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Detection of the *mcr-1* gene in Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) strains isolated from broilers[1]

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ABSTRACT.- Lopes H.P, Costa G.A., Pinto A.C.L.Q., Machado L.S., Cunha N.C., Nascimento E.R., Pereira V.L.A. & Abreu D.L.C. 2020. **Detection of the *mcr-1* gene in Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) strains isolated from broilers**. *Pesquisa Veterinária Brasileira 40(3):165-169*. Departamento de Saúde Coletiva Veterinária e Saúde Pública, Universidade Federal Fluminense, Rua Vital Brazil Filho 64, Santa Rosa, Niterói, RJ 24230-340, Brazil. E-mail: hugop_lopes@hotmail.com

Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) strains are among the major pathotypes found in poultry and their products, which are capable of causing human enteric infections. Colistin has been claimed the drug of choice against diseases caused by multidrug-resistant Gram-negative bacteria (MDRGN) in humans. The *mcr-1* gene was the first plasmidial gene that has been described to be responsible for colistin resistance and has also been detected in birds and poultry products. Our study aimed to detect the *mcr-1* gene in enteropathogenic strains of *E. coli* in order to evaluate the resistance to colistin in broilers. The material was obtained from 240 cloacal samples and 60 broiler carcasses. The strains were isolated by the conventional bacteriological method and by the virulence genes, which characterize the enteropathogenic strains and resistance, and the samples were detected by polymerase chain reaction (PCR). Of the 213 isolated strains of *E. coli*, 57 (26.76%) were characterized as atypical EPEC and 35 (16.43%) as STEC. The *mcr-1* gene was found in 3.5% (2/57) of the EPEC strains and 5.7% (2/35) of the STEC strains. In this study, it was possible to confirm that the *mcr-1* resistance gene is already circulating in the broiler flocks studied and may be associated with the pathogenic strains.

INDEX TERMS: Detection, *mcr-1*, genes, enteropathogenic, Shigatoxigenic, *Escherichia coli*, strains, broilers, chicken, EPEC, STEC.

RESUMO.- [Detecção do gene *mcr-1* em estirpes de *Escherichia coli* Enteropatogênicas (EPEC) e Shigatoxigênicas (STEC) isoladas de frangos de corte.] *Escherichia coli* Enteropatogênica (EPEC) e Shigatoxigênica (STEC) estão entres os principais patotipos encontrados em aves e produtos avícolas que são capazes de causar doença entérica no homem. A colistina tem sido preconizada como droga de escolha para o tratamento de doenças causadas por bactérias Gram-negativas multirresistentes em humanos. O gene *mcr-1* foi o primeiro gene plasmidial a ser descrito como responsável pela resistência a colistina e tem sido descrito em aves e produtos avícolas. Este estudo tem como objetivo a detecção do gene *mcr-1* em estirpes de *E. coli* enteropatogênicas a fim de avaliar a resistência a colistina em frangos de corte. O material foi obtido a partir de 240 amostras cloacais e 60 carcaças de frango de corte. As estirpes foram isoladas pelo método bacteriológico convencional e os genes de virulência, que caracterizam as estirpes enteropatogênicas, e resistência foram detectados pela reação em cadeia pela polimerase (PCR). Das 213 estirpes de *E. coli* isoladas, 57 (26,76%) foram caracterizadas como EPEC atípica e 35 (16,43%) como STEC. O gene *mcr-1* foi encontrado em 3,5% (2/57) das estirpes EPEC e 5,7% (2/35) das estirpes STEC. Neste estudo foi possível confirmar que o gene de resistência *mcr-1* já está em circulação nos lotes de frango de corte estudados e pode estar associado às estirpes patogênicas.

TERMOS DE INDEZAÇÃO: Detecção, genes, *mcr*-1, estirpes, *Escherichia coli*, enteropatogênicas, Shigatoxigênicas, frangos de corte, EPEC, STEC.

