the missing areas of previous studies.

In this study, we aspire to fill the above stated gaps by evaluating the zoonotic potential of various ExPEC strains, that were taken from diseased colibacillosis infected chicken. A total of 139 isolates were whole genome sequenced, analyzed using various genomic tools. The genetic diversity, sequence-type, and serotype expression, VAGS of liver *E. coli* strains were studied. Some of the strains were then assessed for their zoonotic potential using chick model and mouse model.

2. Literature Review

2.1 The One Health Triad

The one health approach states that the health of humans and animals is interrelated with each other and their shared environment. It is a three- pronged system and it addresses potential global health threats through collaborations and governmental policies. One Health approach is of prime importance because it addresses several important issues like controlling outbreaks and priority zoonotic diseases and is frequently used to manage, take preventive measures and response initiatives. Similarly, the One Health strategy ensures that several interdependent concerns are dealt with from an interdisciplinary perspective, that include poor healthcare settings, food safety, climate change as well as antimicrobial resistance. Approximately **1500** pathogens are known to infect humans. Out of these 1500, **60%** cause zoonotic infections, E.g., Brucellosis. They are believed to have emerged from wild animal species. **The One Health model is applicable to** *E. coli*, because of its high prevalence, multidrug resistance and high transmission rates between humans, animals, and their environment.

2.2 *Escherichia coli:*

Escherichia coli is one of the most common commensal microbes that normally lives inside the digestive tract of human beings and other warm-blooded animals. It is very famous for its role in maintain the homeostasis of human gut (Belkaid & Hand, 2014). It also serves as an excellent model organism in the field of genetics and microbiology because of its properties like its metabolic capabilities, very fast growth rate and its ability to manipulate (Riley et al., 2010). *E. coli* have various applications and are widely used to produce recombinant proteins. Additionally, they play a critical role in the manufacturing of numerous biotechnological goods, including insulin and vaccines (Bachmann & Low, 2019). This microbe can be categorised using a number of different attributes. traits that are both phenotypic and genetic. Classification of *E. coli* on the bases of its pathotypes is of prime importance. It serves as a very important aspect of *E. coli* classification. Pathotyping is a method that refers to the specification of *E. coli* species and is concerned with the pathogenic capabilities that the bacteria have that results in the

onset of infection. *E. coli*, when classified on the basis of pathotyping, has several pathotypes. Each pathotype is unique and has a set of virulence factors and different clinical manifestations of the infections caused by them. The veery famous pathotypes of *E. coli* includes UPEC, EPEC and EHEC (Kaper et al., 2004). These differences are associated with different infections. The severity of illness ranges from moderate to life threatening diseases and include haemolytic uremic syndrome, gastroenteritis etc.

2.3 Pathogenicity of *E. coli*

DEC or Diarrheagenic *E. coli* and ExPEC or Extraintestinal pathogenic *E. coli* are known to be Pathogenic (Croxen & Finlay, 2010). ExPEC is normally found in the intestinal microflora but does not colonize the tissue but by breaching the intestinal epithelium barrier it can colonize otherorgans and can cause urinary tract infections (UTI) in humans, septicaemia, and meningitis in new-born babies and colibacillosis in birds (Clermont, Christenson, Denamur, & Gordon, 2013) hencecausing a high economic burden both for poultry industry and in terms of human health care costsand loss of productivity (Pitout, 2012). Another aspect of ExPEC epidemiology is the probabilityof it getting transferred in human bodies by the means food chain. This is revealed in a study, during preparation of raw poultry meat for cooking, bacteria which are present on the surface of chicken or inside its organs can colonize the host GI and be excreted for up to ten days after preparation (Linton et al., 1977). Thus, APEC is not just a concern for the poultry sector but it's also significant from a public health point of view. Often non-pathogenic, *E. coli* is the most prevalent facultative anaerobe in the gastrointestinal flora of warm-blooded animals as well as humans. It is the dominant microorganism in the microbiological stool cultures of all mammals and is typically found in the range of 10^7–10^9 CFU/g in the feces. Commonly *E. coli* strains are commensals, that is they do not cause disease normally, but there are some subsets of commensals bacteria that have acquired various virulence factors and various pathogenic mechanisms to cause severe extraintestinal diseases. These subsets can cause disease both in animals and humans.

2.4 Pathotypes of *E. coli*

E. coli can be divided into two major groups based in their ability to cause several infections: the first one is Intestinal pathogenic *Escherichia coli* (IPEC or DEC) and the second one is Extraintestinal Pathogenic *Escherichia coli* (ExPEC).

2.4.1 Diarrheagenic *E. coli*

DEC consists of variety of bacteria species that are associated with causing intestinal illnesses in humans. There are several different types of DEC that cause different diseases. One of the very famous sub pathotype of DEC is ETEC. ETEC is commonly associated with causing traveller's diarrhoea. People travelling to different developing countries are commonly said to be infected with Traveller's diarrhoea.

ETEC strains can survive in harsh conditions. They produce several toxins. They can survive in harsh conditions like low temperatures or very high temperature. ETEC infections lead fever, abdominal cramps, and watery diarrhoea (Harrington & Elliott, 2019).

2.4.2 Extra intestinal pathogenic *E. coli*

Conversely, ExPEC has been linked to the development of infections such as sepsis, meningitis, and UTIs that do not originate from the digestive system. ExPEC have been divided into subgroups according to their phylogenetic groups and virulence factors. Toxins (Luthje & Brauner, 2014), iron acquisition systems (Skaar, 2010), and adhesins are a few of the virulence factors present in ExPEC (Behzadi, 2018). These factors aid the bacteria in adhering to host cells, invading host tissues, and obtaining the nutrients they need to survive (Johnson et al., 2006). Two of the phylogenetic groups associated with ExPEC are B2 and D; strains belonging to these groups usually have a higher potential for virulence than strains belonging to other phylogenetic groups. (Clermont, 2000). ExPEC is known to cause a wide range of diseases, and different pathotypes have been identified depending on the specific disease they cause (Johnson et al). UPEC, which causes urinary tract infections in both people and animals, is one of the pathotypes of ExPEC. It possesses certain characteristics that allow it to colonise the urinary tract and cause illness. (Flores-Mireles et al., 2015). NMEC strains have pathogenicity factors that cause meningitis once they pass the blood-brain barrier, which causes meningitis in

infants. (Kim et al., 2019). SEPEC strains have virulence factors that enable them to cause sepsis, which is linked to sepsis, a potentially fatal condition caused by bacterial infection. (Schiebel et al., 2014). Infections in chickens' respiratory systems and systems have been linked to strains of Avian Pathogenic E. Coli (APEC). Because of the pathogenic qualities of these strains, bacteria can live inside organs and cause illnesses that affect the respiratory system and other organs. Wright & Dziva, 2008). Mastitis, a highly prevalent and economically significant disease in dairy cattle, is thought to be caused by Mammary Pathogenic *E. Coli* (MPEC). Inflammation and tissue damage can result from MPEC strains because they are invasive and can proliferate inside mammary gland epithelial cells, as per a study by Blum et al. (2018). Because these strains often carry genes for toxins, adhesins, and iron-sequestering systems, they are able to colonise and persist in the mammary gland. Over 10 million UTI cases are reported annually in the USA alone as a result of ExPEC infections, according to a study by Russo and Johnson (2019). These infections are linked to antibiotic resistance, high recurrence rates, and high medical costs. Bloodstream infections are another serious illness brought on by ExPEC, and they cause between 20 and 25 percent of sepsis cases in the US. (Tängdén et al., 2019) Furthermore, it has been reported that ExPEC strains can also cause wound infections, meningitis, pneumonia, and other infections. (Johnson et al., 2018). Additionally, these infections can have a high rate of morbidity and mortality, especially in populations that are more susceptible.

