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Occurrence of non-O157 Shiga toxin-producing *Escherichia coli* in dogs with diarrhea

Ocorrência de *Escherichia coli* não–O157 Shigatoxigênica em cachorros com diarréia

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ABSTRACT

*Shiga toxigenic **Escherichia coli** (STEC) and Attaching and effacing **E. coli** (AEEC) have been associated with diarrhea illness in dogs. From January to December 2006, 92 **E. coli** isolates from 25 diarrheic dogs were analyzed, by screening for the presence of Shiga toxin-producing (stx 1 and stx 2) and intimin (eae) genes. Twelve isolates were detected by PCR to harbor the Shiga toxin genes (7 the stx 1 (7.6%); 5 the stx 2 (5.4%); and none both of them). Nine (9.8%) of the **E. coli** isolates studied were eae positive non Shiga toxin-producing. Thirteen (62.0%) isolates, carrying stx or eae gene, also showed α hemolysin production. The strains with virulence genes were also examined for resistance to 12 antimicrobial agents. Resistances to cephalothin (85.7%), streptomycin (81.0%), amoxicillin (71.4%) and gentamicin (71.4%) were predominantly observed.*

Key words: ***Escherichia coli**, STEC, eae gene, antimicrobial resistance.*

RESUMO

***Escherichia coli** Shiga toxigênica (STEC) e **E. coli** Attaching- effacing (AEEC) têm sido associadas à doença diarréica em cachorros. Entre janeiro e dezembro de 2006, 92 cepas de **E. coli** isoladas de 25 cachorros diarréicos foram examinadas. As cepas foram analisadas para a detecção dos genes produtores de Shiga toxina (stx 1 e stx 2) e da intimina (eae). Por meio de PCR foi observado que sete cepas (7,6%) portavam o gene stx 1, cinco cepas (5,4%) carregavam o gene stx 2 e nenhum cepa apresentou ambos os genes associados. Nove cepas de E. coli (9,8%) apresentaram o gene eae isoladamente. Treze das cepas (62,0%) que apresentaram os genes stx ou eae também apresentaram a produção de α hemolisina. As cepas que apresentaram genes de virulência foram também examinadas em relação à resistência a 12 agentes antimicrobianos. As resistências mais comuns foram para cefalotina (85,7%), estreptomicina (81,0%), amoxicilina (71,4%) e gentamicina (71,4%).*

Palavras-chave: ***Escherichia coli**, STEC, gene eae, resistência antimicrobiana.*

INTRODUCTION

Escherichia coli is a predominant component of the intestinal microbiota of humans and other mammals. Some ***E. coli*** strains represent primary pathogens having an enhanced potential to cause diseases, especially diarrhea (NATARO & KAPER, 1998). An emergent pathogen, Shiga toxin-producing ***E. coli*** (STEC) of the serogroup O 157, designated as enterohemorrhagic ***E. coli*** (EHEC), has been considered to be responsible for many outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (PATON & PATON, 1998). Two types of Shiga toxins, Stx1 and Stx 2, are known and constitute the main virulence factors in STEC strains (PATON & PATON, 1998). Domestic ruminants have been implicated as being the major reservoirs of STEC strains that cause human infections (CHAPMAN et al., 2001). However, other domestic animals like cats and dogs have also been found to carry STEC strains (BEUTIN et al., 1993; HAMMERMULLER et al., 1995; STAATS et al., 2003; BENTANCOR et al., 2007). In these cases, transmission pathways were the direct contact between humans and

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animals as well as fecal contamination (BENTANCOR et al., 2007). The STEC strain most frequently associated with clinical disease is the serotype O157:H7; nevertheless there are over one hundred of different serotypes of STEC strains, some of which are associated with human diseases (LAW, 2000). Since non-O157 strains are more prevalent in animals and as contaminants of food, humans are probably more frequently exposed to these strains (BLANCO et al., 2004).

Pets can be natural reservoirs of several organisms potentially able to cause disease to humans; children in special are central players in this cross-transfer game in view of their frequent nonobservance of proper hygiene habits (RODRIGUES et al., 2004). The eventual role of dogs as reservoirs of STEC strains has not been totally elucidated.

