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Antimicrobial susceptibility and pathogenicity of *Escherichia coli* strains of environmental origin

Suscetibilidade antimicrobiana e patogenicidade de amostras de *Escherichia coli* de origem ambiental

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ABSTRACT

The study aimed to evaluate the antimicrobial susceptibility of 109 samples of *Escherichia coli* (*E. coli*) of environmental origin and to characterize these isolates according to the degree of pathogenicity in vivo, verifying a possible relationship between this variable and susceptibility to the active principles tested. The isolates were subjected to disc diffusion test to 14 antibiotics. From 16.5% to 90% of the samples were sensitive; 1 - 28.5% showed intermediate degree of susceptibility and between 9 to 78% of *E. coli* analyzed were resistant. The highest resistance percentages were seen in the class of quinolones and tetracyclines (>75%), and for sensitivity in the class of amphenicols (68.8%). By inoculating 1- day - old chicks, the isolates were classified as highly pathogenic (2.7%), intermediate (10.1%), low (42.2%) and apathogenic (45%). It was observed a wide variation in the susceptibility profile of isolates in relation to antimicrobials. It was also found that most of the samples had pathogenic potential (55%), thus being considered as APEC (avian pathogenic *E. coli*). No relationship between pathogenicity and antimicrobial susceptibility ($P \leq 0.05$) was observed.

Key words: *Escherichia coli*, resistance, antimicrobials, pathogenicity, poultry.

RESUMO

O estudo teve como objetivo avaliar a suscetibilidade antimicrobiana de 109 amostras de *Escherichia coli* (*E. coli*) de origem ambiental frente a antibióticos e caracterizar esses isolados quanto ao grau de patogenicidade in vivo, verificando-se uma possível relação entre esta variável e a suscetibilidade aos princípios ativos testados. Os isolados foram submetidos ao teste de disco-difusão para 14 antibióticos. Entre 16.5% a 90% das amostras foram sensíveis, 1-28.5% apresentaram grau de suscetibilidade intermediário e entre 9-78% das *E. coli* analisadas foram resistentes. Os maiores percentuais de resistência foram

encontrados para a classe das quinolonas e das tetraciclínas (>75%), e de sensibilidade para a classe dos amfenicóis (68.8%). Por meio da inoculação em pintinhos de um dia de idade, os isolados foram classificados como sendo de patogenicidade alta (2.7%), intermediária (10.1%), baixa (42.2%) e apatogênicos (45%). Foi observada uma ampla variação no perfil de suscetibilidade das amostras frente aos antimicrobianos. Verificou-se também que a maioria apresentou potencial patogênico (55%), sendo, portanto, consideradas APEC (*E. coli* patogênica para aves). Não foi observada relação entre a patogenicidade e a suscetibilidade aos antimicrobianos ($P \leq 0.05$).

Palavras-chave: *Escherichia coli*, resistência, antimicrobianos, patogenicidade, avicultura.

INTRODUCTION

The *E. coli* is a normal inhabitant of the gastrointestinal tract of mammals and birds. This microorganism is a Gram negative bacterium, within the family *Enterobacteriaceae*, and able to grow under aerobic and anaerobic conditions (BARNES et al., 2008). It is assumed that approximately 10^6 *E. coli* CFU per gram of feces might be detected in the aviaries, making it practically impossible to be eliminated from poultry environment (GROSS, 1994). Of this total, between 10 to 20 percent could be potentially pathogenic, excreted in the feces and, then contaminating the environment (FERREIRA & KNÖBL, 2009).

The *E. coli* strains that can play an important role on avian diseases are collectively

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denominated APEC. It is believed that only specific strains of *E. coli* are virulent, thus triggering the onset of the disease (DELICATO et al., 2003), resulting in local or systemic infection, referred, generally, as avian colibacillosis (FERREIRA & KNÖBL, 2009).

