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MICROBIOLOGY

Detection of virulence genes and the phylogenetic groups of *Escherichia coli* isolated from dogs in Brazil

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ABSTRACT: *This study identified the virulence genes, pathovars, and phylogenetic groups of Escherichia coli strains obtained from the feces of dogs with and without diarrhea. Virulence genes and phylogenetic group identification were studied using polymerase chain reaction. Thirty-seven E. coli isolates were positive for at least one virulence factor gene. Twenty-one (57.8%) of the positive isolates were isolated from diarrheal feces and sixteen (43.2%) were from the feces of non-diarrheic dogs. Enteropathogenic E. coli (EPEC) were the most frequently (62.2%) detected pathovar in dog feces and were mainly from phylogroup B1 and E. Necrotoxigenic E. coli were detected in 16.2% of the virulence-positive isolates and these contained the cytotoxic necrotizing factor 1 (cnf1) gene and were classified into phylogroups B2 and D. All E. coli strains were negative for the presence of enterotoxigenic E. coli (ETEC) enterotoxin genes, but four strains were positive for ETEC-related fimbriae 987P and F18. Two isolates were Shiga toxin-producing E. coli strains and contained the toxin genesStx2 or Stx2e, both from phylogroup B1. Our data showed that EPEC was the most frequent pathovar and B1 and E were the most common phylogroups detected in E. coli isolated from the feces of diarrheic and non-diarrheic dogs.*
Key words: *dogs, Escherichia coli, pathovars, phylogroups.*

Detecção de genes de virulência e grupos filogenéticos de amostras *Escherichia coli* isoladas de cães no Brasil

RESUMO: *Este estudo pesquisou genes de virulência, patovares e grupos filogenéticos de amostras de E. coli isoladas de fezes de cães com e sem diarreia. Os genes de virulência e a identificação de grupos filogenéticos foram estudados pela técnica de reação em cadeia da polimerase (PCR). 37 isolados de E. coli foram positivos para pelo menos um fator de virulência na análise de PCR. Destes, 21 (57,8%) foram isolados de fezes de cães com diarreia e 16 (43,2%) de fezes de cães não diarreicos. E. coli enteropatogênica (EPEC) (23/37, 62,2%) foi o patovar mais frequente detectado em fezes de cães e foram classificados principalmente como filogrupos B1 e E. E. coli necrotoxigênica (NTEC) positivos para CNF1 foram detectados (6/37, 16,2%) e classificados como B2 e D. Todas as amostras de E. coli foram negativas quanto à presença de genes de enterotoxinas de E. coli enterotoxigênica (ETEC), mas quatro amostras foram positivas para fimbrias relacionadas ao ETEC, 987P (2) e F18 (2). As amostras de E. coli (STEC) produtora de toxina Shiga foram positivas para a toxina Stx2 (1/37) e Stx2e (1/37), ambas do filogrupo B1. Nossos resultados indicaram que EPEC foi o patovar mais frequente e B1 e E foram os filogrupos mais comuns detectados em amostras E. coli isoladas de fezes de cães diarreicos e não diarreicos.*
Palavras-chave: *cães, Escherichia coli, filogrupos, patovares.*

INTRODUCTION

Escherichia coli are a component of normal intestinal microbiota in humans and other animals. Phenotypic and genotypic characteristics allow the identification of *E. coli* pathogenic strains or pathovars (COURA et al., 2014). Dogs are colonized by *E. coli* during the first days of their life and some strains can cause enteric or extra-intestinal infections (BEUTIN, 1999).

Diarrheagenic *E. coli* strains are classified as pathotypes or pathovars according to their virulence

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factors, pathogenesis, and clinical signs or symptoms present in the host. It is believed that different pathovars can cause diarrhea in dogs. These included enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), and necrotoxigenic *E. coli* (NTEC) (BEUTIN, 1999). Other pathovars are associated with diarrhea in other animals, namely enteroaggregative *E. coli* (EAEC) and enteroinvasive *E. coli* (EIEC) (MAINIL, 2013). Because of the close contact with humans, dogs could increase the risk of transmitting potentially zoonotic microorganisms, such as EPEC and STEC (BEUTIN, 1999).