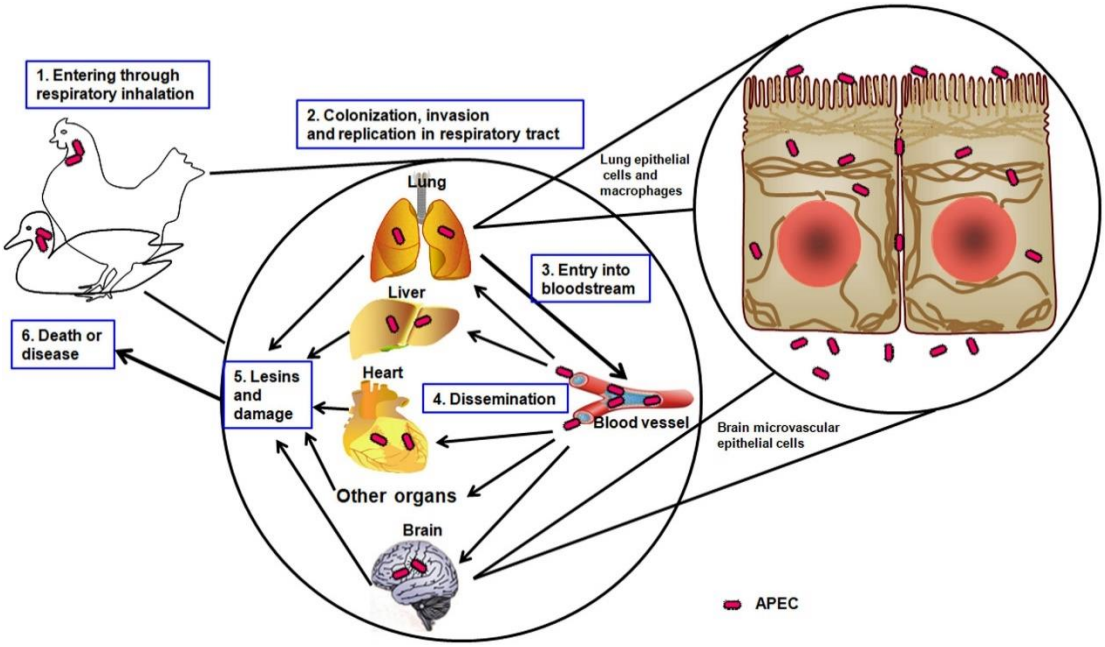

Figure 3: Molecular mechanism of ExPEC infections

2.5 Zoonotic potential of ExPEC strains

In recent times, zoonotic illnesses have gained more attention. To alert the public, the Centres for Disease Control and Prevention (CDC) has published reports on the zoonotic potential of ExPEC. Initial reports showed that human ExPEC and APEC both have similar phylogenetic background and share the same virulence genes, this explains the zoonotic potential of APECO1 somehow (Manges & Johnson, 2012). Sequencing of APECO1:K1:H7 strain revealed that it is highly like human UPEC and NMEC (T. J. Johnson et al., 2007).

2.6 APEC; etiologic agent of colibacillosis

APEC, the sub pathotype of ExPEC is reported to be a causative agent of colibacillosis and an important pathogen of the poultry industry thus resulting in significant diseases, mortality, and subsequent economic losses. Colibacillosis affects all age groups among the poultry and is caused by poor environmental conditions and stress due to concurrent infections. Depending on the organ affected, signs are diverse, non-specific, and sudden death may occur. Other symptoms may include poor growth, lethargy, diarrhoea, weakness, reduced appetite etc. Several conditions can arise including coli septicaemia, yolk sac infection, egg peritonitis and many others. Clinical presentations may vary

accordingly. The severity of disease is determined by age of chicken, co-existing infections, duration of infection and management conditions (Kazibwe et al., 2020). The determinant conditions linked with colibacillosis include cellulitis, coli- granuloma, omphalitis, swollen head syndrome, enteritis, septicaemia resulting in the death of the chicken in severe cases. The genome of *E. coli* holds diversity to a greater extent as it constitutes housekeeping genes that are important for basic cellular functionality and maintenance, resistome that have antibiotic resistance genes, pathogenicity islands etc. (Raimondi, Righini, Candeliere, Musmeci, Bonvicini, Gentilomi, Starčič Erjavec, Amaretti, & Rossi, 2019) and there are still number of genes whose pathogenicity needs to be investigated and the reasons of higher pathogenic behaviour of gram-negative bacteria than the bacteria having gram-positive properties lies in this fact; that the membrane that is outside the gram-negative bacteria has been made up ofan endotoxin i.e. LPS and it has been considered important for gram-negative bacteria survival (Alexander & Rietschel, 2001).

APEC resides in a dry environment and is transmitted to chickens from garbage or faecal contamination. Newborn chicks can get this infection by transmission of pathogen through ovaries (Panth, 2019). This pathogen can spread quickly from chickens to people, infecting them when it colonises their digestive tracts. (Linton et al., 1977). Numerous virulence characteristics have been found to enable these pathogens to proliferate, take over, and infect areas of the body other than the gastrointestinal tract. People can contract this infection by eating undercooked poultry meat or by breathing in contaminated dust.

2.7 Virulence Factors of ExPEC Strains

Virulence factors include presence of large virulence plasmids and the abilities to:

- Resist phagocytosis and serum killing
- To gain iron in low-iron conditions
- Binds to host structure

The factors that contribute to the virulence ability of ExPEC can be categorized into Six classes: secretion system, adhesins, iron uptake factors, protectins, invasins and toxins.

Secretion system: there are four different types of secretion mechanisms known as Type I, Type II, Type III, and Type IV secretion systems. With the help of these secretion systems, *E. coli* release multiple virulence factors that modify the metabolism of host cells, ultimately leading to the development of disease conditions.

Adhesins: These cell surface adhesins, which enable tight contact between the bacteria and the host cell membrane, increase the pathogenic potential of pathogenic *Escherichia coli.*

Iron Uptake Factors: siderophores bind iron and help to stimulate the growth and development of bacteria. These factors facilitate the acquisition of iron. Iron is an essential nutrient for bacterial growth and is often limited in the human body as a defence mechanism. These allows *E. coli* to scavenge iron from its environment.

Protectins: protectins help bacteria in serum survival. The three main factors influencing resistance are *TraT*, capsular antigen *K*, and *OmpA.* The *kpsM* gene is involved in the synthesis of the capsule in *E. coli*. They are protective structures around bacteria that help them evade the host's immune system.

Autotransporters: These genes are involved in the transport of various molecules and proteins across the bacterial cell membrane

Toxins: these function by destroying the membrane of host cells, triggering secondary messenger molecules, and by preventing the host cell from synthesizing proteins. hlyE gene in *E. coli* plays a role in its pathogenesis by encoding a hemolysin protein. Hemolysins are virulent factors that help bacteria infect their host.

2.7 Economic importance of ExPEC:

In Pakistan, poultry industry contributes to 1.3% GDP of the country (Hussain et al., 2015) and is the second largest industry of Pakistan (Memon, 2012). According to economic survey of 2019- 2020, Pakistan produces 1,163 million grill chickens annually, ranking it as the eleventh largest poultry producer in the world. Twenty-eight percent of the meat produced in the nation comes from poultry. (Jan et al., 2018). One of the most devastating diseases to affect chickens globally is colibacillosis, which raises mortality and causes

significant financial losses. (Dho-Moulin & Fairbrother, 1999). This disease causes significant economic losses in the broiler industry every year. Swollen head syndrome (SHS) one form of colibacillosis, can reduce egg production by 2-3% and has a mortality rate of 3-4% (Kabir, 2010). Pathogenic bacteria affecting the poultry are risk for both economy as well as humans due to reduced production and transmission of pathogens to humans via contaminated poultry products (Mitchell et al., 2015)

3. Materials and Methods

3.1 Collection of samples

A total of 139 *E. coli* isolates from the liver of chicken infected with colibacillosis were already collected by the AntiBacter lab (Jalil *et al*., 2023) of National University of Sciences and Technology (NUST), Pakistan was used for this study. The samples were obtained from poultry farms of Rawalpindi Division and Islamabad district and kept at a temperature of 28°C during transport. They were stored at 4°C in refrigerators, prior to isolation.

3.1.1 Screening for *E. coli* and Confirmation Through PCR

All of the isolated strains were streaked MacConkey agar plate (used for growing bacteria on) using streak-plate method and incubated at 37°C overnight. Following this, a single lactose-fermenting colony was carefully picked and streaked onto EMB agar. Green colonies with metallic sheen were obtained. Using boiled DNA extracts from the isolates, To further verify that the isolates are indeed *E. coli*, PCR was utilised to identify the housekeeping gene, *uidA,* in *E. coli*. (Jalil et al., 2022; Jalil et al., 2023).

3.2 DNA Extraction and Quantification

After being streaked on a MacConkey agar plate, the *E. Coli* isolates were incubated at 37°C for the entire night. From the plate, one pure colony was chosen, and it was cultured for the entire night at 37°C with constant agitation in Luria-Bertani (LB) broth. (200 rpm). Following the manufacturer's instructions, genomic DNA was extracted by means of Qiagen DNeasy® Blood & Tissue kit (Qiagen, Valencia, CA, USA). Using a Qubit® 3.0 fluorometer (Thermo Fisher Scientific, MA, USA) and NanoDropTM (Thermo ScientificTM, DE, USA), it was confirmed that the genomic DNA was both pure and concentrated. The genomic DNA with an A260/A280 ratio of ≥1.8 was stored at -20◦C prior to use.

3.3 Whole Genome Sequencing

To prepare the DNA library for whole genome sequencing, genomic DNA was diluted to 0.2 ng/µl and prepped using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA). A quantitation-based process was used to normalise the library before it was combined at the same volume. Using an Illumina MiSeq sequencer and a MiSeq reagent v3 kit, the 600 µl pooled library was denatured and sequenced with 500 (2 x 250) cycles.