In order to reduce mortality associated to avian colibacillosis, the preventive use of antimicrobials is one of the measures used to minimize the economic impact caused by *E. coli* (CHAUVIN et al., 2007). However, this pathogen is becoming increasingly resistant, a fact that has generated great concern in the poultry industry over the years (PESSANHA & GONTIJO FILHO, 2001; ZANATTA et al., 2004; SMITH et al., 2007). One of the reasons for the increased resistance is the antibiotic therapy for colibacillosis, which does not result in recovery of the birds before slaughter and further exacerbates the problem of colibacillosis, as well as other bacterial diseases. The avian *E. coli* are frequently multi-drug resistant and the practice of indiscriminate use of antimicrobials in poultry industry is the most important factor to promote selection and dissemination of drug resistant microorganisms (GYLES, 2008).

The phylogenetic relationships found among ExPEC (Extraintestinal pathogenic *E. coli*) APEC, UPEC (Uropathogenic *E. coli*) and NMEC (Newborn meningitis-causing *E. coli*) strains highlight the zoonotic potential of strains of avian origin (JOHNSON et al., 2007). Thus, the study of antimicrobial susceptibility of these isolates becomes an important health public issue.

About the pathogenicity of *E. coli*, inoculation assays using chicks have been performed in order to evaluate the *in vivo* expression of virulence factors of APEC (SOUZA, 2010; BARBIERI et al., 2012; BARBIERI et al., 2013). Nevertheless, there are few studies correlating the degree of pathogenicity of the microorganism to the findings of resistance or sensitivity to antimicrobial agents, especially when it comes to samples of environmental origin.

In this context, the present study aimed to evaluate the antimicrobial susceptibility profile of *E. coli* isolated from broiler houses, as well as to characterize the isolates concerning their degree of pathogenicity *in vivo*, in order to investigate a possible relationship between these two variables.

MATERIAL AND METHODS

Sample collection and laboratory processing

During November 2011 and April 2012, overshoe swab samples from broiler houses in Rio

Grande do Sul state were processed in the Poultry Health Laboratory & Technological Innovation of the Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF). Sterile boot socks were put over the shoes and collectors walked throughout a large area of the broiler houses. Later, the boot socks were placed inside sterile bags, sealed, identified and stored in cool boxes. Sample bags were sent to laboratory after collection.

When samples arrived at the laboratory, 225mL buffered peptone water were added to each plastic bag and incubated for 18-24 hours at 37°C. After this, each culture was streaked in MacConkey Agar (MAC) plates and then, incubated under the same conditions described previously. Colonies suspected to be *E. coli* (lactose positive) were re-isolated on MAC and submitted to biochemical test to confirm the genus *Escherichia* (lysine decarboxylase test, Simmon's cytrate, sulphite indol mobility, triple sugar iron, urea hydrolysis), as proposed by MARTINEZ & TRABULSI (2008). Altogether, 109 *E. coli* samples were isolated, each one coming from a single broiler house.

Antimicrobial susceptibility assay

All the *E. coli* isolates (n=109), were submitted to the disc diffusion test according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012), using *E. coli* strain ATCC 25922 as a control. The antimicrobial agents tested were: neomycin (30µg), enrofloxacin (5µg), florfenicol (30µg), ciprofloxacin (5µg), tetracycline (30µg), gentamicin (10µg), nalidixic acid (30µg), doxycycline (30µg), nitrofurantoin (300µg), chloramphenicol (30µg), sulphonamide (300µg) norflaxocin (10µg), ampicillin (10µg) and trimethoprim-sulphonamide combinations (25µg). The interpretation categorized each result as sensitive, intermediate or resistant. For each *E. coli* isolate, Multiple Antibiotic Resistance (MAR) Index was calculated as the number of antimicrobials to which test isolate displayed resistance divided by total number of antimicrobials to which the test organism has been evaluated for sensitivity (KRUMPERMAN, 1983).

In vivo pathogenic evaluation

Groups of 10 1-day-old chicks were inoculated subcutaneously with 0.1mL (~1.5x10⁷ CFU) of an overnight culture (a McFarland 0.5 standard suspension) containing ~1.5x10⁸CFU mL⁻¹ of each isolate. A control group was inoculated with BHI (brain heart infusion) broth. The animals were observed over five days, assessing the lethality rate,

in order to classify the bacterial strains as being apathogenic (absence of lethality), low (<50% of lethality), intermediate (≥ 50 -<80% of lethality) or of high virulence (≥ 80 % of lethality). Chick deaths by *E. coli* were confirmed after postmortem examinations looking for gross lesions related to colibacillosis (airsacculitis, pericarditis, perihepatitis, peritonitis and/or cellulitis). APEC isolates were considered to be those capable to cause lethality of, at least, one bird within the group.

