Comparative genotypic and phenotypic characteristics of human associated extraintestinal *Escherichia coli* isolated from cats and dogs

Judith Anne Bourne

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge, it contains no material previously published or written by another person, except where due reference is made in the text.

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Abstract

Escherichia coli extraintestinal infections (ExPEC) cause significant disease in humans and companion animals (cats and dogs) predominantly urinary tract infections, but also sepsis and neonatal meningitis. *E. coli* pathotypes, avian pathogenic *E. coli* (APEC), are also a very important cause of mortality and morbidity in poultry. *E. coli* also causes avian pathogenic *E. coli* (APEC), a very important cause of mortality andmorbidity in poultry. *E. coli* inhabiting the hindgut of healthy humans, companion animals and chickens is regarded as a potential source of ExPEC and APEC strains. The common, close relationshipof cats and dogs with people and the frequent consumption of chicken meat by people has raised concerns that companion animals and chicken meat may serve as reservoirs for ExPEC in humans, as well as a source of antibiotic resistant strains. This study showed that the most important human ExPEC sequence types (ST) (ST69, ST73, ST95 and ST131) within phylogroups B2 and D, could be isolated from faeces collected from healthy domestic cats and dogs in Canberra. This was especially the case for cats and ST73. This ST was isolated from a greater proportion of cat faecal samples than faecal and clinical samples from humans. Genotypic analysis of these isolates and comparison with genomes of strains isolated from people and poultry meat showed that within each sequence type there were discrete host related clusters. In depth analysis of the cat and human ST73 strains indicated sharing between hosts had occurred. Some differences in gene carriage by strains and phenotypic differences could be explained as host adaptations. The findings from this study support the hypothesis that transmission of ExPEC strains occurs between humans, chicken meat and cats, but also provides evidence of host specificity. Thus, further study of isolate attributes which contribute to zoonotic potential and host adaptation must go beyond studying isolates at the phylogroup or sequence type level and examine WGS subgroups to understand the relatedness of *E. coli* isolated from different hosts.

List of Abbreviations

The following abbreviations have been used in the thesis:

%	Percentage
˚C	Degrees Celsius
APEC	Avian pathogenic *E. coli*
AMR	Antimicrobial resistance
BLAST	Basic Local Alignment Search Tool
CC	Clonal complex
CIA	Critically important antibiotic
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
ECOR	*E. coli* collection of reference strains
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Adherent invasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBL	Extended-spectrum beta-lactams
ESC	Extended-spectrum cephalosporin
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extra-intestinal pathogenic *E. coli*
FQ	Fluoroquinolone
HGT	Horizontal gene transfer
IS	Insertion sequences
Mb	Megabase pair
MDR	multidrug-resistant
ml	Millilitre
MLST	Multi-locus sequence typing
mm	Millimetre
MGE	mobile genetic elements
nm	Nanometre
NMEC	Neonatal meningitis- associated *E. coli*
NS	Not significant
OD	Optical Density
PAI	Pathogenicity island
PCR	Polymerase chain reaction
PFGE	Pulsed-field electrophoresis
PMQR	Plasmid-mediated quinolone-resistant
rep-PCR	repetitive extra-genic palindromic PCR
SNP	Single nucleotide polymorphism
ST	Sequence type
µL	Microlitre
WGS	Whole genome sequencing
VAG	Virulence associated genes
WHO	World Health Organization
UPEC	Uropathogenic *E.coli*
UTI	Urinary tract infection

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Chapter 1 General introduction

Introduction

Escherichia coli (*E. coli*) is a member of the family Enterobacteriaceae, in the phylum Gammaproteobacteria. This Gram negative, prototrophic, facultative anaerobic bacterium can respire oxygen or ferment substrates, depending on electron acceptor availability (Conway and Cohen 2015). It is a highly diverse species with a number of life-styles; environmental, commensal or pathogen, colonising numerous niches. Environmental sites include soil, sediment, and water (Somorin, et al. 2016). These have been regarded as secondary, non-host environments resulting from faecal contamination (Winfield and Groisman 2003). But analysis of genomic traits of isolates from water strongly suggest *E. coli* populations are capable of long-term survival and growth without host involvement (Hartl and Dykhuizen 1984; Ishii and Sadowsky 2008; Somorin, et al. 2016; Jang, et al. 2017; Touchon, et al. 2020). As a commensal, *E. coli* colonises the gut as part of the normal flora of warm-blooded animals. The lower intestine, especially of mammals, and less commonly, birds and reptiles can be regarded as the primary habitat of *E. coli* (Savageau 1983; Gopee, et al. 2000; Gordon and Cowling 2003). Healthy humans and other mammals, including dogs and cats harbour *E. coli* in the gastrointestinal tract as a minor representative in numerical terms of the microbiota but as the most abundant facultative anaerobe (Jaureguy, et al. 2008). As a pathogen *E. coli* infections are regarded as a serious international public health concern associated with significant morbidity, mortality and consequent economic impact. The majority of these infections are within the digestive system (intestinal) and less commonly in the urinary tract and other internal organs (extraintestinal). This study considers only *E. coli* isolated from these extra intestinal infections in humans and their relatedness to commensal strains from companion animals and poultry meat.

Commonality between commensal strains isolated from canine and feline faeces and human and companion animal clinical *E. coli* isolates has been demonstrated. These findings together with the close, regular physical contact between companion animals and people has led to concerns that companion animals may serve as a possible reservoir for extra intestinal pathogenic *Escherichia coli* (ExPEC) in humans. This concern extends to the potential for transfer of antibiotic resistant strains (Johnson, Stell, Delavari 2001; Johnson, Kuskowski, et al. 2003).

This study adds to the research to date, by initially determining the prevalence of potentially pathogenic strains of *E. coli* in faeces collected from companion animals. These isolates are compared to a collection of human and poultry meat strains to assess relatedness. Findings from this study will contribute to a better understanding of the relationship between potentially pathogenic

poultry meat, companion animal and human strains. Findings from this study can contribute to a better understanding of whether companion animal *E. coli* strains are potential human pathogens, that is, zoonotic, or are largely host specific and benign.

Genetic Diversity of *E. coli*

E. coli is a highly diverse and adaptive species, continuously evolving into a wide variety of biotypes. These range from commensal strains within the gut flora and strains adapted to the external environment to highly virulent strains that cause both intestinal and extra-intestinal infections (Jang, et al. 2017).

E. coli have a genome size of about 5 Mb (ranging from 4.5 to 5.5Mb) comprising approximately 4700 protein encoding genes (Gordon 2010; Tenaillon, et al. 2010). Approximately half this number of genes are common to all strains of the species and constitute the core genome. Genes comprising the core genome encode 'housekeeping' genes or gene clusters show a relatively low mutation rate and direct basic functions such as replication, transcription and translation and metabolism (Hacker and Kaper 2000; Monk, et al. 2013).

The remaining genes known as the variable, accessory or dispensable genome are drawn from a gene pool (an open pan-genome) of more than 75,000 gene families (if insertion sequences, phage and phage-like genes are included) in strains from a range of different environments (Rasko, 2008; Medini, 2005; Tenaillon, 2010; Touchon, 2020).

These collections of genes contribute to species diversity and can encode non-canonical biochemical pathways, antibiotic resistance and other functions not essential for bacterial growth. Where mobile genetic elements (MGE) carry genes encoding one or more virulence factors these have been called pathogenicity islands (Hacker and Kaper 2000). *E. coli* increases genetic diversity by co-opting these genes via horizontal gene transfer (HGT); taking advantage of the surrounding gene pool offered by other *E. coli* strains in the gut microbiota, including commensals in other phylogroups, to enhance survival and colonisation in the gut (Brzuszkiewicz, et al. 2006; Touchon, et al. 2020).

Accessory genes from the large open pan-genome pool are exchanged as MGEs such as plasmids and bacteriophages, within and between bacterial species by transformation, transduction, and conjugation (Medini, et al. 2005). Sequences of these sections of the genome differ from the core genome in G and C content indicating their derivation via HGT.

Insertion or deletion of genes via HGT largely explains differences of up to 1Mb in genome size. This varies with phylogroup (Lloyd, et al. 2007) so that strains in phylogroups B2 with more MGEs generally have larger genomes than strains in phylogroups A and B1 strains (Touchon, et al. 2020).

HGT provides a high level of plasticity allowing bacteria to adapt to environmental challenges, like different niches and specific environmental conditions (Tenaillon, et al. 2010). A study of a large numbers of *E. coli* isolates found that the highest rate of gene content diversification with fewer diverse MGEs were characteristic of isolates in the phylogroups with the smallest genomes. This led the authors to suggest that smaller genomes are associated with a higher turnover of genetic information (Touchon, et al. 2009). This diversity and the overlap in gene content with related members in *Enterobacteriaceae* suggest the species *E. coli* is an indefinite continuum (Lukjancenko, et al. 2010).

Genetic structure of *E. coli*

Despite the diversity of *E. coli*, the species is considered to have a mainly clonal genetic structure, with a moderate level of recombination (Selander and Levin 1980; Desjardins, et al. 1995) and a strong phylogenetic structure. The species is divided into eight main phylogroups (Clermont, et al. 2019). Of these, A, B1, B2 and D represent the majority of strains and the remaining four (C, E, F and G) are rare (Touchon, et al. 2020). These phylogroups are represented in the standard *E. coli* collection of reference (ECOR) strains. Strains within these phylogroups differ in their capacity to colonise ecological niches and propensity to cause disease (Gordon 2010). The phylogenetic tree splits *E. coli* into two main branches comprising B2, F and G on one branch and the remaining phylogroups on the other (Touchon, et al. 2020).

Groups B2 and D strains are more likely to be recovered from ExPEC infections in humans (Picard, et al. 1999; Johnson, Delavari, Kuskowski, et al. 2001; Nowrouzian, et al. 2005) and companion animals (Salvarani, et al. ; Selander, et al. 1986; Le Gall, et al. 2007; Turchetto, et al. 2015; Liu, Thungrat, et al. 2016; Hutton, et al. 2018), whereas most other pathogenic strains are not B2 (Escobar-Páramo, Clermont, et al. 2004). Group B2 strains are also more likely to be recovered from the faeces of asymptomatic humans, especially in industrialised countries (Zhang, et al. 2002; Escobar-Páramo, et al. 2004). These strains have the most distinctive gene repertoire with a greater number and diversity of virulence-associated genes compared to group A and B1 strains (Picard, et al. 1999; Johnson, Delavari, Kuskowski, et al. 2001; White, et al. 2011; Touchon, et al. 2020). It is important to note that earlier studies identified a limited number of phylogenetic groups (A,B1,B2,D) (Clermont 2000), compared with studies which used more recent methods (Clermont 2019). This would be expected to devalue chronological comparisons of phenotype prevalence.

E. coli in the gut

Gut microbiota

The microbial ecosystem within the gut of companion animals, humans and other animals is called the gut microbiota. The caecum and colon contain the most dense and diverse communities of all microbial body habitats in human adults consisting of 10^{11} bacteria per ml (Sender, et al. 2016). This community is usually dominated by strictly anaerobic species within the *Bacteroidetes* and *Firmicutes* phyla in humans (Marchesi, et al. 2016) and in adult dogs and cats (Minamoto, et al. 2012; Barko, et al. 2018).

E. coli within the *Proteobacteria*, is a component (albeit minor) of this microbiota and each strain competes with other strains of *E. coli* and other species for attachment sites by producing growth suppressants like bacteriocins. Bacteriocins are peptides that inhibit other members of the same or closely related species and are associated with successful gut colonization, prerequisite to translocation and invasion by ExPEC (Martinson and Walk 2020).

E. coli also deplete essential substrates from the environment by encoding efficient mechanisms to scavenge scarce nutrients, such as iron. *E. coli* colonises the colon by penetrating the mucus layer of the epithelium (Freter, et al. 1983) using nutrients acquired from mucus as well as shed epithelial cells and dietary fibre (Conway and Cohen 2015). Colonic mucus comprises mucosal polysaccharides which are degraded by the dominant anaerobes in the colonic microbiota, (Hoskins, et al. 1985) releasing sugars, especially gluconate (Chang, et al. 2004). *E. coli* are ultimately shed with the degraded mucus into the intestinal lumen and form a component of the host's faeces.

Diet affects composition of this microbiome in dogs (Legrand-Defretin 1994). As example, a raw meat diet was found to promote a higher abundance of *E. coli* and *Clostridium perfringens* than the commercial extruded diet (Schmidt, et al. 2018). Similarly in the cat, both diet and age were found to affect faecal microbial composition (Bermingham, et al. 2018).

Ecological structure within the host

Ecological structure of the *E. coli* population in the host depends mainly on two factors; the strains available to the host and whether the type of 'environment' within the host suits colonisation briefly, or long term. Companion animals and humans are constantly exposed to *E. coli* in food, drinking water and fomites that have the potential to colonise the gut.

Thus, geographic location, diet (omnivore, herbivore, carnivore) and proximity to human habitation (level of domestication in animals) affect the range of strains presented to the gut. Exposure alone, though, cannot fully explain why a particular strain of *E. coli* occupies an intestinal niche. Host body size, age, sex, normal core body temperature and structure of the gastrointestinal tract enable varying levels of microbial fermentation and determine the rate of flow through the tract (Gordon and Cowling 2003; Gordon, et al. 2005). This in turn affects the 'environment' within the tract and consequent likelihood of colonisation (Gordon and FitzGibbon 1999).

Pathogenic *E. coli*

E. coli and disease

Of the diseases in humans and other animals attributed to *E. coli,* the most notorious are the diarrhoeal and enterotoxic diseases caused by intestinal pathogenic *E. coli* (IPEC). These are a significant cause of human mortality globally (Troeger, et al. 2018) and are represented by six pathotypes; enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC) (e.g., enterohemorrhagic *E. coli* [EHEC]), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), *Shigella*/enteroinvasive *E.coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper 1998) as well as a new pathotype, adherent invasive *E.coli* (AIEC) (Croxen, et al. 2013). EPEC, EHEC and ETEC can also cause disease in animals(Moon, et al. 1983; China, et al. 1996; Nagy and Fekete 1999) including dogs and cats (Beutin 1999).

Other *E. coli* pathotypes are implicated in extraintestinal infections and these isolates can cause disease at multiple anatomical sites in humans and animals. Historically these *E. coli* have been defined according to isolation site. Strains isolated from UTIs were designated uropathogenic *E. coli* (UPEC); those isolated from bacteraemia/sepsis were described as sepsis-causing *E. coli* (SEPEC) and strains isolated from neonatal meningitis were described as (NMEC). Strains isolated from colibacillosis in poultry were separated as avian pathogenic *E. coli* (APEC) (Russo and Johnson 2000; Kaper, et al. 2004; Dale and Woodford 2015). Syndrome-specific designations were deemed too narrow and misleading as there do not appear to be site-specific VAGs that equip strains to cause infection at a particular anatomic site. The term extra-intestinal pathogenic *E. coli* (ExPEC) was suggested and defined as strains usually belonging to phylogroup B2 or D and having a designated number and type of virulence associated genes (VAG) (Russo and Johnson 2000).

There is considerable variation in the number and types of VAG (also called virulence determinants, or virulence factors (VFs)) which must be carried by isolates in order to be classified as an ExPEC (Johnson, Delavari, Kuskowski,et al. 2001; Riley 2014; Dale and Woodford 2015). Those isolates positive for at least two of five virulence associated genes, that is, *papA* and/or *papC, sfa/foc, afa/dra, iutA, and kpsM* II (Johnson, Kuskowski, et al. 2003) have been defined as ExPEC. Others define ExPEC isolates as those with greater numbers of virulence factors, ranging from nine (*papC, papGII, papGIII, sfa/foc, hlyC, cnf1,iucC, fyuA* and *iroN)* (Jaureguy, Landraud, Passet, Diancourt, Frapy, Guigon, Carbonnelle, Lortholary, (Clermont and Denamur 2008), to over 50 VAG genes (Jørgensen, et al. 2019). Some authors have calculated ‘virulence scores’ to classify isolates as ExPEC (Moreno, et al. 2008), and others have developed a virotyping scheme to

differentiate subgroups within ST131 (Johnson and Stell 2000). A‘virulence score’ has been defined as the number of unique virulence factors detected (Johnson, Kuskowski, et al. 2003). Virotypes were defined generally as *E. coli* isolates with the same combination of virulence factors. Virulence factors associated with pathotypes have also been defined, for example, those in *E. coli* isolated from cases of urosepsis (Johnson and Stell 2000) and recurrent UTIs (Clermont, Lavollay, etal. 2008). Thus, the presence of specific sets of virulence genes is assumed to define a strain as ExPEC and has been linked to pathogenicity (Pitout 2012). Even though there is a strong correlation between VF and phylogeny, there was found to be no association between VF carriage and clinical outcome (Tourret and Denamur2015). The VF profile of APEC strains from poultry have been compared with avian faecal commensal *E. coli* (AFEC) isolates in an effort to define the key VF associated with APEC (Johnson et al., 2008). Five genes were identified as indicative of APEC strains but overlapping representation in strains from the 2 sources was present. Epidemiological and animal experimental evidence of specific genes as virulence factors has been documented (Johnson and Russo 2005). However, the specific virulence gene profiles of isolates from any particular ExPEC can be extremely diverse and, to date, no set of VAGs can be linked to clonal dissemination of any ExPEC lineage or specific toUPEC, APEC, or NMEC. There are likely many alternative strategies used by ExPEC to adapt to particular extra-intestinal niches, invade and cause tissue damage (Croxen and Finlay 2010; Breland,et al. 2017). Many of the genes considered to be virulence factors have not been proven to be essential for the *E.coli* to cause disease and these genes and these gene associations can also be explained as microbial adaptation to particular niches (Tourret and Denamur 2015)

UPEC are by far the most common ExPEC, as the usual cause of community acquired, uncomplicated UTIs in humans (Stamm and Norrby 2001). UTIs are estimated to affect more than 50% of females on at least one occasion, causing significant morbidity and presenting a huge public health cost (Johnson 1991; Foxman 2002; Flores-Mireles, et al. 2015a).

Asymptomatic bacteruria (ABU) is the presence of high numbers of bacteria in the bladder in the absence of symptoms. The prevalence of ABU varies with age, gender, sexual activity, and anomalies of the genitourinary system. ABU in identified risk groups, eg. pregnant women and the elderly may progress to symptomatic UTI or pyelonephritis if undetected (Stork, et al. 2018). UTI recurrence is common and arises either from re-inoculation of the urinary tract or flare-up of infections that are not cleared from the urinary tract (Bower, et al. 2005).

The other infections caused by ExPEC in humans are much more serious and include bloodstream infections (bacteraemia and septicaemia), sepsis, neonatal meningitis and pneumonia (Micenková, et al. 2017; Bogema, et al. 2020). Any normally sterile extraintestinal site can be infected, causing a range of infections, including meningitis, myositis, osteomyelitis and epididymoorchitis (Vila, et al. 2016) with serious sequelae such as meningitis and death. Death rates can be around 10% and up to 40% in infants (Kennedy, et al. 2008).

ExPEC also cause important diseases in companion animals and poultry. UTIs are common in female dogs and *E. coli* is the most common cause (Ling, et al. 2001; Thompson, et al. 2011; McMeekin, et al. 2017; Oh, et al. 2017). *E. coli* is also the most common cause of canine pyometra (Wadås, et al. 1996). Close phylogenetic and pathotypic correspondence has been used to suggest commonality between canine and human ExPEC (Johnson, Stell, Delavari, et al. 2001). Persistent and recurrent UTIs have been reported less commonly in dogs, more often those which are middle-aged or older (Norris, et al. 2000). These infections can lead to serious or life-threatening sequelae in spite of treatment with antibiotics (Drazenovich, et al. 2004). *E. coli* has also been isolated from a range of other non-enteric infections in dogs, including from cases of haemorrhagic cystitis, otitis, mastitis and prostatitis (Oluoch, et al. 2001; Handt, et al. 2003; Siqueira, et al. 2009; Zogg, et al. 2018).

Bacterial UTIs are rare in otherwise healthy cats compared with dogs, with only 1–2% of cats suffering from UTIs in their lifetime (Freitag, et al. 2005; Bailiff, et al. 2008; Litster, et al. 2011; Dorsch, et al. 2019). However, urinary infections are regarded as an important infection in older female cats (>10 years), usually in association with chronic disease such as diabetes mellitus, chronic kidney disease and uncontrolled hyperthyroidism. In these cases, the clinical history may include haematuria, dysuria and frequency, or the infection may be occult (Litster, et al. 2009). Many cats have subclinical bacteriuria and *E. coli* is amongst the most common bacterial species isolated from urine in these cases (Litster, et al. 2007; Bailiff, et al. 2008; Litster, et al. 2011). Few other ExPEC infections in cats have been reported isolated from cases of pneumonia (Sura, et al. 2007, Brooks 2013, Highland 2009).

ExPEC pathotypes in poultry, grouped together as APEC are major pathogens within the poultry industry (Mora, et al. 2013). The main syndrome, colibacillosis, is characterised by septicaemia with multiple organ lesions, including salpingitis and peritonitis. A great diversity of strains of *E. coli* can be isolated from these diseased birds and, although certain genes associated with pathogenicity are

common in APEC, they are not found in all isolates.

E. coli carrying these APEC associated virulence factors can also be isolated from the intestines of healthy birds, suggesting disease mainly results as opportunistic infections in susceptible hosts (Collingwood, et al. 2014).

ExPEC pathogenesis

Unlike pathogenic intestinal *E. coli* strains, ExPEC colonise the healthy human or animal's lower intestine and/or vagina (less commonly) as commensals. ExPEC are usually phylogroup B2 or D and possess virulence factors which enable bacterial cells to attach to and invade host cells (Mora-Rilo 2015). These same factors may also promote faecal abundance, clonal dominance and pauciclonality (Moreno, et al. 2008). In a host with a UTI, the faecal and UTI isolates are closely related (Nielsen, et al. 2017) and the urine isolate tends to be the host's predominant faecal strain(Yamamoto, et al. 1997; Vejborg, et al. 2011; Johnson, et al. 2016). From the gut, *E.coli* gain entry to the normally sterile urinary tract following presumed faecalcontamination of the peri-urethral area. Bacteria ascend the urethra and may be removed by micturition shortly after introduction into the bladder or attach to and colonise the bladder epithelium. *E.coli* then invade and replicate in the urothelial cell cytoplasm forming large biofilm like intracellular bacterial communities (IBCs) (Klein and Hultgren 2020).

Most ExPEC strains attach to, and colonise, urinary tract cells by means of virulence genes called adhesins. The best characterized adhesins are fimbrial adhesins, pyelonephritis-associated pilus (encoded by the *pap* genes) and the type 1 fimbria (encoded by *fim* genes). Others include Dr binding adhesins (*afa/dra*), S fimbriae (*sfa*) and Type F1C (*foc*) (Dale and Woodford 2015). Whereas the PapG adhesin binds to a receptor on kidney cells, the FimH adhesin at the tip of the type 1 fimbria binds receptors on urothelial cells prior to colonization and invasion of the bladder epithelium (Klein and Hultgren 2020). Attachment and colonisation via biofilm formation also depends on type 1 fimbria as well as cell surface appendages such as curli (Van Houdt 2005).

ExPEC causing cystitis may ascend the urinary tract to infect the kidneys leading to pyelonephritis. If untreated further spread from the kidney into the bloodstream may occur causing bacteraemia and septicaemia.

Avian pathogenic *E. coli* (APEC) cause severe infections when colonization of the upper respiratory tract and air sacs occur and is followed by the dissemination of APEC to internal organs causing pericarditis, perihepatitis. APEC may also enter the bloodstream causing septicemia which is often fatal (Dho-Moulin and Fairbrother, 1999). APEC strains, which usually belong to serotypes O78, O1 and O2, have been known to harbor various virulence factors, most of which are also characteristic of human extraintestinal strains.

Host integrin proteins, expressed throughout the urinary tract epithelium, are key receptors for type 1 pili–expressing UPEC. Attachment causes the host plasma membrane to envelop bound bacteria initiating invasion of bladder epithelial cells. This provides UPEC with a protective environment in

which bacteria can either replicate or persist in a quiescent state forming intracellular bacterial communities (Mulvey, et al. 2000). These quiescent bacteria may serve as reservoirs for recurrent (or relapsing) UTIs and kidney colonization and bacteremia/septicaemia (Terlizzi, et al. 2017).

An array of toxins cause tissue damage, dissemination and release of host cell nutrients and include hemolysins (*hyl*), secreted autotransporter toxin (*sat*) and cytotoxic necrotizing factor 1 (*cnf1*) (Wiles, et al. 2008). Iron acquisition systems (siderophores) enable UPEC to source limited iron stores in the host and include yersiniabactin (*fyuA*) and aerobactin (*iuc, iut*) (Torres, et al. 2001; Micenková, et al. 2017).

Iron is essential yet toxic with intense competition between bacteria and host for iron. Most of the iron in humans is unavailable to ExPEC and other invading microbes. As part of the innate immune reaction, concentrations of iron in extracellular fluid and plasma dramatically decrease within hours of infection via hepcidin. This peptide hormone regulates host iron homeostasis. UPEC repress hepcidin to evade renal host defenses during urinary tract infection. Mammalian neutrophils also produce lipocalin-2 that binds one of the siderophore, enterobactin, preventing iron uptake by *E. coli*. However, phylogenetic group B2 strains have numerous siderophores which evade host bindingand provide a greater ability to acquire iron (Martin, et al. 2017).

Sequence types usually associated with ExPEC

Even though there are many diseases associated with *E. coli* and thousands of clonal complexes (CCs) comprising many sequence types (STs), only a very small subset of STs are responsible for most ExPEC infections. These are represented by STs 69 (phylogroup D), 73, 95, 127 and 131 (phylogroup B2) (Riley 2014; Doumith, et al. 2015; Salipante, et al. 2015; Kallonen, et al. 2017). Of these, ST73 is considered to be the oldest CC whereas ST69 and ST131 have emerged relatively recently (Kallonen, et al. 2017). ST117 is also considered in this study as this sequence type is a major cause of APEC infections in poultry, and consequently represents an important avian ExPEC. These lineages are rare in native Australian vertebrates (Gordon 2013) but occur in poultry, other livestock and companion animals (Platell, Johnson, et al. 2011; Ewers, et al. 2012; Manges and Johnson 2012; Maluta, et al. 2014b; Manges 2016).

ST69

ST69 *E. coli* were originally included within a group designated CgA comprising a number of STs resistant to trimethoprim-sulfamethoxazole (Manges, et al. 2001; Johnson, Owens, et al. 2004; Tartof, et al. 2005; Skjøt-Rasmussen, et al. 2013). Isolates of ST69 were relatively recently identified in human clinical samples from the 1990s in the US and from 2002 in blood stream isolates in the UK (Blanco, et al. 2011; Kallonen, et al. 2017). Since then ST69 strains have been isolated from both community-acquired and healthcare-associated ExPEC infections in humans in many countries (Blanco, et al. 2011; Manges, et al. 2019; Riley 2020) as well as from healthy humans (Trobos, et al. 2009). ST69 strains have been isolated from other species, including a cow (ST408), poultry, pig, dogs, and horse (Ramchandani, et al. 2005; Tartof, et al. 2005; Ajiboye, et al. 2009; Grönthal, etal. 2018; Riley 2020) (http://enterobase.warwick.ac.uk/species/ecoli/search_strains?query=st_search).

Diversity

There appears to be considerable heterogeneity amongst isolates identified as ST69, given the number of serogroups that have been observed, including O11, O15, O17, O44, O73, O77, O86, O125ab, and O25b (Tartof, et al. 2005; Riley 2014; Ciesielczuk, et al. 2016). Most ST69 (77%) isolateswere O17:H18 in one study of 83 blood stream infections (Kallonen, et al. 2017).

Spread

Prevalence data from a collection of bacteraemia isolates collected in England from 2001 to 2011 revealed ST69 to be an emerging lineage, together with ST131 (Kallonen, et al. 2017). Investigation of

the structure and history of this emergence showed the most recent common ancestor for ST69 was dated to 1956 and that two large lineages emerged in 1977 with several increases in population size occurring from the late 1970s (Kallonen, et al. 2017). ST69 was a major lineage in a study of isolates from urinary sepsis or bloodstream infection over a similar time period in Ireland (Miajlovic, et al. 2016) and from bloodstream infections between 2011 and 2013 in China (Zhao, et al. 2015). A metanalysis of global published reports of isolates collected from ExPEC infections or the gut between 1995 to 2018 concluded that the prevalence of ST69 has remained stable during this period (Manges, et al. 2019).

Virulence

High virulence scores were observed among a collection of *E. coli* ST69 strains isolated from human clinical samples and poultry meat. Most of these isolates carried *pap* alleles *(papA, papC, papEF, papG II), iha, kps*MTII-K5 and *ompT* (Novais, et al. 2013). However, the reported virulence gene profiles in this sequence type differ between studies (Riley 2014; Kallonen, et al. 2017).

Virulence factors have been identified as specific to ST69 blood stream infections (*foc, tsh, cfaD* and *espC*) (Kallonen, et al. 2017). Virulence genes *papAC, fyu, tra* and *malX* were carried by a ST69 dog clinical isolate (Liu, Liu, et al. 2016). Prototypic (O11; ATCC BAA-457) virulence factor genes for ST69 were listed as *papA* alleles, *sfa/focDE, focG, iha, hlyD, cnf1, fyuA, iutA, iroN, malX*, and afa/dra (Riley 2014) but this assemblage is not specific to ST69 and covers all STs formerly classified as CgA.

Antibiotic resistance

ST69 is associated with resistance to trimethoprim-sulfamethoxazole (Johnson, Menard, et al. 2009; Platell, Trott, et al. 2012), aminoglycosides (Novais, et al. 2013) and beta-lactams (Gibreel, et al. 2010; Hansen, et al. 2014), and are described as a multidrug-resistant (MDR) lineage (Adams-Sapper, et al.
2013; Manjarrez-Hernandez, et al. 2016). MDR isolates are defined variously. Those resistant to 3 or more of the following drug classes: beta-lactams, fluoroquinolones, TMP-SMX, nitrofurantoin, and aminoglycosides (Magiorakos, et al. 2012; Banerjee, Johnston, Lohse, Chattopadhyay, et al. 2013; Sweeney, et al. 2018) and those resistant to one or more antibiotics in three or more (unspecified) classes (Adams-Sapper, et al. 2013). Resistance to tetracycline (Zhao, et al. 2015), chloramphenicol, fluoroquinolones (including PMQR gene *aac(6′)-Ib-cr*), gentamicin and carbapenems (OXA-48) has been reported in human isolates (Ajiboye, et al. 2009; Adams-Sapper, et al. 2013; Novais, et al. 2013; Liu, Liu, et al. 2016). Genes encoding extended spectrum beta-lactamase (ESBL) and AmpC

beta-lactamse production have also been reported in isolates from human urine (CMY-2) and canine urine and faeces (CTX-M-55, CTX-M-9, CTX-M-1, CTX-M-15, CMY-9) (Queiroz, et al. 2012;

Novais, et al. 2013; Schaufler, et al. 2015; Liu, Thungrat, et al. 2016; Roer, et al. 2017; LeCuyer, et al. 2018; Zhang, et al. 2018), canine clinical isolates (Liu, et al. 2016) and sludge samples from a Swedish city including from a university hospital (Fagerström, et al. 2019). Others have reported absence of ESBL in their collections of ST69 isolates (Adams-Sapper, et al. 2013; Zhao, et al. 2015; Manjarrez-Hernandez, et al. 2016).

Human animal comparisons

The relatedness of human and animal ST69 isolates is unclear. Disparate results suggesting both host specificity and strain sharing have been reported. However, these are from very few studies with low numbers using techniques with relatively low discrimination. Host specificity was suggested based on the finding that two ST69 poultry isolates cluster separately to human blood and urine ST69 isolates and displayed a different virulence gene pattern (Novais, et al. 2013). The isolation of *E. coli* ST69 strains identical on pulsed-field gel electrophoresis (PFGE) and antibiotic resistance pattern, from two dogs and two people in the same family is proposed as evidence of strain sharing (Grönthal, et al. 2018). Close relatedness was also proposed on finding that a single ST69 human blood stream isolate differed by 15 or less single nucleotide polymorphisms (SNP)s in the core genome from two ST69 turkey faecal isolates (Ludden, et al. 2019).

ST73

Significance

Strains within clonal complex 73 (CC73) belong to ECOR phylogroup B2 and are major clonal pandemic lineages associated with ExPEC in humans (Riley 2014; Yahiaoui, et al. 2015). Within the CC73 most isolates belong to ST73. Related STs include ST74, ST1404, and ST968 (Salvador, et al. 2012). Most MLST-based studies of ExPEC have concentrated on ST131, due to recent dramatic increases inprevalence with global spread and multi-drug resistance of several clones (Johnson, Porter, et al. 2017). Comparatively little is known about ST73 isolates apart from the ExPEC reference strains CFT073 and 536 which are human pyelonephritis isolates (Welch, et al. 2002; Johnson, Owens, et al.2006).

ST73 remains a leading cause of human extraintestinal infections, commonly associated with UTIs in

both women and men (Salvador, et al. 2012; de Souza da-Silva, et al. 2017; Matsukawa, et al. 2019) and other community and hospital acquired ExPEC infections (Gibreel, et al. 2010) such as bacteraemia, pyelonephritis, hepatobiliary and gastrointestinal infections (Johnson, Johnston, et al.

2008; Horner, et al. 2014). Large studies in the UK, sampling during 2001 to 2013, found ST73 to be the most common sequence type isolated from bacteraemic ExPEC infections (Gibreel, et al. 2010; Horner, et al. 2014; Day, et al. 2016; Kallonen, et al. 2017; Ludden, et al. 2019). ST73 was reported to be a dominant isolate from EXPEC infections in the United States of America (US) (Tartof, et al. 2005; Adams-Sapper, et al. 2013; Banerjee, Johnston, Lohse, Chattopadhyay, et al. 2013), Europe (Brisse, et al. 2012; Riley 2014; Kallonen, et al. 2017) and Australia (Bogema, et al. 2020). Studies have found a similar proportion of ST73 isolates in urine and blood infections (Manges, et al. 2019).

ST73 prevalence in different human environments and populations varies. In hospital and age-care facilities in Seattle, US ST73 was not observed as frequently as ST131 (Banerjee, Johnston, Lohse, Porter, et al. 2013). A recent Canadian survey found ST73 was associated with community infections in the elderly (Holland, et al. 2020). A US study of ExPEC isolates revealed that even though ST131 was more common in clinical isolates from young children and the elderly, and among health care-associated isolates, ST73 was more commonly associated with community infections in adolescents and young adults (Banerjee, Johnston, Lohse, Chattopadhyay, et al. 2013). ST73 isolates were the predominant cause of community associated UTIs in Brazil (Dias, et al. 2009; de Souza da-Silva, et al. 2017) and community acquired blood stream infections in Australia (Hastak, et al. 2020). ST73 can be commonly isolated from asymptomatic bacteruria (ABU) cases and (Salvador, et al. 2012; Stork, et al. 2018) one ABU ST73 strain is promoted as an UTI preventative (Stork, et al. 2018).

ST73 can be isolated from non-clinical sites, that is, as an apparent commensal. A survey of *E. coli* faecal isolates from adult humans in Paris in 2010 found B2 subgroup was the most prevalent phylogroup and ST73 was the most prevalent sequence type in this phylogroup (Massot, et al. 2016). Some studies have found a similar proportion of ST73 strains among clinical and commensal isolates (Clermont, et al. 2017; Nielsen, et al. 2017) based on MLST, SNP-based phylogeny, single-nucleotide variant counts and accessory genome analyses. Other studies found a greater proportion of ST73 strains in UPEC isolates compared with faecal samples (Nielsen, et al. 2017; Matsui, et al. 2020).

Diversity

ST73 strains are genetically diverse with different combinations of plasmids and virulence associated and antibiotic resistance genes (Alhashash, et al. 2015). Compared with ST131, ST73 strains were heterogeneous and less closely related based on single nucleotide polymorphisms (SNPs) (Clark, et al. 2012; Alhashash, et al. 2015; Kallonen, et al. 2017). Consequently, clonal spread cannot explain the success of ST73 (Manges, et al. 2019).

These diverse strains within the large clonal complex CC73, can be divided into eight sequence types with ST73 predominant (Gibreel, et al. 2010). ST73 can be further separated into at least 8 groups based on PFGE (Gibreel, et al. 2010) and average pairwise SNP distance (Kallonen, et al. 2017). A recent study found ST73 blood stream isolates from Australian hospital patients clustered into four major groups which correlated with serotype (Bogema, et al. 2020). Another study of uropathogenic ST73 *E. coli* isolates separated strains into 4 subtypes based on *fimH* type (f-3, f-8, f-9, and f-60) (Tarlton, et al. 2019).

The level of genome heterogeneity (Gibreel, et al. 2010; Alhashash, et al. 2015) and association with UTIs in women from widely separated geographic areas over many years (Manges and Johnson 2012) suggest that ST73 is a relatively older, human-adapted ExPEC lineage (Kallonen, et al. 2017). There is also some evidence of convergence with pathogenic intestinal (IPEC) strains. ST73 strains from dog, pig and cat faeces and one UPEC isolate from a dog showed phenotypic characteristics typical of adherent-invasive E. *coli* (AIEC) (Martinez-Medina, et al. 2009).

Spread

ST73 is regarded as a long-standing ExPEC lineage (Kallonen, et al. 2017), remaining dominant even after 2005 when newly emerging lineages, such as ST131 became much more commonly isolated from blood infections (Roer, et al. 2017) and UTIs (Banerjee, Johnston, Lohse, Chattopadhyay, et al. 2013; Hertz, et al. 2016). A recent rise in the incidence of MDR ST73 strains in the UK could not be attributed to the emergence of a dominant clone as isolates were genetically diverse and carried different plasmids (Bogema, et al. 2020). There appears to be no evidence of spread of a few specific clones of ST73, such as the case withST131. Even within one hospital, no evidence was found of a dominant outbreak strain of ST73 (Bogema, et al. 2020).

Virulence

The emergence of antibiotic resistant lineages, such as ST131, does not seem to have displaced ST73 as a major ExPEC (Kallonen, et al. 2017) and so the reasons for the continuing predominance of ST73 as an ExPEC pathogen are still unclear (Alhashash, et al. 2015). ST73 isolated from human clinical cases carry a broad range of virulence genes including adhesins (*fim, foc, pap, sfa*), iron acquisition (*fyuA, ireA, iro, irp, iuc, sit*), toxins (*cnf1, hly, senB, usp, sat)* and proteases (*ibe, pic, iss*) (Kallonen, et al. 2017; Bogema, et al. 2020). However, the presence of virulence factors cannot explain the successfulestablishment and maintenance of ST73. Many putative virulence factor determinants in CFT073 were also present in two avirulent ST73 strains; the gut commensal strain (Nissle 1917) used as a probiotic and ABU83972 (an asymptomatic bacteriuria strain) (Vejborg, et al. 2010).

Antimicrobial resistance

ST73 strains are generally regarded as susceptible to commonly used antibiotics in contrast to other important ExPEC sequence types such as ST69 and ST131 (Adams-Sapper, et al. 2013; Kallonen, et al. 2017; Matsukawa, et al. 2019; McNally, et al. 2019). Absence of pronounced antimicrobial resistance in ST73 isolates appears to rule this out as a major factor in the successful establishment and maintenance of this sequence type (Kallonen, et al. 2017).

Surveys of ST73 clinical isolates sourced from Europe, US and Egypt found no ESBL producers (Brisse, et al. 2012; Yahiaoui, et al. 2015) or resistance to fluoroquinolones (Yahiaoui, et al. 2015) as well as no MDR strains (Banerjee, Johnston, Lohse, Chattopadhyay, et al. 2013). A UK study noted that the level of phenotypic antibiotic resistance of ST73 was largely unchanged from 2008 to 2012, except for an increase in ampicillin resistance (Kallonen, et al. 2017). Other studies have shown increased levels of resistance, including to extended spectrum cephalosporins (ESC) and fluoroquinolones. A 2008-2009 survey of community and hospital derived UTIs in elderly patients detected more than 20% ST73 isolates as ESBL producers (Croxall, et al. 2011). A survey of bacteraemia cases in the same region of the UK in 2013 found the prevalence of ESBL producing ST73 isolates had increased to displace ST131 as the most common ExPEC isolate (Alhashash, et al. 2015). In Egypt CTX-M-15, and Japan CTX-M-14-producing *E. coli* of group B2 isolates were typed as ST73 (Suzuki, et al. 2008; Fam, et al. 2011). Susceptibility to most antibiotics is also common in ST73 faecal and clinical isolates from companion animals (Wagner, et al. 2014; Skurnik, et al. 2015). In the United States 33% of ST73 isolates from cats were MDR but only one (n=12) was an ESBL producer (Liu, et al. 2015). There is a single reportof ST73 sporadically associated with the spread of ESBL-producing *E. coli* ($bla_{\text{CTX-M-1}}$) in Germany in cats and cattle (Ewers, et al. 2012). Clinical samples from dogs in China yielded an MDR

ST73 isolate carrying *aac(6')-Ib-cr,* the plasmid-mediated quinolone resistance determinant (Liu, Liu, et al. 2016). ST73 isolates from infections in dogs in Australia were infrequently MDR (Kidsley, O'Dea, Saputra, et al. 2020). ST73 isolates from dogs in France were not usually MDR, or ESBL producers (Valat, et al.2020). A survey of clinical cases in cats in Australia determined that 39% of ST73 isolates were MDRand fluoroquinolone susceptible (Kidsley, O'Dea, Ebrahimie, et al. 2020). These companion animal isolates were susceptible to ESCs as were clinical isolates from dogs and cats in Switzerland (Zogg, Zurfluh, et al. 2018), United States (LeCuyer, et al. 2018) and Taiwan (Huang, et al. 2020).

