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Genotypic profile and antimicrobial resistance of avian pathogenic *Escherichia coli*

Perfil genotípico e resietência antimicrobiana de *Escherichia coli* patogênica aviária

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) is responsible for several anatomopathological conditions in poultry, which cause great damage to the poultry sector. This study aimed to evaluate the pathogenicity of *E. coli* strains isolated from broiler chickens and to perform in vitro tests on strains classified as APEC to identify their capacity to form biofilms and sensitivity to antimicrobials routinely used in the poultry breeding process. Sixty *E. coli* poultry isolated were used in this study. The real-time Polymerase Chain Reaction (PCR) test identified that 100% of the isolates had the *hly*F and *omp*T genes, 98.3% the *iro*N and *iss* genes, and 83.33% the *iut*A gene, being all the evaluated strains classified as APEC. In the *in vitro* evaluations regarding the formation and characterization of biofilms, the APEC samples were characterized as 71.66% weakly biofilm-forming. The antibiotic sensitivity test showed that the highest resistance percentages were found in the molecules of oxytetracycline, with 33%, and doxycycline, with 31.66%, but about 58,33% of the samples had a profile of multi-resistance to antimicrobials. Further studies are needed to better characterize APEC virulence genes and multi-drug resistance, given their impact on poultry health/production and potential risk to human health.

KEYWORDS: poultry; bacterium; epidemiology; susceptibility.

RESUMO

Escherichia coli patogênica aviária (APEC) é responsável por diversas condições anatomopatológicas em aves, que causam grandes prejuízos ao setor avícola. Este estudo teve como objetivo avaliar a patogenicidade de cepas de *E. coli* isoladas de frangos de corte e realizar testes in vitro em cepas classificadas como APEC para identificar sua capacidade de formação de biofilmes e sensibilidade aos antimicrobianos rotineiramente utilizados no processo de criação de aves. Sessenta cepas de *E. coli* isoladas de aves foram utilizadas neste estudo. O teste de Reação em Cadeia da Polimerase (PCR) em tempo real identificou que 100% dos isolados possuíam os genes *hly*F e ompT, 98,3% os genes *iro*N e iss e 83,33% o gene *iut*A, sendo todas as cepas avaliadas classificadas como APEC. Nas avaliações *in vitro* quanto à formação e caracterização de biofilmes, as amostras da APEC foram caracterizadas como 71,66% fracamente formadoras de biofilme. O teste de sensibilidade aos antibióticos mostrou que os maiores percentuais de resistência foram encontrados frente às moléculas de oxitetraciclina, com 33%, e doxiciclina, com 31,66%, mas cerca de 58,33% das amostras apresentaram perfil de multirresistência aos antimicrobianos. Mais estudos são necessários para melhor caracterizar os genes de virulência e a resistência a múltiplos medicamentos da APEC, dado o seu impacto na saúde/produção avícola e o risco potencial para a saúde humana.

PALAVRAS-CHAVE: aves, bactérias, epidemiologia, susceptibilidade

INTRODUCTION

All over the world, diseases caused by microorganisms have attracted the attention of researchers from different areas, due to the great economic losses, as well as the high capacity of mortality of animals and humans. Avian pathogenic *Escherichia coli* (APEC) strains, which carry the *iutA, hlyF, iss, iroN*, and *ompT* genes, have a high potential to cause avian colibacillosis, classified as a serious disease in broilers (JOHNSON et al. 2008, RODRIGUES et al. 2019, ALBER et al. 2020).

Avian pathogenic *E. coli* (APEC) is a causative agent of severe respiratory and systemic disease in chickens, commonly called colibacillosis. (ALBER et al. 2020). Among the main characteristic clinical signs caused by this bacterium is the infection of the air sacs, known as aerosacculitis, septicemia, cellulitis, omphalitis, and swollen head syndrome, which, in addition to causing high mortality in broilers, it is also related to total condemnation of the animals (ROSA et al. 2019).

Escherichia coli infections are responsible for starting different pathological conditions in poultry, which cause significant economic losses both on the farm and at the slaughterhouse (MAIORKI & FUKUMOTO 2021). In the survey carried out from 2016 to 2019, evaluating the condemnations of 144 Brazilian slaughterhouses, it was observed that 0.22% of the total condemnations of broilers were due to airsacculitis, 0.019% due to cellulitis, and 0.09% due to septicemia, these data further emphasize the sector's concern with this topic (COLDEBELLA et al. 2021).