3.4 Quality Assessment of Raw Reads

The quality of Illumina paired end reads of all sequenced isolates was checked using the MicroRunQC workflow in GalaxyTrakr (v2.0) (Timme *et al*., 2020). Following are the criteria used to assess the paired end raw reads:

- an average coverage > 40,
- mean quality score > 30,
- number of contigs < 500 and,
- total assembly length between 4.5 and 5.9 Mb

3.5 Submission to NCBI

Raw reads that passed the quality check were then submitted to NCBI under the BioProject PRJNA357722.

3.6 Genome Assembly

Quality evaluated raw reads; adapter sequences were eliminated from the raw reads by trimming them using the Trimmomatic (v0.30) tool. Following quality clearance, raw reads from every isolate that was sequenced were assembled from scratch using Shovill and Trimmomatic on Galaxy v1.0.4 to produce the draught genome (Seemann, 2017).

3.7 WGS based Typing

3.7.1 Serotype Prediction

Serotyping was performed using Enterobase from the Enterobase Tool Kit (EnteroBase v1.1.5) that uses assembled genomes and identifies O and H serotypes. This also follows mapping of O and H antigen alleles. Llipopolysaccharide and flagellar antigen are used for O and H serotyping. O antigen is a surface antigen, a gene cluster is responsible for

the synthesis of O antigen. This prediction is based on O-antigen cluster having genes; *wzx, wzy, wzm, wzt.*

3.7.2 Phylotype Determination

Phylotyping refers to the identification of sequences that has a certain level of similarity. For phylotype determination, Enterobase (v1.1.5) was used that follows the Clermont scheme. The presence or lack of specific genes determines the phylotype of a given strain. *TspE4, chuA, yjaA, and arpA* are these genes. *E. coli* strains are divided into eight phylogroups based on their phylotypes: phylogroups A, B1, B2, C, D, E, F, G, and the recently discovered Clade I.

3.7.3 Multi-Locus Sequence Typing (MLST)

Multilocus sequence typing was performed on Enterobase that follows the Achtman 7 gene MLST sequence archive at EnteroBase v1.1.5. There are seven housekeeping genes of E. coli (*adk*, *fum*C, *gyr*B, *icd*, *mdh*, *pur*A and *rec*A), 7 gene MLST scheme is based on the allelic differences between these 7 housekeeping genes. Based on the different sequences, different STs are assigned to the strains.

3.8 Virulence Assessment

3.8.1 Identification of Virulence Associated Genes

ABRicate (Galaxy v1.0.1) (Seemann, 2016) was used to identify the virulence associated genes by aligning each draft genome assembly against the VFDB (Liu *et al*., 2019), respectively This study included VAGs that met the default VFDB threshold values of ≥90% nucleotide identity and ≥80% coverage. Virulence associated genes of bacteria are of prime importance because they provide us with significant knowledge about bacterial pathogenesis and its mechanisms to evade the immune system of its host.

3.8.2 Pathotyping of Liver *E. coli* isolates

The presence of at least two of the ExPEC defining genes, *sfa/foc, afa/dra, fyuA/iutA, tsh*, *cvi/cvaC, iss/traT, papA and/or papC, and kpsM,* is required to define ExPEC. ExPEC status was checked on every *E. coli* isolate (Mitchell et al., 2015). In order to find the ExPEC isolates' subpathotypes, additional screening was carried out. If a strain is an ExPEC and has at least five of the defining genes for APEC (*papA/papC/papEF/sfa/foc,*

vat/hlyF, traT/iss, and ompT), it is classified as an APEC. To be designated as an NMEC, an ExPEC must carry the *ibeA and kpsM* genes.

Any ExPEC that possesses the *iss and/or traT* gene and exhibits complement resistance phenotype is eligible to be classified as SEPEC. Any ExPEC that wants to be called a UPEC has to be an ExPEC and be able to withstand exposure to human urine.

	Selection based criteria	
ExPEC	detection of ≥ 2 of the following genes: *sfa/foc, afa/dra*, *fyuA/iutA*, *tsh*, *cvi/cvaC*, *iss/traT*, *papA* and/or *papC,* and *kpsM*	
Sub-pathotypes of ExPEC	**Phenotype**	**Genotype**
APEC	None	ExPEC plus ≥5 of 8 genes *iucD/iroN, fyuA/iutA, cvi/cvaC, tsh, papA/papC/papEF/sfa/foc, vat/hlyF, traT/iss*, and *ompT*.
UPEC	Growth in urine	Must be ExPEC
NMEC	None	ExPEC plus *ibeA* and *kpsM* gene
SEPEC	Complement resistant	ExPEC plus *traT* and/or *iss* gene

Table 1. Criteria for pathotype determination of ExPEC and DEC strains

3.8.3 Virulence Associated *in vitro* Pathogenicity Analysis

ExPEC isolates underwent various virulence associated phenotypic analysis mainly growth in human urine and complement resistance.

3.8.3.1 Growth in Human Urine

Growth in urine was done to see if the *E. coli* isolates could survive in the high urea and nitrogenous environment of human urine. Some studies suggest that *E. coli* isolates with UPEC genes that grow in human urine (*in vitro*) could potentially infect humans when they come into contact with them. The procedure previously mentioned was used to conduct the assay. (Jalil et al., 2023). Filter-sterilized urine samples were obtained from

male and female healthy subjects using a 0.2µm sterile filter. After that, the samples were combined and kept in aliquots at -20°C. To put it briefly, the OD of the tested isolates was set to 1.0. The isolates were added to microtiter plates after being diluted 1:100 in sterile urine. After eight hours of incubation at 37°C in a static setting, the absorbance at 600 nm was determined. UPEC strain CFT073 and *E. coli* K-12 MG1655 were used as the positive and negative controls, respectively.

3.8.3.3 Complement Resistance to Fetal Bovine Serum

With some modifications, a standard quantitative microtiter plate method was used to determine complement resistance (Lee et al., 1991; Jalil et al., 2023). In a nutshell, 200µL of the bacterial dilution containing 104 CFU was put into each well of a 96-well microtiter plate after mixing it with an equal volume of 50% serum in 100µl of PBS. Using spectrophotometry, the plate was incubated for 4 hours at 37°C in a static environment. The OD492 was then measured. If the OD492 in wells containing serum was greater than or equal to that of the well without serum, then the ExPEC isolates were deemed to be complement-resistant. As a control, heat-inactivated sera was employed. *E. coli* K-12MG1655 and UPEC strain CFT073 were the positive and negative controls, respectively.

	Growth in Urine	Complement Resistance	Biofilm Formation
Liver Isolates	46 (85%)	42 (78%)	39 (72%)

Table 2: *in vitro* pathogenicity analysis

3.9 Virulence in Chick Model

3.9.1 Experimental birds

From the hatchery, 160 one-day-old Ross breed specific pathogen-free (SPF) broiler chicks were collected. The twelve groups of ten birds each were randomly assigned to contain chicks. They were housed in a secluded space under carefully monitored circumstances. Relative humidity (60–70%) and temperature (28–30 C) were maintained. The chicks were given access to unlimited water and fed on organic soybean feed. The

chicks were fed a diet devoid of antibiotics. Additionally, vitamin and nutrient supplements were given to them**.**

3.9.2 *E. coli* strains and inoculum preparation

Twelve strains of *Escherichia coli* were chosen and incubated in brain heart infusion (BHI) for the entire night at 37°C in order to achieve a final concentration of 108 CFU/ml. Ten groups of three to four-day-old chicks were subcutaneously injected in the abdomen with 0.1 mL of the inoculum solution. On the first day following the inoculation, the chicks were checked every six, twelve, and twenty-four hours. Starting on the second day, the chicks were checked every twelve hours. Every day, the dead chicks were gathered for necropsy, and the following lesions were examined under a microscope: pericarditis, cellulitis, swollen head syndrome, airsacculitis, and perihepatitis. The chicks that endured until the experiment's conclusion were put to death. We then determined the pathogenicity rate of them. On EMB agar, chicks that perished on the first and second day without showing any microscopic lesions were additionally exposed to bacterial isolation from their organs. An organ is considered positively impacted by bacteria if typical metallic green colonies are seen on EMB.

3.9.3 Establishment of Individual Pathogenicity Index (IPI)

The pathogenicity index (IPI) of each chick in a group that received the same *E. coli* strain vaccination is correlated. For an inoculated chick, the maximum pathogenicity rate was determined to be 10, and the lowest pathogenicity rate was found to be 0. Five of these ten points are the scores for the macroscopic lesions that were assigned during the necropsy. For every lesion that was present, a score of 1 was assigned, and when none was, a score of 0. The remaining five points correspond to the bird's time of death. Chicks that died on the first day following bacterial inoculation and showed distinctive green metallic sheen colonies on EMB agar were likely dead from septicaemia, and they were assigned a death time value of 1, which is the highest value assigned to the chick that perished on the first day. Based on the values of the death time and the scores of each macroscopic lesion, a formula was created to determine the IPI. (Souza *et al*., 2016):

IPI= (Td X 5) + Pc + Ph + SHS + C + A

Where: IPI = Individual pathogenicity index; Td = Death time value; Pc = Pericarditis; Ph = Perihepatitis; SHS = Swollen head syndrome; A = Airsacculitis; C = Cellulitis

3.9.4 Establishment of Pathogenicity Index (PI) of *E. coli* strains

The pathogenicity index for each inoculated *E. coli* strain was established according to the following formula.

