











■ Author(s)

Kanabata B¹  <https://orcid.org/0000-0001-6814-7322>
Menck-Costa MF¹  <https://orcid.org/0000-0002-6788-7182>
Souza M¹  <https://orcid.org/0000-0002-9909-6115>
Justino L¹  <https://orcid.org/0000-0002-6552-0635>
Rangel IG¹  <https://orcid.org/0000-0003-3555-8277>
Kobayashi RKT¹  <https://orcid.org/0000-0001-7170-2155>
Nakazato G¹  <https://orcid.org/0000-0001-8624-795X>
Baptista AAS¹  <https://orcid.org/0000-0003-0699-7246>

¹ Department of Preventive Veterinary Medicine, Laboratory of Avian Medicine, State University of Londrina, Paraná, Brazil.

¹ Department of Microbiology, State University of Londrina, Paraná, Brazil.

■ Mail Address

Corresponding author e-mail address
Ana Angelita Sampaio Baptista
Universidade Estadual de Londrina – UEL
Rodovia Celso Garcia Cid - Pr 445, Km 388.
Campus Universitário. Cx. Postal 10011,
CEP 86057-970, Londrina – PR, Brasil.
Phone: +55 (43) 33714766
Email: anaangelita@uel.br

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Extended-Spectrum Beta-Lactamase Producing Strains of *Escherichia Coli* Isolated from Avian Cellulitis Lesions

ABSTRACT

Avian cellulitis causes significant losses to the poultry industry. Avian-pathogenic *Escherichia coli* (APEC) is the etiological agent of that disease. This microorganism has zoonotic potential and may act as reservoir of antimicrobial-resistance genes. In this context, the production of extended-spectrum B-lactamase (ESBL) is one of the main antimicrobial resistance mechanisms. The objective of this study was to determine the production of ESBL in an *Escherichia coli* (*E. coli*) strain isolated from avian cellulitis lesions. Twenty-two *E. Coli* isolates were harvested from cellulitis lesions in chicken carcasses in a commercial processing plant. Isolates were then submitted to virulence genotypic profile (*iutA*, *hlyF*, *iss*, *ironN*, *ompT*) analysis, antimicrobial susceptibility test, and detection of ESBL production. The results showed that 22.7% of the isolates presented five virulence genes, 9.1% four genes, 36.4% three genes, 13.6% two genes, and 18.2% one gene. The tested isolates showed resistance to ampicillin (90.9%), ceftiofur (54.5%), gentamicin (45.5%), tetracycline (72.1%), sulfamethoxazole/trimethoprim (54.5%), and enrofloxacin (54.5%). Furthermore, 77.3% of the isolates presented multidrug resistance (MDR) profile and 72.7% were positive for ESBL production. This study is the first description of ESBL-producing APEC isolated from avian cellulitis lesions, which suggests the need to establish efficient APEC control measures and programs to prevent flock productivity losses due to colibacillosis and public health risks.

INTRODUCTION

Avian cellulitis is an emerging disease that has caused significant economic losses to the poultry industry (Quel *et al.*, 2013). It is a multifactorial disease resulting from management failures, immunosuppressive diseases, and skin lesions. The lesions, often referred to as “plaques,” are typically located in the skin on the abdomen or between the thigh and the midline, and therefore, are identified only after slaughter (Barnes *et al.*, 2008).

Escherichia coli (*E. coli*) is considered a heterogeneous group of pathogens with wide diversity of virulence factors (Cunha *et al.*, 2014) and may cause different clinical manifestations, such as airsacculitis, perihepatitis, pericarditis, sepsis, salpingitis, coligranuloma, and cellulitis (Barnes *et al.*, 2008).

Avian pathogenic *Escherichia coli* (APEC) belongs to the pathotype extraintestinal pathogenic *Escherichia coli* (ExPEC) (Lestrangle *et al.*, 2017). Studies indicate that APEC has zoonotic potential, considering its profile of virulence genes and its capacity of acting as a reservoir of antimicrobial resistance genes, which can be transmitted to humans through the food chain (Aslam *et al.*, 2014; Cunha *et al.*, 2014).



