

## Article

# Risk Factors Associated with the Carriage of Pathogenic *Escherichia coli* in Healthy Commercial Meat Chickens in Queensland, Australia <sup>†</sup>

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**Simple Summary:** At the farm-level, all 40 farms sampled were positive for avian pathogenic *E. coli* (APEC), and the mean within-farm prevalence was 63%. 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 waterline after each flock, farm visitors not showering before entering the shed, distances greater than 20 m between the car park and the poultry shed, and the presence of wild birds within 50 m of the shed. Chlorinating the drinking water combined with automatic water filtration reduced within-farm bird-level APEC prevalence. 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.

**Abstract:** Avian pathogenic *E. coli* (APEC) can cause avian colibacillosis, an economically important disease that contributes to bird mortality and the high costs associated with prevention and treatment. Little is known about APEC in the context of Australian conditions. The current study aimed to estimate the prevalence of APEC and determine the risk factors associated with cloacal carriage of APEC among commercial healthy meat chickens in Queensland. Cloacal swabs were collected at slaughter from 400 healthy meat chickens (ten per farm) originating from 40 farms. A total of 2200 *E. coli* isolates were selected from cultured swabs and screened for the presence of five APEC-associated virulence genes (VGs). Farm-level data were collected using a questionnaire. Binomial general linear models were used to identify farm-level risk factors associated with bird-level APEC prevalence. Thirty-four per cent of the cultured *E. coli* isolates ( $n = 751$ ) were classified as APEC, with all farms testing positive for APEC, and the overall bird-level prevalence of APEC was 63.0%. 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 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 disinfection after each flock cycle (OR = 2.2, 95% CI: 1.4, 3.5,  $p = 0.001$ ); the presence of wild birds within 50 m of the poultry shed (OR = 2.3, 95% CI: 1.4, 3.7,  $p = 0.001$ ). 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 factors associated with APEC and showed that improving biosecurity and water treatments might reduce the prevalence of APEC. The notable high APEC prevalence on all farms requires further epidemiological investigations.



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**Keywords:** avian pathogenic; *Escherichia coli* (APEC); colibacillosis; virulence genes (VGs); risk factors; meat chicken; poultry farm; Australia

## 1. Introduction

Avian colibacillosis is considered one of the most common diseases that affect the poultry industry and results in significant economic losses and increased welfare concerns [1–7]. Despite 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, APEC remains one of the most significant pathogens, posing a considerable challenge to the global poultry industry [1,3,5,6]. In addition, 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 [3,8–14].

APEC can act as a primary or secondary pathogen [6,15–18] and can result in localised and/or systemic infections in birds [5,19,20]. The severity of the disease depends on the virulence of APEC strains, chicken age and immune status, and the presence of predisposing risk factors [1,20,21]. Healthy chickens can harbour APEC and avian faecal *E. coli* (AFEC) in their gut flora, which may harbour various virulence genes [7,19]. Hence, healthy chickens can act as a reservoir for disseminating APEC to other more susceptible chickens (due to age and or immune status) and their surrounding environment, potentially causing recurring infections in new flocks [19,20].

A number of studies have indicated that various virulence genes (VGs) are useful markers for identifying APEC and that they can be used to differentiate between APEC and AFEC [9,12,22–24]. The combinations of different VGs are likely associated with APEC pathogenicity [9,22,25]. Thus, Johnson et al., (2008) [26] developed a PCR targeting five VGs, namely, (haemolysin gene (*hlyF*), increased serum survival gene (*iss*), outer membrane protease gene (*ompT*), and two iron acquisition system genes (*iutA* and *iroN*), which have been used as a tool for the identification of APEC [26–32].

A range of risk factors are associated with likelihood of APEC infection [26–30]. Kemmett et al., (2013) reported a higher APEC prevalence in the intestinal.

Flora of one-day-old healthy chicks compared to adult chickens at slaughter-age [25]. Contrarily, Kwon et al., (2008) [33] found a higher APEC prevalence (31%) in layer birds (approximately 21 to 70 weeks of age) compared to slaughter-age meat chickens (up to 12 weeks of age, APEC prevalence 14%) in Korea. However, these differences might be related to farming practices rather than age, as layer and meat management are very different.

Farm-level risk factors associated with a high prevalence of avian colibacillosis relate to poultry management practices [5,21]. Impaired biosecurity protocols, for example, might result in APEC entry into the chicken sheds and contribute to increased APEC prevalence [34]. 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 [21].

