OAHN FINAL REPORT

Project #: OAHN-02

Project Title: Evaluating virulence genes and antimicrobial susceptibility of avian pathogenic *Escherichia coli* from Ontario broiler and broiler breeder flocks

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Executive Summary

Avian pathogenic *Escherichia coli* (**APEC**) is the causative agent of colibacillosis in poultry, an economically-important disease worldwide. Based on surveillance data collected through the Ontario Animal Health Network, early and late systemic bacterial infections due to APEC continue to be the most commonly reported diseases in broiler chickens in Ontario. This study identified the most common virulence-associated genes and antimicrobial resistance patterns among APEC isolates from Ontario broilers and broiler breeders with systemic lesions consistent with colibacillosis, and identified significant relationships between virulence and antimicrobial resistance in the isolates. Significant associations between several virulence-associated genes and resistance to multiple antimicrobials in the isolates highlight the importance of prudent antimicrobial use to preserve the efficacy of antimicrobials that effectively treat colibacillosis. The age of the birds and the time of sample collection were not associated with virulence or antimicrobial resistance in the isolates, suggesting that environmental contamination of the barn environment and infection of subsequent flocks with APEC might play a role in the epidemiology of colibacillosis. This study provides a benchmark from which to measure changes in the antimicrobial susceptibility and virulence gene patterns of APEC as the industry moves toward reduced antimicrobial use, and offers critical information for the treatment and prevention of colibacillosis in broilers and broiler breeders in Ontario.

Objectives

The objectives of this study were to identify the most common virulence-associated genes of avian pathogenic *E. coli* strains in Ontario broiler and broiler breeder flocks, determine the antimicrobial resistance patterns of these strains, and evaluate potential relationships between virulence genes and antimicrobial resistance.

Materials and methods

The study was conducted between January 1 and December 31, 2016. Clinical cases of broiler and broiler breeder chickens with a high suspicion of colibacillosis were selected by eight Ontario poultry veterinarians (from their regular caseload) for inclusion in the study. The study period was divided into four quarters, such that a total of 75 laboratory submission forms were distributed to the participating veterinarians every three months; larger veterinary practices received more forms to account for higher caseloads. Cases were proportionally selected by the participating veterinarians based on the age of the birds, which were grouped into one of four categories: chicks up to 14 days of age from flocks in which the mortality was > 1%; chickens 15 to 28 days of age; chickens 29 to 140 days of age; and broiler breeders of lay age (> 140 days of age). The participating veterinarians conducted the postmortems, and from each case with gross lesions highly suspicious for colibacillosis, a swab of the affected organ or of the exudate, or an affected organ sample, was sent to the Animal Health Laboratory for testing.

Samples were plated on Columbia blood agar and MacConkey agar plates, and incubated using standard laboratory protocols for *E. coli*. Bacterial identity was confirmed using spectrometry. If a mix of morphologically different *E. coli* colonies was recovered, a single predominant morphotype was selected for susceptibility and genetic testing. Antimicrobial susceptibility to ampicillin (10 µg), apramycin (15 µg), ceftiofur (30 µg), gentamicin (10 µg), kanamycin (30 µg), spectinomycin (100 µg), trimethoprim-sulfamethoxazole (25 µg), and tetracycline (30 µg) was determined by disc diffusion assays following the Clinical and Laboratory Standards Institute guidelines. Isolates were classified as susceptible (which included isolates with intermediate susceptibility) or resistant, as per the measurement of inhibition zones. An isolate was defined as being *multidrug resistant* if it was non-susceptible to at least 1 agent in ≥ 3 antimicrobial classes. Bacterial DNA was extracted from pure bacterial cultures. Multiplex polymerase chain reaction was used to detect 13 virulence-associated genes.