Statistical analysis

Student's t-test was used to verify differences among MAR mean values of groups classified, according to their pathogenicity scores. In order to check a possible relationship between pathogenicity and resistance/sensitivity to the antimicrobials analyzed, the Chi-square test (χ^2) was carried out. All statistical analysis was performed with the Statistical Package for the Social Sciences (IBM SPSS v.18) and statistical significance was accepted at $P \leq 0.05$.

RESULTS

Antibiotic resistance among the isolates

The susceptibility profile of all 109 *E. coli* isolates to antimicrobial agents is shown in figure 1A. With the exception of two isolates, the others were resistant to, at least, one of the antimicrobials. About 42% of the isolates showed MAR index ranging from 0.25-0.5 (resistance from 4 to 7 antimicrobials); 21% with an MAR index of 0-0.25 (resistance to 3 antimicrobials) and only 5.5% showing MAR > 0.75 (resistance to more than 11 antimicrobials). There was no isolate resistant to all of the antimicrobials tested.

When analyzing the classification by chemical group (Figure 1B), it was observed that more than 75% of the isolates were resistant to the tetracycline and quinolone groups. Nonetheless, this percentage drops to 3.7% and 0.9% when excluding nalidixic acid and tetracycline, respectively. Overall, multi-resistance patterns were present in approximately 91% of the *E. coli* isolates. Regarding the sensibility, the greatest percentages were observed in the amphenicols class (68.8%).

Concerning the sites of antibiotic action (Figure 1C), more than 70% were resistant to, at least, one of the antimicrobials that block or inhibit protein synthesis (gentamicin, neomycin, tetracycline, doxycycline, chloramphenicol, florfenicol); nucleic acid synthesis (enrofloxacin,

norfloxacin, ciprofloxacin and nalidixic acid); and folate synthesis (sulphonamide and trimethoprim-sulphonamide combinations). Nitrofurantoin, a drug with a broad mechanism of action, presented the highest percentage of sensibility (47.7%).

Pathogenicity tests

Table 1 shows the classification of the 109 *E. coli* isolates according to their pathogenicity index calculated from the lethality percentages in 1-day-old chicks. It was observed that more than 87.2% of the isolates were classified as low pathogenic and apathogenic strains. Only 10.1% and 2.7% of the isolates presented intermediate and high pathogenicity, respectively.

Pathogenicity versus antimicrobial susceptibility

Apart from the three isolates classified as high pathogenic, the other ones presented antibiotic resistance superior to 78% against quinolones. All of the highly pathogenic strains were resistant to at least one antimicrobial belonging to the beta-lactam and sulphonamides classes; however, the sensitivity was 100% regarding nitrofurans. All groups showed levels of resistance for tetracycline (>65%), amphenicols (20-67%), trimethoprim - sulphonamide (39-67%). Only the isolates of intermediate pathogenicity had resistance superior to 70% for aminoglycosides, whereas in the isolates from other groups, the resistance did not exceed the rate of 48% for this class of antimicrobial.

In relation to MAR indices, no statistical differences were observed, as values ranged from 0.42 to 0.46 (Table 1) among the different groups of pathogenicity. Grouping the strains in pathogenic (high, intermediate and low) and apathogenic, as well as excluding samples with intermediate degree of susceptibility to antibiotics, no statistically significant relationship was observed between variables pathogenicity and sensitivity/resistance to the antimicrobials tested.

DISCUSSION

There was a wide variation in susceptibility profiles among the 109 isolates of *E. coli* to the 14 antimicrobials tested. Between 16.5% and 90% of the isolates were sensitive; 1 to 28.5% of the isolates showed intermediate degree of susceptibility and among 9 to 78% of *E. coli* analyzed were resistant.