PI = ∑ (IPI)/N

PI = Pathogenicity index; ∑ (IPI) = Sum of individual pathogenicity index of all chicks in the same group; N = Total number of inoculated chicks in the same group. Following is the reference table, made while calculating pathogenicity index.

				PE-43					
Chick	Time of Death	SBF	Td	Pathological Features					IPI
				A	Pc	Ph	SHS	C	
1	1	0	1	0	0	1	1	1	8
2	1	0	1	0	0	1	1	1	8
3	1	0	1	0	1	1	1	0	8
4	2	0.143	0.857	0	1	1	0	0	6.285
5	2	0.143	0.857	0	1	1	0	1	7.285
6	2	0.143	0.857	0	0	1	1	1	7.285
7	3	0.286	0.714	0	0	1	0	1	5.57
8	4	0.429	0.571	0	1	1	0	1	5.855
9	4	0.429	0.571	0	1	1	0	1	5.855
10	5	0.572	0.428	0	0	1	1	1	5.15
				PI = 6.72					

Table 3 : Table representing the establishment of PI of PE-43 inoculated in 10, 3-4 days old chicks.

3.9.5 Classification of *E. coli* strains in pathogenicity groups

Based on their pathogenicity index, tested *E. coli* strains were categorized into four pathogenicity groups:

- The pathogenicity index ranging from 7 to 10 indicates a high pathogenicity group.
- Pathogenicity index for the intermediate pathogenicity group, which ranges from 4 to 6.99.
- Pathogenicity index: 1 to 3.99; low pathogenicity group.
- Apathogenicity group: 0 to 0.99 is the pathogenicity index range.

3.10 Virulence in Mammals

To examine the virulence potential of the *E. coli,* isolate for humans, mouse models of human ExPEC infections were employed.

3.10.1 *E. coli* strains and inoculum preparation

Nine different bacterial strains were chosen, and they were all cultivated for an entire night at 37°C in Brain Heart Infusion medium. The ultimate concentration of 108 CFU/ml was achieved by preparing the primary and secondary inoculum. From the cultured LB, aliquots containing 0.1 mL of the final inoculum were prepared. The culture's optical density is measured periodically until the required OD of 0.6 is attained.

3.10.2 Potential to Cause Sepsis in Mouse Models

Four to five-week-old BALB/c mice were used in this experiment. Each of the control and experimental groups included five mice. Nine distinct strains of *Escherichia coli* were identified from the liver of chickens infected with colibacillosis. Out of them, two were non-ExPEC, two were positive and negative controls, and five were ExPEC strains. A 0.1 mL intraperitoneal injection of the inoculum solution was administered to the mice. The negative and positive controls were *E. coli* K-12MG1655 and CFT073, respectively. After receiving the dose, the mice were monitored every day for five days, and the severity of the disease was graded using a previously developed scoring system (Johnson et al., 2012) as follows: 1. Well; 2. Mildly sick; 3. Moderately sick; 4. Severe sick; and 5. Dead. Mice that lived to the end of the experiment were put to death by breathing in carbon dioxide.

3.10.3 Potential to Cause Urinary Tract Infection in Mouse Models

As previously mentioned, *E. coli* isolates' capacity to cause UTIs was examined in mouse models (Thai et al., 2010). BALB/c mice that were 7 to 8 weeks old were used in this experiment. Each of the control and experimental groups included five mice. Nine distinct strains of *Escherichia coli* were identified from the liver of chickens infected with colibacillosis. Out of them, two were non-ExPEC, two were positive and negative controls, and five were ExPEC strains. The negative and positive controls were *E. coli* K-12MG1655 and CFT073, respectively. The mice's bladders were emptied prior to inoculation in order to prevent washing out during the process. Then, 0.1 mL of the inoculum solution was administered to the mice via the transurethral route. After that, the mice were observed twice a day for two days. The CFU/g of the bladder, kidney, liver, and spleen were determined by serial dilution plating of organ homogenates on LB agar after the mice were put to sleep 48 hours after infection. Prior to the experimental endpoint, no mice passed away.

4. Results

4.1 ExPEC and its sub pathotypes

Based on the VAGs screening, 54 out of 139 *E. coli* isolates from liver source were designated as ExPEC. The rest of the 85 isolates were designated as non-ExPEC. Among the ExPEC isolates 45% were APEC, 29.9% were UPEC, 17.7% were SEPEC while none as NMEC. Combined pathotype groups with overlapping traits included APEC/UPEC/SEPEC and were 7.3%.

Sub pathotypes of ExPEC [a]	**Selection based criteria**		**No. (%)** [c]
	Phenotype	**Genotype** [b]	
Main sub pathotypes			
APEC	None	ExPEC plus ≥5 of 8 selected APEC genes	45%
UPEC	Growth in urine	ExPEC	29.9%
NMEC	None	ExPEC plus *ibeA* and *kpsM* gene	0
SEPEC	Compliment resistant	ExPEC plus *traT* and/or *iss* gene	17.7%
Undefined	**---**	**ExPEC**	**0**
With overlapping traits			
APEC/UPEC/SEPEC	Growth in urine, Complement resistance	ExPEC plus ≥5 of 8 selected APEC genes	7.3%

Table 4: Table representing the selection criteria and prevalence of extraintestinal pathogenic E. coli (ExPEC) sub pathotypes among liver E. coli isolates

4.2 Prevalence of phylogenetic groups, serotypes, and sequence types among ExPEC and non-ExPEC isolates

The most prevalent **phylogenetic group** among ExPEC isolates was phylogroup B1, D and B2.

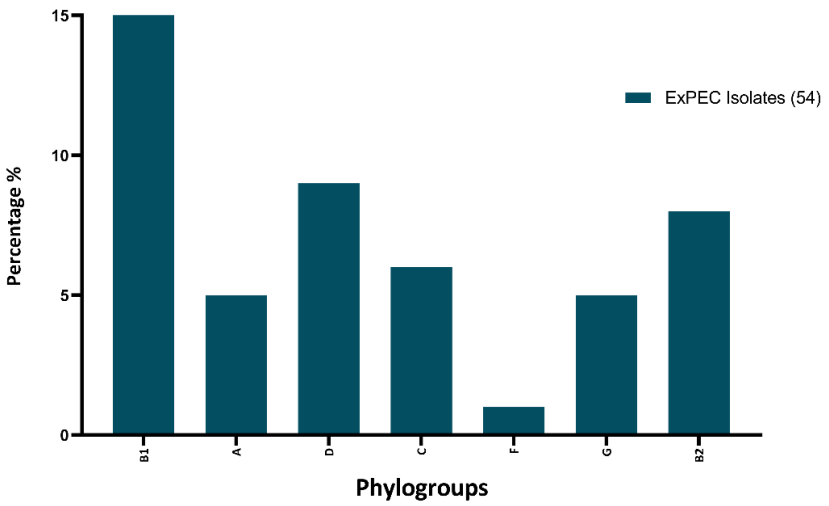

Figure 4:representing the phylogroups of liver *E. coli* isolates

For ExPEC, 12 different **sequence types** were observed, most of the ExPEC falls under ST-131, ST-155. Some of them also belonged to ST-69.

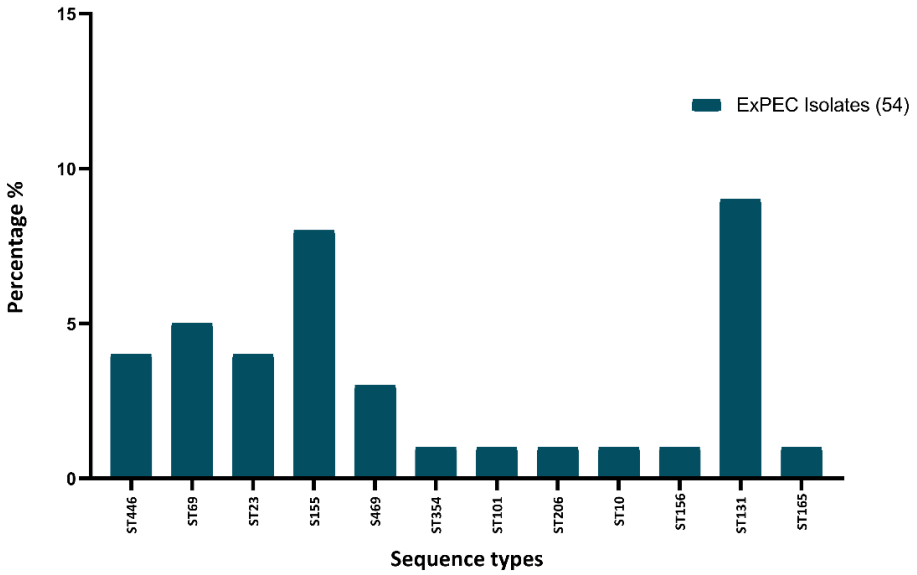

Figure 5:representing the sequence types of liver *E. coli* isolates.

