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Original Article

Detection of enterotoxin and adhesin genes of *Escherichia coli* strains isolated from feces of healthy dogs

Detecção de genes de enterotoxinas e adesinas em cepas de *Escherichia coli* isoladas de fezes de cães saudáveis

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A B S T R A C T

This study aimed to investigate the presence of genes encoding the enterotoxins STa and Stx1 and the adhesins K99 and Intimin in *E. coli* strains isolated from feces of dogs who appeared to be healthy. Rectal swab samples were collected from 50 dogs who visited the Veterinary Hospital of the University of Brasilia and 48 *E. coli* isolates were obtained. No positive isolates were found for STa and K99. However, positive results were found in 21 isolates (43.7%) for Stx1 and 14 isolates (29%) for the Intimin gene (*eae*). The antimicrobial sensitivity profile was also assessed for the following antibiotics: sulfazothrim, azithromycin, enrofloxacin, ceftiofur, amoxicillin + clavulanate, doxycycline, ampicillin, and cephalexin. The antibiotics on which the isolates showed the highest resistance were ampicillin (25%), doxycycline (22.9%) and cephalexin (20.8%). As for sensitivity, the isolates were most sensitive to sulfazothrim (87.5%), azithromycin (85.41%) and enrofloxacin (77%). Healthy dogs can carry multidrug-resistant *E. coli* strains that can also carry enterotoxin and adhesin genes, thus indicating that, the proximity between dogs and humans may contribute to possible zoonotic transmission of these microorganisms.

R E S U M O

Palavras-chave:
Fatores de virulência
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PCR
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Este estudo teve como objetivo investigar a presença de genes que codificam as enterotoxinas STa e Stx1 e as adesinas K99 e Intimina em cepas de *E. coli* isoladas de fezes de cães aparentemente saudáveis. Amostras de swab retal foram coletadas de 50 cães que visitaram o Hospital Veterinário da Universidade de Brasília e 48 isolados de *E. coli* foram obtidos. Não foram encontrados isolados positivos para STa e K99. No entanto, resultados positivos foram encontrados em 21 isolados (43,7%) para Stx1 e 14 isolados (29%) para o gene Intimina (eae). O perfil de sensibilidade antimicrobiana também foi avaliado para os seguintes antibióticos: sulfazotrim, azitromicina, enrofloxacina, ceftiofur, amoxicilina + clavulanato, doxiciclina, ampicilina, e cefalexina. Os antibióticos nos quais os isolados apresentaram maior resistência foram a ampicilina (25%), doxiciclina (22,9%) e cefalexina (20,8%). Quanto à sensibilidade, os isolados foram mais sensíveis ao sulfazotrim (87,5%), azitromicina (85,41%) e enrofloxacina (77%). Cães saudáveis podem carrear cepas de *E. coli* multirresistentes que por sua vez também podem carrear genes codificadores de enterotoxina e adesina, indicando assim que a proximidade entre cães e humanos pode contribuir para a possível transmissão zoonótica desses microrganismos.

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INTRODUCTION

Escherichia coli (*E. coli*) is one of the main enteric pathogens among the *Enterobacteriaceae* family. It is found in the intestinal tract of humans and animals as part of the normal microbiota and sometimes produces enteric diseases, such as gastroenteritis (KAPER et al., 2004; QUINN et al., 2005).

E. coli can be spread by direct or indirect contact, since contaminated feces, urine and discharge can contaminate vegetation, soil, and water, allowing the emergence of new infections (KAPER et al., 2004; QUINN et al., 2005; DA COSTA et al., 2013).

This microorganism expresses many different mechanisms and virulence factors related to gastroenteritis and may cause a variety of other extraintestinal conditions such as mastitis, cystitis, and metritis (KAPER et al., 2004; QUINN et al., 2005).

Classification of *E. coli* pathotypes is based not only on the pathogenesis of the disease, but also on the molecular detection of virulence factors such as the production of hemolysins, thermolabile toxins (LT-I and LT-II), thermostable toxins (STa and STb), Shiga-like toxins (Stx1 and Stx2), necrotizing cytotoxic factors (CNF), adhesins, and others (DEBROY; MADDOX, 2001; KAPER et al., 2004; QUINN et al., 2005).

Dogs have been identified as potential reservoirs of *E. coli* strains that cause infections in humans, mainly because of the phylogenetic proximity of some serogroups and due to the expression of similar virulence factors (JOHNSON et al., 2001; NAKAZATO et al., 2004; RODRIGUES et al., 2004; DAMBORG et al., 2009; STENSKE et al., 2009; DE ALMEIDA et al., 2012). Additionally, humans have also been identified as transmitters of *E. coli* strains for animals (RODRIGUES et al., 2004; DAMBORG et al., 2009).

