THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Studies on avian pathogenic *Escherichia coli* in commercial broiler Chicken in South East Queensland

Leena Awawdeh

Doctor of Veterinary Science and Medicine

Post Grad. Dip Veterinary Public Health

Bachelor of Science in Veterinary Medicine

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2017

School of Veterinary Science

Abstract

Avian pathogenic *Escherichia coli* (APEC) is the causative agent of avian colibacillosis, a localised or systemic infection resulting in clinical diseases such as colisepticemia, chronic respiratory disease and swollen-head syndrome. Globally, avian colibacillosis is the leading cause of morbidity and mortality in poultry, and it has been associated with massive economic losses and welfare problems. This organism is of public health significance as APEC is communicable to humans. The diagnosis of avian colibacillosis relies on clinical signs, typical pathological lesions and culture of *E. coli* from affected tissue(s). Antimicrobial therapy is often used both for treatment and control. Previous overseas studies have characterised APEC and identified virulence genes (VGs) that can be used as molecular markers for the identification of APEC. Little is known about APEC in broiler chickens in Australia.

The aim of this thesis was to gain a better understanding of the epidemiology of APEC in Australian broiler flocks and how factors including presence of VGs and phylogenetic group can improve the identification of this pathotype in Australia. Firstly, three faecal DNA extraction methods were evaluated. Faeces were collected from healthy chickens and chickens with colibacillosis from commercial broiler farms in South East Queensland (SEQ). The extracted DNA was screened by a pentaplex-PCR for five APEC-associated VGs (*iroN*, *iutA*, *iss*, *hlyF* and *ompT*). DNA extracted from *E. coli* isolates cultured from the cloaca and organs of the birds were screened using the same PCR. Repeated bead beating plus column elution was the preferred DNA extraction method, as it yielded good PCR quality and adequate quantity DNA. However, identifying APEC by direct detection of the five VGs from the faecal material was not feasible as all of these genes were also detected in all of the birds. However, the VGs were more commonly detected in *E. coli* isolates cultured from birds with colibacillosis.

A cross-sectional study was performed to estimate APEC farm-level prevalence in healthy broiler chickens in SEQ and to identify potential risk factors associated with the carriage of APEC. At the farm-level, all of the 40 farms sampled were positive for APEC, that is at least one bird per farm carried APEC, while the within-farm prevalence was 63% (95% Confidence Interval: 55.8, 70.2). Higher APEC within-farm bird-level prevalence was significantly associated with the usage of well water as a source of drinking water, failure to disinfect the water line after each flock, farm visitors not showering before entering the shed, distances greater than 20 metres between the car park and the poultry shed and the presence of wild birds within 50 metres of the shed. Chlorinating the drinking water combined with automatic water filtration reduced within-farm bird-level APEC prevalence.

Therefore, based on the results concluded from the multivariable model, improving biosecurity and water treatments might reduce APEC prevalence, decrease the risk of colibacillosis, reduce the use of antimicrobials, improve food safety and positively influence poultry and public health.

The antimicrobial susceptibility profile, phylogenetic group, virulence and plasmid replicon (PR) profiles of 50 clinical *E. coli* (CEC) and 187 faecal *E. coli* (FEC) cultured from broiler chickens were compared. Isolates showed genetic diversity regardless of the *E. coli* isolates' pathogenicity, suggesting that there exists a substantive reservoir of associated antimicrobial resistance, VGs and PRs among *E. coli* from chickens. Resistance to older as well as newer antimicrobial drugs was detected among clinical and faecal *E. coli*. Despite no history of use of extended-spectrum cephalosporins (ESCs) and/or fluoroquinolones (FQs) in the Australian broiler chicken industry, resistance to these antimicrobials of public health importance were detected in Australian chickens for the first time. Sequence type (ST) 354 was the most common ST associated with FQ resistance and this ST has previously been identified in FQ resistant *E. coli* from humans, other domestic animals and wild birds. We hypothesise the source of FQ and ESC resistant *E. coli* may be external to the production facility. The identification of FQ and ESC resistance and globally disseminated STs in broiler chickens suggests the need for further studies to identify how poultry is included within the broader epidemiology of resistance amongst extraintestinal pathogenic *E. coli* (ExPEC) and the potential significance to public health.

Finally, a subset of 29 CEC and 59 FEC, representing 88 enterobacterial repetitive intergenic consensus clusters and the most resistant isolates, were selected to determine the distribution of 35 ExPEC-associated VGs. The study detected 34 VGs, with prevalence ranging from 3.4% (*focG*) in FEC to 100% (*astA*) among CEC. All the tested isolates were positive for at least four VGs. This study identified a set of VGs: *iroN*; *iss*; *iutA*; *tsh*; *fimC*; *papC*; *papEF*; *vat*; *hlyF*; *astA*; *ibeA*; *feoB*; *ireA*; *cvi/cvaC* and *ompT* that were significantly more likely to be found in CEC isolates. Future investigations may be able to use these VGs to detect APEC in Australia assisting in the diagnosis, control and prevention of colibacillosis in poultry.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the policy and procedures of The University of Queensland, the thesis be made available for research and study in accordance with the Copyright Act 1968 unless a period of embargo has been approved by the Dean of the Graduate School.

I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis and have sought permission from co-authors for any jointly authored works included in the thesis.

Publications during candidature

Published journal articles

No publications

Papers accepted for publication

No publications

Additional published works by the author relevant to the thesis but not forming part of it

Conference proceedings

Oral presentations (names of presenting author in bold)

Leena Awawdeh, Conny Turni, Justine Gibson, Rowland Cobbold, Joerg Henning, Joanne Mollinger and Rachel Allavena. (2013) An optimised protocol for molecular screening of avian pathogenic *E. coli* from broiler chickens. Australian Veterinary Poultry Association Scientific Meeting, Adelaide, Australia, 3rd - 4th of October 2013.

Leena Awawdeh, Conny Turni, Justine Gibson, Rowland Cobbold, Joerg Henning, Joanne Mollinger and Rachel Allavena. (2014) Optimised protocol for molecular screening of avian pathogenic *E. coli* from broiler chickens. Tenth Annual Meeting of the Australian Association of Veterinary Laboratory Diagnosticians, Adelaide, Australia, 27th - 28th of November 2014.

L. Awawdeh, C. Turni, J. Mollinger, J. Henning, R. Cobbold, M. Groves, D. Wakeham, F. Shapter and **J. Gibson**. (2016) Low-level presence of resistance to extended-spectrum cephalosporins and fluoroquinolones in *Escherichia coli* in the highly regulated Australian poultry industry. Australian Society for Antimicrobials, Melbourne, Australia, 25th - 27th of February 2016.

L. Awawdeh, C. Turni, J. Mollinger, J. Henning, R. Cobbold, M. Groves, D. Wakeham, F. Shapter and J. Gibson. (2016) Associations between multidrug resistance, plasmid replicons, phylogenetic groups and virulence potential among pathogenic and commensal *Escherichia coli* from broiler chickens in Australia. Molecular Diagnostic Words Summit, London, United Kingdom, 19th - 20th of May 2016.

L. Awawdeh, J. Henning, C. Turni, R. Cobbold, J. Mollinger and J. Gibson. (2016) Risk factors associated with the intestinal carriage of avian pathogenic related virulence genes in *Escherichia coli* isolates from broiler chickens in South East Queensland (2016). Australian Veterinary Poultry Association Scientific Conference, Gold Coast, Australia, 1st - 2nd of June 2016.

Poster presentations (names of presenting author in bold)

Leena Awawdeh, Conny Turni, Justine Gibson, Rowland Cobbold, Joerg Henning, Joanne Mollinger and Rachel Allavena. (2014) An optimised protocol for molecular screening of avian pathogenic *E. coli* from broiler chickens. QAAFI Animal Science Olympics, The University of Queensland, Australia, 14th of September 2014.

Publications included in this thesis

No publications

Contributions by others to the thesis

Chapter 3: An optimised protocol for molecular screening of avian pathogenic *Escherichia coli* from broiler chickens in South East Queensland, Australia.

Leena Awawdeh contribute 80% to the designed and performed all the experiments, data analysis, manuscript writing and editing, Justine Gibson contribute 4% to the advisory role, experimental design and manuscript editing, Conny Turni contribute 4% to the advisory role, experimental design, sample collection and manuscript editing, Joerg Henning contribute 4% to the advisory role, experimental design, data analysis and manuscript editing, Rowland Cobbold contribute 2% to the advisory role and manuscript editing, Rachel Allavena contribute 4% to the advisory role, reviewed histopathology and manuscript editing and finally Joanne Mollinger contribute 2% to the advisory role and manuscript editing.

Chapter 4: Risk factors associated with the carriage of pathogenic *Escherichia coli* from healthy commercial broiler chickens in South East Queensland, Australia.

Leena Awawdeh contribute 80% to the designed and performed all the experiments, data analysis, manuscript writing and editing, Justine Gibson contribute 3% to the advisory role, experimental design and manuscript editing, Conny Turni contribute 3% to the advisory role, experimental design, sample collection and manuscript editing, Joerg Henning contribute 9% to the advisory role, experimental design, data analysis and manuscript editing, Rowland Cobbold contribute 3% to the advisory role and manuscript editing, Rachel Allavena contribute 1% to the advisory role, reviewed histopathology and manuscript editing and finally Joanne Mollinger contribute 1% to the advisory role and manuscript editing.

Chapter 5: Antimicrobial susceptibility, plasmid replicon typing, phylogenetic grouping and virulence potential of *Escherichia coli* cultured from Australian broiler chickens with and without colibacillosis.

Leena Awawdeh contribute 80% to the designed and performed all the experiments, data analysis, manuscript writing and editing, Justine Gibson contribute 6% to the advisory role, experimental design and manuscript editing, Conny Turni contribute 4% to the advisory role, experimental design, sample collection and manuscript editing, Joerg Henning contribute 4% to the advisory role, experimental design, data analysis and manuscript editing, Rowland Cobbold contribute 3% to the

advisory role and manuscript editing, Rachel Allavena contribute 1% to the advisory role, reviewed histopathology and manuscript editing and finally Joanne Mollinger contribute 2% to the advisory role and manuscript editing.

Chapter 6: Virulence associated gene in faecal and clinical *Escherichia coli* isolates cultured from broiler chickens in Australia

Leena Awawdeh contribute 80% to the designed and performed all the experiments, data analysis, manuscript writing and editing, Justine Gibson contribute 4% to the advisory role, experimental design and manuscript editing, Conny Turni contribute 4% to the advisory role, experimental design, sample collection and manuscript editing, Joerg Henning contribute 4% to the advisory role, experimental design, data analysis and manuscript editing, Rowland Cobbold contribute 4% to the advisory role and manuscript editing, Rachel Allavena contribute 2% to the advisory role, reviewed histopathology and manuscript editing and finally Joanne Mollinger contribute 2% to the advisory role and manuscript editing.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

Research Involving Human and Animal Subjects

The samples collection for chapter three were done under animal ethics approval number: QAAFI /478/12/ POULTRY CRC (copy of the ethics approval letter can be found in the thesis's appendix).

The questionnaire for chapter four was done under human ethics approval number: SVS /2014000327/ POULTRY CRC (copy of the ethics approval letter can be found in the thesis's appendix).

The samples collection for chapter four, five and six were done under animal ethics approval number: SVS /323/13/ POULTRY CRC (copy of the ethics approval letter can be found in the thesis's appendix).

Acknowledgements

The work presented in this thesis would not have been possible without the guidance, encouragement and support of my principal supervisor Dr Justine Gibson and co-supervisor Dr Conny Turni, Associate Professor Rowland Cobbold, Dr Joerg Henning, Dr Joanne Mollinger and Dr Rachel Allavena. I am sincerely grateful to you all for giving me an opportunity to embark on this very insightful and eventful journey and for everything that I have learnt on the way.

I would like to thank Dr Patrick Blackall for encouraging my research and for allowing me to grow as a research scientist. Your advice on both research as well as on my career have been invaluable.

I also want to thank all the past and present members of the University of Queensland laboratory for providing me with feedback and constant support, for managing the laboratory efficiently and for making sure that my experiments could run smoothly. I also want to thank all the staff at the School of Veterinary Science who helped me navigate the administrative quagmire during my candidature. I would like to acknowledge the contribution of all those working in the poultry industry for all their support.

I am honoured to have the privilege of being granted a Poultry Cooperative Research Centre scholarship and to top-up award funded by Zoetis Australia. This support allowed me to be free of all financial concerns and helped me give my best to my research.

I would like to take this opportunity to record my deep gratitude and sincere thanks to all my friends who helped me up every time I was down and always stood by my side through good times and bad.

A very special word of appreciation is extended to my family in particular "Mum" for believing in me and providing such amazing encouragement.

I am extremely grateful to my lovely family especially my three beautiful kids Sarah, Yamin and Hannah- without you all I would surely have not made it to the end- your continuous love, smiling faces reminding me every day of the important things in life, unconditional support and encouragement through the years without complaining. Thank you. I am lucky to have such a supportive and loving family without whom my life would not be complete.

Finally, I pay tribute to my Dad who was and still my real hero and biggest inspiration. I owe a great depth of gratitude for your unstinting support, prayers, wisdom and understanding over the years. Without your devotion, this would have been impossible. Forever in my heart.

Financial support

This PhD project was funded by the Poultry Cooperative Research Centre and Australian Society of Antimicrobials.

A top-up to the scholarship was funded by Zoetis Australia.

Keywords

Avian pathogenic *Escherichia coli*, DNA extraction, risk factors, virulence genes, antimicrobial resistance, phylogenetic groups and plasmid replicon.

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 070707 Veterinary Microbiology (excl. Virology), 60%

ANZSRC code: 060503 Microbial Genetics, 30%

ANZSRC code: 070704 Veterinary Epidemiology, 10%

Fields of Research (FoR) Classification

FoR code: 0707 Veterinary Sciences, 60%

FoR code: 0605 Microbiology, 40%

Table of Contents

List of Figures

List of Tables

List of Appendices

Appendices to Chapter 4

Appendices to Chapters 5

List of Abbreviations used in the thesis

Adk	Adenylate kinase gene
afa/drab	Afimbrial/Dr antigen-specific adhesin gene
AFEC	Avian faecal *Escherichia coli*
AIC	Akaike Information Criterion
AMC	Amoxicillin/clavulanic acid
Amp	Ampicillin
AmpC	AmpC β-lactamase
AMR	Antimicrobial resistance
APEC	Avian pathogenic *E. coli*
APM	Apramycin
APVM	Australian Pesticides and Veterinary Medicine Authority
ARGs	Antimicrobial resistance genes
astA	Heat-stable toxin gene
BECS	Brilliance *E. coli*/coliform selective agar
BHI	Brain heart infusion
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
Cdt	Cytolethal distending toxin gene
CEC	Clinical *E. coli*
CFU	Colony forming units
CHL	Chloramphenicol
chuA	Heme receptor gene
CI	Confidence interval
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
Cma	Colicin M-resembles B-lactam gene
cnf1	Cytotoxic necrotising factor 1 gene
CTX	Cefotaxime
cvi/cva	Structural genes of colicin V operon
DAEC	Diffusely adherent *E. coli*
DAFF	Department of Agriculture and Fisheries, Queensland, Australia

DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EFT	Ceftiofur
EHEC	Enterohaemorrhagic *E. coli*
ehxA	Enterohaemolysin gene
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ENR	Enrofloxacin
EPI	Efflux Pump Inhibitor
ERIC	Enterobacterial repetitive intergenic consensus
ESC	Extended-spectrum cephalosporin
ESBL	Extended-spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Union Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal pathogenic *E. coli*
FEC	Faecal *E. coli*
fimC	Type 1 fimbriae gene
fimH	Adhesive subunit of type 1 fimbriae gene
FAO	Food Agriculture Organisation
feoB	Ferrous iron transport protein B gene
focG	F1C fimbriae gene
FOX	Cefoxitin
fumC	Fumarate hydratase
FQ	Fluoroquinolone
fyuA	Ferric yersiniabactan uptake gene
GEE	General Estimation Equation
GN	Gentamicin
Gyr	DNA gyrase gene
HGT	Horizontal gene transfer
hlyA	Haemolysin A gene
hlyF	Putative haemolysin gene

Hra	Heat-resistant agglutinin gene
ibeA	Invasion of brain endothelium gene
Icd	Isocitrate/isopropylmalate dehydrogenase gene
Inc	Incompatibility
IQR	Interquartile range
iroN	Salmochelin siderophore receptor gene
ireA	Iron-responsive element gene
irp2	Iron-repressible protein gene
Iss	Increased serum survival gene
iucD	Aerobactin gene
iutA	Aerobactin siderophore receptor gene
Kbp	Thousand base pair
KF	Cephalothin
KpsMT KI	Group I capsule antigen gene
kpsMT II	Group II capsule antigen gene
M	Meter
maxI	Pathogenicity-associated island marker
MCA	MacConkey agar
Mdh	Malate dehydrogenase gene
MDR	Multidrug resistant
MHA	Mueller Hinton agar
MGE	Mobile genetic elements
MIC	Minimum inhibitory concentration
Ml	Millilitre
MLST	Multilocus sequence typing
NMEC	Neonatal meningitis associated *E. coli*
N	Neomycin
NARMS	National Antimicrobial resistance Monitoring System
NCBI	National Center for Biotechnology Information
neuC	Capsular polysaccharide gene
ND	None detected
Ng	Nana gram
NT	Not tested
OIE	World Organisation for Animal Health

OMP	Outer Membrane Protein
ompT	Outer membrane protease gene
OR	Odd ratio
PAIs	Pathogenicity islands
pAmpCs	Plasmid-mediated AmpC β-lactamase
papC	P-fimbriae gene
pARG	Plasmid-mediated antimicrobial resistance genes
papAH	P fimbriae structural subunit gene
pAPEC	Potential avian pathogenic *E. coli*
PBPs	Penicillin binding proteins
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pMLST	Plasmid multilocus sequence typing
PubCRIS	Public Chemical Registration Information System Search
purA	Adenylosuccinate dehydrogenase gene
BB+P/C	Phenol-dependent bead-beating methods
QC	Quality Control
QRDR	Quinolone Resistance-Determining Region
RBB+C	repeated bead beating plus column
recA	ATP/GTP binding motif gene
RNA	Ribonucleic acid
Sat	Secreted autotransporter toxin gene
SB	Sodium borate buffer
SBA	Sheep blood agar
SEQ	South East Queensland
SePEC	Septicaemic associated *E. coli*
sfa/focDE	S fimbriae and F1C fimbriae genes
sfaS	S fimbriae (sialic acid-specific) gene
SH	Spectinomycin
sitA	Putative iron transport gene
SNPs	Single-nucleotide polymorphisms
spp.	Species
ST	Sequence type
STR	Streptomycin

SXT	Trimethoprim-sulfamethoxazole
TE	Tetracycline
traT	Serum survival factor gene
Tsh	Temperature-sensitive haemagglutinin gene
µl	Microlitre
Mm	Micrometre
UK	United Kingdom
UPEC	Uropathogenic *E. coli*
USA	United States of America
V	Volts
Vat	Vacuolating autotransporter toxin gene
VGs	Virulence genes
VP	Voges Proskauer
WGS	Whole Genome Sequencing

Chapter 1: Literature review

Avian colibacillosis is considered one of the most common diseases that affect the poultry industry. It results in significant economic losses and welfare concerns worldwide (Dho-Moulin and Fairbrother, 1999; Ewers *et al.*, 2004; Nolan *et al.*, 2013). Economic losses are the result of the high costs associated with treatment and vaccination, decreases in growth rate and egg production, high mortality and carcass condemnation at the abattoir (Nolan *et al.*, 2013).

Avian pathogenic *Escherichia coli* (APEC), the causative agent of avian colibacillosis (Antão *et al.*, 2008; Dho-Moulin and Fairbrother, 1999; Ewers *et al.*, 2004; Kabir, 2010; Nolan *et al.*, 2013), is a subpathotype of extraintestinal pathogenic *E. coli* (ExPEC). It is considered a potential zoonotic agent (Johnson *et al.*, 2008b; Nolan *et al.*, 2013; Rodriguez-Siek *et al.*, 2005a). APEC coexists in the gut microbiota of healthy chickens with avian faecal *E. coli* (AFEC) (Dho-Moulin and Fairbrother, 1999; Ewers *et al.*, 2004; Kabir, 2010; Nolan *et al.*, 2013). Clinical signs and pathology in the bird population, isolation of *E. coli* from a lesion or faeces, bacterial virulence genotype, phylogenetic group and serotype have been used by several studies to differentiate APEC from AFEC (Johnson *et al.*, 2008b; Lynne *et al.*, 2012; Pfaff-McDonough *et al.*, 2000; Skyberg *et al.*, 2003).

Avian colibacillosis has been reported as common in all age groups of commercial broiler flocks (Nolan *et al.*, 2013). Reported prevalence ranges between 30% in the United States of America (USA) (Johnson *et al.*, 2008b) and 14% in Bangladesh (Badruzzaman *et al.*, 2015). Currently, there is no Australian data in regards to the prevalence of avian colibacillosis in broiler flocks.

The severity of colibacillosis in chickens depends on the pathogenicity of the APEC, the chicken's immune status and the presence of predisposing risk factors, such as poor chicken welfare, immunosuppression and co-infections that can increase host susceptibility (Dho-Moulin and Fairbrother, 1999; Ewers *et al.*, 2004; Guabiraba and Schouler, 2015; Nolan *et al.*, 2013; Vandekerchove *et al.*, 2004a). A number of overseas studies identified vectors humans (farm worker and visitors), wild birds and rodents, source and type of treatment(s) applied to the drinking water and unfavourable housing conditions as major risk factors associated with avian colibacillosis (Kemmett *et al.*, 2013; Nolan *et al.*, 2013; Vandekerchove *et al.*, 2004a). Low levels of APEC on overseas poultry farms, is associated with strict farm biosecurity and management practices, such as control of vectors by minimising bird interaction with other animals and hygienic cleaning protocols (Nolan *et al.*, 2013; Vandekerchove *et al.*, 2004a; Zanella *et al.*, 2000). However, little knowledge is available in regards to the potential predisposing risk factors (biosecurity and farm management

protocols and environmental factors) associated with APEC among commercial broiler chicken flocks in Australia.

Avian colibacillosis is traditionally diagnosed by clinical signs, pathology and the culture of *E. coli* from the infected lesion(s) (Nolan *et al.*, 2013). However, these traditional methods are nonspecific and time consuming in comparison with being able to identify APEC directly from sample(s) utilising molecular methods. Previous studies have used molecular techniques to identify pathogens and/or genes directly from samples. These methods have proven to be reliable, cost-effective, user friendly, sensitive and specific as well as providing a distinct advantage in regards to time saving over conventional culture methods (Blessmann *et al.*, 2002; Eyigor and Carli, 2003; Garofalo *et al.*, 2007; McOrist *et al.*, 2002).

Antimicrobial agents are widely used in the poultry industry for the treatment and control of avian colibacillosis (Morley *et al.*, 2005). The extensive use of antimicrobial drugs among humans, companion and production animals has led to the emergence of antimicrobial resistant strains among commensal and pathogenic *E. coli* (Zhao *et al.*, 2005). The risk of antimicrobial resistant bacteria is a major concern for public health and veterinary medicine (Barton *et al.*, 2003). In Australia, there is limited knowledge of the antimicrobial susceptibility of APEC to guide veterinarians in developing successful prevention and treatment schemes. Numbers of overseas studies have reported geographical variations in the antimicrobial susceptibility of APEC (Alvarez-Fernandez *et al.*, 2013; Dheilly *et al.*, 2013; Sola-Gines *et al.*, 2015). The geographical variation in susceptibility of APEC highlights the importance of regional knowledge to control, prevent and treat avian colibacillosis (Aarestrup, 2005).

The current review will give an overview of the global poultry industry in general and the Australian industry, specifically. General information in regards to colibacillosis, such as clinical signs, diagnosis, treatment and the causative agent will be reviewed. Identification and molecular characterisation of APEC are also included in this literature review. Furthermore, potential risk factors and preventive and applied control measures associated with avian colibacillosis are explored. Antimicrobial agents of public health and veterinary importance in Australia and worldwide are discussed, along with antimicrobial resistance (AMR) detected in APEC in previous studies. Finally, this literature review covers the current knowledge in regards to the molecular characterisation of commensal and pathogenic *E. coli* in broiler chickens globally.

1.1 The poultry industry

Globally, the poultry industry has experienced massive growth in response to consumer demand for poultry products over the past few decades (Mulder and Nan, 2011). The Food and Agriculture Organisation of the United Nations (FAO) has reported a three-fold increase in poultry meat production in comparison to total population growth over the last five decades (Thornton, 2010). Whilst the largest poultry production growth was experienced in countries such as India and Vietnam with 217% and 136% increases respectively, other countries such as North and Central America and Europe experienced a steady growth over the same period of time (Windhorst, 2006).

The substantial technical and scientific growth in different aspects of the poultry industry in many countries has been accompanied by several challenges in regards to food safety, animal welfare, efficiency of flock production, housing environment and the management of current and emerging diseases (Gororo and Kashangura, 2016; Penz and Bruno, 2011; Scanes, 2007). Respiratory and enteric diseases are considered the major diseases facing the poultry industry globally and cause massive economic and production losses (Kabir, 2010; Nolan *et al.*, 2013). These diseases are due to viruses, such as avian influenza (Bertran *et al.*, 2016; El Houadfi *et al.*, 2016), bacteria, such as *E. coli* (Nolan *et al.*, 2013), *Salmonella* species (Kabir, 2010) and parasites, such as coccidia (Nolan *et al.*, 2013).

1.2 The Australian poultry industry

In 2010, the Australian Bureau of Agricultural and Resource Economics and Sciences reported the growth of the Australian poultry industry was steady and mirrored the global trends (ABARES, 2011). The volume of Australian poultry meat production was estimated to be three million birds in 1951, compared with 600 million birds in 2013 (Australian Chicken Meat Federation, 2014). This expansion in production was due to a combination of competitive chicken pricing compared with other meat and an increase in the diversity of chicken products (ABARES, 2011).

In Australia, the majority of poultry operations are intensively concentrated due to their close proximity to urban populations (Australian Chicken Meat Federation, 2014). The broiler industry is highly vertically integrated with large companies owning or controlling all of the supply and production aspects such as sources of birds, feed mills, farms, veterinary services as well as slaughter and processing facilities (Australian Chicken Meat Federation, 2014). Currently, there are seven large integrated companies in Australia which supply up to 95% of the total Australian chicken meat, while the remaining 5% is supplied by small privately owned farms (Australian Chicken Meat Federation, 2014). Ingham Enterprises Pty Ltd and Baiada Poultry Pty Ltd are the two largest integrated meat

companies supplying 70% of the total domestic Australian poultry meat (Australian Chicken Meat Federation, 2014). Broilers are typically raised in tunnel ventilated sheds containing 10,000 – 20,000 birds (Chinivasagam *et al.*, 2009). A standardised management protocol in regards to farm management, drug administration and biosecurity protocols are followed on all of the chicken farms owned by the company due to the integrated system (ABARES, 2011).

1.3 Avian colibacillosis

Colibacillosis mainly affects birds four to eight weeks of age, though it can affect adults, as a primary or secondary pathogen (Cheville and Arp, 1978; Dhillon and Jack, 1996; Nolan *et al.*, 2013; Rashid *et al.*, 2013). The disease is a complex syndrome that can occur as a localised (subacute form) or systemic infection (acute form) (Nolan *et al.*, 2013; Paixao *et al.*, 2016). The disease can manifest itself in different forms, such as, yolk sac infections (omphalitis), respiratory tract infections (air sacculitis), swollen-head syndrome, septicaemia, polyserositis, coligranuloma, enteritis, coliform cellulitis, salpingitis, pericarditis and perihepatitis in chickens, turkeys and other commercial avian species (Dho-Moulin and Fairbrother, 1999; Moulin-Schouleur *et al.*, 2006; Paixao *et al.*, 2016).

1.4 Economic significance of avian colibacillosis

Avian colibacillosis is considered the most common type of bacterial infection affecting the poultry industry. Globally, *E. coli* infections are considered the number one cause of carcass condemnation at slaughterhouses (Jakob *et al.*, 1998; Santos *et al.*, 2014). For example, 45.2% of poultry carcass rejection in Brazil, one of the world's largest exporters of chicken meat, was due to APEC infections (Fallavena *et al.*, 2000). Globally, 36% – 43% of the broiler carcasses disposed of at slaughter are due to lesions consistent with *E. coli* infections (Fallavena *et al.*, 2000; Norton *et al.*, 1997). A mortality rate of 3.3% was reported in birds due to colibacillosis in Bangladesh (Rashid *et al.*, 2013) and mortality rates of up to 10% have been reported in Denmark and Italy (Stokholm *et al.*, 2010; Zanella *et al.*, 2000). The disease is responsible for 2% – 3% decreases in egg production and the costs associated with prevention/control, vaccination and treatment are substantial (Antão *et al.*, 2008; Ewers *et al.*, 2004; Gross, 1957; Kabir, 2010; Nolan *et al.*, 2013; Stokholm *et al.*, 2010; Zanella *et al.*, 2000).

1.5 Clinical signs and the pathological lesions

The severity of the disease depends on the presence of predisposing factors, the immune status of the bird, the pathogen's route of entry, virulence of the *E. coli* strain and the duration of exposure (Cloud *et al.*, 1985; Dziva and Stevens, 2008). The clinical signs associated with avian colibacillosis are nonspecific and vary with age, species, type of infection and whether it is localised or systemic (Kabir,

2010; Nolan *et al.*, 2013). Affected birds demonstrate signs of respiratory distress, reduced appetite, poor growth, depression and fever, which may also be accompanied by a high mortality rate (Kabir, 2010; Paixao *et al.*, 2016). Characteristic fibrinous lesions, such as, airsacculitis, pericarditis, perihepatitis, peritonitis and salpingitis are commonly found during post-mortem examination of the internal organs (Nolan *et al.*, 2013; Vandekerchove *et al.*, 2004b) (see Figure 1-1).

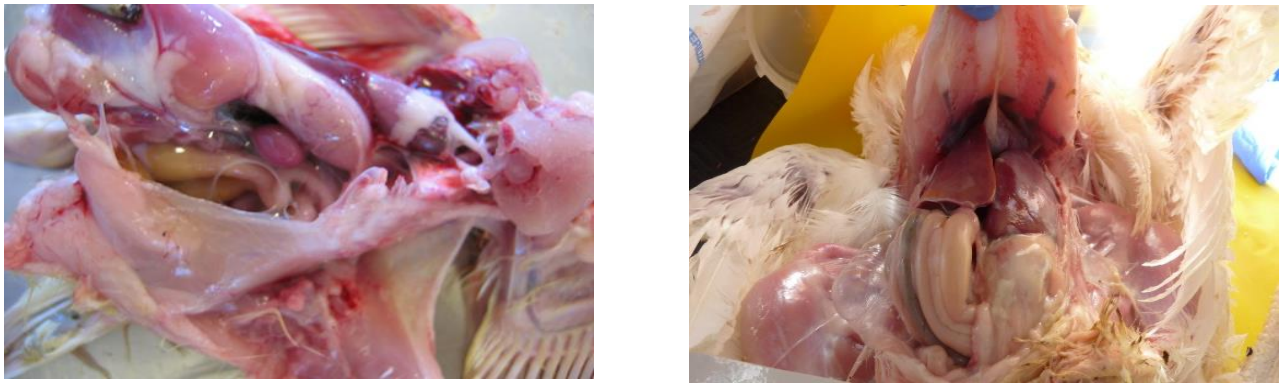


Figure 1.1: Macroscopic lesions characteristic of avian colibacillosis in broiler chickens. (A) Clear air sac of a healthy chicken from http://www.poultryhub.org/health/disease/types-of-disease/colibacillosis; (B) Characteristic fibrinous lesions of airsacculitis, pericarditis and perihepatitis from necropsy at poultry farm in South East Queensland.

1.6 Diagnosis of avian colibacillosis

Currently, the identification of avian colibacillosis is based on the traditional diagnostic methods composed of clinical signs, characteristic macroscopic lesions found during post-mortem examination (Nolan *et al.*, 2013; Whiteman *et al.*, 1979) and isolation and identification of *E. coli* from the affected lesion(s) of chickens (Kabir, 2010). *E. coli* is isolated using selective media such as MacConkey agar and/or eosin-methylene blue agar or other selective media (Quinn *et al.*, 2011). The majority of *E. coli* can ferment lactose on MacConkey agar and eosin-methylene blue agar producing distinctive pink and blue black with or without a green metallic sheen colonies, respectively (Quinn *et al.*, 2011). Further biochemical tests, such as indole, Voges Proskauer (VP), methyl red, citrate and/or commercial assays such as: Microbact™ GNB 24E (Oxoid); API® 20E (bioMerieux) and matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) may be used to confirm the identification of *E. coli* isolates (Quinn *et al.*, 2011). Culture methods are considered to be the gold-standard for isolation and identification of *E. coli* (Kabir, 2010; Nolan *et al.*, 2013). Molecular methods can also be used following phenotypic identification of bacterial isolates using species-specific polymerase chain reactions (PCRs). Pathotypes of *E. coli* can be

further identified based on the presence of virulence genes (VGs) (Ewers *et al.*, 2005; Johnson *et al.*, 2008b).

Culture methods however, are labour intensive and time consuming in comparison with molecular methods such as PCR, which can be used to detect the unique nucleic acids (either RNA or DNA) of pathogens (Ishmael and Stellato, 2008) directly from faecal samples (Blessmann *et al.*, 2002; Garofalo *et al.*, 2007). Pathogen specific PCR tests can be applied on extracted DNA from faecal samples to rule-in or-out targeted pathogens (Nechvatal *et al.*, 2008). However, the direct application of molecular techniques to faecal samples has been hampered by the inability to extract PCR quality DNA that is free from PCR inhibitors, such as bile salts, haemoglobin, degradation products and complex polysaccharides (Abu Al-Soud and Radstrom, 1998; McOrist *et al.*, 2002).

Variation in the composition and consistency of faecal samples from different animal species affects the quality (purity) and the quantity (concentration) of the extracted DNA (McOrist *et al.*, 2002) and therefore, the ability to successfully amplify DNA and detect pathogens (Hart *et al.*, 2015). A number of studies have highlighted the need for careful consideration of DNA extraction methods from different animal species (Goldberg *et al.*, 2016; Hart *et al.*, 2015; Rapp, 2010). For example, DNA was successfully extracted from fish faecal samples using the isopropanol method, while the MoBio Power Fecal DNA kit (Qiagen Pty Ltd, Chadstone, Victoria, Australia) was needed to extract quality DNA from equine faecal samples (Hart *et al.*, 2015).

Generally, chicken faeces contain less moisture compared with other animal species (Akhtar *et al.*, 2013), which leads to difficulties in dissolving the faeces in a buffer (Barnard *et al.*, 2011). Chickens may have additional inhibitors compared to other mammalian faeces as they excrete urinary waste in the faeces via a common cloaca (Chambers *et al.*, 2001). Therefore, testing is required to detect the best method(s) for DNA extraction from faeces for chickens, rather than simply extrapolating from other species.

There are several methods, such as physical, mechanical, chemical extraction and commercial kits, specifically designed for DNA extraction from faecal samples. DNA extraction methods must produce repeatable and accurate PCR results (McOrist *et al.*, 2002; Yu and Morrison, 2004). However, the complex matrix of faecal samples make it a challenging job to choose the most suitable extraction protocol as some common methods, such as the cell lysis by boiling method, are incapable of removing faecal inhibitors (Rapp, 2010; Wilson, 1997).

To evaluate the purity and the contamination level of DNA, the ratio of the absorbance at 260 and 280 nm (A260/280) is used. Samples are considered relatively free from contamination if the ratio of

A260/280 is 1.80 – 2.00 (Desloire *et al.*, 2006). Several studies have reported that QIAamp DNA Stool Mini Kit (Qiagen Pty Ltd) gave superior results (increased purity and DNA concentration and minimised the PCR inhibitors) compared with other commercial kits (FastDNA™ SPIN Kit (MP Biomedicals, Santa Ana, California, USA); NucleoSpin® (Macherey-Nagel, Germany) and Quantum Prep® AquaPure™ genomic DNA isolation kit (Bio-Rad Laboratories Pty Ltd, Gladesville, New South Wales, Australia) when extracting DNA from faecal samples of ruminants and humans (Desloire *et al.*, 2006; McOrist *et al.*, 2002; Nicklas and Buel, 2003; Verweij *et al.*, 2004; Verweij *et al.*, 2007).

Yu and Morrison (2004) evaluated four different DNA extraction methods from faecal samples from cows. The methods tested were the QIAamp DNA Stool Mini Kit (Qiagen); the FastDNA™ SPIN Kit (MP Biomedicals); phenol-dependent bead-beating method (BB+P/C) (Whitford *et al.*, 1998) and repeated bead beating plus column (RBB+C) which is a modified BB+P/C. The study reported that the RBB+C gave a 1.5 to 6-fold higher yield than the other methods evaluated, as well as, providing a higher quality of DNA, free from PCR inhibitors.

Gioffre *et al*. (2004) reported that direct culture from the faeces of bovines gave the highest sensitivity when compared with the QIAamp DNA Stool Mini Kit (Qiagen) and the heat lysis method for the detection of *E. coli*. However, in agreement with Yu and Morrison (2004) they found that the QIAamp DNA Stool Mini Kit extraction method resulted in fewer PCR inhibitors compared to the heat lysis protocol. The commercial kit was able to rapidly detect VGs by PCR from bovine faecal samples. For human stool samples, the extraction using the QIAamp DNA Stool Mini Kit (Qiagen) yielded statistically greater PCR sensitivity in comparison to the Chelex methods (Cordova *et al.*, 2010)

Overall, molecular methods to detect pathogens or VGs directly from faeces have proven to be reliable, cost-effective, user friendly and accurate in other animal species, therefore, providing a distinct advantage over conventional methods (Blessmann *et al.*, 2002; Eyigor and Carli, 2003; Garofalo *et al.*, 2007; Gioffre *et al.*, 2004; McOrist *et al.*, 2002). However, currently, there is a lack of information pertaining to the quality and quantity of extracted DNA from chicken faecal samples using different DNA extraction methods (Barnard *et al.*, 2011).

1.7 Risk and predisposing factors associated with avian colibacillosis

Predisposing factors for colibacillosis can be loosely categorised into three different categories, those related to the bird susceptibility, environment and agent (Barnes, 2013). The interaction between these factors determines if disease occurs in the chicken flocks (Barnes, 2013; Dho-Moulin and Fairbrother, 1999).

The breed of broiler chicken, as well as, the quality of the egg at the hatchery can play a crucial part in relation to the bird's susceptibility to APEC infection (Barnes, 2013; Kabir, 2010). Immunocompromised and/or young birds are more prone to infection and disease in comparison with healthy older birds (Barnes, 2013; Dziva and Stevens, 2008; Kabir, 2010). Furthermore, primary respiratory infections due to other pathogens (e.g. viruses (Newcastle disease virus, infectious bronchitis virus, infectious laryngotracheitis (herpesvirus)) and bacteria (*Mycoplasma gallisepticum, Pasteurella multocida*) might increase the birds' susceptibility to APEC infection (Dho-Moulin and Fairbrother 1999; Kabir, 2010).

Generally, stress can increases the susceptibility of the birds to all diseases (Barnes, 2013; Vandekerchove *et al*., 2004) and colibacillosis in particular (Antao *et al*., 2008; Johnson *et al*., 2008). The occurrence of stress among the chickens could be induced by different infectious agents and/or inappropriate husbandry and management, such as inadequate feeding and poor climatological farm conditions (Barnes, 2013; Dziva and Stevens, 2008; Kabir, 2010). A previous study suggested that the presence of a systemic avian colibacillosis infection can indicate the presence of physiological stressors (Zanella *et al*., 2000).

The fact that APEC is found in the gastrointestinal tract of healthy and unhealthy birds means it is commonly disseminated in faecal material (Circella *et al*., 2012; Johnson *et al*., 2008; McPeake *et al*., 2005; Rodriguez-Siek *et al*., 2005a), which means that APEC are widely distributed in the bird's surrounding environment through faeces, litter, water, dust and feeders (Barnes, 2013; Dziva and Stevens, 2008). Furthermore, APEC can persist for a long period of time under dry conditions, meaning birds may be continually exposed to APEC leading to a higher risk of colibacillosis (Whiteman *et al*., 1979). Several studies have reported constant high levels of *E. coli* among litter in the chicken sheds (Macklin *et al*., 2006; Runge *et al*., 2007). The common practice in Australian poultry production, similar to American poultry production in regards to the litter, is to reuse the litter for several poultry production cycles (Macklin *et al*., 2006; Runge *et al*., 2007). Thirty percent of farms in study by Runge *et al*. (2007) reused their litter multiple times. Poultry bedding is considered as a rich source for pathogens originating from poultry faeces (Runge *et al*., 2007).

Environmental pressures are positively correlated with increased susceptibility to APEC infection among broiler chickens (Kabir, 2010). These environmental pressures can lead to physical damage to the respiratory system due to previous respiratory infections, excessive ammonia levels and/or high faecal contaminated dust (Barnes, 2013; Kabir, 2010; Vandekerchove *et al*., 2004). High flock density, inadequate ventilation and poor litter quality in the broiler flock especially during winter with restricted ventilation are major environmental stressors (Barnes, 2013; Kabir, 2010; Leitner and

Heller, 1992). Proper ventilation is important to reduce the levels of *E. coli* on a farm. Wathes et al. (1986) found warm and dry temperatures of more than 30 °C increased the rapid death of aerosol *E. coli* (Wathes *et al.* 1986). Studies on Australian poultry farms have reported a seasonal pattern for *E. coli* survival, where survival rates increase in the colder months compared to summer months (Chinivasagam *et al.* 2009; Wathes, 1996). Another study by Chinivasagam *et al*. (2009) suggested that high temperatures and direct sunlight reduced the level of bacteria.

One of the main sources of APEC infection in poultry flocks is the acquisition of infections from APEC contaminated aerosols. The majority of the Australian farms are mechanically ventilated and this is a major risk factor for colibacillosis and can also contribute to transmission of large volumes of contaminated air to the outside environment (Barnes, 2013; Davis and Morishita 2005; Ginns *et al. 1998*; Kabir 2010; Vaicionis, 2006).

The normal intestinal flora of birds could be disturbed due to factors such as: general enteritis, antimicrobial administration, poor water quality and sudden feed changes (Barnes, 2013). This disturbance can lead to the introduction of APEC infection through the gastrointestinal route by contaminated water, feed and litter (Barnes, 2013).

Impaired biosecurity protocols on farms which allow birds to contact wild birds, rodents, insects or any other wild animals are potential risk factors for APEC infections (Jiang *et al*. 2014; Wang *et al*. 2013). Generally, the animals and insects act as vectors to introduce and/or spread the disease among flocks (Barnes, 2013; Vandekerchove *et al*. 2004a). It is not only the wild animal that can introduce colibacillosis, but infectious diseases can also be introduced through feed, hence strict biosecurity measures for feed are paramount (Vandekerchove *et al.*, 2004; Wang *et al.*, 2013). The more frequently carcasses are removed is another important factor associated with reducing the introduction and/or spread of APEC within the same flocks or different sheds in the farm by reducing the exposure time and dose of the agent (Vandekerchove *et al.*, 2004a).

Farm visitors or worker can act as a vector to introduce and spread APEC into the farms either directly or indirectly. The human role as a vector can be minimised by following hygiene procedures in regards to foot and hand sanitisation and wearing protective clothes. The lack of disinfection of equipment before and after use in selected sheds can act as a predisposing factor for APEC infection in the chicken flocks (Barnes, 2013).

Vandekerchove *et. al* (2004a) reported that the distance between chicken farms can play an important part as a potential risk factor associated with APEC infection. The closer the chicken farms the higher the prevalence of APEC detected on the chicken farms.

Previous studies have suggested an association between APEC infection and the source of drinking water as well as the treatment applied to the water and feed (Vandekerchove *et al*., 2004a). Several studies have reported that adding chlorine to the drinking water was an effective measure to reduce the economic losses associated with APEC infection in chickens (Vandekerchove *et al*., 2004a).

The literature review highlighted the absence of data regarding the potential risk associated with APEC infection in broiler flocks in Australia. There was no specific information in regards to the associations between farm management and the prevalence of APEC among commercial broiler chickens.

1.8 Intervention strategies for avian colibacillosis

1.8.1 Management procedures to control and/or prevent avian colibacillosis

Commercial broiler poultry in Australia are raised in intensive farming systems; therefore, effective flock management and strict biosecurity protocols are crucial to prevent and control avian colibacillosis (Kabir, 2010). Effective control and prevention of colibacillosis depends on the ability to identify and eliminate any potential predisposing factors that are associated with APEC infection (Kabir, 2010). The main goal is to reduce the level of APEC exposure by reducing any stress related factors and the number of APEC in the chicken's production environment (feed, drinking water, litter, dust) (Barnes, 2013). This goal can be achieved by improving husbandry and biosecurity, ventilation, nutrition, and immune status among the chicken flocks (Dhillon and Jack, 1996; Hasan *et al*., 2011; Nolan *et al*., 2013). Recommendations include the addition of chlorine into drinking water to reduce the introduction and spread of disease and decrease the mortality rates associated with APEC outbreaks (Vandekerchove *et al*., 2004).

Applying and maintaining a high biosecurity level can improve the flock's health, as well as, increase the productivity (Laanen *et al*., 2014; Moore, 1992; Ribbens *et al*., 2008). Strict biosecurity measures at the hatchery are considered the first step to preventing avian colibacillosis, as eggs contaminated with faecal materials can act as vehicles to introduce and/or spread APEC between different flocks (Barnes, 2013). Disinfecting and fumigating of all the eggs, grading the eggs on the basis of quality and discarding cracked or eggs heavily contaminated with faecal materials can reduce APEC contamination of broiler breeder (Kabir, 2010).

Daily removal of bird carcasses is very important to control the spread of avian colibacillosis within the same flocks or the farm (Vandekerchove *et al*., 2004). In the case of an outbreak with APEC infection among the chicken flocks, the usage of strict biosecurity procedures including the use of foot and hand sanitizers and disinfecting of all equipment before and after use in the selected shed

will help reduce the spread of the disease to other chicken flocks (Barnes, 2013).

The disposal of the litter and manure at the end of the flock cycle and the following of regular cleaning protocols on the farm after each flock is sent to slaughter will help to prevent the spread of the disease among future flocks (Barnes, 2013; Kabir, 2010; Vandekerchove *et al.*, 2004).

To minimise the introduction and spread of the disease strict biosecurity protocols should be applied to the farm to prevent contact between the farm birds and wild birds, rodents and insects, as well as restricting human movement between the farms by applying suitable farm management protocols (Barnes, 2013; Wang *et al.*, 2013). The Australian government outlines biosecurity guidelines for bird producer in order to limit the spread of infectious diseases and pests, both within a farm and from one farm to others (Australian Chicken Meat Federation, 2014).

1.8.2 Vaccinations

The need for an effective vaccine to control this economically important poultry disease has become more significant after a European study reported an increase in the prevalence of the disease despite applying strict husbandry measures, as well as, advanced biosecurity rules (Vandekerchove et al., 2005).

However, controlling colibacillosis through an effective vaccine is faced by many challenges such as the ability of the vaccine to provide cross protection against different strains of APEC; the economic feasibility of the vaccine; methods of vaccine delivery, public safety and the age of the chickens at the time of vaccination (Ghunaim *et al*., 2014a). Examples of these challenges include administration of the vaccine at young ages, where the birds are vulnerable to APEC infection, will only provide a protective immune response for the chickens by the age of 21 days and the majority of the birds go to slaughter by 35 days (Ghunaim *et al.*, 2014b). There are also the effects of immune-compromise by other diseases, which could be countered by the vaccination of the broiler or breeder flocks against other common respiratory pathogens to help decrease the occurrence of APEC infection in the flock (Kabir, 2010).

Identification of successful vaccine candidates that can provide protection against the majority of APEC outbreaks may follow investigation of the expression of virulence genes. Several APEC associated virulence genes such as adhesins (de Pace *et al.*, 2010; Ewers *et al*., 2004), iron-acquisition (Janben *et al.*, 2001; McPeake *et al*., 2005), serum resistance (Nolan *et al*., 2003; Tivendale *et al.*, 2004) and VGs encoded in the ColV plasmids have been implicated in virulence through genetic analysis (Antao *et al.*, 2008). In particular, the serum resistance APEC linked VGs such as *traT* and *iss* play a significant role in APEC pathogenicity (Lynne *et al.*, 2006a; Lynne et al., 2012; Nolan *et*

al., 2003; Pfaff-McDonough *et al*., 2000; Rodriguez-Siek *et al*., 2005a).

Early attempts to develop an effective vaccine that provided protection against APEC were focused mainly on using bacteria (Dho-Moulin and Fairbrother, 1999; Ghunaim *et al*., 2014a). An early study by Kariyawasam *et al*. (2002) identified Type 1 fimbriae, P-fimbriae, aerobactin receptor and lipopolysaccharide (LPS) as suitable vaccine candidates against APEC**.** Furthermore, a number of investigations took place to develop an effective vaccine that provided a heterologous protection against different APEC strains (Bao *et al*., 2013; Chaudhari *et al*., 2013; Lynne *et al*., 2006b). The continuing development of new vaccines that have the ability to control APEC infections is supported by the ongoing discovery of new APEC linked VGs combined with the analysis of *E. coli* whole genome sequences. Despite ongoing vaccine development there is no commercial vaccine available in Australia for avian pathogenic *E. coli* in poultry

1.9 *Escherichia coli*

Escherichia coli was named after the German physician Theodor Escherichia, who was the first to describe this bacterium at the end of nineteenth century after isolating it from faecal samples from neonates (Escherich, 1988). *E. coli* is a gram-negative, facultative anaerobic, non-sporulating, rod shaped bacteria that belongs to the *Enterobacteriaceae* family (Berg, 1996; Quinn *et al*., 2011). The bacteria co-exist normally in the gastrointestinal microbiota of healthy humans, mammalian animals and birds and their surrounding environment (Belanger *et al*., 2011; Wirth *et al*., 2006). While the majority of *E. coli* are non-pathogenic, though they may be linked with opportunistic infections in people, animals and birds, some strains are capable of causing intestinal or extraintestinal diseases (Ewers *et al*., 2009; Johnson and Russo, 2002; Russo and Johnson, 2009).

1.9.1 Commensal *E. coli*

The vast majority of *E. coli* are considered commensal or non-pathogenic (Kamada *et al*., 2013; Linton and Hinton, 1988; Russo and Johnson, 2000). Commensal *E. coli* protect the host by competing with pathogenic microorganisms for nutrients and receptors to ferment non-digestible dietary residues in the gastrointestinal tract helping to breakdown food, assist in food absorption and waste production. They also synthesise vitamin K (O'Hara and Shanahan, 2006). Some non-pathogenic *E. coli* can cause disease if the animals are immunocompromised, the mucosal barrier is compromised, or if there is a change in the habitat of the organism, for example urinary tract infections caused by *E. coli* from the gastrointestinal tract which have been able to ascend into the bladder (Packey and Sartor, 2009). Microbiota that is altered, for example, after antimicrobial therapy, enhances the growth of pathogenic bacteria; therefore, the balance between beneficial and

detrimental intestinal bacteria species is disturbed potentially having a negative influence on health (Langdon *et al.*, 2016). Furthermore, commensal *E. coli* can acquire VGs that allow them to adapt to and survive in new niches where they may be able to cause disease (Johnson *et al.*, 2008a; Kaper *et al.*, 2004).

In birds, commensal *E. coli* are known as avian faecal *E. coli* (AFEC) (Kaper *et al.*, 2004; Nolan *et al.*, 2013). Globally, several studies have aimed to define AFEC and differentiate them from APEC (Johnson *et al.*, 2008b; McPeake *et al.*, 2005; Rodriguez-Siek *et al.*, 2005a). Previous studies have also considered AFEC as potentially pathogenic because they may harbour large numbers of APEC-associated VGs. This population of gut *E. coli* that contain APEC-associated VGs pose a risk for birds as they have the potential to cause avian colibacillosis (Ewers *et al.*, 2009; Kemmett *et al.*, 2013; McPeake *et al.*, 2005). In the one Australian study, which screened 251 AFEC isolates cultured from healthy chickens for the presence of eight ExPEC-associated VGs (p*apAH, papC, afa/draBC, sfa/focDE, sfaS, aerJ, kpsMT II* and *focG*), the authors reported that 10% of AFEC isolates carried two or more of the eight VGs and 68.1% harboured none of the VGs (Obeng *et al.*, 2012). The detection of ExPEC-associated VGs among Australian AFEC isolates may pose a public health concern, as these isolates may act as a reservoir for the VGs.

1.9.2 Intestinal pathogenic *E. coli*

Intestinal pathogenic *E. coli* are found at a lower prevalence to commensal *E. coli* in the gastrointestinal tract and are capable of causing intestinal disease (Belanger *et al.*, 2011). Intestinal pathogenic *E. coli* strains can be distinguished from commensal or ExPEC based on the clinical signs and VG profile (Leimbach *et al.*, 2013). Intestinal *E. coli* are classified into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Belanger *et al.*, 2011; Johnson and Russo, 2002).

1.9.3 Extraintestinal pathogenic *E. coli* (ExPEC)

Similar to intestinal *E. coli*, ExPEC can be found in the gastrointestinal tract (Russo and Johnson, 2003). However, unlike intestinal pathogenic *E. coli*, this pathotype of *E. coli* is not capable of causing gastrointestinal disease (Russo and Johnson, 2003). ExPEC are responsible for a diverse spectrum of extraintestinal infections, which may lead to septicaemia in humans, mammals and birds (Johnson and Russo, 2002; Mokady *et al.*, 2005). They have acquired specific VGs that enable them to overcome or challenge the host defence mechanisms and cause diseases at multiple sites outside the gastrointestinal habitat (Ewers *et al.*, 2007; Johnson and Russo, 2002; Mokady *et al.*, 2005). Virulence

genes are often located on mobile genetic elements (MGEs) such as plasmids, bacteriophages or pathogenicity islands (PAIs) and can be transferred to different strains of *E. coli* or different bacterial species (Gyles and Fairbrother, 2010; Russo and Johnson, 2000).

Extraintestinal pathogenic *E. coli* can be classified into different subgroups: septicaemia associated *E. coli* (SePEC); uropathogenic *E. coli* (UPEC) and neonatal meningitis associated *E. coli* (NMEC) in mammals and APEC in birds; based on the target host and the clinical manifestation they cause (Belanger *et al.*, 2011; Kaper *et al.*, 2004). However, this classification is limited and it is not absolute as all ExPEC subgroups demonstrate overlapping phylogenetic groups and VGs (Ewers *et al.*, 2007; Johnson and Russo, 2002).

Johnson *et al.*(2009) proposed screening *E. coli* isolates for eight VGs to allow for the identification of ExPEC. In ExPEC two or more of the VGs: *papAH* (P fimbriae structural subunit); *papC* (P fimbriae assembly); *sfa/foc* (S and F1C fimbriae subunits); *sfaS* (S fimbriae (sialic acid-specific); *afa/dra* (Dr-antigen-binding adhesins); *kpsMTII* (group 2 capsular polysaccharide units); *iutA* (aerobactin receptor) and *focG* (F1C fimbriae) could be detected.

1.9.4 Avian pathogenic *E. coli* (APEC)

Escherichia coli that is capable of causing localised or systemic extraintestinal infections in chickens, turkeys, geese, ducks and other species of birds is known as APEC (Circella *et al.*, 2012; Guabiraba and Schouler, 2015; Nolan *et al.*, 2013). APEC can cause infections in all age groups of birds (Guabiraba and Schouler, 2015; Nolan *et al.*, 2013), acting as a primary (Vandekerchove *et al.*, 2004b) or secondary pathogen (Guabiraba and Schouler, 2015; Kabir, 2010; Kaper *et al.*, 2004; Nolan *et al.*, 2013). Although APEC is present in the intestinal microbiota, the main route of entry into birds is through the respiratory system via inhalation of APEC contaminated aerosols. The bacteria can then translocate from the respiratory tract (air sac and lungs) to the blood stream and other internal organs (Delicato *et al.*, 2003; Dissanayake *et al.*, 2014). Systemic infection can also occur after *E. coli* in the cloaca or the intestinal tract gain access to the bloodstream (Kabir, 2010; Landman and Cornelissen, 2006).

The pathogenicity of APEC strains are multifactorial, based on the presence and expression of different groups of VGs (Won *et al.*, 2009). Globally, the economic, public health and animal welfare significance of avian colibacillosis, has led to a number of studies from United States of America aiming to define APEC and to identify pathogenicity mechanisms (Jeffrey *et al.*, 1999; Kariyawasam and Nolan, 2009; Norton *et al.*, 2000). Studies have identified a large number of VGs in *E. coli* cultured from birds with avian colibacillosis (Antão *et al.*, 2008; Dissanayake *et al.*, 2014; Guabiraba

and Schouler, 2015; Johnson *et al.*, 2008b; Paixao *et al.*, 2016; Wang *et al.*, 2010). The involvement and relevance of these VGs in the pathogenicity of avian colibacillosis is still poorly understood and the majority of studies on APEC and APEC-associated VGs are descriptive (Dozois *et al.*, 1994; Jeffrey *et al.*, 1999; Norton *et al.*, 2000; Obeng *et al.*, 2012; Zhao *et al.*, 2009). Few *in vivo* experimental studies have enhanced the understanding of the APEC pathogenicity mechanisms (Antão *et al.*, 2008; Ask *et al.*, 2006; Musa *et al.*, 2009). However, no single or specific set of VGs have been systematically linked with APEC pathogenicity. It is very rare to find the same set of VGs in all APEC, in the same study and/or different studies (Kawamura-Sato *et al.*, 2010; Kemmett *et al.*, 2013; Paixao *et al.*, 2016; Rodriguez-Siek *et al.*, 2005a; Zhao *et al.*, 2005). Nevertheless, APEC harbour several VGs that enable the bacteria to invade, colonise, evade the immune system and cause avian colibacillosis (Guastalli *et al.*, 2013; Horn *et al.*, 2012; Kemmett *et al.*, 2014; Pires-dos-Santos *et al.*, 2013).

The genetic diversity displayed by APEC makes it very challenging to diagnose, prevent and treat avian colibacillosis. Many *E. coli* strains are not virulent and a fast, accurate diagnostic test to distinguish whether an avian *E. coli* isolate is pathogenic or non-pathogenic, is required. However, several studies have suggested that the prevalence of certain VGs among *E. coli* isolates obtained from chickens with colibacillosis were useful to identify and characterise APEC and distinguish it from AFEC (Ewers *et al.*, 2005; Johnson *et al.*, 2008b; Moulin-Schouleur *et al.*, 2007; Rodriguez-Siek *et al.*, 2005a).

A study by Ewers *et al.* (2005) suggested that eight VGs: P-fimbriae (*papC*); aerobactin (*iucD*); iron-repressible protein (*irp2*); temperature-sensitive hemagglutinin (*tsh*); vacuolating autotransporter toxin (*vat*); enteroaggregative toxin (*astA*); increased serum survival protein (*iss*) and colicin V plasmid operon genes (*cva/cvi*) contributed to the pathogenicity of APEC. The authors proposed that the presence of four of these eight VGs could identify APEC and differentiate between APEC and AFEC. However, only 14 APEC isolates were included in this study reducing the strength of their conclusions. In a study by Kemmet *et al.* (2013) a larger number of faecal *E. coli* isolates (n = 3,360) obtained from apparently healthy chickens and 324 *E. coli* isolates from birds with colibacillosis were screened for the presence of ten VGs (*astA, iss, irp2, iucD, papC, tsh, vat, cvi, sitA* and *ibeA*). The authors reported that 24% of the AFEC isolated from one-day-old healthy chickens harboured more than five of these VGs in comparison with 1% of AFEC sourced from chickens at slaughter. They found *irp2, papC, iucD, cvi, sitA* and *ibeA* genes were significantly associated with APEC.

Rodriguez-Siek *et al.* (2005a) investigated the prevalence of 38 APEC-associated VGs among 451 APEC and 104 AFEC isolates obtained from birds in the USA. They reported that the majority of

APEC isolates harboured *ompT*, several iron acquisition genes (*iroN*, *iutA*, *sitA*, *fyuA* and *irp2*) and other VGs genes that are carried in the APEC plasmid pTJ100 (*cvaC*, *iss*, *tsh*, *iroN*, *iutA* and *sitA*).

Another study in the USA conducted by Johnson *et al*. (2008b) aimed to identify a minimum number of VGs that could identify APEC. In this study, 124 *E. coli* isolates of known pathogenicity underwent extensive genotyping and were screened for the presence of 46 VGs. The author identified five VGs (*iutA*, *iss*, *ompT*, *iroN* and *hlyF*) that were significantly associated with APEC strains. Johnson *et al*. (2008b) developed a pentaplex-PCR, targeting these five VGs, and subsequently screened 994 *E. coli* (794 APEC and 200 AFEC) to validate the selection of the five VGs. The results showed that highly pathogenic APEC strains harboured an average number of 4.6 VGs while an average of 4.3 and 3.9 were found among medium and low pathogenic APEC strains, respectively. The average number of VGs in AFEC isolates was 1.3.

Subsequently, studies have used the pentaplex-PCR described by Johnson *et al*. (2008b) and confirmed that it is a rapid diagnostic tool to differentiate between APEC and AFEC (de Oliveira *et al*., 2015; Dissanayake *et al*., 2014; Hussein *et al*., 2013). Despite the geographical variations of the studies, Kobayashi *et al*. (2011) (68% – 72%), Hussein *et al*. (2013) (90% – 94%) and de Oliverira *et al*. (2015) (82% – 95%) reported similar frequencies of the five VGs as Johnson *et.al*. (2008b) (78% – 85%) among APEC isolates cultured from lesions of birds affected with colibacillosis.

However, Dissanayake *et al*. (2014) found that only 25% of the *E. coli* isolates (14 of 55), cultured from the lesions of birds with colibacillosis in Sri Lanka, harboured the five VGs. These authors identified four VGs (*sitA*, *ompT*, *hlyF* and *iroN*) that were significantly associated with APEC in their region. These four genes were possessed by 54.5% (30 of 55) of the APEC isolates in Sri Lanka.

A study by Schouler *et al*. (2012) examined 1,491 *E. coli* (1,307 APEC and 184 AFEC). They identified 13 VGs (*sitA*, *F1*, *iutA*, *tsh*, *frzorf4*, *tkt1*, *aec4*, P(F11), *aec26*, *neuC*,*sfa-focDE*, *cdt* and *eae*) more frequently found in APEC and four different VG associations (A [*iutA*+, P(F11)+], B [*iutA*+, P(F11)−, *frzorf4*+], C [*iutA*+, P(F11)−, *frzorf4*−, O78+] and D [*iutA*−, *sitA*+, *aec26*+]). These four different VG associations identified 70.2% (247 of 352) of the pathogenic strains, based on a lethality test.

In a more recent study, in Brazil, de Oliveria *et al*. (2015) investigated the association between the APEC VGs proposed by Johnson *et al*. (2008b) and the pathogenicity of the APEC strains. In partial agreement with Johnson *et al*. (2008b), they identified a positive association between the number of VGs and the pathogenicity score of avian colibacillosis. However, the authors found that 51% of the

AFEC harboured between two and four of the five VGs (average of three), compared to the average of 1.3 VGs identified in AFEC by Johnson *et al.* (2008b).

Only two studies have investigated VGs associated with APEC in Australia. The first study investigated the presence of three VGs (*iucA, tsh* and *iss*) among the virulent pVM01 plasmid and their association with APEC pathogenicity. The authors verified the presence of the three VGs in the plasmid and identified a strong association between APEC and *iss* and *iucA*, which represents the entire aerobactin operon, but not the *tsh* gene (Tivendale *et al.*, 2004).

The second Australian study aimed to determine the frequency of eight ExPEC-linked VGs (*iutA, papAH, papC, Afa/draBC, sfa/focDE, sfaS, kpsMTII* and *focG*) among 251 *E. coli* isolates cultured from faeces of healthy chickens (Obeng *et al.*, 2012). The authors found *iutA, papC* and *kpsMTII* genes were common among the AFEC, whilst none of the *E. coli* isolates harboured the *sfa/foc* gene.

In summary, despite the genetic diversity of APEC, all the results obtained by different studies demonstrated the ability to differentiate APEC from AFEC based on a combination of frequently occurring VGs. Johnson *et al.* (2008b) used a large sample size, combined with extensive genotyping to select VG markers for APEC. Despite the variation in frequency of these five VGs in different overseas studies, a significant association between them and APEC has been reported (de Oliveira *et al.*, 2015; Dissanayake *et al.*, 2014; Hussein *et al.*, 2013).

Selecting a specific array of APEC VGs to use as markers to identify APEC in Australia would require regional studies to be conducted due to the large number of VGs associated with both APEC and AFEC, as well as the worldwide diversity in the VGs associate with avian colibacillosis. Individual VGs will be discussed in Section 1.10.6.

1.10 Classification of *E.coli*

1.10.1 Serological classification

A serological classification scheme for *E. coli* was first described in the 1940s and was based on the immunogenicity of the bacterial surface structures. Initially, it consisted of somatic antigen 'O', capsular antigen 'K' and flagella antigen 'H' (Orskov and Orskov, 1992). A fimbrial antigen 'F' was included later (Wray and Woodward, 1994). Serotyping is widely used and is a well-established type of *E. coli* classification for pathogenicity detection and epidemiological studies (Nolan *et al.*, 2013; Scheutz *et al.*, 2004). Serotyping based on the O and H antigens are considered the 'gold-standard' as only limited laboratories have the ability to type the K-group (DebRoy *et al.*, 2011).

Overall, an association between ExPEC pathogenicity and serogroup 'O' antigens has been reported (Orskov and Orskov, 1992). ExPEC, and in particular APEC, have been associated with certain O-

types particularly O78, followed by O1, O87, O2 and to a lesser extent O15, O8, O18, O53 and O55 serogroups (Blanco *et al.*, 1997; Dozois *et al.*, 1992; Ewers *et al.*, 2007; Gomis *et al.*, 1997; Ngeleka *et al.*, 1996; Vandekerchove *et al.*, 2004b). Studies in Germany and the United Kingdom (UK) by Vandekerchove *et al.* (2004b) and McPeak *et al.* (2005) respectively, reported an association between avian colibacillosis and serogroup O78. Other studies have cautioned that the presence of a specific serogroup does not reflect the pathogenicity of APEC strains (Delicato *et al.*, 2003; Ewers *et al.*, 2004; La Ragione and Woodward, 2002). Rodriguez-Siek *et al.* (2005a) proposed that plasmid related genes (pTJ100: large conjugative R plasmid) are more suitable than a serogroup to define APEC.

Overall, serotyping is complex as currently there are 186 different O-groups as well as 53 H-groups among *E. coli* (Fratamico *et al.*, 2016a). Furthermore, serotyping is labour intensive, time consuming and expensive (Fratamico *et al.*, 2016a). Inaccuracy due to cross reactivity of the antisera with different serogroups can also be considered a disadvantage (Fratamico *et al.*, 2016a). Traditional serotyping can only be performed in specialised laboratories, although some laboratories are now also using a PCR based typing system (Fratamico *et al.*, 2016a; Iguchi *et al.*, 2015; Lacher *et al.*, 2014). In addition, many *E. coli* stains are non-typable (Fratamico *et al.*, 2016b; Sharma *et al.*, 2016).

1.10.2 Enterobacterial repetitive intergenic consensus (ERIC)

Enterobacterial repetitive intergenic consensus (ERIC) typing was first described in *E. coli* but has now been used for other members of the *Enterobacteriaceae,* as well as, *Vibrio cholerae* (Hulton *et al.*, 1991; Sharples and Lloyd, 1990). The ERIC sequence is a repeated, short, conserved region of only 126 base pairs (bp) (Hulton *et al.*, 1991; Sharples and Lloyd, 1990; Versalovic *et al.*, 1991). ERIC-PCR typing detects these repeated DNA sequences that are distributed throughout the genome. Genetic relatedness is evaluated using the profile patterns, or "fingerprints", that were created by the separation of the PCR products. The sole usage of this protocol to identify any genetic relatedness is not recommended due to interexperimental variation of ERIC-PCR results by computer software and laboratory variations (Meacham *et al.*, 2003). However, this typing method is commonly used as it is rapid, cost-effective and can classify *E. coli* into different clonal groups for the purpose of epidemiological studies (Cherifi *et al.*, 1991; Gillings and Holley, 1997; Meacham *et al.*, 2003; Ranjbar *et al.*, 2017; Ugorski and Chmielewski, 2000; Wilson and Sharp, 2006). Several studies have recommended the usage of ERIC-PCR for *E. coli* strains obtained from different sources due to it is power of differentiation (Dhanashree and Shrikara, 2012 ; Diab and Al-urk, 2011; Maryam, 2016)

In addition, several other PCR based typing methods have been developed based on the repeated DNA sequence elements distributed throughout the genome. These include randomly amplified polymorphic DNA and restriction fragment length polymorphism (Jonas *et al.*, 2003).

1.10.3 Phylogenetic classification

Phylogenetic grouping provides a very popular method of classification that is based on the presence/absence of two genes *chuA* and *yjaA* as well as TspE4.C2, a DNA fragment. Originally, *E. coli* were classified into four phylogenetic groups (A, B1, B2 and D) (Clermont *et al.*, 2000). This classification has been extended to identify additional groups C, E, F and *Escherichia* cryptic clade I (termed *E.* clade 1) using the Clermont phylogenetic grouping quadruplex PCR plus the follow up PCRs (Clermont *et al.*, 2013). *E. coli* from specific biological niches are more likely grouped into certain phylogroups (Clermont *et al.*, 2013). With ExPEC strains being more likely to be B2, D and F whereas, *E. coli* belonging to phylogroups A, B1, C and E are less likely to be associated with extraintestinal disease. Using the original classification scheme, human ExPEC derive mainly from *E. coli* phylogenetic group B2 and to a lesser extent group D, while groups B1 and A are the common groups found in commensal *E. coli* (Bingen *et al.*, 1998; Ewers *et al.*, 2007; Johnson *et al.*, 2001; Johnson and Russo, 2002). Obeng *et al.* (2012) found the majority of Australian AFEC belonged to the commensal groups A (39.4%) and B1 (32.3%) with less in virulent groups B2 (11.2%) and D (17.1%). Previous research has reported a positive association between phylogenetic group B2 and the presence of larger numbers of VGs among ExPEC, APEC and AFEC (Danzeisen *et al.*, 2013). However, APEC significantly differs in their phylogenetic assignment as they cluster predominantly in phylogenetic group A (Sola-Gines *et al.*, 2015; Zhu Ge *et al.*, 2014).

Caution has to be taken when comparing studies that have used different classification schemes. Logue *et al.* (2017) found when comparing assignment of phylogenetic group using the old and revised Clermont schemes that 53.8% (243 of 452) of APEC strains were reclassified. There were significant changes from phylogenetic groups A to C and from D to E and F.

1.10.4 Multilocus sequence typing (MLST)

Currently, the global gold-standard for the clonal analysis of *E. coli* is multilocus sequence typing (MLST), which was introduced by Maiden *et al.* (1998). There are several MLST schemes available (Ahmed *et al.*, 2016; Chaudhuri and Henderson, 2012; Jaureguy *et al.*, 2008; Reid *et al.*, 2000; Wirth *et al.*, 2006), which classify *E. coli* into different sequence types (STs) (Larsen *et al.*, 2012). This method amplifies short nucleotide sequences about (450 – 500 bp) by PCR of five to seven of the following housekeeping genes: *adk* (adenylate kinase); *fumC* (fumarate hydratase); *gyrB* (DNA gyrase); *icd* (isocitrate/isopropylmalate dehydrogenase); *mdh* (malate dehydrogenase); *purA* (adenylosuccinate dehydrogenase) and *recA* (ATP/GTP binding motif). The fragments are then sequenced and compared to an MLST database to obtain a sequence type (ST).

Multilocus sequence typing is a uniformly powerful typing method of classification that is standardised between different laboratories and the database can be accessed easily on a net-based computer enabling the global exchanging of molecular typing data (Maiden *et al.*, 1998). Besides the classical MLST scheme, several other MLST classifications have been established targeting fewer genes (Sahl *et al.*, 2012; Tchesnokova *et al.*, 2013; Weissman *et al.*, 2012). The most common STs detected among APEC and AFEC are globally disseminated and have emerged among different species of animals, regardless of pathogenicity, such as ST354 and ST10 (Abraham *et al.*, 2015; Dissanayake *et al.*, 2014; Kim *et al.*, 2011; Maluta *et al.*, 2014; Manges and Johnson, 2012; Mora *et al.*, 2011; Pires-dos-Santos *et al.*, 2013; Schaufler *et al.*, 2015).

Furthermore, new classification schemes using informative single-nucleotide polymorphisms (SNPs) collected from the MLST data are being used for *E. coli* typing (Fernandez-Romero *et al.*, 2011). Hommais *et al.* (2005) reported similar results using the SNP method and other typing methods to differentiate particular *E. coli* strains.

1.10.5 Whole genome sequence (WGS)

The ability of whole genome sequencing (WGS) to provide massive data in regards to a pathogen's fingerprint could potentially make this typing method the new "gold-standard" for classification. At present, there are no standardised methods to analyse the enormous amount of data produced by WGS (Franz *et al.*, 2014; Oulas *et al.*, 2015). Regardless of the large amount of information that can be provided by the whole genome analyses, the fact that the predicted products can't be associated to a function or phenotype is considered as one of the major limitation of this type of analysis. A complete genome sequence for a virulent APEC strain detected several virulence and antimicrobial resistance genes (ARGs) among several plasmids (Wang *et al.*, 2016). This finding reinforces the important role that plasmids play in distributing virulence and resistance genes among *E. coli* populations through horizontal gene transmission (Wang *et al.*, 2016). There is a need for whole genome and plasmid sequencing studies to accurately determine the genetic variation within and between ExPEC, as well as, commensal *E. coli*.

1.10.6 Virulence genes (VGs)

Avian pathogenic *E. coli* harbours an assortment of VGs (Table 1.1) including, but not limited to, adhesins, iron uptake systems, increased serum survival and toxins, which contribute to APEC pathogenicity (Dissanayake *et al.*, 2014; Ewers *et al.*, 2007; Johnson and Russo, 2002; La Ragione and Woodward, 2002; Moulin-Schouleur *et al.*, 2007; Rodriguez-Siek *et al.*, 2005a). The presence of certain VGs enables APEC colonisation and infections to occur in extraintestinal locations. Virulence

genes may be encoded on PAIs, which harbour several VGs (Schubert *et al.*, 1998). Furthermore, VGs can be found on plasmids such as R plasmids or CoIV plasmids in APEC (pAPEC-OI, pAPEC-O2-CoIBM and Ptj100) and other MGEs enabling horizontal transmission of the VGs among *E. coli* isolates (Rankin *et al.*, 2011; Rodriguez-Siek *et al.*, 2005a). The plasmid-encoded genes play an important role in the virulence of APEC as they have been shown to be involved in toxin synthesis, iron acquisition systems, outer membrane proteins and adhesins (Skyberg *et al.*, 2006; Toma *et al.*, 2004).

1.10.6.1 Colicins

Colicin *E. coli* producers can inhibit bacterial growth and interfere with membrane formation from the same or related species (Cascales *et al.*, 2007). Colicins can be encoded by genes located on plasmids. These plasmids are frequently called Col plasmids. Colicins are encoded by several genes such as: *Ia; Ib; E1; E2; E3; I; K; B* and *V*, which have been shown to be more prevalent among APEC compared with AFEC (Dias da Silveira *et al.*, 2002; Fantinatti *et al.*, 1994).

