"Obtaining transconjugant *Escherichia coli* costs isolated from chicken litter and chicks"

"Obtenção de cepas transconjugantes de *Escherichia col*i isoladas de cama de frango e pintainhos"

DOI:10.34117/bjdv7n2-474

Recebimento dos originais: 10/01/2021
Aceitação para publicação: 10/02/2021

Bruna Carolina Gonçalves
Mestranda em Microbiologia pelo Programa de Pós-graduação em Microbiologia da Instituição Universidade Estadual de Londrina (UEL)
Endereço: Rodovia Celso Garcia Cid, PR-445, Km 380 - Campus Universitário, PR, 86057-970

Miriam Dibo
Mestranda em Microbiologia pelo Programa de Pós-graduação em Microbiologia da Instituição Universidade Estadual de Londrina (UEL)
Endereço: Rodovia Celso Garcia Cid, PR-445, Km 380 - Campus Universitário, PR, 86057-970

Luís Eduardo de Souza Gazal
Pós-Doc em Microbiologia pelo Programa de Pós-graduação em Microbiologia da Instituição Universidade Estadual de Londrina (UEL)
Endereço: Rodovia Celso Garcia Cid, PR-445, Km 380 - Campus Universitário, PR, 86057-970
mail: gazal_26@hotmail.com

Leonardo Pinto Medeiros
Pós-Doc em Microbiologia pelo Programa de Pós-graduação em Microbiologia da Instituição Universidade Estadual de Londrina (UEL)
Endereço: Rodovia Celso Garcia Cid, PR-445, Km 380 - Campus Universitário, PR, 86057-970

Maísa Fabiana Menck Costa
Mestranda em Microbiologia pelo Programa de Pós-graduação em Microbiologia da Instituição Universidade Estadual de Londrina (UEL)
Endereço: Rodovia Celso Garcia Cid, PR-445, Km 380 - Campus Universitário, PR, 86057-970

Renata Katsuko Takayama Kobayashi
Doutora em Microbiologia pelo Programa de Pós-graduação em Microbiologia da Instituição Universidade Estadual de Londrina (UEL)
Endereço: Rodovia Celso Garcia Cid, PR-445, Km 380 - Campus Universitário, PR, 86057-970

Benito Guimarães Brito
Doutor no Instituto de Pesquisa Veterinárias Desidério Finamor/DDPA/SEAPDR

Endereço: Estrada do Conde, 6000 - Sans Souci, Eldorado do Sul - RS, 92990-000

Gerson Nakazato
Doutor em Microbiologia pelo Programa de Pós-graduação em Microbiologia da Instituição Universidade Estadual de Londrina (UEL)
Endereço: Rodovia Celso Garcia Cid, PR-445, Km 380 - Campus Universitário, PR, 86057-970

ABSTRACT
It is known that chicken litter is home to many pathogenic and antimicrobial resistant bacteria, and in many cases, it is reused without prior treatment in the creation of subsequent batches. Thus, the work aimed to verify whether *Escherichia coli* strains that produce Extended Spectrum Beta-lactamases (ESBL) isolated from chicken beds in the state of Paraná, were able to transfer their genes to *E. coli* isolates from the intestinal microbiota of one day old chicks. For this study, four strains of *E. coli* producing ESBL were used, presenting the $bla_{CTX\text{-}M1}$ gene isolated from chicken litter, to be the plasmid donors, and three strains isolated from chicks newly arrived at the shed, which showed resistance to only gentamicin. For the conjugation assays, the donor and recipient strains were used in a proportion of 1: 2 respectively, and the transconjugants selected for resistance to gentamicin and cefotaxime in specific concentrations. Transconjugant colonies were selected and their phylogenetic groups identified by the PCR technique. Of the 12 conjugations performed, three presented transconjugants harboring the $bla_{CTX\text{-}M1}$ gene, indicating that there is a possibility that this gene could be transferred to *E. coli* of the microbiota. Thus, in the environment of the farms, the transmission of plasmids of resistance from bacteria present in the chicken litter to bacteria from the microbiota of the newly arrived chicks may occur. The fact that chicken litter is reused without proper treatment contributes to the maintenance and dissemination of genetic determinants for ESBL in broiler chickens.

Keywords: Conjugation; Bacterial resistance; APEC.