Considering the easy spread of *E. coli* between different animal species and through different mechanical vectors (KAPER et al., 2004; KANTERE et al., 2014) and the possible transfer of antimicrobial resistance genes (GUARDABASSI et al., 2004; HEUER et al., 2005; WEDLEY et al., 2017), this study aimed to investigate the presence of the genes encoding the enterotoxins STa and Stx1 and the adhesins K99 and Intimin using DNA polymerase chain reaction (PCR) on *E. coli* strains isolated from healthy and unhealthy dogs at the Veterinary Hospital of the University of Brasilia, as well as to determine the antimicrobial resistance profile of these isolates.

MATERIAL AND METHODS

Fecal samples were collected from 50 dogs who attended their first consultation at the Veterinary Hospital of the University of Brasilia. Samples were collected directly from the rectal ampulla using sterile swabs, conditioned in Stuart transport medium (Oxoid/Unipath, Basingstoke, Hampshire, England) and sent directly to the Laboratory of Veterinary Microbiology for processing. The selection included male and female dogs who were not hospitalized but were either healthy (non-diarrheal dogs) or unhealthy (diarrheal dogs).

Fecal samples were inoculated on MacConkey agar (Merck KGaA, Darmstadt, Germany) and incubated for 24 hours at 37 °C to select for lactose fermenting colonies. Selected colonies were collected on 5% sheep blood agar to verify the presence of hemolysis and to proceed to biochemical identification according to QUINN et al. (1994). From the 50 samples, 48 *E. coli* isolates were obtained.

After biochemical identification, the isolates were cultured in blood agar at 37 °C for 24 hours and three colonies of each isolate were diluted in 300 μl of Milli-Q water in microtubes and homogenized in a vortex. The microtubes were placed in a water bath at 100 °C for 10 minutes to lyse the cells and release the DNA and then centrifuged for 15 minutes at 13,000 × g and 4 °C (Centrifuge: SIGMA 2K15, Osterode am Harz, Germany). The supernatant was collected and the quality and yield of the DNA isolates obtained at NanoVue™ Plus Spectrophotometer (GE Healthcare, Germany), by measuring absorbance at 230, 260, and 280 nm, and determining absorbance 260/280 and 260/230 ratios. The DNA integrity was assessed by 1.0% agarose gel electrophoresis. Appropriated DNA isolates were selected according to purity, concentration and integrity for use in PCR amplification.

Reactions contained 5 μl of extracted DNA, 2.5 μl of buffer (10X; Phoneutria, Belo Horizonte, MG, Brazil), 1.5 μl of dNTP (10 mM; Invitrogen®, Waltham, MA, USA), 0.25 μl of Taq DNA polymerase (5 U/μl; Phoneutria, Belo Horizonte, MG, Brazil), and 0.5 μl of each primer at 10 pmol/μl dilution. The final volume was adjusted to 25 μL. For the negative control experiment, a sample of sterile Milli-Q® water was used. The following *E. coli* reference strains were used as positive controls: EDL933 (O157:H7, eaeA, stx1, stx2, ehxA, iha, toxB, efa1), 2568 (stb, STaP, F18, Stx2e), 2571 (StaP, K99, F41) and E2348/69 (bfpA, eae). Amplification conditions and primers were chosen according to SALVADORI et al. (2003), using a Techne® thermal cycler (Stone, Staffordshire, UK). Amplification products were electrophoresed on 1.0% agarose gel and subsequently stained with ethidium bromide.

E. coli colonies were inoculated in Müeller-Hinton broth (Himedia®, Mumbai, India) and incubated at 37 °C until they presented 0.5 turbidities on the McFarland scale. They were then inoculated into Müller-Hinton agar (Bio-Rad®, Hercules, CA, USA), according to the modified Kirby-Bauer method, as recommended by the Clinical and Laboratory Standards Institute. Strains were tested for the following antibiotics: Cephalexin 30 μg, Doxycycline 30 μg, Enrofloxacin 5 μg, Sulfazothrim (Sulfamethoxazole + Trimethoprim) 25 μg, Ampicillin 10

μg, Azithromycin 15 μg, Amoxicillin + clavulanate 30 μg, and Ceftiofur 30 μg. The plates were incubated at 37 ºC for 18 hours and then the inhibition halo was interpreted according to the CLSI (2011) table.

Ethical approval. Consent was obtained from dog's owners before sampling. This study was approved by the Ethical Committee of the Institute of Biology from the University of Brasilia registered under the protocol 134069/2012.

RESULTS

From the 48 *E. coli* isolates, 14 (29%) were positive for the Intimin gene (*eae*), 21 (43.7%) were Stx1 positive and 12 were positive for both Stx1 and Intimin gene. Among the 48 isolates of *E. coli,* only 1 in which the Intimin gene was detected, was originated from a dog with clinical signs of diarrhea. No positive results were found for the genes encoding the STa thermostable toxin and the K99 adhesin, as shown in table 1.

Table 1. *Escherichia coli* virulence factors isolated from healthy and unhealthy dogs.