The majority of APEC strains harbour Colicin V plasmids (Wray and Woodward, 1994). These plasmids also contain other pathogenicity related genes, such as those that encode for the aerobactin iron uptake system and serum resistance (Mokady *et al.*, 2005; Russo and Johnson, 2003; Valvano and Crosa, 1988). It has been demonstrated that mutations to ColV plasmids are linked with decreased APEC pathogenicity, suggesting that some VGs associated with ColV plasmids are involved in APEC pathogenicity mechanisms (Skyberg *et al.*, 2008). Similar to other VGs, geographical location influences the prevalence of CoIV plasmid linked genes among chickens with colibacillosis. A variation in the prevalence of ColV plasmids of 99.1% and 66.8% were reported among APEC isolates in the UK and USA, respectively (McPeake *et al.*, 2005; Rodriguez-Siek *et al.*, 2005a). While a lower frequency was documented in Brazil (22%) and Spain (23%) (Blanco *et al.*, 1997; Rocha *et al.*, 2008).

1.10.6.2 Toxins

Cytotoxic activity in APEC was first described by Fantinatti *et al.* (1994) and by Parreira and Yano (1998). APEC is capable of producing several toxins such as: vacuolating autotransporter toxin (*vat*); enterohaemolysin (*ehxA*); cytotoxic necrotising factor 1 (*cnf1*); microcin ColV (*cvaC*); cytolethal distending toxin (*cdt*); haemolysin (*hly*) and secreted autotransporter toxin (*sat*) (Johnson *et al.*, 2001; Toth *et al.*, 2003).

Originally, Vat, which is a serine protease autotransporter, was discovered in a septicaemic *E. coli* strain (Parreira *et al.*, 1998; Parreira and Gyles, 2003). The *vat* gene encodes for the Vat

protein, which has cytotoxic activity against the chicken embryo fibroblast and kidney cells (Parreira and Gyles, 2003). Several studies have reported that the *vat* gene is encoded in the APEC PAI and occurs at a higher frequency among APEC compared with AFEC (Ewers *et al.*, 2004; Ewers *et al.*, 2005).

The presence of *ehxA*, *sat* and *cnf1* have also been reported in APEC strains (Braga *et al.*, 2016; Cunha *et al.*, 2017). However, their role in pathogenesis is not fully elucidated. Furthermore, some of the toxin genes (*hly*, *cdt* and *cvaC*) have been linked with large transmissible plasmids indicating that these VGs could be easily transferred to other strains and bacterial species (Ewers *et al.*, 2010; Johnson *et al.*, 2010; Mellata *et al.*, 2012).

Several studies have reported that *hlyF*, one of the genes carried on the ColV plasmid, is a molecular marker for APEC (Dissanayake *et al.*, 2014; Johnson *et al.*, 2008b; Mbanga and Nyararai, 2015). Currently, little information is available in regards to the exact role of *hlyF* and how it contributes to pathogenicity. In an infectious chick embryo model, the transcription of *hlyF* was strongly upregulated, which could suggest an essential role of this gene in the establishment of avian colibacillosis (Skyberg *et al.*, 2008). A recent study investigated the role of *hlyF* genes in establishing an extraintestinal infection and reported that this gene was directly involved in the production of outer membrane vesicles (Murase *et al.*, 2016). This outer membrane acts as a channel to deliver bacterial virulence factors into the host, leading to infection (Murase *et al.*, 2016).

1.10.6.3 Serum resistance

Resistance to complement-mediated lysis and opsonophagocytosis play an important role in APEC pathogenicity (Nolan *et al.*, 2003; Vidotto *et al.*, 1990; Wooley *et al.*, 1992). Serum resistance VGs enable the bacteria to survive outside the gastrointestinal tract and overcome the host defence mechanisms (Johnson *et al.*, 2002b; Lynne *et al.*, 2007; Miajlovic and Smith, 2014; Rocha *et al.*, 2008; Tivendale *et al.*, 2004). These VGs are frequently associated with septicaemia as they assist the bacteria in avoiding being killed by the essential defence mechanisms present in the blood, including complement, antimicrobial peptides and other serum components (Boerlin and Reid-Smith, 2008; Jacobson *et al.*, 1992; Jeffrey *et al.*, 2002; Nolan *et al.*, 2003). Several investigations have reported serum resistance VGs are widespread among APEC in comparison with AFEC and have suggested that these VGs contribute to APEC pathogenicity (Delicato *et al.*, 2003; Dozois *et al.*, 1992; Dziva and Stevens, 2008; Lynne *et al.*, 2007; Mellata *et al.*, 2003; Mellata *et al.*, 2012; Nolan *et al.*, 2002; Ozawa *et al.*, 2008; Rocha *et al.*, 2008; Rodriguez-Siek *et al.*, 2005a; Skyberg *et al.*, 2008; Tivendale *et al.*, 2004; Wooley *et al.*, 1992; Yaguchi *et al.*, 2007; Yang *et al.*, 2004; Zhao *et al.*, 2005). Several VGs have been linked with serum resistance mechanisms and the association of

each of the serum resistance VGs with APEC pathogenicity mechanisms have also been independently studied (Nakazato *et al.*, 2009; Rocha *et al.*, 2008). However, limited information is known in regards to the presence of different serum resistance VG combinations and their contribution to APEC pathogenicity (Jeffrey *et al.*, 2002; Nolan *et al.*, 2002; Nolan *et al.*, 2003; Sello, 2012).

The bacterial outer membrane, ColV colicin and/or outer membrane proteins can facilitate resistance to serum complement (Fantinatti *et al.*, 1994; Gross, 1957; Gross, 1990; Lynne *et al.*, 2007; Mellata *et al.*, 2003; Nolan *et al.*, 2003). Pfaff-McDonough *et al.* (2000) screened 294 *E. coli* isolates obtained from chickens with colibacillosis and 75 *E. coli* isolates sourced from healthy chickens to determine the associations between four serum resistance VGs (*iss* (increased serum survival, which is a cytotoxic inhibitor), *traT* (surface exclusion protein), *cvaC* (structural genes of colicin V operon) and *ompA* (outer membrane protein A)) and APEC. While the authors linked all four of the serum resistance VGs with APEC pathogenicity, *iss* was significantly associated with APEC in comparison with AFEC. They also suggested that *iss* was on the conjugative ColV plasmids, which occur in higher frequency among APEC compared to AFEC regardless of the serotype and the origin of the isolate.

The *traT* and *iss* genes were first linked to APEC pathogenicity and serum resistance in 1979, with a 100-fold increase in APEC pathogenicity reported in the presence of *iss* and *traT* (Binns *et al.*, 1979). Both VGs are found on ColV plasmids and encode for outer membrane proteins (McPeake *et al.*, 2005; Mellata *et al.*, 2003; Nolan *et al.*, 2003; Rodriguez-Siek *et al.*, 2005a; Sorsa *et al.*, 2003; Tivendale *et al.*, 2004; Vandekerchove *et al.*, 2005). The role of these two genes in the APEC pathogenicity mechanism was identified by insertional mutagenesis (Wooley *et al.*, 1992). Global variations in the prevalence of the *iss* gene in APEC have been reported, with a high prevalence of 80.5% to 100% found in isolates from the USA, Germany and Egypt (Ewers *et al.*, 2004; Hussein *et al.*, 2013; Johnson *et al.*, 2008b; Rodriguez-Siek *et al.*, 2005a). However, Delicato *et al.* (2003) reported a lower prevalence (38.5%) of the *iss* gene among APEC isolates obtained from poultry with colibacillosis in Brazil. This variation in the prevalence of the *iss* gene among different APEC strains sourced from different countries may indicate a geographical effect on the presence of this VG.

Several studies have reported a positive association between complement resistance, virulence and the presence of the *iss* gene in APEC (Johnson *et al.*, 2008b; Lynne *et al.*, 2012; Nolan *et al.*, 2003; Tivendale *et al.*, 2004). However, it is unknown whether the sole presence of the *iss* gene contributes to the pathogenicity or whether the *iss* gene is a marker for the presence of the plasmids associated with APEC pathogenicity (Hussein *et al.*, 2013; Nilsson *et al.*, 2014; Rodriguez-Siek *et al.*, 2005a).

Chaffer *et al.* (1999) reported the *iss* gene was needed for complement resistance, but not for APEC pathogenicity. However, Mellata *et al.* (2003) reported that the absence of the *iss* gene did not influence the ability of APEC to resist serum complement. In addition to *iss*, APEC have other serum resistance mechanisms that enable them to survive in host serum. For example, Mellata *et al.* (2003) demonstrated that O78 polysaccharide and the K1 capsule increased bacterial serum resistance.

The production of extracellular polysaccharide capsules are considered to be one of the *E. coli* serum resistance mechanisms that can lead to bacteraemia (Guabiraba and Schouler, 2015; Miajlovic and Smith, 2014). The somatic O-antigens and capsular K-antigens act as a barrier to the host defence mechanisms and were described in the 1960s and 1980s, respectively (Boerlin and White, 2013; Perry *et al.*, 2014). Variation in serum resistance mechanisms among APEC could be due to the presence of different types, as well as, combinations of capsules (Mainil, 2013). Overall, the wide variation in the presence of certain serum resistance VGs and the prevalence of these VGs could be reflected in the different clinical symptoms and pathological changes seen with avian colibacillosis (Guabiraba and Schouler, 2015; Mellata *et al.*, 2003).

1.10.6.4 Iron acquisition mechanisms

Iron is an essential element for the survival and growth of *E. coli* in the host and the external environment (Dho and Lafont, 1984; Searle *et al.*, 2015). It is involved in several cellular activities such as electron transport and storage, gene regulation, nucleotide biosynthesis and is a cofactor of various enzymes (Heinemann *et al.*, 2008; Raymond *et al.*, 2003; Rouault and Tong, 2008). Since the concentration of free iron in the host physiological fluids is not enough for the survival of the pathogenic bacteria and much higher concentrations are needed for their metabolic activities. Pathogenic bacteria have been forced to develop multiple strategies to enable them to acquire iron (Belanger *et al.*, 2011). The bacteria are equipped with efficient iron acquisition mechanisms, which enable them to compete with the host iron-binding proteins, such as transferrins, lactoferrin and for haemoglobin free iron. Several different iron acquisition systems, such as aerobactin, yersiniabactin, Sit system and ChuA (encoding heme transport protein) have been found among APEC isolates (Gao *et al.*, 2012). The aerobactin iron acquisition system is considered the most common system in APEC. Previous studies have indicated that the aerobactin system is found in more than 72% of APEC isolates (Emery *et al.*, 1992; Linggood *et al.*, 1987). The aerobactin iron acquisition system consists of hydroxamate siderophore aerobactin and ferric aerobactin uptake proteins, which are encoded by *iucA* and *iucC* genes that are located on ColV plasmids (Boerlin and White, 2013).

Avian pathogenic *E. coli* can up take iron indirectly utilising the shuttle mechanism, which is based on small-molecule compounds called siderophores (high-affinity ferric chelators) that are able to

capture ferric iron (Dho and Lafont, 1984; Gibson and Magrath, 1969; Ratledge and Dover, 2000; Wooldridge and Williams, 1993). Different types of siderophores have been identified: enterobactin; salmochelin; hydroxamate aerobactin and yersiniabactin (Binns *et al.*, 1979; Searle *et al.*, 2015). Each system is involved in different steps of APEC pathogenicity including: synthesis in the cytoplasm; secretion; reception of the ferri-siderophore at the outer membrane surface; internalisation and iron release in the cytoplasm (Belanger *et al.*, 2011). An early study conducted on one-day-old chickens suggested a positive association between low iron levels, APEC survival and the pathogenicity of the tested APEC strains (Lafont *et al.*, 1987). Furthermore, several studies have suggested that the expression of an iron acquisition system occurs mainly in APEC compared with AFEC (Dias da Silveira *et al.*, 2002; Dozois *et al.*, 1992; Emery *et al.*, 1992; Linggood *et al.*, 1987). Among APEC strains, iron acquisition systems can be encoded by genes located on plasmids (Sabri *et al.*, 2008; Skyberg *et al.*, 2006) or by chromosomal PAIs (Kariyawasam *et al.*, 2006a).

1.10.6.4.1 Salmochelin

The first siderophore discovered was the enterobactin molecule, salmochelin, which was identified in *Salmonella enterica* (Hantke *et al.*, 2003). The salmochelin biosynthesis and transport genes have generally been found on large pathogenic plasmids; ColV or ColBM, as well as, on the chromosome in APEC strains (Johnson *et al.*, 2006). The salmochelin system is involved in the virulence mechanism of APEC strains (Sabri *et al.*, 2008; Sabri *et al.*, 2006) and includes *sitABCDE* in combination with other iron uptake systems. It can contribute to iron acquisition and to bacterial survival (Sabri *et al.*, 2006). The salmochelin system contains five genes (*iroB, iroC, iroD, iroE* and *iroN*), which have been reported among APEC (Zhu *et al.*, 2005). The presence of all five genes is required for APEC virulence (Caza *et al.*, 2008). The *iroN* gene codes for an outer membrane siderophore and is considered as the main receptor for ferric salmochelin transport (Hantke *et al.*, 2003).

1.10.6.4.2 Yersiniabactin

The siderophore yersiniabactin was first recognised in *Yersinia enterocolitica* (Koebnik *et al.*, 1993). The yersiniabactin system is encoded by *fyuA* (ferric yersiniabactin uptake) and *irp-2* (iron-repressible) genes (Karch *et al.*, 1999; Pelludat *et al.*, 1998; Schubert *et al.*, 1998). A number of studies have demonstrated an association between the presence of these VGs and APEC pathogenicity (Janssen *et al.*, 2001; Jeong *et al.*, 2012; Mokady *et al.*, 2005; Paixao *et al.*, 2016).

1.10.6.4.3 Aerobactin

Aerobactin is a hydroxamate siderophore, that was first isolated from *Aerobacter aerogenes* cultured in low iron selective medium (Gibson and Magrath, 1969). Aerobactin is synthesised by the *iucABCD* encoded gene (iron uptake chelate) and is taken up by the IutA-encoded receptor protein (Bindereif and Neilands, 1985; Ling *et al.*, 2013). When compared to AFEC, aerobactin biosynthetic genes are more frequently detected in APEC and their incidence correlates with highly pathogenic strains (Dozois *et al.*, 1992; Lafont *et al.*, 1987; Linggood *et al.*, 1987). Compared to the wild type, the virulence of an APEC strain deficient in aerobactin synthesis and uptake was reduced in a chicken systemic infection model (Dozois *et al.*, 2003).

Aerobactin uptake proteins are encoded by *iucC* and *iucD* genes, which are located on a conserved region of ColV plasmids (Dziva and Stevens, 2008; Johnson *et al.*, 2006) or ColBM virulence plasmids, but can also be located on the chromosome in some strains (de Lorenzo and Neilands, 1986; Johnson *et al.*, 2006; Oves-Costales *et al.*, 2009). Skyberg *et al.* (2006) demonstrated that ColV plasmids in APEC, which contain an aerobactin system, can transfer to AFEC and enable the commensal isolate to cause mortality to chicken embryos. Originally, the *iutA* gene was found to be encoded on the pColV-K30 plasmid (Warner *et al.*, 1981; Williams and Warner, 1980) and later it was detected in the chromosome (Crosa, 1989). A study in China investigated the contributions of the *iutA* gene to APEC pathogenicity mechanisms by deleting the *iutA* gene (Ling *et al.*, 2013). This study reported a significant decrease in APEC pathogenicity of the mutant strains, which indicates that the *iutA* gene plays an important role in pathogenicity (Ling *et al.*, 2013).

The presence of more than one of the iron acquisition systems may indicate an adoption mechanism of the bacteria to allow it to survive in different environmental situations (De Carli *et al.*, 2015; Janssen *et al.*, 2001). Global studies have highlighted the higher prevalence rate of iron acquisition VGs such as: *chuA*; *fyuA*; *irp2* and *iucD* among APEC in comparison to AFEC (Ewers *et al.*, 2005; Horne *et al.*, 2000; Janssen *et al.*, 2001; Karch *et al.*, 1999; Nakazato *et al.*, 2009; Rodriguez-Siek *et al.*, 2005a).

1.10.6.5 Adhesin

The bacterial adhesin to the host epithelium tissues is considered to be an important step for pathogenicity of many bacteria since it allows the bacteria to bind to and maintain close contact with the host epithelial tissues (Meyer *et al.*, 1997; Moon, 1990). Specific surface receptor proteins in the host mediate the adhesin process of the pathogen. Initially, Arp and Jensen. (1980) suggested an association between APEC adherence capacity and pathogenicity of the bacteria, and reported a higher prevalence of fimbriated strains compared to afimbriated strains in the trachea of sick turkeys. The role of adhesin VGs in regards to establishing an attachment of APEC to chicken receptors both

in vitro and *in vivo* and its effect on the severity of APEC infections was first reported in 1984 (Naveh *et al.*, 1984).

Early studies suggested a positive association between the presence of adhesin VGs (fimbrial or afimbrial) and the successful ability of APEC to cause infections (Arp and Jensen, 1980; Fantinatti *et al.*, 1994; Naveh *et al.*, 1984) (Kariyawasam and Nolan, 2009; Moon, 1990). Further studies extensively characterised these VGs and their association with APEC and whether they could be used as a marker to identify and differentiate between APEC and AFEC (Ewers *et al.*, 2007; Musa *et al.*, 2009; Nolan *et al.*, 2003). However, the sole presence of adhesin VGs will not result in an infection, as other virulence factors are required for disease to occur (Marc *et al.*, 1998; Ramirez *et al.*, 2009).

1.10.6.5.1 Fimbrial adhesins

It is not surprising that several adhesin genes have been associated with APEC, including Type 1 fimbriae (*fimC, fimH*), P fimbriae (*papC*), curli fibres (*crl*), S fimbriae (*sfa/focCD*), F1C fimbriae, Dr fimbriae, afimbrial (*afa/drab*) adhesins and many novel adhesin genes that have not yet been fully characterised (Antao *et al.*, 2009; Dho-Moulin and Fairbrother, 1999; Ewers *et al.*, 2005; Ewers *et al.*, 2007; Kariyawasam and Nolan, 2009; Knöbl *et al.*, 2004; La Ragione and Woodward, 2002; Monroy *et al.*, 2005; Moon, 1990; Pourbakhsh *et al.*, 1997; Stordeur *et al.*, 2004; Vandekerchove *et al.*, 2004b; Vidotto *et al.*, 2004; Vidotto *et al.*, 1990; Vidotto *et al.*, 1997). Understanding the function of each of the APEC linked adhesin VGs is very important as the presence of each of these VGs have a unique function that enables APEC to recognise a specific receptor in the host where they are able to attach and colonise (Antao *et al.*, 2009; Arp and Jensen, 1980; Naveh *et al.*, 1984).

1.10.6.5.2 F1 fimbriae (Type 1 pili)

F1 fimbriae or Type 1 pili are non-flagella, hair-like proteinaceous long filaments or surface appendages anchored to the cell wall (Dziva and Stevens, 2008; La Ragione and Woodward, 2002). F1 fimbriae were first linked to APEC virulence in 1980 based on a study that revealed less fimbriated strains were easily removed from the respiratory tract of the chicken compared to virulent fimbriated strains (Arp and Jensen, 1980). Both fimbriae types 1 and P adhere to tracheal epithelial cells of chickens (La Ragione *et al.*, 2000; Yerushalmi *et al.*, 1990). Type 1 fimbriae can be found on more than 70% of APEC cultured from the respiratory tract of birds with colibacillosis (Dho-Moulin and Fairbrother, 1999; Dozois *et al.*, 1994; Janssen *et al.*, 2001; Pourbakhsh *et al.*, 1997). In contrast, P fimbriae were expressed by less than 30% of APEC strains that were colonising the respiratory system and internal organs (Pourbakhsh *et al.*, 1997; Stordeur *et al.*, 2004). Furthermore, the expression of P

fimbriae *in vivo* suggests that these fimbriae are most likely not important for the initial colonisation of the upper respiratory tract, but are needed in the latter stages of infection (Pourbakhsh *et al.*, 1997).

The F1 fimbriae are encoded by nine *fim* genes and include a major protein known as FimA and minor proteins known as FimF, FimG and the adhesin FimH. Dozois *et al.* (1994) and Pourbakhsh *et al.* (1997) reported that the expression of type 1 fimbriae only take place in the upper respiratory tract and not in any other internal organs, which suggests they have a role in initial colonisation. An early study of APEC infection by La Ragione *et al.* (2000) was able to demonstrate the significant role of type 1 fimbriae for the adherence, colonisation and invasion of APEC into the host epithelial cells. In contrast, Marc *et al.* (1998) suggested that the presence of type 1 fimbriae alone had a limited role in APEC pathogenicity. Further, Arne *et al.* (2000) reported the failure of an APEC *fimH* mutant strain to adhere to the epithelial cells of the chicken trachea *in vitro*.

A large number of studies have suggested that type AC/I fimbriae is associated with the adherence to the upper respiratory tract and the initial colonisation (de Pace *et al.*, 2010; Delicato *et al.*, 2003; Dozois *et al.*, 1992; Dozois *et al.*, 1995; Janssen *et al.*, 2001; La Ragione *et al.*, 2000; Marc *et al.*, 1998; Pourbakhsh *et al.*, 1997; Vidotto *et al.*, 2004; Wooley *et al.*, 1992). In addition, further studies have reported an association between type AC/I fimbriae and serotype O78 APEC isolates (Babai *et al.*, 1997; Lymberopoulos *et al.*, 2006) indicating the importance AC/I fimbriae in pathogenicity.

P fimbriae or Pap pili (pyelonephritis associated pili) were first defined in UPEC in humans and dogs, and later in APEC (Collinson *et al.*, 1993; Kariyawasam *et al.*, 2006b). The P fimbriae is encoded by the *pap* gene, which is located on the bacterial chromosome (Latham and Stamm, 1984). It is believed that the expression of P fimbriae only takes place in deeper tissues, not the upper respiratory tract, which suggests that P fimbriae are important for systemic infections (Dziva and Stevens, 2008; Pourbakhsh *et al.*, 1997). Although the contribution of P fimbriae to urinary tract infections in humans is well documented, their role in APEC pathogenicity is still debatable. Achtmann *et al.* (1986) found that 52% of APEC isolates sourced from chickens with colibacillosis harboured P fimbriae.

The pap operon, which is located on the bacterial chromosome, harbours different VGs that encode for P fimbriae (Latham and Stamm, 1984). The *papA*, *papI* and *papB* genes encode for the major structural protein (PapA), which is responsible for the phase variation process (Mol and Oudega, 1996). The *papE* gene encodes the fimbriae l structure, *papG* gene encodes for the adhesin and *papD*, *papH*, *papJ*, *papF* and *papK* genes, are responsible for the expression of proteins related to the fimbrial structure (Mol and Oudega, 1996). Kariyawasam *et al.* (2006b) reported that the pap operon is present in a PAI of APEC strain APEC-O1 and it has been shown that the transformation of an avirulent strain into virulent strain could be due to pap horizontal transmission (Stacy *et al.*, 2014).

Vandekerchove *et al.* (2004b) reported a significant association between avian colibacillosis and the detection of F11 fimbriae among the APEC isolates. Furthermore, they reported that the majority of the F11 fimbriae isolates belong to serotype O87. This serotype has been shown to be associated with APEC, as well as, ExPEC isolated from humans (Naveh *et al.*, 1984).

1.10.6.5.3 Curli

Curli fimbriae are thin and curly appendices found on the outer cell surface of *E. coli* (Collinson *et al.*, 1993; Olsen *et al.*, 1989). These outer protein structures are important as they enable the binding of *E. coli* to the extracellular proteins (Collinson *et al.*, 1993; Olsen *et al.*, 1989). Historically, it was assumed that fimbrial adhesins were encoded by the *crl* gene. However, recently the *csgA* gene was recognised as encoding for curli, which have been found to be expressed equally among APEC and AFEC (Amabile de Campos *et al.*, 2005; Cunha *et al.*, 2017; Maluta *et al.*, 2014). This may indicate that curli fimbriae initiate colonisation for both APEC and AFEC. Supporting this hypothesis, McPeake *et al.* (2005) found that the *csgA* gene existed among all *E. coli* isolates regardless of the health status of the chicken. In contrast, Maurer *et al.* (1998) found this gene only in APEC.

The presence of fimbriae F17, Afa and Sfa among APEC strains may indicate that these adhesins can be found on the cell surface of APEC (Amabile de Campos *et al.*, 2005; McPeake *et al.*, 2005; Stordeur and Mainil, 2002) and therefore their presence could indicate virulence of a strain. Although, a study in China by Won *et al.* (2009) did not find Afa, F-fimbriae or AC/I amongst 118 *E. coli* isolates sourced from chickens with colibacillosis.

The Afa/Dr family of adhesins is a diverse group of proteins encoded by the closely related *afa, daa* and *dra* gene clusters (Servin, 2005). The presence of these adhesin VGs were detected in APEC, as well as UPEC, and were found to be associated with infections by enabling adhesin and invasion (Goluszko *et al.*, 1997; Lalioui and Le Bouguenec, 2001).

1.10.6.5.4 Non fimbrial adhesins

1.10.6.5.4.1 Temperature-sensitive hemagglutinin

The temperature sensitive haemagglutination is a protein coded by the *tsh* gene, that can only be expressed at 26 °C and is blocked at 42 °C (Provence and Curtiss, 1994). This protein is an

autotransporter protein with dual function of adhesive and proteolytic activities. This protein remains in the outer membrane and supports the adhesin process during the early stages of the infection (Stathopoulos *et al.*, 1999). Generally, this gene is detected on ColV plasmids at a higher frequency among APEC (Dozois *et al.*, 2000; Johnson *et al.*, 2006; Nakazato *et al.*, 2009; Stehling *et al.*, 2003). An early study by Maurer *et al*. (1998) reported a prevalence of 46% of *tsh* among APEC in comparison to zero among AFEC. Campos *et al.* (2008) reported 25% and 50% of APEC strains isolated from chickens with septicaemia and swollen-head syndrome, respectively, harboured the *tsh* gene and only 6% of AFEC. Similarly, a *tsh* prevalence of 55% was detected in Brazil and 53% in Germany from APEC isolates (*E. coli* isolates obtained from lesions of chicken with colibacillosis) (Ewers *et al.*, 2004; Rocha *et al.*, 2008). Other authors reported an even higher prevalence of *tsh* in APEC of 94% in Ireland (McPeake *et al.*, 2005) and 99% in Canada (Ngeleka *et al.*, 2002). This variation in the prevalence of *tsh* among different studies could be due to geographical variations. However, despite the utilisation of *tsh* as a molecular marker for APEC detection by Ewers *et al.* (2005), future studies are needed to identify the exact role of *tsh* in the pathogenicity of APEC.

1.11 The prevalence of APEC related VGs

Although several studies have suggested that APEC related VGs could be used as molecular markers for the identification and detection of APEC strains (Ewers *et al.*, 2005; Johnson *et al.*, 2008b; Tivendale *et al.*, 2009), a clear discrimination between APEC and AFEC has not yet been achieved. Increased evidence has indicated that APEC-associated VGs are commonly clustered on plasmids, PAIs, or less often, the bacterial chromosome (Ewers *et al.*, 2005; Johnson *et al.*, 2008b; Mellata *et al.*, 2012; Tivendale *et al.*, 2009). Several studies have also found that the prevalence of VGs occurs at a higher rate among APEC in comparison with AFEC (Johnson *et al.*, 2008b; Nolan *et al.*, 2013). However, there are several factors that influence the prevalence of APEC-associated VGs from *E. coli* isolates obtained from healthy chickens and chickens with colibacillosis, such as bird age variations and geographical factors as shown in Table 1.1.

A longitudinal study, where broiler chickens were sampled twice weekly from the day they were placed into the shed to approximately three-days prior to de-population (32 – 35 days) on two farms through two flock cycles, suggested that the chickens age can affect the prevalence of APEC-associated VGs in AFEC. Kemmet *et al.* (2013) reported a wide diversity and higher prevalence of APEC-associated VGs in faecal *E. coli* from one-day-old chicks compared to older chickens. There was a noticeable decrease in the number of APEC-associated VGs in faecal *E. coli* detected at slaughter (Kemmett *et al.*, 2013). However, these variations in the prevalence among young chickens could be as a result of young birds having more diverse microbiota and negative selection in the gut

for virulent strains. Furthermore, horizontal transmission of APEC related VGs could occur from the hatchery or the surrounding environment (Lu *et al.*, 2003; Morris and Potter, 1997).

The prevalence of APEC-associated VGs could be affected by the presence of the plasmids, which may harbour several VGs. For example, an association between the presence of seven APEC-associated VGs (*tsh*, *iucD*, *iroN*, *iss*, *cvi/cva*, *traT* and *iroN*) on plasmids and *E. coli* isolates collected from chickens with colibacillosis have been reported (Ewers *et al.*, 2007).

The absence of an exact definition of APEC and/or AFEC either based on the presence of unique VGs or bird's health status among different studies influences the ability to directly compare studies. The geographical effect on the prevalence of APEC related VGs is documented in several studies (Johnson *et al.*, 2008b; Johnson *et al.*, 2008c; Rodriguez-Siek *et al.*, 2005a). Mbanga *et al.* (2015) detected all of the 12 VGs (*iutA, hlyF, ompT, frz, sitD, fimH, kpsM, sitA, sopB, uvrY, pstB* and *vat*) they screened for in APEC in South Africa. The three most prevalent VGs detected in the 45 APEC were *iutA* (80%), *fimH* (33%) and *hlyF* (24%) with genes *kpsM*, *pstB* and *ompT* being detected in only 2.2% of the isolates. Other studies in the USA and Iran have found higher occurrences of 98% and 62%, respectively, for the *fimH* gene in APEC isolates (Eftekharian *et al.*, 2016; Rodriguez-Siek *et al.*, 2005a). Jin *et al.* (2008) and Wang *et al.* (2010) found *hlyF* was one of the most prevalent genes in *E. coli* sourced from chickens with colibacillosis in China with 77% and 55%, respectively.

Furthermore, variations in the prevalence of the *tsh* gene have been reported ((Delicato *et al.*, 2003; Knobl *et al.*, 2012). The *tsh* gene was detected in 78.3% of APEC and in only 21.7% of AFEC isolates obtained from commercial broiler chickens in Iran (Kafshdouzan *et al.*, 2013). In contrast, *tsh* was reported at a higher prevalence in AFEC (93.3%) and APEC (93.9%) in the UK (McPeake *et al.*, 2005). In Brazil only 4% of the AFEC isolates harboured *tsh* compared to 39.5% of APEC (Delicato *et al.*, 2003). In summary, the geographical variations in the prevalence of APEC-associated VGs reflect the vital role of defining APEC on the basis of VGs from Australian commercial broiler chickens.

1.11.1.1 Current knowledge about APEC related VGs in Australia

In Australia, there are few published studies that assess the prevalence of APEC linked VGs (Obeng *et al.*, 2012; Tivendale *et al.*, 2004). One study showed that among 251 AFEC (*E. coli* isolates obtained from the faeces of healthy chickens) the *iutA* gene was detected in 44% of the isolates followed by 4% for both *kpsMTII* and *sfa* and *papC* (3.2%) while none of the *E. coli* isolates harboured *sfa/foc* (Obeng *et al.*, 2012). Tivendale *et al.* (2004) examined the role of *tsh*, *iucA* and *iss* genes in APEC pathogenicity and confirmed their presence in the pVM01 plasmid from Australian

isolates. The authors reported a positive association of *iss* and *iucA* with high levels of APEC virulence, with either one of the genes causing intermediate levels of virulence. The absence of the *tsh* gene did not affect levels of pathogenicity after aerosol exposure (Tivendale *et al.*, 2004).

Table 1.1: Virulence genes screened and the number (N) of different virulence genes occurring among extraintestinal E. coli (ExPEC), avian pathogenic E. coli (APEC) and avian faecal E. coli (AFEC) among different studies.

Date	Country	APEC (N)	AFEC (N)	ExPEC (N)	Virulence genes screened	Detected virulence genes			Reference
						APEC	AFEC	ExPEC	
1992 - 2000	France, Spain and Belgium	1,491	0	0	*fimA, fimH, neuC, felA, papC, papG, prsG, tsh, iutA, cdt, cnf1, cnf2, VT1, VT2, LT STa, STb, F1, aec26, frz*$_{orf4}$ and F11	*sitA, F1, frz*$_{orf4}$, *aec26* and *iutA*	Not studied	Not studied	(Schouler *et al*., 2012)
1994 and 2002	UK	45	114	0	*fimC, pap, sfa, afa, aer, crl, csg, tsh, hlyE, iss* and *cvaC.*	*fimC, pap, crl, csg, aer, tsh* and *cvaC*	*hlyE* and *tsh*,	Not studied	(McPeake *et al*., 2005)
2005	Germany	14	9	17	*stA, iss, irp2, papC, iucD, tsh, vat* and *cva/cvi*	*stA, iss, irp2, papC, iucD, tsh, vat* and *cva/cvi*	Not studied	*papC, irp2, iss, iucD* and *vat*	(Ewers *et al*., 2005)
2001 – 2006	Sri Lanka	196	0	0	*ompT, hlyF, aec26, iss, frz, cvaC, iroN, iutA, sitAP* (plasmid), *sitAC* (chromosomal), *papC, papG (III), felA, aec4 and iucD*	*iutA, felA, tsh, ompT, iss* and *frz*	Not studied	Not studied	(Dissanayake *et al*., 2014)
2005 - 2008	China	148	0	0	*tsh, iss, iucA, hlyF, sitA, ibeA, traT, cdtB, iroN, cvaC* and *fimH*	*traT, iroN, fimH, iss, tsh, iucA, hlyF, cvaC* and *sitA*	Not studied	Not studied	(Wang *et al*., 2010)
2005	USA	524	0	200	*ApA, fimH, kpsMTIII,, papEF, ireA, ibeA, cnf1, fyuA, iroN, bmaE, sfa, iutA, papGIII, hlyD, rfc, ompT, papGI, kpsMTII, papC, gafD, cvaC, fliC(H7), cdtB, focG, traT, papGII, ihA, afa, iss, sfaS* and *kpsMT(K1)*	*iroN, iss, iucC, iutA, sitA, traT, cvaC* and *tsh*	Not studied	*sit, traT, iss, iroN, iucC, iutA* and *tsh*	(Rodriguez-Siek *et al*., 2005a)
2007	Germany, Brazil, France, Canada, USA, and Netherlands	436	0	90	*afa/dra, crl, fimC, hra, iha, sfa/foc, papC, tsh, iroN, iss, chuA, chuA, ireB, sitB* , chromosomal *sit, irp2, iucD, sitB., cvi/cva, ompA, traT, sat, vat, neuC, EAST-1, cnF1/2, malX, pic, tia, ibeA* and *gimB*	*cvi/cva, sitD., iucD, iss, traT, iroN* and *tsh*	Not studied	*traT, iucD, iss, cvi/cv* and , chromosomal *sit,*	(Ewers *et al*., 2007)
2008	USA	794	200	0	Episomal *iss, iroN, episomal ompT, eitAB, cvaABC, cbi, cma, iutA, hlyF,* and *etsAB,* chromosomal *ompT, ireA, fyuA, papACEFG,* and *vat, gimB, kpsMT1, ibeA, kpsMT2* and *malX,*	*ompT, iss, hlyF, iutA* and *iroN*	Not studied	Not studied	(Johnson *et al*., 2008b)
2010 - 2012	Brazil	138	0	0	*cvaC, iroN, iss, iutA, sitA, tsh, fyuA, irp-2, ompT* and *hlyF*	*hlyF, sitA, ompT, fyuA, cvaC* and *irp-2*	Not studied	Not studied	(De Carli *et al*., 2015)
2008 - 2009	Australia	0	311	0	*papAH, papC, afa/draBC, sfa/focDE, sfaS, aerJ, kpsMTII* and *focG*	*Not studied*	*iutA, papC* and *kpsMTII*	Not studied	(Obeng *et al*., 2012)
2015	South Africa	45	0	0	*frz, sitD, fimH, ompT, iutA, pstB,vat kpsM,, hlyF, uvrY, sitA* and *sopB*	*iutA, fimH, hlyF* and *sopB*	Not studied	Not studied	(Mbanga and Nyararai, 2015)

1.12 The treatment of avian colibacillosis in the poultry industry

The usage of antimicrobial drugs to control and reduce the economic losses associated with avian colibacillosis is considered the main way to control the disease (Dheilly *et al.*, 2011; Nolan *et al.*, 2013). However, the emergence of antimicrobial resistant bacteria is now affecting the way colibacillosis is being treated (Nolan *et al.*, 2013). The majority of current treatment strategies focus on early prevention of the disease in the broiler flocks rather than treatment after infection (Kabir, 2010; Nolan *et al.*, 2013). Historically, tetracycline have been used to treat colibacillosis, but with 90% of *E. coli* isolates now displaying resistance to this drug it is no longer the treatment of choice (Wang *et al.*, 2010). This increasing rate of AMR poses a global threat to the poultry industry as well as the public (Aarestrup, 2005).

1.13 The usage of antimicrobial agents in the Australian poultry industry

Australia has adopted a conservative position regarding the registration of antimicrobials for food-producing animals. Australia is the only country never having permitted the use of fluoroquinolones in livestock (Cheng *et al.*, 2012) and has also placed stringent label restrictions on the use of third generation cephalosporins, such as ceftiofur

All veterinary antimicrobials that can be used in animals in Australia are registered with the Australian Pesticides and Veterinary Medicines Authority (APVMA). Information in regards to those chemicals can be accessed through the Public Chemical Registration Information System Search (PubCRIS) (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015). Few antimicrobials are registered for use in broiler chickens in Australia. Avilamycin is registered as a growth promoter and to aid in the prevention of necrotic enteritis, ionophores as coccidostats and the registered therapeutic antimicrobials are: β-lactams (amoxicillin and ampicillin); macrolides (erythromycin and tylosin); lincosamides (lincomycin); aminoglycosides (apramycin, neomycin and spectinomycin); streptogramin (virginimycin); tetracyclines (chlortetracycline and oxytetracycline); pleuromutilin (tiamulin); trimethoprim sulphonamide and polypeptides (zinc bacitracin) (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015).

The Australian government regulates antimicrobial drug usage in the poultry industry, as well as the withholding period, which is the minimum period of time that must elapse between when the drug is used to use of the animal commodity for human consumption. Withholding periods ensures there is no antimicrobial drug residue in the chicken meat or eggs (van den Bogaard *et al.*, 2001). The antimicrobial regulations cover the dosage, route of administration and the duration of administrated antimicrobial drug.

Despite the emergence of resistant strains among different bacterial species and public health concerns of the use of antimicrobials in food-producing animals, there continues to be a need for antimicrobials to be used as treatments, as well as, for prevention of infections. This highlights the importance of a proper disease diagnosis combined with the identification of the AMR prevalence among commensal and pathogenic bacterial species to allow for identification of specific treatments (Martinez *et al.*, 2014). A national surveillance program is needed to determine the total usage of all antimicrobial drugs among different species of animals and the resistance profile of pathogens to all antimicrobial drugs used. This would inform on the usage effect and selection pressure of these antimicrobials for animals, as well as, for public health, guide antimicrobial stewardship programs and help veterinarians plan therapeutic and prevention/control strategies.

In Australia, antimicrobial usage information can only be obtained from the Australian Pesticides and Veterinary Medicines Authority (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015). However, they only provide limited data in regards to the total drug sales without the exact quantities of the antimicrobial usage in different animal species (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015; Shaban *et al.*, 2014). This is in contrast to antimicrobial monitoring systems applied in other countries, such as Denmark, Germany, Sweden and the UK, where they closely observe and provide detailed information in regards to the consumption of different types of antimicrobials among different animal species. The Danish Integrated AMR Monitoring and Research Program (DANMAP) is an example of such an extensive surveillance system (The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP), 2014).

1.14 Antimicrobial drugs

The word "antimicrobial" is of Greek origin and consists of three components: anti meaning "against", mikros meaning "little" and bios meaning "life" (Gottlieb and Nimmo, 2011; Guardabassi, 2006). Antimicrobial agents are any natural, synthetic or semisynthetic elements that are capable of inhibiting the growth (bacteriostatic) or killing (bactericidal) microorganisms without causing any harm to the host cell (Giguère, 2006; Perry *et al.*, 2014; Sello, 2012). Frequently, the two terms: antimicrobial and antibiotic are used interchangeably (Guardabassi, 2006). The accurate definition of an antibiotic is an agent that is produced by a microorganism and acts against other microorganisms (Perry *et al.*, 2014). While antimicrobial agents are synthetic, as well as, semi-synthetic substances that are originated from different products and are also effective in the control of infections (Guardabassi, 2006).

Antimicrobial agents can be classified, according to their actions, into broad-spectrum (can either inhibit or kill a wide range of microorganisms) and narrow-spectrum (can only kill or inhibit a certain species or genus of bacteria) (Boerlin and White, 2013). Furthermore, antimicrobial agents can also be classified based on their mechanism of action into different groups such as: antimicrobials targeting bacterial cell wall synthesis (β-lactams); inhibiting nucleic acid synthesis (quinolones and fluoroquinolones) or function (sulphonamides and trimethoprim) and inhibiting protein synthesis (aminoglycosides, phenicols, macrolides, lincosamides and tetracyclines) (Boerlin and White, 2013; Mascaretti, 2003; Neu, 1992; Perry *et al.*, 2014)

1.15 Antimicrobial resistance

Antimicrobial resistance is defined as the bacteria's ability to survive and/or grow in the presence of a chemical (drug) that would normally kill them or inhibit their growth (Harbottle *et al.*, 2006). The genetic flexibility of bacteria enables them to adapt, evolve and survive in their environment by developing different resistance mechanisms (Acar *et al.*, 2012). The emergence, selection and the spread of strains also occurs due to selection pressure and antimicrobial consumption in animals, as well as, humans (Boerlin and Reid-Smith, 2008; Boerlin and White, 2013; da Costa *et al.*, 2013; Guardabassi and Kruse, 2008). The complexity of AMR in veterinary medicine is due to different animal species, variations in bacterial species and the diversity of rearing environments (Wasyl *et al.*, 2012; Wasyl *et al.*, 2013). Resistant bacteria can result in serious prolonged infections causing high morbidity and mortality and massive costs associated with prevention, treatment and control (Boerlin and White, 2013; Hawkey and Jones, 2009).

1.15.1 Acquisition and transfer of antimicrobial resistance genes

The majority of virulence and ARGs can be found either on the chromosome or on MGE, such as plasmids. Resistance mechanisms can develop due to mutations (vertical transmission) or through the acquisition of resistance genes from other bacterial strains by horizontal gene transfer (HGT) (Boerlin and Reid-Smith, 2008; Boerlin and White, 2013; da Costa *et al.*, 2013; Davies, 1994; Martinez-Medina *et al.*, 2009). The transmission of genetic material from one generation to the next during replication is known as vertical gene transmission (Rodriguez-Rojas *et al.*, 2013). Horizontal gene transfer is the most important mechanism increasing resistance and is due to the transfer of genes among different bacterial strains and/or species. Horizontal gene transfer occurs mainly through conjugation and MGE (Allen *et al.*, 2013; Boerlin and White, 2013; Davies, 1994; Harbottle *et al.*, 2006).

1.15.2 Mobile genetic elements (MGEs)

Mobile genetic elements can assist HGT. These elements include plasmids, transposons, integrons and phages, which vary in size and structure (Rankin *et al.*, 2011; Wozniak and Waldor, 2010).

1.15.2.1 Integrons

Integrons are genetic units that can capture and express small MGEs (gene cassettes). Integrons can be found on plasmids or transposons as well as on the chromosome (Carattoli, 2001; Domingues *et al.*, 2012). Class 1 integrons are the most ubiquitous having been found in 60% – 70% of gram-negative pathogens (van Essen-Zandbergen *et al.*, 2007). They have also been proven to play a role in distribution and spread of AMR (Deng *et al.*, 2015). Several studies have reported a positive association between integrons and multidrug resistant *E. coli* isolates in food-producing animals (Cavicchio *et al.*, 2015; Dou *et al.*, 2016; Szmolka *et al.*, 2015) as well as in humans (Lavakhamseh *et al.*, 2016).

Class 2 integrons are associated with the Tn7 transposon family and are generally identified at a lower prevalence in *Enterobacteriaceae* (Deng *et al.*, 2015). Class 3 integrons have also been reported in *E. coli* (Deng *et al.*, 2015). Class 1 and 2 integrons have been identified in APEC in poultry worldwide (Awad *et al.*, 2016; Cavicchio *et al.*, 2015; Nogrady *et al.*, 2006).

1.15.2.2 Plasmids

Plasmids play a significant role in the transmission of ARGs and VGs (Allocati *et al.*, 2013; Robicsek *et al.*, 2006a; Schultsz and Geerlings, 2012). Plasmids are self-replicating, consisting of double stranded DNA, are capable of autonomous replication and are present in nearly all bacterial species (Thomas and Nielsen, 2005). They can vary in size from just a few hundred base pairs (bp) to a several kilo bp (Waters, 1999).

In normal conditions, plasmids do not contribute to the host cell growth as they do not carry essential growth genes (Thomas and Nielsen, 2005). Plasmids contribute to the genetic diversity of the bacteria as they harbour a wide spectrum of genes that encode for nonessential functions, such as drug resistance, virulence factors, fitness or adaptation (Bergstrom *et al.*, 2000; Carattoli *et al.*, 2005).

Plasmids can be categorised in different ways. One classification scheme is based on the failure of the complete transmission of the plasmid into new generations, which is known as incompatibility (Inc) (Couturier *et al.*, 1988; Novick, 1987). Many different Inc groups have been identified among *E. coli* such as: IncFIB; IncI1; IncFIIA; IncP; IncB/O; IncN; IncFIC; IncA/C; IncHI2; IncT; IncN; IncW; IncFIA; IncY; IncFrep; IncX; IncHI1 and IncL/M (Carattoli *et al.*, 2005).

The conjugation process, a survival mechanism developed by plasmids, enables the plasmids to maintain their stability during cell division among the bacterial population, regardless of other selective pressure. Plasmids containing ARGs have been shown to be transferred among different bacterial strains, as well as, different species of bacteria (Hayes, 2003). The majority of plasmids include replicon regions, which are responsible for the replication process (Carattoli *et al.*, 2005; Couturier *et al.*, 1988).

Several studies have reported that plasmids contribute to the transfer of resistance genes among *E. coli* isolates (Jones-Dias *et al.*, 2015; Kluytmans *et al.*, 2013). Carattoli *et al.* (2011) described an association between certain types of plasmids and resistance genes such as $bla_{\text{DHA-1}}$ (an AmpC β-lactamase) and plasmid type IncF. However, Mate *et al.* (2012) found this AmpC β-lactamase was also associated with IncL/M plasmids. IncN plasmids have been detected from bacteria cultured from faecal samples from a variety of animals (Dolejska *et al.*, 2013). Several studies have suggested that IncN and InA/C plasmids are associated with dissemination of extended-spectrum cephalosporins (ESC) and plasmid-mediated quinolone resistance (PMQR) genes in *E. coli* and *Salmonella* from animals and humans, as well as, their surrounding environment (Carattoli, 2011; Dolejska *et al.*, 2013; Johnson *et al.*, 2007; Moodley and Guardabassi, 2009; Rodrigues *et al.*, 2013).

The usage of antimicrobials alone is not enough to explain the frequency and spread of the different types of plasmids among *E. coli* isolates sourced from poultry (Svara and Rankin, 2011). However, there is evidence some plasmid replicons, such as IncI1, can survive in *E. coli* isolates without antimicrobial selection pressure with no or little fitness cost to the host (Fischer *et al.*, 2014). Furthermore, the bacteria host can make use of some of the extra elements found on plasmids, such as the adhesin VGs, which help the *E. coli* adhere host cells (Carattoli, 2009).

1.15.2.3 Plasmid related VGs

Avian pathogenic *E. coli* have been found to commonly possess plasmids containing virulence and ARGs (Dobrindt, 2005; Johnson *et al.*, 2002a; Johnson *et al.*, 2007; Rodriguez-Siek *et al.*, 2005a), in particular ColV and ColBM conjugative plasmids (Dias da Silveira *et al.*, 2002; Johnson and Nolan, 2009; Mellata *et al.*, 2003). The ColV plasmid and pTJ100 and their associated VGs are widely distributed among APEC isolates in comparison to AFEC (Johnson *et al.*, 2002a; Mellata *et al.*, 2003; Rodriguez-Siek *et al.*, 2005a). A number of studies have demonstrated a relationship between the possession of these plasmids and the pathogenicity of APEC (Parreira *et al.*, 1998; Pfaff-McDonough *et al.*, 2000; Rodriguez-Siek *et al.*, 2005a). It also should be kept in mind that the occurrence of plasmid associated VGs are not restricted to plasmids as these genes can also be found in the *E. coli* genome (Rodriguez-Siek *et al.*, 2005a). Previous studies have reported that ColV and ColBM

plasmids are significantly associated with the APEC pathotype (Johnson *et al.*, 2007; Rodriguez-Siek *et al.*, 2005a).

Detection of different types of plasmids vary among APEC, with IncFIB being the most common, followed by IncI1, IncFIIA, IncP, IncB/O, IncN and IncFIC (Johnson *et al.*, 2007). These plasmids are associated with the carriage of certain VGs and ARGs (Carattoli, 2013; Johnson *et al.*, 2010). An overlap between the type of plasmid replicon circulating between *E. coli* isolates sourced from humans and birds has also been observed (Fernandez-Alarcon *et al.*, 2011; Johnson *et al.*, 2007).

1.15.3 Antimicrobials targeting the bacterial cell wall

1.15.3.1 ß-lactams

ß-lactams are a broad group of antimicrobials that can be identified by the possession of a ß-lactam ring (Li *et al.*, 2007). They are based on penicillin and its derivatives and are used to treat both human and animal infections caused by gram-positive and gram-negative bacteria (Matagne *et al.*, 1998; Yao and Moellering, 2007). Based on the spectrum of activity and binding affinity, several classes of β-lactams have been synthesised including narrow-spectrum penicillins, narrow and extended-spectrum cephalosporins and broad-spectrum carbapenems (Bush, 2012). Their broad-spectrum ability combined with low toxicity in comparison with other antimicrobials makes these antimicrobials ideal for human and veterinary medicine (Seiffert *et al.*, 2013). For the majority of bacteria, the cell wall requires enzymatic linkage of pentapeptid precursor molecules into the peptidoglycan cell wall in order to synthesise. The enzymes responsible for this reaction are known as penicillin binding proteins (PBPs) (Hollenbeck and Rice, 2012). The structure of β-lactams is an analog of the pentapeptid precursors and therefore, it interferes with bacterial cell wall synthesis in the last stage, as it competes with the precursor molecule that is linking with the peptidoglycan for the PBP. The bacteria die as a result of impaired cell wall synthesis (Bush, 2012; Hollenbeck and Rice, 2012; Sosa, 2009).

Amoxicillin and ampicillin are the only β-lactams used in poultry in Australia (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015)There are no cephalosporins available for use and as discussed in section 1.13, extended-spectrum cephalosporins are not registered for use in poultry in Australia (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015).

The wide usage of β-lactam antimicrobials in both human and veterinary medicine creates a selection pressure (Schwaber *et al.*, 2005) and plays an important role in the development of different resistance mechanisms enabling the bacteria to overcome the drug effects (Babic *et al.*, 2006). There are a number of different resistance mechanisms associated with β-lactam resistance. The most important

mechanism associated with β-lactam resistance in gram-negative bacteria such as *E. coli*, involves the inactivation of the antimicrobial by the production of β-lactamase enzymes that are capable of hydrolysing and inactivating the ß-lactam ring (Allocati *et al.*, 2013; Bradford, 2001; Bush, 2010; Bush, 2012; Li *et al.*, 2007; Seiffert *et al.*, 2013; Sosa, 2009). Resistance may also occur due to mutations in the PBPs, decreased outer membrane permeability and multidrug efflux pumps (Babic *et al.*, 2006; Bush, 2010; Woodford *et al.*, 2007). β-lactam resistance can develop due to vertical transmission of resistance genes from parent to daughter cells or via HGT.

The β-lactamases are a heterogeneous group of enzymes encoded by genes located on chromosomes or plasmids (Bush, 2012; Livermore, 1995; Shah *et al.*, 2004). Identification of the β-lactamase role in the penicillin-inactivating mechanism was first reported in 1940, which was a concern for the continuous usage of β-lactam antimicrobials (Abraham and Chain, 1988). Resistance to broad-spectrum penicillins is usually conferred by plasmid-coded ß-lactamases, mainly of the TEM-type and to a lesser extent the SHV-type. Any single or multiple mutations in the TEM or SHV enzymes will expand their hydrolytic ability to include third- and fourth-generations of β-lactams and cephalosporins; these enzymes are referred to as extended-spectrum β-lactamases (ESBLs). Currently, more than 600 different ESBLs have been described (Bradford, 2001; Bush, 2010). The global distribution of these enzymes among gram-negative bacteria has been linked with MGEs, such as plasmids, and have been associated with the dissemination of MDR (Bush, 2012). β-lactamases TEM1, TEM2 and SHV1 are the major known members of the ESBLs produced by bacteria and are encoded by the genes, bla_{TEM} and bla_{SHV}, that are located on specific plasmids (Bradford, 2001).

Historically, TEM and SHV type β-lactamases were the most common ESBLs (Ewers *et al.*, 2012). Currently, the CTX-M types are replacing TEM and SHV and they are now considered the most common ESBLs in *E. coli* causing significant public health issues (Ewers *et al.*, 2012). High prevalence of ESBL-producing *E. coli* have been detected in livestock in Europe and North America (Dahms *et al.*, 2015; Ewers *et al.*, 2012). In addition, ESBL-producing *E. coli* have been recently identified in Australian livestock, bovines and porcines (Abraham *et al.*, 2015).

AmpC β-lactamases are commonly isolated from extended-spectrum cephalosporin (ESC) resistant gram-negative bacteria. They facilitate resistance to most penicillins, cefoxitin, cefotetan, ceftriaxone and cefotaxime (Abraham and Chain, 1988; Bush *et al.*, 1995). The majority of AmpC β-lactamases are encoded by *bla* genes that are mainly located on the bacterial chromosome, including $bla_{\text{CMY-2}}$, $bla_{\text{ACT-1}}$ and $bla_{\text{DHA-1}}$. To a lesser extent, *bla* genes are found on plasmids (Philippon *et al.*, 2002). Currently, the bla_{CMY} genes in *E. coli* encode the majority of AmpC β-lactamases. There are 99 known CMY alleles (Jacoby, 2009; National Center for Biotechnology Information (NCBI); Taneja *et al.*,

2012). Globally, CMY-2 producing *E. coli* are the most common plasmid-mediated cause of resistance in livestock (Jacoby, 2009). AmpC β-lactamase producing bacteria often harbour other resistance genes resulting in MDR isolates (Chen *et al.*, 2007; Hanson *et al.*, 1999).

CMY-2-producing *E. coli* isolates have been identified from faecal samples of healthy chickens in Spain (Brinas *et al.*, 2003) and from cats, cattle, chickens, dogs, horses, pigs and turkeys (Borjesson *et al.*, 2016; El-Shazly *et al.*, 2017; Jacoby, 2009; Maamar *et al.*, 2016). The $bla_{\text{CMY-2}}$ gene has recently been detected in *E. coli* from Australian livestock (Abraham *et al.*, 2015). Other AmpC β-lactamases have been isolated from *Salmonella* spp. in poultry including DHA-1 (Rayamajhi *et al.*, 2010) and ACC-1 (Dierikx *et al.*, 2010; Hasman *et al.*, 2005). Furthermore, a large number of studies demonstrated the widespread occurrence of plasmid-mediated AmpC β-lactamase producing *E. coli* isolates producing a DHA-1 enzyme, which were significantly associated with increased minimum inhibitory concentration (MIC) of carbapenems (El-Shazly *et al.*, 2017; Lee *et al.*, 2010; Maamar *et al.*, 2016; Pacholewicz *et al.*, 2015; Rasmussen *et al.*, 2015; Rayamajhi *et al.*, 2010; Rayamajhi *et al.*, 2008; Yan *et al.*, 2006).

Mutational and/or alterations of the outer membrane permeability and efflux pumps can decrease entry and/or increase efflux of antimicrobials including β-lactams, which prevent access of the drug to its targets (Li *et al.*, 2007). However, the resistance levels conferred by these mechanisms are low and very often they occur in combinations with β-lactamase production (Bush, 2012; Woodford *et al.*, 2007).

Resistance to β-lactams in *E. coli* is common; and previous studies of Australian *E. coli* poultry isolates have identified resistance to ampicillin (9.4% – 76.6%), potentiated amoxicillin (0% – 3.3%) and cephalothin (0% – 19%) (Abraham *et al.*, 2015; Barton and Wilkins, 2001; Department of Agriculture, Fisheries and Forestry (DAFF), 2007; Obeng *et al.*, 2012). Resistance genes bla_{TEM} and bla_{SHV} have been identified in *E. coli* isolates from Australian poultry (Obeng *et al.*, 2012).

1.15.4 Antimicrobials that inhibit protein synthesis

1.15.4.1 Aminoglycosides

Aminoglycosides are a group of broad-spectrum antimicrobials that were among the first antibiotics discovered. They are primarily active against gram-negative organisms and less active against gram-positive bacteria (Magnet and Blanchard, 2005). Aminoglycosides are isolated from antibiotic producers, such as *Streptomyces* spp. (Craig *et al.*, 2012). They are a group of drugs characterised by the existence of an aminocyclitol ring linked to amino sugars in their structure (Craig *et al.*, 2012). The bactericidal effect of this class is based on irreversibly binding to the aminoacyl site of the 16S

rRNA within the bacterial 30S ribosomal subunits, resulting in mistranslation of the coding protein (Armstrong *et al.*, 2012; Wright, 2010).

Neomycin, apramycin and spectinomycin are the only aminoglycosides permitted for use in broiler chickens in Australia. Gentamicin, an aminoglycoside critically important to human medicine, is not permitted for use in food-producing animals in Australia, because the drug can sequester in the kidneys resulting in residues (Australian Pesticides and Veterinary Medicines Authority (APVMA)).

Escherichia coli can acquire several resistance mechanisms that enable them to survive in the presence of aminoglycosides. These mechanisms include: (1) enzymes that destroy or reduce aminoglycosides activity; (2) target alteration by mutation and methylation of ribosomal proteins and (3) reduction of the intracellular concentration of aminoglycosides by modification of outer membrane permeability, diminished inner membrane transport or increased export outside the cell by active efflux pumps (Craig *et al.*, 2012; Jakobsen *et al.*, 2007; Zarubica *et al.*, 2011).

Aminoglycoside resistance is widespread in *E. coli* isolates worldwide (Armstrong *et al.*, 2012). Low levels of resistance to aminoglycosides: gentamicin (0% – 3.1%); neomycin (2.1% – 17.2%); spectinomycin (8.8% – 27.7%); streptomycin (33.5% – 34%) and apramycin (3.1%) in *E. coli* from poultry have been reported in previous Australian studies (Abraham *et al.*, 2015; Barton and Wilkins, 2001; Department of Agriculture, Fisheries and Forestry (DAFF), 2007). Obeng *et al.* (2012) detected *aph(3')-Ia* and *aadA2* genes from aminoglycoside resistant *E. coli* cultured from chickens from Australian chickens.

1.15.4.2 Tetracycline

Tetracycline was the first broad-spectrum antibiotic that could inhibit gram-positive and gram-negative bacteria and protozoa. Tetracyclines are one of the oldest classes of antibiotics with the clinical usage of this drug introduced in 1952. This drug class inhibits cell growth by blocking protein synthesis (Nelson and Levy, 2011). Tetracyclines normally stop elongation of synthesising proteins, by impairing the stable binding of aminoacyl-transfer (t) RNA to the bacterial ribosomal A-site (Craig *et al.*, 2012). Currently, based on their availability, low cost, low toxicity and broad-spectrum of activity, they are one of the most widely used therapeutic agents and they have been used in human, as well as, veterinary medicine (Castillo, 2013; Thaker *et al.*, 2010). Currently, in Australia, chlortetracycline and oxytetracycline are first line therapeutic drugs for use in broiler chickens.

Tetracycline resistance is usually mediated via the acquisition of resistance genes that confer resistance by one of three main mechanisms: ATP-dependent efflux; enzymatic inactivation of tetracycline; or bacterial ribosomal protection (Roberts, 2012). High tetracycline resistance (39.5% –

56.8%) in *E. coli* isolates sourced from chickens has been documented in Australia (Abraham *et al.*, 2015; Barton and Wilkins, 2001; Obeng *et al.*, 2012) as well as, globally (Hasan *et al.*, 2011; Saenz *et al.*, 2001). Obeng *et al.* (2012) identified *tet(A), tet(B)* and *tet(C)* genes in Australian isolates. This finding is not surprising, because tetracycline has been widely used as a therapeutic and feed additive to improve feed efficiency in animal production systems since its approval in 1948 (Lwinger *et al.*, 2016).

1.15.5 Antimicrobials that affect the synthesis and conformation of nucleic acids

1.15.5.1 Quinolones

Quinolones are a by-product of an anti-malarial drug and are known as 4-quinolones carboxylic acids and fluoroquinolones (Hernandez *et al.*, 2011). Developed in the 1960s, the first quinolone-derived drug, known as nalidixic acid, showed antibacterial activity (Jacoby, 2005; Ruiz, 2003). Since then, many quinolone-derived agents have been developed, especially after the addition of a fluorine atom to the quinolone molecule (Ruiz *et al.*, 2012). In the late 1980s, enrofloxacin was the first approved fluoroquinolone to be used in veterinary medicine (Martineza *et al.*, 2006). The most commonly used quinolones in different animal species and poultry overseas are ciprofloxacin, norfloxacin and enrofloxacin (Al-Mustafa and Al-Ghamdi, 2000; Gouvêa *et al.*, 2015; Rodriguez-Martinez *et al.*, 2006; Van Bambeke *et al.*, 2005).

The quinolones inhibit bacterial growth by interfering with DNA synthesis by binding to the target site and forming complexes between DNA and type II topoisomerases, namely DNA gyrase and topoisomerase IV (Van Bambeke *et al.*, 2005). This target site is known as the quinolone resistance-determining region (QRDR). The bacterial enzymes, DNA gyrase (encoded by *gyrA* and *gyrB*) and DNA topoisomerase IV (encoded by *parC* and *parE*) are necessary for DNA synthesis (Shen *et al.*, 1989). Inactivation of the enzymes due to irreversible attachment of the quinolones to the DNA-topoisomerase complex can cause a rapid bactericidal effect due to the release of DNA double-strand breaks (Cambau and Guillard, 2012; Van Bambeke *et al.*, 2005).

Quinolones are classified into four generations based on their spectrum of activity (Van Bambeke *et al.*, 2005). The first-generation (e.g. nalidixic acid) inhibit only gram-negative bacteria; while the second-generation agents, which were the first of the fluoroquinolones (e.g. ciprofloxacin and enrofloxacin) exhibit a wider spectrum against gram-negative bacteria, as well as, certain gram-positive bacteria (Cambau and Guillard, 2012; Rodriguez-Martinez *et al.*, 2006). The third- and fourth-generation fluoroquinolones have similar characteristics to the second with increased activity against gram-positive bacteria (Cambau and Guillard, 2012; Rodriguez-Martinez *et al.*, 2006).

Globally, the usage of fluoroquinolones has been under strict regulation particularly in food-producing animals because of the potential public health risk (Hernandez *et al.*, 2011; Kools *et al.*, 2008). In Australia, the fluoroquinolone antimicrobials have never been registered for use in food-producing animals (Cheng *et al.*, 2012).

Quinolone resistance is multifactorial and can develop due to one or a combination of mechanisms such as target alteration, reduced uptake and by plasmid-mediated quinolone resistance (PMQR) (Hernandez *et al.*, 2011; Jacoby, 2005; Moudgal and Kaatz, 2009). Primarily, fluoroquinolone resistance develops in gram-negative bacteria due to chromosomal mutations. The chromosomal mutations can result from deletions, substitutions or additions of nucleotide sequence/s in the QRDR.

Target alteration in type II topoisomerase are usually associated with mutations in the *gyrA* or *parC* and to a lesser extend to *gyrB* or *parE* genes (Cambau and Guillard, 2012). The most common mutations reported in *E. coli* are on the codons 83 (Ser83Leu) and 87 (Asp87His) of GyrA, corresponding to the codons 80 and 84 of ParC (Cambau and Guillard, 2012). Increases in quinolone resistance may also be associated with alteration to the outer membrane and/or overexpression of chromosomally encoded efflux pumps (Schultsz and Geerlings, 2012). Efflux pump mechanisms enable the bacteria to pump out quinolones decreasing the susceptibility to quinolones as well as other antimicrobial agents (Cambau and Guillard, 2012). Target alterations and efflux are usually found together in resistant isolates and cause an increase in quinolone MIC (Jacoby, 2005).

Plasmid-mediated quinolone resistance encodes for pentapeptide repeat proteins that bind and protect DNA gyrase and topoisomerase IV from the inhibitory effects of quinolones (Ruiz *et al.*, 2012). The *qnrA1* gene was discovered in 1998 (Martinez-Martinez *et al.*, 1998). Since then, many other quinolone resistance (*qnr*) genes have been reported: *qnrA; qnrB; qnrS; qnrC* and *qnrD* (Cambau and Guillard, 2012; Ruiz *et al.*, 2012). Ten years ago a plasmid-mediated enzymatic inactivation mechanism gene, *aac(6')Ib-cr*, which could contribute to the development of quinolone resistance, was identified (Robicsek *et al.*, 2006b). The *aac(6')Ib-cr* gene encodes for a bifunctional aminoglycoside 6′-N-acetyltransferase enzyme, which has been associated with resistance to kanamycin, as well as, ciprofloxacin in several bacterial species, including *E. coli* (Cambau and Guillard, 2012; Szmolka *et al.*, 2011; Zhao *et al.*, 2015). The PMQR genes, *oqxAB* and *qepA* encode efflux pumps and result in resistance. All of the PMQR mechanisms result in low levels of resistance, but their presence promotes clinical levels of fluoroquinolone resistance to develop and the presence of multiple PMQR mechanisms may result in clinical levels of fluoroquinolone resistance without chromosomal mutations (Jacoby *et al.*, 2014).

Jakobsen *et al.* (2010) reported variation among *E. coli* isolates collected from different sources (chicken meat vs chicken faecal samples). Higher levels of fluoroquinolone resistance were detected in *E. coli* cultured from imported broiler chicken meat (22%), compared to Danish poultry meat (12%) and broiler chicken's faecal samples (7%). The correlation between the geographical source of isolates and resistance detected tends to follow fluoroquinolone usage or selection pressure (Chantziaras *et al.*, 2014). After the USA banned the use of fluoroquinolones in food-producing animals in 2005, fluoroquinolone resistance in *E. coli* cultured from poultry decreased from 1% to 0% (Doyle *et al.*, 2013; Tadesse *et al.*, 2012).

High ciprofloxacin resistance levels (84% and 91.4%) have been detected in *E. coli* from chickens in China and Brazil, respectively (Lima-Filho *et al.*, 2013; Yang *et al.*, 2004). Lower levels of ciprofloxacin resistance have been detected in Iran (7.55%) and Slovakia (29%) in APEC isolates (Kmeť and KmeŤová, 2010; Talebiyan *et al.*, 2014). The variation in resistance reflects the difference in the antimicrobial usage policy of this drug in food-producing animals among the different countries (Kools *et al.*, 2008). China and Brazil use fluoroquinolones for the treatment and prevention of infections in chickens (Gouvêa *et al.*, 2015; Yassin *et al.*, 2017).

In Australia, where fluoroquinolones are not used, resistance to ciprofloxacin (0.4%, 1/269) in AFEC from poultry has been reported only once (Department of Agriculture, Fisheries and Forestry (DAFF), 2007). No ciprofloxacin resistance in *E. coli* from poultry has been reported in other Australian studies (Abraham *et al.*, 2015; Barton and Wilkins, 2001; Obeng *et al.*, 2012).

1.15.5.2 Sulphonamide and trimethoprim

The discovery of sulphonamides, which is a broad-spectrum antimicrobial occurred in Germany in 1932 by Gerhard Domagk and the usage of this antimicrobial in medical practice followed three years later (Sköld, 2010). Trimethoprim was first used in 1962 (Eliopoulos and Huovinen, 2001). The mode of action of sulphonamides and trimethoprim is based on interfering with the synthesis of tetrahydrofolic acid (Sköld, 2010). In 1968 trimethoprim combined with sulphonamides were first registered for clinical use. The combined usage of these two drugs exhibits a wide spectrum of activity against gram-positive and gram-negative bacteria, as well as, protozoa (Prescott, 2013; Sköld, 2010).

The majority of bacteria require folate to enable them to produce nucleic acids as they don't have the ability to absorb folate from the environment (Hollenbeck and Rice, 2012). The mechanism of action of trimethoprim and sulphonamides is based on inhibiting the bacterial growth by interfering with enzyme systems involved in folate synthesis (Eliopoulos and Huovinen, 2001; Sköld, 2010).

Trimethoprim sulphonamides are used in the Australian poultry industry (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015).

Resistance genes to sulphonamides and trimethoprim are normally found on the chromosome, small non conjugative plasmids or large multi-resistance plasmids where it is linked to other resistance genes (Sosa, 2009). Resistance to trimethoprim and sulphonamides are due to efflux pumps and mutations in the target enzymes (Cambau and Guillard, 2012; Eliopoulos and Huovinen, 2001). Acquired resistance is due to the acquisition of *sul* genes for sulphonamides and *dfr* for trimethoprim, genes that encode for alternative dihydropteroate synthase or dihydrofolate reductase. Trimethoprim sulphonamide resistance is common in APEC as class 1 integrons are widely disseminated in *E. coli* and almost always include the genes *sul1* and *dhfr1* (Bass *et al.*, 1999).

In Australian studies on *E. coli* from poultry, resistance to trimethoprim sulphonamides (11.9% – 37.5%) and trimethoprim (36.2% – 46.2%) have been reported (Abraham *et al.*, 2015; Barton and Wilkins, 2001; Department of Agriculture, Fisheries and Forestry (DAFF), 2007; Obeng *et al.*, 2012). Obeng *et al.* (2012) identified *sul1, sulII, dhfrV* and *dhfrXIII* genes in Australian isolates.

1.15.5.3 Phenicols

Florfenicol and chloramphenicol belong to the phenicol family, and are bacteriostatic antimicrobials (Lambert, 2012). This class of drug has a broad-spectrum of activity, which includes gram-positive and gram-negative bacteria (Schwarz *et al.*, 2004). Chloramphenicol, known previously as chloromycetin, was first acquired from *Streptomyces venezuelae* (Schwarz *et al.*, 2004). Amphenicols inhibit bacterial growth by interfering with protein synthesis (Dowling, 2013; Sams, 1994). They prevent protein elongation by inhibiting the peptidyl transferase activity at the bacterial ribosome (Schwarz *et al.*, 2004; Sosa, 2009).

Despite the broad-spectrum activity of chloramphenicol, a severe side effect (bone marrow depression) identified in the mid-1960s, restricted their use in the treatment of human diseases (Lambert, 2012; Schwarz *et al.*, 2004). Historically, chloramphenicol was used in all animals but now most countries, including Australia, have prohibited their use in food-producing animals (Australian Pesticides and Veterinary Medicines Authority (APVMA); Prescott, 2013; Sams, 1994; Yunis, 1988). Florfenicol, a fluorinated synthetic analog of thiamphenicol, is approved for use in veterinary medicine, in particular for controlling respiratory tract infections in cattle and pigs (Schwarz and Chaslus-Dancla, 2001). Florfenicol can cause a dose dependent bone marrow suppression in animals but does not cause idiosyncratic aplastic anaemia in humans (Dowling, 2013). Florfenicol, however,

is not used in Australian poultry (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015; Australian Veterinary Association, 2017).

Multidrug efflux systems and mutations of the target site can contribute to chloramphenicol resistance in *E. coli* (Lambert, 2012; Schwarz *et al.*, 2004). Another important resistance mechanism consists of the presence of resistance genes, such as *floR* gene. These genes are found on plasmids and on the chromosome of *E. coli* from poultry and can cause a combined resistance to chloramphenicol and florfenicol (Schwarz *et al.*, 2004). In Australian studies on *E. coli* from poultry, resistance to chloramphenicol (0% – 1.8%) and florfenicol (0% – 3.4%) has been detected (Abraham *et al.*, 2015; Department of Agriculture, Fisheries and Forestry (DAFF), 2007; Obeng *et al.*, 2012).

1.16 Antimicrobial resistance trend in *E. coli* from poultry

Escherichia coli have the ability to cause several bacterial infections in both humans and animals and are the most prevalent commensal bacteria in the gastrointestinal tract (Johnson and Russo, 2002; Mohammad, 2010). They have great genetic flexibility and can acquire several AMR mechanisms. This is why *E. coli* is often used as an indicator organism to evaluate AMR in the environment and from diverse host species (Guenther *et al.*, 2011; Stedt *et al.*, 2014). Currently, *E. coli* is known as an important reservoir of resistance genes to critically important antimicrobials in human medicine, including ESC, fluoroquinolones, aminoglycosides and trimethoprim sulfamethoxazole (Pitout, 2012). In a longitudinal survey from 1993 to 2013, an increase in APEC resistance to 14 tested antimicrobial drugs in China was observed (Zhang *et al.*, 2017). Similar increases in resistance trends in APEC over eight years (2002 to 2010) were reported in the USA (Tadesse *et al.*, 2012). Increases in the prevalence of AMR in gram-negative pathogens such as *E. coli* have also been reported in Europe (ECDC, 2012).

In 2011, the European Antimicrobial Resistance Surveillance Network (EARS-Net) reported a wide distribution of resistant *E. coli* strains in both human and food-producing animals (ECDC, 2012). These MDR *E. coli* isolates demonstrated a significant increase in resistance, with combined resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides (ECDC, 2012). de Jong *et al.* (2012) reported a higher resistance to older generations of antimicrobials in comparison to newer drugs (such as cefotaxime and ciprofloxacin) in food-producing animals. A systematic study in Germany in 2009 detected AMR in *E. coli* among isolates obtained from faecal samples of healthy food-producing animals (poultry, cattle and pigs) (Kaesbohrer *et al.*, 2012). In summary, the authors reported resistance to newer drugs such as fluoroquinolones and third-generation cephalosporins, as well as, older generation drugs, such as sulphonamides and tetracycline. The *E. coli* isolates obtained

from 49 broiler chickens demonstrated higher resistance rates to ciprofloxacin in comparison to *E. coli* isolates sourced from cattle and pig production chains (Kaesbohrer *et al.*, 2012).

Overall, resistance rate to critically important antimicrobials (especially to quinolones) has increased in *E. coli* sourced from food-producing animals worldwide (Giufre *et al.*, 2012; Halova *et al.*, 2014; Jiang *et al.*, 2011; Lohren *et al.*; Wasyl *et al.*, 2013). The emergence of MDR *E. coli* isolates, in particular resistance to critically important antimicrobials for humans, is alarming and highlights the need for AMR monitoring systems (Canton *et al.*, 2012; Machado *et al.*, 2008). Future studies are needed for every country to identify the prevalence of antimicrobial resistant *E. coli* isolates from food-producing animals and their association with the antimicrobial usage in the veterinary medicine.

1.17 Public health significance of APEC

Bacteria (pathogens and commensals) can be transferred directly either via food (e.g. meat and eggs) or contact with an animal. Indirect transmission can occur through the release of animal waste into the environment (Aidara-Kane, 2012; da Costa *et al.*, 2013; Ewers *et al.*, 2007; Gonzalez-Zorn and Escudero, 2012; Graham *et al.*, 2008). Antimicrobial resistance genes and VGs can be transferred from bacteria found in animals, to bacteria found in humans, through MGE, such as plasmids or transposons (Arias and Carrilho, 2012; da Costa *et al.*, 2013; Johnson *et al.*, 2010; Szmolka and Nagy, 2013).