Spearman's rank correlation with a Bonferroni correction was used to identify correlations between individual virulence-associated genes. A similar approach was used to identify correlations between resistance to individual antimicrobials. Multivariable logistic regression models with a random intercept for participating veterinarian (one model per antimicrobial) were used to identify associations between virulence and resistance in the isolates. Only antimicrobials for which ≥ 5% of the isolates were resistant were considered for this analysis. Poisson regression, with a random intercept for participating veterinarian, was used to evaluate the effect of virulence-associated genes on resistance to multiple antimicrobials in the isolates. The dependent variable was the number of antimicrobials to which an isolate was resistant, irrespective of antimicrobial class; as eight antimicrobials were investigated, this count ranged from zero to eight. Additionally, bivariable logistic regression models were used to

determine the effect of age group (≤ 14 days, 15-28 days, 29-140 days, > 140 days) and time of sample collection (quarter or season) on virulence and resistance in the isolates. Several different dependent variables were investigated: the presence of an individual virulence-associated gene; the presence of four genes previously identified as important for APEC virulence (*iroN*, *iss*, *iutA*, *tsh*) (Johnson et al. 2006) irrespective of the presence of other genes; resistance of an isolate to an individual antimicrobial; and the presence of multidrug resistance in an isolate.

Results

In total, samples from 331 cases were submitted to the lab during the study period. There were 165 cases (49.8%) of chicks up to 14 days of age from flocks in which the mortality was > 1%, 109 cases (32.9%) of chickens 15 to 28 days of age, 42 cases (12.7%) of chickens 29 to 140 days of age, and 15 cases (4.5%) of broiler breeders of lay age. *Escherichia coli* was isolated from all submitted samples.

The percentage of isolates in which virulence-associated genes were detected is shown in **Table 1**, and the most common virulence gene patterns are shown in **Table 2**. The number of genes detected per isolate ranged from as few as zero (2.4% of isolates) to as many as 12 (0.6%). Several correlations of moderate strength (rho ≥ 0.40) were identified between individual virulence-associated genes (**Table 3**).

Table 1. Virulence-associated genes

Gene	Gene Description	n (%)[a]
Adhesins		
papC	Pilus associated with pyelonephritis	34 (10.3)
tsh	Temperature-sensitive haemagglutinin	125 (37.8)
Iron acquisition		
fyuA	Ferric yersinia uptake (yersiniabactin receptor)	158 (47.7)
ireA	Iron-responsive element (putative catecholate)	159 (48.0)
iroN	Catecholate siderophore (salmochelin) receptor	280 (84.6)
iutA	Ferric aerobactin receptor gene; iron transport	262 (79.2)
sitA	Periplasmic iron-binding protein	307 (92.7)
Iron transport		
eitA	Periplasmic binding protein	84 (25.4)
Protectins/Serum resistance		
iss	Increased serum survival	290 (87.6)
kpsII	Group II capsule antigens	76 (23.0)
ompT	Episomal outer membrane protease gene	255 (77.0)
Others		
cvaC	Structural gene of the ColV operon	178 (53.8)
etsB	Putative ABC transport system	222 (67.1)

[a] Number (and percentage) of isolates carrying the gene. Because an isolate can have more than one gene, the total number of isolates in the column is greater than 331.

Table 2. Most common virulence gene patterns

Virulence Gene Pattern	n (%)[a]
11 genes	
cvaC - eitA - etsB - fyuA - iroN - iss - iutA - kpsII - ompT - sitA - tsh	13 (3.9)
10 genes	
cvaC - etsB - fyuA - ireA - iroN - iss - iutA - ompT - sitA - tsh	6 (1.8)
9 genes	
cvaC - etsB - fyuA - ireA - iroN - iss - iutA - ompT - sitA	41 (12.4)
cvaC - eitA - etsB - iroN - iss - iutA - ompT - sitA - tsh	9 (2.7)
eitA - etsB - ireA - iroN - iss - iutA - ompT - sitA - tsh	7 (2.1)
cvaC - etsB - ireA - iroN - iss - iutA - ompT - sitA - tsh	5 (1.5)
8 g*enes*	
cvaC - etsB - fyuA - iroN - iss - iutA - kpsII - sitA	12 (3.6)
cvaC - fyuA - ireA - iroN - iss - iutA - ompT - sitA	12 (3.6)
cvaC - etsB - ireA - iroN - iss - iutA - ompT - sitA	10 (3.0)
etsB - ireA - iroN - iss - iutA - ompT - sitA - tsh	5 (1.5)
7 genes	
etsB - ireA - iroN - iss - iutA - ompT - sitA	7 (2.1)
cvaC- etsB - iroN - iss - iutA - ompT - sitA	5 (1.5)
6 genes	
fyuA - iroN - iss - kpsII - ompT - sitA	7 (2.1)
4 genes	
iroN - iss - ompT - sitA	17 (5.1)
3 genes	
iroN - iss - sitA	6 (1.8)

[a] Number (and percentage) of isolates with each gene pattern. Only patterns with ≥ 5 isolates are shown.