Overall, 21 different **O-serotypes** were observed in liver e. coli isolates. Out of these 21 serotypes, O131 and O2 were prevalent.

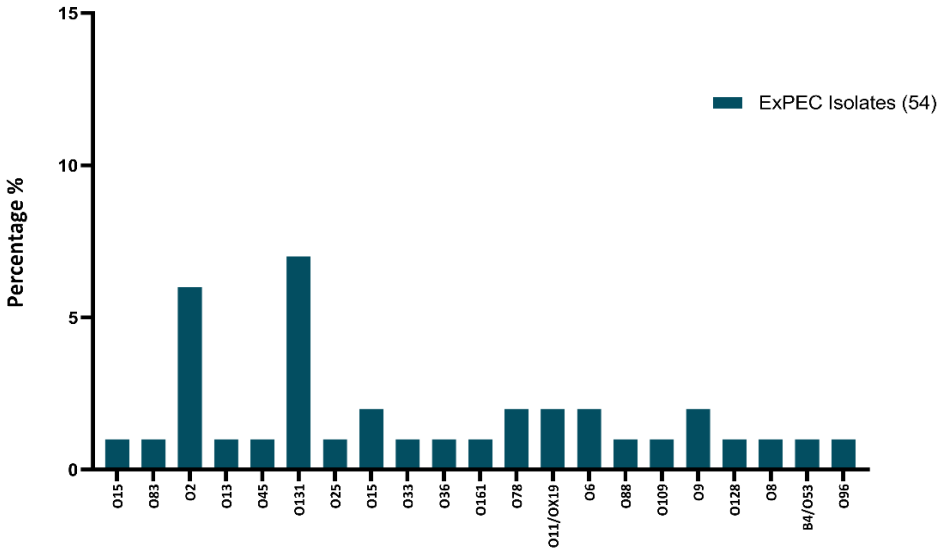

Figure 6:representing the phylogroups of liver *E. coli* isolates.

4.3 Virulence-associated *in vitro* phenotypic analysis

Five virulence-associated phenotypes, such as growth in human urine and complement resistance, were assessed for ExPEC isolates.

In human urine, 85% of the ExPEC isolates showed signs of growth. Among the ExPEC strains, 78% of the isolates exhibited complement resistance.

An ExPEC scatter plot illustrating the increase in urine density. The dots show the optical density (OD) value of each isolate, and the red line shows the standard OD value that the strains must meet in order to be classified as positive for growth in human urine; strains that fall within or above the red lines are considered positive for growth in human urine.

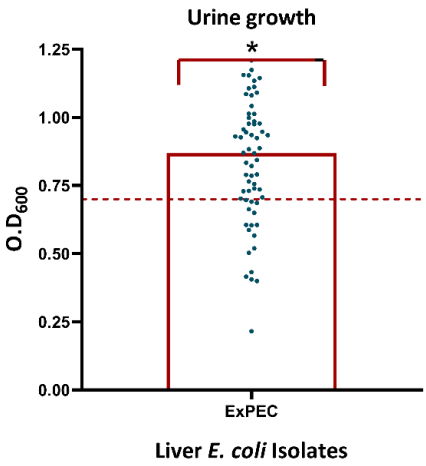

Figure 7:. Scatter plot representing the growth in urine density of liver *E. coli* isolates

4.4 Distribution of virulence genes among ExPEC and non-ExPEC isolates

ExPEC and non-ExPEC isolates were screened for 391 VAGS, commonly associated genes with extraintestinal infections. The genes belonged to 6 different classes including adhesin, toxin, autotransporter, protectin, iron acquisition system and secretion system. All genes belonged to these 6 classes were more prevalent in ExPEC. A heatmap depicting the presence of virulence-associated genes in ExPEC isolates. Besides the ExPEC markers, some of the genes were prevalent in 95-98% isolates these are:

espL: This system allows *E. coli* to inject proteins into host cells and manipulate cellular processes, aiding in its pathogenicity.

iutA: The *iutA* gene encodes the aerobactin siderophore ferric receptor protein, required for the acquisition of iron from the host cell.

kpsM: This gene is involved in the synthesis of the capsule in *E. coli*. They are protective structures around bacteria that help them evade the host's immune system.

ehaB: They are involved in transport of substances that are necessary for the survival of bacteria.

hlyE: This gene encodes a hemolysin protein and helps the bacterium to infect its host.

fimH: this is a lectin protein, and it enables the bacterium to adhere to host cells.

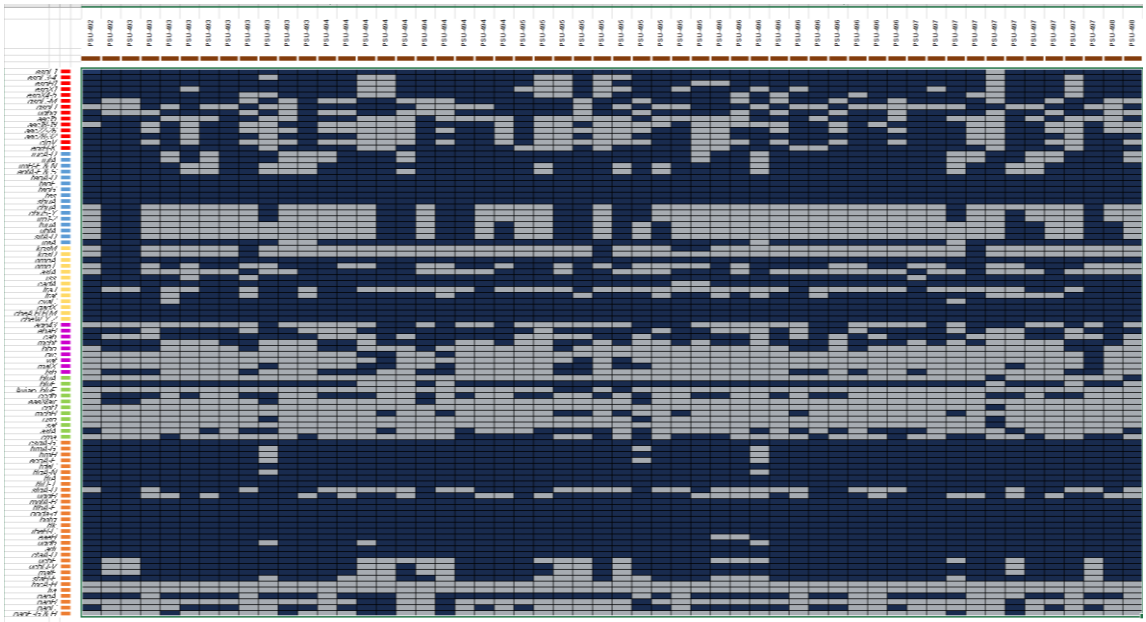

Figure 8: heatmap depicting the presence of VAGS in liver *E. coli* isolates

4.5 Ability of ExPEC/APEC isolates to cause colibacillosis in chicken

During the experiment, 83% of chicks from ExPEC strains died prior meeting the endpoint criteria, due to infection in the form of sepsis or invasion of several organs while the rest were euthanized at 7th day of the experiment. The ExPEC strains showed a mean PI of 6.0. Three of the eight ExPEC isolates exhibited high pathogenicity, and the remaining five displayed intermediate pathogenicity. The pathogenicity of the two non-ExPEC strains was minimal. ExPEC isolates showed significant difference from negative control (P = <0.0001) and some of these ExPEC isolates like PSU-5040 and PSU-5070 equalled or exceeded the positive control for pathogenicity index in chicken model.

Ability of liver *E. coli* isolates to cause virulence in chick models via invasion of the several organs. Every dot stands for a distinct animal. The tested liver *E. coli* isolates are divided from the positive and negative controls by a vertical dashed line. When compared to the negative control, an asterisk (*) indicates the significantly higher values for liver *E. coli* isolates and the positive control that were obtained through an ANOVA and Dunnett's method.