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INTRODUCTION

The Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) strains are pathogens of public health importance due to their ability to cause enteric diseases in humans (Ifeanyi et al. 2016, Fierz et al. 2017, Torres 2017) these pathogens are isolated from poultry and poultry products (Dutta et al. 2011, Alonso et al. 2012, Samanta et al. 2015, Doregiraee et al. 2016, Badi et al. 2018).

The pathotype of EPEC strains are divided into atypical (EPEC-a) and typical (EPEC-t). Both strains have genes that can cause a characteristic lesion to the intestinal epithelium (*eae* gene), called "attaching and effacing" (AE lesion), but only EPEC-t contains the plasmid of *E. coli* adherence factor (EAF) (EPEC adherence factor). The EAF plasmids carry the *bfp* gene that encodes the production of type IV fimbria called the bundle-forming pilus (BFP), which is responsible for the bacterial adherence to enterocytes (Nataro & Kaper 1998). In fact, the EPEC-a pathotypes host humans and a variety of animals, while EPEC-t occurs primarily in humans, but has also been described in some rare cases in captive-bred monkeys, coyotes, and dogs (Souza et al. 2016). These animals are possible reservoirs and sources of infection for humans and the environment. In humans, in most cases, EPEC-induced diarrhea is self-limiting and is treated with rehydration therapy, but requires the use of antimicrobials in persistent infections (Ifeanyi et al. 2016).

Shiga Toxin (STX) is the main virulence factor for the characterization of the STEC pathotype, and the toxins are divided into groups STX 1 and STX 2 and encoded by the genes stx_1 and stx_2, respectively. The STEC strains may present both genes alone or associated with. Patients infected with STX2-producing strains develop more frequently anemia, acute renal failure and thrombocytopenia, characterizing uremic hemolytic syndrome (UHS), than those infected with STX2-producing strains (Nataro & Kaper 1998). Some STEC strains are related to EPEC because they contain the *eae* gene, which is responsible for producing the AE lesion (Alonso et al. 2012). The presence of the *eae* gene increases the virulence of STEC strains carrying both the stx_1 and stx_2 genes, associated or not (Beutin & Martin 2012). As with EPEC, antibiotic therapy is indicated only in severe cases (Menne et al. 2012). These drugs should be avoided in the diarrheal phase of infection of this pathotype, as they may be responsible for the rupture of the bacterial membrane causing the release of STX, which may aggravate the cases of the disease (Wong et al. 2012).

Colistin antimicrobial, from the group of polymyxins, acts on these pathotypes on the bacterial cell wall (Giske 2015). Due to the emergence of resistance of these pathogenic bacteria to multiple antimicrobials, colistin has reappeared as the last treatment choice (Rolain et al. 2014). Evidence of colistin resistance involving chromosomal genes has been described in other bacteria, mainly *Klebsiella pneumoniae* (Cannatelli et al. 2013). However, Liu et al. (2016) were the first research team to highlight the role of the *mcr-1* plasmid gene that encodes an enzyme that alters bacterial cell wall structure by inhibiting the action of the antibiotic on the cell.

The recommended test for phenotypic characterization of colistin resistance is the broth microdilution test. However, due to this antimicrobial characteristic of adhering to various materials, including plastics, the phenotypic characterization using this technique, as well as the disc diffusion technique, does not guarantee reliable results (Karvanen et al. 2017). Given the consequent difficulty in phenotypic detection in clinical laboratories, the Agência Nacional de Vigilância Sanitária (ANVISA) (Brasil 2016a) recommends the detection of the *mcr-1* gene by molecular techniques.

Human contamination by multidrug-resistant (MDR) *E. coli* strains from poultry products has been confirmed by Johnson et al. (2007). In this context, the presence of the *mcr-1* gene becomes relevant due to the possibility of transferring colistin resistance to other bacteria of the human microbiota (Liu et al. 2016). In Brazil, the warning about the presence of these genes in bacteria isolated from animals and their products came from the detection of the *mcr-1* gene in *E. coli* and *Salmonella* spp., isolated from chickens and swine (Fernandes et al. 2016) and also in humans (Rossi et al. 2017).