RESUMO
Sabe-se que cama de frango abriga muitas bactérias patogênicas e resistentes aos antimicrobianos, e em muitos casos ela é reutilizada sem o tratamento prévio na criação de lotes subsequentes. Assim, o trabalho teve como objetivo verificar se cepas de *Escherichia coli* produtoras de Beta-lactamases de Espectro Estendido (ESBL) isoladas de camas de frango no estado do Paraná, eram capazes de transferir seus genes a isolados de *E. coli* da microbiota intestinal de pintainhos de um dia. Para este estudo foram utilizadas quatro cepas de *E. coli* produtora de ESBL apresentando o gene $bla_{CTX\text{-}M1}$ isoladas da cama de frango, para serem as doadoras de plasmídeos, e três cepas isoladas de pintainhos recém-chegados ao galpão, que apresentaram resistência a apenas gentamicina. Para os ensaios de conjugação, as cepas doadoras e receptoras foram utilizadas na proporção de 1:2 respectivamente, e as transconjugantes selecionadas por resistência à gentamicina e cefotaxima em concentrações específicas. As colônias transconjugantes foram selecionadas e seus grupos filogenéticos identificados pela técnica da PCR. Das 12 conjugações realizadas três apresentaram transconjugantes abrigando o gene $bla_{CTX\text{-}M1}$ indicando que existe a possibilidade desse gene ser transferido a *E. coli* da microbiota. Sendo assim, no ambiente das granjas pode ocorrer a transmissão

de plasmídeos de resistência de bactérias presentes na cama de frango para bactérias da microbiota dos pintainhos recém-chegado. O fato de a cama de frango ser reutilizada sem um tratamento adequado contribui para a manutenção e disseminação de determinantes genéticos para ESBL em granjas de frangos de corte.

Palavras-Chave: Conjugação; Resistência bacteriana; APEC.

1 INTRODUCTION

Brazilian poultry production has shown high production rates, conquering a significant space in the world market. Currently, Brazil is the second largest producer of chicken meat in the world, having produced around 13.3 million tons in 2019. Of all this production, around 68% is destined for the domestic market and 32% for export (ABPA , 2020).

This large poultry production results in a large production of chicken litter, necessary for the accommodation of birds on farms, since about 1.75 kg of litter is produced per broiler chicken (Dos Santos et al, 2005). To reduce production costs and environmental impact, a management commonly used in chicken rearing is the reuse of the litter for a variable period of five to six consecutive batches, however the chicken litter contains a high concentration of nutrients, since feed remains, bird feathers and excrement are found, an environment conducive to the spread of microorganisms (Virtuoso et al., 2015).

However, the big problem in relation to the period or number of reuses lots is more related to the sanitary aspect, it is not recommended to reuse the bed when the previous lot has undergone some relevant sanitary challenge. In cases where the aviary has not gone through a period of health challenge, bed reuse can be performed as long as its treatment is adequate in order to reduce the bacterial population present, including possible pathogenic bacteria (Virtuoso et al., 2015).

The composting process is the safest method for reducing bed contaminants. At the beginning of this process, the bio-oxidative phase occurs, in which the microorganisms present carry out the metabolization of the bed components, resulting in CO_2, NH_3, water, organic acids and heat. The heat accumulates inside the bed and promotes an increase in its temperature, causing a reduction in the number of pathogens in the compound (Bernal et al., 2009). However, some studies have identified *Escherichia coli* strains harboring resistance and virulence genes, isolated from chicken beds after the composting process (Puño-Sarmiento et al., 2014; Gazal et al., 2015).

Although the process of antimicrobial resistance is natural, over the years it has become increasingly common to find strains of Enterobacteriaceae that produce the extended spectrum B-lactamase (ESBL) and AmpC due mainly to the increased use of antimicrobials during production avian. These enzymes are mediated by plasmid genes and can be transferred horizontally between bacteria present in the environment and in animals. Thus, the use of antimicrobials in the production, led to the selection of resistant bacteria against sensitive ones (Gazal et al., 2021).

2 MATERIALS AND METHODS

Bacterial samples were collected from farms in the states of Paraná and Rio Grande Sul. Collections were carried out in each state, on the first day of production. Initially samples were collected from 20 chickens by cloacal swab and samples from the aviary environment were collected at 10 different points of the production house. The samples were processed and tested for bacterial sensitivity to antimicrobials and phylogenetic classification were performed.

From the previous isolation of the samples, bacterial strains of the multidrug-resistant chicken litter and commensal strains of the chicks that had resistance to at least one antibiotic were chosen, being necessary that the bacterial strains of the chicken litter were sensitive to the antibiotic in which the chicks were resistant and that the resistance found in the chicks was sensitive in the strains of chicken litter, characteristics necessary for the selection of transconjugants at the end of the test. Thus, for this selection, the strains were grown in Luria-Bertani broth (LB) overnight and sown in plates, only with MacConkey agar, MacConkey supplemented with gentamicin, MacConkey supplemented with cefotaxime and lastly MacConkey supplemented with gentamicin and cefotaxime, in concentrations following the norms of the “Clinical and Laboratory Standards Institute” (CLSI, 2018). Then, selecting four donor strains multiresistant and sensitive to gentamicin: PR01 and PR02 from the phylogenetic group B1, PR09 from group D and PR16 from group A and all samples are positive for $bla_{CTX\text{-}M55}$, $bla_{CTX\text{-}M8}$ and *fosA3*. Three recipient strains resistant only to gentamicin: RS01, RS02 and RS03, all belonging to the phylogenetic group D.