Studies have looked at the direct transfer of bacteria from animals to humans (Alexander *et al.*, 2010; Aslam *et al.*, 2009; Wang *et al.*, 2012). In humans, ExPEC is considered to be an important cause of neonatal meningitis, septicaemia and urinary tract infections (Belanger *et al.*, 2011). Despite the difficulty in identifying the origin of human ExPEC, the genetic overlap reported globally between human ExPEC and avian *E. coli* suggests that APEC may be a zoonotic pathogen (Ewers *et al.*, 2009; Mora *et al.*, 2013; Moulin-Schouleur *et al.*, 2007). The ability of APEC to cause disease in rats, in a study by Tivendale *et al.* (2010), shows APEC can cause disease in mammals. A study in Denmark, by Jakobsen *et al*. (2010), found *E. coli* isolates sourced from human patients, broiler meat and other food-producing animals, including pigs, could not be differentiated on the basis of their VGs nor their AMR profile. They found resistant isolates clustered in the same phylogenetic group regardless of the source of the isolates (Jakobsen *et al.*, 2010). Johnson *et al.* (2012) reported an association between MDR and plasmid content among commensal *E. coli* (AFEC) and ExPEC obtained from humans and poultry.

Human ExPEC and APEC share common VGs and APEC may act as reservoir vehicle for VGs that aid bacteria to cause disease in human (Ewers *et al.*, 2004; Ewers *et al.*, 2007; Mora *et al.*, 2013).

Furthermore, STs such as ST10, ST95, ST131, ST117, ST746 and ST23 have been found to be common between ExPEC, APEC and clonally related isolates from humans, poultry and poultry products. This strengthens the hypothesis that chickens can act as a reservoir for ExPEC infection in humans (Adiri *et al.*, 2003; Danzeisen *et al.*, 2013; Ewers *et al.*, 2007; Johnson *et al.*, 2008c; Johnson *et al.*, 2007; Kohler and Dobrindt, 2011; Rodriguez-Siek *et al.*, 2005a; Tivendale *et al.*, 2010).

The direct food chain is also believed to be a major contributor to the emergence and persistence of AMR in pathogenic, as well as, commensal bacteria (Kluytmans *et al.*, 2013; Liu *et al.*, 2012; Vieira *et al.*, 2011). Several studies have identified the presence of resistant bacterial strains and resistance genes from food animals at all stages of processing (Abdallah *et al.*, 2015; Acar *et al.*, 2012; Marshall and Levy, 2011). The presence of these antimicrobial resistant bacteria within animal products may be due to multi factorial contamination at different stages of food production and provides indirect evidence of transmission of these resistance genes during food handling and/or consumption. Contamination can happen via faecal splash during slaughter, during food processing or at the final stage when the consumer handles the food (Alexander *et al.*, 2010; Verraes *et al.*, 2013).

The emergence of AMR among bacteria of food-producing animals is of public health concern as humans and animals use the same classes of antimicrobials for therapy and AMR mechanisms are the same for human and animal pathogens (Cogliani *et al.*, 2011).

Furthermore, the use of antimicrobials for treatment, as well as, prevention can cause a selective pressure, which contributes to the spread of resistance across different bacteria, including commensals. A positive association has been found between an increase in antimicrobial usage in veterinary medicine and the increasing prevalence of antimicrobial resistant bacteria detected in food-producing animals and food products (Altalhi *et al.*, 2010; Borjesson *et al.*, 2013; Castellanos *et al.*, 2017; da Costa *et al.*, 2010; Hammerum *et al.*, 2012; Hasan *et al.*, 2011; Hordijk *et al.*, 2013; Jiang *et al.*, 2011; Nilsson *et al.*, 2014; Ojer-Usoz *et al.*, 2013).

The poultry industry uses antimicrobials that are classified as highly important or critically important for human medicine by the World Health Organisation, such as tetracycline (chlortetracycline). This potentially provides selective pressure for the maintenance of, or emergence of resistant strains of bacteria in humans. Globally, AMR to critically important antimicrobial drugs has become a public health concern particularly with the global increase in the prevalence of ESC resistant *E. coli* and the mortality associated with it (FAO/WHO/OIE, 2008).

In the Netherlands, the association and distribution of ESBL genes, plasmids and strain genotypes among *E. coli* isolates obtained from human patients, poultry and poultry meat was evaluated. The

authors reported similarities between all of these, which suggests that retail chicken meat and poultry could be a possible source of the ESBL genes in *E. coli* that have transferred from animals to humans or vice versa (Leverstein-van Hall *et al.*, 2011).

1.18 Antimicrobial resistance in *E. coli* and its association with VGs

The significant role of MGE in the transmission of AMR genes makes studies about plasmids the core of the majority of the AMR epidemiological studies. However, the transmission role of MGEs is not limited to ARGs, but also to VGs, as they can be found on the same plasmids (Da Silva and Mendonca, 2012).

Recent studies have reported the correlation between resistance to certain antimicrobials (cefoxitin, gentamicin, kanamycin, streptomycin, tetracycline and ceftiofur) and APEC-associated VGs (*astA*, *iucD, cvaC, iss*, *iutA* and *traT*) in avian-sourced *E. coli* (Lynne *et al.*, 2012; Wang *et al.*, 2010). Despite the evidence that there is an association between virulence and AMR, the interplay between them is still controversial and needs further study to reveal the genetic mechanisms regulating this interplay in order to improve infectious disease management.

1.19 Summary

The present review found that there is not enough information available to define and differentiate between APEC and commensal *E. coli*. The majority of the studies included in the current review were from overseas and there is limited information published on the prevalence of VGs and their association with APEC in Australia. The APEC prevalence and its associated risk factors especially the ones related to the bird's surrounding environment are unknown in the Australian context. The literature review also highlights the need for further studies on the epidemiology and association of AMR, virulence genes and phylogenetic groups to identify risk factors associated with human health.

Chapter 2: Research and project outline

2.1 Knowledge gaps and rationale for the project

Avian colibacillosis is considered one of the most common diseases affecting the global poultry industry (Guabiraba and Schouler, 2015; Nolan *et al.*, 2013). The disease results in substantial economic losses due to impacts on productivity, reductions in the marketability of poultry and poultry products, high mortality, morbidity and the costs associated with prevention and treatment (Cavero *et al.*, 2009; Jakob *et al.*, 1998; Nolan *et al.*, 2013). Avian pathogenic *Escherichia coli* (APEC), the causative agent of avian colibacillosis, is a subgroup of extraintestinal pathogenic *E. coli* (ExPEC), which is capable of causing diseases outside the gastrointestinal tract in poultry and humans (Johnson and Russo, 2002; Mellata, 2013; Russo and Johnson, 2009). Biosecurity, management, as well as the administration of antimicrobial agents (potentiated sulphonamides, penicillin, tetracycline, colistin, spectinomycin, aminoglycosides and fluoroquinolones) are currently considered the most effective measures to treat and control avian colibacillosis (Boerlin and White, 2013; Guardabassi and Kruse, 2008; Nolan *et al.*, 2013). The long-term usage of antimicrobials has contributed to an increase in resistance among APEC to older generation, as well as newer antimicrobials, threatening public health, as well as compromising poultry health and welfare (Barros *et al.*, 2012; Pitout, 2012).

Several studies have aimed to identify APEC isolates and differentiate them from avian faecal *E. coli* (AFEC) isolates based on phylogenetic grouping, virulence genotyping, plasmid profiling, serotyping, sequence typing, genotyping, enterobacterial repetitive intergenic consensus (ERIC) PCR and/or pulsed-field gel electrophoresis (PFGE) and/or whole genome sequencing (Cordoni *et al.*, 2016; Johnson *et al.*, 2008b; Landman *et al.*, 2014; Lynne *et al.*, 2012; Rodriguez-Siek *et al.*, 2005a; Won *et al.*, 2009). The current diagnosis of avian colibacillosis is based on clinical signs, macroscopic lesions and the isolation of *E. coli* from the lesions using traditional culture methods (Guabiraba and Schouler, 2015; Kabir, 2010). However, culture methods are labour intensive and time consuming in comparison to molecular methods (Baron, 2011; Garofalo *et al.*, 2007; Lleo *et al.*, 2005) and without further virulence characterisation, simple isolation of *E. coli* from diagnostic samples does not specifically identify APEC.

The direct application of molecular techniques to faecal samples to identify APEC may be limited by the ability to extract sufficient DNA of a PCR quality that is free from PCR inhibitors (Abu Al-Soud

and Radstrom, 1998; Johnson *et al.*, 2008b; McOrist *et al.*, 2002). Several extraction methods (physical, mechanical and chemical) have been used to extract the DNA from faeces from many animal species and there are specifically designed commercial kits available (McOrist *et al.*, 2002; Yu and Morrison, 2004). However, there is a lack of information pertaining to the quality and quantity of extracted DNA from chicken faecal samples using these different methods (Barnard *et al.*, 2011). Due to the nature of poultry faeces (i.e. combined faecal and urinary wastes), it is not necessarily appropriate to extrapolate methods and results from other species. In addition, no data is available comparing the screening of virulence genes (VGs) in poultry faeces compared to isolation of *E. coli* from birds and screening for VGs in the cultured isolates.

The literature review undertaken in Chapter 1 shows that up-to-date knowledge regarding the molecular, and indeed the general epidemiology of APEC in poultry in Australia, is very limited. There is also no clear definition of APEC based on specific VG patterns or phylogenetic classification, nor any clear knowledge of the association between VGs and phylogenetic groups. The major predisposing risk factors associated with APEC infections are not well understood (Kemmett *et al.*, 2013; Nolan *et al.*, 2013). Additionally, very little knowledge is available regarding antimicrobial resistance (AMR) profiles, as well as plasmid replicon presence in either APEC and/or AFEC in Australia.

In the current research, all of the *E. coli* isolates will be firstly categorised based on the bird's health status and independently from the VG profiles into faecal *E. coli* (FEC) and clinical *E. coli* (CEC). FEC are isolates cultured from the faeces of healthy birds (which are defined as birds that are apparently free from any clinical disease symptoms) and CEC are cultured from the faeces or organs of birds with colibacillosis (which are defined based on the clinical signs and the detection of *E. coli* from the affected lesions). At the molecular level, *E. coli* isolates sourced from anywhere (except from lesions of colibacillosis) that harbour four or more of the APEC VG markers (*iroN, iutA, iss, hlyF* and *ompT*) will be defined as an avian pathogenic *E. coli* (APEC). Therefore, FEC isolates will be molecularly classified into APEC or AFEC, dependant on whether they harbour four or more or less than four of the selected APEC VGs. CEC isolates cultured from lesions, which harbour four or more of the five APEC-associated VGs, will be defined as clinical avian pathogenic *E. coli* (cAPEC). Clinical *E. coli*, which contain less than four of the selected VGs are identified as potential APEC (pAPEC). The VGs were selected because they have previously been identified as successful marker genes for APEC identification (Johnson *et al.*, 2008b).

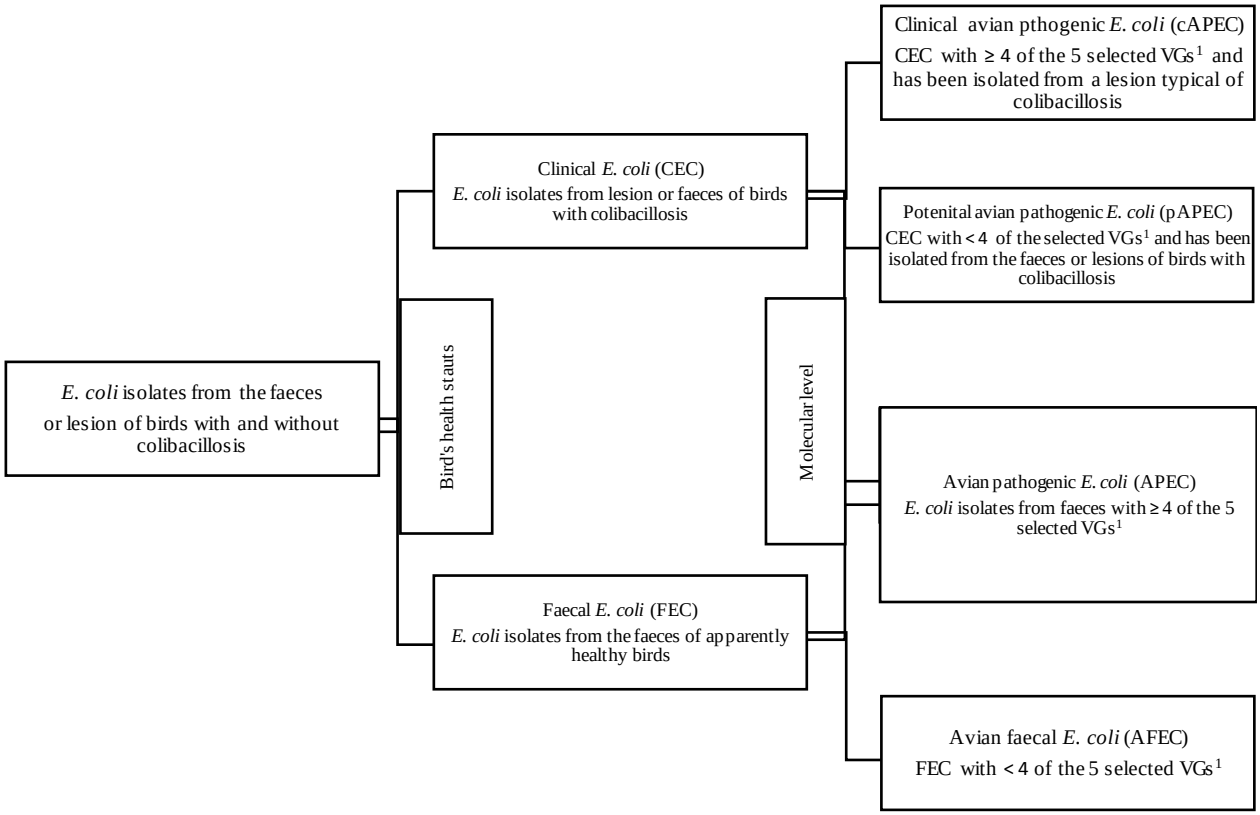


Figure 2.1: Illustration flow chart for the classification applied to all of the *E. coli* isolates included in the current study.

[1] The selected VGs are: *iss; iutA; iroN; ompT* and *hlyF*.

2.2 Aims and objectives

2.2.1 Overall thesis aim

To investigate avian pathogenic *E. coli* in Australian broiler flocks, explore antimicrobial susceptibility and molecular epidemiology to improve the identification of this pathotype.

2.3 Thesis hypotheses

2.3.1 Primary hypothesis

Avian pathogenic *E. coli* can be defined and differentiated from avian faecal *E. coli* based on a specific set of avian pathogenic *E. coli* associated virulence genes, phylogenetic group and plasmid replicons.

2.3.2 Secondary hypothesis

There is a difference in the antimicrobial susceptibility profile between clinical *E. coli* and faecal *E. coli* and certain plasmid replicons are associated with specific antimicrobial resistance profiles.

2.3.3 Risk factor hypothesis

Specific management procedures are associated with an increased risk of avian colibacillosis and avian pathogenic *E. coli* prevalence on commercial broiler chicken farms in South East Queensland, Australia.

2.4 Specific aims, objectives and methodological approaches

2.4.1 Aim 1 (Chapter 3): To compare direct DNA extraction methods from faecal samples and to estimate the prevalence of avian pathogenic *E. coli* between healthy birds and birds with colibacillosis.

2.4.1.1 Objective 1: To compare and evaluate the relative efficiency and effectiveness of three different published approaches for DNA extraction methods from faecal/cloacal swabs and/or faeces of broiler chickens.

2.4.1.2 Methodological approach:

Three different published methods for DNA extraction (a) QIAamp DNA Stool Mini Kit, b) repeated bead beating plus column methods (RBB+C) and c) Chelex methods will be evaluated in triplicate to detect the most effective and practical method to extract high quality and quantity DNA from the cloacal and faecal swabs of broiler chickens. The efficiency of DNA extraction will be assessed by determining the level of DNA yield, the purity of DNA and the presence of impurities, either from the faeces or the extraction process.

2.4.1.3 Objective 2: To investigate the assertion that in healthy chickens *E. coli* is present in the cloacal and intestinal swabs (i.e. intestinal tract) and no other internal organs. To identify if the five APEC related VGs are positively associated with birds affected with colibacillosis and validate their selection as an APEC marker in this research.

2.4.1.4 Methodological approach:

Isolates for this study will be obtained from apparently healthy chickens and chickens with colibacillosis. From each chicken, swabs will be cultured from sites expected to be sterile (heart, liver, spleen, air sac and lung) and the cloaca/intestine. The *E. coli* isolates will be screened by PCR for the presence of five APEC-associated VGs and a statistical comparison will be done on the prevalence of the VGs in healthy birds versus birds with colibacillosis.

2.4.2 Aim 2 (Chapter 4): To estimate the farm-level prevalence of avian pathogenic *E. coli* in clinically healthy chickens from commercial broiler farms in South East Queensland (SEQ), Australia and to identify farm-level risk factors associated with avian pathogenic *E. coli* prevalence.

2.4.2.1 Objective 1: To estimate the farm-level prevalence of APEC related VGs in intestinal *E. coli* from healthy broiler chickens in SEQ, Australia.

2.4.2.2 Methodological approach:

Isolates for this study will be obtained from cloacal swabs of clinically healthy chickens at one abattoir located in SEQ, Australia. Four hundred cloacal swabs will be collected from chickens from 40 poultry farms. Swabs will be cultured and five to ten *E. coli* colonies per chicken will be selected and screened by PCR for the five APEC-associated VGs.

2.4.2.2.1 Objective 2: To identify the farm-level risk factors associated with the prevalence of APEC among the intestinal *E. coli* isolates.

2.4.2.3 Methodological approach:

Information on potential farm-level risk factors (farm management practices, biosecurity measures, vaccination protocols and chicken health) associated with the prevalence of APEC will be obtained through questionnaires and interviews from farms that had supplied birds for slaughter. Binominal general linear models will be used to identify the farm-level risk factors associated with APEC prevalence among the intestinal *E. coli* sourced from broiler chicken farms in SEQ.

2.4.3 Aim 3 (Chapter 5): To determine the antimicrobial resistance profile prevalence and their association with phylogenetic group, avian pathogenic *E. coli* related virulence genes and plasmid replicon profiles among clinical and faecal *E. coli* isolates sourced from Australian commercial broiler chickens.

2.4.3.1 Objective 1: To determine, compare and identify the association between antimicrobial susceptibility, phylogenetic group and plasmid replicon profiles of 50 clinical *E. coli* (CEC) and 187 faecal *E. coli* (FEC) cultured from Australian commercial broiler chickens.

2.4.3.2 Methodological approach:

Fifty CEC and 187 FEC will be collected from chickens with colibacillosis and healthy chickens, respectively. All of the *E. coli* isolates will be subjected to disc diffusion antimicrobial susceptibility testing (20 antimicrobials), phylogenetic grouping, plasmid replicon typing and VG PCR screening for *iroN, iutA, iss, hlyF* and *ompT*.

2.4.3.3 Objective 2: To characterise the extended-spectrum cephalosporin (ESC) and/or fluoroquinolone (FQ) resistant isolates.

2.4.3.4 Methodological approach:

Isolates resistant to ESCs and/or FQs will undergo minimum inhibitory concentration testing (MIC), multilocus sequence typing (MLST) and screening for plasmid-mediated ARGs. The quinolone resistance-determining region (QRDR) of FQ resistant isolates will be sequenced and FQ resistant isolates will also be screened for the presence of an efflux pump using Phe-Arg-β-naphthylamide.

2.4.4 Aim 4 (Chapter 6): To determine the prevalence of 35 extraintestinal pathogenic *E. coli* related virulence genes in *E. coli* circulating in commercial broiler chicken flocks in South East Queensland, Australia and relate these to defining features of the pathotype to derive an improved genetic definition for avian pathogenic *E. coli*.

2.4.4.1 Objective 1: To determine the clonal relatedness between 237 *E. coli* isolates sourced from healthy chickens and chickens with colibacillosis.

2.4.4.2 Methodological approach:

An ERIC-PCR will be performed on the 50 CEC and 187 FEC isolates to identify clonal relationships in the isolates that have been previously characterised (Chapter 5).

2.4.4.3 Objective 2: To determine the association between ExPEC-associated VGs and avian colibacillosis.

2.4.4.4 Methodological approach:

A subset of isolates that display resistance to the most antimicrobials and represent a different ERIC cluster will be selected for further virulence profiling and will be screened for an additional 30 extraintestinal pathogenic *E. coli* related VGs.

2.5 Thesis overview

Chapter 1 reviews the following: literature on avian colibacillosis and its effects on the poultry industry; APEC (the causative agent); diagnosis and treatment of the disease including antimicrobials (and their mechanism of action and resistance mechanisms); VGs associated with APEC; risk factors associated with APEC and the zoonotic potential of APEC. Chapter 2 summarises the overall thesis aims, hypothesis and objectives. Chapter 3 compares and evaluates three different published methods for DNA extraction from cloacal swabs and faecal samples from healthy and unhealthy broiler chickens to identify the most practical and effective methods to extract adequate amounts of PCR quality DNA. Chapter 4 identifies the prevalence of APEC among *E. coli* cultured from the faeces of clinically healthy chickens and the risk factors associated with the carriage of APEC on commercial broiler chicken farms in SEQ, Australia. Chapter 5 characterises the antimicrobial profile pattern and determines the phylogenetic groups, virulence and plasmid replicon profiles of 50 CEC and 187 FEC isolates cultured from Australian commercial broiler chickens. The presence of AmpC and extended-spectrum β-lactamases and/or FQ resistance genes is examined. Furthermore, the presence/absence of the most common and important plasmids from an animal or public health perspective is determined. Finally, the phylogenetic groups, genotypes, AMR patterns, VGs and plasmid replicon profiles of avian clinical and faecal *E. coli* isolates cultured from Australian commercial broiler chickens are compared. Chapter 6 examines the prevalence of 35 ExPEC related VGs among *E. coli* isolates sourced from the cloacal swabs of healthy chickens and chickens with colibacillosis. Chapter 7 summarises all of the concepts and major findings that were identified in chapters 3 to 6, provides a general discussion, summarises the major findings, their implications and progresses to general conclusions and provides suggestions for future work.

2.6 Anticipated deliverables and practical outcomes

Achieving the overall thesis aim would benefit Australia and other parts of the world, which are geographically similar to Australia. Due to the global variation in APEC the outcome of our studies might be most specific to the Australian context; however, these findings do add to the global knowledge of variance between APEC and risk factors associated with colibacillosis and can further be used to explore the epidemiology of APEC strains. The results obtained by the current study should help to improve the identification of APEC by providing a set of VGs that can be used as a marker for the early detection of the presence of APEC on commercial broiler chicken farms in SEQ, Australia. The selection of VGs will allow veterinary diagnostic laboratories to identify APEC. The current thesis explores the possibilities of identifying APEC directly from faecal samples by determining the best extraction methods that can be applied to chicken faeces to obtain PCR quality DNA. The DNA extraction methods that will be recommended by the current thesis can be used to detect other resistance and/or VGs and pathogens. The early detection of APEC directly from faecal samples can reduce the economic losses associated with avian colibacillosis, positively impacting the health and welfare of commercial broiler chickens.

Determining the farm-level prevalence of the intestinal/cloacal carriage of APEC in Australian broiler flocks is one of the outcomes, which should be obtained by the current thesis, and will lead to better recognition of sub-clinical carriage and transition to pathogenicity. Furthermore, the possible associations between potential management risk factors and the farm-level prevalence of carriage of APEC among healthy commercial broiler chicken flocks in SEQ will be investigated and will inform on how to improve control and prevention strategies for avian colibacillosis. The findings of the current thesis should also provide more knowledge of the antimicrobial susceptibility of APEC to guide veterinarians in developing successful prevention and treatment schemes in Australia.

Chapter 3: An optimised protocol for molecular screening of avian pathogenic *Escherichia coli* from broiler chickens in South East Queensland, Australia.

3.1 Foreword

Currently, the diagnosis of avian colibacillosis is based on clinical signs, post-mortem examination and the detection of *Escherichia coli* in the lesion(s), which is time consuming, as well as labour intensive. Establishing a methodology to allow the direct detection of avian pathogenic *E. coli* (APEC) from faecal samples of birds without the requirement for culture of isolates would allow a more rapid diagnostic process to detect the causative agent of avian colibacillosis, an economically important poultry disease.

However, the ability to extract sufficient PCR quality DNA has proved problematic, specifically for bird faecal samples, due to the presence of large amounts of PCR inhibitors. The current chapter compares three published methods for extracting DNA directly from the faecal and cloacal samples of broiler chickens, aiming to detect the most effective and practical method(s) to extract high quantity and quality DNA and determine if APEC can be detected directly from faeces.

Faecal samples and *E. coli* isolates were obtained from apparently healthy chickens and chickens with colibacillosis. The DNA extracted from faecal samples and *E. coli* isolates were screened with a pentaplex-PCR for the detection of five APEC-associated virulence genes (VGs): (haemolysin gene (*hlyF*); increased serum survival gene (*iss*); outer membrane protease gene (*ompT*) and two iron acquisition system genes (*iutA* and *iroN*)). The objectives to confirm that in healthy chickens *E. coli* is present only in the intestinal tract and to validate the pentaplex-PCR for Australian APEC isolates by showing that *E. coli* cultured from the cloaca of healthy chickens have less VGs compared to *E. coli* cultured from chickens with colibacillosis.

3.2 Abstract

Avian pathogenic *Escherichia coli* (APEC) is the causative agent of avian colibacillosis and causes localised and/or systemic infections in poultry. The presence of various virulence genes (VGs) may be a useful marker for the detection of APEC directly from faecal samples. The objectives of this study were to evaluate and compare three different DNA extraction methods from cloacal swabs and faecal samples of broiler chickens and determine if APEC can be detected directly from faeces. The DNA extraction methods were assessed by measuring DNA yield and purity; absence of DNA shearing, 16S ribosomal DNA amplification and reproducibility. Repeated bead beating plus column (RBB+C) was the preferred extraction method, as it yielded an adequate amount of quality DNA for PCR directly from faeces. The DNA extracted from faces, with RBB+C method, and DNA extracted from *E. coli* isolates, of organs and faeces, taken from 23 broiler chickens (10 healthy, 9 with colibacillosis and 4 unhealthy with other infections) were screened with a pentaplex-PCR for the prevalence of APEC-associated VGs: *iroN; ompT*; *iutA*; *iss* and *hlyF*. There was a statistically significant correlation between the presence of the five VGs in *E. coli* cultured from the cloaca, faecal and organs samples and colibacillosis. However, screening extracted DNA from the faeces for the selected VGs was not an effective diagnostic tool to detect APEC as all of the VGs were detected in the extracted faecal DNA from all chickens.

3.3 Introduction

Avian colibacillosis causes multimillion-dollar annual losses for the poultry industry worldwide (Ewers *et al.*, 2004; Hussein *et al.*, 2013; Johnson *et al.*, 2008b; Nolan *et al.*, 2013). Production losses are associated with decreased productivity, high mortality and morbidity and are in addition to the costs associated with treatment and prevention on-farm and abattoir carcass condemnations (Ewers *et al.*, 2004; Kabir, 2010; Nolan *et al.*, 2013). Avian pathogenic *Escherichia coli* (APEC), a subgroup of extraintestinal pathogenic *E. coli* (ExPEC), is the causative agent of avian colibacillosis. Although a number of studies have aimed to identify the virulence mechanisms of APEC, it remains an ill-defined pathotype (Nolan *et al.*, 2013; Rodriguez-Siek *et al.*, 2005a). Healthy chickens and their surrounding farm environment can be colonised by APEC without the chickens displaying any signs of disease (Antão *et al.*, 2008). Recent studies have suggested that the presence of various virulence genes (VGs) were useful markers for the detection of APEC and can differentiate between APEC and avian faecal *E. coli* (AFEC) (Circella *et al.*, 2012; Ewers *et al.*, 2005; Johnson *et al.*, 2008b; Rodriguez-Siek *et al.*, 2005a). Johnson *et al.* (2008b) developed a PCR targeting five VGs:

haemolysin gene (*hlyF*); increased serum survival gene (*iss*); outer membrane protease gene (*ompT*) and two iron acquisition system genes (*iutA* and *iroN*), which could be used as a diagnostic tool for the identification of APEC.

Diagnosis of avian colibacillosis is traditionally based on clinical signs, macroscopic lesions and the isolation of *E. coli* from lesions. Culture methods are considered to be the gold-standard for isolation and identification of *E. coli* (Kabir, 2010). However, culture methods are labour intensive, expensive and time consuming in comparison to molecular methods (Blessmann *et al.*, 2002; Garofalo *et al.*, 2007). The direct application of molecular techniques to identify APEC from faecal samples may be limited by the ability to extract a high quality and quantity DNA that is free from PCR inhibitors, such as bile salts, haemoglobin, degradation products and complex polysaccharides (Abu Al-Soud and Radstrom, 1998; McOrist *et al.*, 2002). There are several methods (physical, mechanical and chemical), as well as specifically designed commercial kits (McOrist *et al.*, 2002; Yu and Morrison, 2004) used to extract DNA from faeces. However, the complex matrix of faecal samples make it a challenging job to choose the most suitable extraction protocol as some methods, such as the cell lysis by boiling method, are incapable of removing faecal inhibitors (Rapp, 2010; Wilson, 1997). Furthermore, there is a lack of information pertaining to the quality and quantity of extracted DNA from chicken faecal samples using these different methods (Barnard *et al.*, 2011).

The aims of this study were to: (i) compare and evaluate three different published methods for DNA extraction from cloacal swabs and faecal samples from broiler chickens (selection of these methods was based on their popularity of use by the veterinary laboratory and/or the poultry industry and ease of use); (ii) identify chickens with colibacillosis by sampling the cloaca and organs and screening cultured *E. coli* for the presence of five APEC-associated VGs to determine if *E. coli* cultured from the cloaca of healthy chickens have less VGs compared to *E. coli* cultured from chickens clinically affected with colibacillosis; (iii) confirm that clinically healthy chickens do not harbour *E. coli* in their organs and (iv) determine if direct application of molecular techniques to faecal samples could identify APEC.

3.4 Materials and methods

3.4.1 Samples collection

Twenty chickens were collected from three different commercial broiler chicken farms located in South East Queensland (SEQ) between June and July 2013 (Animal ethics approval number: QAAFI

/478/12/ POULTRY CRC). The minimum sample size for the pilot study required to detect the difference between probabilities of 0.10 and 0.70 at a two-side significance level of 0.05 and a power of 0.90, requires a replication of 12 for each sample and in order to detect the difference between probabilities 0.10 and 0.80, at a two sided significance level 0.05 and a power of 0.90 requires a replication of 9 for each sample. The farm managers selected nine chickens that appeared to be unhealthy (showing clinical signs of weakness, respiratory distress and ruffled feathers (4 from farm A, 3 from farm B and 2 from farm C) and 11 apparently healthy chickens (4 from farm A, 3 from farm B and 4 from farm C). Additionally, three unhealthy chickens from farm C were collected as part of their daily culling protocol. The age of the chickens ranged between 28 and 48 days. Post-mortem examination and specimen collection were performed on the farms on a total of 23 chickens. A numeric lesion scoring classification scheme representing the severity of each macroscopic gross lesion attributed to *E. coli* was assigned to five organs: heart; liver; spleen; air sac and lung (Antão *et al.*, 2008; Ginns *et al.*, 1998) according to the following scale: 0 = no lesions; 1 = small amount of fibrin on a single small pin size lesion; 2 = small amount of fibrin on two small pin sized lesions; 3 = thin layer of fibrinous exudate covering the organ; 4 = severe, thick and extensive layer of fibrinous exudate covering the organ. Chickens with lesions in the lung and/or air sac were considered to have a respiratory tract infection and chickens with lesions in the liver and/or spleen and/or heart were considered to have a systemic infection. Liver, lung, heart and spleen tissues were collected from each chicken and fixed in an excess of 10% neutral buffered formalin for histopathological processing. From each chicken, nine samples were collected with Amies transport swabs (Sarstedt Australia Pty. Ltd., Technology Park, South Australia, Australia), one from each of the five organs listed previously and four replicate samples from the cloaca (one to be used for *E. coli* culture and three for DNA extraction). Fresh faecal samples were also collected from the cloaca or the end of the large intestine into a sterile container if the cloaca was empty. The swabs and faecal samples were transported on ice to the laboratory for processing within three hours of collection.

3.4.2 Processing of samples

3.4.2.1 Histological examination

Tissues samples were processed from one healthy and five unhealthy chickens overall, representing various health and disease statuses. Each sampled organ underwent routine paraffin-embedding and sectioning (4 μm thickness). Tissues were stained with haematoxylin and eosin while Period Acid

Schiff stains were used for fungi (Gridley, 1957). A board-certified veterinary pathologist reviewed histopathology.

3.4.2.2 Bacterial culture of samples identification and DNA extraction

In order to identify all the bacterial growths all the organ swabs were cultured on 5% sheep blood agar (SBA; Oxoid, Thebarton, South Australia, Australia), MacConkey agar (MCA; Oxoid), chocolate agar (Oxoid), xylose-lysine deoxycholate agar (Oxoid) and brilliant green agar (Oxoid). The plates were incubated aerobically overnight at 37 °C. An additional SBA plate for each sample was incubated anaerobically using the AnaeroGen system (Oxoid) overnight at 37 °C. In addition, the liver swabs were cultured on campylobacter blood-free agar (Oxoid) and incubated under microaerophilic conditions using the CampyGen system (Oxoid) for 48 h at 37 °C. Isolates were single colony picked onto an appropriate agar, incubated under appropriate conditions and then identified using routine veterinary phenotypic diagnostic methods such as Gram stain reaction, morphology and biochemical tests e.g. Microbact ^{24E} (Thermo Fisher Scientific Australia Pty Ltd, Brendale, Queensland, Australia) or API® Staph (bioMerieux, Baulkham Hills, New South Wales, Australia). One cloacal swab from each chicken was cultured onto MCA and incubated aerobically at 37 °C overnight. From each MCA plate, three colonies showing the typical colony morphology of *E. coli* were selected and subcultured onto SBA and incubated aerobically at 37 °C overnight. Isolates (from organs and cloacal swab), which were indole positive and pyrrolidonyl arylamidase negative, were identified as suspected *E. coli* and DNA was extracted (Abraham *et al.*, 2012). An *E. coli* specific PCR targeting the *uspA* gene (Chen and Griffiths, 1998) was performed to confirm identification. All *E. coli* isolates were stored at -80 °C in brain heart infusion (BHI) broth (Oxoid) containing 20% glycerol (Promega, Corporate Park, Sydney, Australia) until further analysis. The extracted DNA (100 µl) was stored at -20 °C for further analysis.

3.4.2.3 DNA extraction from the cloacal and faecal samples

On arrival at the laboratory, each faecal sample was vortexed by adding 1 mL RNA/DNA free water and then six swabs were taken from this homogenate. Three cloacal swabs and the six faecal swabs from each chicken were stored at 4 °C for DNA extraction the next day. All samples were processed within 24 hours of collection. Three different extraction methods were evaluated: 1) QIAamp DNA Stool Mini Kit (Qiagen Pty Ltd, Chadstone, Victoria, Australia); 2) the Chelex DNA extraction protocol and 3) the repeated bead beating plus column (RBB+C) as described below. To test

reproducibility each method was performed using two faecal swabs from each chicken for each DNA extraction method. All extracted DNA was stored at -20 °C for further analysis.

3.4.2.3.1 Method one (QIAamp DNA Stool Mini Kit)

The QIAamp DNA Stool Mini Kit was used according to the manufacturer's protocol with a minor modification; the DNA was eluted with 50 µl of AE buffer instead of 200 µl.

3.4.2.3.2 Method two (Chelex DNA extraction protocol)

Each sample was suspended in 5.0 mL phosphate-buffered saline (pH 7.2) and centrifuged at 100 x *g* for 15 min at 4 °C in order to remove the faecal pellets. The DNA was extracted from each sample using a modified Chelex method (Yang *et al.*, 2008). Briefly, the pellet was suspended in 1.5 mL acetone (Sigma-Aldrich, Castle Hill, New South Wales, Australia) and then centrifuged at 13,000 x *g* for 10 min in order to remove any potential PCR inhibitors. The supernatant was discarded and the pellet dissolved in 200 µL of Chelex-100 (6%) (Sigma-Aldrich) and 0.2 mg protease K (Sigma-Aldrich). The mixture was heated at 56 °C for 30 min, vortexed for 1 min and centrifuged at 10,000 x *g* for 5 min. Supernatant (100 µL) was stored.

3.4.2.3.3 Method three (repeated bead beating plus column (RBB+C)

The RBB+C method was performed as previously described (Yu and Morrison, 2004) with two minor modifications. In this study, the DNA pellets were dried in a biosafety cabinet after washing with 70% ethanol and the DNA was eluted with 50 µl of AE buffer instead of 200 µl as previously described.

3.4.2.3.4 Assessment of DNA extraction

DNA extraction was assessed on the basis of the following criteria: 1) DNA yields; 2) DNA purity and 3) the presence of low-level PCR inhibitors (based on the 16S rDNA PCR amplification results). In addition, the shearing of the DNA and the reproducibility of each method was examined. The quantity (referred to as yield) and quality (referred to as purity) of the extracted DNA was assessed using the Nano Drop ND-1000 Spectrophotometer (Thermo Fisher Scientific Australia Pty Ltd, Scoresby Victoria, Australia). The yield of the extracted DNA was calculated by the amount of light absorbed by 1 µl of the DNA at 260 nm (Desloire *et al.*, 2006) and the purity of DNA was determined by calculating the A260/A280 ratio. DNA was defined as pure if the 260/280 absorbance ratio ranged between 1.8 and 2.0. The shearing of the extracted DNA obtained from the three different methods

was evaluated by running 5 µl of extracted DNA on 1% agarose gel in 1% sodium borate buffer (SB) (Sigma-Aldrich) at 80 volts (V) for 30 min. The DNA fragment size was evaluated using λ DNA cut with HindIII as a DNA marker (Life Technologies Australia, Mulgrave, Victoria, Australia), stained with SYBR Safe (Invitrogen Australia Pty Limited, Mount Waverley, Victoria, Australia) and visualised using the GelDoc System (Bio-Rad Laboratories, Gladesville, New South Wales, Australia). The ability to detect the bacterial 16S rDNA from the extracted DNA, which reflects the presence or absence of PCR inhibitors in the extracted DNA, was evaluated using the 16S rDNA PCR (Sakamoto *et al.*, 2002). Amplified PCR products were electrophoresed on 1% agarose gel in 1% SB buffer at 80 V for 30 minutes stained and visualised using SYBR Safe and the GelDoc System.

3.4.2.4 Molecular detection of virulence genes

A pentaplex-PCR targeting five VGs (*iroN*, *iutA*, *iss*, *hlyF* and *ompT*) was performed as previously described (Johnson *et al.*, 2008b) on the DNA extracted from the cloacal and faecal swabs using the previously determined best extraction method (repeated bead beating plus column) and from the DNA extracted from *E. coli* that were cultured from the organs and cloacal swabs. *E. coli* STJ-1 (Fagan *et al.*, 1999) and *E. coli* ATCC 8739 were used as positive and negative controls, respectively.

3.4.2.5 Spiked faecal samples

The spiking experiments were performed to evaluate the sensitivity for each DNA extraction method. Two faecal samples were collected from two healthy commercial broiler chickens (A and B) and were spiked with six different ten-fold serial dilutions of *E. coli* (STJ-1) ranging between 1.6 × 10 and 1.6 × 10^6 colony forming units (CFU) per mL. One hundred microliters of each serial dilution was added to 20 mg of faeces from each bird, A and B, and the DNA was extracted from the six spiked samples per bird in triplicate using the three different extraction methods.

3.4.2.6 Definition of avian colibacillosis, APEC, cAPEC, pAPEC and AFEC

In the current study, avian colibacillosis was diagnosed based on the presence of clinical signs associated with colibacillosis, macroscopic lesions (assessed on a grading of zero to four) and the isolation of *E. coli* from the affected lesions. A numeric grading representing the severity of the disease was assigned according to the following scale: 0 = no colibacillosis; 1 = mild case of colibacillosis with lesions affecting a single organ; 2 = moderate case of colibacillosis with lesions covering two organs; 3 = moderate case of colibacillosis with lesions covering three organs; 4 = severe case of colibacillosis with lesions covering four or more organs (Antão *et al.*, 2008). For a bird

to be defined as having avian colibacillosis, the bird has to show some clinical signs associated with colibacillosis, a lesion score of 1 or more and had *E. coli* cultured from at least one lesion site.

Clinical avian pathogenic *E. coli* (cAPEC) was defined as an *E. coli* cultured from an organ of a chicken with colibacillosis that harboured four or more of the five APEC-associated VGs. APEC was defined as an *E. coli* isolate sourced from anywhere (except from an organ of a chicken with colibacillosis) that harboured four or more of the five APEC VG markers (*iroN*, *iutA*, *iss*, *hlyF* and *ompT*). Clinical *E. coli*, which contain less than four of the selected VGs are identified as potential APEC (pAPEC). On the other hand, avian faecal *E. coli* (AFEC) was defined as an *E. coli* isolate, cultured from the faeces of chicken, with less than four of the selected APEC VG markers (Johnson *et al.*, 2008b).

3.4.2.7 Statistical analysis

Analyses were performed with Stata software (13th edition, Blackburn North Victoria, Australia, www.stata.com). The mean, median and interquartile range of the yields (ng/µL) and purity for the extracted DNA for faecal and cloacal samples were calculated for the three DNA extraction methods. Normality of yield and purity were examined using histograms and data transformations were performed when necessary. The mean of the normalised DNA yield and purity of the faecal sample was compared to the mean of the duplicate faecal sample for the subgroups (healthy and unhealthy chickens) of the three different DNA extraction methods using t-tests. If the overall mean of the faecal subgroup did not differ from the subgroup duplicate at $p < 0.05$, then the mean of the DNA yield and purity for both faecal samples for each individual chicken was created and used for future analysis. Yield and purity were compared between the three different DNA extraction methods in two separate models using the General Estimation Equation (GEE) procedure (Twisk, 2013). GEE models were chosen to account for clustering of repeated observations within chickens and a negative binomial distribution with a log link function was used in these models. The health status of the chickens (healthy vs unhealthy as previously described) and the interactions between the health status of the chickens and the three types of DNA extractions were added as fixed effects.

Exchangeable correlation structures were used for all analyses. Standard errors were estimated using 'robust' Huber/white/sandwich estimators of variance. The Chelex method was used as the reference category. Data analysis was conducted stratified for the faecal and cloacal samples.

It was also assumed that the purity value of the extracted DNA alone might not be sufficient to characterise the ability of the extraction methods to produce PCR quality DNA. Therefore, a new variable was created ('amplified-PCR') which was a combination of the measured purity and the presence or absence of PCR inhibitors. DNA purity was dichotomized with '1' being assigned for purity measurements between 1.8 and 2.1 and '0' for lower or higher values. Similarly, if the PCR was able to amplify DNA (indicating no PCR inhibitors were present) the PCR test result was coded as '1' and '0' if the DNA failed to amplify (indicating the present of PCR inhibitors). Thus, four combinations of dichotomized purity values and dichotomized PCR inhibitor results indicating the quality of the extracted DNA were derived 1): Amplified-PCR = 1 if both the DNA ratio and the PCR were one; amplified-PCR = 2 if DNA ratio was 0 and the PCR was 1; amplified-PCR = 3 if DNA ratio was 1 and the PCR was 0; amplified-PCR = 4 if the DNA ratio as well as the PCR were 0.

The frequency of the cross-tabulated dichotomized purity and dichotomized PCR inhibitor results for three DNA extraction methods conducted on cloacal and faecal samples collected from APEC infected chickens were compared using the Fisher's exact test.

The total number of VGs from *E. coli* isolates cultured from the cloaca of healthy birds versus birds with colibacillosis (three *E. coli* isolates were selected from each bird) was compared using GEE models to account for clustering of repeated observations within chickens. A Poisson distribution with a log link function was used in this model. Furthermore, to explore if VGs were more common in *E. coli* cultured from organs than from cloacal swabs in chickens with colibacillosis, the total number of VGs detected was compared between *E. coli* cultured from organ swabs (any organ coded as 1) and cloacal swabs (coded as 2) using also a GEE Poisson models.

3.5 Results

3.5.1 Pathology and bacteriology

Of the 11 selected healthy chickens, one chicken (chicken 7) displayed multifocal necrotic lesions on the liver suggestive of necrotising hepatitis and the subcutaneous tissue over the thigh and breast were red and swollen, consistent with cellulitis. As a result of this finding, chicken 7 was reclassified as unhealthy after post-mortem leaving ten healthy chickens. All other healthy chickens displayed no signs of disease. Histopathology on the organs of one healthy chicken confirmed the absence of disease. No macroscopic lesions were observed in any organ of the ten healthy chickens and no

microscopic lesions were detected in the tissues of the one healthy chicken which had histopathology performed. *E. coli* was not cultured from any organs of healthy chickens.

Of the 13 unhealthy chickens (12 selected and chicken 7), four showed lesions consistent with non *E. coli* related systemic infections. Chickens 6, 7 and 16 showed signs of a systemic infection, based on multifocal necrotic lesions on the liver and spleen consistent with staphylococcosis (Nolan *et al.*, 2013). Histopathology of the livers and the lungs of these chickens displayed a marked multifocal granulomatous pneumonia with fibrosis. *Staphylococcus aureus* was isolated from the livers and lungs of the three chickens. Chicken 9 displayed yellow nodules in the air sacs that were consistent with Aspergillus (Nolan *et al.*, 2013; Whiteman *et al.*, 1979). Histopathology of the lung displayed a marked multifocal granulomatous pneumonia with fibrosis and *Aspergillus fumigatus* was cultured from the air sac and the lung.

The remaining nine of 13 unhealthy chickens presented with lesions, which were consistent with colibacillosis (Figure 3.1). All of the nine chickens had lung and/or air sac macroscopic lesions with a lesion score ranging between one and four. Cellulitis and macroscopic lesions of varying severity were detected on the other internal organs of all of the nine chickens. Three chickens (2, 5 and 10) had a thick and extensive layer of fibrinous exudate (heterophilic and fibrinous peritonitis) covering all five organs and signs of severe sepsis or systemic infection; consistent with a colibacillosis grade of 4. Three chickens (23, 22 and 21) displayed a thin layer of fibrin on one or more of the organs, colibacillosis grade 3, while three chickens (1, 4 and 11) showed one or two small pin sized lesions per lung and air sac (colibacillosis grade 1 or 2). Histopathology of three chickens affected with colibacillosis (5, 23, and 11) displayed pathophysiological alterations of the lung, liver, heart and spleen tissues, which is characteristic of systematic infection. The anaerobic and/or microaerophilic growth conditions did not yield any bacterial growth.

Seventy four *E. coli* (68 lactose positive isolates from liver, lung, air sac, spleen and heart of eight chickens and six lactose negative isolates from the liver and spleen of chicken number 12) were obtained and identified, by biochemical and molecular methods, from all five organs from the nine chickens clinically diagnosed with colibacillosis.

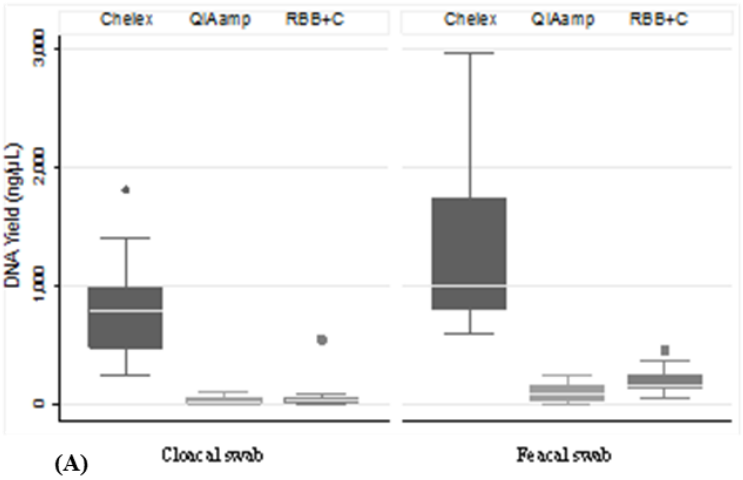
Sixty nine *E. coli* were isolated and identified, by biochemical and molecular methods, from all of the cultured cloacal swabs: 27 *E. coli* isolates were isolated from the cloacal swabs of chickens with colibacillosis (n = 9); 30 *E. coli* isolates were collected from the cloacal swabs of healthy chickens (n

= 10) and 12 *E. coli* isolates from the cloacal swab of chickens with staphylococcosis (n = 3) and aspergillosis (n = 1).

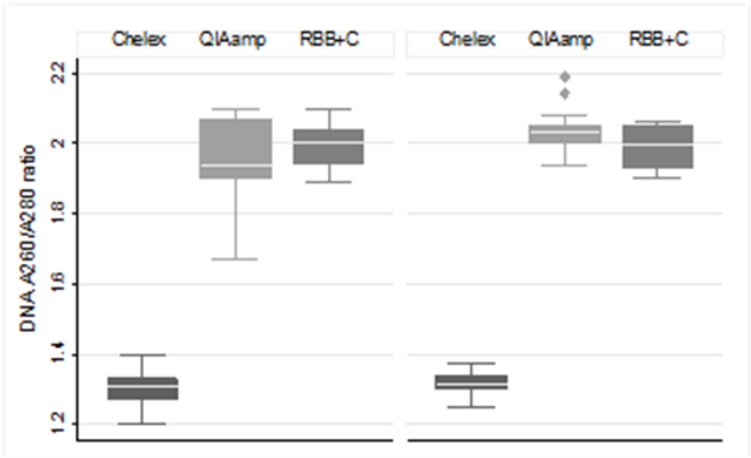
3.5.2 DNA extraction

3.5.2.1 Quantity of the extracted DNA

Overall, the Chelex method produced the highest DNA yields from the cloacal swabs with median DNA concentration of 802 ng/µL (interquartile range [IQR] 249 - 1811), followed by 36 ng/µL (IQR 3.4 - 548) for the RBB+C methods and 15 ng/µL (IQR 1.4 - 112) for the QIAamp DNA Stool Mini Kit. The faecal swabs yielded a comparatively higher DNA quantity in comparison to the cloacal swabs. Nonetheless, the Chelex method again yielded the highest concentration of DNA from the faecal swab with median of 997 ng/µL (IQR 603 - 2963) followed by 171 ng/µL (IQR 72 - 458) for the RBB+C and 90 ng/µL (IQR 7 - 256) for the QIAamp DNA Stool Mini Kit (Figure 3.2).



(A)



(B)

Figure 3.1: Comparison of the three extraction methods: Chelex; QIAamp and RBB+C for DNA extracted from the cloacal swabs (n = 23) and on the mean of duplicate faecal samples (n = 23). (A) Compares the DNA yield (ng/µL). (B) Compares the quality of the DNA extracted (260/280 ratio).

The GEE models of log-transformed DNA yield for comparing the three DNA extraction methods indicated that RBB+C and QIAamp DNA Stool Mini Kit produced lower DNA yield values than the Chelex in both cloacal samples (n observations = 69, n groups = 23, Wald $\chi 2 = 149.21$, d.f. = 2, $p < 0.001$) and faecal samples (n observations = 69, n groups = 23, Wald $\chi 2 = 138.46$, d.f. = 2, $p < 0.001$). Health status and interaction between health status and type of diagnostic test was not associated with the log-transformed DNA yield. The final GEE model results for log2-transformed DNA yield are shown in Table 3.1

Table 3.1: Results from General Estimation Equation Models of log-transformed DNA yield derived from three DNA extraction methods on individual cloacal swabs (n = 23) and on the mean of duplicate faecal samples (n = 23) collected from healthy and unhealthy chickens. The Chelex method was used as the reference group.

Type of sample	DNA extraction method	Ratio of mean yield	95% Confidence Interval	*p*-value
Cloacal	Chelex	Reference group		
	QIAamp	0.037	0.021, 0.063	< 0.001
	RBB+C	0.074	0.034, 0.161	< 0.001
Faecal	Chelex	Reference group		
	QIAamp	0.075	0.048, 0.116	< 0.001
	RBB+C	0.155	0.111, 0.217	< 0.001

3.5.3 Quality of the extracted DNA

In regards to the DNA purity, the RBB+C achieved a similar A260/A280 ratio to the QIAamp DNA Stool Mini Kit extraction with the median of 2 (IQR 1.9 - 2.1) and 1.94 (IQR 1.67 - 2.1) respectively, for faecal swabs and median of 1.99 (IQR 1.9 - 2.06) and 2.03 (IQR 1.94 - 2.19) respectively, for the

cloacal swabs. The Chelex method gave the lowest DNA ratio with median of 1.3 (IQR 1.2 - 1.4) and 1.25 (IQR 1.25 - 1.37) for faecal and cloacal swabs, respectively. The GEE models of log-transformed DNA purity for comparing the three DNA extraction methods indicated that RBB+C and QIAamp DNA Stool Mini Kit produced higher DNA purity values than the Chelex in both cloacal samples (n observations = 69, n groups = 23, Wald $\chi 2$ =1596.87 d.f. = 2, $p < 0.001$) and faecal samples (n observations = 69, n groups = 23, Wald $\chi 2$ = 3435.27, d.f. = 2, $p < 0.001$). Health status and interaction between health status and type of diagnostic test was not associated with the log-transformed DNA purity. The final GEE model results for log2-transformed DNA purity are shown in Table 3.2.

Table 3.2: Results from General Estimation Equation Models of log-transformed DNA purity derived from three DNA extraction methods on individual cloacal swabs (n = 23) and on the mean of duplicate faecal samples (n = 23) collected from healthy and unhealthy chickens. The Chelex method was used as the reference group.

Type of sample	DNA extraction method	Ratio of mean purity	95% Confidence Interval	*p*-value
Cloacal	Chelex	Reference group		
	QIAamp	1.503	1.459, 1.549	< 0.001
	RBB+C	1.521	1.490, 1.554	< 0.001
Faecal	Chelex	Reference group		
	QIAamp	1.543	1.518, 1.569	< 0.001
	RBB+C	1.509	1.487, 1.531	< 0.001

3.5.4 Combination of DNA purity and PCR inhibitor results

The summary of the DNA purity and PCR inhibitor results for the cloacal and faecal swabs obtained from healthy and unhealthy chickens is shown in Table 3.3. There was a significant difference ($p < 0.001$) between dichotomized purity and dichotomized PCR inhibitor results between the three DNA extraction methods for both, cloacal and faecal samples. The RBB+C produced the highest quality DNA and DNA amplification followed by the QIAamp DNA Stool Mini Kit that yielded a similar quality to the RBB+C, however, did not amplify as much DNA. Conversely, the Chelex method produced low ratio DNA that could not be amplified (except for 3 cloacal and 3 duplicate faecal samples).

Table 3.3: Cross-tabulation of dichotomized purity and dichotomized PCR inhibitor results for three DNA extraction methods conducted on individual cloacal swabs (n = 23) and on the mean of duplicate faecal samples (n = 23) collected from healthy and unhealthy chickens.

Type of sample	DNA extraction method	DNA purity[1] = 0		DNA purity[1] = 1	
		PCR[2] = 0	PCR[2] = 1	PCR[2] = 0	PCR[2] = 1
Cloacal	Chelex	20	3	0	0
	QIAamp	1	0	17	5
	RBB+C	0	0	2	21
	Total	21	3	19	26
Faecal	Chelex	20	3	0	0
	QIAamp	0	1	8	14
	RBB+C	0	0	2	21
	Total	20	4	10	35

[1]DNA purity was 1 for purity measurements between A260/A280 absorbance ratio 1.8 and 2.1 and 0 for lower or higher values.

[2]The PCR test result was coded as 1 if the PCR was able to amplify DNA and 0 if the DNA failed to amplify.

The overall integrity of the extracted DNA using the three different methods was of high quality as no shearing was observed on gel electrophoresis. Amplification of the 16S rRNA gene varied between the three different extraction methods utilised. Successful amplification of the 16S rRNA gene was achieved in 91% (63 of 69) of the DNA extracted using the RBB+C, 51% (35 of 69) of the DNA extracted using the QIAamp DNA Stool Mini Kit and 13% (9 of 69) of the DNA extracted using the Chelex method. The three DNA extraction methods yielded reproducible results in regards to the DNA yields, DNA ratio and amplification of the PCR products.

In regards to the spiking experiment, the PCR on the DNA extracted from faeces with the RBB+C was able to amplify all five APEC-associated VGs from all six different spiked dilutions (10^1 to 10^6 CFU/mL). While the DNA extracted using the QIAamp DNA Stool Mini Kit amplified the five APEC-associated VGs from only the two highest spike concentrations. While the DNA extracted by the Chelex method failed to amplify any of the five APEC-associated VGs from DNA extracted from all six dilutions.

3.5.4.1 Molecular detection of APEC virulence genes

All five selected VGs were detected in 100% (63 of 63) of the DNA extracted directly from the cloacal and faecal samples from both healthy chickens and unhealthy chickens using the RBB+C. However, PCR on DNA from *E. coli* cultured from the cloacal swabs (n = 69) failed to detect the same VGs from the same chicken (healthy and unhealthy). The prevalence of the VGs identified from *E. coli* isolates cultured from the cloacal swabs of chickens affected with colibacillosis was statistically significantly higher in comparison to *E. coli* cultured from the cloacal swabs of healthy chickens as shown in Figure 3.3 (GEE model: n observations = 378, n groups = 21, Wald $\chi 2 = 58.78$, d.f. = 1, $p < 0.001$).

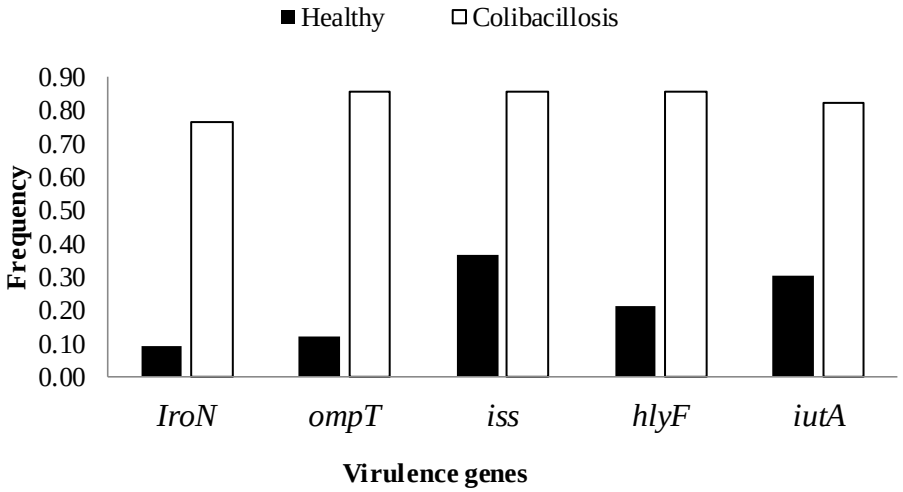


Figure 3.2: The frequency of virulence genes in *E. coli* cultured from the cloacal swabs of ten healthy chickens (n = 30) and nine chickens with colibacillosis (n = 27).

The frequency of the VGs in the 27 *E. coli* isolates that were cultured from the cloacal swabs of nine chickens with colibacillosis were 85% for *iss, hlyF* and *ompT,* 82% for *iutA* and 76% for *iroN.* The frequency of the VGs was lower in the 30 *E. coli* isolates cultured from the cloacal swabs of ten healthy chickens; *iss* (36%) was detected most frequently, followed by *iutA* (30%), *hlyF* (21%), *ompT* (12%) and *iroN* (9%).

Among birds with colibacillosis, significantly more VGs were detected from *E. coli* cultured from organs than *E. coli* cultured from cloacal swabs (GEE model: n observations = 182, n groups = 11,

Wald $\chi 2 = 25.27$, d.f. = 1, $p < 0.001$). PCR data showed that 100% (74 of 74) of the *E. coli* isolates that were cultured from the lesions of chickens affected with colibacillosis harboured all five VGs. Seventy percent (19 of 27) of the *E. coli* that were cultured from the cloacal swabs of chicken affected with colibacillosis harboured all five VGs.

Furthermore, all of the *E. coli* isolates that were cultured from the organs (n = 74) of chickens affected with colibacillosis (n = 9) were classified as cAPEC. While, 85% of the cloacal swabs (23 of 27) of chickens with colibacillosis were classified as pAPEC. However, only 17% of the *E. coli* (5 of 30) isolates cultured from cloacal swabs of apparently healthy chickens were classified as APEC.

All the *E. coli* isolates (n = 9) that were cultured from the organs and the cloacal swabs of chickens with aspergillosis (n = 1) were classified as APEC. None of the *E. coli* isolates (n = 9) from the cloacal swabs of clinically unhealthy chickens with staphylococcosis (n = 3) were classified as APEC.

3.6 Discussion

Currently, the diagnosis of avian colibacillosis relies on clinical signs, typical macroscopic lesions and culture of *E. coli* from the affected tissues (Nolan *et al.*, 2013). Traditional bacterial culture can be labour intensive, expensive and time consuming. While multiple VGs have been defined as useful molecular markers for the detection and characterisation of APEC from cultured *E. coli* obtained from chickens diagnosed with avian colibacillosis (Johnson *et al.*, 2008b) the direct application of molecular techniques to faecal samples has been hampered by the inability to extract high quality DNA (Henderson *et al.*, 2013). There are several methods and commercial kits specifically designed for DNA extraction from faecal samples. However, to be able to obtain repeatable, accurate PCR results, careful consideration must be given to the methodology (McOrist *et al.*, 2002) to ensure adequate yield concentrations are achieved, shearing of the DNA is minimised and the presence of impurities, either from the faeces or the extraction process, is negligible.

The presence of PCR inhibitors in faecal samples is well documented and variation in the composition and consistency of faecal samples from different animal species may affect the quality of extracted DNA (Abu Al-Soud and Radstrom, 1998; Abu Al-Soud and Radstrom, 2000; Gioffre *et al.*, 2004). Therefore, it is necessary to conduct species-specific testing of all extraction protocols. Chicken faeces are low in moisture in comparison with other animals (Akhtar *et al.*, 2013) which leads to difficulty in dissolving the faeces in a buffer (Barnard *et al.*, 2011) and chickens may also have additional inhibitors in comparison to other mammalian faeces as they excrete urinary waste in the

faeces (i.e. common cloaca) (Chambers *et al.*, 2001). Therefore, it is important to test detection methods specifically for chickens, rather than rely on extrapolation from other species. This is the first study to report on such findings.

This study compared three DNA extraction methods directly in order to detect the most effective and practical method(s) to extract a high quantity and quality of DNA from the cloacal and faecal swabs of broiler chickens; a) QIAamp DNA Stool Mini Kit, b) repeated bead beating plus column method (RBB+C) and c) Chelex method. Selection of these methods was based on their popularity of use by the veterinary laboratory and/or the poultry industry and ease of use (Garofalo *et al.*, 2007). There was a positive correlation between the purity and quality of DNA achieved during extraction and the subsequent suitability of the extracted DNA as template for PCR amplification. The RBB+C method was determined to be the best method to extract an adequate yield of PCR quality DNA from the cloacal and faecal samples. The high quality DNA obtained using the RBB+C has been well documented in previous studies where DNA was extracted from the rumen digesta and faecal samples of cattle (Henderson *et al.*, 2013; Yu and Morrison, 2004). The enhanced performance of the RBB+C on chicken faecal extractions, in comparison with the other two methods, may be due to the two additional purification steps, which may minimise the presence of PCR inhibitors, in conjunction with a reduced final elution volume, which may maximise the DNA concentration. The increased sensitivity could also been seen in the spiking experiment, where DNA extracted by the RBB+C yielded positive results for all dilutions tested compared to the other two methods which yielded positive results for two of the six dilutions at best. However, the RBB+C was more laborious and time consuming method in comparison with QIAamp DNA Stool Mini Kit and the Chelex. The cost of the RBB+C was another disadvantage, as it was the most expensive method followed by the QIAamp DNA Stool Mini Kit, while the Chelex method was the most economical.

The QIAamp DNA Stool Mini Kit method also produced pure DNA with similar A260/280 ratio to the RBB+C, in accordance with previous studies (Gioffre *et al.*, 2004; Monteiro *et al.*, 1997). However, amplification of the 16S rDNA failed in 49% of the extracted DNA samples. Monteiro *et al.* (1997) also reported a similar limitation and concluded this was due to the QIAamp DNA Stool Mini Kit eliminating some but not all PCR inhibitors. However, other DNA extraction studies suggest that the use of the QIAamp DNA Stool Mini Kit (in human, cattle and horse faeces) can reduce the presence of PCR inhibitors by 98% in the extracted DNA (Verweij *et al.*, 2004; Verweij *et al.*, 2007).

While the Chelex method gave a superior DNA yield, as also reported by others (Miller *et al.*, 1999; Yang *et al.*, 2008), the quality of DNA produced in this study was low with an A260/280 ratio range between 1.2 - 1.4 and an 87% failure of this DNA to produce PCR product using the 16S rDNA PCR. The limitations of the Chelex method have been observed previously in several other species (Desloire *et al.*, 2006; Hill and Gutierrez, 2003). However, Yang *et al.* (2008) reported that the Chelex method extracted a relatively pure DNA, free from contamination (with absorbance ratio A260/280 of 1.80 - 2.00) from 10-day-old healthy gosling faecal samples.

This variation in the result obtained by the current study and that of Yang *et al.* (2008) could be due to the fact that the faeces of geese contain more water, as geese consume large amounts of water with their food (Janet Kear, 1963). The increased water content of the faeces mean less faecal inhibitors are present, which may result in better quality extracted DNA as seen by the higher quality DNA obtained by Yang *et al.* (2008) in comparison to the poor quality DNA obtained in the current study from the chicken faecal samples.

DNA yields did differ between the cloacal swabs and faecal samples. This could be attributed to the quantity of original sample, as in some cases the cloacal swabs contained only small quantities of faeces. Other studies have also reported a correlation between the amount of the faecal material and DNA yield (Ariefdjohan *et al.*, 2010; Zhang *et al.*, 2006).

Previous studies have identified an association between the prevalence of different combinations of VGs and the pathogenicity of APEC (da Rocha *et al.*, 2002; Ewers *et al.*, 2004; Johnson *et al.*, 2008b; Rocha *et al.*, 2008). Further, they have reported that certain VG combinations are a useful tool to differentiate between APEC and AFEC (Johnson *et al.*, 2008b; Rodriguez-Siek *et al.*, 2005a; Schouler *et al.*, 2012) when the *E. coli* was isolated from the lesions or the cloacal swabs of chickens affected with colibacillosis. In the present study, a PCR was used to screen *E. coli* isolates (obtained from cloaca and organs) and DNA directly extracted from faeces and the cloaca for the presence of five APEC-associated VGs (Johnson *et al.*, 2008b). All five VGs were detected from *E. coli* cultured from both healthy and unhealthy chickens when the pentaplex-PCR was applied to the DNA extracted directly from faecal and/or cloacal samples. However, in contrast, the VGs occurred in lower frequency in the *E. coli* cultured from healthy chickens. As well, the PCR of *E. coli* cultured from cloacal swabs of the same chickens failed to detect the same VGs as those detected in the direct PCR examination of the cloacal swabs. These VGs can be possessed by other bacterial species that are

often found in the intestines of chickens, suggesting that these VGs may not necessarily only be associated with APEC when identified from a faecal sample.

In summary, the presence of the five VGs in all birds highlights the need to improve biosecurity practices, such as restriction of the movement and entry of farm visitors as well as workers to assist in minimising the spread and transmission of APEC between and among farms. Furthermore, early detection of these genes (which may indicate the presence of APEC) using the pentaplex PCR highlights the need to evaluate the management policies priority in order to minimise the presence of these genes and associated bacteria in the sheds. The reduction of these bacteria will help to reduce the risk of inhaling dust contaminated with APEC and therefore, reduce respiratory disease that is often followed by systematic infections characterised by poor flock performance and high mortality.

Data obtained from this study suggests that the presence of these VGs was more likely associated with *E. coli* isolates cultured from lesions in the organs of birds with colibacillosis. This finding agrees with previous studies where they also found associations between the presence of these five VGs and *E. coli* isolates cultured from lesions in the organs of birds affected with colibacillosis (Circella et al., 2012; Rodriguez-Siek et al., 2005a).

All chickens with colibacillosis in this study were assigned a macroscopic as well as a microscopic lesion (for three chickens) score of three and above in the lung and/or air sac and had lesions (macroscopic and microscopic) in one or more of the following organs: liver; spleen and heart (scores of three and above). An association has been found between the presence of four or more of the selected APEC VGs in all of the *E. coli* isolates that were cultured from organs (n = 74) of chicken with colibacillosis. Similarly, Ask *et al*. (2006) reported a direct correlation between the colibacillosis lesions and the occurrence of similar VGs. Of the 74 *E. coli* isolates cultured from organs, 8% (n = 6; isolates from liver and spleen of chicken 12) were lactose negative and harboured all of the five selected VGs. The occurrence of lactose negative APEC has been previously reported by Rodriguez-Siek *et.al* (2005a).

With the exception of chicken 7 (which was diagnosed with staphylococcus), all chickens that were identified by the farmers as healthy had no macroscopic or microscopic lesions on the internal organs and all organs were sterile when cultured.

The main limitation of this study was the small sample size of commercial broiler chickens. However, the fact that this is an exploratory study that aims to assist with the future thesis experiments justifies

the sample size, furthermore, three farms were selected to be sampled to increase the bird variation coming from different management and health backgrounds. Another limitation of this study is that while the faecal samples were processed in duplicate, only one cloacal sample was processed. This was due to the small amount of sample obtained from the cloacal swabs in some birds. However, our sample size was large enough to see a statistical difference.

3.7 Conclusion

Avian pathogenic *E. coli* is a significant disease for the poultry industry and is potentially of public health concern. This study aimed to identify a method to detect APEC directly from the faeces of chickens, which could be used as a rapid diagnostic test. The repeated bead beating plus column method was the preferred DNA extraction method, as it yielded adequate PCR quality and quantity DNA directly from the faecal material of chickens. However, identifying APEC directly, by detecting the five selected VGs (*iroN, iutA, iss, hlyF* and *ompT*) from the faecal material was not feasible because these five VGs that were most commonly found among *E. coli* from birds with colibacillosis were also detected in *E. coli* from healthy birds and are possibly associated with other bacterial species present in the digestive tract of broiler chickens. Therefore, although the RBB+C method is adequate and this study can recommend it for DNA extraction from poultry faeces and/or cloacal swabs, other genetic markers will need to be investigated to identify APEC directly from faecal material.

.

Chapter 4: Risk factors associated with the carriage of pathogenic *Escherichia coli* from healthy commercial broiler chickens in South East Queensland, Australia

4.1 Foreword

Avian pathogenic *Escherichia coli* (APEC) have been identified according to the presence of a specific array of APEC-associated virulence genes (VGs). As discussed previously in this thesis , APEC was defined based on the presence of four or more of five selected APEC-associated VGs: haemolysin gene (*hlyF*); increased serum survival gene (*iss*); outer membrane protease gene (*ompT*) and two iron acquisition system genes (*iutA* and *iroN*). Avian faecal *E. coli* (AFEC) was defined as an *E. coli* isolate cultured from a faecal sample from a healthy chicken that harboured less than four of the VGs. In Chapter 3, the five VGs were used to screen DNA extracted directly from faeces to identify APEC. However, the five VGs were detected in faecal DNA from both healthy chickens and chickens with colibacillosis, and it was concluded that these VGs might have originated from other bacteria and not only from APEC. Hence, in further studies, including this one, faeces were cultured and APEC status based on screening for VGs among selected *E. coli* isolates rather than use DNA extracted from faecal or cloacal samples.

The primary selection of four or more VGs to identify APEC was validated by the results obtained by the pilot study (Chapter 3). That study showed that 85% of the 27 *E. coli* isolates cultured from the faeces of nine chicken with colibacillosis harboured four or more VGs, while only 17% of isolates cultured from the faeces of healthy birds harboured four or more. The low level of APEC in healthy chickens also suggested their role as a reservoir for APEC-associated VGs and potentially colibacillosis in Australia. The occurrence and severity of avian colibacillosis depends on the pathogenicity of the APEC, the immune status of the chickens and the presence of predisposing risk factors. However, little knowledge is available in regards to APEC prevalence and the potential predisposing risk factors (biosecurity and farm management protocols and environmental factors) associated with APEC among commercial broiler chicken flocks in Australia.

Therefore, the aims of the current study were to estimate APEC prevalence and to determine the possible risk factors associated with intestinal/cloacal carriage of APEC among commercial healthy broiler chickens in South East Queensland.

4.2 Abstract

Avian pathogenic *E. coli* (APEC) is the aetiological agent of avian colibacillosis, an acute extraintestinal disease of poultry. Avian colibacillosis is an economically significant disease globally with losses due to the high costs associated with the preventions and treatments and increased mortality and morbidity. Little is known about APEC in the context of Australian conditions. Accordingly, the current study aims to estimate the APEC prevalence and to determine the risk factors associated with the intestinal/cloacal carriage of APEC among commercial healthy broiler chickens in South East Queensland (SEQ).

In the current study, four hundred cloacal swabs were collected at slaughter from 400 healthy broiler chickens originating from 40 poultry farms in SEQ. A total of ten birds per farm were sampled. Swabs were cultured and 2,200 *E. coli* isolates were selected and screened for the presence of five APEC-associated virulence genes (VGs) by PCR. Data on potential within-farm risk factors associated with APEC prevalence were collected using a questionnaire that was completed by the managers and/or owners of the broiler farms. The questionnaire sought information in regards to farm management practices, biosecurity protocols and chicken health. For the purposes of this study, APEC was defined as an *E. coli* isolate that was cultured from the intestinal/cloacal swab and harboured four or more of the selected five APEC related VGs (*iutA, iss, ompT, hlyF* and *iroN*). At the bird-level, a chicken was considered to be APEC positive if it carried at least one *E. coli* isolate, which harboured four or more of the APEC-associated VGs. A farm was considered as APEC positive if at least one bird was APEC positive. Binominal general linear models were used to identify farm-level risk factors associated with bird-level APEC prevalence within individual broiler farm.

Thirty four percent of the cultured *E. coli* isolates (n = 751) were classified as APEC. All of the tested farms (n = 40) were APEC positive, with the overall bird-level prevalence of APEC being 63.0% (95% Confidence Interval (CI): 55.8, 70.2).

Higher APEC within-farm bird-level prevalence was positively associated with the usage of well water as a source of drinking water (Odd Ratio (OR) = 6.2, 95% CI: 2.3, 16.5, $p < 0.001$); not having shower facilities available for farm visitors (OR = 3.6, 95% CI: 1.8, 7.1, $p < 0.001$), distances greater than 20 m between the car park and the poultry shed (OR = 2.2, 95% CI: 1.4, 3.4, $p = 0.001$), not applying water line disinfections after each flock cycle (OR = 2.2, 95% CI: 1.4, 3.5, $p = 0.001$) and the presence of wild birds within 50 m of the poultry shed (OR = 2.3, 95% CI: 1.4, 3.7, $p = 0.001$). The use of chlorine combined with automatic drinking water filtration reduced within-farm bird-level APEC prevalence (OR = 0.07, 95% CI: 0.02, 0.34, $p = 0.001$). This study identified a number of

important potential risk factors for APEC carriage that need to be addressed by the poultry industry. The surprisingly high APEC prevalence on all farms requires further epidemiological investigations.