Phenotypic antibiotic resistance screening usually includes testing for beta-lactams or ESBL production, resistance to quinolones and any multidrug resistance. Carbapenem resistance screening is much less common (Manges, et al. 2019). Use of these screening methods during isolation may have overestimated the prevalence of multidrug and ESBL resistant lineages, such as ST131, ST405, ST38, ST648, ST410, and ST354, and led to the underreporting of other antibiotic susceptible lineages, eg. ST73, ST12, and ST127. As example, under-representation of ST73 strains among ExPEC isolates in African surveys is explained as probably due to selective isolation techniques (Manges, et al. 2019). The usual failure to isolate ST73 from livestock and companion animals, in common with isolation from human samples, is not unexpected. Most of these surveys, especially those involving companion animals, screen isolates with antibiotic selective media (Schink, et al. 2013; Bogaerts, et al. 2015), possibly selecting against isolation of antibiotic susceptible STs such as ST73 (Manges, et al. 2019).

Antibiotic resistance determinants are often located on plasmids. Several authors reported that a high proportion of ST73 *E. coli* isolated from humans and dogs did not carry plasmids (Bengtsson, et al. 2011; Adams-Sapper, et al. 2013; Wagner, et al. 2014; Gama, et al. 2020). Carriage of plasmids

was found to vary with antibiotic resistance with beta-lactam susceptible ST73 isolates less likely to carry plasmids (Hertz, et al. 2016). Transfer frequency of plasmids carrying ESC ($bla_{\text{CTX-M-15}}$) and carbapenemase (*bla*NDM-1) was less efficient in strains belonging to ST73 indicating that conjugation frequency is dependent on the phylogenetic background of strains (Gama, et al. 2020). These authors suggest that lower plasmid carriage frequency and acceptance of some plasmids may partially explain why ST73 is less usually associated with antibiotic resistance than other successful epidemic clones (Gama, et al. 2020).

Human animal comparisons

Determining the source of ExPEC infections has received much attention. Even though the primary source of UPEC and other ExPEC in people is accepted as the person's faeces (Nielsen, et al. 2017) there remains suspicion that a reservoir may be food-borne or within companion animals. ST73 is regardedas a more host-specific or human adapted lineage of ExPEC (Manges 2016) given recovery has been mainly from human sources. Surveys of *E. coli* isolated from large numbers of livestock species (cow, pig, rabbit, sheep, goat, horse) and meat derived from these species detected ST73 uncommonly or not at all (Clermont, et al. 2011; Dierikx, et al. 2012; Manges, et al. 2015; Johnson, Johnston, et al. 2017a; Ludden, et al. 2019). The results from these studies have led the authors to

conclude that these food animals do not represent a reservoir of ST73 (Ewers, et al. 2012; Manges, et al. 2019). Yet,a review paper has reported isolation of ST73 from cattle (Ewers, et al. 2012). And *Enterobase* does contain reports of ST73 isolated from pigs, equids and ruminants from 1972, even though MLST was not available until 1998 (Zhou, et al. 2019). ST73 strains have also been isolated from miscellaneous sites, eg. killer whales (Melendez, et al. 2019), pinnipeds (Mora, et al. 2018) and surface waters (Johnson, Johnston, et al. 2017b).

Poultry were previously disregarded as a possible food source of ST73 but a recent survey of colibacillosis in poultry in Brazil identified this strain among APEC strains. Gene representation in these strains was atypical of APEC strains but typical of those described in human ExPEC isolates (Cunha, et al. 2017). *Enterobase* also reports ST73 isolates from ducks and turkeys, but not chickens (Zhou, et al. 2019).

The situation in companion animals is different. ST73 was among the most frequent clinical isolate from dogs and cats in the United States (Liu, et al. 2015; LeCuyer, et al. 2018), France (Valat, et al. 2020), Switzerland (Zogg, Zurfluh, et al. 2018), Spain (Flament-Simon, et al. 2020) and Australia (Kidsley, O'Dea, Ebrahimie, et al. 2020). ST73 was also isolated from faecal isolates from dogs (Martinez-Medina, et al. 2011; Flament-Simon, et al. 2020). *Enterobase* contains reports of ST73 isolated from cats and dogs, mainly clinical isolates but also from faeces (Zhou, et al. 2019). Other studies which characterised isolates from cats and dog using sequence typing failed to detect ST73 (Dierikx, et al. 2012; Nebbia, et al. 2014; Damborg, et al. 2016; Karkaba, et al. 2017; Liakopoulos, etal. 2018).

A recent Australian study of whole genome sequencing (WGS)-based phylogenetic comparison of

ST73 isolates from dogs, cats, and humans based on core genome single-nucleotide polymorphisms

showed considerable overall phylogenetic diversity. Although there were some were species-specific clusters, there was also considerable overlap of isolates from the three hosts (Kidsley, O'Dea, Saputra, et al. 2020). Isolation of ST73 strains from dogs, and cats, has led some authors to suggest cross-species transmission of this sequence type (Johnson, Johnston, et al. 2008; Valat, et al. 2020) and that companion animals could be a potential reservoir or vice versa.

ST95

ST95 is one of the most frequently observed globally distributed human ExPEC pandemic lineages (Adams-Sapper, et al. 2013; Manges 2016; Kallonen, et al. 2017), especially in North America (Manges, et al. 2019), as well as a major cause of APEC infections in poultry (Olsen, et al. 2016; Jørgensen, et al. 2019).

ST95 has also been isolated from poultry meat, turkey, honeydew melon, wild birds, cattle, dogs, rats and wastewater samples (Tamang, et al. 2012; Riley 2014; Röderova, et al. 2017)(http://mlst.warwick.ac.uk/ mlst/dbs/Ecoli).

Diversity

ST95 strains are diverse, showing a range of O and fimH types. Serotypes reported for APEC ST95 strains are O1, O2, O45 and O18 (Zhu Ge, et al. 2014; Cummins, et al. 2019); a similar range as that reported for human ST95 strains (Lau, et al. 2008; Mora, et al. 2013; Matsukawa, et al. 2019). At least 8 *fimH* types are reported (Adams-Sapper, et al. 2013). This range of serotypes and *fimH* types indicates several sublineages and substantial substructure (Riley 2014; Gordon, et al. 2017). PFGE comparison of a collection of chicken and human O1 and O45 isolates showed separation into 2 phylogeny groups consistent with serogroups (Mora 2013). Several subgroups have also been identified in collections of ST95 strains based on comparison of genomes. SNP typing of the *fimH* gene alleles separated four major sublineages in isolates from human blood stream infections (Adams-Sapper, et al. 2013; Stephens, et al. 2015).

Further substructure within these *fimH* types was found with variation in the O-antigen loci (Stephens, et al. 2017). A pangenome analysis of a diverse collection of CC95 strains from various sources, separated 5 main groups, which varied in serotype, virulence and antibiotic resistance. A larger group of human isolates from Australia, France and the United States was classified into these 5 groups based on PCR screening with the observed clusters correlating largely with strain serotype

and *fimH* types (Gordon, et al. 2017). A more recent study compared APEC and human ExPEC genomes to investigate whether host-specific sublineages adapted to source hosts. The authors separated 3 groups or subclades based on rooted phylogenetic trees of both core and accessory genomes. Two comprised exclusively human strains and the third was a mix of APEC and human strains. The APEC strains were diverse and generally were closely related to human strains in this mixed cluster (Jørgensen, et al. 2019). Overlap of some human strains with strains cultured from honeydew melon (O2:H7-ST95) has also been reported (Vincent, et al. 2010).

Spread

In humans, ST95 prevalence has remained relatively stable from approximately 2000 to 2015 (Kallonen, et al. 2017; Manges, et al. 2019). Surveys reporting decreases or variability in prevalence (Croxall, et al. 2011; Riley 2014) may result from an actual increase in the other less antibiotic susceptible lineages, eg. ST69 and ST131 or the result of isolation techniques more likely to select MDR ExPEC lineages (Manges, et al. 2019).

Virulence

Both avian and human ST95 strains demonstrate virulence in experimental models and carry an array of virulence determinants (Reeves, et al. 2011; Madigan, et al. 2015; Cummins, et al. 2019). Experimental studies have shown that some ST95 O18 APEC strains cause disease in mammals and a human UPEC ST95 serotype O18:K1:H7 (UTI89) and ST95 O18 NMEC strains cause mortality in day old chicks and embryos, and salpingitis in chickens (Tivendale, et al. 2010; Mortensen, et al. 2019).

A study of 200 human CC95 isolates found all strains have *fimH, fyuA,* and *ompT* whereas *afdA*, as well as *focG , lpfA* and *terC*, were absent (Gordon, et al. 2017). A large number of virulence determinants are shared between APEC and human ExPEC strains, including *iroN, iss, kpsMT(II), iucD, iutA, ompT, papGII, neuC* and *usp* (Nandanwar, et al. 2014; Cummins, et al. 2019; Poulsen, et al. 2020). However, there were some differences in representation of these genes with *eitA, ireA, iucD* and *iutA* carried by all APEC isolates but absent or less prevalent in human ExPEC isolates (Nandanwar, et al. 2014). There is also variation in gene representations correlating with sublineage membership (Gordon, et al. 2017; Jørgensen, et al. 2019). One study of strain phenotypes indicated that this genotypic variation may not always be expressed. Assays measuring adhesion, invasion, serum survival and biofilm formation failed to distinguish 55 human and 61 avian ST95 isolates (Nandanwar, et al. 2014).

Antibiotic resistance

MDR is described as rare or less common in ST95 strains compared with other important ExPEC strains (Vincent, et al. 2010; Manges 2016) indicating again that antimicrobial resistance is not always required to become a common ExPEC sequence type (Adams-Sapper, et al. 2013; Matsukawa, et al. 2019). However, resistance to sulphonamides and trimethoprim is widespread in this ST and there are several reports of isolates resistant to fluoroquinolones, ESCs and aztreonam (Riley 2014), including carriage of the $bla_{CTX\text{-}M\text{-}3}$ gene by a ST95 *E. coli* dog isolate (Tamang, et al. 2012). A study of human ST95 isolates found antibiotic resistance gene carriage varied with the subgroup (Gordon, et al. 2017).

Human animal comparisons

The recent comparative genomic analysis of APEC and human ST95 genomes showed that APEC isolates in one of 3 separate clusters were closely related, if not nearly identical, to human ExPEC (Jørgensen, et al. 2019). The close relatedness of human and poultry strains in the combined cluster is further supported by the presence of other genes (*cvaA , cvaB , cvaC, cvi , etsA , hlyF, iucC, iucD , iutA* and *ompT*) in the majority of APEC isolates and also the majority of the human ExPEC strains belonging to the same cluster. These genes were encoded on transferable ColV plasmids, closely related to APEC-associated plasmids (Jørgensen, et al. 2019).

Based on MLST typing and RFLP profile of isolates it was concluded that the same ST95 *E. coli* strain was isolated from a woman with cystitis and her dog and zoonotic transfer implied (Johnson, et al. 2008). Conclusions relating to the degree of relatedness and hence the likelihood of strain sharing require the use of finer discrimination methods than those used by these researchers, with WGS as a starting point.

ST117

Sequence type ST117 is the major ST within CC117 in the newly recognised phylogroup G (Clermont,et al. 2019). Isolates of ST117 have been previously classified as phylogroup D (Homeier, et al. 2010;Maluta, et al. 2014b) and B2 (Fernandes, et al. 2018) based on an earlier phylotyping method (Clermont, et al. 2000) and as phylogroup F (Abraham, et al. 2015; Vangchhia 2017) using an improved PCR protocol (Clermont, et al. 2013).

Isolates identified as ST117 have been recovered from healthy chickens, turkey and chicken meat (Vincent, et al. 2010; Hansen, et al. 2016), cattle (Kim, et al. 2017), pigs (Bergeron, et al. 2012), humans (Maluta, et al. 2014a; Kim, et al. 2017; Ronco, et al. 2017), dogs (Dorado-García, et al. 2018) and cats (http://mlst.warwick.ac.uk/ mlst/dbs/Ecoli)(Wu, et al. 2012).

Diversity

ST117 isolates show extensive O diversity but little H-type and fimH allele diversity. The most commonly observed serogroups of ExPEC ST117 in humans are O24 and O143 (Clermont, et al. 2019) and in APEC ST117 isolates in chickens are O1, O111, O2, O18 and O78 (Cummins, et al. 2019). Most strains have H4 flagella (Ronco, et al. 2017) and are mainly fimH97 type (Clermont, et al. 2019).

Spread

ST117 is an important globally disseminated lineage causing APEC infections in poultry (Mora, et al. 2012; Maluta, et al. 2014b). ST117 is also a sporadic cause of human ExPEC infections in a number of countries (Abraham, et al. 2015) and considered an emerging global human pathogen (Cummins, et al. 2019). A survey of ExPEC literature indicated that the prevalence of ST117 isolates from human ExPEC infections and faeces appeared to rise and fall between 1995 and 2018 (Manges, et al. 2019).

Virulence

ST117 isolates carry many of the characteristic ExPEC genes, including those associated with iron uptake systems *(iuc/iutA, ireA, iroN, irp, sitA, fyuA)*. Other virulence associated genes carried by isolates from chickens (APEC), calf, dog and human sources include *etsA, ibeA, iss, ompT, traT, vat, sat* and *pic* (Homeier, et al. 2010; Wu, et al. 2012; Clermont, et al. 2019; Cormier, et al. 2019; Cummins, et al. 2019).

Antibiotic resistance

Antibiotic resistance genes present in human, canine, bovine and avian ST117 isolates include *bla*$_{\text{CTX-M-1}}$*, bla*$_{\text{CTX-M-14}}$*, bla*$_{\text{TEM-1B}}$ *, blaCMY-2*, *tetB, sul1* and *sul2* (Vincent, et al. 2010; Leverstein-van Hall, et al. 2011; Zhang, et al. 2018; Zogg, Simmen, et al. 2018; Courtice 2019). Antibiotic resistance to fosfomycin and phenicols has been detected, but infrequently (Abraham, et al. 2015; Kim, et al. 2017; Fernandes, et al. 2018; Ludden, et al. 2019). A recent study of ST117 isolates from a range of hosts and sources reported almost 50% of isolates with genes conferring resistance to beta-lactam, trimethoprim/sulphonamide and tetracycline classes of antibiotics (Clermont, et al. 2019). ST117 producing *mcr-1* which encodes resistance to colistin (polymyxin E) have been isolated from chickens

and pigs (Olaitan, et al. 2015; Ding, et al. 2018) and from human infections overseas (Kuo, etal. 2016; Wise, et al. 2018). This is concerning as colistin is now a last-resort drug used to treat MDR infections in humans. It has also been used extensively in veterinary medicine, especially in swine and poultry farming as a prophylactic against colibacillosis overseas (Kempf, et al. 2013).

Human animal comparisons

Given similar serotype, phenotypic and virulence gene profiles, together with similar PFGE patterns of ST117 from chickens and other livestock with isolates from humans, there is some concern of zoonotic transfer (Maluta, et al. 2014b; Nandanwar, et al. 2014). Reports of human ST117 ExPEC strains clustering with animal ST117 strains, including from chicken, bovine and porcine sources lend support to this concern (Maluta, et al. 2014b; Manges, et al. 2015; Kim, et al. 2017; Ronco, et al. 2017).

ST131

ST131 belongs to ECOR phylogroup B2 and was first described as a cause of ExPEC in humans in 2008 (Coque, et al. 2008; Kallonen, et al. 2017; Pitout and Finn 2020). ST131 is widely reported as a frequent UTI isolate, with isolates usually resistant to ESC and fluoroquinolones (Pitout and Finn 2020). Consequently, ST131 is regarded as a major public health problem due to its global distribution, increased transmissibility, number of virulence genes and extensive antibiotic resistance and has been well characterised, especially compared with the other ExPEC sequence types (Nicolas-Chanoine, et al. 2014; Vila, et al. 2016).

The prevalence of ST131 infection increases with the age of the host and has been reported to be 25% or greater in elderly patients in USA and UK (Banerjee, Johnston, Lohse, Chattopadhyay, et al. 2013; Nicolas-Chanoine, et al. 2014; Ludden, et al. 2019). Previous use of antibiotics, healthcare-associated UTI and recurrent and persistent UTI and sepsis have also been associated with increased prevalence (Vila 2016).

ST131 *E. coli* emerged as a human lineage with a relatively low prevalence in non-human isolates (Johnson, Miller, et al. 2009; Banerjee and Johnson 2014) and was considered to be primarily a human rather than animal ST (Nicolas-Chanoine, et al. 2014). It is now recognised that ST131 strains have a broad host range. Infections in animals are presumed to have originated from human sources and ST131 has been isolated from a range of species and environments, including, companion animals and livestock, (Mora, et al. 2010; Platell, et al. 2010; McNally, et al. 2016; Riley 2020).

Diversity

ST131 population structure is divided into multiple lineages with clades A, B, and C dominant

(Stoesser, et al. 2016; Zakour, et al. 2016). The population subtypes are also designated according to their fimH marker alleles which correlate strongly with clades (Petty, et al. 2014). Thus, clade A is fimH41, clade B is fimH22 and clade C is fimH30 (Birgy, et al. 2019). Some authors include an additional fimH22 lineage as an important cause of community-acquired UTI (Liu, et al. 2018). Poultry, in common with humans, can be infected with this sublineage (Liu, et al. 2018).

Dates for adaptive evolution of the clades have been estimated using phylogenetics. These studies suggest that ST131 was detected in association with blood stream infections in the UK in 2003 (Kallonen, et al. 2017).

Clade A represents a small subset of ST131 isolates of serotype O16:H5 and is globally distributed, accounting for 1 to 5% of *E. coli* isolates overall (Johnson, et al. 2014). ST131 phylogeny reconstructed using SNP analysis shows this clade well separated from the other more prominent ST131 clades, including O25b:H4 (Banerjee and Johnson 2014). According to the Pasteur Institute (but not the Achtman) MLST schema the O16 and O25b clades are identified as representing distinct STs with the O16 clade designated ST506 (Matsumura, et al. 2012).

Clade B fimH22 isolates which are susceptible to cephalosporin and fluoroquinolone antibiotics gave rise in the 1980s to clade B_0 and a fluoroquinolone resistant Clade C with chromosomal alleles for the genes *gyrA* and *parC* (Zakour, et al. 2016). This clade has become established in poultry populations around the world (Liu, et al. 2018).

Clade C strains evolved from clade B, and then around 1990, C clade further split into two subclades identified as C1 (fimH30-R) and C2 (fimH30-Rx) (Johnson, et al. 2013; Stoesser, et al. 2016; Zakour, et al. 2016). A distinct cluster, C1-M27 within the C1 subclade, has now emerged in humans, firstly in 2006 in Japan, then rapidly spreading to other parts of Asia, North America, Australia and Europe (Matsumura, et al. 2016; Birgy, et al. 2019; Chen, et al. 2019; Melo, et al. 2019). The C2 subclade, also called ST131 H30-Rx, often carries $bla_{\text{CTX-M}}$ gene and was reported as the dominant clone in the United States and south west England in 2017 to 2018 (Critchley, et al. 2019; Findlay, et al. 2020)

Antibiotic resistance

ST131 is regarded as more resistant to most antimicrobials compared with other ExPEC associated

STs (especially ST73), based on carriage of a wide range of antibiotic-resistance genes (Kallonen, et al. 2017). This characterisation has arisen with the emergence of ST131 C1 and C2 clades as important causes of ExPEC infections with a rapid increase in prevalence of MDR clades. Successive reports document resistance to aminoglycosides, trimethoprim, amoxicillin/ clavulanic acid, gentamicin (Horner, et al. 2014) and fluoroquinolones (Platell, et al. 2011; Kidsley, et al.2020) in many of these ST131 clades.

The relatively constant proportion of these isolates over a 5 year period in companion animals compared with rising prevalence in human infections (Platell, Trott, et al. 2012; Kidsley, O'Dea, Ebrahimie, et al. 2020) has led authors to suggest that ST131 infections in companion animals represent repeated infections from humans (''spillover'') rather than these hosts constituting a reservoir of ExPEC (Kidsley, White, et al. 2020).

Isolation of the *bla*$_{\text{CTX-M-15}}$ positive B2-O25:H4-ST131 *E. coli* clone from dogs has been reported widely (Pomba, et al. 2009; Ewers, et al. 2010; Albrechtova, et al. 2012; Dierikx, et al. 2012; Harada, et al. 2012a; Nicolas-Chanoine, et al. 2014; Karkaba, et al. 2016). ST131 strains carrying other ESBL markers have been identified in faecal samples from dogs and cats in Australia (CTX-M-15, CTX-M-27)(Rusdi, et al. 2018) and clinical samples in China (CTX-M-15, CTX-M-123) (Liu, Liu, et al. 2016; Chen, et al. 2019), Japan (CTX-M-3, CTX-M-14, CTX-M-15, CTX-M-27) (Maeyama, et al. 2018), Switzerland (Zogg, Simmen, et al. 2018), USA (CMY-2) (LeCuyer, et al. 2018) and France (Dahmen, et al. 2013).

ST131 *E. coli* H30Rx carrying carbapenemase genes (*bla*OXA) have been isolated from dogs in Germany, the United States and China (Stolle et al., 2013; Liu et al., 2016). Plasmid-mediated quinolone-resistant (PMQR) genes (*aac(6')-Ib-cr*) have also been detected in clinical isolates from dogs in China, Portugal, Switzerland and Germany (Pomba, et al. 2009; Ewers, et al. 2010; Liu, Liu, et al. 2016; Zogg, Simmen, et al. 2018). PMQR genes can co-exist on the same plasmid with genes encoding ESBLs and be co-transferred to recipients. This has been reported in ST131 *E. coli* dog isolates which carried IncFII plasmids with a multidrug resistance region containing the *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1B}}$ *, blaOXA-1, aac(6)-Ib-cr*, and *qnrB2* genes on IncFII plasmids (Pomba, et al. 2009; Ewers, etal. 2010).

E. coli ST131 isolates producing CTX-M-9 have been recovered from poultry faeces (Cortés, et al. 2010), chicken meat and APEC cases (Mora, et al. 2010). Colistin (*mcr*-1-3) resistance has also been detected in ST131 strains, first as a poultry commensal in China (Liu, Wang, et al. 2016), then in European sourced chicken meat in Denmark (Hasman, et al. 2015) and in a septicaemic chicken in Germany (Ewers, et al. 2016).

However, the antibiotic resistance profile of ST131 isolates varies with clade membership. A survey of blood stream isolates found that clade A or B strains did not carry *bla*$_{CTX\text{-}M\text{-}15}$ but ESBL encoding

genes (*bla*$_{TEM\text{-}1}$*, blaOXA-1, bla*$_{CTX\text{-}M\text{-}15}$) were present in both C1 and C2 clades (Morris, et al. 2012; van der Bij and Pitout 2012; Kallonen, et al. 2017). Human isolates of sublineages B/B$_0$/C from a range of countries carried few antibiotic resistance genes; just those conferring resistance to beta-

lactams, sulfonamides and aminoglycosides (Liu, et al. 2018). Very few of the human clinical *fimH*22 isolates from a Canadian survey of UTI *E. coli* carried antibiotic resistance genes. Usually onlya single isolate carried genes conferring resistance to aminoglycosides, trimethoprim, sulfonamides, beta-lactams or macrolides (Fibke, et al. 2019). Another study which analysed genomes globally recorded most clade B strains did not carry allele combinations associated with fluoroquinolone resistance (Zakour, et al. 2016).

Clade B *fimH*22 chicken isolates also carried few antimicrobial resistance genes. ST131 APEC poultry isolates of B clade O25:H4 from Danish flocks were almost devoid of antibiotic resistance genes except for one isolate with *sul* (Poulsen, et al. 2020). Similarly, Clade B strains in another collection of poultry isolates carried few antibiotic resistance genes and lacked genes conferring ESC and FQ resistance (Zakour, et al. 2016). Less than 50% of chicken isolates carried genes conferring resistance to beta lactams, sulfonamides and aminoglycosides (Liu, et al. 2018).

Virulence

Even though ESC and fluoroquinolone resistance was associated with a large increase in the ST131 population globally it is proposed that the acquisition of virulence factors by ST131 was a necessary precursor (Zakour, et al. 2016). However, reports establishing heightened virulence in this ST are conflicting. ST131 isolates do have proven virulence, in that isolates are from clinical cases in humans. Experimental studies in mice have indicated both no greater virulence (Johnson, et al. 2012; Riley 2014) and enhanced virulence when compared with other *E. coli* (Vimont, et al. 2012). Some studies conclude that the virulence gene profile of ST 131 is only moderate, comparable to other group B2 ExPEC strains (Croxall, et al. 2011; Nicolas-Chanoine, et al. 2014; Pitout and Finn 2020). But others conclude that ST131 isolates have a significantly higher virulence scores than other non-ST131 isolates (Johnson, et al. 2010; Blanco, et al. 2011; Colpan, et al. 2013). Specific virulence genes have appeared to be more frequent in ST131 than in non-ST strains (Johnson, et al. 2010; Petty, et al. 2014). One study of United States isolates found three virulence factor genes (*iha, sat* and *iutA*)were

more prevalent among H30 than among nonH30 ST131 isolates (Banerjee, Robicsek, et al. 2013) and a United Kingdom study identified one gene, *esp*C as almost specific for ST131 isolates(Kallonen, et al. 2017) .

The prototype virulence factor profile for human ST131 isolates is given as *iha, fimH, sat, fyuA, irp2, iutA, iucD , kpsM II, usp, traT, ompT* and *malX* (Riley 2014). Other virulence genes, *papGIII, cdtB, sat,* and *kpsMII K5*, have been associated with human rather than poultry strains (Mora, et al. 2010).

ST131 isolates from clinical samples from dogs carry a range of virulence associated genes, including *celb, cnf1, gad, ireA, iroN, iss, mchB, mchC, mchF, mcmA, vat* (Valat, et al. 2020), *fimC, iha, mat, fyuA, irp2, iutA, iucD, sitA, kpsMII , ompA, traT, sat, malX, afa/draB , hra , pap, focG , cnf , hlyA , puvA, ironN , ibeA* (Platell, Cobbold, et al. 2011) and *papAHEF*, *yfcv, hlyA, fyuA, traT* (Zogg, Simmen, et al. 2018). Many of these virulence factors are also present in poultry ST131 strains (Johnson, Siek, et al. 2006; Mora, et al. 2010).

Spread

Spread of ESBL resistant ST131 clones, mainly by expansion of Clade C2 after 2000 (ST131 H30-Rx), has occurred globally (Price, et al. 2013). Prevalence has increased over time in most countries so that CTX-M variants have become dominant in most regions (Manges, et al. 2019). Even though there are multiple circulating subtypes of the major drug–resistant clade of ST131 (McNally, et al. 2016), the most widespread ESBL-producing ST131 sub-clone, H30-Rx is widespread in both hospital and community settings (Nicolas-Chanoine, et al. 2014; Stoesser, et al. 2016; Zakour, et al. 2016). The prevalence of specific *E. coli* ST131 subclones depends on whether the infection was detected in the community or hospital, geographic location, the age of the patient, and whether antibiotic screening was used during isolation (Nicolas-Chanoine, et al. 2014).

A combination of factors has been suggested to explain the successful spread of the key ST131 subclones, including increased transmissibility, greater ability to colonise and/or persist in the intestine or urinary tract, enhanced virulence associated with specific virulence genes and more-extensive antimicrobial resistance compared to other *E. coli.* (Banerjee and Johnson 2014; Kallonen, et al. 2017; Pitout and Finn 2020).

Spread of ST131 (Clades 1 and 2) within families has been reported, including between pets, between pets and humans and between family members (Johnson, et al. 2016b).

Human animal comparisons

Comparison of human and poultry O25b:H4-ST131-B2, producing CTX-M-9 strains based on virulence gene and PFGE profiles has shown some similarity as well as clustering together of isolates from the two sources (Cortés, et al. 2010; Mora, et al. 2010; Vincent, et al. 2010). Principal components analysis of ESBL/AmpC genes and associated plasmid replicons in ST131 isolates from poultry and humans showed minimal overlapping or clustering of strains from the two different sources (Dorado-García, et al. 2018). Core genome SNP-based phylogenetic analyses have shown that human clinical and chicken meat the ST131-H22 strains intermingled (Liu, et al. 2018; Saidenberg, et al. 2020).

Genetic similarity of *E. coli* ST131 isolates from humans and dogs has been shown by RAPD (Guo, et al. 2013), PFGE (Ewers, et al. 2010; Pomba, et al. 2014), and virulence factor profiles (Platell, Cobbold, et al. 2011). Core genome phylogenetic studies showed that apart from a clustering of cat and dog isolates in Clade A, companion animal ST131 isolates were distributed throughout the phylogenetic tree (McNally, et al. 2016).

Antimicrobial resistance

Infectious disease remains a world-wide burden and is increasing particularly in low and middle income countries and in susceptible populations (Jee, et al. 2018; Nellums, et al. 2018). The effectiveness of treatment for these infections, and the sustainability of health systems generally, are severely compromised by antibiotic resistance particularly given the limited development of new antimicrobial agents (Jee, et al. 2018). There is also increasing concern that the consequences of this increasing resistance will include adverse effects on the environment and food production, further increasing global poverty and reducing the prospects for sustainability.

Antibiotic therapy is the primary treatment for UTIs in both humans and companion animals and UTIs represents the second-most-common reason for antibiotic prescription worldwide (Foxman 2010). Globally the incidence of antibiotic resistant community acquired UTIs continues to increase (Yamaji, et al. 2018) associated with the acquisition of genes encoding resistance to a wider range of critical antibiotics (Manges, et al. 2019). HGT of antibiotic resistant gene loci is likely to occur in a wide range of microbial communities, including within the hospital as well as the gut (Lerminiaux and Cameron 2019; McInnes, et al. 2020). It is surmised that intense localized use of antimicrobial agents, for example in the general hospital environment and intensive animal production, selects for bacteria which can incorporate and express AMR genes. Others contend that the primary forces shaping populations of bacteria are due to competition within the gut commensal niche in the broader human population, where antibiotic use is more sporadic rather than in the hospital population (Kallonen, et al. 2017).

Aside from concern that antibiotic use promotes the development of resistance, antimicrobial treatment of UTIs in humans and animals risks the development of complications such as profoundly disrupting the microbial community of the vaginal cavity and gastrointestinal tract, vaginal yeast infections, and *Clostridium difficile* colitis (Foxman 2010; Flores-Mireles, et al. 2015b) resulting in prolonged morbidity.

Antimicrobial use in humans

Australia ranks as one of the highest antimicrobial users in human medicine. International surveys have found high usage rates for many classes of antimicrobials in Australia, but markedly lower rates for others (ACSQHC 2016). Treatment of urinary tract infection and sepsis are rated amongst the five most common indications for prescribing antimicrobials in Australian hospitals (ACSQHC 2018) and the second-most-common reason worldwide (Foxman 2010). The antimicrobial agents commonly

used to treat uncomplicated urinary tract infections include sulfamethoxazole, trimethoprim, β-lactams, fluoroquinolones, nitrofurantoin and fosfomycin. ExPEC resistance to these agents now occurs as well as to third generation cephalosporins, aminoglycosides, tetracyclines and FQs (Maslow, et al. 2004; Smith, et al. 2007). The resultant increased rates of treatment failure with standard antibiotic therapies has lead to increased use of second- and third-line therapies (given when initial treatment or first-line therapy doesn't work, or stops working). This then limits treatment options for ExPEC infections (Moriel, et al. 2016). The WHO has classified antimicrobials as important, highly important and critically important antibiotics (CIA)s. The last category includes 3rd, 4th and 5th generation cephalosporins (ESCs), polymixins, rifampicin and quinolones (WHO 2019).

Antimicrobial use in agriculture in Australia

Use of antimicrobials in Australia's agriculture sector is amongst the lowest in the world and Australia has been very conservative in registering antibiotics for use in food animals, particularlythose of importance to human health. There is a very limited number of antimicrobials that can be used in meat chickens and even fewer in layers (Gray, et al. 2021). Certain classes of antibiotics are not approved for use andwhen registering antibiotics, AMR potential is taken into account (Schipp 2017). According to industry, 3rd and 4th generation cephalosporins, polymyxins and quinolones have never been registered for use in chicken flocks in Australia (ACMF 2018).

Antimicrobial use in companion animals in Australia

Humans and companion animals share many of the same micro-organisms and antibiotics used in humans are also routinely used in companion animals, such as amoxicillin (with and without clavulanate), cephalosporins, doxycycline and trimethoprim /sulphonamide (Hardefeldt, et al. 2018; Shaban 2014). Antimicrobials commonly used to treat initial urinary tract infections in dogs and cats include amoxicillin, amoxicillin/clavulanic acid, cephalexin and trimethoprim/sulphonamides (Weese, et al. 2019; Barzelai and Whittem 2017).

There are specific Australian guidelines for the treatment of companion animals with antibiotics (Holloway 2013) available to veterinary practitioners. This guide provides recommendations for first line and subsequent antibiotics use in treating common infections in companion animals. Even with these guidelines surveys have shown CIAs are used in companion animal practice. A recent 4-year study of antibiotic usage found that fluoroquinolones, predominantly enrofloxacin, and 3rd generation cephalosporins, predominantly cefovecin, (Hardefeldt, et al. 2018) comprised 8% of all the antimicrobials prescribed. However, this level of

antimicrobial use in dogs and cats was less than half that used in humans over the survey period (Hardefeldt, et al. 2018).

A large study of UTI isolates from dogs and cats across 14 European countries over 16 years found a significant increase in MDR in *E. coli* isolated in southern Europe. This was attributed to the absence of relevant regulations and heightened surveillance operating in the northern European countries (Marques, et al. 2016).

Resistance to CIAs such as ESCs, carbapenems and fluoroquinolones can be found in ExPEC isolated from companion animals, (Ewers, et al. 2012; Guo, et al. 2013; Stolle, et al. 2013; Kidsley,O'Dea, Ebrahimie, et al. 2020). *E. coli* resistant to third generation cephalosporins have been isolated from healthy dogs (Costa, et al. 2008; Ljungquist, et al. 2016) and both cats anddogs with UTIs (Carattoli, et al. 2005; Huber, et al. 2013; Ewers, et al. 2014; LeCuyer, et al. 2018). Prevalence of ESBL producing *E. coli* in companion animals has increased in the last 20 years. These resistant bacteria have been reported in dogs in all continents and in cats in all continents, except Oceania (Salgado-Caxito, et al. 2021). This likely results from global variation in the use of CIAs use in companion animal veterinary practice (Hardefeldt, et al. 2018). Fluoroquinolone resistance has beenreported in *E. coli* isolated from healthy cats (Johnson, Miller, et al. 2009) and dogs (Costa, et al. 2008; Guo, et al. 2015) and clinical cases in dogs (Gottlieb, et al. 2008; Johnson, Kuskowski, et al. 2009; Platell, Cobbold, et al. 2012; Platell, Trott, et al. 2012; Guo, et al. 2015). Carbapenem resistant *E. coli* have been isolated from UTI infections in dogs and a cat (Shaheen, et al. 2013) and non UTI ExPEC infections in dogs (Stolle, et al. 2013).

Surveys of prevalence of antibiotic resistant *E. coli* isolates in faeces from healthy dogs and cats provide disparate results. *E. coli* resistance to ampicillin ranged from 7.7% to 85.7% in dogs and was 16.7% in cats (Costa, et al. 2008; Carvalho, et al. 2016b; Wedley, et al. 2017). Proportions of faecal *E.coli* resistant to tetracycline ranged from 17% to 50% for dog isolates (De Graef, et al. 2004; Costa, etal. 2008; Courtice, et al. 2016) and was 18.2% for cat isolates (Costa, et al. 2008). Higher levels of resistance to these antibiotics is attributed to the frequent use of these and related drugs in dogs and cats (van den Bogaard 2000; Wedley, et al. 2017). The two main methodologies used in these reports are the US based Clinical Laboratory Standards Institute (CLSI) standard and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These antimicrobial susceptibility testing (AST) methods determine minimum inhibitory concentrations (MICs) to determine whether an isolate is expected to be susceptible to an antibioticin a clinical situation.

The majority of these surveys derive resistance data for antibiotics including third generation cephalosporins, quinolones and carbapenams from clinical specimens or a mix of samples from clinical cases and healthy animals plated onto media containing the antibiotics of interest (Carvalho, et al. 2016b). The significance of these surveys in relation to the hazard associated with transfer of resistant strains from companion animals to people is uncertain. This is because selective plating detects strains that may represent less than 1% of the total *E. coli* population (Gordon, et al. 2002). Thus the role of ExPEC isolates from companion animals with respect to the dissemination of antibiotic resistance and virulence in natural environments is currently not well understood (Jang, et al. 2017).

ExPEC epidemiology

ExPEC epidemiology in humans

In humans UTIs are generally considered sporadic illnesses within the community with E. coli regarded as an opportunistic pathogen (Singer 2015). Strains of E. coli often associated with urinary tract infections are commonly isolated from the faeces of healthy humans. WGS has been used to provide strong evidence that faecal and UTI isolates are closely related and can only be distinguished, if at all, by their accessory genome (Nielsen, et al. 2017). That is, the source of a person's UTI is usually the person's gut. Similarly, in dogs the intestinal (rectal) or faecal *E. coli* appear to be nearly identical to the *E. coli* isolated from the urinary tract (Low, et al. 1988; Johnson, Kaster, et al. 2003).

Host risk factors for UTI in humans, include being female (shorter urethral length), anatomical abnormalities of the urinary tract, a pre-existing diagnosis of diabetes mellitus and the presence of an indwelling urinary catheter. Host risk factors for increasing severity of infection include age over 65 years and the presence of medical co-morbidity. Host risk factors for ExPEC bacteraemia secondary to UTI include respiratory tract and gastro-intestinal infections (Dale and Woodford 2015).

Genomic epidemiology has been used to assess other potential ExPEC transmission routes and the source and likely spread of infection in clinical settings (Pightling, et al. 2018). These techniques have provided evidence linking urine and blood isolates (mainly ST131) from participants at health care institutions supporting probable transmission within and between facilities (Salipante, et al. 2015; Brodrick, et al. 2017). An apparently identical pair of isolate genomes were recovered from cases over 1 year apart. With no evidence of an epidemiological link this suggests retention of the clone in a reservoir located in the facility (Salipante, et al. 2015).

Zoonotic ExPEC significance in companion animals

Australia has one of the highest rates of pet ownership in the world. Cats and dogs probably represent the two most popular companion animals; with dogs the most common (39% households) and cats the second most common pet (29% of households). There are estimated to be 3.3 million pet cats in Australia. South Australia has highest household ownership ofdogs (48%) and Victoria/Tasmania the highest household ownership of cats 36%. A 2013 report found that Victoria/Tasmania had 1.3 million dogs and 1.2 million cats (Richmond 2013). This level of ownership provides abundant opportunity for transfer of both bacteria and antibiotic resistance genes between human and animal hosts.

As detailed above, human associated ExPEC sequence types have been isolated from healthy and diseased companion animals, especially ST131 (Johnson, Miller, et al. 2009; Pomba, et al. 2009; Platell, Cobbold, et al. 2011; Guo, et al. 2013). The few reports of the other STs include ST69 in dogs, ST73 in cats and dogs, ST127 in dogs and ST95 isolate in a dog (Johnson, Johnston, et al. 2008; Ewers, et al. 2012; Tamang, et al. 2012; Liu, et al. 2015). Even though the immediate source of human ExPEC infection is usually the infected person's gut (Johnson, Kaster, et al. 2003) and other humans (Foxman, et al. 1997), there is concern that dogs andcats may be a source of strains of *E. coli* capable of causing ExPEC infections in humans, especially in relation to the continuing development of antibacterial resistance in *E.coli* strains (Abraham, et al. 2014). Implicated sources are companion animal faeces (Johnson, Stell and Delavari 2001) and clinical cases, especially UTIs (Johnson, Delavari, Stell, et al. 2001; Johnson, Stell, Delavari, et al. 2001; Freitag, et al. 2005).

Companion animals are assumed to increase the exposure of humans to bacterial species typically encountered in the environment; that is, air, water or soil-borne microbes, with their habits and movement between indoors and outdoors. Sharing of *E. coli* isolates is biologically plausible given the close physical contact between household pets and humans and transmission of other pathogenic microorganisms from dogs and cats to humans has been documented (Elliot, et al. 1985).

Evidence has been presented to show sharing of *E. coli* strains may occur within households between humans and pets. This included sharing of strains associated with urinary infections with other members of the household (Johnson and Clabots 2006; Johnson, Owens, et al. 2008; Damborg, et al. 2009b). Characteristics used to demonstrate that *E. coli* strains from different hosts were identical included common phylogroup representation, sets of virulence genes, sequence types, AMR and PFGE profiles (Caugant, et al. 1984; Johnson, et al. 2000; Guardabassi, et al. 2004). However, in these studies evidence presented derives from very low numbers of isolates, which are selected based on the antibiotic susceptibility of the UTI strain using low-resolution methods. More recently genome analysis has been used to demonstrate a close relatedness of ExPEC strains isolated from both the family dog and it's owners (Reeves, et al. 2011; Damborg, et al. 2018). These studies provides a more accurate comparison and indicate strain sharing can occur but does not prove cross-species transmission or exclude the possibility that humans and animals acquire the same *E. coli* types from common external sources (Singer 2015).

Zoonotic ExPEC significance in food producing animals

Similarly, ExPEC colonization and infections in food producing animals gives rise to fears that a food-borne reservoir of human ExPEC exists (Manges and Johnson 2012). Extraintestinal infections with *E. coli* in these animals include UTIs, newborn meningitis, polyserositis, septicaemia and mastitis (Dezfulian, et al. 2003). This concern has largely focussed on poultry and poultry products (Manges, et al. 2015).

