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Resistance profile and virulence characterization of Escherichia coli isolated from diarrheic neonatal farm animals

Perfil de resistência e caracterização de virulência de Escherichia coli isoladas de animais de produção neonatos com diarreia

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Highlights

More than 69% of the isolated E. coli were characterized as MDR.
In the 301samples, 36 DEC were detected, of which 28 were ETEC and eight were EPEC.
MDR profiles were detected in 33.33% of DECs, two of which were ESBL producers.

Abstract

Neonatal diarrhea is the main cause of early mortality and morbidity in farm animals and the source of huge, direct and indirect, economic husbandry losses. Escherichia coli, a common harmless commensal bacterium, can turn into a main diarrheal pathogen through antibiotic resistance and the expression of genetically acquired virulence factors. In this study, fecal samples obtained from eight farms of animals with clinical signs characteristic of diarrhea were subjected to culture and bacterial isolation. Colonies suggestive of E. coli were identified through morphological and biochemical characteristics. Susceptibility tests to the main veterinary antibacterial agents were conducted using agar disk diffusion followed by phenotypical detection of extended-spectrum β-lactamase (ESBL). A total of 301 colonies were characterized as E. coli and, out of the 192 that were tested, 134 showed resistance to three or more classes of antimicrobial drugs

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and were classified as multidrug resistant (MDR), and 14 were ESBL positive. Bacterial DNA was extracted for multiplex PCR (mPCR) using primers to detect ten different genes of diarrheagenic E. coli (DEC). Thirty-six bacterial strains were positive in the mPCR assay, 28 of which were classified as enterotoxigenic E. coli (ETEC) and eight as enteropathogenic E. coli (EPEC). The high prevalence of MDR strains and the detection of ESBL denote the presence of resistance genes in animal husbandry; thus, it is important to isolate and characterize those pathogens and test antimicrobial sensitivity in vitro to avoid ineffective treatments and the spread of antimicrobial resistance, which are the major concerns of Public Health and One Health..
Key words: ESBL. E.coli. Farm animals. Multidrug resistant bacteria. mPCR. Neonatal diarrhea.

Resumo

Diarreias neonatais são as principais responsáveis por uma precoce mortalidade e morbidade em animais de criação, causando grandes perdas econômicas, direta e indiretamente, a qualquer fazenda de produção. Escherichia coli, normalmente uma bactéria comensal inofensiva, podem ser um dos principais patógenos diarreicos a partir da resistência a antimicrobianos e da expressão de fatores de virulência geneticamente adquiridos. Neste estudo, amostras de fezes obtidas de oito fazendas, de animais apresentando quadro diarreico, foram submetidas à cultura e isolamento bacteriano, e, as colônias sugestivas de E. coli foram identificadas através de características morfológicas e bioquímicas. Os testes de suscetibilidade aos principais agentes antibacterianos veterinários foram realizados por disco difusão em ágar seguida pela detecção fenotípica de β-lactamase de espectro estendido (ESBL). Foram isoladas 301 colônias caracterizadas como E. coli e, de 192 testadas, 134 apresentaram resistência a três ou mais classes de antimicrobianos e 14 foram positivas para ESBL. O DNA bacteriano foi extraído para avaliação multiplex de PCR (mPCR) utilizando primers para detectar dez diferentes genes de E. coli diarreiogênicas. Trinta e seis bactérias atestaram positivas na mPCR, com 28 delas classificadas como E. coli enterotoxigênicas (ETEC) e oito E. coli enteropatogênicas (EPEC). A prevalência de cepas multirresistentes e a detecção de ESBL alertam para a presença de genes de resistência na criação animal, assim, é importante o isolamento e a caracterização desses patógenos, principalmente o teste da sensibilidade antimicrobiana in vitro, para evitar tratamentos ineficazes e a disseminação da resistência antimicrobiana que é uma das principais preocupações dentro da Saúde Pública e Saúde Única.
Palavras-chave: ESBL. E. coli. Animais de produção. Bactérias multirresistentes. mPCR. Diarreia neonatal.