A positive association between the accumulation of the *E. coli* pathogen in the faeces and the surrounding environment with an increase in the birds' susceptibility to APEC infection among meat chickens has been found [20,34]. The main risk factors associated with the bird's susceptibility were the duration of exposure, the virulence of the APEC strain, breed, and the immune status of the bird [34]. There were positive associations between an increased risk of avian colibacillosis and increased environmental infection pressure [5]. Such environmental pressures can result from unfavourable husbandry management, poor biosecurity protocols, and seasonal changes [5,34–37].

Farm-level risk factors for APEC among commercial meat chicken flocks in Australia are unknown. Therefore, the current study aimed to: (i) determine the farm-level and bird-level prevalence of the gastroenteric carriage of APEC and (ii) investigate the asso-

ciations between management risk factors and the within-farm APEC prevalence among healthy commercial meat chicken flocks to identify potential farm-level risk factors for avian colibacillosis.

## 2. Materials and Methods

### 2.1. Study Design

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

### 2.2. Sample Collection

Assuming a high farm-level APEC prevalence of 90%, with a 95% confidence interval (CI) and a precision of 10%, a sample size of 40 farms was required. The required sample size for bird-level prevalence was calculated to be 400 based on an unknown prevalence of birds carrying APEC (the assumed prevalence was set to 50%), a precision of 5% for the prevalence estimate, and a 95% CI. Thus, we sampled ten birds from each of 40 commercial meat farms. Also, a sample size of about 380 birds is needed to achieve 80% power for detecting an OR of 2 for the risk of APEC, if the expected exposure to the risk factor in the group of birds without APEC is about 20%.

The chicken meat farms included in the study belonged to the same commercial company and were randomly selected by the company. The selected company was willing to participate in this research project and provided access to their farm premises. The 40 commercial chicken meat farms were operating in different geographical locations.

Samples were collected at slaughter between October 2013 and July 2014 in Queensland, Australia (animal ethics approval number: SVS /323/13/ POULTRY CRC).

The commercial chicken meat company owned one slaughterhouse, and chickens were sampled at that location to reduce the disturbance of on-farm production. The slaughterhouse processed up to 15 commercial chicken meat 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. The 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 were submitted to the slaughterhouse in flocks, with chickens from each flock kept together in cages (up to 20 chickens per cage). Only one bird was selected and restrained from each cage whilst a cloacal swab (Sarstedt Australia Pty. Ltd., Technology Park, Mawson Lakes, South Australia, Australia) was collected. In this way, a total of ten chickens from ten cages representing one meat farm were sampled. The swabs were transported on ice to the School of Veterinary Science laboratory at the University of Queensland within two hours of collection.

### 2.3. Collection of Risk Factor Data

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

The questionnaire focussed on risk factors previously reported to be associated with APEC in commercial meat chickens [5,21,34] and included questions on the number of sheds and chickens kept on the farm, the farm's location concerning other livestock farms, specific management practices and biosecurity measures on the farm, and general flock health. Management risk factors evaluated included the following: the age structure of meat 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 greater than 50 m); the frequency of litter removal. Biosecurity measures

were assessed in terms of the following: routine cleaning practices (bird disposal, shed and equipment cleaning); precautionary measures used by farmworkers and/or visitors before and after entering the chicken sheds; the frequency of using foot baths; use of protective clothing; use of showers and hand sanitisers; cleaning and disinfecting protocols used; the distance between the meat shed and the car park; the frequency of cleaning and disinfection of the tyres of transport vehicles and of equipment (feeder, drinker, ladder, fixing tools, etc.) before entering the farm; ownership of livestock or pets by the farm manager and workers. Potential flock health data collected comprised information on previous colibacillosis infections and other diseases on the farm, mortality rates per shed, frequency of removing dead bird carcasses from the sheds, and the administration of antimicrobial drugs.

The questionnaire was piloted in February 2014 in face-to-face interviews with three individual meat farm managers, and five questions were revised to increase their clarity. The final questionnaire contained 52 binary, ordinal, and open-ended questions (Supplementary Table S1).

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 first author of this paper were mailed out to the managers of the chicken meat farms in April 2014. In June 2014, a reminder was posted followed by a phone call to remind the farm managers to complete the questionnaire. The managers of ten chicken meat farms were contacted either face-to-face or by phone to clarify some responses after receiving the completed questionnaires.