One colony of each chosen strain was added in LB broth under agitation at 36ºC for overnight growth. Then, 1 ml of each strain was pipetted, placed in a microtube, and centrifuged at 12,000 g for two minutes at 25ºC, the supernatants were discarded and resuspended in 1 mL of LB broth. Then, a volume of 200 µL of a recipient was added

with 100 µL of a donor in a new microtube already containing 4 mL of new LB broth, totaling nine pairs of conjugation, the tubes were incubated in an oven without shaking at 36º during 24 hours. After that, 100 µL of the bacterial strain were seeded with a Drigalski loop on LB agar supplemented with gentamicin at a concentration of 10 µg / mL and cefotaxime with 6 µg / mL and the plates were incubated at 36ºC during 24 hours.

Colonies grown on the selection plates were re-isolated on LB agar supplemented with gentamicin (10 µg / mL) and cefotaxime (6 µg / mL) and placed in the oven at 36ºC for growth, overnight. Then, 3 to 4 colonies of each culture were added in 200 µL of deionized water in a microtube and put to boil for 10 minutes in a water bath at 100ºC, then they were centrifuged for 10 minutes at 12,000 g and 100 µL of the supernatant was stored in a new microtube for use in PCR.

The genetic material obtained was used to identify the $bla_{CTX\text{-}M55}$, $bla_{CTX\text{-}M8}$ and *fosA3* genes and the *chu*A and *TspE4* and *yja*A genes for the phylogenetic classification of transconjugated strains, according to Clermont et al. (2000).

3 RESULTS AND DISCUSSION

After the conjugation assay, seven transconjugants strains were obtained, which were named “T1” to “T7”. When isolating the transconjugants, one growth had two types of colony and they are named “T5a” and “T5b”, whereas strains PR09 + RS01 and PR09 + RS03 in which the donor and recipient had the same group phylogenetic did not show colonies growth.

In all cultures with transconjugants strains, it was possible to observe a high rate of conjugation, because in all cases the growth in the culture medium with the selection marks, the growth occurred throughout the plate, requiring re-isolation for the separation of colonies to be studied.

With the PCR it was possible to classify which phylogenetic group the transconjugants belonged to, since the group obtained at the end would represent which of the strains (donor or recipient) had received the plasmid, because despite the exchange of mobile DNA the chromosomal DNA would remain the same and guarantee the preservation of the phylogenetic group.

Thus, the sample T1, T2, T4 and T6 presented phylogenetic classification D, which shows the transfer of the resistance plasmid from the sample that we classified as a donor to the one that we classified as a recipient, whereas samples T3 belonged to phylogenetic group A and the sample T7 also belongs to the phylogenetic group A

demonstrating that the transfer of the resistance plasmid occurred from the "recipient" to the "donor". The sample T5a, on the other hand, belongs to the phylogenetic group B1 and T5b belongs to group D, which demonstrates that in this case the exchange of plasmids occurred in both directions: "recipient" to "donor" and "donor" to recipient ", respectively. In all cases, the transconjugant strains became resistant to gentamicin and cefotaxime.

The PCR for the $bla_{CTX\text{-}M55}$, $bla_{CTX\text{-}M8}$ and *fosA3* genes demonstrated that Two out of four transconjugants, of which the plasmid was passed from donor to recipient, were positive for these genes.

With the results of the conjugation, it was possible to affirm that it is possible to spread resistance genes within avian production (Gazal et al., 2021) in addition, other studies have indicated the possible transmission between avian strains and strains of human microbiota, characterizing a possible zoonotic problem (Cyoia et al., 2019).

In addition, it was also possible to observe that resistant transconjugants occur without the transfer of the $bla_{CTX\text{-}M55}$, $bla_{CTX\text{-}M8}$, fosA3 genes, indicating that the samples had other plasmids and they had different resistance genes, reinforcing that multidrug-resistant strains are able to spread resistance plasmids in the environment of farmers

4 CONCLUSIONS

With this work, it can be observed that in the farm environment, the transfer of resistance plasmids from bacteria present in the chicken bed to bacteria from the microbiota of the newly arrived chicks and vice versa can occur. The fact that chicken litter is reused without proper treatment contributes to the maintenance and dissemination of genetic determinants for ESBL-producing bacteria in broiler chickens.