The objective of this study was to evaluate the presence of the *mcr-1* gene in *E. coli* strains of EPEC and STEC pathotypes isolated from commercial broilers.

MATERIALS AND METHODS

The experimental protocol of this study followed the Ethical Principles in Animal Experimentation of the "*Sociedade Brasileira de Ciência em Animais de Laboratório"* (SBCAL) and obtained the approval of the Animal Use Ethics Committee (AUEC) of the Universidade Federal Fluminense (UFF), under no. 697.

Material collection. The samples analyzed were obtained from six slaughterhouses supervised by the State Inspection Service (SIE), in the eastern and southern regions of the state of Rio de Janeiro.

In the reception area of each slaughterhouse, 40 broiler chickens were selected to collect cloacal material. The material was collected as the aid of swabs, which were placed in groups of four swabs in tubes containing Cary Blair (OXOID®) medium, in a total of 10 tubes per batch. From the same batch, ten carcasses were randomly selected and removed from noria after dripping and individually wrapped in sterile bags. All samples were transported in recycled ice isotherms.

Conventional bacteriological isolation. In the laboratory, each pool of swab was washed and grown in tubes containing 10mL peptone saline (SSP) and 400mL of this solution added to the bags. Ten milliliters (10mL) from each carcass was removed and transferred to sterile tubes. All samples were incubated for 24h at 37°C according to the method recommended by the United States Department of Agriculture (USDA 1998).

After this period, all samples were seeded on MacConkey agar (HIMEDIA®) with subsequent incubation for 24h at 37°C. From each culture, three colonies with *Escherichia coli* compatible characteristics were selected for biochemical characterization using Triple Sugar Iron (TSI) (PRODIMOL®), Sulphide Indole Motility (SIM) (HIMEDIA®), Methyl Red Broth media (MR) and Voges Proskauer (VP) (MICRO MED®) and Citrate Agar (HIMEDIA®) (MacFaddin 2000).

PCR. All *E. coli* positive samples were subjected to DNA extraction by the thermal method (Andreatti Filho et al. 2011) and subsequently sent to the polymerase chain reaction (PCR) analysis for the detection of the virulence genes *eae*, stx_1, stx_2 and *bfp*, using gene-specific primer pairs (Table 1).

For the amplification reaction of the *eae* gene, 1X Buffer 10X was added to each 100ng of DNA extracted in the previous step; 1.5mM MgCl2, 0.2mM dNTP, 0.4μM of each primer (Table 1), 1U Taq Polymerase; totaling the final volume of 25μL.

In the reaction for detection of *stx-1* and *stx-2* genes, 1X of 10X Buffer was added to each 100ng of extracted DNA, 2mM MgCl2, 0.4mM

Table 1. Primer oligonucleotide sequence and size of PCR products obtained for detection of virulence genes of Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) pathotypes and colistin resistance gene in broiler chickens

Primer oligonucleotides	Sequences	Size of PCR products	References
eae-F	5' GACCCGGCACAAGCA TAAGC 3'	384pb	Dutta et al. (2011)
eae-R	5' CCACCTGCAGCAACAAGAGG 3'		
stx_1-F	5' ATA AATCGCCATTCGTTGACTAC 3'	180pb	Dutta et al. (2011)
stx_1-R	5' AGAACGCCCACTGAGATCATC 3'		
stx_2-F	5' GGCACTGTCTGAAACTGCTCC 3'	255pb	Dutta et al. (2011)
stx_2-R	5' TCGCCAGTTATCT GACATTCTG 3'		
Bfp-F	5' GGAAGTCAAATTCATGGGGGTAT 3'	300pb	Vidal. (2005)
Bfp-R	5' GGAATCAGACGCAGACTGGTAGT 3'		
mcr1	5' CGGTCAGTCCGTTTGTTC 3'	309pb	Liu et al. (2016)
mcr1	5'CTTGGTCGGTCTGTA GGG-3'		

dNTP, 0.4μM of each specific primer (Table 1), 1U Taq Polymerase; totaling the final volume of 25μL.