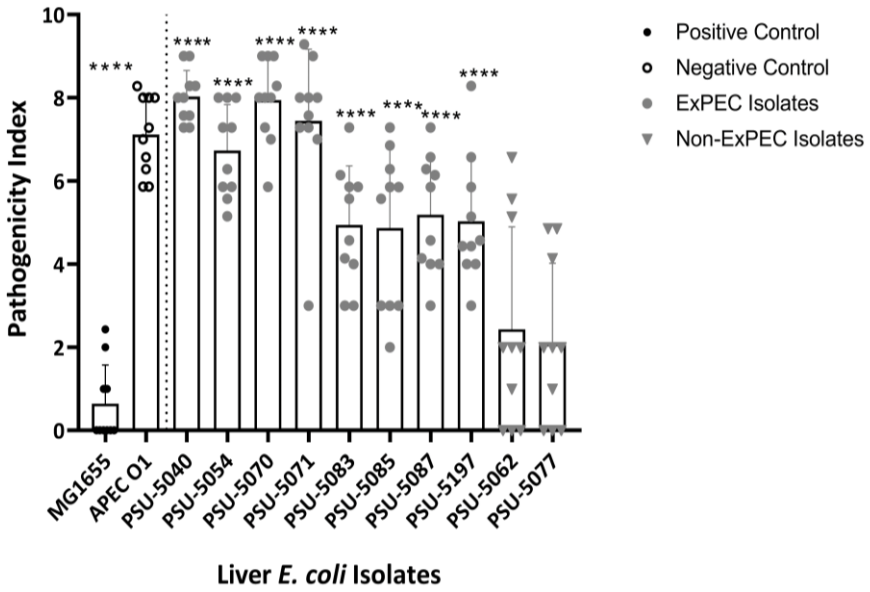

Figure 9: This graph shows the potential of ExPEC isolates to cause virulence in chick models via invasion of several organs

Following dissection, the infected organs were examined histopathological to determine how pathogenesis affected the tissues. There was no tissue damage observed in the negative control group. PSU-5040, the ExPEC test group and the positive control, demonstrated severe tissue-level pathogenesis. Severe vascular congestion (VC), necrosis (N), cellular infiltration (CI), and dilated sinusoids with leukocytes (DS) are visible.

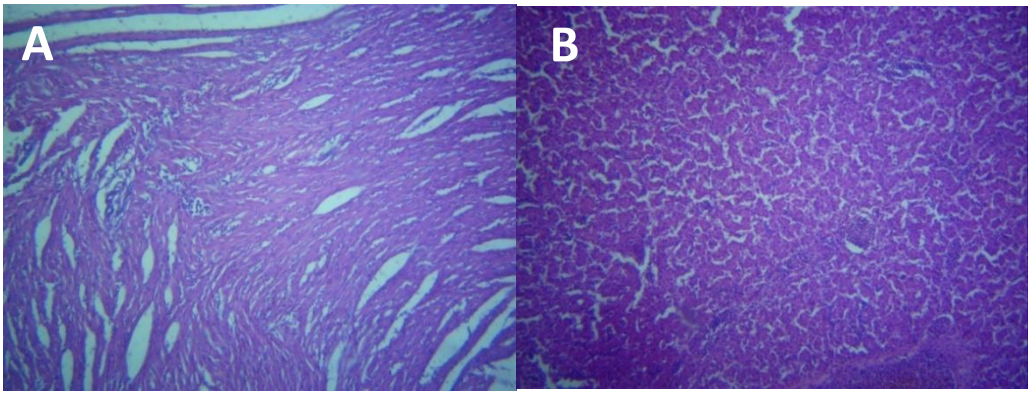

Figure 10: (A) Histopathological analysis of heart tissue. (B) Histopathological analysis of liver tissue of negative control

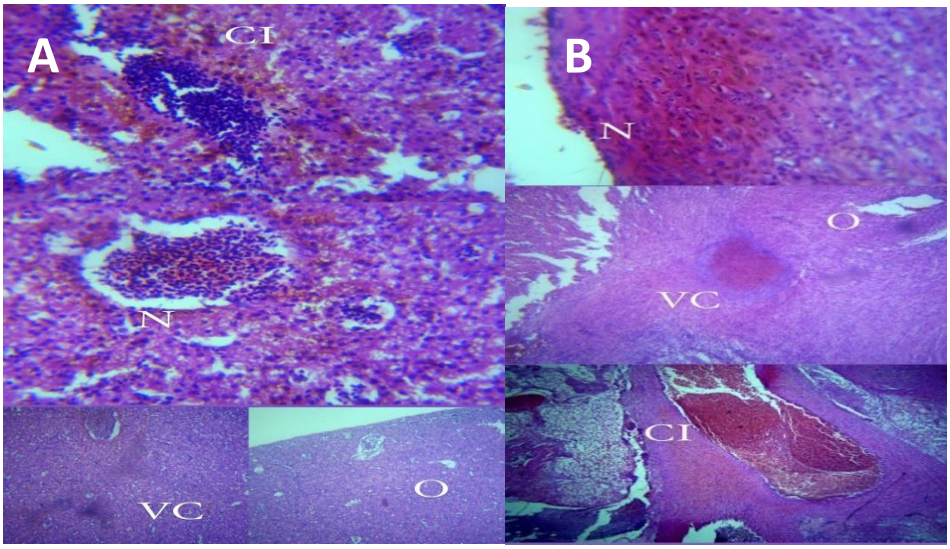

Figure 11:(A) Histopathological analysis of heart tissue. (B) Histopathological analysis of liver tissue of positive control APEC O1

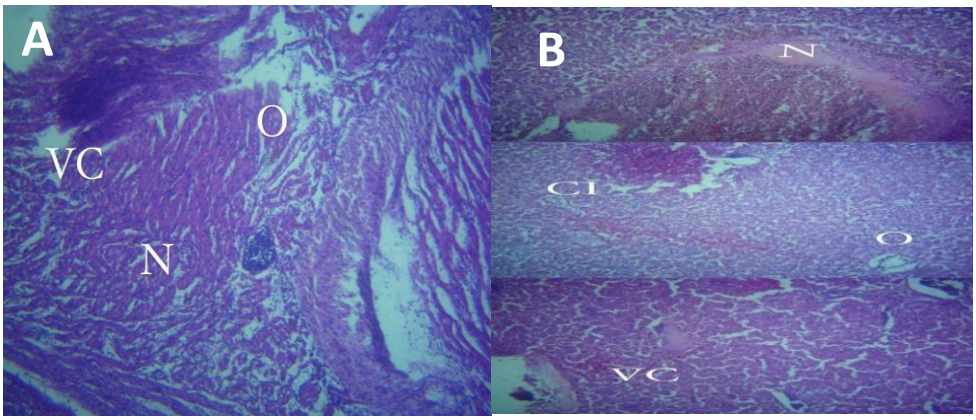

Figure 12:(A) Histopathological analysis of heart tissue. (B) Histopathological analysis of liver tissue of test strain PSU-5040

4.6 The ability of ExPEC/SEPEC isolates to cause lethal sepsis in mice

ExPEC and non-ExPEC isolates from liver sources were chosen, and using a mouse sepsis model, they were compared with negative control MG1655 and positive control CFT073 for the illness severity score. and ExPEC isolates showed significant difference from negative control (P = <0.0001) (Figure 10). During the experiment, 21/25 (88.6%) mice from liver ExPEC strains died prior meeting the endpoint criteria, due to infection in the form of sepsis and from non-ExPEC strains only 1/10 (10%) mice died prior meeting the

endpoint criteria. Most of the ExPEC isolates caused the lethal sepsis in mice along with oedema. The survival curves and illness severity scoring of experimental *E. coli* isolates along with positive control CFT073 and negative control MG1655.

Ability of liver *E. coli* isolates to induce sepsis in mouse models, which results in virulence. Every dot stands for a distinct animal. The tested liver *E. coli* isolates are divided from the positive and negative controls by a vertical dashed line. When compared to the negative control, an asterisk (*) indicates the significantly higher values for liver *E. coli* isolates and the positive control that were obtained through an ANOVA and Dunnett's method.

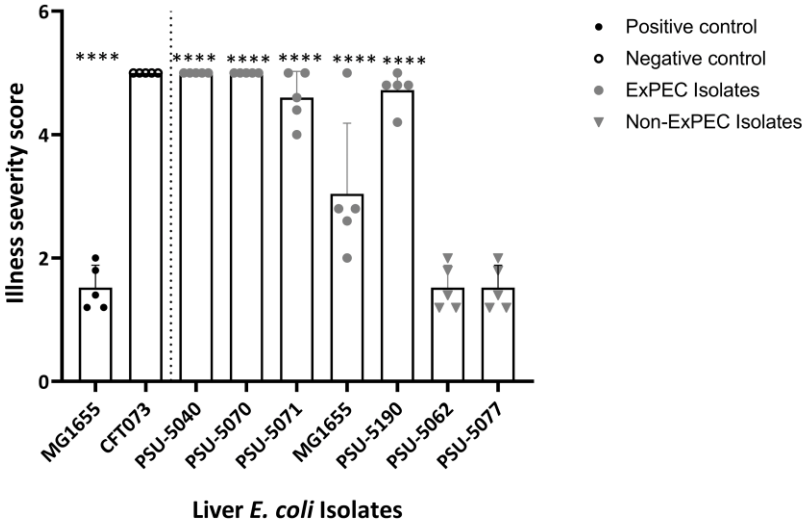

Figure 13: This graph shows the potential of ExPEC isolates to cause sepsis in mouse models.

