

Virulence Genes and Resistance Profile of *Escherichia coli* Isolated in Meat Meal Samples*

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ABSTRACT

Background: Feed is the main route of transmission of pathogenic microorganisms and is responsible for a large part of the cost of poultry production, so the inclusion of alternative foods in diets for monogastrics has been a constant. Among alternative foods most used in modern poultry farming are animal meal, however, when contaminated they constitute a route of transmission of several pathogenic agents, including *Escherichia coli*. In addition, there is a zoonotic potential, as poultry products are intended for human consumption. The objective of this research was to detect virulence genes, as well as to evaluate the resistance profile of *Escherichia coli* isolates from meat meal samples.

Materials, Methods & Results: A total of 40 *Escherichia coli* isolates were analyzed and the virulence genes surveyed *iss*, *ompT*, *hlyF*, *iutA*, and *fimA* identified by Polymerase Chain Reaction (PCR). The antimicrobial agents tested were: amoxicillin (30 µg), ceftiofur (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), florfenicol (30 µg), fosfomicin (200 µg), gentamicin (10 µg), norfloxacin (10 µg) and oxacillin (1 µg). It was possible to observe the occurrence of the *iss* resistance gene in 100% of *Escherichia coli* isolates, followed by *hlyF* (85%), *fimA* (75%), *ompT* (17.5%) and *iutA* (5%). Regarding the simultaneous detection for the genes, a greater association between the genes *iss*, *hlyF* and *fimA* (60%) was verified. All isolates showed resistance to oxacillin (100%), followed by doxycycline (25%), amoxicillin (22.5%), norfloxacin (17.5%), ceftiofur (15%), florfenicol (12.5%), fosfomicin (12.5%), ciprofloxacin (10%) and gentamicin (2.5%). In this study, a variation of the multiple antimicrobial resistance index (IRMA) was observed between 0.22 and 0.77.

Discussion: The indiscriminate use of antimicrobials as performance enhancers in production animals, may have contributed to the increase in antimicrobial resistance, with the occurrence of multiresistant *Escherichia coli* carrying virulence genes. Virulence genes present in *Escherichia coli* isolates are studied to understand the degree of influence they exert in the establishment of the disease, one of the most researched genes is the *iss* gene, involved in the processes that promote serum resistance. In this study, *iss* (100%) was present in all the isolates analyzed, although it is not the only mechanism used by these bacteria to reach internal organs and trigger an infection, this gene encodes an important mechanism associated with high levels of virulence. The second highest prevalence found was of the *hlyF* gene (85%), the high prevalence of *hlyF* suggests virulence potential, involved with the production of hemolysin and improvement of outer membrane vesicles associated with the release of toxins. The *fimA* gene (75%) was detected in a slightly lower percentage when compared to *iss* and *hlyF*. With the second lowest prevalence, the *ompT* gene (17.5%), is involved in a process that includes the proteolytic degradation of antimicrobial peptides and with the lowest prevalence the *iutA* gene (5%). Certain combinations of virulence genes make the strains easier to survive, adhere to, colonize and even the ability to develop septicemic conditions. Multiresistant *E. coli* strains, is a fact of concern for both animal and human health, since the presence of multiresistant strains, originating from poultry, can be transmitted from chicken carcasses. In this sense, the importance of sanitary control of the inputs used in animal feed is emphasized, as well as the prudent use of antimicrobials in animal production, with a view to producing a safe food, minimizing not only the economic losses, but also the risks to human health.

Keywords: antimicrobial, bacterial resistance, colibacillosis, poultry.

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INTRODUCTION

The alternative foods most used in modern poultry farming are animal meal, which is an important source of protein and energy. However, when contaminated, it becomes a transmission route for several pathogenic agents, including bacteria responsible for serious damage to animal and human health, such as *Salmonella* spp., *Campylobacter* spp. and *Clostridium* spp., with emphasis on *Escherichia coli* [33].