Virulence Factor	Healthy Dogs	Unhealthy Dogs
	Non diarrheal dogs	*Diarrheal Dogs*
Stx1	21 (43,7%)	0
Sta	0	0
K99	0	0
Intimin	13 (27%)	1 (2%)
Stx1+Intimin	12 (25%)	0
Total	**22**	**1**

E. coli isolates showed the following percentages of antibiotic sensitivity: sulfazothrim, 87.5%; azithromycin, 85.4%; enrofloxacin, 77.1%; ceftiofur, 64.6%; amoxycillin + clavulanate, 62.5%, doxycycline, 50%; ampicillin, 45.83%; and cephalexin, 33.3%. Percentage of *E. coli* isolates resistant to antibiotics were: ampicillin, 25%; doxycycline, 22.9%; cephalexin, 20.8%; enrofloxacin, 12.5%; sulfazothrim, 12.5%; amoxycillin + clavulanate, 10.4%; ceftiofur, 8.3%; and azithromycin, 2.1%.

DISCUSSION

Considering the proximity between pet animals and humans, studies involving the detection of microorganism's pathogenicity factors are important from the epidemiological and preventive point of view.

In Brazil, the detection of the Intimin gene in feces of diarrheic dogs and healthy dogs was reported by two studies in São Paulo (NAKAZATO et al., 2001; NAKAZATO et al., 2004), one in Rio de Janeiro (DE ALMEIDA et al., 2012), and one in Paraná (PUÑO-SARMIENTO et al., 2013), which is in line with the findings of this study. Another study in São Paulo (PAULA, 2007) showed the same results but only in the feces of diarrheic dogs.

Though the present study found healthy dogs with positive Stx1 toxin samples, NAKAZATO et al. (2001) and NAKAZATO et al. (2004) found no positive samples. Nevertheless, positive samples were found by PAULA (2007) and DE ALMEIDA et al. (2012). The simultaneous presence of the Intimin and Stx1 genes was reported by DE ALMEIDA et al. (2012) in the sample of a dog with diarrhea. On the other hand, PAULA (2007) detected positive samples for Stx1 and Intimin alone.

Two surveys conducted in the United States reported higher frequencies of Intimin and STa detection, with no positive samples for Stx1 (HOLLAND et al., 1999; DEBROY; MADDOX, 2001). One of those studies also found positive samples for K99 (DEBROY; MADDOX, 2001).

Although no positive samples for the two virulence factors (STa and K99) were found in the present study, *E. coli* from dogs may harbor coding genes for the four investigated factors (STa, Stx1, K99, and Intimin). Detection of these virulence factors, especially in healthy dogs, reinforces their involvement as reservoirs of enteropathogens for humans (JOHNSON et al., 2001; NAKAZATO et al., 2004; RODRIGUES et al., 2004; DAMBORG et al., 2009; STENSKE et al., 2009; BÉLANGER et al., 2011; DE ALMEIDA et al., 2012).

Concerns over the possible transfer of diarrheagenic *E. coli* from companion animals to humans should also include the possibility of sharing antimicrobial resistance genes (GUARDABASSI et al., 2004; HEUER et al., 2005; BÉLANGER et al., 2011; WEDLEY et al., 2017). Transfer of enteropathogenic *E. coli* between dogs and humans as well as the occurrence of the same resistance genes in *E. coli* from dogs and humans have already been

reported (RODRIGUES et al., 2004; LJUNGQUIST et al., 2016; WEDLEY et al., 2017).

The same antimicrobial agents used as first choice compounds for the treatment of human infections such as cephalosporins and fluoroquinolones, are often used in companion animals, particularly for dogs (GUARDABASSI et al., 2004; HEUER et al., 2005).

In this study, *E. coli* isolates were analyzed for antimicrobial resistance. The resistance percentages were variable and similar to other studies with *E. coli* carrying virulence factor genes conducted by PAULA (2007) and PUÑO-SARMIENTO et al. (2013). *E. coli* strains resistant to ampicillin, amoxicillin + clavulanate, and enrofloxacin were also found. However, what stands out in these studies is the presence of *E. coli* strains resistant to antibiotics that are most commonly used in human treatment and the presence of multiresistant strains (PAULA, 2007; PUÑO-SARMIENTO et al., 2013). The resistance to antimicrobials rarely used in veterinary practice, such as imipenem, nalidixic acid, and aztreonam, suggests a possible acquisition of resistance genes from human strains (GUARDABASSI et al., 2004; SANNES et al., 2004; PUÑO-SARMIENTO et al., 2013).

Detection of multiresistant *E. coli* strains must be viewed with concern, since these microorganisms can be easily transmitted among different hosts and spread throughout the environment. This contributes to the generalized increase of antimicrobial resistance (GUARDABASSI et al., 2004; DA COSTA et al., 2013).

CONCLUSIONS

In this study, enterotoxin (Stx1) and adhesin (Intimin) genes were found in strains of *E. coli* isolated from dogs who appeared to be healthy, indicating the circulation of these genes without the clinical signs of diarrhea. The proximity between dogs and humans contributes to the establishment of a possible zoonotic chain in the transmission of multiresistant microorganisms and carriers of genes encoding virulence factors.

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