Introduction

Diarrhea in the first three months of life is the cause of considerable economical loses among animal farmers, either in therapy costs or in lower zootechnical performance due to weight loss and poor use of dietary nutrients, and leads to death in many cases (Aref, Abdel-Raheem, Kamaly, & Hussien 2018). The disease, although well-known, is expected in most farms. While the condition is easy to recognize, it presents treatment and prevention problems due to its wide nature, with viral, bacterial, and parasitic pathogens (with co-infection being far from uncommon), and environmental influences, such as feed quality and ambiance hygiene, that contribute to its development (Olivo et al., 2016).

Among all diarrheic pathogens, *Escherichia coli* deserve some focus. This gram-negative, oxidase-negative bacillus is a

heavily distributed commensal microorganism in most mammals and is present in the intestine in the first hours of life. In most cases, the bacteria is inoffensive (Kaper, Nataro, & Mobley, 2004); however, some strains have gene-related virulence factors that cause epithelia adherence and enterotoxin production, and such strains are labeled as DEC - diarrheagenic E. coli. (Gomes et al., 2016). These bacteria are subdivided, according to virulence mechanisms, into enteropathogenic (EPEC), enteroinvasive (EIEC), shiga toxin-producer (STEC), enteroaggregative (EAggEC) and enterotoxigenic *E. coli* (ETEC) classifications, with the last being the most common cause of E. coli-mediated diarrhea. ETEC produces two types of enterotoxins, LT (heat-labile toxin) and ST (heat-stable toxin), as well as epithelium adherence adhesines (Dubreuil, Isaacson, & Schifferli, 2016). The differentiation between DECs is usually performed through a multiplex polymerase chain reaction (mPCR) assay, with primers designed to amplify virulence related-genes (Fujioka, Otomo, & Ahsan, 2013).

The antibiotic resistance crisis is an outstanding topic of investigation, in both human and animal health (Mobarki, Almerabi, & Hattan, 2019). The massive use of antibiotics to treat and prevent diarrhea contributes to the selection and spread of resistant bacteria (Jafari et al., 2009). One drug-resistance mechanism is the production of extended-spectrum β-lactamases (ESBLs), which are enzymes that render β-lactam antibiotics, such as cephalosporins, penicillins, carbapenems, and monobactams, useless. The most common ESBL producer is *E. coli,* which is high-capable of donating ta plasmid that contains resistance genes to other bacteria (Valentin et al., 2014). Granting resistance to at least three classes of antibiotics confers ESBL bacteria the classification of Multidrug-Resistant (MDR), as defined by Magiorakos et al. (2012), and thus, a risk for One Health (Basak, Singh, & Rajurkar, 2016).

In this study, fecal samples from neonatal farm animals with diarrhea, underwent bacterial isolation, and identification, followed by classic antibiogram and ESBL detection evaluations. The isolated *E. coli* also underwent mPCR, with primers to detect ten different DEC genes.

Materials and Methods

Fecal Samples

Samples from 61 animals with signs of diarrhea (49 calves, six piglets, and six foals) from nine different farms were collected and sent to the laboratory of Veterinary Bacteriology for routine diagnosis. The material was collected directly from the rectum of each animal and stored at 4-8ºC until analysis (Table 1).

Table 1
Number of animals with fecal samples from each farm and the number of isolated *E. coli*

Farm Code	Species	Number of Animals	Isolated *E. coli*
1	Equine	5	28
2	Swine	6	31
3	Bovine	5	25
4	Bovine	5	21
5	Bovine	12	52
6	Bovine	7	35
7	Bovine	7	35
8	Bovine	7	39
9	Bovine	7	35
		61	301

Bacterial isolation and identification

For fecal bacterial culture, we use the method described by Miles and Misra (1938), with adaptations. Approximately 0.5 g of each fecal sample was diluted in 4.5 mL of a 0.85% saline solution and subsequently diluted serially, 10 times on scale, and sown in MacConkey agar. Plates were incubated aerobically, at 37ºC for 24 h.

