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

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A healthy chicken's intestinal flora harbours a rich reservoir of *Escherichia coli* as part of the commensal microbiota. However, some strains, known as avian pathogenic *E. coli* (APEC), carry specific virulence genes (VGs) that enable them to invade and cause extraintestinal infections such as avian colibacillosis. Although several VG combinations have been identified, the pathogenic mechanisms associated with APEC are ill-defined. The current study screened a subset of 88 *E. coli* isolates selected from 237 pre-existing isolates obtained from commercial poultry flocks in Australia. The 88 isolates were selected based on their enterobacterial repetitive intergenic consensus (ERIC) and antimicrobial resistance (AMR) profiles and included 29 *E. coli* isolates cultured from chickens with colibacillosis (referred to as clinical *E. coli* or CEC) and 59 faecal *E. coli* (FEC) isolates cultured from clinically healthy chickens. The isolates were screened for the presence of 35 previously reported VGs. Of these, 34 were identified, with *iucA* not being detected. VGs *focG*, *hlyA* and *sfa/foc* were only detected in FEC isolates. Eight VGs had a prevalence of 90% or above in the CEC isolates. Specifically, *astA* (100%); *feoB* (96.6%); *iutA*, *iss*, *ompT*, *iroN* and *hlyF* (all 93.1%); and *vat* (89.7%). The prevalence of these were significantly lower in FEC isolates (*astA* 79.7%, *feoB* 77.9%, *iutA* 52.5%, *iss* 45.8%, *ompT* 50.9%, *iroN* 37.3%, *hlyF* 50.9% and *vat* 42.4%). The odds ratios that each of these eight VGs were more likely to be associated with CEC than FEC ranged from 7.8 to 21.9. These eight VGs may be used to better define APEC and diagnostically detect APEC in Australia. Further investigations are needed to identify the roles of these VGs in pathogenicity.

Keywords antibiotic resistance profile; avian colibacillosis; avian pathogenic *Escherichia coli*; enterobacterial repetitive intergenic consensus; extraintestinal pathogenic *E. coli*; polymerase chain reaction; virulence genes

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Avian colibacillosis is caused by a subgroup of extraintestinal pathogenic *Escherichia coli* (ExPEC), known as avian pathogenic *E. coli* (APEC), which have the ability to invade various internal organs and cause systemic disease.[1–3] Most *E. coli*, however, are commensals that coexist in healthy birds' gut microbiota, do not cause disease and are known as avian faecal *E. coli* (AFEC).[4–7] A large number of overseas studies have aimed to define and differentiate APEC from AFEC based on whole-genome sequencing (WGS), phylogenetic grouping, virulence genotyping, serotyping, as well as fingerprinting methods such as enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR), randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP).[4–8] 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 APEC pathogenicity have been identified.[9,10] Several overseas studies have differentiated APEC and AFEC based on the presence of five VGs that Johnson et al.[6] identified as having a significant association with APEC.[4,9,11] These VGs are *iss* (increase serum survival gene), *ompT* (outer membrane proteinase gene), *hlyF* (putative avian hemolysin gene), *iroN* (salmochelin siderophore receptor gene) and *iutA* (aerobactin receptor gene).[6] Johnson et al. concluded that *E. coli* could be considered an APEC if cultured from a lesion in an internal organ of a chicken with colibacillosis and possessed at least four of these five APEC-associated VGs.[6]

In a previous study, the clonal relatedness between 237 *E. coli* isolates comprised of 50 clinical isolates cultured from chickens with colibacillosis (CEC) and 187 faecal *E. coli* (FEC) isolates cultured from healthy chickens in Australia has been determined using ERIC-PCR.[12] ERIC-PCR is a molecular typing method that has been employed to study genetic diversity due to its simplicity, enabling the examination of relationships among a large number of isolates.[11,12] This method offers advantages such as speed, cost-effectiveness and the ability to categorise *E. coli* into distinct clonal groups and clusters for epidemiological research.[13,14] However, it has limitations, including a lack of repeatability and lower discriminatory power compared with other fingerprinting methods, such as multi-locus sequence typing (MLST) and pulse-field gel electrophoresis (PFGE).[15] In addition, no studies report a clear link between VG and ERIC-PCR profiles. This suggests the existence of distinct VG profiles within clonal groups or clusters, hinting at past genetic exchanges between different strains.