One the hypothesis for the selection of resistant microorganisms is the extensive use of antimicrobials in animal production (Cunha *et al.*, 2014, Hoelzer *et al.*, 2017). The phenomenon of antimicrobial resistance poses a serious problem to human as well as veterinary medicine. The production of extended-spectrum beta-lactamase (ESBL) is one of the main resistance mechanisms of bacteria to antibiotics. This enzyme hydrolyzes the β -lactam ring of several antibiotics, including cephalosporin of third and fourth generations, penicillin, cephalosporin (ceftazidime, cefotaxime, ceftriaxone) and monobactam (aztreonam), inactivating them and, consequently, reducing treatment options (Casella *et al.*, 2017; Shaikh *et al.*, 2015).

Determining the virulence profile is essential to differentiate APEC isolates from non-pathogenic *E. coli* residing in the natural microbiota. In addition, the detection of virulence factors and antimicrobial resistance helps understanding the epidemiology and allows designing measures for the prevention and control of the disease. This study aimed at establishing the profile of virulence genes, phenotypic resistance to antimicrobials, and ESBL-producing capacity of APEC isolates obtained from avian cellulitis lesions.

MATERIALS AND METHODS

Bacterial isolates

Swabs of lesions [n=42] compatible with cellulitis were collected from broiler carcasses in the slaughter line of a broiler processing plant in northern Paraná State, Brazil. Samples were collected at three different times at 7-d intervals in order to obtain isolates from flocks from different sources. Sterile swabs were rubbed on the lesions, refrigerated (2-8°C) in Cary Blair medium and submitted, under refrigeration, to the Laboratory of Avian Medicine, State University of Londrina, for processing.

Swabs were incubated in BHI broth (Brain Heart Infusion, Difco®, France) at 37°C for 18-24 h, and streaked on MacConkey agar (Acumedia®, USA) plates and incubated at 37°C for 18-24 h. The isolated colonies were subjected to biochemical tests, and the colonies compatible with *E. coli* (positive for indole, negative for H₂S, negative for citrate and urease, gas production in TSI (triple sugar iron) agar with acid slant and acid butt, sorbitol-fermenting) were stored at -20°C in BHI broth supplemented with glycerol (20%) until processing, totaling 22 *E. coli* isolates.

Antimicrobial susceptibility test

The disk diffusion test was applied to determine the antimicrobial susceptibility profile of the isolate, according to the protocol of the Clinical and Laboratory Standards Institute (CLSI, 2015). The *Escherichia coli* strain ATCC 25922 was used as control. The following antimicrobials were tested: ampicillin (10 µg), ceftiofur (30 µg), gentamicin (10 µg), tetracycline (30 µg), sulfamethoxazole/trimethoprim (23.75/1.25 µg) + enrofloxacin (5µg). Isolates showing resistance to three or more classes of antimicrobials were considered multidrug-resistant (MDR) phenotypes (Magiorakos *et al.*, 2012).

Phenotypic profile of ESBL production

The production of extended-spectrum beta-lactamase (ESBL) was determined by the disk approximation test (D-test), according to the Clinical and Laboratory Standards Institute (CLSI, 2015). Four antimicrobial agents were tested using the double-disk synergy test: amoxicillin + clavulanic acid (AMX/C-20/10 µg), ceftazidime (CAZ-30 µg), ceftriaxone (CRO-30 µg), cefotaxime (CTX-30 µg). The AMX/C disc was applied at the center of the plate containing Mueller Hinton (MH; Difco®) agar and the others at a distance of 20 mm from the edge of the central disk. Isolates in plates showing any distortion or increase in the area of the amoxicillin-clavulanate disk was considered positive for ESBL production.