The United States is one of the world's main suppliers of broiler chickens (CHAN et al. 2022). The value of broiler chicken production in the US in 2022 was US$50.4 billion, 60% higher than in 2021 (USDA 2022). As the demand for chicken meat increases, there is a need to improve production (CHAN et al. 2022). However, in the USA there is wide acceptance of the No Antibiotic Ever (NAE) system, so controlling diseases in poultry flocks has become a major challenge. Diseases related to poultry production are causing an economic loss of around US$20.3 billion annually. Therefore, it is essential to develop effective control measures against bacterial diseases such as colibacillosis, which causes a great loss to the industry (SCHARFF et al. 2020). In Canada, cellulite is the main cause of condemnations, followed by hepatitis and airsacculitis (CANADA 2019). In Germany in 2018, cellulite was responsible for 25% of all convictions (BERND et al. 2020).

APEC infection, in addition to causing great financial losses, can represent a high risk to public health (UGWU et al. 2020). The genetic similarity between strains of avian pathogenic *E. coli* (APEC) and strains associated with urinary tract infections (UPEC) and meningitis (NMEC) has become a target of the investigations, revealing a health concern in the poultry production chain (CUNHA et al. 2013).

ExPEC strains of human origin have been shown to share characteristics with those of avian origin, including some factors associated with virulence, such as adhesins, iron acquisition systems, toxins, and hemolysins (SORSA et al. 2003). Adhesins are responsible for bacterial adhesion to epithelial tissues, being an important factor for the establishment of *E. coli* infection, while type 1 fimbriae are related to adherence to the upper respiratory tract, and fimbrial adhesin *Pap* (pyelonephritis associated pili) with the establishment of the bacteria in internal organs (EUSÉBIO et al. 2016). The P fimbria was first associated with urinary tract infections in humans but is also found in APEC strains (BARNES et al. 2008).

APEC samples usually sequester iron through the production of aerobactin (MONROY et al. 2005). The aerobactin system is necessary for bacterial growth in hosts; the *iutA* gene is involved in the acquisition of iron and is known to be expressed during poultry infection (CHOUIKHA et al. 2008). The salmochelin gene (*iroN*) is also involved in iron acquisition, being one of the most common virulence genes among APEC strains isolated from chickens with colibacillosis (JEONG et al. 2012).

The ability to resist to serum inhibitory factors allows bacteria to escape to the action of the complement system and phagocytosis in systemic infection processes, and one of the main genes related to the serum resistance of bacteria is the *iss* gene (MONROY et al. 2005). Some genes responsible for the expression of toxins have a higher incidence in APEC strains, such as the genes that encode avian hemolysin (*hlyF*) and the vacuolizing toxin (*vat*) that commonly occurs in human ExPEC (BARNES et al. 2008). Therefore, concerns that the transfer of antibiotic-resistant APEC through the food chain may result in risks of extra-intestinal infection in humans should be considered (CHRISTENSEN et al. 2021).

In addition to the hypothesis about the zoonotic character of samples of avian origin, there is a growing worldwide concern regarding the increase in antimicrobial resistance among Enterobacteriaceae (CUNHA et al. 2013). One of the main reasons is the indiscriminate use of antibiotics in animal production, causing antimicrobial resistance that has worsened over the last few years, contributing to the emergence of

superbugs that are not restricted to animals, as they are spread through various pathways, reaching the environment and humans (REPIK et al. 2022).

Due to its highly diverse genome, *E. coli* can survive in various hosts and environments (OVI et al. 2023). The persistence and survival of APEC strains, both in the environment and in the host, are related to the ability to produce bacterial biofilm (RODRIGUES et al. 2019). Biofilm formation offers multiple advantages for bacteria, as it is an important determinant of pathogenicity, increasing the ability of bacteria to survive in the environment external to the host and providing an ideal environment for the exchange of genetic material (MELLATA et al. 2012).

Therefore, the objective of this study was to identify the *E. coli* strains isolated from broilers as APEC, evaluating the biofilm production capacity, as well as the sensitivity profile to antimicrobials routinely used in poultry.