4.3 Introduction

Avian colibacillosis is considered one of the most common diseases to affect the poultry industry, and results in significant economic losses and increased welfare concerns (Collingwood *et al.*, 2014; Dho-Moulin and Fairbrother, 1999; Ewers *et al.*, 2004; Guabiraba and Schouler, 2015; Johnson and Russo, 2002; Nolan *et al.*, 2013). Despite all of the improvements and modernisation in poultry production systems and the fact that avian pathogenic *Escherichia coli* (APEC) has been a recognised cause of avian colibacillosis for over a century (Collingwood *et al.*, 2014; Dho-Moulin and Fairbrother, 1999; Johnson and Russo, 2002; Nolan *et al.*, 2013), it remains as one of the major pathogens posing a considerable challenge to the global poultry industry (Collingwood *et al.*, 2014; Dho-Moulin and Fairbrother, 1999; Johnson and Russo, 2002; Nolan *et al.*, 2013). The genetic overlap between APEC and other extraintestinal pathogenic *E. coli* (ExPEC) that cause neonatal meningitis, septicaemia and urinary tract infections in humans suggests that APEC is a zoonotic pathogen (de Pace *et al.*, 2010; Ewers *et al.*, 2009; Ewers *et al.*, 2007; Johnson and Russo, 2002; Johnson *et al.*, 2008b; Manges and Johnson, 2012; Tivendale *et al.*, 2010; Zhao *et al.*, 2009).

Avian pathogenic *E. coli* can act as a primary or secondary pathogen (Antão *et al.*, 2008; Bauchart *et al.*, 2010; Kemmett *et al.*, 2013; Kohler and Dobrindt, 2011; Maluta *et al.*, 2014) and can result in localised and/or systemic infections in birds (Guabiraba and Schouler, 2015; Nolan *et al.*, 2013; Rodriguez-Siek *et al.*, 2005a). The severity of the disease depends on the virulence of APEC strains, chicken age and immune status and the presence of predisposing risk factors (Dho-Moulin and Fairbrother, 1999; Guabiraba and Schouler, 2015; Vandekerchove *et al.*, 2004a).

Recent studies have indicated that various virulence genes (VGs) are useful markers for the definition of APEC and that they can be used to differentiate between APEC and avian faecal *E. coli* (AFEC) (Circella *et al.*, 2012; Ewers *et al.*, 2005; Johnson *et al.*, 2008b; Rodriguez-Siek *et al.*, 2005a). It is likely that the combinations of different APEC VGs are associated with APEC pathogenicity (Ewers *et al.*, 2005; Johnson *et al.*, 2008b; Kemmett *et al.*, 2013). Thus Johnson *et al.* (2008b) developed a PCR targeting five VGs, which have been used as a tool for the identification of APEC (de Oliveira *et al.*, 2015; Dissanayake *et al.*, 2014; Hussein *et al.*, 2013; Johnson *et al.*, 2008b; Kobayashi *et al.*, 2011).

Healthy chickens can harbour both APEC and avian faecal *E. coli* (AFEC) in their gut flora (Guabiraba and Schouler, 2015; McPeake *et al.*, 2005; Rodriguez-Siek *et al.*, 2005a). Hence, healthy chickens can act as a reservoir for the dissemination of APEC to other chickens, as well as their surrounding environment, potentially causing recurring infections in new flocks (Guabiraba and Schouler, 2015; Rodriguez-Siek *et al.*, 2005a; Schouler *et al.*, 2012).

Bird-level risk factors are likely to be associated with APEC infection. Kemmett *et al.* (2013) described an association between the prevalence of APEC and age of birds. The authors reported a higher APEC prevalence in the intestinal flora of one-day-old healthy chicks compared to adult chickens at slaughter-age. Contrarily, Kwon *et al.* (2008) found a higher APEC prevalence (31%) in layer birds (approximately 21 to 70 weeks of age), compared to slaughter-age broiler chickens (approximately up to12 weeks of age) (APEC prevalence 14%) in Korea. However, these differences might be related to farm management practices rather than age, as layer and broiler management are very different. Bird breed, and therefore genetic resistance, also differs between broiler and layer production systems. Hassan *et al.*(2004) showed that some breeds of chickens had lower mortalities, while others were highly susceptible to virulent infectious bursal disease virus and Newcastle disease virus.

A positive association between the build-up of the *E. coli* pathogen in the faeces, and birds' surrounding environment, and the increase in bird's susceptibility to APEC infection among broiler chickens has been found (Guabiraba and Schouler, 2015; Kabir, 2010). The main risk factors that were associated with bird's susceptibility were the duration of exposure, virulence of the APEC strain, breed and the immune status of the bird (Kabir, 2010). There were positive associations between increased risk of avian colibacillosis and increased infection pressure in the environment (Nolan *et al.*, 2013). Such environmental pressures can result from unfavourable husbandry management, poor biosecurity protocols and seasonal changes (Gross and Siegel, 1997; Kabir, 2010; Leitner and Heller, 1992; Nolan *et al.*, 2013).

Farm-level risk factors associated with a high prevalence of avian colibacillosis relate to poultry management practices (Nolan *et al.*, 2013; Vandekerchove *et al.*, 2004a; Wang *et al.*, 2013b). Impaired biosecurity protocols for example might result in the entry of APEC into the chicken sheds and can contribute to increased APEC prevalence (Kabir, 2010). On the other hand, good biosecurity practices, such as frequent carcass removal and the use of disinfectants for cleaning decreased the prevalence and the spread of avian colibacillosis (Vandekerchove *et al.*, 2004a).

The potential predisposing farm-level risk factors such as: biosecurity measures; farm management protocols and environmental factors are associated with APEC among commercial broiler flocks in Australia are unknown. Therefore, the aims of the current study were to: (i) determine the farm-level and bird-level prevalence of the intestinal/cloacal carriage of APEC and to (ii) investigate the possible associations between potential management risk factors and the within-farm APEC prevalence among healthy commercial broiler chicken flocks.

4.4 Materials and methods

4.4.1 Study design

A cross-sectional study was conducted to estimate APEC prevalence and identify farm-level risk factors associated with APEC prevalence.

4.4.2 Sample collection

The sample size was calculated based on an unknown prevalence of birds carrying APEC (thus prevalence was set to 0.5), a precision of 5% for the prevalence estimate, and a 95% confidence interval (CI) (Dohoo et al., 2009). A total of 400 samples were needed to yield a CI up to 0.10 wide if the observed prevalence was between 0.3 and 0.7. A total of 400 chickens from 40 commercial broiler farms were sampled at slaughter between October 2013 and July 2014 in South East Queensland (SEQ), Australia (Animal ethics approval number: SVS /323/13/ POULTRY CRC). The sampling focussed on a 2-stage prevalence study, with birds nested within farms; thus, we aimed to sample 40 farms with 10 birds to be sampled within a farm.

The broiler farms sampled belonged to the same commercial broiler company. The selected broiler company was willing to participate in this research project and provided access to their farm premises. The 40 commercial broiler farms were operating in different spatial locations.

The broiler company owned one slaughterhouse and chickens were sampled at that premise to reduce disturbance of on-farm production. The slaughterhouse processed up to 15 commercial broiler flocks from different farms within a day. The slaughterhouse was visited every Monday for eight consecutive weeks and birds from four to six farms were sampled at each visit. Sampling of birds from a particular farm was completed within a day – i.e. birds from the same farm were not sampled across days.

Chickens are submitted to slaughterhouses in flocks, with each flock representing a farm and flocks are clearly distinguished in the slaughterhouse (they were clearly labelled as different flocks/farms). Thus, birds from each farm were kept together in cages (up to 20 chickens per cage). From each cage, only one bird was selected and restrained whilst a cloacal swab (Sarstedt Australia Pty. Ltd., Technology Park, South Australia, Australia) was collected. In this way, a total of ten chickens from ten cages representing one broiler farm were sampled. The swabs were transported on ice to the laboratory at the School of Veterinary Science, The University of Queensland, within two hours of collection.

4.4.3 Collection of risk factor data

A questionnaire was developed to collect data on potential risk factors associated with intestinal/cloacal carriage of APEC in the sampled broiler chickens (Human ethics approval number: SVS /2014000327/ POULTRY CRC).

Questionnaire questions focussed on risk factors previously reported to be associated with APEC in commercial broiler chickens (Kabir, 2010; Nolan *et al.*, 2013; Vandekerchove *et al.*, 2004a). The surveyed included questions on the number of sheds and chickens kept on the farm, the location of the farm in relation to other livestock farms, and on specific management practices and biosecurity measures on the farm and general flock health. Management risk factors evaluated included: the age structure of broiler flocks; restocking practices; sources of drinking water used; number of workers on the farm; type of visitors; reasons for visits and frequency of their visit(s) to the farm; the presence and the frequency of unwanted animals (rats, mice, wild birds, domestic animals such as dogs, cats, cattle or pigs, stray/feral animals, amphibians, reptiles, kangaroos, possums) inside or outside the chicken shed within a categorised distance (less than and equal to 50 m and more than 50 m) and the frequency of litter removal. Biosecurity measures assessed related to routine cleaning practices (bird disposal, shed and equipment cleaning), precautionary measures used by farm worker and/or visitors before and after entering chicken sheds, the frequency of using foot baths, use of protective clothing, use of showers and hand sanitisers, cleaning and disinfecting protocols used, distance between the broiler shed and the car park; and the frequency of cleaning and disinfection of transport vehicles' tyres and of equipment (feeder, drinker, ladder, fixing tools, etc.) before entering the farm, and ownership of livestock or pets by the farm manager and workers. Potential flock health data collected comprised information on previous colibacillosis infections and others diseases on the farm, mortality rates per shed, frequency of removing dead bird carcasses from the sheds, and on the administration of antimicrobial drugs.

The questionnaire was piloted in February 2014 in face-to-face interviews with three individual broiler farm managers and five questions were revised to increase their clarity. The final questionnaire contained 52 questions of binary, ordinal and open-ended responses (Supplementary Table 1).

The questionnaire, information on the background and purpose of the research study, a consent form, and a pre-addressed stamped envelope for posting the completed questionnaire to the researcher were mailed out to the farm manager of the broiler farms in April 2014. In June 2014, a reminder was posted and followed by a phone call to remind the farm managers to complete questionnaires. With managers of ten broiler farms, face-to-face or a phone interviews were conducted after receiving the completed questionnaires to clarify some responses.

4.4.4 Bacterial culture of samples, identification and DNA extraction

All samples were processed within 24 hours of sampling. Each cloacal swab was cultured onto Brilliance™ *E. coli* coliform selective agar (BECS; Oxoid, Thebarton, South Australia, Australia) (Wohlsen, 2011) and incubated aerobically overnight at 37 °C. From each agar plate, five or ten presumptive *E. coli* colonies (for each broiler farm, five colonies were selected from nine chickens and ten colonies from one chicken) were collected. If the selected isolate was not confirmed as *E. coli*, further isolates were selected from the BECS plate until 2,200 *E. coli* isolates were collected from the 40 farms.

Presumptive *E. coli* isolates were subcultured onto sheep blood agar (SBA; Oxoid) and incubated aerobically at 37 °C overnight. Isolates, which were indole positive and pyrrolidonyl arylamidase negative, were presumed to be *E. coli* and DNA was extracted (Abraham *et al.*, 2012). An *E. coli* specific PCR targeting the *uspA* gene (Chen and Griffiths, 1998) was performed to confirm *E. coli* identification. All *E. coli* isolates were stored at -80 °C in brain heart infusion (BHI) broth (Oxoid) containing 20% glycerol until further analysis. The extracted DNA (100 µl) was stored at -20 °C for further analysis.

4.4.5 Molecular detection of virulence genes

All 2,200 *E. coli* isolates were screened for the presence of the five APEC related VGs (*iroN*, *iutA*, *iss*, *hlyF* and *ompT*) using the pentaplex-PCR. *Escherichia coli* STJ-1 (Fagan *et al.*, 1999) and *E. coli* ATCC 8739 were used as positive and negative controls, respectively. A total of 400 *E. coli* isolates were then selected, with each isolate representing one chicken. The *E. coli* selected to represent each bird was the *E. coli* cultured from that bird which contained the most VGs in order to increase the sensitivity of identifying APEC positive birds. If more than one *E. coli* isolate from the same chicken

carried the same number of VGs, random selection was applied using a random number generator by Excel Microsoft (Microsoft Corporation, Sydney, NSW, Australia, www.microsoft.com).

4.4.6 Case definition

For the purpose of this study, APEC was defined as an *E. coli* isolate that was cultured from the intestinal/cloacal swab and harboured four or more of the selected five APEC related VGs (*iutA, iss, ompT, hlyF* and *iroN*) (Johnson *et al.*, 2008b). AFEC was defined as an *E. coli* isolate that was cultured from the intestinal/cloacal swab of healthy chickens and harboured less than four of the selected five APEC related VGs.

At the bird-level, a chicken was considered APEC positive if at least one *E. coli* isolate of the five or ten cultured *E. coli* isolates from that bird harboured four or more of the selected APEC-associated VGs. The farm was considered as APEC positive if at least one bird was APEC positive.

4.4.7 Number of *E. coli* colonies that need to be screened to detect APEC VGs

To identify the number of *E. coli* colonies that needed to be screened to detect four or more of the selected five APEC related VGs, five colonies (obtained from nine chickens within a single farm) and ten colonies (obtained from one chicken within a single farm) were selected from Brilliance™ *E. coli* coliform selective agar (BECS; Oxoid). The *E. coli* colony selected to represent each bird was the *E. coli* colony, which contained the most number of VGs. This *E. coli* isolate (and the individual bird) was then classified as either APEC positive or negative. We used the Kappa statistic to evaluate the agreement in classifying an isolate APEC positive or negative when either five or ten colonies were selected from the agar plates(Landis and Kochi, 1977a; Landis and Kochi, 1977b).

4.4.8 Virulence gene prevalence in APEC positive and negative birds

The prevalence of the five VGs (with 95% Confidence Interval (CI)) among APEC positive and negative birds was summarised. As samples were clustered within broiler farms, survey estimation commands were used with farm being defined as the primary sampling unit (Rao and Cott, 1984; Rao and Scott, 1981). Hence the clustering was accounted for in the standard error and confidence interval estimation. The prevalence of each VG was compared between APEC positive and negative birds using the Pearson chi square statistic with Rao and Scott second order correction (Rao and Scott, 1981, 1984). Thus the Pearson chi square statistic was converted into a survey-design adjusted F statistic (Rao and Cott, 1984; Rao and Scott, 1981).

4.4.9 APEC farm-level, bird-level and within-farm prevalence

The farm-level prevalence was the proportion of farms that had at least one bird that was APEC positive. The farm-level prevalence was the proportion of farms that had at least one bird that was APEC positive. Overall, bird-level prevalence was calculated as the number of APEC positive birds out of the total number of birds (n = 400) sampled in this study. Within-farm prevalence was calculated as the number of APEC positive birds out of the ten birds sampled per farm.

4.4.10 Risk factors for APEC within-farm prevalence

Questionnaire data were entered into a Microsoft Access 2013 database (Microsoft Corporation) and examined for errors and missing values. Missing information or errors were discussed with farm managers over the phone and corrected. Data analyses were performed using Stata (13th edition, Blackburn North Victoria, Australia, www.stata.com).

A total of 120 individual risk factors were derived from the 52 questions in the questionnaire. Binominal general linear models with a log-link function were used to investigate the association of APEC within-farm prevalence and potential APEC farm-level risk factors (Barros and Hirakata, 2003; Skov *et al.*, 1998). The mean was the number of birds positive for APEC for the farms associated with the level of the risk factor. The risk factors with $p < 0.15$ in the univariate analysis were included in the multivariable model. For variables with more than two levels, the overall significance of the levels of the categorical variable was evaluated using the Wald test (Harrell, 2001). A forward stepwise model building strategy was used to develop the multivariable model. Models were compared using the Akaike Information Criterion (AIC) (Akaike, 1973; Akaike, 1974; Dohoo *et al.*, 2009). The analysis was continued by successively re-fitting models with explanatory variables that were not significant at $p < 0.15$ in the univariate analysis and applying the same rules for model comparisons until all remaining variables in the multivariable model were statistically significant at $p < 0.05$. In addition, potential confounding variables was explored by identifying if adding or removing a variable would result in at least a 20% relative change in the coefficients of variables in the multivariable model (Dohoo *et al.*, 2009). If this was the case, such a variable was included in the final multivariable model (Dohoo *et al.*, 2009). Interactions between the different explanatory variables in the final model were also tested. Overall assessment of how well the final multivariable model fitted the observed data was conducted by exploring residuals and covariate patterns and by calculating Pearson X^2, deviance and conducting the Hosmer-Lemeshow tests (Hosmer, 2013).

4.5 Results

4.5.1 Overview about broiler farm management and biosecurity practices

Ninety seven percent of the questionnaires (n = 39) were completed by broiler farm managers and 2.5% by a broiler farm owner (n = 1). Variation in the shed numbers among the participating farms was noted, with the smallest farm consisting of only two sheds that produced up to 50,000 chickens per broiler growing cycle (range 30 to 60 days). The largest farm was composed of 19 sheds producing up to 180,000 chickens per broiler growing cycle. Fifty percent of the surveyed farms were composed of five to eight sheds.

The integrated company livestock managers (which are independent from the farm manager and/or owner) visited each farm weekly to check on farm management, birds' health and the progress of the broiler chickens. The livestock manager supported and supervised all of the participating 40 broiler farms, all of which followed the same biosecurity protocols that were developed by the integrated company.

Twenty five percent of the broiler farms were housing broiler flocks of the same age (range 1 - 50 days-old) in the same shed at the same time when an all-in all-out system of rearing was used. Sixty five percent of broiler farms housed flocks of different ages within the same shed. Ten percent of the farms housed single and multiple aged birds at different sheds at one time.

Fifty percent of the sampled farms used tunnel ventilation (n = 20), 47.5% used natural ventilation (n = 19) and 2.5% (n = 1) used negative pressure ventilation. About 63% of farms (n = 25) used fans as a second source of ventilation.

All farm workers (including manager/owner) and visitors (on all farms) used footbaths. Hand sanitisers were used by 42.5% of farm workers (n = 17) and 55% of farm visitors (n = 22). The majority of farm workers (97.5%, n = 39) and farm visitors (80%, n = 32) did not wear protective overalls when entering the chicken sheds. Only one farm failed to disinfect equipment such as repaired tools or ladders used by farm workers and visitors prior to the equipment re-entering the sheds.

Seventy five percent of farms (n = 30) used town water as the only source of drinking water for chickens, 7.5% of the farms (n = 3) used a well as the only source of drinking water, while 17.5% of farms (n = 7) used a combination of town and dam water as the source of drinking water. Seventy five percent of the farms treated the drinking water either by chlorine alone or in combination with other treatment methods such as automatic filtration.

On all broiler farms, used litter was removed after each batch and sheds were cleaned with a pressure hose using a mild detergent before sheds were restocked with new batches of chickens. Ninety seven percent of the farms washed the walls as part of the shed cleaning protocols and shed sanitisation was conducted by 80% of the farms (n = 32). As part of their cleaning protocols, all farms used insecticides inside the shed after cleaning.

Thirteen of the broiler farms were located within 500 m of other farms housing animals (9 cattle, 2 horse and 2 camel farms). Only 10% of the farm managers/owners owned a dog and/or a cat, however, no contact between their pet(s) and the broiler chickens were reported.

Avian colibacillosis had been diagnosed previously, in the last year, on 40% (n = 16) of the surveyed farms on the basis of clinical signs, post-mortem examination and the isolation of *E. coli* from the affected lesions. Antimicrobial treatment (trimethoprim-sulfamethoxazole) had only been administered to bird flocks in four of these 16 farms. Furthermore, no other respiratory or any other diseases had been diagnosed on any of the farms within the period the sampled flock were kept on the farm.

4.5.2 Number of *E. coli* colonies that need to be screened to detect APEC VGs

Twenty out of the 2,200 *E. coli* isolates that were cultured had to be reselected, as the original isolate selected as presumptive *E. coli* on phenotype was not identified as *E. coli* by molecular testing. Of the 2,200 *E. coli* isolates cultured, 34% (n = 751) carried four or more VGs (average: 4.9 VGs) and were classified as APEC. While 66% (n = 1,449) were classified as AFEC with an average of 2.2 VGs per isolate.

The kappa statistics data comparing the agreement of using five or ten *E. coli* colonies for classifying a chicken as APEC positive or negative is shown in Table 4.1. The agreement varies between almost perfect and moderate agreement (Landis and Kochi, 1977a) and provides confidence that the selection of five colonies was sufficient to correctly classify birds as APEC positive or negative.

Table 4.1: The Kappa statistics comparing detection of APEC virulence genes following the selection of five or ten *E. coli* colonies cultured from broiler chickens from South East Queensland.

Virulence genes	Observed agreement %	Expected agreement %	Kappa	Standard Error	*p-value*
iutA	97.5	88.25	0.787	0.155	< 0.001
Iss	97.5	69.5	0.918	0.158	< 0.001
hlyF	92.5	79.75	0.629	0.147	< 0.001
ompT	92.5	83.75	0.539	0.140	< 0.001
iroN	97.5	72.75	0.908	0.157	< 0.001

4.5.3 Virulence gene prevalence in APEC positive and negative birds

At the bird-level, 63.0% of birds were identified as APEC positive (n = 252), based on at least one of the isolates harbouring four or more VGs. The prevalence of individual VGs in the 400 bird-level isolates (each isolate representing one sampled chicken) is shown in Table 4.2. Overall, the prevalence of APEC related VGs was significantly higher in APEC positive birds in comparison to APEC negative chickens. The *iutA* VG was the most frequently occurring VG in APEC negative chickens with 75.7% prevalence.

Table 4.2 The prevalence of five virulence genes (VGs) with 95% Confidence Interval (CI) among 252 avian pathogenic *Escherichia coli* (APEC) positive chickens and 148 APEC negative chickens that were sampled between October 2013 and July 2014 from commercial broiler farms in South East Queensland.

Virulence Genes	Number of *E. coli* isolates with VG	Prevalence of VG in APEC positive chickens (95% Cl)	Prevalence of VG in APEC negative chickens (95% Cl)	*p*-value
iss	269	0.99 (0.97, 0.99)	12.5 (7.52, 20.1)	< 0.001
iroN	259	0.99 (0.95, 0.99)	7.2 (3.52, 14.3)	< 0.001
ompT	322	0.10	46.1 (0.34, 0.58)	< 0.001
hlyF	318	0.99 (0.97, 0.99)	44.1 (31.4, 57.6)	< 0.001
iutA	352	0.94 (0.90, 0.97)	75.7 (63.9, 84.5)	< 0.001

4.5.4 The prevalence of birds with Avian pathogenic *E. coli*

All of the 40 farms in the current study, were positive for APEC (i.e. at least one bird per farm was APEC positive). At bird-level, the overall prevalence of APEC in commercial broiler chickens (n = 400) was 63.0% (95% CI: 55.8, 70.2). The frequency of APEC positive birds sampled per farm across the 40 commercial farms is shown in Figure 4.1. Fifty eight percent of the farms (n = 23) had six or more APEC positive birds (of 10 sampled birds). Only one farm had only one bird (of 10 sampled birds) identified as APEC positive while four farms had all 10 sampled chickens identified as APEC positive.

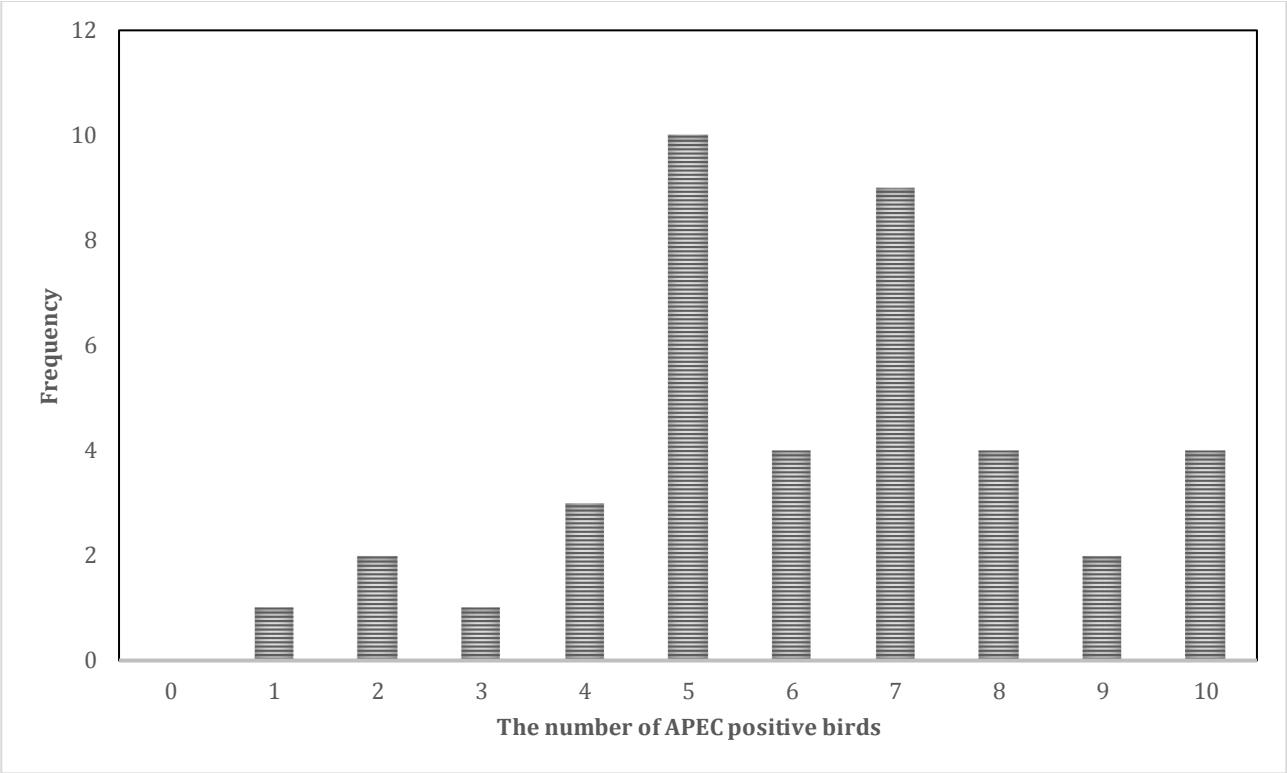


Figure 4.1: Frequency of boiler farms with number of broiler chickens tested as avian pathogenic *E. coli* (APEC) positive (out of 10 birds sampled per farm) in South East Queensland.

4.5.5 Risk factors for APEC bird-level prevalence

The univariate analysis in this study, showed that four risk factor variables were associated with decreased APEC within-farm prevalence ($p < 0.15$) (Table 4.3) while eight risk factors were associated with increased APEC farm prevalence ($p < 0.15$) (Table 4.4).

The final multivariable model describing the association between the farm-level risk factors and within-farm APEC prevalence is shown in Table 4.5. Higher APEC within-farm bird-level prevalence was positively associated with the usage of well water as a source of drinking water (OR 6.2, 95% CI: 2.3, 16.5, $p < 0.001$); not having shower facilities available for farm visitor (OR = 3.6, 95% CI: 1.8, 7.1, $p < 0.001$), distances greater than 20 m between the car park and the poultry shed (OR = 2.2, 95% CI: 1.4, 3.4, $p = 0.001$), the failure to disinfect the waterline after each flock (OR = 2.2, 95% CI: 1.4, 3.5, $p = 0.001$) and the presence of wild birds within 50 m of the poultry shed (OR = 2.3, 95% CI: 1.4, 3.7, $p = 0.001$). The use chlorine combined with the automatic drinking water filtration reduced within-farm bird-level level APEC (OR = 0.07, 95% CI: 0.02, 0.34, $p = 0.001$).

Table 4.3: Univariate analysis of possible farm-level risk factors associated (*p-value* < 0.15) with decreased within-farm prevalence of avian pathogenic *E. coli* (APEC). Data were collected on commercial broiler farms in South East Queensland between October 2013 and July 2014. Risk factor coefficents are presented as Odds ratios (OR) with 95% confidence intervals (CI). The number (N) of farms represents the number of broiler farms that possess or do not possess the risk factor of interest. The APEC prevalence represents the mean number of APEC positive birds within the farms that have or do not have the risk factor of interest.

Risk factor group	Number (%)	APEC prevalence	OR (95% CI)	*p*-value	Wald test *p-value*
Separate age groups of birds housed in a farm at the same time					
No	10 (25)	0.62	Reference		
Yes	30 (75)	0.46	0.52 (0.32 - 0.82)	0.005	
Drinking water treatment					0.01
None	10 (25)	0.53	Reference		
Chlorine	28 (70)	0.51	0.94 (0.44 - 1.99)	0.87	
Chlorine and automatic water filtration	2 (5)	0.10	0.09 (0.05 - 0.19)	0.01	
Number of days per week external (casual) farm worker were present on the farm					0.01
None	16 (40)	0.59	Reference		
Category 1 ≤ 5 days	9 (22.5)	0.43	0.52 (0.31 - 0.88)	0.02	
Category 2 > 5 days	15 (37.5)	0.43	0.52 (0.33 - 0.82)	0.01	
Use of protective overalls by farm workers					
Never	39 (97.5)	0.50	Reference		
Always	1 (2.5)	0.40	0.67 (0.48 - 0.93)	0.02	

Table 4.4: Univariate analysis of possible farm-level risk factors associated (*p-value* < 0.15) with increased within-farm prevalence of avian pathogenic *E. coli* (APEC). Data were collected on commercial broiler farms in South East Queensland between October 2013 and July 2014. Risk factor coefficents are presented as Odds ratios (OR) with 95% confidence intervals (CI). The number (N) of farms represents the number of broiler farms that possess or not possess the risk factor of intrest. The APEC prevalence represents the mean number of APEC positive birds within the farms that have or have not the risk factor of intrest.

Risk factors	Number of farms (%)	APEC prevalence	OR (95% CI)	*p*-value	Wald test *p-value*
The usage of water well as source of drinking water					
No	37 (92.5)	0.48	Reference		
Yes	3 (7.5)	0.66	1.81(1.19, 2.74)	0.005	
The animal species found outside the shed					
None	39 (97.5)	0.49	Reference		
Horses	1 (2.5)	0.89	9.47 (6.88, 13.0)	0.03	
Availability of a shower facility on the farm					
Yes	7 (17.5)	0.37	Reference		
No	33 (82.5)	0.52	1.86 (1.09, 3.17)	< 0.001	
Frequency of water line disinfection after each flock					
Always	21 (52.5)	0.43	Reference		
Often	19 (47.5)	0.57	1.72 (1.16, 2.56)	0.007	
The mortality variations between the sheds within the last 12 months					
No	9 (22.5)	0.39	Reference		
Yes	31 (77.5)	0.53	1.77 (0.88, 3.55)	0.10	
Presence of wild birds within 50 m of the broiler shed(s)					
No	17 (42.5)	0.49	Reference		
Yes	23 (57.5)	0.51	1.15 (0.63, 1.45)	0.11	

Risk factor group	**Number of farms (%)**	**APEC prevalence**	**OR (95% CI)**	***p*-value**	**Wald test *p-value***
Number of rats outside the shed within 50 m					
None	8 (20)	0.33	Reference		0.01
Category 1 ≤ 5	6 (15)	0.46	1.27 (0.85, 1.91)	0.24	
Category 2 >5	26 (65)	0.56	3.39 (2.26, 5.10)	0.01	
Distance between the car park and the shed					
Category 1≤ 20	23 (57.5)	0.44	Reference	0.007	
Category 2 > 20 m	17 (42.5)	0.58	1.73 (1.16, 2.59)		

Table 4.5: Final multivariable model of possible farm-level risk factors associated (*p-value* < 0.05) with within-farm prevalence of avian pathogenic *E. coli* (APEC). Data were collected on commercial broiler farms in South East Queensland between October 2013 and July 2014. Risk factor coefficents are presented as Odds ratios (OR) with 95% confidence intervals (CI). The number (N) of farms represents the number of broiler farms that possess or not possess the risk factor of intrest. The APEC prevalence represents the mean number of APEC positive birds within the farms that have or have not the risk factor of interest.

Risk factors	Number of farms (%)	APEC prevalence	OR (95% CI)	*p*-value	Wald test *p-value*
The usage of a water well as a source of drinking water					
No	37 (92.5)	0.48			
Yes	3 (7.5)	0.66	6.20 (2.32, 16.5)	< 0.001	
Drinking water treatment					0.01
None	10 (25)	0.53			
Chlorine only	28 (70)	0.51	0.77 (0.46, 1.26)	0.317	
Chlorine and automatic water filtration	2 (5)	0.10	0.07 (0.02, 0.34)	0.001	
Distance between the car park and the shed					
Category 1≤ 20	23 (57.50)	0.44			
Category 2 > 20 m	17 (42.5)	0.58	2.16 (1.38, 3.38)	0.001	
Availability of a shower facility on the farm					
Yes	7 (17.5)	0.37			
No	33 (82.5)	0.52	3.59 (1.75, 7.11)	< 0.001	
Frequency of water line disinfection after each flock					
Always	21 (52.5)	0.43			
Often	19 (47.5)	0.57	2.21 (1.41, 3.47)	0.001	
Presence of wild birds within 50 m of the broiler shed(s)					
No	17 (42.5)	0.49			
Yes	23 (57.5)	0.51	2.28 (1.39, 3.72)	0.001	

4.6 Discussion

The current study has revealed a high APEC prevalence in healthy commercial broiler chickens in SEQ. However, direct comparisons between research studies is difficult due to: the high genetic diversity of APEC; the variety of VGs associated with and used to classify APEC; large variability in sampling strategies and microbiological methods (Ewers *et al.*, 2009; Johnson *et al.*, 2008b; Mbanga and Nyararai, 2015).

The selection of five VGs that were proposed by Johnson *et al.* (2008b) as an APEC marker was based on the significant association between these VGs and APEC isolates found in the previous chapter of the current study. Johnson *et al.* (2008b) reported such an association, as did a number of other overseas studies (Ahmed *et al.*, 2013; Hasan *et al.*, 2011; Kobayashi *et al.*, 2011). These genes (*iutA, hlyF, iss, iroN* and *ompT*) are carried by plasmids that typify the APEC pathotype (Johnson *et al.*, 2008b; Rodriguez-Siek *et al.*, 2005a). The current study reported an average number of 2.2 genes in *E. coli* cultured from the faeces of healthy broiler chickens in Australia in comparison to 1.3 genes found in faecal *E. coli* Johnson *et al.* (2008b).

The current study reported that the most prevalent of the tested VGs was *ompT* (100%) followed by *iss, hylF, iroN* and *iutA*, which were all detected in more than 94% of the *E. coli* isolates from APEC positive chickens. Johnson *et al.* (2008b) reported a lower prevalence of these five VGs ranging between 21% to 36% in 200 *E. coli* isolates sourced from the faecal samples of healthy broiler chickens in the United States of America (USA). A higher prevalence of *ompT* (42.5%) and a lower prevalence of *iss* (18.3%) and *iroN* (13.5%) were found by Rodriguez-Siek *et al.* (2005a) in *E. coli* isolates sourced from the faecal samples of healthy birds from the USA. The selection of five to ten *E. coli* isolates per bird may result in a better representation of the *E. coli* populations found in the bird's gastrointestinal tract. The results obtaiend by the current study, showed that the selection of five colonies was sufficient to identify the strains of *E. coli* carried by the birds with the maximum number of VGs, allowing birds to be correctly identified as APEC positive or negative. Additionally, only one *E. coli* isolate, with the highest number of VGs, was chosen to identify APEC status per bird, which may explain the high prevalence of APEC found at farm-level in the current study in comparison with other studies.

Furthermore, the geographical locations, climate, age variables and number of VGs used as APEC markers may contribute to variations in the prevalence of APEC between different studies. For example, despite bird age similarity between the current study and Kemmett *et al.* (2013), considerable APEC prevalence variations were found. A lower APEC prevalence of 1% was reported

by Kemmett *et al.* (2013) in 160 *E. coli* isolates sourced from broiler chickens in the United Kingdom. However, the usage of larger number, and a different range, of VGs (*astA* (heat-stable cytotoxin), *iss*, *irp2* (iron-repressible protein), *iucD* (aerobactin), *papC* (fimbriae), *tsh* (temperature-sensitive hemagglutinin), *vat* (vacuolating autotransporter toxin), *cvi* (colicin V plasmid operon), *sitA* and *ibeA* (invasion of brain endothelium)) could explain the lower APEC prevalence they reported. The same authors identified higher bird-level prevalence of APEC (harboured five or more of the ten VGs) of 24.1% in one-day-old chickens compared to 1% prevalence at slaughter-age (Kemmett *et al.*, 2013). At the bird-level, a 14% APEC prevalence was reported in a Korean study where they used a different set of eight APEC VGs (*astA, iss, irp2, papC, iucD, tsh, vat* and *cva/cvi*) to screen 216 *E. coli* isolates sourced from chickens and environmental samples at the hatcheries (Kwon *et al.*, 2008). Thus, studies that screen for a greater number of VGs and define APEC with more VGs generally identify a lower prevalence of APEC than those studies using fewer VGs. The development of a defined set of VGs for the definition of APEC would aid in the direct comparison of multiple studies.

Understanding the risk factors associated with the increased prevalence of APEC can assist managers to implement strategies to minimise the presence of the pathogen on the farm. Previous studies have suggested that good biosecurity and management protocols are crucial in controlling and reducing environment contamination of APEC and preventing colibacillosis (Collingwood *et al.*, 2014; Dho-Moulin and Fairbrother, 1999; Guabiraba and Schouler, 2015; Kabir, 2010). The current study identified risk factors that are in line with results of a number of studies described in various countries such as poor quality water sources, no treatment of drinking water and direct and/or indirect contact of wild birds with the broiler chickens (Shobrak and Abo-Amer, 2014; Vandekerchove *et al.*, 2004a; Wang *et al.*, 2013b).

The ability of bacteria to survive in water makes drinking water used on broiler farms a potential source of infections (Amaral, 2004). Droppings and secretions of wild birds and other animals could contaminate water sources on poultry farms. It is common on poultry farms for a single water source to supply a large number of birds (Amaral, 2004). Applying basic biosecurity to the drinking water source, such as chlorinated water mains, covered water tanks, cleaning and disinfection of tanks and water lines between flocks, are important practices to assist in reducing the presence of APEC and/or other pathogens in the drinking water (Dhillon and Jack, 1996).

Water wells have been previously been linked to carriage of APEC (Coleman *et al.*, 2012) and *Campylobacter coli* (Whiley *et al.*, 2013). The use of a water wells as a source of drinking water in

three of the surveyed farms in the current study, was associated with higher prevalence of APEC (OR = 6.2) compared to farms that used chlorinated mains water and/or dam water. Interestingly, one of the three farms that used a well did not use any treatment on the drinking water and the other two farms treated the water with chlorine only. The combination of using a water well and the absence of water treatment may have contributed to the observed high APEC prevalence. The current study highlighted the benefit of using a combined water treatment of chlorination and filtration to reduce the prevalence of APEC carriage in broiler chickens. Arsenault *et al.* (2007) also reported the addition of chlorine to the drinking water helped reduce the risk of *Campylobacter* and *Salmonella* colonisation of the intestinal flora. Similarly, other studies have also reported the benefits of chlorination in reducing prevalence of *E. coli* and/or other pathogens and/or decreasing mortality associated with disease (Dhillon and Jack, 1996; Guerin *et al.*, 2007; Nather *et al.*, 2009; Vandeplas *et al.*, 2010). In this study, not disinfecting the water line between each flock increased the APEC prevalence by 2.2-fold. This reflects the role of appropriate infection control measures for drinking water in reducing the prevalence of APEC as well as other infectious agents (Henry *et al.*, 2011; Nather *et al.*, 2009). Hence, the usage of untreated water can be considered as a potential risk factor for broiler flock carriage of APEC, and potentially, other infectious agents. It can be recommended that farms which utilise a well as a source of drinking water should disinfect the water with chlorine and use automatic filtration in order to reduce the prevalence of APEC carriage. Disinfection of the water lines after each flock should also be included as a best practise management technique on-farms.

One concerning finding of the current thesis was that the majority of farm workers and/or visitors did not implement appropriate on-farm biosecurity measures whilst conducting daily work. It was reported that employees were not allocated specific personal protective equipment per shed. Rather, the same pair of overalls and gumboots were utilised across sheds on the same farm. This can facilitate the spread of *E. coli* as well as other infectious agents from one shed to another or introduce agents to the shed from the surrounding environment (Newell *et al.*, 2011). This policy should be re-evaluated in order to minimise the incidence and spread of infectious agents. Each shed should have specific allocated gumboots and overall that are worn exclusively when only working within that shed.

Little data is available regarding the importance of facilities locations and layouts on-farms. The current study identified that a distance greater than 20 m between car park and sheds was associated with 2.2-fold increase in the APEC prevalence. This finding relates not so much to the proximity of

the car park to the sheds, but more to how much farm personnel need to move around the farm before undertaking biosecurity procedures (e.g. showering, use of dedicated clothing) and entering the sheds. A number of studies showed that APEC is found in the surrounding environment around farms (Anza *et al.*, 2014; Cortes *et al.*, 2010; Sola-Gines *et al.*, 2015). The reported overlap between APEC strains isolated from farm workers clothes, hands and boots, and poultry farms, suggest that people may act as a significant vehicle for the introduction and spread of APEC and other pathogens to the poultry farms from the external environment (Belanger *et al.*, 2011; Cho *et al.*, 2012; Johnson *et al.*, 2008b; Lynne *et al.*, 2012; Zhu Ge *et al.*, 2014). Movement of staff and visitors between sheds that potentially harbour APEC and/or other pathogens may also pose a source of infection (Anza *et al.*, 2014). Essentially, parking should be close to the changing area, and stringently imposed requirements for the changing of protective overalls and boots is imperative for infection control (Vandekerchove *et al.*, 2004a; Wang *et al.*, 2013b). Further studies should aim to investigate these potential risk factors and identify the extent that greater distances from car parking to farm facilities has in the increased prevalence of pathogens. Guidelines for the design of future poultry farms should consider the location of parking facilities relative to the farm offices and change room amenities. In addition to this, procedures for all staff and visitors to shower in and out of the facilities can reduce the presence of APEC on-farms. This study found that not having shower facility available on the farm premises was associated with a 3.6 increase in APEC carriage prevalence on seven farms. Hence, this is a management procedure that can be implemented on all farms to reduce the presence of pathogens, such as APEC, within the poultry farm boundaries.

Wild birds present a source of contamination for the poultry flock environment. The presence of wild birds within 50 m of the broiler sheds in this study was significantly associated with increased APEC prevalence. A previous Australian study investigating Newcastle virus and overseas studies on APEC and other infectious agents also identified wild birds as an important biosecurity risk (East, 2007; Keawcharoen *et al.*, 2008; Wang *et al.*, 2013b). Wild birds can directly or indirectly encounter broiler flocks in their sheds, and they can function as mechanical and/or biological vectors for introducing and/or spreading APEC and other avian diseases. The similarity between APEC strains that are extracted from wild birds' faeces and broiler flocks may implicate wild birds as a potential source of risk (Belanger *et al.*, 2011; Oh Jae-Young, 2016). Furthermore, the presence of wild birds can induce stress, which is a known predisposing factor for APEC (Nolan *et al.*, 2013). Therefore, there is a need to control the presence of wild birds near broiler sheds. This can be achieved by applying restricted

biosecurity measures, such as cleaning feed spills and minimising the amount of surface water on the farms, to discourage the presence of wild birds (Darrell *et al.*, 2014).

All farms that were surveyed in this research tested positive for APEC, providing further evidence that APEC is common even in the gut of healthy broiler chickens. Exposure to APEC might be a contributing factor in cases of avian colibacillosis (Kabir, 2010). Thus, there is a need to implement strict biosecurity to reduce the level of APEC exposure on-farms and decrease the prevalence of APEC. It is known that the occurrence and severity of avian colibacillosis depends on the pathogenicity of the APEC strain, the chicken's immune status, and the presence of predisposing risk factors (Kabir, 2010; Nolan *et al.*, 2013). This study has highlighted some of these risk factors including management practices that could currently be contributing to an increased carriage of APEC in healthy chickens. Nevertheless, this study has not taken into consideration that there might be some interplay between farm management and the immune status of the birds. Further research might look at farm management practices to prevent damage to the birds' immune systems and to mitigate the negative impacts of APEC risk. It is also recommended that further investigation should seek to identify how the potential risk factors identified in this study contribute to APEC prevalence. Other biosecurity questions, such as farm infrastructure, location and surrounding environment, could also be further investigated.

The current study was able to overcome some of the difficulty associated with self-reporting questionnaires by conducting piloted face-to-face interviews with three individual broiler farm managers to help simplify any unclear questions and as a result, five questions were revised to increase their clarity. Face-to-face interviews took place on all of the farms that did not respond to overcome the low response rate associated with self-reporting questionnaires. By doing this, we achieved 100% responce rate from all the sampled commercial broiler farms.The main limitation of this study is that only one integrated large commercial broiler company participated. The broiler farms that were sampled belonged to the same company that had implemented the same biosecurity practices recommend by the integrated large commercial broiler company. There are currently three commercial broiler companies operating in Queensland, and future research could compare biosecurity practices with APEC prevalence across these different companies. The commercial consolidation of the poultry sector renders it feasible to have an overall biosecurity plan for an integrated poultry company that is representative of all Australian poultry production, because these three major companies produce 95% of the total poultry produced (Australian Chicken Meat Federation, 2014). Another limitation of this study is that it was conducted as a cross-sectional study,

and causal associations are difficult to detect with this study type (e.g. whether management interventions lead to increased/decreased APEC prevalence, or whether management interventions were implemented in response to increased/decreased APEC prevalence). Furthermore, it is difficult to compare the prevalence findings between studies accurately based on a cross-sectional research design, because there are too many uncontrolled variables. Future investigations could use longitudinal or cohort approaches to explore temporal patterns of APEC prevalence and associated risk factors.

4.7 Conclusions

In summary, the current study identified a high prevalence of APEC carriage in healthy broiler chickens in SEQ. Practices such as disinfecting the water lines after each flock, the availability of a shower facility on the farm, treatment of water sources, decreased distance between the car park and the sheds and/or providing a buffer area close to each shed, where visitors or workers can change into protective clothes, are recommended to decrease the risk of APEC carriage and potentially infections. Particular attention is needed to control risk factors related to impaired biosecurity protocols on the farm, such as direct and indirect contact between chickens and wild birds.

Chapter 5: Antimicrobial susceptibility, plasmid replicon typing, phylogenetic grouping and virulence potential of *Escherichia coli* cultured from Australian broiler chickens with and without colibacillosis

5.1 Foreword

The emergence of multidrug resistance (MDR) (resistance to three or more classes of antimicrobial agents) among avian *Escherichia coli* challenges the treatment of avian colibacillosis and other bacterial poultry diseases. Currently, antimicrobials such as amoxicillin and trimethoprim/sulfamethoxazole are commonly used to treat and prevent avian colibacillosis. Antimicrobials are also commonly used to treat and prevent other bacterial pathogens. The continuous use of veterinary growth promoters and antimicrobials in veterinary and human medicine has contributed to increased antimicrobial resistance among pathogenic and faecal *E. coli* strains in poultry, causing significant economic and health concerns, for humans and the poultry industry (Allocati *et al*., 2013).

Chapter 4 identified several risk factors that were associated with increased avian pathogenic *E. coli* (APEC) carriage among commercial broiler chickens. Addressing these risk factors may reduce APEC carriage in healthy chickens, decreasing the risk of colibacillosis and hence lowering the need for antimicrobials, improving food safety and positively influencing poultry and public health. However, data is still required on the current antimicrobial susceptibility of avian *E. coli* to guide veterinarians in the development of appropriate treatment and control strategies. Overseas studies have characterised APEC and avian faecal *E. coli* (AFEC) and described their antimicrobial susceptibility (Yang *et al.*, 2004; Zhao *et al.*, 2005). In Australia, prior to this research, few studies have investigated the antimicrobial susceptibility of avian *E. coli* (Abraham *et al.*, 2015; Obeng *et al*., 2012).

The first aim of the current chapter was to determine and compare the antimicrobial susceptibility profile, phylogenetic group, virulence and plasmid replicon profiles of clinical *E. coli* (CEC) and faecal *E. coli* (FEC) cultured from Australian commercial broiler chickens between 2006 and 2014. The second aim was to identify any associations between antimicrobial resistance (AMR) and

plasmid replicons, and/or APEC related VGs and/or the phylogenetic groups of the CEC and FEC. Further molecular investigation of a subset of these isolates is described in Chapter 7.

5.2 Abstract

Avian colibacillosis is a common disease affecting the poultry industry globally. However, little is known about *Escherichia coli* from healthy or diseased poultry in Australia. The aim of the current study was to determine and compare antimicrobial susceptibility, phylogenetic group and the profile of virulence genes (VGs) and plasmid replicon types of avian clinical *E. coli* (CEC) and avian faecal *E. coli* (FEC) isolates cultured from Australian commercial broiler chickens.

Fifty CEC cultured from chickens with colibacillosis and 187 FEC from healthy chickens were subjected to antimicrobial susceptibility testing, phylogenetic grouping, plasmid replicon typing and VG profiling. Isolates resistant to extended-spectrum cephalosporins (ESCs) and/or fluoroquinolones (FQs) underwent further characterisation.

Twenty six percent of CEC (13/50) and FEC (49/187) were susceptible to all antimicrobials. Resistance was most commonly detected to sulfamethoxazole/trimethoprim (44%) and (40%), tetracycline (54%) and (28%), streptomycin (24%) and (22%) and ampicillin (28%) and (31%) for the CEC and FEC, respectively. Multidrug resistance (resistance to three or more antimicrobial classes) was detected in 18% of the CEC and 14% of the FEC. Six percent of the CEC and 4% of the FEC were resistant to FQs and/or ESCs. FQ resistant isolates (n = 9) had chromosomal mutations in the quinolone resistance-determining region (QRDR), including those within *gyrA* (83Ser-Leu; 87Asp-Asn), *parC* (80Ser-Ile; 84Glu-Gly), *parE* (458Ser-Ala) and *gyrB* (476Cys-Met; 477Gly-Val; 460Lys-Glu; 465Gln-Asp). Thirty three percent of the FQ resistant isolates (n = 3) contained the *qnrS* gene and 44% (n = 4) were sequence type (ST) 354 with other STs (10, 224, 2705 and 6053) also represented. One of the ESC isolates (which was also FQ resistance) was ST10 and contained $bla_{\text{CMY-2}}$ while the other ESC resistant isolate harboured the $bla_{\text{DHA-1}}$ gene and was ST624.

Clinical *E. coli* were 65 times more likely to contain five avian pathogenic *E. coli* associated VGs (haemolysin gene (*hlyF*), increased serum survival gene (*iss*), outer membrane protease gene (*ompT*) and two iron acquisition system genes (*iutA* and *iroN*)) compared to FEC. The majority of CEC and FEC were phylogenetic group A (50% and 16%) and C (16% and 29%), respectively. The most common plasmid replicon types observed in CEC and FEC were IncFIB (90% and 64%) and IncFrep (64% and 61%), respectively.

The current study showed diversity in antimicrobial phenotypic resistance, VGs and plasmid replicon profiles regardless of the pathogenicity of the *E. coli* isolates, suggesting that there exists a substantive reservoir for associated resistance, VGs and plasmid replicons among *E. coli* populations from commercial broiler chickens. Resistance to older as well as newer antimicrobial drugs was reported among the CEC and FEC. Despite no history of use of FQs or ESCs in the Australian broiler chicken industry, resistance of *E. coli* to ESCs and FQs was detected in Australian chickens for the first time. ST354 was the most common ST associated with FQ resistance and this sequence type has been previously identified in FQ resistant *E. coli* from people, other domestic animals and wild birds. The source of FQ and ESC resistant *E. coli* may be external to the production facility. The identification of resistance to critically important antimicrobials and globally disseminated STs in broiler chickens suggests the need for further studies to identify how poultry is included within the broader epidemiology of resistance amongst extraintestinal pathogenic *E. coli*, and the potential significance to public health. This approach will assist in guiding improvements in infection control practices at the broiler farm production level and optimise both bird health, welfare and public health outcomes.

5.3 Introduction

Avian pathogenic *Escherichia coli* (APEC), a subgroup of extraintestinal pathogenic *E. coli* (ExPEC), is the causative agent of avian colibacillosis in chickens and other poultry species (Collingwood *et al.*, 2014; Nolan *et al.*, 2013). Avian colibacillosis is one of the most common systemic infections in poultry and is responsible for significant economic losses worldwide (Ewers *et al.*, 2003; Nolan *et al.*, 2013). The use of antimicrobial drugs for the treatment as well as the prevention of colibacillosis contributes to increased antimicrobial resistance (AMR) among pathogenic and commensal *E. coli* strains (known as avian faecal *E. coli* (AFEC) in poultry) (Allocati *et al.*, 2013). Avian pathogenic *Escherichia coli* is considered a potentially zoonotic pathogen (Ewers *et al.*, 2007). It has a similar phylogenetic background and virulence gene (VG) suite as human ExPEC and, beyond direct pathogenic potential, can act as a reservoir for VGs and antimicrobial resistance genes (ARGs), which via plasmids or other mobile integrative elements may transfer to other ExPECs (da Costa *et al.*, 2010; Diarra *et al.*, 2007; Manges and Johnson, 2012; Partridge, 2011) and other pathogenic or commensal bacteria (Carattoli, 2013).

Globally, there is increasing discussion about antimicrobial usage in animals and its impact on public health (Landers *et al.*, 2012). Of greatest concern is the use of what the World Health Organisation classify as critically important antimicrobials for human medicine (World Health Organization (WHO), 2016) in livestock production. Among these are extended-spectrum cephalosporins (ESC)

and their potential to select for plasmid-encoded AmpC β-lactamases and CTX-M extended-spectrum β-lactamases (ESBLs) in gram-negative pathogens and commensals (Trott, 2013) and subsequent transfer of the resistance genes or bacteria to humans via the food chain (Ewers *et al.*, 2012; Hammerum and Heuer, 2009; Trott, 2013). A similar concern exists with respect to fluoroquinolone (FQ) resistant gram-negative pathogens (Poole, 2000).

The majority of APEC VGs and ARGs are commonly found on plasmids (Johnson *et al.*, 2007). Despite the critical role of plasmids in promoting the spread of VGs and ARGs, only a limited number of studies have screened for these plasmids in *E. coli* in Australia (Abraham *et al.*, 2012; Moran *et al.*, 2015) and these have been in isolates originating from pigs and humans. Few Australian studies have performed molecular characterisation of virulence, phylogroups and AMR in *E. coli* strains isolated from broiler chickens (Obeng *et al.*, 2012; Obeng *et al.*, 2014) and none have screened for the presence of the plasmid replicons among CEC and FEC populations.

Australia has adopted a conservative position regarding the registration of antimicrobials for food-producing animals and is the only country to never have permitted the use of FQs in food-producing animals (Cheng *et al.*, 2012; Joint Expert Advisory Committee on Antibiotic Resistance (JETACAR), 1999). Australia also has stringent label restrictions on the use of ESCs in livestock (Australian Commission on Safety and Quality in Health Care, 2013 ; Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015). Consequently, the level of resistance to all antimicrobials in commercial livestock in Australia is low (Abraham *et al.*, 2014; Abraham *et al.*, 2015; Department of Agriculture, Fisheries and Forestry (DAFF), 2007). In addition, very few antimicrobials are registered for use in broiler chickens in Australia (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015).

The first aim of the current study was to determine and compare the antimicrobial susceptibility profile, phylogenetic group, virulence and plasmid replicon profiles of CEC and FEC isolates cultured from Australian commercial broiler chickens. The second aim was to identify the association between AMR and plasmid replicons, APEC related VGs and the phylogenetic groups of CEC and FEC.

5.4 Materials and methods

5.4.1 Bacterial isolates

The 237 *E. coli* study isolates, comprised 50 CEC and 187 FEC, cultured from commercial broiler chickens between 2006 and 2014 (Table 5.1) (Animal ethics approval number: Queensland Alliance

for Agriculture and Food Innovation/478/12/ Poultry Cooperative Research Centre). All FEC samples were from intensive broiler farms located in South East Queensland.

One hundred and ninety six isolates were previously identified as *E. coli* by standard biochemical tests and PCR amplification of the *uspA* gene (Chen and Griffiths, 1998) in Chapter 3 (9 CEC and 5 FEC isolates) and in Chapter 4 (182 FEC isolates). In the current chapter, 41 CEC were confirmed as *E. coli* using the same methods. Isolates were stored in Luria–Bertani broth (Thermo Fisher Scientific Australia Pty Ltd, Thebarton, South Australia 5031 Australia) with 20% (v/v) glycerol (Thermo Fisher) at -80 °C until further analysis.

5.4.2 Antimicrobial susceptibility testing and phenotypic testing

Antimicrobial susceptibility testing was performed by disc diffusion as per Clinical and Laboratory Standards Institute guidelines (CLSI) for 20 antimicrobials of veterinary and/or human health importance: amikacin (30 µg); amoxicillin/clavulanic acid (30 µg); ampicillin (10 µg); apramycin (15 µg); cefoxitin (30 µg); ceftazidime (30 µg); ceftiofur (30 µg); cefovecin (30 µg); cephalothin (30 µg); chloramphenicol (30 µg); ciprofloxacin (5 µg); florfenicol (30 µg); gentamicin (10 µg); imipenem (10 µg); neomycin (30 µg); spectinomycin (100 µg); streptomycin (10 µg); sulfamethoxazole/trimethoprim (1.25/23.75 µg); tetracycline (30 µg) and ticarcillin/clavulanic acid (75/10 µg) (Clinical and Laboratory Standards Institute (CLSI), 2013; Clinical and Laboratory Standards Institute (CLSI), 2015a; Clinical and Laboratory Standards Institute (CLSI), 2015b). All antimicrobial discs were obtained from Thermo Fisher Scientific Australia Pty Ltd. The quality control organism used was *E. coli* ATCC 25922. As none of the antimicrobials tested has a veterinary specific breakpoint for poultry, interpretative criteria was extrapolated from the CLSI guidelines for other animal species (including human) for Enterobacteriaceae as recommended (Clinical and Laboratory Standards Institute (CLSI), 2015a; Clinical and Laboratory Standards Institute (CLSI), 2015b). For antimicrobials that lacked published CLSI break points, apramycin (Bayer Australia, Pymble, NSW, 2073, Australia) and neomycin (Zoetis, West Ryde, NSW, 2114, Australia), breakpoint information was obtained directly from the respective manufacturers. For cefovecin breakpoints, the interpretative criteria proposed by Šeol *et al.* (2011) were applied. For antimicrobials florfenicol and spectinomycin, which have no CLSI breakpoints for *E. coli*, breakpoints established for bovine *Pasteurella multocida* were used (Clinical and Laboratory Standards Institute (CLSI), 2015a). For the purpose of the current study, *E. coli* isolates with intermediate susceptibility were defined as not resistant and isolates were considered multidrug resistant (MDR) if they were resistant to three or more antimicrobial classes.

Isolates that demonstrated resistance to ciprofloxacin by disc diffusion underwent minimum inhibitory concentration (MIC) testing by broth dilution to enrofloxacin and ciprofloxacin as per CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI), 2013; Clinical and Laboratory Standards Institute (CLSI), 2015a; Clinical and Laboratory Standards Institute (CLSI), 2015b). The MIC was defined as the lowest antimicrobial concentration that inhibited bacterial growth. *E. coli* ATCC 25922 was used as the quality control organism and the MIC interpretation was based on the breakpoints provided by the CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI), 2015a; Clinical and Laboratory Standards Institute (CLSI), 2015b). In addition, isolates underwent ciprofloxacin and enrofloxacin MIC testing in the presence of the efflux pump inhibitor, Phe-Arg-β-naphthylamide (Sigma-Aldrich, Castle Hill, NSW, 1765, Australia) at 64 mg/L (Platell *et al.*, 2011). *Pseudomonas aeruginosa* ATCC 27853, *E. coli* A100, *E. coli* AG100A, *E. coli* AG112 (Kern *et al.*, 2006) were used as quality control organisms. All antimicrobial powders were obtained from Sigma-Aldrich. Isolates that were resistant to ESCs underwent MIC testing by broth dilution to cefoxitin and ceftiofur as per CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI), 2013; Clinical and Laboratory Standards Institute (CLSI), 2015a; Clinical and Laboratory Standards Institute (CLSI), 2015b). The quality control strain used was *E. coli* ATCC 25922. All ESC resistant isolates were examined for the presence of extended-spectrum and plasmid-mediated AmpC β-lactamases using the MAST DISCS ™ ID AmpC and ESBL (D68C, Mast Group Ltd, UK).

Table 5.1: Number, source, samples site, location and date of isolation of 50 clinical *E. coli* (CEC) and 187 faecal *E. coli* (FEC) isolates cultured from commercial broiler chickens in Australia.

Type of isolates	Number of isolates	Source and samples site	Location	Date of isolation	Reference
FEC	182	Cloacal swab of healthy chickens	Various locations within South East Queensland	2013 - 2014	Chapter 4
FEC	5	Cloacal swab of healthy chickens	3 chicken farms within South East Queensland	2013	Chapter 3
CEC	9	Colibacillosis site in chicken liver (n = 2), air sac (n = 1), lung (n = 2), heart (n = 2), spleen (n = 1) and cloacal swabs (n = 1)	3 chicken farms within South East Queensland	2013	Chapter 3
CEC	30	Colibacillosis site in chicken abdomen (n = 2), air sac (n = 1), cloaca (n = 3), heart (n = 1), infraorbital sinus (n = 1), intestine (n = 1), liver (n = 4), lung (n = 7), pericardium (n = 1) peritoneum (n = 3), pleura (n = 1), nasal cavity (n = 1), trachea (n = 1) and yolk sac (n = 3)	Various locations around Australia	2006 - 2013	Chapter 5
CEC	11	Colibacillosis site in chicken liver (n = 1), lung (n = 3), pericardium (n = 1), air sac (n = 1) intestinal (n = 1), peritoneum (n = 2), duodenum (n = 1), and subcutaneous (n = 1)	Various locations around Australia	2013 - 2014	(Abraham *et al.*, 2015)

5.4.3 Identification of virulence genes

In the current Chapter, 41 CEC isolates were screened for the presence of five APEC related VGs (*iroN, iutA, iss, hlyF* and *ompT*) using a pentaplex-PCR developed by Johnson *et al*. (2008b). While previously, in Chapter 3, nine CEC and five FEC and in Chapter 4 182 FEC were screened for the presence of these APEC related VGs using the same pentaplex-PCR. The positive and negative controls for the pentaplex-PCR were *E. coli* STJ-1 (Fagan *et al*., 1999) and *E. coli* ATCC 8739, respectively.

5.4.4 Phylogenetic analysis, plasmid replicon typing and plasmid multilocus sequence typing

Each of the *E. coli* isolates were assigned to one of the eight distinct phylogenetic groups A, B1, B2, C, D, E, F and *Escherichia* cryptic clade I (termed *E*. clade 1) using the Clermont phylogenetic grouping quadraplex PCR plus the follow up PCRs (Clermont *et al*., 2013). Isolates were also examined for the presence of 18 plasmid replicons: IncFIB; IncI1; IncFIIA; IncP; IncB/O; IncN; IncFIC; IncA/C; IncHI2; IncT; IncN; IncW; IncFIA; IncY; IncFrep; IncX; IncHI1 and IncL/M, using three multiplex panels (Johnson *et al*., 2007). Plasmid multilocus sequence typing (pMLST) based on pill; sogS; ardA; repI1 and trbA was performed on ten isolates (3 CEC and 7 FEC) which contained the Incl1 plasmid [://pubmlst.org/plasmid/] (Garcia-Fernandez *et al*., 2008).

5.4.5 Molecular characterisation of isolates resistant to FQs and/or ESCs

All ESC and FQ resistant isolates underwent multilocus sequence typing (MLST) according to the University of Warwick MLST scheme, which is based on seven housekeeping genes: *adk; fumC; gyrB; icd; mdh; purA* and *recA* (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Polymerase chain reaction and subsequent amplicon-sequencing of the QRDR of *gyrA, gyrB, parC* and *parE* genes and screening for plasmid-mediated quinolone resistance (PMQR) genes: *qnrA; qnrB; qnrS; qepA* and *aac(6')-Ib-cr* were performed on all FQ resistant isolates as previously described (Gibson *et al*., 2010; Platell *et al*., 2011). Amplified PCR products were sequenced at the Animal Genetic Laboratory Facility, University of Queensland, Gatton, Australia on a 3130xl DNA Analyser. Identification of the sequenced regions was completed using the GenBank DNA database using the BLAST alignment (http://blast.ncbi.nlm.nih.gov).

All isolates resistant to ESCs were screened for the presence of AmpC β-lactamase genes as previously described (Perez-Perez and Hanson, 2002). The primer pair CMY25F1 and CMYDR1 was used to amplify and sequence the entire structural gene of $bla_{\text{CMY-2}}$ (Hanson *et al*., 2002). The primer

pair DHA-1A and DHA-1B was used to amplify and sequence the entire structural gene *bla*$_{DHA-1}$ (Mata *et al.*, 2012).

5.4.6 Statistical analysis

Analyses were performed in Stata software (13th edition, Blackburn North Victoria, Australia, www.stata.com). Odds ratios (OR) with a 95% confidence interval (CI) for AMR, phylogenetic group, VGs and plasmid replicon status relative to CEC and FEC designation were calculated using logistic regression. Differences in the prevalence and association of AMR, different phylogenetic groups, plasmid replicons and VGs were assessed between FEC and CEC using the Fisher exact test. Statistical significance was set at a *p*-value of < 0.05.

5.5 Results

5.5.1 Antimicrobial susceptibility and phenotypic results

The prevalence of antimicrobial resistance for CEC and FEC isolates are shown in Table 5.2. All of the isolates were susceptible to amikacin, ceftazidime, ceftiofur, imipenem and florfenicol. Twenty six percent of both the CEC (13/50) and FEC isolates (48/187) were susceptible to all antimicrobials. Clinical *E. coli* isolates demonstrated the most resistance to tetracycline (54%), sulfamethoxazole/trimethoprim (44%), ampicillin (28%) and streptomycin (24%). Faecal *E. coli* isolates demonstrated resistance to sulfamethoxazole/trimethoprim (40%), ampicillin (31%), tetracycline (28%), cephalothin (25%) and streptomycin (22%). One CEC isolate was resistant to ESCs, one to FQs and one CEC was resistant to both ESCs and FQs. Seven FEC were resistant to FQs. Compared to FEC, CEC isolates had a significantly greater prevalence of resistance ($p < 0.05$) to tetracycline, gentamicin, apramycin and spectinomycin (Table 5.2). Faecal *E. coli* were more resistant to cephalothin ($p < 0.002$). Nine resistance patterns were identified among the CEC isolates and 28 different resistance patterns in the FEC isolates. Multidrug resistance was detected in 18% of the CEC isolates (n = 9) and 14% of the FEC isolates (n = 26) ($p < 0.997$).

All isolates resistant to ciprofloxacin by disc diffusion were resistant to ciprofloxacin and enrofloxacin by MIC. The ciprofloxacin and enrofloxacin MICs showed a two to four-fold decrease in the presence of the efflux inhibitor in FQ resistant clinical and FEC isolates (Table 5.6).

Two CEC isolates were resistant to ESCs, one isolate (B1) was resistant to both ceftiofur and cefoxitin on MIC, while B3 was resistant to cefoxitin and showed intermediate susceptibility to ceftiofur. An AmpC β-lactamase was detected in both of these isolates using the MAST DISCS ™. All quality control values were within the stated range for all tests.

Table 5.2: Number (N), prevalence (%) and Odd ratio (OR) with 95% confidence interval (CI) of antimicrobial resistance by disc diffusion among 50 clinical *E. coli* (CEC) and 187 faecal *E. coli* (FEC) isolates from commercial broiler chickens in Australia.

Drug classes	Drug sub classes	Antimicrobial drug	CEC N (%)	FEC N (%)	OR (95% CI)	*p*-value
β-lactams	Penicillin	Ampicillin	14 (28)	59 (31)	0.84 (0.39, 0.75)	0.765
	Potentiated penicillins	Amoxicillin/clavulanic acid	2 (4)	8 (4)	0.93 (0.19, 4.89)	1.000
		Ticarcillin/clavulanic acid	1 (2)	2 (1)	1.88 (0.31, 36.8)	1.000
	1st generation cephalosporin	Cephalothin	3 (6)	47 (25)	0.19 (0.36, 0.64)	0.002
	3rd generation cephalosporin	Ceftazidime	0	0		
		Cefoxitin	2 (4)	0	9.18 (0.71, +∞)	0.087
		Cefovecin	2 (4)	0	9.18 (0.71, +∞)	0.087
		*Ceftiofur	0	0		
	Carbapenem	Imipenem	0	0		
Fluoroquinolones		Ciprofloxacin	2 (4)	7 (4)	1.03 (0.96, 1.01)	0.841
Amphenicols		Chloramphenicol	1 (2)	2 (1)	1.85 (0.03, 36.2)	1.000
		Florfenicol	0	0		
Aminoglycosides		Gentamicin	6 (12)	5 (3)	4.91 (1.19, 21.4)	0.026
		Apramycin	4 (8)	1 (0.5)	15.7 (1.51, 792)	0.015
		Neomycin	4 (8)	4 (2)	3.95 (0.71, 22.0)	0.127
		Spectinomycin	4 (8)	0	20.9 (2.57, +∞)	0.003
		Streptomycin	12 (24)	41 (22)	1.12 (0.48, 2.45)	0.887
		Amikacin	0	0		
Potentiated sulphonamide		Sulfamethoxazole/trimethoprim	22 (44)	75 (40)	1.17 (0.59, 2.31)	0.734
Tetracycline		Tetracycline	27 (54)	52 (28)	3.01 (1.51, 6.05)	< 0.001

* One isolate was intermediately susceptible to ceftiofur on disc diffusion, therefore was identified as not resistant. However, on minimum inhibitory concentration (MIC) testing this isolate was resistant.

5.5.2 Identification of virulence genes and relationships between resistance and virulence genes

The prevalence of the five APEC related VGs detected in the CEC and FEC isolates are presented in Table 5.3. Four percent (n = 2) of the CEC isolates contained none of the five VGs (the two FQ resistant isolates), compared with 19% (n = 36) of the FEC isolates (Table 5.3). An association between certain VGs and some phenotypic resistances were detected among the CEC. This included: *iroN* with resistance to ampicillin and *iss*, *iutA*, *ompT*, *hlyF* and *iroN* with resistance to gentamicin (Supplementary Table 2). An association between *iutA* and *iroN* with resistance to cephalothin and *iss* with sulfamethoxazole/trimethoprim was observed among the FEC (Supplementary Table 2).

There was no association between MDR isolates and the APEC related VGs: 77.8% of the CEC MDR (7 of 9) contained all five APEC related VGs while two harboured none ($p < 0.412$). Four percent of the FEC MDR isolates (1 of 26) contained no APEC related VGs in comparison with 8% that harboured all five APEC related VGs (2 of 26) ($p < 0.2493$).

One of the FQ resistant FEC isolates didn't harbour any APEC related VGs. However, different VGs patterns were observed among the other six FQ resistant FEC isolates. One isolate harboured four VGs (*iss*, *iutA*, *ompT* and *hlyF*), one harboured two (*iss* and *iutA*), three harboured only *iutA* and one only contained *iss* (Table 5.6). One of the ESC resistant CEC isolates contained all five VGs while the other ESC resistant isolate (which was also FQ resistant) harboured none of the CEC related VGs (Table 5.6).

Table 5.3: Number (N), prevalence (%) and Odd ratio (OR) with 95% confidence interval (CI) of five virulence genes among 50 clinical *E. coli* (CEC) isolates and 187 faecal *E. coli* (FEC) isolates from commercial broiler chickens in Australia.

Category	CEC N (%)	FEC N (%)	OR (95% CI)	*p*-value
Virulence Genes				
iroN	45 (90)	71 (38)	14.55 (5.43, 49.2)	< 0.001
ompT	48 (96)	106 (57)	18.19 (4.63, 162)	< 0.001
iss	48 (96)	82 (43)	30.4 (7.58, 265)	< 0.001
hlyF	48 (96)	102 (56)	19.83 (4.94, 173)	< 0.001
iutA	48 (96)	110 (58)	16.67 (4.06, 142)	< 0.001

5.5.3 Identification of plasmid replicons and relationships between resistance and plasmid replicons

Multiplex PCR analysis for 18 plasmid replicon types for the CEC (n = 50) and FEC isolates (n = 187) are presented in Table 5.4. Fifteen plasmid types were identified with at least one or more plasmids detected among 94% and 92% of the CEC and FEC isolates, respectively. None of the isolates contained plasmid replicons IncT, IncFIC and IncW. The most frequently observed plasmid replicons were IncFIB (90% and 64%, CEC and FEC, respectively), followed by IncFrep (64% and 61%, respectively). IncFIB was three times more likely to be found in CEC than FEC isolates ($p < 0.003$). The IncB/O ($p < 0.001$) and IncK/B ($p < 0.018$) replicons were detected in a higher frequency among the FEC isolates in comparison with CEC isolates. Three CEC (6%) and 15 FEC isolates (8%) had no plasmid replicons detected. The plasmid replicons IncP, IncK/B, IncFIIA and IncL/M were not found in the CEC isolates, while the replicons IncA/C and IncX were not found in the FEC isolates. Other plasmid replicons were identified infrequently among FEC and CEC isolates. Seventy percent of CEC isolates (n = 35) contained two plasmid replicon types and 24% (n = 12) contained one plasmid replicon. Nineteen percent of FEC isolates (n = 35) contained two plasmid replicon types; 30% (n = 56) contained three plasmid replicons, six percent (n = 12) contained four plasmid replicons and one percent (n = 2) contain more than five plasmid replicons.

The plasmid replicons that were linked with a higher prevalence of resistance among CEC included IncB/O (resistance to ampicillin and gentamicin), IncK/B (neomycin) and IncFIB (cephalothin) ($p < 0.05$). Plasmid replicon IncFIB was significantly associated with cephalothin resistance among FEC ($p < 0.003$). The faecal isolates demonstrate a significant association ($p < 0.05$) between plasmid replicon IncN, IncA/C and IncL/M and resistance to gentamicin, streptomycin and chloramphenicol, respectively. While the FQ resistant CEC shows a significant association with plasmid replicon IncY ($p < 0.004$).

The plasmid replicon profiles for the FQ and ESC resistant CEC and FEC are presented in Table 5.6. None of the CEC isolates had the same profile and no replicon profile was common across the CEC and FEC isolates. Only two of the seven FEC isolates shared the same replicon profile.

Table 5.4: Number (N), prevalence (%) and Odd ratio (OR) with 95% confidence interval (CI) of plasmid replicon among 50 clinical *E. coli* (CEC) isolates and 187 faecal *E. coli* (FEC) isolates from commercial broiler chickens in Australia.

Category	CEC N (%)	FEC N (%)	OR (95% CI)	*p-value*
IncB/O	3 (6)	65 (35)	0.12 (0.02, 0.40)	< 0.001
IncA/C	3 (6)	0	14.9 (1.58, +∞)	0.018
IncP	0	1 (0.5)	3.74 (0, 145)	1.000
IncK/B	0	18 (10)	0.12 (0, 0.75)	0.018
IncFIIA	0	1 (0.5)	3.74 (0, 145)	1.000
IncFIA	4 (8)	9 (5)	1.73 (0.33, 6.64)	0.676
IncFIB	45 (90)	120 (64)	3.41 (1.42, 9.50)	0.003
IncY	2 (4)	5 (3)	1.53 (0.14, 9.59)	0.909
IncI1	5 (10)	30 (16)	0.56 (0.16, 1.57)	0.355
IncFrep	32 (64)	114 (61)	1.10 (0.55, 2.24)	0.913
IncX	1 (2)	0	3.74 (0.09, + ∞)	0.422
IncHI1	3 (6)	1 (0.5)	11.7 (0.91, 626)	0.060
IncN	5 (10)	9 (5)	2.19 (0.54, 7.70)	0.296
IncHI2	1 (2)	1 (0.5)	2.18 (0.54, 7.70)	0.378
IncL/M	0	2 (1)	1.54 (0, 20.1)	1.000

[1] Plasmid replicons IncT, IncFIC and IncW were not detected.

