An Optimized Protocol for Molecular Screening of Avian Pathogenic *Escherichia Coli* From Broiler Chickens in South East Queensland, Australia

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Primary Audience: Veterinarians, Poultry scientists, and/or researcher

SUMMARY

Avian pathogenic *Escherichia coli* (APEC) is the causative agent of avian colibacillosis and causes localized 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 fecal samples. The objectives of this study were to evaluate and compare 3 different DNA extraction methods from cloacal swabs and fecal samples of broiler chickens and determine if APEC can be detected directly from feces. 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 feces. The DNA extracted from feces, with RBB+C method and DNA extracted from *E. coli* isolates of organs and feces, 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 5 VGs in *E. coli* cultured from the cloaca, fecal, and organs samples from chicken affected with colibacillosis. However, screening extracted DNA from the feces for the selected VGs was not an effective diagnostic tool to detect APEC as all of the VGs were detected in the extracted fecal DNA from all chickens.

Key words: avian colibacillosis, avian pathogenic *E. coli*, PCR, DNA extraction, poultry

DESCRIPTION OF PROBLEM

Avian colibacillosis causes multimillion-dollar annual losses for the poultry industry worldwide [1–4]. 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 [1, 4, 5]. Avian pathogenic *Escherichia coli* (APEC), a subgroup of extraintestinal pathogenic *E. coli* (ExPEC), is the causative agent of avian

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colibacillosis. Although a number of studies have aimed to identify the virulence mechanisms of APEC, it remains an ill-defined pathotype [4, 6]. Healthy chickens and their surrounding farm environment can be colonized by APEC without the chickens displaying any signs of disease [7]. 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 fecal *E. coli* (AFEC) [3, 6, 8, 9]. Johnson *et al.* [3] developed a PCR targeting 5 VGs: hemolysin gene (*hlyF*), increased serum survival gene (*iss*), outer membrane protease gene (*ompT*), and 2 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* [5]. However, culture methods are labor intensive, expensive, and time consuming in comparison to molecular methods [10, 11]. The direct application of molecular techniques to identify APEC from fecal 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, hemoglobin, degradation products, and complex polysaccharides [12, 13]. There are several methods (physical, mechanical, and chemical), as well as specifically designed commercial kits [13, 14] used to extract DNA from feces. However, the complex matrix of fecal 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 fecal inhibitors [15, 16]. Furthermore, there is a lack of information pertaining to the quality and quantity of extracted DNA from chicken fecal samples using these different methods [17].

The aims of this study were to: (i) compare and evaluate 3 different published methods for DNA extraction from cloacal swabs and fecal 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 5 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 harbor *E. coli* in their organs; and (iv) determine if direct application of molecular techniques to fecal samples could identify APEC.

MATERIALS AND METHODS

Sample Collection

A total of 20 chickens were collected from 3 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) [18]. The farm managers selected 9 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, 3 unhealthy chickens from farm C were collected as part of their daily culling protocol. The age of the chickens ranged between 28 and 48 d. 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 5 organs: heart, liver, spleen, air sac, and lung [7, 19, 20]. From each chicken, 9 samples were collected with Amies transport swabs [21], 1 from each of the 5 organs listed previously and replicate samples from the cloaca (1 to be used for *E. coli* culture and 3 for DNA extraction). Fresh fecal 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 fecal samples were transported on ice to the laboratory for processing within 3 h of collection.

Processing of Samples

Histological examination Tissues samples were processed from 1 healthy and 5 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 [22]. A board-certified veterinary pathologist reviewed histopathology.

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) [23], MacConkey agar (MCA) [23], chocolate agar [23], xylose-lysine deoxycholate agar [23], and brilliant green agar [23]. The plates were incubated aerobically overnight at 37°C. An additional SBA plate for each sample was incubated anaerobically using the AnaeroGen system [23] overnight at 37°C. In addition, the liver swabs were cultured on campylobacter blood-free agar [23] and incubated under microaerophilic conditions using the CampyGen system [23] 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 [24] or API® Staph [25]. One cloacal swab from each chicken was cultured onto MCA and incubated aerobically at 37°C overnight. From each MCA plate, 3 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 [26]. An *E. coli* specific PCR targeting the uspA gene [27] was performed to confirm identification. All *E. coli* isolates were stored at −80°C in brain heart infusion (BHI) broth [23] containing 20% glycerol [28] until further analysis. The extracted DNA (100 μl) was stored at −20°C for further analysis.

