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MICROBIOLOGY

Molecular identification of pathogenic *Escherichia coli* virulence genes and histopathological analysis in chickens condemned for airsacculitis

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ABSTRACT: The present study identified virulence genes and pathological changes caused by *Escherichia coli* in chicken carcasses condemned for airsacculitis and assessed if the histopathological examination and polymerase chain reaction (PCR) were effective for studies like this. Trachea, liver, and lung were collected from 30 chickens with suspected airsacculitis that has been condemned in the inspection line. The samples were analyzed by PCR to simultaneously identify two virulence genes (*iss* and *tsh* genes) and for histopathological testing. PCR efficiently genotypically characterize the *E. coli* isolates, where the virulence genes *iss* and *tsh* were found in three birds simultaneously. The histopathological examination detected a predominance of heterophils and mononuclear cells in the trachea (100%), lung (90%), and liver (13.3%). The liver was the organ where practically no alteration was diagnosed. The results of multiplex PCR for the *tsh* and *iss* virulence genes indicate the great potential of the approach in the characterization of *E. coli* isolates. Unspecific identification did not occur, thus making it necessary to use technologies for the identification and prevention of this agent in aviaries and poultry abattoirs.
Key words: poultry health, inspection, microbiological, DNA, histopathological.

Identificação molecular de genes de virulência de *Escherichia coli* e análise histopatológica em frangos condenados por aerossaculite

RESUMO: O objetivo do presente estudo foi identificar genes de virulência e alterações patológicas provocadas por *Escherichia coli* em carcaças de frango condenadas por aerossaculite e se o exame histopatológico e uma Reação em Cadeia da Polimerase (PCR) são eficazes para os estudos dessa natureza. Para isso, foram coletados traqueia, fígado e pulmão de 30 frangos condenados na linha de inspeção, com suspeita de aerossaculite. As amostras foram submetidas a PCR para a identificação de dois genes de virulência de modo simultâneo (genes *iss* e *tsh*) e ao teste histopatológico. A PCR foi eficiente para caracterizar genotipicamente os isolados de *E. coli*, em que se constatou em três aves os genes de virulência *iss* e *tsh* simultaneamente. No exame histopatológico detectou-se a predominância de heterófilos e mononucleares na traqueia 100%, no pulmão 90% e no fígado 13,3%. O fígado foi o órgão onde praticamente não foi diagnosticada nenhuma alteração. Diante dos resultados obtidos, foi possível observar que a PCR multiplex para os genes de virulência *tsh* e *iss* apresenta um grande potencial na caracterização de isolados de *E. coli*, já que não gerou identificação inespecífica, com isso faz-se necessária a utilização de tecnologias para identificação e prevenção desse agente nos aviários e matadouros avícolas.
Palavras-chave: sanidade avícola, inspeção, microbiológico, DNA, histopatológico.

INTRODUCTION

The main causes of condemnation of birds observed by the State Inspection Service (SIE) are related to the presence of pathological changes in the respiratory tract, particularly condemnation due to airsacculitis (FERREIRA et al., 2012). One of the main etiological agents of airosacculitis is strains of *Escherichia coli*, which are a frequent infectious cause of carcass condemnation (SCHOULER et al., 2012). This pathology is characterized by a localized or systemic infection, with numerous forms of manifestations. The most common is the respiratory tract infection that progresses to colisepticemia (LUTFUL KABIR, 2010). In addition to the increase in condemnations, birds with airsacculitis may have lower weight, leading to uneven batches sent to the slaughter line, which increases the risk of failures in the process during evisceration and, consequently, contamination of carcasses with pathogens from the intestinal tract (MACHADO et al., 2012).

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According to the Regulation for Industrial and Sanitary Inspection of Products of Animal Origin (RIISPOA), partial or total condemnation carcasses with an intense process of airsacculitis depends on the type and level of injury they may present (BRASIL, 2017). The severity of the pathological process of airsacculitis is related to the pathogenicity of the strains of *E. coli* pathogenic for birds (APEC) involved and their respective virulence factors, which favor the permanence and multiplication of this microorganism in the host (SCHOULER et al., 2012; HUJA et al., 2015).