After incubation, at least five colonies (when possible, due to some samples producing a small number of bacterial colonies) from each sample were selected for *E. coli* identification by morphological and biochemical characteristics (Koneman & Winn, 2008). Next, three of the five colonies that were positive for *E. coli* were submitted for antimicrobial susceptibility testing (except for farm 1, which evaluated all five colonies) with different antibiotics classes, including: aminoglycosides, penicillin and beta-lactams, fluoroquinolones, cephalosporins, sulphonamides, amphenicols, tetracycline, and carbapenems. Antibiotics were requested by the producer, according to availability on the farm and/or animal species indications. The presence of resistance to antibiotics that belonged to three different classes defined the respective strain as multidrug-resistant bacteria (Schwarz et al., 2010). Lastly, a phenotypical ESBL detection test was performed using the disc approximation method according to the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2015).

For bacterial isolation and identification, 301 colonies from 61 stool samples were characterized, and 192 antimicrobial sensitivity and ESBL tests were performed. Each isolate was stored at -20°C in brain heart infusion (BHI) broth + glycerol until the time of DNA extraction.

DNA extraction and mPCR identification of DEC genes

The 301 colonies that were isolated and classified as *E. coli* were incubated in BHI broth at 37ºC overnight. The resulting strains were

sown on defibrinated 5% sheep blood agar at 37ºC for 24h. These suspensions were boiled for 15 minutes, at 100 ºC, and centrifuged at 12,000 rpm for 3 minutes, and the supernatants (DNA) were collected (Blanco et al., 1997). The DNAs were submitted for mPCR using primers for 10 different DEC genes, according to the methodology of Fujioka et al. (2013), and the primers are shown in Table 2. All of the PCR products were visualized after electrophoresis in a 2% agarose gel that was stained with SYBR® Safe DNA Gel Stain (Invitrogen™) and evaluated using ultraviolet light. The molecular size of the PRC products were estimated by comparisons with a 100 bp scale.

Table 2
Primers used in the mPCR analysis

Target Gene	Sequence (5' to 3')	PCR product size (bp)
stx	AGTTAATGTGGTGGCGAAGG CACCAGACAATGTAACCGC	347
stx2	TTCGGTATCCTATTCCCGG CGTCATCGTATACACAGGAG	589
eae	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	881
bfpA	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	324
aggR	GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC	254
elt	AACGTTCCGGAGGTCTTATG CAACCTTGTGGTGCATGATG	511
esth	TTCACCTTTCCCTCAGGATG ATAGCACCCGGTACAAGCAG	172
estp	ACTGAATCACTTGACTCTTCA TCACAGCAGTAAAATGTGTTGT	120
invE	GCAGGAGCAGATCTTGAAG GAAAGGCACGAGTGACTTTC	208
astA	CCATCAACACAGTATATCCG ACGGCTTTGTAGTCCTTCCA	101

(adapted from Fujioka et al., 2013).

Results and Discussion

A total of 301 different E. coli colonies were isolated in the first step of our experiment (Table 1). Of the isolated colonies, 192 were submitted to antimicrobial susceptibility and ESBL testing, resulting in the detection of 134 MDR E. coli (69.79%) and 14 β-lactamase producers (full list of results is shown in Supplementary Table 1, and resumed in Table 3). A comparative analysis of the antibiotic resistance profile of each property revealed distinct individual portraits. Comparing the same animal species and the same percentage

of MDR isolates from Farm 6, which presented 19.1% gentamicin resistance; while in Farm 7, no isolates showed resistance to this antibiotic. These comparative rates become more discrepant when we compare Farms 1 with 2 (different species) and Farm 7 with 8 (same species but different percentages of isolated MDR bacteria).

