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Characterization and antimicrobial resistance patterns of *Escherichia coli* isolated from feces of healthy broiler chickens

Padrões da caracterização e resistência a antimicrobianos de **Escherichia coli** *isolada das fezes de frangos de corte saudáveis*

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ABSTRACT: Avian pathogenic *Escherichia coli* (APEC) strains are isolated from lesions of poultry presenting colibacillosis, which is a disease that causes either systemic or localized clinical signs. Such strains share many characteristics with *E. coli* strains that cause extra-intestinal illness in humans. There is not a consensus on how to define the APEC pathotype with regard to the presence of virulence traits. On the other hand, in the past few years, five minimal predictors for APEC detection were proposed. The *E. coli* isolates in this work were tested through polymerase chain reaction (PCR) to the five proposed minimal predictors and *cva*C. The strains presenting them were categorized as potential APEC. The APEC and non-APEC categories showed high resistance (> 50%) to cephalotin, erythromycin, streptomycin, sulphametoxazol/trimethoprim, ampicillin, and amoxicillin. Potential APEC strains were significantly more resistant to cephalotin ($p < 0.05$) and neomcycin ($p < 0.01$) than non-APEC. These latter were significantly more resistant to tetracycline ($p < 0.01$) than the potential APEC strains. These results demonstrate that feces of poultry present *E. coli* strains with resistant features, showing or not the potential of causing colibacillosis in poultry. Because APEC and extra-intestinal illness in humans may be similar, these resistant strains are of interest to public health.

KEYWORDS: *Escherichia coli*; avian pathogenic *Escherichia coli*; antimicrobial resistance, broiler chickens.

RESUMO: Cepas de *Escherichia coli* patogênica para aves (APEC) estão isoladas das lesões de frangos com colibacilose, uma doença que causa sinais clínicos sistêmicos ou localizados. As APEC compartilham algumas características com as cepas de *Escherichia coli* que produzem doenças extraintestinais nos seres humanos. Ainda não há um consenso sobre a definição de patotipos das cepas de APEC, no que diz respeito à presença das características de virulência. Entretanto, nos últimos anos, foram definidos cinco indicadores mínimos para a identificação de patotipos das cepas de APEC. Os isolados de *E. coli* utilizados neste trabalho foram testados por meio de reação em cadeia de polimerase (PCR) para os cinco indicadores mínimos e para *cva*C. Os isolados que possuíam os cinco indicadores mínimos foram definidos como potenciais cepas de APEC. As categorias APEC e não APEC apresentaram alta resistência (> 50%) à cefalotina, eritromicina, estreptomicina, sulfametoxazol mais trimetoprim, ampicilina e amoxicilina. Possíveis cepas de APEC foram significativamente mais resistentes à cefalotina ($p < 0,05$) e neomicina ($p < 0,01$) do que as cepas não-APEC. Estas foram significativamente mais resistentes à tetraciclina ($p < 0,01$) do que as possíveis cepas de APEC. Esses resultados demonstram que as fezes dos frangos de corte albergam cepas de *E.coli* com características de resistência, apresentando ou não potencialidade de causar colibacilose. Em função das características de similaridade entre APEC e doenças extraintestinais nos seres humanos, estas cepas resistentes são de interesse à saúde pública.

PALAVRAS-CHAVE: *Escherichia coli*; *Escherichia coli* patogênica para aves; resistência a antimicrobianos; frangos.

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INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) is a large infectious agent present in the modern poultry industry worldwide. Every year, economic losses, in order of millions, due to APEC in the poultry chain (Alekshun; Levy, 1997). The development of diseases caused by *E. coli* in chickens depends on the agent's interaction with the environment and the host. The relevant virulence factors of APEC include the resistance to components of the complement system, adhesins, and the ability to sequester the iron in the host blood and tissues.

