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In-vivo evaluation of pathogenicity and antimicrobial profile susceptibility from *Escherichia coli* samples isolated from commercial layer hens

Avaliação da patogenicidade **in vivo** *e do perfil de resistência antimicrobiana de amostras de* **Escherichia coli** *isoladas de galinhas de postura comercial*

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ABSTRACT: Antimicrobial sensitivity and pathogenicity level of 90 strains of *Escherichia coli* isolated from livers and intestines from commercial layer hens presenting diarrhea were analyzed. To evaluate the antimicrobial susceptibility, all samples were subjected to antimicrobial susceptibility testing using 11 commercial drugs. The results have showed none of the strains was susceptible to all antibiotics tested. All samples showed resistance to two or more drugs. According to the mortality rate of the birds, the in-vivo pathogenicity test classifies the strains into four classes: high, intermediate, low and nonpathogenic. The test has showed 23 (25.5%) of the samples were highly pathogenic, 21 (23.3%) of intermediate pathogenicity, 23 (25.5%) low pathogenic, and 23 (25.5%) nonpathogenic. When the results of the classes of pathogenicity from isolates have been associated with antimicrobial susceptibility, nonpathogenic strains were less sensitive to the antibiotic ampicillin and increased sensitive to streptomycin antimicrobial compared to the others classes of pathogenic. Nonpathogenic strains showed resistance to many antimicrobials, an alert for poultry, since these bacteria might acquire the virulence genes and infect birds, others animals and even human beings.

KEYWORDS: antimicrobial susceptibility; *Escherichia coli*; pathogenic.

RESUMO: Foram verificados a sensibilidade antimicrobiana e o índice de patogenicidade de 90 amostras de *Escherichia coli* isoladas do fígado e do intestino de pintainhas de postura comercial com diarreia. Para avaliar a sensibilidade antimicrobiana, todas as amostras foram submetidas ao teste de susceptibilidade antimicrobiana por meio de 11 drogas comerciais. Os resultados demonstraram que nenhuma das estirpes foi sensível a todos os antimicrobianos testados. Todas as amostras apontaram resistência a duas ou mais drogas. De acordo com o índice de mortalidade das aves, o teste de patogenicidade *in vivo* classificou as estirpes em quatro classes: alta, intermediária, baixa e não patogênica. O teste revelou que 23 (25,5%) das amostras foram de alta patogenicidade, 21 (23,3%) de patogenicidade intermediária, 23 (25,5%) de baixa patogenicidade e 23 (25,5%) não patogênicas. Quando os resultados das classes de patogenicidade dos isolados foram associados à sensibilidade antimicrobiana, estirpes não patogênicas apresentaram menor sensibilidade ao antimicrobiano ampicilina e maior sensibilidade ao antimicrobiano estreptomicina, quando comparadas com as estirpes das demais classes de patogenicidade. Estirpes não patogênicas exibiram resistência a vários antimicrobianos, representando um alerta para a avicultura, uma vez que essas bactérias podem adquirir genes de virulência e, assim, infectar aves, outros animais e até mesmo o seres humanos.

PALAVRAS-CHAVE: Sensibilidade antimicrobiana; *Escherichia coli;* patogenicidade.

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INTRODUCTION

Escherichia coli is a facultatively anaerobic gram-negative bacillus and belongs to the family Enterobacteriaceae, which is part of the enteric microbiota of mammals and most birds (FERREIRA; KNÖBL, 2009). It is an important pathogen for human and veterinary medicine due to the innumerable health problems caused by intestinal pathotypes — enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic. *E. coli* (EHEC), enteroaggregative *E. coli* (EaggEC) and extra-intestinal pathogenic *E. coli* (ExPEC) (CUNHA et al., 2013). The pathotype that affects the birds is called Avian pathogenic *E. coli* (APEC) and belongs to the category of ExPEC (FERREIRA; KNÖBL, 2009).

In birds, *E. coli* is responsible for different infectious conditions, acting as primary and secondary agent. The bacteria can affect virtually all of the bird's organs, causing intestinal and extra-intestinal infections, known as colibacillosis (BARNES et al., 2003).

In the digestive tract of birds, *E. coli* can be found in concentrations above 10^6 colony forming units per gram of feces. This number may be even higher in young birds, since they do not have established microbiota (LEITHNER; HELLER, 1992). Out of this total, 15 to 20% is potentially pathogenic to birds, because they possess antigenic determinants capable of causing disease (FERREIRA; KNÖBL, 2009).

Stress factors such as deprivation of food and water or exposure to heat may lead to the propagation of *E. coli* from the gut of the birds into the bloodstream, resulting in significant mortality (LEITHNER; HELLER, 1992).

In the available literature, the intestinal infection of the *E. coli* is abundantly mentioned as an unusual disease. However, there are reports that affirm the bacterium is responsible for histopathological lesions in the intestines of pigeons and chicks (WADA et al., 1995; SUEYOSHI et al., 1997).

