THE CHARACTERISATION AND EPIDEMIOLOGY OF AVIAN PATHOGENIC *ESCHERICHIA COLI* IN UK BROILER CHICKENS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

Kirsty Ann Kemmett

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Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the University's Regulations and Code of Practice for Research. Except where indicated, the work is my own. Work done in collaboration, or with the assistance of others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed: **Kirsty Kemmett** Date: **August 2013**

Abstract

The characterisation and epidemiology of avian pathogenic *Escherichia coli* in UK broiler chickens

Kirsty A. Kemmett

Poultry health and welfare are important for maintaining sustainable and safe food production. In the UK alone, in excess of 900 million broiler chickens are farmed annually with demand continuously increasing. Avian pathogenic *Escherichia coli* (APEC) is the causative agent of the extraintestinal syndromic poultry disease colibacillosis, which has a major impact on poultry health and is a considerable economic burden to the global poultry industry. The need for disease control is of paramount importance. Little is known about the epidemiology, population dynamics and infection biology of APEC in UK broiler chickens. This knowledge would contribute to the implementation of control measures.

This investigation comprised: one longitudinal field study in commercial flocks aimed at simultaneously identifying potential APEC (pAPEC) in the intestinal tract of healthy birds and extraintestinal *E. coli* in diseased birds, one study determining the contribution of *E. coli* to early flock mortalities and a series of *in vitro* experiments and genetic analyses characterising both extraintestinal and avian faecal *E. coli* isolated from UK broiler chickens. *E. coli* were subjected to virulotyping, phylogenetic typing, macro-restriction pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The extraintestinal *E. coli* isolates from diseased birds represent a diverse genetic population. Furthermore, as broiler chickens age, the prevalence of pAPEC in the gastrointestinal tract decreases. The intestinal tract of day-old chicks contains considerable levels of pAPEC (24.05% of the faecal population sampled), while ~70% of early mortalities relate to extraintestinal *E. coli* infections, possibly originating from the gut. *In vitro*, pAPEC did not appear to invade intestinal epithelial cells, suggesting the respiratory route is likely to be favourable for dissemination, although pAPEC are cytotoxic and may disrupt epithelial integrity prior to dissemination. There were no significant differences in the intracellular persistence of APEC and faecal *E. coli* in cultured avian macrophages and survival in serum; challenges encountered by *E. coli* during dissemination. Overall, this investigation highlights the diverse spectrum of *E. coli* associated with extraintestinal disease in commercial broiler production and the need to determine the contribution of host susceptibility to disease manifestation.

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Despite only one name being on the front page of this thesis, it is in fact a collective effort and summarises an experience I will never forget.

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Abbreviations

AFSF	Antibiotic-free serum-free media
APEC	Avian pathogenic *Escherichia coli*
dNTP	Deoxynucleotide triphosphates
EAEC	Enteroaggregative *Escherichia coli*
EMBA	Eosin methylene blue agar
ExPEC	Extraintestinal pathogenic *Escherichia coli*
GIT	Gastrointestinal tract
LB	Luria-Bertani
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MLVA	Multiple-locus VNTR analysis
mPCR	Multiplex polymerase chain reaction
NMEC	Neonatal meningitis *Escherichia coli*
pAPEC	Potential avian pathogenic *Escherichia coli*
PBS	Phosphate buffered solution

PCR Polymerase chain reaction

PFGE Pulsed-field gel electrophoresis

qRT-PCR Quantitative reverse transcriptase polymerase chain reaction

ST Sequence type

STCC Sequence type clonal complex

UPEC Uropathogenic *Escherichia coli*

VNTR Variable number of tandem repeats

Chapter 1

General Introduction

1.1. Introduction

The global population is expected to reach an estimated 9 billion by 2050, increasing the pressure for sustainable and safe food production. In the UK alone, 900 million broiler chickens are reared annually for meat consumption [3]. With poultry (predominately chicken) being the consumers' preferred choice of affordable animal protein, the UK poultry industry is particularly under pressure. Current challenges include minimising production losses resulting from infectious diseases. Farming practices include routine vaccination of broiler flocks against a number of viral pathogens that have proven effective in disease control. Endemic disease caused by an extraintestinal pathogenic *Escherichia coli* (*E. coli*) known as avian pathogenic *E. coli* (APEC) remains a substantial burden and to date little is known about this poorly defined pathogenic group meriting further research.

1.2. The genus *Escherichia coli*

1.2.1 Introduction

E. coli was first identified in 1885 by Theodor Escherich [4]. *E. coli* is one of five members of the genus *Escherichia* and a natural inhabitant of the gastrointestinal tract (GIT) of all vertebrates, generally residing in the mucosal layer [5]. The genus *Escherichia* is in the class of Gamma proteobacteria, order *Enterobacteriales* and family *Enterobacteriaceae*. *E. coli* are one of the best studied bacterial species and often used as model organisms because of their commensal and pathogenic tendencies [6].

1.2.2 Bacteriological characteristics

E. coli is a non-spore forming, Gram negative, rod-shaped facultative anaerobe and while most *E. coli* are flagellated, allowing motility, this is not a uniform feature. Optimal environmental growth conditions are between 37 and 42 ^{0}C with a pH range of 6.0-8.0 [7]. *E. coli* has the ability to adapt to changes in environmental pH in order to maintain cytoplasmic homeostasis allowing survival in environments with extreme pH, such as the upper GIT [8, 9].

E. coli can be readily identified by cultivation on to a differential solid medium such as Eosin Methylene Blue agar (EMBA) because of its ability to ferment lactose. On EMBA typical *E. coli* colonies appear dark violet often encompassed within a metallic green sheen, produced via lactose fermentation and a subsequent drop in pH. *E. coli* can also be identified based on its' biochemical profile including; non-oxidase activity, catalase production, lactose fermentation, inability to utilise citrate as a carbon source and positive indole production. API-20E test strips (BioMerieux, UK) test many of these biochemical properties and are often used by both veterinary and human diagnostic services to identify *Enterobacteriaceae* [10]. *E. coli* confirmation can be readily achieved using a polymerase chain reaction (PCR) targeting the *uidA* gene, a chromosomal gene encoding β-D-glucuronidase (GUD) carried by most *E. coli* [11].

1.3. Commensal and pathogenic *E. coli*

1.3.1 Commensal *E. coli*

E. coli is one of the first species to colonise the GIT of most neonatal warm blooded vertebrates [12]. Colonisation succession is a dynamic process involving both resident *E. coli* (which may colonise the GIT for years) and ingested *E. coli* (which may be transient and only colonise for several days) [13]. Ingested *E. coli* may colonise the GIT providing it can utilise a growth-limiting nutrient better than resident *E. coli* strains or other microbes already present [14]. In poultry, *E. coli* reside predominately in the mucosal layer of the lower GIT (caeca and colon) and are excreted in faeces with degraded mucus and the sloughing of dead epithelial cells. This process allows dissemination into the surrounding environment [15]. The abundance of *E. coli* between host species varies greatly (as much as 6 times) and this is largely influenced by gut architecture, microbiota and diet [16]. The *E. coli*-host relationship is generally described as one of commensalism; *E. coli* obtain a steady and consistent supply of nutrients within a stabilised protected environment and the host remains unharmed [17]. That said, these commensal *E. coli* have been shown to provide protection against intestinal pathogens such as *Salmonella* Typhimurium and enterohaemorrhagic *E. coli* O157 by competing for sites of adherence and nutrients [18, 19]. *E. coli* reportedly stimulate mucus secretion from goblet cells in order to maintain their nutritional supply [20].

1.3.2 Pathogenic *E. coli*

1.3.2.1 Opportunistic pathogens

In a compromised host, resident *E. coli* have been shown to cause disease. Cirrhosis patients often have decreased levels of the mucosal antibody immunoglobulin A (IgA) and sufferers of peritonitis or inflammatory bowel disease have breached and/or weakened intestinal epithelial barriers, both of which have been associated with opportunistic *E. coli* infections [21, 22]. This highlights the importance of the integrity of the mucosal immune system and epithelia in preventing disease.

1.3.2.2 Extraintestinal pathogenic E. coli *(ExPEC)*

Pathogenic *E. coli* are broadly categorised into diarrhoeal and extraintestinal pathogenic *E. coli* (ExPEC) [23, 24]. The most common human ExPEC pathogens are neonatal meningitis *E. coli* (NMEC) and uropathogenic *E. coli* (UPEC) [25]. The focus of this thesis is on the avian ExPEC pathovar; avian pathogenic *E. coli* (APEC).

Pathogenic *E. coli* evolve following the acquisition of virulence-associated genes (VAGs), which allow them to compete with the resident microbiota or colonise new niches such as systemic sites [23]. The *E. coli* genome has a high degree of plasticity mainly from recombination events in genome 'hotspots' yet they appear to retain a level of clonality allowing the identification of particular strains or related groups of pathogenic *E. coli* [6, 26-28].

1.4. Colibacillosis

1.4.1 Introduction to colibacillosis

APEC related disease has multiple manifestations, collectively referred to as colibacillosis. Colibacillosis poses a substantial economic burden on the global poultry industry [29]. Acute outbreaks of colibacillosis have been reported since the mid-1990s [30]. Potential APEC reservoirs are likely to reside in the avian GIT [31].

1.4.2 APEC as primary or secondary pathogens

In the first instance, colibacillosis was thought to be the result of a secondary or opportunistic APEC infection in an infected or stressed host. Examples of physiological stress in poultry production include the hierarchical social stresses within free-range layer hens and the onset of lay [32, 33].

In chickens, primary infection with turkey rhinotracheitis virus (TRTV) (also known as avian metapneumovirus), infectious bronchitis virus (IBV), infectious bursal disease and Newcastle disease virus have all been shown to predispose birds to extraintestinal *E. coli* infections [34-36]. Early *in vivo* studies aimed at determining the infection biology of APEC often used prior oculonasal administration of a viral pathogen [32, 37, 38]. Primary viral infections and colibacillosis have been well studied; a combination of respiratory distress, tissue damage, impaired ciliary action and altered immune responses are likely to increase the likelihood of colibacillosis [39-42]. Co-infection studies have also been used to assess both APEC pathogenesis and potential APEC vaccine candidates [43, 44].

Although prior viral infection has been shown to enhance the severity of colibacillosis, APEC is now recognised as a primary pathogen not requiring a concurrent viral infection and this has been confirmed in a number of *in vivo* APEC infection studies [45-47].

1.4.3 Colibacillosis in broiler chickens

Colibacillosis in broiler chickens typically manifests as a systemic inflammatory infection with fibrinous-based lesions around visceral organs. Airsacculitis, cellulitis, pericarditis, perihepatitis, respiratory distress, splenomegaly and swollen head syndrome are among the most commonly associated signs of colibacillosis in broilers in the sub-acute form [48]. The acute, rapidly progressing, form of colibacillosis is associated with septicaemia and death is also possible [49].

Yolk sac infections and omphilitis are the most common lesions seen among broiler chicks (up to around 7 days of age) and APEC vertical transmission has been hypothesised [50, 51]. Yolk sac infections pose a great risk of septicaemia and ultimately premature death.

1.4.4 Colibacillosis in other avian species

In layer hens, colibacillosis is often associated with infections of the reproductive tract - peritonitis, salpingitis and polyserositis [30, 52]. Such disease manifestations are associated with a reduction in egg production and increased mortality.

Colibacillosis affects most avian species including turkeys, ducks and ostriches, among others. In turkeys, more commonly fast growing adolescent males with poor cell mediated immunity, turkey osteomyelitis complex (TOC), is a manifestation of

APEC extraintestinal disease [32]. TOC is associated with soft tissue abscesses, green discoloured liver, osteomyelitis of the tibia and arthritis [53].

1.5. The UK poultry industry

1.5.1 Introduction to the UK poultry industry

The UK Department for Environment, Food and Rural Affairs (DEFRA) reported that in 2012 there were over 900 million broiler chickens, 17 million turkeys and 100,000 geese farmed for meat in the UK [3]. This equates to a 3.2% increase in broiler production from the previous year with total broiler chicken production reaching 1.6 million tonnes in 2012. Furthermore, breast meat importations are vital in satisfying consumer demand.

Egg production quarterly figures for 2012 show 5 million packs of eggs in England and Wales were produced [54].

1.5.2 The APEC burden on broiler chicken production

The UK broiler industry estimates an annual loss of ~8%, equating to ~50 million chickens in the UK alone, from APEC related disease (T.J. Humphrey, personal communication, August 2012). Losses are the result of the rejection of carcasses at slaughter and heightened mortality rates. Chicken remains one of the consumers' most popular choices of cheap meat with the poultry industry escaping much of the recent economic crisis relatively lightly.

1.6. Introduction to the broiler chicken (*Gallus gallus domesticus*)

1.6.1 Broiler growth performance and commercial production

In an attempt to meet demand, standard broiler chickens reach slaughter weight of approximately 2.2kg in 35-39 days, 60% faster than they did 20 years previously [55]. Under strict regulations, stocking densities at end-of-life are not to exceed 42kg/m^2, although most are reared at 38kg/m^2. Such intense farming is often the focus of animal activist campaigns, although it is difficult to imagine how the industry could meet demand otherwise. As a result of selective breeding and the consequent rapidly changing host, the modern broiler chicken is unlike other commercial avian species.

1.6.2 Broiler genotypes

Standard commercial broiler chickens which reach slaughter weight between 35 and 39 days represent approximately 90% of UK production. The most common genotype in the UK for intensive indoor production is the Ross 308. Genotypic variations of the Ross bird are available including the Ross 708, which reaches slaughter weight a day or two later. The Ross 308 has been selectively bred for its uniformity and high feed conversion efficiency. Cobb-500 birds are an alternative genotype used in standard production.

The Hubbard JA757 and JA787 make up the remaining ~10% of commercial broilers. The Hubbard genotypes are often reared under higher welfare conditions with lower stocking densities (30kg/m^2) or in free-range systems, which allow access to the outdoor environment for at least half of the flock cycle.

This investigation focuses on the standard Ross 308 genotype, because it accounts for the majority of the UK market.

1.6.3 Broiler susceptibility to endemic disease

The selective breeding of commercial broiler chickens for traits attractive for industrial production such as rapid growth and increased feed conversion efficiency, may have impacted on the adaptive humoral immune system and increased the likelihood of detrimental pro-inflammatory responses [56]. Past work comparing the contribution of APEC-related mortalities between different broiler genotypes suggests rapid growth may reduce broiler 'viability' and increase disease susceptibility [56, 57]. The study highlighted infection-based mortalities in standard broilers were more than double that seen in slower growing breeds [57]. The production of broiler genotypes more resistant to endemic disease is an attractive candidate for reducing mortalities; a difficult concept for APEC control as the pathogenesis remains unclear.

1.7. APEC epidemiology and *E. coli* population genetics

E. coli is one of the best studied bacterial species to date and an array of methods have been developed to identify them genetically. Some of the most common methods are described below. Each of the methods described has advantages and disadvantages relating to genetic resolution, cost, time, labour, portability and the reproducibility of results.

1.7.1 Phylogenetic typing

1.7.1.1 Introduction to the four E. coli *phylogenetic groups*

E. coli can be divided broadly into four main phylogenetic groups (A, B1, B2 and D) with numerous sub-phylogenetic groups [58-60]. Research suggests that phylogroup B2 represents the *E. coli* 'ancestral' lineage as this group exhibits the highest diversity at nucleotide and gene level, suggesting early emergence, evolution and diversification [28, 61]. Phylogroup D was the first lineage to branch from B2, while sister groups A and B1 emerged later [28]. Wirth *et al.* (2006) suggested the 4 main phylogroups reflect 4 ancestral sources prior to the bottle necking of the *E. coli* population 10-30 million years ago [62].

Generally speaking, sister groups A and B1 represent environmental, resident non-pathogenic *E. coli*, whereas groups B2 and, to a lesser extent, D are associated with pathogenic strains [63, 64]. Additional differences in phenotypic traits including antibiotic-resistance, sugar metabolism, growth rate and genome size (groups B2 and D tend to have larger genomes) have been observed between the groups [63].

1.7.1.2 Determining the phylogenetic group

The *E. coli* phylogenetic group can be determined using several techniques, which differ in both cost and labour. The mid-1980s saw the development of multi-locus enzyme electrophoresis (MLEE) and ribotyping. MLEE involves the analysis of relative electrophoretic mobility of 38 water-soluble housekeeping enzymes. Variants in mobility correspond to different alleles (termed electrophoretic type) and relatedness can be mapped using dendrograms [65]. MLEE is both labour intensive and time consuming.

In 2000, Clermont *et al.* published a simple triplex polymerase chain reaction (PCR), which has now been used in over 150 different studies [1, 66]. Clermont's PCR targets 2 genes*; i) chuA*, a gene encoding an outer-membrane hemin receptor *ii) yjaA,* identified following the complete genome sequencing of *E. coli* K-12 and now known to encode part of a lipase esterase gene [67-69]. The third target of Clermont's triplex PCR is a DNA fragment referred to as TSPE4.C2 [68]. Table 1.1 summarises the dichotomous approach used to determine the phylogenetic group, this is based on the combinations of the three DNA targets successfully detected by PCR.

Clermont *et al*. (2000) initially reported an accuracy of 99%, although in a more recent study Gordon *et al.* (2008) suggested that the accuracy was no more than 80-85%. While the exact accuracy was dependent on the phylogroup, highest rates were seen among the B1 and B2 isolates [66].

Originally, isolates negative for all three targets, were grouped into phylogenetic group A by default. However, later work suggested only 17% of said isolates was truly phylogroup A when determined by other methods and despite this group of isolates being non-random, it does not form a monophyletic group [66].

Table 1. 1 The Dichotomous approach to *E. coli* phylogenetic typing

Group	Triplex PCR result
Unassigned	No genes
A	*yjaA*$^+$ only
B1	*TSPE4.C2*$^+$ only
B2	*chuA*$^+$*yjaA*$^+$; *chuA*$^+$*yjaA*$^+$*TSPE4.C2*$^+$
D	*chuA*$^+$; *chuA*$^+$*TSPE4.C2*$^+$

E. coli *may be assigned to one of four major phylogenetic groups based on the presence or absence of 2 genes (chuA, yjaA) and one DNA fragment (TSPE4.C2) [1].*

1.7.1.3 APEC phylogenetic groups

Phylogroups B2 and D are more associated with extraintestinal disease than groups A and B1 [70-72]. Past studies report >80% representation by phylogroups B2 and D [73-75]. Not all studies concur, Rodriguez-siek *et al.* (2005) reported 38% of APEC

(n=524) were in fact phylogroup A isolates and an Italian study conducted by Giufre *et al.* (2012) found most APEC belonged to group A with B2 and D phylogroups representing 18 and 28% of the population respectively [76, 77]. The well characterised APEC O78 (chi7122) isolate is also of B1 origin [78, 79]. The phylogenetic typing of APEC is poorly discriminative and often used in conjunction with other typing methods. Defining APEC by phylogroup may help indicate the nature and origin of *E. coli*, but should not be used conclusively.

1.7.2 Serotyping

1.7.2.1 Introduction to serotyping

Serotyping was first described by Kauffmann *et al.* in the 1940s [80]. This method of typing is based on the allelic combinations of 3 surface antigens of which there are currently, 173 O (somatic lipopolysaccharide), 80 K (capsular) and 56 H (flagellar) antigens. The different O, K and H antigens are associated with strains of the same lineage and are not geographically restricted [6, 81]. Most antigenic combinations have been identified in nature resulting in an extremely high number of *E. coli* serotypes (50,000 – 100,000), thus as with phylogenetic typing serotyping is often used in conjunction with other typing methods.

1.7.2.2 Serotyping methodology

A number of techniques exist for serotyping *E. coli* including the standard slide agglutination test, the use of K-phages and PCR based assays.

Antigens are specific moieties recognised by antibodies and this concept forms the basis of the standard slide agglutination test. Bacterial cell suspensions are exposed

to antisera containing specific antibodies against the different O, H and K antigens. Antibody recognition of specific antigens result in the agglutination and aggregation of bacterial cells [81]. This agglutination protocol provides a rapid result and commercial antisera against common serogroups are available. The disadvantage of this method is that each different antiserum is tested individually. Prior isolate knowledge relating to host species and disease status may help narrow down the expansive antisera to test against [81].

Bacteriophages have evolved to recognise specific polysaccharide capsules coating bacterial cells including those capsules consisting of the K antigen (polysialic acid) [82]. This protocol takes approximately 24 hours and involves the co-incubation of a K antigen-specific bacteriophage and the test isolate on a solid trypticase-soy agar; positive detection yields visually lysed *E. coli* [83]. The main disadvantage of phage assays is the limited number of commercially available phages and the reproducibility of results between laboratories.

Finally, PCR primers targeting different O, H and K antigens have been published and are widely used as a rapid method for determining the presence of certain serotypes associated with disease including K1 (*kpsMT K1* or *neuC*), K54 (*kpsMT KIII*) and H7 (*fliC*) [72, 77]. In 2010, Wang *et al.* (2010) published a PCR designed to detect certain O-serogroups associated with human diarrhoeal disease [84]. PCR based approaches are rapid, cost effective and accurate and possibly the most common test used today in serotyping.

1.7.2.3 APEC serotyping

As with phylogenetic typing, certain serotypes have been associated with pathogenic strains of *E. coli*; these can be subdivided into those which cause diarrhoeal disease

(for example *E. coli* O157:H7 which causes watery diarrhoea and haemolytic anaemia) and those that cause extraintestinal disease [81].

Predominant APEC serogroups include O1, O2 and O78, while O8 and O35 have also been identified [29, 32]. On average, these O-serotypes represent no more than 50% of APEC tested [77, 83, 85].

The K1 capsule is poorly immunogenic because of its similarity to polysialic acid moieties on host cell surface glycoproteins and has previously been associated with increased serum survival via complement inactivation, such traits allow K1 positive *E. coli* to avoid detection by the host immune system and promote systemic survival [86, 87]. The K1 capsule does not seem to be essential for APEC pathogenesis, with as few as 2% of isolates testing positive for the capsule [83, 88]. Finally, H7 antigen has been associated with ExPEC, although, again, the prevalence varies greatly [77]. APEC O1:K1:H7, O2:K1:H5 and O78:H9 serogroups are well characterised strains thought to be representative of APEC and thus have been used as APEC prototypes [31, 78, 79, 89].

These studies also report a significant number of untypable APEC (usually ~30%) and expansive, diverse arrays of other serotypes representing the remaining APEC. Serotyping may not be the most effective typing scheme for APEC. Serogroups O1, O2, O78, and more specifically O1:K1:H7, have been identified among avian faecal *E. coli* populations, supporting the hypothesis that the gut is a potential APEC reservoir [31, 90].

1.7.3 Multi-locus sequence typing (MLST)

1.7.3.1 Introduction to MLST

Multilocus sequence typing (MLST) was first described by Maiden *et al.* in 1998 [91]. Maiden described a lack of available molecular techniques suitable for detecting slowly accumulating genetic change, despite numerous schemes available for detecting rapid genetic change (ribotyping and pulsed field gel electrophoresis) available at the time. High-resolution methods are ideal during disease outbreaks, but can be misleading when studying microbial evolution. On the other hand, phylogenetic typing is very broad and offers little discrimination. In other words, this can be described as local versus global molecular epidemiology, with global epidemiology requiring lower resolution. MLST analysis aims to provide a balance between these extremes.

MLST is a DNA sequencing-based technique involving the sequencing of ~470 nucleotides of 5-10 housekeeping genes (genes essential for normal cell function and under neutral selection). Mutations in single housekeeping genes are slow to accumulate as many genetic mutations are lethal and would result in detrimental non-functional proteins and therefore are not maintained in bacterial populations. The analysis of multiple housekeeping loci increases genetic discrimination and such sequencing data allow for the analysis of linkage disequilibrium between alleles. The determined allelic combinations give rise to sequence types (ST) and STs can then be clustered into sequence type clonal complexes (STCC) based on their level of genetic relatedness. Sequence data can be uploaded to species-specific online databases producing an expanding portable database, which can be exchanged between laboratories. This is the first real molecular technique that is truly portable and

reproducible [92]. A disadvantage of MLST over the previously described molecular typing techniques, serotyping and phylogenetic typing, is the need for DNA sequencing. High throughput MLST protocols have been described with the aim of making it more cost effective, although it is likely that techniques requiring sequencing will become less costly as whole genome sequencing (WGS) becomes more popular [93].

1.7.3.2 E. coli *MLST methodology*

A number of *E. coli* MLST schemes have been published. In 2003, Adiri *et al.* published a protocol based on the amplification of 6 housekeeping genes: *adk* (adenylate kinase), *gcl* (glycoxylate carboligase), *gdh* (glucose-6-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *metA* (homoserine transsuccinylase) and *ppk* (polyphosphate kinase) [94]. This 6 loci gene set was used by Moulin-Schouleur *et al.* in a 2007 study focusing on possible zoonotic risks of APEC by comparing human ExPEC and APEC [75].

An alternative *E. coli* MLST protocol was published in 2006 by Wirth *et al.* and is the more popular scheme [62]. This 7 gene MLST scheme targets *adk*, *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase) and *recA* (ATP/GTP binding motif) and the protocol is publically available online (http://mlst.ucc.i.e./mlst/dbs/Ecoli).

In brief, housekeeping gene targets are amplified by PCR and sequenced using an automated sequencer and the nucleotide sequences are analysed using computer software such as Ridom SeqSphere (Ridom GmbH, Wűrzburg, Germany) or ChromasPro version 1.5 (Technelysium, Australia) and MEGA 5.05 [95]. Analysed

sequences are submitted to the Achtman *E. coli* online database (http://mlst.ucc.ie/mlst/dbs/Ecoli) where ST and STCC are assigned accordingly.

1.7.3.3 APEC MLST sequence types

MLST has proven to be a popular global molecular tool for pathogenic *E. coli* analysis and is potentially more suited to the study of pathogens, which tend to acquire mutations and undergo recombination at a faster rate than other bacteria [62]. Numerous STs have been associated with avian *E. coli* including: ST-23, ST-88, ST-95, ST-162, ST-347, ST-358, ST-369 and ST-1353 [96]. To date, the most common APEC STCCs include STCC-23, STCC-95 and STCC-117, which have been shown to represent around 50% of isolates while they are not geographically restricted and have also been associated with antibiotic resistance [76, 89, 96, 97]. The previously mentioned prototype APEC strain O2:K1:H5 is a ST-140 isolate and found within the STCC-95 [31]. MLST of *E. coli* O78 clones from different hosts demonstrated a highly related clonal structure in *E. coli* populations and links between virulence and ST [94].

MLST has been an important tool in the comparison of human ExPEC (mainly UPEC) and APEC isolates and the potential zoonotic risk of avian *E. coli* [76, 89]. ST-117 and strains within the STCC-117 have been described as potentially zoonotic and have been associated with chicken at retail [98, 99]. ST-117 also represented ~17% of Japanese *E. coli* O78 [96].

As with all the molecular techniques mentioned so far, caution is needed. No single ST solely associated with pathogenic *E. coli* or APEC has been identified. The O1:K1:H7 avian faecal *E. coli* previously mentioned is an ST-95 isolate, thus clustering with APEC prototypes. Ewers *et al.* (2009) utilised MLST when

comparing avian faecal and systemic *E. coli* and highlighted the potential intestinal APEC reservoir, but this also highlights the lack of complete discrimination of pathogenic and supposed commensal *E. coli* and further adds to the problem in deciphering the molecular background of APEC [31].

1.7.4 Macro-restriction analysis of genomic DNA and pulsed field gel electrophoresis

1.7.4.1 Introduction to pulsed-field gel electrophoresis

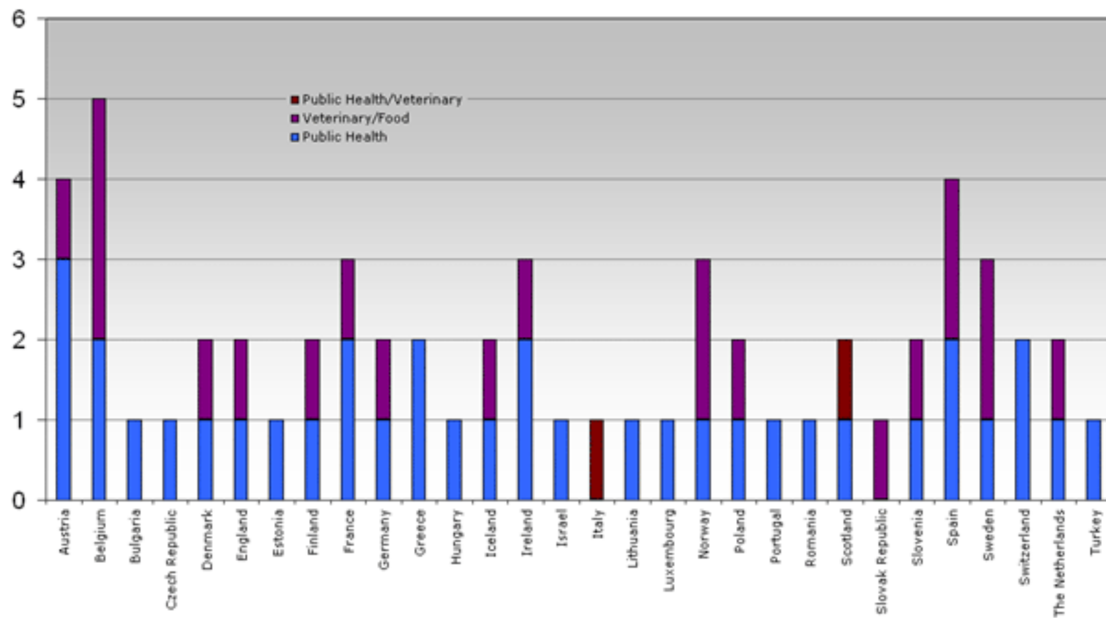
Macro-restriction analysis of genomic DNA using pulsed-field gel electrophoresis (PFGE) involves the cleavage of DNA by restriction endonucleases targeting short specific nucleotide sequences yielding high molecular weight DNA fragments (fingerprints). These fragments are separated using gel electrophoresis by changing the direction of the current through the gel and the observed banding pattern is referred to as the 'pulsotype'. PFGE analysis allows for the detection of microvariation in genomic sequences between strains. PFGE protocols are available for a wide range of bacterial species including those which cause food-borne illness; *E. coli*, *Salmonella*, *Listeria*, *Shigella* and *Campylobacter* [100-103].

PFGE analysis has been available for over 20 years and has been proven to be a valuable tool during epidemics, a good example of this is a large multistate *E. coli* O157:H7 outbreak in 1993, which was later traced back to contaminated beef [104, 105]. A major challenge incurred when using PFGE is the reproducibility of results within and between laboratories. In 2006, Ribot *et al.* published a standardised PFGE protocol which carefully evaluated existing PFGE methodology, highlighting steps

responsible for lab to lab result variation and shortened protocols from 3-4 days to 1-2 days – a time lapse more appropriate during disease outbreaks [103].

This standardised, more rapid, protocol became the 'gold standard' PulseNet reference PFGE protocol for a United States and European national molecular subtyping network for foodborne disease surveillance established and controlled by the United States Centers for Disease Control (CDC) and Prevention and European CDC respectively (see Figure 1.1) [103, 106]. PFGE allows discrimination of strains circulating within the same geographical location, which MLST would fail to achieve [107].

Figure 1. 1 Countries and institutes involved in PulseNet Europe (Pezzoli *et al* (2008))



Bar chart representing the European countries involved in PulseNet European food-borne pathogen surveillance. Chart taken from Pezzoli *et al* (2008)[2].

Such high specificity is perhaps a disadvantage during long-term epidemiological studies. Bacteria mutate and/or acquire and/or lose genetic information, thus, over

time, highly related strains could produce very different pulsotypes. Additionally, bacterial pathogens tend to change at a faster rate than other bacteria. Other disadvantages of PFGE include *i)* it is labour intensive ii) a limited number of isolates can be tested at one time *iii)* time-consuming, requiring at least 24 hours (although MLST is likely to require sending amplified DNA away for sequencing therefore likely to take longer) *iv)* DNA may be subject to endogenous nucleases altering pulsotype analysis *v)* banding patterns can be subject to variation based on individual interpretation even with the use of software packages such as Bionumerics V 4.0 software [108].

1.7.4.2 E. coli *PFGE methodology*

The revised and standardised protocol published by Ribot *et al.* (2006) describes a 2-day process with one day being highly labour intensive. In brief, bacterial cell suspensions with comparable optical densities are incorporated into agarose plugs, which are subsequently washed and digested with an *XbaI* restriction endonuclease (Roche products Ltd, UK) for ~2hrs. *XbaI* (originally isolated from *Xanthomonas badrii*) specifically nicks (∨) the nucleotide motif 5'...T∨CTAGA...3' (reverse strand 3'...AGATC∧T...5'). The lysis to restriction steps should take no longer than 8-10 hours, a great improvement on original protocols which required overnight lysis [109]. Nicked DNA is separated using gel electrophoresis and pulsotypes are visualised and compared.

1.7.4.3 PFGE and APEC characterisation

Timothy *et al.* (2008) demonstrated the usefulness of PFGE analysis in APEC molecular epidemiology following an outbreak of a reproductive tract infection, salpingitis and peritonitis in a layer breeder flock originally reported by Jordan *et al.*

(2005) [52, 110]. Thirty-five of 45 isolates analysed using PFGE had the same pulsotype and were recognised as a single clonal group. Furthermore, an environmental isolate also produced the same pulsotype, perhaps suggesting a source of infection or dissemination into the environment. PFGE has frequently been used for the evaluation of genetic relatedness between ExPEC, APEC and suggested commensal *E. coli*, with studies most frequently using the *XbaI* restriction enzyme [85, 97, 111-113]. The combination of PFGE and MLST may be useful in future APEC epidemiological studies.

1.7.5 Multiple-locus variable number of tandem repeat analysis

Multiple-locus variable number of tandem repeat (MLVA) analysis utilises the naturally occurring polymorphisms found in the genomes of microorganisms. Variations in the number of tandem repeated DNA sequences found at multiple loci in the genomes are compared using MLVA. These loci are often subject to rapid evolutionary change. Multiple loci are amplified by PCR and the number of repeats at each loci can then be determined using either conventional electrophoresis or by using a genetic analyser and fluorescent labels. [114-116]. The generated profiles are uploaded to online reference databases and used to compare profiles.

In European union (EU) countries the only major prokaryote with a wholly accepted standardised MLVA protocol is *Salmonella* Typhimurium, which is used by surveillance centres to monitor potential outbreaks, similar to PulseNet for PFGE based surveillance [117, 118]. For the past 10 years, scientists in France have been trying to establish standardised national surveillance protocols for a range of pathogens including those relating to food-borne disease such as *Listeria*.

As discussed, PFGE is highly discriminative and thus has proven successful during disease outbreaks. However, there are a number of advantages of MLVA over PFGE and MLST. Like MLST, results obtained from MLVA are objective thus less prone to subjective variation like PFGE. Secondly, the data generated from MLVA are highly amenable and can be rapidly uploaded to online databases and shared between laboratories. Some studies have shown MLVA to be even more discriminative than PFGE, easier to perform with fewer decreasing false-positive results [119].

Noller *et al.* (2003) first described an MLVA protocol for *E. coli* [120]. Advantages of using MLVA for the study of *E. coli* molecular epidemiology include *i)* less labour intensive than PFGE *ii)* more rapid *iii)* highly discriminative. Currently there are no published reports of using MLVA in APEC characterisation and epidemiology.

1.7.6 Whole genome sequencing (WGS)

WGS has the potential to allow researchers to decipher APEC molecular pathogenesis by identifying VAGs that have previously gone undetected and assess the phylogenetic relatedness of strains using a larger genome scale. The more publically available whole genomes available there are, the more likely researchers are to further our understanding of APEC. To date there are 4 APEC genome sequences publically available; APEC O1:K1:H7 isolated from a turkey, APEC SCI-07 (untypable O antigen) isolated from a layer hen in Brazil, APEC O78 (chi7122) isolated from an infected turkey and another APEC O78 [78, 89, 121, 122]. The first of these genomes to be made available was APEC ST-95 O1:K1:H7 (APEC O1), which was chosen for sequencing, as it appeared to represent other APEC strains based on the authors' previous work showing its similarities in VAG carriage and

genetic typing to other APEC strains [89]. APEC O1 was originally isolated from the lung of an infected chicken [89]. The same research group also published the most recently available APEC O78 genome which consists of 1 chromosome and 2 plasmids [121]. The full genome sequencing of APEC O78 (chi7122) (and APEC IMT2125) was used to decipher the evolutionary genetic lineages of APEC [78]. Results from this study suggest APEC strains are likely to originate from multiple different lineages and those of ST-23 (chi7122 and IMT2125) appear distinct from APEC O1. To date, there are no broiler chicken associated APEC genomes available.

1.8. Challenges faced during APEC characterisation

The APEC pathotype remains poorly defined; no single molecular typing technique is 100% discriminative for APEC and non-APEC isolates. Multiple molecular typing methods are used in conjunction, perhaps allowing strengthened discrimination of isolates. Our understanding of the APEC evolutionary background remains incomplete. Correlation between PFGE pulsotype clusters and specific serogroups were reported by Ewers *et al.* (2004) [85]. Furthermore, serogroups O1, O2 and O78 mostly cluster into MLST ST-23 and ST-95 [123, 124]. On the other hand, these same studies report a diverse APEC phylogenetic background.

As discussed, WGS comparisons suggest that APEC have evolved from *E. coli* of multiple genetic lineages through the acquisition of distinct VAGs [78]. Furthermore, distinguishing strains by their disease manifestations (i.e. extraintestinal or diarrheagenic) is not wholly supported by genetic analysis. A similar scenario has recently been described for EAEC and UPEC capable of causing urosepsis [125, 126].

1.9. APEC pathogenesis

1.9.1 APEC dissemination

The major APEC reservoir is thought to be the avian GIT [31]. The route of extraintestinal spread has not been fully elucidated, although the oral and upper respiratory routes appear to be the most important [43, 127]. APEC has been reported to persist on poultry house dust particles at levels exceeding 10^6 colony forming units per gram of dust and inhalation of contaminated particles is thought to be an important route of infection [128]. Using the chicken as a model for natural infection researchers have shown that administration of 10^6 and 10^9 CFU into the respiratory tract produces local respiratory and systemic infections respectively [127].

Oral infection and subsequent intestinal transepithelial migration has also been proposed as methods of extraintestinal dissemination [129, 130]. Furthermore, infections of superficial and subcutaneous abrasions have been suggested as alternative mechanisms for dissemination [131, 132].

Vertical transmission may play a role in broiler chicken infection [51, 52]. Olsen *et al* (2012) reported that bacterial infections, primarily *E. coli*, accounted for ~50% of layer flock mortalities during the first week [133]. Omphalitis and/or yolk sac infections, with or without septicaemia, were reported. Such infections may originate from infected breeder hens with salpingitis where the yolk sac becomes infected *in ovo,* or through the hatchery environment [30, 134]. Additionally, Petersen *et al* (2006) demonstrated potential vertical transmission of fluoroquinolone resistant *E. coli* [135]. Vertical transmission is supported by studies demonstrating the contribution of extraintestinal *E. coli* infections in early flock mortality [133].

1.9.2 Introduction to APEC virulence-associated genes (VAGs)

Some *E. coli* pathotypes are associated with certain factors associated with virulence, which aid their identification and characterisation. An example of this is enterohaemorrhagic *E. coli* (EHEC) (or shiga-toxin producing *E. coli*), which carry *stx1* and *stx2* genes encoding shiga-like toxins [136]. EHEC are associated with diarrheal disease, haemolytic colitis and haemolytic uraemic syndrome [137]. Enteropathogenic *E. coli* (EPEC) utilise bundle forming pili encoded by EPEC adherence factor plasmids [138].

Over the past decade, deciphering APEC infection biology has been a major research target; this would ultimately allow better identification of APEC and the opportunity for disease control and intervention.

To date, scientists have failed to identify a single or group of VAGs, which can be found in all APEC isolates. Based on the literature below there are various modes of host-pathogen interactions.

1.9.3 Methods of identifying virulence-associated genes: Comparative genomics

Comparative genomic and mutational studies can be used to evaluate the role of suspected VAGs in APEC pathogenesis. Systemic *E. coli* from diseased birds have previously been compared to those from apparently healthy birds (often isolated from the intestinal microbiota) or to lab-attenuated strains. Genomic suppression subtractive hybridisation analysis has proven a valuable tool in allowing such comparisons to take place [139]. Suppression subtractive hybridisation has helped identify an array of potential APEC virulence genes involved in adhesion, invasion,

iron metabolism and plasmid-encoded genes in APEC strains, yet absent in non-pathogenic strains such as *E. coli* K-12 MG1655 [85, 134, 140-142].

Selective capture of transcribed sequences (SCOTS) allows for the identification of genes expressed by isolates taken from avian infected tissues [143]. SCOTS was used by Dozois *et al.* (2003) to identify essential APEC genes required during infection [144]. An advantage of SCOTS is that it does not require expansive knowledge of the genome in question.

Signature tagged transposon mutagenesis (STM) allows for large scale screening of mutant libraries to identify genes by function involved in pathogenesis [145]. Li *et al.* (2005) used STM to identify VAGs important in an APEC *in vivo* infection model which used an O2:H5 APEC (IMT5155); a series of extracellular polysaccharides and lipopolysaccharides were identified using this technique [47]. STM is perhaps one of the most powerful tools, as it allows for large-scale screening and the identification of unknown genes. Furthermore, mutants are directly linked with attenuation. A disadvantage of STM is the possibility of overlooking moderate attenuation of mutants, although this would be dependent on the sensitivity of the protocol. For example, toxins may not be required for survival *in vivo* but may be required for pathogenesis [146].

1.9.4 Adhesion

ExPEC are known to colonise the avian intestinal tract asymptomatically, accounting for as much as 20% of the *E. coli* population [24, 147]. The gastrointestinal *E. coli* population is a known reservoir of APEC strains [31]. APEC causes disease at various systemic sites, including the respiratory tract, liver, heart and reproductive

tract [48]. Colonisation is the first step in APEC pathogenesis and the bacteria are not thought to be particularly invasive pathogens[148].

Type 1 fimbriae have been shown to play a role in adhesion to a number of different sites including the intestinal mucus layer, enterocytes, the tracheal epithelia and lung tissue [149-151]. Type 1 fimbriae are encoded by the *fim* operon, a 9 gene cluster within the *E. coli* genome. Encoded within the *fim* operon is the fimH protein, which mediates mannose-sensitive binding while recombinases allow phase variation in fimbriae expression. Type 1 fimbriae have also been shown to contribute to UPEC pathogenesis [152].