Among the essential pathogenicity factors for the action of APEC strains, the thermosensitive hemagglutinin type adhesins (*tsh*) and serum resistance proteins (*iss*) stand out. According to DISSANAYAKE et al., (2014), *tsh* and *iss* may be involved in the development of systemic infection, since these genes were detected in APEC that cause septicemia outbreaks. The *tsh* gene expresses an autotransporter protein that adheres to red blood cells and associates with extracellular matrix proteins, being responsible for infections that cause agglutination of erythrocytes (HUJA et al., 2015; PAIXÃO et al., 2016). The gene responsible for expression of the serum resistance protein (*iss*) generates a polypeptide responsible for inhibiting the deposition of the membrane attack complex in the nervous system of birds (DZIVA & STEVENS, 2008).

Thus, it is necessary to characterize the bacterial virulence genes related to airsacculitis in birds and their association with the pathological changes presented. Studies that correlate macroscopic and histopathological lesions and the identification of virulence factors in this infectious disease are scarce. In this context, molecular methods, especially the polymerase chain reaction (PCR), can be an important tool for the diagnosis of APEC. Histopathological examination is a relevant resource to assess the action of bacterial invasion, lesions caused, as well as immunoprotective responses induced by the strains involved in the infection (DOS SANTOS et al., 2018).

Based on the above, the present study identified the relationship of virulence genes involved in serum resistance (*iss*) and hemagglutinin (*tsh*) through a multiplex PCR (mPCR) together with histopathological examination in the organs of chicken carcasses condemned for airsacculitis in a slaughterhouse by the State Inspection Service.

MATERIALS AND METHODS

Thirty chicken carcasses condemned for suspected airsacculitis were collected in a abattoirs under the State Inspection Service of the Defense and Inspection Agency of the State of Pará - ADEPARÁ, located in the northeast region of the state of Pará. The post-mortem inspection procedure of the animals was performed as determined by the Regulation of Industrial and Sanitary Inspection of Products of Animal Origin - RIISPOA (BRASIL, 2017) and Ordinance No. 210 (BRASIL, 1998), based on the macroscopic visual examination and by palpating the carcasses on the so-called “inspection lines”. Trachea, lung, and liver were removed from each bird, collected, placed in duly identified bottles and transported in refrigerated isothermal boxesto the Microbiology Laboratory - Institute of Veterinary Medicine (IMV) - UFPA/Castanhal.

In the laboratory, the organs were fragmented into samples for microbiological, histopathological, and molecular tests. For the isolation of *E. coli*, the samples were cultivated in Brain Heart Infusion (BHI) broth at 37 ºC for 24h and subsequently in blood agar and MacConkey agarat 37 ºC for 24-48h. After the isolation of the microorganisms present, biochemical tests were carried out to identify these microorganisms. Microorganisms that showed the following characteristics in biochemical media were considered positive for *E. coli*: triple sugar iron (acid peak/acid bottom), negative hydrogen sulfide, negative phenylalanine deaminase, positive indole, positive motility, negative urease, methyl positive, Voges Proskauer negative, and citrate negative.

For the histopathological examination, the samples were stored in 10% formaldehyde for 48 h and sent to the Pathology Laboratory (IMV - UFPA). The samples were dehydrated, cleared, and immersed in liquid paraffin. The tissues were embedded in paraffin and sectioned to 0.5 µm in thickness using a model RM 2125 RT rotating microtome (Leica). The sections were placed on glass slides for staining with hematoxylin and eosin in an automatic stainer (SLEE MSM) and mounted with a glass coverslip and Entellan mounting medium.

For molecular analysis, bacterial DNA was initially extracted using the thermal lysis technique following the protocol by ROCHA et al. (2008). For this, the samples were added to the culture medium in 1:10 BHI previously incubated for 24 hours at 37 ºC. One milliliter of bacterial culture suspension was collected and centrifuged for 5 min at 13,200rpm. The supernatant was discarded and 800µL of milliQ water was added. After homogenization, the samples were centrifuged as just described. The supernatant was discarded and 80µL of milliQ water was added. After this step, the samples were heated at 96 ºC for 10

min. Each supernatant was collected and kept frozen in polypropylene tubes at -20 °C until analysis. The extracted DNA was quantified by spectrophotometry in a 260nm filter and the purity was evaluated based on the ratio of the readings at 260nm/280nm.