5.5.4 Identification of phylogenetic group and relationships between resistance and phylogenetic group

The distribution of phylogenetic groups of the 50 CEC and the 187 FEC is summarised in Table 5.5. The clinical *E. coli* isolates were three times more likely to be phylogenetic group A compared to FEC ($p < 0.001$). No other significant differences between phylogenetic group and CEC or FEC were detected (Supplementary table 3).

The association between phenotypic antimicrobial resistance and phylogenetic groups among CEC and FEC is show in Supplementary Table 3. A significant association was detected between phylogenetic group B2 and CEC isolates resistance to trimethoprim-sulfamethoxazole ($p < 0.02$). Phylogenetic group F was significantly associated with FQ resistant faecal isolates ($p < 0.03$).

All FQ and ESC resistant CEC belonged to phylogenetic group A. Seventy one percent of the FQ resistant FEC isolates (n = 5) belonged to group F and 29% (n = 2) group B1 (Table 5.6).

Table 5.5: Number (N), prevalence and odd ratio with 95% confidence interval (CI) of phylogentic group[1] among 50 clinical *E. coli* (CEC) isolates and 187 faecal *E. coli* (FEC) isolates from commercial broiler chickens in Australia.

Category	CEC N (%)	FEC N (%)	OR (95% CI)	*p*-value
		Phylogenetic Group		
A	25 (50)	30 (16)	4.79 (2.30, 10.0)	< 0.001
B1	2 (4)	18 (10)	0.39 (0.04, 1.73)	0.325
B2	5 (10)	12 (6)	1.61 (0.42, 5.25)	0.550
C	8 (16)	54 (29)	0.13 (0.003, 0.89)	0.321
D	1 (2)	24 (13)	0.486 (0.172, 1.19)	0.128
E	0	12 (6)	0.232 (0, 1.46)	0.138
F	9 (18)	31 (17)	1.25 (0.50, 2.91)	0.703
***E*. clade 1**	0	5 (3)	0.547 (0, 4.10)	0.605

All of the *E. coli* isolates were assigned to one of the eight distinct phylogenetic groups using the Clermont phylogenetic grouping quadraplex PCR plus the followup PCRs (Clermont *et al.*, 2013).

5.5.5 Plasmid multilocus sequence typing

The results for the pMLST sequence typing for the 10 MDR avian isolates (seven FEC and three CEC) that contained IncI1 are present in Table 5.7. In summary, there was no common ST between the faecal and CEC isolates. The three CEC isolates each had a unique ST - ST3, ST183 and ST200. Of the seven FEC isolates, two isolates were allocated to each of ST21 and ST201 while the remaining three isolates had a unique ST: ST119; ST148 and ST202. In terms of clonal complex (CC), ST3 belongs to CC3 and ST21 belongs to CC5.

5.5.6 Molecular characterisation of isolates resistant to FQs and/or ESCs.

One of the CEC FQ resistant isolates was ST10 while the others were ST224 and ST624 (Table 5.6). The majority of the FQ resistant FEC isolates (n = 4) were ST345, while the others were ST57, ST2705 and ST6053 (Table 5.6).

The two FQ resistant CEC isolates demonstrated known mutations in the QRDRs, including a double mutation in *gyrA* (83Ser-Leu; 87Asp-Asn) and a single mutation in *parC* (80Ser-Ile). A single mutation in *parE* (458Ser-Ala) was also detected in both isolates. Both isolates had two mutations in *gyrB*, B1 (476Cys-Met, 477Gly-Val) and B2 (460Lys-Glu, 465Gln-Asp), although these mutations are not in known quinolone resistance-determining sites. No PMQR genes were identified. The seven FQs resistant FEC isolates demonstrated known mutations in the QRDRs, including a double mutation in *gyrA* (83Ser-Leu; 87Asp-Asn) and a double mutation in *parC* (80Ser-Ile; 84Glu-Gly). No mutations were detected in *parE*. Isolates 107D, 368B and 255H had one mutation in *gyrB* (460Lys-Glu), although this mutation is not in a known quinolone resistance-determining site. Three out of the seven FEC FQ resistant isolates harboured the *qnrS1* gene.

AmpC β-lactamase genotyping showed that one of the ESC resistant isolates (B1) contained $bla_{\text{CMY-2}}$ and belonged to ST10. The other ESC resistant isolate (B3) harboured the $bla_{\text{DHA-1}}$ gene and was ST624 (Table 5.6).

Table 5.6: Characterisation of extended-spectrum cephalosporin and/or fluoroquinolone resistant CEC (n = 3) and FEC (n = 7) cultured from commercial broiler chickens.

Location of the isolate	Isolate ID	Sample/ Farm	MIC mg/L						Antibiogram	Phylo-genetic group	ST	Plasmid replicon	Virulence genes	pARG
			CIP	CIP EPI	ENR	ENR EPI	EFT	FOX						
Clinical *E. coli* isolates														
Various locations around Australia	B1	Nasal Cavity	64	16	64	8-16	8	32	FOX, CIP AMC, AMP, KF, STR,GN, SH, SXT, TE	A	10	IncY, IncI1	ND	bla_{CMY2}
Various locations around Australia	B2	Air Sac	64	16	128	8-16	NT	NT	CIP, AMP, STR, KF, CHL, GN, N, SH, SXT, TE	A	224	ND	ND	
Various locations around Australia	B3	Cloaca	NT	NT	NT	NT	2-4	256	FOX, AMP, STR AMC, KF, N, SXT, TE,	A	624	IncFIA, IncFIB, IncFrep	*iss, iutA, ompT, hlyF, iroN*	bla_{DHA1}
Faecal *E. coli* isolates														
SEQ	107D	9	64	32	128	8	NT	NT	CIP, GN, TE	F	6053	IncFIA, IncFIB	*iss, iutA*	
SEQ	131D	11	64	32	128	8	NT	NT	CIP, GN, TE,	F	354	IncB/O, IncK/B, IncFIA, IncFIB	*iutA*	*qnrS1*
SEQ	196B	15	64	32	128	8	NT	NT	AMP, KF, CIP, GN, SXT, STR	F	354	IncB/O, IncK/B, IncFIA, IncFIB, IncFrep	*iss*	*qrnS1*
SEQ	255H	24	64	32	128	8	NT	NT	AMP, CIP, TE, APM	F	354	IncFIA, IncFIB, IncI1	*iutA*	
SEQ	412A	39	64	32	128	8	NT	NT	KF, GN, CIP	F	354	IncB/O, IncK/B, IncFIA, IncFIB	*iutA*	
SEQ	56E	4	64	32	128	8	NT	NT	AMP, CIP, SXT, TE, STR	B1	2705	IncFIB	*iss, iutA, ompT, hlyF*	
SEQ	368B	35	64	32	64	8	NT	NT	AMP, KF, CIP, GN, SXT, TE, STR	B1	57	IncFrep, IncN	ND	*qnrS1*

MIC, minimum inhibitory concentration, EPI, Efflux Pump Inhibitor (PaβN) 64mg/L; ST, sequence type; pARG, Plasmid-mediated antimicrobial resistance genes; ND, not detected; NT, not tested; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; KF, cephalothin; STR, streptomycin; GN, gentamicin; SEQ, South East Queensland; SH, spectinomycin; SXT, trimethoprim-sulfamethoxazole; ENR, enrofloxacin; EFT, ceftiofur; FOX, cefoxitin; TE, tetracycline; CIP; ciprofloxacin; CHL, chloramphenicol; N, neomycin; APM, apramycin.

Table 5.7: Plasmid multilocus sequence typing (pMLST) of the IncI1 from three clinical (CEC) and seven faecal *E. coli* isolates (FEC) from broiler chickens in Australia.

Isolate ID	Allele					IncI1 typing
	Repl1	ardA	trbA	SogS	PilL	Sequence typing
	Clinical *E. coli* isolates					
B1	1	2	8	10	10	183
B3	2	1	4	1	2	3
B4	1	2	17	3	2	200
	Faecal *E. coli* isolates					
29C	1	2	17	6	18	119
62C	1	2	11	3	3	21
84E	1	4	3	4	3	148
200C	1	9	3	4	3	201
177D	1	2	11	3	3	21
295F	1	9	3	4	3	201
365C	1	3	5	4	3	202

5.6 Discussion

This study investigated antimicrobial susceptibility of CEC and FEC from Australian broiler chickens and looked for associations between AMR, VGs, phylogenetic groups and plasmid replicons. AMR was low, though resistance to important antimicrobials was detected at very low levels. Clinical *E. coli* harboured more APEC linked VGs in comparisons with FEC. A significant association between plasmid replicons type IncFIB and IncA/C occurred among CEC in comparison with FEC. Clinical *E. coli* isolates were also three times more likely to belong to phylogenetic group A compared to FEC.

The majority of CEC and FEC isolates which were resistant to antimicrobials were resistant to well established antimicrobial drugs such as ampicillin, tetracycline and trimethoprim/sulfamethoxazole that have been registered and used for many years in the poultry industry in Australia. Previous studies in Australia and further afield for both pigs and poultry have shown similar (Abraham *et al.*, 2015; Department of Agriculture, Fisheries and Forestry (DAFF), 2007). The long and continuous usage of these antimicrobials in Australia, as well as residual environmental contamination, may explain the persistence of AMR among *E. coli* isolates sourced from broiler chickens (Walsh and Fanning, 2008). The current study showed that compared to FEC, CEC had a significantly greater prevalence of

resistance ($p < 0.05$) to tetracycline, gentamicin, apramycin and spectinomycin. This may be due to the chickens from which CEC isolates were cultured being treated with antimicrobials prior to sampling. Although, gentamicin is no longer registered for use in food-producing animals, other aminoglycosides drug such as apramycin, neomycin and spectinomycin are still used in the Australian broiler industry. The use of these aminoglycoside drugs can cause cross-resistance to gentamicin (Choi *et al.*, 2011; Jensen *et al.*, 2006). Co-resistance, may also occur and selection pressure associated with the commonly used antimicrobials such as amoxicillin, tetracycline and trimethoprim/sulfamethoxazole may select for gentamicin resistance (Schwarz and Chaslus-Dancla, 2001).

The low levels of AMR detected in the current study reflects the strict regulation of antimicrobials used in food-producing animals in Australia and can be compared to similar studies undertaken in other countries, such as Denmark and the Netherland which also have restricted antimicrobial use and low levels of AMR (NORM/NORM-VET, 2007; Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM), 2007). In comparison, higher levels of resistance were detected in countries with less stringent antimicrobial use regulations such as Egypt, Korea and China (Aarestrup, 2005; Hussein *et al.*, 2013; Kim *et al.*, 2007; Yang *et al.*, 2004).

Very low level of resistance to critically important antimicrobials, ESCs and FQs, were detected among the clinical and FEC isolates. Extended-spectrum cephalosporins and FQs have never been used in the poultry industry in Australia (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015). In fact, Australia is the only country that has never permitted the use of fluoroquinolones in food-producing animals (Cheng *et al.*, 2012; Ndi and Barton, 2012). Furthermore, cephalosporins are not used in poultry in Australia (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015). However, resistance to FQs and ESCs have recently been reported in Australia in other food-producing animals (Abraham *et al.*, 2015). Identification of resistance in the absence of antimicrobial selection may indicate that these resistant isolates originated from humans (Meyer *et al.*, 2010), wild birds (Smith *et al.*, 2014) and/or from the environment (Alves *et al.*, 2014; Sjolund *et al.*, 2008) and gained access to the poultry production environment. Further screening and characterisation of antimicrobial resistant *E. coli* isolates from wild bird species, wild animals and the environment as occurred in some studies already (Guenther *et al.*, 2011; Radhouani *et al.*, 2014) could provide evidence of a potential risk pathway for the transmission of bacteria as well as the resistance genes. Furthermore, vertical and/or horizontal co-selection pressure could be considered as a possible explanation for the FQ and ESC resistance

reported in the current study (Oz *et al.*, 2014). An efflux pump was identified phenotypically in all FQ resistant isolates. Efflux pumps may select for resistance to FQs and other antimicrobial drugs simultaneously. Additionally, a PMQR gene (*qnrS*) was detected in three of the FEC isolates. This finding is in agreement with a previous study in dogs and humans in Australia (Tagg *et al.*, 2015) as well as globally (Cremet *et al.*, 2011; Rodriguez-Martinez *et al.*, 2006; Yang *et al.*, 2014), where *qnrS* were detected either alone or in combination with other PMQR gene. The dissemination of the PMQR genes among some of the FQ resistant faecal isolates suggests horizontal co-selection pressures due to the use of other antimicrobial drugs (Liu *et al.*, 2013; Osinska *et al.*, 2016).

The current study has, for the first time, reported the detection of $bla_{\text{CMY-2}}$ and $bla_{\text{DHA-1}}$ in ESC resistant *E. coli* isolates cultured from broiler chickens in Australia. The $bla_{\text{CMY-2}}$ and $bla_{\text{DHA-1}}$ genes have been previously reported in other animals and other bacterial species in Australia (Abraham *et al.*, 2014; Cherif *et al.*, 2015; Rogers *et al.*, 2014; Sidjabat *et al.*, 2007). This is of great public health significance as those genes can be transmitted directly (via the food chain or environment) or indirectly (via mobilised plasmid) to other bacterial species

The $bla_{\text{CMY-2}}$ gene is the most frequently detected AmpC β-lactamases in food-producing animals worldwide (Jacoby, 2009; Sidjabat *et al.*, 2007). This gene has been detected among *E. coli* isolates sourced from animals as well as humans and may be transmitted via horizontal gene transfer via conjugation of plasmids such as IncI1 (Carattoli, 2009; Johnson *et al.*, 2007). The $bla_{\text{DHA-1}}$ gene has recently increased in prevalence, which may suggest the acquisition of new plasmids among AmpC β-lactamases harbouring *E. coli* sourced from broiler chickens (Mata *et al.*, 2012; Tagg *et al.*, 2015).

The majority of the sequence types (ST10, ST57 ST 224, ST354 and S624) identified in the current study from FQ and ESC resistant isolates have been previously described and are globally distributed amongst humans as well as animals (Fernandes *et al.*, 2016; Kim *et al.*, 2011; Maluta *et al.*, 2014; Sola-Gines *et al.*, 2015; Zhao *et al.*, 2014). ST2705 and ST6053 are host restricted and have been previously isolated from poultry and wild birds (Dahms *et al.*, 2015; Day *et al.*, 2016; Jones-Dias *et al.*, 2015). The majority of the FQ resistant FEC were identified as ST354 belonging to phylogenetic group F. This ST has been previously identified in pathogenic *E. coli* from chickens, dogs, cats and pigs as well as in non-pathogenic *E. coli*, worldwide (Kim *et al.*, 2011; Mora *et al.*, 2011; Schaufler *et al.*, 2015). Furthermore, ST354 has been recently reported in Australia from FQ resistant faecal and pathogenic *E. coli* isolates sourced from humans, birds, canines and the environment (Guo *et al.*, 2015; Vangchhia *et al.*, 2016).

Genetic diversity was reported among the FQ resistant isolates with none of the FEC belonging to phylogenetic group A, only CEC were group A. The current study reported a variation in the ST among the clinical FQ resistant isolates (keeping in mind that there was only two clinical FQ resistant isolates) with one assigned as ST224 while the other was ST10. Both STs have previously been identified in horses, dogs, buffalos and human pathogenic and non-pathogenic *E. coli* (Abraham *et al.*, 2015; Aizawa *et al.*, 2014; Dissanayake *et al.*, 2014; Maluta *et al.*, 2014; Manges and Johnson, 2012; Pires-dos-Santos *et al.*, 2013; Vignaroli *et al.*, 2012; Zhao *et al.*, 2014).

Determining the origin of the STs, which are common among humans and birds is difficult, but the identification of these STs could imply a possible transmission link between humans and poultry. Chickens may also act as a source for the transmission of bacteria into the surrounding environment (Fernandes et al., 2016). Wild birds and other animals, such as vermin and rodents, (Dahms et al., 2015) could also disseminate resistant *E. coli* (Guenther et al., 2010; Laube et al., 2014). Similarly, humans could act as a direct or indirect source of transmission to poultry and the environment (Marathe et al., 2013). On the other hand, poultry meat contaminated with antimicrobial resistant bacteria and or ARGs can be transmitted to humans directly through the food chain (Radhouani et al., 2014).

Similar to other studies worldwide, the majority of the FEC isolates were phylogenetic group C, A and B1 (Hiki *et al.*, 2014; Jakobsen *et al.*, 2010; Sola-Gines *et al.*, 2015). In this current study, fifty percent of the CEC isolates belonged to phylogenetic group A, which is typically identified as a commensal, non-pathogenic *E. coli* (Clermont *et al.*, 2013). Other studies have also reported that the majority of CEC belong to group A (Rodriguez-Siek *et al.*, 2005a; Sola-Gines *et al.*, 2015). However, there are reports that the majority of CEC are group D (Campos *et al.*, 2008; Clermont *et al.*, 2000). Variation in identified phylogenetic grouping between studies may be due to geographical and temporal variations. Geographical variations have been reported in a recent study where 35.3% and 56.1% of APEC isolates belonged to phylogroup B2 in Germany and the United Kingdom, respectively. While in Italy, 53.3% of APEC isolates belonged to phylogroup A (Cordoni *et al.*, 2016).

The current study also used the new phylogenetic group determination methods that reassigns group A and D that failed to correctly assign in the old Clermont method (Clermont *et al.*, 2013). The most significant advantage of the new Clermont quadruplex PCR method for *E. coli* phylogenetic grouping assignment is its ability to classify the *E. coli* strains into four further groups: C; E; F; and *E.* clade 1 (Clermont *et al.*, 2013). The association between phylogenetic group B2 and pathogenicity has been

well documented (Carattoli, 2009; Lynne *et al.*, 2012). In this study, only 10% of the CEC and 6% of the FEC were phylogenetic group B2.

In the current study, there was no association between AMR phenotype and phylogenetic group. This finding could suggest that resistance among *E. coli* cultured from broiler chickens in Australia, regardless of their pathogenicity, was not due to clonal spread but most likely due to the transmission of ARGs (Chah *et al.*, 2010). However, not all of the isolates were pathogenic, 187 were cultured from the faeces of healthy birds. Forty one CEC were submitted from a diagnostic laboratory, where only one isolate per sample submitted from a clinically sick bird was further investigated. The isolate selected may not have been the pathogenic strain. This might explain why, as seen in this study, not all CEC isolates contained APEC-associated VGs. It must also be noted that the current study only involved screening for five VGs. Finally, the isolate selected by the diagnostic laboratory may also have a different antimicrobial susceptibility profile then the isolate responsible for disease.

Plasmids play a major role in the spread of $bla_{\text{CMY-2}}$ and other resistance genes in *E. coli* and other bacterial species. Similar to other studies screening for plasmids in chicken meat and FEC, IncFIB and IncFrep were the most common plasmids found (Da Silva and Mendonca, 2012; Lynne *et al.*, 2012). These plasmids and others including IncFIB, IncFrep, IncHI1, IncHI1, IncY, IncI, IncFIA, IncN and IncL/M have been shown to encode for multidrug resistance in previous studies among *E. coli* and other *Enterobacteriaceae* from humans and food-producing animals (Carattoli, 2009). These replicons are often linked with ESBLs (Garcia-Fernandez *et al.*, 2008; Woodford *et al.*, 2007).

The detection of IncA/C plasmids were of concern as previous studies (Guo *et al.*, 2015; Mataseje *et al.*, 2010) have shown that IncA/C plasmids are associated with horizontal gene transfer and they have been implicated in transfer of ARGs between Salmonella and *E. coli* from food animals, the environment and humans, as well as being associated with MDR and the AmpC β-lactamase gene $bla_{\text{CMY-2}}$, specifically (Fernandez-Alarcon *et al.*, 2011). However, the three isolates that were identified in the current study harbouring an IncA/C plasmid were susceptible to all antimicrobials. And the isolates with the bla_{CMY}-detected did not have an IncA/C plasmid.

The IncI1, IncFIB, IncY and IncN plasmids have been identified in poultry previously and were associated with MDR and ESBLs producing *E. coli* (Bortolaia *et al.*, 2010; Garcia-Fernandez *et al.*, 2008; Kluytmans *et al.*, 2013; Lynne *et al.*, 2012; Seni *et al.*, 2016; Wang *et al.*, 2013a). One of the ESC resistant isolates contained IncY and IncI1 plasmid types, while the other contained IncFIA, IncFIB and IncFrep plasmids. IncFrep plasmids have previously been isolated from the environment,

animals and humans, harbouring ESBLs and PMQR mechanisms in addition to other ARGs (Dolejska *et al.*, 2013).

Overall, the usage of antimicrobials, disinfectants and/or heavy metals in Australian poultry is not sufficient to explain the prevalence and dissemination of the tested plasmid replicons in the poultry isolates in the current study. However, there is evidence that some plasmid replicons, such as IncI1, can survive in *E. coli* isolates without antimicrobial selection pressure with no or little fitness cost to the host (Fischer *et al.*, 2014). Furthermore, the host bacteria can use some extra elements encoded by plasmids for their survival (Carattoli, 2009). Several studies (Carattoli, 2013; Lynne *et al.*, 2012) have shown a low prevalence of MDR encoding plasmids among human sourced *E. coli* isolates in comparison with the avian *E. coli* isolates. Which may suggest that the transfer of these plasmids from poultry to humans might be uncommon.

Several studies (Carattoli, 2009; Mata *et al.*, 2012) have reported a strong association between $bla_{\text{DHA-1}}$ and IncF plasmids. However, the isolate in our study which, contained $bla_{\text{DHA-1}}$ did not carry the IncF plasmid (Carattoli, 2009; Mata *et al.*, 2012). Globally, an association between plasmid replicon types N, L/M and HI2 among *E. coli* and *Salmonella* spp. and *qnrS1* from human and animals were reported which are consistent with the finding of the current study (Carattoli, 2013).

Johnson *et al*. (2008b) suggest that CEC isolates can be distinguished from FEC isolates by their possession of five APEC related VGs. The current results show that the presence of the five genes predicting pathogenicity was significantly higher in the CEC isolates compared with the FEC isolates (Johnson *et al.*, 2003; Johnson *et al.*, 2008b). However, the presence of APEC-associated VGs among the FEC suggests a possible reservoir of the VGs in the commensal *E. coli* population.

Overall, the CEC also showed more resistance to antimicrobials compared to the FEC. This may be due the fact that the CEC isolates were sourced from farms with disease outbreaks and were thus more likely to have been exposed to antimicrobials than those FEC isolates that were sourced from healthy flocks. Alternatively, it could be that resistant isolates are more virulent. Interestingly, the current study suggested that the FEC isolates exhibit more diversity in their resistance profiles than CEC isolates. It has been previously suggested that commensal *E. coli* flora are a source of emerging and developing *E. coli* resistant strains and/or resistance genes (da Costa *et al.*, 2013).

The FQ resistant FEC in this study have various VG patterns and none of the two FQ resistant CEC isolates harboured any APEC related VGs. Similar findings have occurred in earlier studies in other parts of the world where low prevalence of VGs were detected among FQ resistant CEC isolates

(Horcajada *et al.*, 2005; Huang *et al.*, 2009; Kawamura-Sato *et al.*, 2010). These isolates may have been opportunistic pathogens that acquired FQ resistance (Drews *et al.*, 2005; Vila *et al.*, 2002) or they may be CEC isolates that lost their VGs after they developed mutations in the QRDR (Horcajada *et al.*, 2005; Moreno *et al.*; Sawma-Aouad *et al.*, 2009). Furthermore, the virulence profile of the AmpC harbouring *E. coli* isolates also varied. The first ESC resistant *E. coli* harboured none of the CEC related VGs, while the other ESC resistant isolate harboured all five VGs. The fact that all of the FQ and ESC resistant isolates do not belong to phylogenetic group B2, the most common phylogenetic group of pathogenic ExPEC, could support the theory of low pathogenic opportunistic isolates.

There are a number of limitations that apply to the current study. Firstly, all of the FEC isolates were obtained from one geographical area (SEQ), while the CEC isolates were sourced from across Australia, so the FEC isolates examined may not be representative of the larger population. There was a limited number of resistant isolates, especially ESC and FQ resistant, limiting the power of statistical comparisons. There was also no available history of previous antimicrobial use for the clinical and faecal isolates. The fact that the current study did not screen the resistant isolates for the presence of antimicrobial resistance genes was a limitation of the current study.

Another limitation was the lack of clinical break points available for interpretation of antimicrobial susceptibility in *E. coli* cultured from poultry, and as such, interpretations had to be extrapolated from human *E. coli* data or from other pathogens from other host species. A high level of resistance was detected to cephalothin in the current study, despite the fact that this antimicrobial has never been used in the poultry industry, calling into question the validity of using human breakpoints, as there is no clinical breakpoint for cephalothin available for *E. coli* in poultry.

Future antimicrobial and resistance surveillance investigations are needed using structured sampling strategies involving much larger sample sizes, and not relying on laboratory-acquired isolates with limited history in order to highlight and identify resistance mechanisms with public health potential.

5.7 Conclusion

The key finding of the current study is that a low level of resistance to both well established and critically important antimicrobials such as FQs and ESCs were reported. This demonstrates that the strict Australian regulations around the use of antimicrobials in Australian poultry have provided some protection to the industry. Therefore, the observed prevalence of FQ and ESC resistance, despite the nil usage of those antimicrobial drugs, may indicate they have been the acquired by the result of

co-selection and/or co-resistance pressure mediated by the usage of other antimicrobials and/or disinfectants or clones introduced from sources outside the sheds.

A high diversity of plasmid replicons, phylogenetic groups and APEC related VGs were detected among CEC and FEC of broiler chickens in Australia, suggesting that *E. coli* from broiler chickens could act as reservoirs for virulence and resistance genes for *E. coli* or other bacterial species and contribute to the spread of these genes.

Chapter 6: Virulence associated genes in faecal and clinical *Escherichia coli* isolates cultured from broiler chickens in Australia

6.1 Forward

Escherichia coli organisms are genetically diverse and both commensal and avian pathogenic *Escherichia coli* (APEC) harbour a wide variety of virulence genes (VGs). Several studies have demonstrated that not all APEC-associated VGs are present in isolates cultured from birds with colibacillosis (Ewers *et al.*, 2003; Johnson *et al.*, 2008b; Qabajah *et al.*, 2014) and it is not known which VGs or combinations of VGs are required for colibacillosis to occur.

Studies from different countries have aimed to define and differentiate APEC from avian faecal *E. coli* (AFEC) in order to establish an association between the carriage of several VGs and disease. In Australia, prior to this investigation, the only available information on APEC was published in 2004 and identified the roles of *tsh, iss* and *iucA* and the pVM01 plasmid in pathogenicity (Tivendale *et al.*, 2004). However, that study did not screen for the presence of APEC VGs among faecal isolates nor compare AFEC and APEC on the basis of VG carriage.

In this thesis, five VGs (*iutA, ompT, hlyF, iss* and *iroN*) were selected to define APEC (Johnson *et al.*, 2008b). However, these five genes were detected with a high prevalence (63%) among faecal isolates (Chapter 4), highlighting the need to extend screening to identify additional VGs associated with APEC in Australia. The phylogenetic groups and the presence of five APEC related VGs were examined among all the clinical and faecal isolates in the previous chapter (Chapter 5). In this chapter a sub set of avian clinical and faecal *E. coli* isolates were selected based on genetic diversity and antimicrobial susceptibility profile for further analysis. In total, 88 *E. coli* isolates were selected and screened for the presence of 30 additional extraintestinal pathogenic *E. coli* (ExPEC) associated VGs to identify the association between these VGs and avian colibacillosis.

6.2 Abstract

The healthy bird's intestinal flora harbours a rich reservoir of *Escherichia coli* as part of the commensal microbiota. However, some *E. coli* strains known as extraintestinal pathogenic *E. coli* (ExPEC) carry certain virulence genes (VGs) that enable them to invade and cause extraintestinal infections, such as avian colibacillosis. In birds, the causative agent for avian colibacillosis is a subgroup of ExPEC known as avian pathogenic *E. coli* (APEC). The pathogenic mechanisms associated with APEC are ill-defined, although several VG combinations have been identified which are believed to enable APEC to invade and cause disease outside the gastrointestinal tract.

The current study compared ExPEC-associated VGs in *E. coli* cultured from birds with colibacillosis and healthy birds to determine which genes were more abundant in *E. coli* from Australian birds with colibacillosis. A subset of 88 *E. coli* isolates from commercial poultry flocks in Australia, including 29 *E. coli* isolates cultured from chickens with colibacillosis (referred to as clinical *E. coli*: CEC) and 59 faecal *E. coli* (FEC) from clinically healthy chickens were screened for the presence of 30 ExPEC-associated VGs (in addition to the 5 APEC-associated VGs screened for in previous chapters). The 88 isolates were selected from the 237 *E. coli* isolates that were investigated (in Chapter 5) based on their enterobacterial repetitive intergenic consensus (ERIC) and AMR profile.

The current study identified the presence of 34 of the 35 VGs, with prevalence ranging from 3.4% (*focG*) among FEC to 100% (*astA*) among CEC. All of the tested isolates were positive for the presence of at least four VGs. The nine most prevalent virulence genes in the 29 CEC isolates were: *astA* (100%); *feoB* (96.6%); *iutA; iss; ompT; iroN* and *hlyF* (all 93.1%)*; vat* (89.7%) and *fimC* (86.2%). The *iucA* gene was not detected in any of the *E. coli* isolates.

However, a set of VGs*: iroN; iss; iutA*; *tsh; fimC; papC; papEF; vat*; *hlyF; astA; ibeA; feoB; ireA; cvi/cvaC* and *ompT* were significantly more likely to be found in the CEC isolates in comparison with FEC. Further investigations are needed to identify the roles of these VGs in pathogenicity. These VGs may be able to be used to better define APEC, and diagnostically to detect APEC in Australia.

6.3 Introduction

Avian colibacillosis is caused by a subgroup of extraintestinal pathogenic *E. coli* (ExPEC), known as avian pathogenic *Escherichia coli* (APEC), which have the ability to invade various internal organs and cause systemic disease (La Ragione and Woodward, 2002; Mokady *et al.*, 2005). However, the majority of *E. coli* are commensals and coexist in the gut microbiota of healthy birds, do not cause disease and are known as avian faecal *E. coli* (AFEC) (Johnson *et al.*, 2008b; Nolan *et al.*, 2013; Oh *et al.*, 2011; Rodriguez-Siek *et al.*, 2005a).

A large number of overseas studies have aimed to define and differentiate APEC from AFEC based on phylogenetic grouping, virulence genotyping, serotyping, as well as finger printing methods (such as enterobacterial repetitive intergenic consensus (ERIC) PCR, randomly amplified polymorphic DNA and restriction fragment length polymorphism) (Johnson *et al.*, 2008b; Nolan *et al.*, 2013; Obeng *et al.*, 2012; Oh *et al.*, 2011; Rodriguez-Siek *et al.*, 2005a). Nevertheless, APEC are still not clearly defined. Several virulence genes (VGs) have been found to be associated with APEC, however, no specific VG or set of VGs that contribute entirely to the pathogenicity of APEC have been identified (Hussein *et al.*, 2013; Vandekerchove *et al.*, 2005).

Several overseas studies have differentiated APEC and AFEC on the basis of the presence of five VGs that Johnson *et al.* (2008b) identified as having a significant association with APEC (Dissanayake *et al.*, 2014; Hussein *et al.*, 2013; Nolan *et al.*, 2013). The VGs are: *iss* (increase serum survival); *ompT* (outer membrane proteinase); *hlyF* (putative avian hemolysin)*; iroN* (salmochelin siderophore receptor) and *iutA* (aerobactin receptor) (Johnson *et al.*, 2008b). Johnson *et al.* (2008b) concluded that *E. coli* could be considered an APEC if it was cultured from a lesion in an internal organ of a chicken with colibacillosis and possessed four or more of these five APEC-associated VGs.

The first aim of the current study was to determine the clonal relatedness between 237 *E. coli* (50 clinical and 187 faecal *E. coli*) isolates that were previously characterised (Chapter 5) and sourced from chickens with colibacillosis and healthy chickens. Secondly, a subset of isolates representing diverse clonality and the most resistant isolates were selected to determine the distribution of 35 APEC-associated VGs between clinical and faecal *E. coli* cultured from Australian broiler chickens.

6.4 Materials and methods

6.4.1 Bacterial isolates and subset selection criteria

In total, 237 *E. coli* isolates that were described in Chapter 5 (187 FEC and 50 CEC) underwent fingerprinting using ERIC-PCR and antibiogram analysis to assist in subset selection of isolates. Selection criteria for the *E. coli* isolates for further characterisation included (i) one isolate from all of the clusters (≥ 80% similar) identified by ERIC profile, (ii) antimicrobial resistance profile, the most resistant isolate from each cluster were included (Chapter five) and (iii) bird's health status (the CEC isolates were selected prior to the FEC isolates if both CEC and FEC isolates belonged to the same cluster and had the same resistance profile). Random selections were applied if there was more than one isolate belonging to the same cluster with the same resistance and health profile.

6.4.2 Enterobacterial repetitive intergenic consensus (ERIC)-PCR

Clonality between the 237 *E. coli* isolates that were previously studied in Chapter 5 were determined by ERIC-PCR (Versalovic *et al.*, 1991). Banding patterns were analysed using Gel ComparII (Applied Maths, Sint-Martens-Latem, Belgium). Similarity was estimated with a Dice Coefficient of 0.1% and a tolerance of 1% and cluster analysis was performed with Dice Coefficients and unweighted-pair group method with arithmetic mean (UPGMA). Rooted rendered tree was generated using the UPGMA.

Similar to other studies, isolates with a > 93% similarity in their ERIC profile were assumed to be closely related (a clonal group) (Moreno *et al.*, 2006; Sabate *et al.*, 2008). A cluster was defined as a group of isolates that shared ≥ 80% similarity in their ERIC-PCR profile patterns.

6.4.3 Virulence genotyping

The selected *E. coli* isolates were previously screened for the presence of the five APEC related VGs (*iroN, iutA, iss, hlyF* and *ompT*) (Johnson *et al.*, 2008b) (Chapter 5). Isolates were then screened for a further 30 APEC-associated VGs using published single and multiplex PCR assay panels. The first PCR panel targeted *astA, irp2, papC, iucD, tsh, vat* and *cvalAB* (Ewers *et al.*, 2005). The second panel targeted *chuA* and *traT* (Ewers *et al.*, 2007). The third panel amplified the following genes: *fyuA; papG* and *kpsMT k1* (Johnson and Stell, 2000).The fourth panel screened for *fimH, papEF, ireA* and *ibeA* (Rodriguez-Siek *et al.*, 2005a). Group five targeted *sitA* and *feoB* (Rodriguez-Siek *et al.*, 2005a). Primers for the following genes: *sfaS* and *focG* were included in the sixth panel (Obeng *et al.*, 2012). The seventh group screened the following genes: *cbi; cma* and *cvaC* (Johnson *et al.*, 2006). The

following genes: *kpsMTII; hlyA; fimC; neuC; afa/drab* and *sfa/foc* were amplified using the protocol validated by Ewers *et al*. (2007). The last PCR amplified *iucA* (Johnson *et al.*, 2006).

6.4.4 Case definition

All of the *E. coli* isolates were categorised based on the bird's health status and independently from the VG profile into faecal *E. coli* (FEC) and clinical *E. coli* (CEC) as shown in Figure 6.1. Faecal *E. coli* were isolates cultured from the faeces of healthy birds and CEC were cultured from the faeces or organs of birds with colibacillosis. Faecal *E. coli* and CEC were further characterised as APEC if they were sourced from faeces or the cloaca and harboured four or more of the APEC VG markers (*iroN, iutA, iss, hlyF* and *ompT*). Clinical *E. coli* isolates cultured from lesions from birds with colibacillosis, which harboured four or more of the five APEC-associated VGs, were defined as clinical avian pathogenic *E. coli* (cAPEC). While CEC cultured from lesions or faeces and contained less than four of the selected VGs were identified as potential APEC (pAPEC). Faecal *E. coli* isolates were molecularly classified into avian faecal (AFEC) if they harboured less than four of the selected APEC VG markers.

6.4.5 Statistical analysis

Analyses were performed in Stata software (13th edition, Blackburn North Victoria, Australia, www.stata.com). Comparisons of the associations between VGs for CEC and FEC were carried out using Fisher's exact test (Fisher exact was used instead of the Chi-square in case of small values), odds ratio (OR) and their 95% confidence interval (CI) were calculated. A *p-value* of < 0.05 was considered significant.

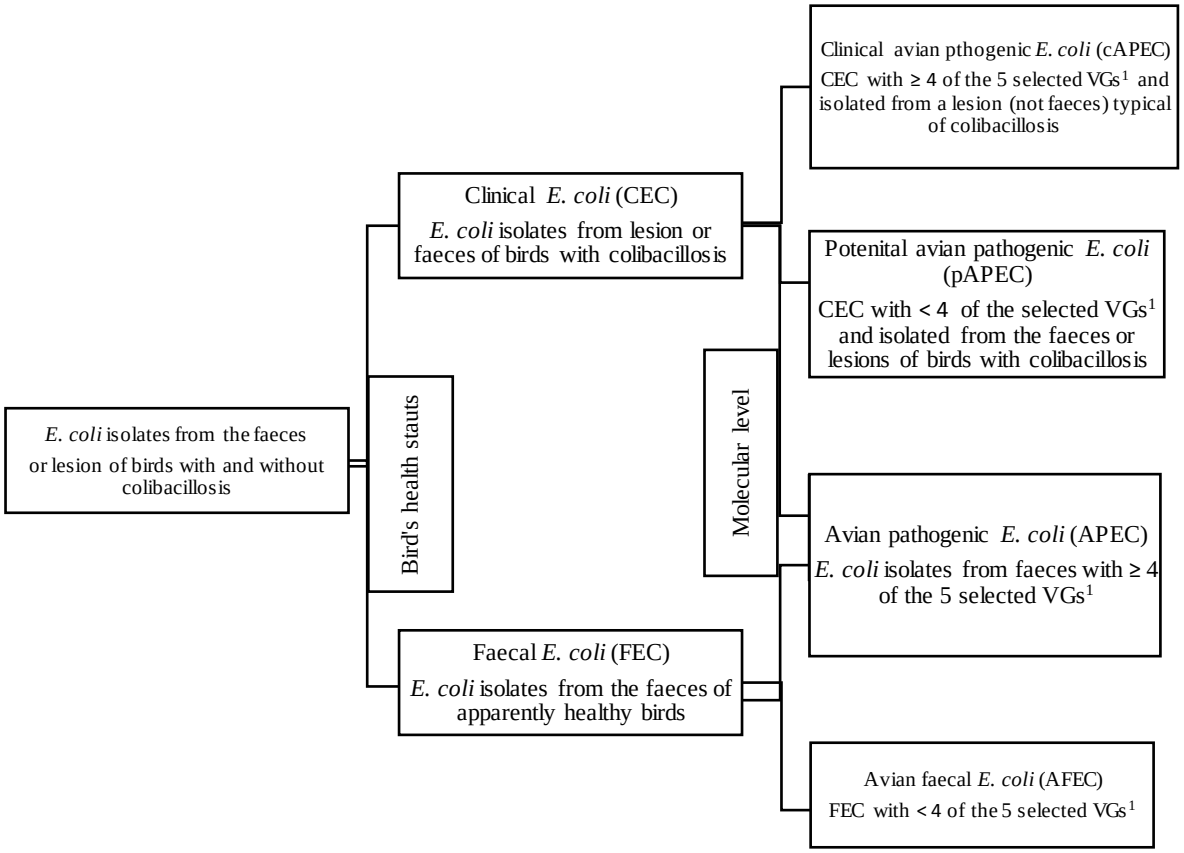


Figure 6.1: Illustration flow chart for the classification applied to all of the *E. coli* isolates included in the current study.

[1] The selected VGs are: *iss; iutA; iroN; ompT* and *hlyF*.

6.5 Results

6.5.1 Bacterial isolates

The *E. coli* isolates (n = 88) investigated in this study consisted of 59 avian faecal isolates (FEC) and 29 clinical *E. coli* isolates (CEC) (Table 6.1).

6.5.2 Enterobacterial repetitive intergenic consensus (ERIC)-PCR

A comparison of the clustering pattern produced by the data obtained by ERIC-PCR for the 237 *E. coli* (50 CEC and 187 FEC) isolates revealed 88 distinct ERIC clusters (Figure 6.2). The 237 *E. coli* did not form distinct clusters with respect to CEC and FEC and/or antimicrobial resistance profile. There were eight larger clusters, which comprised 51.1% (121/237) of the *E. coli* isolates (clusters 3, 4, 16, 19, 26, 37, 55 and 69), observed among the isolates. Of all isolates, cluster 16 was the largest with 22 isolates (9.28%) aligned with 88% similarity. Cluster 69 contained 19 (8.01%)

isolates, cluster 3 and 4 both contained 16 (6.75%) isolates, cluster 26 and 37 both contained 14 (5.91%) isolates, cluster 19 contained 11 (4.67) isolates and cluster 55 contained nine (3.80%) isolates.

Within cluster 4, four isolates were clonal and were cultured from chickens from one farm. Within cluster 7, three isolates (out of eight) were clonal, with all three isolates being > 95% similar in their ERIC profile. Two out of these three isolates were collected from the same farm at the same time, although the third isolate was cultured from a chicken from a different Australian state. Similarly, five isolates in cluster 27 (out of seven) and four (out of eight) in cluster 42 were clonal; however, isolates did not appear to be related in time or space (these isolates were collected from different Australian states at different time).

Thirty of the 88 *E. coli* isolates, each representing one cluster, were MDR, resistant to three or more antimicrobial classes; 42 were resistant to two classes; while 16 were resistant to only one antimicrobial class. Out of the 29 CEC isolates, two did not harbor any VGs, while 27 were previously classified as cAPEC. Nineteen of the FEC were previously classified as APEC, while 40 were AFEC.

6.5.3 Virulence genotyping

Figure 6.3 illustrates the virulence genotype for the 88 isolates. Table 6.2 shows the number and prevalence of the 31 ExPEC related VGs among the 29 CEC and 59 FEC isolates. Overall, none of the CEC or FEC isolates harboured *iucA*. The following VGs: *sfa/foc*; *focG* and *hlyA* couldn't be found among the CEC isolates. The following VGs were identified with significant (*p-value* of less than 0.05) prevalence among CEC: *astA* (100%); *foeB* (96.6%); *iroN* (93.1%); *ompT* (93.1%); *iss* (93.1%); *iutA* (93.1%); *hlyF* (93.1%); *vat* (89.7%); *fimC* (86.2%); *cvi/cvaC* (79.3%), *tsh* (55.2%); *ireA* (51.7%); *papC* (44.8%); *papEF* (41.4%) and *ibeA* (37.9%). The following VGs: *papG; irp2; chuA; kpsMT11; kpsMTK1; cbi; cma; sitA; traT*; *fimH*; *afa-drab; fyuA; sfaS; iucD; neuC* and *maxI* were found with a similar prevalence among the CEC and FEC isolates. Seven percent, 51.7% and 41.4% of the CEC harbored 10, 20 and 30 VGs in comparison with 22%, 71.2% and 6.7% of the FEC.

Table 6.1: Number, source, samples site, location and date of isolation of 29 clinical *E. coli* (CEC) and 59 faecal *E. coli* (FEC) isolates obtained from commercial broiler chickens in Australia.

Type of isolates	Number of Isolates	Source and samples site	Location	Date of isolation	Reference
FEC	59	Cloacal swab of healthy chickens	Various locations within Southeast Queensland	2013 - 2014	Chapter 4
CEC	9	Colibacillosis site in chicken liver (n = 2), air sac (n = 1), lung (n = 2), heart (n = 2), spleen (n = 1) and cloacal swabs (n = 1)	3 chicken farms within Southeast Queensland	2013	Chapter 3
CEC	15	Colibacillosis site in chicken abdomen (n = 2), air sac (n = 1), heart (n = 1), infraorbital sinus (n = 1), intestine (n = 1), liver (n = 1), lung (n = 3), pericardium (n = 1) peritoneum (n = 1), pleura (n = 1), nasal cavity (n = 1) and trachea (n = 1)	Biosecurity Sciences Laboratory	2006 - 2013	Chapter 5
CEC	5	Colibacillosis site in chicken liver (n = 1), lung (n = 1), pericardium (n = 1), air sac (n = 1) and subcutaneous (n = 1)	Australian diagnostic laboratories	2013 - 2014	(Abraham *et al.*, 2015)

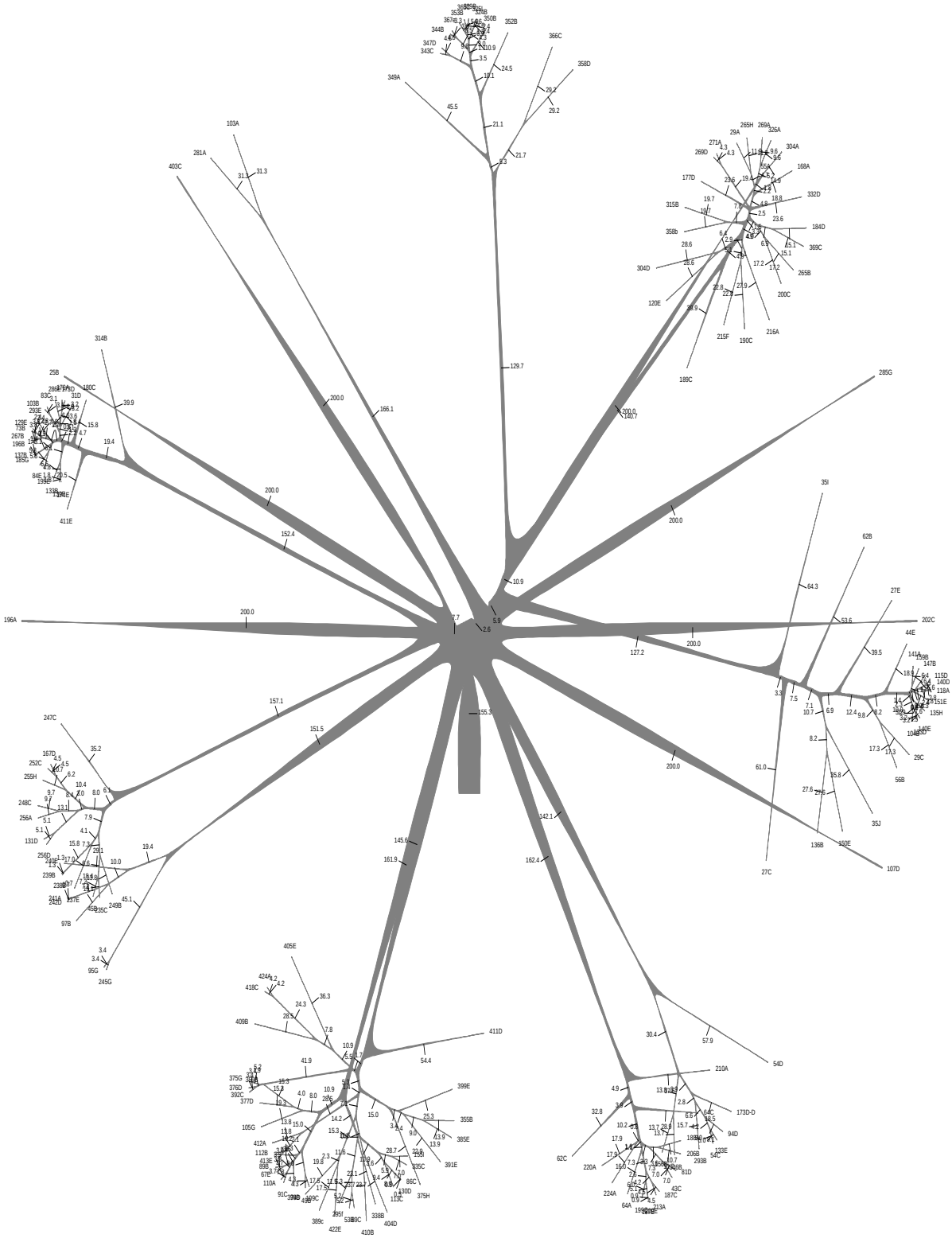


Figure 6.2: Rooted rendered tree by enterobacterial repetitive intergenic consensus *PCR* profile of 237 *E. coli* study isolates, comprised of 50 clinical *E. coli* (CEC) isolates and 187 faecal *E. coli* (FEC) cultured from commercial broiler chickens between 2006 and 2014.

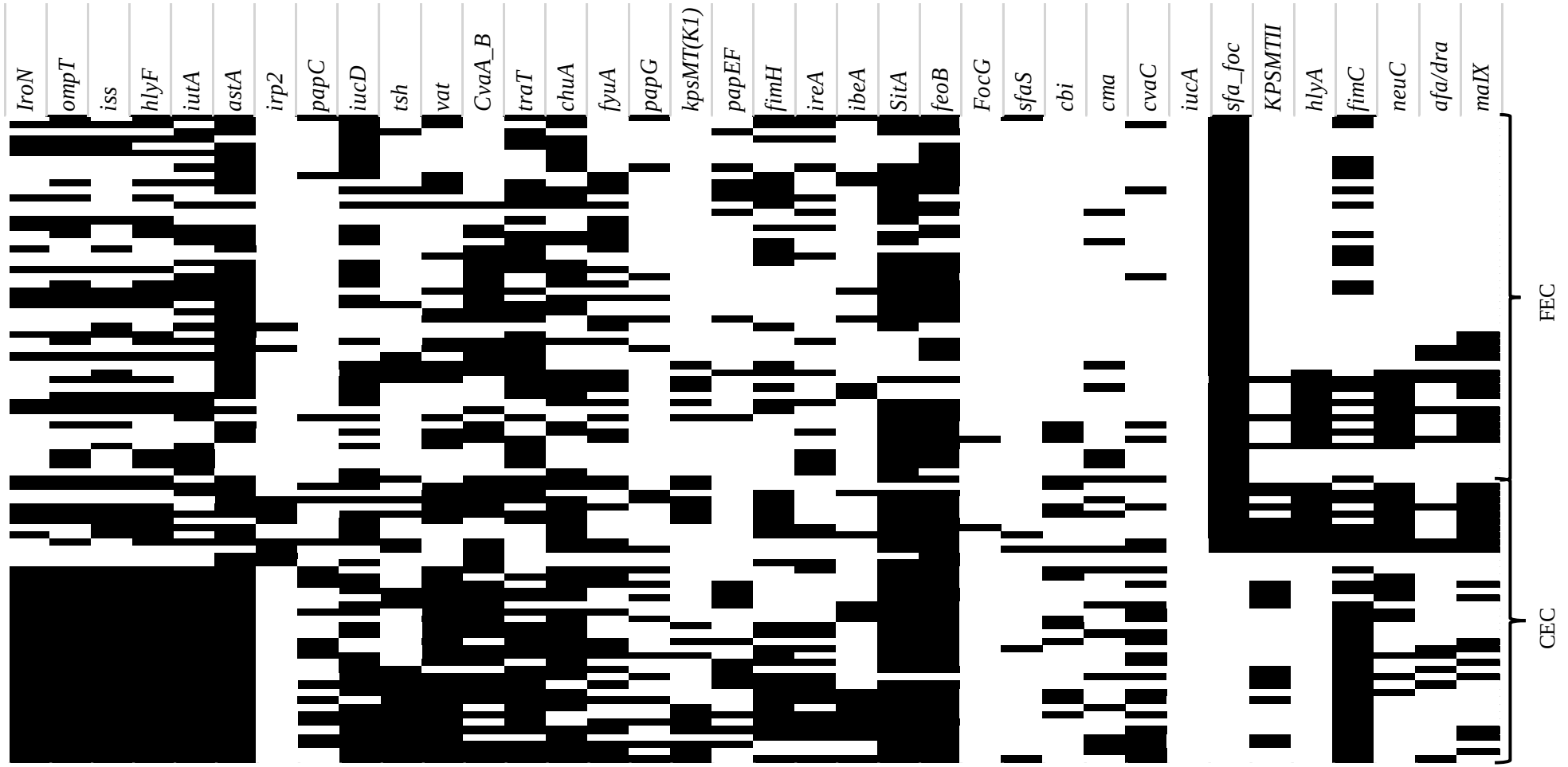


Figure 6.3: Virulence genotype of each of 29 clinical *E. coli* (CEC) and 59 faecal *E. coli* (FEC) from broiler chickens in Australia.

Table 6.2: Number (N), prevalence (%) and Odd ratio (OR) with 95% confidence interval (CI) of 31 virulence genes among 29 clinical *E. coli* (CEC) and 59 faecal *E. coli* (FEC) from broiler chickens in Australia.

Putative Gene Function	Virulence gene[1]	OR (95% CI)	CEC N (%)	FEC N (%)	*p*-value
PTJ100 related genes	*cvi/cvaC*[1]	3.42 (1.14, 11.8)	23 (79.3)	31 (52.5)	0.026
	iroN[2]	21.9 (4.76, 208)	27 (93.1)	22 (37.3)	< 0.001
	iss[2]	15.6 (3.39, 147)	27 (93.1)	27 (45.8)	< 0.001
	traT	1.45 (0.50, 4.45)	22 (72.4)	38 (64.4)	0.616
	iutA[2]	11.9 (2.59, 112)	27 (93.1)	31 (52.5)	0.002
	Tsh	4.27 (1.51, 12.6)	16 (55.2)	13 (22.1)	0.005
	sitA	4.38 (0.94, 43.9)	27 (93.1)	44 (74.6)	0.064
Adhesins	*fimC*	7.24 (2.13, 32.2)	25 (86.2)	27 (45.8)	0.004
	papC	6.98 (2.08, 26.3)	13 (44.8)	6 (10.2)	0.001
	papG	2.88 (0.89, 9.46)	10 (34.5)	9 (15.3)	0.079
	papEF	3.85 (1.24, 12.4)	12 (41.4)	9 (15.3)	0.017
	fimH	1.55 (0.58, 4.22)	16 (55.2)	26(44.1)	0.451
	afa/drab	1.16 (0.27, 4.36)	5 (17.3)	9 (15.3)	1.000
Iron acquisition	*irp2*	0.48 (0.05, 2.62)	2 (6.9)	8 (13.6)	0.591
	chuA	2.10 (0.69, 7.33)	23 (79.3)	38 (64.4)	0.242
	fyuA	1.27 (0.47, 3.41)	15 (51.7)	27 (45.8)	0.761
	ireA	2.84 (1.03, 8.04)	15 (51.7)	16 (27.1)	0.043
	feoB	7.80 (1.06, 348)	28 (96.6)	46 (77.9)	0.041
	sfaS	1.38 (0.11, 12.8)	2 (6.9)	3 (5.08)	1.000
Protectins/serum resistance	*iucD*	1.61 (0.54, 5.22)	22 (75.9)	39 (66.1)	0.496
	kpsMTII	2.27 (0.73, 7.05)	10 (34.5)	11 (18.6)	0.173
	neuC	0.82 (0.28, 2.29)	9 (31.1)	21 (35.6)	0.86
Invasions	*kpsMTK1*	2.27 (0.73, 7.05)	10 (34.5)	11 (18.6)	0.173
Toxins	*ibeA*	3.34 (1.06, 10.8)	11 (37.9)	9 (15.3)	0.038
	Vat	11.5 (3.02, 65.7)	26 (89.7)	25(42.4)	< 0.001
Colicin genes	*hlyF*[2]	12.7 (2.78, 120)	27 (93.1)	30 (50.9)	< 0.001
	Cbi	2.41 (0.68, 8.46)	8 (27.6)	8 (13.6)	0.139
	Cma	2.09 (0.61, 7.11)	8 (27.6)	9 (15.3)	0.276
Miscellaneous	*maxI*	0.71 (0.24, 1.97)	9 (31.1)	23 (38.9)	0.627
	ompT[2]	12.7 (2.78, 120)	27 (93.1)	30 (50.9)	< 0.001
	astA	9.99 (1.55, ∞)	29 (100)	47 (79.7)	0.011

[1] *afa/draB*, afimbrial/Dr antigen-specific adhesin; *astA*, EAST1 (heat-stable cytotoxin associated with enteroaggregative *E. coli*); *cbi*, corresponding immunity; *cma*, Colicin M-resembles B-lactam; *chuA*, heme receptor gene (heme uptake); *cvi/cva*, structural genes of colicin V operon (microcin ColV);feoB, ferrous iron transport protein B *fimC*, type 1 fimbriae (d-mannose-specific adhesin); *fimH*, adhesive subunit of type 1 fimbriae; *fyuA*, ferric yersiniabactin uptake (yersiniabactin receptor); *hlyA*, haemolysin A; *ibeA*, invasion of brain endothelium; *ireA*, iron-responsive element (putative catecholate siderophore receptor); *iroN*, catecholate siderophore (salmochelin) receptor; *irp2*, iron-repressible protein (yersiniabactin synthesis); *iss*, increased serum survival; *iucD*, aerobactin synthesis; *iutA*, ferric aerobactin receptor (iron uptake/transport); *kpsMT*I, group I capsule antigens; *kpsMT K1*, group I capsule antigens; *kpsMT* II, group II capsule antigens; *maxI*, pathogenicity-associated island marker; *neuC*, K1 capsular polysaccharide; *ompT*, outer membrane

protease; *papC*, P-fimbriae; *sitA*, putative iron transport gene; *sfa/focDE*, *sfa* (S fimbriae) and *foc* (F1C fimbriae; *traT*, surface exclusion protein (serum survival factor); *tsh*, temperature-sensitive haemagglutinin; *vat*, vacuolating autotransporter toxin.

[2]The PCR for these genes was performed in Chapter 4 and 5.

Virulence gene *iucA* was not detected in CEC and FEC while *hlyA*, *focG* and *sfa/foc* were not detected in CEC.

6.6 Discussion

Globally, colibacillosis is considered one of the major problems that affects the poultry industry and translates into multimillion-dollar losses annually (Guabiraba and Schouler, 2015; Johnson *et al.*, 2008b; Nolan *et al.*, 2013). Interestingly, although there has been some development of vaccines to alleviate this problem, none have been effective in fully controlling this infection or disease (Lynne *et al.*, 2012; Lynne *et al.*, 2007). Despite the intensive research globally, the genetic diversity of APEC make it hard to reach a consensus on definition of APEC (Johnson *et al.*, 2008b; Lynne *et al.*, 2007; Rodriguez-Siek *et al.*, 2005a).

In the current study, the *E. coli* isolates (29 CEC 59 and FEC) carried an assortment of VGs, with some genes being significantly associated with CEC. The prevalence of VGs reported in this study are similar to a number of overseas studies, which have reported variation in VG frequency and combinations in CEC and FEC isolates (Johnson *et al.*, 2008b; Lynne *et al.*, 2012; Maturana *et al.*, 2011; Olsen *et al.*, 2012; Rodriguez-Siek *et al.*, 2005a).

In the current study, ERIC-PCR was used to investigate the genetic diversity of isolates, since it is a simpler method to identify relationships in comparison to other molecular typing methods and allows processing of a large number of isolates. It has the benefits of being rapid, cost-effective and can classify *E. coli* into different clonal groups or clusters for the purpose of epidemiological studies (Cherifi *et al.*, 1991; Gillings and Holley, 1997; Meacham *et al.*, 2003; Ranjbar *et al.*, 2017; Ugorski and Chmielewski, 2000; Wilson and Sharp, 2006). The lack of repeatability and discriminatory power and lower type ability compared to other finger printing methods, such as multilocus sequence typing (MLST) and pulse field gel electrophoresis (PFGE), are some of the disadvantages of this protocol (Meacham *et al.*, 2003). Ewers *et al*. (2004) and Knobl *et al.* (2012) have reported that none of the molecular typing methods have the ability to differentiate between clinical and FEC isolates. In this study, ERIC-PCR was not able to differentiate between clinical and FEC and there was not a clear association between VG pattern and ERIC-PCR profile. This may suggest independent VG patterns

within clonal groups or clusters, highlighting the potential of previous genetic transfers between different strains.

Phylogenetic analysis using whole genome sequencing (WGS) is recommended to provide rapid, accurate and valuable genetic data (Franz et al., 2014; Oulas et al., 2015; Wang et al., 2016). This data has the discriminatory power for future APEC pathogenesis studies and epidemiological investigations that will improve the bird's welfare and help in the development of prevention and control measures against avian colibacillosis (Wang et al., 2016). The ability of WGS to compare and link with international bacterial typing databases is considered one of the advantages in comparison with other genetic typing methods such as ERIC where data is not standardised and cannot be compared between laboratories.

The frequency of APEC related VGs among *E. coli* strains vary and VG profile may be influenced by several factors such as geographical locations, season, bird immune status, variation in the sampling sites, different husbandry and vaccination protocols (Johnson *et al.*, 2008b; Qabajah *et al.*, 2014; Wang *et al.*, 2013a). Therefore, APEC VG markers specific to Australian conditions need to be defined in order to better identify APEC and reduce the economic losses associated with the disease in the poultry industry.

Statistical analysis of the CEC and FEC VG characterisation showed a significant association ($p < 0.05$) between clinical *E. coli* and 15 VGs: *cvi/cvaC*; *iroN*; *iss*; *iutA*; *tsh*; *fimC; papC*; *papEF*; *ireA*; *feoB; ibeA*; *vat;*, *hlyF*; *ompT* and *astA*. The presences of each of these VGs were more than twice as likely to be detected among CEC than among the FEC in the current study. The positive association between avian colibacillosis and the presence of these VGs either individually or in different combinations has been previously documented in other parts of the world (Circella *et al.*, 2012; Delicato *et al.*, 2003; Guastalli *et al.*, 2013; Jeong *et al.*, 2012; Johnson *et al.*, 2008b; Rodriguez-Siek *et al.*, 2005a).

The current study reported 93.1% of CEC carried the five VGs (*iutA, iss, ompT, iroN* and *hlyF*) that were used in earlier chapters of this thesis to define APEC and were identified by Johnson *et al.* (Johnson *et al.*, 2008b) as a group of VGs that could be screened to predict APEC. Studies in Brazil (82% – 95%) and Egypt (90% – 94%) have reported a similar frequency of these five VGs among APEC isolates cultured from lesions of birds with colibacillosis, supporting the selection of the pentaplex-PCR to identify APEC (Dissanayake *et al.*, 2014; Hussein *et al.*, 2013).

The combination of APEC linked VGs that were identified in the current study play roles in various aspects of the extraintestinal pathogenesis of APEC and can be categorised into different groups according to their functions and contribution to the APEC pathogenicity mechanisms (Dziva and Stevens, 2008; Nolan *et al.*, 2013). At different stages of infection, alternative VGs could be involved in the pathogenicity mechanism of APEC, including colonisation (*fimH, fimC, papC, papEF* and *tsh*), invasion (*ibeA, vat*), iron acquisition (*iutA, iroN, IreA, feoB*), serum complement resistance (*iss*) and putative iron transport (*sitA*). Resistance to serum complement can be facilitated by *iss*, which encodes for a lipoprotein of the bacterial outer membrane (McPeake *et al.*, 2005; Mellata *et al.*, 2003; Nolan *et al.*, 2013; Rodriguez-Siek *et al.*, 2005a; Sorsa *et al.*, 2003; Tivendale *et al.*, 2004; Vandekerchove *et al.*, 2005). In the current study the prevalence of *iss* in APEC was 93.1%, in other countries such as the USA (80.5%), Germany (82.7%), Spain (91%), Egypt (72%) and Brazil (51%) prevalence varied (De Carli *et al.*, 2015; Ewers *et al.*, 2004; Hussein *et al.*, 2013; Johnson *et al.*, 2008b; Sola-Gines *et al.*, 2015).

The current study reported a significant association between CEC and adhesin genes, with 86.2% and 44.8% prevalence of *fimC* and *papC* genes, respectively. These genes mediate the adherence of *E. coli* to host epithelial cells to enable colonisation (Pourbakhsh *et al.*, 1997). The reported prevalence of *fimC* in this study (86.2%) is similar to published data from Germany (92%), Brazil (86%) and Korea (90.1%) from *E. coli* obtained from chickens with colibacillosis (Ewers *et al.*, 2004; Jeong *et al.*, 2012; Knobl *et al.*, 2012). The positive association of *papC* as well as *iss, tsh, cva/cvi* and *vat* with APEC were found in *E. coli* isolates sourced from chickens affected with colibacillosis in Germany. Ewers *et al.* (2005) found that those five VGs as well as *iucD, irp2* and *astA* could identify and differentiate APEC from AFEC.

The prevalence of the *papC* gene, which enables the adherence and the survival of *E. coli* in the internal organs (Pourbakhsh *et al.*, 1997), was 44.8% in the current study. Lower prevalence were found in *E. coli* isolates sourced from broiler chickens with colibacillosis from the United States of America (USA) (24.8%) and Germany (22.7%) (Ewers *et al.*, 2004; Johnson *et al.*, 2008b).

There was a significant association between CEC isolates and the *tsh* gene which contributes mainly to the respiratory form of avian colibacillosis by mediating bacterial adhesin to the respiratory tract (Dozois *et al.*, 2000; Maurer *et al.*, 1998; Servin, 2005; Stehling *et al.*, 2003). In this study, 55% of CEC isolates carried this gene. Other studies have also identified this association (Dozois *et al.*, 2000; Maurer *et al.*, 1998), though these studies reported prevalence of *tsh* as low as 19% (Dozois *et al.*, 2003) and as high as 85.3% in CEC (Delicato *et al.*, 2003; Zhao *et al.*, 2005).

The *vat* gene encodes for the Vat protein with has cytotoxic activity for the chicken embryo fibroblast and kidney cells (Parreira and Gyles, 2003). Several studies have reported that the *vat* gene is encoded in the APEC PAI and occurs in a higher frequency among APEC compared with AFEC (Ewers *et al.*, 2004; Ewers *et al.*, 2005).

The current study also found that seven pTJ100-plasmids related genes (*iroN, traT, iutA, sitA, iss, cvaC* and *tsh*) were widely distributed among clinical isolates (Johnson *et al.*, 2008b; Rodriguez-Siek *et al.*, 2005a; Zhao *et al.*, 2009). This finding reflects the role of plasmids in the pathogenicity and the transfer of certain VGs among the pathogenic as well as commensal *E. coli* isolates. This highlights the need for further molecular studies to identify the role of plasmids and other forms of horizontal gene transfer in APEC pathogenicity (Won *et al.*, 2009; Zhao *et al.*, 2009).

The following VGs: *papG; irp2; chuA; kpsMT11; kpsMT K1; cbi; cma; traT*; *fimH*; *afa-drab; fyuA; sfaS; iucD; neuC* and *maxI* were detected at a similar prevalence among both CEC and FEC. The presence of those APEC linked VGs in FEC indicate that FEC may act as reservoir for some VGs (Janssen *et al.*, 2001; Mokady *et al.*, 2005; Vandekerchove *et al.*, 2005).

In the current study, the absence of the *iucA* gene, which is one of the iron acquisition systems from both the clinical and faecal *E. coli* isolates, differs from the previous Australian study by Tivendale *et al*. (2004). Tivendale *et al*. (2004) identified the presence of *iss*, *tsh* and *iucA* in plasmids found in *E. coli* strains isolated from birds with severe colibacillosis in Australia. This difference in the detection of *iucA* between the studies could be correlated with variation of the levels of virulence of the *E. coli* isolates in the Tivendale *et al*. (2004) study in comparison with the current study. Tivendale *et al*. (2004) demonstrated the ability of the three most virulent APEC strains to amplify the *iucA* gene in comparison with less virulent APEC strains. Other studies have also suggested that *iucA* plays an important role in the pathogenicity of APEC (Ling *et al.*, 2013; Xiong *et al.*, 2012).

Despite the fact that the large number of VGs suggested in the current study to define APEC are more sensitive than previous protocols, there are some limitations. The fact that three PCRs were needed to investigate the presence of APEC makes it not a practical approach and highlights the need to use faster and more accurate methods to screen for the presence of the APEC associated VGs. A number of methods may be suitable for multiplex analysis in one assay, such as Luminex that allows multiplexing of up to 100 analytes in a single well of a microtiter plate (Sheikholvaezin, et al, 2011). In addition to PCR and genotyping methods, DNA microarrays are able to characterise bacteria by detecting large numbers of VGs and antimicrobial resistance genes (Anjum et al., 2007). Furthermore,

microarrays can identify genomic variations between different strains of bacteria. With the advances seen in recent years, whole genome sequencing will become feasible for practical routine screening to rapidly characterise and identify large numbers of virulence genes (Fratamico et al., (2016; Pallen, 2016).

Although the current thesis identified a set of APEC related VGs (*iroN, iss, iutA, tsh, fimC, papC, papEF, vat, hlyF, astA, ibeA, feoB, ireA, cvi/cvaC* and *ompT*) that were more likely associated with CEC, time limitations restricted further work into the pathogenicity of these VGs. The inability to screen all 237 isolates of *E. coli* collected during this thesis and the fact that FEC isolates were obtained from poultry farms located in a restricted geographically area (SEQ) during a limited time were also considered as limitations. However, the testing of representative isolates from 88 clusters, and the fact that the CEC isolates were collected from diverse geographical locations were strengths of the current research.

6.7 Conclusion

In conclusion, there was a wide diversity of VG profiles detected among CEC and FEC cultured from Australian broiler chickens. Overall, the VG profiles were similar with those previously reported in other countries. The detection of VGs among the faecal isolates may suggest their role as a reservoir for ExPEC-associated VGs. The current study proposes an APEC molecular screening tool composed of VGs (*iroN, iss, iutA, tsh, fimC, papC, papEF, vat, hlyF, astA, ibeA, feoB, ireA, cvi/cvaC* and *ompT*) that were significantly more likely to be found in the avian *E. coli* strains sourced from chickens with colibacillosis in Australia. Five of these VGs were ones that had been selected earlier in this thesis to identify APEC, and their selection as an APEC marker has now been validated. VGs are often located on plasmids, such as PTJ100 related genes *iroN, traT, iutA, sitA, iss, cvaC* and *tsh*, which highlights the association between the pathogenicity of avian *E. coli* strains and the possession of MGE. Further studies are required to investigate the possibility of utilising these VGs in the field and in diagnostic laboratories to identify avian colibacillosis in Australia. Rapid detection of avian colibacillosis could minimise the economic losses and welfare effects associated with the disease. A study to investigate the role of these VGs and their contribution to the pathogenicity of avian pathogenic *E. coli* is also recommended.

Chapter 7: Discussion

7.1 Introduction

The overarching aim of this thesis was to gain a better understanding of the epidemiology of avian pathogenic *Escherichia coli* (APEC) in Australian broiler flocks, and the potential risk factors associated with higher carriage of APEC in Australian broilers. Of particular interest was the identification of virulence genes (VGs) and how these genes could be used to improve the identification of this pathotype in Australia. In addition, the ability to diagnose APEC directly from faeces by detecting a particular set of VGs was investigated. In this thesis, *E. coli* isolates were categorised based on the health status of the bird from which they were cultured and their carriage of five APEC-associated VGs: *iss* (increase serum survival); *ompT* (outer membrane proteinase); *hlyF* (putative avian hemolysin); *iroN* (salmochelin siderophore receptor) and *iutA* (aerobactin receptor). *E. coli* containing these five VGs were previously identified by Johnson *et al.* (2008b) and others as having a strong association with avian colibacillosis.

Prior to this research, little was known about APEC in Australia, with only two investigative studies on the prevalence of APEC-associated VGs in *E. coli* cultured from healthy chickens and chickens with colibacillosis having been undertaken (Obeng *et al.*, 2012; Tivendale *et al.*, 2004). Neither study differentiated APEC from avian faecal *E. coli* (AFEC), nor was there any data in regards to the prevalence of APEC carriage in healthy chickens or associated risk factors. Very few studies had investigated the antimicrobial susceptibility of commensal and clinical *E. coli* isolates sourced from broiler chickens (Abraham *et al.*, 2015; Obeng *et al.*, 2012; Shaban *et al.*, 2014) and none had compared the susceptibility of clinical and faecal *E. coli* isolates. Investigations of the prevalence of plasmid replicons and their associations with antimicrobial resistance was absent from all studies.

A pilot study (Chapter 3) evaluated methods for DNA extraction directly from cloacal swabs or faeces of broiler chickens and screened faecal DNA, as well as DNA from isolated *E. coli*, for the five APEC-associated VGs. This pilot study identified the most effective method for DNA extraction from avian faeces; defined as yielding high quantity and quality DNA. The presence of the five VGs previously identified as significant in APEC were identified in Australian isolates, validating the use of the pentaplex-PCR published by Johnson *et al*. (Johnson *et al.*, 2008b). This pilot study also confirmed healthy chickens only harbour *E. coli* in their cloaca/faeces and intestines, and not in other internal organs. As screening for VGs in DNA extracted directly from faecal samples could not

confirm the presence of APEC, a cross-sectional study (Chapter 4) was conducted, which cultured *E. coli* from faecal samples to then screen for VGs to confirm the presence of APEC. In this chapter, the prevalence of carriage of APEC among healthy commercial broiler chickens was estimated, and potential risk factors identified. Furthermore, the association between antimicrobial susceptibility, phylogenetic group, virulence and plasmid replicon profiles of clinical *E. coli* (CEC) and faecal *E. coli* (FEC) isolates cultured from Australian commercial broiler chickens was investigated (Chapter 5). Lastly, subgroups of CEC and FEC isolates were screened for additional VGs to further characterise the pathotype of APEC in Australia (Chapter 6).

7.2 Major findings and future directions

7.2.1 Investigating the optimised protocol for molecular screening of avian pathogenic *Escherichia coli* from broiler chickens in Australia (Chapter 3)

Extracting DNA directly from faeces and performing molecular identification of pathogens offers many advantages over conventional cultural methods, which may be time consuming and laborious (Zhao *et al.*, 2014). Molecular methods have high sensitivity, specificity and are generally time efficient (Law *et al.*, 2014) for rapid detection of pathogens. Therefore, the objective of this study was to determine if direct application of molecular techniques to faecal samples could identify APEC, which would save time (compared to screening cultured isolates) and thereby decrease economic losses associated with colibacillosis through more rapid diagnosis and response.

This study found that the repeated bead beating plus column (RBB+C) method extracted a high quantity and quality of DNA from cloacal and faecal swabs from broiler chickens ($p < 0.001$) in comparison to the QIAamp DNA Stool Mini Kit and the Chelex method. DNA yields differed between cloacal swabs and faecal samples, with faecal samples yielding a higher DNA quantity. This finding could be attributed to more faeces being contained in the faecal samples compared to cloacal swabs. There was no variation in the purity of the DNA extracted from faecal samples and cloacal swabs with both producing DNA at a quality suitable for molecular investigations. Therefore, collecting faeces by swabbing the cloaca is an adequate sampling method, causes minimal stress to the bird and little opportunity for sample contamination.

The detection variation of APEC-associated VGs between the DNA extracted from cloacal swabs and/or faecal samples of a chicken in comparison with DNA obtained from the *E. coli* isolates was a particularly interesting finding. All five VGs were detected from DNA extracted directly from faecal and/or cloacal samples from all birds, both healthy birds and birds with colibacillosis. However, when

screening the *E. coli* cultured from cloacal swabs, differences between CEC and FEC were observed, with more VGs detected in *E. coli* isolates cultured from birds with colibacillosis compared to healthy birds ($p < 0.001$).