E. coli are not thought to be particularly invasive [148]. Studies suggest translocation of the intestinal epithelium by APEC provides an alternative route for dissemination, but this only occurs when birds are predisposed to stress [129, 130]. A number of studies have identified ExPEC factors associated with epithelial invasion including Outer membrane protein A (OmpA), invasion barrier epithelia proteins (IbeA, B and C), fimbriae and temperature sensitive haemagglutinin (Tsh), but the exact mechanisms in many cases remain unknown [131, 149, 153-155].

Ibe proteins were first described in NMEC pathogenesis for their role in invasion of the blood-brain barrier [156, 157]. Johnson *et al.* (2001) estimated 33-44% of NMEC carry *ibe* genes [158]. Germon *et al.* (2005) reported 24% of APEC carry *ibeA*, compared to 0% of non-APEC strains tested, despite a negative correlation with O78 strains [159]. The exact role of Ibe proteins is not clear, some authors suggest *ibeA* encodes an extracellular protein capable of binding a 55KDa ibeA receptor (ibe10R) on bovine and human microvascular endothelial cells (BMECS/HMECS) [160]. However, Cortes *et al.* (2008) later proposed that *ibeA* may in fact encode a

cytoplasmic protein with enzymatic activity as no signal secretion sequence has been identified yet a putative flavin adenine dinucleotide binding domain has been found [154]. Cortes suggests that *ibeA* regulates type 1 fimbriae expression and thus indirectly contributes to the adhesion-invasive properties of ExPEC. APEC *ibeA*$^{-}$ mutants have been shown to have decreased biofilm formation potential and decreased invasiveness and virulence in both *in vitro* BMEC, chicken embryo DF-1 cell models and *in vivo* using 3-week old chickens [157, 159, 161, 162].

Autotransporter proteins are a distinct family of secreted proteins of Gram-negative bacteria. They possess an overall unifying structure composing of an N-terminal signal sequence, a C-terminal outer-membrane pore-forming translocator domain and a passenger (secreted protein) domain. The first autotransporter to be identified in APEC chi7122 was the 106KDa Tsh autotransporter protein [163]. Dozois *et al.* (2000) demonstrated that the Tsh protein contributed to the adherence of APEC to avian air sacs during the early stages of infection [155]. The *tsh* gene is carried by 46-85% of APEC and is located on a number of virulence plasmids including the pAPEC-O2-ColV plasmid [26, 164]. Ewers *et al.* (2007) reported a prevalence of 4-4.5% in UPEC isolates [165].

The APEC autotransporter adhesin (*aatA* gene) and aatB contribute to biofilm formation and adherence to chicken embryo fibroblasts (DF-1 cells) [166, 167]. In a recent study *aatB* was carried by ~27% of 273 tested APEC (predominately of the B2 and D phylogroup) from China [167]. *aatB* was discovered following the genome sequencing of APEC DE205B, originally isolated from the brain of a Duck. *aatB* perhaps plays a redundant role in colonisation by some APEC, given its relatively

low prevalence and Δ*aatB* mutants still colonised infected birds but at lower capacity [167].

Pyelonephritis-associated pili (P pili) encoded by the *pap* operon may also contribute to APEC pathogenesis [29, 87, 168]. P pili are not thought to be important in the early stages of infection, but play a role during later stages leading to septicaemia and organ failure [87]. The complete *pap* operon is carried by the well characterised pathogenicity associated island (PAI) $I_{APEC\text{-}01}$ [169]. The PAI $I_{APEC\text{-}O1}$ *pap* operon, excluding *papA,* which showed 99% homology to porcine septicaemic *E. coli*, shows high sequence homology to that of UPEC CFT073 [170]. The prevalence of *pap* genes among APEC has been reported to be between 18.5 and 40% [171, 172].

In all, an array of adhesins has been shown to contribute to APEC pathogenicity and many are likely to still be unknown. It is possible that a combination of adhesins is required in pathogenesis and with certain adhesins playing a role at specific points during infection.

1.9.5 Iron Acquisition

Iron is an essential requirement for most metabolic pathways involving electron transportation and in nucleotide biosynthesis [173, 174]. The concentration of extracellular free iron is low in extraintestinal sites of infection; iron tends to be chelated by host proteins (transferrin or lactoferrin) or bound to host haem-containing proteins (haemoglobin), while, intracellularly, iron is often associated with ferritin. Bacteria have evolved direct and indirect mechanisms for sequestering iron from the host to ensure their survival. Hijacking iron from haem-containing proteins (direct acquisition) is an effective mechanism of iron acquisition; haem is

the most abundant source of iron *in vivo*. Some bacteria possess outer-membrane protein receptors such as ChuA, which are capable of binding haemproteins and allow uptake into the bacterial periplasm. Then, once within the periplasm, ATP-binding protein cassettes such as ChuT allow transfer of iron into the cytoplasm [175].

Indirect mechanisms of iron acquisition involve shuttling iron to the bacterial cell with the aid of bacterial siderophores; high affinity ferric iron chelators. These include catecholates, enterobactins, salmochelins and yersiniabactins [176]. Salmochelins were first identified in *Salmonella enterica* and are encoded by the *iro* locus (*iroBCDEN*) [177]. Salmochelins have been associated with the virulence of NMEC, UPEC and APEC, and the *iro* locus has been identified on ColV and ColBM plasmids suggesting that ExPEC share similar iron uptake mechanisms [178-181]. Salmochelins are C-glycosylated derivatives of enterobactins and appear to be better iron chelators and to potentially contribute more to APEC virulence than enterobactins [180]. Chicken infection studies revealed that deletion of the *iro* locus, decreased the ability of APEC chi7122 to persist and cause deep tissue lesions [144].

Hydroxamate aerobactin, encoded by the *iucABCD* locus, is estimated to be carried by 63-98% of APEC [182, 183]. Like the *iro* locus, the *iucABCD* locus has been identified on APEC transmissible plasmids [26]. Mutational studies have helped reveal the importance of *iucA* and *iucC* in APEC E058 pathogenesis [184]. Iron repressible protein 2 (Irp2) is involved in the synthesis of yersiniabactin and that, and another siderophore receptor (*ireA*), have both previously been associated with human ExPEC virulence and located to APEC plasmids and PAIs [170, 182, 185,

186]. Ferric yersiniabactin uptake protein encoded by the *fyuA* gene has been identified in 66% of APEC [187].

The *SitABCD* operon, encodes an iron and manganese transport system and was shown to contribute to the virulence of APEC chi7122 in a chicken infection model with a possible additional role in protection against oxidative stress [26, 188]. In one study, the *sitABCD* operon was identified in over 85% of APEC [189].

It is perhaps the case that APEC strains possess multiple redundant mechanisms to ensure iron acquisition is achieved because of its essential requirement [180, 182]. Iron is known to be the key regulator in siderophore gene expression, but Valdebenito *et al.* (2006) also suggest that other environmental factors such as pH influence siderophore gene expression [190].

The avian innate immune system appears to have developed strategies to minimise the effectiveness of bacterial siderophores by producing Ex-FABP homologous to mammalian siderocalins. Ex-FABP binds ferric iron-siderophore complexes to prevent bacteria from acquiring iron [191]. Ex-FABP inhibited *E. coli* K12 growth *in vitro* but pathogenic *E. coli* carrying siderophores such as salmochelins, aerobactin and yersiniabactin grew normally in the presence of Ex-FABP [191].

Despite its importance, too much iron can be lethal to *E. coli*; high iron concentrations in the blood require tight regulation and factors involved in such regulation (AraC-like regulator and YbtA) are associated with increased expression in septicaemic APEC and deletion of these factors is detrimental to survival in blood [192].

1.9.6 Overcoming the host immune system

To infect extraintestinal sites, APEC must evade the avian immune system. The respiratory tract lacks a resident innate cellular defence system with very few macrophages and heterophils (the chicken orthologue of mammalian polymorphonuclear neutrophils) residing in the respiratory tissue [193]. Thus, the respiratory tract must rely on the infiltration of peripheral blood monocytes along with macrophages and heterophils residing in bronchus-associated lymphoid tissues (BALT) [193-195]. APEC may reside free in the air sac lumen or in close contact with macrophages, with some speculation over the ability of APEC to replicate intracellularly [196]. Pourbakhsh *et al* (1997) correlated virulence with the ability of APEC to resist killing by macrophages [196]. APEC infection studies using STM or specific gene knockouts have been used to test molecular Koch's postulates in a number of different studies to evaluate the contribution of VAGs to APEC colonisation and persistence in respiratory tissues [47, 155, 197].

The K1 capsular antigen N-acetylneuraminic acid shows little immunogenic capacity. In epidemiological and mutational studies, the pathogenic association of K1 has previously been associated with NMEC and APEC, although not all studies support the beneficial effects of the K1 capsule in pathogenesis [47, 198, 199]. Mellata *et al.* (2003) showed that the absence of P-fimbriae, K1 and the O78 antigen promoted association of APEC with phagocytic cells [200].

Once within the bloodstream, APEC are faced with the bactericidal effects of the complement system, a constituent of the innate immune system found in sera. A number of VAGs have been associated with complement resistance including *iss*, which encodes the 10-11KDa increased serum survival (Iss) outer-membrane

lipoprotein [201]. Iss reportedly contributed to a 100 fold increase in *E. coli* virulence [202]. High sequence homology between *iss* and the phage lambda *bor* gene, also involved in serum resistance, suggests *iss* evolved from a *bor* precursor [201]. Johnson *et al.* (2008) described the presence of 3 *iss* alleles associated with ColV/BM plasmids and at least 2 encoded on the *E. coli* chromosome [26, 203]. Between 38 and 82.7% of APEC possess the *iss* gene and past studies have focused on this gene as a potential vaccine target offering homologous and heterologous protection [182, 204]. Not all research concurs with the importance of *iss*, suggesting *iss* may play only subtle role in virulence [200, 205].

Other proteins associated with serum survival include the outer membrane proteins TraT [88, 202]. TraT inhibits complement by preventing the deposition of C3 and the formation of the C5b6 complex in bacterial cells, which ultimately leads to bacterial lysis [206, 207]. TraT has also been associated with EAEC pathogenesis [208].

Lysozyme is another bactericidal component of the innate immune system, non-specifically destroying bacterial cell walls by hydrolysing the peptidoglycan layer. Two genes have been associated with APEC resistance to lysozyme and increased virulence *in vivo* using knock-out mutants; an inhibitor of vertebrate lysozyme (*ivy*) and a membrane bound lysozyme inhibitor (*MLi-C*) of C-type lysozyme[209].

1.9.7 Toxin production

The exact contribution of toxins to APEC virulence remains to be fully elucidated, although various toxins may be involved [43]. The first PAI of APEC was the VAT-PAI encoding a vacuolating autotransporter toxin (Vat) [210]. VAT induces the formation of intracellular vacuoles having an overall cytotoxic effect. Parreira *et al.*

(2003) observed a decline in virulence following the deletion of the *vat* gene in a broiler chicken respiratory infection model [210]. This autotransporter toxin shares 75% sequence homology to the *tsh* gene described earlier and is found in approximately 38% of APEC, although it has been reported in over 50% of ExPEC [47, 182]. Johnson *et al.* (2007) even reported the presence of *vat* in the backbone genome of ExPEC strains [89].

Toxins previously associated with other ExPEC have been suggested to contribute to APEC virulence but existing data are contradictory. The enteroaggregative heat stable enterotoxin 1 (EAST1), encoded by the *astA* gene, was originally identified in EAEC and later associated with both ETEC and 20-30% of APEC pathotypes and induces diarrhoea [187, 210, 211]. Olsen *et al.* (2011) demonstrated that although EAST1 may be associated with pathogenicity it is not found in all outbreak APEC strains [123].

Recently cellulitis was induced following the subcutaneous inoculation of purified *E. coli* vacuolating factor (ECVF) into 40-day old broiler chickens confirming its role in the initiation of inflammation and pathogenesis [212].

Other toxins associated with ExPEC pathogenesis, include the haemolysin (hlyE), heat-labile enterotoxin (a homologue of EAST1) and shiga toxins, although their exact role in APEC pathogenesis remains unclear [213-216].

1.9.8 Plasmid encoded genes and pathogenicity islands

A number of conjugation and mutational studies have highlighted the ability to transfer virulence using virulence plasmids or PAI [217-220]. The avian gut has been

described as a 'mixing vessel' allowing the transfer for virulence encoded genetic elements and is a known source of APEC [221].

1.9.8.1 Colicin V plasmids

Colicin V (ColV) plasmids are large ones (80-180kb) associated with ExPEC [189, 205, 222, 223]. These plasmids mainly fall into incompatibility groups IncFIB and IncFIIA (defined by the replication mechanism used) [224]. ColV plasmids are named because they carry the bacteriolysin colicin V; this phenotype was first described as transmissible in the early 1960s and was later associated with F-type plasmids [225, 226]. In 1980, Williams and Warner reported that the colicin phenotype did not actually contribute to virulence of ExPEC and it was merely associated with large plasmids encoding other VAGs [227].

Numerous APEC ColV plasmids have been fully sequenced including: 1) pAPEC-O2-ColV carried by a particularly virulent strain of APEC from a turkey with colibacillosis 2) pEco588 from an O45 NMEC isolate 3) PCVM29188_146 originally isolated from a *Salmonella spp.* from retail chickens *4) pChi7122-1* carried by the model APEC chi7122 [26, 28, 164, 228]. Johnson *et al.* (2006) published the first fully sequenced ColV plasmid (pAPEC-O2-ColV) which became available in 2006 [26].

Iron acquisition systems, serum resistance and even selective growth advantages under acidic conditions are associated with ColV plasmid carriage [229]. The pAPEC-O2-ColV plasmid is 180kb with a 93kb region encoding VAGs containing both 'constant' and 'variable' regions. The constant region focuses around the plasmid replicon along with the aerobactin operon, salmochelin siderophore, *sit* operon, ompT, Ets (an ABC transporter) and *iss*. The variable region contained genes

for the synthesis of Tsh and the transport system Eit [224]. Virulence of this plasmid was confirmed when it was transferred into non-pathogenic commensal *E. coli* [220].

pChi7122-1 also contains many of the expected APEC VAGs previously described and has been shown to be a major contributor to virulence [164].

1.9.8.2 Colicin B & M plasmids

Colicin B and M (ColBM) plasmids (named so because they encode Colicin B and Colicin M) are thought to have evolved from ColV plasmids. ColBM plasmids are within the same incompatibility group IncFIIB as ColV plasmids. One such sequenced plasmid (called the pAPEC-ColBM) encodes similar VAGs to those found on the ColV plasmids and is of a similar size and was identified in ~28% of APEC tested [221]. Often, ColBM plasmids are associated with multidrug resistance, particularly to tetracycline, ampicillin and streptomycin [228]. Another ColBM plasmid, which has been fully sequenced, is the pAPEC-O103-ColBM [230].

Overall, the colicin plasmid pangenome is large and plasmids are highly heterogeneous whilst maintaining conserved structures, suggesting differences in plasmids arise from larger insertions and deletions of genetic material as opposed to single nucleotide polymorphisms and mutations.

1.9.8.3 Other APEC associated plasmids and PAIs

Non-ColV plasmids have also been linked to virulence in APEC. Mellata *et al.* (2012) recently described the contributing roles of pChi7122-2, -3 and the small cryptic plasmid pChi7122-4 in APEC chi7122 pathogenesis in addition to the already well defined pChi7122-1 plasmid [231]. This work highlighted that combinations of plasmids and their nature are important and indeed determine the outcome of

bacterial behaviour. pChi7122-2 and -3 do not encode the common ExPEC VAGs and were shown to contribute to acid tolerance, biofilm formation and bacterial interaction with the epithelium, possibly due to the encoding of Type IV fimbriae and the eitABCD transporter. These plasmids play minor roles in virulence contributing more to APEC persistence in stressful (low iron) environments.

A transmissible IncHI2 plasmid (pAPEC-O1-R) encoding for multidrug resistance to eight antibiotics and heavy ion resistance has also been fully sequenced [232]. The prevalence of this plasmid amongst ExPEC (including APEC and UPEC) was determined to be low but highlights the potential for the emergence of large drug resistance phenotypes.

A number of chromosomally embedded APEC associated PAI have been identified amongst pathogenic *E. coli* that are missing from non-pathogenic strains. Such chromosomal regions vary in size from 20-200kb and can be horizontally transferred within a bacterial population [233]. The $PAI_{APEC-O1}$ was sequenced to reveal the *pap* operon [169]. Furthermore, the *vat* encoding PAI was the first to be identified and deletion of this PAI in broiler respiratory models reduced virulence [210].

1.9.9 VAG summary

The molecular basis of APEC pathogenesis is yet to be fully understood but it is apparent there are many modes of host-pathogen interaction. Many genes have been identified to have a contributing role in APEC pathogenesis, yet no defining factor has been identified to date. A recent *in vivo* APEC infection study focusing on APEC with faecal *E. coli* infection comparisons revealed that both groups were capable of

producing inflammation in infected lung tissue and despite efforts it is difficult to draw a line that separates pathogenic and non-pathogenic avian *E. coli* [234].

1.10. Chicken Immunology

1.10.1 Introduction to chicken immunology

The divergence of mammals and birds from a common reptilian ancestor occurred 200 million years ago. Despite this evolutionary time period, the fundamental principles of both the innate and adaptive immune systems of mammals and birds are the same. The availability of the chicken genome has helped improve our understanding of the avian immune system [235].

1.10.2 The chicken innate immune system

The first non-specific arm of the avian immune system is known as the innate system. As in mammals, the most well characterised family of non-specific pattern recognition receptors (PRRs) are membrane-bound Toll-like receptors (TLRs) expressed by various cell types including epithelial cells and sentinel cells such as antigen presenting cells (APCs), dendritic cells and macrophages. In the chicken, TLRs are also expressed by heterophils, a polymorphonuclear leukocyte and homologue of mammalian neutrophils [236]. TLRs detect structurally conserved microbial specific motifs. Thirteen TLRs have been described in the chicken; 11 are also present in mammals while two are chicken-specific (TLR-15 and TLR-21) [237, 238]. Despite differences in TLR families, similar microbial motifs are recognised by both TLR repertoires.

Invading *E. coli* are likely to be recognised by TLR-2, -4, -5 and -21 which recognise peptidoglycan, LPS, flagellin and unmethylated CpG DNA motifs respectively [237-239]. Receptor-ligand binding triggers proinflammatory intracellular signalling pathways (NFκB and mitogen activated protein kinase pathways) initiating the activation and recruitment of phagocytic cells and lymphocytes through the expression of pro-inflammatory cytokines and chemokines (molecular messengers and immunomodulators responsible for coordinating cells of the innate and adaptive immune system).

An invaluable tool in starting to understand the chicken cytokine responses is the availability of quantitative real-time reverse transcription PCR assays for many of the chicken cytokine messenger RNAs [240]. Pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, IL-8 and chemokines CXCLi 1, 2 and 4 are involved in the recruitment of heterophils and macrophages to the intestinal epithelium during chicken infection with *Salmonella* Typhimurium [241]. The respiratory tract and air sacs are thought to be one of the primary sites for APEC infection. The air sacs possess no resident cellular defences and rely on the rapid pro-inflammatory influx of heterophils and then macrophages [193]. An initial pro-inflammatory avian response is common following pathogen exposure. IL-2 induces heterophil activation and T lymphocyte activation (a cell of the adaptive immune system) [242]. For example, exposure of chicken peripheral blood mononuclear cells (PBMC) to *S.* Enteritidis can induce a rapid change in IL-6, CXCLi2 and anti-inflammatory transforming growth factor – β4 (TGF-β), although some reports are contradictory [240].

An association between *E. coli* and innate immune cells has been reported. Mellata *et al*. (2003) suggested that Type 1 fimbriae and the absence of P fimbriae, K1 antigen and the O78 antigen promoted the association of *E. coli* and innate cells. However, the presence of Type 1 and P fimbriae protected *E. coli* from the subsequent bactericidal effects of phagocytes [243]. Pourbaksh *et al*. (1997) reported that following *in vivo* infection of chickens, *E. coli* were detected within macrophages in the air sacs and extracellular *E. coli* were associated with heterophils, fibrin or epithelial cells [87]. An increase in the number of phagocytes and levels of IL-6 in the lung and blood were recorded following the administration of a recombinant pro-inflammatory interferon-y (IFN-y) during an *E. coli in vivo* infection [244].

1.10.3 The adaptive immune system

The second arm of the chicken immune system is the adaptive one involved in both cellular and humoral (antibody) responses, as well as the production of memory cells. The avian antigenic repertoire is more compact than that of the mammalian system; one reason for this is that birds only possess 2 of the Major Histocompatability complex class (MHC) 1 alleles, compared to the 6 of mammals [245]. MHC class 1 is found on almost all nucleated cells and their function is to present antigen peptides to $CD8^+$ cytotoxic T lymphocytes (T-cells). Despite this more limited repertoire, birds are still effective at mounting an adaptive immune response and clear pathogens such as *S.* Enteritidis by T Helper 1 $CD8^+$ cells [241].

1.10.4 APEC vaccine production

A recent study identified that approximately 50% of first week layer hen mortalities were related to infection, with *E. coli* being responsible for a significant proportion

of deaths [133]. The major limiting factor in designing an APEC vaccine is the inability to define APEC by a single or even a combination of factors. Olsen *et al.* (2012) found that the first week mortalities related to *E. coli* were due to a polyclonal population further adding to the problems faced in vaccine production. Some attempts in vaccine production have failed to overcome this problem, resulting in poor protection against heterogeneous challenge [246].

Liposomal inactivated APEC vaccines have shown some promise [247]. Following eye drop administration of the inactivated APEC, levels of IgG in sera and IgA at mucosal sites increased. A similar observation was made using a recombinant iss protein vaccine [204, 248]. Lynne *et al.* (2012) showed that the recombinant APEC vaccine of multiple different serogroups provided sufficient protection against subsequent challenge [248]. Absolute protection from this vaccine was not seen, corresponding with the idea that the Iss protein may only play a minor role in APEC virulence [200, 205].

Live virus vaccines would allow for mass vaccination as they can be administered in feed and water or as an aerosol spray. A number of live APEC vaccines have been described in recent years, including mutant O2 and O78 strains [249, 250]. These vaccine candidates contained mutations in genes involved in intracellular signalling, bacterial transcription (*E. coli* cyclic AMP binding protein (*crp*)), sugar fermentation and amino acid biosynthesis.

1.11. The aims of this thesis

APEC research has become increasingly popular in the past decade but there is still much to be done. To date, very little work has focused on the epidemiology of APEC in UK commercial broiler chickens. This investigation addresses the epidemiology, population dynamics, and (subsequently) the *in vitro* phenotypic behaviour of both avian faecal and extraintestinal *E. coli* populations isolated from UK commercial broiler chickens using several innovative approaches. Such information may help elucidate the complex interaction between host and microbe and contribute to the formation of effective control measures in the poultry industry. The specific aims are:

1. To explore the population genetics and VAG carriage of *E. coli* found in the avian GIT.
2. To explore the population genetics and VAG carriage of extraintestinal *E. coli* in diseased broiler chickens.
3. To compare the genetic background and VAG carriage of intestinal and extraintestinal *E. coli* from the same flock cycle.
4. To determine the changes/dynamics of *E. coli* populations found intestinally and extraintestinally as birds age.
5. To determine the contribution of *E. coli* to first week mortalities of UK broiler chickens.
6. To compare the *in vitro* behaviour of avian extraintestinal and intestinal *E. coli* isolated from the field.
7. To compare the avian innate immune response to avian extraintestinal and intestinal *E. coli.*

Chapter 2

General Materials and Methods

2.1 Longitudinal field study

2.1.1 Commercial poultry farms

Two commercial poultry farms were involved in this investigation. These poultry farms are located in North East Wales, approximately 30 miles from the University of Liverpool (Leahurst campus) and approximately 15miles from one another. One of the two farms consisted of three sheds, while the other had four. All poultry, irrespective of farm, were reared to conform to the standard commercial end-of-life stocking density of 38kg/m^2. Farm managers are required to perform 3-4 welfare inspections daily.

The broiler chickens included in this thesis were a standard commercial breed, representative of approximately 90% of the total industry. All flocks involved were routinely vaccinated against avian pneumovirus (7 days old), infectious bronchitis virus (14 days old) and infectious bursal disease (16 days old).

2.1.2 Sample collection

For the longitudinal component of the investigation 2 flock cycles from each of the two farms were included. Thus 4 flock cycles were sampled in total.

2.1.2.1 Faecal E. coli

Once a week (every 7th day where possible), 20 fresh faecal samples were collected using sterile swabs from one broiler shed on each of the two farms. The same shed was sampled throughout the study. Sampling commenced the day the chicks were placed in the sheds and concluded when the birds reached 5 weeks old, coinciding with the depopulation of the flock. During depopulation events, approximately 30%

of the flock is removed and sent to slaughter to allow farmers to conform to end-of-life stocking density legislation.

2.1.2.2 Extraintestinal E. coli

From week 2 of production and alongside faecal sampling, dead birds were collected during the first welfare walk of the day and examined by post-mortem. To minimise the detection of systemic *E. coli* resulting from a loss of intestinal integrity following death, birds were only selected for post-mortem examination if they did not show signs of extensive trauma (pecking, broken legs). For all birds, signs of disease/tissue lesions were recorded including: ascites, airsacculitis, cellulitis, splenomegaly, pericarditis, perihepatitis and lesions within respiratory tissue.

For each bird, up to 1 gram of the following tissues were collected; heart, kidney, liver, lung and spleen using sterile forceps and scalpels. An equal volume of sterile phosphate buffered saline (PBS) was added to each tissue sample and homogenised using a Biomaster Micro-stomacher 80 (Steward, UK) for 60 seconds at high speed.

2.1.3 Isolation and resuscitation of *E. coli*

Each faecal swab (or 50µl of tissue homogenate) was cultured onto eosin-methylene blue agar (EMBA) and incubated overnight at 37°C. From each plate, eight randomly selected colonies typical of *E. coli* were sub-cultured onto nutrient agar to obtain pure cultures and incubated overnight at 37°C. On EMBA, *E. coli* are typically dark colonies with a metallic green sheen, the result of lactose fermentation and acid production. Not all *E. coli* produce the green sheen and identification was confirmed using a polymerase chain reaction (PCR) targeting the *uidA* gene, see section 2.4.3

for assay conditions [11]. *uidA* encodes a β-glucuronidase enzyme carried by ~96% of *E. coli* [251]. All media were obtained from LabM (IDG) Ltd (Bury, UK).

The *E. coli* culture collection obtained during this study was stored long term at -80°C in Microbank vials (Pro-Lab Diagnostics, UK). When required, bacterial isolates were resuscitated from -80°C storage by streaking onto nutrient agar and incubating overnight at 37°C.

2.2 Defining and identifying pAPEC

In the following investigation, potential APEC (pAPEC) are defined as *E. coli* isolated from the faeces of broiler chickens and which carry ≥ 5 virulence-associated genes (VAGs) identified using PCR based assays, see section 2.4.1.

2.2.1 Identifying pAPEC among entire faecal *E. coli* population

To screen the faecal population for pAPEC, the eight *E. coli* isolated from each swab were pooled and screened for 4 VAGs (*cvi, iss, iucD* and *tsh*) using a published multiplex PCR (mPCR), see section 2.4.1 [252]. PCR positive pools for ≥ 3 VAGS were subsequently separated into their 8 individual isolates and virulotyped individually. *E. coli* pools with < 3 positive genes were discarded. This screening protocol is illustrated as a flowchart in Figure 2.1.

2.3 DNA extractions

The pooled mPCR and the virulotyping mPCR DNA extractions were performed using Chelex-100 (in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (Bio-Rad, Hertfordshire, UK) based protocols, described by Walsh *et al* (1991) [253].

2.3.1 DNA extraction for screening pooled faecal *E. coli*

One colony of each of the 8 faecal *E. coli* isolated from the same swab was pooled into 600µl of Chelex-100 and incubated at 95^{0}C for 10 min. At each time point there were 20 pools of 8 *E. coli*. Samples were centrifuged at 10,000rpm for 2 min and 50µl of supernatant was added to 250µl of sterile double distilled water. Pooled DNA preparations were stored short term at 4°C.

2.3.2 DNA extraction for virulotyping individual *E. coli*

To prepare individual *E. coli* isolates for virulotyping by PCR analysis, extractions were made as above (section 2.3.1) with a few modifications. One loopful of bacterial cells was suspended in 300µl of Chelex-100 solution. The solution was heated at 95°C for 10 minutes and then centrifuged at 10,000rpm for 2 min. 50µl of the clear supernatant was added to a new eppendorf tube containing 450µl sterile distilled water [253]. Pooled DNA preparations were stored short term (weeks) at 4°C and for long term (months) at -80°C.

2.4 Polymerase Chain Reaction (PCR) assays

Multiple PCR assays were used during the course of this investigation. All PCR constituents were supplied by Thermo Scientific Ltd, Surrey and primers were obtained from Eurofins MWG operon (Germany).

2.4.1 Screening faecal *E. coli*

As a means of screening faecal *E. coli,* largely expected to be non-pathogenic*,* each pooled DNA extract was screened for four VAGs previously associated with APEC pathogenesis; *iss, tsh, iucC* and *cvi,* using a mPCR [252].

2.4.1.1 Screening faecal E. coli: *the reaction*

Primer sequences are shown in Table 2.1[252]. Each 50µl reaction contained: 12µl of 25mM $MgCl_2$, 21.3µl sterile water, 5µl 10x PCR buffer, 4µl of 20mM dNTPs, 0.3µl of each 100pmol forward and reverse primer, 0.3µl 5U/µl Taq polymerase and 5µl template DNA.

Table 2.1 Gene target, primer sequences, accession number and product length for pooled multiplex PCR

Gene	Primer sequence (5' - 3')	Gene Bank Accession no.	Size (bp)	Reference
iss	GTGGCGAAAACTAGTAAAACAGC	AF042279	760	[254]
	CGCCTCGGGGTGGATAA			
tsh	GGGAAATGACCTGAATGCTGG	AF218073	420	[255]
	CCGCTCATCAGTCAGTACCAC			
iucC	CGCCGTGGCTGGGGTAAG	X76100	541	[252]
	CAGCCGGTTCACCAAGTATCACTG			
cvi	GGGCCTCCTACCCTTCACTCTTG	AF062858	366	[252]
	ACGCCCTGAAGCACCACCAGAA			

2.4.1.2 Screening faecal E. coli: *thermocycler conditions*

Thermocycler conditions were: initial denaturation 95^{0}C for 5 min; nine cycles of 95^{0}C for 60 sec, 55^{0}C for 30 sec, 72^{0}C for 60 sec; twenty eight cycles of 94^{0}C for 30 sec, 55^{0}C for 30 sec, 72^{0}C for 30 sec with a final extension 72^{0}C for 7 min. The mixture was cooled and held at 4^{0}C until visualised.

2.4.2 Virulotyping *E. coli* by PCR

Individual *E. coli* passing the screening threshold or those isolated directly from extraintestinal sites were virulotyped based on the presence of 10 VAGs; *astA*, *iss*, *irp2*, *papC*, *iucD*, *tsh, cvi*, *vat*, *sitA* and *ibeA*.

In order to do this, 3 PCR assays were required: one multiplex and two single PCRs. The published mPCR targets *astA*, *iss*, *irp2*, *papC*, *iucD*, *tsh*, *cvi* and *vat* [182]. The two single PCRs target *sitA* and *ibeA* and have been previously described by Timothy *et al* (2008) [110].

2.4.2.1 Virulotyping E. coli: *the reaction*

Primer sequences are shown in Table 2.2. Reactions were performed in 25µl containing: 4µl of 25mM $MgCl_2$, 13.9µl sterile water, 2.5µl 10x PCR buffer, 0.5µl 20mM dNTPs, 0.1µl of each 100pmol forward and reverse primers, 0.5µl 5U/µl Taq polymerase and 2µl DNA template.

sitA and *ibeA* PCR assays contained 1µl DNA template, 1µl of each primer (100pmol) and 22µl of 1.1 x Reddymix PCR mastermix with 1.5mM $MgCl_2$.

2.4.2.2 Virulotyping E. coli*: thermocycler conditions*

Thermocycler conditions were as follows: initial denaturation 94^{0}C for 3 min and 25 cycles of; 94^{0}C for 30 sec, 58^{0}C for 30 sec, 68^{0}C for 3 min; final extension 72^{0}C for 10 min; hold at 4^{0}C until visualised.

Thermocycler conditions for *sitA* and *ibeA* were identical; 95^{0}C for 12 min; 25 cycles of: 94^{0}C for 30 sec, 63^{0}C for 30 sec, 68^{0}C for 3 min with a final 72^{0}C for 10 min. The mixture was held at 4^{0}C until visualised.

Table 2.2 Gene target, primer sequences, accession number and product length for virulence-associated genes targeted in second stage of virulotyping

Gene	Primer sequence (5' - 3')	Gene Bank Accession no.	Size (bp)	Reference
astA	TGCCATCAACACAGTATATCC TCAGGTCGCGAGTGACGGC	AF143819	116	[256]
iss	ATCACATAGGATTCTGCCG CAGCGGAGTATAGATGCCA	X52665	309	[171]
irp2	AAGGATTCGCTGTTACCGGAC AACTCCTGATACAGGTGGC	L18881	413	[171, 187]
papC	TGATATCACGCAGTCAGTAGC CCGGCCATATTCACATAA	Y00529	501	[257]
iucD	ACAAAAAGTTCTATCGCTTCC CCTGATCCAGATGATGCTC	M18968	714	[257]
tsh	ACTATTCTCTGCAGGAAGTC CTTCCGATGTTCTGAACGT	AF218073	824	[171]
vat	TCCTGGGACATAATGGTCAG GTGTCAGAACGGAATTGT	AY151282	981	[171]
cvi/cva	TGGTAGAATGTGCCAGAGCAAG GAGCTGTTTGTAGCGAAGCC	AJ223631	1181	[171]
ibeA	AGGCAGGTGTGCGCCGCGTAC TGGTGCTCCGGCAAACCATGC	L42624	171	[72]
sitA	TGGTGCTCCGGCAAACCATGC AGGGGGCACAACTGATTCTCG	AY126440	608	[77]

2.4.3 *E. coli uidA* PCR

E. coli identification was confirmed by the presence of *uidA* [11]. Each reaction was performed in 25µl containing: 1µl of template DNA, 1µl of each 20pmol forward and reverse primers and 22µl of 1.1 x Reddymix PCR mastermix with 1.5mM $MgCl_2$. Thermocycler conditions were as follows: 25 cycles of 1 min of each 94, 58 and 72°C with a final extension at 72°C for 7 min. Forward primer (5'to 3') CCAAAAGCCAGACAGAGT and reverse primer (5' to 3') GCACAGCACATCAAAGAG [11]. PCR amplicon was 623bp.

2.4.4 Phylogenetic typing PCR based assay

2.4.4.1 Phylogenetic typing; the reaction

E. coli were assigned to 1 of 4 phylogenetic groups (A, B1, B2 or D) using a mPCR targeting *chuA, yjaA* and the DNA fragment TSPE4.C2 [1]. Phylogenetic classification was based on the combination of *chuA*, *yjaA* and TSPE4.C2: A (*chuA*$^-$, TSPE4.C2$^-$, *yjaA*$^+$), B1 (*chuA*$^-$, TSPE4.C2$^+$, *yjaA*$^-$), B2 (*chuA*$^+$, TSPE4.C2$^{-/+}$, *yjaA*$^+$) and D (*chuA*$^+$, TSPE4.C2$^{-/+}$, *yjaA*$^-$).

Each 25µl PCR reaction contained: 1µl of template DNA extract, 1µl of each forward and reverse 100pmol primer and 22µl of 1.1 x Reddymix with 1.5mM $MgCl_2$.

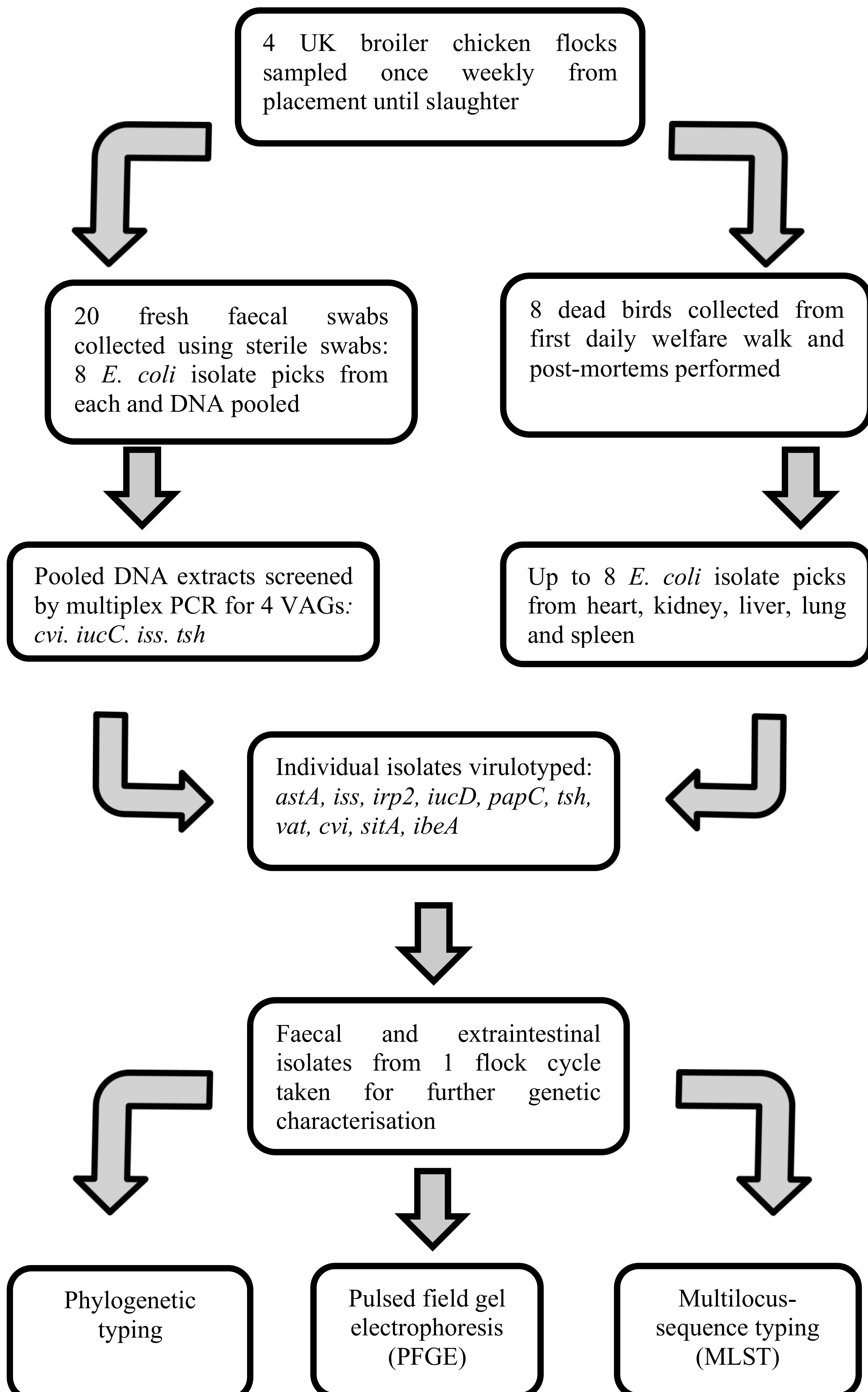
2.4.4.2 Phylogenetic typing; thermocycler conditions

Thermocycler conditions were as follows: initial denaturation at 94^{0}C for 4 min; 30 cycles of; 5 sec at 94^{0}C and 10 sec at 59^{0}C with a final extension at 72^{0}C for 5 min. The reaction mixture was held at 4^{0}C until visualised.

2.4.5 Visualisation of PCR products

All mPCR products were visualised on a 1.5% agarose gel while single PCR products were visualised on a 2% agarose gels made using agarose (Biorad, UK) in 1 x Tris-borate ethanoate (TBE) buffer (Sigma-Aldrich) with 10µl/ml of ethidium bromide. For all PCR assays, 15µl of PCR product was loaded into the gel wells alongside10µl of 100bp molecular weight marker (Superladder low 100bp, Thermo scientific). Gels loaded with mPCR products were run in 1 x TBE buffer at potential difference of 120V and current 500mA for a maximum of 1.5 hours and checked periodically. Single PCR products were run at 150V and current 500mA for 30-40 minutes. Product bands were visualised using a gel documentation system (UviTee Gel Documentation system, UVItec, Cambridge, UK) under ultraviolet (UV) transillumination and images recorded using the UVIProMV computer program (UVItec).

Figure 2. 1 Flow diagram for sampling *E. coli* on broiler chicken farms



2.5 Gentamicin Invasion Assay

The gentamicin invasion assay was used to analyse the invasive potential of pAPEC using the human colonic carcinoma cell line (Caco-2) and the intracellular persistence of avian faecal and extraintestinal *E. coli* in the avian macrophage cell line (HD11) [258]. The protocol for both experiments was based on that previously described [259, 260].

2.5.1 Cell line seeding protocols

2.5.1.1 Caco-2 cell line

The human colonic carcinoma cell line (Caco-2 cells) was sourced from Dr Barry Campbell from the Gastroenterology Department, University of Liverpool, UK (ATCC®, Number HTB-37). Cells were grown in Dulbecco's minimum essential medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich, UK), 1% GlutaMAX™ (100x concentration) (Invitrogen, UK), 1% MEM non-essential amino acid solution (Sigma-Aldrich, UK) and penicillin–streptomycin (100 U/ml) antibiotics (Sigma-Aldrich, UK). Cells were grown at 37°C 5% CO_2.

Twelve days before infection, cells were seeded into 24-well tissue culture plates at a density of 3 x 10^5 cells per well and incubated at 37°C in 5% CO_2 to give a final density of 1 x 10^6 cells. The DMEM was changed every 2-3 days during this time [259]. Three days before the experiment, the DMEM with the above supplementation was substituted for antibiotic-and serum-free medium (AFSF DMEM).

2.5.1.2 HD11 cell line

The avian macrophage HD11 cell line [258] was grown in RPMI-1640 media (Sigma-Aldrich, UK) with the same supplementation as that of the Caco-2 cell line described in section 2.5.1.1. Cells were grown at 37°C. 5% CO_2 [258].