In addition to the pathotype and pathovar classification, *E. coli* strains can be assigned to one of the seven phylogenetic groups, including A, B1, B2, C, D, E, and F (CLERMONT et al., 2013). *E. coli* genomic structure has shown that the strains belonging to different phylogroups are associated with the disease state and source of isolation (CLERMONT et al., 2013). The extraintestinal pathogenic *E. coli* (ExPEC) strains are clustered mostly in groups B2 and D and intestinal pathogenic *E. coli* are mostly in phylogroups A, B1, and E (ESCOBAR-PÁRAMO et al., 2004; CLERMONT et al., 2011). Although phylogenetic characterization is an important tool to improve the understanding of *E. coli* populations and the relationship between strains and disease (CLERMONT et al., 2011; COURA et al., 2015), few studies have determined phylogenetic groups of *E. coli* isolated from dogs worldwide, and of those that did, the most frequently used method was a triplex PCR method developed by CLERMONT in 2000. This method is only capable of determining phylogroups A, B1, B2, and D (HARADA et al., 2012; SALVARANI et al., 2012; SCHMIDT et al., 2015) instead of the seven phylogroups that were detected in this study. In Brazil, only two studies identified *E. coli* pathovars associated with diarrhea in dogs (ALMEIDA et al., 2012; PUÑO-SARMIENTO et al., 2013), but none determined the phylogenetic groups of *E. coli*. Therefore, this study aimed to detect the virulence genes, pathovars, and phylogenetic groups of *E. coli* strains isolated from the feces of dogs with and without diarrhea.

MATERIALS AND METHODS

Stool samples

Stool samples were collected from 154 dogs, of which 92 were diarrheic and 62 were without diarrhea. Samples from diarrheic dogs were obtained directly from the rectum, at the Veterinary Hospital of Universidade Federal de Minas Gerais (Belo Horizonte city), upon admission. Collections were performed from dogs that were undergoing a consultation for the occurrence of diarrhea. With owner's consent, fecal samples from non-diarrheic animals were obtained from the dogs of students attending the Universidade Federal de Minas Gerais. For non-diarrheic dogs, fecal samples were either taken directly from the rectum or after spontaneous defecation, sampling the upper portion of the fecal content that did not have contact with the environment.

E. coli isolation and DNA extraction

Fecal samples were directly plated onto MacConkey agar and incubated for 18 to 24h at 37°C. At least one colony with the characteristics of *E. coli* (lactose-fermenting colonies) per animal were selected for molecular analysis. Suggestive colonies were screened for the uidA gene to confirm the presence of *E. coli* (MCDANIELS et al., 1996). Bacterial DNA was obtained by suspending the colonies in 50µL of sterile water and heating at 100°C for 10min in a thermoblock.

Detection of virulence genes and characterization of phylogenetic groups

The presence of virulence genes was determined by PCR. Strains were screened for the following genes: the transcriptional regulator aggR (TOKUDA et al., 2010); cnf1 and cnf2 (BLANCO et al., 1996); the toxin genes sta, Stx1, Stx2 (multiplex PCR) (FRANCK et al., 1998); intimin (eae) (BLANCO et al., 2006); bundle-forming pili (bfpA) (GUNZBURG; TORNIEPORTH; RILEY, 1995); ipaH for EIEC (ARANDA et al., 2007); Stx2e, the most frequent pathovar identified, F41, stb, LT, 987P, F18, K88, and K99 (multiplex PCR) (MACÊDO et al., 2007). The PCR reaction conditions were performed according to each author, with no modifications. The following *E. coli* reference strains were used as positive controls: The O157:H7 strain EDL 933 (eaeA, Stx1, Stx2, ehxA, iha, toxB, efa1), EAEC O42 (astA, aggR, aaf, pet), S5 (F17,cnf2), E2348/69 (bfpA, eae), 2568 (stb, STaP, F18, Stx2e), 2569 (stb, LT, K88), 2570 (987P, StaP), and CNF1 strain 2571 (StaP, K99, F41). Sterile water was used as a negative control. Amplified DNA was resolved on a 1.5% agarose gel, stained with 0.5µg/mL of ethidium bromide, and photographed under UV light. A 100bp DNA size marker (100bp DNA ladder, New England Biolabs, USA) was used to estimate the size of the amplified product.

E. coli strains positive for one or more virulence genes were tested by PCR for

characterization of the phylogenetic groups A, B1, B2, C, D, E, and F, according to CLERMONT et al. (2013). If a phylogenetic group was not identified, then the *E. coli* strains were classified as unknown.