MATERIAL AND METHODS

Sample collection

The isolates came from the strain bank of the Animal Health laboratory obtained from clinical cases of colibacillosis in broilers. The broilers were raised in farms linked to an integration system and after they were slaughtered in two slaughterhouses under federal supervision, located in the West of Santa Catarina, Brazil, in 2020.

In this study, 60 *E. coli* isolates were used from poultry samples: 17 liver isolates; 13 tonsil isolates; 09 heart isolates; 10 spleen isolates, and 11 swab isolates from organs (O.S.) (heart, liver, and spleen). For the isolation and identification of *Escherichia coli*, organ samples from broilers were subcultured onto MacConkey Agar (Merck, Darmstadt, Germany) and incubated for 18 to 24 hours at 37 °C. The samples that showed morphological characteristics compatible with *E. coli* were confirmed through the biochemical test described by the Instituto Adolfo Lutz (IAL) (RUGAI & ARAÚJO 1968).

After the confirmation, the strain was subcultured on nutrient agar (Merck, Darmstadt, Germany) and incubated again at 37 °C for 18 to 24 hours for further analysis of virulence factors, biofilm production capacity, and sensitivity to antimicrobials.

Molecular detection of virulence factors by PCR.

DNA extraction from the 60 selected strains was performed using a commercial kit (MagAttract 96 Cador Pathogen Kit®, Qiagen, Germany). Sample amplification was performed using a commercial kit (New Gene APECamp® (SIMBIOS 2019), both methods were performed following the manufacturer's recommendations.

For this procedure, three reactions were performed: the first to detect the *hlyF* and *ompT* genes; the second to detect the *iutA* and *ironN* genes, and the third to detect the *iss* gene, which determines the virulence factors of avian pathogenic *Escherichia coli* (Table 1).

Table 1. Virulence factors of avian pathogenic *Escherichia coli*.

Gene	Description
	Toxin
*hly*F	Supposed avian hemolysin gene
	Iron acquisition
*iut*A	Aerobactin (receptor)
*iron*N	Salmochelin (receptor)
	Protectins and serum resistance
Iss	Serum resistance
	Other
*omp*T	Episome external membrane protease

Adapted from BARNES et al. (2003).

The five genes (*hlyF, ompT, ironN, iutA* and *iss*) were selected according to the oligonucleotide sequence (Table 2).

Table 2. Oligonucleotide sequence of the *iss, iut*A, *hly*F, *omp*T, and i*ro*N genes of *Escherichia coli* strains isolated from samples of broiler organs with clinical signs of colibacillosis.

GENE	OLIGONUCLEOTIDE SEQUENCE (5'-3')	BASE PAIRS
Iss	F- CGGGAATTGGACAAGAGAAAAC R-TTTCTGCACCGCCACAAA FAM-TTTGGCTGCATCAAC-ZEN-IOWA BLACK FQ	57
*iut*A	F-CGGTGGCGTACGCTATCAGT R-GCGCGTAGCCGATGAAAT VIC-CACTGAAAACAAGATTGAT-MGB	59
*hly*F	F-GGTTGCCCGACCATCAATT R-ACTGGTTGAAGGTAAGCACCCTAA FAM-TTGTTGGCCACAGTCG-MGB	61
*omp*T	F-GGTTCCGGGATTGCTCGTAT R-GGTCGTGGAGGCAATATGGT VIC-CAGCCAGTCCCTGTC-MGB	57
*iro*N	F-CCGTTGGTGCAGAGTGGAA R-CAGGCTGGTAGAGGAAGGATCA FAM-CGCGATAAGCTCG-MGB	53

IKUTA et al. (2014).

For the sample to be considered as APEC, at least three of the genes investigated *(hlyF, ompT, ironN, iutA* and *iss*) should be detected.