The identification of overlapping characteristics such as serogroups, virulence genotypes and phylogenetic group assignments in some strains of APEC and UPEC has led to concern that APEC contaminated poultry can be a source of ExPEC infection in humans (Mora, et al. 2013; Maluta, et al. 2014b). The detection of antibiotic resistant ExPEC strains in poultry has heightened this concern (Bélanger, et al. 2011). However, the evidence supporting sharing and hence the role of food transmission in the dissemination of the *E. coli* strains that cause community acquired UTIs is questioned by some authors (Giufrè, et al. 2010; Singer 2015).

A study comparing many human and food animal isolate sequences concluded that human ExPEC infections were not derived recently from livestock, and that host-specific *E. coli* lineages were partitioned according to source, that is hospitals and farms. Using long-read sequencing and analysis of mobile genetic elements, this group also failed to find strong evidence of sharing of AMR genes between livestock and humans (Ludden, et al. 2019). Overall, the evidence supporting pork/pigs, and beef/cattle as sources of ExPEC for humans is weak (Manges and Johnson 2015).

Host specificity

Defining the level of host adaptation or host specificity is important in any evaluation of the zoonotic significance of dogs, cats and poultry *E. coli* isolates. Host specificity in pathogen terms, means infection is restricted to a particular host (e.g., humans) or defined host group (e.g., mammals) (Pan, et al. 2014). Specialists evolve in homogeneous environments that remain relatively constant and generalists evolve in heterogeneous variable environments (Kassen 2002). A clear example of specialisation by *E. coli* is strains specifically adapted to waste water environments (Touchon, et al. 2020). Populations in the faeces of animals are described as a mixture of host-specialists and host-generalists (Bäumler and Fang 2013).

Understanding the genetic basis of host specificity in pathogenic bacteria can increase understanding of pathogenic mechanisms, especially in relation to potential impact on human health. Interspecies transmission can often be traced to modification of key microbial factors that facilitate the formation of particular host associations. Examples of this specificity have been documented but the genetic

mechanisms governing it are poorly characterized. Genome-level studies have the potential to enhance understanding of the effect of these changes on host specificity (Reeves, et al. 2011).

Host-specific adaptation has been attributed to changes in regions or domains of specific genes, so-called biomarkers, and is determined by multiple molecular interactions between the pathogen and host (Pan, et al. 2014). Identification of host-specific genetic determinants in microbial populations can be a challenge requiring complicated analyses as adaptation to a specific host can involve a number of genes as well as their regulatory genes. Host specific genetic determinants can be encoded in a group of genes (gene repertoire, region, domains or set of particular genes), a specific gene, or SNPs within a single gene. Differences in nucleotide sequence in groups of genes encoding allelic variants of *fimH* adhesins of *Salmonella enterica* Typhimurium serovars were correlated with host specificities, for example pathogens in sheep and pigs (Yue, et al. 2015). Host specificity of *Yersinia* species can be attributed to gene loss (Reuter, et al. 2014). Conversely, the addition of a single gene (*rscS*) converted a strain of *Vibrio fischeri,* a specialist isolated from fish, to a generalist capable of infecting squid (Rotman, et al. 2019). Examples where SNPs have been shown to alter the host-specific nature of bacteria have been described in *Salmonella enterica* serovars, Typhi, Paratyphi A and Sendai (Tracz et al., 2006; Eswarappa et al., 2008). Beyond changes in gene presence or absence and in the single nucleotide, it is surmisedthat finer-scale changes such as methylation, may also influence host specificity (Kirzinger and Stavrinides 2012).

E. coli as a species has a broad host range infecting many animal species and various environmental niches, such as water bodies, septic tanks, foodstuffs (Zhi, et al. 2016). There are, however, very few examples of host-specific *E. coli* pathogens; *Shigella* species, which only infect humans and closely related primates (Hershberg, et al. 2007) and arabbit specific enteropathogenic *E. coli* (strain RDEC-1) (Berendson, et al. 1983).

Several studies have shown the effect the type of host has on the representation of *E. coli* phylogroups. But findings vary. For example, in one study of a large collection of 2,300 ~~of~~ *E. coli* isolates from native Australian animals, representatives of phylogroup B1 were prevalent in fish, reptiles, birds and marsupial carnivores and representatives of phylogroup B2 were more prevalent in native Australian marsupial hosts with a hindgut fermentation chamber. Phylogroup D strains were isolated more frequently from birds and native Australian mammals than from other vertebrates (Gordon and Cowling 2003). This is consistent with another survey of *E. coli* isolates in European and South American birds which were also predominantly B1/D. Non-human mammals carried mainly phylogroups A and B1 whereas B2 was more common in humans (Escobar-Páramo, et al. 2006; Carlos, et al. 2010). However, a survey of *E. coli* in faeces from humans and animals in and around a remote South American village found B2 representation in humans was low but high in nearby wild animals (Lescat, et al. 2013). Another survey found *E. coli* isolated from cattle were predominantly phylogroup B1, but *E. coli* isolated from water buffalo were mainly phylogroups B2 and D (Morcatti Coura, et al. 2015). A study found wild porcine, ovine and caprine derived isolates were more likely to be phylogroup B2 or D compared with isolates from domesticated counterparts (Escobar-Páramo, et al. 2006). Phylogroups A and B1 have been regarded as generalists and can be isolated from all vertebrates andoften secondary habitats, eg. water bodies (Touchon, et al. 2009). Phylogroups B2 and D are more likely to be specialists or host adapted (Gordon and Cowling 2003; White, et al. 2011).

Within the B2 phylogroup there is some evidence of host adaptation/specificity. Based on a comparison of promoter sequences controlling production of curli fimbriae, flagella, and nutrient import, B2 isolates were shown to be more likely to be host adapted than isolates in the other major phylogroups (White, et al. 2011). As example, a phylogroup B2 *E. coli* clone was found to be widely distributed in humans but could not be recovered from animal samples and is considered human specific (Clermont, Lescat, et al. 2008).

Efforts directed at demonstrating *E. coli* host specificity have been driven largely by the need to determine potential sources of faecal bacteria found in surface water to aid in monitoring water quality and source attribution after outbreaks of human illness from foodborne microbiological hazards. This practice derives from the hypothesis that specific *E. coli* strains are more prevalent in, or specific to, particular hosts. Evidence supporting this hypothesis, however, is limited (Johnson, Brown, et al. 2004). To demonstrate host specificity a host origin database must be developed to differentiate among isolates, ideally drawing on large numbers of isolates collected from a wide geographic area (EFSA 2008).

A range of typing techniques are used to develop host specific databases and have included the use of phenotypic and genotypic library-based methods to detect patterns unique to the *E. coli* of the targeted host species.

Typing methods.

The aims of typing isolates can be several. Typing using biochemical methods and serotyping is used to identify suspect pathogens in clinical samples, as a basis for classification and during epidemiological investigations to link outbreaks of disease or food poisoning to a common causative agent or clone. Traditionally these methods relied on phenotypic attributes of isolates but currently most methods are DNA and WGS based. Typing is also important as a means of determining *E. coli* population substructure and constructing the evolutionary history and hence clonal relatedness of various isolates (Van Belkum, et al. 2007). Initial identification and basic classification is still prerequisite prior to use of these newer molecular methods. There is a plethora of typing methods available so only those related to or used in this study are described here.

Phenotypic methods

Typical phenotypic methods used to identify *E. coli* strains of interest as first level testing include Gram staining, morphological characteristics, antibiogram-based typing, biochemical characterisation, bacteriophage and plasmid typing, serotyping and more recently mass spectroscopy (MALDI-TOF) (Van Belkum, et al. 2007). Biochemical characterisation or biochemical profiles differentiate isolates based on enzyme activity. Serotyping, plasmid analysis, bacteriocin production and multilocus enzyme electrophoresis provide additional levels of discrimination.

Phenotypic library-based methods include serotyping, antibiotic resistance profiles, and growth patterns of isolates on substrates (Souza, et al. 1999; Hagedorn, et al. 2003; Escobar-Páramo, et al.

2006).

E. coli have traditionally been serotyped using antisera against three antigens: somatic (O), capsular (K) and flagellar (H) as certain serotype combinations were associated with disease. Even though more than 170 distinct O antigens and 53 H-flagellar antigens have been identified, only a small number of these serotypes are associated with disease. There was early recognition that only specific O types e.g., O4, O6 and O18, were associated with ExPEC infections in humans (Russo and Johnson 2000). ExPEC strains often possess group antigens O4 and O6 and are frequently isolated from normal faecal flora of in dogs and cats (Beutin 1999). Serotyping using antisera panels is very complex, generally time consuming and not always accurate (Fratamico, et al. 2016).

In summary, the discriminatory power of phenotypic methods using low-resolution typing methods is limited, due to low sensitivity at the intraspecies level, and limited specificity as phenotypic characteristics can be unstable.

Genotypic methods

Genetic-based subtyping methods include polymerase chain reaction (PCR), repetitive extra-genic palindromic PCR (rep-PCR), multi-locus sequence typing (MLST) and genome analysis following WGS. PCR uses primer sequences of DNA to detect and amplify specific genes or groups of genes and is used to assign strains to particular phylogroups. The original multiplex PCR protocol assigned ExPEC isolates to one of four phylogroups of (A, B1, B2, and D) (Clermont, et al. 2000). The accuracy of this method was improved and at present there are nine recognized phylogroups of *E. coli*. Phylogroups A, B1, B2, C, D, E, F, G belong to *E. coli sensu stricto* and one corresponding to *Escherichia* clade I (Clermont, et al. 2013; Clermont, et al. 2019). Of these, four (A, B1, B2 and D) represent most of the *E. coli* strains (Clermont, et al. 2021).

Rep-PCR is a type of PCR which uses oligonucelotide primers complementary to specific repetitive non-coding sequences interspersed between coding sequences throughout the genome of *E. coli* to amplify the regions of DNA flanked by these repeated sequences (Versalovic, et al. 1991). This yields an amplicon pattern reasonably specific for an individual *E. coli* strain. There are four main types of repetitive sequences named Repetitive Extragenic Palindromic (REP), Enterobacterial Repetitive Intergenic Consensus (ERIC), the BOX sequences and the polytrinucleotide $(GTG)_5$ sequences (Versalovic, et al. 1994; Mohapatra, et al. 2007).

Genotypic library-based methods which identify patterns in the genetic material of *E. coli* isolates include PCR detection of virulence associated genes and rep-PCR (Stoeckel, et al. 2004). Data derived from these methods, have been analysed to attribute host-specificity to strains. These statistical methods include discriminant analysis and logistic regression (Carlos et al., 2010).

Phylogroups show considerable genetic heterogeneity, and multilocus sequence typing (MLST) can be used to further characterize sub-groups as sequence types (ST). There are different MLST databases for *E. coli* using different loci. The largest scheme (Achtman database available at https://cge.cbs.dtu.dk/services/MLST/#ref04) uses the seven housekeeping loci *adk*, *fumC*, *gyrB*, *icdA*, *mdh*, *purA*, and *recA* representing over 1000 STs (Wirth, et al. 2006; Gordon 2010). The alternative, lesser used Pasteur MLST scheme uses eight different housekeeping genes (*dinB, icdA, pabB, polB, putP, trpA, trpB and uidA*) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html). The different nucleotide sequences of each locus are assigned different allele numbers. Each strain is then assigned an allelic profile based on the alleles of the seven loci. Groups of STs related by descent have been grouped

into clonal complexes (CC), (Riley 2014) and members of a clonal complex can share similar ecological traits (Gordon 2010). MLST can be used to determine the population structure of a collection of *E. coli* isolates; those with similar or identical MLST genotypes are considered to be closely related.

However, there is still a high level of diversity in the pathogenic potential of strains of the same sequence type. As example, both CFT073 associated with UTI and the probiotic strain Nissle1917 are ST73. Even though MLST has been the usual recent means of identifying ExPEC associated lineages (Manges, et al. 2019), partial-or whole-genome sequencing is increasingly used to reveal the substructure within sequence types. A study of a large number of strains found those within a sequence type had smaller intra-sequence type genetic distances compared to inter-sequence type genetic distances but also had extensive genetic diversity at the genome level (Touchon, et al. 2020).

WGS together with a multitude of sequence analysis tools provide a higher resolving power than the above subtyping methods for comparing isolates (Clermont, et al. 2015; Fratamico, et al. 2016). Analysis of SNPs of the core genome and differences in gene content of the variable genome allows source attribution and evaluation of relatedness.

Non library-based genetic methods (DNA sequence based) detect genetic markers or unique allelic profiles assumed to be host specific. These methods operate at the population rather than the isolate level requiring a large number of markers to be screened simultaneously (Hamilton, et al. 2006; Fu and Li 2014). Methods include the detection of host-specific PCR, and DNA specific markers (Griffith, et al. 2003; Zhi, et al. 2015), MLST and SNP analysis (Hommais, et al. 2005; Le Gall, et al. 2007) or through phylogenetic analysis (Clermont et al., 2011; Ivanetich et al., 2006).

Machine learning models, supervised and unsupervised, have been used to investigate host specificity in *E. coli* (Lupolova 2019). Supervised learning logic-regression-based analysis of DNA sequence variability in intergenic regions (ITGR) has been used to identify single nucleotide polymorphism (SNP) biomarker patterns correlated with host origin. A unique ITGR biomarker pattern gave 98% and 99% specificity respectively to human and deer *E. coli* isolates (Zhi, et al. 2016).

Research Aims

E. coli in companion animals has been well studied, but these studies have mainly focussed on the isolation and characterisation of antibiotic resistant strains. Given the same classes of antimicrobials are used in human and veterinary medicine, there is general concern about antimicrobial use and the development of antimicrobial resistance in *E. coli* carried by companion animal (Guardabassi, et al. 2004; Lloyd 2007;Costa, et al. 2008; Shaban, et al. 2014). Transfer of resistance may further limit treatment options for ExPEC infections in humans and pets. Implied ExPEC sharing and transfer of antibiotic resistance from companion animals to humans has relied largely on reports of detection of antibiotic resistant *E. coli* in faeces from healthy animals and ExPEC infections in dogs and cats. Similarities in the virulence factor profiles of resistant strains from animals have been interpreted as evidence that resistance develops in animals because of antibiotic use and is then transferred to people (Pomba, et al. 2017).

No studies to the best of my knowledge, to date, have compared *E. coli* isolated from companion animal faecal samples with faecal and clinical samples from humans in an effort to determine the level of host preference or adaptation. The aim of this research project is to determine the prevalence of human associated ExPECphylogroups and sequence types in faecal samples from over 200 dogs and cats in the Canberra, Australian Capital Territory, Australia region. The initial plan was to characterise isolates (phenotype and genotype) and explore any association between isolates and host characteristics, such as size, location, sex, neuter status and age in dogs and cats. However, sample collection proved more difficult than anticipated and complete host information was often not available.

Having isolated and characterised strains in dogs and cats which were significant human ExPEC strains, REP typing was used to select a set of unique types for WGS. This study used sequence data for *in silico* characterization, including MLST, serotyping, presence of virulence associated and antimicrobial resistance genes, metabolic pathways, plasmid, bacteriophage and bacteriocin content as well as the genetic structure and diversity of isolates. Early work (Chapter 2) indicated that sequence types ST69, ST73, ST95, ST117 and ST131 were represented to a greater extent in cats compared to dogs, notably ST73, so subsequent WGS investigation only included cats strains. Sequence data of these sequence types from cats was compared with sequence data from poultry meat isolates and commensal and clinical *E. coli* isolates from people living in the Canberra region.

These human strains had been isolated and characterised previously by other researchers in the Gordon Group (https://biology.anu.edu.au/research/research-groups/honorary-groups/gordon-group-population-biology-micro-organisms). The research aim has been to characterise and compare isolates and their derived genomes with those from humans and chickens to investigate relatedness and any host associations. This study can then add to the assessment of the potential for sharing of strains between these hosts.

Sequence data of ST73 strains, isolated at high prevalence from cat faeces, will be compared in detail with ST73 commensal and clinical strains previously isolated from humans in the Canberra region. The role of different attributes in ExPEC that contribute to fitness in the human colon and in other reservoirs is still not fully understood (Manges, et al. 2019). This comparison study will be carried out to identify any differences in characteristics which would indicate an increase in the fitness and representation in the large intestines of human and cats. In addition, this study will seek to identify characteristics which contribute to colonisation of the gastrointestinal tract which are differentially expressedby cat and human strains. Apart from the globally distributed clones of ST131, most WGS-based studies have focused primarily on ST131. We still know little about the different genomic determinants which maintain the few clinically prevalent STs as successful pathogens and the mechanisms of clonal dissemination. Specifically, the roles of antimicrobial resistance, virulence factors, metabolism, regulators, transporters etc. which allow human and animal strains to become pathogens. This comparison study aims to use high-resolution typing methods to discriminate cat and human strains, identifying any evidence of host preference or specificity, and hence provide further evidence to support or refutethe hypothesis that transmission of *E. coli* strains occurs between the two hosts.

This first chapter provides an overview of relevant literature. Chapter 2 largely presents data and analysis drawn from a paper published part way through my research. The findings here have directed subsequent research described in the following chapters. The prevalence of important human and poultry associated sequence types in samples obtained from cats supported the decision to restrict subsequent detailed genetic and phenotypic studies to cat strains, excluding dog strains. Thus Chapter 3 describes a detailed study of strains of ST69, 95 117 and 131.This largely involved comparison of cat strains of sequence types ST69, ST95, ST117 and ST131 with corresponding human and poultry meat strains characterised and sequenced by other worker from the Gordon group. Chapter 4 compares cat and human ST73 strains. Discussion of results presented within a context of current literature is presented in Chapter 5. Methods and reagents used to obtain samples, data and analysis are given in Chapter 6.

Chapter 2 A study of *Escherichia coli* in Dogs and Cats

This chapter is a shortened version of work previously published (Bourne et al 2019). It describes and characterises strains isolated from samples collected up to the time of submission for publication of the paper. Experimental methods have been deleted to avoid duplication within the thesis.

Summary

Extraintestinal pathogenic *Escherichia coli* (ExPEC) cause clinical infections in humans. Understanding the evolution and dissemination of ExPEC strains via potential reservoirs is importantdue to associated morbidity, health care costs and mortality. This survey has examined isolates recovered from the faeces of 221 healthy dogs and 427 healthy cats. The distribution of phylogroupsvaried with host species, and depended on if the animal was living in a shelter or a home. The human associated STs 69, 73, 95, 131 and 127 were prevalent, with 30.5% of cat isolates and 10.3% of dog isolates representing these ExPEC sequence types. Resistance to the antibiotics ampicillin and tetracycline was common, but resistance to other antimicrobials was negligible.

Introduction

Escherichia coli is a common member of the microbiota of the lower intestine of mammals and to a lesser extent birds (Gordon and Cowling, 2003). The species is genetically diverse and exhibits considerable genetic substructure (Tenaillon et al., 2010). The species has been partitioned into eight phylogroups (Clermont et al., 2013). *E. coli* isolates are most likely to belong to phylogroups A, B1, B2 and D, while strains belonging to phylogroup C, E, F and Clade I are less frequently observed (Clermont et al., 2013). Strains belonging to the various phylogroups differ in their genome size, variable gene content, ecological niche, and life history characteristics (Gordon, 2004).

Although most strains of *E. coli* behave as commensals of the lower intestine of vertebrates, some can cause intestinal and extra-intestinal disease. Strains responsible for intestinal disease have diverse phylogenetic origins (Croxen et al., 2013). However, most strains responsible for extra-intestinal diseases such as urinary tract infections (UTIs) or septicaemia are members of phylogroup B2 and to a lesser extent phylogroup D (Picard et al., 1999; Johnson et al., 2001). The technique of multi-locus sequence typing (MLST) (Maiden et al., 1998) has revealed that there are hundreds, if not thousands, of sequence types (STs) within phylogroups B2 and D. However, despite this diversity, a very small fraction of STs, are responsible for the great majority of extra-intestinal infections. These are phylogroup B2 STs, ST73, ST95, and ST131 and the phylogroup D ST, ST69 (Doumith et al., 2015), which are largely human associated. At least one (ST 95) is rarely isolated from livestock, wild birds or mammals (Gordon et al., 2017). The frequency with which these common human associated STs are observed in companion animals is unclear.

Extra-intestinal infections also occur in dogs and cats, primarily as uncomplicated UTIs. As in humans, *E. coli* is the most common cause of such infections in dogs (Thompson et al., 2011). Urinary tract infection is relatively uncommon in cats (Litster et al., 2011), but when it does occur, *E. coli* is also the most common cause. As in humans, isolates belonging to phylogroup B2 and D are more likely to be recovered from extra-intestinal infections of companion animals (Clermont et al., 2011; Hutton et al., 2018). The four STs commonly responsible for extra-intestinal infection in humans (STs 69, 73, 95 and 131) have been isolated from healthy or diseased companion animals. ST131 is commonly reported in dogs and cats (Ewers et al., 2010; Platell et al., 2011; Guo et al., 2013) but there are few reports of the other STs: ST69 in dogs, ST73 in cats and dogs, ST127 in dogs and a single ST95 isolate in a dog (Johnson et al., 2008c; Ewers et al., 2012; Tamang et al., 2012; Liu et al., 2015). What studies have been undertaken have largely been based on clinical isolates exhibiting

resistance to particular antimicrobials, such as fluoroquinolones (Ewers et al., 2010; Platell et al., 2010; Guo et al., 2013).

Although the human associated STs 69, 73, 95 and 131 have been observed in companion animals, there is relatively little data with which to assess the extent to which companion animals represent a zoonotic reservoir of these human associated STs. Family members, including the family pet, have been shown to be more likely to share *E. coli* strains than individuals not living in the same household (Johnson et al., 2008b). While, in one study, an ST73 strain was found to be shared by family members, including the dog, and persisted in the family for more than three years (Reeves et al., 2011).

The aim of the present study was to assess the genetic structure, diversity, as well as the frequency of the human associated STs of *E. coli* in the faeces of dogs and cats living in the Canberra region of Australia, and to determine the antibiotic sensitivity of the isolates recovered.

Results

E. coli diversity

E. coli was detected in 334 (78.2%) of 427 cats and in 203 (91.9%) of 221 dogs. Thus *E. coli* was significantly less likely to be detected in cats as compared to dogs (Contingency Table Analysis: $P>X^2$ <0.001). The relative abundance of the phylogroups varied with host type (cat or dog) and differed dependingon if the animal was a pet or residing in an animal shelter (Nominal Logistic Regression: Cat/Dog, $P>X^2 < 0.01$; Pet/Shelter $P>X^2 <$ 0.001: Cat/Dog*Pet/Shelter $P>X^2 < 0.82$) (Table 1). There was little difference in the relative abundance of phylogroup B1, D, E or F strains in cats compared to dogs, butphylogroup B2 strains were overrepresented in cats, while phylogroup A were overrepresented in dogs (Table 1). Contrasting cats and dogs living in an animal shelter with those living in a home, reveals that individuals living in a shelter have lower frequency of phylogroup A strains and an increased frequency of B1 strains compared to animals not living in a shelter (Table 1). The relative abundance of phylogroups B2, D, E and F did not differ between animals living in a home versus those residing in a shelter.

For cats residing in an animal shelter the relative abundance of the phylogroups did not vary with the sex of the animal (Contingency Table Analysis: $P>X^2 < 0.16$)(data not presented). For dogs, the relative abundance of the phylogroups did not differ between the sexes (Nominal Logistic Regression: Sex, $P>X^2 < 0.051$; Pet/Shelter $P>X^2 < 0.12$) (data not presented).

Of the 334 isolates from cats, 30.5% represented one of the human associated STs (STs 69, 73, 95, 127, 131), a significantly greater frequency of human associated STs than was observed in dogs, as only 10.3% of the 203 isolates from dogs represented these STs (Contingency Table Analysis: $P>X^2 <$ 0.0001).

If either a cat or dog harboured a phylogroup D strain, then the likelihood that the strain was an ST69 strain did not depend on the host from which the isolate was recovered (Contingency Table Analysis; $P>X^2 = 0.17$)(Table 2). Similarly, if either a cat or dog harboured a phylogroup B2 strain, then the likelihood that phylogroup B2 strain was an ST95, ST127 or ST131 strain did not depend on the host from which the isolate was recovered (Contingency Table Analysis; ST95, $P>X^2 = 0.09$; ST127, $P>X^2 =$ 0.19; ST131, $P>X^2 = 0.15$)(Table 2). However, a phylogroup B2 strain was significantly more likely to be an ST73 strain if it was recovered from a cat rather than isolated from a dog (Contingency Table Analysis; $P>X^2 < 0.0001$)(Table 2).

Overall genotype (REP-type) diversity was high, indicating that most animals hosted a different genotype (Table 3). There were two exceptions to this pattern, phylogroup B2 and F isolates from cats. The lower levels of diversity observed among the B2 isolates was largely due to the high frequency of ST73 strains, while a single genotype represented 70% of phylogroup F isolates detected.

Antimicrobial sensitivity

Excepting ampicillin and tetracycline, most isolates were susceptible to all of the antibiotics tested (Table 4). Among cats and dogs with a home, 62.5% of 64 isolates from cats were susceptible to both ampicillin and tetracycline, while 70.5% of 173 dog isolates were susceptible and there was no difference in the ampicillin/tetracycline sensitivity profiles of isolates from cats or dogs with a home (Contingency Table Analysis: $P>X^2 = 0.30$), and resistance to both ampicillin/tetracycline was uncommon (3.4 %). Among the dogs and cats residing in an animal shelter 65.2 % of 270 isolates from cats were sensitive to both ampicillin and tetracycline, while 83.3 % of 30 dog isolates were susceptible. There was a significant difference in the ampicillin/tetracycline sensitivity profiles of isolates residing in a shelter (Contingency Table Analysis: $P>X^2 = 0.034$), with dog isolates more likely to be sensitive to both antibiotics, and while 10% of isolates from cats were resistant to both ampicillin and tetracycline, none of the dog isolates were resistant to both antibiotics.

The frequency of isolates susceptible to both ampicillin and tetracycline declined significantly with the number of days the cat spent in the animal shelter, while the frequency of isolates resistant to both antibiotics increased (Nominal Logistic Regression: $P>X^2 = 0.004$)(Fig. 1). There was a modest increase in the frequency of isolates resistant to just tetracycline (Fig. 1).

Discussion

Few other studies have examined the relative abundance of the *E. coli* phylogroups in the faeces of healthy dogs and cats, and comparison of results here with a few other studies reveals little concordance (Table 5). The relative abundance of phylogroups in human faecal samples has been shown to vary with geographic locality (Escobar-Páramo et al., 2004). However, in samples taken from humans living in temperate climates/developed countries, isolates belonging to phylogroup B2 are most common, while phylogroup A or B1 strains dominate in samples from people living in tropical climates/developing countries (Escobar-Páramo et al., 2004; Blyton et al., 2014). All of the studies investigating the genetic structure of *E. coli* in healthy dogs were in temperate climate/developed country locations. While, typical dog food diets may vary between Japan and the other countries, it seems unlikely that that there are large differences among the diets fed to dogs in Australia, Canada and the United Kingdom. The present study has shown differences in phylogroup structure depending on if the dogs were household pets or residing in an animal shelter. The dogs sampled in the Canadian and UK studies were household pets. In healthy cats living in Toronto, Canada, faecal *E.coli* phylogroup representation was 76.2%, 14.3%, 9.5% and 21% respectively for A, B1, B2 and D phylogroups (White et al., 2011). Phylogroup representation of *E. coli* isolated from rectal swabs of healthy cats in Iran (n=90) of groups A, B1, B2 and D was 66.7%, 1.2%, 13.4% and 18.9% (Akhtardanesh et al., 2016). By contrast, the frequency of phylogroup B1 and B2 strains in Australian cats was much higher (Table 1). Further studies are required if we are to understand the differences in phylogroup structure of *E. coli* populations isolated from a single host species sampled from different localities.

REP typing revealed a high diversity amongst dominant isolates. Overall there were 185 unique DNA fingerprints among 334 cat isolates, and 158 unique types among the 201 dog isolates. This result is comparable with that reported by Johnson et al. (2004) with 48 unique types among 108 isolates from 37 cats, and 106 types among 196 isolates from 71 dogs.

The human associated STs represent about a third of all *E. coli* isolates recovered from human faecal samples in the Canberra region (Gordon et al., 2017) (Table 6). Dogs are as likely as people to carry ST127 strains, but substantially less likely to carry strains belonging to the other human associated STs (Table 6). By contrast, while cats are less likely to harbour ST95, ST131 or ST69strains, they are as likely to harbour ST127 strains and are almost twice as likely to carry ST73 strains compared to carriage of these STs in humans (Table 6).

All of the human associated STs have been isolated from healthy or diseased dogs, but the great majority of the previous studies focused on clinical isolates, used antibiotic selection (especially fluoroquinolones), or both. ST131 is the most common ST reported in dogs and cats (Johnson et al., 2009; Platell et al., 2011; Guo et al., 2013) but there are a few reports of the other STs: ST69 in dogs, ST73 in cats and dogs, ST127 in dogs and a single ST95 isolate in a dog (Johnson et al., 2008c; Ewers et al., 2012; Tamang et al., 2012).

There is concern that antimicrobial resistance in *E. coli* harboured by companion animal strains may transfer to humans further limiting treatment options for ExPEC infections in humans. While admitting that there is no direct evidence of transmission of antimicrobial resistance from *E. coli* in pets to humans, several authors (Guardabassi et al., 2004; Lloyd, 2007; Costa et al., 2008) point out that the close relationship of humans and their companion animals provides opportunities for sharing strains; that resistant bacteria and genes have been recovered from healthy pets (Costa et al., 2008); that the same classes of antimicrobials are used in human and veterinary medicine (Shaban et al., 2014); and cases of possible ExPEC transmission from pet animals to humans have been reported (Johnson et al., 2008a; Damborg et al., 2009).

Screening of dominant *E. coli* isolates in this study found phenotypic resistance to ampicillin in 31.1% cat isolates and 20.2% of dog isolates and to tetracycline in 13.2% cat isolates and 9.9% dog isolates. Resistance to ampicillin and tetracycline is common and this finding is consistent with most previous studies. A recent survey of *E. coli* faecal isolates from dogs visiting veterinarians in the UK revealed that 37.2% of isolates were resistant to ampicillin and 30% were resistant to tetracycline (Wedley et al., 2017). A survey of dogs and cats in Portugal found a much lower prevalence of ampicillin resistance in *E. coli* faecal isolates with 16.7% of cat isolates resistant and 7.7% of dog isolates resistant; levels of resistance to tetracycline were 20.5% for faecal isolates from dog isolates and 18.2% for cat faecal isolates (Costa et al., 2008). A survey of healthy kennel dogs in Belgium found 12% resistance to ampicillin and 17% resistance to tetracycline and very low or no detectable levels of resistance to chloramphenicol, enrofloxacin and gentamicin (De Graef et al., 2004). A Canadian study of clinical *E. coli* isolates from canine UTI infections found low levels of resistance to all antibiotics, with ampicillin at 8.8% and tetracycline at 7.1% and no ESBLs were detected (Courtice et al., 2016). Australian surveys report low levels of resistance to fluoroquinolones in clinical strains from dogs (9.1%) and cats (3.2%) (Saputra et al., 2017), and strains isolated using selective media from dog faeces (Guo et al., 2013).

Direct contact with companion animals living in close contact with their owners, has been implicated as a source of human infection with extended spectrum beta-lactamase (ESBLs) producing *E. coli* (Johnson et al., 2008b; Marques et al., 2017). However, in this study very few *E. coli* isolates from dogs and cats were resistant to third generation cephalosporins, based on screening using ceftazidime. This antibiotic is considered one of the third generation cephalosporins with the highest sensitivity for ESBL detection (Harwalkar, et al. 2017). The ESBL susceptibility found in this study contrasts with reports overseas of high levels of ESBL resistance in *E. coli* isolated from both dogs and cats (Ewers etal., 2010; Huber et al., 2013; Rubin and Pitout, 2014; Carvalho et al., 2016; Ljungquist et al., 2016; Pomba et al., 2017; Wedley et al., 2017). In common with another report which didn't use antibiotic selective media (Nam et al., 2010), there was no evidence of resistance to carbapenems among this collection of isolates from Canberra dogs and cats.

The frequency of *E. coli* isolates from cats resistant to both ampicillin and tetracycline, increased with the time animals had resided in the animal shelter prior to being sampled. Tetracyclines (doxycycline) were commonly used to empirically treat cats residing in the shelter showing symptoms of feline upper respiratory infection (causal agents include feline herpesvirus, calicivirus, *Mycoplasma felis, Chlamydophila felis* and *Bordatella bonchiseptica*) and secondary bacterial infection as recommended by the *Australian Infectious Diseases Advisory Panel - Antibiotics*

(Holloway 2013). Isolates resistant to ampicillin, tetracycline or both were observed among strains belonging to phylogroups A, B1, B2, D and F. Such outcomes suggest that the evolution of isolates resistant to both antibiotics may have been due to the transfer of an R plasmid among isolates belonging to different lineages, rather than independent evolution events occurring in isolates belonging to each of the phylogroups. Sequential sampling of the same individuals through their stay at the animal shelter coupled with whole genome sequencing of the isolates would be required to determine howthe frequency of isolates resistant to both antibiotics arises.

This study has shown that companion animals do carry *E. coli* isolates belonging to one of the human associated lineages. However considerable substructure is known to occur within clonal complexes 73, 95, and 131 (Zakour et al., 2016; Gordon et al., 2017; Kallonen et al., 2017). Clonal complex 95 consists of at least five well-defined subgroups and while CC95 strains can be isolated from poultry and humans (Gordon et al., 2017), the great majority of isolates from poultry belong to just one of the CC95 subgroups. Such an outcome suggests that there may be some degree of 'host preference' exhibited by isolates belonging to the same clonal complex. Substructure is also present in ST131 (Zakour et al., 2016) and ST73 (Kallonen et al., 2017) but clear host specificities have not been

described to date (de Been et al., 2014)(McNally et al., 2016). Whole genome sequencing studies are required in order to determine the degree of difference exhibited by isolates belonging to the same clonal complex but isolated from different host species.

Supporting information

Table 1 The relative abundance of *E. coli* phylogroups

(203 *E. coli* isolates from 221 dogs and 334 isolates from 427 cats).

Phylogroup	Cat		Dog	
	Pet % (n)	Shelter % (n)	Pet % (n)	**Shelter % (n)**
A	15.6 (10)	5.6 (15)	31.8 (55)	**13.3 (4)**
B1	26.6 (17)	41.6 (112)	33.0 (57)	**46.7 (14)**
B2	40.6 (26)	41.6 (112)	24.3 (42)	**33.3 (10)**
D	6.2 (4)	7.4 (20)	6.4 (11)	**6.7 (2)**
E	0 (0)	0.3 (1)	1.2 (2)	**0 (0)**
F	10.9 (7)	3.7 (10)	3.5 (6)	0 (0)

Table 2 Frequency of major human associated *E. coli* sequence types (STs)

(with respect to either the number of phylogroup D isolates (ST69), the number of B2 isolates (STs 73, 95, 127, 131) or the total number of *E. coli* isolates recovered from the host group)

Phylogroup	ST	Cat n	% of D or B2 isolates	% of *E. coli* isolates	Dog n	% of D or B2 isolates	% of *E. coli* isolates
D	69	8	33.3	2.4	2	15.4	1.0
B2	73	64	46.4	19.1	8	15.4	3.9
B2	95	24	17.4	6.9	4	7.7	2.0
B2	127	6	4.3	1.8	5	9.6	2.5
B2	131	1	0.7	0.3	2	3.8	1.0

Table 3 *E. coli* genotype (REP=types) richness and diversity

(*E. coli* isolated from cats and dogs with respect to the phylogroup membership of the isolate).

	Cat			**Dog**		
Phylogroup	Number of Isolates	Number of Rep Types	Simpson Diversity	Number of Isolates	Number of Rep Types	Simpson Diversity
A	25	22	0.99	59	38	0.98
B1	129	64	0.98	71	67	0.99
B2	138	71	0.94	52	35	0.97
D	24	21	0.99	13	12	0.99
F	17	6	0.51	6	6	1.00

Table 4 Antibiotic disc diffusion test results

(334 *E. coli* isolates from cats and 203 isolates from dogs).

Antibiotic	Cat % resistant (n	Dog % resistant (n)
Ampicillin	31.1 (104)	20.2(41)
Tetracycline	13.2 (44)	9.9 (20)
Chloramphenicol	0.3 (1)	0
Ceftazidine	0.3 (1)	0.5 (1)
Nalidixic acid	0.3 (1)	0.5 (1)
Gentamicin	0	0.5 (1)
Ciprofloxacin	0	0.5 (1)
Ertapenem	0	0

Table 5 Examples of relative abundance of *E. coli* phylogroups

(isolated from healthy dogs living in different countries)

Phylogroup	Phylogroup (%)			
A	7.1	7.4	12.2	29.1
B1	53.6	25.0	58.8	35.0
B2	14.2	41.2	23.7	25.6
D	21.4	26.4*	5.3	10.3*
Number of dogs	28	34	73	203
Locality	Canada	Japan	United Kingdom	Australia
Reference	(White et al., 2011)	(Harada et al., 2012b)	(Schmidt et al., 2015)	This study

*Published studies used the Clermont (2000) method. Phylogroup E and F strains detected using the Clermont (2013) method would be identified as D strains using Clermont (2000).

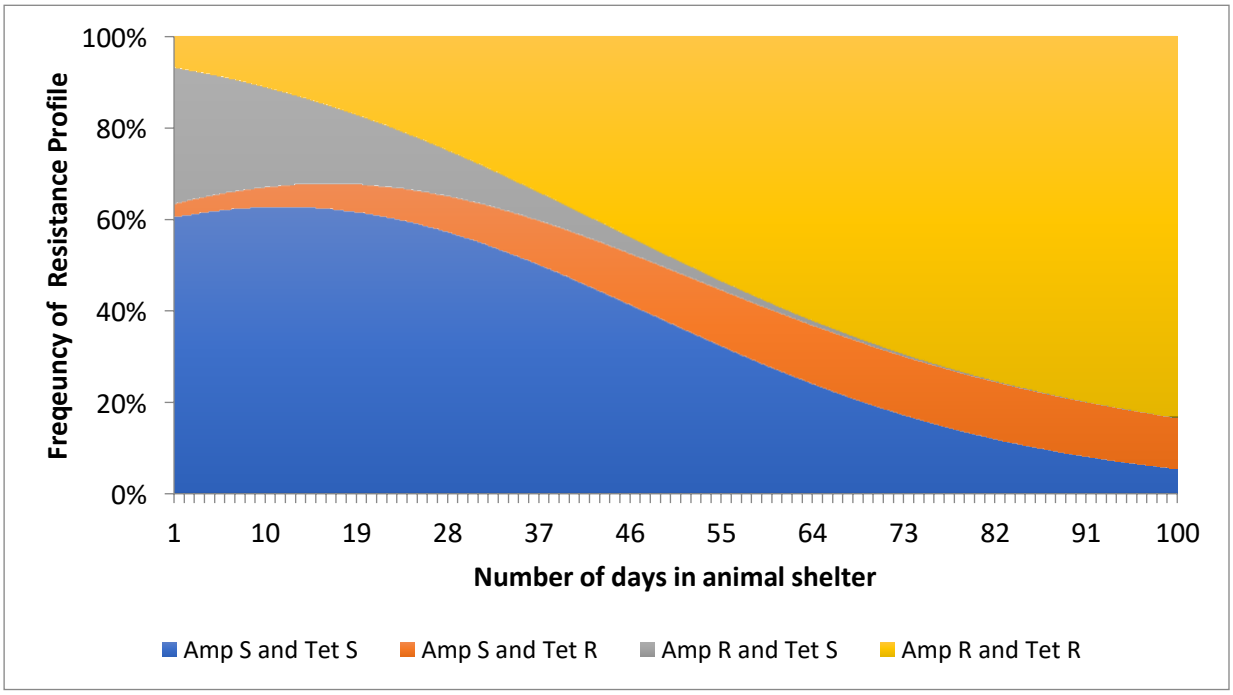
Table 6 Frequency with which STs 69, 73, 95, 127, and 131 are detected

in cats, dogs and people living the in Canberra, ACT region of Australia

Phylogroup	Sequence Type	Human % of *E. coli* isolates	Cat % of *E. coli* Isolates (n=333)	Dog % of *E. coli* Isolates (n=201)
D	69	4.6	2.4	1.0
B2	73	10.0	19.1	3.9
B2	95	9.8	7.2	2.0
B2	127	2.0	1.8	2.5
B2	131	7.8	0.3	1.0

Fig. 1 Predicted change in the frequency of *E. coli* isolates from cats resistant to ampicillin, tetracycline, or both antimicrobials as function of the numbers of days the cat had resided in the animal shelter.

(Amp S = strain sensitive to ampicillin; Tet S = strain sensitive to tetracycline; Amp R = strain resistant to ampicillin; Tet R = strain resistant to tetracycline)



Chapter 3 Genotypic comparison of *Escherichia coli* of ST69, ST95,ST117, ST131 isolated from cats, chicken meat and humans

Introduction

E. coli strains commonly associated with ExPEC in humans include sequence types ST69, ST73, ST95 and ST131 (Doumith, et al. 2015; Fibke, et al. 2019). ST117, a major cause of disease in poultry, is considered an emerging human pathogen globally (Cummins, et al. 2019). Of these common ExPEC sequence types, ST73, ST95 and ST131 belong to phylogroup B2. ST69 is a member of phylogroup D (originally named clonal group A) and ST117 representatives belong to the recently described phylogroup G (Clermont, et al. 2019).