Some bacteria, especially *E. coli*, have the ability to transmit the virulence genotype to other bacteria through mobile genetic elements [29]. In this sense, studies correlate avian strains with *E. coli* strains that cause disease in humans and suggest a zoonotic potential of APEC strains [6,27]. Another concern is the use of antimicrobials as performance enhancers in production animals, as it favors the increase of antimicrobial resistance, in addition, it is possible that these bacteria and their virulence genes are transmitted and incorporated into the human microbiota, thus reducing the effectiveness of antimicrobial treatment [10].

In view of the above, we highlight the relevance of the knowledge about the occurrence of virulence genes in *E. coli* isolates and the sensitivity profile of the most commonly used antimicrobials in industrial poultry, since the rations and their raw materials, especially those of animal origin, represent an important route of transmission when contaminated by potentially pathogenic agents, a fact that causes serious damage to the poultry sector and is relevant for public health.

MATERIALS AND METHODS

Samples

A total of 40 *Escherichia coli* isolates, stored at -20°C in 20% Brain Heart Infusion (BHI)¹, belonging to a biological sample bank of the Animal Diagnostic Laboratory - LADA, located in the state of Pernambuco, Brazil, were analyzed.

Reactivation of *Escherichia coli* isolates

The isolates were transferred to BHI broth¹ and incubated at 37°C for 16-18 h then were seeded in agar Levine² and subjected to the same incubation conditions. The colonies were confirmed by Gram staining and biochemical tests: triple sugar iron (TSI) citrate, methyl red (VM), Voges Proskauer (VP) indole, motility, hydrogen sulphide production in agar Sulphate/

Indol/Motility (SIM) [8]. For all tests, the *E. coli* strain ATCC (25922) was used as a positive control.

Determination of the resistance profile

The resistance profile was determined using the disk diffusion test according to the protocol described in the Clinical and Laboratory Standards Institute [11]. The tested antibiotics³ were: amoxicillin (30 µg), ceftiofur (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), enrofloxacin (5 µg), florfenicol (30 µg), fosfomicin (200 µg), gentamicin (10 µg), nalidixic acid (30 µg), norfloxacin (10 µg) and oxacillin (1 µg). The multiple antimicrobial resistance index (IRMA), this index was determined by the ratio between the number of antimicrobials in which isolates exhibited resistance and the total number of antimicrobials [22].

Determination of virulence genotype

For determination of virulence genotype of the isolates, the DNA was extracted by the thermo-extraction method [12]. After extraction, the genetic material was submitted to the Polymerase Chain Reaction (PCR) technique uniplex. The primers and amplification cycles were according to the references, described in Table 1. For the negative control of the reactions, ultrapure water was used and as positive controls, well-known and sequenced samples from the Federal Rural University of Pernambuco (UFRPE) were used. The amplified DNA was subjected to agarose electrophoresis gel² (1%), stained with Blue Green⁴, visualized under UV light and documented by an image capture system⁵.

RESULTS

The occurrence of the *iss* virulence gene in 100% (40/40) of *Escherichia coli* isolates was observed, followed by *hlyF* 85% (34/40), *fimA* 75% (30/40), *ompT* 17.5% (7/40) and *iutA* 5% (2/40). The data for the isolated or simultaneous detection for the genes under study are set out in Table 2, with a higher occurrence of association between the *iss*, *hlyF* and *fimA* genes with 60% (24/40).

The resistance profile of *E. coli* isolates against commercial antimicrobials is shown in Table 3. All isolates showed resistance to oxacillin (100%), followed by doxycycline (25%), amoxicillin (22.5%). The most effective antimicrobial among the isolates tested was gentamicin (97.5%); ceftiofur, ciprofloxacin and fosfomicin had the same percentage of sensitivity (85%). All

isolates tested were resistant to at least 1 antimicrobial. Regarding the multiresistance profile, 27.5% (11/40) of the strains were resistant to a minimum of 3 and a

maximum of 7 antimicrobials. In this study, a variation of the multiple antimicrobial resistance index (IRMA) was observed between 0.22 and 0.77.

