Detection of *Escherichia coli* O157:H7 and APEC in water samples from the poultry industry

Detecção de *Escherichia coli* O157:H7 e APEC em amostras de água da produção avícola

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Abstract: *Escherichia coli* are commensal bacteria and are present in the env*iroN*ment. However, some pathotypes can cause disease in animals and humans. *E. coli* APEC causes colibacillosis in chickens, inducing respiratory and systemic symptoms. Enterohemorrhagic serotype O157:H7 causes foodborne infections in humans, with hemorrhagic diarrhea and hemolytic-uremic syndrome. The objective was to detect the *E. coli* APEC and O157:H7 serotypes in water samples from different points from the poultry production, and to evaluate the antimicrobial resistance profiles. PCR analysis was performed to detect the patterns of genes *hlyF*, *iutA*, *iroN*, iss and *ompT* from *E. coli* APEC and the H7 flagellar antigen and the *Stx1* and *Stx2* toxins from *E. coli* O157:H7. Thirty-three water samples from different points were positive for *E. coli*, among them, eight for APEC and none for O157:H7. APEC isolates showed different degrees of susceptibility to antimicrobials.

Keywords: *E. coli*; colibacillosis; pathotypes; chickens; production.

Resumo: *Escherichia coli* são bactérias comensais e estão presentes no ambiente. Entretanto, alguns patotipos podem causar doenças em animais e humanos. *E. coli* APEC causa a colibacilose em aves, provocando sinais respiratórios e sistêmicos. O sorotipo enterohemorrágico O157:H7 causa infecções transmitidas por alimentos em humanos, provocando quadros de diarreia hemorrágica e síndrome hemolítico-urêmica. O objetivo do estudo foi detectar os sorotipos *E. coli* APEC e O157:H7 em amostras de água em diferentes pontos da produção avícola e avaliar os perfis de resistência antimicrobiana. A análise de PCR foi realizada para detectar os padrões dos genes *hlyF, iutA, iroN, iss* e *ompT* de *E. coli* APEC e o antígeno flagelar H7 e as toxinas *Stx1* e *Stx2* de *E. coli* O157:H7. Trinta e três amostras de água de diferentes pontos foram positivas para *E. coli*, entre elas, oito para APEC e nenhuma para O157:H7. Os isolados de APEC apresentaram diferentes graus de suscetibilidade aos antimicrobianos.

Palavras-chave: *E. coli*; colibacilose; patotipos; aves; produção.

1. Introduction

Although most *Escherichia coli* (*E. coli*) strains are considered commensal bacteria, and are present in the environment; however, some strains, classified as extra-intestinal pathogenic *E. coli* (ExPEC) or zoonotic intestinal pathogens (IPEC), express virulence factors and can cause disease in hosts [1–4]. In addition to the pathogenicity of some *E coli* serotypes, another factor of concern for public health is that this bacterium is highly associated with antibiotic resistance, especially to those used in animal production [1,5].

There are six subpathotypes of ExPEC: UPEC (Uropathogenic *E. coli*), NMEC (neonatal meningitis *E. coli*), APEC (Avian pathogenic *E. coli)*, SePEC (Sepsis-associated *E. coli*), MPEC (Mammary pathogenic *E. coli*), and EnPEC (Endometrial pathogenic *E. coli*)[4]. APEC is a subpathotype that causes respiratory and systemic infections known as colibacillosis in chickens. The pathogenicity of this pathotype may be linked to previously studied virulence factors, of which five have been identified: *hlyF* (toxin production), *iutA* and *iroN* (iron acquisition), *iss* and *ompT* (protectins) genes [6,7]. These genes are prevalent in isolates from different parts of the world, including the United States, Thailand, Korea, and Australia. Despite this, other methods, such as genetic sequencing, have become a tool to complement with more relevant information, including other genes as virulence factors [8]. APEC is one of the most common and economically-devastating bacterial diseases affecting poultry. It manifests in diverse ways, including peritonitis, salpingitis, arthritis, and airsacculitis, and may be related to high morbidity and mortality in the flock[8].

Among the IPEC pathotypes, there is a group of diarrheagenic *E. coli* (DEC), which includes the enterohemorrhagic serotype O157:H7, capable of causing food infections in humans, presenting as hemorrhagic diarrhea and hemolytic-uremic syndrome (HUS), which are considered to be of great importance to public health[4,5]. The O157:H7 serotype produces a verocytotoxin (resulting in the moniker 'VTEC') known as Shiga toxin (*Stx*), a potent bacteriophage-encoded cytotoxin, and the main virulence marker of this serotype. Serotype O157 is frequently associated with the H7 antigen (encoded by *fliC* H7)[9]. This microorganism can colonize the gastrointestinal tract of birds without causing disease; however, it can serve as a source of contamination for chicken carcasses in slaughterhouses, and consequently pose a risk to human health[5].