Some environmental situations such as high concentrations of ammonia in the shed, poor ventilation, extreme temperatures, wet poultry litter, high density of animals, and inefficient disinfection can act as predisposing factors (Ferreira et al., 2009). APEC belongs to the extra-intestinal pathogenic *E. coli* category and is associated with colibacillosis. This term refers to colisepticemia, peritonitis, pneumonia, pleuropneumonia, sacculitis, pericarditis, cellulitis, swollen head syndrome, among others diseases in poultry (Barnes et al., 2008). Transmission in poultry can occur either by horizontal via (contact with other birds, feces, food, air, and water) or by vertical via (during laying and salpingitis) (Nakazato et al., 2009). There is a continuous excretion of *E. coli* with the potential of causing diseases in birds by poultry faces, contaminating the environment and consequently other birds. Therefore, the main reservoir of *E. coli.*is the poultry's intestine (Rodriguez-Siek et al., 2005).

There is not a consensus on how to define the APEC pathotype regarding its virulence traits. However, a very compressive work has shown the presence of five genes that act as minimal predictors (*iut*A, *hly*F, *iss*, *iro*N, and *omp*T), and can indicate if an *E. coli* strain has a strong potential of causing extra-intestinal diseases in birds (Johnson et al., 2008). Thus, in the present work, we classified the strains containing these five traits as potential APEC. Many of them also harbor colicins (encoded by the gene *cva*), which are proteins expressed by *E. coli* that inhibit the bacterial growth from the same or related species (Nakazato et al., 2009).

Recent studies have indicated a close relationship between APEC and *E. coli* isolates associated with extra-intestinal disease in humans, then a better understanding of APEC can yield benefits not only for animals' health, but also for humans' health (Johnson et al., 2008). Through the continuous antimicrobial selection, microorganisms that are resistant not only to the ingested drugs, but also to other structurally unrelated drugs, may colonize the intestinal microbiota. In addition, in poultry, multidrug-resistant microorganisms can emerge after the application of sub-therapeutic amounts of tetracycline (growth promoter) in their feed (Levy; Marshall, 2004). Populations of *E. coli* in the gut of poultry play an important role in the colonization and spread of pathogenicity characteristics and antimicrobials resistance. Thus, this work aimed at detecting *E. coli* in the feces of healthy broilers, and further at characterizing the isolates, regarding their antimicrobials resistance and genetic profiles.

MATERIAL AND METHODS

Samples were collected from 80 Cobb broilers aged 21 days that represented 25% of a poultry flock. After sampling, the cloacal swabs were immediately placed in tubes containing peptone water. In the laboratory, 0.5 mL of peptone water from each tube was put in tubes with brilliant green broth (HiMedia Laboratories, India) and incubated for 24 hours at 37°C. After incubation, the content was streaked out on MacConkey agar (Oxoid, United Kingdom). Then, it was incubated for 24 hours at 37°C. The colonies (five per plate) with typical *E. coli* characteristics (lactose positive) were biochemically identified through the indole production, methyl red and Voges-Proskauer reactions, citrate utilization, production of urease and hydrogen sulfide (H_2S) after incubation for 24 and 72 hours at 37°C (Koneman et al., 2001).

The microbial DNA template was obtained based on a thermal lysis technique (Keskimaki et al., 2001). Isolates identified as *E. coli* were subjected to polymerase chain reaction (PCR) for detection of *iut*A, *hly*F, *omp*T, and *cva*C (Johnson; Stell, 2000). Furthermore, *iss* and *iro*N were detected using primers and the conditions previously described (Rodriguez-Siek et al., 2005). The isolates were tested for resistance to ampicillin (10 μg), amoxicillin (20 μg), cephalothin (30 μg), enrofloxacin (5 μg), erythromycin (15 μg), streptomycin (10 μg), gentamicin (10 μg), neomycin (30 μg), tetracycline (30 μg), nitrofurantoin (300 μg), and sulfamethoxazole + trimethoprim (1.25 + 23.75 μg). The method used was disk diffusion described by the National Committee for Clinical Laboratory Standards (NCCLS, 2003). Fisher's exact test was employed to compare the antimicrobial resistance of potential APEC *versus* non-APEC.

RESULTS AND DISCUSSION

From the 80 sampled animals, it was possible to detect *E. coli.* in 48 (60%) of them. Five isolates of each positive animal were tested through biochemical reaction, yielding 91 *E. coli* strains. Table 1 presents that more than half of the isolates harbored at least one of the targeted genes. The most frequent profile found was *iro*N + *omp*T + *iss* + *iut*A + *hly*F + *cva*C, followed by *omp*T + *iut*A + *hly*F and *omp*T + *hly*F. Individually, the five most frequent minimal predictor genes detected was *hly*F (49.4%) followed by *omp*T (46%), *iut*A (43%), *iss* (31.7%), and *iro*N (22%). The individual prevalence of *cva*C was 18.7%. We identified 15 (16.5%) APEC

isolates with at least five predictors (either with or without the additional gene *cva*C) in 10 animals.