Table 3
Number of animals with fecal samples from each farm and the number of isolated *E. coli*

Farm	Samples	MDR	ESBL	Clo	Flo	Ami	Gen	Neo	Cef	Enr	A/C	Cip	Sul	Tet
1	28	28	3	100	-	35.7	78.6	-	46.4	-	-	35.7	100	100
2	20	20	2	-	100	-	25.0	50.0	15.0	95.0	-	-	60.0	100
3	15	13	2	-	66.7	-	20.0	80.0	20.0	40.0	-	-	73.3	93.3
4	13	0	0	0	0	0	0	-	0	0	-	0	-	53.9
5	32	26	3	53.1	-	0	50.0	-	81.3	-	90.6	-	62.5	100
6	21	4	0	14.3	-	-	19.1	52.4	4.8	0	-	-	38.1	42.9
7	21	4	0	19.0	-	-	0	76.2	9.5	4.8	-	-	19.0	33.3
8	21	18	1	71.4	61.9	-	57.1	-	9.5	85.7	-	-	71.4	95.2
9	21	21	3	100	-	85.7	100	-	100	-	-	85.7	100	100
Total	192	134	14											

Clor: Chloramphenicol; Flo: Florfenicol; Ami: Amikacin; Gen: Gentamicin; Neo: Neomycin; Cef: Ceftiofur; Enr: Enrofloxacin; A/C: Amoxicillin/Clavulanate; Cip: Ciprofloxacin; Sul: Sulfazotrim; Tet: Tetracycline
MDR: Multidrug Resistant; ESBL: extended-spectrum β-lactamase
*Resistance to an antibiotic is expressed in percentage (%) of the samples tested. A '-' means that the antibiotic was not tested in that farm.

These differences could result from different selection pressures to which the bacteria were exposed, different treatments that were previously employed, the introduction of animals from different origins carrying MDR pathogens, and the different chemical and physical conditions at each property. MacFadden, McGough, Fisman, Santillan, & Brownstein (2018) stated that temperature can influence the antibiotic resistance of local bacteria, while another study demonstrated that *E. coli* samples collected from different spots along one river showed different antibiotic resistance profiles, according to the activities that surrounded the location and human intervention (Souza, Pinto, Fruet, Piana, & Moura, 2014).

Thus, when studying different farms with different environments and different activities, we can expect a huge difference in antibiotic resistance profiles. Therefore, there is need for antimicrobial susceptibility testing before treatment with antimicrobial drugs in every bacteriosis-suspected case; however, this is not current practice (Landers, Cohen, Wittum, & Larson, 2012). Pairing constant

monitoring (to identify the resistance profile of local pathogens) with drug usage alterations are the most important strategies for diminishing the global antibiotic resistance problem (Windels, Michiels, Bergh, Fauvart, & Michiels, 2019). We should note that in all farms with an elevated number of MDR bacteria (1, 2, 3, 5, and 8) there was greater than 60% resistance against sulfamethoxazole/trimethoprim and tetracycline, which makes us question the current efficacy and use of those drugs against bacterial infections.

Antibiotic resistance has become a major problem for One Health and is recognized by the World Health Organization (WHO) as a threat to modern medicine. A global action plan is currently underway to increase the monitoring of antimicrobial resistance, reduce the incidence of infections, rationalize the use of antimicrobials, and expand investments in alternative drugs and/or new diagnostic tools (World Health Organization [WHO], 2015). Sulfamethoxazole/trimethoprim and tetracycline are the most used antimicrobials in veterinary medicine, being recommended in several treatments without previous sensitivity tests (Capasso & Supuran, 2014; Pereira-Maia et al., 2010). Due to this lack of awareness of the importance of conducting antimicrobial sensitivity tests, the number of multi-resistant bacteria has been increasing dramatically (Yu et al., 2020).