Biofilm formation ability test

For biofilm formation assays, the methodology developed by STEPANOVIĆ et al. (2007) was utilized. The 60 APEC samples stored in Nutrient Agar (Merck, Germany) were subcultured onto MacConkey Agar (Merck, Germany) again to verify their purity and incubated at 37 °C for 18 to 24 hours. Afterward, one colony of each sample was subcultured onto Tryptone Soy Agar (TSA) (Merck, Germany) without glucose and incubated for 18 to 24 hours at 37 °C. The samples were standardized on the 0.5 turbidity scale on the Mc-Farland scale, which corresponds to approximately 1.5x108 CFU/mL. 20µL of each bacterial suspension was inoculated in triplicate and three replicates were performed for each isolate in 96-well flat-bottom polystyrene (PS) microtiter plates. 180 µL of Tryptone Soy Broth (TSB) (Merck, Germany) without glucose were added to each well and incubated for 24 hours at 25 °C to simulate room temperature.

After incubation, the bacterial suspension was removed by washing the plate with 250 µL of 0.9% saline solution. The plate was oven-dried at 25 °C to simulate room temperature. After drying the plate, 200 µL of methanol (Merck, Germany) was placed in each well for 15 minutes. After methanol removal, the microplates were dried at 25 °C for 15 minutes. The plates were stained with 200µL of 1% (m/v) crystal violet solution (Laborclin, Brazil) for five minutes at 25 °C. The microplates were washed in distilled water and 200 µL of 33% glacial acid (Merck, Germany) was added. After that, the absorbance results were verified in a microplate reader (Biotek, USA) at 550nm. To determine the degree of biofilm formation, the following classification was used, concerning the absorbance value of the negative control (OD): non-biofilm forming: optical density of the sample ODa ≤ OD; weakly biofilm-forming: OD < ODa ≤ 2.OD; moderately biofilm-forming: 2.OD < ODa ≤ 4.OD, and strongly biofilm-forming: 4.OD < ODa.

Disk diffusion test

The test was performed with 60 APEC isolates in triplicate. To determine the sensitivity profile of the bacteria, disk diffusion tests were performed on Mueller Hinton Agar (Merck, Germany) according to the methodology of KIRBY & BAUER (1966). The antibiotics routinely administered to broilers were used: amoxicillin (AML), kanamycin (K), colistin (CT), doxycycline (DO), fosfomycin (FOS), lincomycin+spectinomycin (LS), neomycin (N), oxytetracycline (OT), tetracycline (TE). The strains were subcultured with a sterile bacteriological loop on TSA agar, incubated at 37 °C for 18-24 hours, and then three to five colonies were selected from each plate for standardization in sterile saline solution (NaCl) 0.9% (m/v) by the 0.5 Mc-Farland scale.

Subsequently, the strains were seeded on Mueller Hinton Agar, in 90mmx15mm Petri plates, in all ways and directions to ensure uniform distribution of inoculum. After adding the disks, the plates were inverted and incubated at 37 °C for 18 to 24 hours. Results were measured by measuring the diameters (mm - millimeters) of zones of complete growth inhibition. The values of inhibitory halos obtained were compared with those of reference from the *Clinical and Laboratory Standards Methods Institute* (CLSI 2018), which determine the susceptibility profile of the microorganism in resistant, intermediate, and sensitive.

The classification regarding bacterial multi-resistance was performed by evaluating the percentage of strains that were classified as resistant and intermediate against at least three or more antimicrobials tested according to the CLSI classification (2018).

Statistical analysis

Comparisons of the frequencies of the genes investigated among the organs were performed using Fisher's exact test, at 5% probability. The antimicrobial resistance profile was expressed as a percentage of the total strains isolated. All data were analyzed using the SAS software.

RESULTS

Molecular detection of virulence factors by PCR

All 60 samples tested were confirmed as APEC, showing at least 3 of the virulence genes in the PCR virulence factor molecular detection test (Table 3).

Table 3. Percentage of detection of the *hly*F, *omp*T, *iro*N, *iss,* and *iut*A genes of *Escherichia coli* strains isolated from samples of organs from broilers with clinical signs of colibacillosis.

	*hly*F	*omp*T	*iut*A	*iro*N	*Iss*
Organs	Pos. n (%)^{ns}	Pos. n (%)^{ns}	Pos. n (%)^{ns}	Pos. n (%)^{ns}	Pos. n (%)^{ns}
Spleen	10 (100%)	10 (100%)	9 (90%)	10 (100%)	10 (100%)
Heart	9 (100%)	9 (100%)	8 (88.88%)	9 (100%)	9 (100%)
Liver	17 (100%)	17 (100%)	14 (82.35%)	17 (100%)	16 (94.1%)
Tonsils	13 (100%)	13 (100%)	9 (69.23%)	12 (92.3%)	13 (100%)
O.S.	11 (100%)	11 (100%)	10 (90.90%)	11 (100%)	11 (100%)
Total	60 (100%)	60 (100%)	50 (83.33%)	59 (98.3%)	59 (98.3%)

n = number, Pos: = positive, O.S. = organ swab (heart, liver, and spleen). ns = non-significant difference in frequency of positives among organs by Fisher's exact test, at 5% probability.