#### 2.4. Bacterial Culture of Samples, Identification and DNA Extraction

All samples were processed within 24 h of collection. Each cloacal swab was cultured on Brilliance™ *E. coli* coliform selective agar (BECS; Oxoid, Thebarton, South Australia, Australia) [38] and incubated aerobically overnight at 37 °C. From each agar plate, five or ten presumptive *E. coli* colonies (for each 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 2200 *E. coli* isolates were collected from the 40 farms.

Presumptive *E. coli* isolates were subcultured on sheep blood agar (SBA; Oxoid) and incubated aerobically at 37 °C overnight. Isolates that were indole positive and pyrrolidonyl arylamidase negative were presumed to be *E. coli* and DNA was extracted [39]. An *E. coli*-specific PCR targeting the *uspA* gene [40] 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 until further analysis.

#### 2.5. Molecular Detection of Virulence Genes

All 2200 *E. coli* isolates were screened for the presence of five APEC-related VGs (*iroN*, *iutA*, *iss*, *hlyF*, and *ompT*) using the pentaplex-PCR [26] *Escherichia coli* STJ-1 [41] and *E. coli* ATCC 8739 were used as positive and negative controls, respectively. The *E. coli* isolate selected to represent each bird was the *E. coli* cultured from that bird that contained the most VGs 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](http://www.microsoft.com) (accessed on 11 February 2014)).

#### 2.6. Case Definition

For the purpose of this study, APEC were defined as an *E. coli* isolate that was cultured from cloacal swabs and harboured four or more of the five selected APEC-related VGs (*iutA*, *iss*, *ompT*, *hlyF*, and *iroN*) [26]. AFEC was defined as an isolate that was cultured

from the cloacal swab of a healthy chicken and harboured less than four of the five selected APEC related VGs.

A chicken was considered APEC positive at the bird-level if at least one of its *E. coli* harboured four or more of the selected APEC-associated VGs. The farm was considered APEC positive if at least one bird on that farm was APEC positive.

### 2.7. Validation of the 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 five selected 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 containing the most VGs. This *E. coli* isolate (and the individual bird) was 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 [42].

### 2.8. Virulence Gene Prevalence in APEC Positive and Negative Birds

The prevalence of the five VGs (with 95% CI) among APEC positive and negative birds was summarised. As samples were clustered within meat farms, survey estimation commands were used, with the farm being defined as the primary sampling unit [43,44]. Hence, the standard error and CI estimation accounted for the clustering. 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 [43,44]. Thus the Pearson chi-square statistic was converted into a survey-design adjusted F statistic [43,44].

### 2.9. Farm-Level, Bird-Level and within-Farm APEC Prevalence

Overall 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.

### 2.10. 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. Data analyses were performed using Stata (13th edition, Blackburn North Victoria, Australia, [www.stata.com](http://www.stata.com) (accessed on 20 October 2014).

A total of 120 individual risk factors were derived from the 52 questions in the questionnaire. Logistic regression models were used to investigate the association of APEC within-farm prevalence and potential APEC farm-level risk factors [45,46]. Risk factors with  $p < 0.15$  in the univariate analysis were included in the multivariable model. A forward stepwise model building strategy was used to develop the final model. The analysis was continued by successively re-fitting models with explanatory variables that were not significant at  $p < 0.15$  in the univariate analysis until all remaining variables in the final model were statistically significant at  $p < 0.05$  [47–50]. In addition, potential confounding variables were 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 [50]. Interactions between the different explanatory variables in the final model were also tested. For variables with more than two levels, the overall significance of the categorical variable levels was evaluated using the Wald test [47]. Models were compared using the Akaike information criterion (AIC) [48–50]. 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  $\chi^2$ , deviance, and conducting the Hosmer–Lemeshow tests [51].

### 3. Results

#### 3.1. Overview of Meat Farm Management and Biosecurity Practices

The majority (97.5%) of the questionnaires ( $n = 39$ ) were completed by farm managers and 2.5% by a farm owner ( $n = 1$ ). Variation in 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 meat growing cycle (range 30 to 60 days). The largest farm was composed of 19 sheds producing up to 180,000 chickens per meat growing cycle. Half (50%) of the surveyed farms were composed of five to eight sheds.