These sequence types have been isolated from a range of non-human sources including companion animals (LeCuyer, et al. 2018) and livestock (Ewers, et al. 2007; Olsen, et al. 2011; Mora, et al. 2012; Riley 2014). Given the close relationship of humans with cats and dogs, this has raised concerns that inter-species transmission of ExPEC to humans can occur through contact. Several authors have reported apparent sharing within households of ExPEC clones that cause UTI in humans. A dog and cat shared an *E. coli* strain based on matching MLST and RFLP profiling (Murray, et al. 2004; Johnson, et al.
2008). In another study transfer of ESBL and AmpC-producing *E. coli* strains between humans and dogs was assumed in two separate households based on multilocus variable number of tandem repeats analysis (MLVA) and carriage of the same antibiotic resistance genes by hosts in the respective households. In one household the child and one of the dogs carried *E. coli* with the beta-lactamase gene $bla_{\text{CTX-M-27}}$; in the second household the child and one of the dogs carried *E. coli* with the beta-lactamase genes *blaCMY-2* and $bla_{\text{TEM-1}}$)(Ljungquist, et al. 2016).

This concern extends to food derived from livestock, especially poultry. Even though it is acknowledged ExPEC may be acquired by many routes, the knowledge that food is the main source of intestinal pathogenic *E.coli* (IPEC), has given rise to the hypothesis that food, especially poultry meat, is also a source of humanExPEC infection (Manges 2016). The heightened suspicion that chicken meat may be a source of ExPEC has been initiated by finding similarities, including genetic, with APEC strains and that these APEC strains can cause disease in experimental animal models (Manges 2016). Chickens, either diseased with avian pathogenic *E. coli* (APEC) or healthy are considered to represent potential reservoirs of human ExPEC (Ewers, et al. 2007; Bergeron, et al. 2012; Maluta, et al. 2014; Manges 2016), mainly of ST95 and ST117 (Olsen, et al. 2011). APEC cause

an economically important global disease of chickens (colibacillosis) and are regarded as chicken

ExPEC strains that have similar attributes to human ExPEC strains. These include similar phylogroup representation, serotypes, ST commonality, PFGE and antibiotic resistance patterns and VAG presence (Mora, et al. 2009; Cortés,et al. 2010; Bergeron, et al. 2012; Manges 2016).

However, these concerns largely derive from strain comparisons of relatively small numbers of single sequence types (STs) using low-resolution typing methods which may not provide the level of discrimination required to determine if strain sharing actually occurs. The few WGS analyses comparing human ExPEC and poultry-associated isolates of the same ST reported disparate results. One WGS analysis revealed considerable heterogeneity between human and poultry-associated ST117 and were judged not to be closely related based on an analysis of core genome SNPs (de Been, et al. 2014). Another core genome SNP based study of ST117 isolates found that a single human isolate did cluster with poultry isolates (Ronco, et al. 2017). Another comparative genomic analysis found APEC isolates were genetically diverse and there was multiple overlapping of some ST95 human ExPEC isolates with APEC isolates (Jørgensen, et al. 2019). Core genome SNP-based phylogenetic analysis of ST131 sublineage (ST131-H22) isolates showed close clustering of a subset of human clinical isolates with poultry meat (chicken and turkey) isolates (Liu, et al. 2018).

Reviews of foodborne ExPEC linkages remind that linking a food source to ExPEC infections in humans is difficult due to the time delay between colonisation in the gut and expression of disease and the failure of studies to take into consideration and sample other sources of ExPEC (Singer 2015; Manges 2016; Muloi, et al. 2018). Following a meta-analysis of relevant literature, these authors suggested that there are more opportunities for humans to cause reverse zoonoses (zooanthroponoses) (Messenger, et al. 2014).

To the best of my knowledge, no large cross-sectional study has been carried out to compare ExPEC isolated from healthy companion animal faeces with isolates of the same ST to explore relatedness and hence the likelihood of ExPEC sharing in Australia. In this study, we compared the whole genome data of *E. coli* strains belonging to ST69, ST95 and ST117 collected from human clinical samples (H), faecal samples from healthy cats and chicken meat (PM) samples, in Canberra, ACT, Australia. Dog isolates were not sequenced as the prevalence of these sequence types in the dogs sampled was much less than in cats (Refer Chapter 2). Chicken meat samples were collected during 2013 to 2015, cat faecal samples between 2015 and 2017 and human urine, blood and faecal samples were collected between 2014 and 2016 (refer Chapter 6 Methods for source of these isolates).

All isolates of ST69 and ST117 sequenced from cat faeces were included in this study for comparison with all isolates of the corresponding sequence type from humans and chicken meat. However. as cat isolates of ST95 as well as chicken meat isolates were predominantly subgroup C (Gordon 2017) they were compared with a sub-set of human samples (Table 7).

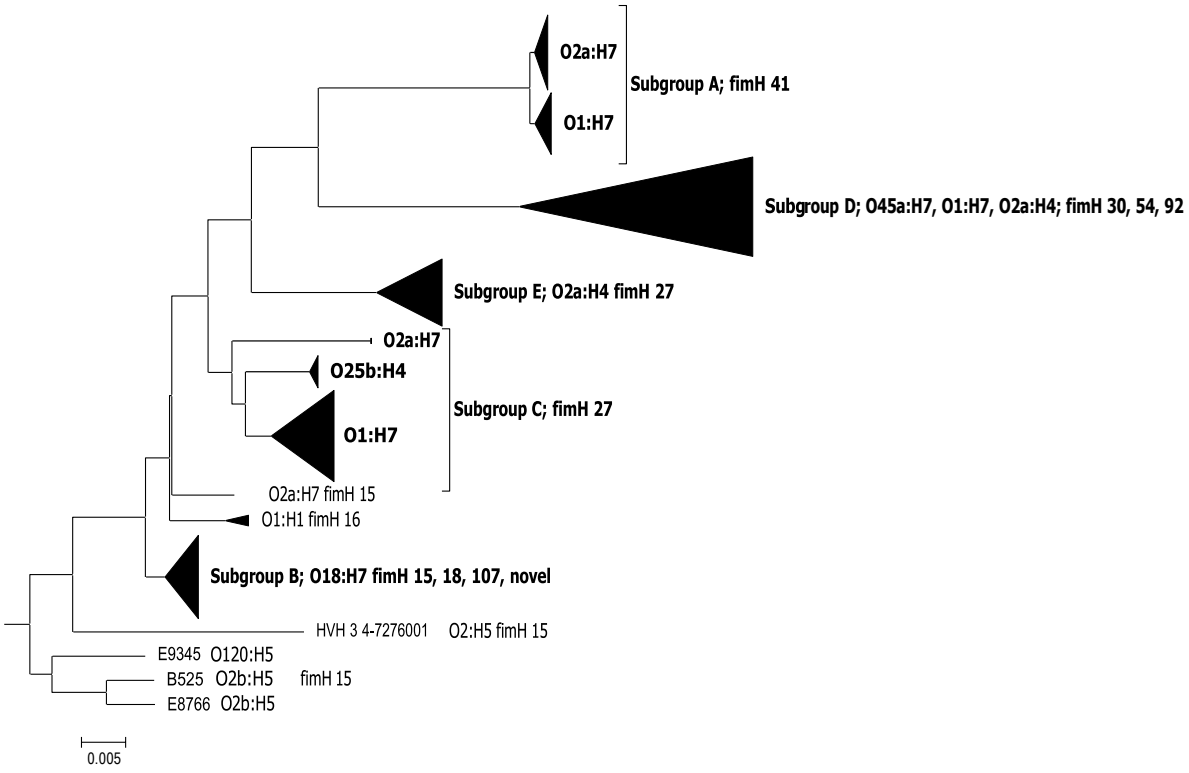


Fig. 6 Phylogeny of ST95 Subgroups (Gordon, et al. 2017)

Subgroups within CC95 were proposed by Gordon et al (Gordon, et al. 2017) based on their analysis of 200 ST95 isolates from Australian humans and poultry (Fig. 6). They showed that most chicken meat isolates were classed as subgroup C but a very low proportion of human isolates were within this subgroup (Table 7) (Gordon, et al. 2017). Within another collection of ST95 strains a subset of strains were identified as fimH27 (assumed to be in subgroup C). These strains were exclusively in the cluster containing both avian and certain human strains, as distinct from the 2 clusters containing only human strains (Jørgensen, et al. 2019).

Source	n	Subgroup C
Poultry Meat	17	88.2%
Cats	12	75.0%
Humans	147	14.3%

Table 7 ST95 Subgroup membership

(Gordon, et al. 2017)

As most cat isolates in this study were fimH27 (except for 2 excluded isolates) and either O-:H7[3] or O1:H7 these were compared with a collection of fimH27 human and chicken meat isolates classed as belonging to subgroup C (Gordon, et al. 2017). WGS data were available for 11 cat, 12 chicken meat and 12 human ST95 isolates. The two cat isolates excluded from the analysis were classed as subgroup A and D (fimH41 and 18), and both were *svg* negative (Bidet, et al. 2007).

Results

ST69

WGS data was available for 9 cat, 7 chicken meat and 15 human ST69 isolates. The source of these isolates is described in Chapter 6 Methods. These isolates belonged to 8 different serotypes (Table 9; Fig. 2) with O17/O77:H18 and O15:H18 the most common. The tree derived from the core genome of ST69 strains shows two main branches with thesecond branch divided into multiple sub-branches.

Serotypes O17/O77:H18 and O17/O44:H18 were represented in strains from the 3 sources (Fig. 2). These serotypes are typical of ST69 strains reported elsewhere (Tartof, et al. 2005; Riley 2014; Ciesielczuk, et al. 2016). Only cat and human strains were of the O15 serotype and these strains were on a separate branch forming 2 clusters. Isolates of O17/O77 were interspersed with clusters containing other serotypes.

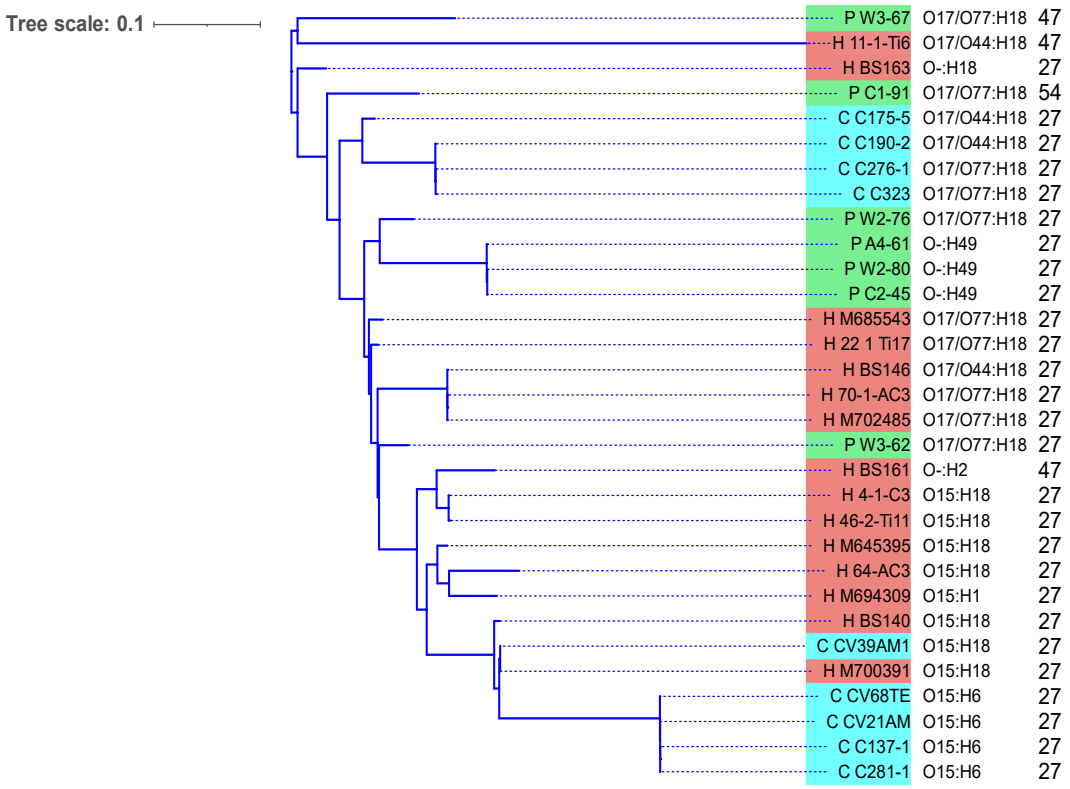


Fig. 2 Phylogenetic tree derived from the core genome of ST69 strains
Inferred phylogenetic relationships of 31 *E. coli* ST69 strains. The SNPs were extracted using the Harvest suite of tools (Treangen et al., 2014). Strains were collected from chicken meat (green), humans (red) and cats (blue) (Refer Table 9). The first column ring gives the strain name and source of the strains, the next column the serotype and the last

column shows the fimH type. Visualization of the phylogenetic tree was performed using iTOL (Letunic and Bork, 2016).

The most common *fimH* type amongst this group of strains was *fimH*27, with only 3 *fimH*47 and one *fimH*54 (Fig. 2) strain. These 4 strains with different *fimH* types did not group together on the tree (Fig. 2). The diversity of strains in this collection is consistent with other ST69 isolate surveys (Tartof, et al. 2005), indicating the collection of ST69 strains in this study is broadly representative of this sequence type.

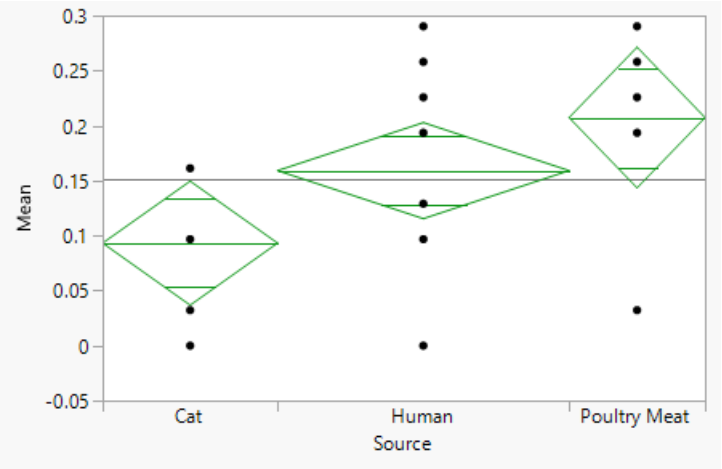


Fig. 3 Comparison of ST69 strains

(Fit X by Y: source vs ARG, VAG, bacteriocin and plasmid genes)

Pairwise comparison using just antibiotic resistance, virulence associated, bacteriocin and plasmid associated gene content shows that only cat and chicken meat strains are significantly different (p=0.0105; t-test).

Analysis of the accessory genome by PCO shows clustering according to source more clearly (Fig. 4). Both ANOSIM and PERMANOVA confirm that this separation of strains is significant (p=<0.05). Several human isolates do cluster with the chicken meat isolate and one human isolate is close to the cat isolate cluster. However, no overlap between cat and chicken meat occurs with these isolates.

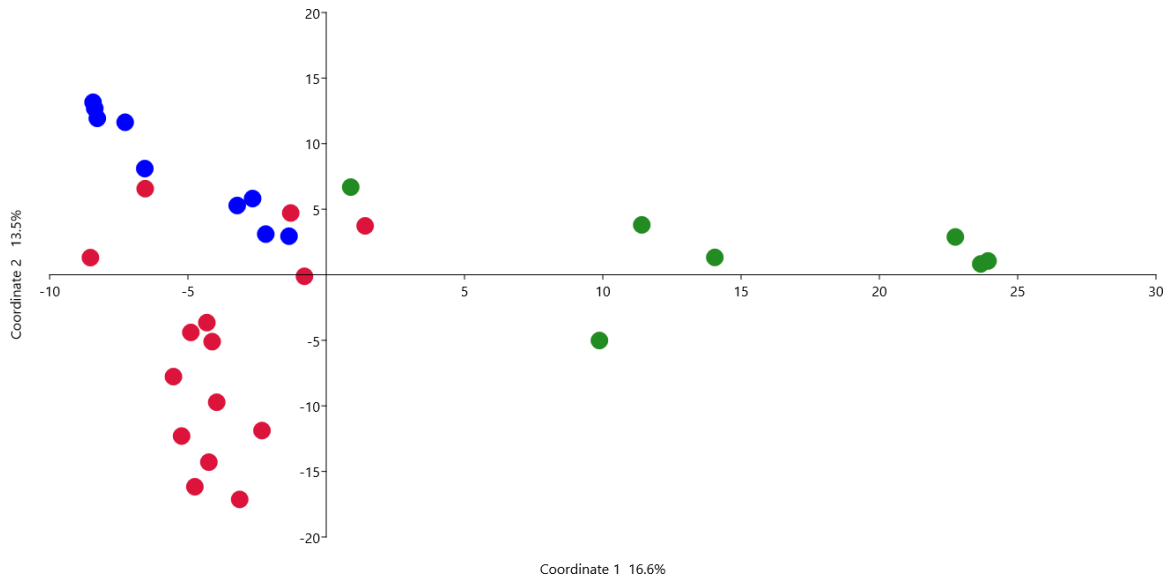


Fig. 4 PCO of accessory genome of ST69

(Green dots = poultry strains, red dots = human strains, blue dots = cat).

SIMPER analysis shows significant differences in genes representation, especially between cat and chicken meat strains, but most of the genes are hypothetical proteins or prophage elements. Of the identified genes *hpaB*, is present in most human and chicken meat strains but not cat strains and encodes 4-hydroxyphenylacetate 3-monooxygenase (EC 1.14.14.9). This enzyme allows growth of *E. coli* on 4-hydroxyphenylacetate (4HPA), a gut microbiota metabolite of aromatic amino acids associated with consumption of higher protein diets in humans (Ross, et al. 2013). Several genes are present in most chicken meat genomes, but not those of human or cats. An allele of the gene *amiC* is present in all chicken meat but no human or cat genomes. This gene encodes the enzyme N-acetylmuramoyl-L-alanine amidase which facilitates septum cleavage during cell division (Kerff, et al. 2010). The gene *mazF* is present in most chicken meat but no human or cat genomes and encodes the stable toxin MazF when *E. coli* is subjected to stress triggered by amino acid starvation (Nigam, et al. 2019) independent of the (p)ppGpp alarmone (Fraikin, et al. 2020). The gene *sodC,* together with associated gene *ydhB*, is present in most chicken meat but absent from human and cat genomes and is a superoxide dismutase (Cu-Zn).

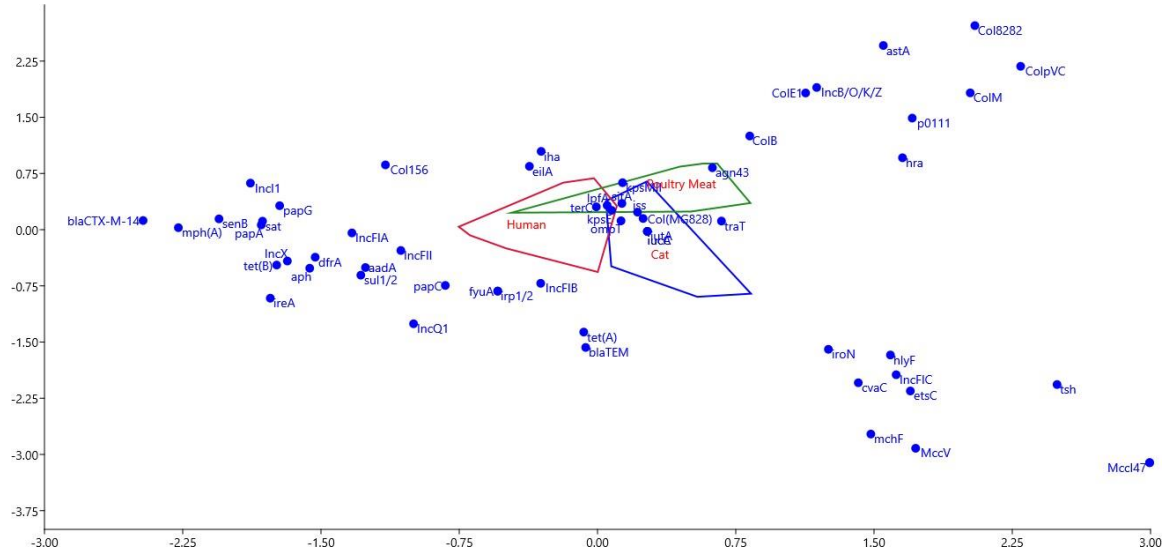


Fig. 5 Correspondence analysis – ST69

(Antibiotic resistance, virulence and plasmid associated and bacteriocin genes (blue dots) in human, cat and chicken meat. Green outline = chicken meat strains, red outline = human strains, blue outline = cat).

Correspondence analysis of antibiotic resistance, bacteriocin, virulence and plasmid associated gene representation in ST69 genomes (Fig. 5) shows cat strains are more likely to encode antibiotic resistance genes $bla_{\text{TEM-1B}}$ (p=0.0077) . Cat strains are more likely to carry ABC transport system gene, *etsC (p=0.0217),* haemolysin and haemagglutinin genes *hlyF* (p=0.0062) and *tsh* (p=0.003) and iron acquisition gene, *iroN* (p= 0.025). Cat strains are also more likely to encode genes for microcin V (p=0.012) and carry incFIC. By contrast human strains are more likely to encode antibiotic resistance gene *tet(B)* (p=0.0158) and adhesins genes *papAC* (p<0.05), siderophore *irp2* (p=0.0109) and toxin genes, *sat* and *senB* (both p=0.0002) and encode genes for plasmid replicons IncI (p=0.0399), incFII (p=0.0158) and col156 (p=0.001). Chicken meat strains were more likely to encode toxin genes *astA* (p=0.0001) and *hra* (p<0.01) and carrygenes for colicins E1 (p=0.0001), M (p=0.004) and B (p=0.0025) and incB/Q/K/Z (p=0.0062), colpVC (p=0.0396) and col8282 (p=0.004) plasmid replicons.

ST95

In this study, the proportion of ST95 in cat faeces was 6.9% of all isolates processed. Comparable proportions (5.6%) were reported for isolates from Australian commercial chicken meat samples (Gordon, et al. 2017). These proportions are much higher though, than estimates (<0.06%) for CC95 strains in the faeces of nonhuman "native" vertebrates (Gordon, et al. 2017) and in clinical samples from cats in Australia (0%) (Kidsley, O'Dea, et al. 2020).

Of the 24 ST95 cat strains isolated, only 11 were sequenced. The 33 ST95 isolates studied belonged to 2 different serotypes (Supplementary Table 9; Fig. 7) either O1:H7 or NA:H7, with O1:H7 the most common. The tree derived from the core genome of ST95 strains showsthat these serotypes were represented in strains from each of the 3 sources (Fig. 7).

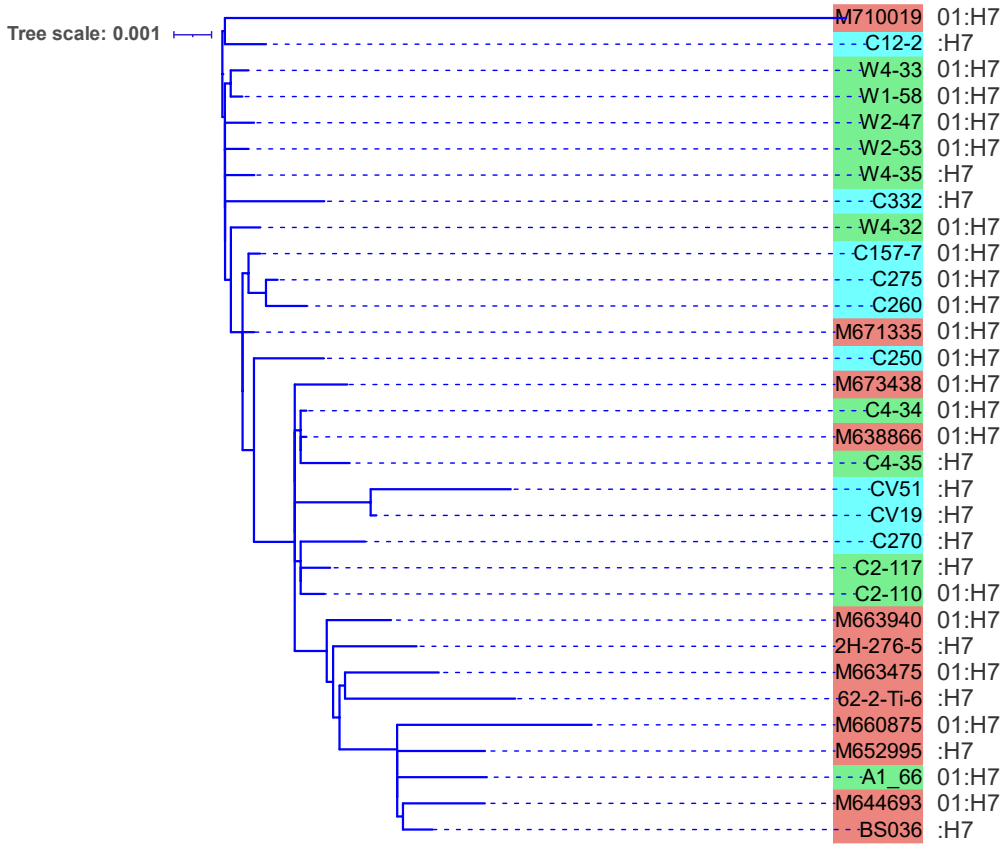


Fig. 7 Phylogenetic tree derived from the core genome of ST95 isolates strains
Inferred phylogenetic relationships of 33 *E. coli* ST95 strains. The SNPs were extracted using the Harvest suite of tools (Treangen et al., 2014). Strains were collected from chicken meat (green), humans (red) and cats (blue). The first column gives the strain name and source of the strains, and the next column the serotype (O or O-like). All strains are *fimH27*. Visualization of the phylogenetic tree was performed using iTOL (Letunic and Bork, 2016).

Serotype O1:H7 is typical of Subgroup C but also Subgroup A (Gordon, et al. 2017) and ST95 strains reported elsewhere (Riley 2014; Jørgensen, et al. 2019). Strains from cats and humans were more likely to be :H7 than chicken meat strains.

PCO analysis of the variable gene content of ST95 strains shows that poultry and human strains clustered together, but cat strains did not form a discrete cluster and were separate from chicken meat and human strains (Fig. 8). Analysis of this data by ANOSIM and PERMANOVA indicates that chicken meat isolates were separate from human isolates, but this separation was borderline significant (p= 0.0485 ANOSIM: p=0.0509 PERMANOVA). Cat strains were significantly separate from both the other sources (p<0.005).

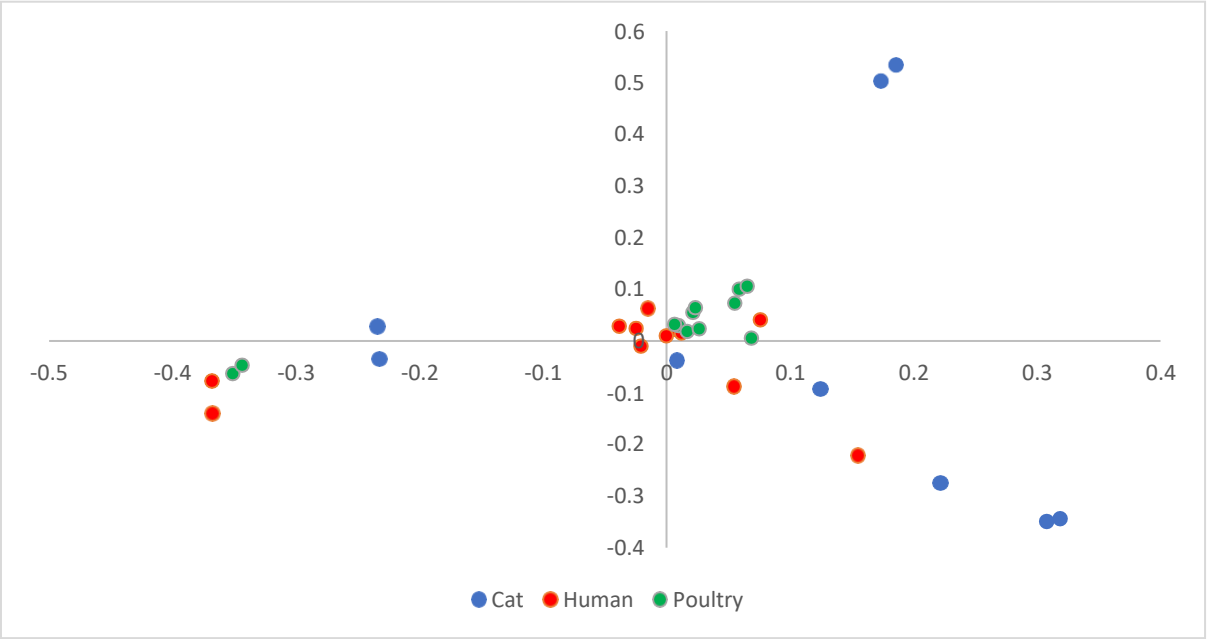


Fig. 8 PCO of the accessory genome of ST95 human, cat and chicken meat strains
(Green dots = chicken meat strains, red dots = human strains, blue dots = cat strains)

According to SIMPER analysis, most genes that predominantly differentiate strains from the 3 sources either encoded prophages or were of unknown function. Siderophore genes (*yeeS* and *fhuC*) were present in most chicken meat genomes but absent or uncommon amongst cat and human genomes.

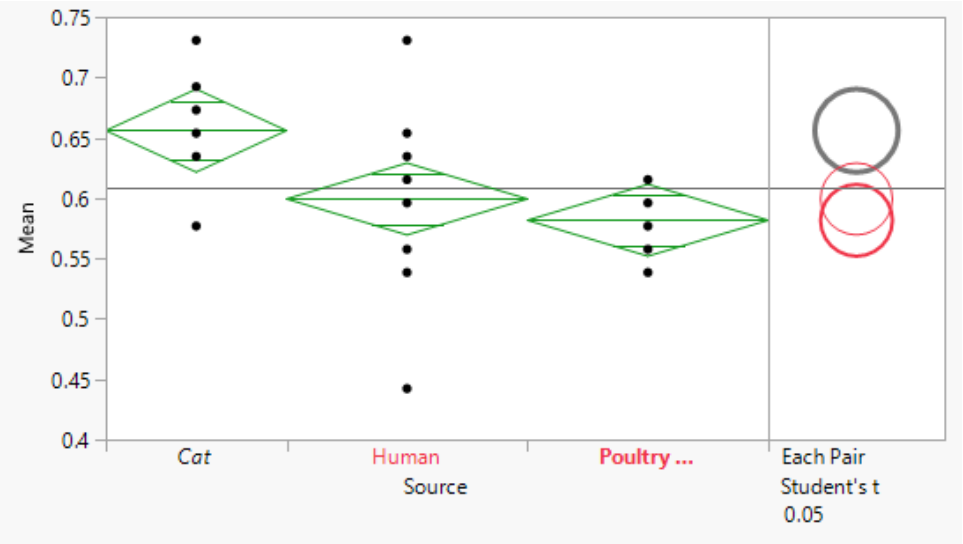


Fig. 9 Comparison of ST95 strains

(Fit X by Y: source vs antibiotic resistance, virulence associated, bacteriocin and plasmid associated gene content) Pairwise comparison of ST95 strains using just antibiotic resistance, virulence associated, bacteriocinand plasmid associated gene content shows that cat and chicken meat strains (p=0.0023) and cat and human strains are significantly different (p=0.0164).

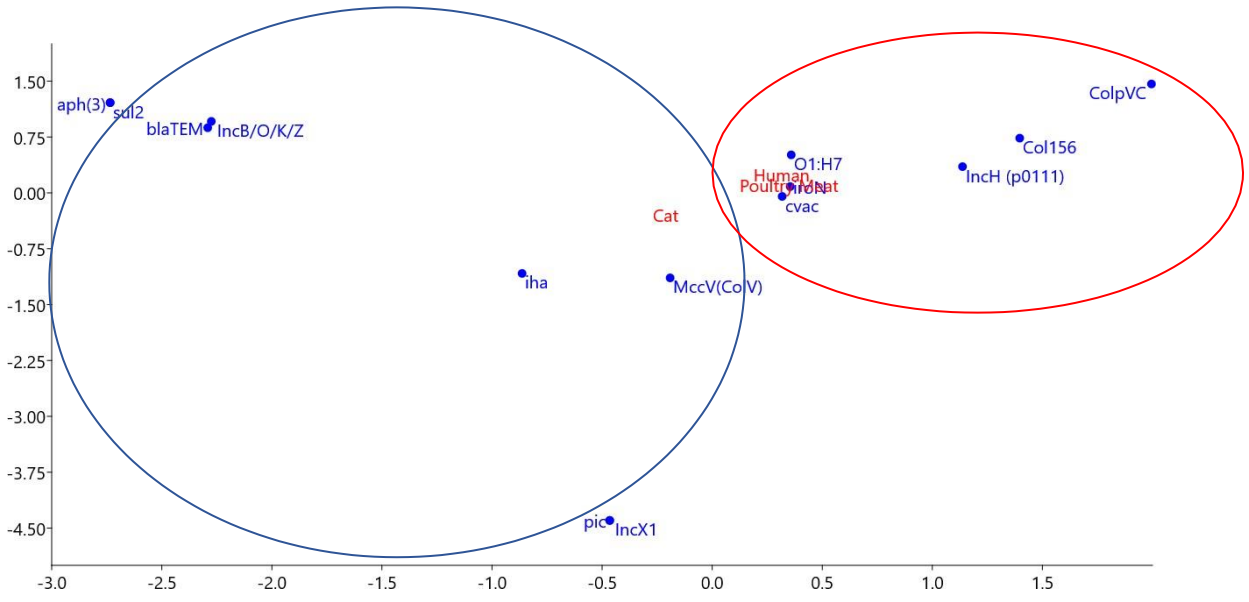


Fig. 10 Correspondence analysis ST95

(Antibiotic resistance, virulence associated, bacteriocin and plasmid associated genes (blue dots) in human, cat and chicken meat genomes; red outline = human and chicken meat strains, blue outline = cat.)

Correspondence analysis of antibiotic resistance, virulence associated, bacteriocin and plasmid associated genes in human and poultry meat ST95 strains shows very few genes partition according to source (Fig. 10). Cat strains are significantly more likely to encode adhesin gene *iha* (p=0.0008) and microcin V (p=0.0489) and plasmid replicons incX1 and p0111 (both NS). Human strains are more likely to be of O1:H7 serotype (NS) and encode plasmid replicon Col156 (p= 0.037).

ST117

Two cat isolates were sequenced and both were ST117, now recognised as within phylogroup G (Clermont, et al. 2019). There were 6 genomes from humans and 9 genomes from chicken meat available for comparison. All strains were ST117 apart from one strain, which was typed as ST657; one of the other STs included in Phylogroup G (Clermont, et al. 2019).

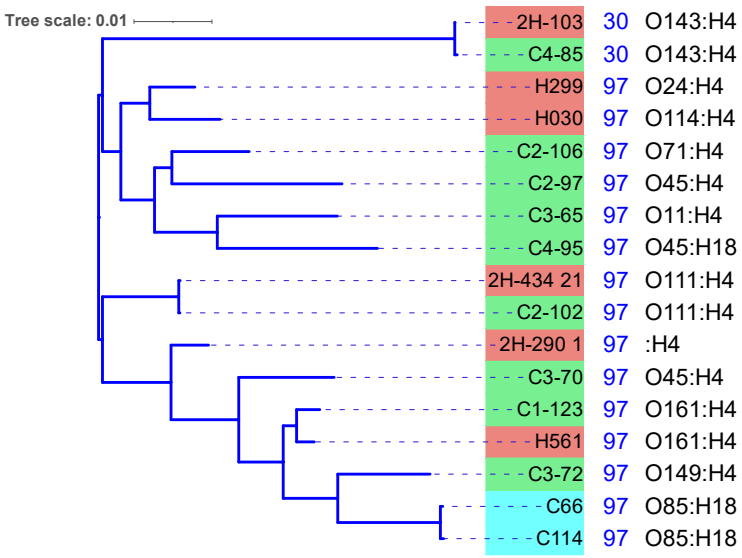


Fig. 11 Phylogenetic tree of ST117 strains derived from the core genome

Serotype of each strain is given adjacent to strain name (Green = poultry strains, red = human strains, blue = cat.) Inferred phylogenetic relationships of 17 *E. coli* ST117 strains. The SNPs were extracted using the Harvest suite of tools (Treangen et al., 2014). Strains were collected from chicken meat (green), humans (red) and cats (blue). The first column ring gives the strain name and source of the strains, and the next column the serotype All strains are *fimH27*. Visualization of the phylogenetic tree was performed using iTOL (Letunic and Bork, 2016).

The tree derived from the core genome of ST117 strains shows very short internal branches with cat strains on a separate subbranch, and chicken meat and human strains intermingled and interspersed over the two main branches which are fimH97 (Fig.11). The strains in this study were of diverse serotypes, apart from the cat isolates which were both O85 (Table 9). Strains showed high diversity in O-types (11 types), but little H-type diversity (3 types) typical of ST117 strains (Clermont, et al. 2019). The serotype of the cat isolates was not shared with isolates from humans or poultry.

Serotypes shared between human and chicken meat isolates were O111:H4, O161:H4 and O143:H4 (Table 9). The diversity of *fimH* alleles was also low with most strains *fimH97* and only 2 were *fimH30* (Fig. 11).

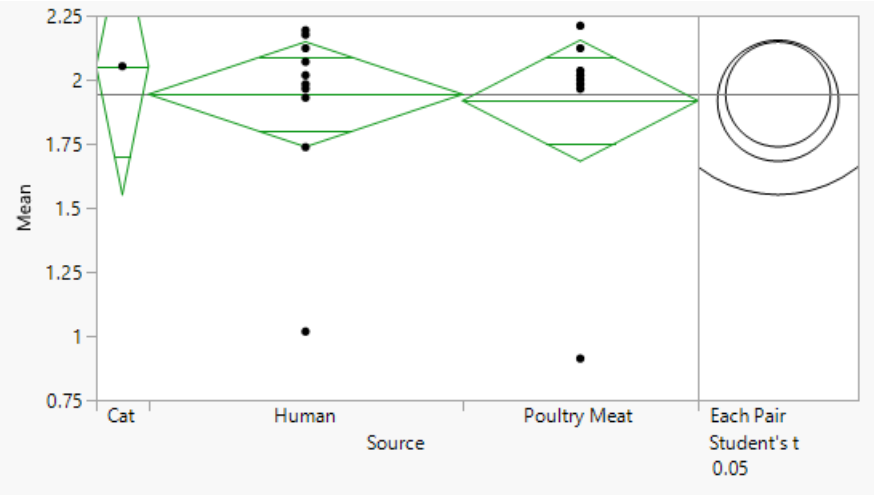


Fig. 12 Comparison of ST117 strains

(Fit X by Y; source vs ARG, VAG, bacteriocin and plasmid genes)

Pairwise comparison of ST117 strains using just antibiotic resistance, virulence associated, bacteriocin and plasmid associated gene content shows that strains from the 3 sources are not significantly different (Student's t test) (Fig. 12).

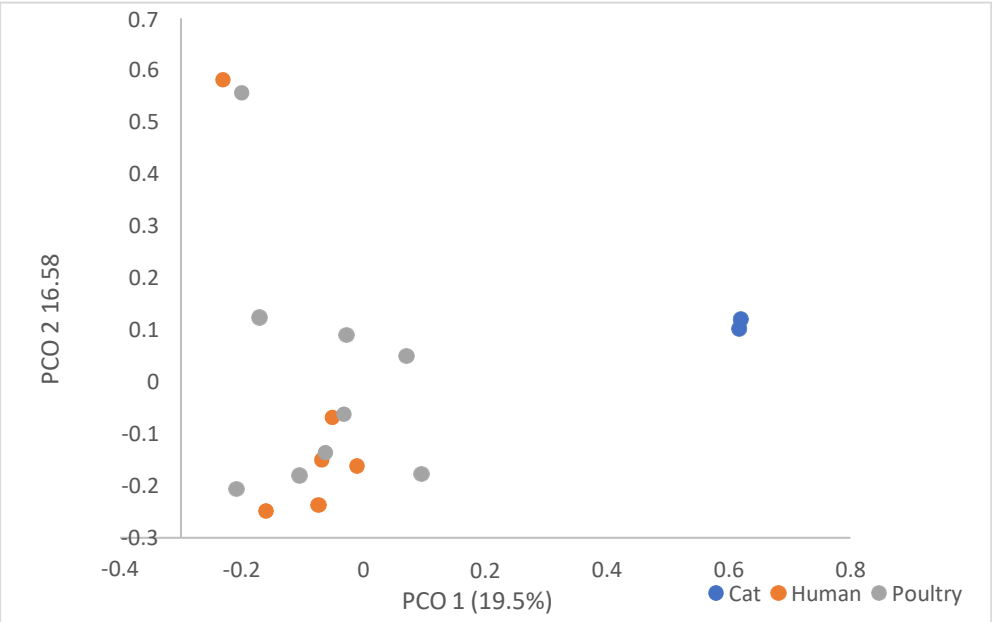


Fig. 13 PCO variable gene content ST117 human, cat and poultry strains

(Green dots = chicken meat strains, red dots = human strains, blue dots = cat)

Analysis of the variable gene content of CC117 strains by PCO (Fig. 13) shows the 2 cat strains clustered together and separately from chicken meat and human strains. Strains from chicken meat and humans mainly clustered together. Statistical analysis with ANOSIM (p= p=0.913) and PERMANOVA (p=0.793) showed no significant difference between human and poultry isolates. The same outcome is seen for the PYSEER GWAS analysis. These analyses also showed that cat isolates differ from both human and poultry isolates (both p<0.05). Given the two cat isolates are very closely related to one another and quite distantly related to the other CC117 isolates, any differences between the two isolates are likely to be artifacts. More isolates would be required to determine if cat isolates are different.