The quality of water on farms is essential to control pathogens of interest to human and animal health and may be a source of *E. coli* infection through the oro-fecal route for chickens[1]. These findings provide valuable insights into the transmission of APEC and O157:H7, and their potential public health threats through antimicrobial resistance. This study therefore aimed to evaluate the presence of *E. coli* APEC and O157:H7 in raw and treated water from different cycles of poultry production and to test their sensitivity to antibiotics.

2. Material and methods

2.1 *E. coli* isolation

Raw and treated water samples were collected from different locations in the poultry production chain in São Paulo, Minas Gerais, Paraná, and Mato Grosso, Brazil. Water samples (100 mL) were tested using a chromogen substrate (Colilert; IDEXX Laboratories). Following confirmation of positivity for *E. coli*, 100 µL of the sample were plated on MacConkey agar, and incubated for 18 to 24 hours at 37ºC. The characteristic colony morphology of *E. coli* was cultivated on biochemical media for confirmation, and then on nutrient agar plates.

2.2 DNA extraction

Three colonies from each sample were inoculated into 200 µL of PBS buffer (Phosphate Buffered Saline 1x). The DNA of the samples was extracted using the Indimag Pathogen kit in Indimag 48, in accordance with the manufacturer's specifications.

2.3 Detection of *E. coli* O157:H7

Primers for the detection of the H7 flagellar antigen [10] and *Stx*1 and *Stx*2 toxins [11] from *E. coli* O157:H7 were synthesized, as shown in Table 1. The PCR mix was prepared using the QuantiFast SyBR Green PCR kit (Qiagen®), primers, and the sample, following the cycling profile, in accordance with the manufacturer's specifications. The following steps were followed: PCR initial activation step (5 minutes at 95ºC) and two-step cycling; 35 cycles of denaturation (10 seconds at 95ºC) and combined annealing/extension (30 seconds at 60ºC). After amplification, samples with Ct ≤32 were analyzed through evaluation of the melting curve and temperature, and comparison with those of the positive control (ATCC 43888).

Table 1. Primers and TaqMan probes used in real-time PCR.

Gene	Oligonucleotide sequence (59–39)	Product size (base pairs)	References
fliC	F: ATAATCTACGCCGCCAACT R: GACTCCATCCAGGACGAAA	381	Goma et al., 2019
Stx1	F: GTCACAGTAACAAACCGTAACA R: TCGTTGACTACTTCTTATCTGGA	95	Jothikumar & Griffiths, 2002
Stx2	F: CGACCCCTCTTGAACATA R: GATAGACATCAAGCCCTCGT	108	Jothikumar & Griffiths, 2002
iss	F: CGGGAATTGGACAAGAGAAAC R: TTTCTGCACCGCCACAAA FAM: TTTGGCTGCATCAAC - ZEN-IOWA BLACK FQ	57	Ikuta et al., 2014
iutA	F: CGGTGGCGTACGCTATCAGT R: GCGCGTAGCCGATGAAAT VIC: CACTGAAAACAAGATTGAT - MGB	59	Ikuta et al., 2014
hlyF	F: GGTTGCCCGACCATCAATT R: ACTGGTTGAAGGTAAGCACCCTAA FAM: TTGTTGGCCACAGTCG - MGB	61	Ikuta et al., 2014

	F: GGTTCCGGGATTGCTCGTAT		
ompT	R: GGTCGTGGAGGCAATATGGT	57	Ikuta et al., 2014
	VIC: CAGCCAGTCCCTGTC - MGB		
	F: CCGTTGGTGCAGAGTGGAA		
iroN	R: CAGGCTGGTAGAGGAAGGATCA	53	Ikuta et al., 2014
	FAM: CGCGATAAGCTCG - MGB		

F: primer forward; R: primer reverse; FAM and VIC: TaqMan reporter dye labels; MGB and ZEN-IOWA BLACK FQ, TaqMan quencher dye labels.

2.4 Detection of E. coli APEC

Primers/TaqMan for the detection of *hlyF, iutA, iroN, iss,* and *ompT* virulence genes [7] were synthesized, as shown in Table 1. The mix was prepared using the QuantFast Probe PCR kit (Qiagen®), primers/TaqMan and the sample, following the cycling profile, and in accordance with the manufacturer's specifications. The following steps were followed: PCR initial activation step (3 minutes at 95ºC) and two-step cycling; 35 cycles of denaturation (3 seconds at 95ºC) and combined annealing/ extension (30 seconds at 60ºC). After cycling, genes with Ct ≤32 were considered positive, and the sample was considered *E. coli* APEC when it tested positive for 4 or more genes in the amplification.