Overall, the potential APEC isolates were slightly more resistant to the tested antimicrobials than the remaining isolates (non-APEC). There was absolute resistance (100%) in potential APEC isolates to cephalothin, erythromycin, streptomycin, neomycin, ampicillin, and amoxicillin while, for non-APEC, the resistance for these antimicrobials was 73.7, 96.1, 88.2, 42.1, 81.6, and 80.3% respectively. For sulphametoxazol plus trimethoprim, the potential APEC showed a resistance level of 86.7% while the non-APEC indicated 64.5%. However, for the antimicrobials enrofloxacin, nitrofurantoin, tetracycline and gentamicin, the non-APEC isolates were more resistant (27.6, 5.3, 77.6, and 10.5%, respectively) than the potential APEC (6.7, 0, 13.3, and 6.7%, respectively). Potential APEC strains were significantly more resistant to cephalotin ($p < 0.05$) and neomcycin ($p < 0.01$) than non-APEC strains. The non-APEC strains were significantly more resistant to tetracycline ($p < 0.01$) than the potential APEC strains.

In general, all isolates were less resistant to the antimicrobials nitrofurantoin (APEC: 0%; non-APEC: 5.13%) and gentamicin (APEC: 6.7%; non-APEC: 10.5%). More differences between groups were observed for antimicrobials neomycin (APEC: 100%; non-APEC: 42.1%) and tetracycline (APEC: 13.3%; non-APEC: 77.6%). Regarding multiresistance, no isolate was sensitive or resistant to the 11 tested antimicrobials. The potential APEC showed higher multiresistance levels than non-APEC (Table 2).

In this work, it was possible to detect *E. coli* strains in poultry feces presenting the minimal predictors for the APEC patothype. All these strains were multiresistant to antimicrobials. Many strains without the five minimal APEC predictors were also multiresistant.

The gene related to aerobactin (*iut*A), which encodes a ferric aerobactin receptor, was present in 43% of the *E. coli* detected in this work. In a previous investigation (Wooley et al., 1992), the presence of aerobactin was reported in 95% of *E. coli* isolates from chickens with colisepticemia, demonstrating the probable role of such gene on this disease pathogenesis. The ability to capture iron from the environment is associated with the pathogenic ability of strains. The genes *iut*A and *iro*N (other iron uptake related gene) present in 43 and 22% of the isolates of this investigation reinforce this role, since a previous work (Silveira et al., 2002) showed that APEC strains may express iron sequester system, while non-pathogenic may not.

The increased serum survival (ISS) factor is associated with the pathogenicity of APEC, because it is more frequently found among pathogenic strains than in nonpathogenic ones (Pfaff-McDonough et al., 2000). Our results indicate the presence of *iss* gene in 31.7% of the isolates. Its occurrence in the plasmid ColV suggests that there is a relation between the factor ISS and APEC pathogenicity, as previously suggested (Mellata *e*t al., 2003).

The most common genes in the strains were *hly*F (49.4%) and *omp*T (46%), which are linked to hemolytic capacity and production of proteolytic enzymes, respectively. These are important features for the colonization and establishment of ecological niches. The majority of APEC strains have a number of genes such as *cvaC*, *iro*N, *iss*, and *iut*A. Many of them are located on a plasmid known as pTJ100. It has been suggested this group of genes might be useful in defining the APEC pathotype (Rodriguez-siek et al., 2005). Clearly, the pathogenicity of APEC isolates reflects a coordinated action of multiple genes, frequently present in plasmids, which facilitates the ecological dispersion of pathogenic characteristics.

Table 1. Genetic profiles of *E. coli* strains isolated from poultry feces.