Two days before infection, cells were seeded into 24-well tissue culture plates at a density of 4.5 x 10^5 cells per well and incubated at 37°C to give a final density of 1 x 10^6 cells. One day before the experiment, the RPMI with the above supplementation was substituted for AFSF RPMI.

2.5.2 Bacterial culture preparation

All bacterial isolates were resuscitated from the -80°C culture collections by streaking onto nutrient agar and incubating overnight at 37°C. For all assays, *S.* Typhimurium 4/74 was used as an invasive positive control and APEC O78 was used as a reference APEC control. The APEC O78 reference strain was kindly donated by Professor Mark Stevens of The University of Edinburgh Roslin Institute, Scotland, UK. APEC O78 is a spontaneous mutant of the EC1 strain isolated from the liver of a diseased turkey [79].

One day prior to the invasion assays, 2-3 colonies of each overnight culture were used to inoculate 2ml of sterile LB broth. Bacterial suspensions were incubated overnight at 37°C. On the day of the infection studies, 100µl of overnight culture was added to 10ml of fresh LB broth and incubated at 37°C for 3.5 hours at 150rpm. After 3.5 hours the OD_{600} was adjusted using sterile PBS to 0.27 +/- 0.05 to reduce

differences in multiplicity of infection (MOI) between bacterial samples. These adjusted suspensions (MOI of ~28) were used to infect the immortalised cell lines.

2.5.2.1 In vitro *infection*

The AFSF growth medium was removed from the cultured cell lines and the cells were washed 3 times with sterile PBS and replaced with 1ml of heated (37°C) AFSF growth medium. Cell monolayers were incubated for 2 hours at 37°C 5% CO_2.

100µl of bacterial samples (with adjusted OD_{600}) were added to the monolayers in triplicate. Infected monolayers were re-incubated at 37°C for one or two hours for HD11 and Caco-2 cells respectively. After the initial incubation time, the supernatant was removed. One ml of growth media containing 100µg/ml gentamicin sulphate (Invitrogen, UK) was added to each well and incubated at 37°C for 1 hour. This was removed and cells washed once with sterile PBS. Cells were lysed with 0.5% Triton X-100 in 1ml PBS incubated at 37°C for 5 minutes. Cell lysates were serially diluted (1:10) using PBS and intracellular bacteria enumerated on nutrient agar. For enumeration, 3 x 20µl of each dilution (neat to 10^{-8}) was plated onto nutrient agar and the average for each dilution was calculated.

To assess the level of persistence at later time points (refer to Chapter 5 and 6 for details) following the initial incubation periods described above, 1ml of fresh growth medium containing 20µg/ml of gentamicin sulphate was added to the infected monolayers instead of lysing the cells with PBS-Triton X-100. Cells were then incubated at 37°C 5% CO_2 until the pre-defined point (up to 24 hours post-infection). At this time, the supernatant was removed, lysed with PBS-Triton X-100 and enumerated as previously described.

Chapter 3

Manuscript 1

A longitudinal study simultaneously exploring the carriage of APEC virulence-associated genes and the molecular epidemiology of faecal and systemic *E. coli* in commercial broiler chickens

K. Kemmett[1], T. Humphrey[1], S. Rushton[2], A. Close[2], P. Wigley[1], N. J. Williams[1]

[1]University of Liverpool, Leahurst Campus, Neston, CH64 7TE, UK

[2]Newcastle University, Newcastle upon Tyne, Tyne and Wear NE1 7RU, UK

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ABSTRACT

Colibacillosis is an economically important syndromic disease of poultry caused by extraintestinal avian pathogenic *Escherichia coli* (APEC) but the pathotype remains poorly defined. Combinations of virulence-associated genes (VAGs) have aided APEC identification. The intestinal microbiota is a potential APEC reservoir. This study simultaneously investigates intestinal *E. coli* VAG carriage in apparently healthy birds and characterises systemic *E. coli* from diseased broiler chickens from the same flocks. Four flocks were sampled longitudinally from chick placement until slaughter. Phylogrouping, macro-restriction pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) were performed on an isolate subset from one flock to investigate the population structure of faecal and systemic *E. coli*.

Early in production, VAG carriage among chick intestinal *E. coli* populations was diverse (average Simpson's D value = 0.73); 24.05% of intestinal *E. coli* (n=160) from day-old chicks were carrying ≥ 5 VAGs. Generalised Linear models demonstrated VAG prevalence in potential APEC populations declined with age; 1% of *E. coli* carrying ≥5 VAGs at slaughter and demonstrated high strain diversity. A variety of VAG profiles and high strain diversity were observed among systemic *E. coli*; polyclonal infections were also identified within organs of the same bird. Thirty three new MLST sequence types were identified among 50 isolates and a new sequence type (ST-2999) representing 22.2% (ST-2999) of the systemic population was found, differing from the pre-defined pathogenic ST-117 at a single locus. For the first time, this study takes a longitudinal approach to unravelling the APEC paradigm. Our findings, supported by other studies, highlight the difficulty in defining the APEC pathotype.

3.1 Introduction

Colibacillosis is an avian syndromic disease characterised by fibrinous lesions around visceral organs caused by a group of extraintestinal pathogenic *Escherichia coli* (ExPEC) known as avian pathogenic *E. coli* (APEC). Airsacculitis, cellulitis, pericarditis, perihepatitis and respiratory distress are among the most commonly associated signs of colibacillosis in broiler chickens [48].

The domestic chicken (*Gallus gallus domesticus*) is the most abundant domestic bird species on the planet while chicken remains the most popular meat of choice. The UK Department for Environment, Food and Rural Affairs (DEFRA) report annual production of over 900 million broiler chickens, 17 million turkeys and 100,000 geese in the UK [3]. These 2012 figures show a 3.2% increase in broiler chicken production since 2011. Endemic infections are a massive economic burden on the global poultry industry. *E. coli* infections heighten levels of flock mortality and morbidity and rejection of carcasses at slaughter.

The *E. coli* genome has a high degree of plasticity whilst retaining a degree of clonality, resulting from recombination events of short mobile elements in genome 'hotspots' [6, 26-28]. This clonal nature is advantageous in deciphering the genetic relatedness of different strains. With *E. coli* being one of the most studied microbes to date it is no surprise that an array of molecular genetic techniques exist. The *E. coli* reference collection (ECOR) originally described by Ochman and Selander shows the species *E. coli* is divided into four phylogenetic groups (A, B1, B2 and D) and a number of sub-phylogenetic groups [57-59]. The original ancestral group (B2) and D are more frequently associated with ExPEC than sister groups A and B1, which are often associated with environmental sources and commensalism [63, 64].

Allocating *E. coli* to a phylogenetic group involves a simple triplex polymerase chain reaction (PCR) [1]. Multi-locus sequence typing (MLST) allows finer resolution of the relatedness of strains than phylotyping and for the detection of slowly evolving housekeeping genes. MLST generally involves the sequencing of ~420 nucleotide bases of 6-7 housekeeping genes.

Certain traits have been identified in aiding ExPEC pathogenesis and systemic survival, including those involved in adhesion, invasion, toxin production, serum survival and iron acquisition. All have been shown to contribute to APEC pathogenesis [83, 159, 182, 185, 189, 252]. It is likely that combinations of virulence-associated genes (termed VAGs profiles or virulotypes) are needed to give rise to pathogenic *E. coli*, as no single virulence gene has been identified exclusively in APEC. A recent study demonstrates that APEC strains arise from multiple *E. coli* lineages following the acquisition of distinct VAGs, highlighting the potential high genetic diversity among these bacteria [78]. Serotyping has been used as a method for identifying APEC but several authors suggest it fails to discriminate APEC and avian faecal *E. coli* and a significant proportion of *E. coli* is untypable [31].

Previous studies have identified the gastrointestinal microbiota as a potential reservoir for APEC infection [31, 83]. It has been shown that infection follows either inhalation of contaminated faecal dust followed by dissemination into the bloodstream or possibly via active bacterial gut translocation across the epithelial barrier [129, 261]. Intestinal *E. coli* carrying numerous VAGs maybe referred to as 'potential' APEC (pAPEC) populations and their presence is likely to pose an increased risk to systemic disease.

Commercial broiler chickens are selectively bred for efficient and uniform growth. Despite commercial importance, relatively little work has exclusively focused on colibacillosis in broiler chickens [26, 31, 83, 189]. The gastrointestinal tract of a young animal is a rich ecological niche ideal for bacterial colonisation and subsequent microbial succession. The outcomes of host-microbial interactions are influenced by host (age, immunity), microbial (microbiota, VAGs) and environmental factors [262, 263]. Initially, commercial broiler gut colonisation can be influenced by: vertical transmission, the hatchery environment, handling and transportation [135, 264, 265]. Once on farm, birds are exposed to a different rearing environment, dietary changes and a series of routine vaccinations (see materials and methods).

This chapter uses virulotyping, phylogenetic typing, macro-restriction pulsed field gel electrophoresis (PFGE) and MLST to:

a. Determine temporal changes in the intestinal pAPEC reservoir with age
b. Determine whether certain VAGs and/or VAG profiles are selected for in the intestinal environment
c. Determine the population dynamics of systemic *E. coli* in diseased broiler chickens
d. Determine if there is an association between faecal and systemic *E. coli* populations

3.2 Materials and methods

3.2.1 Ethics statement

The following protocol involved the (non invasive) collection of faecal samples (using sterile cotton swabs) following excretion; no approval under the Animals (Scientific Procedures) Act (1986) was needed. No birds were culled for the purpose of this study and all dead birds intended for post-mortem examination were collected on the first daily welfare walk conducted by farmers. The study was approved by the University of Liverpool Committee on Research Ethics: Physical Interventions sub-committee (reference RETH000448), with the mandatory condition that any serious adverse events be reported to the sub-committee within 24 hours. The study was conducted in strict accordance with the University of Liverpool Research Governance policies and permission for sampling on the broiler farms was granted by the farms.

3.2.2 Sample collection

Two consecutive flock cycles on two standard commercial broiler chicken farms in the UK were visited once or twice weekly. The sampling described below commenced from the day the chicks were placed in rearing houses and was completed approximately 3 days before the first de-population event (~32-35 days). Approximately 30% of the flock is removed at first depopulation to allow farmers to conform to end-of-life stocking density standards. The flocks used in this study were routinely vaccinated as industrial practice in the UK. All isolates collected during the course of this study are available upon request.

3.2.2.1 Gut E. coli *population VAG carriage*

At each visit, 20 fresh faecal swabs were collected at random from different areas of the broiler house floor. Each swab was cultured onto eosin-methylene blue agar (EMBA) and incubated overnight at 37°C. From each plate, eight randomly selected colonies typical of *E. coli* were sub-cultured onto nutrient agar to obtain pure cultures and incubated overnight at 37°C. All media used were obtained from LabM (IDG) Ltd (Bury, UK). *E. coli* identification was confirmed using a PCR targeting the *uidA* gene [11]. One colony, representing each of the eight isolates, was pooled in 600µl of Chelex-100 and the DNA extracted for PCR analysis, see Chapter 2 for details [253].

As a means of screening faecal *E. coli,* largely expected to be non-pathogenic *E. coli,* each pooled DNA extract was screened for four VAGs previously associated with avian *E. coli* pathogenesis; *iss, tsh, iucC* and *cvi,* using a multiplex PCR [252]. Primer sequences, thermocycler conditions and PCR product analysis can be found in Chapter 2 and were originally described by Skyberg *et al* (2003) [252]. When a sample pool was positive for ≥ 3 of the 4 genes, a new Chelex-100 preparation was made for each individual isolate within the pool. Pooled samples with fewer than 3 VAGs were discarded.

The individual isolate DNA templates were then screened for 10 VAGs; *astA, iss, irp2, iucD, papC, tsh, vat, cvi, sitA* and *ibeA*. Three separate PCR assays were performed; one multiplex PCR previously described by Ewers *et al.* [182] and two single PCR assays for *ibeA* and *sitA* outlined by Timothy *et al.* [110]. Primer sequences and reaction conditions are described in Chapter 2 The presence or

absence of the 10 VAGs produced a series of 10 numbers, which denoted the VAG profile for each isolate (presence ‘1’ or absence ‘0’).

3.2.2.2 Post-mortem examination of dead broiler chickens

As well as faecal sample collection throughout rearing, from week 2 onwards, at each faecal sampling time point, 8 dead birds were collected from the first welfare walk of the day for post-mortem examination. To minimise the detection of systemic *E. coli* resulting from a loss of intestinal integrity following death, only birds identified as recently dead were included. Birds were only selected for post-mortem examination if they did not show signs of extensive pecking, had not been trodden on (flattened appearance) and/or did not have broken legs or other obvious injury. For all birds, any observed classic colibacillosis characteristics were recorded including; ascites, airsacculitis, cellulitis, enlarged spleen, pericarditis and perihepatitis [48]. For each bird, up to 1 gram of the following tissues were collected; heart, kidney, liver, lung and spleen using sterile forceps and scalpels. An equal volume of sterile phosphate buffered saline (PBS) was added to each sample and tissues were homogenised using a Biomaster Micro-stomacher 80 (Steward, UK) for 60 seconds at high speed. 50µl of the homogenate was streaked onto EMBA and incubated overnight at 37°C. Eight *E. coli* colonies were picked, re-plated onto nutrient agar and incubated overnight at 37°C. All isolates were immediately subjected to a full screen of all 10 virulence genes using the assays described previously and each isolate was given a corresponding VAG profile.

3.2.2.3 Statistical analysis

Collected data were analysed using multiple statistical tests. Intestinal *E. coli* VAG profile diversity at each sampling time point was calculated using Simpson's diversity index (D). Generalised linear models (GLMs) were used to investigate the relationship between VAG profile diversity and time. Several different statistical measures were used a) the Pearson's correlation coefficient from VAG profile diversity data and the detection of potential APEC isolates b) the P-value obtained from the Fisher's exact test to assess the distribution of VAG genes between faecal and systemic *E. coli* population. Associations were considered statistically significant if the calculated P-value was < 0.05.

3.2.3 Phylogenetic typing

Faecal and systemic isolates collected from one of the four flock cycles underwent further molecular analysis by phylogenetic typing. Two hundred and sixteen faecal and 35 systemic *E. coli* were analysed. Isolates were assigned to 1 of 4 *E. coli* phylogenetic groups (A, B1, B2 or D) using a triplex PCR targeting *chuA, yjaA* and the DNA fragment TSPE4.C2 [1].

3.2.4 Macro-restriction pulsed-field gel electrophoresis

Two hundred and twenty two faecal and 48 systemic *E. coli* were analysed using PFGE. The PFGE protocol used was based on the standardised Pulsenet Rapid *E. coli* method [103] with slight modifications. During sample preparation, plugs were incubated for 2h at 54^{0}C with vigorous shaking at 175rpm and for sample digestion; each sample was incubated for 2h with 50U of *Xba*1 restriction enzyme (Roche

products Ltd, Hertfordshire) at 37^{0}C. Samples were run on a 1% 0.5X Tris-Borate running buffer (TBE) (Life technologies, UK) agarose universal (alpha laboratories, Hampshire) with 0.5X TBE running buffer for 20 hours at 14^{0}C, at 6V/cm^{2} with the initial switch time of 2.2s and final switch time of 54.2s in a CHEF-DRIII PFGE system. A Lambda ladder PFGE marker (New England Biolabs, Ipswich, MA, USA) was run on each gel. The gel was stained in an ethidium bromide solution (500µl ethidium bromide in 500ml 0.5X TBE running buffer) for 25 mins and visualised under UV using a transilluminator. Samples which failed PFGE analysis, were re-tested with a longer proteinase K incubation period; 24h at 54^{0}C with vigorous shaking at 175rpm. Image analysis was performed using BioNumerics version 4.0 and Dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

3.2.5 Multi-Locus sequence typing (MLST)

Fifty *E. coli* isolates from intestinal and systemic sites were analysed by MLST. Genomic template DNA was prepared using the Chelex 100 DNA extraction method as previously described [253]. Seven house-keeping genes were targeted for PCR; adenylate kinase (*adk*), fumarate hydratase (*fumC*), DNA gyrase (*gyrA*), isocitrate dehydrogenase (*icd*), malate dehydrogenase (*mdh*), adenylosuccinate dehydrogenase (*purA*) and the ATP/GTP binding motif (*recA*) [62]. All primer sequences and a detailed protocol are given by Wirth *et al.* [62]. For this present study, the PCR based protocol was modified slightly and each 25µl reaction contained: 0.5µl of each forward and reverse primer (20pmol), 23µl of 1.1 x Reddymix with 1.5mM $MgCl_2$ and 1µl of template DNA (Chelex 100 extractions). The PCR conditions included an initial denaturation at 95^{0}C for 2 mins, 30 cycles of; 95^{0}C for 1 mins, target specific

primer annealing temperature for 1 mins (outlined in Wirth *et al.* , 2006) and a final extension at 72^{0}C for 5 mins. PCR success was confirmed by running products on a 1.5% agarose gel in TAE buffer for 30mins at 150v. The remaining product was cleaned using a 20% (w/v) polyethylene glycol (PEG_{8000}), 2.5M NaCl (Yorkshire Bioscience Ltd, UK) precipitation protocol. Cleaned PCR products were sequenced commercially (Macrogen, Korea) with 1:15 diluted sequencing primers (same as amplification primers). Sequences were analysed using ChromasPro version 1.5 (Technelysium, Australia) and MEGA 5.05 [95] and submitted to the Achtman *E. coli* MLST online database (http://mlst.ucc.ie/mlst/dbs/Ecoli). To determine the genetic relatedness of our STs and those previously submitted to the online database, eBurst (version 3) diagrams were constructed following the online instructions (http://eburst.mlst.net/).

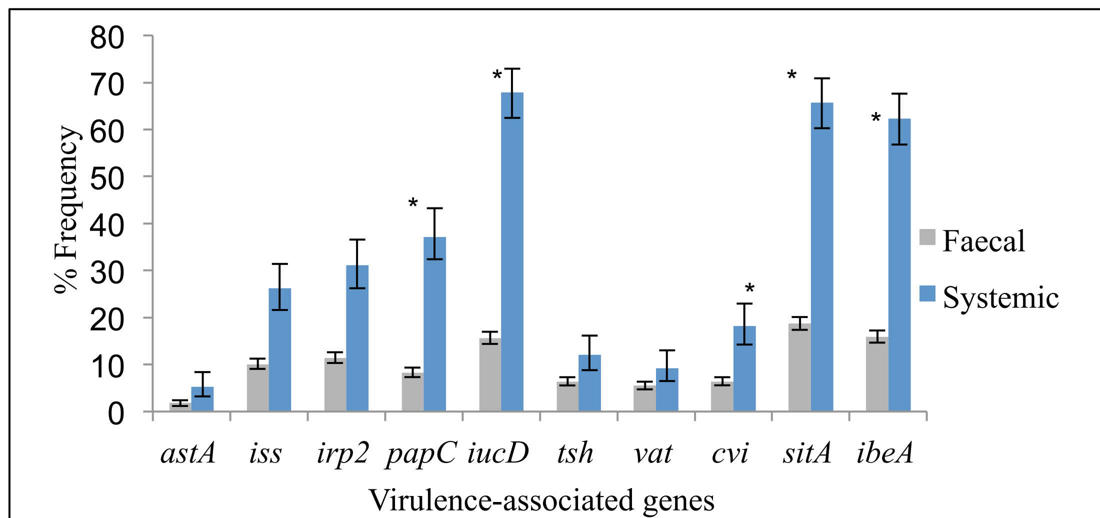
3.3 Results

3.3.1 *E. coli* carriage of virulence-associated genes in healthy broiler chickens

A total of 420 *E. coli* pools were obtained from apparently healthy birds from two flocks on two farms, between May and July 2011 and following initial screening, 119 were positive for ≥ 3 of the 4 targeted VAGs. Thus a total of 952 isolates were assigned a VAG profile out of 3360. Generally, fewer pooled samples met the threshold as birds aged. Overall, VAGs were more frequently associated with systemic *E. coli* populations than faecal ones (Figure 3.1). For individual intestinal *E. coli* isolates, the *sitA* gene was the most commonly detected VAG, ranging between 0.68% and 20.57% prevalence, with an average detection of 8.51% over each sampling point for all flocks. Toxin encoding genes (*astA* and *vat*) were the least frequently detected in these populations; 0.00-11.25% (average 1.12%) and 0.00-9.38% (average 2.11%) for *astA* and *vat* respectively over the four flock cycles. Genes associated with iron acquisition, *sitA*, *iucD* and *irp2*, were commonly carried by individual isolates averaging 5.10% and 7.34% for *irp2* and *iucD* respectively.

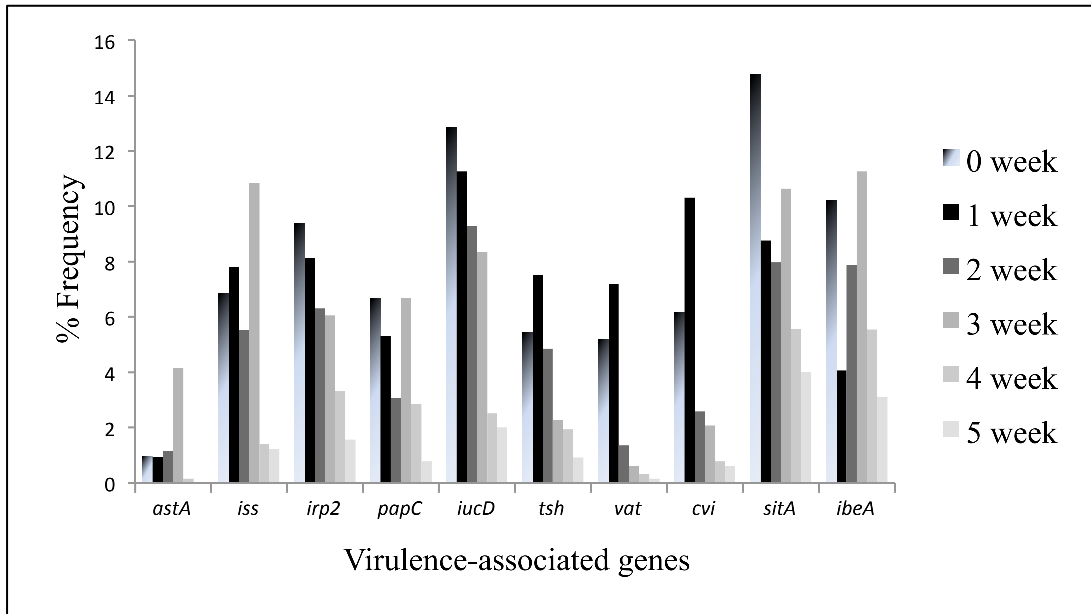
The frequency at which the invasion-related gene, *ibeA*, was detected varied, ranging from 0.6% to 17.73% over the 4 flock cycles. At t=0, *ibeA* detection ranged from 5 - 14.10%. Over the first week, the level of *ibeA* detection decreased, before peaking between weeks 2-3 (approximately 13%) and then declining once again (to 3.12%) towards week 5. A similar trend was also observed for *iss* detection (Figure 3.2).

Figure 3.1 Comparison of faecal and systemic *E. coli* VAG carriage



Upper and lower bound 95% confidence intervals indicate statistically significant differences between VAG carriages within the two populations. Fisher's exact test indicates that irp2, papC, iucD, cvi, sitA and ibeA are significantly more associated with systemic E. coli.

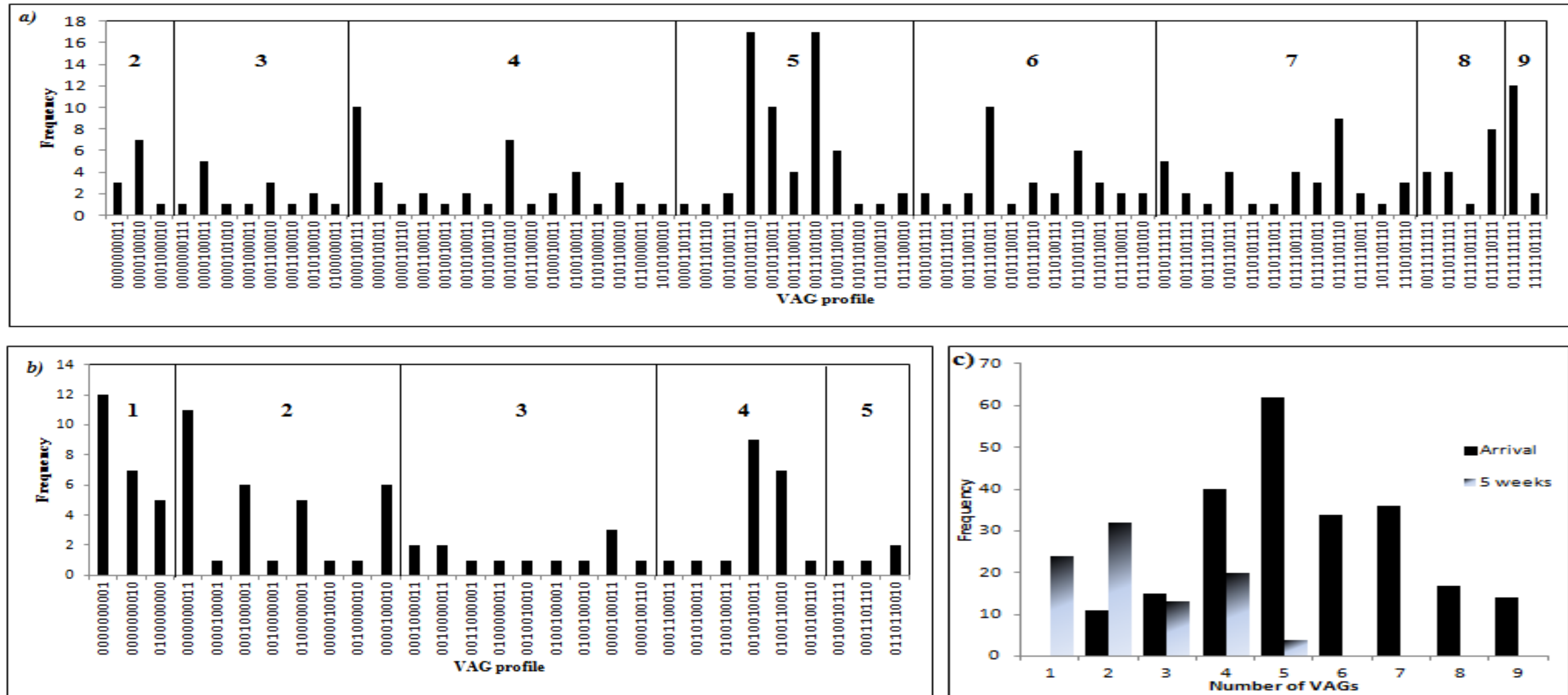
Figure 3.2 Average percentage frequency of VAGs



Average percentage frequencies of 10 VAGs were calculated and plotted against time, from t = 0 (arrival) to t = week 5 (depopulation). Overall, VAGs appear to decline with time, with a peak in detection at week 3 for iss, sitA and ibeA. Iron acquisition genes irp2 and iucD consistently decline with time.

VAG profiles (P-) were created based on observed combinations of the 10 different VAGs targeted (a systematic numbering system). A total of 206 different unique profiles were observed in the faeces of apparently healthy broiler chickens; P-1 (astA, iss, irp2, iucD, papC, tsh, cvi, vat, sitA, ibeA: 0000000001) represents the carriage of ibeA only, whereas P-206 is assigned to isolates carrying none of the targeted genes. P-206 was the most common profile detected in all flocks and its level of detection increased with time perhaps suggesting a positive selection for non-pathogenic traits within an intestinal population. Figure 3.3 shows the frequencies of detection for different profiles at t=0 and t=5.

Figure 3.3 Virulence-associated gene profile diversity for all flocks



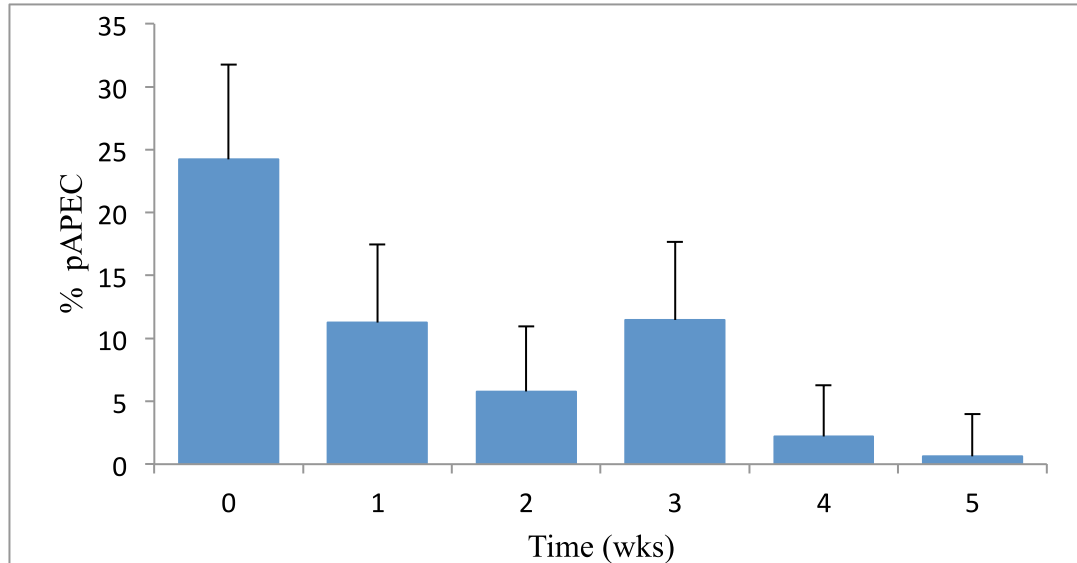
a) Shows the VAG profiles identified at t = 0. Profiles consisting of 4 VAGs were the most diverse, with differences in iron acquisition genes being the most abundant, while profiles 0010101110 (irp2^{+}, papC^{+}, vat^{+},cvi^{+}, sitA^{+}) and 0011101010 (irp2^{+}, iucD^{+}, papC^{+}, vat^{+}, sitA^{+}) were the most common profile b) Shows the VAG profiles identified at t = week 5. VAG profile diversity had declined over time.. No isolates carried more than 5 VAGs c) Comparison of total number of VAGs carried by E. coli at t = 0 and 5. Profile 206 (0000000000) excluded from both graphs.

3.3.2 Changes in VAG profile diversity with respect to farm/flock and time

When the VAG data were analysed for individual farms and flocks; F1C1 (farm 1; cycle1), F2C1, F1C2 and F2C2, a total of 57, 45, 86 and 112 different VAG profiles were identified, respectively. Sixty two out of 206 different profiles (30.10%) were detected on >1 farm/flock, while 69.9% were only identified on one farm. Despite farm/flock individual VAG profile frequency differences, a common trend was observed with respect to time.

On average, 24.05% of *E. coli* isolates screened from the gastrointestinal tract of chicks at t=0 (placement) carried at least 5 of the 10 VAGs (termed pAPEC) (Figure 3.4). The *sitA* gene was consistently the most frequently detected VAG from all four flock cycles on the two farms.

Figure 3.4 Average percentage of pAPEC with respect to time



At weekly intervals the average percentage of pAPEC, defined by the carriage of ≥5 VAGs, from the total faecal E. coli *population was calculated. At each time point, 160 faecal* E. coli *were assessed. 95% upper confidence interval error bars shown.*

Simpson's diversity index (D) was used to compare the profile diversity at each week of production for the second flock cycles on both farms; D values are shown in Table 3.3. Generalised linear models confirmed the significant effect of time on VAG profile diversity ($p<0.05$). Overall, VAG profile diversity declined through time, with a common peak at week 3 of production (Table 3.1).

Table 3.1 Simpson's diversity index for VAG profile diversity through time

	D value	
Week	**F1**	**F2**
0	0.683	0.779
1	0.683	0.359
2	0.438	0.582
3	0.704	0.686
4	0.070	0.307
5	0.391	0.200

Simpson's diversity index (D) was used to compare VAG profile diversity through time in the second flock cycles of farm 1 (F1) and farm 2 (F2). Overall, profile diversity decreases with time, with a peak at week 3.

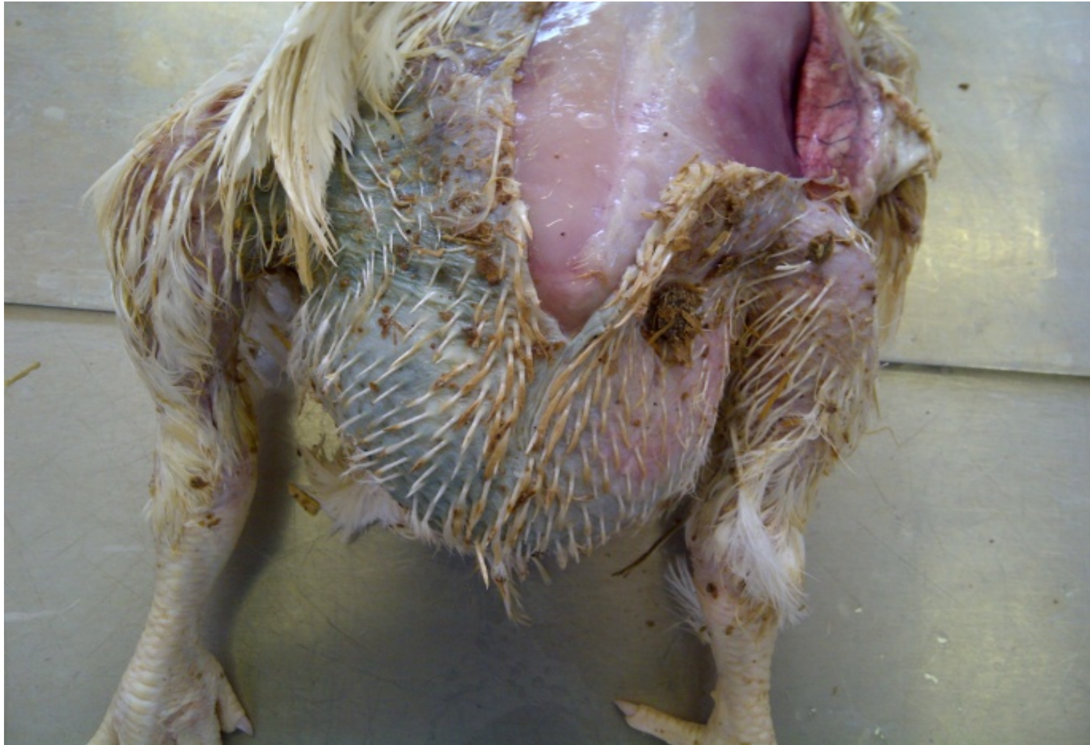
As birds aged, the percentage frequency of pAPEC in the gastrointestinal tract declined. Prior to the first depopulation event at 5 weeks of production, only 1% of *E. coli* carried ≥5 VAGs.

An average decrease of 12.97% in pAPEC from t=0 to week 1 was detected, followed by a further 5.47% decrease between weeks 1 and 2 (Figure 3.4).

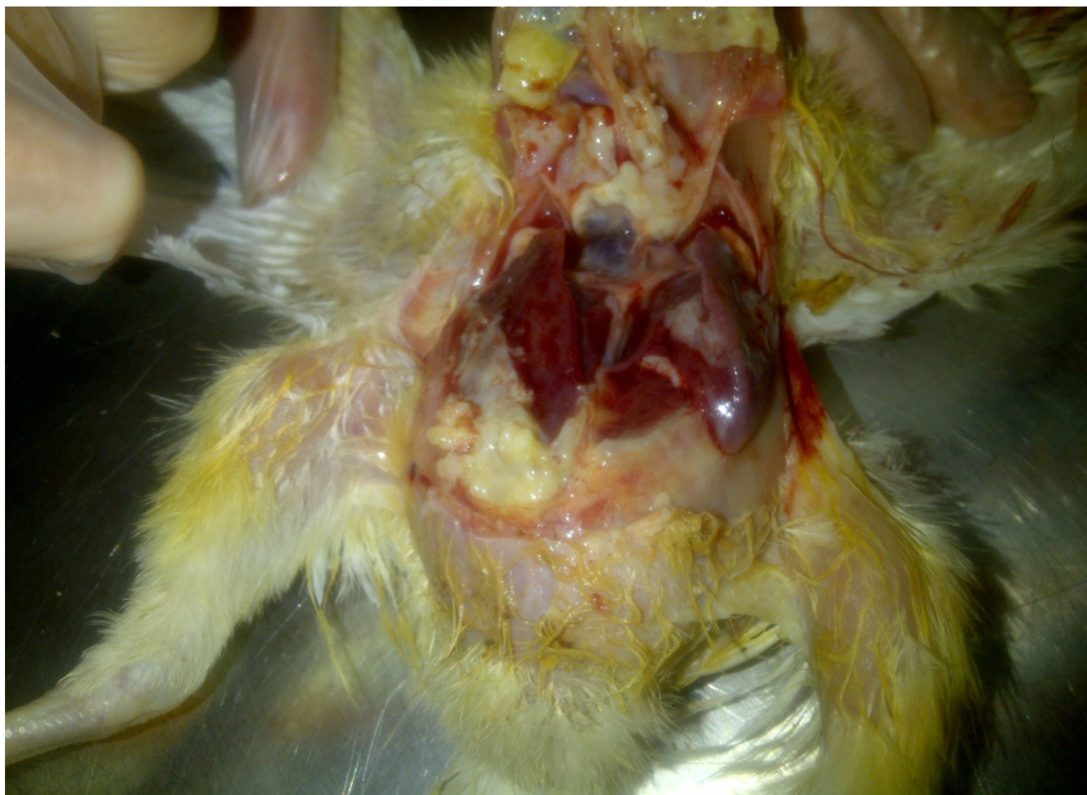
3.3.3 Longitudinal analysis of systemic *E. coli* carriage of virulence-associated genes

On average, over the four flocks, 39.1% of dead birds (n=128) collected on the first daily welfare walk showed signs of colibacillosis and systemic *E. coli* was identified, see Figure 3.5. Three hundred and twenty four isolates were virulotyped. Figure 3.1 shows the distribution of VAG frequencies between both faecal and systemic *E. coli* populations. Fisher's exact test was used to assess the frequency differences between the faecal and systemic populations; *irp2, papC, iucD, cvi, sitA* and *ibeA* genes were significantly associated with systemic *E. coli* populations ($p<0.05$); *astA*, *vat*, *iss* and *tsh* were not ($p>0.05$). Sixty-three different VAG profiles were identified among systemic *E. coli*. Thirteen of the 63 profiles (20.63%) were found on more than one farm. Fifty-eight of 324 isolates (17.90%) carried no VAGs (P-206). P-15 (*ibeA*$^{+}$, *iucD*$^{+}$, *sitA*$^{+}$) was the second most frequent profile (9.88%). However, this was only identified on F1C1. Of the profiles that were found on more than one farm, 46.26% accounted for profiles with ≥4 VAGs; in all these profiles at least 50% of the genes detected were involved in iron acquisition. None of the tested isolates carried more than 7 VAGs. Observed VAG profile diversity was not correlated with the number of *E. coli* investigated ($p>0.05$), suggesting sample size variation has not influenced profile detection and thus the reported level of diversity. Over the four flock cycles, 36.4 – 80% of VAG profiles identified in systemic isolates were also identified at least once in faecal isolates collected before and/or at the same time from apparently healthy birds during the same cycle. Nineteen profiles out of 63 were unique amongst systemic isolates, only one of these profiles was identified on more than one occasion (P-221; *iss*$^{+}$, *irp2*$^{+}$, *papC*$^{+}$, *iucD*$^{+}$). Overall, there were no profiles wholly associated with diseased birds.