The current research focused on a subset of the existing isolates obtained from Australian broiler chickens that exhibited diverse

clonality and were highly antimicrobial resistant.[12] This subset of isolates was used to investigate the presence and distribution of 35 VGs reported to be associated with APEC within both CEC and FEC isolates. The 35 VGs investigated included the five APEC-associated VGs that Johnson et al.[6] identified and encompassed eight VG categories, including PTJ100 related (*cvaC*, *iroN2*, *iss2*, *traT*, *iutA2*, *tsh* and *SitA*), adhesins (*fimC*, *papC*, *papG*, *papEF*, *fimH*, *afa/dra*, *focG*, *sfa/focDE* and *hlyA*), iron acquisition (*irp*, *chuA*, *fyuA*, *ireA*, *feoB*, *sfaS* and *IucA3*), protectins/serum resistance (*iucD*, *kpsMTII* and *neuC*), invasions (*kpsMTK1*), toxins (*ibeA* and *Vat*), colicins (*hlyF*, *cbi* and *cma*) and miscellaneous (*maxI*, *ompT* and *astA*).[13] The aim of the study was to identify an APEC-associated VG profile that distinguished between the CEC and FEC isolate types in Australian broiler chickens.

Materials and methods

Bacterial isolates and subset selection criteria

In total, 237 *E. coli* isolates (187 FEC and 50 CEC) were available from a previous study.[12] For this study, the most resistant isolate from each of the 88 ERIC-PCR profile clusters (see Section 1.2) was selected based on their predetermined AMR profile (see Section 1.3). If more than one isolate from the same cluster had the same AMR profile, then the bird's health status was used to determine which isolate would be included with isolates cultured from chickens with colibacillosis being selected. Random selections were applied if more than one isolate belonged to the same cluster with the same AMR and health status profile.

The subset of *E. coli* isolates ($n = 88$) selected for testing in this study are detailed in Table 1.

Enterobacterial repetitive intergenic consensus (ERIC)-PCR

Clonality between the 237 *E. coli* isolates had been established by ERIC-PCR.[17] The banding patterns were analysed using Gel ComparII (Applied Maths, Sint-Martens-Latem, Belgium). The similarity was estimated with a Dice Coefficient of 0.1% and a tolerance of 1%, and cluster analysis was performed with Dice Coefficients and an unweighted-pair group method with arithmetic mean (UPGMA). A 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).[18,19] A cluster was defined as a group of isolates that shared ≥80% similarity in their ERIC-PCR profile patterns.

Antimicrobial susceptibility testing of bacterial isolates

Antimicrobial susceptibility testing had been performed in a previous study[12] using tryptone soy agar disc diffusion as per Clinical and Laboratory Standards Institute guidelines (CLSI)[20] for 20 antimicrobials of veterinary and/or human health importance. These included amikacin, amoxicillin/clavulanic acid, ampicillin, apramycin, cefoxitin, ceftazidime, ceftiofur, cefovecin, cephalothin, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, imipenem, neomycin, spectinomycin, streptomycin, sulfamethoxazole/trimethoprim, tetracycline and ticarcillin/clavulanic acid.[20] The antimicrobial discs were sourced from Thermo Fisher Scientific Australia Pty Ltd. The quality control organism used was *E. coli ATCC 25922*. Since there were no specific veterinary breakpoints for poultry, interpretative criteria were extrapolated from the CLSI guidelines for other animal species. For antimicrobials without CLSI breakpoints, breakpoint information was obtained from the respective manufacturers. In the context of this study, *E. coli* isolates with intermediate susceptibility were considered not resistant, and isolates were labelled as multidrug-resistant (MDR) if they were resistant to one or more antimicrobial agents in three or more antimicrobial classes.[21]