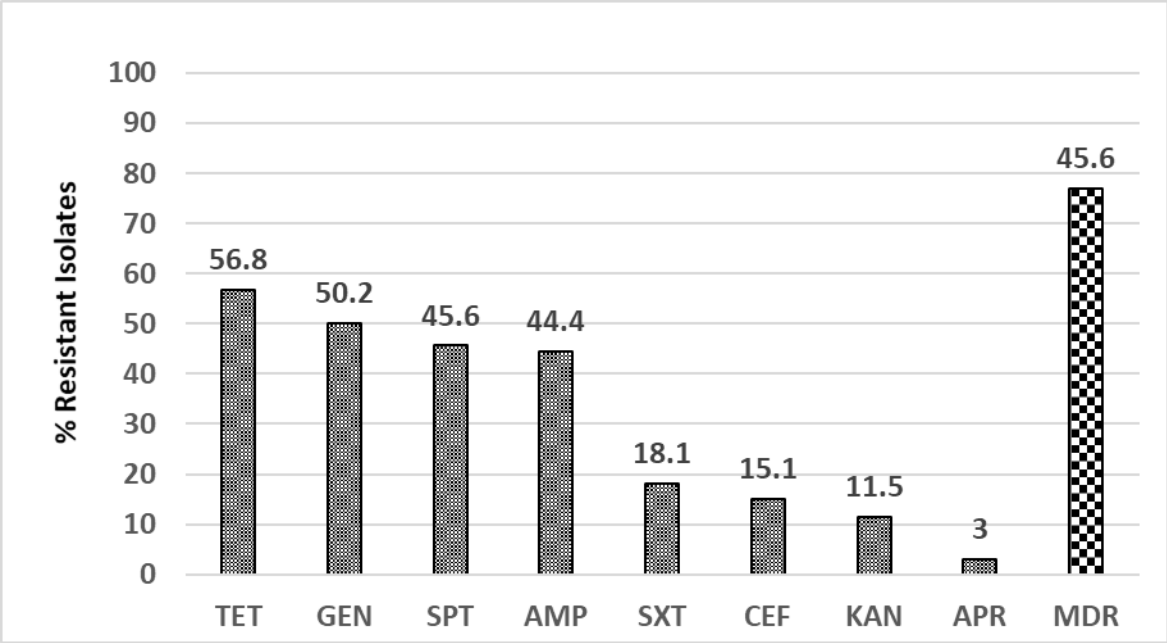
Table 3. Correlations (rho) between pairs of virulence-associated genes

	cvaC	***eitA***	***etsB***	***fyuA***	**ireA**	***iroN***	***iss***	***iutA***	***kpsII***	***ompT***	***papC***	***sitA***	***tsh***
cvaC	X												
eitA		X											
etsB	0.54		X										
fyuA	0.32			X									
ireA	0.22		0.31	0.26	X								
iroN	0.31		0.41			X							
iss	0.35		0.38			0.73	X						
iutA	0.45	0.28	0.59	0.27	0.40		0.24	X					
kpsII		0.26		0.27	-0.35				X				
ompT					0.34	0.32	0.30	0.27		X			
papC											X		
sitA	0.28					0.33	0.35	0.34		0.24		X	
tsh		0.68	0.25					0.35					X

Only statistically significant ($p \leq 0.00385$) Spearman's rank correlation coefficients are shown.

The proportion of isolates that were resistant to each antimicrobial is shown in **Figure 1**. The number of antimicrobials to which an isolate was resistant ranged from as few as zero (15.7% of isolates) to as many as seven (1.2%). Forty-six percent of the isolates were multidrug resistant. The most common antimicrobial resistance patterns are shown in **Table 4**. Moderately strong correlations between ampicillin and ceftiofur resistance, and between gentamicin and spectinomycin resistance, were identified (**Table 5**).