2.5 Antimicrobial susceptibility testing

Strains positive for APEC or O157:H7 were recovered from stock agar and plated on Mueller-Hinton (MH) agar. Colonies grown on MH agar were used for antibiotic susceptibility testing using the Disk Diffusion method, in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI)[12]. The antibiotics tested were Amoxicillin (10 µg), Kanamycin (30 µg), Ceftiofur (30 µg), Doxycycline (30 µg), Enrofloxacin (5 µg), Fosfomycin (50 µg), Gentamicin (10 µg), Lincomycin + Spectinomycin (109 µg), Neomycin (30 µg), Norfloxacin (10 µg), Oxytetracycline (30 µg), Sulfonamide (300 µg), and Tetracyclines (30 µg).

3. Results

Seventy-two water samples were analyzed, of which 33 were positive for *E. coli* (Table 2), eight were positive for APEC by PCR, and none were positive for O157:H7. Seven strains were positive for all five virulence genes detected (*ompT*, *iroN*, *hlyF*, *iutA*, *iss*), and one was positive for four genes (*ompT*, *iroN*, *hlyF*, *iss*).

Table 2. Escherichia coli strains isolated from water at different points in the poultry chain.

Sample	Farm	Age	*E. coli* APEC	*E. coli* O157:H7
Treated water	Grandparent	Shed empty	P	N
Treated water	Grandparent	Shed empty	N	N
Treated water	Grandparent	15 weeks	N	N
Treated water	Broiler breeder	8 weeks	N	N
Treated water	Hatchery	-	N	N
Treated water	Hatchery	-	N	N
Treated water	Broiler	Shed empty	N	N
Treated water	Broiler	Shed empty	N	N
Treated water	Broiler	Shed empty	N	N
Treated water	Broiler	Shed empty	N	N
Treated water	Broiler	Shed empty	N	N
Treated water	Broiler	Shed empty	P	N
Treated water	Broiler	Shed empty	P	N
Treated water	Broiler	Shed empty	N	N
Treated water	Broiler	Shed empty	N	N
Treated water	Broiler	Shed empty	P	N
Treated water	Broiler	Shed empty	N	N
Treated water	Broiler	40 days	N	N
Treated water	Broiler	14 days	P	N
Treated water	Broiler	25 days	N	N
Treated water	Broiler	10 days	N	N
Raw water	Broiler	Shed empty	N	N
Raw water	Broiler	Shed empty	N	N
Raw water	Broiler	Shed empty	N	N
Raw water	Broiler	Shed empty	N	N
Raw water	Broiler	Shed empty	P	N
Raw water	Broiler	Shed empty	N	N
Raw water	Broiler	Shed empty	N	N
Raw water	Broiler	Shed empty	P	N
Raw water	Broiler	Shed empty	N	N
Raw water	Broiler	Shed empty	P	N
Raw water	Broiler	40 days	N	N