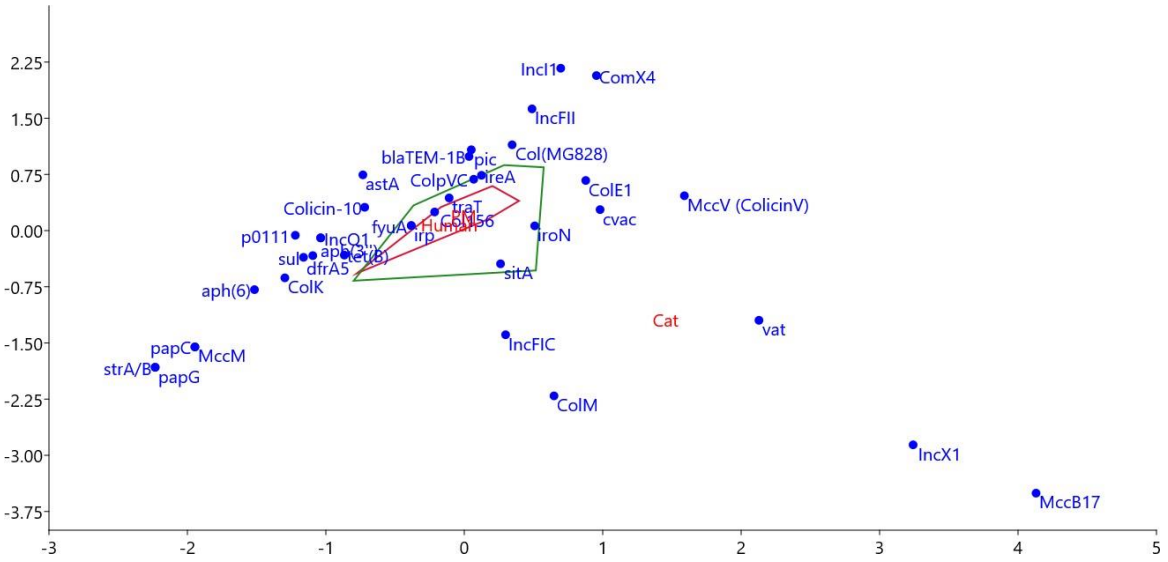


Fig. 14 ST117 Correspondence analysis ST117

(Antibiotic resistance, virulence associated and bacteriocin genes (blue dots) in human, cat and chicken meat green outline = poultry strains, red outline = human strains, black outline = cat.)

Correspondence analysis of antibiotic resistance, virulence associated, bacteriocin and plasmid replicons gene representation in ST117 strains (Fig. 14) shows cat strains are more likely to encode microcins B17 (p=0.0021), M (NS) and V (NS). Given human and chicken meat strains overlap, associations are difficult to discern. However, many of these genes are carried both by human and chicken meat strains but not the 2 cat strains. Included are genes encoding antibiotic resistance (*blaTEM1B, sul, tet, dfr*), virulence factors (*fyuA, ireA, irp1, pic, traT*) and genes encoding colicins 10 and K. Human and chicken meat strains encode genes for plasmid replicons FII and COL 156 whereas cats encode FIC; but these differences were not significant.

Both cat strains, 66% of chicken meat and 83% of human strains carried genes (*iroN, iss, iucC, iutA* and *sitA* and plasmid replicon FIB (Table 9) suggesting presence of a plasmid linked to neonatal meningitis and avian sepsis (Clermont 2019). The combination of *fyuA* and *irp2* genes was present in no cat, all human and 67% of chicken meat genomes, suggested as indicative of a pathogenicity island (Clermont 2019).

ST131

WGS data was available for one cat, 5 chicken meat and 18 human ST131 isolates. All human and chicken meat isolates were serotype O25:H4, *fimH* type 22, whereas the single cat isolate was O16:H5, *fimH* type 41. Human and chicken meat isolates are likely in Clade B and the single cat isolate in Clade A. Data from another study (Gordon pers comm) shows Clade B is primarily associated with poultry and an uncommon human strain (Table 8).

Source	n	O25:H4 fimH22
Poultry Meat	6	100%
Humans	71	14%

Table 8 ST131 Subgroup membership (Vangchhia 2017)

Of these, relatively few ST131 strains carried antibiotic resistance genes and few antibiotic classes were represented. Only one human and 2 chicken meat isolates were MDR, defined in this thesis as resistance to at least 3 antibiotics of different classes. Genes conferring resistance to beta-lactams were present in all chicken meat isolates, but poorly represented in human isolates (Table 9).

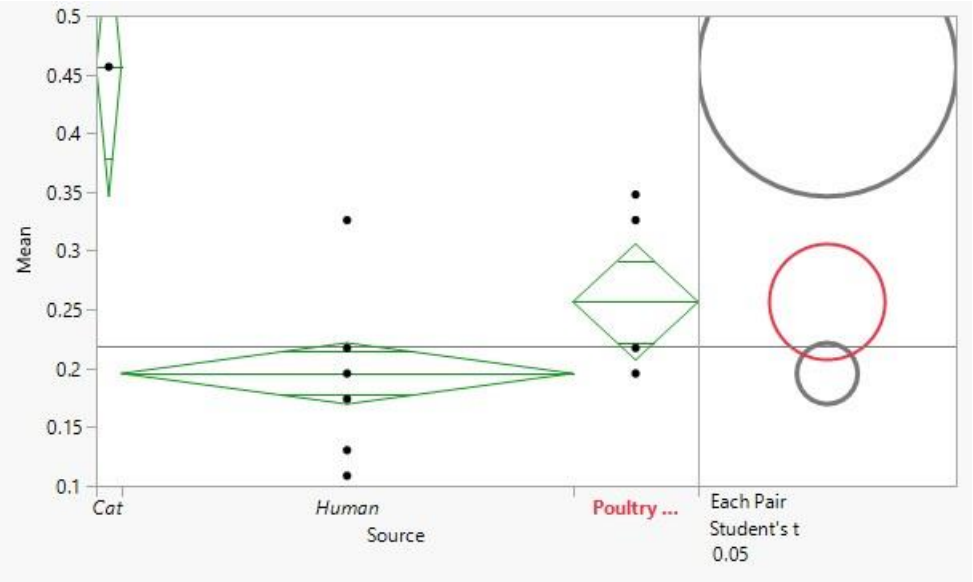


Fig. 15 Comparison of ST131 strains

(Fit X by Y: source vs ARG, VAG, bacteriocin and plasmid genes)

Pairwise comparison of ST131 strains using just antibiotic resistance, virulence associated, bacteriocin and plasmid associated gene content shows that strains from the 3 sources are all significantly different; cat and human ($p<0.0001$), cat and chicken meat ($p=0.0024$), chicken meat and human ($p=0.0337$) (Student's t test) (Fig. 15).

Correspondence analysis based on just AR, VAG, plasmid and bacteriocin genes of ST131 strains shows that the poultry meat and human genomes cluster separately (Fig. 16).

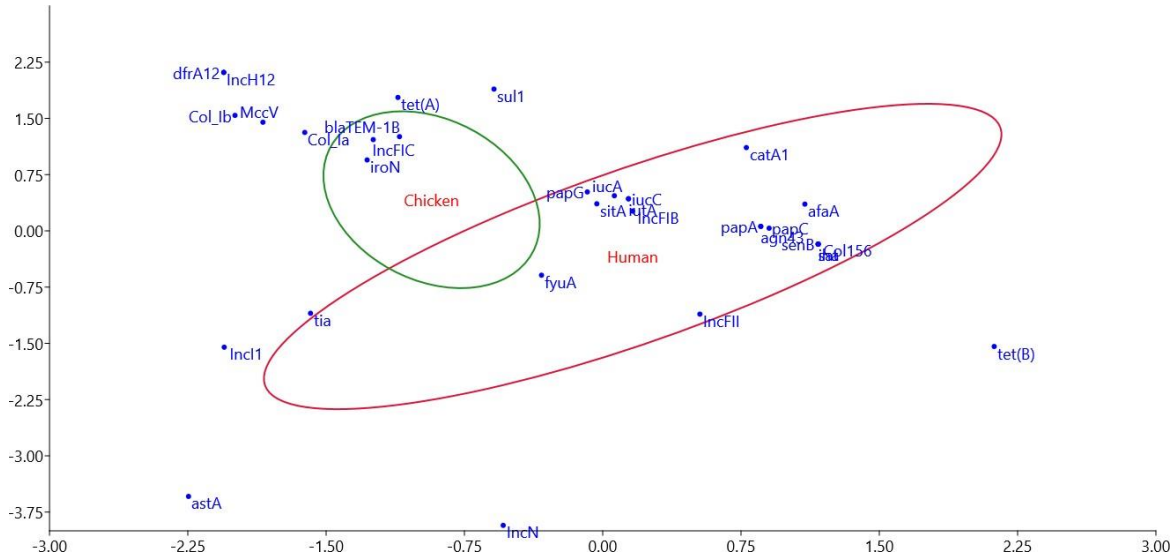


Fig. 16 Correspondence analysis ST131

(Antibiotic resistance, virulence associated, plasmid replicon and bacteriocin genes = blue dots. Green circle (95% elipse) = poultry strains, red circle = human strains.)

In this study human ST131 strains carried a greater number and variety of virulence associated genes compared to chicken meat strains. Correspondence analysis of antibiotic resistance, virulence associated, plasmid replicon and bacteriocin genes in ST131 strains shows human strains are more likely to encode antibiotic resistance genes *tet(B)* and adhesin genes *papAC* and *afaA,* toxin genes *astA, sat, tia* and *senB* and iron acquisition genes *iucC, sitA* and *iutA* and encode genes for plasmid replicon FIB. Chicken meat strains are more likely to encode antibiotic resistance genes *blaTEM* and iron acquisition gene *iroN* and encode genes for microcin V and plasmid replicons FIC.

Discussion

ExPEC strains can be isolated from companion animals and food producing animals, mainly poultry, as explained in Chapter 1. Attributing chicken meat as a source of IPEC during an outbreak is usually straightforward as these *E. coli* strains are localised, specific gut pathogens, causing disease within a short time. However, defining the lag time and hence attribution to gut colonisation and ultimately development of an ExPEC infection is a challenge. Therefore, any non-genomic based epidemiological approach to attribution is not useful as the techniques are insufficiently discriminatory and the time between exposure to the food and the development of infection is poorly defined and understood.

For this reason, genetic comparison of known ExPEC strains in companion animals and food with human ExPEC strains, despite these same time delay limitations, has become the main means of investigating sharing of these *E. coli* between hosts. This limitation is acknowledged, but it is warned that even if the likelihood is extremely small, the dangers associated with the transmission of ExPEC from non-human sources, especially multidrug-resistant lineages, warrant continuing study (Manges 2016).

The aim of this study was to use genomic analysis to compare genomes of some of the main *E.coli* ExPEC and APEC sequence types isolated from cat faeces with genomes of human and chicken meat isolates from the Gordon group at ANU collection. This comparison will add to efforts to assess the likelihood chicken meat and cats represent a reservoir of ExPEC by harbouring *E. coli* of these sequence types. In this chapter important ExPEC sequence types ST131, ST95, ST69 and ST117, from 3 different sources are compared genotypically. The prevalence of ST73 in cats, the other important sequence type, can approach or exceed the prevalence in humans (Liu, et al. 2015; Kallonen, et al. 2017). For this reason, ST73 strains isolated from cats and humans are considered separately in the following chapter.

Examination of the trees based on the core genome of ST69, 95 and 117 strains showed no clear separation of strains from the 3 separate sources. Strains from humans and chicken meat broadly overlapped with many of the human and chicken meat strains difficult to separate with respect to tree distance. Generally, human and chicken meat strains were separate from cat strains but with some overlap.

There are no other reports comparing human and chicken strains to cat strains to the level of detail here. A phylogenetic tree constructed from core genomes of human and poultry ST95 strains did show clear separation of human strains in an identified ExPEC/HExPEC-1 subclade from poultry strains. However, strains within another subclade designated A/E-PEC showed large genetic overlap (Jørgensen 2019). However, the extent of partitioning of strains according to source showed some variation dependent on sequence type. As example, the PCO analysis of the variable gene content of ST69 strains showed that strains of this sequence type generally clustered according to source. This was despite the small sample size for these strains and that strains werediverse. However, chicken meat and human ST95 and 117 strains clustered together and the cat strains generally scattered and were separate to these main clusters (Figs. 8 and 13).

Further analysis was carried out to determine whether this partitioning according to source could be explained by differences in carriage of certain genes. SIMPER analysis showed that differences in variable gene carriage, with several exceptions, were mainly related to differences in carriage of genes with unknown properties or encoding prophages.

Differences in carriage of prophage genes may benefit the host bacteria by increasing the host's response to environmental stress, including antibiotics but also by enhancing growth in various media and enhancing phosphorus and nitrogen utilization (Wang, et al. 2010). As example, prophage genes *ybcON* and *yfdDRT* were present in all ST69 cat strains, but very few human strains. Genes in the *ybc* operon inhibit the growth of competitors for nutrients (Ren, et al. 2004) and *yfd* genes enhance resistance to oxidative stress (Wang, et al. 2010).

Most human, but no cat ST69 strains, carried genes *hpaB, hpcBC* and *badR*. These genes are part of one of several biodegradation gene clusters encoding pathways which catabolise aromatic compounds widely distributed in ecosystems. In this case, these genes catabolise 3,4-hydroxyphenylacetic acid/acetate (HPA), a polyphenol metabolite (Dıaz, et al. 2001; Vázquez-Fresno, et al. 2014; Giskeødegård, et al. 2015). Polyphenols occur widely in plants, and are present in fruits, vegetables, cereals, tea, coffee, and wine (Moco, 2012). The presence of these genes only in human ST69 strains is consistent with selection for strains exposed to these metabolites. The *kilR* gene is present in all chicken meat ST69 strains but absent from cat strains. This gene, which causes cell death in susceptible bacteria (Altieri, et al. 1986) is located on the col E1 plasmid; also predominant in chicken meat ST69 strains.

All ST69 chicken meat strains carried *amiC*, one of three genes which encode the amidase enzyme, N-acetylmuramoyl-L-alanine amidase. Amidase enzymes split the murein septum during cell division, prerequisite to cell division (Kerff, et al. 2010). The three genes are redundant, so the significance of chicken meat strain's disproportionate carriage of just *amiC* is uncertain.

In this study all ST95, 11.1% of ST117, 71.4% of ST69 and no ST131 chicken meat strains carried the virulence gene agn43 (originally identified as *flu*). The product of the agn43 gene, antigen 43 (Ag43) (CP4-44 prophage), is an abundant outer membrane protein in *E. coli* belonging to the autotransporter family (Wallecha, et al. 2014). Ag43 promotes *E. coli* autoaggregation, biofilm formation, and microcolony formation on epithelial cells (De Luna, et al. 2008). According to SIMPERanalysis, a *yeeS* allele, a prophage which encodes Zn metal ion binding proteins, was a predominant gene which differentiated chicken meat strains from both human and cat strains. This gene can be adjacent to the agn43 or *flu* gene together with other *yee* and prophage genes, but the significance of this association is uncertain.

Correspondence analysis showed all sequence types of cat strains carried an antibiotic resistance, virulence factor and bacteriocin gene profile distinct from that in the human and chicken meat strains. Very few antibiotic resistance genes are carried by cat strains, except the single ST131 strain, which carried genes encoding resistance to aminoglycosides, chloramphenicol, macrolides, sulfonamides and tetracyclines (Table 9). *blaTEM1b* genes were carried by some ST69 strains (66%) and *tetB* was carried by all ST95 strains. However, few cat strains were MDR; a low proportion of ST69 and ST95 strains were MDR (both 22%) and the two ST117 strains were not MDR. All ST117 cat strains in this study (n=19) were susceptible to a panel of antibiotics which included fluoroquinolones and 3rd generation cephalosporins. Genes conferring resistance to critical antibiotics were not detected (eg. colistin, ESC, fluoroquinolones).

These results for ST69, ST95 and ST117 cannot be compared with other results published for cat isolates as there are nil or few reports of these sequence types specifically in cats, and of the few reports these do not provide details of sublineage (Liu, et al. 2016). However, the antibiotic resistance of the single cat faecal ST131 isolate in this study can be compared with a singleclade A cat urine isolate (Kidsley, White, et al. 2020). This clinical isolate was also MDR but differed from the isolate studied here in carrying β-lactamase and fluoroquinolone resistance genes.

Few genes conferring antibiotic resistance were present in human strains. The widest range of genes conferring antibiotic resistance was carried by ST69 and ST117 strains (Table 9). In this study, 53% and 67% of ST69 and ST117 strains respectively, were MDR. Others have reported similarly high

levels of MDR in human ST117 (Clermont, et al. 2019) and ST69 strains (Adams-Sapper, et al. 2013; Manjarrez-Hernandez, et al. 2016; Fibke, et al. 2019). However, very few human ST95 isolates carried antibiotic resistance genes apart from *tet(B).* Only 17% of human ST95 strains were MDR. This is consistent with the findings from other surveys (Gordon, et al. 2017; Fibke, et al. 2019; Poulsen, et al. 2020) but not that of a Danish study which found genes encoding resistance to beta-lactams and sulphonamides to be prevalent in human ST95 isolates (Jørgensen, et al. 2019) although the sublineage of these strains is uncertain.

As the antibiotic resistance profile of ST131 strains varies with clade membership, comparison of results from this study for human strains can only be made with those from other studies which give antibiotic resistance profiles specifically for Clade B strains, that is O25:H4, *fimH* type 22 strains. In this study very few human ST131 strains carried antibiotic resistance genes and only one of these strains (5.6%) was MDR (Table 9). This is consistent with other studies of human sublineage B from a range of countries. Few strains carried antibiotic resistance genes, just those conferring resistance to beta-lactams, sulfonamides and aminoglycosides (Liu, et al. 2018). A Canadian survey of UTI *E. coli* strains found few carried antibiotic resistance genes, usually just a single isolate carried genes conferring resistance to aminoglycosides, trimethoprim, sulfonamides, beta-lactams or macrolides (Fibke, et al. 2019).

Another study which analysed genomes from a number of regions recorded that most clade B strains did not carry allele combinations associated with fluoroquinolone resistance (Zakour, et al. 2016). This is because expression of resistance requires strains to have mutations in at least two of a number of key genes (*gyrAB, parCE, marOR, acrR*) and specific allele combinations in *gyr* and *par* genes (Lindgren, et al. 2003; Zakour, et al. 2016; Liu, et al. 2018). Most of the human clinical human *E. coli* isolates (94%) in this study carried chromosomal mutations in only a single gene associated with fluoroquinolone resistance, *parE*. Consequently, antibiotic screening would be expected to show fluoroquinolone susceptibility for all ST131 isolates in this study.

Most human strains analysed in this study did not carry antibiotic resistance genes to the important classes of antibiotics (Table 9), that is, carbapenems (ertapenem), fluoroquinolones or quinolones with just one ST117 isolate carrying an ESC resistance gene (bla_{CTX-M}). This finding is consistent with other studies for ST117 (Clermont, et al. 2019), ST95 (Gordon, et al. 2017; Jørgensen, et al. 2019) and ST131 clade B (Liu, et al. 2018) strains but contrasts with recent reports of ESBL production and resistance to fluoroquinolones in human ST69 (Ajiboye, et al. 2009; Novais, et al. 2013) and ST131 strains (Zakour, et al. 2016; Pietsch, et al. 2018; Fibke, et al. 2019).

Chicken meat isolates in this study carried few antibiotic resistance genes apart from *blaTEM1b* in ST131 and *tetB* in ST95 strains (Table 9). This is consistent with the findings of other surveys (Gordon, et al. 2017; Fibke, et al. 2019; Poulsen, et al. 2020). The results of this study are also consistent with those of a study of ST131 poultry strains (APEC) of O25:H4 in Danish flocks which were almost devoid of antibiotic resistance genes except for one isolate with *sul* (Poulsen, et al. 2020). Similarly, Clade B poultry isolates from North America, India, Australia and Europe (Zakour, et al. 2016) carried few antibiotic resistance genes and lacked ESBL genes and those conferring FQ resistance. Less than 50% of USA Clade B isolates carried genes conferring resistance to beta-lactams, sulfonamides and aminoglycosides (Liu, et al. 2018).

Few chicken meat strains in this study were MDR; only one ST69 strain, no ST95 strains, 22% of chicken meat ST117 strains and 40% of chicken meat ST131 isolates. This contrasts with a US study of chicken meat fimH22 ST131 strains (n=5) which were 100% MDR (Johnson, et al. 2017). Greater prophylactic use of antibiotics during commercial poultry production in the USA compared with Australian practices likely explain this difference (US poultry data show big reductions in key antibiotics).

Virulence factor profiles and even specific genes have been described as lineage-specific (Vila, et al. 2016; Kallonen, et al. 2017; Shaik, et al. 2017) but others have found variations in profiles are common even within a given lineage (Vila, et al. 2016). Variations in carriage of virulence factors can also vary according to source within sequence types, as found in this study.

Comparing gene carriage of specific genes showed cat ST69 strains were less likely to carry key virulence associated genes. However, several genes were carried by significantly more cat strains than other ST69 strains, that is haemolysin gene *hlyF, iroN* and *tsh*. Apart from a significantly greater proportion of cat strains carrying *gad* and *iha* genes, there were no other differences in genes carried by ST95 strains from the 3 sources.

Of genes reported to be characteristic of subgroup C (Gordon, et al. 2017), only *cvaC, iucC, iutA*, *ireA, papC* and *iroN* were present in the majority of strains from all sources in this study. All strains in this study carried *flu (agn43)* yet this gene was absent from subgroup C strains in another study (Gordon, et al. 2017). Whereas *ireA* and *fyuA* are

typically carried by ST117 strains (Clermont, et al. 2019), these genes were absent from cat strains but present in human and chicken meat strains. There were few virulence factors differentiating ST117 strains from the 3 sources. Other genes typically carried by this sequence type, *iroN, iss, iucC, iutA, lpfA* and *sitA,* were present in most or all strains in this study.

ST69 human strains carried a greater range of virulence genes compared with the other sources, including *fyuA, iroN, iha, papAG, irp, sat, senB* and *traT*. Only human strains carried key ExPEC associated virulence genes *papAG, sat* and *senB.*

A greater proportion of chicken meat ST69 strains carried *agn43, astA, hra, iha* and *traT* than other strains. There was little overlap of virulence factors present in human and chicken meat isolates with those reported by others (Novais, et al. 2013; Kallonen, et al. 2017).

Cat ST69 strains were more likely than strains from other sources to carry microcin V. Most (82%) cat ST95 strains carried the plasmid associated gene for colicin E1 and a few cat strains carried microcin V (36%). Only cat strains carried genes encoding microcin B17.

Few ST69 human strains (27%) carried any of the genes encoding bacteriocins. This is consistent with another report of no known bacteriocins detected in human ST69 clinical source strains, despite the resistance of ST69 strains to bacteriocins produced by other sequence type (da Cruz Campos, et al. 2019). Plasmid-encoded bacteriocins colicin Ia and microcin V are typically in subgroup C ST95 human strains (Gordon, et al. 2017). However, in this collection of human strains all carried colicin E1, none colicin Ia, but a few (33%) did have microcin V. Even though human ST117 strains carried a greater number of bacteriocin genes than chicken meat and cat strains, less than 50% of strains carried 3 or more genes. This result contrasts with that of another study comprising much larger numbers which found over half of STc117 encoded 3 or more bacteriocins (Clermont, et al. 2019). Apart from one strain carrying colicin E1 no other human ST131 strains carried bacteriocins. This contrasts with a US finding that all ST131 fimH22 strains carried colicin V plasmids, regarded as a marker of poultry-to-human transmission (Liu, et al. 2018).

Chicken meat strains overall carried a distinctive range of bacteriocins. ST69 strains were significantly more likely to carry Col B, E1 and M than other strains. Compared to other strains a greater proportion of ST117 chicken meat strains carried colicin 10 and chicken meat strains were the only ST131 strains which carried microcin V.

There were very few plasmid associated genes in the strains in this collection. Significant differences in carriage of some genes according to source occurred, but numbers in most cases were very low. A significantly greater proportion of ST69 cat strains harboured incompatibility group FIC(FII) (AP01918) and only ST95 strains from cats carried IncX1(EU370913) and only ST117 strains from catscarried IncX4 (FN543504). IncX plasmids are narrow host range plasmids which encode type IV fimbriae and increase resistance of host bacteria towards antimicrobial agents (Johnson, et al. 2012).

Human ST69 strains were more likely to harbour Col 156 (NC 009781), IncI1(AP0005147) and IncFII(29) (CP003035) than other strains. All or most of the ST95 strains in this study harboured incompatibility groups FIB (IncFIB) (AP001918) and IncFII (AY45016). These replicons are reported in STc95 isolates from humans by others (Bengtsson, et al. 2011; Stephens, et al. 2017; Stork, et al. 2018).

Chicken meat strains also carried a distinctive range of plasmid associated genes. A significantly greater number of ST69 strains carried col 8282 (DQ995353) and IncB/K/O/Z (GQ259888) than other strains. Greater numbers of chicken meat than human strains harboured ColpVC (JX133088) although numbers were very small (n=2).

Plasmids carry most AMR genes, when present, and carriage of the number and types of plasmid may in part explain the association of particular *E. coli* lineages with MDR (Bengtsson, et al. 2011) and carriage of ESC resistance genes (Casella, et al. 2017). In this study there were no clear associations between plasmid types and MDR but there was a significant (p=0.0023) relationship between carriage of $bla_{\text{CTX-M-14}}$ and IncI1 in ST69 human strains, although numbers were low (n=2). This association was identified by others in strains isolated from poultry and companion animals overseas, but not in ST69 (Madec and Haenni 2018).

This study has shown that cat strains of ST69, ST95, ST117 and ST131 are definitely distinct from strains from other sources, based on a number of criteria. Cat strains largely cluster separately from human and poultry strains, forming distinct subpopulations within each sequence type. It appears that, even though these strains are important ExPEC and APEC sequence types, large-scale transmission from cats to humans is highly unlikely and cats are not an important reservoir of these ExPEC sequence type for humans.

However, conclusions from this study regarding the extent of similarity of chicken meat and human strains vary with sequence type. ST69 and ST131 strains are distinct but chicken meat and human ST95 fimH27 strains were very similar, as were ST117 fimH97 strains even though this sequence type is an *E. coli* lineage largely associated with poultry.

Notable is the reported absence of the human ExPEC sequence types ST69 and ST95 in isolates from cat clinical cases sourced in Australia (Kidsley, O'Dea, et al. 2020), the US (Liu, et al. 2015) and Switzerland (Zogg, et al. 2018). My study's isolation of ST95, ST69 and ST117 *E. coli* from cat faeces could be due to the greater number of cat isolates that were typed using MLST (n = 334) compared

with less than 75 isolates from clinical cases in each of the other surveys which failed to detect these STs. Alternatively, the *E. coli* of ST69, ST95 and ST117 which I studied, probably preferentially colonise the cat gut as a separate sub population and cannot cause UTI in cats. This indicates a level of host specificity of subpopulations of these sequence types.

Cats would be expected to acquire ST69, ST95 and ST117 through contact with their human owners and/or the consumption of raw chicken meat or wild birds or a common source in the environment. However, as shown in this study, the cat isolates studied here are separate from isolates from humans. Likewise, this study indicates that poultry meat, whether derived from commercial or free range birds is an unlikely source of the cat strains studied here. Host adaptation has likely occurred within the gut of cats with the evolution of distinct subpopulations of each sequencetype. An alternative source of cat strains could be wild birds but presumably isolates from free range chickens would carry these strains.

In Australia and overseas the broiler industry has a pyramidal structure comprising various layers with breeding stock of pedigree chickens (grandparent flock) at the top and the broiler chickens at the base of the pyramid. *E. coli* isolates on chicken meat are assumed to originate from at least two sources; vertical transmission from breeding stock to chickens, or horizontal transmission in the broiler house environment.

The occurrence of vertical transmission, at least by eggshell contamination, is suggested by the isolation of the same strains from parent and broiler flocks (Giovanardi, et al. 2005; Poulsen, et al. 2017) but considered unlikely by others (Projahn, et al. 2018).There are multiple avenues of *E. coli* horizontal spread into the broiler production shed, which include by humans during transport and husbandry, contaminated litter, water or feed. But this mode of transmission is unlikely given modern husbandry systems which are highly mechanised withminimal exposure to environmental *E. coli* including those strains harboured by humans. Spread of *E. coli* between carcasses occurs during processing from broiler faeces or crop (Hubbard, et al. 2020). One study showed that chicken meat contaminants were more like *E. coli* strains which could be isolated from the crop and gizzard rather than faecal strains (Johnson, et al. 2009). However, contamination can occur anywhere along the supply chain.

Broiler production is an all-in all-out production system, with a very short time span. Evolution of distinct subpopulations through adaptation to the gut environment within the largely clonal broiler chickens is therefore unlikely. Genetic drift therefore is a more likely explanation for any genetic variation occurring in poultry specific strains. This contrasts with the situation in companion animals where the animal and hence it's gut microbiome is presented with continuous, frequent exposure to *E. coli* from the environment, especially from it's human owner or handler. The longer periods that dogs and cats spend in specific environments provide a greater opportunity for host adaptation and the evolution of more host specific strains.

In summary, this study showed clear differences in variable gene content of strains according to source. But the value of this study is limited by the relatively small number of strains available for study. This is especially the case for ST117 and ST131. In addition, despite the literature available on these ExPEC sequence types, only a limited number of publications subtype strains beyond MLST making it difficult to compare the results of this study with others and hence determine the significance.

Supplementary Tables

Table 9 Metadata for ST69, ST95, ST117 and ST131 strains studied

(Sequence type, serotype, fimH type, plasmid replicon type (incompatibility group) and antibiotic resistance gene profile. Strains indicated in green = poultry meat strains; red = human strains; blue= cat strains.)

Strain	Sequence type	Serotype	FimH	Inc type	Antibiotic resistance genes
C137-1	69	O15:H6	27	FIB, FIC	*bla*$_{TEM-1B}$, *tet(A)*
C175-5	69	O17/O44:H18	27		
C190-2	69	O17/O44:H18	27		
C276-1	69	O17/O77:H18	27	FIB, FII	*aadA1*, *bla*$_{TEM-1B}$, *dfrA*, *sul1*, *tet(A)*
C281-1	69	O15:H6	27	FIB, FII	*bla*$_{TEM-1B}$, *tet(A)*
C323	69	O17/O77:H18	27		
CV21AM	69	O15:H6	27	FIB, FIC	*bla*$_{TEM-1B}$
CV39AM1	69	O15 :H18	27	FIB, FII	*bla*$_{TEM-1B}$, *dfrA*, *sul2*
CV68TE	69	O15 :H6	27	FIB, FIC	*bla*$_{TEM-1B}$, *tet(A)*
4-1-C3	69	O15 :H18	27	FIB, FII	*tet(A)*
11-1-Ti6	69	O17/O44 :H18	47		
22_1_Ti17	69	O17/O77 :H18	27	FIA, I1	
46-2-Ti11	69	O15 :H18	27	FIB, B/O/K/Z, FII	
64-AC3	69	O15 :H18	27	FIB, FIA,I1, B/O/K/Z, FII	*aac(3)-Iid*, *bla*$_{CTX-M}$, *bla*$_{TEM-1B}$, *dfrA*, *mph(A)*, *tet(B)*, *tet(A)*
70-1-AC3	69	O17/O77 :H18	27	FIB, FII,FIC	
BS140	69	O15 :H18	27	FIB, Q1	*bla*$_{TEM-1B}$, *dfrA*, *sul1*, *sul2*, *tet(A)*
BS146	69	O17/O44 :H18	27	FIB, FII,I1,Q1	*aadA5*, *bla*$_{TEM-1B}$, *dfrA*, *sul1*, *sul2*
BS161	69	:H2	27	FIB, FIA, FII	*bla*$_{TEM-1B}$, *dfrA*, *mph(A)*, *sul2*, *tet(B)*
BS163	69	:H18	47		
M645395	69	O15 :H18	27	FIB	
M685543	69	O17/O77 :H18	27	FIB, FII,I1,I2,	*bla*$_{CTX-M}$, *dfrA*, *mph(A)*, *sul2*, *tet(B)*, *tet(A)*
M694309	69	O15 :H1	27	FIB, FIA, FII	*aadA1*, *bla*$_{TEM-1B}$, *catA1*, *dfrA*, *sul1*, *tet(B)*
M700391	69	O15 :H18	27	FIB, FIA, FII,Q1	*bla*$_{TEM-1B}$ *B*, *sul2*, *tet(B)*
M702485	69	O17/O77 :H18	27	FIB, FII	
A4-61	69	:H49	27	B/O/K/Z, p0111	
C1-91	69	O17/O77 :H18	54		*dfrA*, *sul2*
C2-45	69	:H49	27	B/O/K/Z, p0111	
W2-76	69	O17/O77 :H18	27	FIB, FIC, B/O/K/Z,	*tet(C)*
W2-80	69	:H49	27	B/O/K/Z, p0111	
W3-62	69	O17/O77:H18	27	FIB,FII, B/O/K/Z	
W3-67	69	O17/O44 :H18	47		
C12-2	95	:H7	27	FIB, FII	*tet(B)*
C157-7	95	O1:H7	27	FIB, FII	*tet(B)*
C250-1	95	O1:H7	27	FIB, FII, B/O/K/Z	*tet(B)*
C260.1	95	O1:H7	27	FIB, FII, B/O/K/Z	*aph(3'')*, *aph(6)*, *bla*$_{TEM-1C}$, *sul2*, *tet(B)*

Strain	Sequence type	Serotype	FimH	Inc type	Antibiotic resistance genes
C270	95	:H7	27	FIB, FII	*tet(B)*
C275.2	95	O1:H7	27	FIB, FII, B/O/K/Z	*aph(3'')*, *aph(6)*, *bla*$_{TEM-1}$, *sul2*, *tet(B)*
C332	95	:H7	27	FIB, FII	*tet(B)*
CV19	95	:H7	27	FIB, FII, X4	*tet(B)*
CV51	95	:H7	27	FIB, FII, X4	*tet(B)*
H-276-5_R	95	:H7	27	FIB, FII, I1	*tet(B)*
62-2-Ti-6_R	95	:H7	27	FIB, FII	*tet(B)*
BS036	95	:H7	27	FIB, FII	*tet(B)*
M638866	95	O1 :H7	27	FIB, FII	*tet(B)*
M644693	95	O1 :H7	27		*tet(B)*
M652995	95	:H7	27	FIB, FII	*bla*$_{TEM-1C}$, *tet(B)*, *tet(A)*
M660875	95	O1 :H7	27	FIB, FII	*tet(B)*
M663475	95	O1 :H7	27	FIB, FII	*tet(B)*
M663940	95	O1 :H7	27	FIB, FII	*tet(B)*
M671335	95	O1:H7	27	FIB, FII	*tet(B)*
M673438	95	O1 :H7	27	FIB, FII	*tet(B)*
M710019	95	O1 :H7	27	FIB, FII, B/O/K/Z	*aph(3'')*, *aph(6)*, *bla*$_{TEM-1C}$, *sul2*, *tet(B)*, *tet(A)*
A1_66_R	95	O1 :H7	27	FIB, FII	*tet(B)*
C2-110	95	O1 :H7	27	FIB, FII	*tet(B)*
C2-117	95	:H7	27	FIB, FII	*tet(B)*
C4-34	95	O1:H7	27	FIB, FII	*tet(B)*
C4-35	95	:H7	27	FIB, FII	*tet(B)*
W1-58	95	O1 :H7	27	FIB, FII	*tet(B)*
W2-47	95	O1 :H7	27	FIB, FII	*tet(B)*
W2-53	95	O1 :H7	27	FIB, FII	*tet(B)*
W4-32	95	O1:H7	27	FIB, FII	*tet(B)*
W4-33	95	O1 :H7	27	FIB, FII	*tet(B)*
W4-35	95	:H7	27	FIB, FII	*tet(B)*
W4-39	95	O1 :H7	27	FIB, FII	*tet(B)*
C66	117	O85 :H18	97	FIB, FIC, X1,X4	
C114	117	O85 :H18	97	FIB, FIC, X1,X4	
2H-103	117	O143 :H4	30	FIB, FII,Q1	*aph(3'')*, *aph(6)*, *bla*$_{TEM-1B}$, *dfrA*, *sul2*, *tet(A)*
2 290	117	O-:H4	97	FIB, FII, I1	*bla*$_{TEM-1B}$, *tet(B)*
2 434_21	117	O111 :H4	97	FIB, FIC,p0111	*aph(6)*, *strA/B*, *dfrA*, *sul1*, *sul2*, *tet(B)*
H030_R	117	O114 :H4	97	FIB, FIC,FII	*aadA5*, *bla*$_{TEM-1B}$, *dfrA*, *sul1*, *tet(A)*
H299	117	O24 :H4	97	FIB, FIC,FII,Q1	*aph(3'')*, *aph(6)*, *bla*$_{TEM-1B}$, *dfrA*, *sul2*, *tet(A)*, *tetC*
H561_R	117	O161 :H4	97	FIB, FII	
C1_123	117	O161 :H4	97	FIB, FII	
C2_102	117	O111 :H4	97	FIB, FIC	*aph(6)*, *strA/B*, *dfrA*, *sul1*, *sul2*, *tet(B)*
C2_106	117	O71 :H4	97	FIB, FII,p0111	*sul2*
C2_97	117	O45:H4	97	FIB, FIC, X1	*bla*$_{CMY-2}$
C3_65	117	O11 :H4	97	FIB, FII	

Strain	Sequence type	Serotype	FimH	Inc type	Antibiotic resistance genes
C3_70	117	O45:H4	97	FIB, FIC,Q1	aph(3''), aph(6), sul2
C3_72	117	O149 :H10	97	FIB, FII,Y	*bla*$_{TEM-1B}$
C4_85	117	O143 :H4	30	FIB, FIC, p0111	dfrA, tet(A)
C4_95	117	O45:H18	97	FIB	*bla*$_{TEM-1B}$, dfrA
C269	131	O16:H5	41	FIB	aadA5, catA1, mph(A), sul1, tet(B)
55-1-TI19	131	O25 :H4	22	FIB, FII	
BS003	131	O25 :H4	22	FIB, FIC	
H005	131	O25 :H4	22	FII, I1, N	
H407	131	O25 :H4	22	FIB, FII	
H392	131	O25 :H4	22	FIB, FII, N	
H346	131	O25 :H4	22	FIB, FII	
H186	131	O25 :H4	22	FIB, FII	
H471	131	O25 :H4	22	FIB, FII	
H183	131	O25 :H4	22	FIB, FII, N	
H136	131	O25 :H4	22	FIB, FII	tet(B)
H114	131	O25 :H4	22	FII, I1, N	
H606	131	O25 :H4	22	FIB, FII	
H712	131	O25 :H4	22	FIC, FII	*bla*$_{TEM-1B}$
M652394	131	O25 :H4	22	FIB, FII	
M683442	131	O25 :H4	22	I1	
M653835	131	O25 :H4	22	FIB, FII	aph(6), *bla*$_{TEM-1B}$, catA1, sul1, tet(A)
H090	131	O25 :H4	22	FIB, FII	
M710059	131	O25 :H4	22	FIB, FII	*bla*$_{TEM-1C}$
W1-65	131	O25 :H4	22	FIB, FIC, I1	*bla*$_{TEM-1B}$
C4-100	131	O25 :H4	22	FIB, FIC, I1	*bla*$_{TEM-1B}$
C3-29	131	O25 :H4	22	FIB, FIC, I1, B/O/K/Z, H12	*bla*$_{TEM-1B}$, tet(A)
A1-49	131	O25 :H4	22	FIB, FIC, I1	*bla*$_{TEM-1B}$
C2-116	131	O25 :H4	22	FIB, FIC, I1, H12	aadA2, *bla*$_{TEM-1B}$, sul1, tet(A)

Table 10 Metadata for ST69,ST95,ST117 and ST131 strains studied (Contd.)

(C% = percentage of cat strains; H% = percentage of human strains; CM% = percentage of chicken meat strains).