Determination of virulence factors

E. coli isolates were grown in BHI broth, and then subjected to DNA extraction. The boiling technique was applied, consisting of centrifugation of 1.5 mL bacterial growth for 1 min at 7500 x g. The precipitate was suspended in 200 µL of ultrapure sterile water, incubated at 100°C for 10 min, and centrifuged again for 5 min at 2800 x g. Next, 150 µL of the supernatant were removed and stored at -20°C until use.

The virulence genes *iutA*, *hlyF*, *iss*, *ironN* and *ompT* were studied in the isolates following the protocol described by Johnson *et al.* (2008).

RESULTS

Phenotypic resistance to antimicrobials and ESBL production

Significant resistance of the bacterial isolates was detected against ampicillin (90.9%), tetracycline (72.1%), ceftiofur (54.5%), enrofloxacin (54.5%),



sulfamethoxazole/trimethoprim (54.5%), and gentamicin (45.5%) (Table 1).

Multiple drug resistance profile (MDR) was detected in 77.3% of the isolates, while ESBL production was observed in 72.7% (Table 1).

Table 1 – Profile of phenotypic resistance to antimicrobials, ESBL production and multidrug resistance (MDR) in *Escherichia coli* isolates from lesions of avian cellulitis.

Antimicrobial susceptibility test	Number (%) of isolates showing resistance n=22
Enrofloxacin - EN	12 (54.5%)
Sulfamethoxazole/Trimethoprim - SUT	12 (54.5%)
Gentamicin – GEN	10 (45.5%)
Ceftiofur - CTF	12 (54.5%)
Tetracycline – TET	16 (72.7%)
Ampicillin - AMP	20 (90.9%)
ESBL phenotype	16 (72.7%)
MDR phenotype	17 (77.3%)

GEN – Gentamicin (10µg); CTF – Ceftiofur (30µg); TET – Tetracycline (30µg); AMP – Ampicillin (10µg); EM – Enrofloxacin (5µg); SUT – Sulfamethoxazole/Trimethoprim (23.75/1.25 µg).

Virulence genes

Twenty-two *E. coli* isolates were obtained from cellulitis lesions in chicken carcasses, out of which 22.7% (5/22) presented five virulence genes (*iss*, *ompT*, *iroN*, *iutA*, *hlyF*), 9.1% (2/22) four genes, 36.4% (8/22) three genes, 13.6% (3/22) two genes, and 18.2% (4/22) only one gene (Table 2).

Table 2 – Relationship of virulence genes and their combinations in samples of *Escherichia coli* isolated from avian cellulitis lesions.

Genes	Number of samples (%)
<i>iss</i>	0/22 (0%)
<i>ompT</i>	0/22 (0%)
<i>iroN</i>	0/22 (0%)
<i>iutA</i>	2/22 (9.1%)
<i>hlyF</i>	2/22 (9.1%)
<i>iss</i> + <i>ompT</i>	1/22 (4.5%)
<i>iroN</i> + <i>iutA</i>	1/22 (4.5%)
<i>iutA</i> + <i>hlyF</i>	1/22 (4.5%)
<i>iss</i> + <i>ompT</i> + <i>iutA</i>	2/22 (9.15%)
<i>iroN</i> + <i>iutA</i> + <i>hlyF</i>	6/22 (27.3%)
<i>iss</i> + <i>ompT</i> + <i>iroN</i> + <i>hlyF</i>	2/22 (9.1%)
<i>iss</i> + <i>ompT</i> + <i>iroN</i> + <i>iutA</i> + <i>hlyF</i>	5/22 (22.7%)

The genes *iss*, *ompT* and *iroN* were not detected in any of the isolates evaluated. In the present study, 27.3% (6/22) of isolates showed the combination of *iroN*, *iutA* and *hlyF* genes. Conversely, the presence of individual genes was observed in 18.2% of the isolates, with 9.1% prevalence of *iutA* and *hlyF*.