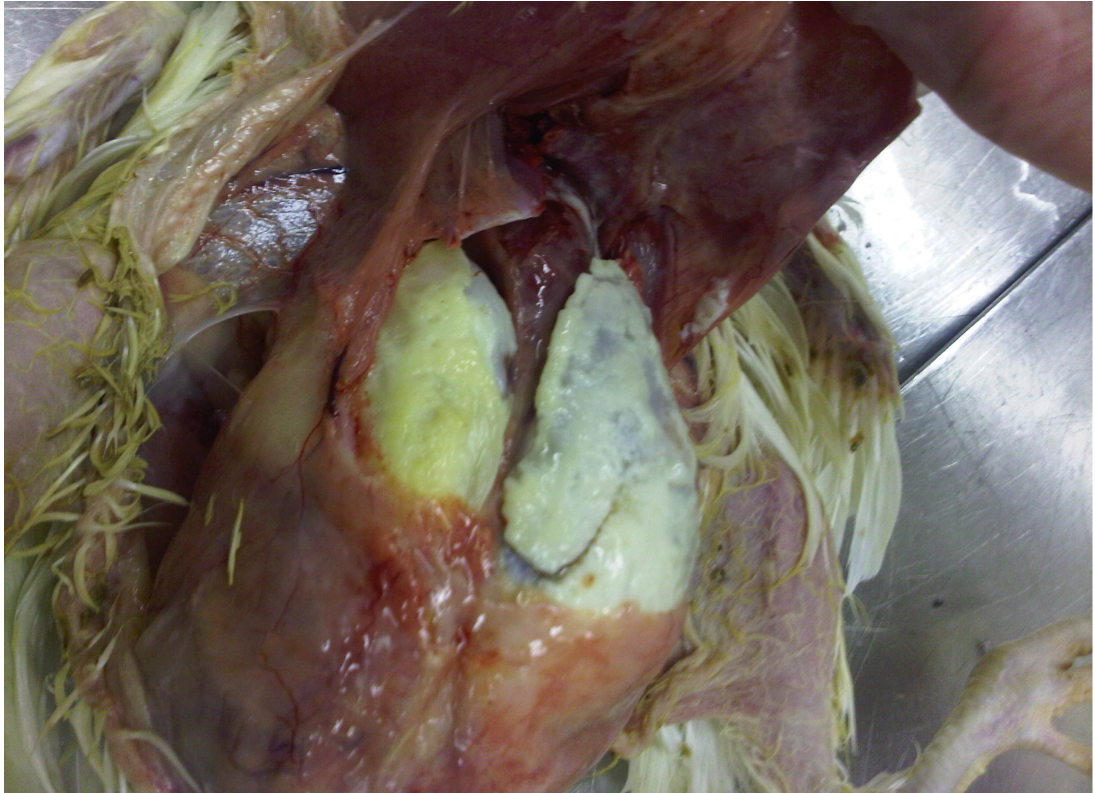
Figure 3.5 The clinical manifestations of colibacillosis



a) 35 day old broiler chicken with ascites (accumulation of fluid in the abdominal cavity)

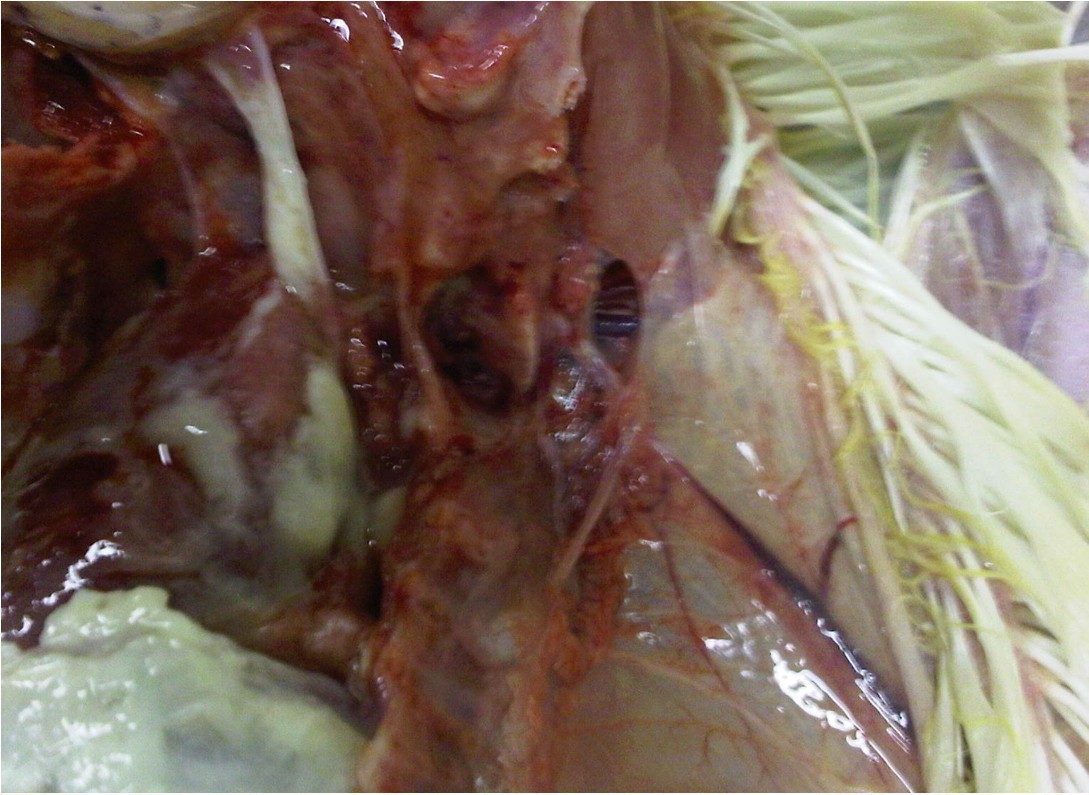


b) 14 day old broiler chicken with cellulitis



c) 35 day old broiler chicken with perihepatitis (fibirn lesions coating the liver)

d) 28 day old broiler chicken with perihepatitis



e) 14 day old broiler chicken with pericarditis (fibrin based inflammatory lesions of the pericardium)

3.3.4 Phylogenetic analysis

Table 3.2 shows the assignment of 216 faecal and 35 systemic *E. coli* collected from F1C2 to the four phylogenetic groups and 1 subgroup. If no amplification occurred for any of the 3 targets, isolates were assigned to subgroup A_0 [266, 267].

Table 3.2 Assignment of faecal and systemic *E. coli* to phylogenetic groups

	Number of isolates (% frequency)				
Source	**A**	**A0**	**B1**	**B2**	**D**
Faecal	85 (39.35)	21 (9.72)	1 (0.46)	5 (2.31)	104 (48.15)
Systemic	11 (31.43)	14 (40.00)	0 (0.00)	1 (2.86)	9 (25.71)
Total	96	35	1	6	113

216 faecal and 35 systemic E. coli *were typed using the Clermont* et al *triplex PCR and assigned to 1 of 5 phylogenetic groups. Those isolates that showed no amplification of any of the 3 targets were assigned to subgroup A0. Group D was the most frequently detected phylogenetic group among faecal population, while A0 (untypable) was the most common group among systemic isolates. B2 and D have been previously associated with more pathogenic* E. coli *but they only accounted for 28.57% of systemic isolates in this study.*

With the exception of week 2 (22.50%; individual data not shown), group D was the most frequently detected phylogenetic group among faecal isolates. In week 2, group A was the most frequently detected phylogenetic group (58.75%). The screening-based protocol of faecal isolates would have led to sampling bias towards ones

containing VAGs and therefore possibly group B2 and D isolates. There are no obvious changes in phylogenetic groups through time.

Fourteen of 35 (40%) systemic isolates grouped into phylogenetic group A_0. Pathogenic associated phylogenetic groups D and B_2 represented 25.71% and 2.86% of systemic isolates respectively. Results suggest that no distinct phylogenetic group accounts for systemic *E. coli*.

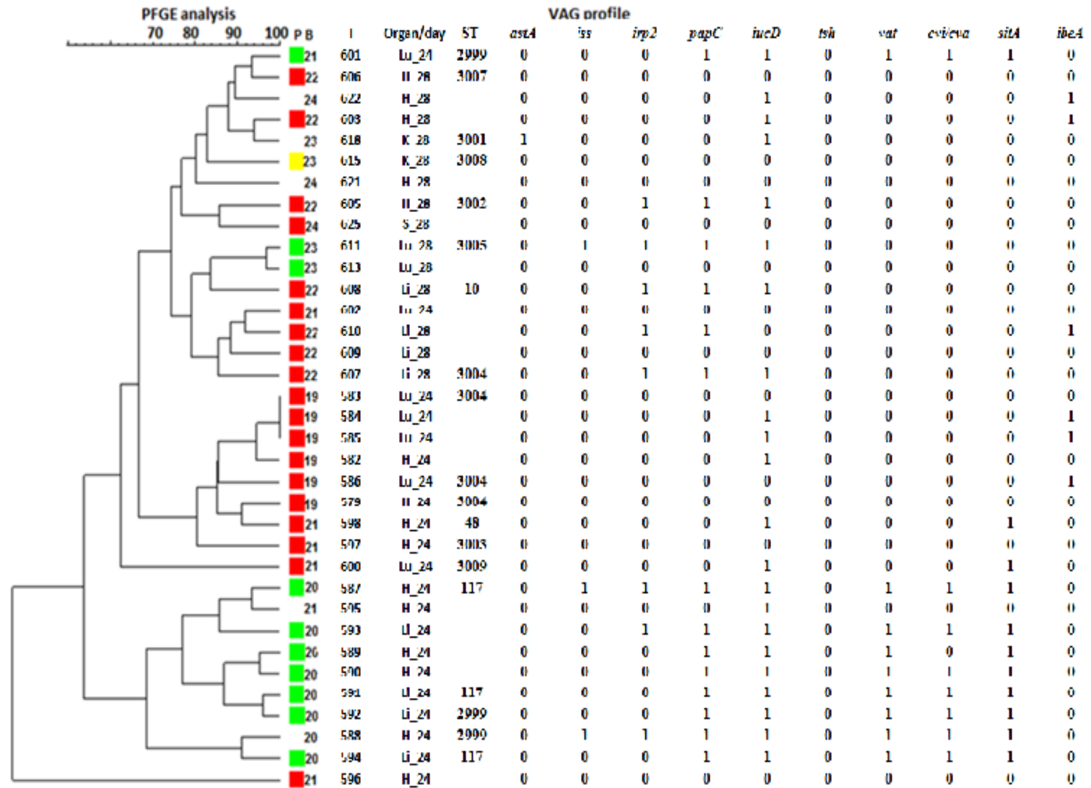
3.3.5 Macro-restriction PFGE analysis

Two hundred and twenty two faecal and 48 systemic *E. coli* isolated from the same flock were analysed by PFGE to look for changes in gut population through time, common strain types associated with systemic *E. coli* and to relate genetic background to the carriage of VAGs. One hundred and sixty six faecal and 35 extraintestinal isolates were successfully digested and dendrograms constructed.

A dendrogram constructed from the pulsotypes of 48 *E. coli* isolated from faeces at t=0 shows large strain diversity and no apparent association between phylotype and VAG carriage. The 48 isolates fell into 5 groups with 80% similarity. There appears to be no retained strain type correlated with time.

Thirty-five systemic *E. coli* belonged to 10 groups with 80% similarity (Figure 3.6), suggesting a diverse strain population amongst systemic isolates. The dendrogram also highlights the isolation of multiple strain types from individual diseased birds and the presence of similar strain types amongst faecal and systemic isolates.

Figure 3.6 Dendrogram constructed using DICE for systemic *E. coli using XbaI PFGE*



(tolerance 5%) (minimum height >0.0%, minimum surface >0.0%)(0.0-100% coefficient). A dendrogram showing the relatedness and strain diversity amongst systemic E. coli *harbouring APEC VAG using BioNumerics software by unweighted pair group method with Arithmetic mean. The dendrogram also shows; phylogenetic group (P) (green = D; red = A; yellow = B2), isolate (I), organ and age of bird at isolation (H = heart: K = kidney: Li = liver: Lu = lung; S = spleen), MLST sequence type (ST) and VAG profiles. The dendrogram shows the clustering of ST 117 and 2999 isolates (excluding 601) which by PFGE analysis are ~60% different from other isolates. Several ST 3004 were identified and these potentially show the acquisition of 2 Iron acquisition genes (irp2 and iucD) while other ST 3004 isolates have no VAGs (isolates 579 and 583).*

3.3.6 MLST analysis

To assess the underlying clonal association between isolates with VAGs in faecal and diseased bird populations, 24 faecal *E. coli* (8 with ≥5 VAGs, 8 with <5 VAGs and 8 with 0 VAGs) and 23 extraintestinal *E. coli* (11 with ≥5 VAGs, 7 with <5 VAGs and 5 with 0 VAGs) were submitted to the MLST online database (http://mlst.ucc.ie/mlst/dbs/Ecoli). All results are shown in Table 3.3.

In total, 33 new sequence types (ST) were identified, 6 of which were single locus variants (SLV) of ST-10. This was the only clonal complex (CC-10) in which faecal and systemic isolates were clustered.

Interestingly, 3 of the 24 faecal isolates were identified as ST-352, all 3 isolates carried more than 5 VAGs 1) *astA*$^+$, *irp2*$^+$, *papC*$^+$, *iucD*$^+$, *vat*$^+$, *cvi*$^+$, *sitA*$^+$ 2) *iss*$^+$, *irp2*$^+$, *papC*$^+$, *iucD*$^+$, *vat*$^+$, *cvi*$^+$, *sitA*$^+$ 3) *iss*$^+$, *irp2*$^+$, *papC*$^+$, *iucD*$^+$, *vat*$^+$, *cvi*$^+$, *sitA*$^+$. ST-352 did not cluster with any of the other faecal or systemic isolates in the constructed eBURST diagrams.

Four ST-2999 isolates (representing 22.22% of systemic isolates tested) were isolated from two diseased birds, ST-2999 is a SLV of the emerging pathogenic clone ST-117 [98]. All four ST-2999 isolates carried ≥5 VAGs and no ST-2999 isolates were identified among the faecal population (Figure 3.7). Furthermore, the genetic relatedness of ST-2999 and ST-117 is highlighted by their general clustering in constructed PFGE dendrograms (Figure 2.6). ST-48 (CC-10) and ST-10 (CC-10) were also among those already known STs identified in systemic populations. ST-3004 was identified only among systemic isolates. ST-3004 isolates were found to differ in the number of VAGs they carried; no VAGs (isolate 579 and 583), 1 (isolate

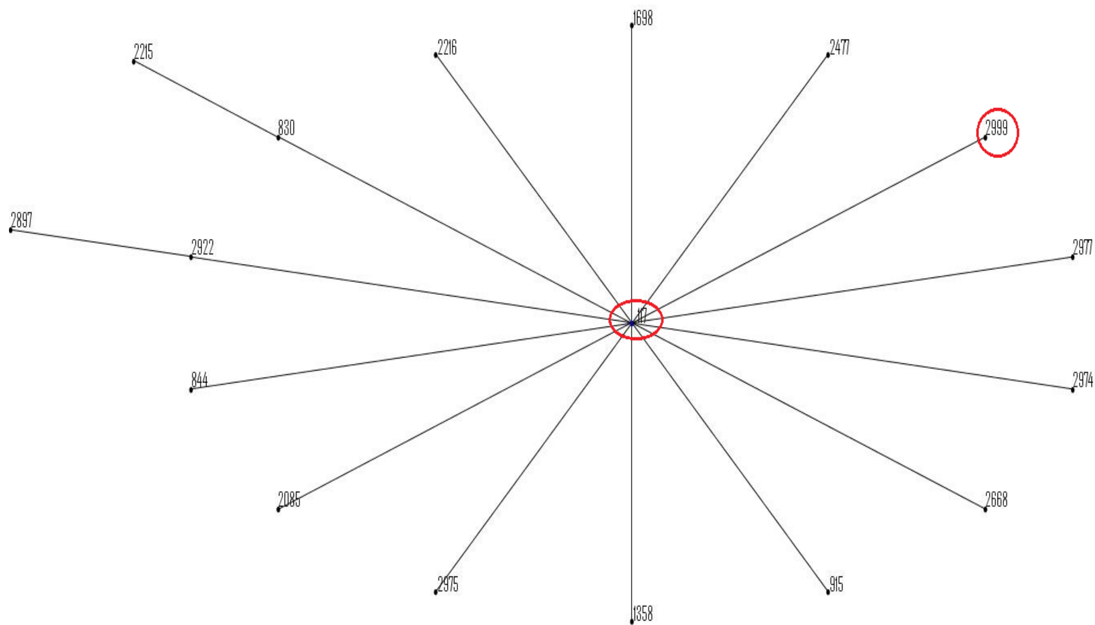
586) and 3 (isolate 607). Two out of the 3 VAGs are involved in iron acquisition (*irp2* and *iucD*) (see Figure 3.6).

Table 3.3 Observed faecal and systemic *E. coli* MLST Sequence types categorised by VAG carriage

VAGs	Site of isolation	
	Faeces	**Systemic**
0	2990, 2991, 2992, 2993, 2994, 2995, 2996, 2997	3003, 3004 (2), 3007, 3008
< 5	2980, 2981, 2982, 2983, 2987, 2988, 2989	3001, 3002, 3004 (2), 3005, 3006, 3009, 10
≥ 5	352 (3), 2978, 2984, 2985, 2986, 3010	117 (3), 2998, 2999 (4), 3000

(n) = ST observation frequency. All faecal E. coli *belonged to newly identified sequence types (ST) excluding ST-352. Interestingly, all ST-352 isolates harboured more than 5 VAGs with the following profiles:1) astA$^+$, irp2$^+$, papC$^+$, iucD$^+$, vat$^+$, cvi$^+$, sitA$^+$ 2) iss$^+$, irp2$^+$, papC$^+$, iucD$^+$, vat$^+$, cvi$^+$, sitA$^+$ 3) iss$^+$, irp2$^+$, papC$^+$, iucD$^+$, vat$^+$, cvi$^+$, sitA$^+$ and they did not group with other* E. coli *in the online database. Systemic* E. coli *analysis identified 3 ST–117 and 4 ST- 2999 isolates; however ST-2998 and ST-3000 did not cluster with the other two STs in this category.*

Figure 3.7 Multi-locus sequence typing Eburst diagram showing clustering of the new sequence type (ST-2999) and emerging pathogenic ST-117



Multi-locus sequence typing (MLST) identified 4 systemic E. coli *that belonged to the new sequence type (ST): ST-2999. All isolates carried ≥ 5 virulence-associated genes. ST-2999 is a single locus variant of the previously identified emerging pathogenic ST- 117.*

3.4 Discussion

To our knowledge, this is the first study to address the longitudinal diversity of intestinal *E. coli* populations with a focus on APEC VAG carriage, while simultaneously characterising systemic *E. coli* isolated from visceral organs of diseased birds in UK broiler flocks.

Previous work suggests that the clonal nature of *E. coli* makes it possible to associate certain lineages with ExPEC status that could help elucidate a “typical” APEC [74, 85, 89, 96, 268]. The *E. coli* genome has a high degree of plasticity whilst retaining a level of clonality resulting from recombination events of short mobile elements in genome ‘’hotspots’’. These elements often contain VAGs [6, 26-28]. A similar observation was made recently regarding the clonality of extended β-lactamase producing *E. coli* [112]. Research suggests that APEC arise from the acquisition of VAGs and certain lineages may be more accepting of incoming genetic elements and thus pathogenic [217, 269, 270]. In the current study, MLST identified a new sequence type (ST-2999) among the systemic isolates carrying ≥ 5 VAGs. ST-2999 is an SLV of ST-117, a potentially emerging pathogenic ST previously associated with retail chicken and human disease [98, 99]. ST-117 was also identified among the systemic isolates. PFGE allows for more refined comparisons between isolates and here confirmed the genetic relatedness between these isolates compared to the other systemic ones. However, the overall high level of strain diversity among systemic *E. coli* isolated from diseased birds; the lack of correlation with VAG carriage and the identification of multiple strains as opposed to a single clone in one bird perhaps suggests the opportunistic nature of certain *E. coli* [32]. Additionally, this perhaps suggests that differences in broiler susceptibility were identified [26, 78,

89]. As only a subset of isolates were subjected to genetic analysis, it is possible to have underestimated the level of diversity present. However, it is clear a high level of diversity is present.

The intestinal *E. coli* population of birds has previously been identified as an APEC reservoir [31, 83]. The findings from this study further support this with 36.4 - 80.0% of systemic VAG profiles also being identified among faecal *E. coli* of the same flock.

E. coli is one of the first bacterial species to colonise the neonatal gut before succession [12, 271, 272]. A large proportion of pAPEC contributed to early colonisation of the neonatal chick (24.05% of tested population). Sources of such *E. coli* include: parent flock (vertical transmission), hatchery environment, human handling, and transportation equipment [135, 264, 265]. Yassin *et al.* (2009) correlated first week chick mortalities with hatchery and breeder age, highlighting the potential important influence of these factors [273]. Interestingly, despite all four flocks in this study being sourced from different hatcheries, the level of observed pAPEC at this stage was comparable. Past studies have shown that that the possession of VAGs could be advantageous in microbial gut populations offering commensalism fitness advantages [274-276]. The positioning of VAGs on mobile genetic elements would allow for their selective maintenance within populations [6, 26, 185].

As birds aged, both VAG profile diversity and the detection of pAPEC declined; by the last week of production, 1% of the population sampled were classified as pAPEC. Furthermore, as birds exceeded 3 weeks of age there was a noticeable decline in the proportion of pooled samples reaching the 3 VAG threshold outlined in our sampling

protocol, suggesting a negative selection in the avian gut. Younger birds have been shown to possess a more diverse microbiota compared to that of older birds, likely to be due to rapid initial opportunistic colonisation of an available ecological environment; with age microbial succession and microbial bottle necking occur [263, 277]. The bottle necking of VAG diversity and pAPEC with microbial succession may represent the persistence of stronger colonisers and the loss of more transient strains. One hypothesis is that different VAGs offer selective advantages at different stages of development [6]. A note of caution is required, as our list of VAGs is not an exhaustive list of APEC-associated virulence genes.

Irrespective of time, *sitA* was the most frequently detected VAG in this study. The *sitABCD* encoded transporter regulates iron and manganese transport and provides increased resistance to oxidative stress [188]. This mechanism could be advantageous among competing gastrointestinal populations and during inflammation [263]. Additionally, a redundancy of iron acquisition systems is thought to be advantageous in environmental survival [278]. Interestingly, our study identified multiple ST-3004 isolates which differed in their possession of VAGs namely ones involved in iron acquisition (*irp2* and *iucD*). Could this be the result of gene transfer and acquisition?

The *ibeA* gene was detected among intestinal *E. coli* populations of young birds. The *ibeA* gene encodes a 50kDa protein thought to aid microvascular epithelial adherence and invasion in the brain [159]. The exact mechanism of IbeA remains to be determined but it has been shown to modulate type 1 fimbriae [154]. The advantage of possessing *ibeA* while in the gut remains unknown; it could relate to the increased

survival of attached *E. coli,* particularly in a transient inflammatory environment [156, 159].

The 10 VAGs selected for this study do not represent an exhaustive list of APEC determinants [279]. For future work, an investigation published after this study was carried out presents a new virulotyping protocol offering vastly improved error margins in APEC detection, ideal for epidemiological studies [132]. Based on the literature, the APEC pathotype is likely to contain a mix of iron acquisition genes and those encoded on plasmids [31, 189]. This was reflected in our chosen panel of VAGs. It was necessary to add a level of bias to the faecal sampling given the ubiquitous nature of *E. coli* in the gastrointestinal tract allowing practical detection of the proportion of the population that are potentially pathogenic. Such sampling is technically demanding and labour intensive. The 4 VAGs used in the initial screening were selected based on their high prevalence among APEC strains; *iss* (~83%), *iucC* (75%), *tsh* (53-63%) and *cvi* (63%) [77, 165]. This panel allowed for the detection of as many pAPEC as possible given the limitations in screening the large number of samples. All calculations regarding the 'proportion of potentially pathogenic *E. coli'* were calculated using the entire population sampled, i.e. the original number of *E. coli* picked before initial PCR screening.

The avian host also contributes to shaping the microbiota. Lu *et al.* (2003) described a more stable microbiota between 2 and 4 weeks of age in fast growing birds, reflecting the current study which observed more consistent levels of pAPEC between weeks 4 and 5 [263]. Immunological changes during host development are likely to contribute to changes in the microbiota; heterophil function (avian polymorphonuclear neutrophils (PMNs)) has been shown to be lacking in day old

chicks [280]. Crhanova *et al.* (2011) reported transient gut physiological inflammation in 4 day old chicks, while the cellular immune responses to *Salmonella* Typhimurium of 1 day old and 1 week old chicks have been shown to be markedly different, suggesting rapid immunological changes in early life [241, 263, 277, 281]. It is likely that a combination of host (immunity and vaccination), microbial (microbiota composition, VAG carriage) and environmental (feed, production systems) changes has contributed to the changes in pAPEC observed in this study, highlighting the importance of host-microbial interactions [282]. This warrants further investigation.. It would be of interest to determine causes of death in the first 48-72 hours of life; a period of limited heterophil function, often the point of highest mortality during commercial rearing and as noticed in this study the point in production where APEC VAGs are at the greatest prevalence in the avian gut [273].

In summary, we have shown colonisation of the broiler gut by pAPEC may occur before chicks are placed and as broilers age these populations shift while VAG diversity bottlenecks. The reasons for this remain to be determined. Our work supports that of others, identifying the avian gut as an APEC reservoir, but did not find a predominant APEC pathotype in the flocks studied. The identification of highly diverse systemic *E. coli* populations rather than single or highly related clones perhaps suggests the broiler chicken and its susceptibility is a major contributor to disease manifestation. Further work is required (i.e. molecular analysis on more isolates, elucidation of contributing impacting factors to pAPEC dynamics), but this study offers the first insight into the temporal movement and dynamics of *E. coli* in the avian host and offers a new approach to deciphering APEC.

Chapter 4

Manuscript 2

The contribution of systemic *Escherichia coli* infection to the early mortalities of commercial broiler chickens

K. Kemmett, N. J. Williams, G. Chaloner, S. Humphrey, P. Wigley, T. Humphrey

University of Liverpool, Leahurst Campus, Neston, CH64 7TE, UK

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) are a substantial burden to the global poultry industry. APEC cause a syndromic poultry infection known as colibacillosis, which has been previously associated with broiler chickens over 2-weeks old. We previously described the intestinal tracts of day-old broilers to harbour a rich reservoir of potentially pathogenic *E. coli*. Prior infections of the reproductive tract of breeders, egg hygiene and transportation all contribute to early colonisation of the neonatal gut. Up to half of all flock deaths can occur in the first week of production, but few data are available describing the contribution of *E. coli* to this. In the present study, all dead birds collected on the first daily welfare walk 48 and 72 hours after chick placement underwent post-mortem examination. Diseased tissues were selectively cultured for *E. coli* and isolates subsequently virulotyped using 10 APEC virulence-associated genes (VAGs): *astA, iss, irp2, iucD, papC, tsh, vat, cvi, sitA* and *ibeA.* Approximately 70% of birds displayed signs of colibacillosis. Thirty distinct virulence profiles were identified among 157 *E. coli*. Isolates carried between 0 and 7 VAGs; ~30% of *E. coli* carried 5-7 VAGs, 12.7% shared the same VAG profile: *astA, iss, irp2, iucD, tsh, cvi* and *sitA*. Overall, this study demonstrates the significant contribution of *E. coli* infections to early broiler mortalities. The identification of a diverse *E. coli* population is unsurprising based on our previous findings. This work emphasises the need for effective control measures that should target early stages of production, possibly including early vaccination programmes or the use of probiotics.

4.1 Introduction

Avian pathogenic *Escherichia coli* (APEC) is an ill-defined pathotype of the extraintestinal pathogenic *E. coli* (ExPEC) group. APEC is the aetiological agent of an avian syndromic disease characterised by fibrinous lesions around visceral organs collectively termed colibacillosis. Airsacculitis, cellulitis, pericarditis, perihepatitis respiratory distress and septicaemia are among the most commonly associated signs of colibacillosis [48]. The broiler (meat chicken) industry is substantial, with over 900 million broiler chickens reared annually for consumption in the UK alone [3]. Colibacillosis is an endemic disease in commercial flocks and responsible for substantial economic losses globally.

The APEC pathotype shows high diversity. Recent work suggests APEC evolve from multiple *E. coli* lineages following the acquisition of virulence-associated genes (VAGs), often encoded on mobile genetic elements, explaining the high genetic diversity within this pathogenic group [78]. Genes involved in adhesion, invasion, toxin production, serum survival and iron acquisition have been shown to contribute to APEC pathogenesis [83, 159, 169, 182, 189, 252]. Such diversity has hindered the production of an effective vaccination programme capable of protecting against heterologous challenge.

The avian intestinal *E. coli* population has been identified as a potential APEC (pAPEC) reservoir and described as a “mixing vessel” allowing VAG acquisition [31]. In the previous chapter, I described the intestinal tract of day-old commercial broiler chicks to be rich in pAPEC [283]. These pAPEC were identified by their possession of at least 5 VAGs previously identified as contributors to APEC pathogenesis [182, 189, 252]. Given the identification of such a potential pathogenic

threat it was of interest to determine the prevalence of *E. coli*-related systemic disease in broiler chicks.

First week mortalities can account for up to 50% of total flock loses [133, 273]. Early mortalities reportedly reflect overall flock performance, which has led to contracts between hatcheries and farmers often stating an adjusted cost per chick based on flock performance during the first week [273, 284]. Chick survival during this initial period has been associated with the breeder farm and hatcheries with emphasis on flock management (nutrition, age, lighting) [285]. Early mortalities also correlate with the extreme breeder ages, egg storage length, breeder feed and the hatchery used [286-289].

Olsen *et al* (2012) reported that bacterial infections, primarily *E. coli*, accounted for ~50% of layer flock mortalities during the first week of life [133]. In this instance, omphalitis and/or yolk sac infections, with or without septicaemia, accounted for the majority of the observed clinical manifestations. Such infections may originate from infected breeders (subsequently infecting the yolk sac *in ovo*), or the hatchery environment [30, 134]. In support of vertical transmission, Petersen *et al* (2006) demonstrated the potential vertical transmission of fluoroquinolone resistant *E. coli* [135].

Investigations into broiler flock infection mortalities are rarely conducted and there is no existing published data regarding UK broilers. This present study reports on the contribution of *E. coli* to chick mortalities in the first 72-hours of production.

4.2 Materials and methods

4.2.1 Ethics statement

The following protocol did not involve any invasive procedures; no approval under the Animals (Scientific Procedures) Act (1986) was needed. No birds were culled for the purpose of this study and all dead birds intended for post-mortem examination were collected on the first daily welfare walk conducted by farmers. The study was conducted in strict accordance with the University of Liverpool Research Governance policies and permission for sampling on the broiler farms was granted by the farms and companies.

4.2.2 Standard commercial broiler farm

In commercial production, day-of hatch and 1-day old broiler chicks are transported to the broiler farm for rearing. At the hatchery, birds were vaccinated against the infectious bronchitis virus. No prior veterinary treatment was undertaken.

4.2.3 Post-mortem examination of dead broiler chickens

Dead chicks were collected during the first daily welfare walk on a standard commercial broiler chicken farm at 48 and 72 hours after placement. To minimise the detection of systemic *E. coli* resulting from a loss of intestinal integrity following death, only birds displaying minimal physical trauma were included. All birds were examined for classic signs of colibacillosis, including: ascites, airsacculitis, cellulitis, pericarditis and perihepatitis and yolk sac infection [48]. For each bird, up to 1 gram of each the following tissues were collected; heart, kidney, liver, lung and spleen

using sterile forceps and scalpels. Any other clinical manifestations were swabbed using sterile swabs. Enough sterile phosphate buffered saline (PBS) was added to each sample for homogenisation using a Biomaster Micro-stomacher 80 (Steward, UK) for 60 seconds at high speed. 50µl of each homogenate was streaked onto eosin-methylene blue agar (EMBA) and incubated overnight at 37°C. All media used were obtained from LabM Ltd (Bury, UK). Two to 3 *E. coli* colonies per positive tissue sample were picked, re-plated onto nutrient agar and incubated overnight at 37°C.

4.2.4 Virulotyping of extraintestinal *E. coli*

The DNA of each isolate was extracted using Chelex-100 (Bio-Rad, Hertfordshire, UK) [253]. *E. coli* identification was confirmed using a polymerase-chain reaction (PCR) assay targeting *uidA* [11]. Assay details are outlined in Chapter 2.

All isolates were subjected to a full screen of 10 VAGs and subsequently given a corresponding VAG profile, depending on the presence and absence of VAGs (presence '1' or absence '0'). The VAG targets were: *astA, iss, irp2, iucD, papC, tsh, vat, cvi, sitA* and *ibeA*. Three separate PCR assays were performed; one multiplex PCR previously described by Ewers *et al.* [182] and two single PCR assays for *ibeA* and *sitA* outlined by Timothy *et al.* (2008) [110]. All primers were obtained from Eurofins MWG operon (Germany) and all molecular reagents from Thermo Scientific (Surrey, UK). Primer sequences and assay conditions are described in detail in Chapter 2.

4.2.5 Phylogenetic analysis

E. coli were assigned to 1 of 4 phylogenetic groups (A, B1, B2 or D) using a triplex PCR targeting *chuA*, *yjaA* and the DNA fragment TSPE4.C2 [1]. All reagents were obtained from Thermo Scientific (Surrey, UK). Each 25µl PCR reaction contained: 3µl of template DNA extract, 0.2µl of each forward and reverse 100pmol primer (Eurofins MWG operon, Germany), 2.5µl dNTPs, 4µl $MgCl_2$, 2µl 10x PCR buffer and 0.25µl 5U/µl Taq polymerase. Thermocycler conditions were as follows: initial denaturation at 94°C for 5mins; 30 cycles of; 30 secs at 94°C, 30 secs at 59°C and 30 secs 72°C with a final extension at 72°C for 7 mins. PCR products were separated by electrophoresis. Phylogenetic group classification was based on the combination of *chuA, yjaA* and TSPE4.C2 as described in the Materials and Methods chapter section 2.5.4.

4.3 Results

4.3.1 Post mortem analysis

At placement, 25,700 chicks were placed in the rearing shed. The overall flock mortality rate for the sampled flock at the point of slaughter was 4.36%, while flock mortality in the first week was recorded as 1.03% and 0.44% for the first 72 hours.

Overall, 37 birds (n=14 at 48 hours and n=23 at 72 hours after placement) were collected on the first daily welfare walk and subject to post-mortem. Twenty-six out of 37 birds (70.27%) showed clinical signs associated with colibacillosis (n=10 at 48 hours and n=16 at 72 hours after placement) (illustrated in Figure 4.1). *E. coli* was isolated by pure culture from all pathological lesions tested, although quantification was not undertaken. Table 4.1 summarises the pathology observed at post-mortem.

4.3.2 Virulotyping

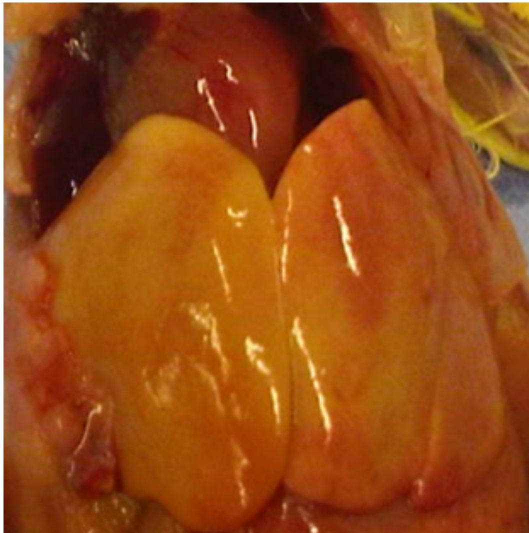
One hundred and fifty seven extraintestinal *E. coli* were screened for 10 VAGs. The overall presence of each VAG is represented in Figure 4.2 and a summary of the distribution of VAG profiles is shown in Table 4.2.

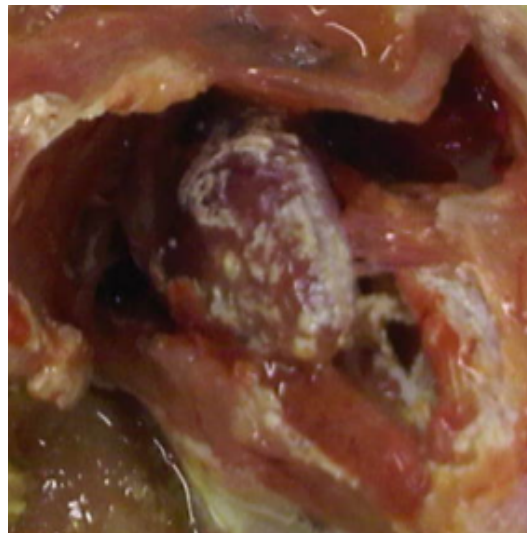
Three of the four most prevalent genes identified among the extraintestinal *E. coli* are ones involved in iron acquisition (*sitA*, *iucD* and *irp2,* positive in 98.09, 41.40 and 37.58% of the population respectively). The *iss* gene is involved in serum survival, an important trait during septicaemia, and was detected in 38.22% of the isolates tested. In the present study only 2.55% of isolates carried the pyelonephritis-associated pili gene (*papC*). Toxin-producing genes *astA* and *vat* were identified in 21.02 and 17.83% of isolates respectively.

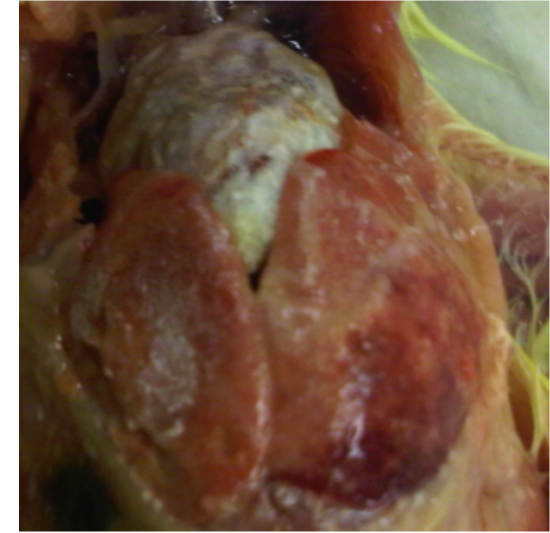
4.3.3 Phylogenetic analysis

Table 4.3 summarises the distribution of *E. coli* into the five phylogenetic groups (A, A0, B1, B2 and D). The most common phylogenetic group was group A (29.94%), while phylogenetic groups largely expected to represent pathogenic *E. coli* (B2 and D) represented 12.74 and 15.29% of *E. coli* respectively. *E. coli* with the VAG profile *astA, iss irp2, iucD, tsh, cvi* and *sitA* mentioned above, were assigned to A or A0 (untypable). Other *E. coli* with 7 VAGs (distinct to the described profile) were classified as A, B2 or D *E. coli*, suggesting these isolates were not all clonal. There appeared to be no association between the number of VAGs carried and the phylogentic group; only 75% of B2 *E. coli* carried < 5 VAGs and phylogroup A represented the majority of *E. coli* carrying 7 VAGs.

Figure 4. 1 Post-mortem examination of broiler chicks with colibacillosis







a) Accumulation of fluid around the heart and discoloured liver of a 72hr-old chick. b) Pericarditis (fibrin based lesions around the pericardium) in a 48hr old chick. c) Severe pericarditis in a 72hr old chick. E. coli *was cultured from all tissue samples.*

Table 4. 1 Prevalence of pathological lesions associated with colibacillosis identified during post-mortem examination of broiler chicks

	Percentage prevalence (%)		
Pathology	**48 hours (n=14)**	**72hours (n=23)**	**Overall (n=37)**
Pericarditis	20.00	43.75	24.32
Perihepatitis	10.00	56.25	27.03
Discoloured liver	60.00	25.00	27.03
Ascites	30.00	12.50	13.51
Cellulitis	0.00	18.75	8.11
Yolk sac Infection	10.00	18.75	10.81

Figure 4. 2 The percentage prevalence of virulence-associated genes among *E. coli* isolated from broiler chicks within 72 hours of placement.

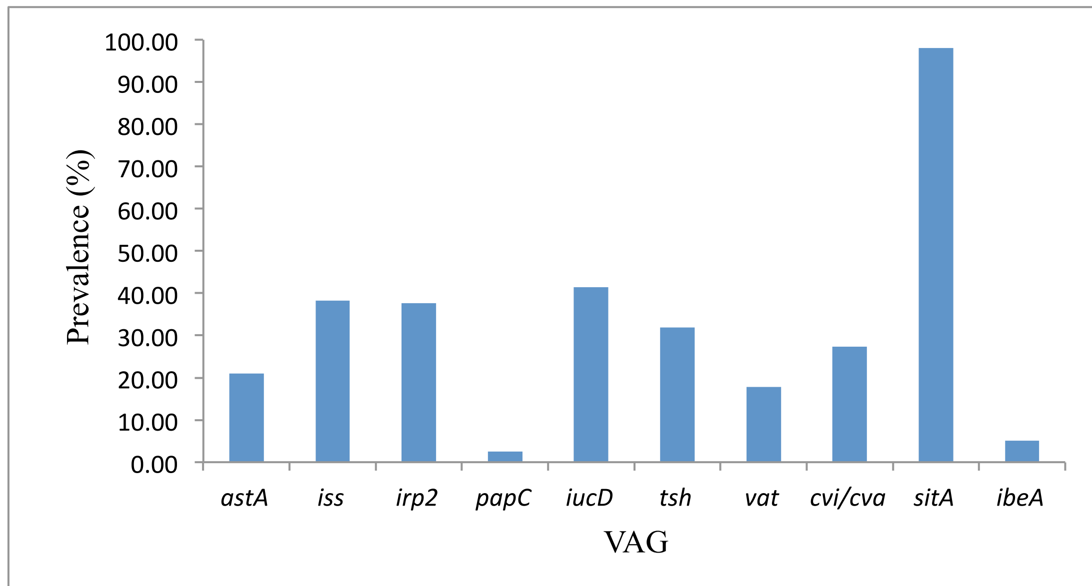


Table 4. 2 Frequency of VAG profiles in extraintestinal *E. coli* isolates.

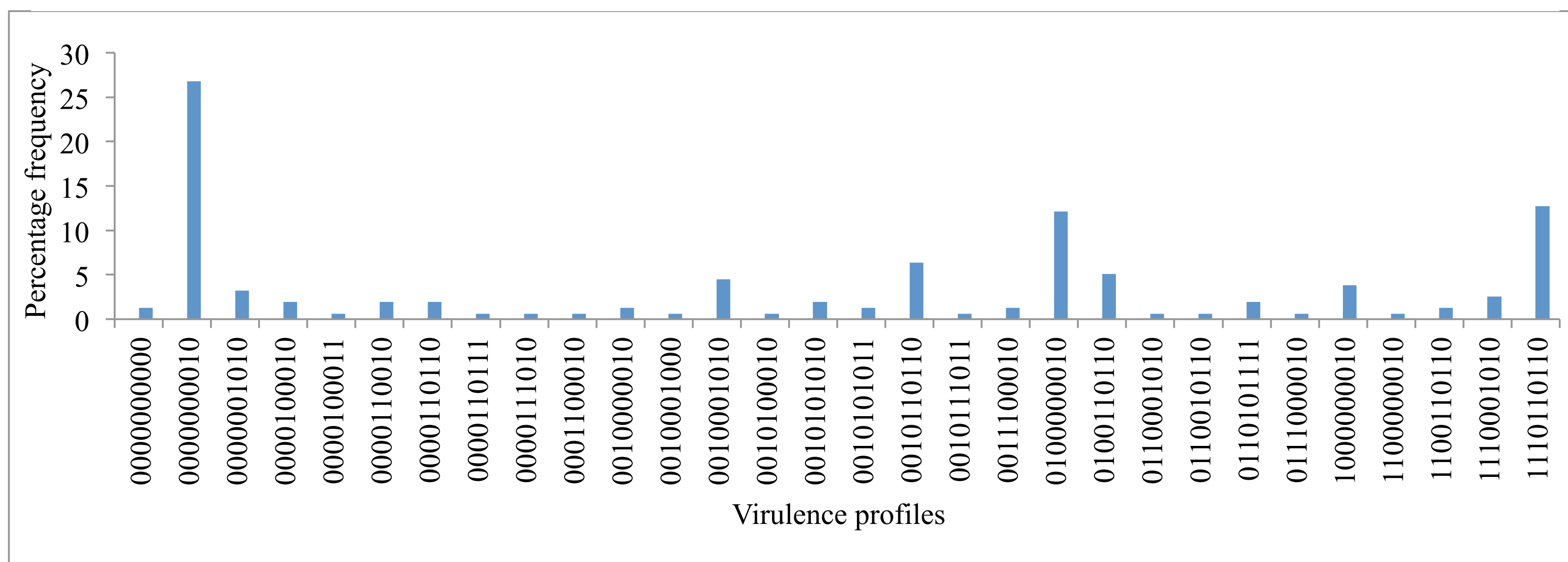
VAGs (10)	Frequency (%)
0	1.27
1	26.75
2	22.93
3	8.92
4	7.01
5	16.56
6	1.91
7	14.65
≥ 8	0.00

Thirty distinct virulence profiles were identified (Figure 4.3). Isolates with 0/10 VAGs accounted for 1.27% of the total, while the maximum number of VAGs identified in an individual isolate was 7/10 (14.65%). Approximately 30% of *E. coli* carried 5 - 7 VAGs. 12.7% of *E. coli* harboured the same VAG profile, positive for *astA, iss irp2, iucD, tsh, cvi* and *sitA,* and negative for *papC*, *vat* and *ibeA*. These *E.coli* were isolated from multiple birds and organs (heart, liver and cellulitis swab). The most abundant virulence profile, representing 42/157 isolates (26.8%) was positive for *sitA* whilst negative for the remaining 9 genes. Multiple VAG profiles were carried by *E. coli* isolated from the same bird, supporting our previous findings [283]. Simpson's diversity index (D = 0.915) indicates a large degree of profile diversity.