Case definition

All *E. coli* isolates were categorised as shown in Figure 1. The bird's health status from which the isolate was obtained was determined independently from the isolate's VG profile. FEC were further characterised as APEC if sourced from faeces or the

Table 1. Sampling details of 29 clinical *Escherichia coli* (CEC) and 59 faecal *E. coli* (FEC) isolates obtained from commercial broiler chickens in Australia

Type of isolates	Number of isolates	Sampling site and sample number	Location	Date of isolation	Reference
FEC	59	A cloacal swab of healthy chickens	Various locations within Southeast Queensland	2013–2014	14
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 ($n = 1$)	Three chicken farms within Southeast Queensland	2013	15
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 from Queensland	2006–2013	15
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	16

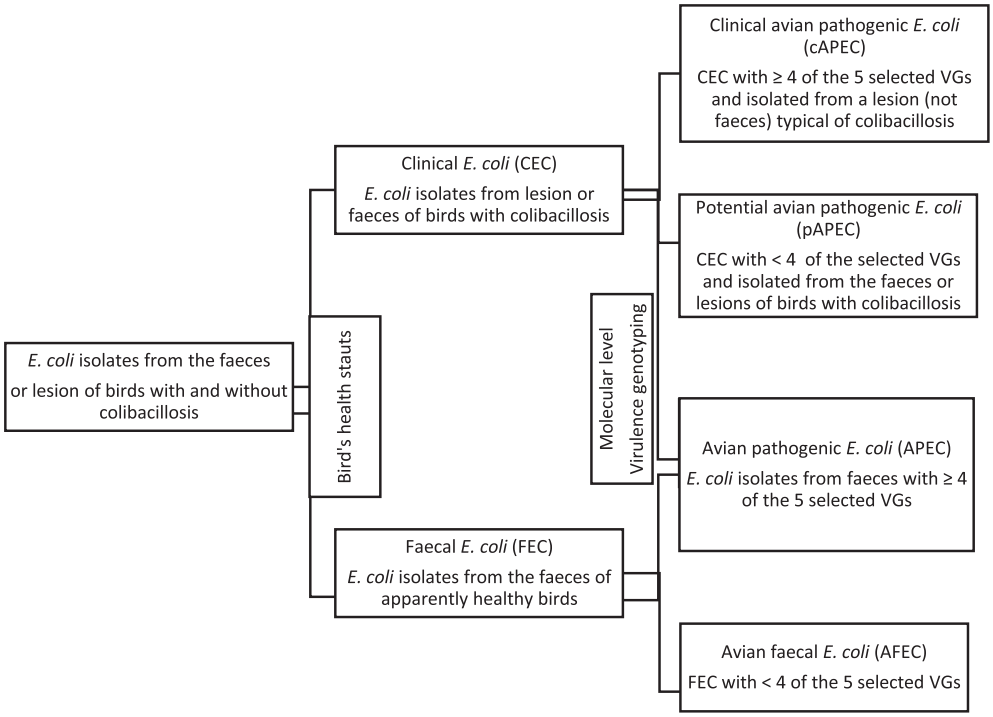

Figure 1. **Flow chart detailing the case classification applied to all *Escherichia coli* isolates ($n = 88$) included in the current study. The five selected VGs were *iss; iutA; iroN; ompT* and *hlyF*.[6]**

cloaca and harboured four or more of the APEC-associated VGs (*iroN*, *iutA*, *iss*, *hlyF* and *ompT*) defined by Johnson et al[6] or AFEC if they did not.[6] Upon genotyping, CEC isolates, which harboured four or more of the five APEC-associated VGs, were defined as clinical avian pathogenic *E. coli* (cAPEC) and those that contained less than four of the selected VGs were identified as potential APEC (pAPEC).