DNA extraction from the cloacal and fecal samples On arrival at the laboratory, each fecal sample was vortexed by adding 1 mL RNA/DNA free water and then 6 swabs were taken from this homogenate. Three cloacal swabs and the 6 fecal swabs from each chicken were stored at

4°C for DNA extraction the next day. All samples were processed within 24 h of collection. Three different extraction methods were evaluated: 1) QIAamp DNA Stool Mini Kit [29]; 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 2 fecal swabs from each chicken for each DNA extraction method. All extracted DNA was stored at −20°C for further analysis.

Method 1 (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.

Method 2 (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 fecal pellets. The DNA was extracted from each sample using a modified Chelex method [30]. Briefly, the pellet was suspended in 1.5 mL acetone 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%) and 0.2 mg protease K. 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.

Method 3 (repeated bead beating plus column) The RBB+C method was performed as previously described [14] with 2 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.

Assessment of DNA extraction DNA extraction was assessed based on the following criteria: DNA yields, DNA purity, and 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 [24]. The yield of the extracted DNA was calculated by the amount of light absorbed by 1 μl of the

DNA at 260 nm [31] 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 3 different methods was evaluated by running 5 μl of extracted DNA on 1% agarose gel in 1% sodium borate buffer (SB) [32] at 80 volts (V) for 30 min. The DNA fragment size was evaluated using λ DNA cut with HindIII as a DNA marker [33], stained with SYBR Safe [34] and visualized using the GelDoc System [35]. 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 [36].

Molecular detection of virulence genes A pentaplex-PCR targeting 5 VGs (*iroN*, *iutA*, *iss*, *hlyF,* and *ompT*) was performed as previously described [3] on the DNA extracted from the cloacal and fecal swabs using the previously determined best extraction method (RBB+C) and from the DNA extracted from *E. coli* that were cultured from the organs and cloacal swabs. *E. coli* STJ-1 [37] and *E. coli* ATCC 8739 were used as positive and negative controls, respectively.

Spiked fecal samples The spiking experiments were performed to evaluate the sensitivity for each DNA extraction method. Two fecal samples were collected from 2 healthy commercial broiler chickens (A and B) and were spiked with 6 different 10-fold serial dilutions of *E. coli* (STJ-1) ranging between 1.6×10 and 1.6×10^6 CFU per mL. One hundred microliters of each serial dilution was added to 20 mg of feces from each bird, A and B, and the DNA was extracted from the 6 spiked samples per bird in triplicate using the 3 different extraction methods.

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 0 to 4) and the isolation of *E. coli* from the affected lesions. A numeric grading representing the severity of the disease was assigned according to previously published scale [7, 18]. 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 harbored 4 or more of the 5 APEC-associated VGs. APEC was defined as an *E. coli* isolate sourced from anywhere (except from an organ of a chicken with colibacillosis) that harbored 4 or more of the 5 APEC VG markers. Clinical *E. coli,* which contain less than 4 of the selected VGs are identified as potential APEC (pAPEC). On the other hand, avian fecal *E. coli* (AFEC) was defined as an *E. coli* isolate, cultured from the feces of chicken, with less than 4 of the selected APEC VG markers [3].

Statistical Analysis

Analyses were performed with Stata software [38]. The mean, median and interquartile range of the yields (ng/μL) and purity for the extracted DNA for fecal and cloacal samples were calculated for the 3 DNA extraction methods. Normality of yield and purity were examined using histograms and data transformations were performed when necessary. The mean of the normalized DNA yield and purity of the fecal sample was compared to the mean of the duplicate fecal sample for the subgroups (healthy and unhealthy chickens) of the 3 different DNA extraction methods using *t*-tests. If the overall mean of the fecal subgroup did not differ from the subgroup duplicate at $P < 0.05$, then the mean of the DNA yield and purity for both fecal samples for each individual chicken was created and used for future analysis. Yield and purity were compared between the 3 different DNA extraction methods in 2 separate models using the General Estimation Equation (GEE) procedure [39]. 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 3 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 fecal and cloacal samples.