Table 4.3 Phylogenetic analysis of extraintestinal *E. coli*

Phylogenetic group	Frequency (%)
A0	24.20
A	29.24
B1	17.83
B2	12.74
D	15.29

Figure 4. 3 The frequency of virulence profiles identified among extraintestinal *E. coli*



Thirty different virulence profiles were identified among 157 E. coli *isolates, based on their carriage of 10 VAGs (positive carriage is indicated by '1' and negative carriage of gene is indicated by '0'. VAG order: astA, iss, irp2, papC, iucD, tsh, vat, cvi/cva, sitA, ibeA.*

4.4 Discussion

Previously, we investigated the carriage of VAGs associated with APEC pathogenicity in intestinal populations of *E. coli*. We identified the gut of 1-day old chicks to be a reservoir rich in potentially pathogenic *E. coli*. In the present study, we report on the contribution of extraintestinal *E. coli* infections to flock mortalities in the first few days of production. Approximately 70% of birds in the present study showed signs of extraintestinal *E. coli* infection. Olsen *et al* (2012) recently reported that approximately 50% of mortalities in commercial Danish layer flocks was related to *E. coli* or *Enterococcus faecalis* infection [133].The present study supports similar findings to those observed in layer flocks in which *E. coli* infections represent many strains of *E. coli* [133]. Colibacillosis is an economically important poultry infection resulting in increased mortality rates and higher rejections of carcasses at slaughter. Colibacillosis has previously been associated with a disease of older broiler chickens (> 2 weeks old), but several studies have taken an 'integrated poultry production' approach and suggested breeders and hatcheries pose significant risks to broiler chickens either via environmental contamination or vertical transmission of APEC [51, 135].

Poor flock performance has been correlated with increased early mortality rates while increased early mortalities have been related to hatchery practices and breeders [273, 284, 290]. Young breeders often produce smaller eggs containing increased levels of albumen producing lower live chick weights [288, 289]. Older breeders are associated with increased navel to yolk sac infections and eggs frequently hatch sooner leading to increased chick dehydration at the hatchery [287]. Acting as a key

indicator, reasons behind early flock mortalities appear central to flock management and reducing overall mortality.

Thirty different virulence profiles were identified from 157 *E. coli*. The most prevalent virulence profile was positive for *sitA* and negative for the other nine genes (0000000010) and *sitA* was carried by ~98% of all the isolates. The *sitABCD* operon encodes an iron and manganese transport system and was shown to contribute to the virulence of APEC chi7122 in a chicken infection model, with a possible additional role in protection against oxidative stress [26, 188]. The *sitABCD* operon has been identified in over 85% of APEC populations previously and has been located to ColV plasmids, associated with APEC pathogenicity [189].

Around 30% of isolates carried 5 - 7 VAGs. A conserved profile of *astA, iss, irp2, iucD, tsh, cvi* and *sitA* was observed in 12.7% of isolates. The identification of this profile in more than one bird and organ at the same time point (72 hours) may suggest a common or related *E. coli* strain. Phylogenetic analysis using a widely accepted protocol suggests these *E. coli* fall into phylogroup A or were untypable (A0) [1]. Phylogroup A has previously largely been associated with non-pathogenic and environmental *E. coli [189].* This profile was not identified in the faeces of 1-day old broilers in the previous study [283]. Colonisation of chicks by this pathogenic *E.coli* may have occured *in ovo* as a consequence of a reproductive tract infection, or alternatively acquired post-hatch from the hatchery or via faceal contamination [291, 292]. Phylogenetic analysis highlights the high genetic diversity seen among extraintestinal *E. coli* in diseased broiler chicks, with all four major phylogenetic groups represented in the 157 isolates.

The intestinal tract of newly hatched chicks is relatively immuno-incompetent and lacks a stable microbiota, both provide ideal conditions for pathogen colonisation [293]. Industrial use of probiotics, or direct-fed microbials, to reduce infectious enteric pathogen colonisation is not new and it is an industry expected to grow in the near future [294]. The introduction of beneficial bacteria including *Bacillus* and *Lactobacillus* species to enhance growth performance, feed conversion efficiencies while reducing the APEC burden and strengthening the intestinal mucosal immune system in broiler chickens has attracted interest recently. This concept requires further investigation based on exisiting conflicting results likely to be due to differences in host status and the avian microbiota between studies [295, 296]. Targeting newly hatched chicks or breeders with such measures to manipulate the neonatal gut could be beneficial in reducing the prevalence of *E. coli* and subsequently extraintestinal disease.

Alternatively, the identification of key VAGs involved in extraintestinal infections could be used in developing recombinant vaccines. Lynne *et al* (2012) previously tested a recombinant Iss-based vaccine, which showed some promise leading to both serum and mucosal humoural immune responses [248]. In the present study, the *iss* gene was carried by almost 40% of *E. coli*, other targets would therefore need to be sought. The vaccination of broiler breeders and the subsequent transfer of maternal antibodies would be a valuable control measure due to the early detection of extraintestinal *E. coli* infection, the short life span of a broiler chicken and the substantial loss associated with early mortalities. There is some promise in the ability to transfer protective egg yolk ImmunoglobulinY (IgY) and other immunomodulatory peptides to chicks, although further work is required [297-299].

The panel of VAGs used in this investigation is not an exhausted list of potential targets. Genes involved in bacterial adhesion, invasion, toxin production, serum survival and iron acquisition have all been associated with APEC pathogenesis. The 10 genes used in the current investigation reflect these traits.

To our knowledge, this is the first study to assess the contribution of extraintestinal *E. coli* to early broiler deaths. The identification of diverse population based on VAG carriage supports the work of others. These findings are unsurprising based on previous findings of a rich reservoir of potentially pathogenic *E. coli* in the intestinal tract of day-old chicks. This work emphasises the need for effective control measures and provides the foundations for future work. Additionally, improved egg and chick hygiene at the hatchery and during transportation is likely to be highly valuable. Further work including the sampling of hatcheries and breeders will strengthen our current understanding of integrated poultry production.

Chapter 5

Manuscript 3

Assessing the growth characteristics, intestinal invasion and cytotoxicity of potentially pathogenic *Escherichia coli* isolated from UK broiler flocks

K. Kemmett, T. Humphrey, G. Chaloner, S. Humphrey, A. Close, P. Wigley, N. J. Williams

University of Liverpool, Leahurst Campus, Neston, CH64 7TE, UK

ABSTRACT

Avian pathogenic *E. coli* causes a syndromic disease in poultry called colibacillosis. Colibacillosis is responsible for significant economic losses to the global poultry industry. Poultry health is crucial in allowing for the sustainable production of safe food that meets consumer demand. Potential APEC (pAPEC) populations reside in the avian intestinal tract and extraintestinal dissemination is primarily via the inhalation of contaminated faecal dust but active gut translocation or migration up the reproductive tract may also be important. 10 pAPEC isolated from UK broiler chickens were used to assess invasion and cytotoxicity to the human colonic carcinoma cell line, Caco-2. The pAPEC were not invasive in intestinal epithelial cells. No more than 0.05% of the initial inoculum invaded 2 hours and 24 hours post-infection. There were no significant differences between the pAPEC ($p>0.05$) yet all isolates were significantly poorer invaders compared to *S*. Typhimurium 4/74 ($p<0.05$). There were no significant differences in pAPEC cytotoxicity in Caco-2 cells measured at 2 and 24 hours post-infection. Cytotoxicity as high as ~60% (average 40%) was observed 24 hours post-infection, such damage may allow bacterial translocation during infection. Further work is required in an attempt to expand on the dynamics of infection, including the contribution of physiological stress and the microbiota that may be encountered in the intestinal tract of commercial broiler chickens. Our efforts are limited, as we await the development of a reliable and reproducible chicken intestinal cell line.

5.1 Introduction

Avian pathogenic *Escherichia coli* (APEC) are an ill-defined pathotype of the extraintestinal pathogenic *E. coli* (ExPEC) group. APEC cause an avian syndromic disease often characterised by fibrinous lesions around visceral organs, often referred to as colibacillosis, which is associated with airsacculitis, cellulitis, pericarditis, perihepatitis, respiratory infections, infections of the reproductive tract and septicaemia [48]. The broiler (meat chicken) industry is substantial, with over 900 million broiler chickens reared annually for consumption in the UK alone [3]. Colibacillosis is an endemic disease within commercial flocks and responsible for substantial economic losses globally.

APEC is an accepted primary and opportunistic pathogen, yet its pathogenesis remains unresolved. Whole genome analysis suggests that APEC are likely to originate from multiple *E. coli* lineages [78]. A multitude of virulence-associated genes (VAGs) have been described, including those involved in: adhesion, growth and avoidance of the host immune system, although not all literature concurs with the importance of individual factors [83, 159, 182, 185, 189, 252]. Acquisition of VAGs found on mobile genetic elements are likely to enhance pathogenic potential.

The avian gastrointestinal tract has been identified as an APEC reservoir and described as a ‘mixing vessel’ for the horizontal transfer of VAGs [31, 283]. Faecal shedding provides a mechanism for bacterial dissemination into the surrounding environment. Numerous routes of systemic dissemination have been described including: vertical transmission (originating from broiler breeders), subcutaneous infection, migration up the reproductive tract, oral infection and respiratory infection [43, 51, 52, 127, 131, 132]. Inhalation of *E. coli*-contaminated faecal dust is thought

to be the primary mechanism of infection [43, 127]. *E. coli* have been shown to persist on poultry house dust particles at levels exceeding 10^6 colony forming units per gram [128]. *In vivo* infection studies often mimic the respiratory route through intra-tracheal or intra-air sac inoculation [78, 127, 144]. To mimic natural infection, infection of the upper respiratory tract is perhaps more representative given that incoming pathogens will need to pass through physical barriers not encountered when directly inserted into the air sacs [196, 300].

Earlier studies suggest translocation of the intestinal epithelium by pathogenic *E. coli* provides an alternative route for dissemination, but only when birds have been predisposed to stress [129, 130]. Thus, APEC may be avian-adapted opportunists as opposed to true pathogens. This hypothesis is supported by the isolation of numerous *E. coli* strains from diseased birds [83, 90, 189, 283].

Whether APEC traverse the intestinal, respiratory or reproductive mucosa they must possess the ability to invade lining epithelial cells or be capable of disrupting the epithelial integrity, subsequently allowing transcellular passage. Pathogens have evolved a number of strategies to invade host cells [301]. *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) and *S*. Typhi possess *Salmonella* pathogenicity islands (SPI-1 and SPI-2), both of which encode type three secretion systems (TTSS); needle-like structures which insert proteins required for invasion into target host cells [302]. *Salmonella* species, including *S*. Gallinarum, which causes fowl typhoid, also possess SPI-1-independent invasive mechanisms [260]. Some *E. coli* pathotypes have been associated with intracellular stages [303, 304].

This fundamental step in APEC pathogenesis remains unclear. A number of studies have identified ExPEC factors associated with epithelial invasion including outer

membrane protein A (OmpA), invasion barrier epithelia proteins (IbeA, B and C), fimbriae and temperature sensitive haemagglutinin (Tsh), but the exact mechanisms in many cases remain unknown [131, 149, 153-155]. Past work on the invasive properties of APEC to non-phagocytic cells is minimal and somewhat confusing [87, 151, 305].

One difficulty in studying the interactions of APEC with the chicken intestinal epithelium is the lack of a reliable immortal chicken intestinal cell line [306]. This limitation means human epithelial, non-phagocytic non-epithelial chickens cells (such as chicken fibroblasts) or primary cell cultures are often used as alternatives [151, 307, 308].

This chapter aims to characterise the growth characteristics along with the invasive and cytotoxic nature of 10 pAPEC using the human colonic carcinoma cell line, Caco-2. These *E. coli* were obtained from commercial broiler flocks sampled during the longitudinal field study described in Chapter 3 [283].

5.2 Materials and methods

5.2.1 Bacterial isolates

Nine *E. coli* isolates were originally isolated from the faeces of commercial broiler chickens (Table 5.1) and 1 *E. coli* was isolated extraintestinally (588) [283]. Test isolates were selected based on their carriage of APEC VAGs determined previously [283]. Prior to use, all *E. coli* were stored at -80°C in Microbank vials (Pro-Lab Diagnostics, UK). APEC O78 (chi 7122) was used as a representative APEC control strain and was kindly donated by Professor Mark Stevens of The University of Edinburgh Roslin Institute, Scotland, UK. APEC O78 is a spontaneous mutant of a strain originally isolated from the liver of a diseased turkey [79].

5.2.2 Growth characteristics

All bacterial isolates were resuscitated from -80°C storage, streaked onto nutrient agar and incubated overnight at 37°C. 2-3 colonies of each overnight culture were used to inoculate 2ml of sterile Luria broth (LB broth). Liquid cultures were incubated overnight at 37°C. All media used were obtained from LabM (IDG) Ltd (Bury, UK). Overnight cultures were diluted 1:100 (v/v) using fresh LB broth. 200µl of bacterial suspension was extracted and the optical density at 600nm (OD_{600}) was recorded in triplicate using a spectrophotometer. Sterile LB broth acted as a blank control. Additionally, each bacterial suspension was serially diluted 1:10 (v/v) using sterile phosphate buffered saline (PBS) and plated onto nutrient agar for enumeration. Method of enumeration is outlined in Chapter 2. Plates were incubated overnight at 37°C (Time 0: T.0). Bacterial suspensions were incubated at 37°C in a

shaking incubator (Stuart, Orbital incubator SI 500, UK) at 150rpm. The OD_{600} was recorded periodically at 30 minute intervals and serial dilutions for enumeration were undertaken every hour. This was repeated up to T.6 or until cultures reached stationary phase (OD_{600} plateau). In using the optical density to measure bacterial growth, it was assumed the number of bacterial cells was proportional to the optical density under steady state conditions.

Bacterial growth was assessed on three separate occasions and at 42°C (corresponding with the avian body temperature). The growth rate data for APEC O78 were obtained via a personal communication (Charlotte Collingwood) at the University of Liverpool.

5.2.3 Bacterial motility

E. coli motility was assessed using a basic motility assay. A sterile volume (20-25ml) of 0.35% semi-solid Bactoagar (BD Diagnostics, UK) was poured into standard petri-dishes and left to cool. A sterilised straightened paper clip was used to inoculate the semi-solid agar with overnight liquid cultures of *E. coli* in LB broth and incubated at 37°C overnight. The level of motility was measured using a ruler and recorded in millimetres. The assay was repeated in triplicate and an average motility reading was recorded. *S*. Gallinarum 287 was used as a negative control.

5.2.4 Caco-2 intestinal epithelial cell gentamicin invasion assays

The gentamicin invasion assay protocol was based on that previously described by others [259, 260]. A detailed protocol is outlined in Chapter 2 and is described briefly below.

5.2.4.1 Cell line seeding protocol and cell line preparation

The human colonic carcinoma cell line (Caco-2 cells) was sourced from Dr Barry Campbell from the Gastroenterology Department, University of Liverpool, UK (ATCC®, Number HTB-37). Cells were grown in Dulbecco's minimum essential medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich, UK), 1% GlutaMAX^{TM} (100x concentration) (Invitrogen, UK), 1% MEM non-essential amino acid solution (Sigma-Aldrich, UK) and penicillin–streptomycin (100 U/ml) antibiotics (Sigma-Aldrich, UK). Cells were grown at 37°C 5% CO_2.

Twelve days before the invasion studies, cells were seeded into 24-well tissue culture plates at a density of 3×10^5 cells per well and incubated at 37°C in 5% CO_2 to give a final density of 1×10^6 cells. The DMEM was changed every 2-3 days during this period [259]. Three days before the experiment, the DMEM with the above supplementation was substituted for antibiotic-free, serum-free medium (AFSF DMEM).

5.2.4.2 Bacterial culture preparation

All bacterial isolates were resuscitated from the -80°C culture collections by streaking onto nutrient agar and incubating overnight at 37°C. *S.* Typhimurium 4/74 was used as an invasive positive control and APEC O78 was used as a characterised APEC control. One day prior to the invasion assay, bacterial isolates were prepared for inoculation as described in Chapter 2. Bacterial suspensions were adjusted to give an MOI of ~28.

5.2.4.3 Infection of intestinal epithelial cells

The AFSF DMEM was removed from the Caco-2 cells and cells washed 3 times with sterile PBS and replaced with 1ml of warm (37°C) AFSF DMEM. Cell monolayers were incubated for 2 hours at 37°C.

100µl of prepared bacterial samples (with adjusted OD_{600}) were added to the monolayers in triplicate. Infected monolayers were re-incubated at 37°C for two hours. After two hours, the supernatant was removed and stored at -20°C for cytotoxic analysis. One ml of DMEM containing 100µg/ml gentamicin sulphate (Invitrogen, UK) was added to each well and incubated at 37°C for 1 hour. The DMEM was removed and cells washed once with sterile PBS. Cells were lysed with 0.5% Triton X-100 in 1ml PBS incubated at 37°C for 5 minutes. Cell lysates were serially diluted (1:10) using PBS and invasive bacteria enumerated on nutrient agar.

The level of intracellular persistence was assessed at 24 hours post-infection. For this time point, the protocol followed the same as above. However, instead of adding PBS-Triton X-100, 1ml of fresh DMEM containing 20µg/ml of gentamicin sulphate was added to the infected monolayers. Cells were incubated at 37°C until 24 hours post-infection. At this time, the supernatant was removed and stored at -20°C for cytotoxic analysis and cells were lysed using PBS-Triton X-100 as previously described.

5.2.4.4 Statistical analysis

The difference in percentage invasion between isolates was examined using analysis of variance.

5.2.5 Caco-2 lactate dehydrogenase release cytotoxicity assays

Lactate dehydrogenase (LDH) is a cytosolic enzyme, therefore detection extracellularly can be used as a measure of cell necrosis/death [309]. Supernatants collected from the gentamicin invasion assays above at 2 and 24 hours post-infection were stored at -20°C until required for the Lactate dehydrogenase cytotoxicity assay (LDH assay). Immediately prior to the LDH assay, samples were freeze thawed and 150µl transferred to a sterile round bottom 96-well plate in triplicate.

The following controls were used: Caco-2 spontaneous LDH release control, Maximum LDH release control (Caco-2 cells lysed with Triton X-100 for 1 hour) and a DMEM background control (AFSF DMEM only).

The 96-well plate was centrifuged at 250 x g for 4 minutes to remove cell debris. 50µl of supernatant was transferred to a fresh 96-well plate. 50µl of reconstituted CytoTox-96 Substrate mix was added to each well and mixed gently. Samples were incubated at 22°C for 30 minutes in darkness. Next, 50µl of Stop Solution was added to each well and samples shaken gently for 10 seconds, keeping them protected from the light. The absorbance at 490nm was recorded. The LDH release was calculated as follows:

$$\% \text{ Cytotoxicity} = 100 \text{ x } \frac{\text{Experimental} - \text{Caco} - 2 \text{ spontaneous release}}{\text{Maximum LDH release}}$$

5.2.5.2 Statistical analysis

Significant differences between isolates and controls were identified using analysis of variance.

Table 5. 1 *E. coli* test strains with virulence profiles and phylogenetic groups.

	Virulence Associated Genes (VAGs)										
Isolate	***astA***	***iss***	***irp2***	***papC***	***iucD***	***tsh***	***vat***	***cvi/cva***	***sitA***	***ibeA***	**Total VAGs**	**Phylogenetic group**
284	1	0	1	1	1	0	1	1	1	0	7	B2
292	0	1	1	0	1	0	1	1	1	1	7	D
293	0	0	1	0	1	0	1	1	1	1	6	D
295	0	1	1	1	1	0	1	1	1	0	7	D
297	0	1	0	0	1	0	0	0	1	1	4	A
339	1	1	0	1	1	1	0	1	1	0	7	U
352	0	1	1	1	1	1	1	1	0	0	7	U
356	0	1	1	1	1	1	1	1	0	1	8	U
410	0	0	1	0	1	1	1	0	1	1	6	D
588	0	1	1	1	1	0	1	1	1	0	7	D

U; Unknown phylogenetic group, 1; positive by PCR for VAG, 0; negative by PCR for VAG

5.3 Results

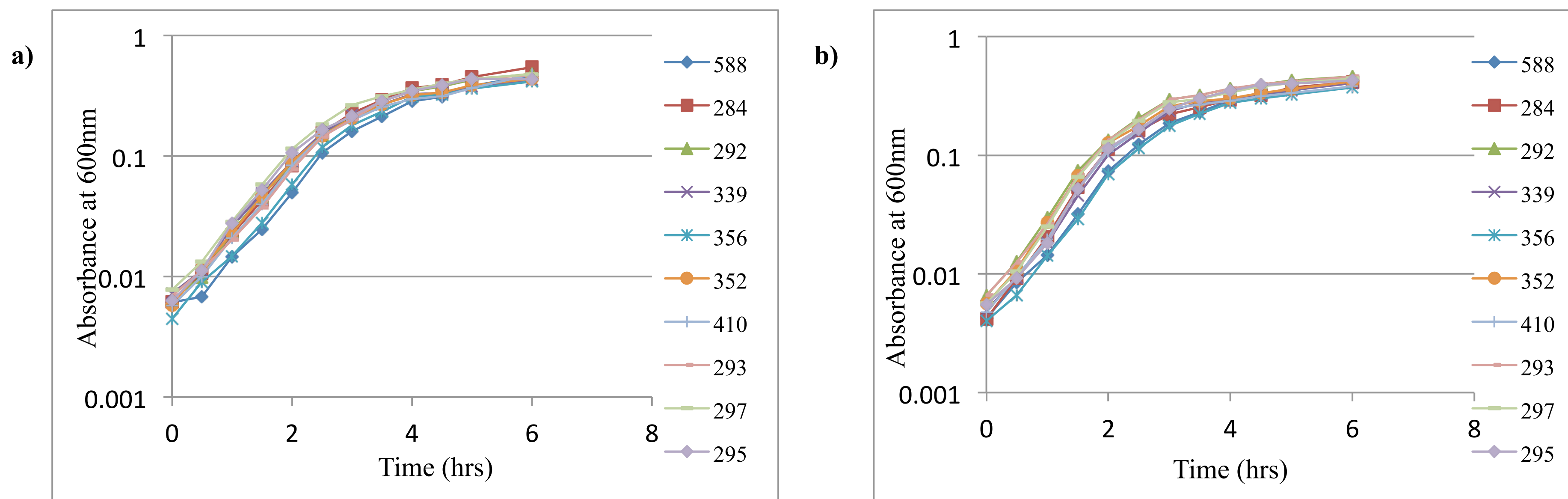
5.3.1 Comparing the growth of *E. coli* strains

The growth rates of 10 *E. coli* strains, isolated from commercial broiler chickens, were assessed in liquid growth media. Figure 5.1 shows the average changes in bacterial densities at 37 and 42°C calculated from three repeat experiments. There were no significant differences in growth rates between the *E. coli* isolates. All isolates entered exponential/log phase at approximately 1.5 hours and reached stationary phase at approximately 4-5 hours. Based on these results the subsequent invasion assays were performed using late log phase *E. coli*, which required 3.5 hours of growth prior to infection of the epithelial Caco-2 cell line.

5.3.2 *E. coli* motility

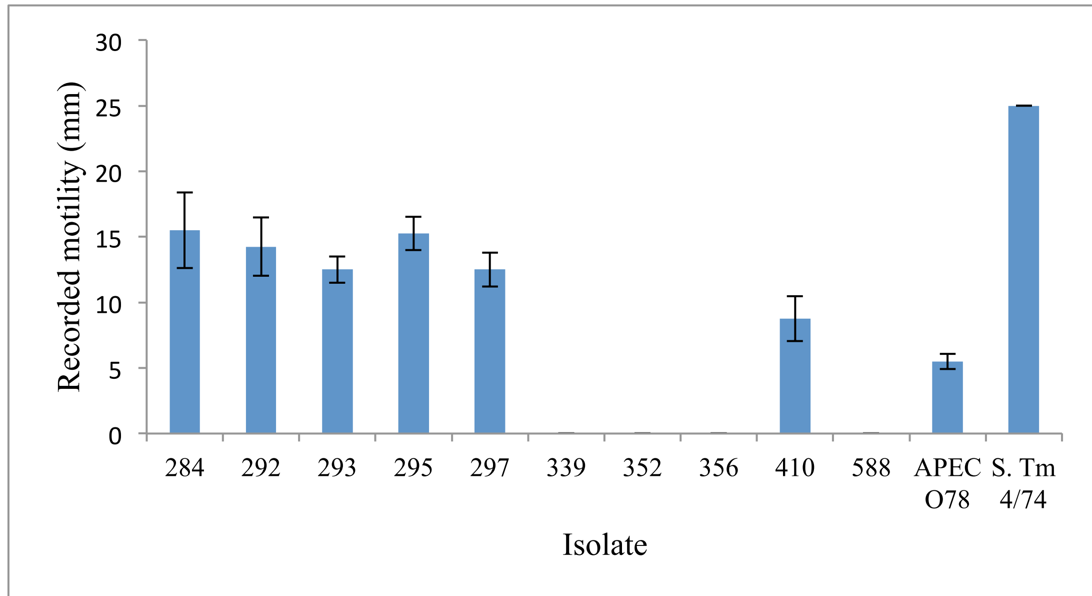
In total, 6/10 *E. coli* were motile. *E. coli* 339, 352, 356 and 588, appeared non-motile. The results for the motility assay can be seen in Figure 5.2. The reference strain APEC isolate O78 appeared less motile than the other motile *E. coli* in this study. None of the *E. coli* isolates were as motile as *S*. Typhimurium 4/74.

Figure 5. 1 Growth of 10 *Escherichia coli* isolates in liquid culture.



Isolates were isolated from commercial broiler chickens. a) Growth at 37°C b) Growth at 42°C. There were no significant differences in growth between the individual E. coli *isolates and temperature was not a significant factor in the growth rate of* E.coli.

Figure 5. 2 Average *E. coli* motility.



Overnight motility was recorded in millimetres and an average was taken over 3 experiments. The standard deviation is also shown

5.3.3 *E. coli* invasion of Caco-2 epithelial cells – 2 hours post-infection

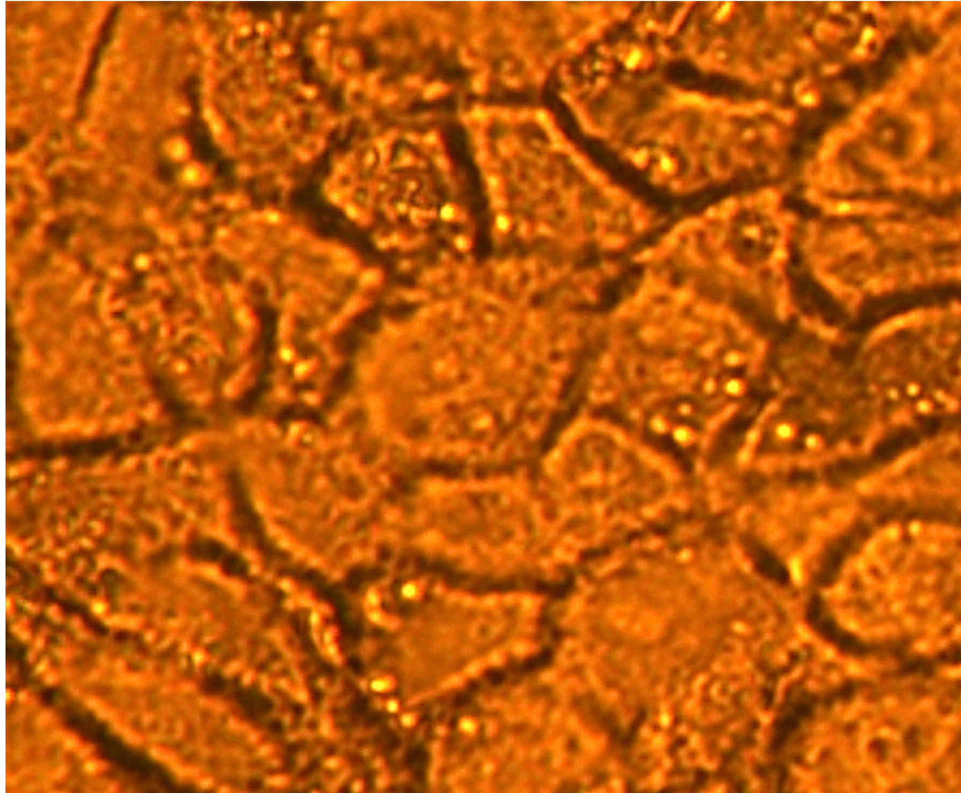
Caco-2 epithelial cells were grown for 12 days prior to infection, Figure 5.3. The initial invasiveness of *E. coli* in human epithelial cells 2 hours post-infection was measured and is represented in Figure 5.4a. *E. coli* 284, 292, 356, 410 and 293 show comparable invasiveness to the APEC reference strain O78, with approximately 0.02% of the inocula being successfully recovered in each case. All *E. coli* were significantly poorer invaders in comparison to *S.* Typhimurium 4/74, where 0.51% of the inoculum had invaded 2 hours post-infection ($p<0.05$). Isolates 588, 339, 352 and 297 were non-invasive during the 2 hour infection period. The percentage invasion of

E. coli 295 was significantly different to all other *E. coli* ($p<0.05$) and the invasive potential of *E. coli* 284 and 292 was significant compared to the non-invasive isolates 588, 339 and 352 ($p<0.05$).

5.3.4 *E. coli* net replication in Caco-2 epithelial cells – 24 hours post-infection

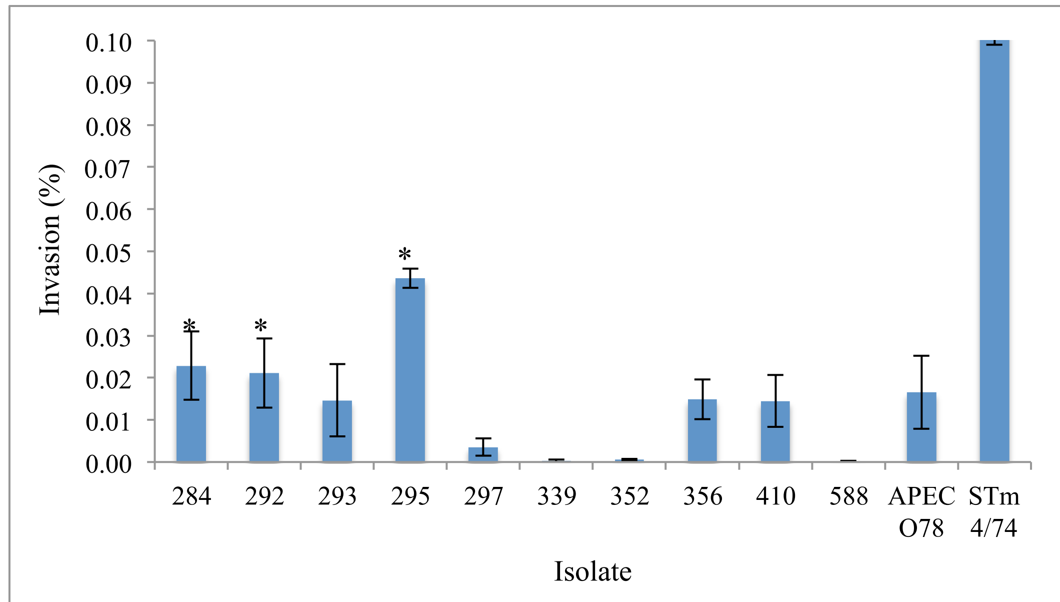
The intracellular persistence of *E. coli* was measured following the 24 hour incubation with the Caco-2 monolayer. Only 2 isolates, 588 and 295, showed persistence within epithelial cells (0.01 and 0.04% of the inoculum respectively) (Figure 5.4b). All other *E. coli* failed to persist at the limit of theoretical detection for this study (less than 5 CFU per ml). This level of persistence was insignificant compared to that of *S*. Typhimurium 4/74, where an average of 2.19% of the inoculum had persisted 24 hours after infection ($p<0.05$). There were no significant differences between the *E. coli* ($p<0.05$).

Figure 5. 3 Cultured Caco-2 human colon carcinoma cells

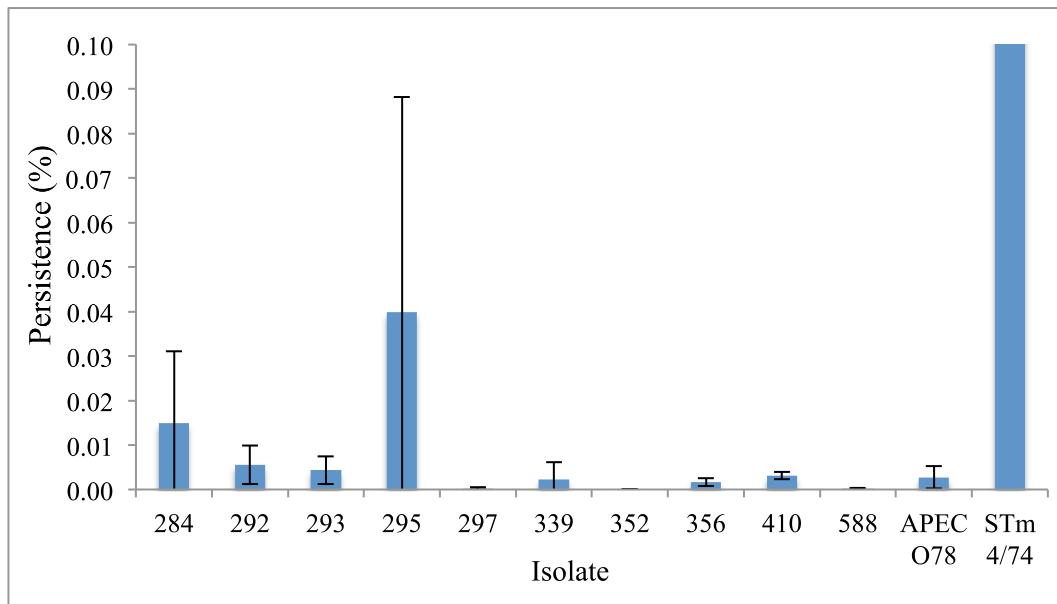


3 x 10^5 Caco-2 cells were added to each 24-well plate and incubated at 37°C in 5% CO_2 for 12 days to give a final density of 1 x 10^6 cells. The photograph illustrates confluent cells with tight junctions formation.

Figure 5. 4a Invasive ability of *E. coli* in human epithelial cell line Caco-2: 2 hours post infection.



4b) Persistence/ net replication 24 hours post infection

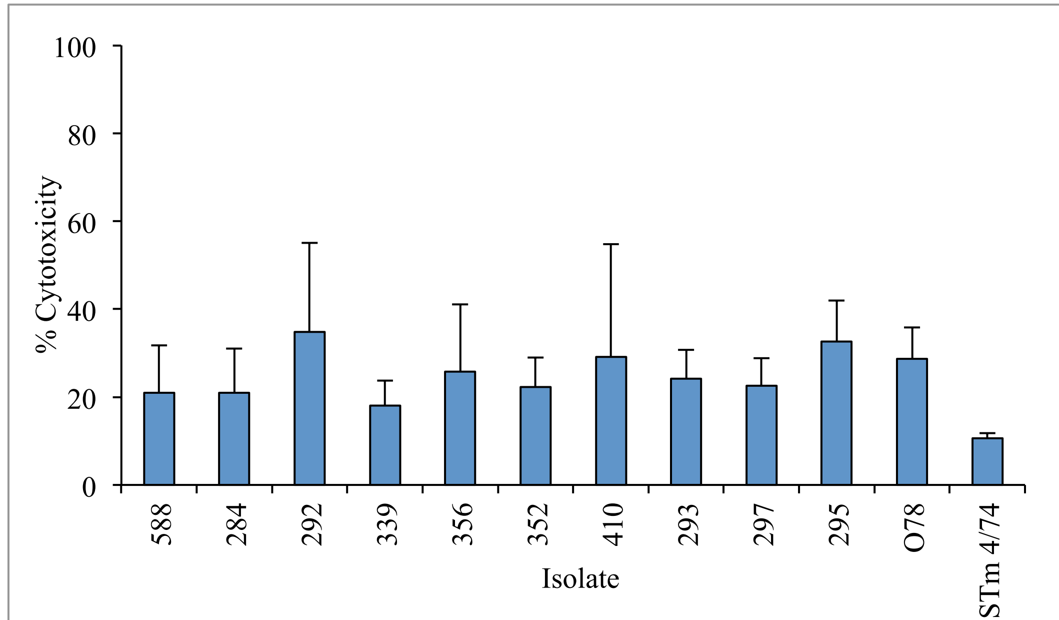


Invasion was assessed using the gentamicin invasion assay. Calculations were based on the percentage of the original inoculum. An average over three repeat experiments (undertaken in triplicate on each occasion) was used. S. *Typhimurium was used as a positive invasive control and APEC O78 was used as an APEC control. The standard deviation is shown and * indicates statistically significant differences (p<0.05): refer to section 5.3.3 for details.*

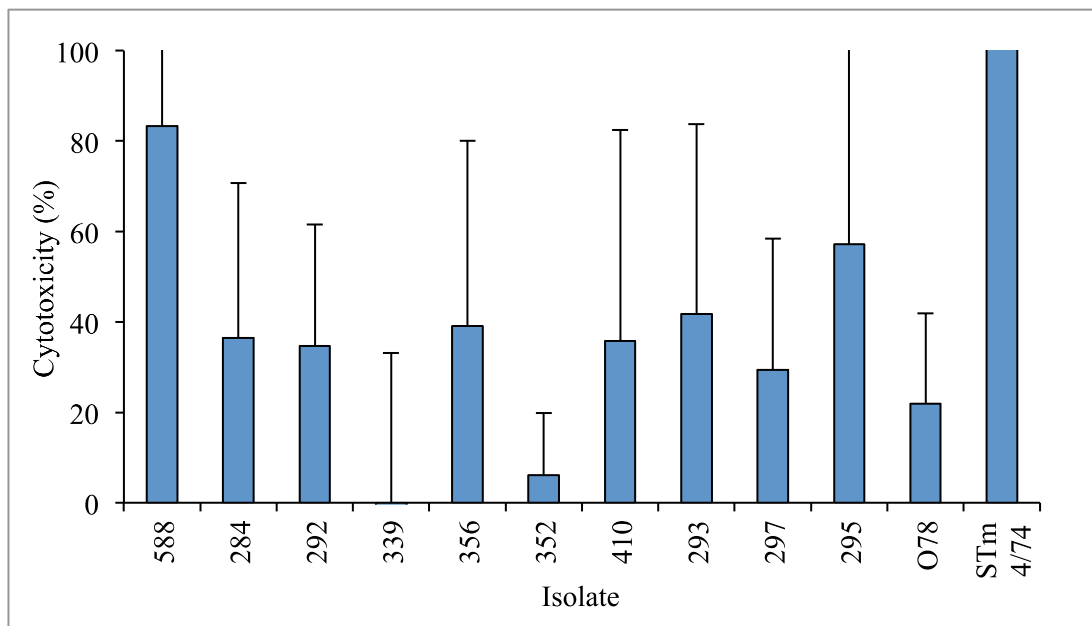
5.3.5 Cytotoxicity of *E. coli* to Caco-2 epithelial cells

The cytotoxicity of the *E. coli* isolates was determined by the quantification of LDH in the cell culture growth media collected during the invasion studies. All *E. coli* appear similar in their cytotoxic impact and that of the field isolates was comparable to that of the APEC reference strain O78. All *E. coli* were 20-40% cytotoxic, although cytotoxicity varied between each repeat. The cytotoxicity of *S.* Typhimurium 4/74 was comparably lower (10.60%). The results for 2 hour and 24 hour post-infection are illustrated in Figure 5.5. There were no significant differences between the *E. coli* ($p<0.05$).

Figure 5. 5a The average percentage of cytotoxicity of *E. coli* to Caco-2 epithelial cells 2 hours post-infection.



4b) 24 hours post-infection



The averages were taken from the three repeats. Standard deviation is also shown. Cytotoxicity was measured using a lactate dehydrogenase assay ($p>0.05$).

5.4 Discussion

This series of experiments aimed at elucidating the invasiveness and cytotoxicity of 10 *E. coli* isolates, 9 of which were isolated from the gastrointestinal tract of UK broiler chickens during a previous study [283]. Overall, these pAPEC were not invasive in human colon carcinoma epithelial cells. This concurs with previous work conducted by Matter *et al* (2011) who tested 8 APEC strains, only one of which appeared invasive at comparable levels to *S.* Typhimurium 4/74 [305].

Other work suggests APEC are capable of intracellular invasion. Silveira *et al* (2002) used human cell lines to compare a panel of diverse APEC and described invasive behaviour. However, they found no correlation between VAG carriage, serogroups and invasiveness [308]. Prior to this, Pourbakhsh *et al* (1997) administered 10^8 colony forming units of APEC into the air sacs of broiler chicks and identified intracellular APEC 6 hours post-infection using electron microscopy [196].

The primary route of APEC dissemination is thought to be via the respiratory tract [43, 127]. Infection models have shown that as few as 3 hours after intra-air sac administration, air sac epithelial cells appear swollen, vacuolated and the space between cells increased [196]. The integrity of the epithelium is then jeopardised. Damaged air sacs are quickly vascularised, given they possess no cellular defence mechanisms of their own and rely on the incoming of innate immune cells such as heterophils and macrophages [193, 310]. Vascularisation would favour bacteraemia.