N: Negative; P: Positive

The antibiotic susceptibilities of the APEC isolates are shown in Figure 1. The APEC strains showed different degrees of susceptibility to the antibiotics amoxicillin, kanamycin, ceftiofur, doxycycline, enrofloxacin, lincomycin + spectinomycin, neomycin, oxytetracycline, sulphonamide, tetracycline, fosfomycin, gentamicin, and norfloxacin. Two isolates (25%) were resistant to doxycycline, oxytetracycline, sulphonamide, and tetracycline, and one strain (12.5%) was resistant to Amoxicillin and Lincomycin + Spectinomycin.
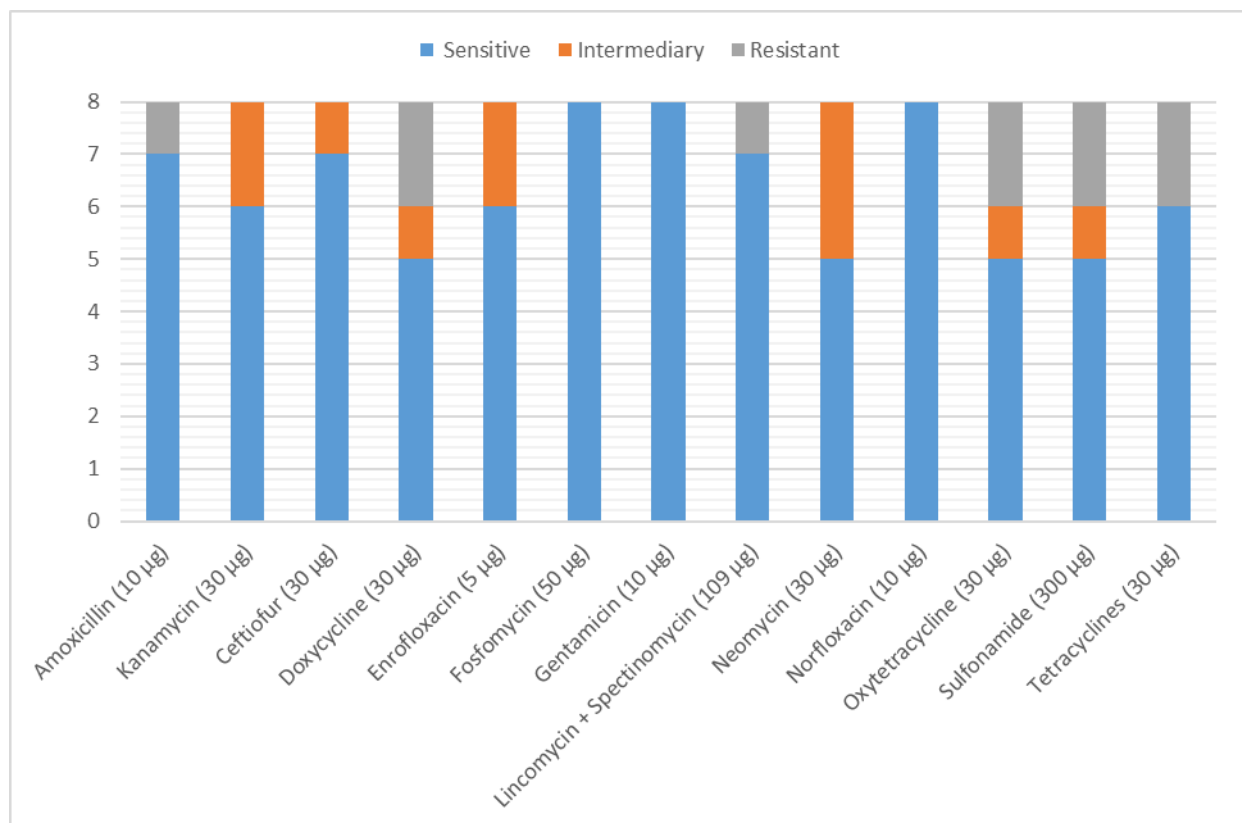
Figure 1. Profile of the antibiotic sensitivities of *Escherichia coli* APEC isolates.

4. Discussion

The virulence genes selected for this study (*hlyF, iutA, iroN, iss,* and *ompT*) encompassed those previously shown to be the most common in APEC strains; however, other sets of genes were also detected, including *tsh*, *iuc*, *fim*, *neuS*, *cvi*, *astA*, *vat*, *irp2*, *iucD*, *papC*, *cva-cvi*[7,13]. The absence of the *iutA* gene, as seen in one of the samples, has been commonly observed in other studies, and may be related to the geographical origin or even the pathogenic profile of the isolate[14]. *E. coli* O157:H7 is one of the primary pathogens that can cause foodborne diseases (FDB). Studies have demonstrated a positive correlation between drinking water contamination with *E. coli* O157:H7 [15] and the presence of this organism in poultry feces. Contamination of the carcass during slaughter can lead to significant economic losses due to the blocking of products[16]. In the present study, *E. coli* O157:H7 was not detected in any of the samples. However, it is important to understand the current positive scenario of our production of this pathogen.

The APEC strains isolated in this study demonstrated varying degrees of susceptibility to the 13 antibiotic tested molecules. Only 25% of the isolates were resistant to Doxycycline, Oxytetracycline, Sulfonamide and Tetracycline, while 12.5% were resistant to Amoxicillin and Lincomycin + Spectinomycin. Despite the low levels of antimicrobial resistance of the *E. coli* APEC strains isolated in the study, it is known that this is a topic of concern worldwide, and therefore monitoring should be carried out to control the resistance rates that occur in

production. The uncontrolled use of antibiotics as growth promoters, or in an inadequate and incorrect manner, can result in the selection of multiresistant bacteria, which can share resistance genes with other bacteria, spreading the problem [14].

5. Conclusion

In conclusion, the present study showed that *E. coli* APEC strains with different degrees of susceptibility to antimicrobials are widespread in the poultry chain, meaning that infection in chickens can originate from different environmental contaminants, including raw and treated water. Although raw water undergoes treatment, it is worth noting that biosecurity deviations can result in the contamination of drinking water and the subsequent infection of birds. *E. coli* O157:H7 serotype was not isolated from the water samples, indicating that it was a positive control for this pathogen.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

Author contributions

Conceptualization: P.D. Lopes. *Data curation*: P.D. Lopes. *Methodology:* P. D. Lopes*. Project management:* P.D. Lopes. *Investigation*: T. Hass. and M. Ó.. Rosa. *Formal analysis*: T. Hass and M. Unk. Rosa. *Writing (original draft)*: P. D. Lopes. *Writing (proofreading and editing)*: P.D. Lopes.

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