Figure 1 shows the distribution of virulence genes in the *E. coli* isolates evaluated. The most prevalent genes were *iutA* and *hlyF*, in 77.3% and 72.7% of the isolates, respectively, *iroN* was observed in 63.6%, while the *ompT* and *iss* genes were detected in only 45.4% of the isolates.

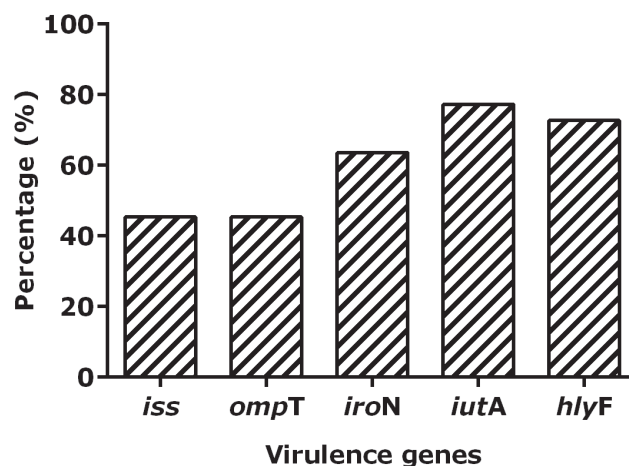


Figure 1 – Distribution of virulence genes (*iss*, *ompT*, *iroN*, *iutA*, *hlyF*) in samples of *Escherichia coli* isolated from avian cellulitis lesions.

DISCUSSION

Escherichia coli is a natural resident of the intestinal microbiota of birds and mammals; however, some pathotypes designated avian pathogenic *E. coli* (APEC) may invade different organs and cause systemic disease or colonize individual tissues, such as the case of cellulitis (Li *et al.*, 2015).

The present study determined the virulence gene profile, phenotypic resistance to antimicrobials, and extended-spectrum beta-lactamase (ESBL) production of APEC isolates collected from avian cellulitis lesions. The objective was to demonstrate that the antimicrobial resistance profile of a microorganism may cause disease in broilers and pose a public health risk through the food chain, transferring resistance genes to potential human pathogens.

To the best of our knowledge, this is the first study to describe ESBL-producing APEC isolates in avian cellulitis lesions, although other studies have isolated ESBL-positive *E. coli* strains in live chickens and poultry products (Lim *et al.*, 2015; Hussain *et al.*, 2017; Silva *et al.*, 2017).

Antimicrobial therapy is one of the primary control measures to reduce morbidity and mortality caused by APEC (Barbieri *et al.*, 2013). However, the frequent use of antimicrobials allows the selection of resistant isolates, which is a global health concern (Nhung *et al.*, 2017).



In the present study, 77.3% of the isolates evaluated presented the MDR phenotype, which presented high resistance to ampicillin (90.9%) and tetracycline (72.1%). Similarly, Barbieri *et al.* (2013) found a high percentage of resistance to tetracycline (69.4%) in *E. coli* isolated from avian cellulitis in broilers in southern Brazil. Wu *et al.* (2014) found 84.4 and 71.1% of resistance to tetracycline and ampicillin, respectively, in *E. coli* isolates from poultry in China.

In our study, 54.5% of the isolates showed resistance to sulfamethoxazole/trimethoprim. This profile is alarming because these drugs are the first choice for the treatment of human patients in intensive care units (Brown & Foxman, 2002). Chen *et al.* (2014) observed that 90.6% APEC isolate were resistant to tetracycline, and suggest a co-resistance relationship among tetracycline, trimethoprim-sulfamethoxazole, and ampicillin.

In addition to public health aspects, the presence of antimicrobial-resistant pathogenic microorganisms directly affects livestock production, since it undermines treatments and prolong the disease in animals, reducing therapeutic alternatives, increasing production costs, and reducing profitability (El-Shazly *et al.*, 2017).