For the *bpf* gene amplification reaction, every 100ng of DNA extracted in the previous step was added 1X 10X Buffer, 1.5mM MgCl2, 0.2mM dNTP, 0.4 of each primer (Table 1), 1U Taq Polymerase; totaling the final volume of 25μL.

Amplification was performed in a thermal cycler (Programmable Thermal, Controller-PTC-100). After prior denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 45 seconds, 59°C for 45 seconds, 72°C for one minute and a final extension at 72°C for 6 minutes were used (Dutta et al. 2011).

For the amplification reaction of the *mcr-1* gene were added to each 2.0μL of DNA, extracted in the previous step, 1X of 10X Buffer, 1.5mM MgCl2, 0.2mM dNTP, 0.2mM of each primer (Table 1), 1U Taq Polymerase; totaling the final volume of 25μL.

Amplification was performed in a thermal cycler under the following conditions: 94°C for 15 minutes; followed by 25 cycles with denaturation at 94°C for 30 seconds; annealing of the primers at 58°C for one minute and 30 seconds and extension at 72°C for one minute. After these cycles, a final step of 72°C was followed for 10 minutes and the samples were kept for 30 minutes at a temperature of 4°C.

The PCR products were submitted to 1.5% agarose gel electrophoresis and observed in a transilluminator under ultraviolet light.

Statistical analysis. Fisher's exact test, with a significance level of 0.05, was used to evaluate the frequency association between EPEC and STEC strains and the presence of the *mcr-1* gene in cloaca and carcass.

RESULTS AND DISCUSSION

A total of 213 strains of *Escherichia coli* were isolated from the analyzed samples, being 107 isolated from carcasses and 106 isolated from cloacas.

EPEC and STEC characterization

Of the total strains isolated, 26.8% (57/213) were characterized as EPEC harboring only the *eae* gene. Of these, 53% (30/57) were isolated from the cloacas and 47% (27/57) from carcasses. None of the EPEC strains harbored the *bfp* gene and were therefore characterized as EPEC-a (Girón et al. 1993, Gomes & Trabulsi 2008), which was expected since only humans have been identified as EPEC-t hosts (Kaper et al. 2004) (Table 2). Probably the strains found in the study have reduced dissemination capacity in the poultry environment due to the absence of the *bfp* gene, also responsible for the adhesion of the bacteria at the binding site (Nataro & Kaper 1998).

The EPEC-a strains have been detected in birds reared in the conventional system and its products (Dutta et al. 2011, Alonso et al. 2012, Samanta et al. 2015, Doregiraee et al. 2016, Badi et al. 2018), as occurred in the present study.

Of the isolated strains, 16.4% (35/213) were characterized as STEC, being 54% (19/35) isolated from carcasses, and 46% (16/35) of the cloacas. The presence of both stx_1 and stx_2 genes characterizes the strains of the STEC pathotype, and genes may be present alone or associated, including the *eae* gene (Gomes & Trabulsi 2008).

Of the isolated STEC strains, 66% (23/35) presented the *stx1* gene associated with the *eae* gene, 20% (7/35) presented the stx_1 gene associated with the stx_2, 5.6% (2/35) presented only the stx_1 gene, 5.6% (2/35) presented the stx_2 gene and only 2.8% (1/35) the *stx2* gene associated with *eae* gene. No single strain had the stx_1 and stx_2 genes associated with the *eae* gene (Table 2). Among the STEC strains, the stx_1 gene in

Table 2. Frequency of virulence genes (*eae*, *bfp*, stx_1, stx_2) and colistin resistance gene (*mcr-1*) in strains of Enteropathogenic *Escherichia coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) pathotypes isolated from broiler chickens