It was also assumed that the purity value of the extracted DNA alone might not be sufficient to characterize 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, 4 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 1; 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; and 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 3 DNA extraction methods conducted on cloacal and fecal 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 vs. birds with colibacillosis (3 *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.

RESULTS AND DISCUSSION

Pathology and Bacteriology

Of the 11 selected healthy chickens, 1 chicken (chicken 7) displayed multifocal necrotic lesions on the liver suggestive of necrotizing 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 10 healthy chickens. The remaining healthy chickens were morphologically normal on necropsy. Histopathology on the organs of one representative healthy chicken confirmed the absence of disease. *E. coli* was not cultured from any organs of healthy chickens.

Of the 13 unhealthy chickens (12 selected and chicken 7), 4 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 in the liver and spleen consistent with staphylococcosis [4]. 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 3 chickens. Chicken 9 displayed yellow nodules in the air sacs that were consistent with Aspergillus [4, 40]. Histopathology of the lung demonstrated a marked multifocal granulomatous pneumonia with fibrosis and *Aspergillus fumigatus* was cultured from the air sac and the lung.

The remaining 9 of 13 unhealthy chickens presented with lesions that were consistent with colibacillosis. All of the 9 chickens had lung and/or air sac macroscopic lesions with a lesion score ranging between one and 4. 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 5 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 1 or more of the organs, colibacillosis grade 3, while 3 chickens (1, 4, and 11) showed one or 2 small pin sized lesions per lung and air
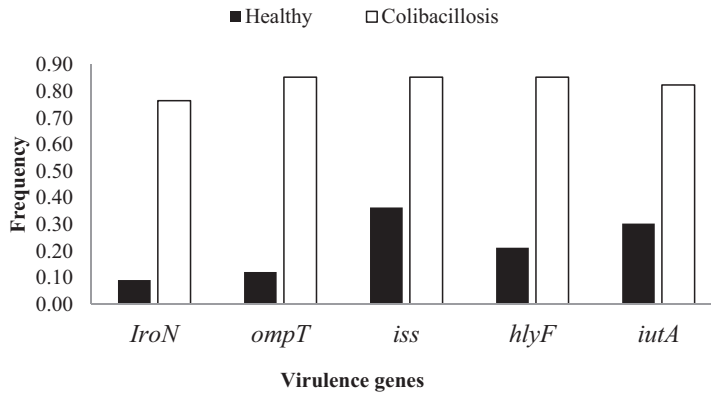
Figure 1. The frequency of virulence genes in *Escherichia coli* cultured from the cloacal swabs of 10 healthy chickens (n = 30) and 9 chickens with colibacillosis (n = 27).

sac (colibacillosis grade 1 or 2). Histopathology of 3 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.

A total of 74 *E. coli* (68 lactose positive isolates from liver, lung, air sac, spleen, and heart of 8 chickens and 6 lactose negative isolates from the liver and spleen of chicken number 12) were obtained and identified, by biochemical and molecular methods, from all 5 organs from the nine chickens clinically diagnosed with colibacillosis. The occurrence of lactose negative APEC has been previously reported by Rodriguez-Siek *et al.* [6].

A total of 69 *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).

DNA Extraction

Quantity of the extracted DNA This study compared 3 DNA extraction methods directly in order to detect the most effective and practical method(s) to extract a high quantity and qual-

ity of DNA from the cloacal and fecal swabs of broiler chickens: a) QIAamp DNA Stool Mini Kit, b) 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 [11]. 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 fecal swabs yielded a comparatively higher DNA quantity in comparison to the cloacal swabs (Supplementary Figure 1). Nonetheless, the Chelex method again yielded the highest concentration of DNA from the fecal 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 1).

The GEE models of log-transformed DNA yield for comparing the 3 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 fecal 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 1.