Virulence genotyping

The selected *E. coli* isolates ($n = 88$) had been previously screened for the presence of the five APEC-associated VGs (*iroN*, *iutA*, *iss*, *hlyF* and *ompT*).[6,15] The 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* and *vat*[13]; the second panel targeted *chuA* and *traT*[22]; the third panel amplified *fyuA*; *papG* and *kpsMT k1*[23]; the fourth panel screened for *fimH*, *papEF*, *ireA* and *ibeA*[7]; the fifth panel targeted *sitA* and *feoB*[7]; *sfaS* and *focG* were included in the sixth panel[8]; the seventh panel amplified *cbi*; *cma* and *cvaC*[24]; the eighth panel included *kpsMTII*; *hlyA*; *fimC*; *neuC*; *afa/drab*, *maxI* and *sfa/foc*[22] and the ninth panel amplified *iucA*.[24]

Statistical analysis

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

Results

Isolate case classification

Two of the 29 CEC isolates did not harbour any of the 35 APEC-related VGs and were classified as pAPEC, whereas 27 were classified as cAPEC. Nineteen of the FEC were classified as APEC, whereas 40 were AFEC.

Virulence genotyping

Figure 2 illustrates the 35 APEC-related VG profile for each of the 88 isolates. Table 2 shows the number and prevalence of the 35 APEC-related VGs among the 29 CEC and 59 FEC isolates. None of the CEC or FEC isolates harboured *iucA*. The APEC-related VGs *sfa/foc*; *focG*, and *hlyA*, were not present in the CEC isolates. Table 2 provides the odds ratio that a particular VG was associated with a CEC rather than an FEC isolate, along with the prevalence of the VG in both the CEC and FEC isolates. The following VGs were identified as being significantly (p-value ≤0.05) associated with CEC isolates: *astA* (100%); *foeB* (96.6%); *iroN* (93.1%); *ompT* (93.1%); *iss* (93.1%); *iutA* (93.1%); *iutA* (93.1%); *vat* (89.7%); *fimC* (86.2%); *cvaC* (79.3%), *tsh* (55.2%); *ireA* (51.7%); *papC* (44.8%); *papEF* (41.4%) and *ibeA* (37.9%). Those with a prevalence of 90% or above (namely, *astA*, *foeB*, *iroN*, *omp*, *iss*, *iutA*, *iutA*, *vat*) were associated with odds ratios ranging from 7.8 to 21.9 reflecting that these VGs were much more likely to be associated with CEC than FEC (Table 2). For the following VGs, a significant difference between the CEC and FEC prevalence was not detected: *afa-drab*, *cbi*, *chuA*, *cma*, *fimH*, *fyuA*, *irp2*, *iucD*, *kpsMT11*, *kpsMTK1*, *maxI*, *neuC*, *papG*, *sfaS*, *sitA* and *traT* (Table 2). Seven per cent, 51.7% and 41.4% of the CEC harboured 10, 20 and 30 VGs in comparison with 22%, 71.2% and 6.7% of the FEC.

Figure 2. Rooted rendered virulence gene profiles of 29 clinical *E. coli* (CEC) and 59 faecal *E. coli* (FEC) isolates obtained from broiler chickens in Australia. Virulence genes screened ($n = 35$): *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*, //−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.

Discussion

Globally, colibacillosis or APEC infection is considered a significant problem affecting the poultry industry and translates into multimillion-dollar losses annually.[4,6,25] Interestingly, although vaccines have been developed to alleviate this problem, none have effectively controlled this infection or disease.[26,27] Despite the intensive research globally, the genetic diversity of APEC makes it hard to reach a consensus on the definition of APEC.[6,7,26] In the current study, the *E. coli* isolates (29 CEC and 59 FEC) carried an assortment of APEC-associated VGs, some significantly associated with CEC. The varying prevalence of the VGs reported in this study is comparable with several overseas studies, which have reported variations in VG frequency and combinations in CEC and FEC isolates.[6,7,27–29]