Attaching and effacing ***E. coli*** (AEEC) are characterized as leading to diarrhea due to their ability to cause A/E lesions in the gut mucosa of human and animal hosts. A/E lesions are initiated by the intimate attachement of bacteria to enterocytes, mediated by intimin, an adhesin encoded by the *eae* gene (NATARO & KAPER, 1998). Detection of this gene has been taken as an indication of the presence of the A/E pathogenicity factor in a given bacterial strain (KNUTTON et al., 1991). Natural infections with AEEC in dogs have also been described (BEAUDRY et al., 1996; NAKAZATO et al., 2004).

It has been shown that antimicrobials present in animals destined for alimentary purposes may contribute to selective antimicrobial resistance, posing risks to humans due to the transmission of resistant zoonotic bacteria via the food chain (WEGENER et al., 1999). Such resistant bacteria could be acquired by humans via alternative pathways including person-to-person transmission, environmental and/or direct exposure to animals. Cats and dogs represent potential sources for the spreading of antimicrobial resistance in view of the extensive use of antimicrobial agents in these animals, and their close contact with human beings (GUARDABASSI et al., 2004).

The aim of the present study was to study the occurrence of STEC strains in diarrheic dogs, as well as of their antimicrobial susceptibilities to 12 antimicrobial agents, as means to assess their importance as sources of infection.

MATERIAL AND METHODS

Sample collection

In order to establish STEC occurrence as well as their antimicrobial susceptibility to 12 antimicrobial agents, 25 diarrheic dogs were randomly selected by order of presentation to a private clinic in the city of Ituverava, State of São Paulo, between January and December 2006. Animals of any age, sex or breed were chosen on the following clinical inclusion criteria: acute hemorrhagic or not diarrhea, inappetence, no previous antimicrobial drugs treatment, with no other gastrointestinal disease or evidence of surgical approach. Samples collected by rectal swabbing with a sterile cotton swab under veterinary supervision were placed in Stuart transport medium and taken to immediate laboratory processing.

Culture

Samples were transferred to MacConkey agar (Mac-Difco) and incubated for 24h at 37ºC. At least five colonies were selected from each plate for analysis. Biochemical confirmation of the strains as ***E. coli*** was performed according to KONEMAN et al. (1997).

Determination of stx genes

Bacterial strains (***E. coli*** isolates)grown overnight in nutrient broth (Sigma Chemical Co, St Louis, USA) at 37ºC, were tested for the presence of *stx* genes (*stx* 1 and *stx* 2) using the polymerase chain reaction (PCR) protocol of ORDEN et al. (1998). DNA templates were prepared by pelleting 1ml of culture enriched by centrifugation at 12000g. The cell pellet was resuspended in 250µl of sterile distilled water and boiled for 10min at 100ºC, re-centrifuged and supernatants were subjected to PCR in an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). The amplified DNA products were separated by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and examined for detection under ultraviolet light. Reference ***E. coli*** strains used as controls were EDL 933 (O157:H7, *stx*1, *stx* 2, *eae*) and DH5a (negative control), both from Dr. Tânia A. Tardeli Gomes (Department of Microbiology, Immunology and Parasitology, Escola Paulista de Medicina, São Paulo, Brasil).

Characterization of isolates

Isolates were confirmed as stx+ and tested for the accessory virulence marker *eae*, using the PCR protocol of CHINA et al. (1996).

O157 latex agglutination

STEC isolates were typed for O serotype O157 using the O157 Latex Agglutination test kit (Oxoid, Basingstoke, Hampshire, UK). The EDL 933 strain was used as a positive control. Strains negative to agglutination were considered non-O157 strains.

Susceptibility Testing

Antimicrobial disk susceptibility tests were performed using the disk diffusion method, according to the standards of the Clinical Laboratory Standards Institute (CLSI- formerly National Committee for Clinical Laboratory Standards-NCCLS, 2002,2003). Drug-impregnated disks (CEFAR, São Paulo, BR) were placed on the surface of the Mueller Hinton agar using a disk dispenser. The following twelve antimicrobial agents were tested: ampicillin (AMP,10µg); amoxicillin (AMO,10µg), amoxicillin/clavulanic acid (AMC,30µg); amikacin (AMK,30µg); cephalothin (CFL,30µg); ceftriaxone (CEF,30µg); gentamicin (GEN,5µg); streptomycin (STR,10µg); nalidixic acid (NAL,30µg); cotrimoxazole (SUT, 25µg); ciprofloxacin (CIP,5µg), tetracycline (TET, 30µg). ***E. coli*** ATCC 25922 and ATCC 35218 were included as recommended quality control strains, both from Adolfo Lutz culture collection (São Paulo, SP)