Statistical analysis

Categorical variables were examined using a chi-square test or Fisher's exact test. For the variable age, the Mann-Whitney U or Kruskal-Wallis test was used.

RESULTS AND DISCUSSION

Out of 154 stool samples tested, 37 (24%) were positive for *E. coli* isolates, with at least one virulence gene detected in PCR analysis. Of these positive isolates, 21 (57.8%) were obtained from dogs with diarrhea and 16 (43.2%) were obtained from non-diarrheic dogs. Statistical analysis showed no association between pathovars and diarrhea or age. Table 1 shows the distribution of strains, according to the pathovar and its correlation with virulence genes. The distribution of phylogenetic groups among the pathogenic *E. coli* is shown in table 2.

In our study, EPEC was the most frequent pathovar detected and accounted for 62.1% of the PCR-positive strains. This pathovar was isolated from 14.9% of the dogs, and the frequency was higher in diarrhea samples than in those from apparently healthy animals. EPEC is characterized by the production of intimin (eae) and it can be classified as typical or atypical based on the presence or absence of bundle-forming pili (bfp), respectively (MAINIL, 2013). Our results are in accordance with other studies and suggested that EPEC are important diarrheagenic *E. coli* in dogs, especially atypical EPEC (NAKAZATO et al., 2004; ALMEIDA et al., 2012; PUÑO-SARMIENTO et al., 2013). In addition, EPEC were mainly classified in phylogroups B1 and E, as reported in previously published studies in zoo animals, dogs, pigs, and calves (BALDY-CHUDZIK et al., 2008; TRAMUTA et al., 2008; COURA et al., 2017). Moreover, intestinal associated *E. coli* are mainly in phylogroups B1 and E (CLERMONT et al., 2011). These findings are important since EPEC was detected more frequently in diarrheic dogs than in non-diarrheic dogs. The EPEC can complicate the clinical signs of diarrhea and should be considered as a possible differential diagnosis in dogs with enteric infections (KJAERGAARD et al., 2016).

In the present study, seven dogs (4.5%) were positive for strains containing cnf1, including one hybrid strain with eae and cnf1. In dogs, CNF1 is the most frequent toxin of NTEC detected from dogs (BEUTIN, 1999). Moreover, in our study, cnf1 strains were usually classified in phylogroups B2 and D (Table 2). In previous studies, it was shown that ExPEC strains are clustered mostly in groups B2 and D (ESCOBAR-PÁRAMO et al., 2004). Interestingly, *E. coli* strains isolated from septicemic human patients belonged mainly to groups B2 and D (ČUROVÁ et al., 2014). Furthermore, cnf1 strains in phylogroup B2 were associated with diarrhea and mortality of puppies (TURCHETTO et al., 2015) and phylogroup B2 was associated with urine samples that were positive for cnf1 (OSUGUI et al., 2014). Collectively, these results suggested that NTEC from dogs possess ExPEC characteristics.

All *E. coli* strains isolated and tested by PCR were negative for the presence of heat labile and heat stable enterotoxins genes. Two isolates were positive for fimbriae genes, including 987P (F6) and F18, and both are generally related to ETEC strains of animal origin (MAINIL, 2013). Other studies did

Table 1 - Distribution of pathovars and virulence factors of *Escherichia coli* isolated from non-diarrheic and diarrheic dogs (n=37).

Pathovars (virulence factors)	----------Number of isolates (%)----------		
	Non-diarrheic	Diarrheic	Total
HYBRID (*eae*, *sta*)	0 (0)	1 (2.7)	1 (2.7)
NTEC (*cnf1*)	3 (8.1)	3 (8.1)	6 (16.2)
HYBRID (*eae*and*cnf1*)	1 (2.7)	0 (0)	1 (2.7)
Atypical EPEC (*eae*)	9 (24.3)	13 (35.1)	22 (59.4)
Typical EPEC (*eae*, *bfp*)	1 (2.7)	0 (0)	1 (2.7)
987P fimbria	1 (2.7)	1 (2.7)	2 (5.4)
F18 fimbria	0 (0)	2 (5.4)	2 (5.4)
STEC (*Stx2e*)	0 (0)	1 (2.7)	1 (2.7)
STEC (*Stx2*)	1 (2.7)	0 (0)	1 (2.7)

Table 2 - Distribution of phylogenetic groups and virulence factor genes of *Escherichia coli* obtained from the feces of dogs (n=37).