The genetic diversity and relatedness of isolates used in the current study was previously determined using ERIC-PCR[20] as it is a simpler when compared with other molecular typing methods and allows the processing of a large number of isolates.[30,31] It has the benefits of being rapid, cost-effective and able to classify *E. coli* into different clonal groups or clusters for epidemiological studies.[30–35] The lack of repeatability and discriminatory power and lower typing ability compared with other fingerprinting methods, such as MLST and PFGE, are some of the disadvantages of this protocol.[30] However, Ewers et al.[36] and Knobl et al.[37] have reported that none of the molecular typing methods have the ability to differentiate between CEC and FEC isolates. Consistent with this, the ERIC-PCR profiles did not differentiate between CEC and FEC in the current study, and there was no clear association between VG pattern and ERIC-PCR profile. This may suggest the presence of distinct VG profile patterns within clonal groups or clusters and past genetic exchanges between various strains. Phylogenetic analysis using WGS is recommended to provide rapid, accurate and valuable genetic data.[38–40] Unlike ERIC-PCR which only amplifies a small portion of the genome and can only be used to distinguish between strain of bacteria, WGS can be used to not only differentiated between strains of bacteria but also to identify genetic variations between strains and identify genes associated with specific traits. These data have the discriminatory power for future APEC pathogenesis studies and epidemiological investigations to improve the bird's welfare and help develop prevention and control measures against avian colibacillosis.[40] 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 are not standardised and cannot be compared between laboratories.[41]

The frequency of APEC-related VGs among the *E. coli* isolates varied as reported in Table 2. VG profiles may be influenced by several factors such as geographical locations, season, the bird's immune status, the sampling sites and different husbandry and vaccination protocols.[6,42,43] Therefore, APEC-associated VG markers specific to Australian conditions need to be determined to better define and identify APEC in order to reduce the economic losses associated with the disease in the poultry industry. The positive association between avian colibacillosis and the presence of APEC-associated VGs, either individually or in different combinations, has been previously documented in other parts of the world.[6,7,44–47] In the current study, 15 VGs were found to be significantly associated with CEC when compared with FEC: *cvi/cvaC*; *iroN*; *iss*; *iutA*; *tsh*; *fimC*; *papC*; *papEF*; *ireA*; *feoB*; *ibeA*; *vat*; *hlyF*; *ompT* and *astA* (Table 2). The odds ratios for those VGs that had a CEC prevalence of 90% or above (*astA*, *foeB*, *iroN*, *ompT*, *iss*, *iutA*, *hlyF* and *vat*) ranged from 7.8 to 21.9 (Table 2). Thus, using these eight VGs in combination has a very high likelihood of predicting CEC isolates from FEC isolates and therefore can be used to define APEC in Australian broiler chickens. Not surprisingly, the five VGs (*iutA*, *iss*, *ompT*, *iroN* and *hlyF*) identified by Johnson et al.[6] were among these eight high prevalence APEC-associated VGs. 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.[9,11] Ewers et al.[13] found that the five VGs described by Johnson et al.,[6] as well as *iucD*, *irp2* and *astA* could identify and differentiate APEC from AFEC, of which *astA* was among the eight high prevalence VGs identified in this study (Table 2). Interestingly, *iucD* and *irp2* were not found to be significantly associated with CEC in Australian broiler chickens which supports the notion that there is variation in the genes underlying the pathogenicity of *E. coli* strains making it difficult to identify a comprehensive VG profile that defines APEC globally.

The present study investigated 35 APEC-associated VGs to also facilitate a better understanding of the pathogenic mechanisms associated with APEC. Each of the APEC-associated VGs investigated serve distinct roles in the extraintestinal pathogenicity of APEC and can be categorised into different groups according to their functions and contribution to the APEC pathogenicity mechanisms.[4,48] At different stages of infection, alternative VGs could be involved in the pathogenicity mechanism of APEC; therefore, for functions where multiple VGs have been identified such as 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*), their prevalence may vary between APEC strains.[7] Consistent with this notion are the findings with regard to *iss*, which encodes for a lipoprotein of the bacterial outer membrane that facilitates resistance to serum complement.[4,7,10,49–52] In the current study, the prevalence of *iss* in APEC was 93.1% but this varies in other countries such as the USA (80.5%), Germany (82.7%), Spain (91%), Egypt (72%) and Brazil (51%).[6,9,36,53,54] This indicates that APEC strains exhibit genetic diversity underpinning their pathogenic mechanisms, highlighting the needs for additional research to elucidate the prevalence of APEC-associated VGs in different countries.