Figure 1. Antimicrobial resistance



AMP = ampicillin; APR = apramycin; CEF = ceftiofur; GEN = gentamicin; KAN = kanamycin; SPT = spectinomycin; SXT = trimethoprim-sulfamethoxazole; TET = tetracycline; MDR = multidrug resistance (APR, GEN, KAN and SPT are in the same antimicrobial class)

Table 4. Most common antimicrobial resistance patterns

Antimicrobial Resistance Pattern	Number of Antimicrobial Classes in Pattern (Multidrug Resistant)	n (%)[a]
GEN-SPT-TET	2 (No)	30 (9.1)
AMP-SXT-TET	3 (Yes)	9 (2.7)
SPT-SXT-TET	3 (Yes)	7 (2.1)
AMP-GEN-SPT-TET	3 (Yes)	11 (3.3)
AMP-CEF-GEN-SPT	3 (Yes)	8 (2.4)
AMP-KAN-SXT-TET	4 (Yes)	5 (1.5)
GEN-KAN-SPT-TET	2 (No)	5 (1.5)
AMP-CEF-GEN-SPT-TET	4 (Yes)	22 (6.7)

[a] Number (and percentage) of isolates with each antimicrobial resistance pattern. Only patterns with ≥ 5 isolates are shown.

Table 5. Correlations (rho) between antimicrobials with respect to resistance

	AMP	APR	CEF	GEN	KAN	SPT	SXT	TET
AMP	X							
APR		X						
CEF	0.47		X					
GEN	0.30		0.20	X				
KAN					X			
SPT			0.24	0.55		X		
SXT				-0.28			X	
TET					0.28	0.24	0.22	X

Only statistically significant ($P \leq 0.00625$) Spearman's rank correlation coefficients are shown.

Several statistically significant associations between antimicrobial resistance and the presence of virulence-associated genes were identified (**Table 6**). In general, individual genes tended to be positively associated with ceftiofur, gentamicin, kanamycin, spectinomycin, and tetracycline resistance, and negatively associated with ampicillin and trimethoprim-sulfamethoxazole resistance. However, there were no common patterns among antimicrobials with respect to the genes associated with resistance. The number of antimicrobials to which an isolate was resistant significantly increased with the presence of *eitA* (RR = 1.37), *iroN* (RR = 1.24), *papC* (RR = 1.34), and *sitA* (RR = 1.77), and significantly decreased with the presence of *tsh* (RR = 0.79). Age group and time of sample collection were not significantly associated with the presence of individual virulence-associated genes, the presence of *iroN*, *iss*, *iutA*, and *tsh*, resistance to individual antimicrobials, or the presence of multidrug resistance.

Table 6. Associations (odds ratios) between antimicrobial resistance and virulence genes

	AMP	**CEF**	**GEN**	**KAN**	**SPT**	**SXT**	**TET**
cvaC							2.12
eitA							2.15
etsB		2.98					
ireA	0.32				2.50		
iutA					3.15		
kpsII	1.88	2.61					
ompT			3.89			0.14	
papC				50.10			8.27
sitA			3.54				
tsh	0.46					0.31	

Only statistically significant ($p \leq 0.05$) associations are shown.

Applications

The results from this study provide a benchmark from which to measure changes in the antimicrobial susceptibility and virulence gene patterns of avian pathogenic *Escherichia coli* as the industry moves toward reduced antimicrobial use, and offers critical information for Ontario poultry veterinarians for making decisions related to the treatment of colibacillosis in broilers and broiler breeders. The results can be used to guide molecular studies on APEC, and potentially, to guide research into alternatives to antimicrobials, such as the development of a candidate vaccine or alternative product to reduce or eliminate infections.