Table 1. Description of the genes, primers and references used in the study to identify the virulence profile of *Escherichia coli* isolates

Genes	Sequence of the primers (5'-3')	Nº of base pairs	Reference
<i>iss</i>	CAGCAACCCGAACCACTTGATG AGCATTGCCAGAGCGGCAGAA	323	[19]
<i>ompT</i>	ATCTAGCCGAAGAAGGAGGC CCCGGGTCATAGTGTTTCATC	559	[32]
<i>hlyF</i>	GGCGATTTAGGCATTCCGATACTC ACGGGGTCGCTAGTTAAGGAG	599	[18]
<i>iutA</i>	GGCTGGACATCATGGGAACCTGG CGTCGGGAACGGGTAGAATCG	302	[19]
<i>fimA</i>	GTTGATCAAACCGTTCAG AATAACGCGCCTGGAACG	540	[24]

Table 2. Occurrence of association of the genes *iss*, *hlyF*, *fimA*, *ompT* and *iutA* in 40 *Escherichia coli* isolates from meat meal.

Genes	n	%
1		
<i>iss</i>	5	12.5
2		
<i>hlyF</i> + <i>iss</i>	2	5
<i>fimA</i> + <i>iss</i>	1	2.5
3		
<i>iss</i> + <i>hlyF</i> + <i>ompT</i>	2	5
<i>iss</i> + <i>hlyF</i> + <i>fimA</i>	24	60
<i>iss</i> + <i>hlyF</i> + <i>iutA</i>	1	2.5
4		
<i>iss</i> + <i>hlyF</i> + <i>ompT</i> + <i>fimA</i>	4	10
All genes		
<i>iss</i> + <i>hlyF</i> + <i>ompT</i> + <i>iutA</i> + <i>fimA</i>	1	2.5
Total	40	100

Table 3. Resistance profile of 40 *Escherichia coli* isolates from meat meal.

Pharmacological Class	Antimicrobial	Resistance profile		
		Sensitive	Intermediate	Resistant
Cephalosporins	Ceftiofur	34 (85%)	-	6 (15%)
	Ciprofloxacin	34 (85%)	2 (5%)	4 (10%)
Quinolones	Norfloxacin	33 (82.5%)	-	7 (17.5%)
	Enrofloxacin	29 (72.5%)	2 (5%)	9 (22.5%)
	Nalidixic Acid	22 (55%)	2 (5%)	16 (40%)
Fosfomycins	Fosfomicin	34 (85%)	1 (2.5%)	5 (12.5%)
Tetracycline	Doxycycline	22 (55%)	8 (20%)	10 (25%)
Aminoglycosides	Gentamicin	39 (97.5%)	-	1 (2.5%)
Penicillin	Amoxicillin	27 (64.5%)	4 (10%)	9 (22.5%)
	Oxacillin	-	-	40 (100%)
	Florfenicol	33 (82.5%)	2 (5.0%)	5 (12.5%)

DISCUSSION

In this study, some of the most frequent virulence genes were investigated, in which an occurrence of 100% was obtained for the *iss* gene in the isolates analyzed. This gene also showed high prevalence in comparative studies between APEC strains and isolates from healthy birds [16,31]. The gene *iss*, is one of the genes most searched in APEC strains, involved in mechanisms that allow the escape of the bacteria from the action of the complement system and the process of phagocytosis, during infection [1].

Although not the only mechanism used by *E. coli* to target organs and trigger infection, the *iss* gene has been associated with high levels of virulence. A survey conducted in different regions of Brazil, with *E. coli* isolates from suspected colibacillosis in birds, obtained prevalences of 78.6%, 63.2%, 58.8% and 46.2% of the *iss* gene in the South-West, South-East and Northeast regions respectively [7]. Due to the high prevalence of colibacillosis, the presence of this gene in isolates from flours of animal origin may serve as a risk indicator for the use of this raw material in the preparation of feed.