The integrated company livestock manager (independent from the farm manager and/or owner) visited each farm weekly to check on-farm management, health of the bird, and progress of the meat chickens. The livestock manager supported and supervised all of the participating 40 farms, all of which followed the same biosecurity protocols that the integrated company had developed.

A quarter (25%,  $n = 10$ ) of the farms housed chicken meat flocks of the same age (range 1–50 days-old) in the same shed at the same time using an all-out all-in system of rearing. Sixty-five per cent ( $n = 26$ ) of the farms housed flocks of different ages within the same shed. Ten per cent of the farms ( $n = 4$ ) housed single and multiple aged birds in 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 the manager/owner) and visitors on all farms ( $n = 40$ ) used footbaths. Hand sanitisers were used by 42.5% of farmworkers ( $n = 17$ ) and 55% of farm visitors ( $n = 22$ ). Most 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 farmworkers and visitors prior to the equipment re-entering the sheds.

Seventy-five per cent of farms ( $n = 30$ ) used reticulated 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, and 17.5% of farms ( $n = 7$ ) used a combination of reticulated and dam water as the source of drinking water. Seventy-five per cent ( $n = 30$ ) of the farms treated the drinking water with either chlorine alone or in combination with other treatment methods such as automatic filtration.

Every farm removed used litter after each batch of chickens and the sheds were cleaned with a pressure hose using a mild detergent before they were restocked. Most of the farms (97.5%,  $n = 39$ ) washed the walls as part of the shed cleaning protocol, 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 farms (33%) were located within 500 m of other farms housing animals (nine cattle, two horse and two camel farms). Only 10% ( $n = 4$ ) of farm managers/owners owned a dog and/or a cat. However, no contact between their pet(s) and the meat chickens was reported.

Avian colibacillosis had been diagnosed in the last year for 40% ( $n = 16$ ) of the surveyed farms based on clinical signs, post-mortem examination, and the isolation of *E. coli* from the affected lesions. Antimicrobial treatment (trimethoprim-sulfamethoxazole) had been administered to bird flocks on 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 was kept on the farm.

#### 3.2. Validation of the Number of *E. coli* Colonies That Need to Be Screened to Detect APEC VGs

Of the 2200 *E. coli* isolates cultured, 34% ( $n = 751$ ) carried four or more VGs (mean = 4.9 VGs) and were classified as APEC positive. In contrast, 66% ( $n = 1449$ ) were classified as AFEC positive with a mean of 2.2 VGs per isolate.

The kappa statistics data on the agreement of using five or ten *E. coli* colonies for classifying a chicken as APEC positive or negative are shown in Table 1. The agreement varied between almost perfect and moderate [42] and provided confidence that five colonies were sufficient for classifying birds as APEC positive or negative correctly.

**Table 1.** Agreement, kappa statistics and *p*-value for the detection of APEC virulence genes after selecting five or ten *E. coli* colonies cultured from meat chickens from Queensland.

Virulence Genes	Observed Agreement %	Expected Agreement %	Kappa	<i>p</i> -Value
<i>iutA</i>	97.5	88.25	0.787	<0.001
<i>Iss</i>	97.5	69.5	0.918	<0.001
<i>hlyF</i>	92.5	79.75	0.629	<0.001
<i>ompT</i>	92.5	83.75	0.539	<0.001
<i>iroN</i>	97.5	72.75	0.908	<0.001

### 3.3. Virulence Gene Prevalence in APEC Positive and Negative Birds

The prevalence of individual VGs in the 400 bird-level isolates (each isolate representing one sampled chicken) is shown in Table 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 a 75.7% prevalence.

**Table 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 meat chicken farms in Queensland.

Virulence Gene	Number of <i>E. coli</i> Isolates with VG.	Number of APEC with VG.	Number of AFEC with VG.	Prevalence of VG in APEC Positive Chickens (95% CI)	Prevalence of VG in APEC Negative Chickens (95% CI)	<i>p</i> -Value
<i>iss</i>	269	250	19	99.2 (0.97, 0.99)	12.8 (7.52, 20.1)	<0.001
<i>iroN</i>	259	248	11	98.4 (0.95, 0.99)	7.4 (3.52, 14.3)	<0.001
<i>ompT</i>	322	252	70	100	47.3 (0.34, 0.58)	<0.001
<i>hlyF</i>	318	251	67	99.6 (0.97, 0.99)	45.3 (31.4, 57.6)	<0.001
<i>iutA</i>	351	237	114	94.1 (0.90, 0.97)	77.0 (63.9, 84.5)	<0.001