In recent years, the genetic similarity between APEC and strains has associated with human infections and has become the target of research and health concern in the poultry production chain (MALUTA et al. 2016; MARYVONN et al. 2006; RON, 2006). According to CUNHA et al. (2013), the hypothesis that production animals could serve as reservoirs of potentially pathogenic strain to humans may be strong enough to make avian colibacillosis a new sanitary barrier and impose restrictions on animal-exporting countries.

In poultry, antimicrobials are used to prevent infections to minimize the damage caused by bacterial infections and the incidence of mortality, thus reducing economic losses. However, misuse, its use in animal feed for therapeutic, prophylactic and growth promoting purposes are primarily responsible for the presence of resistance to antibiotics by pathogenic bacteria for animals and for humans (SILVA, 2008).

In turn, these resistant bacteria can be transferred from animals to humans, especially in individuals who work directly with animals or in the processing industries of products of animal origin (BARTON, 2000).

This work aimed to associate pathogenicity in vivo with the antimicrobial resistance profile of strains of *E. coli* isolated from laying hens with diarrhea.

MATERIALS AND METHODS

This work is in accordance with the Ethical Principles in Animal Experimentation, adopted by the Colégio Brasileiro de Experimentação Animal (COBEA), and was also approved by the Comissão de Ética Bioética Bem-estar Animal (CEBEA) from the Comitê de Ética em Experimentação Animal da Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), protocol no. 026702-08.

Twenty flocks of seven-day-old white and brown commercial layer hens were used in this study. The birds were purchased from commercial farms from Bastos city and region, state of São Paulo.

Fifteen birds of each flock presenting smaller size and with diarrhea were selected for the investigation. The birds were taken to the Bastos Research and Development Unit, where they were necropsied, and a pool of fragments of liver and intestine (in separate flasks) of each flock for the isolation of *E. coli* was collected aseptically.

For the isolation and identification of *E. coli*, followed by the technique described by FERREIRA; KNÖBL (2009). All the isolated samples were conserved in Luria Bertani agar (LB) and submitted to the in-vivo pathogenicity test and to the antimicrobial susceptibility test. The pathogenicity test in one-day-old chicks was carried out following the methodology described by KNÖBL (2005) and MONROY et al. (2005). For each sample, ten-day-old male chicks from a commercial source were used, which were inoculated with 0,1 mL of bacterial culture, containing approximately 10^7 colony forming units/mL (UFC/mL) in the left thoracic air sac. The chicks were observed for ten days, and the strains were classified according to the following mortality index: highly pathogenic (mortality ≥ 80%), intermediate pathogenicity (mortality > 50% but < 80%), low pathogenicity (mortality ≤ 50%) and non-pathogenic (no mortality).

For the antimicrobial susceptibility test, the technique of diffusion of the antibiotic impregnated in filter paper disks was used, following the methodology described by

National Committee for Clinical Laboratory Standards (NCCLS, 2008).

All isolates from *E. coli* were transferred to tubes containing 2 mL of saline solution 0,85%, in sufficient quantity to establish a turbidity compatible with the grade 0.5 of the McFarland scale. With a cotton swab, the culture was seeded on plates containing Mueller-Hinton agar (CM 0337). After drying, the plates were placed on the surface of the medium discs of the following antimicrobials: ampicillin (10 mcg), enrofloxacin (5 mcg), erythromycin (15 mcg), spectinomycin (100 mcg), streptomycin (10 mcg), fosfomicina (200 mcg), kanamycin (30 mcg), lincomycin (2 mcg), norfloxacin (10 mcg), sulfa + trimethoprim (25 mcg) and tetracycline (30 mcg). The plates were incubated at 37ºC for 24h. The reading was performed, classifying the strains under analysis according to the following items: sensitive, intermediate or resistant to the antimicrobial tested.

RESULTS AND DISCUSSION

The in-vivo pathogenicity test revealed 23 (25.5%), 21 (23.3%), 23 (25.5%) and 23 (25.5%) of the 90 *E. coli* strains studied were of high, intermediate, low and none pathogenicity, respectively.

The results of the susceptibility test are shown in Figure 1. The test was performed using 11 commercial drugs. The results showed that none of the isolated strains was sensitive to all tested antimicrobials, and all of them were resistant to two or more drugs.

Spectinomycin and fosfomycin were the most efficient antimicrobials; the samples of *E. coli* presented 92.2% and 91.1%, respectively, of sensitivity to these antibiotics.