There was no difference in the percentage of positive samples among the organs evaluated (P>0.05). The majority (80%) of the isolated strains had the five virulence genes investigated by PCR, and the remainder (20%) had four genes confirmed for APEC.

Biofilm formation ability test

Among the 60 APEC strains analyzed, 71.66% (43) were characterized as weakly biofilm-forming and a percentage of 28.33% (17) no biofilm-forming. No strains were identified as moderately or strongly biofilm-forming.

Disk diffusion test

Regarding the sensitivity to the active ingredients used in the disk diffusion test with APEC isolates, 100% (60/60) of the strains tested were sensitive to colistin, and 93.34% (56/60) were sensitive to fosfomycin. Lower percentages were obtained for neomycin, showing 26.67% (16/60) of sensitive strains and 33.33% (20/60) of sensitivity compared to kanamycin (Figure 1).

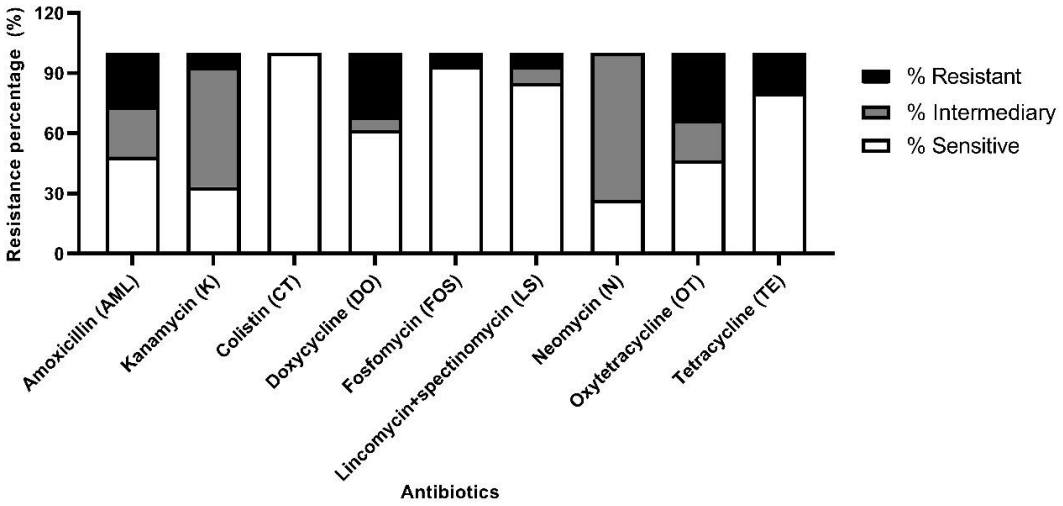

Figure 1. Percentages of bacterial resistance of avian pathogenic *Escherichia coli* to the tested antimicrobials.

As for the strains classified as intermediate, 73.33% (44/60) showed this characteristic compared to neomycin and 60% (36/60) compared to kanamycin. The percentage of strains resistant to oxytetracycline was 33.33% (20/60), and 31.66% (19/60) of resistance was found for doxycycline. Furthermore, it was observed that 58.33% (35/60) of the analyzed strains presented a multi-resistance profile and 48.33% (29/60) were resistant to at least one tested antimicrobial. Comparing the resistance profile of the tested APEC isolates in relation to the different classes of antimicrobials, the strains were more sensitive to the peptide group, with the highest sensitivity percentage of 96.66% (58/60), followed by lincosamides + aminocyclitols with 85% (51/60) of sensitivity. The highest percentage of strain resistance was observed in the group of tetracyclines -31.66% (19/60) - and beta-lactams -26.66% (16/60) (Figure 2).