Number (%) of strains positive for the indicated genes	Pathotype genes
1 (1.1)	*iss*
2 (2.2)	*iutA*
1 (1.1)	*hlyF*
2 (2.2)	*iroN+ompT+iss+iutA*
2 (2.2)	*iroN+ompT+iss+iutA+hlyF*
13 (14.3)	*iroN+ompT+iss+iutA+hlyF+cvaC*
1 (1.1)	*iroN+ompT+iss+hlyF*
2 (2.2)	*iroN+ompT+iss+hlyF+cvaC*
2 (2.2)	*ompT+iss+iutA*
1 (1.1)	*ompT+iss+iutA+hlyF+cvaC*
1 (1.1)	*ompT+iutA*
11 (12.0)	*ompT+iutA+hlyF*
7 (7.7)	*ompT+hlyF*
1 (1.1)	*iss+iutA*
1 (1.1)	*iss+iutA+hlyF*
1 (1.1)	*iss+iutA+hlyF+cvaC*
3 (3.3)	*iss+hlyF*
2 (2.2)	*iutA+ hlyF*
54 (59.5)	

Table 2. Multiresistance levels of potential avian and non-avian pathogenic *Escherichia coli* strains isolated from the poultry cloaca.

Number of antimicrobials	Number (%) of resistant potential APEC strains	Number (%) of resistant non-APEC strains
0	0	0
1 to 3	0	7 (9.21)
4 to 5	0	10 (13.15)
6 to 10	15 (100)	59 (77.64)
11	0	0

APEC: avian pathogenic *Escherichia coli*.

No single factor can be related unequivocally to APEC pathogenicity (Kariyawasam et al., 2006). Therefore, it is essential to identify microbial populations that potentially may cause intestinal imbalances and diseases affecting the health and the weight gain of poultry. Increasing resistance to first-line antimicrobial agents among *E. coli* isolates represents a potential threat both to animal's and human's health (Diarra et al., 2007). In this study, antimicrobial resistance was more generalized among potential APEC isolates; however, there were variations, and for some antimicrobials, such as enrofloxacin, gentamicin and nitrofurantoin, the non-APEC isolates were more resistant. Furthermore, for tetracycline, the non-APEC isolates showed to be statistically more resistant than the potential APEC. The high resistance found among non-APEC isolates in this work is in accordance with a previous report (Bonnet et al., 2009), in which although potentially virulent *E. coli* isolates tended to carry few antibiotic resistance genes, broiler chickens act as a reservoir for commensal *E. coli* strains carrying large numbers of antibiotic resistance genes. It is noteworthy that all potential APEC strains showed multiresistance, as well as many non-APEC strains. This reinforces the possibility of *E. coli* of avian origin to be a reservoir of resistant genes, raising a concern in public health.

The impact of the drug selection process can be largely confined to the individual taking the antibiotic if widespread antibiotic usage is absent. After therapy, the selected resistant commensal strains will eventually be 'diluted out' and their growth will be suppressed by the return of drug-susceptible and natural competitors (Levy; Marshall, 2004). The problem of widespread resistance among bacterial populations in poultry is that they continually receive antibiotics in feed as growth promoters. If, however, whole populations are treated with the same class of antibiotic, susceptible strains will have little opportunity to recolonize their niche, and resistant strains will acquire an important advantage (Levy; Marshall, 2004). The resulting ecological imbalance produces a potentially serious environmental pool of resistant genes (Alekshun; Levy, 1997), which eventually can be used by APEC strains.

Nonetheless, some studies have tracked a decline in resistance frequencies when an antibiotic is removed (Barbosa; Levy, 2000). Replacement by susceptible flora represents a chief contribution to a decrease in resistant strains (Levy; Marshall, 2004). Thus, one way to reduce the number of resistant strains would be to mix poultry-resistant flora with those who have sensitive flora, causing a dilution of resistant strains among populations. The findings suggest that the fastest way to eliminate resistant strains is to outnumber them with susceptible strains (Levy; Marshall, 2004).

CONCLUSIONS

The strains characterized in this work reflect a resistant and potential pathogenic population that can be spread on the environment by poultry feces, contaminating agricultural workers, agribusiness, and foods. Finally, the profile of these multiresistant isolates is of concern because APEC and human extra-intestinal illness may be similar, and so the zoonotic risk of APEC is increased when the strains show multiresistance.

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