	ST69			ST131			ST117			ST95		
Virulence factor	**C%**	**H%**	**CM%**	**C%**	**H%**	**CM%**	**C%**	**H%**	**CM%**	**C%**	**H%**	**CM%**
afaABCD	0	13	0	0	24	0	0	0	0	0	0	0
agn43	22	27	71	20	65	17	0	0	11	100	100	100
air	100	100	86	0	0	0	0	0	0	0	0	0
astA	0	13	86	20	18	33	0	17	22	0	8	0
cvaC	56	7	0	40	0	83	100	83	44	78	75	92
eilA	44	87	86	0	0	0	0	0	0	0	0	0
etsC	0	0	0	0	0	0	0	0	0	100	92	100
fyuA	56	80	14	70	88	100	0	100	67	100	100	100
hlyE	0	0	0	90	0	0	100	100	100	78	0	0
hlyF	67	7	29	0	0	0	0	0	0	0	0	0
hra	22	7	71	0	0	0	0	0	0	0	0	0
iha	0	60	100	10	71	0	0	0	0	78	0	17
ireA	0	7	0	70	0	0	0	83	78	100	100	100
iroN	67	13	29	60	12	100	100	100	67	100	92	100
irp1	56	80	14	60	0	0	0	83	67	100	100	100
irp2	56	80	14	70	0	0	0	100	67	100	100	100
iss	100	87	86	50	6	0	100	50	56	100	100	100
iucA	56	67	86	100	65	100	100	100	100	100	92	100
iucC	56	67	86	100	71	100	100	100	100	100	92	100
iutA	56	67	86	100	71	100	100	100	100	100	92	100
kpsE	100	93	100	10	0	0	0	0	0	0	0	0
kpsMII	67	67	86	10	100	100	0	0	0	0	0	0
lpfA	100	100	100	90	0	0	100	100	100	0	0	0
ompT	100	87	71	100	0	0	100	83	100	0	0	0
papA	0	67	0	10	65	17	0	17	0	100	100	100
papC	0	53	0	10	71	17	0	33	11	100	100	100
papG	0	67	0	10	6	17	0	17	11	100	100	100
pic	0	0	0	60	0	0	0	50	67	22	0	0
sat	0	60	0	10	71	0	0	0	0	0	0	0
senB	0	40	0	10	71	0	0	0	0	0	0	0
sitA	100	100	100	70	65	100	100	100	67	100	100	100
terC	78	93	100	0	29	100	0	0	0	0	0	0
tsh	33	0	14	0	0	0	0	0	0	0	0	0
traT	0	67	86	90	0	0	0	83	89	100	92	100
vat	0	0	0	30	0	0	50	0	22	100	100	100

	ST69			ST131			ST117			ST95		
Bacteriocins	**C%**	**H%**	**CM%**	**C%**	**H%**	**CM%**	**C%**	**H%**	**CM%**	**C%**	**H%**	**CM%**
Colicin-10	0	0	0	83	0	0	0	100	100	11	0	0
Colicin_B	0	20	71	0	0	17	0	0	0	0	0	0
Colicin_E1	0	13	86	75	6	0	50	100	100	100	100	100
Colicin_E6	0	0	0	50	0	0	0	0	100	0	0	0
Colicin_K	0	0	0	50	0	0	0	100	100	0	0	0
Colicin_Ia	0	0	0	0	0	33	0	100	0	0	0	8
Colicin_Ib	0	0	0	100	0	50	0	0	100	11	8	0
Colicin-M	11	0	71	67	0	0	100	100	100	0	0	0
MicrocinB17	0	0	0	0	0	0	100	0	0	22	0	0
MicrocinH47	0	0	0	0	0	0	0	100	0	0	8	8
MicrocinI47	11	0	0	0	0	0	0	0	0	0	0	0
MicrocinM	0	0	0	50	0	0	0	100	100	0	0	0
MicrocinV (ColicinV)	44	7	0	50	0	83	50	75	100	44	33	8
ComX4	0	0	0	75	0	0	0	100	100	11	0	0

Chapter 4 Comparison of ST73 from humans and cats

Introduction

In this chapter, the genotypic and phenotypic attributes of ST73 strains isolated from cats and from humans are compared. As detailed in Chapter 1, ST73 is a major pandemic lineage associated with ExPEC infections in humans, mainly as a leading cause of UTIs, but also bacteraemias. ST73 is considered to be one of the oldest lineages associated with ExPEC and represents an important and successful lineage within phylogroup B2. This sequence type includes the prototype UPEC strain *E. coli* CFT073 and the nonpathogenic *E. coli* strain Nissle 1917; highlighting the diversity among members of this sequence type and providing an example of the genome plasticity of *E. coli* (Zdziarski, et al. 2008).

ST73 has been long considered a predominantly human associated sequence type. However, as a preliminary finding in this study (Chapter 2), the proportion of strains typed as ST73 recovered from faecal samples collected from cats was almost double that found for ST73 *E. coli* recovered from samples collected from people living in the same Canberra region of Australia (refer Table 6). This high prevalence of ST73 incats has been reported in several other studies of clinical isolates (Kidsley, et al. 2019; Liu, et al. 2020). For these reasons, the comparisonof phenotypic and genotypic properties of ST73 strains isolated from cats and humans is considered separately in this chapter.

The aim of this chapter is to demonstrate that cat and human isolates can be differentiated using phenotypic and genotypic testing and that these clear differences can be explained as adaptations to different hosts. In addition, any attributes of strains which are clearly cat-adaptations, but have been isolated from humans, will be examined to determine factors which may explain these instances of strain sharing.

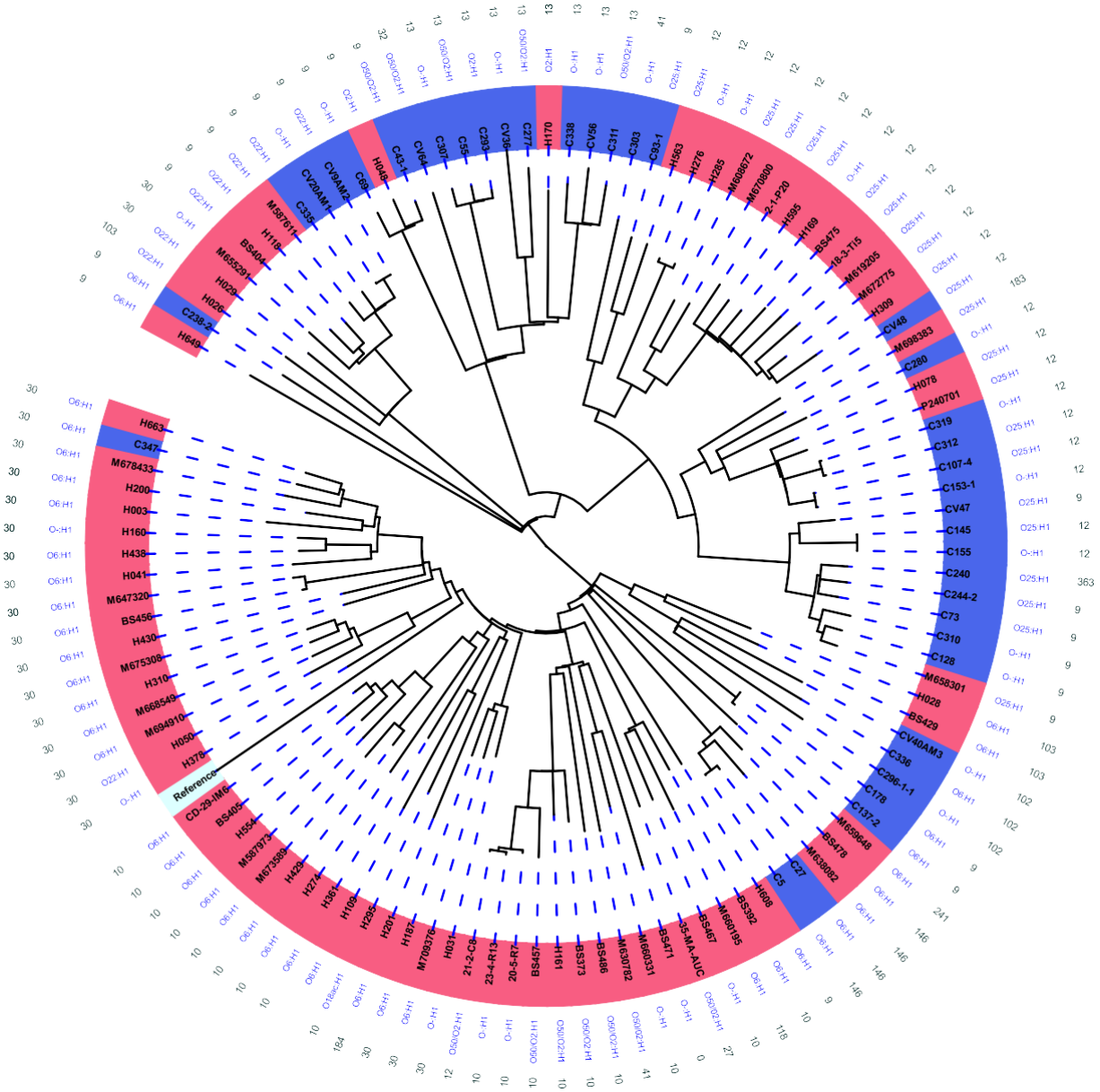
Results

Genotypic assays

In this study the 115 ST73 isolates from humans (n=75) and cats (n=39) were diverse, belonging to 7 different serotypes with O6:H1 and O25:H1 the most common (Table 19; Fig. 17). Most of the human isolates were in the large O6:H1, *fimH*30/10 cluster and most of the cat isolates were untyped O-:H1 and fimH9. Inspection of the core genome phylogeny reaffirms the typical diversity ofthis phylogroup. Isolates separated into at least 8 separate clusters closely aligned with common serotypes including O6:H1, O22:H1 and O50/02:H1 and *fimH* types, including *fimH* types 30,12 and 10. Several clusters were exclusively or almost exclusively comprised of human or cat strains. Other clusters were of strains from mixed sources but with common serotypes and *fimH* types (Fig. 17).

Fig. 17 Phylogeny of ST73 *E. coli* strains (core genome)

The phylogenetic tree was constructed using core genome SNPs inferred by HARVEST tools of the whole genome sequences of 115 *E. coli* ST73 strains (H=75, C=39) isolated in the Canberra region. The innermost ring depicts the source of samples with blue = cat strains; red = human strains. The tree was rooted on the phylogroup B2 reference strain CFT073 and annotated using the web interface Interactive tree of life (iTOL) (Letunic and Bork 2019).



Sub-groups defined

Analysis of the variable gene content data (Jaccard distance matrix) with ANOSIM and PERMANOVA showed that, on average, isolates from cats had a significantly different variable gene content compared to the variable gene content of isolates from humans (AMOSIM and Permanova: $p < 0.0001$). Principal coordinates analysis (PCoA) of the variable gene content of genomes from cats and humans separates strains into 3 clear clusters. One of predominantly isolates from humans, another of prominently cat isolates and one group of strains being intermediate between the other two groups and comprising mainly isolates from humans (Fig. 18).

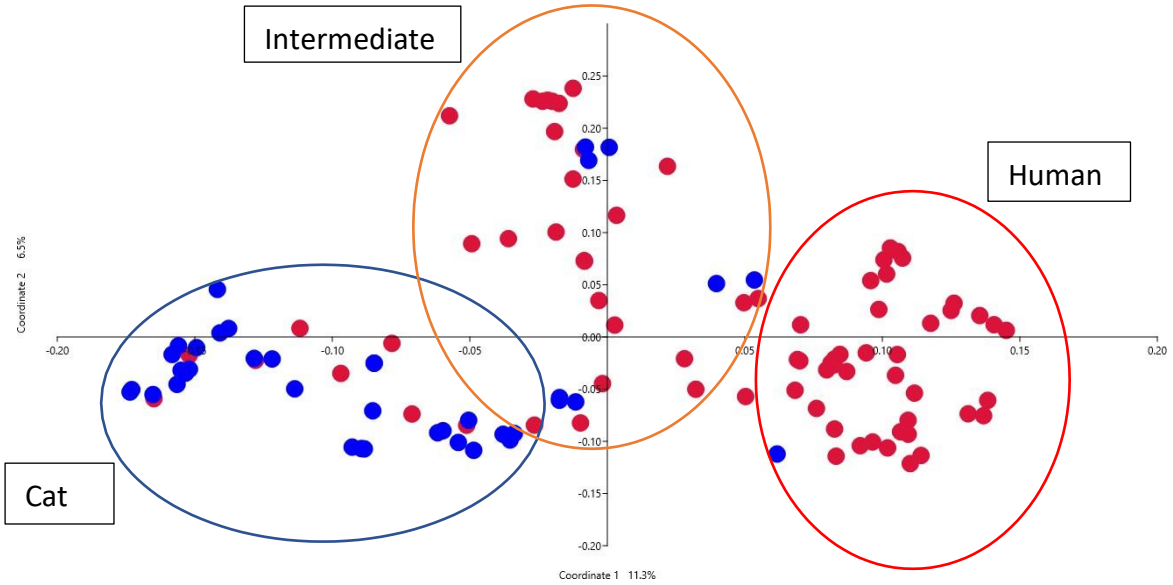


Fig. 18 Principal coordinates analysis of variable gene content of ST73 genomes.
(Blue dots = cat strains, red dots = human strains (PAST))

The machine learning algorithm Random forest (RF) (Chen and Ishwaran 2012; Mageiros, et al. 2021) was used to generate the probability of a strain being isolated from a human based on the variable gene content data. A plot of these probabilities with respect to the actual source of the isolates clearly shows that a large proportion of the isolates from humans were predicted to be from humans, and conversely a large proportion of the isolates from cats had a low probability of being isolated from humans (Fig. 19).

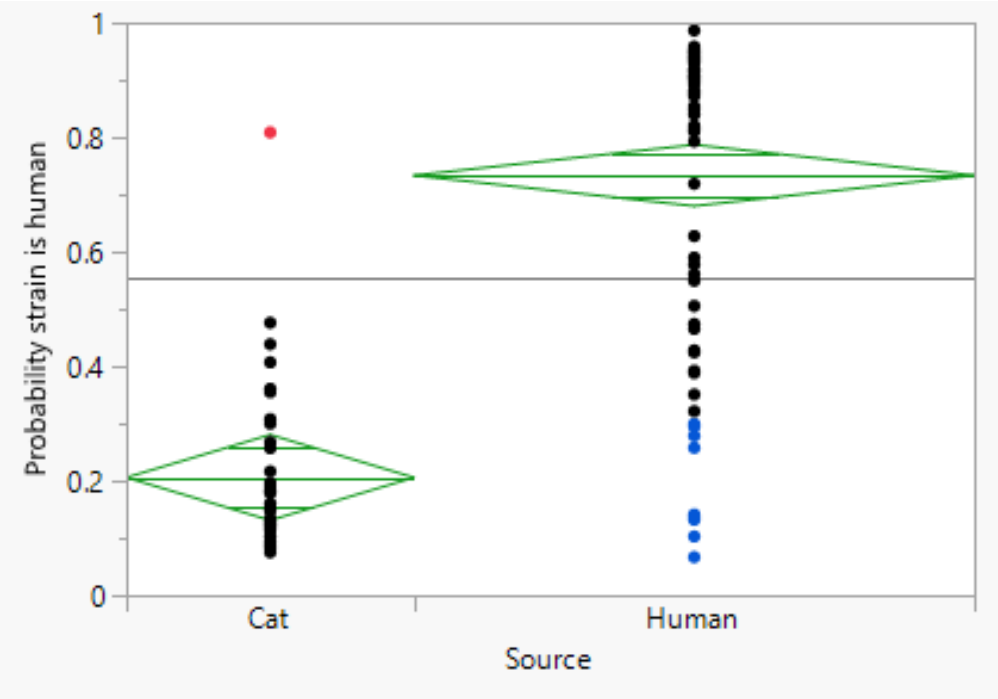


Fig. 19 ST73 strains: source vs predicted '' probability strain is human" strains

(Fit X by Y graph of all ST73 strains; the red symbol indicates a cat strain predicted to be an isolate from a human and the blue dots are isolates from humans with a very low probability of resembling other isolates from humans (that is, predicted to be cat strains))

Cat-like isolates were defined as any isolate with a Random Forest probability of < 0.28 (the upper 95% CI for the isolates recovered from cats) (Fig. 19). Human-like isolates were defined as any isolate with a Random Forest probability of > 0.68 (the lower 95% CI for the isolates recovered from humans) (Fig. 19). Any isolate with a Random Forest probability > 0.280 and < 0.68 were classified as 'other'. One isolate from a cat had a variable gene content that matched those of strains isolated from humans with a high probability. Nine isolated from humans had a variable gene content that matched that of strains isolated from cats (Fig. 19). These cat-like strains were isolated from human faecal (n=3), urine (n=4) and blood (n=2) samples.

Identifying isolates on the PCO graph of the variable gene content based on the assignments generated by the Random Forest probabilities (cat-like, human-like, other) revealed that, as expected, most of the isolates in the group called 'Intermediate' in Fig. 18 were classed as 'Other'. Subsequent analyses were then restricted to human-like (n=53) and cat-like (n=41) classes, excluding strains classified as "other" (n=21) (Fig.20).

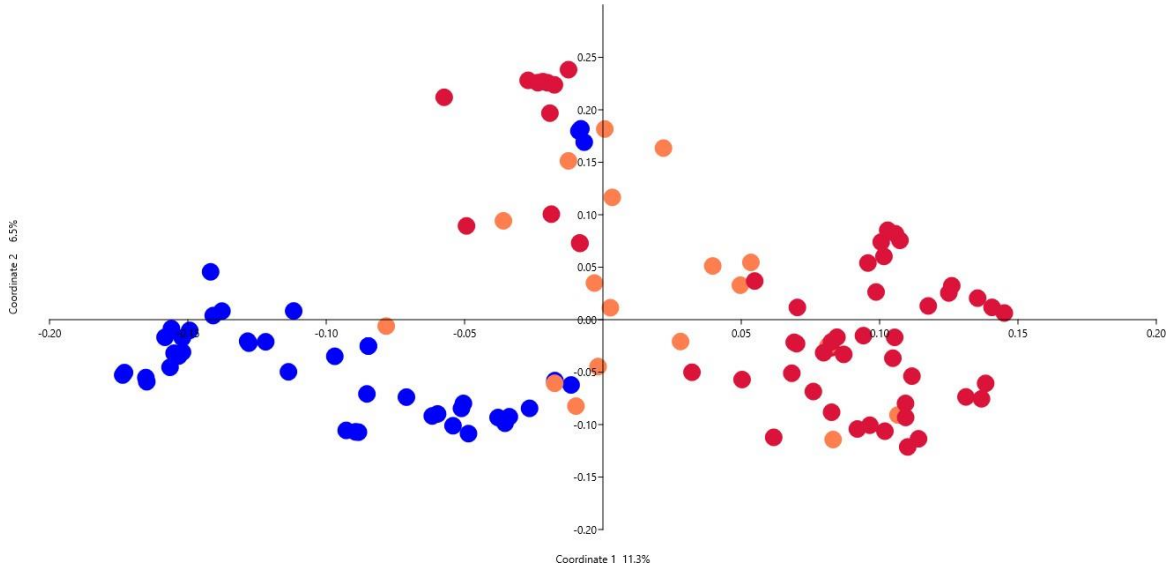


Fig. 20 Principal coordinates analysis of variable gene content of ST73 genomes

(Red dots = human-like cluster (predicted); orange dots = intermediate cluster (predicted); blue dots = cat-like cluster (predicted).)

Identification of cat-like and human-like genome specific genes

Excluding strains classified as ''other'', was predicted to more accurately identify genes which are non randomly distributed between cat-like and human-like strains.

SIMPER, JMP modelling and Random Forest analysis of the variable gene content of cat-like and human-like genomes were used to identify genes which were significantly over-represented in each group. A list of genes present in greater than 70% of cat-like strains but absent or present in a very low proportion of human-like strains is at Table 21.

Discounting insertion sequences and prophages elements the following genes are more prevalent in human-like than cat-like strains; *iraM (elbA), papA, iucA, iucB* and *iucC* and the following genes more prevalent in cat-like strains; *epsM, hisP, ttgI, dcuA_2* and *yjiE_2*.

Correspondence analysis of fewer genes with established associations, that is, antibiotic resistance, virulence, bacteriocin and plasmid associated genes, showed differences in representations in cat-like and human-like genomes (Fig. 21).

Very few genes conferring antibiotic resistance were identified in both cat-like and human-like strains, but the diversity of genes present was greater inhuman-like than in cat-like strains (Table 11).

Antibiotic resistance gene	Cat-like % (n=41)	Human-like % (n=53)
bla $_{\text{TEM-1B}}$	29	55
bla $_{\text{TEM-1C}}$	0	4
SHV-2	2	0
tetA	0	2
tetB	7	11
aph(3')-1c	5	0
aadA	10	23
aac(3)-11d	0	2
catA1	0	4
sul1	10	21
sul2	0	15
strAB	0	8
mph	0	4
dfrA	0	9

Table 11 AR associated genes in ST73 cat-like and human-like genomes

Most cat-like strains lacked antibiotic resistance genes apart from $bla_{\text{TEM-1B}}$. Only 55% of human-like and 29% of cat-like strains carried this gene (p=0.0061). Only 7% of cat-like strains and 30% of human-like strains could be classified as MDR. This is consistent with the general susceptibility to antibiotics that is characteristic of ST73 human clinical isolates (Bengtsson, et al. 2011; Yahiaoui, et al. 2015; Campos, et al. 2018).

In this study, most virulence associated genes, if present, were carried by both human-like and cat-like ST73 isolates. Correspondence analysis shows a human-like strains were more likely to carry the genes *afaD, iha, iucA, iutA, papA, focI, tcpC, traT, sat* and *senB*. Cat-like strains were more likely to carry *cnf1, hlyA, hra, ireA, iroN* and *sfaD* compared to human-like strains (Fig. 21).

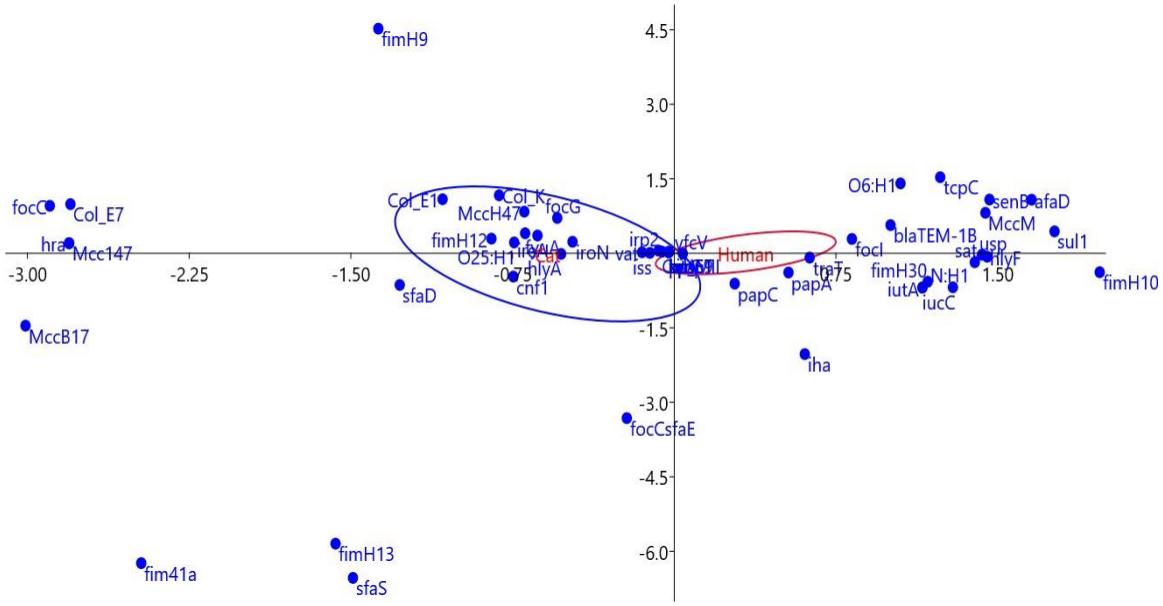


Fig. 21 Correspondence analysis of human and cat-like ST73 variable genes

There are many types of siderophores, or iron chelators, which bind to iron with high affinity. Most of the genes encoding siderophores (eg. *chu, fyuA*) are present in strains from both hosts. However, aerobactin encoding genes (*iucABCD*) partition according to source, as did the genes *iroN* and *ireA*, although to a lesser extent (Table 12).

Gene	Cat-like %	Human-like %	P>χ^2
iucA	21	96	<0.0001
iucB	21	94	<0.0001
iucC	21	98	<0.0001
iucD	14	73	<0.0001
iutA	22	98	<0.0001
ireA	68	30	0.0002
fyuA	100	100	0.0320
chuA	100	100	1.00
iroN	98	72	0.0003

Table 12 Genes associated with iron acquisition in cat-like and human-like genomes

At least one bacteriocin gene was detected in all cat-like and human strains. A significantly greater proportion of cat-like strains had genes for colicins E1 and E7 and microcins I47 and B17 (Table 13). A significantly greater proportion of human-like strains carried microcin M.

Bacteriocin	Cat-like %	Human-like %	P>χ^2
Colicin E1	66	36	0.0036
Colicin E7	51	0	<0.0001
Colicin K	5	2	0.0251
Microcin M	15	53	<0.0001
Microcin H47	54	34	0.0287
Microcin B17	27	6	0.0038
Microcin J25	7	0	0.0238
Microcin 147	68	2	<0.0001

Table 13 Genes associated with bacteriocins in cat-like and human-like genomes

Correspondence analysis shows human strains were more likely to be O6:H1 and cat-like strains O25:H1 (Fig. 21). A diverse range of *fimH* types were identified in all isolates with no single type dominant (Table 19) however, correspondence analysis shows human strains were more likely to be *fimH10* or *fimH30* and cat strains *fimH9* or *fimH12.* No *fimH10* types were detected in cat-like isolates but comprised almost 25% of human isolates in this study. *fimH30* was the most prevalent *fimH* type amongst human isolates but was poorly represented in cat-like genomes in this study.

Plasmid replicons in cat-like and human-like genomes

Searches using Plasmid finder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) identified at least one plasmid incompatability group (InC Group) in most human-like and cat-like genomes (Table 14).

Number of InC Groups detected/strain	0	1	2	3
Cat-like %	32	34	27	7
Human-like %	26	21	28	25
Total %	29	27	28	17

Table 14 Plasmid replicons detected in human-like and cat-like genomes

The most abundant plasmid replicon in both human-like and cat-like genomes was Col156 (Table 15) with no significant difference in proportions of genomes with this replicon. A significantly greater proportion of human-like genomes were IncF (FIB and FII) and a significantly greater proportion of cat-like genomes carried Col RNA type replicons.

Replicon	Cat-like %	Human-like %	P>χ2
Col156	42	57	0.0163
ColRNA	22	2	0.0011
IncFIB	15	42	0.0037
IncFII	27	45	0.0276

Table 15 Proportion of human-like and cat-like genomes with plasmid replicons types

Phenotypic assays

A sub-set of isolates (n=20) from each of the human-like and cat-like groups were subjected to tests todetermine their phenotypic characteristics.

Antibiotic resistance screening

The results of antibiotic screening of all sequenced cat-like isolates (n=39) were almost identical to the antibiotic resistance gene *in silico* findings. One each of ampicillin and tetracycline resistant isolates did not apparently have the corresponding gene. However, this result may be due to short read sequencinginterrupting the respective antibiotic resistance gene.

BIOLOG assays

Of the strains screened with the BIOLOG GENIII and PMB3 systems (n=40) there were few differences between cat-like and human-like strains (Table 22). The majority of both human-like and cat-like strains reduced the carbohydrates D-sorbitol, D-mannose, D-maltose, D-melibiose, D-fructose, D-trehalose, D-galactose, L-lactic acid, glycerol, L-fucose, D-saccharic acid, and the amino acids, L-aspartic acid, D-serine and L-serine. Other organic compounds such as, L-galactonic acid, protein metabolites, eg. guanidine, were reduced by most or all ST73 strains in this collection. Most cat-like and human-like strains reduced the substrate in the presence of stressors, such as osmotic agents, eg. 4% NaCl; surfactants eg. Niaproof 4 and antibiotics, eg. vancomycin, troleandomycin and lincomycin.

The only GENIII substrates with significantly greater mean 24 hour OD 600 nm readings between cat-like strains and human-like strains were D-aspartate and 1% sodium lactate (Table 16). Human-like strains achieved a significantly greater mean 24 hour OD reading for D-gluconic acid, compared to cat-like strains.

Substrate	Average OD(600nm) at 24 hours		P>F
	Cat-like strains	Human-like strains	
D-gluconic	0.560	0.599	0.0295
L-aspartate	0.284	0.276	0.5556
D-aspartate	0.291	0	<0.0001
1% sodium lactate	0.879	0.812	0.0229
pH5	0.611	0.563	0.2343
pH6	0.906	0.859	0.0116
Fusidic acid	0.274	0.404	0.0001
Minocycline	0	0.157	0.0363
Rifamycin	0.981	0.937	0.0413

Table 16 Average OD(600nm) result in a selection of GENIII substrates

Cat-like strains had a significantly higher mean 24 hour OD reading than human-like strains growing in pH6 (Table 16). This difference could be an anomaly as mean OD of cat-like strains in the presence of pH5 was greater but not significantly different. Significantly more human-like strains had a higher 24 hour OD reading than cat-like strains growing in the antibiotic fusidic acid (Table 16).

Adhesion assays

Two techniques were used to assay phenotypic expression of genes associated with adhesion of cat-like and human-like ST73 strains. Biofilm formation detected by crystal violet staining was greater in cat-like than human-like strains. Mean OD 590nm of cat-like strains was 0.475 and of human-like strains was 0.328 after 48 hours static growth. This difference was significant (P>F, p = 0.0009).

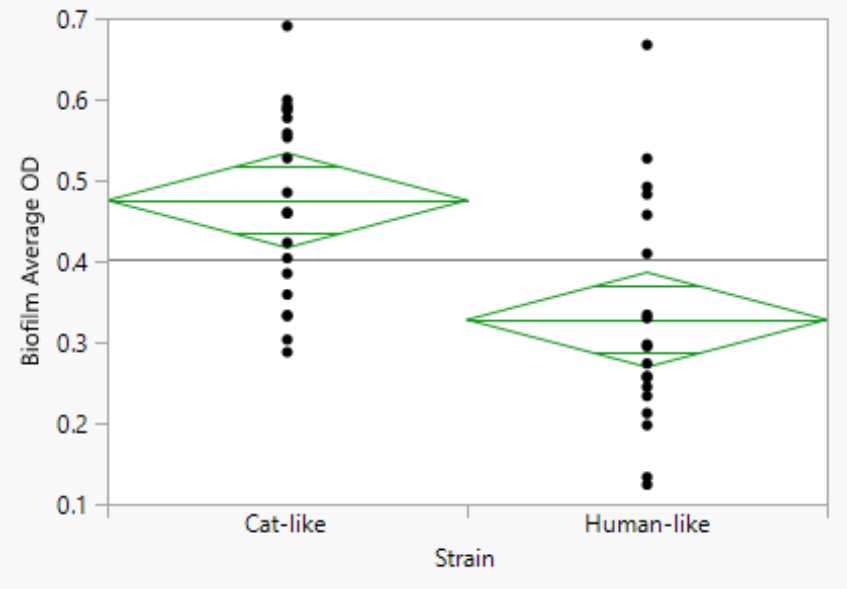


Fig. 22 Biofilm OD(590nm) for cat-like and human-like strains

Bacterial cell adhesion was also assessed by testing the capacity of cells from overnight shaking cultures of human-like strains (n=20) and cat-like strains (n=20) to agglutinate yeast cells, according to the standard method for monitoring the production of functional type 1 fimbriae (Totsika, et al. 2011). Human-like isolates were usually negative for yeast agglutination (90%) whereas cat-like isolates were usually positive (90%) ($p<0.0001$) indicating type 1 fimbriae expression. This result strongly negativelycorrelates with *papA* presence in the strains tested (human-like 95%; cat-like 0%) but less so with *papB, fimH* type or serotype (Table 17).

Strains	Predicted Source	Serotype	*fimH* type	Yeast Agglutination	*papA*	*papB*	*sfa genes*
BS373	Human	050/02:H1	10	+	+	+	0
BS392	Human	O6:H1	10	-	+	+	0
BS405	Human	O6:H1	10	-	+	+	0
BS456	Human	O6:H1	30	-	+	+	1
BS475	Human	O25:H1	12	-	+	-	0
H187	Human	O6:H1	30	-	-	+	0
H309	Human	O25:H1	30	-	+	+	0
H310	Human	O6:H1	30	-	+	+	0
H429	Human	O6:H1	10	+	+	-	2
H554	Human	O6:H1	10	-	+	+	0
M587973	Human	O6:H1	10	-	+	-	0
M619205	Human	O25:H1	12	-	+	+	0
M668549	Human	O6:H1	30	-	+	+	0
M672775	Human	O25:H1	12	-	+	+	1
M673589	Human	O6:H1	10	-	+	+	0
H285	Human	O-:H1	10	-	+	+	0
M694910	Human	O6:H1	30	-	+	-	0
M709376	Human	O6:H1	30	-	+	+	0
G_20-5-R7	Human	O-:H1	10	-	+	+	0
H050	Human	O6:H1	30	-	+	-	0
H078	Cat	025:H1	12	+	-	+	4
P240701	Cat	O25:H1	12	+	-	-	5
M658301	Cat	O25:H1	9	+	-	+	1
C128	Cat	O-:H1	9	+	-	+	2
C145	Cat	O25:H1	12	+	-	-	5
C153	Cat	O-:H1	12	+	-	-	3
C155	Cat	O-:H1	12	+	-	-	5
C240	Cat	O25:H1	363	+	-	+	3
C293	Cat	O2:H1	13	+	-	+	3
C296	Cat	O-:H1	102	+	-	-	1
C303	Cat	O-:H1	41	-	-	-	1
C307	Cat	O-:H1	13	+	-	-	1
C310	Cat	O-:H1	9	+	-	-	3
C311	Cat	O50/O2:H1	13	+	-	+	3
C312	Cat	O25:H1	12	+	-	-	6
C319	Cat	O-:H1	12	+	-	-	6
C338	Cat	O-:H1	13	+	-	+	3
C55	Cat	O50/O2:H1	13	-	-	-	3
CV36	Cat	O50/O2:H1	13	+	-	+	2
CV47	Cat	O25:H1	9	+	-	-	4

Table 17 Yeast agglutination results

Average of 3 assays of a subset of human-like strains and cat-like strains with corresponding *fimH* type and serotype of each strain. *Sfa* genes = number of *sfa* alleles present (- =no agglutination, +=agglutination)

Human-like and cat-like strains differed in carriage of *pap* and other adhesin genes (Table 17). Of the adhesin genes identified using the CGE virulence finder tool, *sfaD* and *focCG* explained the positive yeast agglutination result whereas *afaD, focI* and *papA* explained the negative result (Table 18).

Adhesin gene	No agglutination (%)	Yeast agglutination (%)	P>F	Number of isolates with gene
afaD	100	0	p<0.0001	10
focC	7	93	p<0.0001	14
focCsfaE	56	44	0.0747	9
focG	48	52	0.0747	31
focI	71	29	0.0009	24
iha	44	56	0.5182	16
papC	50	50	1.00	22
papA	82	18	0.0003	17
papG	80	20	0.1394	5
sfaD	14	86	0.0006	14
sfaS	17	83	0.0659	6

Table 18 **Proportion of strains with adhesin genes vs yeast agglutination result** (CGE search)

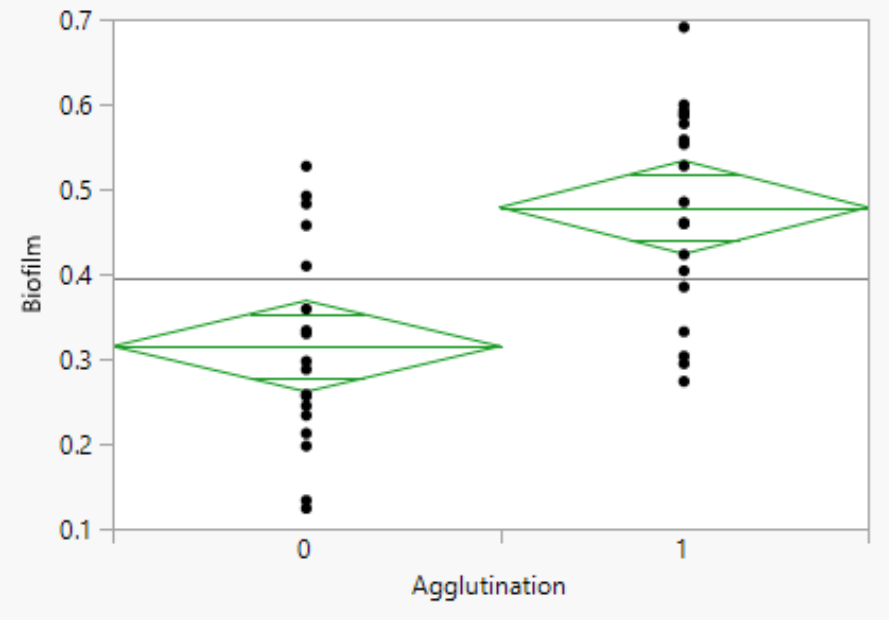


Fig. 23 Biofilm OD (590nm) vs yeast agglutination result - cat-like and human-like strains

Strains which were positive in the yeast agglutination assay were significantly more likely to form more biofilm as measured at OD590nm) (P>F, p = 0.0001) than negative strains.

Testing growth of isolates in D-aspartate

Given the clear difference in the metabolism of the amino acid, D-aspartate by human-like strains (n=20) and cat-like strains (n=20) as shown by the GENIII Biolog assay, it was decided to measure growth of a greater number of isolates in minimal media supplemented with 0.1% D-aspartate (7.5 mM). In this assay, D-aspartate was the only carbon source available. The OD reading after 24 hours growth under aerobic conditions for the cat-like (n=34) and human-like (n=43) strains confirmed this difference between the clusters (Fig. 24) (P>t, p = 0.0001).

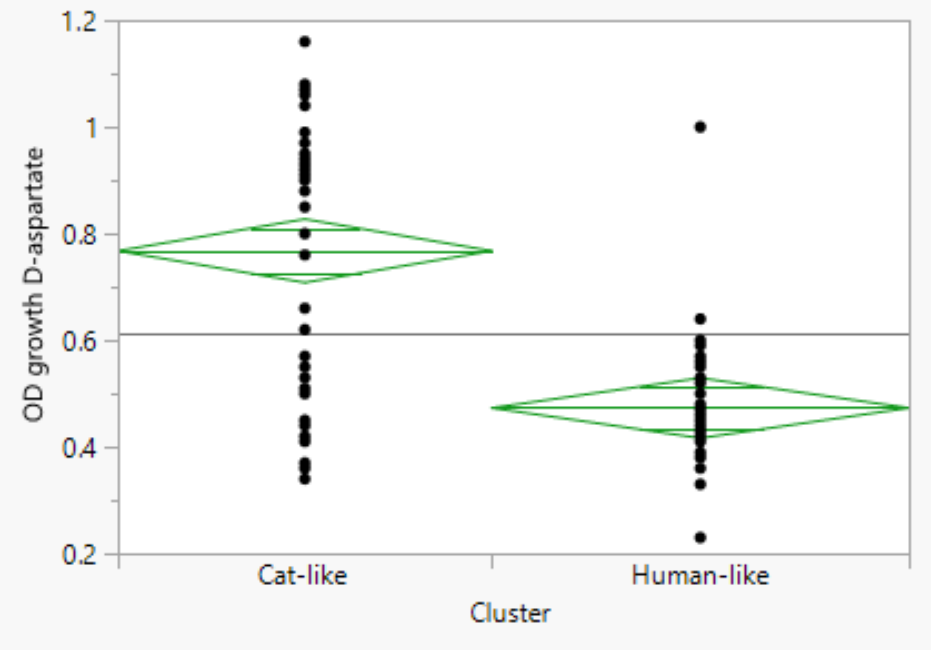


Fig. 24 Growth of cat-like and human-like strains in 0.1% D-aspartate
(Supplemented in minimal media (7.5 mM), aerobic conditions)

Strains which reduced D-aspartate in the BIOLOG GENIII assay were significantly more likely to grow in 0.1% D-aspartate supplemented in minimal media (7.5 mM) (prob>F 0.0001) (Figs. 25 and 26).

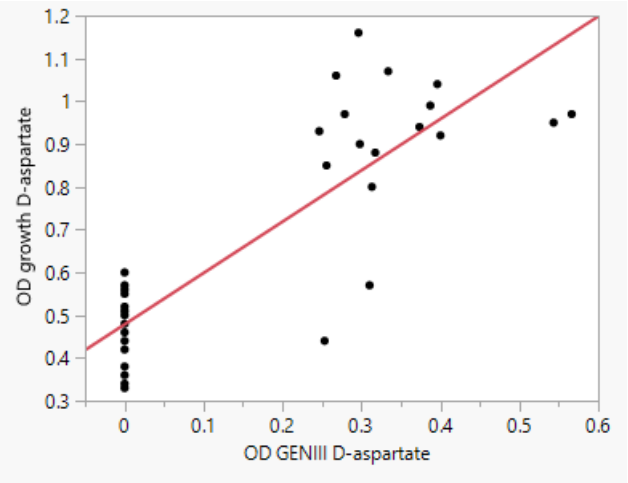


Fig. 25 Growth in 0.1% D-aspartate by OD in BIOLOG GENIII D-aspartate

(Bivariate fit OD (590nm): cat-like and human-like ST73 strains)

A search of the variable gene content of all ST73 strains for genes associated with aspartate metabolism and transport found that every strain carried one copy of the *dcuA* gene but significantly more cat-like strains (67%) than human-like strains (6%) ($P>X^2$, $p<0.0001$) carried a second copy of this gene, *dcuA_2*).

Carriage of the *dcuA_2* gene is significantly correlated with both growth in the presence of D-aspartate supplemented in minimal media (P>F, p<0.0001) (Fig. 26) and the GENIII OD result (P>F, p<0.0001) (Fig. 27).