The highest levels of resistance were found for nalidixic acid and tetracycline. These findings were also reported by other authors (PESSANHA &

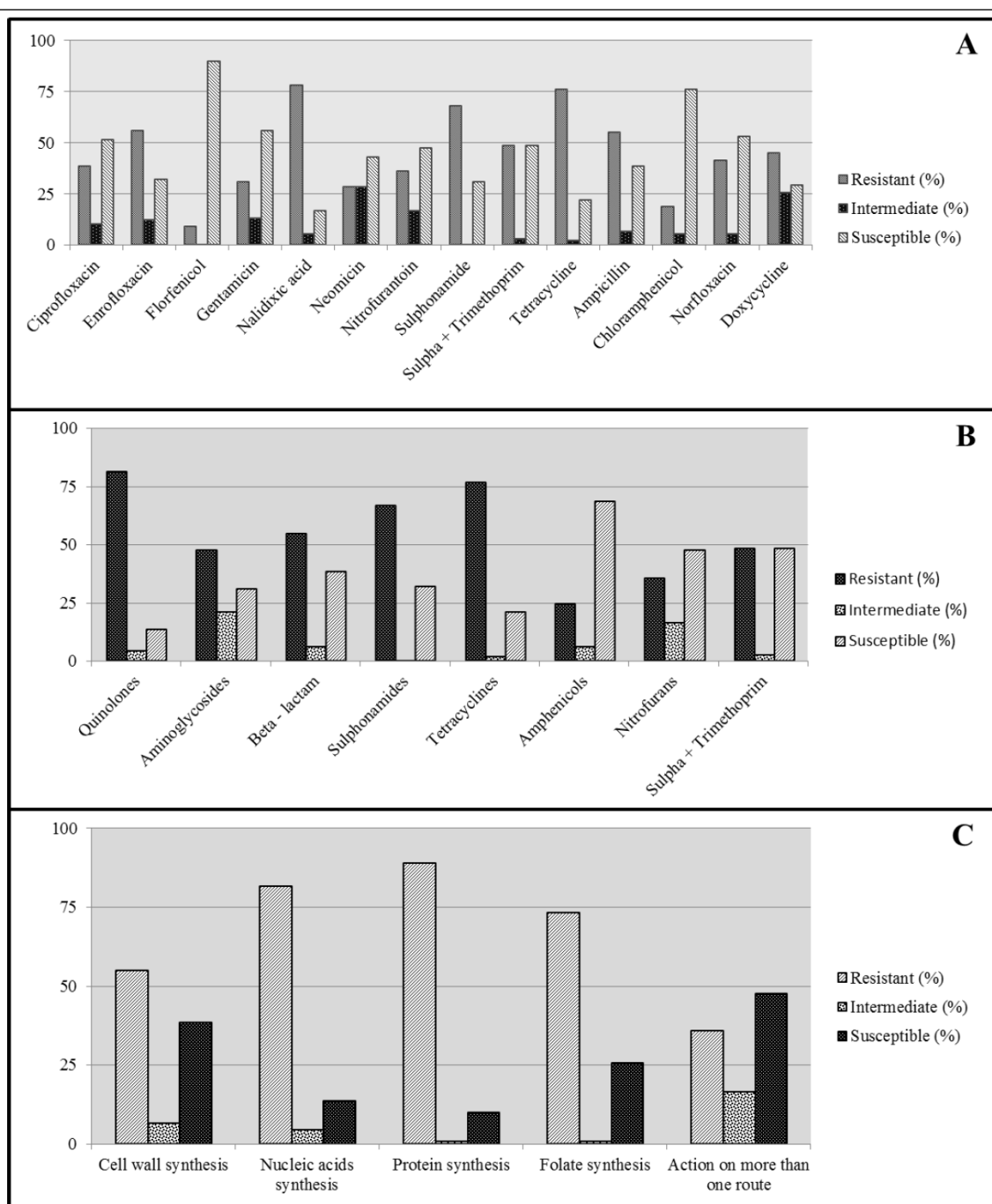


Figure 1 - A: classification of 109 *E. coli* isolates, according to their susceptibility to the 14 antimicrobials tested. B: according to their susceptibility to different classes of antimicrobials. C: according to their susceptibility to different antimicrobials grouped by the action mechanism.