### 3.4. Prevalence of Avian Pathogenic *E. coli*

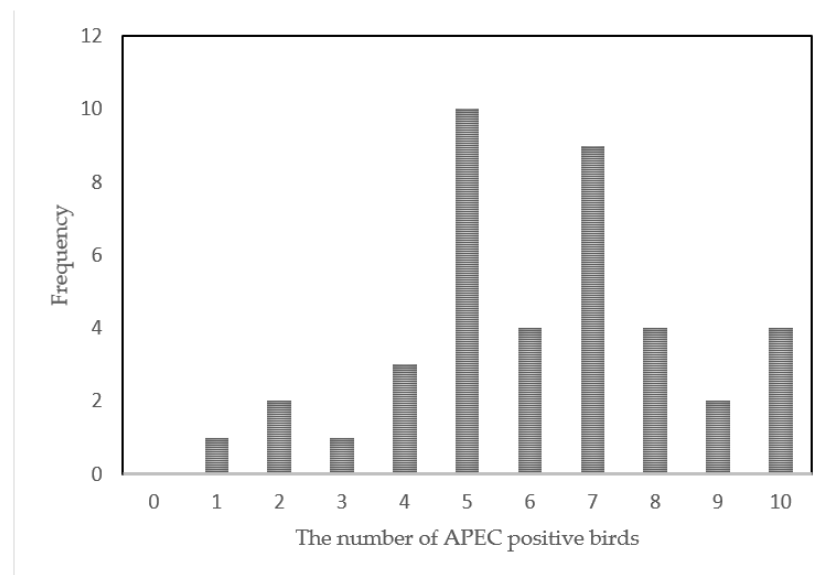
All of the 40 farms were positive for APEC (i.e., at least one bird per farm was APEC positive). At the bird-level, the overall prevalence of APEC in commercial meat chickens ( $n = 400$ ) was 63.0% (95% CI: 55.8, 70.2). The frequency of APEC positive birds sampled per farm across the 40 commercial farms is shown in Figure 1. More than half (58%) of the farms ( $n = 23$ ) had six or more APEC positive birds (of 10 sampled birds). Only one farm had one bird (of 10 sampled birds) identified as APEC positive, and four farms had all ten sampled chickens identified as APEC positive.

### 3.5. Risk Factors for APEC Bird-Level Prevalence

The univariate analysis showed that four risk factor variables were associated with decreased APEC within-farm prevalence ( $p < 0.15$ ) (Table 3), and eight risk factors were associated with increased APEC farm prevalence ( $p < 0.15$ ) (Table 4).

The final multivariable model describing the association between the farm-level risk factors and within-farm APEC prevalence is shown in Table 5. Higher APEC within-farm bird-level prevalence was positively associated with the following: 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,  $p = 0.001$ ); failure to disinfect the waterline after each flock (OR = 2.2, 95% CI: 1.4, 3.5,  $p = 0.001$ ); 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,  $p = 0.001$ ).



**Figure 1.** Frequency of meat farms with the number of meat chickens that tested as avian pathogenic *E. coli* (APEC) positive (out of 10 birds sampled per farm) in Queensland.

**Table 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 from commercial meat chicken farms in Queensland between October 2013 and July 2014. Risk factor coefficients are presented as odds ratios (OR) with 95% confidence intervals (CI). The number (N) of farms represents the number of meat farms that possess or do not possess the risk factor of interest. 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) farmworkers were present on the farm					0.01
None	16 (40)	0.59	Reference		
Category 1 $\leq 5$ 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 farmworkers					
Never	39 (97.5)	0.50	Reference		
Always	1 (2.5)	0.40	0.67 (0.48–0.93)	0.02	