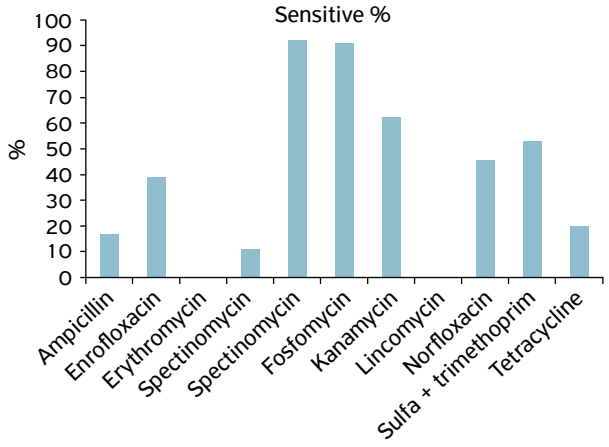

Figure 1. Result of the antimicrobial susceptibility test of 90 strains of *Escherichia coli* against 11 commercial drugs.

Kanamycin, sulfa + trimethoprim and norfloxacin showed moderate antibacterial activity (from 62.2 to 45.6% of sensitive strains). For the antimicrobials enrofloxacin, tetracycline, ampicillin and streptomycin, the sensitivity was lower (38.9 to 11.1%).

Table 1 demonstrates the in-vivo pathogenicity results associated with antimicrobial susceptibility. The results showed that the strains classified in the pathogenicity test as non-pathogenic had lower sensitivity for three antimicrobials: ampicillin (13.04%), enrofloxacin (34.78%) and fosfomycin (86.96%), when compared to those with high pathogenicity (30.43, 39.13 and 95.65%, respectively). In relation to the higher sensitivity presented by non-pathogenic strains, when compared to those with high pathogenicity, it was presented against antimicrobials: streptomycin, kanamycin, norfloxacin, sulfa + trimethoprim and tetracycline, when compared to the high and to the intermediate pathogenicity classes (0%).

None of the four classes of pathogenicity for the strains of *E. coli* has antimicrobial sensitivity for the drugs erythromycin and lincomycin.

Although the use of probiotics and prebiotics contributes to intestinal colonization by bacteria beneficial to intestinal health, those drugs are not always effective in eliminating pathogenic samples from the intestine (CUNHA et al., 2013). These pathogenic strains present in the intestinal tract, when eliminated in feces, promote intense environmental contamination and lead to the occurrence of extra-intestinal and systemic infections, resulting in increased mortality of young birds (PHILIPS et al., 2004).

Non-pathogenic strains of *E. coli* from intestines of animals are important reservoirs of resistant genes to antimicrobial drugs (MARSHALL et al. 1990). According to DAVIES, (1994) and BUERIS (2005), the transmission of virulence genes and plasmid resistance between different genera and bacterial species occur widely.

In this study, non-pathogenic strains isolated from both the liver and the intestine presented resistance to several antimicrobials, representing an alert for poultry farming, since the transmission of virulence genes may occur and infect birds, other animals and even humans. So, the results are important not only for poultry, but also for human medicine, reinforcing the importance of performing the antibiogram before the prescription of the treatment.

CONCLUSION

- None of the strains, no matter the classification degree of pathogenicity, presented sensitivity to all tested antimicrobials;

Table 1. Percentage of *Escherichia coli* strains sensitive to antimicrobials tested, according to pathogenicity classification.

Antimicrobials	% of sensitive strains in each class of pathogenicity			
	High (n = 23)	Intermediate (n = 21)	Low (n = 23)	Non-pathogenic (n = 23)
Ampicillin	30.43	9.52	4.35	13.04
Enrofloxacin	39.13	28.57	47.83	34.78
Erythromycin	0	0	0	0
Streptomycin	0	0	21.74	21.74
Spectinomycin	86.96	100	82.61	100
Fosfomycin	95.65	85.71	95.65	86.96
Kanamycin	60.87	47.62	65.22	69.56
Lincomycin	0	0	0	0
Norfloxacin	39.13	28.57	65.22	47.87
Sulfa + Trimethoprim	52.17	38.09	60.87	60.87
Tetracycline	17.39	9.52	30.43	21.74
Antimicrobials	**% of sensitive strains in each class of pathogenicity**			
	High (n = 23)	**Intermediate (n = 21)**	**Low (n = 23)**	**Non-pathogenic (n = 23)**
Ampicillin	30.43	9.52	4.35	13.04
Enrofloxacin	39.13	28.57	47.83	34.78
Erythromycin	0	0	0	0
Streptomycin	0	0	21.74	21.74
Spectinomycin	86.96	100	82.61	100
Fosfomycin	95.65	85.71	95.65	86.96
Kanamycin	60.87	47.62	65.22	69.56
Lincomycin	0	0	0	0
Norfloxacin	39.13	28.57	65.22	47.87
Sulfa + Trimethoprim	52.17	38.09	60.87	60.87
Tetracycline	17.39	9.52	30.43	21.74

- Strains classified as non-pathogenic in the in-vivo test had a less sensitive profile to the antimicrobial ampicillin;
- *E. coli* samples studied showed higher sensitivity to the antimicrobial spectinomycin;
- It was not possible to claim that more pathogenic samples have higher resistance to the antimicrobials evaluated.

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