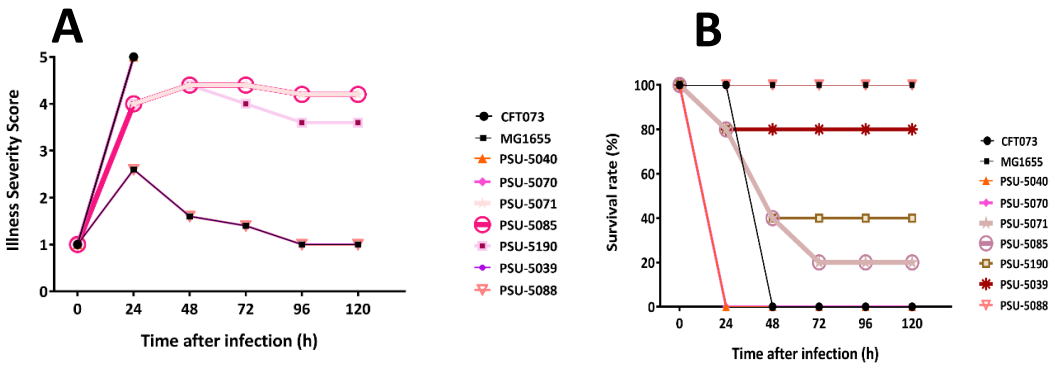

Figure 14: (A) This graph shows the illness severity score of mice (B) this graph shows the survival rate of mice.

4.7 Ability to ExPEC/UPEC isolate to cause UTI in mice

Bacterial loads (cfu/g) in the bladder, kidney, liver, and spleen of the mice in the ascending UTI model were measured 48 hours after the test strains were injected into the bladder through a urinary catheter. Regardless of whether they were ExPEC or not, every *E. coli* isolate demonstrated a notable deviation from the negative control and some level of bladder colonisation in mice. In the bladders of inoculated mice, certain liver ExPEC isolates matched or surpassed the positive control for bacterial counts. Similar to the bladder, every *E. coli* isolate demonstrated some level of colonisation in the kidneys of inoculated mice; however, only the ExPEC isolates exhibited a noteworthy deviation from the negative control. Despite not exhibiting any notable variations, the non-ExPEC isolates were capable of colonising the kidneys of mice. In the kidneys of inoculated mice, some liver ExPEC isolates matched or surpassed the positive control for bacterial counts.

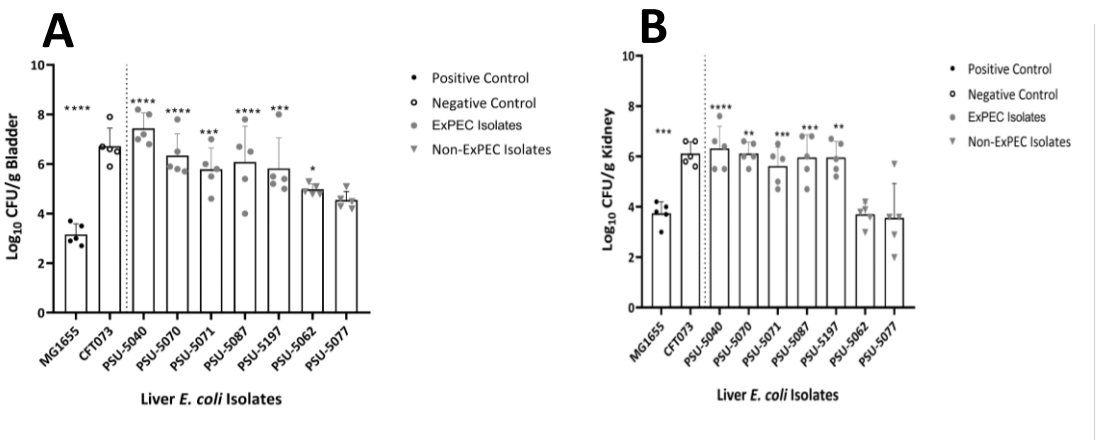

Figure 15: (A) This graph shows the potential of ExPEC to colonize bladder(B) this graph shows the potential of ExPEC to colonize kidney

Unlike colonization in bladders and kidneys of mice, only ExPEC isolates were able to invade liver and spleen of inoculated mice thus showed significant difference from negative control. Non-ExPEC isolates were not able to invade liver and spleen thus didn't show any quantifiable bacterial load. Most of the ExPEC isolates equalled or exceeded the positive control for bacterial counts in livers and spleens of the inoculated mice.

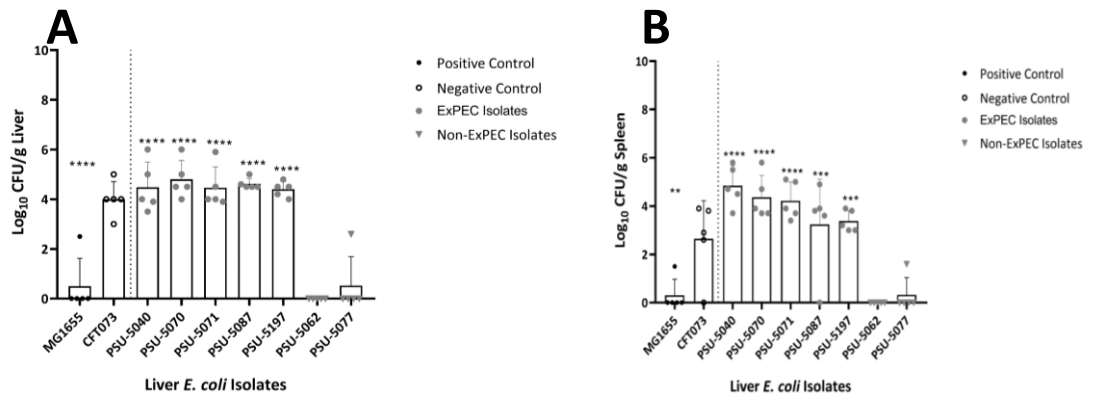

Figure 16: (A) This graph shows the potential of ExPEC to invade liver (B) this graph shows the potential of ExPEC to invade spleen

5. Discussion

The existence and traits of pathogenic *E. coli* colonising various organs in ExPEC infections may be of prime importance for the health of humans and animals and represent a significant advancement in One-Health research. In Pakistan, poultry is one of the most important and growing industries, and the health of poultry is linked with the health of human from so many perspectives, it is important to understand the genetic characteristics linked to the virulence of Avian ExPEC isolates. For this reason, we isolated 139 strains from liver source to assess their zoonotic potential using chick model and mouse model. Hence, we performed genomic analysis and comparison of 139 *E. coli* isolated from infected poultry to study their potential risk to public health, including their potential to cause disease in animal models.

Concerns regarding the zoonotic potential of ExPEC isolates were raised by a number of international research projects. Therefore, we genotypically and phenotypically characterized 139 *E. coli* strains that were isolated from the liver of chickens infected with colibacillosis in this study. In summary, 85 (61%) of the 139 *E. coli* isolates were classified as non-ExPEC isolates, and 54 (39%) of the isolates were ExPEC isolates. Diverse studies have reported varying frequencies of ExPEC isolation; these differences are probably due to differences in isolation protocols, classification schemes, geographic locations, and management approaches.

Numerous investigations have demonstrated that most of the *E. coli* from phylogroups A and B1 are frequently linked to diarrheagenic infections. Conversely, according to Liu et al. (2014), ExPEC infections are linked to ExPEC from phylogroups B2 and D. The majority of the ExPEC isolates in our investigation are from phylogroups B2, D, and B1. When it comes to APEC infections, Phylogroup D is the most significant phylogroup. The most prevalent sequence type among ST-133, ST-151, and ST-69 among ExPEC isolates was ST-131 is associated with bloodstream infections of *E. coli* isolates. It is also associated with ESBL production. ST-155 is associated with the spread of antibiotic resistance. ST-69 is the most common sequence type found in bloodstream isolates of *E. coli* (Goswami *et al.*, 2018) and is also associated with antibiotic resistance mainly

aminoglycosides, fluoroquinolones etc. The most common serotype among ExPEC isolates was O2 and O131. Serotype O2 has genetic similarities with human ExPEC.

The most pathogenic isolate from this study PSU-5040 belonged to phylogroup D and ST-69.