The search for virulence genes was carried out using a previously standardized mPCR (unpublished data), where oligoprimers were used that amplify sequences of 620 bp and 760 bp specific for the respective fragments of the *tsh* and *iss* genes, previously described by ROCHA et al. (2008). The reverse (Rev) and forward (For) sequences were:*tsh* Rev: 5'GGT GGT GCA CTG GAG TGG3' and *tsh* For: 3'AGT CCA GCG TGA TAG TGG5'; *iss* Rev: 5'GTG GCG AAA ACT AGT AAA ACA GC3' and*iss* For: 3'CGC CTC GGG GTG GAT AA5'. The mPCR protocol was calculated for a final volume of 25 µL for each reaction. For this, 50 mM MgCl and 10 mM Tris-HCl (10× buffer), 10 mM DNTP mix, approximately 60 ng of template DNA, 1 U Taq DNA polymerase, and 20 pmol of each primer were used, and the volume was completed with water ultra pure sterilized.

A VERITI® 96thermal cycler (Applied Biosystems) was programmed for 30 cycles, in which the temperatures and times used for denaturation, annealing and extension were, respectively: 94 °C for 5 min, 94 °C for 1 min, and 58 °C for 1 min, plus initial denaturation at 72 °C for 2 min and final extension at 72 °C for 10 min. The amplified product was analyzed by 1.5% agarose gel electrophoresis in 0.5 Tris Borate EDTA (TBE) buffer and stained with Gelred (non-mutagenic dye). The analysis of the results was carried out using a photo documentation equipment on ultraviolet light associated with the Total Lab version 5.2 software.

The data obtained were stored in Microsoft Excel® software spreadsheets and also exported to Bioestat 5.0. Descriptive and inferential statistics analyses were performed based on the construction of contingency tables and the application of Kappa-Cohen's concordance tests and Fisher's Exact, for a significance level of 5%.

RESULTS

During the sanitary inspection, the 30 birds collected showed macroscopic alterations, mainly in the trachea, lung and liver, with characteristic lesions of airsacculitis. The lesions present in these animals that were observed at the slaughter line manifested as changes in color (tending to reddish-yellow) and irregularities on the skin surface. The tracheal mucosa was diffusely reddish, lungs and livers had whitish spots, with thickened edges and increased volume. All carcasses with these characteristics were condemned and suspected of airsacculitis.

In the microbiological analysis, *E. coli* was isolated in all birds investigated in at least one of the evaluated organs. The highest frequency of *E. coli* was in the trachea, where the percentage of positivity was 100% (30/30), followed by 97% (27/30) in the lung and 33.3% (10/30) in the liver.

In the histopathological examination, in 90% of the carcasses, lesions were identified in the organs of the respiratory system (trachea and lungs). An inflammatory process characterized by the association of two main cell types (heterophiles and mononuclear cells) was evident. In addition, mixed infiltrates of heterophils and lymphocytes were identified, especially present in respiratory organs, with foci of necrosis surrounded by macrophages and multinucleated giant cells in response to bacterial tissue invasion.

In tracheal lesions, thickening of the mucosa due to epithelial hyperplasia was observed, in a diffuse and accentuated form with absence of cilia, metaplasia of mucous glands, and discreet lymphoplasmacytic infiltrate (24/30 samples) (Figure 1A). Lung lesions were also observed, characterized by stratified epithelium generating squamous metaplasia, marked multifocal fibrinonecrotic bronchopneumonia, surrounded by infiltrates of heterophils, lymphocytes, plasmocytes, macrophages and multinucleated giant cells (22/30 samples) with intralesional bacterial colonies (Figure 1B). However, only 4/30 liver samples showed rare foci of hepatocyte necrosis in random distribution, thick capsule, mild diffuse cytoplasmic vacuolation of hepatocytes, proliferation of fibrous connective tissue, suggestive of bacterial hepatitis (Figure 1C).