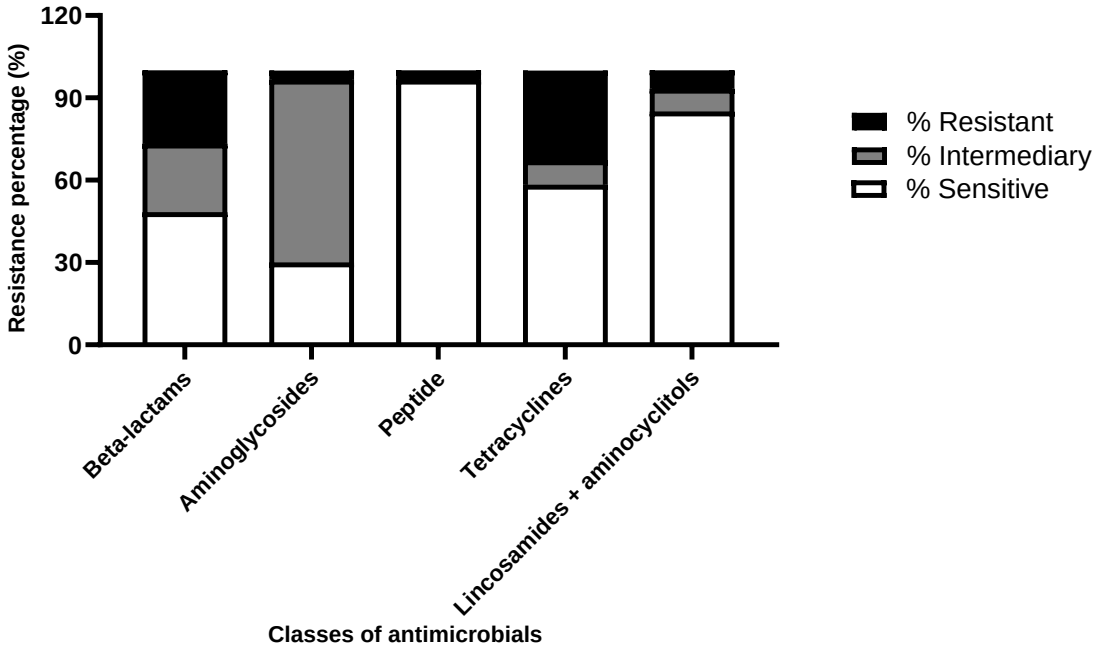

Figure 2. Percentage of bacterial resistance of avian pathogenic *Escherichia coli* according to different classes of antimicrobials.

DISCUSSION

Research reports many virulence-associated genes highly prevalent only in APEC isolates that are used as a prerequisite for establishing APEC infections in chickens (OVI et al. 2023). The molecular characterization results confirm that the strains were APEC, causing concern for the poultry sector due to the

high pathogenicity of the bacteria in chickens. There was no difference in the frequency of positivity among the analyzed organs, and the *hlyF, ompT, iss,* and *ironN* genes were detected in more than 98.33% of the samples and *iutA* in 83.33%. These results corroborate a study carried out by JOHNSON et al. (2008), in which virulence patterns were defined for APEC, such as those loaded on colicin V plasmids (*iss, tsh, iucC, cvi, iutA, hylA, iss, iroN,* and *ompT*). CARLI et al. (2015) reported that in 79 isolates characterized as APEC, a significant frequency of these virulence genes was also detected: *iutA* (81.5%), *iss* (96.3%), *ompT* (100%), and *hlyF* (100%). Frequencies also varied for *iroN* (43.4% to 95%), *iutA* (40.8% to 68.1%), *iss* (93%), and *hlyF* (68.8%) (KOBAYASHI et al. 2011, MALUTA et al. 2014). KIM et al. (2020) detected a higher frequency of *hlyF* genes (93.7%), followed by *iutA* (91.9%).

The persistence and survival of APEC in the environment and the host may be a consequence of the biofilm production capacity (RODRIGUES et al. 2019). In this study, there were no strains characterized as moderately or strongly biofilm-forming. In previous studies, such as in HUSSAIN et al. (2017), isolates of avian *E. coli* that were involved in the development of the biofilm demonstrated the presence of virulence genes *fimA*, *fimH* and *fimC*, and siderophore. NAVES et al. (2008) found the alleles *papC* and *papG*, *sfa* / *focDE*, *focG*, *hlyA*, and *cnf1*, while CRECENCIO et al. (2020) showed that the strongly biofilm-forming strains expressed the *fimC*, *papG,* and *crl* genes. OOSTERIK et al. (2014a) found that biofilm formation by APEC was affected by the serogroup they belonged to, where strains in serogroup O2 were often moderate or strong biofilm producers, while most O78 strains were weak biofilm producers.