Virulence factor genes	Phylogroup (%)					
	A	B1	B2	D	E	Unknown
987P	0 (0)	2 (5.4)	0 (0)	0 (0)	0 (0)	0 (0)
eae/sta	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.7)
cnf1	0 (0)	0 (0)	4 (10.8)	1 (2.7)	0 (0)	1 (2.7)
eae/cnf1	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.7)	0 (0)
eae	1 (2.7)	4 (10.8)	3 (8.1)	1 (2.7)	7 (18.9)	6 (16.2)
eae/bfp	0 (0)	0 (0)	0 (0)	1 (2.7)	0 (0)	0 (0)
F18	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (5.4)
Stx2e	0 (0)	1 (2.7)	0 (0)	0 (0)	0 (0)	0 (0)
Stx2	0 (0)	1 (2.7)	0 (0)	0 (0)	0 (0)	0 (0)
Total	1 (2.7)	8 (21.6)	7 (18.9)	3 (8.1)	8 (21.6)	10 (27)

not find enterotoxins in *E. coli* obtained from dogs (HAMMERMUELLER et al., 1995; NAKAZATO et al., 2004; PUÑO-SARMIENTO et al., 2013). Fimbriae F18 and 987P are commonly reported in ETEC isolated from piglets (MACÊDO et al., 2007), but their importance as adherence fimbriae for *E. coli* isolated from dogs should be further investigated. Our results from fimbrial-positive strains are difficult to compare to other studies since only two isolates were positive and both were negative for the toxin STa. It is also important to note that, according to the owners, both dogs positive for 987P and F18 were housed without contact with other animals.

Two STEC strains were detected. One was positive for toxin Stx2 and another was positive for Stx2e, with both strains from phylogroup B1. The STEC from dogs are usually positive for Stx2 (HAMMERMUELLER et al., 1995). Regarding the phylogenetic group, STEC strains of phylogroup B1 were also detected in calves (COURA et al., 2017). Because of the low number of STEC isolates, the importance of this pathovar in dogs and its phylogenetic group placement is difficult to assess.

Two *E. coli* strains identified during this investigation could not be classified into a phylogenetic group, both containing the eae gene. This finding emphasized the dynamics of gene transfer between *E. coli* strains of different pathovars, resulting in the development of new pathovars and contributing to the emergence of pathogenic strains (MÜLLER et al., 2007), such as *E. coli* O104:H4.

E. coli characterized by PCR for virulence genes obtained from the feces of dogs were mostly from phylogroups B1 and E. This finding reinforces the possible connection between *E. coli* strains and diarrhea in dogs, since intestinal pathogenic *E. coli* are mostly B1 and E (CLERMONT et al., 2011) and the virulence genes identified are associated with diarrhea in humans and other animals (MAINIL, 2013; COURA et al., 2014; COURA et al., 2017).

Some of the *E. coli* strains detected in this study could not be classified into phylogroups using the Clermont method. This occasionally occurs because it uses a combination of the presence and absence of certain genes. According to CLERMONT et al. (2013), some *E. coli* strains cannot be assigned to a phylogroup due to the extremely rare occurrence of the phylogroup, the strain is the result of large-scale recombination between two different phylogroups, or the highly variable *E. coli* genome content was driven by the gain and loss of genes.

This study provided important information regarding pathogenic *E. coli* isolated from the feces of dogs. The presence of virulence genes and the phylogenetic classification of *E. coli* were determined and are important in understanding canine *E. coli* infections and associated syndromes. There was no association between virulence genes or phylogroups and any of the study's epidemiological aspects, including age, breed, or gender. Our results indicated that EPEC were the most frequently identified pathovar in the feces of dogs and are mainly classified into the intestinal origin phylogroups, B1 and E. NTEC positive for cnf1 were also identified and were mostly in phylogroups B2 and D, which are associated with ExPEC. Detection of other pathovars was very low, but the ones identified were associated with disease in humans and other animal species. Because of the close contact between dogs and humans and the likely contact with other animal species, these results emphasized the need for further studies to clarify the role of *E. coli* in dogs.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

All animal procedures were approved by the Comitê de Ética em Experimentação Animal (CEUA/UFMG), protocol 51/2015.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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