ESBL production is an important resistance mechanism to antimicrobials (Daehre *et al.*, 2017). In our study, the ESBL phenotype was present in 72.1% of the isolates, which is significant. This result is consistent with those reported by Casella *et al.* (2017), who observed 96.1% (74/77) positivity for ESBL in *E. coli* isolates from poultry in France. Our study is the first description of ESBL-producing APEC isolated from avian cellulitis lesions.

According to Johnson *et al.* (2008), the *iutA*, *hlyF*, *iss*, *iroN* and *ompT* genes are associated with plasmids and are considered good APEC markers, although other markers have been described. Our findings are consistent with those of Hiki *et al.* (2014), who determined that *iutA* and *hlyF* as the first and second most prevalent virulence genes in *Escherichia coli* isolates from healthy broilers in Japan.

Ozaki *et al.* (2017) observed that *iss* gene was present in 93% of APEC isolates from perihepatite and pericarditis lesions in broilers. These data differ from our results, since *iss* was observed in 45% of the isolates. This difference may be attributed to the fact that we analyzed isolates from cellulitis lesions, whereas Ozaki *et al.* (2017) worked with isolates causing septicemia. According to Silveira *et al.* (2016), the presence of the *ompT*, *iroN*, and *iss* genes is correlated with disease severity, while the *hlyF* and *iutA* genes are involved

with the capacity of causing disease. Therefore, we can infer that 27% of the isolates in the present study had capacity of causing severe disease.

CONCLUSIONS

The identification of APEC isolates from avian cellulitis lesions containing virulence genes with capacity of producing ESBL and MDR suggests the need to establish efficient APEC control measures and programs in order to reduce flock productivity losses due to colibacillosis and public health risks.

REFERENCES

- Aslam M, Toufeer M, Narvaez Bravo C, Lai V, Rempel H, Manges A, et al. Characterization of extraintestinal pathogenic *Escherichia coli* isolated from retail poultry meats from Alberta, Canada. *International Journal of Food Microbiology* 2014;177:49-56.
- Barbieri NL, Oliveira AL, Tejkowski TM, Pavanelo DB, Rocha DA, Matter LB, et al. Genotypes and pathogenicity of cellulitis isolates reveal traits that modulate APEC virulence. *PLoS ONE* 2013;8(8):e72322.
- Barnes HJ, Vaillancourt J, Gross WB. Colibacillosis. In: Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE, editors. *Diseases of poultry*. 12th ed. Ames: Blackwell Publishing; 2008.
- Brown PD, Freeman A, Foxman B. Prevalence and predictors of trimethoprim-sulfamethoxazole resistance among uropathogenic *Escherichia coli* isolates in Michigan. *Clinical Infectious Diseases* 2002;34(8):1061-1066.
- Casella T, Nogueira MCL, Saras E, Haenni M, Madec JY. High prevalence of ESBLs in retail chicken meat despite reduced use of antimicrobials in chicken production, France. *International Journal Food Microbiology* 2017;257(18):271-275.
- Chen X, Zhang W, Yin J, Zhan N, Geng S, Zhou X, et al. *Escherichia coli* isolates from sick chickens in China: Changes in antimicrobial resistance between 1993 and 2013. *Veterinary Journal* 2014;202(1):112-115.
- CLSI – Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 25th ed. Wayne; 2015.
- Cunha MPV, Oliveira MGX, Oliveira MCV, Silva KC, Gomes CR, Moreno AM, et al. Virulence profiles, phylogenetic background, and antibiotic resistance of *Escherichia coli* isolated from turkeys with airsacculitis. *Scientific World Journal* 2014; 2014 (2):8.
- Daehre K, Projahn M, Semmler T, Roesler U, Friese A. Extended-spectrum beta-lactamase-ampc beta-lactamase-producing enterobacteriaceae in broiler farms: transmission dynamics at farm level. *Microbiology Drug Resistance* 2017;24(4):511-518.
- El-Shazly DA, Nasef SA, Mahmoud FF, Jonas D. Expanded spectrum β -lactamase producing *Escherichia coli* isolated from chickens with colibacillosis in Egypt. *Poultry Science* 2017;96(7):2375-2384.
- Hiki M, Usui M, Akiyama T, Kawanishi M, Tsuyuki M, Imamura S, et al. Phylogenetic grouping, epidemiological typing, analysis of virulence genes, and antimicrobial susceptibility of *Escherichia coli* isolated from healthy broilers in Japan. *Irish Veterinary Journal* 2014;67(1):14.
- Hoelzer K, Wong N, Thomas J, Talkington K, Jungman E, Coukell A. Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence? *BMC Veterinary Research* 2017; 13(1):211.