Discussion / Suggestions for next steps

The majority (94%) of the *E. coli* isolates possessed at least three virulence-associated genes, and some carried as many as 12 genes. Among adhesins, *tsh* was the most common gene. Among the iron acquisition and iron transport genes, *sitA*, *iroN*, and *iutA* were the most common. Among protectins and serum resistance genes, *iss* and *ompT* were the most common. Other common virulence-associated genes included *etsB* and *cvaC*. Some of the more common virulence gene patterns included *sitA*, *iss*, *iroN*, *iutA* and *ompT*, which have previously been associated with plasmid pathogenicity islands (Ahmed et al. 2013, Johnson et al. 2006, Johnson et al. 2008a) and shown to be important for APEC virulence. This finding agrees with studies from several other countries (Ahmed et al. 2013, Barbieri et al. 2013, De Carli et al. 2015, Delicato et al. 2003, Ewers et al. 2007, Jeong et al. 2012, Johnson et al. 2008a, Paixão et al. 2016, Solà-Ginés et al. 2015), where researchers identified a high prevalence of these genes in APEC isolates from broiler chickens with colibacillosis. Several statistically significant correlations between individual genes were identified. Although the biological significance of these correlations is unknown, the stronger relationships (e.g., between *cvaC* and *etsB*, *cvaC* and *iutA*, *eitA* and *tsh*, *etsB* and *iroN*, *etsB* and *iutA, ireA* and *iutA*, and *iroN* and *iss*) might indicate co-location of genes on chromosomes or plasmids. Molecular studies are needed to investigate this further. Of note, a small proportion (2%) of the isolates did not possess any virulence-associated genes. Potentially, this could have been due to the presence of other unknown virulence genes that were not included in the multiplex PCR, or to the submission of swabs or harvesting of colonies that represented commensal, rather than pathogenic strains of *E. coli*. Further studies aimed at understanding the pathogenesis of APEC are needed, as it not fully understood which genes (or combination of genes) are necessary for clinical disease to occur.

It is commonly accepted that antimicrobial use is an important factor for antimicrobial resistance development in commensal and pathogenic enteric bacteria of food animals. In Ontario, the first drug of choice for the treatment of colibacillosis is typically a potentiated sulfa (e.g., trimethoprim-sulfadiazine), while the second is typically an aminopenicillin (e.g., ampicillin) or tetracycline (Agunos et al. 2012). In this study, a high proportion of isolates were resistant to tetracycline, gentamicin, spectinomycin, and ampicillin. A high prevalence of resistance to antimicrobials frequently used to treat colibacillosis has also been shown in other countries (Ahmed et al. 2013, Dou et al. 2016). Of note, almost half of the isolates in this study were multidrug resistant, and 7% belonged to a single pattern (ampicillin - ceftiofur - gentamicin - spectinomycin - tetracycline) that included four different antimicrobial classes. The moderately strong, positive correlation between ampicillin and ceftiofur resistance can be explained by

the co-resistance provided by the blaCMY-2 gene, which has been shown to be widespread in poultry in Canada, and in Québec and Ontario in particular (Chalmers et al. 2017, Zhang 2017). Further, the moderately strong, positive correlation between gentamicin and spectinomycin resistance is likely related to the co-location of the aadA gene for spectinomyin resistance and the aac(3)VI gene for gentamicin resistance on the same plasmid, more precisely, as gene cassettes in an integrin (Chalmers et al. 2017). This finding is of particular importance, as it supports a recent study in Québec, in which spectinomycin-lincomycin use was associated with the development of gentamicin resistance in APEC isolates from broiler chickens (Chalmers et al. 2017). Thus, the responsible use of antimicrobials in poultry must continue to be an important part of an effective antimicrobial resistance control program.

Several significant relationships between virulence and antimicrobial resistance were identified. Although there were no common patterns among antimicrobials with respect to the genes linked to resistance, there were positive associations between the presence of *kpsII* and ampicillin resistance, *etsB* and *kpsII* and ceftiofur resistance, *ompT* and *sitA* and gentamicin resistance, *papC* and kanamycin resistance, *ireA* and *iutA* and spectinomycin resistance, and *cvaC*, *eitA*, and *papC* and tetracycline resistance. Further, the number of antimicrobials to which an isolate was resistant significantly increased with the presence of *eitA*, *iroN*, *papC*, and *sitA*. Associations between resistance to multiple antimicrobials and the presence of several genes considered important for APEC virulence is of concern. Infections with multidrug resistant and multi-virulent APEC strains could cause substantial economic losses in broiler flocks due to increased morbidity, mortality, and a lack of effective antimicrobial treatment options. Molecular epidemiological studies aimed at understanding the emergence and spread of multidrug resistant APEC that also possess several virulence-associated genes should be a focus of future research, as resistance and virulence could develop in the absence of selection pressure of antimicrobial use, through co-selection (Chalmers et al. 2017, Fang et al. 2016) or horizontal gene transfer (Stevenson et al. 2017).