Such attack on the integrity of the host mucosal surfaces and the inconsistent invasive behaviour of APEC strains may suggest the bacteria transverse the epithelial barrier only after they have weakened it. The current study assessed the cytotoxicity

of pAPEC to colonic epithelial cells and found that at 2 hours post-infection the epithelial cells showed an average of 25% cytotoxicity. This was greater than the invasive *S.* Typhimurium control (~11%). This is also approximately double the cytotoxicity that Matter *et al* (2011) reported [305]. There were no significant differences between the pAPEC isolates at this time point. At 24 hours post-infection, there were also no significant differences between the pAPEC isolates but the average cytotoxicity was ~40%. This may sufficiently disrupt the epithelial integrity allowing *E. coli* to transverse and disseminate *in vivo*. Inflammation and host stress are known to reduce epithelial integrity [259]. *E. coli* display certain antigens including flagellin and lipopolysaccharide (LPS) which are recognised by a family of host receptors known as Toll-like receptors [311]. *E. coli* may utilise the avian inflammatory response to subsequently reduce the transepithelial resistance allowing migration intercellularly, as demonstrated by *Shigella* [312]. This study observed large variation in cytotoxicity between repeat experiments. Reasons for this could be due to differences in the cultured cells between experiments. Caution must be taken when considering the intracellular *E. coli* counts at 24 hours post-infection. The gentamicin invasion assay relies on the impermeable cellular barrier to prevent gentamicin from gaining access to the cytosol. Our cytotoxicity results suggest this barrier is jeopardised at this time point and this intracellular counts for net replication/persistence may be somewhat inaccurate.

APEC pathogenesis is associated with a number of toxins and other molecular molecules associated with inducing cell death. The first genomic pathogenicity associated island (PAI) of APEC was the VAT-PAI encoding a vacuolating autotransporter toxin (vat), which induces the formation of cytotoxic intracellular vacuoles [210]. Parriera *et al.* (2003) reported a decline in virulence following the

deletion of *vat* in a broiler chicken respiratory infection model [210]. This autotransporter toxin shares 75% sequence homology to *tsh* and is found in approximately 38% of APEC, although it has been reported in over 50% of ExPEC [47, 182]. The Enteroaggregative heat stable enterotoxin 1 (EAST1), encoded by the *astA* gene, was originally identified in enteroaggregative *E. coli* (EAEC) and later associated with both enterotoxigenic *E. coli* (ETEC) and 20-30% of APEC [187, 210, 211]. Olsen *et al.* (2011) demonstrated that although EAST1 may be associated with pathogenicity it is not found in all outbreak APEC strains [123]. In the current study, 8/10 and 2/10 *E. coli* carried the *vat* and *astA* gene respectively but no significant differences in cytotoxicity were observed.

Other toxins associated with ExPEC pathogenesis include the haemolysin (hlyE), heat-liable enterotoxin (a homologue of EAST1) and verocytotoxins/shiga toxins (*vtx1* and *vtx2*) [213-216] although their exact role in APEC pathogenesis remains unclear and fell outside this study.

Generally speaking, *E. coli* are not typically intracellular pathogens, but a number of ExPEC pathovars are associated with invasive behaviour, including uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC). Both pathotypes show a high level of genetic similarities to APEC [89].

NMEC is responsible for meningitis and sepsis in humans. The pathogenic pathway of NMEC involves bacteraemia and the subsequent crossing of the blood-brain barrier. NMEC invade the brain microvascular endothelial cells (BMEC) via a zipper-like mechanism involving the rearrangement of endothelial cell actin microfilaments [304]. NMEC invasion can be inhibited with the use of actin inhibitors such as cytochalasin D [313]. OmpA and type 1 fimbriae have been shown

to be important adhesins required for invasion of BMEC [304]. NMEC do not transverse the brain endothelium intercellularly by endothelial disruption [313]. Ibe proteins have been shown to play a role in invasion of BMEC and Δ*IbeA* NMEC BEN2908 were significantly less invasive than wild type strains [159]. It was originally thought that *ibeA* encoded an outermembrane protein involved in invasion. However, *Cortes et al* (2008) later suggested that *ibeA* encoded an intracellular bacterial protein involved in the regulation of type 1 fimbriae required for adhesion to endothelial cells [154]. Ibe proteins have also been shown to aid APEC pathogenicity in some cases with approximately 25% of APEC carrying this VAG, although it has been reported to be higher in isolates from UK broiler chickens [159, 283]. *IbeA* aids invasion but does not appear to be essential [159]. The *ibeA* gene was carried by 4 of the *E. coli* used in this study but this appeared insignificant to invasion.

Human urinary tract infections (UTIs) are the most common of extraintestinal *E. coli* infections and are caused primarily by the UPEC pathotype [23]. UPEC form protective ‘pod-like’ polysaccharide rich intracellular bacterial communities (IBCs) on the surface of the uroepithelium [303]. Within IBCs, UPEC interact with uroplakin using type 1 fimbriae to form stable structures [303]. *E. coli* contained within IBCs are protected against host surveillance and antibiotics, explaining why some UTIs are recurrent. One route of APEC systemic dissemination could be via the migration of APEC up the avian reproductive tract.

Enteroinvasive *E. coli* (EIEC) cause invasive inflammatory colitis and differ from other *E. coli* pathotypes as they are obligate intracellular bacteria. EIEC and *Shigella* are considered to have the same pathogenicity mechanisms but because of the

disease *Shigella* causes (shigellosis) it retains its own genus [23]. Most of the pathogenesis of EIEC is mediated by a TTSS encoded on a ~220kb virulence plasmid; pWR100 [312]. The Mxi-Spa locus of this plasmid encodes effector proteins IpaA-C and IpgD. IpaB and IpaC are inserted into specialised antigen sampling epithelial cells known as Microfold cells (M-cells). This initiates intracellular actin microfilament rearrangements at the host:bacterial interface, promoting the membrane ruffling required for bacterial invasion [314]. Once inside epithelial cells, bacteria are able to hijack the host machinery to avoid host detection and promote their intercellular dissemination [25]. *Shigella* can exit the basolateral surface of M cells and enter the submucosa [312]. Unlike EHEC, *Shigella* can also pass through the intercellular spaces of the epithelial cells created following destruction of tight junctions during inflammation [312, 314]. There are some examples demonstrating *E. coli* and related bacterial species intracellular nature.

Bacterial motility is considered an important virulence factor of ExPEC aiding bacterial migration and contact with the host epithelium. This is particularly true in the case of UPEC pathogenesis, where flagella mediated motility enables ascendance of the urinary tract [315]. In the current study, 6/10 *E. coli* were considered motile but no correlation between invasion and motility was observed probably because once *E. coli* were added to the epithelial cells, they were gently mixed insuring the bacteria came into contact with the cells during the invasion assay. EIEC are, in fact, non-motile pathogens and, as mentioned, utilise the host machinery to ensure migration [312]. Wooley *et al* (1993) showed that systemic *E. coli* were more likely to be motile than intestinal strains [199]. Non-motile intestinal and extraintestinal pathogens are well documented suggesting means of overcoming the non-motile

phenotype and perhaps advantages to being non-motile (to avoid host recognition of flagella for example) [316, 317].

These examples illustrate how certain *E. coli* pathotypes have evolved mechanisms to allow invasion. The host is also likely to play an important role in bacterial:host interactions. Some studies suggest APEC are only invasive under host physiological stress conditions. Oral inoculation of turkeys and broiler chickens with approximately 10^8 *E. coli* resulted in colonisation of turkey spleens and increased mortality in the presence of an environmental stressor (feed withdrawal or heat) [130]. In a similar study, the predisposition of axenic turkeys to stress prior to oral administration of pathogenic *E. coli* resulted in greater colonisation of liver rather than lung tissue [129]. Stress has been described as one of the leading causes of APEC dissemination [46]. The Caco-2 epithelia used in this study was not subjected to any stressful conditions but this has been simulated previously with the introduction of noradrenaline to cultured epithelial cells [259].

Noradrenaline has been shown to increase the expression of *E. coli* O157 virulence factors including adhesion factors, which led to increased adherence and colitis in infection models [318, 319]. Pre-incubation of noradrenaline and Caco-2 cells has been shown to increase the breakdown of epithelial tight junctions and noradrenaline increases intestinal vascularisation to aid the infiltration of host immune cells. Such physiological changes could allow APEC to enter the bloodstream [259].

In an attempt to meet consumer demands, modern commercial broilers now reach slaughter weight (1.8-3.0kg) 60% faster than in the 1970s, with an annual increase in growth of 5% [55]. The growth rate stressor may facilitate systemic dissemination of APEC. *In vitro* experiments are used in an attempt to achieve the three Rs

(reduction, replacement and refinement) of licensed animals but they do not allow for an accurate understanding of the complex interactions involved in the pathogenesis of bacterial pathogens. Such experiments would also allow consideration for the surrounding microbiota that is likely to influence bacterial behaviour and the gut environment [320].

This chapter reports the apparent non-invasive phenotype of pAPEC but suggests other mechanisms may be used to disseminate throughout the host, including disruption of the epithelial integrity. The *E. coli* strains used in this study showed comparative biological characteristics to the reference *E. coli* strain, O78. Further work is required in an attempt to expand on the *in vitro* conditions assessed including the simulation of physiological stress, conditions which may be encountered in the intestinal tract of commercial broiler chickens. Efforts are limited as we await the development of a reliable and reproducible chicken intestinal cell line.

Chapter 6

Identifying differences in the interactions of avian pathogenic and avian faecal *E. coli* with avian macrophages and survival in chicken serum

K. Kemmett, T. Humphrey, G. Chaloner, S. Humphrey, P. Wigley, N. J. Williams

[1]University of Liverpool, Leahurst Campus, Neston, CH64 7TE, UK

ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) is a massive economic burden to the global poultry industry, which is currently struggling to meet consumer demand. APEC is associated with fibrin-based lesions around visceral organs and septicaemia in poultry. The molecular mechanisms involved in APEC pathogenesis are not fully understood. In order to survive extraintestinally, APEC must resist killing by the avian immune system. Colonisation of the respiratory tract following the inhalation of faecal dust initiates the influx of innate defence cells including phagocytic macrophages. To disseminate via the blood, APEC must resist killing by the complement system. The current study aimed to compare the intracellular persistence and survival of APEC (n=5) and avian faecal *E. coli* (n=4) in cultured avian macrophages (HD11) and in chicken serum. There were no significant differences ($p>0.05$) in the uptake and persistence of APEC and avian faecal *E. coli* by HD11 macrophages. At 24 hours post-infection, avian faecal *E. coli* 396 and 495 showed the greatest amount of persistence with 2.44 and 3.04% of the original inocula detected intracellularly, respectively, compared to 10.89% with the *Salmonella* Typhimurium control. All isolates successfully stimulated proinflammatory responses in HD11 cells (quantification of IL-6 and CxCLi2). There were no significant differences between APEC and avian faecal *E. coli* survival in serum; all isolates showed at least 60-70% survival following 3 hours incubation with 10% serum. Three APEC isolates carried *iss* and demonstrated 95-109% survival. This supports the hypothesis that opportunistic infection by non-pathogenic *E. coli* may be possible.

6.1 Introduction

Poultry health and welfare are important in allowing for sustainable and secure food production. In the UK alone, over 900 million broiler chickens are currently farmed annually and worldwide poultry production is expected to increase rapidly to meet both the rise in the global population and the increasing demand for meat in an increasingly rich populations in countries such as India and China [3]. Meeting this demand, whilst retaining or improving animal health and welfare, is a challenge. Furthermore, practices such as the use of antibiotics as growth promoters are unsustainable and have been banned in the EU and there is an increased reliance on other disease control methods including vaccination and breeding for disease resistance.

Avian pathogenic *Escherichia coli* (APEC) are an ill-defined pathotype of the extraintestinal pathogenic *E. coli* (ExPEC) group. APEC cause an avian syndromic disease characterised by fibrinous lesions around visceral organs often referred to as colibacillosis, which is commonly associated with airsacculitis, cellulitis, pericarditis, perihepatitis, respiratory distress and septicaemia in broiler chickens [48]. Colibacillosis is an economic burden resulting in mortalities during production and the rejection of carcasses at slaughter.

The APEC pathotype originates from diverse *E. coli* lineages and, although APEC pathogenesis is unclear, a range of virulence-associated genes (VAGs) has been described [78, 83, 159, 182, 185, 189, 252].

Potential APEC (pAPEC) reside in the gastrointestinal tract and systemic infection is likely to be the result of inhalation of contaminated faecal dust [43, 127]. *E. coli* have

been shown to persist on poultry house dust particles at levels exceeding 10^6 colony forming units per gram [128]. *In vivo* studies suggest 10^6 and 10^9 CFU are sufficient in causing lung and systemic infections respectively [127]. Previous chapters support the presence of pAPEC in the avian gut and the high diversity among extraintestinal *E. coli*.

The respiratory tract lacks a resident innate cellular defence system with very few macrophages and heterophils (the chicken orthologue of mammalian polymorphonuclear neutrophils) residing in the respiratory tissue awaiting challenge [193]. Thus, the respiratory tract must rely on the infiltration of peripheral blood monocytes along with macrophages and heterophils residing in bronchus-associated lymphoid tissues (BALT) [193-195].

Heterophils are polymorphonuclear cells and are considered to be the orthologue of the mammalian neutrophil. They are rapid responders to infection but only have a short life span of around 7-10 days once released from bone marrow. Macrophages are large mononuclear phagocytic cells that may be found in tissues or as progenitor forms such as monocytes and pro-monocytes within the circulatory system. Their life span is longer than that of heterophils, typically around 80 days. Heterophils and macrophages are considered to be the main effector cells of the induced innate immune system [195].

The availability of the chicken genome has helped improve our understanding of the avian immune system [235]. Pattern recognition receptors (PRR) found on the surface of phagocytic cells recognise a diverse set of microbial associated molecular patterns (MAMPs). The most common PRR are Toll-like receptors (TLRs) and currently 13 have been described in the chicken (two of which are chicken specific:

TLR-15 and TLR-21) [321]. Chicken TLRs recognise a similar pool of MAMPs to the mammalian repertoire including: lipopolysaccharide (LPS), peptidoglycan, flagellin and nucleic acids. Receptor-ligand binding triggers proinflammatory intracellular signalling pathways (NFκB and mitogen activated protein kinase pathways) initiating the activation and recruitment of phagocytic cells and lymphocytes. This is reviewed elsewhere [311]. Macrophages produce bactericidal reactive oxygen species (hypochlorous acid, superoxide and hydrogen peroxide) using oxidative burst reactions within the phagolysosome allowing bacterial degradation and clearance. Immune responses are co-ordinated by molecular messengers known as cytokines and chemokines [322].

APEC infection studies using signature tagged mutagenesis or specific gene knock-outs have been used to test molecular Koch's postulates in a number of different studies to evaluate the contribution of VAGs to APEC colonisation and persistence in respiratory tissues [47, 155, 197]. These studies have highlighted the possible importance of temperature sensitive haemagglutinin (Tsh), P-fimbriae and Type 1 fimbriae in colonisation of the respiratory tract. Previous work has also looked at the contribution of these factors to the interaction of APEC with avian macrophages whereby some *E. coli* factors repel such interactions (P-fimbriae) while others appear to promote them yet prevent degradation once bacteria reside intracellularly (Type 1 fimbriae) [243].

In order to cause systemic infection and gain entry into the bloodstream, APEC must overcome infiltrating macrophages. It has been shown *in vivo* that APEC may reside free in the air sac lumen or in close contact with macrophages, with some speculation over the ability of APEC to replicate intracellularly [196]. Pourbakhsh *et al* (1997)

correlated virulence with the ability of APEC to resist killing by macrophages [196]. Persistence in macrophages could provide *E. coli* with systemic transport mechanism within a protected environment.

Previously, this thesis has shown that extraintestinal *E. coli* represents a diverse population in terms of both their genetic background and VAG carriage [283]. However, we speculate that all successful extraintestinal pathogenic *E. coli* must possess mechanisms allowing their survival and non-pathogenic *E. coli* would not harbour such traits.

Recently Horn *et al* (2012) compared 3 previously isolated APEC strains, including APEC MT78, and 1 avian faecal *E. coli* [234]. Examination of pathological lesions could not distinguish APEC from the non-APEC strain but only a limited number of isolates were included in the study.

Examining such interactions can be done in the laboratory using an immortalised avian macrophage cell line known as HD11. This HD11 cell line has been well characterised and shown to be highly representative of primary macrophages in terms of cytokine production [323, 324].

During septicaemia (often associated with APEC infection) APEC must overcome the bactericidal effects of the complement immune system. Two complement pathways in serum can result in the killing of APEC; the antibody-mediated classical pathway or the surface polysaccharide recognition alternative one [325, 326]. APEC survival in serum has been described since the early 1990s and a number of virulence factors have been identified in contributing including; *pap* operon, O78 antigen, OmpA, TraT and Iss [171].

To start to address whether the diversity seen among extraintestinal *E. coli* in previous chapters is a result of opportunistic infection, or whether these *E. coli* possess mechanisms required to survive systemically, this chapter compares the survival of UK isolated extraintestinal (APEC) and avian faecal *E. coli* following exposure to macrophages and commercial broiler serum.

6.2 Materials and methods

6.2.1 Bacterial isolates

The *E. coli* isolates used in this study were isolated from the faeces or visceral organs of 'healthy' or diseased commercial broiler chickens respectively (Table 6.1) [283]. Test isolates were selected based on their carriage of VAGs and site of isolation, as determined previously [283]. A summary of isolate information can be found in Table 6.1. Prior to use, all *E. coli* were stored at -80°C in Microbank vials (Pro-Lab Diagnostics, UK). APEC O78 was used as a representative APEC strain and was kindly donated by Professor Mark Stevens of The University of Edinburgh Roslin Institute, Scotland, UK.

Table 6. 1 List of test *E. coli* strains from various sources including VAG carriage and phylogroup data

		Virulence Associated Genes (VAGs)											
Isolate	***Source***	***astA***	***iss***	***irp2***	***papC***	***iucD***	***tsh***	***vat***	***cvi/cva***	***sitA***	***ibeA***	**Total VAGs**	**Phylogenetic group**
601	Lung	0	0	0	1	1	0	1	1	1	0	5	D
588	Heart	0	1	1	1	1	0	1	1	1	0	7	D
24B	Liver	0	1	1	0	1	0	1	1	1	1	7	B2
24F	Heart	0	0	1	0	1	1	1	0	1	1	6	D
18C	Heart	1	1	1	0	1	1	0	1	1	0	7	D
317	Faeces	0	0	0	0	0	0	0	0	0	0	0	D
396	Faeces	0	0	0	0	0	0	0	0	0	0	0	A
495	Faeces	0	0	0	0	0	0	0	0	0	0	0	A
571	Faeces	0	0	0	0	0	0	0	0	0	0	0	A

1; positive by PCR for VAG, 0; negative by PCR for VAG

6.2.2 Avian macrophage gentamicin invasion assays

The gentamicin invasion assay protocol was based on that previously described by others [259, 260].

6.2.2.1 Cell line seeding protocol and cell line preparation

The avian macrophage cell line (HD11) [258] was grown in RPMI-1640 media (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich, UK), 1% GlutaMAX^{TM} (100x concentration) (Invitrogen, UK), 1% MEM non-essential amino acid solution (Sigma-Aldrich, UK) and penicillin–streptomycin (100 U/ml) antibiotics (Sigma-Aldrich, UK). Cells were grown at 37°C. Two days before the infection studies, cells were seeded into 24-well tissue culture plates at a density of 4.5 x 10^5 cells per well and incubated at 37°C to give a final density of 1 x 10^6 cells. One day before the experiment, the RPMI with the above supplementation was substituted for antibiotic-free, serum-free medium (AFSF RPMI).

6.2.2.2 Bacterial culture preparation

All bacterial isolates were obtained from the -80°C culture collections, streaked onto nutrient agar (NA) (LabM, UK) and incubated overnight at 37°C. *S.* Typhimurium 4/74 was used as an invasive positive control and APEC O78 as a characterised APEC positive control. One day prior to the invasion assay, 2-3 colonies of each overnight culture were used to inoculate 2ml of sterile LB broth. Bacterial suspensions were incubated overnight at 37°C. On the day of the experiment, 100µl of overnight culture was added to 10ml of fresh LB broth and incubated at 37°C for 3.5 hours at 150rpm. After 3.5 hours, the OD_{600} was adjusted using sterile phosphate

buffered saline (PBS) to 0.27 +/- 0.05 to reduce differences in multiplicity of infection (MOI) between bacterial samples. These adjusted suspensions (MOI of ~28) were used to infect the HD11 cell line.

6.2.2.3 Infection of avian macrophages

The intracellular survival of *E. coli* in HD11 macrophages was assessed at 1, 4 and 24 hours post-infection.

Prior to infection, the AFSF RPMI was removed from the HD11 cells. Cells were washed 3 times with sterile PBS, which was replaced with 1ml of 37°C AFSF RPMI. Cell monolayers were incubated for 2 hours at 37°C in 5% CO_2.

100µl of bacterial samples (with adjusted OD_{600}) were added to the monolayers in triplicate. Infected monolayers were re-incubated at 37°C in 5% CO_2 for one hour. The supernatant was removed and stored at -20°C to assess pathogen-mediated cytoxicity. One ml of RPMI containing 100µg/ml gentamicin sulphate (Invitrogen, UK) was added to each well and incubated at 37°C for 1 hour. The RPMI was removed and cells washed once with sterile PBS. Cells were lysed with 0.5% Triton X-100 in 1ml PBS incubated at 37°C for 5 minutes. Cell lysates were serially diluted (1:10) using PBS and invasive bacteria enumerated on NA.

The level of bacterial invasiveness was also analysed at 4 and 24 hours post-infection. However, instead of adding PBS-Triton X-100 after 1 hour post infection, 1ml of fresh RPMI containing 20µg/ml of gentamicin sulphate was added to the infected monolayers. Cells were then incubated at 37°C until 4 or 24 hours post-infection. At this time, the supernatant was removed and stored at -20°C for

cytotoxic analysis and cells were lysed using PBS-Triton X-100 as previously described.

6.2.2.4 Statistical analysis

Differences in the percentage invasion were examined using analysis of variance.

6.2.3 Real time quantitative reverse transcriptase polymerase chain reaction for avian cytokines

6.2.3.1 Isolation of RNA

After 1 hour co-incubation of avian macrophage HD11 cells with *E. coli* (as described above), 350μl of Buffer RLT was added directly to the cells [327]. Cells were then expelled from wells and stored in 1.5ml eppendorf tubes at -80°C until required. RNA was eluted into 50μl of RNase-free water using a Qiagen RNeasy Mini kit following the manufacturer's instructions (Qiagen, West Sussex, UK). The total RNA was quantified by measuring light absorption at 260 and 280 nm with a Nanodrop (ND-1000) spectrophotometer.

6.2.3.2 Quantification of pro-inflammatory cytokine RNA

The expression of the pro-inflammatory cytokine Interleukin 6 (IL-6) and the pro-inflammatory chemokine CXCLi2 by avian macrophages in response to exposure to *E. coli* was quantified using real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). All reagents were obtained from Qiagen, West Sussex, UK. The quantification of 28S rRNA was used as a standard to allow comparisons. Primers and probes were based on those previously described and are outlined in

Table 6.2 [328]. Twenty µl-reaction mixtures for one-step qRT-PCR were prepared using the RotorGene Probe RT-PCR kit and contained: 1 µl of total RNA, 10 µl of RotorGene Probe RT-PCR Master Mix, 0.2 µl RotorGene RT Enzyme Mix, 1.6 µl of each primer (at 10 µM), 0.8 µl of probe (at 5 µM) and 4.8 µl of RNase-free water. Reactions were set up using an automated QIAgility system. Real-time RT-PCR was performed on a RotorGene Q system with the following reaction conditions: 50°C for 10 minutes (RNA to cDNA reaction), then 95°C for 5 minutes, and 40 cycles of the two stage reaction of 95°C for 5 seconds and 60°C for 10 seconds. Each reaction was performed in triplicate and each run included non-template controls and non-probe controls.

6.2.3.3 qRT-PCR analysis

The levels of IL-6 and CXCLi2 expression are shown measured as the average threshold cycle value (Ct) from the triplicate PCRs. The Ct value is the PCR cycle number at which the reporter dye passes a significance threshold (this is half way up the exponential phase and varies depending on the level of RNA). The level of target gene expression was assessed relative to the standard reference gene 28S rRNA by calculating the difference (ΔC_T) between the comparative threshold (C_T)values of the target genes and the 28S rRNA. To determine the effect of *E. coli* exposure on the expression of pro-inflammatory messengers, the relative expression of the target genes in a sample ($\Delta\Delta C_T$) was calculated by finding the difference between each sample target gene ΔC_T and the uninfected control ΔC_T for each cytokine/chemokine. Finally, the fold change in expression between the infected and uninfected cells was calculated ($2^{-\Delta\Delta CT}$) for each isolate.

To clearly illustrate the differences between the infected and uninfected expression and the individual isolates, the results have further been analysed in the 40-Ct format. This represents the maximum cycle number (40) minus the threshold detection value (Ct). The higher the 40-Ct value the greater the gene expression.

6.2.3.4 Statistical analysis

The PCRs were repeated in triplicate for each *E. coli* isolate and the infection experiments were also repeated three times. The average level of expression and standard deviation was calculated for each of the 12 bacterial isolates. Significant differences between individual isolates were determined using analysis of variance (one way ANOVA) with the post-hoc Tukey test. Differences between systemic and faecally derived *E. coli* were assessed using the Student's t-test. Differences were considered significant at $p<0.05$.

Table 6. 2 Real time quantitative RT-PCR primers and probes

RNA target	Primer sequence (5' - 3')	Gene Bank Accession no.
28s	F: GGCGAAGCCAGAGGAAACT R: GACGACCGATTTGCACGTC P: (FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)	X59733
IL-6	F: GCTCGCCGGCTTCGA R: GGTAGGTCTGAAAGGCGAACAG P: (FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)	AJ250838
CXCLi2	F: GCCCTCCTCCTGGTTTCAG R: TGGCACCGCAGCTCATT P: (FAM)-TCTTTACCAGCGTCCTACCTTGCGACA-(TAMRA)	AJ009800

6.2.4 Serum survival assay

The serum survival assay was based on that previously described with slight modifications [171]. Serum was collected from 3-week-old commercial broiler chickens and stored at -20°C. *E. coli* and *S.* Typhimurium 4/74 were grown in 2ml LB broth overnight at 37°C in a shaking incubator at 150rpm. Mid-log phase cultures were obtained by diluting overnight cultures 1:100 into fresh LB broth and incubated at 37°C at 150rpm for 1 hour 45 minutes. The optical density at 600nm was measured and cultures adjusted accordingly in gelatin-veronal buffered saline solution with magnesium and calcium ions (GVB^{2+}), pH 7.35 (TCS Biosciences limited, UK) to give final inocula containing approximately 10^6 colony forming units (CFU) ml^{-1} mid-log phase bacteria. 100μl of serum was added to 900μl of inoculum (1:10) with gentle mixing and incubated at 37°C for 3 hours. At 1 hour intervals, 20μl of culture was removed, serially diluted in PBS and plated onto NA for bacterial enumeration. Results are displayed as percentage survival of the original inoculum. The same assay was repeated using stationary phase bacteria (4 hours incubation). The differences between APEC and avian faecal *E. coli* were assessed using the student's t test.

6.3 Results

6.3.1 Comparing the invasive phenotypes and persistence of APEC and avian faecal *E. coli* in avian macrophages

This study focused on comparing the invasion/phagocytosis and persistence of APEC and avian faecal *E. coli* in cultured avian macrophages at 1, 4 and 24 hour post-infection.

6.3.1.1 Invasion/uptake in avian macrophages – 1 hour post-infection

At 1 hour post-infection, the highest level of intracellular bacteria was observed for the avian faecal isolate 495 (0.41% of inoculum). The highest intracellular count by systemic *E. coli* isolates was that of 24B (0.26% of original inoculum). The positive control *S*. Typhimurium 4/74 averaged 1.69%. The data are illustrated in Figure 6.1. The bacterial counts 1hour post-infection for *E. coli* 24B, 24F, 495 and 571 were not significantly different compared to the *S*. Typhimurium control ($p<0.05$). There were no significant differences between APEC and avian faecal *E. coli* at this time point ($p>0.05$).

6.3.1.2 Invasion/persistence in avian macrophages – 4 hours post-infection

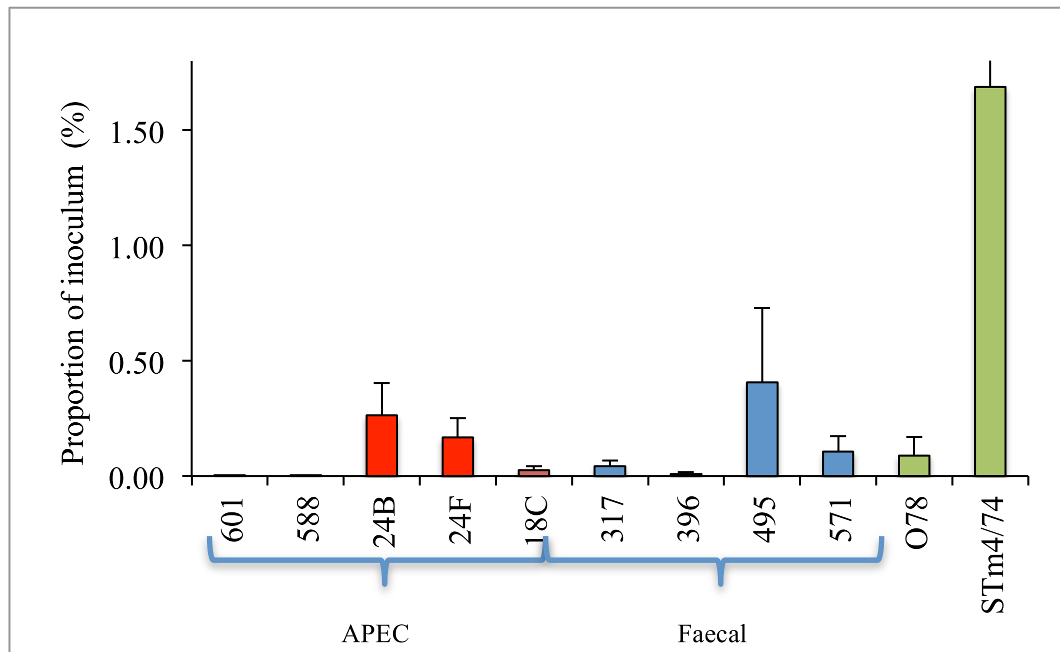
At 4 hours post-infection, there was no overall statistically significant difference between APEC and avian faecal *E. coli* ($p>0.05$). There were also no significant differences between APEC 601 and 588 ($p>0.05$) and *S*. Typhimurium 4/74, suggesting these isolates show similar levels of intracellular bacteria at this intermediate time point. Intracellular APEC 601 and 588 (0.84% and 1.04%

respectively) were identified at higher levels than the APEC O78 (0.34%). Results are displayed in Figure 6.1b.

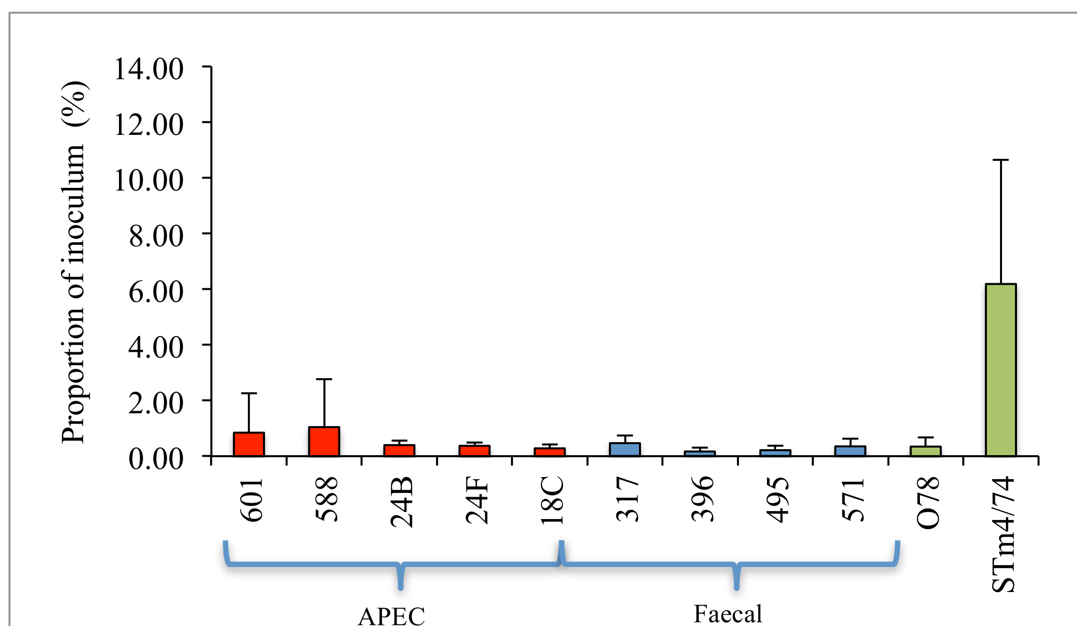
6.3.1.3 Net replication/persistence in avian macrophages – 24 hours post-infection

The ability of APEC and avian faecal *E. coli* to persist in avian macrophages (as a possible mechanism of systemic spread) was evaluated following 24 hour incubation of the HD11 cells. Avian faecal *E. coli* 396 and 495 showed the highest level of persistence among the test strains with 2.44 and 3.04% of the inoculum being detected 24 hours post-infection but this was not statistically significant ($p>0.05$). There were no significant differences between the *E. coli* used in this study and all strains, including APEC O78, were significantly poor persisters in comparison to *S.* Typhimurium 4/74 (10.89%). Results are displayed in Figure 6.1c. The cytotoxic effects of exposure to *E. coli* and time was not measured, however caution must be taken when considering the bacterial counts obtained here; any increase in permeability would allow for gentamicin access to intracellular bacteria.

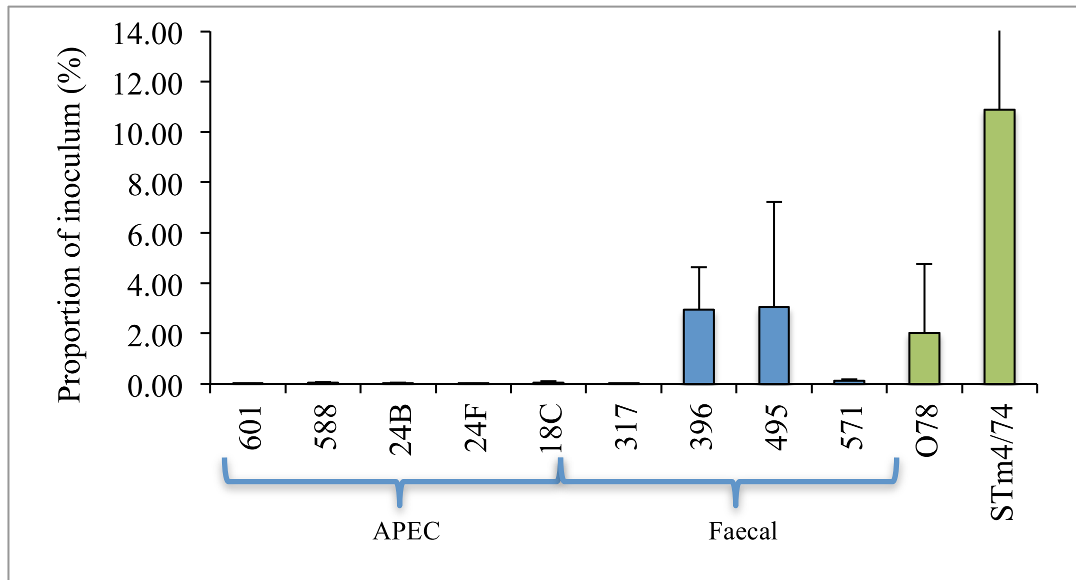
Figure 6. 1a Measure of intracellular *E. coli* in avian macrophages (gentamicin invasion assay) 1 hour post-infection.



b) 4 hour post-infection



c) 24 hour post-infection

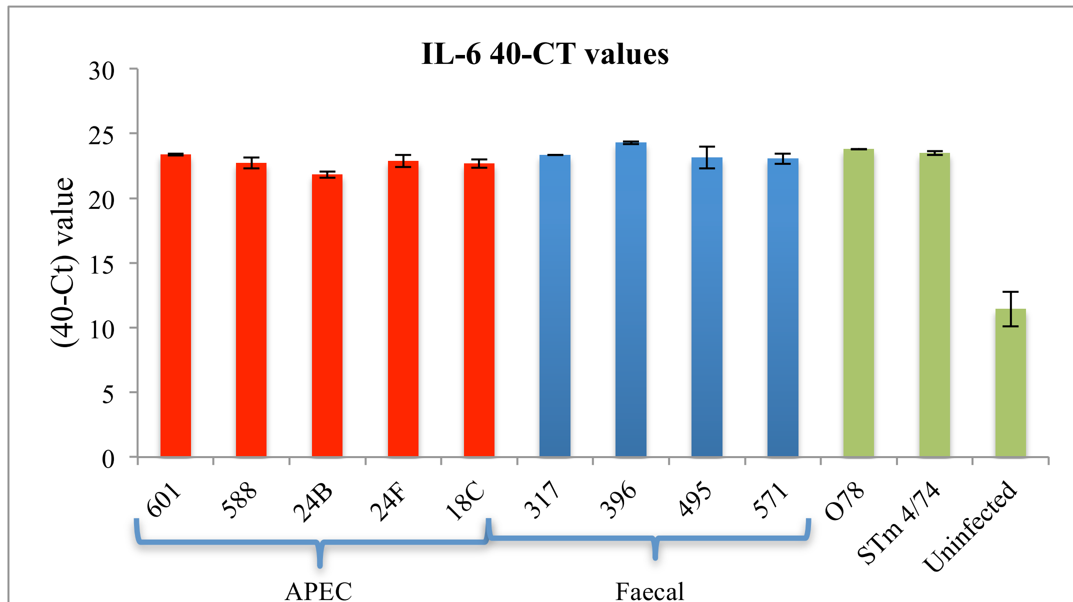


Values are shown as the percentage of the original inoculum. The standard deviation of the three experiments is represented by the error bars. Analysis of variance identified no significant differences between APEC and avian faecal E. coli. S. *Typhimurium 4/74 was used as a positive invasive control and APEC O78 was used as an APEC reference strain.*

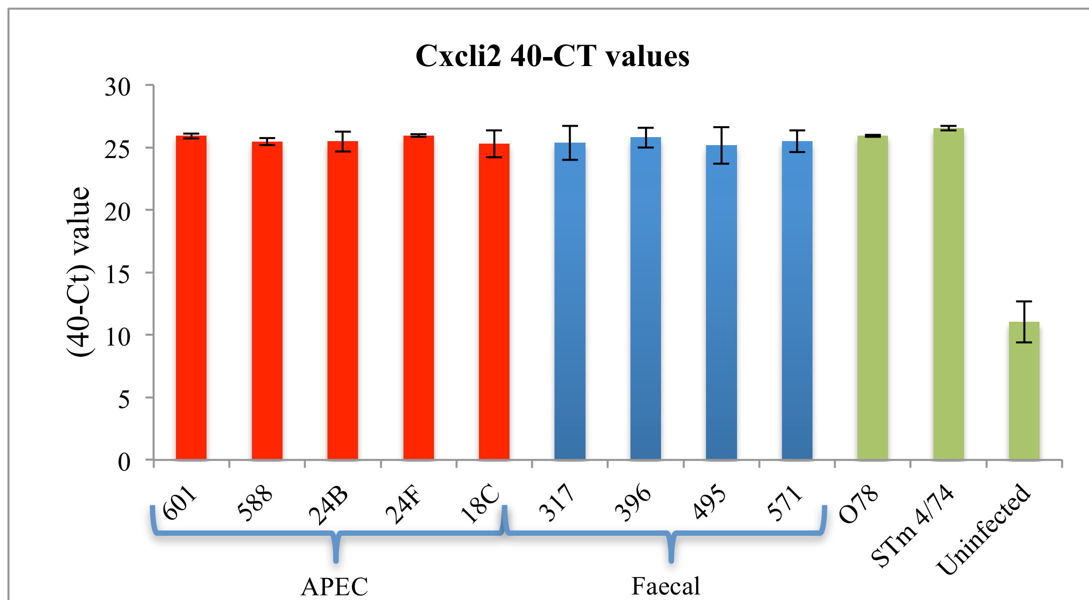
6.3.2 Pro-inflammatory cytokine production in response to exposure to *E. coli*

Infection with all *E. coli* elicited a rapid pro-inflammatory response in the avian macrophage HD11 cell line when compared to non-infected macrophages ($P<0.05$) (Figures 6.2). There were no significant differences in the expression fold change between those isolates classified as APEC and avian faecal ($p>0.05$). Furthermore, there were no significant differences between the individual isolates. Both macrophage IL-6 and CXCLi2 expression was increased following exposure to *E. coli*.