E. coli isolates require various virulent genes to survive in the extra intestinal environment of its host. For this purpose, *E. coli* must acquire certain nutrients like iron to survive. Apart from the acquisition of iron, *E. coli* have to survive the harsh environment of host cells, for this purpose protectins are used to protect the bacteria. *E. coli* must adhere to host cells to cause infections, adhesins help the bacteria for this cause. The majority of the ExPEC isolates' capacity to endure in serum and adhere to or colonise extraintestinal environments is demonstrated by the high frequency of these gene classes in the isolates. Although iss is expressed in both commensal and ExPEC environments, its frequency is significantly higher in the latter, where it confers resistance to complement-mediated killing on pathogenic *Escherichia coli* (Johnson and Wannemuehler, 2008). These ExPEC isolates may be involved in zoonosis due to their high bacteriuria potential and *fimH* prevalence, as the gene they carry codes for an adhesin that mediates binding with α-mannoside, which is present on the epithelial lining of various organs, including the human upper urinary tract. (Vandemaele *et al*., 2003).

The isolates were also assessed for the presence of 5 virulence associated phenotypes including growth in urine, complement resistance, biofilm formation, swimming motility and swarming motility. 78% of the isolates were able to survive in serum and were complement resistant mainly by the high prevalence of various genes like *iss, traT* and *omp* genes. The *iss* gene helps the bacteria to survive in serum by producing phagocytosis protection factor; *omp* gene encodes a protein present in the outer membrane of bacteria that helps the bacteria to evade the host's immune response, hence, ensure the survival of bacteria. And the *traT* gene plays a key role in inhibiting the activity of complement system.

Growth in human urine was evident for 85% of isolates, this predicts the potential of these isolates to cause UTI in humans. 72% of the isolates were able to form biofilms. 85%

were motile in swimming motility media and 78% were motile in swarming motility media, hence ensuring their pathogenicity.

We used these virulence profiles to categorize ExPEC isolates to their sub pathotypes. These sub pathotypes were APEC (45%), UPEC (29.9%), SEPEC (17.%).

The selected isolates were then further tested on chick model for the establishment of pathogenicity index. In this study we found out that ExPEC isolates from liver source, were able to cause colibacillosis in 3-4 days old chicks and possessed several macroscopic lesions like pericarditis, perihepatitis, cellulitis, airsacculitis etc. 83% of the chick inoculated with the ExPEC strains died prior meeting the endpoint criteria due to infection in the form of sepsis or the invasion of several organs. Out of the 8 tested trains, 3 of the strains showed high pathogenicity which is indicative of their zoonotic potential. Test strains PSU-5040 and PSU-5070 exceeded the positive control APEC O1 for their pathogenicity rate.

The study isolates were further investigated for their potential to cause sepsis and UTI in mouse models. In a previous study, APEC isolates from infected poultry were able to cause meningitis and bacteraemia in mouse models (Tivendale *et al.,* 2010).

In this study, we found out that ExPEC/SEPEC isolates from liver of colibacillosis infected chicken were capable of inducing sepsis in mouse models. 84% of the mice inoculated with ExPEC strains died prior meeting the endpoint criteria with infection in the form of sepsis and the most lethal strains PSU-5040 and PSU-5070 showed lethal sepsis along with oedema. These strains caused lethal sepsis in $\leq$ 20h, like human urosepsis strain CFT073. However, the non-ExPEC isolates failed to caused sepsis in mouse models. Sepsis also known as blood poisoning triggers an inflammatory response in the body that results in multi organ failure leading to the death of the organism within few hours post infection.

In mouse models of ascending UTI, the tested ExPEC/UPEC isolates were able to cause urinary tract infection and invade different organs of mice like liver and spleen. ExPEC strains showed quantifiable bacterial loads in mice. Strains like PSU-5040 and PSU-5070 exceeded the positive control uroseptic strain CFT073 for their bacterial load in infected

mice kidney. The non-ExPEC isolates from this study were not able to cause UTI infection in mouse models.

These findings suggest that ExPEC present in infected poultry are a real threat to human health, and food safety because they can cause human UTI and sepsis. Along with that high degree of genetic diversity was observed among the ExPEC strains which is consistent with the fact that ExPEC is a heterogenous group of bacteria.

The findings of this study highlight *E. coli* bacteria from the infected poultry harbor virulence factors and resistance genes that can spread to other bacteria through horizontal gene transfer and eventually be passed on to humans through the food supply chain or direct contact.

6. Conclusion

In conclusion, this study thoroughly assessed the virulence potential of ExPEC strains isolated from liver source. Detailed genomic background of ExPEC isolates was analyzed. There was a high prevalence of genes belonging to adhesins, siderophores and protectins, highlighting the potential of *E. coli* to survive in serum and cause extra-intestinal infections. Various virulence associated phenotypes were analyzed, including the assessment of virulence in chick model and mouse model.

The results were indicative of the fact that some of the Avian ExPEC harbors virulent factors that can cause sepsis and UTI in humans and thus have a zoonotic potential. Most of the ExPEC from liver source was able to cause lethal sepsis and UTI in mice. The majority of ExPEC derived from bovine sources demonstrated high or intermediate pathogenicity index and were capable of inducing lethal sepsis and UTI infection in mice models. A few ExPEC isolates were also successful in inducing colibacillosis in chicken models. These results are significant for food safety because they imply that animals free of ExPEC infections may infect *E. coli* and cause ExPEC-related infections. ExPEC isolates were screened for also screened for AMR genes. Most of the AMRs harboured by ExPEC isolates belonged to tetracycline, and aminoglycoside and macrolide classes.

The most pathogenic strain PSU-5040, belonged to the phylogroup D and ST69, which has previously been reported to be highly pathogenic.

7. Future Prospects

First, formulation of an effective vaccine against Avian Pathogenic *E. coli* can be an effective strategy to combat avian infections that results in huge economic losses. Detailed study of the strains harboring zoonotic potential, for example AMR and stress genotypes because they can be passed to other bacteria through zoonosis. Analysis of plasmids and mobile genetic elements can be carried out to study the role of horizontal gene transfer in the spread of virulence and resistance genes. Zoonotic potential of Diarrheagenic *E. coli* (DEC) strains also needs to be investigated to identify their potential risk to human health. Lastly, gene knockout to study the genes involved in zoonosis.

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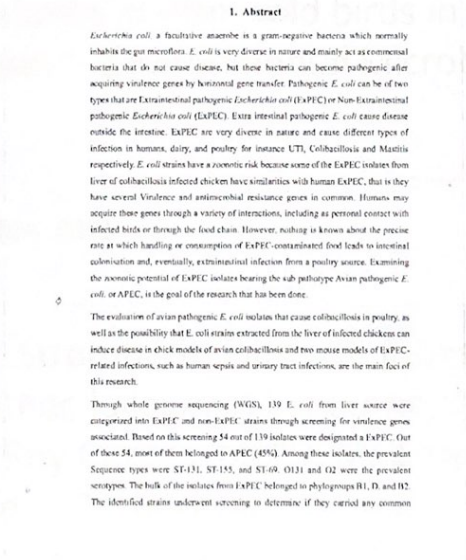

1. Abstract

Escherichia coli, a facultative anaerobe is a gram-negative bacteria which normally inhabits the gut microflora. *E. coli* is very diverse in nature and mainly act as commensal bacteria that do not cause disease, but these bacteria can become pathogenic after acquiring virulence genes by horizontal gene transfer. Pathogenic *E. coli* can be of two types that are Extraintestinal pathogenic *Escherichia coli* (ExPEC) or Non-Extraintestinal pathogenic *Escherichia coli* (ExPEC). Extra intestinal pathogenic *E. coli* cause disease outside the intestine. ExPEC are very diverse in nature and cause different types of infection in humans, dairy, and poultry for instance UTI, Colibacillosis and Mastitis respectively. *E. coli* strains have a zoonotic risk because some of the ExPEC isolates from liver of colibacillosis infected chicken have similarities with human ExPEC, that is they have several Virulence and antimicrobial resistance genes in common. Humans may acquire these genes through a variety of interactions, including as personal contact with infected birds or through the food chain. However, nothing is known about the precise rate at which handling or consumption of ExPEC-contaminated food leads to intestinal colonisation and, eventually, extraintestinal infection from a poultry source. Examining the zoonotic potential of ExPEC isolates bearing the sub pathotype Avian pathogenic *E. coli*, or APEC, is the goal of the research that has been done.

The evaluation of avian pathogenic *E. coli* isolates that cause colibacillosis in poultry, as well as the possibility that E. coli strains extracted from the liver of infected chickens can induce disease in chick models of avian colibacillosis and two mouse models of ExPEC-related infections, such as human sepsis and urinary tract infections, are the main foci of this research.

Through whole genome sequencing (WGS), 139 *E. coli* from liver source were categorized into ExPEC and non-ExPEC strains through screening for virulence genes associated. Based on this screening 54 out of 139 isolates were designated a ExPEC. Out of these 54, most of them belonged to APEC (45%). Among these isolates, the prevalent Sequence types were ST-131, ST-155, and ST-69. O131 and O2 were the prevalent serotypes. The bulk of the isolates from ExPEC belonged to phylogroups B1, D, and B2. The identified strains underwent screening to determine if they carried any common

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