The papC gene is part of an operon encoding for a pilus/adhesin involved in the pathogenesis of pyelonephritis in humans. In this study, the presence of *papC* was strongly associated with resistance to both kanamycin and tetracycline, suggesting that the genes encoding *papC* and resistance to these two antimicrobials are likely located on the same genetic element. Further, several studies have shown that mobile genetic elements that encode for virulence and antimicrobial resistance can be exchanged between human and animal origin extra-intestinal pathogenic *E. coli* isolates (Ewers et al. 2004, Johnson et al. 2008b, Mitchell et al. 2015). However, as this study was limited to the phenotypic evaluation of resistance (i.e., antimicrobial susceptibility testing) rather than detection of resistance genes, further molecular-level studies are needed to elucidate this finding.

Age group and time of sample collection were not significantly associated with resistance to individual antimicrobials or the presence of multidrug resistance, or to the presence of virulence-associated genes, suggesting that APEC isolates are widespread across all ages and seasons. Contamination of the barn environment with APEC, and persistence of the pathogen after cleaning and disinfection might play a role in continuous APEC infections of broilers and broiler breeders. Further studies are needed to elucidate the effectiveness of cleaning and disinfection practices, and other factors, which influence the on-farm survival of APEC.

Communications (presenting author*)

COMPLETED

Guerin M, Varga C, Slavic D, Boerlin P, Brash M, Martin E, Ouckama R, Weisz A, Petrik M, Philippe C, Barham M. Evaluating virulence genes and antimicrobial susceptibility of avian pathogenic Escherichia coli from Ontario broiler and broiler breeder flocks. Animal Health Laboratory Newsletter, Mar 2016; 20(1):9.

Varga C*, Slavic D, Boerlin P, Brash M, Martin E, Ouckama R, Weisz A, Petrik M, Philippe C, Barham M, Guerin M. Evaluating virulence genes and antimicrobial resistance of avian pathogenic *Escherichia coli* from Ontario broiler and broiler breeder flocks. 16th Annual Meeting of the Canadian Animal Health Laboratorians Network (CAHLN), Jun 4-7, 2017; Guelph, ON, Canada (oral presentation)

Varga C, Brash M, Slavic D, Boerlin P, Ouckama R, Weisz A, Petrik M, Philippe C, Guerin M. Evaluating virulence-associated genes and antimicrobial resistance of avian pathogenic *Escherichia coli* from Ontario broiler and broiler breeder flocks. 5th Annual Workshop of the Ontario Animal Health Network (OAHN), Jan 31, 2018; Guelph, ON, Canada (poster)

PLANNED OR IN-PROGRESS

Varga C, Brash ML, Slavic D, Boerlin P, Ouckama R, Weisz A, Petrik M, Philippe C, Guerin MT. Evaluating virulence-associated genes and antimicrobial resistance of avian pathogenic *Escherichia coli* from Ontario broiler and broiler breeder flocks. Prepared for submission to *Avian Diseases*

Guerin M* (Invited), Varga C, Brash M, Slavic D, Boerlin P, Ouckama R, Weisz A, Petrik M, Philippe C. Virulence and antimicrobial resistance of avian pathogenic *Escherichia coli* from broiler and broiler breeder flocks in Ontario. Jefo Poultry Tour, Aug 28-29, 2018; Québec City, QC, Canada (oral presentation)

<u>Other potential communications</u>

Information sharing with veterinarians through the Ontario Association of Poultry Veterinarians

Information sharing with producers through a research summary in the Canadian Poultry magazine

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