Figure 6. 2 Expression of IL-6 (a) and CXCLi2 (b) by avian macrophages in response to APEC and avian faecal *E. coli.*



b) CXCLi2

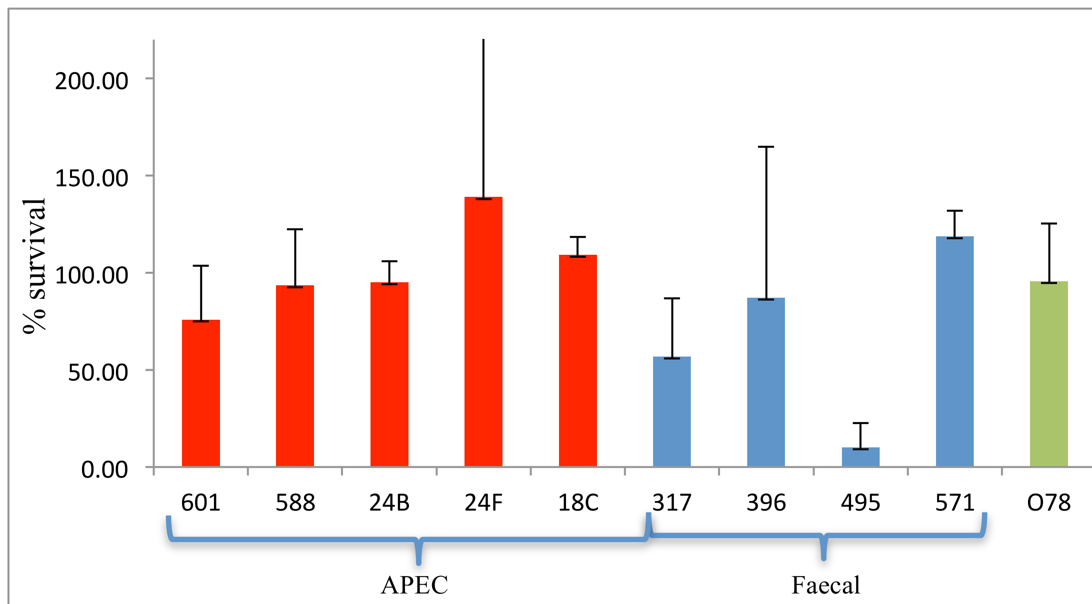


All E. coli *elicited a significant IL-6 and CXCLi2 response in avian macrophages compared to uninfected controls ($p<0.05$). Data are represented as 40-Ct values. 40 is the maximum number of repeat cycles and the Ct is the cycle number at which the detection of a probe reaches a threshold value. The higher the 40-Ct value the greater the expression.*

6.3.3 Serum survival

There were no significant differences in the survival of *E. coli* in mid-log and stationary phase. There were also no significant differences between APEC and avian faecal *E. coli* (p>0.05). Of the isolates carrying the *iss* gene (588, 24B and 18C), approximately 95-109% of the original inoculum was present 3 hours after exposure. The results for the 3 hour exposure time point are presented as percentage survival of original inoculum for each isolate (mid-log) (Figure 6.4). A number of the isolates appeared to be capable of growth in the presence of serum (*E. coli* 24F, 18C and 571). All other isolates, excluding 396, were identified at levels of approximately 60-70% of the original inoculum following 3 hours exposure to 10% serum.

Figure 6. 4 Serum survival of APEC and avian faecal *E. coli*.



Results are presented as the percentage survival of the original inoculum. Error bars represent the standard deviation from the three repeats. There were no significant differences between APEC and avian faecal E. coli *(p>0.05).*

6.4 Discussion

In order for APEC to spread systemically it must overcome several challenges imposed by the avian immune system. One of the initial challenges is the infiltration of innate immune cells at the site of infection [43, 127, 128]. The avian respiratory tract harbours low levels of resident heterophils and macrophages and relies heavily on cellular infiltration [194]. Following infection, air sacs are quickly vascularised to support this [193, 310].

This study found no significant differences between APEC and avian faecal *E. coli* in their ability to persist inside phagocytic cells. A recent study comparing a smaller panel of APEC and avian faecal *E. coli* reported no differences in the histopathology of infected birds and comparable colonisation levels between avian faecal and APEC isolates [234]. Such results highlight the differences between APEC isolates themselves and make deciphering APEC pathogenesis more difficult.

Electron microscopy of chicken air sacs 24 and 48 hours post inoculation revealed an average of 18 and 29 intracellular CFU per macrophage respectively [196]. Furthermore, APEC virulence was correlated with resistance to macrophage killing. Similarly, invasive adherent *E. coli* (IAEC) are capable of replicating in J774 murine macrophages with cell counts increasing more than 74 fold in 48 hours [329]. There is speculation over the ability of APEC to replicate intracellularly, but the mechanisms are not understood [196]. In the current study, both APEC and avian faecal isolates failed to persist intracellularly in macrophages following 24 hours infection. This study did not assess the extracellular survival of *E. coli* in the presence of macrophages, but this may be an important trait in allowing systemic spread considering APEC fail to persist intracellularly.

Key cells of the innate immune system (heterophils and macrophages) are associated with non-specific immune responses. PRRs, such as TLRs, recognise a variety of MAMPs associated with both non-pathogenic and pathogenic bacteria [311]. Despite this, macrophages from inbred chicken lines reportedly contribute to *Salmonella* resistance [327]. One hypothesis for this is a rapid proinflammatory cytokine and chemokine response, involving significant fold increases in IL-6 and IL-18 (lymphocyte and macrophage inducing cytokines) levels [327]. These cytokines have also been shown to be important in the differential susceptibility to Marek's Disease Virus [330].

The current study looked at the fold change in the proinflammatory cytokine IL-6 and the proinflammatory chemokine CXCLi2. There were no significant differences in fold change between APEC and avian faecal isolates. This is most likely because of the non-specific responses induced by macrophages. Some pathogens actively dampen proinflammatory responses to remain undetected by the host [327]. My work suggests that this panel of APEC, failed to dampen proinflammatory responses and therefore this is not a pathogenic mechanism used by these isolates. The availability of the chicken genome continues to be a valuable tool in improving our understanding of the chicken immune system, an absolute paramount in allowing improvements in poultry health and welfare and subsequently food safety, security and improved financial return [235].

Some pathogens have evolved mechanisms to avoid clearance by phagocytic cells. Mutations in the *Salmonella* pathogenicity island (SPI-2) type 3 secretion system reduce the ability of these bacteria to resist killing by macrophages [260].

Mutational and gene knock out studies have aided the identification of APEC colonisation factors in the respiratory system. Type 1 fimbriae bind D-mannose residues located in the trachea, lung and air sacs but play little role in the colonisation of deeper tissues [300]. Variation also occurs within different sites of the respiratory tract itself: P-fimbriae (expressed by the *pap* operon) are expressed by APEC in the air sac and lungs but not in the trachea [331]. Tsh has also been shown to contribute to colonisation of the trachea [155]. IbeA is an invasive factor involved in the pathogenesis of neonatal meningitis *E. coli* (NMEC) and APEC [159]. Deletion of *ibeA* impairs Type 1 fimbriae and reduces APEC biofilm formation [154, 162]. The assembly of biofilms contributes to resistance against macrophages [332]. Additionally, large amounts of iron are required for biofilm formation and many APEC VAGs relate to iron acquisition (*irp2, iucD, sitA*) [32, 43]. Biofilm formation was not assessed in the current study, although a number of isolates carried genes that may contribute to this. Past work has shown that APEC harbouring very similar VAG profiles differ in their infection biology, suggesting other factors, currently unidentified, may contribute to pathogenesis [234].

The K1 capsule and P-fimbriae have been negatively associated with macrophage interactions [243, 333]. The authors suggested that the PapG component of P-fimbriae produces an electrostatic repulsive effect to macrophages as shown in human *E. coli*. The same authors suggest Type 1 fimbriae promote the association of APEC and macrophages but protect against subsequent killing. It is clear that the interaction between APEC and macrophages is complex and not fully understood, although it involves a network of factors.

Many Gram negative bacteria possess conserved environmental sensory systems allowing them to detect changes and adapt accordingly to promote survival [334, 335]. One system associated with APEC pathogenesis is the BarA-UvrY two-component system [336]. Functional mutations in this system in APEC O78 (chi7122) reduced its ability to survive in HD11 macrophages and in serum. Mutant APEC O78 were more rapidly engulfed and digested. The authors suggested that this was perhaps because of reduced catalase production associated with such a mutation, subsequently impairing the ability of APEC to resist the oxidative burst induced by macrophages.

The second part of this chapter assessed the survival of APEC and avian faecal *E. coli* in serum. This study suggests there were no significant differences in the ability of APEC and avian faecal *E. coli* to resist destruction by the complement system. Resistance to complement has long been associated with systemic pathogens [337, 338]. Two complement pathways found in serum can result in the killing of APEC; the antibody-mediated classical pathway and the surface polysaccharide recognition alternative pathway [325, 326]. APEC survival in serum has been described since the early 1990s and a number of contributing factors have been identified including; P pili, O78 antigen, OmpA, TraT and Iss [171]. The *iss* gene encodes a 10-11KDa Increased serum survival (Iss) outermembrane lipoprotein [201]. Iss reportedly contributes to as much as a 100 fold increase in *E. coli* virulence [202]. High sequence homology between *iss* and the phage lambda *bor* gene, also involved in serum resistance, suggests *iss* evolved from a *bor* precursor [201]. Johnson *et al.* (2008) described the presence of 3 *iss* alleles associated with ColV/BM plasmids and at least 2 encoded on the *E. coli* chromosome [26, 203]. Between 38 and 82.7% of APEC possess *iss* and past studies have even focused on this gene as a potential

vaccine target [182, 204]. Not all research concurs with the importance of *iss* [200, 205]. In the current study, despite differences in VAG carriage (2 APEC possessed *iss*), there were no significant differences in serum survival. Furthermore, survival of the APEC isolated from the field was similar to that of the reference strain O78. Perhaps Iss plays a subtle role in virulence and is associated with a larger pathogenicity entity possibly being required for complete virulence.

Other proteins have been associated with APEC serum resistance, including TraT, another outer membrane protein [88, 202]. TraT inhibits complement by preventing the deposition of C3 and the formation of the C5b6 complex [206, 207]. TraT has also been associated with Enteroaggregative *E. coli* (EAEC) pathogenesis [208]. It is unknown whether this panel of *E. coli* encoded TraT.

Due to the increased levels of iron found in blood, bacteria must tightly regulate its uptake, as too much is toxic. Factors involved in such regulation (AraC-like regulator and YbtA) are increasingly expressed when in serum and deletion of these proteins reduces APEC survival [192].

Overall, no definitive differences were seen between APEC and avian faecal strains, potentially relating back to the possible opportunistic nature of APEC described first in Chapter 3 following the identification of diverse extraintestinal *E. coli* in broiler chickens [283]. It appears other contributing factors, including the state of the host, may be important and future work will need to focus on the broiler chicken specifically.

Chapter 7

Concluding Discussion

7.1 General Discussion

The primary objective of the investigation was to provide insight into the epidemiology of avian pathogenic *Escherichia coli* (APEC) on UK commercial broiler chicken farms. Knowledge about the epidemiology of APEC in broiler chickens is scarce, previous literature has largely focused on other production birds including layer hens and turkeys [85, 110, 123]. Particularly, little is known about the epidemiology and population biology of APEC burdening UK broiler production [85, 172, 339, 340].

The current investigation has contributed to our understanding of extraintestinal *E. coli* and the risk of infection, with the simultaneous identification and characterisation of potentially pathogenic *E. coli* (pAPEC) residing in the avian gastrointestinal tract (GIT) and extraintestinal *E. coli* from diseased broilers. This was achieved by screening 3360 avian faecal *E. coli* (952 of which were virulotyped) and the virulotyping of 324 extraintestinal *E. coli*. In the current investigation, pAPEC were defined as faecal *E. coli* harbouring ≥ 5 APEC virulence-associated genes (VAGs) (genes which aid APEC dissemination and systemic survival). The avian GIT has previously been identified as an APEC reservoir [31]. The avian GIT provides both a stable environment and the opportunity for APEC to disseminate into the external environment through faecal shedding. Over 10^6 *E. coli* can reside on poultry house dust particles, providing ample opportunity for the inhalation of APEC contaminated dust [127, 128]. Furthermore, as the digestive tract and the reproductive tract of poultry meet in the cloaca, another potential opportunity for *E. coli* to disseminate extraintestinally is by migration up the reproductive tract.

Chapter 3 included the longitudinal sampling of faecal *E. coli* populations. This offered a valuable snap shot of the changing intestinal *E. coli* population as well as the prevalence of pAPEC colonising the gut. Results obtained here suggest that as birds age the prevalence of pAPEC declines and the population diversity bottlenecks; with pAPEC accounting for almost 25% of the screened population in day-old chicks, compared to only 1% in 5 week-old birds. *E. coli* is a rapid coloniser of the neonatal gut, with such bacteria likely to originate from breeding flocks, the hatchery and human handling during transportation [12, 135, 264, 265, 341, 342]. The carriage of VAGs among intestinal colonisers has proven advantageous when competing with other members of the microbiota or within the hosts' external environment [274-276].

The findings from the first study prompted the investigation of extraintestinal *E. coli* related disease in chicks. Similar studies on Italian (broiler chicken) and Danish (layer hen) poultry farms confirmed the presence of extraintestinal disease at this early stage, often manifesting as omphilitis and yolk sac infections [50, 51, 123]. Furthermore, infected breeders may be a potential source for infection as well as a source for pAPEC colonising the chick GIT [30, 134]. The investigation undertaken in Chapter 4 supports that of others and suggests a proportion of early flock mortalities are the result of extraintestinal *E. coli* infections, with the isolation of extraintestinal *E. coli* from 70% of chicks subject to post mortem. This study did not look at alternative contributors to early mortalities, but work by Olsen *et al* (2012), among others, found dehydration, kidney related disease (nephropathy and visceral gout) and other infectious agents such as *Enterococcus faecalis* and *E. hirae* to be responsible for early mortalities (although *E. coli* infections were the primary cause) [133, 343].

Newly hatched chicks and breeders could be valuable targets in preventative therapies aimed at inhibiting or minimising pAPEC colonisation. The introduction of beneficial bacteria including *Bacillus* and *Lactobacillus* species to enhance growth performance and feed conversion efficiencies while reducing the APEC burden and strengthening the intestinal integrity in broiler chickens has recently attracted interest [295, 344]. This requires further investigation based on exisiting conflicting results likely to be due to differences in host status and differences in the avian microbiota between studies [295, 296].

A key finding in the current investigation is the high population diversity of extraintestinal *E. coli* isolated from diseased birds, which provides new insights into the infection biology of APEC related disease. Extraintestinal pathogenic *E. coli* (ExPEC) are a group of pathogenic variants capable of causing extraintestinal disease. Research suggests ExPEC strains cannot be discriminated from non- ExPEC bacteria using molecular epidemiological methods [345]. Much of the research focus has been on identifying the accessory genome (VAGs) promoting pathogenic status [74, 85, 89, 96, 268]. The identification of diverse lineages among APEC suggests that multiple lineages may give rise to APEC through the acquisition of VAGs, ultimately providing the machinery required to cause systemic infection [78, 269]. The availability of molecular techniques such as multi-locus sequence typing (MLST) has allowed for the identification of several sequence types (STs)/ clonal complexes (CC) associated with APEC including ST10, 23, 95 and 117 complexes [89, 96, 97]. Serotyping has identified O1, O2 and O78 as APEC associated serotypes, although these 3 groups represent only ~50% of APEC [29, 32], and take little account of genetic variation or potential for virulence within a serotype.

Although this investigation reports the high overall diversity among extraintestinal *E. coli*, findings, to an extent, concur with those from previous work, as a new ST (ST-2999) representing 22% of the extraintestinal *E. coli* analysed and multiple ST-117 isolates were identified. All of these isolates carried more than 5 VAGs. ST-2999 is a single locus variant of ST-117, an ST previously associated with human disease and chicken meat at retail [98, 99]. There remains an underlying concern for zoonotic disease here. This concern is elevated with the association of APEC with ExPEC variants including uropathogenic *E. coli* (UPEC), the primary cause of human urinary tract infections [89, 141]. As emphasised, the wider picture suggests the extraintestinal population diversity is high with overall little correlation between clonality or pulsotypes and VAG carriage. The identification of multiple strains, as opposed to a single clone in one bird, perhaps suggests infection may be opportunistic [32]. Since the undertaking of this investigation a study was published suggesting most *E. coli* found in human urinary infections are capable of bacteraemia, a similar scenario to the one described here [125].

Furthermore, the inability to determine an APEC pathotype is a major limiting factor in disease control on commercial poultry farms. This work found no significant correlation between VAG carriage and clonality. Sixty-three different VAG profiles amongst 324 extraintestinal *E. coli* from broilers aged 2 weeks and over were identified. A similar level of diversity was seen during the early mortality study of Chapter 4 where 30 VAG profiles were observed amongst 157 *E. coli*. Some studies have shown that virulence plasmids and PAIs, particularly colicin plasmids, contribute to APEC pathogenesis and are relatively abundant among these bacteria [217, 218, 224, 230, 231]. Linkages and co-inheritance of known VAGs have also been described. For example, *iss, tsh* and *cvi* have all been found on an APEC

associated ColV plasmid [26]. This investigation failed to find similar linkages and interestingly almost 18% of systemic isolates carried none of the 10 VAGs used in this investigation, but this list is not exhaustive. At the time of the study, constraints in finance and labour, as well as the large scale screening undertaken, prevented further VAG analysis.

Another hypothesis suggests there may be key host factors contributing to the susceptibility of birds to extraintestinal disease [56, 57]. The modern broiler chicken is far removed from both laying hens and traditional chicken breeds; the result of selective breeding for certain characteristics including high feed conversion ratio, rapid weight gain and high carcass conformity, potentially negatively selected against key host defence systems (such as innate immunity) [26, 56, 78, 89]. The host genetic background may be an important contributing factor in disease outcome, APEC pathogenesis and opportunistic microbial behaviour. Past work comparing APEC-related flock mortalities of different broiler genotypes suggests that rapid growth reduces broiler 'viability' [56, 57]. It is difficult to comment further, as broiler susceptibility was not the focus of this investigation.

It is impossible to truly simulate host-microbe dynamics in an *in vitro* environment, but such studies are valuable tools in elucidating potentially pathogenic and non-pathogenic bacteria by ultimately removing the host factor. Furthermore, *in vitro* analysis aims at reducing the use of animals in research (the 3 R's).

The identification of genetically diverse extraintestinal *E. coli* led the investigation to compare certain traits between extraintestinal and avian faecal *E. coli*. One hypothesis is extraintestinal *E. coli*, despite their diversity, all harbour mechanisms for overcoming components of the avian immune system while commensal *E. coli* do

not. Chapter 5 and 6 focused on a series of *in vitro* experiments addressing this. There were no significant differences between extraintestinal (carrying > 5 VAGS) and avian faecal *E. coli* (carrying 0 VAGs) to; persist in avian macrophages, elicit an innate inflammatory cytokine and chemokine response in macrophages, or resist the bactericidal effects of complement found in serum. Key to these experiments was the use of *E. coli* isolated from UK poultry farms in Chapter 3 and 4.

APEC survival in serum has been described since the early 1990s and a number of virulence factors have been identified to contribute; *pap* operon, O78 antigen, OmpA, TraT and Iss [171]. Li *et al* (2011) reported transcriptome data from APEC O1 grown in chicken serum (the study did not state whether this was from broilers or layer hens), revealing the occurrence of adaptive responses regulated by genes encoded on virulence plasmids and pathogenicity islands to subsequently aid survival and growth in the presence of serum [192]. One criticism of this study was the lack of comparison to a non-pathogenic control bacterium. In the current investigation, not only did some avian faecal *E. coli* resist the bactericidal effects of complement in serum, but in the case of *E. coli* 571, they appeared capable of replicating, similar to that described by Li *et al* (2011) for APEC O1 [192]. Interestingly, as with the current investigation, not all research concurs with the importance of *iss,* suggesting it only plays a subtle role in virulence [200, 205]. It may, however, be the case that minimal genes are required for septicaemia and most *E. coli* are capable of causing this, as has recently been described for human urosepsis [125].

Other work suggests that APEC are capable of residing and potentially replicating in avian macrophages *in vivo* [196]. Electron microscopy of avian air sacs 24 and 48 hours post APEC infection revealed an average of 18 and 29 intracellular colony

forming units per macrophage respectively, while virulence was correlated with macrophage resistance [196]. This did not appear to be the case for the isolates used in the current investigation.

A limitation to this study was the screening for only 10 VAGs. It is unknown whether the *E. coli* carried OmpA, TraT, type 1 Fimbriae, iron regulators (AraC-like regulator and YbtA) and the BarA-UvrY two-component system, all of which have been related to either APEC survival in sera or persistence in macrophages [26, 171, 192, 202, 207, 243, 336]. Since this work, further mPCRs reporting apparent accurate minimal markers for APEC detection have been published, although in the defence of this study these PCRs do not target the above VAGs. It would be of interest to know the profiles of the isolates collected during this investigation [132].

The results from the *in vitro* studies support the hypothesis developed in the early stages of the investigation; extraintestinal *E. coli* infections in broiler chickens may be the result of competent opportunists and the insignificant differences seen during the *in vitro* experiments are the result of avian adapted *E. coli* (with the absence of truly pathogenic *E. coli*).

Some ExPEC subtypes are associated with non-phagocytic invasive behaviour including NMEC and UPEC [303, 304]. Some studies show APEC possess similar invasive potential, although this was not confirmed in this study, which compared a panel of 10 pAPEC isolates using the human Caco-2 cell line [305]. Such invasion would allow pAPEC to transverse the intestinal tract in order to disseminate. Alternatively APEC may migrate up the reproductive tract and invade the lining epithelia in order to disseminate. Conflicting results between the current study and that of Matter *et al* (2011) could be due to the genetic and phenotypic diversity of

APEC. Furthermore, there are limitations when using *in vitro* analysis that eliminates physiological stress, inflammatory markers and the microbial population found in the avian gut. The availability of a reliable immortal chicken intestinal cell line would be useful.

This work highlights the complex relationship between the modern broiler chicken (as seen with the field studies) and the true pathogenic mechanisms of APEC (as attempted to be shown *in vitro*). Previous studies have used criteria such as virulence in day-old chicks, specific-pathogen free birds, or the intra-air sac inoculation of $10^8/10^9$ APEC to assess virulence [196, 223, 234]. Such criteria have resulted in conflicting results. For example, Horn *et al* (2012) found the virulence of UEL17 to be far less severe in 5 week-old chickens than was previously described by Vidotto *et al* (1990) [223, 234]. Host-microbial interactions are highly complex and caution must be taken when classifying “pathogens”. As shown in the current investigation and as largely expected, faecal *E. coli* are capable at eliciting similar pro-inflammatory responses as ones deemed pathogenic based on VAG carriage and are therefore likely to elicit similar damage in a chicken infection model [234].

7.2 Future work

This study provides the first insight into the epidemiology and population biology of APEC-related disease on UK broiler farms but it raises a number of questions that could be addressed by further research.

7.2.1 Field studies

- The farm contribution to VAG profile diversity obtained during the longitudinal sampling phase was interesting and it would be of interest to sample more farms in a similar manner, possibly considering different broiler genotypes and disease susceptibility. Furthermore, the rapid succession of the avian microbiota during bird development prevented the current investigation from recording the dynamics of the intestinal *E. coli* carriage, thus more sampling time points would be valuable.
- Following the identification of a high level of pAPEC in day old chicks and the contribution of extraintestinal *E. coli* to early mortalities, it would be of interest to assess the population biology of *E. coli* in both the hatchery environment and in broiler breeders. Previous work in Denmark reported a significant association between flock performance (measured partly by flock mortalities), the hatchery and breeder age [273]. Can the same be said for the UK production chain? It may be that certain breeder flocks or hatcheries harbour more pAPEC, which pose an increased risk to the performance of broiler flocks; a problem industry continue to face. Such information would be highly sensitive to the poultry industry and engaging industry in such studies would be challenging.

7.2.2 In the laboratory

This was the first investigation to simultaneously assess the avian faecal and extraintestinal *E. coli* populations in a longitudinally manner, thus a valuable culture collection was formed.

- A limitation to the current investigation was the virulotyping of *E. coli* based on the presence of only 10 VAGs. The availability of other mPCR schemes would allow for the further characterisation of the *E. coli* culture collection collected during this investigation [132, 279].
- To date, a limited number of APEC have been analysed by whole genome sequencing, existing isolates include APEC O1, chi 7122 and IMT2125 [78, 89]. The end of the current investigation saw the submission of 103 avian faecal, pAPEC and APEC (extraintestinal *E. coli* with > 5 VAGs) submitted for whole genome sequencing. It would be valuable to compare the genome sequences of such a population to one another and to the existing APEC genomes in an attempt to identify genetic markers associated with extraintestinal status among *E. coli* in UK broiler flocks. Whole genome sequencing is becoming increasingly popular as the cost continues to decrease and such a technique offers a wealth of information. However, caution must be taken, as with techniques such as PFGE and MLVA, the high resolution produced can in itself be problematic when deciphering the relatedness of micro-organisms.
- The broiler chicken has undergone substantial commercial genetic selection, and is far removed from traditional chicken breeds, reaching slaughter weight in approximately 5 weeks and this has been related to disease susceptibility

[56]. Furthermore, conflicting results relating to the virulence of APEC highlight errors in the current methodologies used to assess virulence (virulence in day old chicks, the use of SPF chickens and high inoculation doses). It would be valuable to compare the outcome of both avian faecal, pAPEC and extraintestinal *E. coli in vivo* infections using today's commercial broiler chickens. This would allow clarification of the results obtained from the *in vitro* studies performed in the current work, which found no significant difference in avian faecal and pathogenic *E. coli* infections.

- Focus on the use of probiotics to manipulate the intestinal microbiota of chicks or breeders could prove highly beneficial in reducing the pAPEC reservoir.

7.3 Conclusions

From the results collected in this investigation I extrapolate that extraintestinal *E. coli* pose a significant burden on the UK poultry industry. The population structure of extraintestinal *E. coli* has been shown to be so diverse that an APEC pathotype may not exist, a similar scenario to that of the ill-defined EAEC pathotype which currently lacks a strict pathotype and discriminating traits [126].

APEC pathogenesis remains to be determined and disease mechanisms are not simplistic. The entire broiler production chain merits investigation, including breeder flocks and hatcheries to bring about disease management; the identification of pAPEC in day-old chicks and the contribution of extraintestinal *E. coli* in early mortalities confirm this. The broiler chicken should also be considered on its own principles.

Overall, although some *E. coli* appear to fit an APEC pathotype, they reside within a large and highly diverse spectrum, thus the concept of a definitive APEC pathotype in UK broiler chickens looks unlikely.

Chapter 8

Appendices

Appendix I

(Chapter 3 published manuscript)

Kemmett K, Humphrey T, Rushton S, Close A, Wigley P, et al. (2013) A Longitudinal Study Simultaneously Exploring the Carriage of APEC Virulence Associated Genes and the Molecular Epidemiology of Faecal and Systemic *E. coli* in Commercial Broiler Chickens.PLoS ONE 8(6): e67749. doi:10.1371/journal.pone.0067749

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A Longitudinal Study Simultaneously Exploring the Carriage of APEC Virulence Associated Genes and the Molecular Epidemiology of Faecal and Systemic *E. coli* in Commercial Broiler Chickens

Kirsty Kemmett[1]*, Tom Humphrey[1], Steven Rushton[3], Andrew Close[3], Paul Wigley[1], Nicola J. Williams[2]

1 Institute of Infection and Global Health, Department of Infection Biology, School of Veterinary Science, The University of Liverpool, Neston, Cheshire, United Kingdom, **2** Institute of Infection and Global Health, Department of Epidemiology and Population Health, School of Veterinary Science, The University of Liverpool, Neston, Cheshire, United Kingdom, **3** School of Biology, Newcastle University, Newcastle Upon Tyne, United Kingdom

Abstract

Colibacillosis is an economically important syndromic disease of poultry caused by extra-intestinal avian pathogenic *Escherichia coli* (APEC) but the pathotype remains poorly defined. Combinations of virulence-associated genes (VAGs) have aided APEC identification. The intestinal microbiota is a potential APEC reservoir. Broiler chickens are selectively bred for fast, uniform growth. Here we simultaneously investigate intestinal *E. coli* VAG carriage in apparently healthy birds and characterise systemic *E. coli* from diseased broiler chickens from the same flocks. Four flocks were sampled longitudinally from chick placement until slaughter. Phylogrouping, macro-restriction pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) were performed on an isolate subset from one flock to investigate the population structure of faecal and systemic *E. coli*. Early in production, VAG carriage among chick intestinal *E. coli* populations was diverse (average Simpson's D value =0.73); 24.05% of intestinal *E. coli* (n = 160) from 1 day old chicks were carrying ≥5 VAGs. Generalised Linear models demonstrated VAG prevalence in potential APEC populations declined with age; 1% of *E. coli* carrying ≥5 VAGs at slaughter and demonstrated high strain diversity. A variety of VAG profiles and high strain diversity were observed among systemic *E. coli*. Thirty three new MLST sequence types were identified among 50 isolates and a new sequence type representing 22.2% (ST-2999) of the systemic population was found, differing from the pre-defined pathogenic ST-117 at a single locus. For the first time, this study takes a longitudinal approach to unravelling the APEC paradigm. Our findings, supported by other studies, highlight the difficulty in defining the APEC pathotype. Here we report a high genetic diversity among systemic *E. coli* between and within diseased broilers, harbouring diverse VAG profiles rather than single and/or highly related pathogenic clones suggesting host susceptibility in broilers plays an important role in APEC pathogenesis.

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* E-mail: K.Kemmett@liv.ac.uk

Introduction

Colibacillosis is a syndromic disease of birds characterised by fibrinous lesions around visceral organs caused by a group of extraintestinal pathogenic *Escherichia coli* (ExPEC) known as avian pathogenic *E. coli* (APEC). Airsacculitis, cellulitis, pericarditis, perihepatitis and respiratory distress are among the most commonly associated signs of colibacillosis in broiler (meat producing) chickens [1]. Extraintestinal *E. coli* infections are a considerable economic burden on the global poultry industry due to increased mortality rates during rearing and rejection of carcasses at slaughter. Despite a number of studies aimed at elucidating the APEC pathotype, it remains poorly defined. Genes involved in bacterial adhesion, invasion, toxin production, serum survival and iron acquisition have all been shown to contribute to APEC pathogenesis [2,3,4,5,6,7]. It is likely that combinations of virulence associated genes (termed VAG profiles or virulotypes) are needed to give rise to pathogenic *E. coli*, as no single gene has been exclusively associated with APEC. A recent study demonstrates APEC strains arise from multiple *E. coli* lineages following the acquisition of distinct VAGs, highlighting the potential high genetic diversity among these bacteria [8]. Serotyping has been used as a method for identifying APEC but several authors suggest it fails to discriminate APEC and avian faecal *E. coli* and a significant proportion of *E. coli* is untypable [9].

Previous studies have identified the gastrointestinal microbiota as a potential reservoir for APEC infection [5,9]. It has been shown that infection follows either inhalation of contaminated faecal dust followed by septicaemia or via active gut translocation [10,11]. Intestinal *E. coli* carrying numerous VAGs maybe referred to as 'potential' APEC (pAPEC) populations and their presence is likely to pose an increased risk to systemic disease.

Commercial broiler chickens are selectively bred for their efficient and uniform growth. Despite its' commercial importance,

relatively little work has exclusively focused on colibacillosis in broiler chickens [5,6,9,12]. The gastrointestinal tract of a young animal is a rich ecological niche ideal for bacterial colonisation and subsequent microbial succession. The outcomes of host-microbial interactions are influenced by host (age, immunity), the microbe (microbiota, VAGs) and environmental factors [13,14]. Initially, gut colonisation of production birds can be influenced by: vertical transmission, the hatchery environment, handling, transportation [15,16,17]. Once on farm, birds are exposed to a different rearing environment, dietary changes and vaccinations (see materials and methods).

It is currently unknown how APEC VAG carriage among intestinal *E. coli* populations change with respect to bird age. Thus, the current study aims to address several new questions: to determine whether there are significant changes with time in the intestinal pAPEC reservoir among avian faecal *E. coli* populations; if certain *E. coli* strains, VAGs and/or profiles are selected for in the gastrointestinal tract through time and how this relates to the strains and VAG profiles seen among systemic *E. coli* isolated from birds, which die as a consequence of APEC infection.

Materials and Methods

Ethics statement

The following protocol involved the (non invasive) collection of faecal samples (using sterile cotton swabs) following excretion and no approval under the Animals (Scientific Procedures) Act (1986) was needed. No birds were culled for the purpose of this study and all dead birds intended for post-mortem examination were collected on the first daily welfare walk conducted by farmers. The study was approved by the University of Liverpool Committee on Research Ethics: Physical Interventions sub-committee (reference RETH000448) with the mandatory condition that any serious adverse events be reported to the sub-committee within 24 hours. The study was conducted in strict accordance with the University of Liverpool Research Governance policies and permission for sampling on the broiler farms was granted by the farms.

2.1 Longitudinal sample collection

Two consecutive flock cycles on two standard commercial broiler chicken farms in the UK were visited once to twice weekly. The sampling described below commenced from the day the chicks were placed in rearing houses and was completed approximately 3 days before the first de-population event (~32–35 days). Approximately 30% of the flock is removed at depopulation to allow farmers to conform to end-of-life stocking density standards. The flocks used in this study were routinely vaccinated as industrial practice in the UK: Avian Pneumovirus (7 days old), Infectious Bronchitis virus (14 days old) and Infectious Bursal Disease (16 days old). The flocks did not receive any veterinary treatment. All *E. coli* isolates collected during the course of this study are available upon request.

2.1.1 Gut E. coli population VAG carriage. At each visit, 20 fresh faecal swabs were collected at random from different areas of the broiler house floor. Each swab was cultured onto eosin-methylene blue agar (EMBA) and incubated overnight at 37°C. From each plate, eight randomly selected colonies typical of *E. coli* were subcultured onto nutrient agar to obtain pure cultures and incubated overnight at 37°C. All media used were obtained from LabM (IDG) Ltd (Bury, UK). *E. coli* identification was confirmed using a PCR targeting the *uidA* gene [18]. One colony, representing each of the eight isolates, was pooled in 600 µl of 20% (w/v) Chelex-100 in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (Bio-Rad, Hertfordshire, UK). DNA was extracted from each pooled sample using a modified protocol described previously [19]. Briefly, 600 µl of Chelex 100 containing pooled colonies was incubated at 95°C for 10 min. Samples were centrifuged at 10,000 rpm for 2 min and 50 µl of supernatant was added to 250 µl of sterile double distilled water.

As a means of screening faecal *E. coli*, largely expected to be non-pathogenic, each pooled DNA extract was screened for four VAGs previously associated with avian *E.coli* pathogenesis; *iss, tsh, iucC* and *cvi*, using a multiplex PCR [2]. Primers were obtained from Eurofins MWG operon (Germany); all PCR constituents used in this study were supplied by Thermo Scientific, Surrey. All four primer sequences are given in Skyberg *et al* [2]. Briefly, each 50 µl reaction contained: 12 µl of 25 mM $MgCl_2$, 21.3 µl sterile water, 5 µl 10×PCR buffer, 4 µl of 20 mM dNTPs, 0.3 µl of each 100 pmol forward and reverse primer, 0.3 µl 5 U/µl Taq polymerase and 5 µl template DNA. Thermocycler conditions were: initial denaturation 95°C for 5 min; nine cycles of 95°C for 60 sec, 55°C for 30 sec, 72°C for 60 sec; twenty eight cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec with a final extension 72°C for 7 min. The mixture was held at 4°C. PCR products were subject to electrophoresis on a 2% agarose gel in tris–acetate buffer (TAE) at 150 volts for 60 min alongside a Superladder-Low 100 bp ladder (Thermo Scientific, Surrey). When a sample pool was positive for ≥3 of the 4 genes, a new Chelex-100 preparation was made for each individual isolate within the pool as described above. Pooled samples with fewer than 3 VAGs were discarded. The individual isolate DNA templates were then screened for 10 VAGs; *astA, iss, irp2, iucD, papC, tsh, vat, cvi, sitA* and *ibeA*. Three separate PCR assays were performed; one multiplex PCR previously described by Ewers *et al* [3] and two single PCR assays for *ibeA* and *sitA* outlined by Timothy *et al* [20]. Briefly, for a 25 µl multiplex PCR, 4 µl of 25 mM $MgCl_2$, 13.9 µl sterile water, 2.5 µl 10×PCR buffer, 0.5 µl 20 mM dNTPs, 0.1 µl of each 100 pmol forward and reverse primers, 0.5 µl 5 U/µl Taq polymerase and 2 µl DNA template were used. Multiplex PCR thermocycler conditions were as follows: initial denaturation 94°C for 3 mins followed by 25 cycles of: 94°C for 30 secs, 58°C for 30 secs, 68°C for 3 mins with a final extension 72°C for 10 mins. The mixture was held at 4°C. Each individual PCR contained 1 µl DNA template, 1 µl of each primer (100 pmol) and 22 µl of 1.1×Reddymix PCR mastermix with 1.5 mM $MgCl_2$. Thermocycler conditions for *sitA* and *ibeA* were identical; 95°C for 12 min and 25 cycles of: 94°C for 30 sec, 63°C for 30 sec, 68°C for 3 min; 72°C for 10 min with a final hold 4°C. PCR products were subject to electrophoresis as above. The presence or absence of the 10 VAGs produced a series of 10 numbers, which denoted the VAG profile for each isolate (presence '1' or absence '0'). Isolates carrying ≥5 VAGs were classified as pAPEC.

2.1.2 Post-mortem examination of dead broiler chickens. As well as faecal sample collection throughout rearing, from week 2 onwards, at each faecal sampling time point, 8 dead birds were collected from the first welfare walk of the day for post-mortem examination. To minimise the detection of systemic *E. coli* resulting from a loss of intestinal integrity following death, only birds identified as recently dead were included. Birds were only selected for post-mortem examination if they did not show signs of extensive pecking, had not been trodden on (flattened appearance) and/or did not have broken legs or other obvious injury. For all birds, any observed classic colibacillosis characteristics were recorded including; ascites, airsacculitis, cellulitis, enlarged spleen, pericarditis and perihepatitis [1]. For each bird, up to 1 gram of the following tissues were collected;

heart, kidney, liver, lung and spleen using sterile forceps and scalpels. An equal volume of sterile phosphate buffered saline (PBS) was added to each sample and tissues were homogenised using a Biomaster Micro-stomacher 80 (Steward, UK) for 60 seconds at high speed. 50 µl of the homogenate was streaked onto EMBA and incubated overnight at 37°C. Eight *E.coli* colonies were picked, re-plated onto nutrient agar and incubated overnight at 37°C. All isolates were immediately subjected to a full screen of all 10 virulence genes using the assays described above and each isolate was given a corresponding VAG profile.

2.1.2.2 Statistical analysis. Collected data were analysed using multiple statistical tests. Intestinal *E. coli* VAG profile diversity at each sampling time point was calculated using Simpson's diversity index (D). Generalised linear models (GLMs) were used to investigate the relationship between VAG profile diversity and time. Several different statistical measures were used a) the Pearson's correlation coefficient from VAG profile diversity data and the detection of potential APEC isolates b) the P-value obtained from the Fisher's exact test to assess the distribution of VAG genes between faecal and systemic *E. coli* population. Associations were considered statistically significant if the calculated P-value was <0.05.

2.2 Phylogenetic typing

Faecal and systemic isolates collected from one of the four flock cycles underwent further molecular analysis by phylogenetic typing. Two hundred and sixteen faecal and 35 systemic *E. coli* were analysed.

Isolates were assigned to 1 of 4 *E. coli* phylogenetic groups (A, B1, B2 or D) using a triplex PCR targeting *chuA, yjaA* and the DNA fragment TSPE4.C2 [21]. Each 25 µl PCR reaction contained: 1 µl of template DNA extract, 1 µl of each forward and reverse 100 pmol primer (Eurofins MWG operon, Germany) and 22 µl of 1.1×Reddymix with 1.5 mM $MgCl_2$. Thermocycler conditions were as follows: initial denaturation at 94°C for 4 mins; 30 cycles of; 5 secs at 94°C and 10 secs at 59°C with final extension at 72°C for 5 mins. The reaction mixture was held at 4°C. PCR products were subject to electrophoresis as stated above. Phylogenetic group classification was based on the combination of *chuA*, *yjaA* and TSPE4.C2: A (*chuA*$^-$, TSPE4.C2$^-$, *yjaA*$^+$), B1 (*chuA*$^-$, TSPE4.C2$^+$, *yjaA*$^-$), B2 (*chuA*$^+$, TSPE4.C2$^{-/+}$, *yjaA*$^+$) and D (*chuA*$^+$, TSPE4.C2$^{-/+}$, *yjaA*$^-$).

2.3 Macro-restriction pulsed-field gel electrophoresis

Two hundred and twenty two faecal and 48 systemic *E. coli* were analysed using PFGE. The PFGE protocol used was based on the standardised Pulsenet Rapid *E. coli* method [22] with slight modifications. During sample preparation, plugs were incubated for 2 h at 54°C with vigorous shaking at 175 rpm and for sample digestion each sample was incubated for 2 h with 50U of *Xba*1 restriction enzyme (Roche products Ltd, Hertfordshire) at 37°C. Samples were run on a 1% 0.5X Tris-Borate running buffer (TBE) (Life technologies, UK) agarose universal (alpha laboratories, Hampshire) with 0.5X TBE running buffer for 20 hours at 14°C, at 6 V/cm^2 with the initial switch time of 2.2 s and final switch time of 54.2 s in a CHEF-DRIII PFGE system. A Lambda ladder PFGE marker (New England Biolabs, Ipswich, MA, USA) was run on each gel. The gel was stained in an ethidium bromide solution (500 µl ethidium bromide in 500 ml 0.5X TBE running buffer) for 25 mins and visualised under UV using a transilluminator. Samples which failed PFGE analysis, were re-tested with a longer proteinase K incubation period; 24 h at 54°C with vigorous shaking at 175 rpm. Image analysis was performed using BioNumerics version 4.0 and Dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

2.4 Multi-Locus sequence typing (MLST)

Fifty *E. coli* isolates from intestinal and systemic sites were analysed by MLST. Genomic template DNA was prepared using the Chelex-100 DNA extraction method as previously described [19]. Seven house-keeping genes were targeted for PCR; adenylate kinase (*adk*), fumerate hydratase (*fumC*), DNA gyrase (*gyrA*), isocitrate dehydrogenase (*icd*), malate dehydrogenase (*mdh*), adenylosuccinate dehydrogenase (*purA*) and the ATP/GTP binding motif (*recA*) [23]. All primer sequences and a detailed protocol are given by Wirth *et al* [23]. For this present study, the PCR based protocol was modified slightly and each 25 µl reaction contained: 0.5 µl of each forward and reverse primer (20 pmol), 23 µl of 1.1×Reddymix with 1.5 mM $MgCl_2$ and 1 µl of template DNA (Chelex 100 extractions). The PCR conditions included an initial denaturation at 95°C for 2 mins, 30 cycles of; 95°C for 1 mins, target specific primer annealing temperature for 1 mins (outlined in Wirth *et al*, 2006) and a final extension at 72°C for 5 mins. PCR success was confirmed by running products on a 1.5% agarose gel in TAE buffer for 30 mins at 150 v. The remaining product was cleaned using a 20% (w/v) polyethylene glycol (PEG_{8000}), 2.5 M NaCl (Yorkshire Bioscience Ltd, UK) precipitation protocol. Cleaned PCR products were sequenced commercially (Macrogen, Korea) with 1:15 diluted sequencing primers (same as amplification primers). Sequences were analysed using ChromasPro version 1.5 (Technelysium, Australia) and MEGA 5.05 [24] and submitted to the Achtman *E. coli* MLST online database (http://mlst.ucc.ie/mlst/dbs/Ecoli). To determine the genetic relatedness of our STs and those previously submitted to the online database, Burst (version 3) diagrams were constructed following the online instructions (http://eburst.mlst.net/).