The adhesin genes, *fimC* and *papC* genes, were significantly associated with CEC in the isolates obtained from Australian broiler chickens. These genes mediate the adherence of *E. coli* to host epithelial cells to enable colonisation.[55] The prevalence of *fimC* in this study was reported as 86.2% (Table 2), which is comparable with Brazil (86%) but lower than that reported for Germany (92%) and Korea (90.1%) for *E. coli* obtained from chickens with colibacillosis.[36,37,47] 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.[22] The prevalence of *papC*, which enables the adherence and the survival of *E. coli* in the internal organs,[55] was 44.8% in the current study. A lower prevalence in *E. coli* isolates from broiler chickens with colibacillosis was also observed in the United States of America

Table 2. Number (N), prevalence (%) and odds ratio (OR) with 95% confidence interval (CI) for 35 avian pathogenic *Escherichia coli* (APEC)-associated virulence genes among 29 clinical *E. coli* (CEC) and 59 faecal *E. coli* (FEC) from broiler chickens in Australia

Putative gene function	Virulence gene[a,b]	OR[c] (95% CI)	CEC N (%)	FEC N (%)	*p*-value
PTJ100 related genes	*cvaC*	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/dra	1.16 (0.27, 4.36)	5 (17.3)	9 (15.3)	1.000
	focG		-	2 (3.4)	
	sfa/foc		-	2 (3.4)	
	hlyA		-	1 (1.7)	
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
	lucA		-	-	
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**

[a] *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*, //-responsive element (putative catecholate siderophore receptor); *iroN*, catecholate siderophore (salmochelin) receptor; *irp*2, 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.

[b] The PCR for these genes was performed in previous study.[15]

[c] Virulence gene *iucA* was not detected in CEC and FEC, whereas *hlyA*, *focG* and *sfa/foc* were not detected in CEC; therefore, ORs for these could not be calculated.

Bolded *p* values are significant using the $\alpha = 0.05$ level.

(USA) (24.8%) and Germany (22.7%).[6,36] Consistent with a role in virulence, the prevalence of *papC* in *E. coli* collected from healthy chickens was very low, with rates of (0%) in the USA and (7.13%) in Japan.[56,57]

The APEC-associated VG *tsh* contributes mainly to the respiratory form of avian colibacillosis.[58–61] In this study, 55% of CEC isolates carried this gene; however, respiratory tract sample sites only accounted for 11 of the 29 isolates (38%, Table 1) suggesting that in Australian broiler chickens *tsh* may also contribute to non-respiratory forms of colibacillosis. Other studies have reported the association between *tsh* and CEC,[45,62] though these studies reported a prevalence of *tsh* as low as 19%[63] and as high as 85.3%,[45,62] which may indicate the dominant form of avian colibacillosis (non-respiratory versus respiratory, respectively) in these studies.

The APEC-associated VG *vat* encodes for the Vat protein with cytotoxic activity for the chicken embryo fibroblast and kidney cells.[64] Several studies have reported that the *vat* gene is encoded in the APEC pathogenicity island (PAI) and occurs in a higher frequency among APEC compared with AFEC.[13,36,65] Consistent with this, *vat* had a prevalence of 89.7% in CEC isolates compared with 42.4% in FEC isolates in this study.