The mPCR detection of the *iss* and *tsh* virulence factors demonstrated the simultaneous amplification of fragments of the *E. coli* genes, characteristic of APEC (Figure 2).

Amplification of at least one of the virulence genes was observed in 55.5% of the samples (50/90), 13.33% (4/30) in the liver, 73.33% (22/30) in the lung, and 80% (24/30) in the trachea. The most frequently amplified gene was*tsh* (18/30) and the least frequent was *iss* (12/30). In birds 15, 18, 19, and 22, the *tsh*virulence factor was amplified in the three organs, suggesting septicemia by *E. coli*, given the presence of the bacterium in different organs.

In respiratory organs of birds 1, 2, 8, 13, 23, 27, and 28, simultaneous amplification of the two *E. coli* virulence genes was observed. Birds 3, 4, 5, and 30 had both factors in only the trachea.

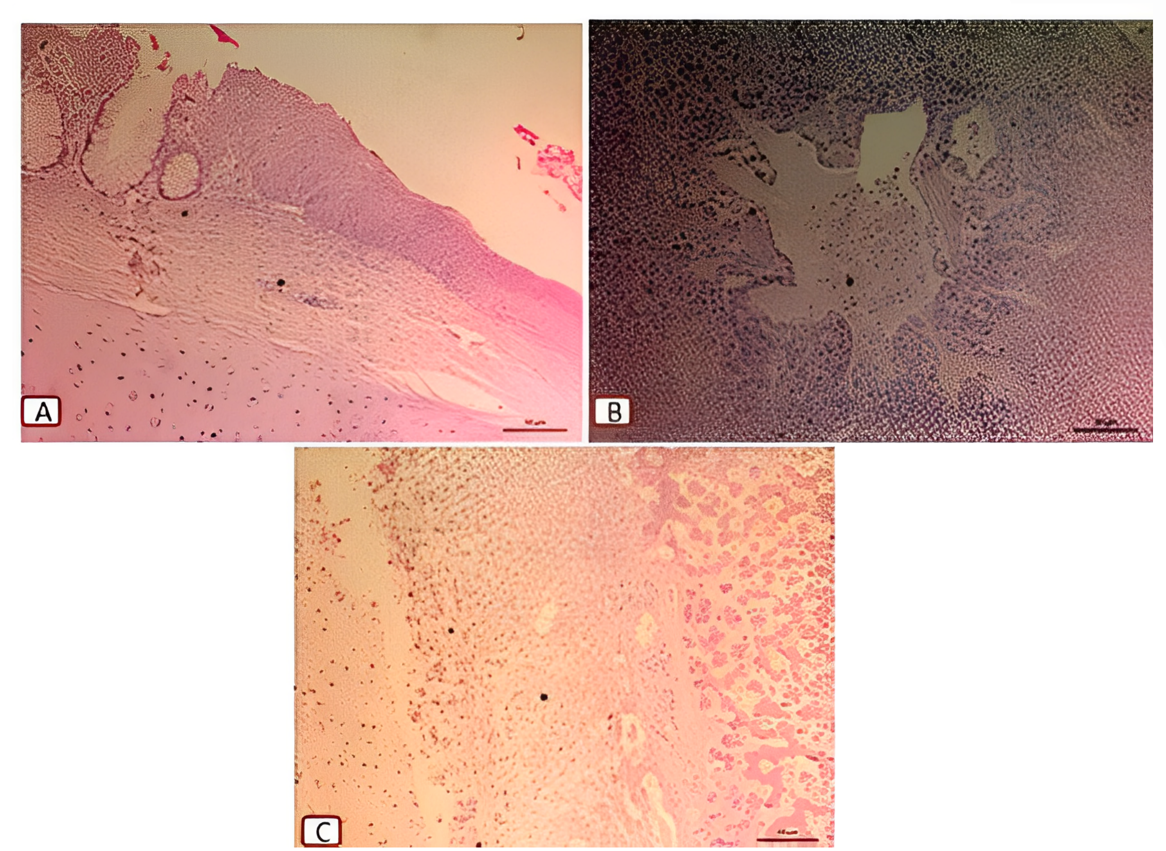

Figure 1 - Tissues from birds condemned for airsacculitis. A: transition/metaplasia on the surface (trachea), B: (lung) exudates within the parenchyma necrotic debris, below the pleura and C: inflammatory infiltrate, thickened capsule, proliferation of fibrous connective tissue with heterophils on the surface, (liver).