- Hussain A, Shaik S, Ranjan A, Nandanwar N, Tiwari SK, Majid M, et al. Risk of transmission of antimicrobial resistant *Escherichia coli* from commercial broiler and free-range retail chicken in India. *Frontier in Microbiology* 2017; 8:2120.
- Johnson TJ, Wannemuehler Y, Doetkott C, Johnson SJ, Rosenberger SC, Nolan LK. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. *Journal Clinical Microbiology* 2008;46(12):3987-3996.
- Lestrang K, Markland SM, Hoover DG, Sharma M, Kniel KE. An evaluation of the virulence and adherence properties of avian pathogenic *Escherichia coli*. *One Health* 2017;4:22-26.
- Li Y, Chen L, Wu X, Huo S. Molecular characterization of multidrug-resistant avian pathogenic *Escherichia coli* isolated from septicemic broilers. *Poultry Science* 2015;94(4):601–611.
- Lim JS, Choi DS, Kim YJ, Chon JW, Kim HS, Park HJ, et al. Characterization of *Escherichia coli*–producing extended-spectrum β -lactamase (esbl) isolated from chicken slaughterhouses in South Korea. *Foodborne Pathogens and Disease* 2015;12(9):741-748.
- Magiorako AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection* 2012;18:268–281.
- Nhung NT, Chansiripornchai N, Carrique-Mas JJ. Antimicrobial resistance in bacterial poultry pathogens: a review. *Frontiers in Veterinary Science* 2017; 4:126.
- Ozaki H, Matsuoka Y, Nakagawa E, Murase T. Characteristics of *Escherichia coli* isolated from broiler chickens with colibacillosis in commercial farms from a common hatchery. *Poultry Science* 2017;96(10):3717-3724.
- Quel NG, Aragão AZ, Salvadori MR, Farias AS, Joazeiro PP, Santos LM, et al. Cellulitis lesions in broiler chickens are induced by *Escherichia coli* Vacuolating Factor (ECVF). *Veterinary Microbiology* 2013;162(2-4):866–872.
- Shaikh S, Fatima J, Shakil S, Rizvi SM, Kamal MA. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. *Saudi Journal of Biological Sciences* 2015;22(1):90–101.
- Silva KC, Cunha MP, Cerdeira L, de Oliveira MG, de Oliveira MC, Gomes CR, et al. High-virulence CMY-2-and CTX-M-2-producing avian pathogenic *Escherichia coli* strains isolated from commercial turkeys. *Diagnostic Microbiology and Infectious Disease* 2017; 87(1):64–67.
- Silveira F, Maluta RP, Tiba MR, de Paiva JB, Guastalli EA, da Silveira WD. Comparison between avian pathogenic (APEC) and avian faecal (AFEC) *Escherichia coli* isolated from different regions in Brazil. *Veterinary Journal* 2016;217:65–67.
- Wu Q, Xi M, Lv X, Xu Y, Feng Y, Li Q, et al. Presence and antimicrobial susceptibility of *Escherichia coli* recovered from retail chicken in China. *Journal of Food Protection* 2014;77(10):1773-1777.