GONTIJO FILHO, 2001; ZANATTA et al., 2004). These results reflect the high levels of resistance found in the class of drugs that act by inhibiting protein and nucleic acids synthesis, encompassing tetracycline and nalidixic acid, respectively.

As reported by MARIETTO GONÇALVES & ANDREATTI FILHO (2010), the low sensitivity to these active principles can be explained, in part, because they have a low cost and are easily obtained, which leads to an indiscriminate and incorrect use

Table 1 - Classification of 109 *E. coli* samples according to the pathogenicity index and MAR mean values obtained for the different antimicrobials.

Pathogenicity index (% lethality)	Samples (%)	MAR
High (>80)	2.7	0.44 ^a
Intermediate (=50 - <80)	10.1	0.42 ^a
Low (<50)	42.2	0.42 ^a
Apathogenic (0)	45	0.46 ^a

Indicated mean values using the same letter do not differ (P=0.05).

of antimicrobial drugs. According to KHAN et al. (2014) the use of tetracyclines with therapeutic purpose in poultry production is very common, which is generating a significant increase in resistance findings. SILVA & HOLLENBACH (2010) claim that susceptibility reduction to quinolones has been observed frequently in different areas of veterinary medicine, mainly for the first generation drugs, as in the case of nalidixic acid. The class of amphenicols and nitrofurans obtained the highest percentages of sensitivity among all antimicrobials tested. This result is possibly due to the banning of these classes as a food additive and for therapeutic purposes in Brazil since 2003 (BRASIL, 2003).

It was noted that no antibiotic was 100% effective, with most of the isolates (91%) presenting multidrug - resistance, even in the case of environmental samples, which, in theory, was expected a greater sensitivity. These findings reflect at MAR indices, being that most of the samples were resistant to four or more antimicrobials. CHINWE et al. (2014), when assessing *E. coli* samples isolated from birds, also reported high levels of resistance in samples of environmental origin. The multidrug resistance's feature has been observed in *E. coli* samples from avian origin in Brazil and other American countries (ZANATTA et al., 2004; SMITH et al., 2007), as well as in Europe (GUERRA et al., 2003), Asia (SAHOO et al., 2012), Africa (OGUNLEYE et al., 2008) and Oceania (INGRAM et al., 2011). This fact demonstrates that the use of antimicrobials in poultry industry over the years has increasingly been favoring the emergence of resistant isolates. According to PESSANHA & GONTIJO FILHO (2001), broilers can act as reservoirs of important antibiotic resistance genes in veterinary and human medicine and the use of growth promoters in animal feed may contribute to the occurrence of multidrug - resistant isolates as well.

Regarding pathogenicity, it was noted that despite being of environmental origin samples, more than 50% of them were pathogenic to a lesser

or greater degree, therefore, being regarded as APEC. This finding is in contrast with data from FERREIRA & KNÖBL (2009), which consider that within *E. coli* of environmental origin; only 10 to 20% are pathogenic. In this study, it was also observed that the pathogenicity of the samples did not interfere on the results of susceptibility to antimicrobials. Hence, the pathogenicity of a sample cannot be considered as primary factor for resistance or sensitivity to a particular drug, under the conditions of this study. Pathogenicity, as well as the high levels of antimicrobial resistance can be explained, likely, by the exchange of genetic material between different bacterial strains, where genes involved in virulence and resistance are constantly transferred, mostly mediated by plasmids. As reported by FLORIAN FRICKE et al. (2009), a single plasmid can possess genes responsible for both antimicrobial resistance and pathogenicity of APEC isolates. The transfer of genetic elements leads an apathogenic strain to present some pathogenic potential and, similarly, isolates that are sensitive to certain antimicrobials can become resistant.

CONCLUSION

There was a large variation in the antimicrobial susceptibility profiles tested. The highest levels of resistance were found for tetracycline and nalidixic acid, as well as the largest percentage of sensitivity for amphenicols. Concerning the pathogenicity, most of the samples presented some pathogenic potential, but this variable had no influence on the degree of susceptibility of *E. coli* regarding the antimicrobials tested.

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