Some animals (11, 21, 24, and 26) only presented amplification of the *iss* gene in respiratory organs and another seven carcasses (9, 12, 17, 25, and 29) only amplified the *tsh* gene in the respiratory organs. It is noteworthy that only four of the carcasses (birds 6, 7, 10, and 16) did not show amplification of any of the virulence genes in the analyzed organs, which does not rule out the possibility of infection by strains with other virulence factors.

It was possible to observe a close correlation between the three tests used to identify *E. coli* and their virulence factors in birds condemned with typical airsacculitis lesions (Table 1).

The histopathological lesions reported in the organs of birds were directly related to the presence of bacterial virulence factors. The infiltrate focus was present in the samples that presented at least one virulence factor detected by the molecular method. In addition, four of the birds analyzed showed histopathological alterations and the amplification of virulence genes in the three target organs, suggesting a chronic or septicemic nature of the disease (Table 2).

DISCUSSION

The levels of agreement between the tests used ranged from moderate to almost perfect, in which it was observed that the detection of bacteria in the microbiological examination was not always associated with the existence of virulence factors and the manifestation of histopathological changes (moderate agreement). The microbiological screening performed revealed strains of *E. coli* in general, but only through molecular and histopathological tests was it possible to assess the pathogenicity of the isolates. Conversely, the presence of virulence factors was closely linked to histopathological alterations, indicating the considerable agreement between these two tests (Table 1).

Results obtained in the macroscopic examination of the condemned birds were similar to