The current study also found that seven pTJ100-plasmids-related genes (*iroN*, *traT*, *iutA*, *sitA*, *iss*, *cvaC* and *tsh*) were more prevalent among CEC isolates than FEC isolates (Table 2) which is consistent with previous studies.[6,7,66] 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.[66,67] The prevalence of the following VGs: *papG*; *irp2*; *chuA*; *kpsMT11*; *kpsMT K1*; *cbi*; *cma*; *traT*; *fimH*; *afa-drab*; *fyuA*; *sfaS*; *iucD*; *neuC* and *maxI* was not found to be different between CEC and FEC isolates (Table 2). The presence of APEC-associated VGs in FEC isolates indicates that AFEC may act as a reservoir for some VGs.[1,10,68]

In the current study, *iucA* which is involved in one of the iron acquisition systems from both CEC and FEC isolates, was not detected. This differs from a previous study by Tivendale et al.[51] who identified the co-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 in the levels of virulence of the *E. coli* isolates in the Tivendale et al.[51] study compared with the current study. Tivendale et al.[51] demonstrated the ability of the three most virulent APEC strains to amplify the *iucA* gene compared with less virulent APEC strains. Other studies have also suggested that *iucA* plays a vital role in the pathogenicity of APEC.[69,70]

Collectively the result from this study indicates that a VG profile consisting of eight genes, namely, *astA*, *foeB*, *iroN*, *ompT*, *iss*, *iutA*, *hlyF* and *vat* could offer a highly sensitive approach for detecting APEC strains in Australian broiler chickens. Despite this finding, it is essential to recognise the existing limitations within this methodology. The fact that three PCRs would be needed to investigate the presence of the VGs to determine APEC status is 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, which allows multiplexing of up to 100 analytes in a single well of a microtiter plate.[71] In addition to PCR and genotyping methods, DNA microarrays are able to characterise bacteria by detecting large numbers of VGs and antimicrobial resistance genes.[72] 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.[73,74]

Although the current study identified a set of APEC-associated 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 the pathogenicity of these VGs still has to be explored. Furthermore, whereas this study provides important information about APEC-associated VG profiles for isolates obtained between 2006 and 2014, it needs to be acknowledged that there is the potential for shifts in *E. coli* strains and, therefore VG profiles, over time. Further research using more recent data is needed to ascertain the current prevalence of APEC in broiler chickens in Australia along with their VG profiles. Comparing more recent data with that of this study will allow a better understanding of the epidemiology associated with colibacillosis so that the Australian poultry industry is better equipped to track and combat this disease.

Conclusions

The current study identified 15 specific VGs (*iroN*, *iss*, *iutA*, *tsh*, *fimC*, *papC*, *papEF*, *vat*, *hlyF*, *astA*, *ibeA*, *feoB*, *ireA*, *cvi/cvaC* and *ompT*) that had a significantly higher prevalence in CEC than in FEC isolates; however, they were present in both indicating that AFEC may act as a reservoir for VGs. These results confirm that the set of five VGs (*iroN*, *iutA*, *iss*, *hlyF* and *ompT*) previously used to define APEC in other countries can also be applied in Australian broiler chickens as they had a significantly higher prevalence in CEC compared with FEC isolates. An additional ten APEC-associated VGs were also identified (*tsh*, *fimC*, *papC*, *papEF*, *vat*, *astA*, *ibeA*, *feoB*, *ireA and cvi/cvaC*) that were significantly more likely to be found in the avian *E. coli* strains sourced from chickens with colibacillosis in Australia. Of these, eight VGs (*astA*, *feoB*, *iutA*, *iss*, *ompT*, *iroN*, *hlyF* and *vat*) have been identified as a VG profile for the identification of APEC in Australian broiler chickens. VGs are often located on plasmids, such as PTJ100-related genes *iroN*, *traT*, *iutA*, *sitA*, *iss*, *cvaC* and *tsh* highlighting the association between avian pathogenicity *E. coli* strains and the possession of mobile genetic elements. Given *iroN*, *iutA and iss* were identified as APEC-associated VGs in Australian broiler chickens, further studies are required to investigate the possibility of using these VGs in the field and 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.

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