Results

3.1 E. coli carriage of virulence-associated genes in healthy broiler chickens

A total of 420 *E. coli* pools was obtained from apparently healthy birds from two crops on two farms, between May and July 2011 and following initial screening, 119 were positive for ≥3 of the 4 targeted VAGs. Thus a total of 952 isolates were assigned a VAG profile out of 3360. Generally, fewer pooled samples met the threshold as birds aged.

Overall, VAGs were more frequently associated with systemic *E. coli* populations than faecal ones (Figure 1). For individual intestinal *E. coli* isolates, the *sitA* gene was the most commonly detected VAG, ranging between 0.68% and 20.57% prevalence, with an average detection of 8.51% over each sampling point for all flocks. Toxin encoding genes (*astA* and *vat*) were the least frequently detected in these populations; 0.00–11.25% (average 1.12%) and 0.00–9.38% (average 2.11%) for *astA* and *vat* respectively over the four flock cycles. Genes associated with iron acquisition, *sitA*, *iucD* and *irp2*, were commonly carried by individual isolates averaging 5.10% and 7.34% for *irp2* and *iucD* respectively.

The frequency at which the invasion-related gene, *ibeA*, was detected varied, ranging from 0.6% to 17.73% over the 4 flock cycles. At t = 0, *ibeA* detection ranged from 5–14.10%. Over the first week, the level of *ibeA* detection decreased, before peaking between weeks 2–3 (approximately 13%) and then declining once again (to 3.12%) towards week 5. A similar trend was also observed for *iss* detection (Figure 2).

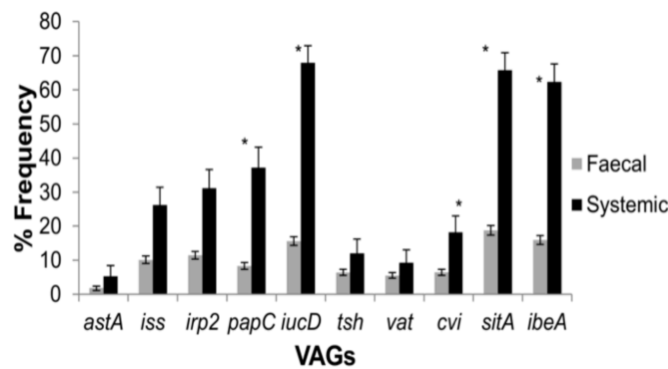

Figure 1. Comparison of faecal and systemic *E. coli* VAG carriage. Upper and lower bound 95% confidence intervals indicate statistically significant differences between VAG carriages by the two populations. Fisher's exact test indicates that *irp2, papC, iucD, cvi, sitA* and *ibeA* are significantly more associated with systemic *E. coli* ($p<0.05$) denoted in figure by *.
doi:10.1371/journal.pone.0067749.g001

VAG profiles (P-) were created based on observed combinations of the 10 different VAGs targeted (a systematic numbering system). A total of 206 different unique profiles were observed in the faeces of apparently healthy broiler chickens; P-1 (*astA, iss, irp2, iucD, papC, tsh, cvi, vat, sitA, ibeA*: 0000000001) represents the carriage of *ibeA* only, whereas P-206 is assigned to isolates carrying none of the targeted genes. P-206 was the most common profile detected in all flocks and its level of detection increased with time perhaps suggesting a positive selection for non-pathogenic traits within an intestinal population. Figure 3 shows the frequencies of detection for different profiles at t = 0 and t = week 5.

3.1.1 Changes in VAG profile diversity with respect to farm/flock and time. When the VAG data were analysed for individual farms and flocks; F1C1 (farm 1; cycle1), F2C1, F1C2 and F2C2, a total of 57, 45, 86 and 112 different VAG profiles were identified, respectively. Sixty two out of 206 different profiles (30.10%) were detected on >1 farm/flock, while 69.9% were only identified on one farm. Despite farm/flock individual VAG profile frequency differences, a common trend was observed with respect to time.

On average, 24.05% of *E. coli* isolates screened from the gastrointestinal tract of chicks at t = 0 (placement) carried at least 5 of the 10 VAGs (termed pAPEC) (Figure 4). The *sitA* gene was consistently the most frequently detected VAG from all four flock cycles on the two farms.

Simpson's diversity index (D) was used to compare the profile diversity at each week of production for the second flock cycles on both farms; D values are shown in Table 1. Generalised linear models confirmed the significant effect of time on VAG profile diversity ($p<0.05$). Overall, VAG profile diversity declined through time, with a common peak at week 3 of production.

As birds aged, the percentage frequency of pAPEC in the gastrointestinal tract declined. Prior to the first depopulation event at 5 weeks of production, only 1% of *E. coli* carried ≥5 VAGs.

An average decrease of 12.97% in pAPEC from t = 0 to week 1 was detected, followed by a further 5.47% decrease between weeks 1 and 2 (Figure 4).

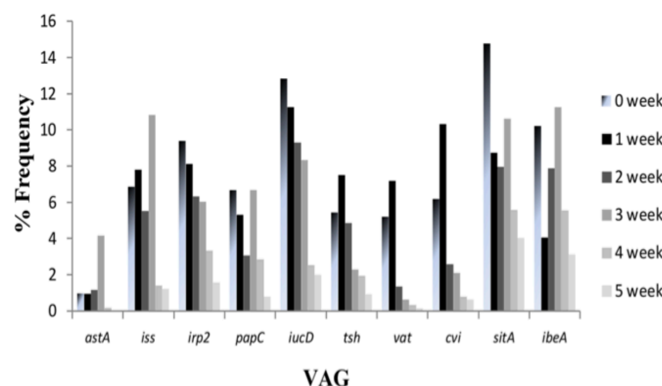

Figure 2. Average percentage frequency of VAGs. (inclusive of all flocks). Average percentage frequencies of 10 VAGs were calculated and plotted against time, from t = 0 (arrival) to t = week 5 (depopulation). Overall, VAGs appear to decline with time, with a peak in detection at week 3 for *iss, sitA* and *ibeA*. Iron acquisition genes *irp2* and *iucD* consistently decline with time.
doi:10.1371/journal.pone.0067749.g002

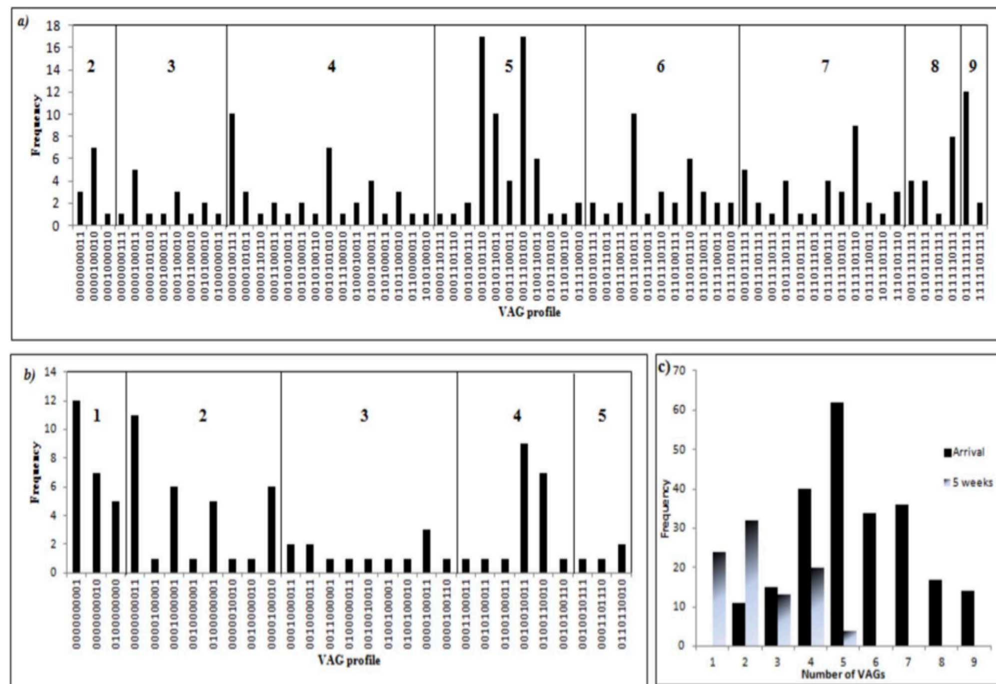

Figure 3. VAG profile diversity for all flocks. *a)* Shows the VAG profiles identified at t = 0 (arrival of chicks). Profiles consisting of 4 VAGs were the most diverse, with differences in iron acquisition genes being the most abundant, while profiles 0010101110 (*irp2*$^+$, *papC*$^+$, *vat*$^+$,*cvi*$^+$, *sitA*$^+$) and 0011101010 (*irp2*$^+$, *iucD*$^+$, *papC*$^+$, *vat*$^+$, *sitA*$^+$) both with 5 VAGs were the most common profile identified *b)* Shows the VAG profiles identified at t = week 5. VAG profile diversity had declined over time. Most diversity was detected with the possession of 3 VAGs. No isolates carried more than 5 VAGs *c)* Comparison of total number of VAGs carried by individually tested *E. coli* at t = 0 and 5. Profile 206 (0000000000) excluded from both graphs. Not all profiles were represented in all four cycles.
doi:10.1371/journal.pone.0067749.g003

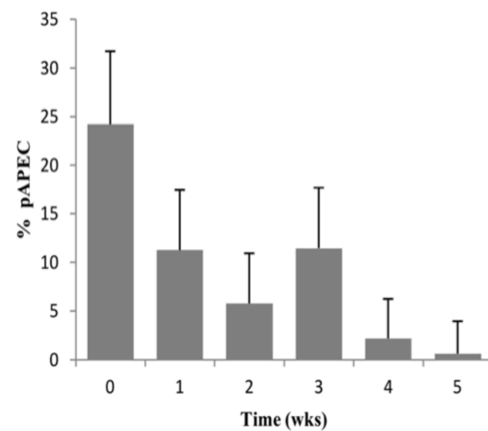

Figure 4. Average percentage of pAPEC with respect to time. At weekly intervals the average percentage of potential APEC, defined by the carriage of ≥5 VAGs, from the total faecal *E.coli* population was calculated. At each time point, 160 faecal *E. coli* were assessed. 95% upper confidence interval error bars shown. Overall, there is a general decline with time; the average detection frequency at placement of chicks (week 0) was 24.05% and only 1% by week 5.
doi:10.1371/journal.pone.0067749.g004

3.2 Longitudinal analysis of systemic E. coli carriage of virulence-associated genes

On average, over the four flocks, 39.1% of dead birds (n = 128) collected on the first daily welfare walk showed signs of colibacillosis and systemic *E. coli* was identified. Three hundred and twenty four isolates were virulotyped. Figure 4 shows the distribution of VAG frequencies between both faecal and systemic *E. coli* populations. Fisher's exact test was used to assess the

Table 1. Simpson's diversity index for VAG profile diversity through time.

	D value	
Week	**F1**	**F2**
0	0.683	0.779
1	0.683	0.359
2	0.438	0.582
3	0.704	0.686
4	0.070	0.307
5	0.391	0.200

Simpson's diversity index (D) was used to compare VAG profile diversity through time in the second flock cycles of farm 1 (F1) and farm 2 (F2). Overall, profile diversity decreases with time, with a peak at week 3.
doi:10.1371/journal.pone.0067749.t001

frequency differences between the faecal and systemic populations; *irp2, papC, iucD, cvi, sitA* and *ibeA* genes were significantly associated with systemic *E. coli* populations ($p<0.05$); *astA, vat, iss* and *tsh* were not ($p>0.05$).

Sixty three different profiles were detected among the systemic *E. coli* collected. Thirteen of the 63 profiles (20.63%) were found on more than one farm. Fifty eight of 324 isolates (17.90%) carried no VAGs (P-206). P-15 (*ibeA*$^+$, *iucD*$^+$, *sitA*$^+$) was the second most frequent profile (9.88%). However, this was only identified on F1C1. Of the profiles which were found on more than one farm, 46.26% accounted for profiles with ≥4 VAGs; in all these profiles at least 50% of the genes detected were involved in iron acquisition. None of the tested isolates carried more than 7 VAGs. Observed VAG profile diversity was not correlated with the number of *E. coli* investigated ($p>0.05$), suggesting sample size variation has not influenced profile detection and thus the reported level of diversity. Over the four flock cycles, 36.4–80% of VAG profiles identified in systemic isolates were also identified at least once in faecal isolates collected before and/or at the same time from apparently healthy birds during the same cycle. Nineteen profiles out of 63 were unique amongst systemic isolates. However, only one of these profiles was identified on more than one occasion (P-221; *iss*$^+$, *irp2*$^+$, *papC*$^+$, *iucD*$^+$). Overall, there were no profiles, which appeared to be wholly significantly associated with diseased birds; the large VAG profile diversity seen amongst systemic isolates suggests that specific VAG profile alone are not responsible for disease in commercial broiler chickens.

3.3 Phylogenetic analysis

Table 2 shows the assignment of 216 faecal *E. coli* and 35 systemic *E. coli* collected from F1C2 to the four phylogenetic groups and 1 subgroup. If no amplification occurred for any of the 3 targets, isolates were assigned to subgroup A_0 [25,26].

With the exception of week 2 (22.50%), group D was the most frequently detected phylogenetic group among faecal isolates. In week 2, Group A was most frequently detected phylogenetic group (58.75%). The screening-based protocol of faecal isolates would have led to sampling bias towards ones containing VAGs and therefore possibly group B2 and D isolates. There are no obvious changes in phylogenetic groups through time.

Fourteen of 35 (40%) systemic isolates grouped into phylogenetic group A_0. Previously pathogenic associated phylogenetic groups D and B_2 represented 25.71% and 2.86% of systemic isolates respectively. Results suggest that no distinct phylogenetic group accounts for systemic *E. coli*.

Table 2. Assignment of faecal and systemic *E. coli* to phylogenetic groups.

	Number of isolates (% frequency)				
Source	**A**	**A0**	**B1**	**B2**	**D**
Faecal	85 (39.35)	21 (9.72)	1 (0.46)	5 (2.31)	104 (48.15)
Systemic	11 (31.43)	14 (40.00)	0 (0.00)	1 (2.86)	9 (25.71)
Total	96	35	1	6	113

216 faecal *E. coli* and 35 systemic *E. coli* from vital organs were typed using the Clermont *et al* triplex PCR and assigned to 1 of 5 phylogenetic groups. Those isolates which showed no amplification of any of the 3 targets, yet confirmed to be *E. coli* were assigned to subgroup A0. Group D was the most frequently detected phylogenetic group among faecal population, while A0 (untypable) was the most common group among systemic isolates. B2 and D have been previously associated with more pathogenic *E. coli*, however they only accounted for 28.57% of systemic isolates in this study.
doi:10.1371/journal.pone.0067749.t002

3.4 Macro-restriction PFGE analysis

Two hundred and twenty two faecal and 48 systemic *E. coli* isolated from the same flock were analysed by PFGE to look for changes in gut population through time, common strain types associated with systemic *E. coli* and to relate genetic background to the carriage of VAGs. One hundred and sixty six faecal and 35 extraintestinal isolates were successfully digested and dendrograms constructed.

A dendrogram constructed from the pulsotypes of 48 *E. coli* isolated from faeces at t = 0 shows large strain diversity and no apparent association between strain type and VAG carriage. The 48 isolates fell into 5 groups with 80% similarity. There appears to be no retained strain type correlated with time.

Thirty-five systemic *E. coli* belonged to 10 groups with 80% similarity (Figure 5), suggesting diverse strain diversity amongst systemic isolates. The dendrogram also highlights the isolation of multiple strain types from individual diseased birds and the presence of similar strain types amongst faecal and systemic isolates.

3.5 MLST analysis

To assess underlying clonal association between isolates with VAGs in faecal and diseased bird populations, 24 faecal *E. coli* (8 with ≥5 VAGs, 8 with <5 VAGs and 8 with 0 VAGs) and 23 extraintestinal *E. coli* (11 with ≥5 VAGs, 7 with <5 VAGs and 5 with 0 VAGs) were submitted to the MLST online database (http://mlst.ucc.ie/mlst/dbs/Ecoli). All results are shown in table 3.

In total, 33 new sequence types were identified, 6 of which were single locus variants (SLV) of ST-10. This was the only clonal complex (CC-10) in which faecal and systemic isolates were shown to be related.

Interestingly, 3 of the 24 faecal isolates were identified as ST-352 and all carried more than 5 VAGs 1) *astA*$^+$, *irp2*$^+$, *papC*$^+$, *iucD*$^+$, *vat*$^+$, *cvi*$^+$, *sitA*$^+$ 2) *iss*$^+$, *irp2*$^+$, *papC*$^+$, *iucD*$^+$, *vat*$^+$, *cvi*$^+$, *sitA*$^+$ 3) *iss*$^+$, *irp2*$^+$, *papC*$^+$, *iucD*$^+$, *vat*$^+$, *cvi*$^+$, *sitA*$^+$. ST-352 did not cluster in any of the other faecal or systemic isolates in the constructed burst diagrams.

Four ST-2999 isolates (representing 22.22% of systemic isolates tested) were isolated from two diseased birds, ST-2999 is a SLV of the emerging pathogenic clone ST-117 [27]. All four ST-2999 isolates carried ≥5 VAGs and no ST-2999 isolates were identified among the faecal population. Furthermore, the genetic relatedness of ST-2999 and ST-117 is highlighted in their general clustering in constructed PFGE dendrograms (Figure 5). ST-48 (CC-10) and ST-10 (CC-10) were also amongst those already known STs identified in systemic populations. ST-3004 was identified only among systemic isolates. ST-3004 isolates were found to differ in the number of VAGs they carried; no VAGs (isolate 579 and 583), 1 (isolate 586) and 3 (isolate 607). Two out of the 3 VAGs are involved in iron acquisition (*irp2* and *iucD*) (Figure 5).

Discussion

To our knowledge, this is the first study to address the longitudinal diversity of intestinal *E.coli* populations with a focus on APEC VAG carriage, while simultaneously characterising systemic *E. coli* isolated from visceral organs of diseased birds in UK broiler flocks.

Previous work suggests the clonal nature of *E. coli* makes it possible to associate certain lineages with ExPEC status which

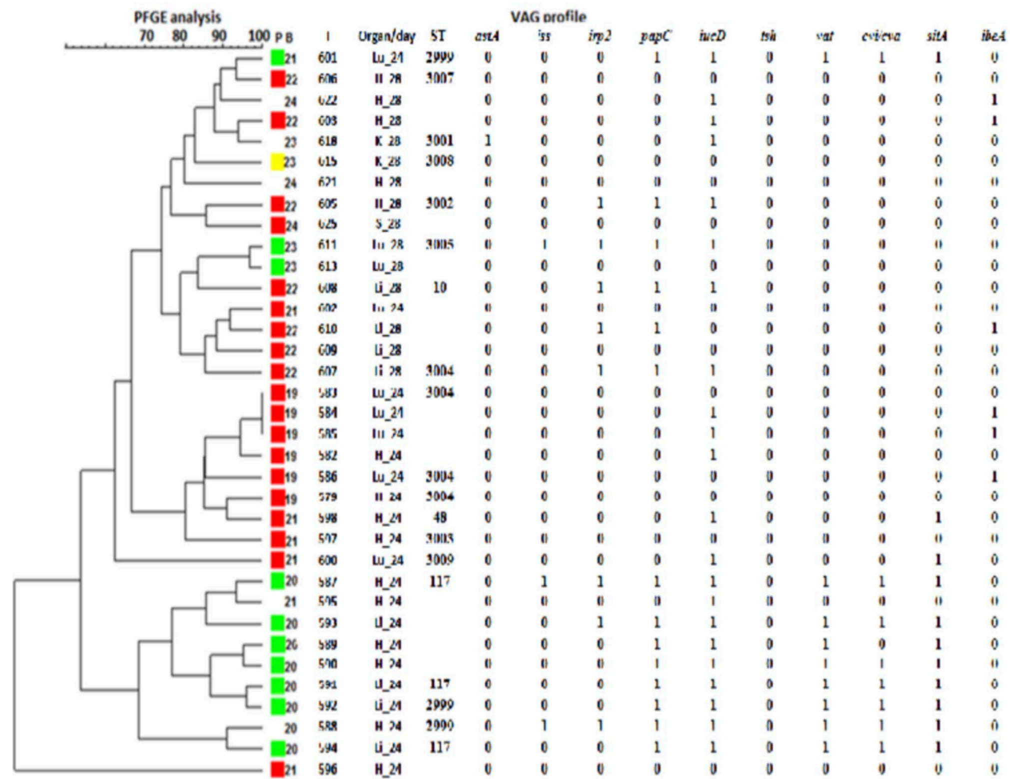

Figure 5. Dendrogram constructed using DICE for systemic *E. coli*. (tolerance 5%) (minimum height >0.0%, minimum surface >0.0%)(0.0–100% coefficient) for XbaI PFGE. A dendrogram showing the strain diversity amongst systemic E. coli harbouring APEC VAGs constructed using BioNumerics software by unweighted pair group method with Arithmetic mean. The dendrogram also shows; phylogenetic group (P) (green = D; red = A; yellow = B2; blue = B1), isolate (I), organ and age of bird at isolation (H = heart: K = kidney: Li = liver: Lu = lung; S = spleen), MLST sequence type (ST) and VAG profiles. The dendrogram shows the clustering of ST 117 and 2999 isolates (excluding 601) which by PFGE analysis are ~60% different from other isolates. Several ST 3004 were identified and these potentially show the acquisition of 2 Iron acquisition genes (*irp2* and *iucD*) while other ST 3004 isolates have no VAGs (isolates 579 and 583).
doi:10.1371/journal.pone.0067749.g005

could help elucidate a "typical" APEC [28,29,30,31,32]. The *E. coli* genome has a high degree of plasticity whilst retaining a level of clonality resulting from recombination events of short mobile elements in genome "hotspots", often these elements contain VAGs [12,33,34,35]. A similar observation was made recently regarding the clonality of extended β-lactamase producing *E. coli* [36]. Research suggests APEC arise from the acquisition of VAGs and certain lineages may be more accepting of incoming genetic elements and thus pathogenic [37,38,39]. In the current study, MLST identified a new sequence type (ST-2999) among the systemic isolates carrying ≥5 VAGs. ST-2999 is an SLV of ST-117, a potentially emerging pathogenic ST previously associated with retail chicken and human disease [27,40]. ST-117 was also identified among the systemic isolates. PFGE allows for more refined comparisons between isolates and here confirmed the genetic relatedness between these isolates compared to the other

Table 3. Observed faecal and systemic *E. coli* MLST Sequence types categorised by VAG carriage.

VAGs	Site of isolation	
	Faeces	**Systemic**
0	2990, 2991, 2992, 2993, 2994, 2995, 2996, 2997	3003, 3004 (2), 3007, 3008
<5	2980, 2981, 2982, 2983, 2987, 2988, 2989	3001, 3002, 3004 (2), 3005, 3006, 3009, 10
≥5	352 (3), 2978, 2984, 2985, 2986, 3010	117 (3), 2998, 2999 (4), 3000

(n) = ST observation frequency. All faecal *E. coli* belonged to newly identified sequence types (ST) excluding ST-352. Interestingly, all ST-352 isolates harboured more than 5 VAGs with the following profiles:1) *astA*$^+$, *irp2*$^+$, *papC*$^+$, *iucD*$^+$, *vat*$^+$, *cvi*$^+$, *sitA*$^+$ 2) *iss*$^+$, *irp2*$^+$, *papC*$^+$, *iucD*$^+$, *vat*$^+$, *cvi*$^+$, *sitA*$^+$ 3) *iss*$^+$, *irp2*$^+$, *papC*$^+$, *iucD*$^+$, *vat*$^+$, *cvi*$^+$, *sitA*$^+$ and they did not group with other *E. coli* in the online database. Systemic *E. coli* analysis identified 3 ST–117 and 4 ST- 2999 isolates; however ST-2998 and ST-3000 did not cluster with the other two STs in this category.
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systemic ones. However, the overall high level of strain diversity among systemic *E. coli* isolated from diseased birds; the lack of correlation with VAG carriage and the identification of multiple strains as opposed to a single clone in one bird perhaps suggests the opportunistic nature of certain *E. coli* [41]. Additionally, this perhaps suggests differences in broiler susceptibility compared to other avian species (layer hens and turkeys) where single clones of APEC have been identified [8,12,29]. As only a subset of isolates were subjected to genetic analysis, it is possible that we have underestimated the level of diversity present. It is clear a high level of diversity exists.

The intestinal *E. coli* population of birds has previously been identified as an APEC reservoir [5,9]. The findings from this study further support this with 36.4–80.0% of systemic VAG profiles also being identified among faecal *E. coli* of the same flock.

E. coli is one of the first bacterial species to colonise the neonatal gut before succession [42,43,44]. A large proportion of pAPEC contributed to early colonisation of the neonatal chick (24.05% of tested population). Sources of such *E. coli* include: parent flock (vertical transmission), hatchery environment, human handling and transportation equipment [15,16,17]. Yassin *et al* (2009) correlated first week mortalities with hatchery and breeder age, highlighting the potential important influence of these factors [45]. Interestingly, despite all four flocks in this study being sourced from different hatcheries, the level of observed pAPEC at this stage was comparable. Past studies have shown that the possession of VAGs could be advantageous in microbial gut populations offering commensalism fitness advantages [46,47,48]. The positioning of VAGs on mobile genetic elements would allow for their selective maintenance within populations [7,12,34].

As birds aged, both VAG profile diversity and the detection of pAPEC declined; by the last week of production, 1% of the population sampled were classified as pAPEC. Furthermore, as birds exceeded 3 weeks of age there was a noticeable decline in the proportion of pooled samples reaching the 3 VAG threshold outlined in our sampling protocol, suggesting a negative selection in the avian gut. Younger birds have been shown to possess a more diverse microbiota compared to that of older birds, likely to be due to rapid initial opportunistic colonisation of an available ecological environment; with age microbial succession and interspecies convergence occur [14,49]. The bottle necking of VAG diversity and pAPEC with microbial succession may represent the persistence of stronger colonisers and the loss of more transient strains. One hypothesis is that different VAGs offer selective advantages at different stages of development [34]. A note of caution is required, as our list of VAGs is not an exhaustive list of APEC-associated virulence genes.

Irrespective of time, *sitA* was the most frequently detected VAG in this study. The *sitABCD* encoded transporter regulates iron and manganese transport and provides increased resistance to oxidative stress [50]. This mechanism could be advantageous among competing gastrointestinal populations and during inflammation [14]. Additionally, the redundancy of iron acquisition systems is thought to be advantageous in environmental survival [51]. Interestingly our study identified multiple ST-3004 isolates which differed in their possession of VAGs namely ones involved in iron acquisition (*irp2* and *iucD*). Could this be the result of gene transfer and acquisition?

The *ibeA* gene was frequently detected among intestinal *E. coli* populations of young birds. The *ibeA* gene encodes a 50 kDa protein thought to aid brain microvascular epithelial adherence and invasion [4]. The exact mechanism of Ibe proteins remains to be determined but it has been shown to modulate type 1 fimbriae [52]. The advantage of possessing *ibeA* while in the gut remains unknown; it could relate to the increased survival of attached *E. coli*, particularly in a transient inflammatory environment [4,53].

The 10 VAGs selected for this study do not represent an exhausted list of APEC determinants [54]. For future work, an investigation published after this study was carried out presents a new virulotyping protocol offering vastly improved error margins in APEC detection, ideal for epidemiological studies[55]. Based on the literature the APEC pathotype is likely to contain a mix of iron acquisition genes and those encoded on plasmids [6,9]. This was reflected in our chosen panel of VAGs. It was necessary to add a level of bias to the faecal sampling given the ubiquitous nature of *E. coli* in the gastrointestinal tract allowing practical detection of the proportion of the population that are potentially pathogenic. Such sampling is technically demanding and labour intensive. The 4 VAGs used in the initial screening were selected based on their high prevalence among APEC strains; *iss* (~83%), *iucC* (75%), *tsh* (53–63%) and *cvi* (63%) [56,57]. This panel allowed for the detection of as many potential APEC as possible given the limitations in screening the large number of samples. All calculations regarding the 'proportion of potentially pathogenic *E. coli*' was calculated using the entire population sampled, i.e. the original number of *E. coli* picked before initial PCR screening.

The avian host also contributes to shaping the microbiota. Lu *et al* (2003) described a more stable microbiota between 2 and 4 weeks of age in fast growing birds, reflecting the current study which observed more consistent levels of pAPEC between weeks 4 and 5 [14]. Immunological changes during host development are likely to contribute to changes in the microbiota; heterophil function (avian polymorphonuclear neutrophils (PMNs)) has been shown to be lacking in day old chicks [58]. Crhanova *et al* (2011) reported transient gut physiological inflammation in 4 day old chicks, while the cellular immune responses to *Salmonella* Typhimurium of 1 day old and 1 week old chicks have been shown to be markedly different, suggesting rapid immunological changes in early life [14,49,59,60]. It is likely that a combination of host (immunity and vaccination), microbial (microbiota composition, VAG carriage) and environmental (feed, production systems) changes has contributed to the changes in pAPEC observed in this study, highlighting the importance of host-microbial interactions [61], although this needs to be looked at more closely,. It would be of interest to determine causes of death in the first 48–72 hours of life; a period of limited heterophil function, often the point of highest mortality during commercial rearing and as noticed in this study the point in production where APEC VAGs are at the greatest prevalence in the avian gut [45].Exploratory analysis to elucidate the contribution of environmental factors to the observed changes in pAPEC is ongoing.

In summary, we have shown colonisation of the broiler gut by pAPEC to occur before chicks are placed and as broilers age the populations shift while appearing to bottle neck in VAG carriage diversity. The reasons for this remain to be determined. Our work supports that of others, identifying the avian gut as an APEC reservoir, but did not find a predominant APEC pathotype in the flocks studied. The identification of highly diverse systemic *E. coli* populations rather than single or highly related clones perhaps suggests the broiler chicken and its susceptibility is a major contributor to disease manifestation. Further work is required (i.e. molecular analysis on more isolates, elucidation of contributing impacting factors to pAPEC dynamics), but this study offers the first insight into the temporal movement and dynamics of *E. coli* in the avian host and offers a new approach to deciphering APEC.

To conclude, the concept of an APEC pathotype is arguably fundamentally flawed in broilers.

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Author Contributions

Conceived and designed the experiments: KK NJW TH PW. Performed the experiments: KK. Analyzed the data: KK SR AC NJW. Contributed reagents/materials/analysis tools: NJW TH AC SR. Wrote the paper: KK NJW SR PW TH.

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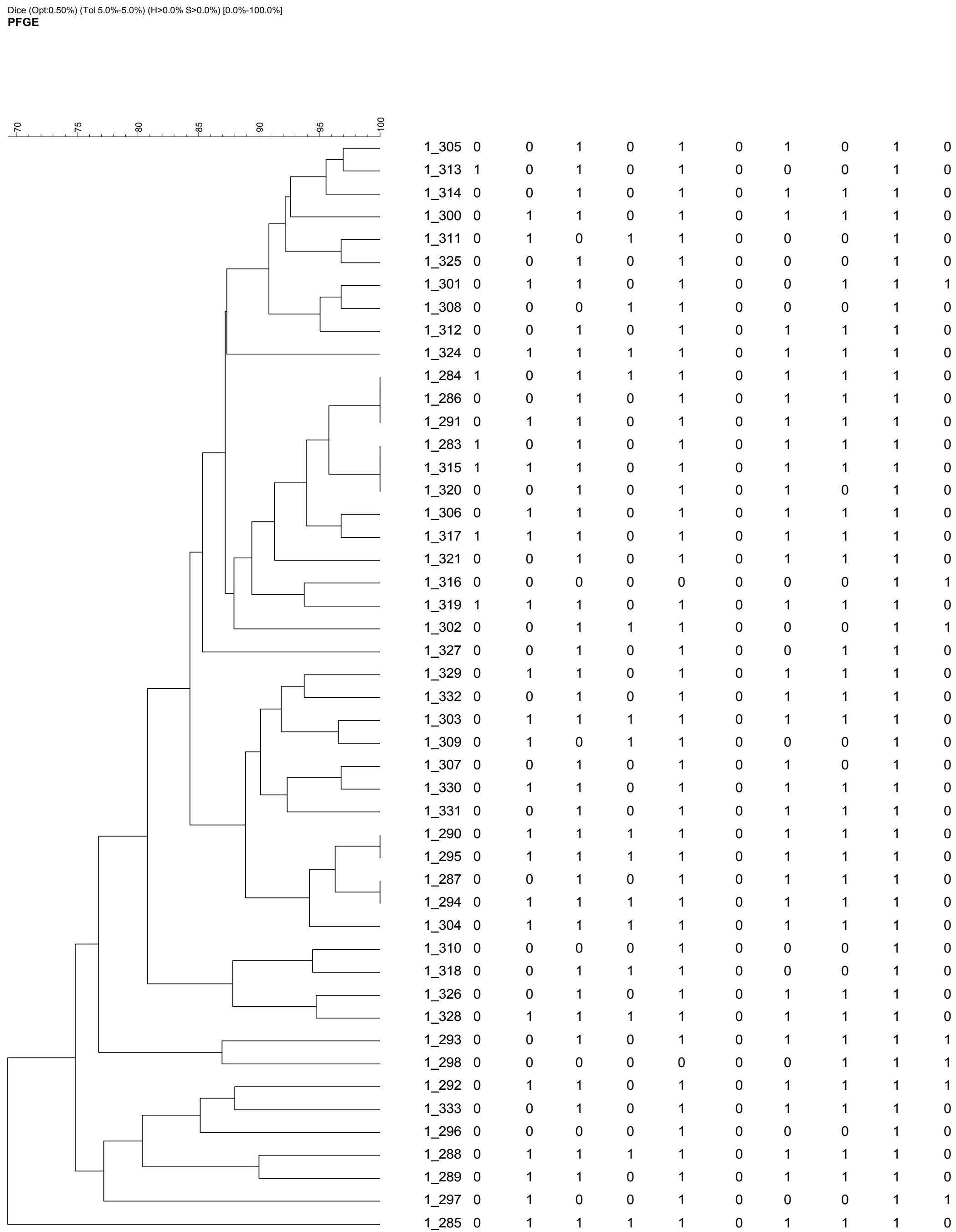
Appendix II

(Chapter 3 genetic analysis material)

Week 0 (day of placement) faecal *E. coli* macro-restriction pulsed field gel electrophoresis dendrogram



(tolerance 5%) (minimum height >0.0%, minimum surface >0.0%)(0.0-100% coefficient). A dendrogram showing the relatedness and strain diversity amongst faecal E. coli *collected from chicks at placement harbouring APEC VAG using BioNumerics software by unweighted pair group method with Arithmetic mean.*

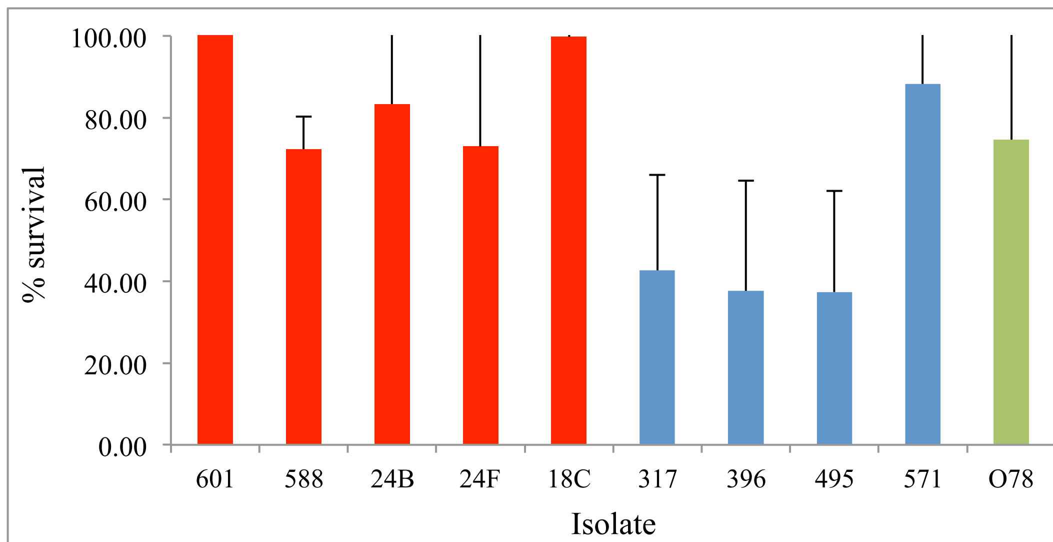
Multi-locus sequence typing (MLST) spreadsheet for systemic and faecal *E. coli*

		Allele							
Source	**No.VAGs**	***adk***	***fumC***	***gyrB***	***icd***	***mdh***	***purA***	***recA***	**ST**
Faeces	>5	232	11	4	8	92	8	2	3010
Faeces	>5	76	96	19	89	17	1	10	352
Faeces	>5	111	11	5	1	7	8	2	2978
Faeces	>5	76	96	19	89	17	1	10	352
Faeces	>5	232	11	4	323	20	8	2	2984
Faeces	>5	187	96	19	323	92	8	2	2985
Faeces	>5	76	96	19	89	17	1	10	352
Faeces	>5	179	11	22	1	20	89	205	2986
Systemic	>5	232	11	278	55	8	8	49	2998
Systemic	>5	20	45	41	43	5	32	221	2999
Systemic	>5	57	336	230	43	7	8	2	3000
Systemic	>5	20	45	41	43	5	32	2	117
Systemic	>5	20	45	41	43	5	32	221	2999
Systemic	>5	20	45	41	43	5	32	221	2999
Systemic	>5	20	45	41	43	5	32	221	2999
Systemic	>5	20	45	41	43	5	32	2	117
Systemic	>5	20	45	41	43	5	32	2	117
Systemic	>5	6	11	5	8	8	8	2	48
Systemic	>5	10	11	4	8	8	8	170	3004
Faeces	<5	76	96	12	339	20	211	2	2979
Faeces	<5	179	96	22	8	8	8	10	2980
Faeces	<5	76	82	12	89	17	1	10	2981
Faeces	<5	56	4	116	316	20	12	173	2982
Faeces	<5	6	4	12	316	20	12	7	2983
Faeces	<5	10	11	230	238	8	8	173	2987
Faeces	<5	6	7	5	238	8	18	2	2988
Faeces	<5	200	11	4	278	12	8	2	2989
Systemic	<5	20	4	230	83	8	8	6	3001
Systemic	<5	20	45	230	8	8	8	6	3002
Systemic	<5	10	11	4	8	8	8	170	3004
Systemic	<5	34	36	39	87	67	16	39	3005
Systemic	<5	10	11	4	8	92	8	2	3006
Systemic	<5	10	11	4	8	8	8	2	10
Systemic	<5	6	11	4	1	8	8	144	3009
Faeces	0	232	7	4	278	8	8	2	2990
Faeces	0	232	11	4	278	8	8	2	2991
Faeces	0	6	282	230	238	8	8	173	2992
Faeces	0	232	11	4	278	8	250	2	2993
Faeces	0	10	11	4	278	8	251	2	2994
Faeces	0	272	282	230	238	8	1	173	2995
Faeces	0	179	282	4	238	8	8	144	2996
Faeces	0	23	282	230	274	8	8	173	2997
Systemic	0	6	45	230	8	8	8	6	3003
Systemic	0	10	11	4	8	8	8	170	3004
Systemic	0	10	11	4	8	8	8	170	3004
Systemic	0	232	282	230	8	8	8	2	3007
Systemic	0	84	4	5	8	8	8	6	3008

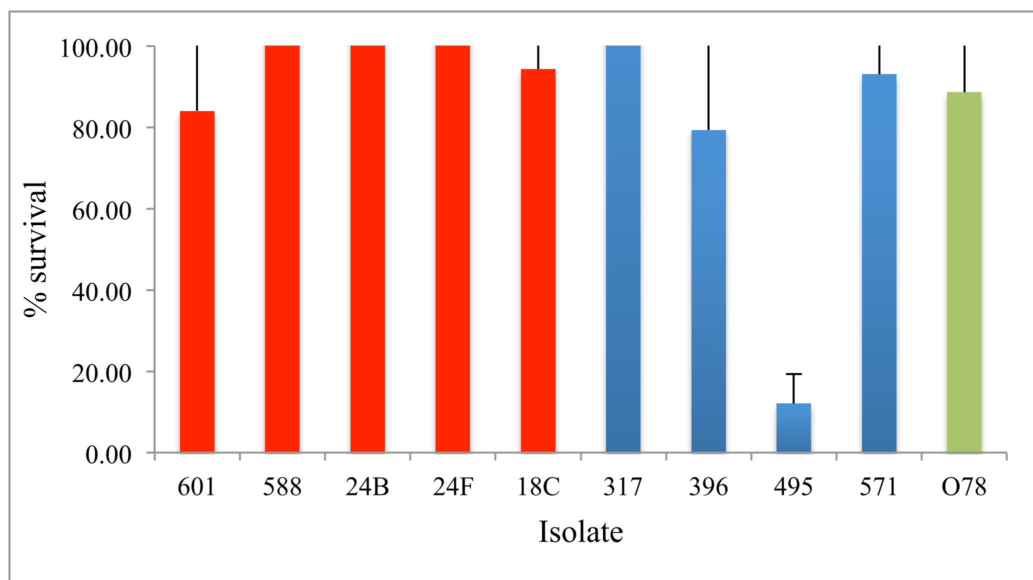
Appendix III

(Chapter 6 additional data)

Serum survival of APEC and avian faecal *E. coli* – 1 hour incubation



2 hour incubation



Results are presented as the percentage survival of the original inoculum. Error bars represent the standard deviation from the three repeats. There were no significant differences between APEC and avian faecal E. coli *($p>0.05$).*

Chapter 9

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