Although the ESBL number identified in this study was low (14 of 192 samples) compared with other studies (Ibrahim, Dodd, Stekel, Ramsden, & Hobman, 2016; Valentin et al., 2014), its relevancy to One health is undeniable. After being first described in 1983, ESBL-producing enterobacteriaceae infections have been identified in several patients around the world (Palmeira & Ferreira, 2020), due to its action against β-lactamic drugs, especially cephalosporins, which are largely used to treat bacterial infections (Abayneh, Tesfaw, & Abdissa, 2018). Enterobacteriaceae, such as *E. coli,* are very common environmental bacteria and are present in both human and animal natural microbiomes. Notably, the presence of resistance genes in its genetic material, especially in plasmids, is easily acquired by other bacteria in its surrounding environment (Tsang, 2017). This is a warning signal for the need to mitigate indiscriminate antibiotics use.

The mPCR results identified 36 DEC bacteria (from the 301 isolates), 28 of which were ETEC and eight were EPEC (Table 4), which suggests that those bacteria where the diarrheic agents in factors that cause diarrhea in these animals. This finding was confirmed by those of Dubreuil et al. (2016) and Cho and Yoon (2014), who showed that the most common E. coli strain responsible for diarrhea is ETEC. The adhesion factors of ETEC assist the initial intestinal colonization, while the production of heat-labile and/or heat-stable toxins induce rapid dehydration and an electrolyte imbalance in the host (Dubreuil et al., 2016).

Table 4
Diarrheagenic Escherichia coli from each farm detected using mPCR and antimicrobial resistance profiles

Farm	Bacteria Code[1]	MDR	ESBL	DEC type
1	C1, C2, C3, C4	Yes	No	ETEC
2	A3	Yes	Yes	ETEC
6	A1, A3, A5, F1, F3, F5	No	No	ETEC
	A2, A4, D4, F4, G4	Susceptibility Test Non-realized		ETEC
	B1	No	No	EPEC
	D3	Yes	No	ETEC
7	A1, A3, E1, E2, E3	No	No	EPEC
	E4	No	No	ETEC
8	A2, G2	No	No	ETEC
	A4, A5, A6, B6	Susceptibility Test Non-realized		ETEC
	B2	Yes	No	ETEC
	F3	Yes	Yes	ETEC
9	C1, C3	Yes	No	EPEC
	F1, F3	Yes	No	ETEC
Total	36	12	2	28 ETEC 8 EPEC

[1]Bacteria with the same letter within a farm were isolated from the same animal.
MDR: Multidrug Resistant; ESBL: extended-spectrum β-lactamase.

EPEC has variable prevalence and symptoms in calve diarrheal outbreaks (Coura, Lage, & Heinemann, 2014), and differences in ETEC strains has been notable due to their inability to produce heat-labile and heat-stable toxins. The virulence mechanism mainly involves adhesion factors that function in lesion formation in epithelial cell microvilli (Gomes et al., 2016). Additionally, Thiry et al. (2017) found that the prevalence of EPEC strains is growing in Europe and can be a potential problem for human health. Notably, all farms with positive EPEC (and Farm 7) also had positive ETEC results. Thus, the presence of both bacterial types will result in elimination of both bacteria through the feces, which is a more aggressive symptomatology and causes diarrhea in farm animals.

Two DEC-ESBL (one from a calf and another from a piglet) where detected in this study. Those bacteria have high multiplication in the host, and, as a diarrheic pathogen, are eliminated to the external environment in greater quantities. ESBL positivity grants these microorganisms an MDR status. Furthermore, since the β-lactamase genes were commonly found in plasmids, the chance of passing these genes to other pathogenic bacteria rises (Li, Chang, Zhang, Hu, & Wang, 2019). These samples were stored for further reference and studies.

Conclusion

This study shows that MDR and ESBL pathogenic E. coli are present in diarrheic farm animals. Due to the high multiplication and elimination of these bacteria in the environment, such organisms are predisposed to pass antibiotic resistance to other pathogens in the gastrointestinal tract and other locations. Furthermore, E. coli has zoonotic potential, being a risk for both human and animal health. The elevated sulfamethoxazole/trimethoprim and tetracycline resistance make us question the efficacy of these antibiotics for treating bacterial diarrhea and the increase in number of multi-resistant pathogens. This is of great relevance for public health, and further studies on this topic are needed.

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