		EPEC	STEC
Virulence genes	*eae*	57 (100%)	-
	bfp	0	0
	stx_1	0	2 (5.6%)
	stx_2	0	2 (5.6%)
	stx_1 + stx_2	0	7 (20%)
	stx_1 + *eae*	0	23 (66%)
	stx_2 + *eae*	0	1 (2.8%)
	stx_1 + stx_2 + *eae*	0	0
TOTAL		57 (100%)	35 (100%)
Resistance gene	*mcr-1*	2/57 (3.5%)	2/35 (5.7%)

association with the *eae* gene is more frequent than reported by Dutta et al. (2011) and Samanta et al. (2015), demonstrating that these genes are in greater circulation in the STEC of birds and products studied. Although stx_2 gene is associated with higher virulence of STEC strains, the association of stx_1 gene with *eae* gene has been responsible for increased virulence of STEC strains (Beutin & Martin 2012).

There was a significant difference (p=0.0099), by Fisher's test, between the presence of EPEC and STEC strains presented in this study, being isolated a higher percentage of EPEC-a pathotype strains (26.8%) in relation to STEC (16.4%), corroborating with Alonso et al. (2012) who observed 3.9% and 3.3% percentages of EPEC-a and STEC in carcasses and 11.9% and 0.1% of these same pathotypes in cloacas, respectively. Regardless of the detected pathotype, both are of great public health importance because they are responsible for human enteric diseases (Blanco et al. 2006, Rasko et al. 2011, Canizalez-Roman et al. 2016).

Detection of the *mcr-1* gene. Of the strains isolated, 9/213 presented the *mcr-1* gene by PCR, 6/9 from carcasses, and 3/9 from the cloacas. There was no statistical difference between the percentage of strains carrying the *mcr-1* carcass or the cloaca gene (p=0.4984). Fernandes et al. (2016) and Irrgang et al. (2016) reaffirmed the circulation of the *mcr-1* gene in percentages from 0 to 5.3% in live and broiler chicken meat.

Although the first description of the *mcr-1* gene was in 2015 in China by Liu et al. (2016), this gene was detected in isolated *E. coli* strains in Germany in 2010 (Irrgang et al. 2016). Current work corroborated this study by describing the circulation of this gene in several countries to date (Fernandes et al. 2016, Trung et al. 2017, Clemente et al. 2019, Dominguez et al. 2019). Therefore, it is likely that the selective pressure of gene-bound that confers colistin resistance occurred even before its prohibition on animal production in some countries such as Brazil (Brasil 2016b).

Detection of the *mcr-1* gene in poultry and carcasses in the present study is of concern because sensitive strains present in humans may become resistant if plasmid transfers from strains containing the *mcr-1* gene from birds and products by manipulation or ingestion. Liu et al. (2016), suggested that the origin of plasmid from the *mcr-1* gene favors gene transfer to sensitive bacteria from the environmental microbiota, farm animals and humans; this fact may lead to increased resistance of these strains to colistin.

EPEC and STEC strains with the *mcr-1* gene. In this study, it was found that the *mcr-1* gene was present in 3.5% (2/57) of EPEC strains and 5.7% (2/35) of STEC strains (Table 2). The presence of the *mcr-1* gene in these strains is of concern because it is pathogenic to humans, posing a public health risk. The use of antimicrobials to treat infections caused by the EPEC and STEC pathotypes is recommended only in severe cases (Wong et al. 2012, Ifeanyi et al. 2016). As colistin has been cited as a last resort for the treatment of multidrug-resistant bacteria, resistance to this antimicrobial is very important, as it may be a limiting factor for the successful treatment of infections.

CONCLUSION

It was possible to detect the presence of EPEC and STEC strains and to confirm that the *mcr-1* gene is circulating in these strains in live broilers and carcasses, representing a potential public health risk. This study seems to be the first report of the circulation of the *mcr-1* resistance gene in the state of Rio de Janeiro.

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Conflict of interest statement.- The authors have no competing interests.

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