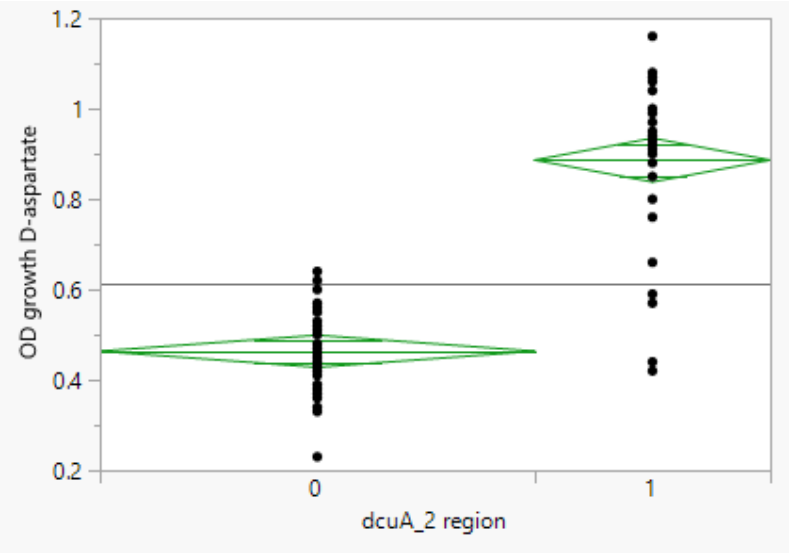


Fig. 26 Growth of ST73 strains in 0.1% D-aspartate vs presence of *dcuA_2* gene
(OD(590nm) of cat-like and human-like strains)

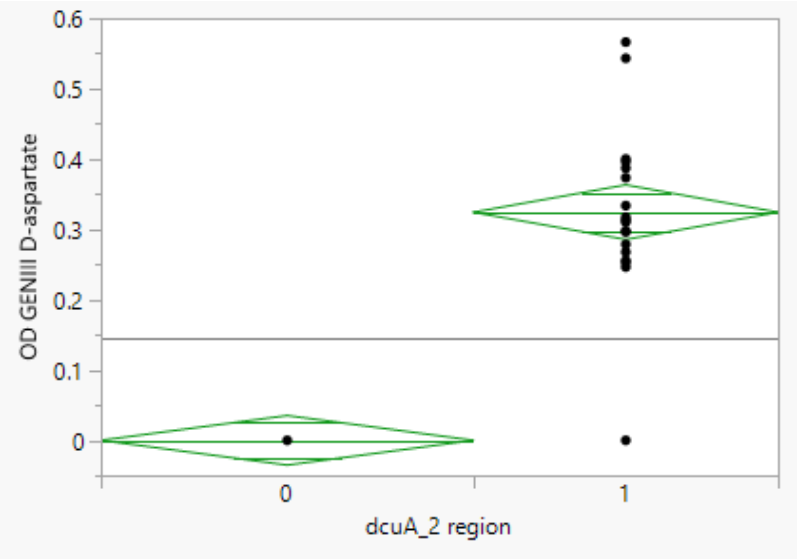


Fig. 27 OD(590nm) for D-aspartate in GENIII assay vs presence of *dcuA_2* gene
(OD(590nm) of cat-like and human-like strains)

The ability to metabolise D-aspartate and the presence of an additional *dcu* gene in many of the cat strains are distinctive source differences. Aspartate, in common with other C4-dicarboxylates, fumarate, maleate, malate and succinate, is an energy source used by *E. coli* during both aerobic and anaerobic growth. The integral inner membrane protein carriers DcuA, and DcuB, encoded by homologous genes *dcuA* and *dcuB* transports these C4-dicarboxylates into the cell (Zientz, et al.

1996; Lourenço, et al. 2016). A two-component regulatory system, designated DcuS-DcuR, regulates the expression of *dcuB* and other genes in response to external C4-dicarboxylates (Reitzer 2004). The frequencies of *dcuS, dsuR* and *dcuB* genes are the same in cat-like and human-like strains.

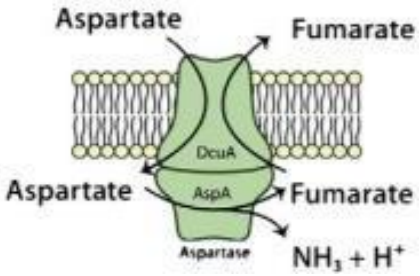


Fig. 28 Diagram of the AspA/DcuA transporter (Beatson, et al. 2015)

The *dcuA* gene is adjacent to the aspartase gene (*aspA*), forming the *dcu/asp* operon (Fig. 29). Both genes were detected in all strains in this study. The *aspA* gene encodes aspartase A (aspartate ammonia-lyase) which converts L-aspartate to fumarate. Aspartate enters the cell via DcuA which is then converted to fumarate (Fig. 28). Fumarate is transported out of the cell in exchange for aspartate, leaving ammonium inside the cell (Moraes 2012). Fumarate enters the cell via DcuB and is converted to malate directly by FumB. Malate then exits the cell via the same transporter in an antiport mechanism.

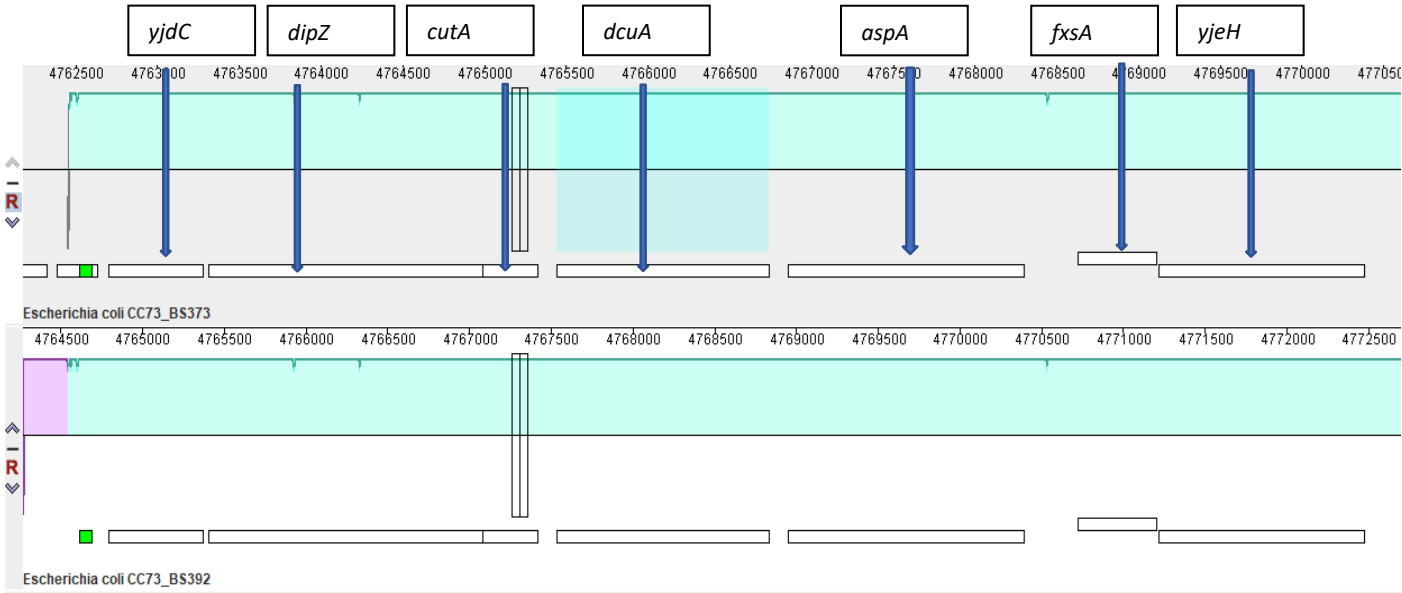


Fig. 29 Genes associated with the *dcuA* gene in ST73 strains

(Progressive Mauve alignment; CC73_BS373 and CC73_BS392 are human-like ST73 strains)

In contrast to the strong regulation of *dcuB*, the *dcuA* gene is expressed constitutively under aerobic and anaerobic conditions independent of the carbon source, virtually unaffected by environmental and regulatory factors (Six, et al. 1994; Golby, et al. 1998). DcuA has a general function in C4-dicarboxylate transport whereas DcuB primarily mediates C4-dicarboxylate transport during anaerobic fumarate respiration (Golby, et al. 1998). DcuB represents the major C4-dicarboxylate/succinate antiporter of fumarate respiration (Zientz, et al. 1999). Expression of this gene is fully repressed under aerobic conditions.

In contrast to the arrangement of genes associated with *dcuA*, different genes are associated with the *dcuA_2* genes, which are mainly present in cat genomes (Fig. 30). The second copy of *dcuA* is flanked by *ygeA* (aspartate racemase gene) which encodes the putative amino acid racemase YgeA (Miyamoto, et al. 2017). This racemase converts a range of amino acids including non-canonical amino acids such as D-aspartate to the respective stereoisomer.

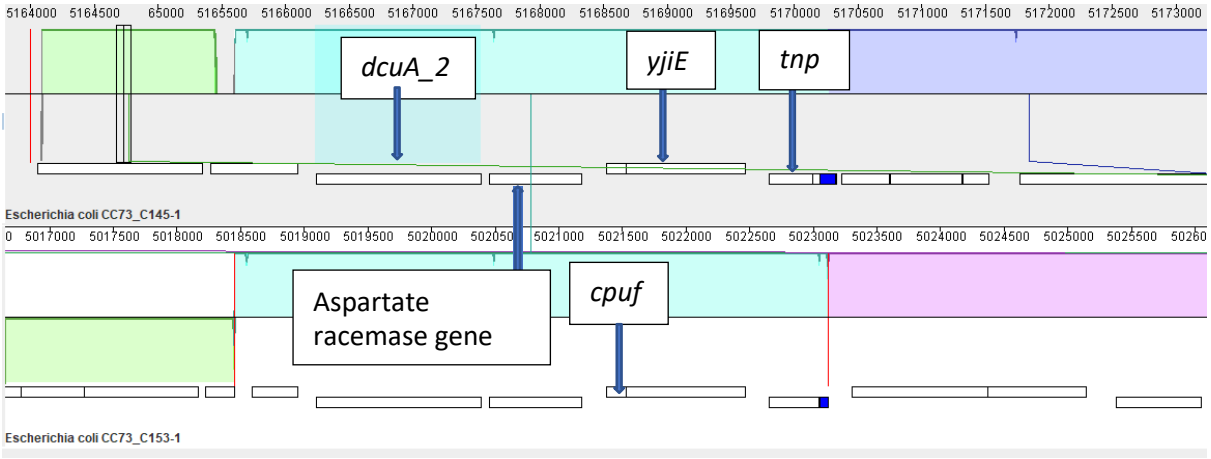


Fig. 30 Genes associated with *dcuA_2* gene

(Progressive Mauve alignment; CC73_C145-1 and CC73_C153 are cat-like strains carrying a second copy of *dcuA* (cpuf = conserved protein of unknown function))

In summary, all cat and human strains carry one copy of each of these transmembrane genes associated with *fumB* and *aspA*, but, significantly more cat strains carry a second copy of the *dcuA* genes in association with an aspartate racemase. These additional genes may represent an adaptation of resident *E. coli* in cats to differences in available nutrients as well as the anaerobic conditions in the hindgut.

Discussion

The data presented here, obtained from isolates collected from clinical and commensal human and commensal feline sources show a highly diverse population of circulating ST73 strains. The strains in this collection of strains can be separated into at least 3 groups based on core genome SNPs inferred relationship amongst all cat and human genomes. Several of the subgroups though are comprised largely of either cat or human strains and the others a mix from both sources (Fig.17). Substructure within ST73 strains has been reported by others. ST73 bacteraemia isolates collected from across England over an 11 year period (n=261) were divergent, and assigned to nine serotypes with different serotypes represented in different lineages. These isolates were not closely related and could be separated into at least eight clades (Kallonen, et al. 2017). Four major groups were identified among an international collection of mainly human sourced ST73 isolates (n=210) including those from a single hospital over a 2 year period (n=16) (Bogema, et al. 2020). Both studies concluded that clusters correlated with serotype. Strains in this study can be grouped into the four broad clusters identified by Bogema (Bogema, et al. 2020) largely correlating with serotype and *fimH* type. Human strains were mainly in two clusters: O6:H1 *fimH10/30* and O25:H1 *fimH*12 groups. Cat strains were mainly O25:H1 *fimH*12/9 and O2/O50:H1 *fimH* 9/13. The cluster containing O22:H1 *fimH* 9/30 strains comprised both cat and human strains. Thus, representation of *fimH* and serotypes in ST73 isolates in this study is similar to that reported for isolates in other collections from humans and companion animals overseas (Kallonen, et al. 2017) and in Australia (Bogema, et al. 2020; Kidsley, et al. 2020). Therefore, the strains studied here are broadly representative of ST73 isolates.

Despite this diversity and substructure there are clear difference at the phenotypic and genotypic level between cat and human isolates. At the genotypic level many of these differences represent hypothetical protein genes and it is only possible to speculate about the function of these genes. One example is the ‘gene’ named Group 2002, which is only present in human-like genomes. A BLASTn search of Group 2002 found an identical sequence match to the gene which encodes a protein in the AAA family ATPase (also ATP binding proteins). AAA stands for ‘ATPases Associated with diverse cellular Activities.’ The diverse functions of this family of ATPases include protein degradation, membrane complex maturation, gene expression, homo-and heterotypic membrane fusion, and microtubule disassembly (Frickey and Lupas 2004). However, there are at least 79 ABC proteins classified, making this the largest paralogous family of proteins in *E. coli* (Linton and Higgins 1998) rendering it difficult to determine what the over-representation of this gene in human-like compared to cat-like isolates might mean in terms of the fitness benefit conferred by this gene.

Speculation about the potential adaptive value of genes which have a known function, and which are present in different proportions of cat and human isolates is possible. This is the case with a greater proportion of strongly ExPEC related virulence associated genes such as *papA* and *iucA* in human-like strains compared to cat-like strains. Also, this study has found clear phenotypic differences between strains from the two sources which invite one to speculate on the genotypic origins explaining these differences. This is the case with the adhesin assay (yeast agglutination and biofilm formation) and D-aspartate growth results. These differences potentially provide a fitness advantage to strains in each host species.

Fim genes

Cat-like strains were more likely than human-like strains to test positive in the yeast agglutination assay (Table 17) and give higher OD 590nm values for biofilm formation. These assays detect the presence and expression of type 1 fimbriae encoded by *fim* genes which bind to mannose-containing glycoprotein receptors like uroplakins on the surfaces of bladder epithelium (Schembri and Klemm 2001; Mulvey 2002) and mannose-containing receptors on human colonic epithelium and intestinal mucus (Wold, et al. 1988). These fimbriae are widely distributed among non-pathogenic, commensal and laboratory strains of *E. coli* strains (Werneburg and Thanassi 2018) and regarded as a major virulence factor of UPEC (Connell, et al. 1996).

Type 1 fimbriae have a complex structure with structural, chaperone/usher and regulatory subunits (Fig. 31).

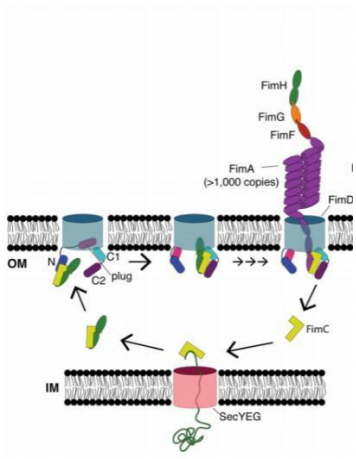


Fig. 31 Model of F fimbria assembly (Werneburg and Thanassi 2018)

The structural, assembly and regulatory components of the type 1 fimbriae are encoded by the chromosomally located *fim* gene cluster (Schembri, et al. 2000). The major fimbrial subunit FimA forms the helical rod proximal to the cell surface. FimB and FimE are regulatory proteins involved in phase variation of type 1 pilus expression. FimC is the pilus chaperone and FimD the usher protein. FimF and FimG are adapter subunits present in the distal tip fibrillum, and FimH is the tip-associated adhesin (Fig. 31). The function of FimI is uncertain but is probably the rod-terminating subunit. All of the genes encoding these subunits were detected in both cat-like and human-like strains and did not partition according to source.

The yeast agglutination assay is regarded as an efficient screening assay for the detecting bacteria that express functional FimH (Schembri, et al. 2000). As shown in Table 17, there are multiple *fimH* alleles but these are present in a similar proportion of predicted human and cat strains. Consequently, differences in assay results cannot be explained as a difference in the proportion of cat-like and human-like strains with any of the *fim* genes.

So rather than look for differences in *fim* gene representation to explain the results in Table 17, I have looked for evidence of differences in genes regulating expression of these genes. Production of fimbriae is tightly regulated as it involves the expenditure of considerable resources by cells. Restricting the expression of these fimbrae during a specific phase of colonisation of the gut or infection would likely conserve protein and energy use by the cell and thus increase fitness of *E.coli* strains (Schembri, et al. 2002). This phase variation is thought to ensure that fimbriae are expressed when attachment is of benefit and avoid disadvantages like an antibody response by the host or incurring the metabolic cost of producing fimbriae (Xia, et al. 2000; Snyder, et al. 2005; Spurbeck and Mobley 2013).

Regulation of *fim* gene expression would be expected to be an advantage to *E.coli* during colonisation of the hindgut. Here, even though mannose-specific type 1 fimbriae are expressed by *E. coli*, the ability to attach to mannose-containing receptors may be a disadvantage as secretory immunoglobulin A, which is abundant in the gut lumen, binds and agglutinates mannose-specific *E. coli* (Wold, et al. 1988). This binding would be expected to reduce the likelihood of survival and thus colonisation of the gut by bacteria expressing type 1 fimbriae.

Bacteria with adhesin molecules specific to gut epithelium therefore would be expected to better colonise the gastrointestinal tract. Duration of colonisation of commensal bacteria has been correlated with type of attachment. *E. coli* in the colon have been classified as resident strains which persist for months or years or transient strains which are rapidly replaced (Sears, et al. 1956).

Resident *E. coli* are more likely to be P-fimbriated compared to transient strains (Tullus, et al. 1992; Wold, et al. 1992; Adlerberth, et al. 1998) likely because receptors for P fimbriae are also found on colonic epithelial cells and resident strains have greater expression of P fimbriae than transient strains (Wold, et al. 1988). That is, P fimbriae, rather than type 1 fimbriae seem to contribute to the ability of the *E. coli* strain to colonise the human intestine (Tullus, et al. 1992).

This phase variation would potentially also be an advantage if opportunistic UPEC gain entry to the urinary tract. During initial stages of urinary tract infections, *fim* gene expression is increased while bacteria invade the surface and underlying superficial umbrella cells of the bladder to form intracellular bacterial communities (IBCs) (Wright, et al. 2007). When bacteria leave the IBCs and disseminate throughout the bladder as free-living (planktonic) cells in urine, expression of *fim* genes is turned off (Stærk, et al. 2016).

Expression of fimbrial components is hypothesized to be transient, controlled through several complex mechanisms mostly by affecting transcription originating at the major promoter of the operon (Xia, et al. 2000; Werneburg and Thanassi 2018). This results in variable (on/off) expression of most or all genes in the *fim* operon (Van Der Woude and Bäumler 2004). Changes in the environment initiate changes in bacteria that affect the level of regulatory proteins, which in turn control this phase switching ability. Transcription of the *fim* operon depends on the orientation of the *fimS* invertible DNA element, which is the promoter for the *fimA* structural gene (Schwan 2011). The orientation of the *fimS* invertible element is controlled by FimB and FimE and PapB. Type 1 fimbriae are formed with FimB formation (switch-on). PapB, in concert with FimE, inhibit phase transition (turn-off). However, these regulatory *fim* genes, *fimB* and *fimE*, were present in all or most strains, so cannot explain the agglutination and biofilm assay results. Presence of one of the *fim* gene expression regulators, *papB*, in strains, was negatively correlated with yeast agglutination (Table 17). However, there was not complete correspondence and almost half (40%) of the cat strains carried *papB*. Another putative regulatory gene, *sfa* was absent from most human-like genomes whereas cat-like strains carried one or more of the *sfa* subunit genes (Table 17) and there was strong positive correlation with yeast agglutination. The S fimbrial adhesin has a structure similar to type 1 and P fimbriae (Mulvey 2002). S pili regulatory proteins SfaB and SfaX, suppress expression of type 1 fimbriae (Marklund, et al. 1992; Xia, et al. 2000; Holden, et al. 2001), however, neither *sfaB* or *sfaX* were detected in human-like or cat-like genomes and if present, a negative correlation would be expected. Many other regulatory genes are described (Van Der Woude and Bäumler 2004), for example, *hns* and *lrp* but these are present in all strains.

Annotated genes which might explain the phenotypic result do not partition according to source. So the precise gene or genes regulating this phase variation cannot as yet be identified but may be amongst the uncharacterised genes present predominantly in human strains. This uncertainty is consistent with others acknowledging that understanding of the regulation of fimbrial expression is still incomplete (Spurbeck and Mobley 2013).

Pap genes

A gene that clearly partitions according to the assay results is *papA* (Table 17). This gene encodes PapA, the major component of P fimbriae which are the other major ExPEC associated adhesins (Werneburg and Thanassi 2018). P fimbriae are transcribed from the *pap* gene operon. *papC* which encodes PapC (Fig. 32), the usher for P fimbriae, is present in all strains. *papI* and *papB* are genes supposedly controlling expression of pap expression however, *papI* was not detected in strains studied and *papB* was only in 75% of human-like strains assayed for agglutination. The fimbrial tip subunit, PapG recognises the Galal 4Gal13 moiety on urinary tract cells surfaces (Werneburg and Thanassi 2018) is present in only 30% of the human-like ST73 genomes.

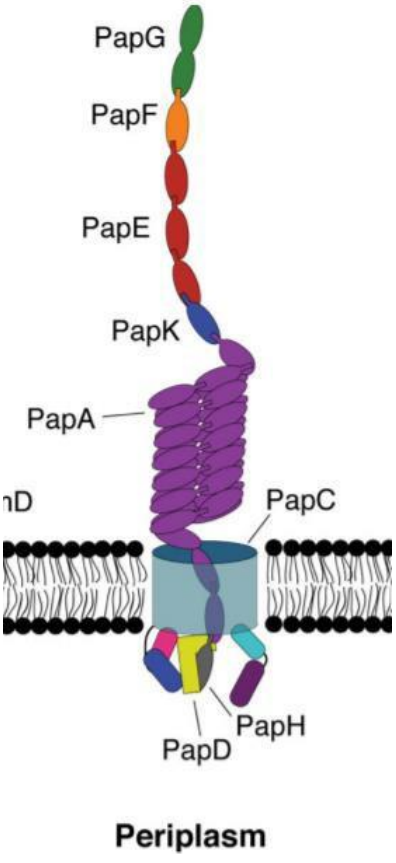


Fig. 32 Model of P fimbria (Werneburg and Thanassi 2018)

Other significant genes encoding the chaperone (PapD) and the rod-terminator that determines pilus length and anchors the fimbrial fiber in the outer membrane (PapH) as well as adapter subunits that form the distal P fimbrial tip fiber (PapK and PapF) were not detected in the strains in this study.

As P fimbriae, rather than type 1 fimbriae seem to contribute to the ability of the *E. coli* strain to colonise the human intestine (Tullus, et al. 1992), the partitioning of the key structural gene *papA* may indicate a host preference. This difference in gene representation applied to all strains studied and there was no significant difference between sample types (faeces, blood, urine). *PapA* was detected in 3% of all cat strains and 59% of all human strains. However, a much higher prevalence of *papA* (75%, n=12) was reported in ST73 UPEC isolates from cats (Liu, et al. 2015) and an unstated proportion in isolates in another study (Kidsley, et al. 2020). Complete absence of *papA* from cat-like strains in this study suggests these genes are host specific, but this finding is not consistent with results from studies of clinical isolates.

Thus, the clear phenotypic difference, that is the adhesin assay results, cannot be functionally associated with carriage of the *papA* gene. Also, neither attribute appears to be associated with any fitness advantage with regard to gut colonisation. However, there are many adhesins available to *E. coli* (Soto and Hultgren 1999) so it is possible that conditions in the hindgut of cats in this study favour strains with alternative means of epithelial adhesion, expressing genes which also promote yeast agglutination.

Other genes identified as adhesins (Sarowska, et al. 2019) were either not detected in the genomes analysed (*dra),* were present in all cat-like and human-like genomes (*iha, mae, csg*) or partitioned according to source and yeast agglutination result. Generally *pap* and *afa* genes were in human-like genomes and *sfa* and *focC* genes were detected in most cat-like genomes. Genes which regulate expression of type 1 fimbrial genes, would be expected to be present in a greater proportion of human isolates. SIMPER testing of strains according to the agglutination result yielded virtually the same suite of genes predicted when isolates were apportioned with respect to agglutination as when apportioned with respect to host of isolation.

Stress response

Sublethal environmental adversity, such as nutrient scarcity or exposure to acid or low oxygen, elicits a stress response in *E. coli.* This response promotes resistance to environmental changes or deficiencies.

The stress response varies between commensal and pathogenic *E. coli* and between strains of pathogenic *E. coli* (Chung, et al. 2006). Bacteria sense and respond to environmental stresses using signal transduction systems to initiate and control expression of genes to protect and/or adapt cells. This response often involves sigma factors. The alternative sigma factor σ^S (RpoS), is the major regulator of the stationary phase or general stress response in *E. coli* and otherenteric bacteria. RpoS is considered to be a key factor in the cellular response to stationary phase and stress.

The stress response gene, *iraM* (alias *elbA*) is another key gene clearly specific to human-like isolates. This gene encodes the anti-adaptor protein IraM, an important component of the stress response to Mg2+ and Ca2+ starvation and acid exposure. Exposure to low extracytoplasmic magnesium detected by the sensor protein PhoQ modifies the phosphorylated state of the DNA-binding protein PhoP. The altered PhoP protein controls the expression of a large number of genes that mediate adaptation to low Mg2+ environments and regulates acid resistance determinants (Zwir, et al. 2005).

IraM also inhibits σ^S proteolysis in response to Mg^{2+} and Ca^{2+} starvation. σ^S is degraded by the protease ClpXP. For σ^S to be recognized as a substrate by the protease, σ^S must interact with RssB, an adaptor protein (Battesti, et al. 2012). IraM is one of at least 3 antiadaptor proteins (IraD, IraP and IraM) which interact with RssB thus preventing binding to σ^S. By this means IraM stabilises σ^S allowing maintenance of the stress response.

IraM is also involved in acid resistance even though this was not expressed in the Biolog assay. Exposure to acid conditions initiates a cascade of reactions. EvgS/EvgA activates the acid resistance gene regulation (EvgA-YdeO-GadE) and the PhoQ/PhoP system via SafA (Fig. 33). This leads to an enhanced level of cellular σ^S via IraM. σ^S induces GadE, the central regulator of acid resistance genes, which together provide the cell with acid resistance (Eguchi, et al. 2011). All human-like and most cat-like genomes have the *evgA/evgS* genes, all have *gadE, phoP/Q* and most (>90%) have *ydeO*.

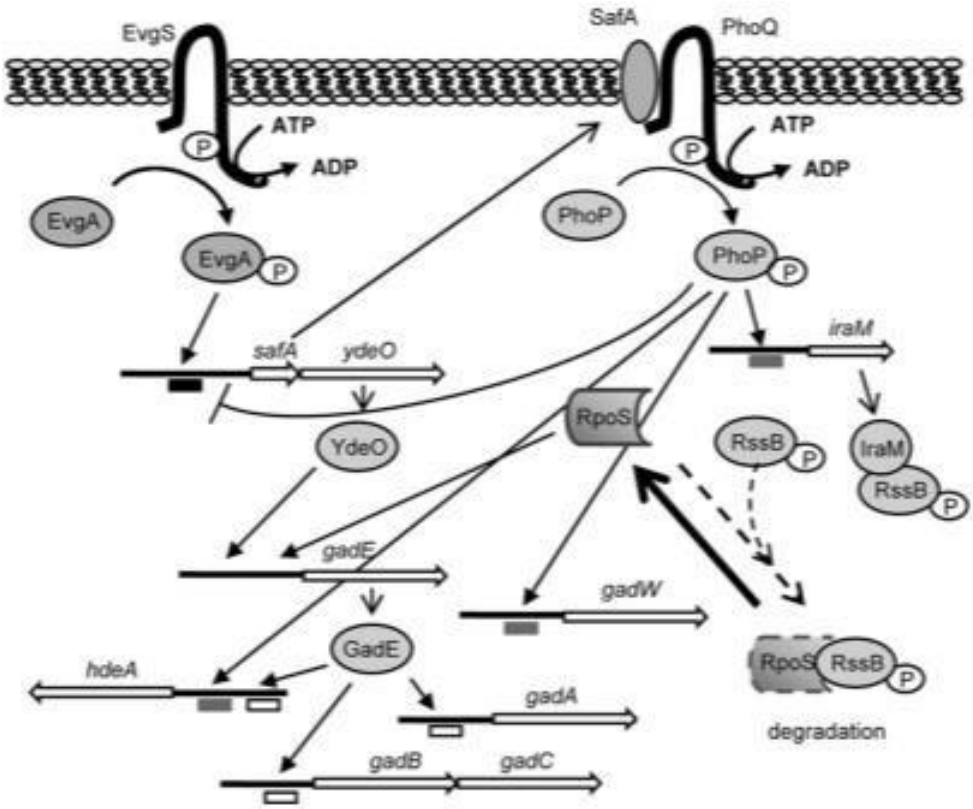


Fig. 33 Model of regulation network of AR genes initiated by the EvgS/EvgA system
(Eguchi 2011)

In addition to the stabilization of σ^S, activation of the PhoQ/PhoP system also induces expression of acid resistance genes *gadW* and *hdeA* (Eguchi, et al. 2011). Both genes are present in all cat-like and human-like genomes

E. coli access to essential substrates, such as amino acids, and metals varies widely. Nutrient availability within the gut depends on host diet and the composition and metabolism of the host microbiota (Fang, et al. 2016). The concentration of both calcium and magnesium is higher in faeces than in urine in humans (Gimblet, et al. 1967; Medicine 1997; Siener, et al. 2004) but survival of *E. coli* in the gut environment with low concentrations of these minerals is most relevant here. One hypothesis is that the concentration of magnesium and calcium in the gut and urine would be expected to vary more in humans compared to cats. The type of food humans eat each day is much more varied than that of most domestic cats. Domestic cats are usually trained as kittens to consume a fairly unvaried diet (Alegría-Morán, et al. 2019). This variation in diet affects the concentration of key nutrients, such as magnesium and calcium, available to *E.coli* in the gut (Alegría-Morán, et al. 2019). For example, humans consume variable amounts of caffeine, protein, fibre, alcohol and sucrose, and this has been shown to cause significant differences in calcium and

magnesium concentration in faeces (Johnson, et al. 1970; Hegsted and Linkswiler 1981; Saunders and Wiggins 1983; Hollingbery 1988; Bergman, et al. 1990; Shah, et al. 2009).

In summary, the over-representation of a key stress response gene *iraM* in human genomes likely represents a fitness advantage to *E. coli* strains colonizing an environment intermittently starved of these ions.

Iron acquisition genes

In this study the genes *iucABC,* which encode the siderophore aerobactin, are significantly more likely to be present in human-like compared to cat-like genomes (Table 21). Siderophores are small, high-affinity iron-chelating compounds that are secreted by bacteria in iron poor environments. As iron is an essential micronutrient for *E. coli,* there are many redundant systems for the acquisition of iron (Reigstad et al., 2007). Siderophores vary in their properties and potentially to promote survival of *E. coli* in different environments and overcome host innate immunity (Garénaux, et al. 2011). Many environmental factors affect solubility and hence availability of iron to gut microbiota in the colonic lumen (Yilmaz and Li 2018).

Similar proportions of cat and human strains carry genes for siderophores other than aerobactin (Table 21), so the difference in gene carriage in the two hosts could be explained as differences in iron availability in the gut of the two hosts. *E. coli* resident in the colon of cats fed a fairly constant diet rich in iron would be expected to carry sufficient genes to acquire enough iron. Given a set amount of iron is absorbed by specific transporters, largely in the small intestine, human *E. coli* strains would probably be exposed to large daily variations in iron availability. Oxygen levels, pH, vitamin C, iron supplements and food components such as plant residues including phytate, polyphenols and tannins can also affect the solubility and hence availability of iron (Yilmaz and Li 2018). Hence genes which provide for variable concentrations of iron could provide a possible fitness advantage for human-like strains and explain the partitioning of aerobactin genes in the two host strains.

Carboxylate transport and metabolism

The clear phenotypic difference in the metabolism of the amino acid, D-aspartate by human-like and cat-like strains may be explained as an adaptive advantage of cat-like strains to the diet fed to domestic cats. Cats are obligatory carnivores (MacDonald and Rogers 1984) requiring a higher concentrations of protein with moderate concentrations of fat and carbohydrates (Zoran 2002; Plantinga, et al. 2011) whereas humans are generally omnivores consuming a wide variety of food

types. Most commercial diets for domestic cats are formulated to take into account these metabolic adaptations. Cat food contains approximately two to three times the protein and double the non-essential amino acids compared to dog and rat foods (Hendriks 1996; Laflamme and Hannah 2005).

In addition, D-aspartate is present in relatively high concentration in commercial cat food even though there is no minimum recommended nutrient levels for this amino acid (Legrand-Defretin 1994; McGorum, et al. 2017). Heating during production of both dry cat food via extrusion cooking and wet cat food in cans (Tran, et al. 2008) causes racemization of L-amino acids to D-isomers (Friedman 1999). Heating also reduces digestibility of most proteins and amino acids, especially that of aspartate (Luzzana, et al. 1996; Hendriks, et al. 1999; Friedman and Levin 2012; Bellagamba, et al. 2015).

In vivo human experiments have shown that L-amino acids are preferentially absorbed from the small intestine rather than the D-amino acids (Kuroda and Gimbel 1954). Also, aspartate, is one of the main endogenous amino acids excreted at the terminal ileum of cats (Hendriks, et al. 1996), so it is likely high concentrations of this residual aspartate enters the large intestine mainly in the D-stereoisomer form. It is thus conceivable that D-aspartate would be present in relatively high concentrations in the domestic cat hindgut.

E. coli isolates in cats with the second *dcuA* and aspartate racemase (*ygeA*) genes would be expected to have an increased capacity to utilize the D-aspartate available in the hind gut and presence of these genes may provide a fitness advantage over strains which cannot utilise this resource.

These additional genes, predominantly in cat strains increase the capacity of *E. coli* to convert the D-form into the L-form of aspartate and glutamate and vice versa. The ability to metabolize D-aspartic acid has been shown to enhance survival of *Shigella flexneri* and attributed to genes coding for an aspartate racemase (*aspA*) and DcuA (Henríquez, et al. 2020). These authors suggest bacterial survival in environments rich in D-aspartate can be attributed to carriage of genes encoding aspartate racemase and *dcuA*. This combination of genes is present in all strains studied but an extra *dcuA* gene is present in significantly more cat-like strains than human-like strains.

Racemization of the presumed high concentrations of D-aspartate in the cat diet to L-aspartate which DcuA, encoded by the second copy of *dcuA,* would translocate into the cell via C4 dicarboxylate fumarate succinate exchange. Bacteria with genes encoding enzymes and transport systems to exploit the presumed high concentrations of D-aspartate in the large intestine would thus be expected to have a growth and survival advantage. The presence of additional genes enabling

conversion and utilisation of this D enantiomer could be explained as selection or adaptation to the specialised environment in the cat gut compared to that in the human.

Conclusions

To my knowledge this is the only study of ST73 isolated from faeces of healthy cats from both shelter and domestic environments. There is one paper describing ST73 isolates from cats with enteritis (Medina, et al. 2011) but the only other published work specifically on ST73 strains is a study of clinical isolates (Kidsley, et al. 2020).

This study shows that ST73 strains from both sources share many phenotypic and genotypic attributes but there are also key differences, some of which suggest host preference.

Host preference is evident in cat-like strains which metabolise D-aspartate and carry an extra *dcuA* gene which are likely adaptations to an environment in the gut with high concentrations of D-aspartate. Host preference in human-like strains is evident with the clear negative results in the adhesin assays, but this could not be attributed to differences in the presence or absence of recognised genes. There is also genotypic evidence that human-like strains show a preference for an environment with reduced or variable nutrient availability in the gut.

In summary, this study comparing strains of ST73 *E. coli* from human and cats collected in Canberra, Australia region presents the following significant findings. Core genome SNP analysis shows strains could be differentiated into 4 broad groupings with more or less separate human and cat host clusters. Identified cat-like strains isolated from humans provide further evidence of host sharing, lending support to concerns that cats may be a reservoir of ExPEC for humans. The greater representation in human strains of genes regarded as characteristic of ExPEC, lends support to the hypothesis that factors present in human strains not only increase the fitness advantage in the gut but also enhance the capacity of these strains to invade and cause disease in the urinary tract. ST73 strains recovered from UTIs in cats conceivably share this capacity but must rely on different, as yet, unidentified factors.

Supplementary Tables

Table 19 Metadata of ST73 cat-like and human-like strains

(Serotype, *fimH* type and antibiotic resistance gene profiles).

Strain	Serotype	FimH type	Cluster	Source	Sample	Antibiotic resistance genes
C107	O25:H1	12	Cat	Cat	Faeces	
C128	O-:H1	9	Cat	Cat	Faeces	*bla*$_{TEM-1B}$
C137	O6:H1	9	Cat	Cat	Faeces	
C145	O25:H1	12	Cat	Cat	Faeces	*bla*$_{TEM-1B}$
C153	O-:H1	12	Cat	Cat	Faeces	
C155	O-:H1	12	Cat	Cat	Faeces	*bla*$_{TEM-1B}$
C178	O6:H1	9	Cat	Cat	Faeces	
C240	O25:H1	363	Cat	Cat	Faeces	*bla*$_{TEM-1B}$*, aph(3')-1c*
C244	O25:H1	9	Cat	Cat	Faeces	*bla*$_{TEM-1B}$*, aph(3')-1c*
C277	O50/O2:H1	13	Cat	Cat	Faeces	
C280	O-:H1	12	Cat	Cat	Faeces	
C293	O2:H1	13	Cat	Cat	Faeces	
C296	O-:H1	102	Cat	Cat	Faeces	
C303	O-:H1	41	Cat	Cat	Faeces	
C307	O-:H1	13	Cat	Cat	Faeces	
C310	O-:H1	9	Cat	Cat	Faeces	
C311	O50/O2:H1	13	Cat	Cat	Faeces	
C312	O25:H1	12	Cat	Cat	Faeces	
C319	O-:H1	12	Cat	Cat	Faeces	
C336	O6:H1	102	Cat	Cat	Faeces	
C338	O-:H1	13	Cat	Cat	Faeces	
C43	O50/O2:H1	9	Cat	Cat	Faeces	
C55	O50/O2:H1	13	Cat	Cat	Faeces	
C69	O-:H1	9	Cat	Cat	Faeces	*bla*$_{TEM-1B}$
C73	O25:H1	9	Cat	Cat	Faeces	
C93	O25:H1	9	Cat	Cat	Faeces	
CV36	O-:H1	13	Cat	Cat	Faeces	
CV40AM3	O-:H1	102	Cat	Cat	Faeces	
CV47	O25:H1	9	Cat	Cat	Faeces	
CV48	O25:H1	12	Cat	Cat	Faeces	
CV56	O-:H1	13	Cat	Cat	Faeces	*bla*$_{TEM-1B}$
CV64	O50/O2:H1	32	Cat	Cat	Faeces	
H078	025:H1	12	Cat	Human	Blood	*bla*$_{TEM-1B}$*, aadA1, sul1*
H563	O25:H1	12	Cat	Human	Blood	
H170	O2:H1	13	Cat	Human	Faeces	*SHV-2, tetB*
M658301	O25:H1	9	Cat	Human	Faeces	*bla*$_{TEM-1B}$*, tetB, sul1, dfrA5*
M698383	O25:H1	183	Cat	Human	Faeces	
H048	O2:H1	9	Cat	Human	Urine	*bla*$_{TEM-1B}$*, tetB, aadA1, dfrA1*

Strain	Serotype	FimH type	Cluster	Source	Sample	Antibiotic resistance genes
H649	O6:H1	70	Cat	Human	Urine	*bla*$_{TEM-1B}$, *aadA1, sul1*
M587611	O22:H1	9	Cat	Human	Urine	*bla*$_{TEM-1B}$, *aadA1, sul1*
P240701	O25:H1	12	Cat	Human	Urine	
C347	O6:H1	30	Human	Cat	Faeces	
BS373	050/02:H1	10	Human	Human	Blood	
BS392	O6:H1	10	Human	Human	Blood	
BS405	O6:H1	10	Human	Human	Blood	
BS456	O6:H1	30	Human	Human	Blood	*bla*$_{TEM-1B}$
BS457	050/02:H1	10	Human	Human	Blood	*bla*$_{TEM-1B}$, *sul2*
BS467	O-:H1	10	Human	Human	Blood	*bla*$_{TEM-1B}$, *tetB*
BS471	O-:H1	0	Human	Human	Blood	*bla*$_{TEM-1B}$
BS475	O25:H1	12	Human	Human	Blood	
BS486	050/02:H1	10	Human	Human	Blood	*bla*$_{TEM-1B}$, *sul2, strA, strB, mph*
H003	O6:H1	30	Human	Human	Blood	*aadA1, dfrA1*
H031	O-:H1	30	Human	Human	Blood	*bla*$_{TEM-1B}$, *sul2, strA, strB, dfrA3*
H041	06:H1	30	Human	Human	Blood	*bla*$_{TEM-1B}$
H309	O25:H1	12	Human	Human	Blood	*bla*$_{TEM-1B}$, *tetB, aadA1*
H310	O6:H1	30	Human	Human	Blood	
H438	O6:H1	30	Human	Human	Blood	*bla*$_{TEM-1B}$, *aadA1, sul1*
H554	O6:H1	10	Human	Human	Blood	
H160	O-:H1	30	Human	Human	Faeces	*bla*$_{TEM-1B}$
H161	050/02:H1	10	Human	Human	Faeces	*bla*$_{TEM-1B}$, *aadA1, sul1*
H169	O-:H1	12	Human	Human	Faeces	
H274	O6:H1	10	Human	Human	Faeces	
H276	O-:H1	12	Human	Human	Faeces	
H285	O-:H1	12	Human	Human	Faeces	*bla*$_{TEM-1B}$
H295	O-:H1	10	Human	Human	Faeces	*bla*$_{TEM-1B}$, *tetB, catA1, sul1, dfrA7*
H378	O-:H1	30	Human	Human	Faeces	*bla*$_{TEM-1B}$, *tetB*
M608672	O25:H1	12	Human	Human	Faeces	*bla*$_{TEM-1B}$
M660195	O6:H1	118	Human	Human	Faeces	
M660331	O-:H1	10	Human	Human	Faeces	
M670800	O25:H1	12	Human	Human	Faeces	
M675308	O6:H1	30	Human	Human	Faeces	*bla*$_{TEM-1C}$
M694910	O6:H1	30	Human	Human	Faeces	
M709376	O6:H1	30	Human	Human	Faeces	*bla*$_{TEM-1B}$, *aadA1, sul1*
G18-3-Ti5	O25:H1	12	Human	Human	Faeces	*bla*$_{TEM-1B}$, *sul2*
G_20-5-R7	O-:H1	10	Human	Human	Faeces	*bla*$_{TEM-1B}$, *sul2*
G21-2-C8	050/02:H1	12	Human	Human	Faeces	*bla*$_{TEM-1B}$, *sul1*
23-4-R13	O-:H1	10	Human	Human	Faeces	*bla*$_{TEM-1B}$, *tetB, aadA5, sul1, dfrA17*
35-MA-AUC	050/02:H1	27	Human	Human	Faeces	*bla*$_{TEM-1B}$, *aadA1, sul1*
CD-29-IM6	O6:H1	10	Human	Human	Faeces	*bla*$_{TEM-1B}$
H050	O6:H1	30	Human	Human	Urine	*bla*$_{TEM-1B}$ *bla*$_{TEM-1B}$
H187	O6:H1	30	Human	Human	Urine	

Strain	Serotype	FimH type	Cluster	Source	Sample	Antibiotic resistance genes
H200	O6:H1	30	Human	Human	Urine	*bla*$_{TEM-1B}$, *tetB, catA1, sul2, strA, strB*
H201	O6:H1	185	Human	Human	Urine	*bla*$_{TEM-1B}$, *aadA1, sul1*
H361	O6:H1	10	Human	Human	Urine	
H429	O6:H1	10	Human	Human	Urine	
H430	O6:H1	30	Human	Human	Urine	*bla*$_{TEM-1B}$, *aadA1, sul1*
H595	O25:H1	12	Human	Human	Urine	
M619205	O25:H1	12	Human	Human	Urine	*bla*$_{TEM-1B}$, *tetA, aadA2, aac(3)-11d, sul1, sul2, strA, strB, mph, dfrA12*
M630782	O50/02:H1	41	Human	Human	Urine	*bla*$_{TEM-1B}$
M647320	O6:H1	30	Human	Human	Urine	
M668549	O6:H1	30	Human	Human	Urine	
M672775	O25:H1	12	Human	Human	Urine	
M673589	O6:H1	10	Human	Human	Urine	*bla*$_{TEM-1C}$, *aadA5, sul2*
M587973	O6:H1	10	Human	Human	Urine	*bla*$_{TEM-1B}$, *aadA1, sul1*

Table 20 Virulence factor distribution of ST73 strains by cluster.