Escherichia coli failed to be cultured from internal organs (excluding the gastrointestinal tract) of healthy chickens. However, *E. coli* was cultured from the intestine and cloaca of all healthy chickens and chickens with colibacillosis, as well as from organ lesions of chickens with colibacillosis. All (n = 74) of the *E. coli* isolates that were cultured from the lesions of chickens with colibacillosis harboured all five VGs. The primary selection of four or more VGs to identify APEC based on the recommendation of Johnson *et al.* (2008b) was validated by the results obtained in this pilot study. That is, 85% of the *E. coli* isolates (23 of 27) cultured from the faeces of chickens with colibacillosis harboured four or more VGs, while only 17% (5 of 30) of isolates cultured from the faeces of healthy birds harboured four or more VGs. This noteworthy finding also suggests that healthy birds could act as a reservoir for APEC-associated VGs in Australia.

A NCBI BLAST search (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi, accessed 16/01/16) found that at least four other bacterial species, *Salmonella enterica, Klebsiella oxytoca, Kluyvera intermedia* and *Enterobacter cloacae* may also harbour these APEC-associated VGs. These bacterial genera are often found in the intestines of chickens (Amit-Romach *et al.*, 2004; Pan and Yu, 2014) suggesting that the VGs of interest in this study may not necessarily be associated with APEC only. Thus, further research is warranted in order to gain a better understanding of their distribution.

7.2.2 Investigating the prevalence of carriage of avian pathogenic *Escherichia coli* from healthy commercial broiler chickens in South East Queensland, Australia and associated risk factors (Chapter 4)

This Chapter estimated prevalence of APEC in commercial broiler chickens, and investigated the possible associations between the detected APEC prevalence and biosecurity measures, flock health and management practices. Methodology of APEC prevalence estimation was validated in Chapter 3. A cross-sectional study involving healthy commercial broiler chickens allowed the comparison and identification of many different potential risk factors simultaneously.

Thirty four percent of the cultured *E. coli* isolates (751 of 2200) were classified as APEC. All of the tested farms (n = 40) were APEC positive and 63% (252 of 400) of birds were identified as APEC positive. APEC-associated VGs (*iss*, *hlyF*, *ompT*, *iroN* and *hlyF*) were commonly detected in *E. coli*

cultured from the faeces of healthy commercial broiler chickens. Therefore, healthy birds may be a reservoir for APEC-associated genes.

The analysis of risk factors revealed many similar contributors associated with the carriage of APEC in Australia compared to overseas studies (Vandekerchove *et al.*, 2004a; Wang *et al.*, 2013b). The risk factors that were significantly associated with higher APEC prevalence in Australia included using water wells as a source of drinking water; not having shower facilities available for farm visitors, distances greater than 20 m between the car park and the poultry shed; not applying water line disinfections after each flock cycle and the presence of wild birds within 50 m of the poultry shed. The use of chlorine combined with automatic filtration of drinking water was identified as a protective factor and reduced farm-level APEC prevalence.

Water is considered a substantial source of various infections on poultry farms (Amaral, 2004; Saidy *et al.*, 2015). Seventy five percent of the farms (n = 30) in this study used treated, reticulated water as the only source of drinking water for chickens, 7.5% of farms (n = 3) used water wells as the only source of drinking water, while 17.5% of farms (n = 7) used a combination of town and dam water. If the same water sources are used to supply the entire farm stock, any contamination with pathogens would result in widespread infection. Although a zero bacterial level is recommended in the drinking water, less than 100 bacteria per millilitre of drinking water is acceptable (Watkins, 2008). The detection of more than 50 coliform bacteria per millilitre reflects faecal contamination of the water (Watkins, 2008). In this study, water wells as a source of drinking water increased the prevalence of APEC by six-fold, which is suggestive of contamination.

It is important to explore where such contamination might originate; at the source of the water well or during the transport of the water to the poultry sheds. Contamination may occur through droppings of wild birds, rats or other animals if the water source is not covered or supply lines not adequately cleaned. Furthermore, poor well design and construction and location of wells in close proximity to sewerage systems might also increase the risk of water contamination (Amaral, 2004). Shallow, aged and/or poorly maintained water wells can lead to contamination (Saidy *et al.*, 2015) therefore, these should be investigated and any potential issues rectified.

This study revealed that failure to disinfect the water lines between each flock contributed to the increased prevalence of APEC by more than two-fold. Avian pathogenic *E. coli* has been previously detected in standing water sources on poultry farms (Amaral, 2004; Pandey *et al.*, 2014), consequently, adequate water treatment is paramount (Pandey *et al.*, 2014; Saidy *et al.*, 2015). Dhillon

and Jack (1996) reported that adding chlorine to drinking water provided control in the spread of avian colibacillosis and decreased bird mortality associated with the disease. Filtration of the water source removes insoluble particulates and further reduces contamination by filtering microorganisms of a certain size from the water. Filtration also helps to prevent dripping and obstruction of the waterline (Folorunso *et al.*, 2014). Seventy percent of farms (n = 28) treated drinking water with chlorine alone, whilst 5% of farms (n = 2) used chlorine in combination with automatic filtration. There was a significant decrease in the prevalence of APEC on-farms that incorporated both chlorination and filtration into their management practices, supporting the benefits of this technique as recommended by Saidy *et al*. (2015) and Watkin *et al*. (2008).

Identifying drinking water as a significant risk factor for contamination enables management mechanisms to be put in place in order to reduce the prevalence of APEC. These include chlorination and filtration of drinking water, covering of water wells and all water sources, as well as cleaning and disinfecting tanks and water lines between each flock. Applying appropriate management measures such as these are essential in disease control on poultry farms (Henry *et al.*, 2011; Nather *et al.*, 2009).

Humans may be either directly or indirectly involved in the introduction and transmission of APEC between farms and the poultry sheds. A distance greater than 20 m between the car park and the shed which can relate to overall design of the farm and human movement, a lack of shower facilities and/or people not showering prior to entering the sheds were risk factors for increased incidence of APEC carriage. It has been highlighted previously that APEC might be common in the surrounding farm environments (Guenther *et al.*, 2011) and that people entering poultry sheds can introduce APEC from the external environment (Anza *et al.*, 2014). Further studies, which investigate the importance and benefits of spatial containment (i.e. distance to car park) and personal hygiene between sheds on disease prevention, have shown these to be crucial (Johnson *et al.*, 2012; Maluta *et al.*, 2014).

Wild birds have been shown to be vectors that can introduce and disseminate APEC on poultry farms (Vandekerchove *et al.*, 2004a). Common sequence types and strains of *E. coli* have been isolated from wild birds' faeces and broiler chickens (Elmberg *et al.*, 2017; Shobrak and Abo-Amer, 2014). In the current study, a higher prevalence of APEC was found to be associated with the presence of wild birds outside the poultry sheds. Furthermore, the presence of wild birds may induce stress and create unfavourable housing conditions, which are also known to be predisposing factors for APEC (Vandekerchove *et al.*, 2004a).

The association between APEC prevalence and the potential risk factors identified in the current thesis highlights the need for further investigation across more regions and with more farms representing different companies to address the associations between risk factors and APEC using different study designs such as a cohort study. The data presented in this thesis indicates a need for careful reassessment of current biosecurity programs and the adoption of a higher level of biosecurity on poultry farms in Australia.

Further investigations exploring the location of the farms, structure of the farm buildings, water sources and surrounding environment are also recommended in order to investigate how these potential risk factors contribute to APEC carriage so that a holistic approach to farm-wide disease prevention and controls can be developed and stringently applied.

7.2.3 Investigation of the prevalence of antimicrobial susceptibility, plasmid replicon typing, phylogenetic grouping and virulence potential of *Escherichia coli* cultured from Australian broiler chickens with and without colibacillosis (Chapter 5)

This study determined the prevalence and association of antimicrobial-resistance profiles with phylogenetic groups, APEC-associated VGs and plasmid replicon profiles among and between CEC and FEC isolates cultured from Australian commercial broiler chickens. The finding of low antimicrobial resistance among all *E. coli* isolates cultured from healthy birds and birds with colibacillosis reflects the strict regulations of antimicrobial registration applied to the poultry industry in Australia (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015; Australian Veterinary Association, 2017). Twenty six percent of both the CEC and FEC were susceptible to all twenty tested antimicrobial agents. All isolates were susceptible to amikacin, ceftazidime, imipenem and florfenicol, which mirror the nil usage of these critically important antimicrobials in the Australian poultry industry (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015; Australian Veterinary Association, 2017).

As expected, the most commonly found resistances were to older antimicrobials that are still in common use in the broiler industry. Resistance in CEC and FEC were identified, respectively, to sulfamethoxazole/trimethoprim (44% and 40%) introduced in 1936; tetracycline (54% and 28%); introduced in 1948 followed by ampicillin (28% and 31%) introduced in 1961 (Tadesse *et al.*, 2012). Previous Australian studies, which investigated antimicrobial resistance in commensal organisms in poultry, identified similar resistances (Abraham *et al.*, 2015; Department of Agriculture, Fisheries and Forestry (DAFF), 2007; Obeng *et al.*, 2012; Obeng *et al.*, 2014). The widespread and continuous

use of these antimicrobials in Australia, as well as residual environmental contamination, may explain the persistence of antimicrobial resistance among *E. coli* isolates sourced from broiler chickens (Walsh and Fanning, 2008). A number of studies have highlighted the fact that resistance to one antimicrobial can result in resistance to other classes of antimicrobials in *E. coli* (Dragana *et al.*, 2016; EFSA European Food Safety Authority, 2015; Velhner and Milanov, 2015). The use of certain antimicrobials will select for resistant organisms and the potential for cross-resistance across different antimicrobial classes is acknowledged, for example, the overlapping of targets and drug efflux (Boerlin and Reid-Smith, 2008). Resistance genes associated with mobile genetic elements (MGE), such as integrons or plasmids, are linked, and the use of any antimicrobial that is a substrate for one of the resistance mechanisms will co-select for resistance to other antimicrobials (Rankin *et al.*, 2011).

Multidrug resistance, (MDR) was identified in 18% of the clinical isolates and 14% of the FEC in the current study. The most common resistances reported in multidrug resistant CEC were to the well established antimicrobials sulfamethoxazole/trimethoprim, aminoglycosides, β-lactams and tetracylines (Tadesse *et al.*, 2012; Zhao *et al.*, 2005). The potential public health risk from the emergence of MDR among *E. coli* from food-producing animals is considered one of the biggest issues facing both human and veterinary medicine (Cogliani *et al.*, 2011). The detection of strains resistant to antimicrobials commonly being used to treat poultry should alert the poultry industry of the need for routine antimicrobial susceptibility testing of isolates, rather than simply using antimicrobials empirically. Reviews of treatment protocols applied across the Australian poultry industry are highly recommended so as to improve animal welfare and ensure public health is protected.

Overall, the CEC isolates were more resistant to antimicrobials in comparison to FEC isolates. The CEC isolates were cultured from birds with colibacillosis that might have been exposed to antimicrobials prior to sampling. However, history of prior antimicrobial use was not available.

Not all antimicrobial resistance detected in the current study reflects antimicrobials used in the Australian poultry industry. A major finding in this study was the identification of a low-level resistance to critically important antimicrobials, extended-spectrum cephalosporins (ESCs) and fluoroquinolones (FQs) among both the clinical and faecal isolates. Australia is the only country to have never permitted the use of fluoroquinolone antimicrobials in food-producing animals (Cheng *et al.*, 2012), and the benefits are evident. Furthermore, ESC are not registered for use in poultry in Australia (Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015). However,

resistance to FQs and ESCs have been reported previously in Australia in other food-producing animals (Abraham *et al.*, 2014; Abraham *et al.*, 2015).

The majority of sequence types found among the FQ and ESC resistant isolates in the current study (ST354, ST10, S648 and ST 224) have been previously described globally among humans as well as animals (Abraham *et al.*, 2015; Aizawa *et al.*, 2014; Dahms *et al.*, 2015; Day *et al.*, 2016; Dissanayake *et al.*, 2014; Kim *et al.*, 2011; Maluta *et al.*, 2014; Manges and Johnson, 2012; Sola-Gines *et al.*, 2015; Vignaroli *et al.*, 2012). Nonetheless, four of the FQ resistant FEC isolates belonged to the same sequence type (ST354), but were cultured from different geographical regions indicating that they did not originate from one shed. However, the same company owned these farms. Thus, birds originated from the same breeding stock and all followed the same management and biosecurity protocols. Therefore, the resistant isolates may not be truly independent. Sampling population of birds from other companies, in other geographical regions and breeding stock is required to determine how widespread resistance is to these critically important drugs in Australia and the clonal linkage across companies, sheds and locations.

Alternatively, the detection of FQ and ESC resistant bacteria without selection pressure could imply that the bacteria have been introduced into the shed from outside (Alves *et al.*, 2014; Kmeť *et al.*, 2013; Smith *et al.*, 2014). The finding of host restricted sequence types (ST2705 and ST6053) in this study, which have been previously isolated from poultry and wild birds (Dahms *et al.*, 2015; Day *et al.*, 2016; Jones-Dias *et al.*, 2015) supports this hypothesis. The globally disseminated sequence types may have been introduced into the sheds by people, fomite or other animal movement.

Wild birds and animals such as vermin and rodents can disseminate resistant *E. coli* (Guenther *et al.*, 2010; Laube *et al.*, 2014). FQ and ESC resistant *E. coli* strains have been identified in wild birds (Allen *et al.*, 2010; Literak *et al.*, 2010) and from Australian wildlife rehabilitation centres (Blyton *et al.*, 2015). Chapter 4 of this thesis identified wild birds as a potential risk factor associated with increased APEC carriage in healthy birds. However, many factors such as strict farm biosecurity will minimise the contact between wild bird and poultry inside the sheds, thus decreasing the transmission of strains. Humans may also act as a direct or indirect (via faeces, water, food) transmission source (Marathe *et al.*, 2013).

The current study reported for the first time the detection of $bla_{\text{CMY-2}}$ and $bla_{\text{DHA-1}}$ in ESC resistant APEC in Australia. These genes have been previously found in ESC resistant *E. coli* isolates cultured from chickens and other farm animals (Abgottspon *et al.*, 2014; Cortes *et al.*, 2010; Pitout, 2012;

Schaufler *et al.*, 2015) and from humans worldwide (Deshpande *et al.*, 2006; Halova *et al.*, 2014; Literak *et al.*, 2010; Woodford *et al.*, 2007). Plasmids play a major role in the spread of virulence and resistance genes in *E. coli* and other bacterial species. Despite extensive studies on plasmids in ExPEC and APEC (Carattoli, 2011; Carattoli, 2013; Carattoli *et al.*, 2002), there is limited information about the role and the impact of these plasmids in APEC.

The presence of the following plasmids in this study were reported in both clinical and faecal isolates: IncHI1; IncHI1; IncY; IncI; IncFIA and IncN. However, it was also found that the most common plasmid replicon types observed in CEC and FEC were IncFIB (90% and 64%, respectively) and IncFrep (64% and 61%). Johnson *et al.* (2007) also reported plasmid IncFIB type as the most prevalent among *E. coli* isolates of avian, human and poultry meat followed by IncI1. The virulence and resistant genes found in APEC have been detected on IncFIB plasmids, such as pAPEC-1 (Mellata *et al.*, 2012). All plasmid replicons reported in the current study have been previously detected in *E. coli* isolates sourced from the environment, other animals and humans (Bortolaia *et al.*, 2010; Garcia-Fernandez *et al.*, 2008; Kluytmans *et al.*, 2013; Lynne *et al.*, 2012; Seni *et al.*, 2016; Wang *et al.*, 2013a; Woodford *et al.*, 2007).

Commonly, ESC resistance is associated with transferable plasmids that harbour resistance genes such as AmpC β-lactamases. In this study, the plasmid profile for the two ESC resistant isolates did not contain the same plasmid replicons, with one isolate containing IncY and IncI1 plasmid types, while the other contained IncFIA, IncFIB and IncFrep plasmids. There is evidence that some types of plasmid replicons, such as IncI1, can survive in *E. coli* isolates without the antimicrobial selection pressure with little to no fitness cost to the host (Carattoli, 2011; Garcia-Fernandez *et al.*, 2008).

Here, APEC was identified by the possession of four out of the five VGs, which have been linked with IncFIB plasmids (also known as ColV plasmids) (Johnson *et al.*, 2006). Several studies have found that ColV plasmids contain a highly conserved region that harbors VGs and/or resistance genes that contribute to the virulence of APEC (Johnson and Russo, 2002; Johnson *et al.*, 2006; Johnson *et al.*, 2005; Kariyawasam *et al.*, 2006a).

IncA/C plasmids are associated with horizontal gene transfer and can be transferred between *Salmonella* and *E. coli* from food-producing animals, the environment and humans (Mataseje *et al.*, 2010), and are associated with MDR and the AmpC $bla_{\text{CMY-2}}$ (Fernandez-Alarcon *et al.*, 2011). Only three isolates harboured the IncA/C plasmid, they did not exhibit multidrug or ESC resistance, but there is a potential for acquired resistance as the plasmid is in the population of *E. coli* on the farms.

In this thesis, 96% of the CEC isolates harboured all five VGs in comparison with 36% of the FEC isolates ($p < 0.001$). Four percent of the clinical isolates (n = 2) contained none of the five VGs compared with 19% of the FEC isolates. Interestingly, the two clinical isolates that contained no VGs were both FQ resistant, supporting the theory that these represent low pathogenic opportunistic isolates. Similar findings have occurred in earlier studies in other parts of the world where low prevalence of VGs were detected in FQ resistant CEC isolates (Horcajada *et al.*, 2005; Huang *et al.*, 2009; Kawamura-Sato *et al.*, 2010). These isolates may have been opportunistic pathogens that acquired FQ resistance (Drews *et al.*, 2005; Vila *et al.*, 2002) or they may be CEC isolates that lost their VGs after they developed mutations in the quinolone resistance-determining region (QRDR) (Horcajada *et al.*, 2005; Moreno *et al.*, 2006; Sawma-Aouad *et al.*, 2009). Furthermore, the virulence profile of the AmpC β lactamase harbouring *E. coli* isolates also varied. One of the ESC resistant *E. coli* isolate harboured none of the CEC related VGs, while the other ESC resistant isolate harboured all five VGs. The fact that all of the FQ and ESC resistant isolates do not belong to phylogenetic group B2, the most common phylogenetic group of pathogenic ExPEC, could support the theory that they are opportunistic. Further investigation of FQ and ESC resistant *E. coli* using whole genome sequencing technology establishing antimicrobial resistance mechanisms is justified. This might be part of a much larger study, which may explore the distribution of AmpC β-lactamase genes in avian *E. coli* isolates at different geographical locations and how similar Australian isolates are to overseas strains.

Overall, the current thesis found that the prevalence of antimicrobial resistance, plasmids and VGs in FEC occur at a lower frequency in comparison with the CEC. However, their presence in faecal isolates suggests that FEC may act as a reservoir for plasmids, antimicrobial resistance and VGs. Interestingly, the current study showed that the faecal isolates exhibited more diversity in their resistance profiles than the clinical isolates, supporting the suggestion that commensal *E. coli* flora are a source of emerging and developing *E. coli* resistant strains and/or resistance genes (da Costa *et al.*, 2013).

Antimicrobial resistance surveillance investigations using structured sampling strategies involving larger sample sizes to represent all food-producing animal species in Australia, which does not rely on laboratory-acquired isolates with limited history, is recommended. The information provided by the surveillance study will be able to identify the antimicrobial consumption in each animal, resistance trends and emergence of pathogens at national levels, thereby highlighting any emerging resistance

characteristics with public health potential. These data will guide and help farm managers and veterinarians in their treatment strategies and ultimately improve bird health, welfare and production.

The VGs and antimicrobial resistance profile detected in the current thesis proposes the need to investigate the role of plasmids in transferring ARGs and VGs among avian *E. coli* isolates and the ability of the plasmids to transfer into other bacteria that are found in the normal gut flora of birds.

7.2.4 Virulence associated genes in faecal and clinical *Escherichia coli* isolates cultured from broiler chickens in Australia (Chapter 6)

This final study aimed to produce a more in-depth understanding of the range of ExPEC-associated VGs present in *E. coli* circulating in commercial broiler chicken flocks and derive a set of VGs which could be used to better define and identify APEC in Australia. Thirty four of 35 VGs were detected in Australian *E. coli* isolates, with prevalence ranging from 3.4% (*focG*) among faecal isolates to 100% (*astA*) among clinical isolates. The following VGs were identified with significant (with *p-value* less than 0.05) prevalence among CEC: *astA* (100%); *foeB* (96.6%); *iroN* (93.1%); *ompT* (93.1%); *iss* (93.1%); *iutA* (93.1%), *hlyF* (93.1%); *vat* (89.7%), *fimC* (86.2%), *cvi/cvaC* (79.3%), *tsh* (55.2%); *ireA* (51.7%); *papC* (44.8%); *papEF* (41.4%) and *ibeA* (37.9%). Previous studies have shown that these VGs are predominantly found on plasmids (Collingwood *et al.*, 2014; da Rocha *et al.*, 2002; Johnson *et al.*, 2005; Nakazato *et al.*, 2009). The presence of ExPEC-associated genes among the FEC and CEC isolates in the current study supports the hypothesis that clinical and faecal avian *E. coli* have zoonotic potential.

Numerous studies (Ewers *et al.*, 2004; Johnson *et al.*, 2008b; McPeake *et al.*, 2005; Rodriguez-Siek *et al.*, 2005a) have compared CEC isolates sourced from birds affected with colibacillosis with faecal isolates from clinically healthy birds. Although the current study identified a set of VGs that were significantly associated with CEC isolates, none of the VGs were associated exclusively with CEC isolates. These VGs were also found in FEC isolates, though at a much lower prevalence.

In conclusion, the five VGs that were used to define APEC in the United States by Johnson *et al.* (2008b) can be used to identify APEC in Australian conditions. However, in addition to those five VGs, the current study identified ten more VGs (*tsh, fimC, papC, papEF, vat, astA, ibeA, feoB, ireA* and *cvi/cvaC*) that were significantly associated with APEC. Further investigation with a larger sample size is recommended to validate the selection of these 15 VGs as future markers for APEC detection in the field and in the veterinary diagnostic laboratory. Using the five initially identified VGs, the detection of APEC directly from faecal material proved to be unreliable. However, utilising

the repeated bead beating plus column (the recommended DNA extraction method validated in the first study) in combination with a more stringent subset of the ten further identified VGs would be a useful tool in investigating the possibility of rapid methods based on detection of APEC directly from faecal material. Furthermore, assessment of the role and contributions to the pathogenicity of avian pathogenic *E. coli* of the ten VGs is warranted.

7.3 General discussion

The current thesis reported resistance to ESCs and FQs from *E. coli* isolates sourced from broiler chickens in Australia for the first time. Extended-spectrum cephalosporin resistance was encoded by $bla_{\text{CMY-2}}$ and $bla_{\text{DHA-1}}$ genes, which have been previously reported in other animals and other bacterial species in Australia (Abraham *et al.*, 2014; Cherif *et al.*, 2015; Rogers *et al.*, 2014; Sidjabat *et al.*, 2007), but not previously in poultry. The $bla_{\text{CMY-2}}$ gene is the most frequently detected AmpC β-lactamase in food-producing animals worldwide (Borjesson *et al.*, 2013; Bortolaia *et al.*, 2010; Cherif *et al.*, 2015; El-Shazly *et al.*, 2017; Ewers *et al.*, 2012; Jacoby, 2009). Furthermore, a large number of studies have recently demonstrated a widespread increase in the prevalence of plasmid-mediated AmpC *E. coli* isolates harbouring the $bla_{\text{DHA-1}}$ gene from livestock (El-Shazly *et al.*, 2017; Lee *et al.*, 2010; Maamar *et al.*, 2016; Mataseje *et al.*, 2010; Pacholewicz *et al.*, 2015; Rasmussen *et al.*, 2015; Rayamajhi *et al.*, 2010; Rayamajhi *et al.*, 2008; Tagg *et al.*, 2015; Yan *et al.*, 2006).

The detection of the plasmid-mediated genes $bla_{\text{CMY-2}}$, $bla_{\text{DHA-1}}$ and *qnrS1* indicates the existence of a potential animal reservoir for these genes in poultry in Australia. This is of public health significance as these genes that encode for critically important antimicrobial resistance can be transmitted to other bacterial species by horizontal gene transfer (HGT) (Allen *et al.*, 2013; Boerlin and Reid-Smith, 2008; da Costa *et al.*, 2013; Davies, 1994; Martinez-Medina *et al.*, 2009). Further investigations are needed to identify the plasmids associated with the ARGs among *E. coli* isolates sourced from chickens in Australia.

The two host restricted sequence types ST2705 and ST6053 found in the current thesis may have originated from the breeder flock (vertical transmission) or via horizontal transmission (Dahms *et al.*, 2015; Day *et al.*, 2016; Jones-Dias *et al.*, 2015). The globally disseminated STs (ST354, ST10, S624, ST57 and ST224) suggest horizontal transmission sources that are likely external to the production facility. The majority of these sequence types have been previously described and are globally distributed amongst humans as well as animals (Fernandes *et al.*, 2016; Kim *et al.*, 2011; Maluta *et al.*, 2014; Sola-Gines *et al.*, 2015). This may suggest that the resistant isolates originated from humans

(Meyer *et al.*, 2010), wild birds (Smith *et al.*, 2014) and/or from the farm environment such as water sources (Alves *et al.*, 2014; Sjolund *et al.*, 2008) and then gained access to the poultry production environment.

The horizontal transmission to the sheds from the external environmental may be facilitated by the presence of wild birds, as a number of studies have reported that wild birds can act as reservoirs and vectors in the global dissemination of APEC-associated VGs and ARGs (Belanger *et al.*, 2011; Foster *et al.*, 2006; Wang *et al.*, 2017). If biosecurity is poor on-farm, faecal droppings of wild birds may contaminate poultry feed and drinking water (Fogarty *et al.*, 2003; Lévesque *et al.*, 2000). Resistance to disinfectants often occurs in conjunction with ARGs (Bragg *et al.*, 2014; Wales and Davies, 2015). Therefore, resistant *E. coli* isolates may be able to survive inside the poultry shed despite the usage of sanitisation products. Further screening and characterisation of *E. coli* isolates from wild bird species, wild animals and the farm environment (Guenther *et al.*, 2011; Radhouani *et al.*, 2014) is required to identify the potential risk pathways for the introduction as well the transmission of APEC and/or antimicrobial resistance genes into the poultry shed.

Biosecurity is critically important - the presence of wild birds and unnecessary human contact on the poultry farm should be minimised to reduce the risk of infection by APEC and other pathogens. This study found that a lack of shower facilities on the farm or people not showering prior to entering the shed were associated with an increase in APEC prevalence. Strict biosecurity measures such as wearing overalls before entering each shed should be in place when human presence cannot be avoided in order to reduce the prevalence of APEC (Tadesse *et al.*, 2012).

The introduction and spread of APEC is not limited to the farm worker, farm visitors play a noteworthy role also. They are a potential risk, especially if they move from one farm to another using their own vehicles, equipment, boots and clothing (Newell *et al.*, 2011). In this study, 80% of the 40-surveyed farms reported that their farm visitors (n = 30) did not wear protective overalls before entering the shed and 17.5% of farms (n = 7) did not have a shower facility on the premises. The lack of protective wear and personal hygiene and/or biosecurity can facilitate the spread of the *E. coli* as well as other infections agents from one shed to another or, introduce agents to the shed from the surrounding environment. It is recommended that the poultry companies re-evaluate this policy and incorporate mechanisms to minimise the incidence and spread of infectious agents.

A distance of more than 20 m between car park and shed was also identified as a potential risk factor for APEC. The greater distance relates not so much to the proximity of the car park to the sheds, but

more to the movement of farm workers/visitors around the farm before reaching the entry point to the shed. Combined with a lack of biosecurity procedures this identified risk factor increase the possibility of environmental contamination introduced to the shed via people. It also points to the importance of design and workflow on the farm to avoid introduction of pathogens into the poultry shed. A review of current biosecurity procedures applied on-farm that identifies areas where farm policy needs to modify in regards to visitors is recommended.

The current study identified three risk factors related to poor quality water sources as well as inappropriate water management in regards to treatment and sanitation of drinking water (Shobrak and Abo-Amer, 2014; Vandekerchove *et al.*, 2004a; Wang *et al.*, 2013b). The identification of water related risk factors suggest that applying strict water biosecurity and management protocols on commercial broiler farms can be improved in Australia and this should contribute to the reduction of the prevalence of APEC within the flock. However, further longitudinal investigation to estimate the carriage of APEC in one-day-old chickens through to slaughter, and measures of carcass rejections in the same flocks at the abattoirs, is necessary to identify associations between the prevalence of APEC in healthy chickens and carcass rejection at the slaughterhouse.

Selecting a specific array of APEC-associated VGs to identify APEC in Australia was difficult due to the lack of regional studies, the large number of VGs associated with APEC and AFEC, as well as the worldwide genetic variations in VGs. This research proposes using VGs (*iroN, iss, iutA, tsh, fimC, papC, papEF, vat, hlyF, astA, ibeA, feoB, ireA, cvi/cvaC* and *ompT*) to identify APEC in Australia. These VGs were significantly associated with avian *E. coli* strains sourced from chickens with colibacillosis. Five of these VGs were those that had been selected in the first chapters of the thesis to define APEC. These five genes have been used worldwide to identify APEC and this study shows they can also be used in Australia. Screening for these and the ten additional VGs will increase specificity. Seven out of the 15 VGs are plasmid related genes, which highlights the association between the pathogenicity of avian *E. coli* strains and the possession of plasmids. Further studies are required to investigate the possibility of utilising these VGs as markers for field practitioners for the early identification of APEC and avian colibacillosis in Australia.

The current study data showed that APEC associated VGs were found in the faecal samples of healthy birds, which could indicate the presence of APEC. Keeping in mind that the presence of these VGs does not automatically mean that those genes are expressed and avian colibacillosis will occur. The occurrence of the disease depends on the interaction outcome between the host, agent and surrounding environment. The pathogenicity of the bacteria depends on the expression of VGs, which has to be

co-ordinated for invasion and disease to occur in the host. Very often, expression involves very complex cascades of component systems and quorum sensing (Thomas and Wigneshweraraj, 2014). However, the early detection of these genes highlights the need to evaluate the priorities of management policies in order to minimise the presence of these genes and associated bacteria in the sheds. The reduction of these bacteria will help to reduce the inhalation risk of dust contaminated with APEC and therefore, reduce respiratory disease that is often followed by systematic infections that are characterised by poor flock performance and high mortality.

Rapid detection of APEC directly from faecal samples could minimise the economic losses associated with the disease as more specific control and treatment measures could be applied earlier, e.g. decreasing mortality and the costs associated with inappropriate therapy and/or rejecting of the carcass at the slaughterhouse. This thesis identified and validated an effective method to extract DNA from faeces. The repeated bead beating plus column method effectively extracted PCR quality DNA. The ability to use the RBB+C to detect APEC directly from the faecal samples using the APEC VGs that were suggested in Chapter 6 (*iroN, iss, iutA, tsh, fimC, papC, papEF, vat, hlyF, astA, ibeA, feoB, ireA, cvi/cvaC* and *ompT*) should be investigated. It needs to be noted that five of those VGs (*iutA, iss, ompT, iroN* and *hly*) have been found to be associated with other bacterial species also found in chicken faeces samples.

The genetic diversity of *E. coli* was demonstrated by the variation in the VG prevalence and pattern as well as the resistance profiles of the *E. coli* isolates cultured from the faecal samples of the birds. In Chapter 4, after cloacal culture, five and ten colonies of *E. coli* were selected and screened for the five VGs. The statistical analysis showed good VG profile agreement in the five versus ten *E. coli* isolates picked. Five *E. coli* colonies were identified as the less-resource intensive method that was able to capture the variation of *E. coli* strains (based on VG profile) in the faecal samples. Hence, it is recommended that further studies use five as a sample size. Birds affected with colibacillosis had a higher prevalence of APEC and were more likely to contain less variation in VG carriage in Chapter 3 and 5. However, avian pathogenic *E. coli* was detected in healthy chickens. Therefore, healthy birds can act as reservoir for VGs and ARGs. The fact that healthy birds carry APEC points to the fact that the occurrence of the disease does not only depend on the presence of APEC.

The ability of APEC to cause localised and systemic avian colibacillosis infection by acting either as a primary or secondary disease agent depends on the interaction outcome between the environment, pathogenicity of the agent and the bird's immunity. The ability of APEC to reach the respiratory tract and reach the blood stream causing systematic infection depends on any challenging conditions in the

environment (such as high dust and ammonia level and poor ventilation), stress and immunocompromised birds (due to pre viral and/or bacterial infection) combined with the pathogenicity and the duration of APEC exposure. Addressing the potential risk factors suggested in the current study can enhance farm biosecurity and improve the production system, which may help to prevent the occurrence and spread of avian colibacillosis. The combination of certain bird's breed, strict biosecurity protocols and farm management as well as an effective vaccine may help to prevent the occurrence of avian colibacillosis or reduce the severity of the disease.

Despite the ability to use the large number of APEC associated virulence genes suggested in the current study to detect the presence of APEC on the farm, the regulation of their expression has not been investigated in the current study. Further studies to explore their role in APEC pathogenicity are needed. Furthermore, the fact that three PCRs were needed to investigate the presence of APEC makes it impractical and highlights the need for faster and more accurate method to screen for the presence of these APEC associated virulence genes. Methods using whole genome sequencing (WGS) are recommended to provide rapid, accurate and valuable genetic data (Wang *et al*., 2016; Franz *et al.*, 2014; Oulas *et al*., 2015). These data hae the discriminatory power for future APEC pathogenesis studies and epidemiological investigations that will improve the bird's welfare and help in the development of prevention and control measures against avian colibacillosis (Wang *et al*., 2016).

7.4 Limitations

Defining and identifying APEC is challenging. Many studies simply identify all *E. coli* cultured from birds with colibacillosis as APEC and all cultured from healthy birds as AFEC, irrespective of VG carriage. Many factors potentially limited the outcomes of this thesis. The geographically diverse sources for the clinical samples was one of the strengths of the current thesis, with the CEC profiles in regards to the VGs, AMR, plasmid replicons and phylogenetic groups representing a large geographical area. However, the FEC isolates were obtained from poultry farms, which were restricted geographically (South East Queensland) at a different point of time to the CEC isolates, limiting the strength of the current thesis when comparing the clinical and faecal isolates. Although this study found a small number of resistant isolates, especially ESC and FQ resistant isolates, limited knowledge of historical farm practices, especially in regards to previous antimicrobial usage, were limiting factors.

The risk factor study was conducted as a cross-sectional study, and therefore it was not possible to conclude that there were causal relationships (e.g. whether management interventions lead to increased/decreased APEC prevalence or if management interventions were implemented in response to increased/decreased APEC prevalence). Further studies that use designs in advance of observational studies are thus indicated to better explore the causal pathways.

Another limitation was the lack of clinical break points available for interpretation of antimicrobial susceptibility in *E. coli* isolates cultured from poultry, and as such, interpretations had to be extrapolated from humans or other pathogens. An example for this limitation was highlighted with the resistance detected to cephalothin among the FEC in the current study. Despite the fact that this antimicrobial is no longer used in the poultry industry, it calls into question the validity of using human breakpoints. Studies are required to set recommended clinical and/or epidemiological breakpoints for common bacteria in all animal species.

Although the current thesis identified a set of APEC-associated VGs, their contributions to pathogenicity were not investigated nor were all isolates screened for all VGs. With the increasing availability of whole genome sequencing, this could be investigated in future studies using a large number of isolates.

7.5 Industry application and practical outcomes

The current study offers several practical outcomes for the poultry meat industry. The identification of a suitable DNA extraction method for bird faecal samples is of importance because chicken faeces has additional faecal inhibitors in comparison with other animals (Akhtar *et al.*, 2013; Barnard *et al.*, 2011; Chambers *et al.*, 2001). Identifying pathogens including APEC directly from samples will lead to faster and more effective treatments of disease whereby improving bird welfare and reducing economic losses associated with disease. The identification of a pathogen's resistance profile can reduce costs associated with treatment and guide the veterinarian to effective therapy. The similarity of the sequence types and VG prevalence in *E. coli* isolates sourced from broiler chickens in the current study with data obtained from overseas studies suggest that poultry products could act as a source of ExPEC in humans (Manges and Johnson, 2012). This finding should emphasise the importance of applying strict biosecurity measures and adequate hygiene practices in the poultry farms in order to protect not just poultry health and productivity, but human health and the safety and marketability of the associated food chain.

At the beginning of the current study, no knowledge was available about the possible associations between risk factors and the prevalence of APEC in commercial broiler chickens in Australia. Reporting the prevalence of APEC among healthy commercial broiler chickens helps to gain a better understanding of the epidemiology of APEC in Australian broiler flocks, which will lead to better recognition of sub-clinical carriage and transition to pathogenicity. Furthermore, the current study reported potential risk factors associated with APEC carriage and suggests that addressing these potential factors will lead to better prevention strategies at the broiler farms. Overall, the study has opened the way to launch into a larger Australian wide prevalence study to help determine farms that harbor APEC and the identified risk factors in this study can be addressed to keep APEC out of the farm environment. APEC is not associated with high disease levels in Australia; however, the fact that antimicrobial resistance to critical human antimicrobials has been found makes the potential of resistance transmission an important incentive to implement strategies to prevent transmission of virulence and resistance genes and bacteria. Antimicrobial resistance varies between countries and the identification of resistance points to the need of an antimicrobial resistance surveillance program for Australia. This study has established the approaches and tools to do large-scale studies and implement biosecurity measures.

7.6 General conclusions

In conclusion, the current thesis contributes significantly to better defining APEC and produces preliminary data on the prevalence of APEC in Australian poultry. A set of VGs that can be used to identify APEC in the field or laboratory has been suggested, and identification and validation of an effective method to extract DNA directly from faecal samples was achieved.

Despite ESCs and FQs not being used in the poultry industry in Australia, resistance to these critically important antimicrobials was detected in *E. coli* isolates from broiler chickens. The identification of resistance to these antimicrobials in globally disseminated STs suggests the need for further studies to identify how poultry is included within the broader epidemiology of resistance amongst extraintestinal pathogenic *E. coli*, and the potential significance to public health. This approach will assist in guiding improvements in infection control practices at the broiler farm production level and optimise bird health, welfare and public health outcomes.

Addressing and controlling the potential risk factors identified can possibly reduce the risk of APEC colonisation on commercial poultry farms across Australia. Australian poultry producers should focus on improving biosecurity and applying strict biosecurity protocols at the farm-level to not only

prevent the transmission of APEC but also to prevent the spread of *E. coli* strains or their genes to humans. It is highly likely that the FQ and ESC resistant *E. coli* isolates originated from the birds' external environment. Additionally, the birds can act as a vehicle to transmit antimicrobial resistance genes to the external environment via people visiting the shed and/or be transmitted by their protective clothing.

Data obtained through the current study suggests a diverse population of antimicrobial resistance genes, plasmid replicons, phylogenetic groups and ExPEC related VGs among avian *E. coli*, regardless of the health status of the broiler chickens in Australia. Many of the VGs reported in the current thesis are human ExPEC genes, suggesting that CEC and FEC may represent a potential health risk (Manges and Johnson, 2012). Detection of antimicrobial resistance supports the general concern that avian *E. coli* can act as a reservoir for the resistance genes and spread resistance to humans through the food chain. The direct and indirect linkage of chicken products as a source of ExPEC infections and antimicrobial resistance to humans will continue to challenge the control and treatment of APEC infections. This ongoing emergence of multidrug resistance, points to the need to enhance the biosecurity strategies on-farms as well as food safety measures to control APEC infections.

Chapter 8: References

Aarestrup, F.M., 2005. Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. Basic Clinical Pharmacology and Toxicology 96, pp 271-281.

ABARES, 2011. Agricultural Commodities: The chicken meat industry in Australia, 1 September Quarter 2011, [Available from http://adl.brs.gov.au/data/warehouse/pe_A20110920.01/AC2011.V1.1_AgCommodities1.2.0_LR.pdf]; Accessed March 2014.

Abdallah, H.M., Reuland, E.A., Wintermans, B.B., Al Naiemi, N., Koek, A., Abdelwahab, A.M., Ammar, A.M., Mohamed, A.A. and Vandenbroucke-Grauls, C.M., 2015. Extended-spectrum β-lactamases and/or carbapenemases-producing Enterobacteriaceae isolated from retail chicken meat in Zagazig, Egypt. PLoS One 10, pp e0136052.

Abgottspon, H., Stephan, R., Bagutti, C., Brodmann, P., Hachler, H. and Zurfluh, K., 2014. Characteristics of extended-spectrum cephalosporin-resistant *Escherichia coli* isolated from Swiss and imported poultry meat. Journal of Food Protection 77, pp 112-115.

Abraham, E.P. and Chain, E., 1988. An enzyme from bacteria able to destroy penicillin. Reviews of Infectious Diseases 10, pp 677-678.

Abraham, S., Chin, J., Brouwers, H.J., Zhang, R. and Chapman, T.A., 2012. Molecular serogrouping of porcine enterotoxigenic *Escherichia coli* from Australia. Journal of Microbiological Methods 88, pp 73-76.

Abraham, S., Groves, M.D., Trott, D.J., Chapman, T.A., Turner, B., Hornitzky, M. and Jordan, D., 2014. *Salmonella enterica* isolated from infections in Australian livestock remain susceptible to critical antimicrobials. International Journal of Antimicrobial Agents 43, pp 126-130.

Abraham, S., Jordan, D., Wong, H.S., Johnson, J.R., Toleman, M.A., Wakeham, D.L., Gordon, D.M., Turnidge, J.D., Mollinger, J.L., Gibson, J.S. and Trott, D.J., 2015. First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant *Escherichia coli* in Australian food-producing animals Journal of Global Antimicrobial Resistance 3, pp 273-277.

Abu Al-Soud, W. and Radstrom, P., 1998. Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-Inhibiting samples. Applied and Environmental Microbiology 64, pp 3748-3753.

Abu Al-Soud, W. and Radstrom, P., 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. Journal of Clinical Microbiology 38, pp 4463-4470.

Acar, J.F., Moulin, G., Page, S.W. and Pastoret, P.P., 2012. Antimicrobial resistance in animal and public health: introduction and classification of antimicrobial agents. Revue Scientifique et Technique 31, pp 15-21.

Achtman, M., Heuzenroeder, M., Kusecek, B., Ochman, H., Caugant, D., Selander, R.K., Vaisanen-Rhen, V., Korhonen, T.K., Stuart, S. and Orskov, F., 1986. Clonal analysis of *Escherichia coli* O2:K1 isolated from diseased humans and animals. Infection and Immunity 51, pp 268-276.

Adiri, R.S., Gophna, U. and Ron, E.Z., 2003. Multilocus sequence typing (MLST) of *Escherichia coli* O78 strains. FEMS Microbiology Letters 222, pp 199-203.

Ahmed, A.M., Shimamoto, T. and Shimamoto, T., 2013. Molecular characterization of multidrug-resistant avian pathogenic *Escherichia coli* isolated from septicemic broilers. International Journal of Medical Microbiology 303, pp 475-483.

Ahmed, S., Besser, T.E., Call, D.R., Weissman, S.J., Jones, L.P. and Davis, M.A., 2016. Evaluation of two multi-locus sequence typing schemes for commensal *Escherichia coli* from dairy cattle in Washington State. Journal of Microbiological Methods 124, pp 57-61.

Aidara-Kane, A., 2012. Containment of antimicrobial resistance due to use of antimicrobial agents in animals intended for food: WHO perspective. Revue Scientifique et Technique 31, pp 277-287.

Aizawa, J., Neuwirt, N., Barbato, L., Neves, P.R., Leigue, L., Padilha, J., Pestana de Castro, A.F., Gregory, L. and Lincopan, N., 2014. Identification of fluoroquinolone-resistant extended-spectrum β-lactamase (CTX-M-8)-producing *Escherichia coli* ST224, ST2179 and ST2308 in buffalo (*Bubalus bubalis*). The Journal of Antimicrobial Chemotherapy 69, pp 2866-2869.

Akaike, H. 1973. Information Theory and an Extension of the Maximum Likelihood Principle. In: Petrov, B.N. and Csaki, F. (Eds.), Proceedings of the 2nd International Symposium on Information Theory, Akademiai Kiado, Budapest, pp 267-281.

Akaike, H., 1974. A new look at the statistical model identification. IEEE Transaction Automatic Control 19, pp 716-723.

Akhtar, S., Shakeel, S., Mehmood, A., Hamid, A. and Saif, S., 2013. Comparative analysis of animal manure for soil conditioning. International Journal of Agronomy and Plant Production 4, pp 3360-3366.

Al-Mustafa, Z.H. and Al-Ghamdi, M.S., 2000. Use of norfloxacin in poultry production in the eastern province of Saudi Arabia and its possible impact on public health. International Journal of Environmental Health Research 10, pp 291-299.

Alexander, T.W., Inglis, G.D., Yanke, L.J., Topp, E., Read, R.R., Reuter, T. and McAllister, T.A., 2010. Farm-to-fork characterization of *Escherichia coli* associated with feedlot cattle with a known history of antimicrobial use. International Journal of Food Microbiology 137, pp 40-48.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J., 2010. Call of the wild: antibiotic resistance genes in natural environments. Nature Reviews. Microbiology 8, pp 251-259.

Allen, S.E., Janecko, N., Pearl, D.L., Boerlin, P., Reid-Smith, R.J. and Jardine, C.M., 2013. Comparison of *Escherichia coli* recovery and antimicrobial resistance in cecal, colon, and fecal samples collected from wild house mice (*Mus musculus*). Journal of Wildlife Diseases 49, pp 432-436.

Allocati, N., Masulli, M., Alexeyev, M.F. and Di Ilio, C., 2013. *Escherichia coli* in Europe: an overview. International Journal of Environmental Research and Public Health 10, pp 6235-6254.

Altalhi, A.D., Gherbawy, Y.A. and Hassan, S.A., 2010. Antibiotic resistance in *Escherichia coli* isolated from retail raw chicken meat in Taif, Saudi Arabia. Foodborne Pathogens and Disease 7, pp 281-285.

Alvarez-Fernandez, E., Cancelo, A., Diaz-Vega, C., Capita, R. and Alonso-Calleja, C., 2013. Antimicrobial resistance in *E. coli* isolates from conventionally and organically reared poultry: A comparison of agar disc diffusion and sensi test Gram-negative methods. Food Control 30, pp 227-234.

Alves, M.S., Pereira, A., Araujo, S.M., Castro, B.B., Correia, A.C. and Henriques, I., 2014. Seawater is a reservoir of multi-resistant *Escherichia coli*, including strains hosting plasmid-mediated

quinolones resistance and extended-spectrum beta-lactamases genes. Frontiers in Microbiology 5, pp 426-410.

Amabile de Campos, T., Stehling, E.G., Ferreira, A., Pestana de Castro, A.F., Brocchi, M. and Dias da Silveira, W., 2005. Adhesion properties, fimbrial expression and PCR detection of adhesin-related genes of avian *Escherichia coli* strains. Veterinary Microbiology 106, pp 275-285.

Amaral, L.d., 2004. Drinking water as a risk factor to poultry health. Revista Brasileira de Ciência Avícola 6, pp 191-199.

Amit-Romach, E., Sklan, D. and Uni, Z., 2004. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. Poultry Science 83, pp 1093-1098.

Antão, E.M., Glodde, S., Li, G., Sharifi, R., Homeier, T., Laturnus, C., Diehl, I., Bethe, A., Philipp, H.-C., Preisinger, R., Wieler, L. and Ewers, C., 2008. The chicken as a natural model for extraintestinal infections caused by avian pathogenic *Escherichia coli* (APEC). Microbial Pathogenesis 45, pp 361-369.

Antao, E.M., Wieler, L.H. and Ewers, C., 2009. Adhesive threads of extraintestinal pathogenic *Escherichia coli*. Gut Pathogens 1, pp 1-12.

Anza, I., Vidal, D., Laguna, C., Diaz-Sanchez, S., Sanchez, S., Chicote, A., Florin, M. and Mateo, R., 2014. Eutrophication and bacterial pathogens as risk factors for avian botulism outbreaks in wetlands receiving effluents from urban wastewater treatment plants. Applied and Environmental Microbiology 80, pp 4251-4259.

Arias, M.V.B. and Carrilho, C., 2012. Antimicrobial resistance in animals and in human being. There is reason for concern? Semina-Ciencias Agrarias 33, pp 775-790.

Ariefdjohan, M.W., Savaiano, D.A. and Nakatsu, C.H., 2010. Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. Nutrition Journal 9, pp 23-28.

Armstrong, E.S., Kostrub, C.F., Cass, R.T., Moser, H.E., Serio, A.W. and Miller, G.H., 2012. Aminoglycosides. In: Dougherty, T.J., Pucci, M.J. (Eds.), Antibiotic Discovery and Developments. Springer Boston, US, pp 229-269.

Arné, P., Marc, D., Brée, A., Schouler, C. and Dho-Moulin, M., 2000. Increased tracheal colonization in chickens without impairing pathogenic properties of avian pathogenic *Escherichia coli* MT78 with a *fimH* deletion. Avian Diseases 44, pp 343-355.

Arp, L.H. and Jensen, A.E., 1980. Piliation, hemagglutination, motility, and generation time of *Escherichia coli* that are virulent or avirulent for turkeys. Avian Diseases 24, pp 153-161.

Arsenault, J., Letellier, A., Quessy, S., Normand, V. and Boulianne, M., 2007. Prevalence and risk factors for *Salmonella* spp. and *Campylobacter* spp. caecal colonization in broiler chicken and turkey flocks slaughtered in Quebec, Canada. Preventive Veterinary Medicine 81, pp 250-264.

Ask, B., van der Waaij, E.H., van Eck, J.H., van Arendonk, J.A. and Stegeman, J.A., 2006. Defining susceptibility of broiler chicks to colibacillosis. Avian Pathology 35, pp 147-153.

Aslam, M., Diarra, M.S., Service, C. and Rempel, H., 2009. Antimicrobial resistance genes in *Escherichia coli* isolates recovered from a commercial beef processing plant. Journal of Food Protection 72, pp 1089-1093.

Australian Chicken Meat Federation, 2014. Industry Facts and Figures. [Available from http://www.chicken.org.au/page.php?id=4]; Accessed 15th September 2016.

Australian Commission on Safety and Quality in Health Care, 2013 Australian One Health Antimicrobial Resistance Colloquium Background Paper July [Available from https://www.safetyandquality.gov.au/wp-content/uploads/2013/07/Briefing-paper-for-One-Health-AMR-Colloquium-participants-Final-Jul-2013.pdf]; Accessed 25 November 2016.

Australian Pesticides and Veterinary Medicines Authority (APVMA), 2015. Public Chemical Registration Information System Search (PubCRIS), [Available from https://portal.apvma.gov.au/pubcris]; Accessed May 2016.

Australian Veterinary Association, 2017. Veterinary use of antibiotics highly important to human health, [Available from http://www.ava.com.au/sites/default/files/AVA_website/pdfs/Veterinary%20use%20of%20antibiotics%20critical%20to%20human%20health.pdf]; Accessed July 2017.

Awad, A., Arafat, N. and Elhadidy, M., 2016. Genetic elements associated with antimicrobial resistance among avian pathogenic *Escherichia coli*. Annals of Clinical Microbiology and Antimicrobials 15, pp 1-8.

Babai, R., Blum-Oehler, G., Stern, B.E., Hacker, J. and Ron, E.Z., 1997. Virulence patterns from septicemic *Escherichia coli* O78 strains. FEMS Microbiology Letters 149, pp 99-105.

Babic, M., Hujer, A.M. and Bonomo, R.A., 2006. What's new in antibiotic resistance? Focus on beta-lactamases. Drug Resistance Update 9, pp 142-156.

Badruzzaman, A., Noor, M., Mamun, M., Husna, A. and Islam, K., 2015. Prevalence of diseases in commercial chickens at Sylhet division of Bangladesh. International Clinical Pathology Journal 1, pp 1-5.

Barnard, T.G., Robertson, C.A., Jagals, P. and Potgieter, N., 2011. A rapid and low-cost DNA extraction method for isolating *Escherichia coli* DNA from animal stools. African Journal of Biotechnology 10, pp 1485-1490.

Baron, E.J., 2011. Conventional versus molecular methods for pathogen detection and the role of clinical microbiology in infection control. Journal of Clinical Microbiology 49, pp S43.

Barros, A.J. and Hirakata, V.N., 2003. Alternatives for logical regression in cross-sectional studies: an empirical comparison of models that directly estimatet the prevalence ratio. BMC Medical Research Methodology 3, pp 1-13.

Barros, M.R., da Silveira, W.D., de Araujo, J.M., Costa, E.P., Oliveira, A.A.D., Santos, A., Silva, V.A.S. and Mota, R.A., 2012. Antimicrobial resistance and plasmidial profile of *Escherichia coli* strain isolated from broilers and commercial layers in the state of Pernambuco, Brazil. Pesquisa Veterinaria Brasileira 32, pp 405-410.

Barton, M. and Wilkins, J. 2001. Antibiotic resistance in bacteria isolated from poultry. A report for the Rural Industries. Research and Development Corporation. RIRDC Publication No 01/105, Project No USA-9A.

Barton, M.D., Pratt, R. and Hart, W.S., 2003. Antibiotic resistance in animals. Communicable Diseases Intelligence 27, pp 121-126.

Bass, L., C.A. , Liebert, M.D., Lee, A.O., Summers, D.G., White, S.G., Thayer, S. and Maurer, J.J., 1999. Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance in avian *Escherichia coli*. Antimicrobial Agents and Chemotherapy 43, pp 2925-2929.

Bauchart, P., Germon, P., Bree, A., Oswald, E., Hacker, J. and Dobrindt, U., 2010. Pathogenomic comparison of human extraintestinal and avian pathogenic *Escherichia coli* search for factors involved in host specificity or zoonotic potential. Microbial Pathogenesis 49, pp 105-115.

Belanger, L., Garenaux, A., Harel, J., Boulianne, M., Nadeau, E. and Dozois, C.M., 2011. *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli*. FEMS Immunology and Medical Microbiology 62, pp 1-10.

Berg, R.D., 1996. The indigenous gastrointestinal microflora. Trends in Microbiology 4, pp 430-435.

Bergstrom, C.T., Lipsitch, M. and Levin, B.R., 2000. Natural selection, infectious transfer and the existence conditions for bacterial plasmids. Genetics 155, pp 1505-1519.

Bertran, K., Lee, D.H., Balzli, C., Pantin-Jackwood, M.J., Spackman, E. and Swayne, D.E., 2016. Age is not a determinant factor in susceptibility of broilers to H5N2 clade 2.3.4.4 high pathogenicity avian influenza virus. Veterinary Research 47, pp 116-127.

Bindereif, A. and Neilands, J.B., 1985. Aerobactin genes in clinical isolates of *Escherichia coli*. Journal of Bacteriology 161, pp 727-735.

Bingen, E., Picard, B., Brahimi, N., Mathy, S., Desjardins, P., Elion, J. and Denamur, E., 1998. Phylogenetic analysis of *Escherichia coli* strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains. Journal of Infectious Diseases 177, pp 642-650.

Binns, M.M., Davies, D.L. and Hardy, K.G., 1979. Cloned fragments of the plasmid ColV,I-K94 specifying virulence and serum resistance. Nature 279, pp 778-781.

Blanco, J.E., Blanco, M., Mora, A. and Blanco, J., 1997. Production of toxins (enterotoxins, verotoxins, and necrotoxins) and colicins by *Escherichia coli* strains isolated from septicemic and healthy chickens: relationship with *in vivo* pathogenicity. Journal of Clinical Microbiology 35, pp 2953-2957.

Blessmann, J., Buss, H., Nu, P.A., Dinh, B.T., Ngo, Q.T., Van, A.L., Alla, M.D., Jackson, T.F., Ravdin, J.I. and Tannich, E., 2002. Real-time PCR for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in fecal samples. Journal of Clinical Microbiology 40, pp 4413-4417.

Blyton, M.D., Pi, H., Vangchhia, B., Abraham, S., Trott, D.J., Johnson, J.R. and Gordon, D.M., 2015. Genetic structure and antimicrobial resistance of *Escherichia coli* and cryptic clades in birds with diverse human associations. Applied and Environmental Microbiology 81, pp 5123-5133.

Boerlin, P. and Reid-Smith, R.J., 2008. Antimicrobial resistance: its emergence and transmission. Animal Health Research Reviews 9, pp 115-126.

Boerlin, P. and White, D., 2013. Antimicrobial resistance and its epidemiology. In: Giguere, S., Prescott, J., Dowling, P. (Ed.) Antimicrobial Therapy in Veterinary Medicine. John Wiley and Sons, Hoboken, New Jersey, USA, pp 21-40.

Borjesson, S., Egervarn, M., Lindblad, M. and Englund, S., 2013. Frequent occurrence of extended-spectrum beta-lactamase- and transferable AmpC beta-lactamase-producing *Escherichia coli* on domestic chicken meat in Sweden. Applied and Environmental Microbiology 79, pp 2463-2466.

Borjesson, S., Ny, S., Egervarn, M., Bergstrom, J., Rosengren, A., Englund, S., Lofmark, S. and Byfors, S., 2016. Limited dissemination of extended-spectrum β-lactamase– and plasmid-encoded AmpC–producing *Escherichia coli* from food and farm animals, Sweden. Emerging Infectious Diseases 22, pp 634-640.

Bortolaia, V., Guardabassi, L., Trevisani, M., Bisgaard, M., Venturi, L. and Bojesen, A., 2010. High diversity of extended-spectrum β-lactamases in *Escherichia coli* isolates from Italian broiler flocks. Antimicrobial Agents and Chemotherapy 54, pp 1623-1626.

Bradford, P.A., 2001. Extended-spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. Clinical Microbiology Reviews 14, pp 933-951.

Braga, J.F.V., Chanteloup, N.K., Trotereau, A., Baucheron, S., Guabiraba, R., Ecco, R. and Schouler, C., 2016. Diversity of *Escherichia coli* strains involved in vertebral osteomyelitis and arthritis in broilers in Brazil. BMC Veterinary Research 12, pp 1-12.

Bragg, R., Jansen, A., Coetzee, M., van der Westhuizen, W. and Boucher, C., 2014. Bacterial resistance to quaternary ammonium compounds (QAC) disinfectants. Advances in Experimental Medicine and Biology 808, pp 1-13.

Brinas, L., Moreno, M.A., Zarazaga, M., Porrero, C., Saenz, Y., Garcia, M., Dominguez, L. and Torres, C., 2003. Detection of CMY-2, CTX-M-14, and SHV-12 β-lactamases in *Escherichia coli* fecal-sample isolates from healthy chickens. Antimicrobial Agents and Chemotherapy 47, pp 2056-2058.

Bush, K., 2010. Bench-to-bedside review: The role of β-lactamases in antibiotic-resistant Gram-negative infections. Critical Care 14, pp 1-8.

Bush, K., 2012. Antimicrobial agents targeting bacterial cell walls and cell membranes. Revue Scientifique et Technique 31, pp 43-56.

Bush, K., Jacoby, G.A. and Medeiros, A.A., 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrobial Agents and Chemotherapy 39, pp 1211-1233.

Cambau, E. and Guillard, T., 2012. Antimicrobials that affect the synthesis and conformation of nucleic acids. Revue Scientifique et Technique 31, pp 65-87.

Campos, T.A.d., Lago, J.C., Nakazato, G., Stehling, E.G., Brocchi, M., Castro, A.F.P.d. and Silveira, W.D.d., 2008. Occurrence of virulence-related sequences and phylogenetic analysis of commensal and pathogenic avian *Escherichia coli* strains (APEC). Pesquisa Veterinária Brasileira 28, pp 533-540.

Canton, R., Gonzalez-Alba, J.M. and Galan, J.C., 2012. CTX-M enzymes: origin and diffusion. Frontiers in Microbiology 3, pp 110-129.

Carattoli, A., 2001. Importance of integrons in the diffusion of resistance. Veterinary Research 32, pp 243-259.

Carattoli, A., 2009. Resistance plasmid families in *Enterobacteriaceae*. Antimicrobial Agents and Chemotherapy 53, pp 2227-2238.

Carattoli, A., 2011. Plasmids in Gram negatives: Molecular typing of resistance plasmids. International Journal of Medical Microbiology 301, pp 654-658.

Carattoli, A., 2013. Plasmids and the spread of resistance. International Journal of Medical Microbiology 303, pp 298-304.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L. and Threlfall, E.J., 2005. Identification of plasmids by PCR-based replicon typing. Journal of Microbiological Methods 63, pp 219-228.

Carattoli, A., Tosini, F., Giles, W.P., Rupp, M.E., Hinrichs, S.H., Angulo, F.J., Barrett, T.J. and Fey, P.D., 2002. Characterization of plasmids carrying CMY-2 from expanded-spectrum

cephalosporin-resistant *Salmonella* strains isolated in the United States between 1996 and 1998. Antimicrobial Agents and Chemotherapy 46, pp 1269-1272.

Cascales, E., Buchanan, S.K., Duche, D., Kleanthous, C., Lloubes, R., Postle, K., Riley, M., Slatin, S. and Cavard, D., 2007. Colicin biology. Microbiology and Molecular Biology Reviews 71, pp 158-229.

Castellanos, L.R., Donado-Godoy, P., Leon, M., Clavijo, V., Arevalo, A., Bernal, J.F., Timmerman, A.J., Mevius, D.J., Wagenaar, J.A. and Hordijk, J., 2017. High heterogeneity of *Escherichia coli* sequence types harbouring ESBL/AmpC genes on IncI1 plasmids in the Colombian poultry chain. PLoS One 12, pp e0170777.

Castillo, J.R., 2013. Tetracyclines. In: Giguere, S., Prescott, J., Dowling, P. (Ed.) Antimicrobial Therapy in Veterinary Medicine. Wiley and Sons, Inc., Hoboken, New Jersey, USA, pp 257-268.

Cavero, D., Schmutz, M., Philipp, H.C. and Preisinger, R., 2009. Breeding to reduce susceptibility to *Escherichia coli* in layers. Poultry Science 88, pp 2063-2068.

Cavicchio, L., Dotto, G., Giacomelli, M., Giovanardi, D., Grilli, G., Franciosini, M.P., Trocino, A. and Piccirillo, A., 2015. Class 1 and class 2 integrons in avian pathogenic *Escherichia coli* from poultry in Italy. Poultry Science 94, pp 1202-1208.

Caza, M., Lepine, F., Milot, S. and Dozois, C.M., 2008. Specific roles of the *iroBCDEN* genes in virulence of an avian pathogenic *Escherichia coli* O78 strain and in production of salmochelins. Infection and Immunity 76, pp 3539-3549.

Chaffer, M., Heller, E.D. and Schwartsburd, B., 1999. Relationship between resistance to complement, virulence and outer membrane protein patterns in pathogenic *Escherichia coli* O2 isolates. Veterianry Microbiology 64, pp 323-332.

Chah, K.F., Agbo, I.C., Eze, D.C., Somalo, S., Estepa, V. and Torres, C., 2010. Antimicrobial resistance, integrons and plasmid replicon typing in multiresistant clinical *Escherichia coli* strains from Enugu State, Nigeria. Journal of Basic Microbiology 50 Suppl 1, pp S18-24.

Chambers, P.A., Duggan, P.S., Forbes, J.M. and Heritage, J., 2001. A rapid, reliable method for the extraction from avian faeces of total bacterial DNA to be used as a template for the detection of antibiotic resistance genes. The Journal of Antimicrobial Chemotherapy 47, pp 241-243.

Chantziaras, I., Boyen, F., Callens, B. and Dewulf, J., 2014. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. The Journal of Antimicrobial Chemotherapy 69, pp 827-834.

Chaudhuri, R.R. and Henderson, I.R., 2012. The evolution of the *Escherichia coli* phylogeny. Infection Genetics and Evolution 12, pp 214-226.

Chen, J. and Griffiths, M.W., 1998. PCR differentiation of *Escherichia coli* from other Gram-negative bacteria using primers derived from the nucleotide sequences flanking the gene encoding the universal stress protein. Letters in Applied Microbiology 27, pp 369-371.

Chen, Y.T., Lauderdale, T.L., Liao, T.L., Shiau, Y.R., Shu, H.Y., Wu, K.M., Yan, J.J., Su, I.J. and Tsai, S.F., 2007. Sequencing and comparative genomic analysis of pK29, a 269-kilobase conjugative plasmid encoding CMY-8 and CTX-M-3 β-lactamases in *Klebsiella pneumoniae*. Antimicrobial Agents and Chemotherapy 51, pp 3004-3007.

Cheng, A.C., Turnidge, J., Collignon, P., Looke, D., Barton, M. and Gottlieb, T., 2012. Control of fluoroquinolone resistance through successful regulation, Australia. Emerging Infectious Diseases 18, pp 1453-1460.

Cherif, T., Saidani, M., Decre, D., Boutiba-Ben Boubaker, I. and Arlet, G., 2015. Cooccurrence of multiple AmpC β-lactamases in *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* in Tunisia. Antimicrobial Agents and Chemotherapy 60, pp 44-51.

Cherifi, A., Contrepois, M., Picard, B., Goullet, P., Orskov, I., Orskov, F. and De Rycke, J., 1991. Clonal relationships among *Escherichia coli* serogroup 06 isolates from human and animal infections. FEMS Microbiology Letters 64, pp 225-230.

Cheville, N.F. and Arp, L.H., 1978. Comparative pathologic findings of *Escherichia coli* infection in birds. Journal of the American Veterinary Medical Association 173, pp 584-591.

Chinivasagam, H.N., Tran, T., Maddock, L., Gale, A. and Blackall, P.J., 2009. Mechanically ventilated broiler sheds: a possible source of aerosolized *Salmonella*, *Campylobacter*, and *Escherichia coli*. Applied and Environmental Microbiology 75, pp 7417-7425.

Cho, S.H., Lim, Y.S. and Kang, Y.H., 2012. Comparison of antimicrobial resistance in *Escherichia coli* strains isolated from healthy poultry and swine farm workers using antibiotics in Korea. Osong Public Health and Research Perspectives 3, pp 151-155.

Choi, M.J., Lim, S.K., Nam, H.M., Kim, A.R., Jung, S.C. and Kim, M.N., 2011. Apramycin and gentamicin resistances in indicator and clinical *Escherichia coli* isolates from farm animals in Korea. Foodborne Pathogens and Disease 8, pp 119-123.

Circella, E., Pennelli, D., Tagliabue, S. and Camarda, A., 2012. Virulence-associated genes in avian pathogenic *Escherichia coli* from laying hens in Apulia, Southern Italy. British Poultry Science 53, pp 465-470.

Clermont, O., Bonacorsi, S. and Bingen, E., 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Applied and Environmental Microbiology 66, pp 4555-4558.

Clermont, O., Christenson, J.K., Denamur, E. and Gordon, D.M., 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environmental Microbiology Reports 5, pp 58-65.

Clinical Laboratory Standards Institute (CLSI), 2013. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard, Fourth Edition. CLSI Document VET01-A4. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

Clinical Laboratory Standards Institute (CLSI), 2015a. Performance Standards for Antimicrobial Disk and dilution susceptibility tests for bacteria isolated from animals; Third Edition. CLSI supplement VET01S. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

Clinical Laboratory Standards Institute (CLSI), 2015b. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. CLSI document M100-S25. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

Cloud, S.S., Rosenberger, J.K., Fries, P.A., Wilson, R.A. and Odor, E.M., 1985. *In vitro* and *in vivo* characterization of avian *Escherichia coli* I. Serotypes, metabolic activity, and antibiotic sensitivity. Avian Diseases 29, pp 1084-1093.

Cogliani, C., Goossens, H. and Greko, C., 2011. Restricting antimicrobial use in food animals: Lessons from Europe. Microbe 6, pp 274-279.

Coleman, B.L., Salvadori, M.I., McGeer, A.J., Sibley, K.A., Neumann, N.F., Bondy, S.J., Gutmanis, I.A., McEwen, S.A., Lavoie, M., Strong, D., Johnson, I., Jamieson, F.B. and Louie, M., 2012.

The role of drinking water in the transmission of antimicrobial-resistant *E. coli*. Epidemiology and Infection 140, pp 633-642.

Collingwood, C., Kemmett, K., Williams, N. and Wigley, P., 2014. Is the concept of avian pathogenic *Escherichia coli* as a single pathotype fundamentally flawed? Frontiers in Veterinary Science 1, pp 1-5.

Collinson, S.K., Doig, P.C., Doran, J.L., Clouthier, S., Trust, T.J. and Kay, W.W., 1993. Thin, aggregative fimbriae mediate binding of *Salmonella enteritidis* to fibronectin. Journal of Bacteriology 175, pp 12-18.

Cordoni, G., Woodward, M.J., Wu, H., Alanazi, M., Wallis, T. and La Ragione, R.M., 2016. Comparative genomics of European avian pathogenic *E. coli* (APEC). BMC Genomics 17, pp 1-21.

Cordova, J., Shiloh, R., Gilman, R.H., Sheen, P., Martin, L., Arenas, F., Caviedes, L., Kawai, V., Soto, G., Williams, D.L., Zimic, M., Escombe, A.R. and Evans, C.A., 2010. Evaluation of molecular tools for detection and drug susceptibility testing of *Mycobacterium tuberculosis* in stool specimens from patients with pulmonary tuberculosis. Journal of Clinical Microbiology 48, pp 1820-1826.

Cortes, P., Blanc, V., Mora, A., Dahbi, G., Blanco, J.E., Blanco, M., Lopez, C., Andreu, A., Navarro, F., Alonso, M.P., Bou, G., Blanco, J. and Llagostera, M., 2010. Isolation and characterization of potentially pathogenic antimicrobial-resistant *Escherichia coli* strains from chicken and pig farms in Spain. Applied and Environmental Microbiology 76, pp 2799-2805.

Couturier, M., Bergquist, P., Bex, F. and Maas, W., 1988. Identification and classification of bacterial plasmids. Microbiology Review 52, pp 375-395.

Craig, M., Lambert, S., Jourdan, S., Tenconi, E., Colson, S., Maciejewska, M., Ongena, M., Martin, J.F., van Wezel, G. and Rigali, S., 2012. Unsuspected control of siderophore production by N-acetylglucosamine in streptomycetes. Environmental Microbiology Reports 4, pp 512-521.

Cremet, L., Caroff, N., Dauvergne, S., Reynaud, A., Lepelletier, D. and Corvec, S., 2011. Prevalence of plasmid-mediated quinolone resistance determinants in ESBL *Enterobacteriaceae* clinical isolates over a 1-year period in a French hospital. Pathologie-Biologie 59, pp 151-156.

Crosa, J.H., 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiological Reviews 53, pp 517-530.

Cunha, M.P.V., Saidenberg, A.B., Moreno, A.M., Ferreira, A.J.P., Vieira, M.A.M., Gomes, T.A.T. and Knobl, T., 2017. Pandemic extra-intestinal pathogenic *Escherichia coli* (ExPEC) clonal group O6-B2-ST73 as a cause of avian colibacillosis in Brazil. PLoS One 12, pp e0178970.

da Costa, P.M., Bica, A., Vaz-Pires, P. and Bernardo, F., 2010. Changes in antimicrobial resistance among faecal enterococci isolated from growing broilers prophylactically medicated with three commercial antimicrobials. Preventive Veterinary Medicine 93, pp 71-76.

da Costa, P.M., Loureiro, L. and Matos, A.J., 2013. Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment. International Journal of Environmental Research and Public Health 10, pp 278-294.

da Rocha, A., da Silva, A.B., de Brito, B.G., Moraes, H.L.D., Pontes, A.P., Ce, M.C., do Nascimento, V.P. and Salle, C.T.P., 2002. Virulence factors of avian pathogenic *Escherichia coli* isolated from broilers from the South of Brazil. Avian Diseases 46, pp 749-753.

Da Silva, G.J. and Mendonca, N., 2012. Association between antimicrobial resistance and virulence in *Escherichia coli*. Virulence 3, pp 18-28.

Dahms, C., Hubner, N.O., Kossow, A., Mellmann, A., Dittmann, K. and Kramer, A., 2015. Occurrence of ESBL-producing *Escherichia coli* in livestock and farm workers in Mecklenburg-Western Pomerania, Germany. PLoS One 10, pp e0143326.

Danzeisen, J.L., Wannemuehler, Y., Nolan, L.K. and Johnson, T.J., 2013. Comparison of multilocus sequence analysis and virulence genotyping of *Escherichia coli* from live birds, retail poultry meat, and human extraintestinal infection. Avian Diseases 57, pp 104-108.

Darrell, W.T., Thomas, G.H. and Richard, K.G., 2014. Integrated farm management to prevent *Salmonella* Enteritidis contamination of eggs. The Journal of Applied Poultry Research 23, pp 353–365.

Davies, J., 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science 264, pp 375-382.

Day, M.J., Rodriguez, I., van Essen-Zandbergen, A., Dierikx, C., Kadlec, K., Schink, A.K., Wu, G., Chattaway, M.A., DoNascimento, V., Wain, J., Helmuth, R., Guerra, B., Schwarz, S., Threlfall, J., Woodward, M.J., Coldham, N., Mevius, D. and Woodford, N., 2016. Diversity of STs, plasmids and ESBL genes among *Escherichia coli* from humans, animals and food in

Germany, the Netherlands and the UK. The Journal of Antimicrobal Chemotherapy 71, pp 1178-1182.

De Carli, S., Ikuta, N., Lehmann, F.K., da Silveira, V.P., de Melo Predebon, G., Fonseca, A.S. and Lunge, V.R., 2015. Virulence gene content in *Escherichia coli* isolates from poultry flocks with clinical signs of colibacillosis in Brazil. Poultry Science 94, pp 2635-2640.

de Jong, A., Thomas, V., Simjee, S., Godinho, K., Schiessl, B., Klein, U., Butty, P., Valle, M., Marion, H. and Shryock, T.R., 2012. Pan-European monitoring of susceptibility to human-use antimicrobial agents in enteric bacteria isolated from healthy food-producing animals. The Journal of Antimicrobial Chemotherapy 67, pp 638-651.

de Lorenzo, V. and Neilands, J.B., 1986. Characterization of *iucA* and *iucC* genes of the aerobactin system of plasmid ColV-K30 in *Escherichia coli*. Journal of Bacteriology 167, pp 350-355.

de Oliveira, A.L., Rocha, D.A., Finkler, F., de Moraes, L.B., Barbieri, N.L., Pavanelo, D.B., Winkler, C., Grassotti, T.T., de Brito, K.C., de Brito, B.G. and Horn, F., 2015. Prevalence of ColV plasmid-linked genes and *in vivo* pathogenicity of avian strains of *Escherichia coli*. Foodborne Pathogens and Disease 12, pp 679-685.

de Pace, F., Nakazato, G., Pacheco, A., de Paiva, J.B., Sperandio, V. and da Silveira, W.D., 2010. The type VI secretion system plays a role in type 1 fimbria expression and pathogenesis of an avian pathogenic *Escherichia coli* strain. Infection and Immunity 78, pp 4990-4998.

DebRoy, C., Roberts, E. and Fratamico, P.M., 2011. Detection of O antigens in *Escherichia coli*. Animal Health Research Reviews 12, pp 169-185.

Delicato, E.R., de Brito, B.G., Gaziri, L.C.J. and Vidotto, M.C., 2003. Virulence-associated genes in *Escherichia coli* isolates from poultry with colibacillosis. Veterinary Microbiology 94, pp 97-103.

Deng, Y., Bao, X., Ji, L., Chen, L., Liu, J., Miao, J., Chen, D., Bian, H., Li, Y. and Yu, G., 2015. Resistance integrons: class 1, 2 and 3 integrons. Annals of Clinical Microbiology and Antimicrobials 14, pp 1-11.