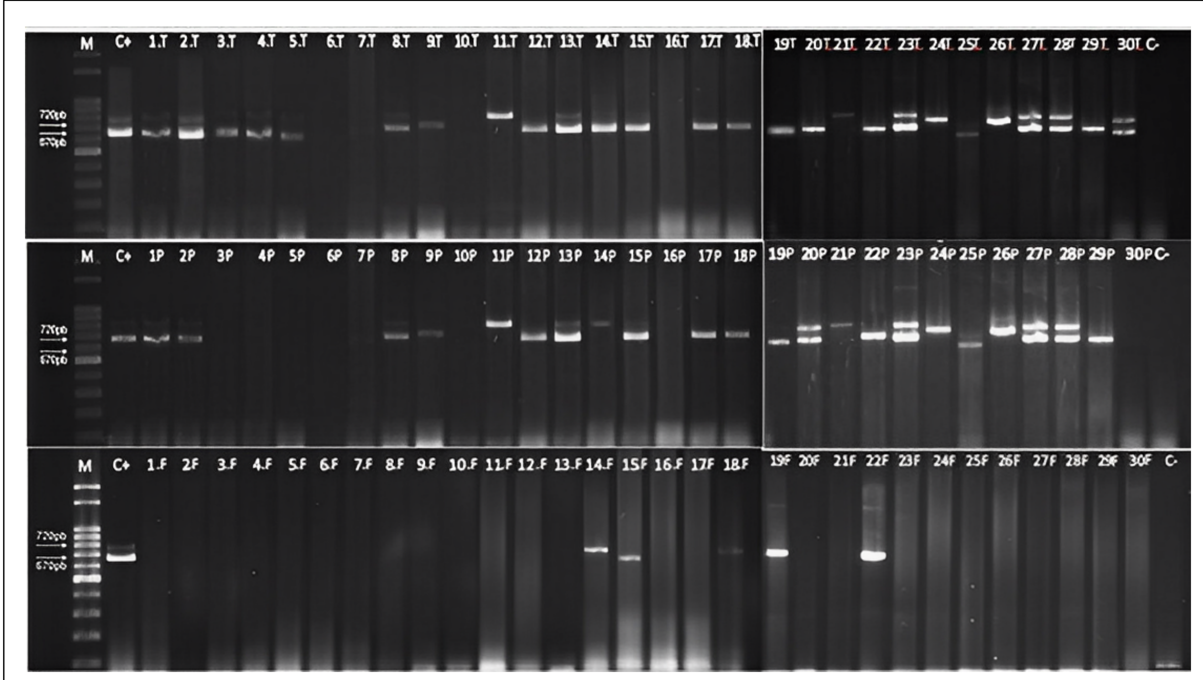

Figure 2 - Agarose gel electrophoresis demonstrating the results obtained from the polymerase chain reaction (PCR) performed on standard strains and contaminated samples where: M: 100pb molecular weight marker; C+: positive control with amplification of two virulence factors *iss* (760pb) and *tsh* (620pb); 1 to 30: birds; T: sample from trachea; P: lung samples, F: liver samples and C-: negative control.

those already reported by other authors. DUARTE et al. (2019) identified the presence of catarrhal secretion in the medial portion of the trachea. CASAGRANDE et al. (2017) recorded the occurrence of macroscopic liver changes (greenish spots) in livers of condemned broilers. In the histopathological examination of the present study, the most affected organs were those of the respiratory tract, as previously elucidated by MACHADO et al. (2012). These authors also described a relationship between the presence of *E. coli* and histopathological changes in affected organs.

The data obtained in our research also corroborated those of CASAGRANDE et al. (2017), who reported that systemic cases of *E. coli* infection usually start with primary infections in the respiratory tract that subsequently spread to various organs, characterized by fibrin exudation, infiltration of heterophils, macrophages, lymphocytes and plasma cells mild to moderate focally extensive, chronic pericarditis and airsacculitis due to fibroblast proliferation, in addition to moderate focal macrophage infiltrate.

Table 1 - Agreement between the microbiological, histopathological, and molecular tests used to identify *Escherichia coli* in birds condemned for airsacculitis.

Tests	Kappa coefficient	Level of agreement	P-value
Microbiological / PCR	K= 0.41	Moderate	0.01.*
Microbiological/ Histopathological	K= 0.41	Moderate	0.01.*
PCR/ Histopathological	K= 1	Almost perfect	0.001.*

*level of significance adopted with $P < 0.05$.

Table 2 - Percentage and absolute frequency of identification of histopathological lesions and *Escherichia coli* virulence factors in chickens condemned for airsacculitis and the relationship between positive results for the tests employed.

Organ	Histopathological	-----------------PCR-----------------		----Histopathological/*ISS*[**]----		--Histopathological/*TSH*[***]--	
	f (%)	*ISS* % f (%)	*TSH* f (%)	f (%)	P-value	f (%)	P-value
Trachea	24/30(80%)	17/30(56.6%)	23/30(76.6%)	17/30(56.6%)	0.0029*	23/30(76.6%)	0.0051*
Lung	22/30(73.3%)	14/30(46.6%)	18/30(60%)	14/30(46.6%)	0.0027*	17/30(56.6%)	0.0001*
Liver	4/30(13.3%)	1/30(3.3%)	4/30(13.3%)	1/30(3.3%)	0.1379	3/30(10%)	0.0038*

*Fisher Exact Test. Significance level of $P < 0.05$.
** PCR pathogenicity factors the serum resistance proteins (iss).
*** PCR pathogenicity factors the thermosensitive hemagglutinin type adhesins (tsh).

The presence of mixed infiltrates, especially containing heterophils and lymphocytes, associated with the presence of *E. coli* was observed in most of the investigated organs. This pattern has also been reported by other authors, who also mentioned the incidence of lesions in the liver causing multifocal heterophilic cholangiohepatitis, followed by hepatitis random necrotizing, with necrotic areas, related to the identification of the bacterium through its virulence factors (BARCELOS et al., 2006).

Primary infections in respiratory organs of birds are manifested mainly by tracheitis, pneumonia, airsacculitis, pericarditis and polyserositis (NOLAN et al., 2013). In our study, in addition to the more pronounced changes in the respiratory tract, virulent bacteria were present in this location, demonstrating the gateway to an infection route that can become systemic. The high frequency of *E. coli* virulence genes in samples collected during necropsy (liver, trachea, lung) is consistent with the research by MACIEL et al. (2017), who described these organs as target tissues for pathogenic *E. coli* infections in birds.