**Table 4.** Univariate analysis of possible farm-level risk factors associated ( $p$ -value < 0.15) with an increased within-farm prevalence of avian pathogenic *E. coli* (APEC). Data were collected from commercial meat chicken farms in Queensland between October 2013 and July 2014. Risk factor coefficients are presented as odds ratios (OR) with 95% confidence intervals (CI). The number (N) of farms represents the number of meat farms that possess or do not possess the risk factor of interest. 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 Factors	Number of Farms (%)	APEC Prevalence	OR (95% CI)	$p$ -Value	Wald Test $p$ -Value
The usage of water well as a 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 meat shed(s)					
No	17 (42.5)	0.49	Reference		
Yes	23 (57.5)	0.51	1.15 (0.63, 1.45)	0.11	
Number of rats outside the shed within 50 m					
None	8 (20)	0.33	Reference		0.01
Category 1 $\leq$ 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 $\leq$ 20 m	23 (57.5)	0.44	Reference	0.007	
Category 2 $>$ 20 m	17 (42.5)	0.58	1.73 (1.16, 2.59)		

**Table 5.** Final multivariable model of possible farm-level risk factors associated ( $p$ -value < 0.05) with a within-farm prevalence of avian pathogenic *E. coli* (APEC). Data were collected from commercial meat chicken farms in Queensland between October 2013 and July 2014. Risk factor coefficients are presented as odds ratios (OR) with 95% confidence intervals (CI). The number (N) of farms represents the number of meat farms that possess or do not possess the risk factor of interest. APEC prevalence represents the mean number of APEC positive birds within the farms that do not have 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	

Table 5. Cont.

Risk Factors	Number of Farms (%)	APEC Prevalence	OR (95% CI)	p-Value	Wald Test p-Value
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 m	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. Discussion

The current study estimated APEC prevalence in commercial broiler chickens and investigated the possible associations between the detected APEC prevalence and biosecurity measures, flock health, and management practices. A cross-sectional study involving healthy commercial broiler chickens simultaneously allowed the comparison and identification of many potential risk factors. The current study has revealed a high APEC prevalence in healthy commercial meat chickens in Queensland. 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 APEC carriage in Australia compared to studies conducted in China and Belgium [21,52]. 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.

The VGs that were proposed by Johnson et al., (2008) [26] as an APEC marker were based on the significant association between these VGs and APEC [27,53,54]. These genes (*iutA*, *hlyF*, *iss*, *iroN*, and *ompT*) are carried by plasmids that typify the APEC pathotype [23,26]. The current study reported an average number of 2.2 genes in *E. coli* cultured from the faeces of healthy meat chickens in Australia compared to 1.3 genes found in faecal *E. coli* in the United States of America [26].

The current study reported that the most prevalent of the tested VGs was *ompT* (100%) followed by *iss*, *hlyF*, *iroN*, and *iutA*, which were all detected in more than 94% of the 252 *E. coli* isolates from APEC positive chickens. Johnson et al., (2008) 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 meat chickens in the United States of America (USA) [26].

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., (2005) in *E. coli* isolates from faecal samples of healthy birds from the USA [23].

This 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 and allow them 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 the farm-level in the current study compared with other studies. No data were collected regarding the whole genomic sequence and phylogenetic groups in the current study, so these molecular comparisons between poultry and human isolates should be the focus of future research.

Despite the age of the birds, there was a similarity between the current study and Kemmett et al., (2013), where considerable APEC prevalence variations were found [25]. A lower APEC prevalence of 1% was reported by Kemmett et al., (2013) [25] in 160 *E. coli* isolates sourced from meat chickens in the United Kingdom. However, the usage of a 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 a higher bird-level prevalence of APEC (that harboured five or more of the ten VGs) of 24.1% in one-day-old chickens compared to 1% prevalence at slaughter-age [25]. 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 [33]. Thus, studies that screen for a larger 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 environmental contamination by APEC and preventing colibacillosis [1,6,20,34]. The current study identified risk factors in line with the results of several 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 meat chickens [37,55,56].

The ability of bacteria to survive in water makes the drinking water used on meat farms a potential source of infection [57]. 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 many birds [57]. Applying essential 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 essential practices to assist in reducing the presence of APEC and/or other pathogens in the drinking water [58].

Water wells have previously been linked to the carriage of APEC [59] and *Campylobacter coli* [60]. The use of water wells as a source of drinking water in three farms in the current study was associated with a 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 of 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. Although there were only three farms involved, the current study highlights the benefit of using a combination of water treatment by chlorination and filtration to reduce the prevalence of APEC carriage in meat chickens. Arsenault et al., (2007) also reported that the addition of

chlorine to the drinking water helped reduce the risk of *Campylobacter* and *Salmonella* colonisation of intestinal flora [61].