Found as the second largest occurrence, the *hlyF* gene was detected in 85% of the isolates analyzed. Similar prevalences were obtained in other studies, with 78.2%, 68.8% and 70.3% [7,17,23]. Coded by ColV plasmid, the *hlyF* gene is used as an epidemiological marker of APEC [14]. Involved with hemolysin production and enhancement of outer membrane vesicles (OMVs), *hlyF* is associated with the release of toxins, responsible for promoting lesions in infected hosts [30]. Complementary studies should be performed to verify if the isolates of this research produce toxins, as well as to verify the pathogenicity of the same, which would imply in a greater risk for the use of these meat meals in the elaboration of rations.

The *fimA* gene was detected in 75% of the isolates, a slightly lower percentage when compared to the *iss* and *hlyF*. A similar result was found in strains of *E. coli* isolated in chickens with a form of colibacillosis, swollen head syndrome, with a prevalence of 64.3%, responsible for adhesion and antigen-binding proteins, this gene plays an important role during infection [25].

With an occurrence of 17.5% the *ompT* gene is involved in a process that includes the proteolytic degradation of antimicrobial peptides (AMPs). Some foodborne pathogens, such as *E. coli*, cause severe

diarrheal diseases, and to colonize the intestine, these pathogens must overcome innate immune defenses, such as antimicrobial peptides (AMPs) [35].

The *iutA* gene had a lower occurrence, this gene plays a relevant individual action, this action was identified in a study that induced suppression of the *iutA* gene, and consequently a significant decrease in the pathogenicity of strains of *E. coli* in the presence of infection in chickens free of pathogens (SPF) [15].

In addition, one study demonstrated an association between the ability to transport the *iutA* gene with antimicrobial resistance, strains capable of transporting *papC* or *iutA* genes were resistant to at least one of the antibiotics tested. In the multivariate analysis, the presence of the *iutA* gene was one of the factors that contributed independently for the strains to be resistant to ampicillin and tetracycline [20].

Among isolates of APEC obtained from birds with colibacillosis in Zimbabwe, the 3 most prevalent virulence genes were *iutA* (80%), *fimH* (33.3%) and *hlyF* (24.4%), *kpsM*, *pstB* and *ompT* had the lowest prevalence, detected in only 2.2% of the isolates [26]. In Egypt, the prevalence of *iroN*, *ompT*, *hlyF*, *iss* and *iutA* genes ranged from 89.5% to 94.7% among APEC strains, while the prevalence among *E. coli* strains isolated in batches of healthy chicken ranged from 10% to 25% and in commensal strains (AFEC) between 46.6% and 53.3% [16]. The *iutA* and *iss* genes are also indicated as molecular markers of virulence for differentiation of strains causing necrotic dermatitis in broilers [9].

In a study carried out with isolates of *E. coli* in Brazil, with broilers and commercial laying hens produced in the State of Pernambuco, it was evidenced that the genes *hlyF* 66.6%, *ompT* 66.6% and *iss* 75% presented higher occurrence in the infraorbital sinuses, while that for the cecal content isolates, the genes *fimA* 52.2% and *iutA* 30.4% were the most frequent in the infraorbital sinuses [4]. Considering the data obtained in the 2 studies carried out in the state of Pernambuco, it can be inferred that the field and input isolates used in the diet present virulence factors that may affect poultry plant health and cause economic losses.

Virulent strains of APEC from different origins showed a higher occurrence of plasmid *colV* genes (*iroN*, *ompT*, *hlyF*, *iss* and *iutA*) when compared to non-pathogenic *E. coli* strains from apparently healthy poultry litter [31]. These studies highlight the relevance

of the genes involved in the infectious processes with *E. coli*, and reinforce the relationship with potentially pathogenic strains.

In this study, the most found genetic combination was *iss+hlyF+fimA*, present in 60% of all isolates and 12.5% showed only the *iss* gene. The combination of *sitAP+ompT+hlyF+iroN* genes with *E. coli* strains isolated from birds with septicemia and absent in commensal strains [14]. This finding highlights the possibility of the identification of pathogenic strains to the detriment of the association of certain virulence genes. In another study, the combinations of *iroN+fimC+ompT+hlyF+iucD+iss+fyuA*, *irp2+tsh+iroN+ fimC+ompT+hlyF+iucD+iss+tsh* and *iroN+fimC+ompT+hlyF+iss* were the most frequent, with 22.8%, 21.8% and 11.9%, respectively [17].