Table 1. Results From General Estimation Equation Models of Log-transformed DNA Yield Derived From 3 DNA Extraction Methods on Individual Cloacal Swabs (n = 23) and on the Mean of Duplicate Fecal Samples (n = 23) Collected From Healthy and Unhealthy Chickens. The Chelex Method was Used as the Reference Group.

Type of sample	DNA extraction method	Median ng/µL	Interquartile range (IQR)	Ratio of mean yield	95% Confidence interval	P-value
Cloacal	Chelex	802	249–1811	Reference group		
	QIAamp	15	1.4–112	0.037	0.021, 0.063	<0.001
	RBB+C	36	3.4–548–112	0.074	0.034, 0.161	<0.001
Fecal	Chelex	997	603–2963	Reference group		
	QIAamp	90	7–256	0.075	0.048, 0.116	<0.001
	RBB+C	171	72–458	0.155	0.111, 0.217	<0.001

Table 2. Results From General Estimation Equation Models of Log-transformed DNA Purity Derived From 3 DNA Extraction Methods on Individual Cloacal Swabs (n = 23) and on the Mean of Duplicate Fecal Samples (n = 23) Collected From Healthy and Unhealthy Chickens. The Chelex Method was Used as the Reference Group.

Type of sample	DNA extraction method	Median ng/µL	Interquartile range (IQR)	Ratio of mean purity	95% Confidence Interval	P-value
Cloacal	Chelex	1.25	1.25–1.37	Reference group		
	QIAamp	2.03	1.94–2.19	1.503	1.459, 1.549	<0.001
	Repeated bead beating plus column (RBB+C)	1.99	1.9–2.06	1.521	1.490, 1.554	<0.001
Fecal	Chelex	1.3	1.2–1.4	Reference group		
	QIAamp	1.94	1.67–2.1	1.543	1.518, 1.569	<0.001
	RBB+C	2	1.9–2.1	1.509	1.487, 1.531	<0.001

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 fecal 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 fecal and cloacal swabs, respectively. The GEE models of log-transformed DNA purity for comparing the 3 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 fecal 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 2.

Combination of DNA purity and PCR inhibitor results The summary of the DNA purity and PCR inhibitor results for the cloacal and fecal swabs obtained from healthy and unhealthy chickens is shown in Table 3. There was a significant difference ($P < 0.001$) between dichotomized purity and dichotomized PCR inhibitor results between the 3 DNA extraction methods for both, cloacal and fecal 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 fecal samples).

The overall integrity of the extracted DNA using the 3 different methods was of high quality as no shearing was observed on gel electrophoresis. Amplification of the 16S rRNA gene varied between the 3 different extraction methods utilized. Successful amplification of the 16S rRNA gene was achieved in 91% (63 of 69) of the DNA

Table 3. Cross-Tabulation of Dichotomized Purity and Dichotomized PCR Inhibitor Results for 3 DNA Extraction Methods Conducted on Individual Cloacal Swabs (n = 23) and on the Mean of Duplicate Fecal 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
	Repeated bead beating plus column (RBB+C)	0	0	2	21
	Total	21	3	19	26
Fecal	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.

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 3 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 feces with the RBB+C was able to amplify all 5 APEC-associated VGs from all 6 different spiked dilutions (10^1 to 10^6 CFU/mL). While the DNA extracted using the QIAamp DNA Stool Mini Kit amplified the VGs from only the 2 highest spike concentrations and the Chelex method failed to amplify any of the VGs from DNA extracted from all 6 dilutions.

The presence of PCR inhibitors in fecal samples is well documented and variation in the composition and consistency of fecal samples from different animal species may affect the quality of extracted DNA [12, 41, 42]. Therefore, it is necessary to conduct species-specific testing of all extraction protocols. Chicken feces are low in moisture in comparison with other animals [43] which leads to difficulty in dissolving the feces in a buffer [17] and chickens may also have additional inhibitors in comparison to other mammalian feces as they excrete urinary waste in their feces (i.e., common cloaca) [44]. 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.