Virulence associated gene	Cat-like % (n=20)	Human-like % (n=20)
afaD	2	34
air	0	0
capU	2	2
cea	88	75
cba	5	0
celb	22	6
chuA	100	100
cma	5	0
clbB	95	100
cnf1	80	47
cvaC	0	2
etsC	0	0
focC	63	0
focC/sfaE	39	6
focG	68	66
focI	32	72
fyuA	83	66
gad	100	100
hlyA	85	47
hlyF	22	96
hra	78	0
iha	41	53
ireA	68	30
iroN	98	72
irp2	100	94
iss	100	94
iucC	22	98
iutA	22	98
kpsE	100	100
kpsMII	98	100
lpfA	0	0
mcbA	5	2
mchB	93	72
mchC	98	72
mchF	98	72
mcmA	98	72
ompT	100	100
papC	63	66
papA	2	77
papB	39	53
papG	0	49
pic	100	100
sat	15	98
senB	10	57

Virulence associated gene	Cat-like %	Human-like %
setB	100	100
sfaD	73	8
sfaS	23	0
sitA	100	98
tcpC	10	68
terC	100	98
traT	34	68
usp	90	96
vat	98	85
yfcV	95	94

Table 21 Genes present in a greater proportion of cat-like ST73 strains

Gene	Human-like strains % (n=53)	Cat-like strains %(>70%)(n=41)	Putative function
insF-1_1	9	98	IS3 element protein InsF
insN-1_1	9	98	CP4-6 prophage; partial regulator of insertion element IS911A
group_4317	9	98	hypothetical protein
group_2323	15	90	hypothetical protein
group_406	8	81	MarR family transcriptional regulator
epsM	13	83	Type II secretion system protein M (Vibrio)
group_5622	9	78	S/F1C fimbrial major subunit operon transcriptional regulator
hisP	23	88	Histidine transport ATP-binding protein HisP
ttgI	23	88	solvent efflux RND transporter outer membrane subunit SrpC
group_2023	13	78	site-specific DNA-methyltransferase
group_4728	0	71	LysR family transcriptional regulator
ygeA_2	0	71	amino acid racemase YgeA
group_7312	0	71	capsid protein
group_7305	0	71	holin
group_7313	0	71	phage portal protein
hmuU_2	0	71	chu, fecD,ABC transporter permease
dcuA_2	0	71	C4-dicarboxylate transporter DcuA
yjiE_2	0	71	HTH-type transcriptional regulator YjiE

Table 22 Percentage of cat-like and human-like ST73 BIOLOG GENIII positive strains (sub-set of strains screened: n=20 human-like, n=20 cat-like)

Substrate	Cat number	Cat %	Human number	Human %
Negative Control	0	0	0	0
D-Raffinose	0	0	0	0
D-Glucose	20	100	20	100
D-Sorbitol	20	100	20	100
Gelatin	0	0	0	0
Pectin	0	0	1	5
p-Hydroxy-Phenylacetic Acid	0	0	0	0
Tween 40	0	0	0	0
Dextrin	2	10	1	5
D-Lactose	20	100	20	100
D-Mannose	20	100	20	100
D-Mannitol	20	100	20	100
Glycyl-L-Proline	19	95	20	100
D-Galacturonic Acid	20	100	20	100
Methy Pyruvate	19	95	20	100
Amino-Butyric Acid	0	0	0	0
D-Maltose	20	100	20	100
D-Melibiose	19	95	18	90
D-Fructose	20	100	20	100
D-Arabitol	0	0	0	0
L-Alanine	19	95	20	100
L-Galactonic Acid Lactone	19	95	20	100
D-Lactic Acid Methy Ester	0	0	0	0
Hydroxy-Butyric Acid	1	5	0	0
D-Trehalose	20	100	20	100
Methyl d-Glucoside	4	20	5	25
D-Galactose	20	100	20	100
myo-Inositol	0	0	0	0
L-Arginine	0	0	0	0
D-Gluconic Acid	20	100	20	100
L-Lactic Acid	20	100	20	100
Hydroy-D,Lbutyric Acid	0	0	0	0
D-Cellobiose	0	0	0	0
D-Salicin	0	0	0	0
3-Methyl Glucose	0	0	0	0
Gycerol	20	100	20	100
L-Aspartic Acid	20	100	20	100
D-Glucuronic Acid	20	100	20	100
Citric Acid	0	0	0	0
Keto-Butyric Acid	0	0	0	0
Gentiobiose	0	0	0	0
N-Acetly-D-Glucosamine	20	100	20	100

Substrate	Cat number	Cat %	Human number	Human %
D-Fucose	0	0	0	0
D-Glucose-6-PO4	20	100	20	100
L-Glutamic Acid	3	15	15	75
Glucuronamide	13	65	15	75
Keto-Glutaric Acid	0	0	0	0
Acetoacetic Acid	17	85	19	95
Sucrose	0	0	0	0
Acetly-B-D-Mannosamine	4	20	11	55
L-Fucose	20	100	20	100
D-Fructose-6-PO4	20	100	20	100
L-Histidine	0	0	0	0
Mucic Acid	20	100	20	100
D-Malic Acid	0	0	0	0
Propionic Acid	17	85	15	75
D_Turanose	0	0	0	0
N-Acetly-D-Galactosamine	20	100	20	100
L-Rhamnose	18	90	20	100
D-Aspartic Acid	17	85	0	0
L-Pyroglutamic Acid	1	5	0	0
Quinic Acid	1	5	0	0
L-Malic Acid	20	100	20	100
Acetic Acid	20	100	20	100
Stachyose	0	0	0	0
N-Acetly-Neuraminic Acid	20	100	20	100
Inosine	20	100	20	100
D-Serine	20	100	20	100
L-Serine	20	100	20	100
D-Saccharic Acid	20	100	20	100
Bromo-Succinic Acid	19	95	20	100
Formic Acid	0	0	0	0
PosCon	20	100	20	100
1% NaCl	20	100	20	100
1% Sodium Lactate	20	100	20	100
Troleandomycin	20	100	20	100
Lincomycin	20	100	20	100
Vancomycin	20	100	20	100
Nalidixic	0	0	0	0
Aztreonam	1	5	1	5
pH 6	20	100	20	100
4% NaCl	20	100	20	100
Fusidic Acid	19	95	20	100
Rifamycin SV	20	100	20	100
Guanidine	20	100	20	100
Tetrazolium Violet	20	100	20	100

Substrate	Cat number	Cat %	Human number	Human %
Lithium Chloride	9	45	9	45
Sodium Butyrate	20	100	19	95
pH 5	20	100	19	95
8% NaCl	2	10	17	85
D-Serine 2	0	0	0	0
Minocycline	0	0	16	80
Niaproof 4	20	100	20	100
Tetrazolium Blue	20	100	20	100
Potassium Tellurite	0	0	18	90
Sodium Bromate	2	10	0	0

Chapter 5 General discussion

Extraintestinal *Escherichia coli*

The focus of this study is the *E. coli* responsible for extra-intestinal infection and the continuing concern that such strains may be found in companion animals and food species. ExPEC cause a range of diseases, mainly UTIs in humans and companion animals, but also more serious blood stream infections and neonatal meningitis. ExPEC are also responsible for many infectious diseases in livestock (Garénaux, et al. 2011). Avian pathogenic *E. coli,* as the equivalent ExPEC in poultry is an important disease affecting poultry production (Russo and Johnson 2000; Kaper, et al. 2004; Dale and Woodford 2015). UTIs are by far the most prevalent infection in humans causing significant morbidity and presenting a significant public health cost (Johnson 1991; Foxman 2002; Flores-Mireles, et al. 2015). ExPEC isolated from humans are usually phylogroup B2 and D and of the thousands of clonal complexes comprising many sequence types. Only ST69 (phylogroup D), 73, 95, 127 and 131 (phylogroup B2) are responsible for most ExPEC infections (Riley 2014; Doumith, et al. 2015; Salipante, et al. 2015; Kallonen, et al. 2017).

Even though other studies have shown B2 and D phylogroups are rare in other mammalian hosts and food, this study found these phylogroups to be prevalent in dogs and cats, and this is consistent with findings from other studies of isolates from companion animals (Salvarani, et al. ; Selander, et al. 1986; Le Gall, et al. 2007; Turchetto, et al. 2015; Liu, Thungrat, et al. 2016; Hutton, et al. 2018). This study also confirms that the major human associated ExPEC sequence types can be isolated from faeces collected from healthy companion animals. The especially high prevalence of ST73 in cat samples is significant and this finding mirrors that found in clinical isolates (Liu, et al. 2015; Kidsley, O'Dea, Ebrahimie, et al. 2020).

Companion animals and poultry meat as a source of ExPEC

Commonality between companion animal and human ExPEC has led to concerns these pets represent a reservoir of infection (Johnson, et al. 2001; Johnson and Clabots 2006; Manges and Johnson 2015; Johnson, et al. 2016). Also, there has been keen interest in implicating food as a reservoir of ExPEC with most concern focussed on poultry meat (Mora, et al. 2013; Collingwood, et al. 2014). This concern extends to the prevalence and diversity of antibiotic resistance genes in clinical and commensal samples from companion animals and poultry (Szmolka and Nagy 2013; Kidsley, White, et al. 2020; Loayza, et al. 2020). It has been suggested that resistance develops in animals because of antibiotic use and is then transferred to people (Johnson, Kuskowski, et al. 2009)

and that transfer of antibiotic resistance from these sources may further limit treatment options for ExPEC infections in humans.

Sharing of gut microbiota between humans and between humans and dogs within households has been shown to occur (Song, et al. 2013) and there is evidence of ExPEC transmission between companion animals and humans (Johnson, Johnston, et al. 2008; Reeves, et al. 2011; Ljungquist, et al. 2016). (Johnson, et al. 2001). Other studies have implicated, but not demonstrated, poultry meat as a source of human ExPEC infections (Overdevest, et al. 2011; Lazarus, et al. 2015).

Cats and dogs frequent both environmental and human household spheres so they have the potential to share environmental *E. coli* with their owners. Transfer of parasites from free-roaming owned dogs to humans occurs (e.g., hydatids, *Echinococcus granulosus;* Jenkins, 2006: *Neospora caninum;* King et al., 2011) and humans and their dogs also share microbiomes (Song, et al. 2013). This suggests that this direct and frequent contact between dogs and owners may increase exposure of humans to the environmental biota with the potential to increase the frequency of exposure to *E. coli* strains. Within the gut of dogs and cats *E. coli* may diversify by acquiring genes and mobile elements from ingested environmental *E. coli*. The greater exposure of pets to environmental *E. coli*, comprising mainly phylogroups A and B1, (Touchon, et al.2020) having larger pan-genomes and more diverse mobile elements possibly gives access to a greater pangenome and hence array of genes which have the potential to transfer to humans. This may shape the composition of human microbial communities including the acquisition of virulence factors and antibiotic resistance genes by this means.

Strain sharing with companion animals is of concern for several reasons. Selective pressures on diverse populations of commensal strains, including those picked up from the environment may lead to the emergence and sharing of virulence factors with conversion to more virulent strains, including ExPEC as well as antibiotic resistant strains (Ahmed, et al. 2015). These more virulent strains potentially arising in the gut of cats and dogs and/or antimicrobial resistant strains may cause disease in humans. This has raised concerns that ExPEC strains in companion animals may be zoonotic. The World Health Organisation defines a zoonosis as ‘’ those diseases and infections naturally transmitted between people and vertebrate animals’’.

Companion animals as a source of antibiotic resistance

Data presented in this study show that very few antibiotic resistance genes were carried by cat strains, except the single ST131 strain, which carried a wide range of genes. Genes conferring resistance to critical antibiotics (ESCs, fluoroquinolones, carbapenems, colistins) were very rare

indicating that companion animals in the Canberra region are unlikely to be a significant source of these genes. There are reports from elsewhere in Australia (Guo, et al. 2013;Gibson 2010) and overseas of strains isolated from humans, chickens and companion animals with genes encoding these last resort antibiotics (Ewers, et al. 2016; Pomba, et al. 2017; de Jong, et al. 2018; Zhang, et al.2018). These resistant strains were isolated from both clinical and faecal samples, with faecal isolates often cultured on media containing the antibiotic of interest (Ortega-Paredes, et al. 2019; Carvalho, et al. 2021). So, the discrepancy between these reports and those reported here may be due to culture methods and regional differences in community antibiotic usage.

However, a relative lack of genes conferring antibiotic resistance, including against fluoroquinolones and 3rd generation cephalosporins is a common finding for clinical cat and dog isolates (LeCuyer, et al. 2018; Zogg, et al. 2018; Kidsley, O'Dea, Ebrahimie, et al. 2020). A survey in Switzerland found ST73 clinical isolates from dogs (n=1) and cats(n=3) to be devoid of plasmid carried resistance genes (Zogg, et al. 2018). Recent surveys have reported low levels of antibiotic resistance with only 15% (n=13) dog ST73 isolates (Kidsley, O'Dea, Saputra, et al. 2020) in Australia and 20% (n=5) dog ST73 isolates in France (Valat, et al. 2020) classified as MDR. A higher prevalence of strains showing MDR was reported in cat clinical ST73 isolates (33%) in the United States (Liu, et al. 2015).

There is however, some recent evidence of emerging antibiotic resistance. A single ST73 strain carrying CTX-M-1, CTX-M-15 and CTX-M-123 genes has been isolated from a dog in China (Liu, Liu, et al. 2016). Also, a recent global review of reports of extended-spectrum β-lactamase producing *E. coli* in dogs and cats recorded ST73 amongst resistant strains in America, Asia and Europe (Salgado-Caxito, et al. 2021).

Host preference and specificity

I have provided here multiple examples of genetic differentiation among strains belonging to the same sequence type from different hosts. These demonstrate some host preference or host specificity. Three types of host specificity have been described for pathogenic bacteria. That is, more broadly causing disease in a range of hosts, infecting a wide range of hosts but only causing disease in a preferred host or very specifically, only infecting and causing disease in a restricted group of hosts. *Shigella* infection and disease is confined to primates, including humans, with no known animal or environmental reservoir, so is an example of the third type of host specificity (Lampel, et al. 2018). An example of the intermediate type of host specificity in ExPEC strains is avian septicaemic *E. coli* strains which is more virulent to chicks than *E. coli* strains isolated from human ExPEC cases (Ron 2006).

Changes in the host preference of animal pathogens, in particular, are often of greatest concern due to their immediate and unexpected impact on human health. Host switching or host jumps can often be traced to modification of key microbial pathogenicity factors that facilitate the formation of particular host associations. This work provides examples of strain sharing, for example ST73 cat-like strains isolated from humans and chicken meat ST95 strains isolated from humans.

Host preference also relates to colonization and adherence to host tissues, not necessarily related to tissue tropisms and disease causation. Clear phylogroup preferences for certain hosts have been found (Gordon 2001; Escobar-Páramo, et al. 2006; Clermont, et al. 2008). Other studies have shown this preference extends to sequence type and subgroups within particular sequence types (McNally, et al. 2016; Gordon, et al. 2017; Jørgensen, et al. 2019)

The present study has presented considerable data to show that *E. coli* are non-randomly distributed with respect to host of isolation and hence show a host preference. This partitioning occurs at the phylogroup and sequence type level. In addition, my data adds to that of others to show that even within a sequence type, strains can partition further with respect to source of isolation (Gordon, et al. 2017). Within the subgroup C of ST95, which are rarely isolated from humans, strains from chicken meat and cats show key differences according to host source.

In addition, this study showed that the one ST131 strain isolated from a cat was clearly very different from the human and chicken isolates available for comparison. The cat strain was in an entirely different subgroup and could not be compared with strains from the other hosts. ST117 strains show a clear preference for poultry as hosts but, even though rare in companion animals and humans, can infect humans and cats, but rarely causes disease in these hosts (Clermont, et al. 2019; Liu, et al. 2015; Kidsley, O'Dea, Ebrahimie, et al. 2020).

E. coli responds to the specific conditions of the gastrointestinal tract

Searching for host specificity determinants by comparing strains in humans and cats can provide a better understanding of genetic determinants that confer a selective advantage in the host gut. This in turn can inform mechanisms which may lead to host transfer and the prospect of disease in humans. This work has shown that ST73 is a dominant *E. coli* sequence type in the faeces of cats and humans, indicating success of this sequence type in colonising the hindgut in both hosts. Nutrients are mostly abundant within the hindgut of humans and other mammals, but access requires competition with the microbiota, potentially creating a functional nutrient scarcity in this niche.

Bacteria in the hindgut must use at least one nutrient which is limiting better than competitors to survive (Freter, et al. 1983). However, discovery of these key metabolic survival factors can be elusive. Screening of ST73 and ST95 isolates with the GENIII metabolite panel showed few differences which could be related to host, apart from more cat than human ST73 strains reducing D-aspartate and lactate and human strains using D-gluconic acid. Screening of strains with a broader range of substrates has allowed differentiation of commensal and ExPEC strains and led to the prediction of nutrients which can give commensal strains of *E. coli* a metabolic advantage (Monk, et al. 2013). Application of this type of screening to strains in this collection could potentially reveal unique host specific metabolic pathways, suggesting adaptation to specific environmental niches, eg. nutrient availability, within the host.

Long-term *in vivo* and *in vitro* evolution experiments show evidence of adaptation.

Compared with strains of the other *E. coli* phylogenetic groups, those of the B2 phylogenetic appear to be well suited to the gut of mammals, including humans and increasingly prefer the gastrointestinal tract of people living in industrialized countries (Tenaillon, et al. 2010). This study and those of others show this preference also extends to *E. coli* in the gastrointestinal tract of our companion animals. To find evidence of adaptation which might explain this preference and determine its molecular bases two main approaches have been used; comparative genomics of natural isolates, as in this study, and experimental evolution.

Long term *in vivo* and *in vitro* evolution experiments have provided insights into the molecular bases that may explain how *E. coli* adapts to environmental conditions. Long term culture on a single carbon source showed an increased ability to use glucose and the decay of unused catabolic functions with a reduced ability to use other carbon sources (Cooper and Lenski 2000). *In vivo* experiments, infecting mice with a streptomycin resistant strain of *E.coli* and maintaining exposure of mice to the antibiotic over the year of the experiment showed recovered strains were much more resistant to the antibiotic. Analysis of genomics data showed that the molecular determinants of these adaptations included mutations in genes involved in streptomycin resistance (Lescat, et al. 2017).

Host specificity derives from a continuum of genetic determinants, and also results from changes in gene presence (Kirzinger and Stavrinides 2012). This study provides an example of a two single genes, which encode the racemisation and translocation of D-aspartate, which is more prevalent in cat rather than human strains. This can be explained as an adaptation to an environment with a high

concentration of D-aspartate, expected to be present in the hindgut of domestic cats. A difference in gene carriage by cat and human isolates may indicate the loss of genes by human pathogens as the genes are not required in an environment with less abundant or absent D-aspartate (Andreatta, et al. 2010).

Another indicator of host preference is the presence of genes associated with intermittent nutrient starvation which potentially activate the stress response in *E. coli.* Human ST73 strains have adapted to not only the competitive environment within the gastrointestinal tract, in common with cat strains, but also carry genes enabling transition to the very different environment in the urinary tract of the host. As shown in this work some of these genes (*iraM, iucAC*) are significantly more likely to be present in human strains than those from cats. Compared to the very varied diet of most people, that of domestic cats is relatively constant. In addition, it is plausible to suggest genes enhancing the stress response in human strains which are ExPEC would improve fitness in adapting to the extreme differences in nutrient availability in the gut and urinary tract.

Probability that ExPEC strains from poultry meat and faeces of companion animal are an important source of ExPEC for humans

There is clear evidence from this study that a significant portion of companion animals carry ExPEC in their faeces, especially cats, showing that human ExPEC can colonise the hindgut of dogs and cats. Predominant ExPEC associated phylogroups B2 and D were isolated at high prevalence, especially from cat faeces, as were all of the key ExPEC sequence types studied. These cat strains carried ExPEC associated virulence factors and antibiotic resistance genes. Poultry meat strains studied were of key ExPEC sequence types also, and carried ExPEC associated virulence factors and antibiotic resistance genes.

However, *E.coli* can be isolated from a multitude of ecological niches, both free-living and host-associated. Large-scale sampling efforts have shown the vastness of the *E. coli* pangenome and environmental and host associations (Touchon, et al. 2020; Gordon and Cowling 2003; Escobar-Páramo, et al. 2006). Other studies have shown *E. coli* of phylogroups B2 and D with ExPEC characteristics can represent approximately 50% of human faecal isolates (Gordon, et al. 2015). Hence, isolating ExPEC from animals is not necessarily proof of transmission (Singer 2015).

This study and the work of others have provided multiple examples of genetic differentiation among strains belonging to the same sequence types from different hosts suggestive of host specificity. This work showed this was especially the case for ST73 and ST69 strains. Other studies have shown host

specific subgroups in ST95 (Gordon, et al. 2017; Jørgensen, et al. 2019), ST117 (Clermont, et al. 2019) and ST131 (Liu, et al. 2018).

Despite evidence of differentiation according to host source, strain sharing does occur. The detailed genomic comparison of ST73 strain in this work provides clear evidence of sharing between humans and cats both ways. One strain isolated from a cat was classified as human-like, and concerningly, nine other strains isolated from humans, including several from sepsis cases, were classified as cat-like (Table 19). Strains studied within the host associated subgroups of ST95, ST117 and ST95 showed human strains within these poultry meat associated subgroups. Other studies have shown sharing between humans in households (Johnson and Clabots 2006), between humans and their pets (Johnson, Owens, et al. 2008; Johnson, Miller, et al. 2009) and between poultry meat and pets (Baede, et al. 2017). Consequently, the findings reported here add to the findings of others in supporting the concern that there is a risk companion animals, especially cats, may act as a reservoir of important ExPEC sequence types, especially ST73 (Zogg, et al. 2018; Kidsley, O'Dea, Ebrahimie, et al. 2020) and that poultry meat strains may be shared with humans (Mora, et al. 2013).

However, proving a causal relationship between a disease which is opportunistic and sporadic, and a wide-spread and diverse pathogen is extremely difficult. And to date, there is little epidemiological evidence derived from large studies to clearly quantify the likelihood of the different potential primary sources of infection for human ExPEC infections. This situation contrasts with diseases causing outbreaks as occurs with IPEC or food poisoning episodes where the source can more often be identified (Manges, et al. 2008).

It is also important to go beyond considering the possibility or potential that pets and poultry meat pose a significant source of ExPEC to attempt to evaluate whether these sources, rather than human sources, pose a significant risk to human health. Pathways of infection, and exposure factors must also be taken into account to evaluate this risk. Considering this, even though there is clear evidence that poultry adapted strains have infected humans, a significant pathway to infection is unlikely as poultry meat is cooked prior to consumption. Consequently, poultry meat must represent an extremely low risk of acquisition of ExPEC compared to the risk posed by human family members.

The exposure pathway for cat and dog strains to infect humans, however, is direct, so that the risk of ExPEC infection of humans by this route would be expected to be high. The relative risk though, must take into account the probability the person themselves, or other people are the source of infection, which must be high. As shown in this study, in the case of ST73, approximately a third of

the human strains were faecal, that is, commensal. Hence the risk from cats vs humans appears comparable.

The findings from this study are consistent with other reports that antibiotic gene carriage by cat strains is very low, so cat strains are not an important source of antibiotic resistance genes for humans.

Conclusions

This study has provided evidence of host adaptation of important ExPEC strains, especially ST73 strains in cats and humans. Sharing of strains between hosts has also been shown in this study. This study speculates that the risk of *E. coli* infection in humans from poultry meat which is cooked prior to consumption isvery low. In contrast, the risk of *E. coli* infection in humans from companion animals is estimated to be much higher. But the most significant source of infection remains other humans, especially familymembers.

This study has also shown sharing of predominantly poultry meat associated sequence types ST117 and ST95 (subgroup C) with cats occurs. The likely exposure pathway could be through the feeding of *E. coli* contaminated raw poultry meat to cats, shown by others to increase the risk of transfer of *E. coli* to cats (Nilsson 2015; Baede, et al. 2017).

Future directions

To further explore factors affecting host adaptation, experiments could be designed to clarify the link between diet and changes in cat ST73 populations in the gut. As example, changes in phylogroup and sequence type of cats fed diets with different protein concentrations could be monitored. Also, diets with various D-aspartate concentrations could show the effect on copy of numbers of *dcuA* in ST73 isolates from cats. Cats are difficult experimental animals to work with however, so alternatively, rats could be fed cat food or other diets with high concentrations of D-aspartate and inoculated with cat ST73 strains to monitor strain persistence.

While strains obtained from companion animals in the Canberra region show low levels of antibiotic resistance, especially to the critically important antibiotics, resistance can spread into this region. Exposure of the hindgut microbiota to antibiotics has the potential to select for bacteria carrying antibiotic resistance genes. These locally adaptive traits could transfer to ExPEC by the incorporation of genes carried on mobile elements and thereby facilitate the acquisition of resistance from environmental bacteria (Touchon, et al. 2020). Hence, further studies are required to determine the

prevalence and dissemination of ExPEC strains in companion animals to monitor the spread of resistant clones in Australia among community-dwelling humans, companion animals, and poultry and whether any increase is restricted to particular lineages.

Sequence data was available for only two of the ST117 strains isolated from cats. It would be useful to compare the genome of the other cat isolates to confirm that ST117 cat strains do cluster separately to human and chicken meat strains and represent a cat specific subgroup within this sequence type.

Ultimately, to assess the zoonotic potential of cats and dogs as reservoirs of ExPEC significantly more samples and much more epidemiological data is required.

Chapter 6
Materials and methods

Strains/Source of samples.

Voided faecal samples from healthy dogs (n=221) and cats (n=427) were collected from animal shelters (dog n=1; cat n=1, short-term boarding facilities (dog n=5; cat=2), households (dog=8; cats= 0) and dog parks (n=3) in the city of Canberra and its surrounds (Australian Capital Territory), Australia between 2015 and 2017. All source material was collected by means of convenience sampling. Animal Ethics approval was not required as samples were collected from the animal's immediate environment and not directly from animals. Host age was not available for most animals, but the isolates were generally from adults. Treatment with antibiotics was only available as metadata for a limited number of samples collected from the catteries so this information was not included.

The human *E. coli* samples were isolatedbetween 2014 and 2016 from urine, blood (clinical samples) and faeces by the Microbiology Unit of the Canberra Hospital, ACT, Australia .
Chicken meat samples were collected during the summer of 2013 (November to December), autumn (April) and winter (August) of 2014. Chicken meat sampling (number, source etc.) as described by Vangchhia (Vangchhia 2017).

E. coli isolation.

Fresh faecal material was dilution streaked onto MacConkey agar plates (Power and McCuen 1988) and incubated overnight at 35^{o}C. From each sample well isolated lactose-positive colonies (one or more) from dogs (n=734) and cats (n=867) were transferred to Simmons citrate and Luria Bertani (LB) agar plates (Power and McCuen 1988) and incubated overnight at 35°C. Putative *E. coli* isolates (lactose positive and citrate negative) were confirmed to be *E. coli* by genetic analysis (see below). A single isolate from the LB plates was inoculated into 5 ml of lysogeny broth and incubated at 36°C with shaking for 18 h. Molecular analysis of urine isolates has shown that in the majority of cases a single isolate was representative of the dominant taxon in the community at both the genus and the strain level (Willner, et al. 2014). A 1 ml aliquot of this suspension was combined with 0.5 ml sterile glycerol and stored at -80°C.

E. coli characterisation.

The isolation procedure yielded 203 isolates from 221 dogs and 334 isolates from 427 cats. DNA was extracted from an overnight LB culture of each isolate using DNAzol genomic DNA isolation reagent (Invitrogen) according to the manufacturer's instructions. Ethanol precipitated DNA was resuspended in Tris-EDTA buffer and this template DNA was stored at -20C.

DNA fingerprints of different strains were obtained using polymerase chain reaction (PCR) to amplify Repetitive Extragenic Palindromic (REP) elements and Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences (Versalovic, et al. 1991; Adamus-Bialek, et al. 2009). PCR amplification was performed in an Applied Biosystems 2720 Thermal Cycler. REP PCR reaction mix (20 μl) contained about 10 ng of DNA template, 1 x PCR polymerisation buffer (Fisher Biotech), $MgCl_2$ at 3.5 mM, $N_6(CGG)_4$ primer (Adamus-Bialek, et al. 2009) at 0.4 mM and 1.0 U MyTaq Red DNA polymerase (Bioline). Cycling conditions were as follows: denaturation at 95°C for 3 min, followed by amplification for 35 cycles of 95°C for 1 min and 72°C for 3 min, followed by a final extension step at 72°C for 8 min. The ERIC PCR reaction mix (20 μl) contained approximately 10 ng of DNA template, 1 x PCR polymerisation buffer (Bioline), ERIC1 and ERIC2 (Versalovic, et al. 1991) each at 0.4 mM and 1.0 U MyTaq Red DNA polymerase (Bioline). ERIC cycling conditions were as follows: 2 min initial denaturation at 95°C, followed by 30 cycles of 3 s at 94°C, 30 s at 92°C, annealing at 50°C for 1 min and extension at 65°C for 8 min. PCR products were separated and visualised on 1.2% agarose gels in 1-Trisborate-EDTA (TBE) buffer using ethidium bromide staining.

Isolates were assigned to a phylogroup using the method of Clermont (Clermont, et al. 2013). The reaction mix (20 μl) contained about 10 ng of DNA template, 1 x MyTaq Red PCR buffer (Bioline), Primers (*arpA.f, arpA.r, Chua.1b, Chua.2, Yja.1b, Yja.2b, Tsp.1b* and *Tsp.2* each at 0.4 mM and 1.0 U MyTaq Red DNA polymerase (Bioline). Cycling conditions were as follows: 5 min initial denaturation at 94°C, followed by 30 cycles of 5 s at 94°C, 25 s at 59°C; with a final extension of 5 min at 72°C. PCR products were separated and visualised on 1.2% agarose gels in 1-Trisborate-EDTA (TBE) buffer using ethidium bromide staining.

Phylogroup B2 and D isolates were screened for the presence of four human associated sequence types (STs) 69, 73, 95 and 131 using the method of Doumith (Doumith, et al. 2015). The method of Clermont (Clermont, et al. 2014) was used to detect whether phylogroup B2 isolates could be assigned to any of the nine main *E. coli* phylogroup B2 lineages involved in human extra-intestinal infection strains (Clermont, et al. 2014).

The ST95 strains were further characterised with respect to O type and *svg* carriage (Bidet, et al. 2007).

Whole genome sequencing

Genomic DNA for whole genome sequencing was isolated using isolate II Genomic DNA kit extraction (Bioline). DNA was quantified using Qubit dsDNA (double stranded DNA) BR (broad range) Assay kit (Invitrogen). DNA (0.5 ng) at 0.2 ng/µL was used for preparing the sequencing libraries using the Nextera XT DNA Sample Preparation kit (Illumina) and the Nextera XT index kit (Illumina). Whole genome DNA sequencing was performed on an Illumina MiSeq platform using a 600-cycle MiSeq Nextera XT version 3-reagent kit (2x300 paired-end reads). The raw genomic sequencing data files were assembled as de novo genome sequences with SPADes and exported as fasta files (Bankevich, et al. 2012). PROKKA was used to annotate preassembled genomic DNA sequences in FASTA format (Seemann 2014) and a pan genome analysis was conducted using Roary (Page, et al. 2015) and Scoary (Brynildsrud, et al. 2016).

Phenotypic screening

Antimicrobial susceptibility

All isolates from cat and dog faeces were screened using the BD BBL Sensi-Disc procedure (http://legacy.bd.com/ds/technicalCenter/inserts/8840621(201107).pdf) on Mueller-Hinton agar plates (Merck kGaA) and using BBL Sensi-Disc antimicrobial susceptibility test discs (Becton, Dickinson and Company). The antimicrobials representing eight key antimicrobial classes were ampicillin (10 μg), ciprofloxacin (5 μg), tetracycline (30 μg), chloramphenicol (30μg), gentamicin (120 μg), ertapenem (10 μg), nalidixic acid (30 μg) and ceftazidine (30 μg). The zone of inhibition diameters were measured using the instrument ProtoCol 3 (Synbiosis). The *E. coli* strains were classified as susceptible, intermediate or resistant to an antimicrobial, based on their zone diametersafter 18 to 24 hours incubation at 35^{o}C using BBL Sensi-Disc breakpoints. No control bacteria were used. Antibiotic susceptibility testing was not conducted on the collection of available human and chicken meat strains.

Yeast agglutination

ST73 isolates were inoculated into LB and incubated at 37°C overnight with shaking (250 rpm). On aglass slide 15 µl of each overnight culture was mixed with 10 µl of a 5% suspension of freeze dried
Baker's yeast dissolved in PBS with stirring for 10-20 min (Mirelman, et al. 1980; Schembri and Klemm 2001). The presence of clumping within 2 to 5 min was recorded as positive agglutination. Ifno agglutination occurred after 2 min, the strain was recorded as negative (Schaeffer, et al. 1980). Tests were repeated 3 times.

To confirm the specificity of agglutination to mannose residues (type 1 fimbriae expression) the assay was repeated for all positive isolates in the presence of D-mannose at a final concentration of
2% (wt/vol). If no agglutination occurs this indicates that D-mannose specifically inhibits adherence of the isolate to the D-mannose or D-mannose-like receptors on yeast cells preventing agglutination(Schaeffer, et al. 1980).

Biofilm formation

ST73 isolates were grown in 150 µL LB broth in microtitre plates at 37°C for 48 hours. Media was removed and plates were washed with water. 150 µL of 0.1% crystal violet was added to each well and the plates held at 4°C for 30 min. The crystal violet solution was discarded and the plate was thoroughly washed with water. Free crystal violet was eluted with 150 µL ethanol/acetone mix. Final absorbance at 595 nmwas measured using an ELISA plate reader BioTek ELx808 Absorbance Microplate Reader with installed KC Junior software (Winooski,VT,USA).

Screening with BIOLOG - GEN III MicroPlate™

Overnight growth of ST73 and ST95 isolates on Davis Minimal Media supplemented with 0.1% D- glucose was suspended in IF-A media to an approximate cell density of 95%. All wells of the BIOLOG -GEN III MicroPlate™ test panel were filled with 100 µL of this suspension. The plate was incubated for 18 h at 37^{o}C with shaking for 1 min every 10 mins (Bochner 1989). Absorbance at 600 nm was recorded every 10 mins using an ELISA plate reader BioTek ELx808 Absorbance Microplate Reader with installed KC Junior software (Winooski,VT,USA).

Screening with BIOLOG - PM3B Nitrogen plates™

Overnight growth of ST73 isolates was suspended in IF-0a GN/GP Base media (supplemented withdye mix, ferric citrate and sodium succinate) to approximately 95%T. All wells of the BIOLOG microtitre panel were filled with 100 µL of this suspension. Absorbance at 600 nm was recorded every 10 mins using an ELISA plate reader BioTek ELx808 Absorbance Microplate Reader with installed KC Junior software (Winooski,VT,USA).

Growth of ST73 isolates in D-aspartate

For the measurement of ST73 isolate growth with D-aspartate as the sole carbon source, 6 cat isolates which gave a positive reaction in Biolog plates and 6 human isolates giving negative reactions were assayed in a range of concentrations of D-aspartate in BD Difco™ Davis Minimal Media (without dextrose (DMM) [Dipotassium Phosphate 7g/L, Monopotassium Phosphate 2g/L, Sodium Citrate 0.5g/L, Magnesium Sulphate 0.1g/L, Ammonium Sulphate 1g/L] (BD Biosciences) to determine the optimum D-aspartate concentration. D-aspartate concentrations of 0.01% and 0.04%

in DMM were used to test all human and cat ST73 isolates. Growth of each isolate was determined by adding 2 μl of overnight growth in LB broth into 100 μl of D-aspartate in DMM in 96 well microtitre plates. Plate were incubated at 37^{o}C with shaking every 10 minutes and absorbance at 600nm after 24h was measured using an ELISA plate reader BioTek ELx808 Absorbance Microplate Reader with installed KC Junior software (Winooski,VT,USA). This assay method draws on that used by Eisenstadt with extensive modification (Eisenstadt, et al. 1959).

Analysis of data

In silico MLST and serotyping

The Centre for Genomic Epidemiology (CGE) website (www.genomicepidemiology.org) was used todetermine the MLST (Wirth, et al. 2006), the virulence determinants using the VirulenceFinder tool (Joensen, et al. 2014), antibiotic resistance genes carriage with ResFinder (Zankari, et al. 2012), serotype with SeroTypeFinder (Joensen, et al. 2015), plasmid content with PlasmidFinder tools, and plasmid Multilocus Sequence Typing (pMLST) for plasmids of the type IncF (Carattoli, et al. 2014) of the cat and dog genomes. The presence of additional extra intestinal virulence factors in cat genomes, beyond those detected by VirulenceFinder was determined using gene searches from ROARY and SCOARY annotations. Bacteriocin content was determined using Bagel automated bacteriocin mining (http://bagel2.molgenrug.nl/index.php/bagel3). Phage presence was determinedusing Phage Search Tool PHASTER (Arndt, et al. 2016).

Random Forests algorithm was used to rate the probability ST73 isolates were more human-like ormore cat-like based on gene presence or absence and hence define the sub-groups subsequently analysed in Chapter 4.

Genoscope (https://www.genoscope.cns.fr/agc/microscope/metabolism/antismash.php?) was usedfor the identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in the 10 most cat and cat like strains and the 10 most human strains (based on Random Forest analysis) using the assembly of pseudomolecules for these strains (Blin, et al. 2019).

The phylogenetic tree was constructed using core genome SNPs inferred by HARVEST

tools of the whole genome sequences of 115 *E. coli* ST73 strains (Treangen, et al. 2014) and displayed using iTOL(Letunic and Bork 2019).
Progressive MAUVE was for used for multiple alignment of genomes to identify gene differencesbetween cat and human genomes (Darling, et al. 2010).
Phandango was used for genome wide association studies (GWAS) (Hadfield, et al. 2017).

Statistical analyses were conducted on raw count data of variable gene presence and established antibiotic resistance, virulence, plasmid associated and bacteriocin associated genes. The variablegene content was analysed using principal components analysis (PCO) and PYSEER GWAS analysis. Abricate (https://github.com/tseemann/abricate) was used to screen contigs for key genes eg. virulence genes.

Differences in variable gene content between strains from the 3 sources were also assessed using Analysis of Variance (ANOVA) and Permutational Multivariate Analysis of Variance (PERMANOVA)(Anderson 2001). The PERMANOVA had two factors; gene content and strain source. Statistical analyses were conducted using the statistical software package PAST (Hammer, et al. 2001) to generate principal coordinates and correspondence analyses. Variable gene data was tested using PAST SIMPER analysis to explore which genes contributed to overall differences between strains from the 3 sources.

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