Certain combinations of virulence genes confer the greatest strains on survival, adherence, colonization, and even the ability to develop septicemia, the differentiation of pathogenic strains of *E. coli* from commensals may be through the identification of specific combinations of genes [2].

Multidrug-resistant strains of *E. coli* show a concern for both animal and human health, since the presence of multiresistant strains from domestic poultry can be transmitted from chicken carcasses [34]. The hypothesis of virulence gene transfer and resistance to human ExPEC suggests the zoonotic ability of strains present in chicken products [13].

A survey conducted in Canada, when evaluating ExPEC strains from chicken meat, obtained a similarity level of 55-70% when compared to ExPEC strains isolated from human cultures, with prevalences of 52.3%, 97.73% and 56.8% for *iss*, *iutA* and *ompT*, respectively [3]. These studies demonstrated a similarity between *E. coli* strains of different origins and emphasize the importance of this fact for public health, as well as the increasing antimicrobial resistance presented by such pathogenic strains.

The percentage of resistance, found in this study, was 100% to oxacillin, this can be justified due to the excessive use of this penicillin in the treatment of infections, in the region studied; as for multiresistance, 57.5% of isolates were resistant to more than 1 antimicrobial drug and 27.5% of resistant isolates from 3 to 7 antimicrobial drugs. In Brazil, the susceptibility of *E. coli* strains from

human sources and food products of animal origin was analyzed in a study developed in Bahia, where 26%, 20.2%, 15.4% and 6% of the isolates were resistant to tetracycline, ampicillin, sulfamethoxazole/trimethoprim and cephalothin, respectively; in addition, 22% of the isolates showed resistance to more than 1 antimicrobial and found that *E. coli* from humans and food are resistant to commonly used antibiotics [28].

In Pernambuco, isolates of *E. coli* from broilers and commercial laying hens, presented 94.2% of multiresistance, being resistant to 3 or more antimicrobials belonging to different groups and the presence of detected plasmids demonstrated a possible association with antimicrobial resistance. In relation to multiple antimicrobial resistance indices, showed a variation between 0.07 and 1 in *E. coli* isolates [5]. Results close to the ones found in this study, with IRMA between 0.22 and 0.77, are worrying, since there is a possibility of transmission of multiresistant strains to humans, through food of animal origin or through direct contact, when refers to risk groups such as field workers and veterinarians.

Before the evaluation by pharmacological class, penicillins and tetracyclines were less effective. Tetracyclines have also been cited in other studies, especially in human isolates, with high resistance to this class of antimicrobials [28]; and chicks with different breeding ages showing a gradual increase in resistance with day-old chicks (67%), 30 days (90%) and 1-day before slaughter (94%) [36].

The selective pressure with the consequent permanence of more resistant strains, to the detriment of the indiscriminate use and in greater quantity, of antimicrobial agents in intensive breeding, was pointed out as justification, in a research done, which compared *E. coli* isolates in subsistence and broiler breeding, and indicated a susceptibility score of 46.6% (28/60) and 81% (49/60), respectively [21]. The difference between the results of antimicrobial resistance in the various researches may be related to the routine use of the different antibiotics, once they are used in the treatment and prevention of diseases, where the active principles are chosen according to the protocols adopted in each farm. It is also emphasized that the choice of the antibiotic is carried out empirically, since in some situations antimicrobial susceptibility tests are not performed.

CONCLUSION

It is concluded that the meat meal is contaminated by *Escherichia coli* carrying virulence genes and multiresistant to the antimicrobial agents most used in industrial poultry farming. In this sense, the importance of sanitary control of the inputs used in animal feed is emphasized, as well as the prudent use of these kind of drugs in animal production, with a view to producing a safe food, minimizing not only the economic losses, but also the risks to human health.

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