Similarly, other studies have also reported the benefits of chlorination in reducing the prevalence of *E. coli* and/or other pathogens and/or decreasing mortality associated with disease [58,62–64]. In this study, not disinfecting the water line between each flock increased 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 [63,65]. Hence, untreated water usage can be considered a potential risk factor for meat flock carriage of APEC, and potentially, other infectious agents. It can be recommended that farms that utilise a well as a source of drinking water should disinfect the water with chlorine and use automatic filtration to reduce the prevalence of APEC carriage. After each flock, disinfection of the water lines should also be included as a best practice management technique on farms.

One concerning finding of the current study was that most farm workers and/or visitors did not implement appropriate on-farm biosecurity measures whilst conducting daily work. For example, the same pair of overalls and gumboots were utilised across sheds on the same farm. It has been shown that 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 [66].

The current study identified that a distance greater than 20 m between car parks and sheds was associated with a 2.2-fold increase in 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 [67–69]. The reported overlap between APEC strains isolated from farmworkers clothes, hands and boots, and poultry farms suggests that people may act as a significant vehicle for introducing and spreading APEC and other pathogens to the poultry farms from the external environment [26,70–73]. Essentially, parking should be close to the changing area, and stringently imposed requirements for changing protective overalls and boots is imperative for infection control [52,55].

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 a shower facility on the farm premises was associated with a 3.6-fold 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 meat 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 [52,74,75]. Wild birds can directly or indirectly encounter meat 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 the faeces of wild birds and meat flocks may implicate wild birds as a potential source of risk [70,76]. Furthermore, the presence of wild birds can induce stress, which is a known predisposing factor for APEC [5]. Therefore, there is a need to control the presence of wild birds near meat sheds. This can be achieved by applying restricted biosecurity measures, such as cleaning feed spills and minimising the amount of surface water on the farm, to discourage the presence of wild birds [77].

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 meat chickens. Exposure to APEC might be a contributing factor in cases of avian colibacillosis [34]. Thus, there is a need to implement strict biosecurity to reduce 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 immune status of the chicken, and the presence of predisposing risk factors [34]. This study has highlighted some of these risk factors, including management practices that could contribute 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 damaging the immune system of the birds and mitigate the negative effects 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 the surrounding environment, could also be further investigated.

The current study was able to overcome some of the difficulties associated with self-reporting questionnaires by conducting piloted face-to-face interviews with three individual meat farm managers to help simplify any unclear questions. As a result, five questions were revised to increase their clarity. Face-to-face interviews took place on all farms that did not respond to overcome the low response rate associated with self-reporting questionnaires. By doing this, we achieved a 100% response rate from all the sampled commercial meat farms. The main limitation of this study is that only one integrated large commercial meat company participated. The sampled chicken meat farms belonged to the same company and had implemented the biosecurity practices recommended by that company. Future research could compare biosecurity practices with APEC prevalence across different companies. 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 accurately compare the prevalence findings between studies based on a cross-sectional research design because there are too many uncontrolled variables. Future investigations could use longitudinal or cohort approaches to explore APEC prevalence and associated risk factor temporal patterns.

## 5. Conclusions

In summary, the current study identified a high prevalence of APEC carriage in healthy meat chickens in Queensland. The current study suggests that practices such as the availability of a shower facility on the farm, a 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 clothing will decrease the risk of APEC carriage and potential infection. Furthermore, our study identified drinking water as a significant risk factor for contamination highlighting the need for water management mechanisms to be put in place in order to reduce the prevalence of APEC. These include chlorination and filtration of drinking water, covering water wells and all water sources, as well as cleaning and disinfecting tanks and water lines between each flock. 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 (for example, through access to the same drinking water).

While this study has identified a high prevalence of APEC at both the bird and farm level in Queensland, longitudinal investigations are needed to estimate the carriage of APEC in one-day-old chickens through to slaughter (or death prior to slaughter), track the associated APEC prevention and treatment costs (and effectiveness/efficacies), and determine the extent of carcass rejections in the same flocks at the abattoirs to ascertain the economic cost of APEC to the chicken meat industry in Australia.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/poultry1020009/s1>: Risk Factors Associated with the Carriage of Pathogenic *Escherichia coli* in Healthy Commercial Meat Chickens in Queensland, Australia; Table S1: Questionnaire for the cross-sectional study to identify risk factors associated with avian pathogenetic *E.coli* on commercial meat chicken farms in Queensland.

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