In the present study, a statistically significant relationship was observed between the presence of *E. coli* virulence factors and histopathological lesions in analyzed organs (Table 2). The results echo those of other authors. GONÇALVES et al. (2012) evaluated the presence of pathogenic *E. coli* in air sacs and tracheas of broiler chickens in 120 samples of birds. The authors reported a 17.8% positive rate, with the air sacs being the most affected organs (66.1% of positive samples).

The *tsh* and *iss* genes investigated in this study were identified simultaneously in seven birds. We suggested that the combination of these virulence factors favors the dissemination of the microorganism, evasion of the host's defensive system and tissue invasion, mainly of the respiratory system, which represents a potential risk for infection and persistence of the pathogen in the host organism. The results are similar to those reported by EMARA et al. (2019), in which all shell-dead chicken embryos harbored the *tsh* and *iss* virulence genes.

Likewise, the *iss* gene was reported in 95.6% and 63.8% of the APEC strains studied by SERRANO et al. (2017) and DOU et al. (2016), respectively. The function of the *iss* is to mediate bacterial resistance to serum complement. According to KWON et al. (2008), the high frequency of this virulence factor reveals the importance in the ability of this bacterium to evade the host's defenses, and even multiply and disseminate, favoring the development of the disease. Despite the smaller number of positive samples for the *iss* gene in the samples of this study, this gene can be considered a marker of virulence, with the *iss* gene cited as the most prevalent in strains from diseased birds (ROCHA et al., 2008; ABREU et al., 2010). Another gene identified in this work was *tsh*, the temperature-regulated adhesin (HUJA et al., 2015). The *tsh* and *iss* genes may be involved in the development of systemic infection, since these genes were detected in strains of *E. coli* that caused outbreaks of septicemia, but were absent in strains isolated from healthy chickens (DISSANAYAKE et al., 2014), reinforcing the data obtained in the present study.

This differentiation of pathogenic strains can be performed through the analysis of virulence genes, given that the method tested in the present study proved to be effective and the results obtained reinforce the possibility of using molecular biology tools to assess bacterial virulence that determine the pathogenic potential of the isolated *E. coli* strains. PERRY et al. (2007) confirmed that in addition to saving on reagents, mPCR reduces the potential for false positives and false negatives in the amplification of two or more DNA sequences associated with the same pathogen.

Although, it is recommended that birds be condemned through macroscopic observation (BRASIL, 1998), it is important to emphasize that there are cases in which, even in the absence of some signs, the presence of pathogens is possible (ZOU et al., 2021), which represents a risk to public health. In this sense, more current legislation (BRASIL, 2017) stipulates that establishments must control their production process through physical, microbiological, physical-chemical, molecular biology, histological, and other analyzes that may be necessary for the evaluation of the conformity of raw materials and products of animal origin, as main measures for the prevention and control of diseases of interest in poultry and public health.

In view of this, there is a strong demand for the standardization and validation of new technologies that are practical, but above all efficient, for the identification and prevention of pathogens, such as APEC. Thus, the present study is of fundamental importance, since it proves the efficiency of histopathological and molecular tests for the identification of virulence factors and *E. coli*.These tools can be used in favor of inspection services, especially mPCR, which isa quick and low-cost method (WEI et al., 2018) to guarantee poultry health and food safety.

CONCLUSION

A large percentage of chickens with lesions associated with pathogenic *E. coli* elucidates the importance of this bacterium as the cuae of infections in broiler chickens. The presence of virulence genes detected in mPCR was directly associated with histopathological changes in the carcass of the analyzed birds. The isolated strains capable of causing histopathological alterations were those that presented one or both of the analyzed genes.

In addition, most lesions and pathogenic strains were concentrated in the respiratory tract, indicating that this is the entry point for the microorganism, which can spread to other organs. Finally, the mPCR used in this study together with the histopathological examination represent important tools to diagnose APEC and ensure poultry health.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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