The RBB+C method was determined to be the best method to extract an adequate yield of PCR quality DNA from the cloacal and fecal 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 fecal samples of cattle [14, 45]. The enhanced performance of the RBB+C on chicken fecal extractions, in comparison with the other 2 methods, may be due to the 2 additional purification steps, which may minimize the presence of PCR inhibitors, in conjunction with a reduced final elution volume, which may maximize 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 2 methods which yielded positive results for 2 of the 6 dilutions at best. However, the RBB+C was a 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 [42, 46]. However, amplification of the 16S rDNA failed in 49% of the extracted DNA samples. Monteiro *et al.* [46] 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 feces) can reduce the presence of PCR inhibitors by 98% in the extracted DNA [47, 48].

While the Chelex method gave a superior DNA yield, as also reported by others [30, 49], 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 [31, 50]. However, Yang *et al.* [30] 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-d-old healthy gosling fecal samples.

This variation in the result obtained by the current study and that of Yang *et al.* [30] could be due to the fact that the feces of geese contain more water, as geese consume large amounts of water with their food [51]. The increased water content of the feces mean less fecal inhibitors are present, which may result in better quality extracted DNA as seen by the higher quality DNA obtained by Yang *et al*. [30] in comparison to the poor quality DNA obtained in the current study from the chicken fecal samples.

DNA yields did differ between the cloacal swabs and fecal samples. This could be attributed to the quantity of original sample, as in some cases the cloacal swabs contained only small quantities of feces. Other studies have also reported a correlation between the amount of the fecal material and DNA yield [52, 53].

Molecular detection of APEC virulence genes All 5 selected VGs were detected in 100% (63 of 63) of the DNA extracted directly from the cloacal and fecal 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) as those detected in the direct PCR examination of the cloacal swabs. Other bacterial species found in the intestine of chickens might harbor these VGs and hence these VGS may not necessarily be associated with APEC when identified directly from DNA extracted from a fecal sample.

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 1 (GEE model: n observations = 378, n groups = 21, Wald $\chi 2$ = 58.78, d.f. = 1, $P < 0.001$).

The frequency of the VGs in the 27 *E. coli* isolates that were cultured from the cloacal swabs of 9 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 harbored all 5 VGs. In all 70% (19 of 27) of the *E. coli* that were cultured from the cloacal swabs of chicken affected with colibacillosis harbored all 5 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. This finding agrees with previous studies where they also found associations between the presence of these 5 VGs and *E. coli* isolates cultured from lesions in the organs of birds affected with colibacillosis [6, 8].

While 85% of the cloacal swabs (23 of 27) of chickens with colibacillosis were classified as pAPEC, 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.

Previous studies have identified an association between the prevalence of different combinations of VGs and the pathogenicity of APEC [1, 3, 54, 55]. Further, it was have reported that certain VG combinations are a useful tool to differentiate between APEC and AFEC [3, 6, 56] 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 feces and the cloaca for the presence of 5 APEC-associated VGs [3]. All 5 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 fecal 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 DNA extracted from a fecal sample.

The main limitation of this study was the small sample size of commercial broiler chickens. However, this was an exploratory study and the selection and sampling of 3 farms with different management and health backgrounds increased the bird variation. Another limitation of this study is that while the fecal samples were processed in duplicate, only 1 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.

CONCLUSION AND APPLICATIONS

1. 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 feces of chickens, which could be used as a rapid diagnostic test.

2. The RBB+C method was the preferred DNA extraction method, as it yielded adequate PCR quality and quantity DNA directly from the fecal material of chickens. However, identifying APEC directly, by detecting the 5 selected VGs (*iroN, iutA, iss, hlyF,* and *ompT*) from the fecal material was not feasible because these 5 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 feces and/or cloacal swabs, other genetic markers will need to be investigated to identify APEC directly from fecal material.

SUPPLEMENTARY DATA

Supplementary data are available at *JAPR* online.

Supplementary Figure 1. Comparison of the 3 extraction methods: Chelex; QIAamp and RBB+C for DNA extracted from the cloacal swabs (n = 23) and on the mean of duplicate fecal samples (n = 23). (A) Compares the DNA yield (ng/μL). (B) Compares the quality of the DNA extracted (260/280 ratio).

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