Deshpande, L.M., Jones, R.N., Fritsche, T.R. and Sader, H.S., 2006. Occurrence of plasmidic AmpC type β-lactamase-mediated resistance in *Escherichia coli*: report from the SENTRY Antimicrobial Surveillance Program (North America, 2004). International Journal of Antimicrobial Agents 28, pp 578-581.

Department of Agriculture, Fisheries and Forestry (DAFF), 2007. Pilot surveillance program for antimicrobial resistance in bacteria of animal origin, Australian Government Department of Agriculture, Fisheries and Forestry, Canberra. [Available from: http://www.agriculture.gov.au/animal/health/amr/antimicrobial-resistance-bacteria-animal-origin]; Accessed May 2015.

Desloire, S., Valiente Moro, C., Chauve, C. and Zenner, L., 2006. Comparison of four methods of extracting DNA from *D. gallinae* Veterinary Research 37, pp 725-732.

Dhanashree, B. and Shrikara, P.M., 2012 Molecular typing of enteropathogenic *Escherichia coli* from diarrheagenic stool samples. Journal of Clinical and Diagnostic Research 6, pp 400-404.

Dheilly, A., Bouder, A., Le Devendec, L., Hellard, G. and Kempf, I., 2011. Clinical and microbial efficacy of antimicrobial treatments of experimental avian colibacillosis. Veterinary Microbiology 149, pp 422-429.

Dheilly, A., Le Devendec, L., Mourand, G., Jouy, E. and Kempf, I., 2013. Antimicrobial resistance selection in avian pathogenic *E. coli* during treatment. Veterinary Microbiology 166, pp 655-658.

Dhillon, A.S. and Jack, O.K., 1996. Two outbreaks of colibacillosis in commercial caged layers. Avian Diseases 40, pp 742-746.

Dho-Moulin, M. and Fairbrother, J.M., 1999. Avian pathogenic *Escherichia coli* (APEC). Veterinary Research 30, pp 299-316.

Dho, M. and Lafont, J.P., 1984. Adhesive properties and iron uptake ability in *Escherichia coli* lethal and nonlethal for chicks. Avian Diseases 28, pp 1016-1025.

Diab, A.M. and Al-urk, I.M., 2011. ERIC and RAPD PCR-based DNA fingerprinting techniques application for microbial source tracking (MST) at Al-Madinah Al-Munwwarah, KSA. Journal of Taibah University for Science 5, pp 31-38.

Diarra, M.S., Silversides, F.G., Diarrassouba, F., Pritchard, J., Masson, L., Brousseau, R., Bonnet, C., Delaquis, P., Bach, S., Skura, B.J. and Topp, E., 2007. Impact of feed supplementation with antimicrobial agents on growth performance of broiler chickens, *Clostridium perfringens* and *Enterococcus* counts, and antibiotic resistance phenotypes and distribution of antimicrobial resistance determinants in *Escherichia coli* isolates. Applied and Environmental Microbiology 73, pp 6566-6576.

Dias da Silveira, W., Ferreira, A., Brocchi, M., Maria de Hollanda, L., Pestana de Castro, A.F., Tatsumi Yamada, A. and Lancellotti, M., 2002. Biological characteristics and pathogenicity of avian *Escherichia coli* strains. Vetrinary Microbiology 85, pp 47-53.

Dierikx, C., van Essen-Zandbergen, A., Veldman, K., Smith, H. and Mevius, D., 2010. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. Veterinary Microbiology 145, pp 273-278.

Dissanayake, D.R., Octavia, S. and Lan, R., 2014. Population structure and virulence content of avian pathogenic *Escherichia coli* isolated from outbreaks in Sri Lanka. Veterinary Microbiology 168, pp 403-412.

Dobrindt, U., 2005. (Patho-)Genomics of *Escherichia coli*. International Journal of Medical Microbiology 295, pp 357-371.

Dohoo, I., Martin, S.W. and Stryhn, H., 2009. Veterinary Epidemiologic Research In: Dohoo, I., Martin, W., Stryhn, H. (Eds.), VER, Incorporated, Charlottetown, Prince Edward Island, Canada.

Dolejska, M., Villa, L., Hasman, H., Hansen, L. and Carattoli, A., 2013. Characterization of IncN plasmids carrying $bla_{\text{CTX-M-1}}$ and *qnr* genes in *Escherichia coli* and *Salmonella* from animals, the environment and humans. The Journal of Antimicrobial Chemotherapy 68, pp 333-342.

Domingues, S., da Silva, G.J. and Nielsen, K.M., 2012. Integrons: Vehicles and pathways for horizontal dissemination in bacteria. Mobile Genetic Elements 2, pp 211-223.

Dou, X., Gong, J., Han, X., Xu, M., Shen, H., Zhang, D., Zhuang, L., Liu, J. and Zou, J., 2016. Characterization of avian pathogenic *Escherichia coli* isolated in Eastern China. Gene 576, pp 244-248.

Dowling, P., 2013. Chlorampehicol, thiamphenicol and florfenicol. In: Giguère, S., Prescott, J.F., Dowling, P. (Eds.) Antimicrobial Therapy in Veterinary Medicine. John Wiley and Sons Inc, Hoboken, New Jersey, USA, pp 269-277.

Doyle, M.P., Loneragan, G.H., Scott, H.M. and Singer, R.S., 2013. Antimicrobial resistance: challenges and perspectives. Comprehensive Reviews in Food Science and Food Safety 12, pp 234-248.

Dozois, C.M., Chanteloup, N., Dhomoulin, M., Bree, A., Desautels, C. and Fairbrother, J.M., 1994. Bacterial colonization and *in vivo* expression of F1 (type 1) fimbrial antigens in chickens experimentally infected with pathogenic *Escherichia coli*. Avian Diseases 38, pp 231-239.

Dozois, C.M., Daigle, F. and Curtiss, R., 2003. Identification of pathogen-specific and conserved genes expressed *in vivo* by an avian pathogenic *Escherichia coli* strain. Proceedings of the National Academy of Sciences of the United States of America 100, pp 247-252.

Dozois, C.M., Dho-Moulin, M., Bree, A., Fairbrother, J.M., Desautels, C. and Curtiss, R., 3rd, 2000. Relationship between the Tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the *tsh* genetic region. Infection and Immunity 68, pp 4145-4154.

Dozois, C.M., Fairbrother, J.M., Harel, J. and Bosse, M., 1992. pap-and pil-related DNA sequences and other virulence determinants associated with *Escherichia coli* isolated from septicemic chickens and turkeys. Infection and Immunity 60, pp 2648-2656.

Dozois, C.M., Pourbakhsh, S.A. and Fairbrother, J.M., 1995. Expression of p-fimbriae and type-1 (F1) fimbriae in pathogenic *Escherichia coli* from poultry. Veterinary Microbiology 45, pp 297-309.

Dragana, L., Maja, V., Dalibor, T., Marko, P. and Dubravka, M., 2016. Tetracycline resistance in *Escherichia coli* isolates from poultry. Arhiv Veterinarske Medicine 9, pp 61-81.

Drews, S.J., Poutanen, S.M., Mazzulli, T., McGeer, A.J., Sarabia, A., Pong-Porter, S., Rzayev, Y., Willey, B., Green, K. and Low, D.E., 2005. Decreased prevalence of virulence factors among ciprofloxacin-resistant uropathogenic *Escherichia coli* isolates. Journal of Clinical Microbiology 43, pp 4218-4220.

Dziva, F. and Stevens, M.P., 2008. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathology 37, pp 355-366.

East, I.J., 2007. Adoption of biosecurity practices in the Australian poultry industries. Australian Veterinary Journal 85, pp 107-112.

ECDC, 2012. Antimicrobial resistance surveillance in Europe 2011. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). European Centre for Disease Prevention and Control (ECDC), Stockholm.

EFSA European Food Safety Authority, 2015. EU summary report on antimicrobial resistance and indicator bacteria from humans, animals and food in 2013. EFSA Journal 13, pp 4036-4012.

Eftekharian, S., Ghorbanpoor, M., Seyfi Abad Shapouri, M.R., Ghanbarpour, R., Jafari, R.J. and Amani, A., 2016. Frequency of selected virulence-associated genes in intestinal and extra-intestinal *Escherichia coli* isolates from chicken. Iranian Journal of Veterinary Medicine 10, pp 91-96.

El-Shazly, D.A., Nasef, S.A., Mahmoud, F.F. and Jonas, D., 2017. Expanded spectrum β-lactamase producing *Escherichia coli* isolated from chickens with colibacillosis in Egypt. Poultry Science 96, pp 2375-2384.

El Houadfi, M., Fellahi, S., Nassik, S., Guerin, J.L. and Ducatez, M.F., 2016. First outbreaks and phylogenetic analyses of avian influenza H9N2 viruses isolated from poultry flocks in Morocco. Virology Journal 13, pp 140-147.

Eliopoulos, G.M. and Huovinen, P., 2001. Resistance to trimethoprim-sulfamethoxazole. Clinical Infectious Diseases 32, pp 1608-1614.

Elmberg, J., Berg, C., Lerner, H., Waldenstrom, J. and Hessel, R., 2017. Potential disease transmission from wild geese and swans to livestock, poultry and humans: a review of the scientific literature from a one health perspective. Infection Ecology and Epidemiology 7, pp 1300450-1300421.

Emery, D.A., Nagaraja, K.V., Shaw, D.P., Newman, J.A. and White, D.G., 1992. Virulence factors of *Escherichia coli* associated with colisepticemia in chickens and turkeys. Avian Diseases 36, pp 504-511.

Escherich, T., 1988. The intestinal bacteria of the neonate and breast-fed infant 1884. Reviews of Infectious Diseases 10, pp 1220-1225.

Ewers, C., Antao, E.M., Diehl, I., Philipp, H.C. and Wieler, L.H., 2009. Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. Applied and Environmental Microbiology 75, pp 184-192.

Ewers, C., Bethe, A., Semmler, T., Guenther, S. and Wieler, L.H., 2012. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clinical Microbiology and Infection 18, pp 646-655.

Ewers, C., Grobbel, M., Stamm, I., Kopp, P.A., Diehl, I., Semmler, T., Fruth, A., Beutlich, J., Guerra, B., Wieler, L.H. and Guenther, S., 2010. Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-β-lactamase-producing *Escherichia coli* among companion animals. The Journal of Antimicrobial Chemotherapy 65, pp 651-660.

Ewers, C., Janssen, T., Kiessling, S., Philipp, H.C. and Wieler, L.H., 2004. Molecular epidemiology of avian pathogenic *Escherichia coli* (APEC) isolated from colisepticemia in poultry. Veterinary Microbiology 104, pp 91-101.

Ewers, C., Janssen, T., Kiessling, S., Philipp, H.C. and Wieler, L.H., 2005. Rapid detection of virulence-associated genes in avian pathogenic *Escherichia coli* by multiplex polymerase chain reaction. Avian Diseases 49, pp 269-273.

Ewers, C., Janssen, T. and Wieler, L.H., 2003. Avian pathogenic *Escherichia coli* (APEC). Berliner und Münchener Tierärztliche Wochenschrift 116, pp 381-395.

Ewers, C., Li, G.W., Wilking, H., Kiessling, S., Alt, K., Antao, E.M., Laturnus, C., Diehl, I., Glodde, S., Homeier, T., Bohnke, U., Steinruck, H., Philipp, H.C. and Wieler, L.H., 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: How closely related are they? International Journal of Medical Microbiology 297, pp 163-176.

Eyigor, A. and Carli, K.T., 2003. Rapid detection of *Salmonella* from poultry by real-time polymerase chain reaction with fluorescent hybridization probes. Avian Diseases 47, pp 380-386.

Fagan, P.K., Hornitzky, M.A., Bettelheim, K.A. and Djordjevic, S.P., 1999. Detection of shiga-like toxin (*stx*$_1$ and *stx*$_2$), intimin (*eaeA*), and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC *hlyA*) genes in animal feces by multiplex PCR. Applied and Environmental Microbiology 65, pp 868-872.

Fallavena, L.C., Moraes, H.L., Salle, C.T., Silva, A.B., Vargas, R.S., Nascimento, V.P. and Canal, C.W., 2000. Diagnosis of skin lesions in condemned or downgraded broiler carcasses - a microscopic and macroscopic study. Avian Pathology 29, pp 557-562.

Fantinatti, F., Silveira, W.D. and Castro, A.F.P., 1994. Characteristics associated with pathogenicity of avian septicemic *Escherichia coli* strains. Veterinary Microbiology 41, pp 75-86.

FAO/WHO/OIE, 2008. Joint FAO/OIE/WHO Expert Meeting on Critically Important Antimicrobials. Report of a meeting held in FAO, Rome, Italy,November 2007, [Available from

http://www.who.int/foodborne_disease/resources/Report%20joint%20CIA%20Meeting.pdf] ; Accessed May 2014.

Fernandes, M.R., McCulloch, J.A., Vianello, M.A., Moura, Q., Perez-Chaparro, P.J., Esposito, F., Sartori, L., Dropa, M., Matte, M.H., Lira, D.P., Mamizuka, E.M. and Lincopan, N., 2016. First report of the globally disseminated IncX4 plasmid carrying the *mcr-1* gene in a colistin-resistant *Escherichia coli* sequence type 101 isolate from a human infection in Brazil. Antimicrobial Agents and Chemotherapy 60, pp 6415-6417.

Fernandez-Alarcon, C., Singer, R.S. and Johnson, T.J., 2011. Comparative genomics of multidrug resistance-encoding IncA/C plasmids from commensal and pathogenic *Escherichia coli* from multiple animal sources. PLoS One 6, pp e23415.

Fernandez-Romero, N., Romero-Gomez, M.P., Gomez-Gil, M.R. and Mingorance, J., 2011. Epidemic population structure of extraintestinal pathogenic *Escherichia coli* determined by single nucleotide polymorphism pyrosequencing. Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases 11, pp 1655-1663.

Fischer, J., Rodriguez, I., Baumann, B., Guiral, E., Beutin, L., Schroeter, A., Kaesbohrer, A., Pfeifer, Y., Helmuth, R. and Guerra, B., 2014. $bla_{\text{CTX-M-15}}$-carrying *Escherichia coli* and *Salmonella* isolates from livestock and food in Germany. The Journal of Antimicrobial Chemotherapy 69, pp 2951-2958.

Fogarty, L.R., Haack, S.K., Wolcott, M.J. and Whitman, R.L., 2003. Abundance and characteristics of the recreational water quality indicator bacteria *Escherichia coli* and enterococci in gull faeces. Journal of Applied Microbiology 94, pp 865-878.

Folorunso, O.R., Kayode, S. and Onibon, V.O., 2014. Poultry farm hygiene: microbiological quality assessment of drinking water used in layer chickens managed under the battery cage and deep litter systems at three poultry farms in South Western Nigeria. Pakistan Journal of Biological Sciences 17, pp 74-79.

Foster, G., Evans, J., Knight, H.I., Smith, A.W., Gunn, G.J., Allison, L.J., Synge, B.A. and Pennycott, T.W., 2006. Analysis of feces samples collected from a wild-bird garden feeding station in Scotland for the presence of verocytotoxin-producing *Escherichia coli* O157. Applied and Environmental Microbiology 72, pp 2265-2267.

Franz, E., Delaquis, P., Morabito, S., Beutin, L., Gobius, K., Rasko, D.A., Bono, J., French, N., Osek, J., Lindstedt, B.A., Muniesa, M., Manning, S., LeJeune, J., Callaway, T., Beatson, S., Eppinger, M., Dallman, T., Forbes, K.J., Aarts, H., Pearl, D.L., Gannon, V.P., Laing, C.R. and Strachan, N.J., 2014. Exploiting the explosion of information associated with whole genome sequencing to tackle Shiga toxin-producing *Escherichia coli* (STEC) in global food production systems. International Jounal of Food Microbiology 187, pp 57-72.

Fratamico, P.M., DebRoy, C., Liu, Y., Needleman, D.S., Baranzoni, G.M. and Feng, P., 2016a. Advances in molecular serotyping and subtyping of *Escherichia coli*. Frontiers in Microbiology 7, pp 644-653.

Fratamico, P.M., DebRoy, C. and Needleman, D.S., 2016b. Editorial: emerging approaches for typing, detection, characterization, and traceback of *Escherichia coli*. Frontiers in Microbiology 7, pp 2089-2025.

Gao, Q., Wang, X., Xu, H., Xu, Y., Ling, J., Zhang, D., Gao, S. and Liu, X., 2012. Roles of iron acquisition systems in virulence of extraintestinal pathogenic *Escherichia coli*: salmochelin and aerobactin contribute more to virulence than heme in a chicken infection model. BMC Microbiology 12, pp 143-112.

Garcia-Fernandez, A., Chiaretto, G., Bertini, A., Villa, L., Fortini, D., Ricci, A. and Carattoli, A., 2008. Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum β-lactamases in *Escherichia coli* and *Salmonella* of human and animal origin. Journal of Antimicrobial Chemotherapy 61, pp 1229-1233.

Garofalo, C., Vignaroli, C., Zandri, G., Aquilanti, L., Bordoni, D., Osimani, A., Clementi, F. and Biavasco, F., 2007. Direct detection of antibiotic resistance genes in specimens of chicken and pork meat. International Journal of Food Microbiology 113, pp 75-83.

Gibson, F.I. and Magrath, D.I., 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-1. Biochim Biophys Acta 152, pp 175-184.

Gibson, J.S., Cobbold, R.N., Heisig, P., Sidjabat, H.E., Kyaw-Tanner, M.T. and Trott, D.J., 2010. Identification of Qnr and AAC(6')-1b-cr plasmid-mediated fluoroquinolone resistance determinants in multidrug-resistant *Enterobacter* spp. isolated from extraintestinal infections in companion animals. Veterinary Microbiology 143, pp 329-336.

Giguère, S., 2006. Antimicrobial Drug Action and Interaction: An Introduction. In: Giguère, S., Prescott, J.F., Dowling, P.M. (Eds.) Antimicrobial Therapy in Veterinary Medicine. Blackwell Publishing Professional, Ames, USA, pp 3-9.

Gillings, M. and Holley, M., 1997. Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. Letters in Applied Microbiology 25, pp 17-21.

Ginns, C.A., Browning, G.F., Benham, M.L. and Whithear, K.G., 1998. Development and application of an aerosol challenge method for reproduction of avian colibacillosis. Avian Pathology 27, pp 505-511.

Gioffre, A., Meichtri, L., Zumarraga, M., Rodriguez, R. and Cataldi, A., 2004. Evaluation of a QIAamp DNA stool purification kit for shiga-toxigenic *Escherichia coli* detection in bovine fecal swabs by PCR. Revista Argentina de Microbiologia 36, pp 1-5.

Giufre, M., Graziani, C., Accogli, M., Luzzi, I., Busani, L., Cerquetti, M. and *Escherichia coli* Study Group, 2012. *Escherichia coli* of human and avian origin: detection of clonal groups associated with fluoroquinolone and multidrug resistance in Italy. Journal of Antimicrobial Chemotherapy 67, pp 860-867.

Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee, A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E. and Taberlet, P., 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution 7, pp 1299–1307.

Goluszko, P., Moseley, S.L., Truong, L.D., Kaul, A., Williford, J.R., Selvarangan, R., Nowicki, S. and Nowicki, B., 1997. Development of experimental model of chronic pyelonephritis with *Escherichia coli* O75:K5:H-bearing Dr fimbriae: mutation in the dra region prevented tubulointerstitial nephritis. The Journal of Clinical Investigation 99, pp 1662-1672.

Gomis, S.M., Goodhope, R., Kumor, L., Caddy, N., Riddell, C., Potter, A.A. and Allan, B.J., 1997. Isolation of *Escherichia coli* from cellulitis and other lesions of the same bird in broilers at slaughter. Canadian Veterinary Journal 38, pp 159-162.

Gonzalez-Zorn, B. and Escudero, J.A., 2012. Ecology of antimicrobial resistance: humans, animals, food and environment. International Microbiology 15, pp 101-109.

Gororo, E. and Kashangura, T., 2016. Broiler production in an urban and peri-urban area of Zimbabwe. Development Southern Africa 33, pp 99-112.

Gottlieb, T. and Nimmo, G.R., 2011. Antibiotic resistance is an emerging threat to public health: an urgent call to action at the Antimicrobial Resistance Summit 2011. The Medical Journal of Australia 194, pp 281-283.

Gouvêa, R., Santos, F.d., Aquino, M.d. and Pereira, VL.de.A., 2015. Fluoroquinolones in industrial poultry production, bacterial resistance and food residues:a review. Brazilian Journal of Poultry Science 17, pp 1-10.

Graham, J.P., Leibler, J.H., Price, L.B., Otte, J.M., Pfeiffer, D.U., Tiensin, T. and Silbergeld, E.K., 2008. The animal-human interface and infectious disease in industrial food animal production: rethinking biosecurity and biocontainment. Public Health Reports 123, pp 282-299.

Gridley, M.F., 1957. Manual of Histologic and Special Staining Technics, In: Armed Forces Institute of Pathology (Ed.) Blakiston Division, McGraw-Hill McGraw-Hill Book Company Inc., New York, USA.

Gross, W.B., 1957. Pathological changes of an *Escherichia coli* infection in chickens and turkeys. American Journal of Veterinary Research 18, pp 724-730.

Gross, W.B., 1990. Factors affecting the development of respiratory-disease complex in chickens. Avian Diseases 34, pp 607-610.

Gross, W.B. and Siegel, P.B., 1997. Why some get sick? Journal of Applied Poultry Research 6, pp 453-460.

Guabiraba, R. and Schouler, C., 2015. Avian colibacillosis: still many black holes. FEMS Microbiology Letters 362, pp 23-30.

Guardabassi, L., 2006. Modes of antimicrobial action and mechanisms of bacterial resistance. In: Aarestrup, F.M. (Ed.) Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington, D.C, pp 1-18.

Guardabassi, L. and Kruse, H., 2008. Principles of prudent and rational use of antimicrobials in animals. In: Guardabassi, L., Jensen, L.B., Kruse, H. (Eds.) Guide to Antimicrobial Use in Animals Blackwell Publishing Ltd, Oxford, UK, pp 1-12.

Guastalli, E.A.L., Guastalli, B.H.L., Soares, N.M., Leite, D.S., Ikuno, A.A., Maluta, R.P., Cardozo, M.V., Beraldo, L.G., Borges, C.A. and Avila, F.A., 2013. Virulence characteristics of *Escherichia coli* isolates obtained from commercial one-week-old layer chicks with diarrhea. African Journal of Microbiology Research 7, pp 5306-5313.

Guenther, S., Ewers, C. and Wieler, L.H., 2011. Extended-spectrum beta-lactamase producing *E. coli* in wildlife, yet another form of environmental pollution? Frontiers in Microbiology 2, pp 246-254.

Guenther, S., Grobbel, M., Beutlich, J., Guerra, B., Ulrich, R. and Wieler, L., 2010. Detection of pandemic B2-O25-ST131 *Escherichia coli* harbouring the CTX-M-9 extended-spectrum β-lactamase type in a feral urban brown rat (*Rattus norvegicus*). Journal of Antimicrobial Chemotherapy 65, pp 255-267.

Guerin, M.T., Martin, W., Reiersen, J., Berke, O., McEwen, S.A., Bisaillon, J.R. and Lowman, R., 2007. A farm-level study of risk factors associated with the colonization of broiler flocks with *Campylobacter* spp. in Iceland, 2001-2004. Acta Veterinaria Scandinavica 49, pp 18-27.

Guo, S., Wakeham, D., Brouwers, H.J., Cobbold, R.N., Abraham, S., Mollinger, J.L., Johnson, J.R., Chapman, T.A., Gordon, D.M., Barrs, V.R. and Trott, D.J., 2015. Human-associated fluoroquinolone-resistant *Escherichia coli* clonal lineages, including ST354, isolated from canine feces and extraintestinal infections in Australia. Microbes and Infection 17, pp 266-274.

Gyles, C.L. and Fairbrother, J.M., 2010. *Escherichia coli*. In: Gyles, C.L., Prescott, J F., Songer, G., Thoen, C.O., Glenn,T., Charles, O P. (Eds.) Pathogenesis of Bacterial Infections in Animals. Wiley-Blackwell, Oxford, UK, pp 267-308.

Halova, D., Papousek, I., Jamborova, I., Masarikova, M., Cizek, A., Janecko, N., Oravcova, V., Zurek, L., Clark, A.B., Townsend, A., Ellis, J.C. and Literak, I., 2014. Plasmid-mediated quinolone resistance genes in *Enterobacteriaceae* from American crows: high prevalence of bacteria with variable *qnrB* genes. Antimicrobial Agents and Chemotherapy 58, pp 1257-1258.

Hammerum, A.M. and Heuer, O.E., 2009. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. Clinical Infectious Diseases 48, pp 916-921.

Hammerum, A.M., Jakobsen, L., Olsen, S.S. and Agerso, Y., 2012. Characterization of CTX-M-14- and CTX-M-15-producing *Escherichia coli* of porcine origin. The Journal of Antimicrobial Chemotherapy 67, pp 2047-2049.

Hanson, N.D., Moland, E.S., Hossain, A., Neville, S.A., Gosbell, I.B. and Thomson, K.S., 2002. Unusual *Salmonella enterica* serotype Typhimurium isolate producing CMY-7, SHV-9 and OXA-30 β-Lactamases. The Journal of Antimicrobial Chemotherapy 49, pp 1011-1014.

Hanson, N.D., Thomson, K.S., Moland, E.S., Sanders, C.C., Berthold, G. and Penn, R.G., 1999. Molecular characterization of a multiply resistant *Klebsiella pneumoniae* encoding ESBLs and a plasmid-mediated AmpC. The Journal of Antimicrobial Chemotherapy 44, pp 377-380.

Hantke, K., Nicholson, G., Rabsch, W. and Winkelmann, G., 2003. Salmochelins, siderophores of *Salmonella enterica* and uropathogenic *Escherichia coli* strains, are recognized by the outer membrane receptor IroN. Proceedings of the National Academy of Sciences of the United States of America 100, pp 3677-3682.

Harbottle, H., Thakur, S., Zhao, S. and White, D.G., 2006. Genetics of antimicrobial resistance. Animal Biotechnology 17, pp 111-124.

Harrell, F.E., 2001. Regression Modeling Strategies With Applications to Linear Models, Logistic Regression, and Survival Analysis, In: Springer Link Content, P. (Ed.). New York, USA.

Hart, M.L., Meyer, A., Johnson, P.J. and Ericsson, A.C., 2015. Comparative evaluation of DNA extraction methods from feces of multiple host species for downstream next-generation sequencing. PLoS One 10, pp e0143334.

Hasan, B., Faruque, R., Drobni, M., Waldenstrom, J., Sadique, A., Ahmed, K.U., Islam, Z., Parvez, M.B.H., Olsen, B. and Alam, M., 2011. High prevalence of antibiotic resistance in pathogenic *Escherichia coli* from large- and small-scale poultry farms in Bangladesh. Avian Diseases 55, pp 689-692.

Hasman, H., Mevius, D., Veldman, K., Olesen, I. and Aarestrup, F.M., 2005. β-lactamases among extended-spectrum β-lactamase (ESBL)-resistant *Salmonella* from poultry, poultry products and human patients in the Netherlands. Journal of Antimicrobial Chemotherapy 56, pp 115-121.

Hassan, M.K., Afify, M.A. and Aly, M.M., 2004. Genetic resistance of Egyptian chickens to infectious bursal disease and Newcastle disease. Tropical Animal Health and Production 36, pp 1-9.

Hawkey, P.M. and Jones, A.M., 2009. The changing epidemiology of resistance. Journal of Antimicrobial Chemotherapy 64, pp 3-10.

Hayes, F., 2003. Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. Science 301, pp 1496-1499.

Heinemann, I.U., Jahn, M. and Jahn, D., 2008. The biochemistry of heme biosynthesis. Archives of Biochemistry and Biophysics 474, pp 238-251.

Henderson, G., Cox, F., Kittelmann, S., Miri, V.H., Zethof, M., Noel, S.J., Waghorn, G.C. and Janssen, P.H., 2013. Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. PloS One 8, pp e74787.

Henry, I., Reichardt, J., Denis, M. and Cardinale, E., 2011. Prevalence and risk factors for *Campylobacter* spp. in chicken broiler flocks in Reunion Island (Indian Ocean). Preventive Veterinary Medicine 100, pp 64-70.

Hernandez, A., Sanchez, M.B. and Martinez, J.L., 2011. Quinolone resistance: much more than predicted. Frontiers in Microbiology 2, pp 1-6.

Hiki, M., Usui, M., Akiyama, T., Kawanishi, M., Tsuyuki, M., Imamura, S., Sekiguchi, H., Kojima, A. and Asai, T., 2014. Phylogenetic grouping, epidemiological typing, analysis of virulence genes, and antimicrobial susceptibility of *Escherichia coli* isolated from healthy broilers in Japan. Irish Veterinary Journal 67, pp 14-15.

Hill, C.A. and Gutierrez, J.A., 2003. A method for extraction and analysis of high quality genomic DNA from ixodid ticks. Medical and Veterinary Entomology 17, pp 224-227.

Hollenbeck, B.L. and Rice, L.B., 2012. Intrinsic and acquired resistance mechanisms in *Enterococcus*. Virulence 3, pp 421-433.

Hommais, F., Pereira, S., Acquaviva, C., Escobar-Paramo, P. and Denamur, E., 2005. Single-nucleotide polymorphism phylotyping of *Escherichia coli*. Applied and Environmental Microbiology 71, pp 4784-4792.

Horcajada, J.P., Soto, S., Gajewski, A., Smithson, A., Jimenez de Anta, M.T., Mensa, J., Vila, J. and Johnson, J.R., 2005. Quinolone-resistant uropathogenic *Escherichia coli* strains from phylogenetic group B2 have fewer virulence factors than their susceptible counterparts. Journal of Clinical Microbiology 43, pp 2962-2964.

Hordijk, J., Wagenaar, J.A., van de Giessen, A., Dierikx, C., van Essen-Zandbergen, A., Veldman, K., Kant, A. and Mevius, D., 2013. Increasing prevalence and diversity of ESBL/AmpC-type β-Lactamase genes in *Escherichia coli* isolated from veal calves from 1997 to 2010. The Journal of Antimicrobial Chemotherapy 68, pp 1970-1973.

Horn, F., Ribeiro Correa, A.M., Barbieri, N.L., Glodde, S., Weyrauch, K.D., Kaspers, B., Driemeier, D., Ewers, C. and Wieler, L.H., 2012. Infections with avian pathogenic and fecal *Escherichia coli* strains display similar lung histopathology and macrophage apoptosis. PloS One 7, pp e0041031.

Horne, S.M., Pfaff-McDonough, S.J., Giddings, C.W. and Nolan, L.K., 2000. Cloning and sequencing of the *iss* gene from a virulent avian *Escherichia coli*. Avian Diseases 44, pp 179-184.

Hosmer, D.W., 2013. Applied logistic regression, In: Lemeshow, S., Sturdivant, R.X. (Eds.) John Wiley and Sons, Inc., Hoboken, New Jersey, USA.

Huang, S.Y., Dai, L., Xia, L.N., Du, X.D., Qi, Y.H., Liu, H.B., Wu, C.M. and Shen, J.Z., 2009. Increased prevalence of plasmid-mediated quinolone resistance determinants in chicken *Escherichia coli* isolates from 2001 to 2007. Foodborne Pathogens and Disease 6, pp 1203-1209.

Hulton, C.S.J., Higgins, C.F. and Sharp, P.M., 1991. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella* Typhimurium and other *Enterobacteria*. Molecular Microbiology 5, pp 825-834.

Hussein, A.H., Ghanem, I.A., Eid, A.A., Ali, M.A., Sherwood, J.S., Li, G., Nolan, L.K. and Logue, C.M., 2013. Molecular and phenotypic characterization of *Escherichia coli* isolated from broiler chicken flocks in Egypt. Avian Diseases 57, pp 602-611.

Iguchi, A., Iyoda, S., Seto, K., Morita-Ishihara, T., Scheutz, F. and Ohnishi, M., 2015. *Escherichia coli* O-genotyping PCR: a comprehensive and practical platform for molecular O serogrouping. Journal of Clinical Microbiology 53, pp 2427-2432.

Ishmael, F.T. and Stellato, C., 2008. Principles and applications of polymerase chain reaction: basic science for the practicing physician. Annals of Allergy, Asthma and Immunology 101, pp 437-443.

Jacobson, S.H., Ostenson, C.G., Tullus, K. and Brauner, A., 1992. Serum resistance in *Escherichia coli* strains causing acute pyelonephritis and bacteraemia. Acta Pathologica, Microbiologica and Immunologica Scandinavica 100, pp 147-153.

Jacoby, G.A., 2005. Mechanisms of resistance to quinolones. Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America 41 Suppl 2, pp 120-126.

Jacoby, G.A., 2009. AmpC β-Lactamase. Clinical Microbiology Reviews 22, pp 161-182.

Jacoby, G.A., Strahilevitz, J. and Hooper, D.C., 2014. Plasmid-mediated quinolone resistance. Microbiology Spectrum 2, pp 1-24.

Jakob, H.P., Morgenstern, R., Albicker, P. and Hoop, R.K., 1998. Reasons for condemnation of slaughtered broilers from two large Swiss producers. Schweizer Archiv für Tierheilkunde 140, pp 60-64.

Jakobsen, L., Kurbasic, A., Skjot-Rasmussen, L., Ejrnaes, K., Porsbo, L.J., Pedersen, K., Jensen, L.B., Emborg, H.D., Agerso, Y., Olsen, K.E., Aarestrup, F.M., Frimodt-Moller, N. and Hammerum, A.M., 2010. *Escherichia coli* isolates from broiler chicken meat, broiler chickens, pork, and pigs share phylogroups and antimicrobial resistance with community-dwelling humans and patients with urinary tract infection. Foodborne Pathogens and Disease 7, pp 537-547.

Jakobsen, L., Sandvang, D., Jensen, V.F., Seyfarth, A.M., Frimodt-Moller, N. and Hammerum, A.M., 2007. Gentamicin susceptibility in *Escherichia coli* related to the genetic background: problems with breakpoints. Clinical Microbiology and Infection 13, pp 830-832.

Janssen, T., Schwarz, C., Preikschat, P., Voss, M., Philipp, H.C. and Wieler, L.H., 2001. Virulence-associated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis. International Journal of Medical Microbiology 291, pp 371-378.

Jaureguy, F., Landraud, L., Passet, V., Diancourt, L., Frapy, E., Guigon, G., Carbonnelle, E., Lortholary, O., Clermont, O., Denamur, E., Picard, B., Nassif, X. and Brisse, S., 2008. Phylogenetic and genomic diversity of human bacteremic *Escherichia coli* strains. BMC Genomics 9, pp 560-566.

Jeffrey, J.S., Chin, R.P. and Singer, R.S., 1999. Assessing cellulitis pathogenicity of *Escherichia coli* isolates in broiler chickens assessed by an *in vivo* inoculation model. Avian Diseases 43, pp 491-496.

Jeffrey, J.S., Nolan, L.K., Tonooka, K.H., Wolfe, S., Giddings, C.W., Horne, S.M., Foley, S.L., Lynne, A.M., Ebert, J.O., Elijah, L.M., Bjorklund, G., Pfaff-McDonough, S.J. and Singer, R.S., 2002. Virulence factors of *Escherichia coli* from cellulitis or colisepticemia lesions in chickens. Avian Diseases 46, pp 48-52.

Jensen, V.F., Jakobsen, L., Emborg, H.D., Seyfarth, A.M. and Hammerum, A.M., 2006. Correlation between apramycin and gentamicin use in pigs and an increasing reservoir of gentamicin-resistant *Escherichia coli*. The Journal of Antimicrobial Chemotherapy 58, pp 101-107.

Jeong, Y.W., Kim, T.E., Kim, J.H. and Kwon, H.J., 2012. Pathotyping avian pathogenic *Escherichia coli* strains in Korea. Journal of Veterinary Science 13, pp 145-152.

Jiang, H.X., Lu, D.H., Chen, Z.L., Wang, X.M., Chen, J.R., Liu, Y.H., Liao, X.P., Liu, J.H. and Zeng, Z.L., 2011. High prevalence and widespread distribution of multi-resistant *Escherichia coli* isolates in pigs and poultry in China. Veterinary Journal 187, pp 99-103.

Jin, W., Zheng, Z., Zhang, Y., Qin, A., Shao, H. and Liu, Y., 2008. Distribution of virulence-associated genes of avian pathogenic *Escherichia coli* isolates in China. Agricultural Sciences in China 7, pp 1511-1515.

Johnson, J.R., Delavari, P., Kuskowski, M. and Stell, A.L., 2001. Phylogenetic distribution of extraintestinal virulence-associated traits in *Escherichia coli*. The Journal of Infectious Diseases 183, pp 78-88.

Johnson, J.R., Johnston, B., Clabots, C.R., Kuskowski, M.A., Roberts, E. and C., D., 2008a. Virulence genotypes and phylogenetic background of *Escherichia coli* serogroup O6 isolates from humans, dogs, and cats. Journal of Clinical Microbiology 46, pp 417-422.

Johnson, J.R., Kuskowski, M.A., Owens, K., Gajewski, A. and Winokur, P.L., 2003. Phylogenetic origin and virulence genotype in relation to resistance to fluoroquinolones and/or extended-spectrum cephalosporins and cephamycins among *Escherichia coli* isolates from animals and humans. The Journal of Infectious Diseases 188, pp 759-768.

Johnson, J.R., McCabe, J.S., White, D.G., Johnston, B., Kuskowski, M.A. and McDermott, P., 2009. Molecular analysis of *Escherichia coli* from retail meats (2002-2004) from the United States

National Antimicrobial Resistance Monitoring System. Clinical Infectious Diseases 49, pp 195-201.

Johnson, J.R. and Russo, T.A., 2002. Extraintestinal pathogenic *Escherichia coli*: "the other bad *E. coli*". The Journal of Laboratory and Clinical Medicine 139, pp 155-162.

Johnson, J.R. and Stell, A.L., 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. Journal of Infectious Diseases 181, pp 261-272.

Johnson, J.R., Stell, A.L., O'Bryan, T.T., Kuskowski, M., Nowicki, B., Johnson, C., Maslow, J.N., Kaul, A., Kavle, J. and Prats, G., 2002a. Global molecular epidemiology of the O15:K52:H1 extraintestinal pathogenic *Escherichia coli* clonal group: Evidence of distribution beyond Europe. Journal of Clinical Microbiology 40, pp 1913-1923.

Johnson, T.J., Giddings, C.W., Horne, S.M., Gibbs, P.S., Wooley, R.E., Skyberg, J., Olah, P., Kercher, R., Sherwood, J.S., Foley, S.L. and Nolan, L.K., 2002b. Location of increased serum survival gene and selected virulence traits on a conjugative R plasmid in an avian *Escherichia coli* isolate. Avian Diseases 46, pp 342-352.

Johnson, T.J., Johnson, S.J. and Nolan, L.K., 2006. Complete DNA sequence of a ColBM plasmid from avian pathogenic *Escherichia coli* suggests that it evolved from closely related ColV virulence plasmids. Journal of Bacteriology 188, pp 5975-5983.

Johnson, T.J., Jordan, D., Kariyawasam, S., Stell, A.L., Bell, N.P., Wannemuehler, Y.M., Fernandez-Alarcon, C., Li, G.W., Tivendale, K.A., Logue, C.M. and Nolan, L.K., 2010. Sequence analysis and characterization of a transferable hybrid plasmid encoding multidrug resistance and enabling zoonotic potential for extraintestinal *Escherichia coli*. Infection and Immunity 78, pp 1931-1942.

Johnson, T.J., Logue, C.M., Johnson, J.R., Kuskowski, M.A., Sherwood, J.S., Barnes, H.J., DebRoy, C., Wannemuehler, Y.M., Obata-Yasuoka, M., Spanjaard, L. and Nolan, L.K., 2012. Associations between multidrug resistance, plasmid content, and virulence potential among extraintestinal pathogenic and commensal *Escherichia coli* from humans and poultry. Foodborne Pathogens and Disease 9, pp 37-46.

Johnson, T.J. and Nolan, L.K., 2009. Pathogenomics of the virulence plasmids of *Escherichia coli*. Microbiology and Molecular Biology Reviews 73, pp 750-774.

Johnson, T.J., Siek, K.E., Johnson, S.J. and Nolan, L.K., 2005. DNA sequence and comparative genomics of pAPEC-O2-R, an avian pathogenic *Escherichia coli* transmissible R plasmid. Antimicrobial Agents and Chemotherapy 49, pp 4681-4688.

Johnson, T.J., Wannemuehler, Y., Doetkott, C., Johnson, S.J., Rosenberger, S.C. and Nolan, L.K., 2008b. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. Journal of Clinical Microbiology 46, pp 3987-3996.

Johnson, T.J., Wannemuehler, Y., Johnson, S.J., Stell, A.L., Doetkott, C., Johnson, J.R., Kim, K.S., Spanjaard, L. and Nolan, L.K., 2008c. Comparison of extraintestinal pathogenic *Escherichia coli* strains from human and avian sources reveals a mixed subset representing potential zoonotic pathogens. Applied and Environmental Microbiology 74, pp 7043-7050.

Johnson, T.J., Wannemuehler, Y.M., Johnson, S.J., Logue, C.M., White, D.G., Doetkott, C. and Nolan, L.K., 2007. Plasmid replicon typing of commensal and pathogenic *Escherichia coli* isolates. Applied and Environmental Microbiology 73, pp 1976-1983.

Joint Expert Advisory Committee on Antibiotic Resistance (JETACAR), 1999. The use of antibiotics in food-producing animals: Antibiotic-resistant bacteria in animals and humans [Report], [Available from http://www.health.gov.au/internet/main/publishing.nsf/content/health-pubs-jetacar-cnt.htm /]; Accessed February 2014.

Jonas, D., Spitzmuller, B., Weist, K., Ruden, H. and Daschner, F.D., 2003. Comparison of PCR-based methods for typing *Escherichia coli*. Clinical Microbiology and Infection 9, pp 823-831.

Jones-Dias, D., Manageiro, V., Sampaio, D.A., Vieira, L. and Canica, M., 2015. Draft genome sequence of a pathogenic O86:H25 sequence type 57 *Escherichia coli* strain isolated from poultry and carrying 12 acquired antibiotic resistance genes. Genome Announcements 3, pp e01107.

Kabir, S.M.L., 2010. Avian colibacillosis and salmonellosis: A closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. International Journal of Environmental Research and Public Health 7, pp 89-114.

Kaesbohrer, A., Schroeter, A., Tenhagen, B.A., Alt, K., Guerra, B. and Appel, B., 2012. Emerging antimicrobial resistance in commensal *Escherichia coli* with public health relevance. Zoonoses and Public Health 59 pp 158-165.

Kafshdouzan, K., Salehi, T.Z., Nayeri, B., Madadgar, O., Yamasaki, S. and Hinenoya, A., 2013. Distribution of virulence associated genes in isolated *Escherichia coli* from avian colibacillosis. Iranian Journal of Veterinary Medicine 7, pp 1-6.

Kamada, N., Chen, G.Y., Inohara, N. and Nunez, G., 2013. Control of pathogens and pathobionts by the gut microbiota. Nature Immunology 14, pp 685-690.

Kaper, J.B., Nataro, J.P. and Mobley, H.L.T., 2004. Pathogenic *Escherichia coli*. Nature Reviews Microbiology 2, pp 123-140.

Karch, H., Schubert, S., Zhang, D., Zhang, W., Schmidt, H., Olschlager, T. and Hacker, J., 1999. A genomic island, termed high-pathogenicity island, is present in certain non-O157 Shiga toxin-producing *Escherichia coli* clonal lineages. Infection and Immunity 67, pp 5994-6001.

Kariyawasam, S., Johnson, T.J., Debroy, C. and Nolan, L.K., 2006a. Occurrence of pathogenicity island $I_{APEC-O1}$ genes among *Escherichia coli* implicated in avian colibacillosis. Avian Diseases 50, pp 405-410.

Kariyawasam, S., Johnson, T.J. and Nolan, L.K., 2006b. The pap operon of avian pathogenic *Escherichia coli* strain O1:K1 is located on a novel pathogenicity island. Infection and Immunity 74, pp 744-749.

Kariyawasam, S. and Nolan, L.K., 2009. Pap mutant of avian pathogenic *Escherichia coli* O1, an O1:K1:H7 strain, is attenuated *in vivo*. Avian Diseases 53, pp 255-260.

Kawamura-Sato, K., Yoshida, R., Shibayama, K. and Ohta, M., 2010. Virulence genes, quinolone and fluoroquinolone resistance, and phylogenetic background of uropathogenic *Escherichia coli* strains isolated in Japan. Japanese Journal of Infectious Diseases 63J, pp 113-115.

Keawcharoen, J., van Riel, D., van Amerongen, G., Bestebroer, T., Beyer, W.E., van Lavieren, R., Osterhaus, A.D., Fouchier, R.A. and Kuiken, T., 2008. Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5N1). Emerging Infectious Diseases 14, pp 600-607.

Kemmett, K., Humphrey, T., Rushton, S., Close, A., Wigley, P. and Williams, N.J., 2013. A longitudinal study simultaneously exploring the carriage of APEC virulence associated genes and the molecular epidemiology of faecal and systemic *E. coli* in commercial broiler chickens. PLoS One 8, pp e67749.

Kemmett, K., Williams, N.J., Chaloner, G., Humphrey, S., Wigley, P. and Humphrey, T., 2014. The contribution of systemic *Escherichia coli* infection to the early mortalities of commercial broiler chickens. Avian Pathology 43, pp 37-42.

Kern, W.V., Steinke, P., Schumacher, A., Schuster, S., von Baum, H. and Bohnert, J.A., 2006. Effect of 1-(1-naphthylmethyl)-piperazine, a novel putative efflux pump inhibitor, on antimicrobial drug susceptibility in clinical isolates of *Escherichia coli*. The Journal of Antimicrobial Chemotherapy 57, pp 339-343.

Kim, J., Bae, I.K., Jeong, S.H., Chang, C.L., Lee, C.H. and Lee, K., 2011. Characterization of IncF plasmids carrying the $bla_{CTXM-14}$ gene in clinical isolates of *Escherichia coli* from Korea. The Journal of Antimicrobial Chemotherapy 66, pp 1263-1268.

Kim, T.E., Jeong, Y.W., Cho, S.H., Kim, S.J. and Kwon, H.J., 2007. Chronological study of antibiotic resistances and their relevant genes in Korean avian pathogenic *Escherichia coli* isolates. Journal of Clinical Microbiology 45, pp 3309-3315.

Kluytmans, J.A., Overdevest, I.T., Willemsen, I., Kluytmans-van den Bergh, M.F., van der Zwaluw, K., Heck, M., Rijnsburger, M., Vandenbroucke-Grauls, C.M., Savelkoul, P.H., Johnston, B.D., Gordon, D. and Johnson, J.R., 2013. Extended-spectrum β-Lactamases-producing *Escherichia coli* from retail chicken meat and humans: comparison of strains, plasmids, resistance genes, and virulence factors. Clinical Infectious Diseases 56, pp 478-487.

Kmeť, V. and KmeŤová, M., 2010. High level of quinolone resistance in *Escherichia coli* from healthy chicken broilers. Folia Microbiology 55, pp 79-82.

Kmeť, V., KmeŤová, M., Drugdova, Z. and Stanko, M., 2013. Virulence and antibiotic resistance of *Escherichia coli* isolated from rooks. Annals of Agricultural and Environmental Medicine 20, pp 273-275.

Knöbl, T., Gomes, T.A.T., Vieira, M.A.M., Bottino, J.A. and Ferreira, A.J.P., 2004. Detection of *pap, sfa* and *fim* adhesin-encoding operons in avian pathogenic *Escherichia coli*. International Journal of Applied Research Veterinary Medicine 2, pp 135-141.

Knobl, T., Moreno, A.M., Paixao, R., Gomes, T.A., Vieira, M.A., da Silva Leite, D., Blanco, J.E. and Ferreira, A.J., 2012. Prevalence of avian pathogenic *Escherichia coli* (APEC) clone harboring *sfa* gene in Brazil. Scientific World Journal 2012, pp 437-342.

Kobayashi, R.K., Aquino, I., Ferreira, A.L. and Vidotto, M., 2011. ECOR phylogenetic analysis and virulence genotyping of avian pathogenic *Escherichia coli* strains and *Escherichia coli* isolates from commercial chicken carcasses in Southern Brazil. Foodborne Pathogen Diseases 8, pp 631-634.

Koebnik, R., Baumler, A.J., Heesemann, J., Braun, V. and Hantke, K., 1993. The TonB protein of *Yersinia enterocolitica* and its interactions with TonB-box proteins. Molecular and General Genetics 237, pp 152-160.

Kohler, C.D. and Dobrindt, U., 2011. What defines extraintestinal pathogenic *Escherichia coli*? International Journal of Medical Microbiology 301, pp 642-647.

Kools, S.A., Moltmann, J.F. and Knacker, T., 2008. Estimating the use of veterinary medicines in the European union. Regulatory Toxicology and Pharmacology 50, pp 59-65.

Kwon, S-G., Cha, S-Y., Choi, E-J., Kim, B., Song, H-J. and Jang, H-K., 2008. Epidemiological prevalence of avian pathogenic *Escherichia coli* differentiated by multiplex PCR from commercial chickens and hatchery in Korea. Journal of Bacteriology and Virology 38, pp 179-188.

La Ragione, R.M., Cooley, W.A. and Woodward, M.J., 2000. The role of fimbriae and flagella in the adherence of avian strains of *Escherichia coli* O78 : K80 to tissue culture cells and tracheal and gut explants. Journal of Medical Microbiology 49, pp 327-338.

La Ragione, R.M. and Woodward, M.J., 2002. Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia. Research in Veterinary Science 73, pp 27-35.

Lacher, D.W., Gangiredla, J., Jackson, S.A., Elkins, C.A. and Feng, P.C., 2014. Novel microarray design for molecular serotyping of shiga toxin- producing *Escherichia coli* strains isolated from fresh produce. Applied and Environmental Microbiology 80, pp 4677-4682.

Lafont, J.P., Dho, M., Dhauteville, H.M., Bree, A. and Sansonetti, P.J., 1987. Presence and expression of aerobactin genes in virulent avian strains of *Escherichia coli*. Infection and Immunity 55, pp 193-197.

Lalioui, L. and Le Bouguenec, C., 2001. *afa-8* Gene cluster is carried by a pathogenicity island inserted into the $tRNA^{Phe}$ of human and bovine pathogenic *Escherichia coli* isolates. Infection and Immunity 69, pp 937-948.

Lambert, T., 2012. Antibiotics that affect the ribosome. Revue Scientifique et Technique 31, pp 57-64.

Landers, T.F., Cohen, B., Wittum, T.E. and Larson, E.L., 2012. A review of antibiotic use in food animals: perspective, policy, and potential. Public Health Reports 127, pp 4-22.

Landis, J.R. and Kochi, G.G., 1977a. The measurement of observer agreement for categorical data. Biometrics 33, pp 159–174.

Landis, J.R. and Kochi, G.G., 1977b. A one-way components of variance model for categorical data. Biometrics 33, pp 671–679.

Landman, W.J., Buter, G.J., Dijkman, R. and van Eck, J.H., 2014. Molecular typing of avian pathogenic *Escherichia coli* colonies originating from outbreaks of *E. coli* peritonitis syndrome in chicken flocks. Avian Pathology 43, pp 345-356.

Landman, W.J.M. and Cornelissen, R.A., 2006. Virulence factors of *Escherichia coli*, with emphasis on avian pathogenic isolates. Tijdschrift Voor Diergeneeskunde 131, pp 822-830.

Langdon, A., Crook, N. and Dantas, G., 2016. The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. Genome Medicine 8, pp 39-12.

Larsen, M.V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R.L., Jelsbak, L., Sicheritz-Ponten, T. and Ussery, D.W., 2012. Multilocus sequence typing of total-genome-sequenced bacteria. Journal of Clinical Microbiology 50, pp 1355-1361.

Latham, R.H. and Stamm, W.E., 1984. Role of fimbriated *Escherichia coli* in urinary tract infections in adult women: correlation with localization studies. The Journal of Infectious Diseases 149, pp 835-840.

Laube, H., Friese, A., von Salviati, C., Guerra, B. and Rosler, U., 2014. Transmission of ESBL/AmpC-producing *Escherichia coli* from broiler chicken farms to surrounding areas. Veterinary Microbiology 172, pp 3-4.

Lavakhamseh, H., Mohajeri, P., Rouhi, S., Shakib, P., Ramazanzadeh, R., Rasani, A. and Mansouri, M., 2016. Multidrug-resistant *Escherichia coli* strains isolated from patients are associated with Class 1 and 2 Integrons. Chemotherapy 61, pp 72-76.

Law, J.W., Ab Mutalib, N.S., Chan, K.G. and Lee, L.H., 2014. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. Frontiers in Microbiology 5, pp 770-779.

Lee, C.H., Su, L.H., Li, C.C., Chien, C.C., Tang, Y.F. and Liu, J.W., 2010. Microbiologic and clinical implications of bacteremia due to extended-spectrum-β-Lactamases-producing *Klebsiella pneumoniae* with or without plasmid-mediated AmpC β-Lactamases DHA-1. Antimicrobial Agents and Chemotherapy 54, pp 5395-5398.

Leimbach, A., Hacker, J. and Dobrindt, U., 2013. *E. coli* as an all-rounder: the thin line between commensalism and pathogenicity. Current Topics in Microbiology and Immunology 358, pp 3-32.

Leitner, G. and Heller, E.D., 1992. Colonization of *Escherichia coli* in young turkeys and chickens. Avian Diseases 36, pp 211-220.

Leverstein-van Hall, M.A., Dierikx, C.M., Cohen Stuart, J., Voets, G.M., van den Munckhof, M.P., van Essen-Zandbergen, A., Platteel, T., Fluit, A.C., van de Sande-Bruinsma, N., Scharinga, J., Bonten, M.J. and Mevius, D.J., 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clinical Microbiology and Infection 17, pp 873-880.

Lévesque, B.t., Brousseau, P., Bernier, F., Dewailly, É. and Joly, J., 2000. Study of the bacterial content of ring-billed gull droppings in relation to recreational water quality. Water Research 34, pp 1089-1096.

Li, X.Z., Mehrotra, M., Ghimire, S. and Adewoye, L., 2007. β-lactam resistance and β-Lactamases in bacteria of animal origin. Veterinary Microbiology 121, pp 197-214.

Lima-Filho, J.V., Martins, L.V., Nascimento, D.C.d.O., Ventura, R.F., Batista, J.E.C., Silva, A.F.B., Ralph, M.T., Vaz, R.V., Rabello, C.B.-V., Silva, I.d.M.M.d. and Evêncio-Neto, J., 2013. Zoonotic potential of multidrug-resistant extraintestinal pathogenic *Escherichia coli* obtained from healthy poultry carcasses in Salvador, Brazil. Brazilian Journal of Infectious Diseases 17, pp 54-61.

Ling, J., Pan, H., Gao, Q., Xiong, L., Zhou, Y., Zhang, D., Gao, S. and Liu, X., 2013. Aerobactin synthesis genes *iucA* and *iucC* contribute to the pathogenicity of avian pathogenic *Escherichia coli* O2 strain E058. PLoS One 8, pp e57794.

Linggood, M.A., Roberts, M., Ford, S., Parry, S.H. and Williams, P.H., 1987. Incidence of the aerobactin iron uptake system among *Escherichia coli* isolates from infections of farm animals. Journal of General Microbiology 133, pp 835-842.

Linton, A.H. and Hinton, M.H., 1988. Enterobacteriaceae associated with animals in health and disease. Society for Applied Bacteriology Symposium Series 17, pp 71-85.

Literak, I., Dolejska, M., Janoszowska, D., Hrusakova, J., Meissner, W., Rzyska, H., Bzoma, S. and Cizek, A., 2010. Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes encoding the extended-spectrum beta-lactamase and QnrS, in waterbirds on the Baltic Sea coast of Poland. Applied and Environmental Microbiology 76, pp 8126-8134.

Liu, B.T., Liao, X.P., Yang, S.S., Wang, X.M., Li, L.L., Sun, J., Yang, Y.R., Fang, L.X., Li, L., Zhao, D.H. and Liu, Y.H., 2012. Detection of mutations in the *gyrA* and *parC* genes in *Escherichia coli* isolates carrying plasmid-mediated quinolone resistance genes from diseased food-producing animals. Journal of Medical Microbiology 61, pp 1591-1599.

Liu, B.T., Yang, Q.E., Li, L., Sun, J., Liao, X.P., Fang, L.X., Yang, S.S., Deng, H. and Liu, Y.H., 2013. Dissemination and characterization of plasmids carrying *oqxAB-bla*$_{\text{CTX-M}}$ genes in *Escherichia coli* isolates from food-producing animals. PLoS One 8, pp e73947.

Livermore, D.M., 1995. β-Lactamases in laboratory and clinical resistance. Clinical Microbiology Reviews 8, pp 557-584.

Lleo, M.M., Bonato, B., Tafi, M.C., Signoretto, C., Pruzzo, C. and Canepari, P., 2005. Molecular vs culture methods for the detection of bacterial faecal indicators in groundwater for human use. Letters in Applied Microbiology 40, pp 289-294.

Logue, C.M., Wannemuehler, Y., Nicholson, B.A., Doetkott, C., Barbieri, N.L. and Nolan, L.K., 2017. Comparative analysis of phylogenetic assignment of human and avian ExPEC and fecal commensal *Escherichia coli* using the (previous and revised) clermont phylogenetic typing methods and its impact on avian pathogenic *Escherichia coli* (APEC) classification. Frontiers in Microbiology 8, pp 1-11.

Lohren, U., Ricci, A. and Cummings, T.S., 2009. Guidelines for antimicrobial use in poultry. In: Guardabassi, L., Jensen, L., Kruse, H. (Eds.) Guide to Antimicrobial Use in Animals. Blackwell Publishing, Oxford, UK, pp 126-142.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J.J. and Lee, M.D., 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Applied and Environmental Microbiology 69, pp 6816-6824.

Lwinger, K., Fisher, C., Jeroch, H., Sauveur, B., Tiller, H. and Whitehead, C., 2016. A brief history of poultry nutrition over the last hundred years. World's Poultry Science Journal 72, pp 701-720.

Lymberopoulos, M.H., Houle, S., Daigle, F., Leveille, S., Bree, A., Moulin-Schouleur, M., Johnson, J.R. and Dozois, C.M., 2006. Characterization of Stg fimbriae from an avian pathogenic *Escherichia coli* O78:K80 strain and assessment of their contribution to colonization of the chicken respiratory tract. Journal of Bacteriology 188, pp 6449-6459.

Lynne, A.M., Kariyawasam, S., Wannemuehler, Y., Johnson, T.J., Johnson, S.J., Sinha, A.S., Lynne, D.K., Moon, H.W., Jordan, D.M., Logue, C.M., Foley, S.L. and Nolan, L.K., 2012. Recombinant Iss as a potential vaccine for avian colibacillosis. Avian Diseases 56, pp 192-199.

Lynne, A.M., Skyberg, J.A., Logue, C.M. and Nolan, L.K., 2007. Detection of Iss and Bor on the surface of *Escherichia coli*. Veterinary Microbiology 102, pp 660-666.

Maamar, E., Hammami, S., Alonso, C.A., Dakhli, N., Abbassi, M.S., Ferjani, S., Hamzaoui, Z., Saidani, M., Torres, C. and Boutiba-Ben Boubaker, I., 2016. High prevalence of extended-spectrum and plasmidic AmpC beta-lactamase-producing *Escherichia coli* from poultry in Tunisia. International Journal of Food Microbiology 231, pp 69-75.

Machado, E., Coque, T.M., Canton, R., Sousa, J.C. and Peixe, L., 2008. Antibiotic resistance integrons and extended-spectrum β-lactamases among *Enterobacteriaceae* isolates recovered from chickens and swine in Portugal. The Journal of Antimicrobial Chemotherapy 62, pp 296-302.

Magnet, S. and Blanchard, J.S., 2005. Molecular insights into aminoglycoside action and resistance. Chemical Reviews 105, pp 477-498.

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M. and Spratt, B.G., 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of

pathogenic microorganisms. Proceedings of the National Academy of Sciences of the United States of America 95, pp 3140-3145.

Mainil, J., 2013. *Escherichia coli* virulence factors. Veterinary Immunology and Immunopathology 152, pp 2-12.

Maluta, R.P., Logue, C.M., Casas, M.R., Meng, T., Guastalli, E.A., Rojas, T.C., Montelli, A.C., Sadatsune, T., de Carvalho Ramos, M., Nolan, L.K. and da Silveira, W.D., 2014. Overlapped sequence types (STs) and serogroups of avian pathogenic (APEC) and human extra-intestinal pathogenic (ExPEC) *Escherichia coli* isolated in Brazil. PLoS One 9, pp e105016.

Manges, A.R. and Johnson, J.R., 2012. Food-borne origins of *Escherichia coli* causing extraintestinal infections. Clinical Infectious Diseases 55, pp 712-719.

Marathe, N., Regina, V., Walujkar, S., Charan, S., Moore, E. and Larsson, D., 2013. A treatment plant receiving waste water from multiple bulk drug manufacturers is a reservoir for highly multi-drug resistant integron-bearing bacteria. PLoS One 8, pp e77310.

Marc, D., Arne, P., Bree, A. and Dho-Moulin, M., 1998. Colonization ability and pathogenic properties of a *fim*(-) mutant of an avian strain of *Escherichia coli*. Research in Microbiology 149, pp 473-485.

Marshall, B.M. and Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. Clinical Microbiology Reviews 24, pp 718-733.

Martinez-Martinez, L., Pascual, A. and Jacoby, G.A., 1998. Quinolone resistance from a transferable plasmid. Lancet 351, pp 797-799.

Martinez-Medina, M., Mora, A., Blanco, M., Lopez, C., Alonso, M.P., Bonacorsi, S., Nicolas-Chanoine, M.H., Darfeuille-Michaud, A., Garcia-Gil, J. and Blanco, J., 2009. Similarity and divergence among adherent-invasive *Escherichia coli* and extraintestinal pathogenic *E. coli* strains. Journal of Clinical Microbiology 47, pp 3968-3979.

Martinez, M., Blondeau, J., Cerniglia, C.E., Fink-Gremmels, J., Guenther, S., Hunter, R.P., Li, X.Z., Papich, M., Silley, P., Soback, S., Toutain, P.L. and Zhang, Q., 2014. Workshop report: The 2012 Antimicrobial Agents in Veterinary Medicine: exploring the consequences of antimicrobial drug use: a 3-D approach. Journal of Veterinary Pharmacology and Therapeutics 37, pp 1-16.

Martineza, M., McDermott, P. and Walker, R., 2006. Pharmacology of the fluoroquinolones: A perspective for the use in domestic animals. The Veterinary Journal 172, pp 10-28.

Maryam, A.R., 2016. Molecular typing of uropathogenic *E. coli* strains by the ERIC-PCR method. Electronic Physician 8, pp 2291-2296.

Mascaretti, O.A., 2003. Antibiotics and synthetic antibacterial agents. In: Mascaretti, O.A. (Ed.) Bacteria versus Antibacterial Agents an Integrated Approach. ASM Press, Washington DC, pp 97-105.

Mata, C., Miro, E., Alvarado, A., Garcillan-Barcia, M.P., Toleman, M., Walsh, T.R., de la Cruz, F. and Navarro, F., 2012. Plasmid typing and genetic context of AmpC β-lactamases in Enterobacteriaceae lacking inducible chromosomal *ampC* genes: findings from a Spanish hospital 1999-2007. The Journal of Antimicrobial Chemotherapy 67, pp 115-122.

Matagne, A.J., Lamotte-Brasseur, J. and Frere, J.M., 1998. Catalytic properties of class A beta-lactamases: efficiency and diversity. Biochemical Journal 330, pp 581-598.

Mataseje, L.F., Baudry, P.J., Zhanel, G.G., Morck, D.W., Read, R.R., Louie, M. and Mulvey, M.R., 2010. Comparison of CMY-2 plasmids isolated from human, animal, and environmental *Escherichia coli* and *Salmonella* spp. from Canada. Diagnostic Microbiology and Infectious Disease 67, pp 387-391.

Maturana, V.G., De Pace, F., Carlos, C., Pires, M.M., De Campos, T.A. and Nakazato, G., 2011. Subpathotypes of avian pathogenic *Escherichia coli* (APEC) exist as defined by their syndromes and virulence traits. Open Microbiology Journal 5, pp 55-64.

Maurer, J.J., Brown, T.P., Steffens, W.L. and Thayer, S.G., 1998. The occurrence of ambient temperature-regulated adhesions, curli and the temperature-sensitive hemagglutinin Tsh among avian *Escherichia coli*. Avian Diseases 42, pp 106-118.

Mbanga, J. and Nyararai, Y.O., 2015. Virulence gene profiles of avian pathogenic *Escherichia coli* isolated from chickens with colibacillosis in Bulawayo, Zimbabwe. The Onderstepoort Journal of Veterinary Research 82, pp 840-850.

McOrist, A.L., Jackson, M. and Bird, A.R., 2002. A comparison of five methods for extraction of bacterial DNA from human faecal samples. Journal of Microbiological Methods 50, pp 131-139.

McPeake, S.J., Smyth, J.A. and Ball, H.J., 2005. Characterisation of avian pathogenic *Escherichia coli* (APEC) associated with colisepticaemia compared to faecal isolates from healthy birds. Veterinary Microbiology 110, pp 245-253.

Meacham, K.J., Zhang, L., Foxman, B., Bauer, R.J. and Marrs, C.F., 2003. Evaluation of genotyping large numbers of *Escherichia coli* isolates by enterobacterial repetitive intergenic consensus-PCR. Journal of Clinical Microbiology 41, pp 5224-5226.

Mellata, M., 2013. Human and avian extraintestinal pathogenic *Escherichia coli*: infections, zoonotic risks and antibiotic resistance trends. Foodborne Pathogens and Disease 10, pp 916-932.

Mellata, M., Dho-Moulin, M., Dozois, C.M., Curtiss, R., 3rd, Brown, P.K., Arne, P., Bree, A., Desautels, C. and Fairbrother, J.M., 2003. Role of virulence factors in resistance of avian pathogenic *Escherichia coli* to serum and in pathogenicity. Infection and Immunity 71, pp 536-576.

Mellata, M., Maddux, J.T., Nam, T., Thomson, N., Hauser, H., Stevens, M.P., Mukhopadhyay, S., Sarker, S., Crabbe, A., Nickerson, C.A., Santander, J. and Curtiss, R., 2012. New insights into the bacterial fitness-associated mechanisms revealed by the characterization of large plasmids of an avian pathogenic *E. coli*. PloS One 7, pp e29481.

Meyer, D.H., Mintz, K.P. and Fives-Taylor, P.M., 1997. Models of invasion of enteric and periodontal pathogens into epithelial cells: a comparative analysis. Critical Reviews in Oral Biology and Medicine 8, pp 389-409.

Meyer, E., Schwab, F., Schroeren-Boersch, B. and Gastmeier, P., 2010. Dramatic increase of third-generation cephalosporin-resistant *E. coli* in German intensive care units: secular trends in antibiotic drug use and bacterial resistance, 2001 to 2008. Critical Care 14, pp 113-222.

Miajlovic, H. and Smith, S.G., 2014. Bacterial self-defence: how *Escherichia coli* evades serum killing. FEMS Microbiology Letters 354, pp 1-9.

Miller, D.N., Bryant, J.E., Madsen, E.L. and Ghiorse, W.C., 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Applied and Environmental Microbiology 65, pp 4715-4724.

Mohammad, K., 2010. Population structure of gut *Escherichia coli* and its role in development of extra-intestinal infections. Iranian Journal of Microbiology 2, pp 59–72.

Mokady, D., Gophna, U. and Ron, E.Z., 2005. Virulence factors of septicemic *Escherichia coli* strains. International Journal of Medical Microbiology 295, pp 455-462.

Mol, O. and Oudega, B., 1996. Molecular and structural aspects of fimbriae biosynthesis and assembly in *Escherichia coli*. FEMS Microbiology Reviews 19, pp 25-52.

Monroy, M.A., Knobl, T., Bottino, J.A., Ferreira, C.S. and Ferreira, A.J., 2005. Virulence characteristics of *Escherichia coli* isolates obtained from broiler breeders with salpingitis. Comparative Immunology, Microbiology and Infectious Diseases 28, pp 1-15.

Monteiro, L., Bonnemaison, D., Vekris, A., Petry, K.G., Bonnet, J., Vidal, R., Cabrita, J. and Megraud, F., 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. Journal of Clinical Microbiology 35, pp 995-998.

Moodley, A. and Guardabassi, L., 2009. Transmission of IncN plasmids carrying $bla_{\text{CTX-M-1}}$ between commensal *Escherichia coli* in pigs and farm workers. Antimicrobial Agents and Chemotherapy 53, pp 1709-1711.

Moon, H.W., 1990. Colonization factor antigens of enterotoxigenic *Escherichia coli* in animals. Current Topics in Microbiology and Immunology 151, pp 147-165.

Mora, A., Blanco, M., Lopez, C., Mamani, R., Blanco, J.E., Alonso, M.P., Garcia-Garrote, F., Dahbi, G., Herrera, A., Fernandez, A., Fernandez, B., Agulla, A., Bou, G. and Blanco, J., 2011. Emergence of clonal groups O1:HNM-D-ST59, O15:H1-D-ST393, O20:H34/HNM-D-ST354, O25b:H4-B2-ST131 and ONT:H21,42-B1-ST101 among CTX-M-14-producing *Escherichia coli* clinical isolates in Galicia, Northwest Spain. International Journal of Antimicrobial Agents 37, pp 16-21.

Mora, A., Viso, S., Lopez, C., Alonso, M.P., Garcia-Garrote, F., Dabhi, G., Mamani, R., Herrera, A., Marzoa, J., Blanco, M., Blanco, J.E., Moulin-Schouleur, M., Schouler, C. and Blanco, J., 2013. Poultry as reservoir for extraintestinal pathogenic *Escherichia coli* O45:K1:H7-B2-ST95 in humans. Veterinary Microbiology 167, pp 506-512.

Moran, R.A., Anantham, S., Pinyon, J.L. and Hall, R.M., 2015. Plasmids in antibiotic susceptible and antibiotic resistant commensal *Escherichia coli* from healthy Australian adults. Plasmid 80, pp 24-31.

Moreno, E., Prats, G., Sabate, M., Perez, T., Johnson, J.R. and Andreu, A., 2006. Quinolone, fluoroquinolone and trimethoprim/sulfamethoxazole resistance in relation to virulence

determinants and phylogenetic background among uropathogenic *Escherichia coli*. The Journal of Antimicrobial Chemotherapy 57, pp 204-211.

Morley, P.S., Apley, M.D., Besser, T.E., Burney, D.P., Fedorka-Cray, P.J., Papich, M.G., Traub-Dargatz, J.L. and Weese, J.S., 2005. Antimicrobial drug use in veterinary medicine. Journal of Veterinary Medical Science 19, pp 617-629.

Morris, J.G. and Potter, M., 1997. Emergence of new pathogens as a function of changes in host susceptibility. Emerging Infectious Diseases 3, pp 435-441.

Moudgal, V. and Kaatz, G.W., 2009. Fluoroquinolone resistance in bacteria. In: Mayers, D.L. (Ed.) Antimicrobial Drug Resistance. Humana Press Inc., Totowa, NJ, USA, pp 195-205.

Moulin-Schouleur, M., Reperant, M., Laurent, S., Bree, A., Mignon-Grasteau, S., Germon, P., Rasschaert, D. and Schouler, C., 2007. Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: Link between phylogenetic relationships and common virulence patterns. Journal of Clinical Microbiology 45, pp 3366-3376.

Moulin-Schouleur, M., Schouler, C., Tailliez, P., Kao, M.-R., Bree, A., Germon, P., Oswald, E., Mainil, J., Blanco, M. and Blanco, J., 2006. Common virulence factors and genetic relationships between O18:K1:H7 *Escherichia coli* isolates of human and avian origin. Journal of Clinical Microbiology 44, pp 3484-3492.

Mulder and Nan, D., 2011. Crossroads for Growth. The International Poultry Sector Towards 2020. Rabobank. Utrecht. The Netherlands.

Murase, K., Martin, P., Porcheron, G., Houle, S., Helloin, E., Penary, M., Nougayrede, J.P., Dozois, C.M., Hayashi, T. and Oswald, E., 2016. HlyF produced by extraintestinal pathogenic *Escherichia coli* is a virulence factor that regulates outer membrane vesicle biogenesis. The Journal of Infectious Diseases 213, pp 856-865.

Musa, H.H., He, S.F., Wu, S.L., Zhu, C.H., Liu, Z.H., Zhang, Z.N., Raj, V.S., Gu, R.X. and Zhu, G.Q., 2009. Genetic engineering of avian pathogenic *E. coli* to study the functions of FimH adhesin. Indian Journal of Experimental Biology 47, pp 916-920.

Nakazato, G., de Campos, T.A., Stehling, E.G., Brocchi, M. and da Silveira, W.D., 2009. Virulence factors of avian pathogenic *Escherichia coli* (APEC). Pesquisa Veterinaria Brasileira 29, pp 479-486.

Nather, G., Alter, T., Martin, A. and Ellerbroek, L., 2009. Analysis of risk factors for *Campylobacter* species infection in broiler flocks. Poultry Science 88, pp 1299-1305.

National Center for Biotechnology Information (NCBI), Beta-lactamase data resources, [Available from https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/]; Accessed September 2017.

Naveh, M.W., Zusman, T., Skutelsky, E. and Ron, E.Z., 1984. Adherence pili in avian strains of *Escherichia coli*: effect on pathogenicity. Avian Diseases 28, pp 651-661.

Ndi, O. and Barton, M., 2012. Antibiotic Resistance in Animals—The Australian Perspective. In: Keen, P., Montforts, M. (Eds.) Antimicrobial Resistance in the Environment. John Wiley and Sons, Inc., Hoboken, New Jersey, USA, pp 265-290.

Nechvatal, J.M., Ram, J.L., Basson, M.D., Namprachan, P., Niec, S.R., Badsha, K.Z., Matherly, L.H., Majumdar, A.P. and Kato, I., 2008. Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces. Journal of Microbiological Methods 72, pp 124-132.

Nelson, M.L. and Levy, S.B., 2011. The history of the tetracyclines. Annals of the New York Academy of Sciences 1241, pp 17-32.

Neu, H.C., 1992. The crisis in antibiotic-resistance. Science 257, pp 1064-1073.

Newell, D.G., Elvers, K.T., Dopfer, D., Hansson, I., Jones, P., James, S., Gittins, J., Stern, N.J., Davies, R., Connerton, I., Pearson, D., Salvat, G. and Allen, V.M., 2011. Biosecurity-based interventions and strategies to reduce *Campylobacter* spp. on poultry farms. Applied and Environmental Microbiology 77, pp 8605-8614.

Ngeleka, M., Brereton, L., Brown, G. and Fairbrother, J.M., 2002. Pathotypes of avian *Escherichia coli* as related to *tsh-*, *pap-*, *pil-*, and *iuc*-DNA sequences, and antibiotic sensitivity of isolates from internal tissues and the cloacae of broilers. Avian Diseases 46, pp 143-152.

Ngeleka, M., Kwaga, J.K., White, D.G., Whittam, T.S., Riddell, C., Goodhope, R., Potter, A.A. and Allan, B., 1996. *Escherichia coli* cellulitis in broiler chickens: clonal relationships among strains and analysis of virulence-associated factors of isolates from diseased birds. Infection and Immunity 64, pp 3118-3126.