ACKNOWLEDGMENTS

To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) that financed the project, to Prof. Dr. Gerson Nakazato for the opportunity to develop this project and to everyone at the Basic and Applied Bacteriology Laboratory (NIP3) who have always been willing to help.

REFERENCES

ABPA. Associação Brasileira de Proteína Animal, Relatório Anual. 2020.

Bernal, M. P; Alburquerque, J. A.; Moral, R. Composting of animal manures and chemical criteria for compost maturity assessment. A review. **Bioresource Technology**, v. 100, n. 22, p. 5444-5453, 2009.

Clermont, O.; Bonacorsi, S.; Bingen, E. Rapid and simple Determination of the Escherichia coli phylogenetic group. **Applied and Enviromental Microbiology**, USA, ano 10, v. 66, p. 4555-4558, 7 jul. 2000.

Cyoia, P. S. et al. (2019). Distribution of ExPEC virulence factors, blaCTX-M, fosA3, and mcr-1 in Escherichia coli isolated from commercialized chicken carcasses. **Frontiers in Microbiolology** 9:3254. doi: 10.3389/fmicb.2018.03254

Dho-Moulin, M.; Fairbrother, J. M. Avian pathogenic *Escherichia coli* (APEC). **Veterinary Research**, v. 30, n. 2-3, p. 299-316, 1999.

Dos Santos, T. M. B.; De Lucas J.; Sakomura, N. K. Effects of broiler stocking density and poultry litter reuse in broiler performance and poultry litter production. **Revista Portuguesa de Ciências Veterinárias**, v. 553, p. 45–52, 2005.

Gazal, L. E. S. et al. Detection of ESBL/AmpC-Producing and Fosfomycin-Resistant Escherichia coli From Different Sources in Poultry Production in Southern Brazil. **Frontiers in Microbioliology** 11:604544. doi: 10.3389/fmicb.2020.604544

Gazal, L. E. S. et al. Presence of pathogenicity islands and virulence genes of extraintestinal pathogenic *Escherichia coli* (ExPEC) in isolates from avian organic fertilizer. **Poultry Science**, v. 94, n. 12, p. 3025-3033, 2015.

Kaper, J. B.; Nataro, J. P.; Mobley, H. L. T. Pathogenic *Escherichia coli*. **Nature Reviews Microbiology**, v. 2, n. 2, p. 123-140, 2004.

Puño-Sarmiento, J. et al. Identification of diarrheagenic *Escherichia coli* strains from avian organic fertilizers. **International Journal of Environmental Research and Public Health**, v. 11, n. 9, p. 8924-8939, 2014.

Virtuoso, M. C. S.; De Oliveira, D. G.; Dias, L. N. S. Reutilização da cama de frango. **Revista Eletrônica Nutritime**, [*S. l.*], ano 02, v. 12, n. 02, p. 3964-3979, 31 mar. 2015. Disponível em: www.nutritime.com.br. Acesso em: 24 ago. 2019.

TABLE 1
Table 1: Conjugation experiment between *E. coli* from poultry litter and *E. coli* from the microbiota of chicks.

Conjugation	Growth	Code
PR01 (B1) + RS01 (D)	+	T1
PR02 (B1) + RS01 (D)	+	T2
PR09 (D) + RS01 (D)	-	-
PR16 (A) + RS01 (D)	+	T3
PR01 (B1) + RS02 (D)	+	T4
PR02 (B1) + RS02 (D)	+	T5a and T5b
PR09 (D) + RS02 (D)	-	-
PR02 (B1) + RS03 (D)	+	T6
PR09 (D) + RS03 (D)	-	-
PR16 (A) + RS03 (D)	+	T7

TABLE 2
Table 2: Results from PCR for genes for Phylogenetic Group.

	Transconjugants strains							
Gene	T1	T2	T3	T4	T5a	T5b	T6	T7
chuA	+	+	-	+	-	+	+	-
TspE4	+	-	-	+	+	-	+	-
yjoA	-	-	+	-	-	-	-	+
Phylogenetic Group	D	D	A	D	B1	D	D	A

TABLE 3
Table 3: Results from PCR for ESBL genes.

	PCR for ESBL Genes			
	T1	T2	T4	T6
bla_{CTX-M5}	+	-	+	-
bla_{CTX-M8}	+	-	+	-
fosA3	+	-	+	-
References	Gazal et al; (2021)	Own author	Gazal et al. (2021)	Own author