The observed differences in biofilm resistance are likely due to many factors, including the types of avian isolates examined, virulence genes involved, the disease status of the birds, and even the medium used for biofilm analysis (NEWMAN et al. 2021). Despite the routine use of antimicrobials and disinfectants on farms, the occurrence of APEC in poultry farms is associated with its ability to form a biofilm, which is further aggravated by various virulence factors and multidrug resistance that help bacteria thrive under different conditions. environmental conditions (GRAKH et al. 2022).

PAVLICKOVA et al. (2017) found a significant correlation between the prevalence of antibiotic resistance and the ability to form biofilms, where the highest prevalence of antibiotic resistance was observed in weakly biofilm-forming strains. This fact corroborates the results observed in this study, where the analyzed strains were mostly weakly biofilm-forming, but with a high percentage of antimicrobial resistance.

Regarding the resistance to the antimicrobials tested, the highest percentages were for oxytetracycline (33.33%), doxycycline (31.66%), amoxicillin (26.66%), and tetracycline (20%). The APEC strains did not show any percentage of resistance to colistin and neomycin antibiotics. These results differ from those reported by BARROS et al. (2012), in which high percentages of resistance were found for oxytetracycline (100%), chlortetracycline (84.50%), and amoxicillin (84.60%). NHUNG et al. (2017) reported that mean resistance levels for ampicillin, amoxicillin, tetracycline, and doxycycline were greater than 70%. In a study carried out by AWAD et al. (2020), resistance to tetracyclines and colistin was 92.31%, and doxycycline was 84.62%. OOSTERIK et al. (2014b) studied the susceptibility of APEC samples and found resistance to ampicillin (35.1%), and tetracycline (53.6%), while KIM et al. (2020) showed high resistance of APEC isolates to ampicillin (83.5%), tetracycline (64.6%), and ciprofloxacin (46.8%).

SUBEDI et al. (2018) evaluated the multi-resistance profile of *E. coli* isolates and found that 94% of the samples were resistant to three or more antimicrobials, with 22% of *E. coli* showing multi-resistance to five different types of antimicrobials. MEGUENNI et al. (2019) reported that 97.2% of the isolates were resistant to at least one antibiotic, and 53.5% demonstrated multi-resistance to three different classes of antimicrobials. The results found in this study differ from the results of SUBEDI et al. (2018) and MEGUENNI et al. (2019) since 58.33% of the analyzed APEC strains showed a multi-resistance profile to more than three antimicrobials tested, and 48.33% to at least one antimicrobial.

When strain resistance was evaluated according to antimicrobial class, *E coli* strains showed greater resistance to tetracyclines (31.66%), followed by the beta-lactam group, with 26.66% resistance. CRECENCIO et al. (2020) also cited the group of beta-lactams with the highest resistance profile (39.5%), as well as MEGUENNI et al. (2019), where the highest percentage of resistance found was in the group of beta-lactam antibiotics.

Avian pathogenic *E. coli* represents a high risk for the poultry sector since all the isolates tested in this study showed the characteristic virulence genes of the bacterium, which increases the concern for the sector because in addition to being a bacterium that demonstrates potential risk to public health, causes huge

losses in poultry production.

Although the APEC did not express the potential for biofilm formation, bacterial multi-resistance is a factor of concern. Regional studies are needed to better understand this resistance profile of the bacteria, because each country/region works with a sanitary program, adopting the use of different antimicrobials, and influenced by market requirements and current legislation.

CONCLUSION

Avian pathogenic *Escherichia coli* was frequently isolated in broiler farms. The virulence factors *hlyF*, *ompT*, *iss*, and *ironN* were found in most of the isolates analyzed. There was no ability of the strains studied to form biofilms classified as moderate or strong. The APEC strains isolated from broilers showed multi-resistance to the tested antimicrobials.

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