Nicklas, J.A. and Buel, E., 2003. Quantification of DNA in forensic samples. Analytical and Bioanalytical Chemistry 376, pp 1160-1167.

Nilsson, O., Borjesson, S., Landen, A. and Bengtsson, B., 2014. Vertical transmission of *Escherichia coli* carrying plasmid-mediated AmpC (pAmpC) through the broiler production pyramid. The Journal of Antimicrobial Chemotherapy 69, pp 1497-1500.

Nogrady, N., Paszti, J., Piko, H. and Nagy, B., 2006. Class 1 integrons and their conjugal transfer with and without virulence-associated genes in extra-intestinal and intestinal *Escherichia coli* of poultry. Avian Pathology 35, pp 349-321.

Nolan, L., K., Barnes, H., Jean Pirre, V., Abdul-Aziz, T. and Louge, C.M., 2013. Colibacillosis. In: Swayne, D., E. (Eds.) Diseases of Poultry. John Wiley and Sons, Ames, Iowa, pp 751-785.

Nolan, L.K., Giddings, C.W., Horne, S.M., Doetkott, C., Gibbs, P.S., Wooley, R.E. and Foley, S.L., 2002. Complement resistance, as determined by viable count and flow cytometric methods, and its association with the presence of *iss* and the virulence of avian *Escherichia coli*. Avian Diseases 46, pp 386-392.

Nolan, L.K., Horne, S.M., Giddings, C.W., Foley, S.L., Johnson, T.J., Lynne, A.M. and Skyberg, J., 2003. Resistance to serum complement, *iss*, and virulence of avian *Escherichia coli*. Veterinary Research Communications 27, pp 101-110.

NORM/NORM-VET, 2007. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway, [Available from http://www.vetinst.no/eng/Research/Publications/Norm-Norm-Vet-Report/The-Norm-Norm-Vet-Report-2006]; Accessed June 2015.

Norton, R.A., Bilgili, S.F. and McMurtrey, B.C., 1997. A reproducible model for the induction of avian cellulitis in broiler chickens. Avian Diseases 41, pp 422-428.

Norton, R.A., Macklin, K.S. and McMurtrey, B.L., 2000. The association of various isolates of *Escherichia coli* from the United States with induced cellulitis and colibacillosis in young broiler chickens. Avian Pathology 29, pp 571-574.

Novick, R.P., 1987. Plasmid incompatibility. Microbiological Reviews 51, pp 381-395.

O'Hara, A.M. and Shanahan, F., 2006. The gut flora as a forgotten organ. EMBO Reports 7, pp 688-693.

Obeng, A.S., Rickard, H., Ndi, O., Sexton, M. and Barton, M., 2012. Antibiotic resistance, phylogenetic grouping and virulence potential of *Escherichia coli* isolated from the faeces of intensively farmed and free range poultry. Veterinary Microbiology 154, pp 305-315.

Obeng, A.S., Rickard, H., Ndi, O., Sexton, M. and Barton, M., 2014. Prevalence of antimicrobial resistance in Enterococci and *Escherichia coli* in meat chicken flocks during a production cycle and egg layer pullets during rearing. International Journal of Poultry Science 13, pp 489-503.

Oh J.Y., Kwon Y.K., Tamang M.D., Jang H.K., Jeong O.M., Lee H.S., Kang M.S. , 2016. Plasmid-mediated quinolone resistance in *Escherichia coli* isolates from wild birds and chickens in South Korea. Microbial Drug Resistance 22, pp 69-79.

Oh, J.Y., Kang, J.M., Song, E.A., Kim, J.Y., Shin, E.G., Kim, M.J., Kwon, J.H. and Kwon, Y.K., 2011. Characterization of *Escherichia coli* isolates from laying hens with colibacillosis on 2 commercial egg-producing farms in Korea. Poultry Science 90, pp 1948-1954.

Ojer-Usoz, E., Gonzalez, D., Vitas, A.I., Leiva, J., Garcia-Jalon, I., Febles-Casquero, A. and Escolano Mde, L., 2013. Prevalence of extended-spectrum β-lactamase-producing *Enterobacteriaceae* in meat products sold in Navarra, Spain. Meat Science 93, pp 316-321.

Olsen, A., Jonsson, A. and Normark, S., 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature 338, pp 652-655.

Olsen, R.H., Christensen, H. and Bisgaard, M., 2012. Comparative genomics of multiple plasmids from APEC associated with clonal outbreaks demonstrates major similarities and identifies several potential vaccine-targets. Veterinary Microbiology 158, pp 384-393.

Orskov, F. and Orskov, I., 1992. *Escherichia coli* serotyping and disease in man and animals. Canadian Journal of Microbiology 38, pp 699-704.

Osinska, A., Harnisz, M. and Korzeniewska, E., 2016. Prevalence of plasmid-mediated multidrug resistance determinants in fluoroquinolone-resistant bacteria isolated from sewage and surface water. Environmental Science and Pollution Research International 23, pp 10818-10831.

Oulas, A., Pavloudi, C., Polymenakou, P., Pavlopoulos, G.A., Papanikolaou, N., Kotoulas, G., Arvanitidis, C. and Iliopoulos, I., 2015. Metagenomics: tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. Bioinformatics and Biology Insights 9, pp 75-88.

Oves-Costales, D., Kadi, N. and Challis, G.L., 2009. The long-overlooked enzymology of a nonribosomal peptide synthetase-independent pathway for virulence-conferring siderophore biosynthesis. Chem Commun 21, pp 6530-6541.

Oz, T., Guvenek, A., Yildiz, S., Karaboga, E., Tamer, Y.T., Mumcuyan, N., Ozan, V.B., Senturk, G.H., Cokol, M., Yeh, P. and Toprak, E., 2014. Strength of selection pressure is an important parameter contributing to the complexity of antibiotic resistance evolution. Molecular Biology and Evolution 31, pp 2387-2401.

Ozawa, M., Harada, K., Kojima, A., Asai, T. and Sameshima, T., 2008. Antimicrobial susceptibilities, serogroups, and molecular characterization of avian pathogenic *Escherichia coli* isolates in Japan. Avian Diseases 52, pp 392-397.

Pacholewicz, E., Liakopoulos, A., Swart, A., Gortemaker, B., Dierikx, C., Havelaar, A. and Schmitt, H., 2015. Reduction of extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing *Escherichia coli* through processing in two broiler chicken slaughterhouses. International Journal of Food Microbiology 215, pp 57-63.

Packey, C.D. and Sartor, R.B., 2009. Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. Current Opinion in Infectious Diseases 22, pp 292-301.

Paixao, A.C., Ferreira, A.C., Fontes, M., Themudo, P., Albuquerque, T., Soares, M.C., Fevereiro, M., Martins, L. and Correa de Sa, M.I., 2016. Detection of virulence-associated genes in pathogenic and commensal avian *Escherichia coli* isolates. Poultry Science 95, pp 1646–1652.

Pallen, M. J. (2016). Microbial bioinformatics 2020. *Microbial Biotechnology*. 9, pp. 681–

686.

Pan, D. and Yu, Z., 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5, pp 108-119.

Pandey, P.K., Kass, P.H., Soupir, M.L., Biswas, S. and Singh, V.P., 2014. Contamination of water resources by pathogenic bacteria. AMB Express 4, pp 51-12.

Parreira, V.R., Arns, C.W. and Yano, T., 1998. Virulence factors of avian *Escherichia coli* associated with swollen head syndrome. Avian Patholgy 27, pp 148-154.

Parreira, V.R. and Gyles, C.L., 2003. A novel pathogenicity island integrated adjacent to the *thrW* tRNA gene of avian pathogenic *Escherichia coli* encodes a vacuolating autotransporter toxin. Infection and Immunity 71, pp 5087-5096.

Partridge, S.R., 2011. Analysis of antibiotic resistance regions in Gram-negative bacteria. FEMS Microbiology Reviews 35, pp 820-855.

Pelludat, C., Rakin, A., Jacobi, C.A., Schubert, S. and Heesemann, J., 1998. The yersiniabactin biosynthetic gene cluster of *Yersinia enterocolitica*: organization and siderophore-dependent regulation. Journal of Bacteriology 180, pp 538-546.

Penz, A. and Bruno, D. 2011. Challenges facing the global poultry industry until 2020. In: Proceedings of 22nd Annual Australian Poultry Science Symposium, Sydney, New South Wales. pp 49-55.

Perez-Perez, F.J. and Hanson, N.D., 2002. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. Journal of Clinical Microbiology 40, pp 2153-2162.

Perry, J.A., Westman, E.L. and Wright, G.D., 2014. The antibiotic resistome: what's new? Current Opinion in Microbiology 21, pp 45-50.

Pfaff-McDonough, S.J., Horne, S.M., Giddings, C.W., Ebert, J.O., Doetkott, C., Smith, M.H. and Nolan, L.K., 2000. Complement resistance-related traits among *Escherichia coli* isolates from apparently healthy birds and birds with colibacillosis. Avian Diseases 44, pp 23-33.

Philippon, A., Arlet, G. and Jacoby, G.A., 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrobial Agents and Chemotherapy 46, pp 1-11.

Pires-dos-Santos, T., Bisgaard, M. and Christensen, H., 2013. Genetic diversity and virulence profiles of *Escherichia coli* causing salpingitis and peritonitis in broiler breeders. Veterinary Microbiology 162, pp 873-880.

Pitout, J.D., 2012. Extraintestinal pathogenic *Escherichia coli*: a combination of virulence with antibiotic resistance. Frontiers in Microbiology 3, pp 1-9.

Platell, J.L., Cobbold, R.N., Johnson, J.R., Heisig, A., Heisig, P., Clabots, C., Kuskowski, M.A. and Trott, D.J., 2011. Commonality among fluoroquinolone-resistant sequence type ST131 extraintestinal *Escherichia coli* isolates from humans and companion animals in Australia. Antimicrobial Agents and Chemotherapy 55, pp 3782-3787.

Poole, K., 2000. Efflux-mediated resistance to fluoroquinolones in Gram-negative bacteria. Antimicrob Agents and Chemotherapy 44, pp 2233-2241.

Pourbakhsh, S.A., Dho-Moulin, M., Brée, A., Desaultels, C., Doize, B.M. and Fairbrother, J.M., 1997. Localization of the *in vivo* expression of P and F1 fimbriae in chickens experimentally inoculated with pathogenic *Escherichia coli*. Microbial Pathology 22, pp 331-341.

Prescott, J.F., 2013. Sulfonamides, diaminopyrimidines and their combinations. In: Giguère, S., Prescott, J.F., Dowling, P.M. (Eds.) Antimicrobial Therapy in Veterinary Medicine. John Wiley and Sons, Inc, Hoboken, New Jersey, USA, pp 279-294.

Provence, D.L. and Curtiss, R., 1994. Isolation and characterization of a gene involved in hemagglutination by an avian pathogenic *Escherichia coli* strain. Infection and Immunity 62, pp 1369-1380.

Qabajah, M., Awwad, E. and Ashhab, Y., 2014. Molecular characterisation of *Escherichia coli* from dead broiler chickens with signs of colibacillosis and ready-to-market chicken meat in the West Bank. British Poultry Science 55, pp 442-451.

Quinn, P.J., Markey, B.K., Bryan, K., Leonard, F.C., Fitz-Patrick, E.S., Fanning, S. and Hartigan, P.J., 2011. Veterinary Microbiology and Microbial Disease. Wiley-Blackwell, Chichester, UK.

Radhouani, H., Silva, N., Poeta, P., Torres, C., Correia, S. and Igrejas, G., 2014. Potential impact of antimicrobial resistance in wildlife, environment and human health. Frontiers in Microbiology 5, pp 1-23.

Ramirez, R.M., Almanza, Y., Gonzalez, R., Garcia, S. and Heredia, N., 2009. Avian pathogenic *Escherichia coli* bind fibronectin and laminin. Veterinary Research Communications 33, pp 379-386.

Ranjbar, R., Tabatabaee, A., Behzadi, P. and Kheiri, R., 2017. Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) genotyping of *Escherichia coli* strains isolated from different animal stool specimens. Iranian Journal of Pathology 12, pp 25-34.

Rankin, D.J., Rocha, E.P. and Brown, S.P., 2011. What traits are carried on mobile genetic elements, and why? Heredity 106, pp 1-10.

Rao, J.N.K. and Cott, A.J.S., 1984. On chi-squared tests for multiway contingency tables with cell proportions estimated from survey data. Annals of Statistics 12, pp 46–60.

Rao, J.N.K. and Scott, A.J., 1981. The analysis of categorical data from complex surveys: chi-squared tests for goodness of fit and independence in two-way tables. Journal of the American Statistical Association 76, pp 221-230.

Rapp, D., 2010. DNA extraction from bovine faeces: current status and future trends. Journal of Applied Microbiology 108, pp 1485-1493.

Rashid, M.H., Xue, C., Islam, M.R., Islam, M.T. and Cao, Y., 2013. A longitudinal study on the incidence of mortality of infectious diseases of commercial layer birds in Bangladesh. Preventive Veterinary Medicine 109, pp 354-358.

Rasmussen, M.M., Opintan, J.A., Frimodt-Moller, N. and Styrishave, B., 2015. β-lactamase producing *Escherichia coli* isolates in imported and locally produced chicken meat from Ghana. PLoS One 10, pp e0139706.

Ratledge, C. and Dover, L.G., 2000. Iron metabolism in pathogenic bacteria. Annual Review of Microbiology 54, pp 881-941.

Rayamajhi, N., Jung, B.Y., Cha, S.B., Shin, M.K., Kim, A., Kang, M.S., Lee, K.M. and Yoo, H.S., 2010. Antibiotic resistance patterns and detection of $bla_{\text{DHA-1}}$ in *Salmonella* species isolates from chicken farms in South Korea. Applied and Environmental Microbiology 76, pp 4760-4764.

Rayamajhi, N., Kang, S.G., Lee, D.Y., Kang, M.L., Lee, S.I., Park, K.Y., Lee, H.S. and Yoo, H.S., 2008. Characterization of TEM-, SHV- and AmpC-type β-lactamases from cephalosporin-resistant Enterobacteriaceae isolated from swine. International Journal of Food Microbiology 124, pp 183-187.

Raymond, K.N., Dertz, E.A. and Kim, S.S., 2003. Enterobactin: an archetype for microbial iron transport. Proceedings of the National Academy of Sciences of the United States of America 100, pp 3584-3588.

Reid, S.D., Herbelin, C.J., Bumbaugh, A.C., Selander, R.K. and Whittam, T.S., 2000. Parallel evolution of virulence in pathogenic *Escherichia coli*. Nature 406, pp 64-67.

Roberts, M.C., 2012. Acquired tetracycline resistance genes. In: Dougherty, T.J., Pucci, M.J. (Eds.) Antibiotic Discovery and Development. Springer, US, Boston, pp 543-568.

Robicsek, A., Jacoby, G.A. and Hooper, D.C., 2006a. The worldwide emergence of plasmid-mediated quinolone resistance. The Lancet. Infectious Diseases 6, pp 629-640.

Robicsek, A., Strahilevitz, J., Jacoby, G.A., Macielag, M., Abbanat, D., Park, C.H., Bush, K. and Hooper, D.C., 2006b. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nature Medicine 12, pp 83-88.

Rocha, A.C.G.P., Rocha, S.L.S., Lima-Rosa, C.A.V., Souza, G.F., Moraes, H.L.S., Salle, F.O., Moraes, L.B. and Salle, C.T.P., 2008. Genes associated with pathogenicity of avian *Escherichia coli* (APEC) isolated from respiratory cases of poultry. Pesquisa Veterinaria Brasileira 28, pp 183-186.

Rodrigues, C., Machado, E., Peixe, L. and Novais, A., 2013. IncI1/ST3 and IncN/ST1 plasmids drive the spread of $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-1/-32}}$ in diverse *Escherichia coli* clones from different piggeries. The Journal of Antimicrobial Chemotherapy 68, pp 2245-2248.

Rodriguez-Martinez, J.M., Velasco, C., Pascual, A., Garcia, I. and Martinez-Martinez, L., 2006. Correlation of quinolone resistance levels and differences in basal and quinolone-induced expression from three *qnrA*-containing plasmids. Clinical Microbiology and Infection 12, pp 440-445.

Rodriguez-Rojas, A., Rodriguez-Beltran, J., Couce, A. and Blazquez, J., 2013. Antibiotics and antibiotic resistance: A bitter fight against evolution. International Journal of Medical Microbiology 303, pp 293-297.

Rodriguez-Siek, K.E., Giddings, C.W., Doetkott, C., Johnson, T.J. and Nolan, L.K., 2005a. Characterizing the APEC pathotype. Veterinary Research 36, pp 241-256.

Rogers, B.A., Ingram, P.R., Runnegar, N., Pitman, M.C., Freeman, J.T., Athan, E., Havers, S.M., Sidjabat, H.E., Jones, M., Gunning, E., De Almeida, M., Styles, K. and Paterson, D.L., 2014. Community-onset *Escherichia coli* infection resistant to expanded-spectrum cephalosporins in low-prevalence countries. Antimicrobial Agents and Chemotheropy 58, pp 2126-2134.

Rouault, T.A. and Tong, W.H., 2008. Iron-sulfur cluster biogenesis and human disease. Trends in Genetics 24, pp 398-407.

Ruiz, J., 2003. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. The Journal of Antimicrobial Chemotherapy 51, pp 1109-1117.

Ruiz, J., Pons, M.J. and Gomes, C., 2012. Transferable mechanisms of quinolone resistance. International Journal of Antimicrobial Agents 40, pp 196-203.

Russo, T.A. and Johnson, J.R., 2000. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. Journal of Infectious Diseases 181, pp 1753-1754.

Russo, T.A. and Johnson, J.R., 2003. Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. Microbes and Infection 5, pp 449-456.

Russo, T.A. and Johnson, J.R., 2009. Extraintestinal Pathogenic *Escherichia coli*. In: Barret, A.D.T., Stanberry, L.R. (Eds.) Vaccines for Biodefence and Emerging and Neclected Diseases Elsevier Ltd., London, pp 939-961.

Sabate, M., Prats, G., Moreno, E., Balleste, E., Blanch, A.R. and Andreu, A., 2008. Virulence and antimicrobial resistance profiles among *Escherichia coli* strains isolated from human and animal wastewater. Research in Microbiology 159, pp 288-293.

Sabri, M., Caza, M., Proulx, J., Lymberopoulos, M.H., Bree, A., Moulin-Schouleur, M., Curtiss, R. and Dozois, C.M., 2008. Contribution of the SitABCD, MntH, and FeoB metal transporters to the virulence of avian pathogenic *Escherichia coli* O78 strain χ7122. Infection and Immunity 76, pp 601-611.

Sabri, M., Leveille, S. and Dozois, C.M., 2006. A SitABCD homologue from an avian pathogenic *Escherichia coli* strain mediates transport of iron and manganese and resistance to hydrogen peroxide. Microbiology 152, pp 745-758.

Saenz, Y., Zarazaga, M., Brinas, L., Lantero, M., Ruiz-Larrea, F. and Torres, C., 2001. Antibiotic resistance in *Escherichia coli* isolates obtained from animals, foods and humans in Spain. International Journal of Antimicrobial Agents 18, pp 353-358.

Sahl, J.W., Matalka, M.N. and Rasko, D.A., 2012. Phylomark, a tool to identify conserved phylogenetic markers from whole-genome alignments. Applied and Environmental Microbiology 78, pp 4884-4892.

Saidy, L.N., Mohamed, R.A. and Abouelenien, F., 2015. Assessment of variable drinking water sources used in Egypt on broiler health and welfare. Veterinary World 8, pp 855-864.

Sakamoto, K., Hayashi, A., Sakamoto, A., Kiga, D., Nakayama, H., Soma, A., Kobayashi, T., Kitabatake, M., Takio, K., Saito, K., Shirouzu, M., Hirao, I. and Yokoyama, S., 2002. Site-

specific incorporation of an unnatural amino acid into proteins in mammalian cells. Nucleic Acids Research 30, pp 4692-4699.

Sams, R. 1994. Florfenicol: chemistry and metabolism of a novel broad spectrum antibiotic. In: Proceedings of the XVIII World Buiatrics Congress, Bologna, Italy, 13-17.

Santos, M.M., Alcântara, A.C., Perecmanis, S., Campos, A. and Santana, A.P., 2014. Antimicrobial resistance of bacterial strains isolated from avian cellulitis. Revista Brasileira de Ciência Avícola 16, pp 13-18.

Sawma-Aouad, G., Hashwa, F. and Tokajian, S., 2009. Antimicrobial resistance in relation to virulence determinants and phylogenetic background among uropathogenic *Escherichia coli* in Lebanon. Journal of Chemotherapy 21, pp 153-158.

Scanes, C.G., 2007. The global importance of poultry. Poultry Science 86, pp 1057-1058.

Schaufler, K., Bethe, A., Lubke-Becker, A., Ewers, C., Kohn, B., Wieler, L.H. and Guenther, S., 2015. Putative connection between zoonotic multiresistant extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in dog feces from a veterinary campus and clinical isolates from dogs. Infection Ecology and Epidemiology 5, pp 25334-25344.

Scheutz, F., Cheasty, T., Woodward, D. and Smith, H.R., 2004. Designation of O174 and O175 to temporary O groups OX3 and OX7, and six new *E. coli* O groups that include Verocytotoxin-producing *E. coli* (VTEC): O176, O177, O178, O179, O180 and O181. Acta Pathologica, Microbiologica and Immunologica Scandinavica 112, pp 569-584.

Schouler, C., Schaeffer, B., Bree, A., Mora, A., Dahbi, G., Biet, F., Oswald, E., Mainil, J., Blanco, J. and Moulin-Schouleur, M., 2012. Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. Journal of Clinical Microbiology 50, pp 1673-1678.

Schubert, S., Rakin, A., Karch, H., Carniel, E. and Heesemann, J., 1998. Prevalence of the "high-pathogenicity island" of *Yersinia* species among *Escherichia coli* strains that are pathogenic to humans. Infection and Immunity 66, pp 480-485.

Schultsz, C. and Geerlings, S., 2012. Plasmid-mediated resistance in *Enterobacteriaceae*: changing landscape and implications for therapy. Drugs 72, pp 1-16.

Schwaber, M.J., Navon-Venezia, S., Schwartz, D. and Carmeli, Y., 2005. High levels of antimicrobial coresistance among extended-spectrum-β-lactamase-producing *Enterobacteriaceae*. Antimicrobial Agents and Chemotherapy 49, pp 2137-2139.

Schwarz, S. and Chaslus-Dancla, E., 2001. Use of antimicrobials in veterinary medicine and mechanisms of resistance. Veterinary Research 32, pp 201-225.

Schwarz, S., Kehrenberg, C., Doublet, B. and Cloeckaert, A., 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiology Reviews 28, pp 519-542.

Searle, L.J., Meric, G., Porcelli, I., Sheppard, S.K. and Lucchini, S., 2015. Variation in siderophore biosynthetic gene distribution and production across environmental and faecal populations of *Escherichia coli*. PLoS One 10, pp e0117906.

Seiffert, S.N., Hilty, M., Perreten, V. and Endimiani, A., 2013. Extended-spectrum cephalosporin-resistant Gram-negative organisms in livestock: An emerging problem for human health? Drug Resistance Updates 16, pp 22-45.

Sello, J.K., 2012. Mining the antibiotic resistome. Chemistry and Biology 19, pp 1220-1221.

Seni, J., Falgenhauer, L., Simeo, N., Mirambo, M.M., Imirzalioglu, C., Matee, M., Rweyemamu, M., Chakraborty, T. and Mshana, S.E., 2016. Multiple ESBL-producing *Escherichia coli* sequence types carrying quinolone and aminoglycoside resistance genes circulating in companion and domestic farm animals in Mwanza, Tanzania, harbor commonly occurring plasmids. Frontiers in Microbiology 7, pp 142-148.

Šeol, B., Matanović, K., Mekić, S. and Starešina, V., 2011. *In vitro* activity of cefovecin, extended-spectrum cephalosporin, against 284 clinical isolates collected from cats and dogs in Croatia. Veterinarski Arhiv 81, pp 91-97.

Servin, A.L., 2005. Pathogenesis of Afa/Dr diffusely adhering *Escherichia coli*. Clinical Microbiology Reviews 18, pp 264-292.

Shaban, R.Z., Simon, G.I., Trott, D.J., Turnidge, J. and Jordan, D., 2014. Surveillance and reporting of antimicrobial resistance and antibiotic usage in animals and agriculture in Australia, Report to the Department of Agriculture, Griffith University, and University of Adelaide, Australia. Department of Agriculture, Canberra, Australia, [Available from http://www.agriculture.gov.au/SiteCollectionDocuments/animal-plant/animal-health/amria.pdf]; Accessed March 2015.

Shah, A.A., Hasan, F., Ahmed, S. and Hameed, A., 2004. Characteristics, epidemiology and clinical importance of emerging strains of Gram-negative bacilli producing extended-spectrum β-lactamases. Research in Microbiology 155, pp 409-421.

Sharma, S., Kaur, N., Malhotra, S., Madan, P., Ahmad, W. and Hans, C., 2016. Serotyping and antimicrobial susceptibility pattern of *Escherichia coli* isolates from urinary tract infections in pediatric population in a tertiary care hospital. Journal of Pathogens 2016, pp 4-10.

Sharples, G.J. and Lloyd, R.G., 1990. A novel repeated sequence located in the intergenic regions of bacterial chromosomes. Nucleic Acids Resarch 18, pp 6503-6508.

Sheikholvaezin, A., Blomberg, F., Öhrmalm, C., Sjösten, A., Blomberg, J., 2011. Rational recombinant XMRV antigen preparation and bead coupling for multiplex serology in a suspension array. Protein Expression and Purification 80, pp 176-184.

Shen, L., Kohlbrenner, W., Weigl, D. and Baranowski, J., 1989. Mechanism of quinolone inhibition of DNA gyrase. Appearance of unique norfloxacin binding sites in enzyme- DNA complexes. Journal of Biological Chemistry 264, pp 2973-2978.

Shobrak, M.Y. and Abo-Amer, A.E., 2014. Role of wild birds as carriers of multi-drug resistant *Escherichia coli* and *Escherichia vulneris*. Brazilian Journal of Microbiology 45, pp 1199-1209.

Sidjabat, H.E., Hanson, N.D., Smith-Moland, E., Bell, J.M., Gibson, J.S., Filippich, L.J. and Trott, D.J., 2007. Identification of plasmid-mediated extended-spectrum and AmpC β-lactamases in *Enterobacter* spp. isolated from dogs. Journal of Medical Microbiology 56, pp 426-434.

Sjolund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., Kahlmeter, G. and Olsen, B., 2008. Dissemination of multidrug-resistant bacteria into the Arctic. Emerging Infectious Diseases 14, pp 70-72.

Sköld, O., 2010. Sulfonamides and trimethoprim. Expert Review of Anti-infective Therapy 8, pp 1-6.

Skov, T., Deddens, J., Petersen, M. and Endahl, L., 1998. Prevalence proportion ratios: estimation and hypothesis testing. International Journal of Epidemiology 27, pp 91-95.

Skyberg, J.A., Horne, S.M., Giddings, C.W., Wooley, R.E., Gibbs, P.S. and Nolan, L.K., 2003. Characterizing avian *Escherichia coli* isolates with multiplex polymerase chain reaction. Avian Diseases 47, pp 1441-1448.

Skyberg, J.A., Johnson, T.J., Johnson, J.R., Clabots, C., Logue, C.A. and Nolan, L.K., 2006. Acquisition of avian pathogenic *Escherichia coli* plasmids by a commensal *E. coli* isolate enhances its abilities to kill chicken embryos, grow in human urine, and colonize the murine kidney. Infection and Immunity 74, pp 6287-6292.

Skyberg, J.A., Johnson, T.J. and Nolan, L.K., 2008. Mutational and transcriptional analyses of an avian pathogenic *Escherichia coli* CoIV plasmid. Microbiology 8, pp 10-18.

Smith, S.G., Wang, J., Fanning, S. and McMahon, B.J., 2014. Antimicrobial resistant bacteria in wild mammals and birds: a coincidence or cause for concern? Irish Veterinary Journal 67, pp 8-14.

Sola-Gines, M., Cameron-Veas, K., Badiola, I., Dolz, R., Majo, N., Dahbi, G., Viso, S., Mora, A., Blanco, J., Piedra-Carrasco, N., Gonzalez-Lopez, J.J. and Migura-Garcia, L., 2015. Diversity of multi-drug resistant avian pathogenic *Escherichia coli* (APEC) causing outbreaks of colibacillosis in broilers during 2012 in Spain. PLoS One 10, pp e0143191.

Sorsa, L.J., Dufke, S., Heesemann, J. and Schubert, S., 2003. Characterization of an *iroBCDEN* gene cluster on a transmissible plasmid of uropathogenic *Escherichia coli*: evidence for horizontal transfer of a chromosomal virulence factor. Infection and Immunity 71, pp 3285-3293.

Sosa, A.D.J., 2009. Antimicrobial Resistance in Developing Countries. In: Aníbal, D.J., SosaDenis, K., Bya rugaba, C.F., Amábile, C., Po-Ren, H., Samuel, K., Iruka, N.O. (Eds.) Springer New York, NY, USA.

Stacy, A.K., Mitchell, N.M., Maddux, J.T., De la Cruz, M.A., Duran, L., Giron, J.A., Curtiss, R., 3rd and Mellata, M., 2014. Evaluation of the prevalence and production of *Escherichia coli* common pilus among avian pathogenic *E. coli* and its role in virulence. PLoS One 9, pp e86565.

Stathopoulos, C., Provence, D.L. and Curtiss, R., 3rd, 1999. Characterization of the avian pathogenic *Escherichia coli* hemagglutinin Tsh, a member of the immunoglobulin A protease-type family of autotransporters. Infection and Immunity 67, pp 772-781.

Stedt, J., Bonnedahl, J., Hernandez, J., McMahon, B.J., Hasan, B., Olsen, B., Drobni, M. and Waldenstrom, J., 2014. Antibiotic resistance patterns in *Escherichia coli* from gulls in nine European countries. Infection Ecology and Epidemiology 4, pp 10-20.

Stehling, E.G., Yano, T., Brocchi, M. and da Silveira, W.D., 2003. Characterization of a plasmid-encoded adhesin of an avian pathogenic *Escherichia coli* (APEC) strain isolated from a case of swollen head syndrome (SHS). Veterinary Microbiology 95, pp 111-120.

Stokholm, N., Permin, A., Bisgaard, M. and Christensen, J., 2010. Causes of mortality in commercial organic layers in Denmark. Avian Diseases 54, pp 1241-1250.

Stordeur, P., Brée, A.M., J. and Moulin-Schouleur, M., 2004. Pathogenicity of *pap*-negative avian *Escherichia coli* isolated from septicaemic lesions. Microbes Infection 6, pp 637-645.

Stordeur, P. and Mainil, J., 2002. Colibacillosis in poultry. Annales De Medecine Veterinaire 146, pp 11-18.

Svara, F. and Rankin, D.J., 2011. The evolution of plasmid-carried antibiotic resistance. BMC Evolutionary Biology 11, pp 130-120.

Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM), 2007. [Available from http://www.sva.se/upload/pdf/Tj%C3%A4nster%20och%20produkter/Trycksaker/svarm2006.pdf]; Accessed August 2016.

Szmolka, A., Fortini, D., Villa, L., Carattoli, A., Anjum, M.F. and Nagy, B., 2011. First report on IncN plasmid-mediated quinolone resistance gene *qnrS1* in porcine *Escherichia coli* in Europe. Microbial Drug Resistance 17, pp 567-573.

Szmolka, A., Lestar, B., Paszti, J., Fekete, P. and Nagy, B., 2015. Conjugative IncF and IncI1 plasmids with *tet*(A) and class 1 integron conferring multidrug resistance in F18(+) porcine enterotoxigenic *E. coli*. Acta Veterinaria Hungarica 63, pp 425-443.

Szmolka, A. and Nagy, B., 2013. Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. Frontiers in Microbiology 4, pp 258-256.

Tadesse, D.A., Zhao, S., Tong, E., Ayers, S., Singh, A., Bartholomew, M.J. and McDermott, P.F., 2012. Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950-2002. Emerging Infectious Diseases 18, pp 741-749.

Tagg, K.A., Ginn, A.N., Jiang, X., Ellem, J., Partridge, S.R. and Iredell, J.R., 2015. Distribution of acquired AmpC β-lactamase genes in Sydney, Australia. Diagnostic Microbiology and Infectious Disease 83, pp 56-58.

Talebiyan, R., Kheradmand, M., Khamesipour, F. and Rabiee-Faradonbeh, M., 2014. Multiple antimicrobial resistance of *Escherichia coli* isolated from chickens in Iran. Veterinary Medicine International 20, pp 1-4.

Taneja, N., Singh, G., Singh, M., Madhup, S., Pahil, S. and Sharma, M., 2012. High occurrence of bla_{CMY-1} AmpC lactamase producing *Escherichia coli* in cases of complicated urinary tract infection (UTI) from a tertiary health care centre in North India. The Indian Journal of Medical Research 136, pp 289-291.

Tchesnokova, V., Billig, M., Chattopadhyay, S., Linardopoulou, E., Aprikian, P., Roberts, P.L., Skrivankova, V., Johnston, B., Gileva, A., Igusheva, I., Toland, A., Riddell, K., Rogers, P., Qin, X., Butler-Wu, S., Cookson, B.T., Fang, F.C., Kahl, B., Price, L.B., Weissman, S.J., Limaye, A., Scholes, D., Johnson, J.R. and Sokurenko, E.V., 2013. Predictive diagnostics for *Escherichia coli* infections based on the clonal association of antimicrobial resistance and clinical outcome. Journal of Clinical Microbiology 51, pp 2991-2999.

Thaker, M., Spanogiannopoulos, P. and Wright, G.D., 2010. The tetracycline resistome. Cellular and Molecular Life Sciences 67, pp 419-431.

The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP), 2014. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark, [Available from www.danmap.org/]; Accessed February 2016.

Thomas, C.M. and Nielsen, K.M., 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nature Reviews Microbiology 3, pp 711-721.

Thomas, M. S., & Wigneshweraraj, S. (2014). Regulation of virulence gene expression. Virulence, 5(8), 832–834.

Thornton, P.K., 2010. Livestock production: recent trends, future prospects. Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences 365, pp 2853-2867.

Tivendale, K.A., Allen, J.L. and Browning, G.F., 2009. Plasmid-borne virulence-associated genes have a conserved organization in virulent strains of avian pathogenic *Escherichia coli*. Journal of Clinical Microbiology 47, pp 2513-2519.

Tivendale, K.A., Allen, J.L., Ginns, C.A., Crabb, B.S. and Browning, G.F., 2004. Association of *iss* and *iucA* but not *tsh*, with plasmid-mediated virulence of avian pathogenic *Escherichia coli*. Infection and Immunity 72, pp 6554-6560.

Tivendale, K.A., Logue, C.M., Kariyawasam, S., Jordan, D., Hussein, A., Li, G.W., Wannemuehler, Y. and Nolan, L.K., 2010. Avian-pathogenic *Escherichia coli* strains are similar to neonatal meningitis *E. coli* strains and are able to cause meningitis in the rat model of human disease. Infection and Immunity 78, pp 3412-3419.

Toma, C., Espinosa, E.M., Song, T.Y., Miliwebsky, E., Chinen, I., Iyoda, S., Iwanaga, M. and Rivas, M., 2004. Distribution of putative adhesins in different seropathotypes of Shiga toxin-producing *Escherichia coli*. Journal of Clinical Microbiology 42, pp 4937-4946.

Toth, I., Herault, F., Beutin, L. and Oswald, E., 2003. Production of cytolethal distending toxins by pathogenic *Escherichia coli* strains isolated from human and animal sources: establishment of the existence of a new *cdt* variant (Type IV). Journal of Clinical Microbiology 41, pp 4285-4291.

Trott, D., 2013. β-lactam resistance in Gram-negative pathogens isolated from animals. Applied and Environmental Microbiology 19, pp 239-249.

Twisk, J.W.R., 2013. Applied Longitudinal Data Analysis for Epidemiology: a Practical Guide. Department of Epidemiology and Biostatistics, Medical Centre and the Department of Health Sciences of the Vrije Universteit, Cambridge University Press, Amsterdam.

Ugorski, M. and Chmielewski, R., 2000. REP and ERIC repetitive DNA sequences in bacteria-diagnostic significance. Postepy Higieny Medycyny Doswiadczalnej 54, pp 3-15.

Valvano, M.A. and Crosa, J.H., 1988. Molecular cloning, expression, and regulation in *Escherichia coli* K-12 of a chromosome-mediated aerobactin iron transport system from a human invasive isolate of *E. coli* K1. Journal of Bacteriology 170, pp 5529-5538.

Van Bambeke, F., Michot, J.M., Van Eldere, J. and Tulkens, P.M., 2005. Quinolones in 2005: an update. Clinical Microbiology and Infection 11, pp 256-280.

van den Bogaard, A.E., London, N., Driessen, C. and Stobberingh, E.E., 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. Journal of Antimicrobial Chemotherapy 47, pp 763-771.

van Essen-Zandbergen, A., Smith, H., Veldman, K. and Mevius, D., 2007. Occurrence and characteristics of class 1, 2 and 3 integrons in *Escherichia coli, Salmonella* and *Campylobacter* spp. in the Netherlands. Journal of Antimicrobial Chemotherapy 59, pp 746-750.

Vandekerchove, D., De Herdt, P., Laevens, H. and Pasmans, F., 2004a. Risk factors associated with colibacillosis outbreaks in caged layer flocks. Avian Pathology 33, pp 337-342.

Vandekerchove, D., Herdt, P.D., Laevens, H., Butaye, P., Meulemans, G. and Pasmans, F., 2004b. Significance of interactions between *Escherichia coli* and respiratory pathogens in layer hen flocks suffering from colibacillosis-associated mortality. Avian Pathology 33, pp 298-302.

Vandekerchove, D., Vandemaele, F., Adriaensen, C., Zaleska, A., Hernalsteens, J.P., De Baets, L., Butaye, P., Van Immerseel, F., Wattiau, P., Laevens, H., Mast, J., Goddeeris, B. and Pasmans, F., 2005. Virulence-associated traits in avian *Escherichia coli*: Comparison between isolates from colibacillosis-affected and clinically healthy layer flocks. Veterinary Microbiology 108, pp 75-87.

Vandeplas, S., Dubois Dauphin, R., Beckers, Y., Thonart, P. and Thewis, A., 2010. *Salmonella* in chicken: current and developing strategies to reduce contamination at farm level. Journal of Food Protection 73, pp 774-785.

Vangchhia, B., Abraham, S., Bell, J.M., Collignon, P., Gibson, J.S., Ingram, P.R., Johnson, J.R., Kennedy, K., Trott, D.J., Turnidge, J.D. and Gordon, D.M., 2016. Phylogenetic diversity, antimicrobial susceptibility and virulence characteristics of phylogroup F *Escherichia coli* in Australia. Microbiology 162, pp 1904-1912.

Velhner, M. and Milanov, D., 2015. Resistance to tetracycline in *Escherichia coli* and *Staphylococcus aureus*: brief overview on mechanisms of resistance and epidemiology. Archives of Veterinary Medicine 8, pp 27-36.

Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., de Schaetzen, M.A., Van Huffel, X., Imberechts, H., Dierick, K., Daube, G., Saegerman, C., De Block, J., Dewulf, J. and Herman, L., 2013. Antimicrobial resistance in the food chain: a review. International Journal of Environmental Research and Public Health 10, pp 2643-2669.

Versalovic, J., Koeuth, T. and Lupski, J.R., 1991. Distribution of repetitive DNA-sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Research 19, pp 6823-6831.

Verweij, J.J., Blange, R.A., Templeton, K., Schinkel, J., Brienen, E.A., van Rooyen, M.A., van Lieshout, L. and Polderman, A.M., 2004. Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in feacal samples by using multiplex real-time PCR. Journal of Clinical Microbiology 42, pp 1220-1223.

Verweij, J.J., Ten Hove, R., Brienen, E.A. and van Lieshout, L., 2007. Multiplex detection of *Enterocytozoon bieneusi* and *Encephalitozoon* spp. in feacal samples using real-time PCR. Diagnostic Microbiology and Infectious Disease 57, pp 163-167.

Vidotto, M.C., Gaziri, L.C.J. and Delicato, E.R., 2004. Virulence-associated genes in *Escherichia coli* isolates from poultry with colibacillosis: correction. Veterinary Microbiology 102, pp 95-96.

Vidotto, M.C., Muller, E.E., Defreitas, J.C., Alfieri, A.A., Guimaraes, I.G. and Santos, D.S., 1990. Virulence factors of avian *Escherichia coli*. Avian Diseases 34, pp 531-538.

Vidotto, M.C., Navarro, H.R. and Gaziri, L.C.J., 1997. Adherence pili of pathogenic strains of avian *Escherichia coli*. Veterinary Microbiology 59, pp 79-87.

Vieira, A.R., Collignon, P., Aarestrup, F.M., McEwen, S.A., Hendriksen, R.S., Hald, T. and Wegener, H.C., 2011. Association between antimicrobial resistance in *Escherichia coli* isolates from food animals and blood stream isolates from humans in Europe: an ecological study. Foodborne Pathogens and Disease 8, pp 1295-1301.

Vignaroli, C., Luna, G.M., Rinaldi, C., Di Cesare, A., Danovaro, R. and Biavasco, F., 2012. New sequence types and multidrug resistance among pathogenic *Escherichia coli* isolates from coastal marine sediments. Applied and Environmental Microbiology 78, pp 3916-3922.

Vila, J., Simon, K., Ruiz, J., Horcajada, J.P., Velasco, M., Barranco, M., Moreno, A. and Mensa, J., 2002. Are quinolone-resistant uropathogenic *Escherichia coli* less virulent? The Journal of Infectious Diseases 186, pp 1039-1042.

Wales, A.D. and Davies, R.H., 2015. Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens. Antibiotics 4, pp 567-604.

Walsh, C. and Fanning, S., 2008. Antimicrobial resistance in foodborne pathogens- a cause for concern? Current Drug Targets 9, pp 808-815.

Wang, H., McEntire, J.C., Zhang, L., Li, X. and Doyle, M., 2012. The transfer of antibiotic resistance from food to humans: facts, implications and future directions. Revue Scientifique et Technique 31, pp 249-260.

Wang, J., Ma, Z.B., Zeng, Z.L., Yang, X.W., Huang, Y. and Liu, J.H., 2017. The role of wildlife (wild birds) in the global transmission of antimicrobial resistance genes. Zoological Research 38, pp 55-80.

Wang, J., Stephan, R., Karczmarczyk, M., Yan, Q., Hachler, H. and Fanning, S., 2013a. Molecular characterization of bla_{ESBL}-harboring conjugative plasmids identified in multidrug resistant *Escherichia coli* isolated from food-producing animals and healthy humans. Frontiers in Microbiology 4, pp 188-113.

Wang, X.-M., Liao, X.-P., Zhang, W.-J., Jiang, H.-X., Sun, J., Zhang, M.-J., He, X.-F., Lao, D.-X. and Liu, Y.-H., 2010. Prevalence of serogroups, virulence genotypes, antimicrobial resistance, and phylogenetic background of avian pathogenic *Escherichia coli* in South of China. Foodborne Pathogens and Disease, pp 1099-1106.

Wang, X., Wei, L., Wang, B., Zhang, R., Liu, C., Bi, D., Chen, H. and Tan, C., 2016. Complete genome sequence and characterization of avian pathogenic *Escherichia coli* field isolate ACN001. Standards in Genomic Sciences 11, pp 13-20.

Wang, Y., Jiang, Z., Jin, Z., Tan, H. and Xu, B., 2013b. Risk factors for infectious diseases in backyard poultry farms in the Poyang Lake area, China. PLoS One 8, pp e67366.

Warner, P.J., Williams, P.H., Bindereif, A. and Neilands, J.B., 1981. ColV plasmid-specific aerobactin synthesis by invasive strains of *Escherichia coli*. Infection and Immunity 33, pp 540-545.

Wasyl, D., Hasman, H., Cavaco, L.M. and Aarestrup, F.M., 2012. Prevalence and characterization of cephalosporin resistance in nonpathogenic *Escherichia coli* from food-producing animals slaughtered in Poland. Microbial Drug Resistance 18, pp 79-82.

Wasyl, D., Hoszowski, A., Zajac, M. and Szulowski, K., 2013. Antimicrobial resistance in commensal *Escherichia coli* isolated from animals at slaughter. Frontiers in Microbiology 4, pp 221-228.

Waters, V.L., 1999. Conjugative transfer in the dissemination of beta-lactam and aminoglycoside resistance. Frontiers in Bioscience 4, pp 433-456.

Watkins, S., 2008. Water identifying and correcting challenges. Avian Advice 10, pp 10-15.

Weissman, S.J., Johnson, J.R., Tchesnokova, V., Billig, M., Dykhuizen, D., Riddell, K., Rogers, P., Qin, X., Butler-Wu, S., Cookson, B.T., Fang, F.C., Scholes, D., Chattopadhyay, S. and Sokurenko, E., 2012. High-resolution two-locus clonal typing of extraintestinal pathogenic *Escherichia coli*. Applied and Environmental Microbiology 78, pp 1353-1360.

Whiley, H., van den Akker, B., Giglio, S. and Bentham, R., 2013. The role of environmental reservoirs in human campylobacteriosis. International Journal of Environmental Research and Public Health 10, pp 5886-5907.

Whiteman, C.E., Bickford, A.A. and Barnes, H.J., 1979. Avian Disease Manual, In: Barnes, H.J., American Association of Avian Pathologists (Eds.) Texas A and M University College Station, Texas, USA.

Whitford, M.F., Forster, R.J., Beard, C.E., Gong, J. and Teather, R.M., 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. Anaerobe 4, pp 153-163.

Williams, P.H. and Warner, P.J., 1980. ColV plasmid-mediated, colicin V-independent iron uptake system of invasive strains of *Escherichia coli*. Infection and Immunity 29, pp 411-416.

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 63, pp 3741-3751.

Wilson, L.A. and Sharp, P.M., 2006. Enterobacterial repetitive intergenic consensus (ERIC) sequences in *Escherichia coli*: Evolution and implications for ERIC-PCR. Molecular Biology and Evolution 23, pp 1156-1168.

Windhorst, H.W., 2006. Changes in poultry production and trade worldwide. Worlds Poultry Science Journal 62, pp 585-602.

Wirth, T., Falush, D., Lan, R.T., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C.J., Ochman, H. and Achtman, M., 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Molecular Microbiology 60, pp 1136-1151.

Wohlsen, T.D., 2011. Comparative evaluation of chromogenic agar CM1046 and mFC agar for detection of *E. coli* and thermotolerant coliform bacteria from water samples. Letters in Applied Microbiology 53, pp 155-160.

Won, G.Y., Moon, B.M., Oh, I.G., Matsuda, K., Chaudhari, A.A., Hur, J., Eo, S.K., Yu, I.J., Lee, Y.J., Lee, Y.S., Kim, B.S. and Lee, J.H., 2009. Profiles of virulence-associated genes of avian pathogenic *Escherichia coli* isolates from chickens with colibacillosis. Journal of Poultry Science 46, pp 260-266.

Woodford, N., Reddy, S., Fagan, E.J., Hill, R.L., Hopkins, K.L., Kaufmann, M.E., Kistler, J., Palepou, M.F., Pike, R., Ward, M.E., Cheesbrough, J. and Livermore, D.M., 2007. Wide geographic spread of diverse acquired AmpC β-lactamases among *Escherichia coli* and *Klebsiella* spp. in the UK and Ireland. The Journal of Antimicrobial Chemotherapy 59, pp 102-105.

Wooldridge, K.G. and Williams, P.H., 1993. Iron uptake mechanisms of pathogenic bacteria. FEMS Microbiology Reviews 12, pp 325-348.

Wooley, R.E., Spears, K.R., Brown, J., Nolan, L.K. and Fletcher, O.J., 1992. Relationship of complement resistance and selected virulence factors in pathogenic avian *Escherichia coli*. Avian Diseases 36, pp 679-684.

World Health Organization (WHO), 2016. Critically important antimicrobials for human medicine: categorization for the development of risk management strategies to contain antimicrobial resistance due to non-human antimicrobial use: report of the second WHO Expert Meeting, Copenhagen, 29–31 May 2007, [Available from http://www.who.int/foodborne_disease/resistance/]; Accessed February 2016.

Wozniak, R.A.F. and Waldor, M.K., 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nature Reviews Microbiology 8, pp 552-563.

Wray, C. and Woodward, M.J., 1994. Laboratory diagnosis of *Escherichia coli* infections. In: Gyles, C.L. (Ed.) *Escherichia coli* in Domestic Animals and Humans. CAB International, Wallingford, UK, pp 595-628.

Wright, G.D., 2010. Q&A: Antibiotic resistance: where does it come from and what can we do about it? BMC Biology 8, pp 123-129.

Xiong, L., Ling, J., Gao, Q., Zhou, Y., Li, T., Gao, S. and Liu, X., 2012. Construction of *iucB* and *iucBiutA* mutants of avian pathogenic *Escherichia coli* and evaluation of their pathogenicity. Veterinary Microbiology 159, pp 420-431.

Yaguchi, K., Ogitani, T., Osawa, R., Kawano, M., Kokumai, N., Kaneshige, T., Noro, T., Masubuchi, K. and Shimizu, Y., 2007. Virulence factors of avian pathogenic *Escherichia coli* strains isolated from chickens with colisepticemia in Japan. Avian Diseases 51, pp 656-662.

Yan, J.J., Hsueh, P.R., Lu, J.J., Chang, F.Y., Shyr, J.M., Wan, J.H., Liu, Y.C., Chuang, Y.C., Yang, Y.C., Tsao, S.M., Wu, H.H., Wang, L.S., Lin, T.P., Wu, H.M., Chen, H.M. and Wu, J.J., 2006. Extended-spectrum β-lactamases and plasmid-mediated AmpC enzymes among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from seven medical centers in Taiwan. Antimicrobial Agents and Chemotherapy 50, pp 1861-1864.

Yang, H., Chen, S., White, D.G., Zhao, S., McDermott, P., Walker, R. and Meng, J., 2004. Characterization of multiple-antimicrobial-resistant *Escherichia coli* isolates from diseased chickens and swine in China. Journal of Clinical Microbiology 42, pp 3483-3492.

Yang, H.Y., Nam, Y.S. and Lee, H.J., 2014. Prevalence of plasmid-mediated quinolone resistance genes among ciprofloxacin-nonsusceptible *Escherichia coli* and *Klebsiella pneumoniae* isolated from blood cultures in Korea. The Canadian Journal of Infectious Diseases and Medical Microbiology 25, pp 163-169.

Yang, J.L., Wang, M.S., Cheng, A.C., Pan, K.C., Li, C.F. and Deng, S.X., 2008. A simple and rapid method for extracting bacterial DNA from intestinal microflora for ERIC-PCR detection. World Journal of Gastroenterology 14, pp 2872-2876.

Yao, J., D.C. and Moellering, R.C., 2007. Antibacterial agents. In: Murray, E.J., Baron, J.J., Orgensen, H., Landry, M.L., Pfaller, M.A. (Eds.) Manual of Clinical Microbiology. ASM Press, Washington DC, pp 1077-1113.

Yassin, A., Gong, J., Kelly, P., Lu, G. and Guardabassi, L., 2017. Antimicrobial resistance in clinical *Escherichia coli* isolates from poultry and livestock, China. PLoS One 12, pp e0185326.

Yerushalmi, Z., Smorodinsky, N.I., Naveh, M.W. and Ron, E.Z., 1990. Adherence pili of avian strains of *Escherichia coli* O78. Infection and Immunity 58, pp 1129-1131.

Yu, Z. and Morrison, M., 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. BioTechniques 36, pp 808-812.

Yunis, A.A., 1988. Chloramphenicol: relation of structure to activity and toxicity. Annual Review of Pharmacology and Toxicology 28, pp 83-100.

Zanella, A., Alborali, G.L., Bardotti, M., Candotti, P., Guadagnini, P.F., Martino, P.A. and Stonfer, M., 2000. Severe *Escherichia coli* O111 septicaemia and polyserositis in hens at the start of lay. Avian Pathology 29, pp 311-317.

Zarubica, T., Baker, M.R., Wright, H.T. and Rife, J.P., 2011. The aminoglycoside resistance methyltransferases from the ArmA/Rmt family operate late in the 30S ribosomal biogenesis pathway. RNA 17, pp 346-355.

Zhang, B.W., Li, M., Ma, L.C. and Wei, F.W., 2006. A widely applicable protocol for DNA isolation from fecal samples. Biochemical Genetics 44, pp 503-512.

Zhang, P., Shen, Z., Zhang, C., Song, L., Wang, B., Shang, J., Yue, X., Qu, Z., Li, X., Wu, L., Zheng, Y., Aditya, A., Wang, Y., Xu, S. and Wu, C., 2017. Surveillance of antimicrobial resistance among *Escherichia coli* from chicken and swine, China, 2008-2015. Veterinary Microbiology 203, pp 49-55.

Zhao, L., Gao, S., Huan, H., Xu, X., Zhu, X., Yang, W., Gao, Q. and Liu, X., 2009. Comparison of virulence factors and expression of specific genes between uropathogenic *Escherichia coli* and avian pathogenic *E. coli* in a murine urinary tract infection model and a chicken challenge model. Microbiology 155, pp 1634-1644.

Zhao, R., Shi, J., Shen, Y., Li, Y., Han, Q., Zhang, X., Gu, G. and Xu, J., 2015. Phylogenetic distribution of virulence genes among ESBL-producing uropathogenic *Escherichia coli* isolated from long-term hospitalized patients. Journal of Clinical and Diagnostic Research 9, pp 1-4.

Zhao, S., Maurer, J.J., Hubert, S., De Villena, J.F., McDermott, P.F., Meng, J., Ayers, S., English, L. and White, D.G., 2005. Antimicrobial susceptibility and molecular characterization of avian pathogenic *Escherichia coli* isolates. Veterinary Microbiology 107, pp 215-224.

Zhao, X., Lin, C.W., Wang, J. and Oh, D.H., 2014. Advances in rapid detection methods for foodborne pathogens. Journal of Microbiology and Biotechnology 24, pp 297-312.

Zhu Ge, X., Jiang, J., Pan, Z., Hu, L., Wang, S., Wang, H., Leung, F.C., Dai, J. and Fan, H., 2014. Comparative genomic analysis shows that avian pathogenic *Escherichia coli* isolate IMT5155

(O2:K1:H5; ST complex 95, ST140) shares close relationship with ST95 APEC O1:K1 and human ExPEC O18:K1 strains. PLoS One 9, pp e112048.

Zhu, M., Valdebenito, M., Winkelmann, G. and Hantke, K., 2005. Functions of the siderophore esterases IroD and IroE in iron-salmochelin utilization. Microbiology 151, pp 2363-2372.

List of Appendices

Appendices to Chapter 4

Supplementary Table 1: Questionnaire for the cross-sectional study to identify risk factors associated with avian pathogenic *E. coli* on commercial broiler chicken farms in South East Queensland.

(Section one)

Details from slaughterhouse visit and farm list (To be completed by the researcher)

1. Date of field investigation: __/__/____
2. Date of slaughterhouse visit: __/__/____
3. Name of poultry slaughterhouse: _____
4. Name of farm: _____
5. Farm code: _____
6. City: _____
7. Farm location saved on the GPS? Yes ☐ No ☐
   a) If yes, farm GPS location saved as: _____
8. Elevation: _____metres; Longitude: _____; Latitude: _____
9. Phone No: _____
10. Fax No.: _____
11. Email: _____
12. Website: _____

Section Two

Farm details

(To be completed by the farmers/managers)

1. Date: __/__/____
2. Surname: _____First name: _____
3. Your role on the farm: Owner / Manager / Both
4. Email: _____
5. Farm address:_____ Post code: _____
6. How chickens are currently housed (tick all boxes that might apply)?
   - ☐ Separate age groups housed on the farm at the same time
   - ☐ Same age groups housed on the farm at the same time
   - ☐ Other (please specify) _____
7. Where does the chickens' drinking water currently come from (tick all boxes that might apply)?
   - ☐ Local water utility
   - ☐ Well on the property
   - ☐ River
   - ☐ Other (please specify):_____

a) Is there any treatment of water currently conducted before it is given to the chickens?
Yes □ No □

If yes, how is the water treated?

8. Are there currently any vehicle tyre disinfection facilities on the farm?
Yes □ No □

9. If yes, how often are vehicle tyres disinfected by 'workers /visitors' when entering the farm?

	Always	**Usually**	**Sometimes**	**Rarely**	**Never**	**Unknown**
Farm worker	□	□	□	□	□	□
Visitors	□	□	□	□	□	□

10. Please specify who currently conducts the spreading service for litter used on the farm?

	Always	**Usually**	**Sometimes**	**Rarely**	**Never**	**Unknown**
Farm worker	□	□	□	□	□	□
Other, please specify	□	□	□	□	□	□

11. Which of these methods are currently used to clean the farm (tick all the boxes that applies)?

	At the removal of birds to the abattoir of each flock	At the removal of birds to the abattoir of every second flock	At the removal of birds to the abattoir of every third flock	At the removal of birds to the abattoir of every fourth flock	Other, please specify frequency
Litter removal	□	□	□	□	□
Sweeping without water	□	□	□	□	□
Pressure hose + mild detergent	□	□	□	□	□
Sanitization of the shed(s)	□	□	□	□	□
Washing the walls	□	□	□	□	□
Other (please, specify)	□	□	□	□	□

12. Are there other farm animal (either backyard or commercial) currently kept within 500 m to this farm?

Yes □ No □ If No, go to next question

If Yes,

Type of farm animal	Number of animals (if known)	Distance to this farm

13. Number of chicken sheds currently on the farm: _____
14. What were the maximum numbers of birds per shed within the last 12 months? _____
15. What were the minimum numbers of birds per shed within the last 12 months? _____
16. What were the average numbers of birds per shed within the last 12 months? _____
17. Did the mortality vary between the shed(s) within the last 12 months?

Yes □ No □

If yes,

a) What was the highest mortality rate per shed within the last 12 months? _____

b) What was the lowest mortality rate per shed within the last 12 months? _____

c) What was the average mortality rate per shed within the last 12 months? _____

18. If you answered yes for Question 17, why do you think there were differences in the mortality rate between sheds?

_____

19. Total number of birds supplied to the farm from the hatchery in the last year (2013): _____
20. Total number of birds send to slaughter in the last year (2013): _____
21. Please specify the type of workers currently working on the farm (for example a) manger or b) causal farm worker c) other, (please specify). A worker is a person who works on the farm on daily basis or has contact with the chickens on the farm on a regular basis).

Type of worker	**Average number of days/week**	**Type of work**	**Does the farm worker own or come into contact with other**	**Specify the animal type and frequency of contact with animals (daily, weekly,**

	working on the farm		animals (pets, or livestock) regularly?	monthly, specify other frequency)
			Yes □ No□ Unknown□	
			Yes □ No□ Unknown□	
			Yes □ No□ Unknown□	
			Yes □ No□ Unknown□	
			Yes □ No□ Unknown□	
			Yes □ No□ Unknown□	

Section Three

Farm ------- shed number……

Farm details (To be completed by the farmers/managers)

22. Please specify any visitors that visited the farm during the period the sampled flock was present on the farm.

Visitors	Average frequency of visits (daily/ Weekly/monthly, as required etc.)	Reasons for visit(s)	Did the visitor own or was into contact with other animals (pets, poultry or livestock) regularly?	Specify the animal type and frequency of contact with these animals (daily, weekly, monthly, specify other frequency)
Repairer			Yes □ No□ Unknown□	
Cleaner			Yes □ No□ Unknown□	
Management advisor			Yes □ No□ Unknown□	
Veterinarian			Yes □ No□ Unknown□	
Researcher			Yes □ No□ Unknown□	
Collector of chickens			Yes □ No□ Unknown□	

23. Have you noticed the presence of any of the following animals inside or within 300 m distance to the outside of the shed during the period the sampled flock was present on the farm?

Type of animals	Inside the shed	Outside the shed (within 300 m distance)

	Number of individuals	Average frequency seen (daily/weekly/monthly, specify other frequency)	Number of individuals	Average frequency seen (daily/weekly/monthly, specify other frequency)
Rats/mice				
Wild birds (specify)				
Amphibians or reptiles				
Domestic dogs				
Domestic cats				
Domestic ruminants (cow/sheep)				
Other domestic animals (specify) 1. 2.				
Stray/feral animal (specify) 1. 2.				
Kangaroos				
Possums				
Other (please specify) 1. 2. 3.				

24. Did farm workers or visitors use footbaths when entering the poultry sheds where the sampled flock was housed?

	Always	**Usually**	**Sometimes**	**Rarely**	**Never**	**Unknown**
Farm worker	□	□	□	□	□	□
Visitors	□	□	□	□	□	□

a) If footbaths were used, what disinfectant was used in these foot baths specified above?

_____

25. Did farm workers or visitors use overalls supplied by the farm when having contact with the chickens of the sampled flock?

	Always	Usually	Sometimes	Rarely	Never	Unknown
Farm worker	□	□	□	□	□	□
Visitors	□	□	□	□	□	□

26. Is there any shower facility located on the farm?
Yes □ No □

27. Did farm workers or visitors use the shower facility located on the farm before entering the sheds where the sampled flock was kept?

	Always	Usually	Sometimes	Rarely	Never	Unknown
Farm worker	□	□	□	□	□	□
Visitors	□	□	□	□	□	□

28. Did farm workers or visitors use hand sanitiser before entering the shed or before handling the chickens from the sampled flock?

	Always	Usually	Sometimes	Rarely	Never	Unknown
Farm worker	□	□	□	□	□	□
Visitors	□	□	□	□	□	□

a) If yes, can you please specify the type of sanitiser used?
_____

29. What is the approximate distance in metres between the car park and poultry shed where the sampled flock was kept? _____

30. Did farm workers or visitors disinfect equipment (ladder, fixing tools, etc.) before entering the shed of the sampled flock?

	Always	Usually	Sometimes	Rarely	Never	Unknown
Farm worker	□	□	□	□	□	□
Visitors	□	□	□	□	□	□

b) If so, what disinfectant was used?

_____

31. How often was the following equipment cleaned in the shed where the sampled flock was kept?

	Always	**Usually**	**Sometimes**	**Rarely**	**Never**	**Unknown**
Pan feeder	□	□	□	□	□	□
Water lines	□	□	□	□	□	□

32. How frequently was litter changed where the sampled flock was kept?

At the removal of birds to the abattoir of each flock	At the removal of birds to the abattoir of every second flock	At the removal of birds to the abattoir of every third flock	At the removal of birds to the abattoir of every fourth flock	Other, specify frequency
□	□	□	□	□

33. Was the shed where the sampled flock was kept, cleaned after the litter removal?

Yes □ No □

34. Was insecticide used in the shed where the sampled flock was kept, after cleaning the shed?

Yes □ No □

a) If so, what type of insecticide was used?

_____

35. How many birds died in the shed where the sampled flock was kept?

Week number	number of deaths
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	

36. How frequently were dead birds removed from the shed(s) during the duration the sampled flock was kept in these sheds?

More than twice a day	Twice a day	Once a day	Every other day	Other, specify frequency
□	□	□	□	□

37. What was the peak density per kilogram per square metres for the shed where the sampled flock was kept?

_____

38. In which week did you reach peak density in the shed(s) where the sampled flock was kept?

_____

39. Can you describe the ammonia odour in the shed where the sampled flock were kept at peak density?

No odour	Slightly annoying (faint)	Annoying (distinct)	Very annoying (strong)	Extremely annoying (very strong)
□	□	□	□	□

40. Can you describe the ammonia odour in the shed of the sampled flock before slaughtering?

No odour	Slightly annoying (faint)	Annoying (distinct)	Very annoying (strong)	Extremely annoying (very strong)
□	□	□	□	□

41. What type of ventilation was used in the shed where the sampled flock was kept, please describe in detail?

_____

_____

_____

42. Were transport vehicles (for transport of chickens to the slaughterhouse) allowed to enter the farm during the sampling period?

Yes □ No □

a) If yes, did the transport vehicles have chickens on them when they arrived at the farm?
Yes □ No □ Sometimes□

Section Five

Farm ------- shed number……..

Farm Management Details (To be completed by the farmers/managers)

43. Were there any second grade eggs 'dirty eggs' used for the sampled flock?
Yes □ No □

44. How many second grade eggs 'dirty eggs' were used for the sampled flock?

_____

45. What was the weekly weight of the sampled flock?

Week number	Average weight for the sampled flock
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	

46. Was there any stress-full events occurring during the period when the sampled flock was present on the farm?
Yes □ No □

If yes, Please provide details

_____

_____

_____

Section Six

Farm ------- shed number……..

47. Has *E. coli* been diagnosed on this farm within the last 12 months?

Yes □ No □

Clinical signs observed	Diagnostic test method(s) used	Date (estimate)	Number of death associated with *E. coli* infection
		__/__/__	
		__/__/__	

48. Has *E. coli* diagnosed on this farm in the sampled flock?

Yes □ No □

If yes, please provide details below.

Clinical signs observed	Diagnostic test method(s) used	Date (estimate)	Number of death associated with the *E. coli* infection
		_/__/__	
		_/__/__	

49. Have any respiratory diseases other than *E. coli* been diagnosed on this farm within the last 12 months?

Yes □ No □

If yes, please provide details below.

Disease	Diagnostic test method(s) used	Date (estimate)	Symptoms of the disease observed
		__/__/__	
		__/__/__	

50. Have any respiratory diseases other than *E. coli* been diagnosed on this farm in the sampled flock?

Yes □ No □

If yes, please provide details below.

Disease	Diagnostic test method(s) used	Date (estimate)	Symptoms of the disease observed
		__/__/__	
		__/__/__	

51. Have any antimicrobial treatments been used on this farm within the last 12 months?
Yes □ No □

If yes, please provide details below.

Type of antimicrobial used	Date (estimate)	Details (use for what problem, administration procedure etc.)
	__/__/__	
	__/__/__	

52. Where any antimicrobial treatments used on this farm in the sampled flock?
Yes □ No □

If yes, please provide details below.

Type of antimicrobial used	Date (estimate)	Details (use for what problem, administration procedure etc.)
	__/__/__	

Appendices to Chapters 5

Supplementary Table 2: Number (N), prevalence and association of the five avian pathogenic *E. coli* related virulence genes among clinical *E. coli* (n = 50) and faecal *E. coli* isolates (n = 187) cultured from Australian broiler chickens.

Antimicrobial drug	Clinical *E. coli*						Faecal *E. coli*					
	iroN **N (%)**	***ompT*** **N (%)**	***iss*** **N (%)**	***hlyF*** **N (%)**	***iutA*** **N (%)**	**Total Number**	***iroN*** **N (%)**	***ompT*** **N (%)**	***iss*** **N (%)**	***hlyF*** **N (%)**	***iutA*** **N (%)**	**Total Number**
Ampicillin	10 (71)$^{+}$	12 (86)	12 (86)	12 (86)	12 (86)	14	23 (39)	33 (56)	27 (46)	34 (58)	37 (58)	59
Amoxicillin/clavulanic acid	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	2	4 (50)	7 (88)	4 (50)	7 (88)	3 (38)	8
Ticarcillin/clavulanic acid	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1	1 (50)	1 (50)	1 (50)	1 (50)	2 (100)	2
Cephalothin	2 (67)	2 (67)	2 (67)	2 (67)	2 (67)	3	24 (51)$^{+}$	27 (57)	24 (51)	25 (53)	21 (45)$^{+}$	47
Cefoxitin	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	2	0	0	0	0	0	0
Cefovecin	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	2	0	0	0	0	0	0
Ciprofloxacin	0	0	0	0	0	2	0	0	3 (38)	1 (13)	5 (75)	7
Chloramphenicol	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1	0	0	0	2 (100)	2 (100)	2
Apramycin	4 (100	4 (100	4 (100	4 (100)	4 (100)	4	0	0	0	0	1 (100)	1
Gentamicin	4 (67)$^{+}$	4 (67)$^{+}$	4 (67)$^{+}$	4 (67)$^{+}$	4 (67)$^{+}$	6	0	0	2 (40)	0	3 (60)	5
Streptomycin	10 (83)	10 (83)	10 (83)	10 (83)	10 (83)	12	16 (39)	19 (46)	19 (46)	19 (46)	20 (49)	41
Spectinomycin	2 (50)	2 (50)	2 (50)	2 (50)	2 (50)	4	0	0	0	0	0	0
Neomycin	3 (75)	3 (75)	3 (75)	3 (75)	3 (75)	4	3 (75)	3 (75)	3 (75)	3 (75)	3 (75)	4
Sulfamethoxazole/trimethoprim	19 (86)	20 (91)	20 (91)	20 (91)	20 (91)	22	33 (44)	45 (60)	40 (53)$^{+}$	47 (63)	44 (59)	75
Tetracycline	23 (85)	25 (93)	25 (93)	25 (93)	25 (93)	27	24 (46)	32 (62)	27 (52)	32 (62)	32 (62)	52

$^{+}$ Indicates a positive association between traits (p <0.05).

Supplementary Table 3: Number (N), prevalence and association of phylogenetic groups among antimicrobial resistant avian clinical *E. coli* (n = 50) and faecal *E. coli* (n = 187) isolates from commercial broiler chickens in Australia.

Antimicrobial drug	Phylogenetic Group															
	A		B1		B2		C		D		E		*E_clade 1*		F	
	CEC N (%)	FEC N (%)	CEC N (%)	FEC N (%)	CEC N (%)	FEC N (%)	CEC N (%)	FEC N (%)	CEC N (%)	FEC N (%)	CEC N (%)	FEC N (%)	CEC N (%)	FEC N (%)	CEC N (%)	FEC N (%)
Ampicillin	9 (64)	8 (14)	0	6 (10)	3 (21)	1 (2)	2 (14)	18 (31)	0	8 (14)	0	3 (5)	0	2 (3)	0	13(22)
Amoxicillin/clavulanic acid	2 (100)	2 (25)	0	0	0	0	0	1 (13)	0	2 (25)	0	1 (13)	0	0	0	2 (25)
Ticarcillin/clavulanic acid	1 (100)	0	0	0	0	0	0	0	0	1 (50)	0	0	0	0	0	1 (50)
Cephalothin	2 (67)	10 (21)	0	4 (9)	0	1 (2)	1 (33)	13 (28)	0	5 (11)	0	2 (4)	0	0	0	12 (26)
Cefoxitin	2 (100)	0	0	0	0	0	0	0		0	0	0	0	0	0	0
Cefovecin	2 (100)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ciprofloxacin	2 (100)	0	0	2 (26)	0	0	0	0	0	0	0	0	0	0	0)	5 (71)[+]
Chloramphenicol	1 (100)	0	0	0	0	0	0	2 (100)	0	0	0	0	0	0	0	0
Gentamicin	3 (50)	1 (20)	0	1 (20)	2 (33)	0	1 (17)	0	0	0	0	0	0	0	0	3 (60)
Streptomycin	2 (50)	5 (12)	0	6 (15)	1 (25)	1 (2)	1 (25)	12 (29)	0	6 (15)	0	2 (5)	0	0	0	8 (20)
Neomycin	4 (100)	1 (25)	0	1 (25)	0	0	0	0	0	1 (25)	0	0	0	0	0	0
Spectinomycin	1 (25)	0	0	0	2 (50)	0	1 (25)	0	0	0	0	0	0	0	0	0
Apramycin	3 (75)	0	0	0	1 (25)	0	0	0	0	0	0	0	0	0	0	1 (100)
Sulfamethoxazole/ trimethoprim	12 (55)	12 (16)	0	6 (8)	5 (23)[+]	5 (7)	3 (14)	24 (32)	0	10 (13)	0	2 (3)	0	2 (3)	2 (9)	13 (17)
Tetracycline	14 (52)	11 (21)	0	5 (10)	3 (11)	1 (2)	6 (22)	16 (31)	0	5 (10)	0	1 (2)	0	2 (4)	4 (15)	11 (21)

[+] Indicates a positive association between traits (P <0.05).

THE UNIVERSITY OF QUEENSLAND

UQ Research and Innovation
Director, Research Management Office
Nicole Thompson

ANIMAL ETHICS APPROVAL CERTIFICATE

27-Nov-2012

Activity Details

Chief Investigator:	Dr Conny Turni, Queensland Alliance for Agriculture and Food Innovation
Title:	Studies on avian pathogenic Escherichia coli
AEC Approval Number:	QAAFI/478/12/POULTRY CRC
Previous AEC Number:	
Approval Duration:	01-Feb-2013 to 01-Feb-2014
Funding Body:	Australian Poultry - CRC
Group:	Production and Companion Animal
Other Staff/Students:	Patrick Blackall, Justine Gibson
Location(s):	Other Queensland Location

Summary

Subspecies	Strain	Class	Gender	Source	Approved	Remaining
Poultry		Adults	Mix	Commercial breeding colony	20	20

Permit(s):

Proviso(s):

Approval Details

Description	Amount	Balance
Poultry (Mix, Adults, Commercial breeding colony)		
21 Nov 2012 Initial Approval	20	20

UQ Research and Innovation
The University of Queensland
Cumbrae-Stewart Building
Research Road
Brisbane Qld 4072 Australia
T +61 7 3365 2925 (Enquiries)
T +61 7 3365 2713 (Manager)
F +61 7 3365 4455
E animalwelfare@research.uq.edu.au
W www.uq.edu.au/research/rid/

100 YEARS AHEAD

THE UNIVERSITY OF QUEENSLAND

UQ Research and Innovation
Director, Research Management Office
Nicole Thompson

ANIMAL ETHICS APPROVAL CERTIFICATE

10-Oct-2013

Activity Details

Chief Investigator:	Dr Conny Turni, Animal Science
Title:	Studies on avian pathogenic Escherichia coli in commercial broiler chickens in Southeast Queensland
AEC Approval Number:	SVS/323/13/POULTRY CRC
Previous AEC Number:	
Approval Duration:	11-Oct-2013 to 11-Oct-2014
Funding Body:	Australian Poultry - CRC
Group:	Production and Companion Animal
Other Staff/Students:	Justine Gibson, Leena Awawdeh, Patrick Blackall
Location(s):	Other Queensland Location

Summary

Subspecies	Strain	Class	Gender	Source	Approved	Remaining
Poultry		Adults	Mix	Commercial breeding colony	400	400

Permit(s):

Proviso(s):

Approval Details

Description	Amount	Balance
Poultry (Mix, Adults, Commercial breeding colony)		
18 Sep 2013 Initial approval	400	400

UQ Research and Innovation
The University of Queensland
Cumbrae-Stewart Building
Research Road
Brisbane Qld 4072 Australia
T +61 7 3365 2925 (Enquiries)
T +61 7 3365 2713 (Manager)
F +61 7 3365 4455
E animalwelfare@research.uq.edu.au
W www.uq.edu.au/research/rid/

100 YEARS AHEAD

Human ethic approval

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Studies On Avian Pathogenic Escherichia Coli In Commercial Broiler Chickens In Southeast Queensland - 24/06/2014 - AMENDMENT
Chief Investigator:	Ms Leena Awawdeh
Supervisor:	Dr Justine Gibson, Dr Conny Turni, Dr Joerg Henning, Dr Rowland Cobbold, Dr Pat Blackall, Dr Joanne Platell, Dr Rachel Allavena
Co-Investigator(s):	Dr Justine Gibson, Dr Conny Turni, Dr Joerg Henning
School(s):	School of Veterinary Science
Approval Number:	2014000327
Granting Agency/Degree:	The Poultry cooperative Research Centre
Duration:	31st March 2015

Comments/Conditions:

Note: if this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
Behavioural & Social Sciences Ethical Review Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Professor Jolanda Jetten
Deputy Chairperson
Behavioural & Social Sciences Ethical Review Committee

Signature _____ Date 3-7-14