RESULTS

A total of 92 ***E. coli*** strains were isolated from 25 dogs with diarrhea. All ***E. coli*** isolates were investigated by PCR for the presence of Shiga-like toxin-producing genes (*stx* 1 and *stx* 2) and of the intimin (*eae*) gene. As it can be seen from table 1, 21 (22.8%) of the strains carried the *stx* or the *eae* genes. PCR showed that 7 (7.6%) of STEC strains carried only the *stx* 1 gene, 5 (5.4%) the *stx* 2 gene, and none carried both *stx* 1 and *stx* 2 genes, 9 (9.8%) strains carried only the *eae* gene. Twelve STEC isolates carrying stx 1 or stx 2 genes were isolated from 10 different dogs (10/25 - 40.0%) (results not shown). All STEC strains isolated were tested by the O157 latex agglutination test kit, and not one O157 isolate was detected. There existed a positive correlation between α-hemolysin production and the presence of *stx* or *eae* (13/21-62.0%).

Among the 21 isolates carrying the *stx* or *eae* genes found, the highest resistance was showed against cephalothin (85.7%), followed by those to streptomycin (81.0%), amoxicillin (71.4%) and gentamicin (71.4%); low resistance to ceftriaxone (0.0%) and to amoxicillin/clavulanic acid (14.4%) was found (Table 2). No one isolate was susceptible to all antimicrobial agents tested.

Table 1 - Virulence gene profile of ***Escherichia coli*** isolates from twenty-five diarrheic dogs (92 isolates) in Ituverava, State of São Paulo, Brazil, between January and December 2006.

Virulence factor profile	Number of isolates (%)	α hemolysin Positive isolate/total
stx 1	7 (7.6)	2/7
stx 2	5 (5.4)	5/5
Eae	9 (9.8)	6/9
Total	21	13/21

DISCUSSION

A variety of ***E. coli*** strains including the attaching and effacing ***E. coli*** (AEEC) and the Shiga toxigenic ***E. coli*** (STEC), has been associated with diarrheic illness in dogs (BEUTIN, 1999). Occasional isolation from both healthy and diarrheic dog feces have been reported (BENTANCOR, 2006)

The occurrence of *stx* genes among diarrheic animals (10/25 - 40.0%) described in the present study, agree with the results reported by HAMMERMULER et al. (1995) showing 44.4% of such an outcome, as well as the presence of *stx* 1 and *stx* 2 genes but not of both together, in STEC isolates. Nevertheless, our results contrast with the report (HAMMERMULER et al., 1995) showing the predominance of the *stx* 2 gene among STEC isolates; in the present study, similar levels of *stx* 1 (7.6%) and of *stx* 2 (5.4%) genes were detected (Table 1).

Although the distribution of *stx* 1 and *stx* 2 genes found in diarrheic dogs agrees with that of other reports (STAATS et al., 2003; BENTANCOR et al., 2007), NAKAZATO et al. (2004) in Brazil did not find STEC strains carrying *stx* 1 or *stx* 2 genes among 146 diarrheic dogs and 36 healthy dogs analyzed. Furthermore, they did not observe a single O157:H7 strain among the animals examined.

The *eae* gene has been demonstrated in AEEC isolated from dogs, and attaching and effacing lesions have been found in dog intestinal tissue (BEAUDRY et al., 1996). Since the presence of the *eae* gene correlates with the attaching and effacing phenotype, the detection of this gene in ***E. coli*** provides sufficient evidence to indicate potential virulence (NATARO & KAPER, 1998). In the present study, four animals (16.0%) presented AEEC strains with the *eae* gene, in agreement with the report of NAKAZATO et al. (2004), but it was less then reported by others (BEAUDRY et al., 1996; BENTANCOR et al., 2007).

KRAUSE et al. (2005) reported the isolation from dogs, of AEEC strains resembling typical enteropathogenic ***E. coli*** (EPEC) (*eae* + *bfp*A+), confirming other published data (GOFFAUX et al., 2000; NAKAZATO et al., 2004). Dogs live in close contact with humans and direct transmission of ***E. coli*** strains is a probable occurrence. RODRIGUES et al. (2004)

Table 2 - Antimicrobial susceptibility testing of 21 ***E. coli*** strains carrying *stx* or *eae* genes (virulence factors) isolated from diarrheic dogs in Ituverava, SP, BR.

	------Phenotype – Percentage------		
Antimicrobial drugs	Resistant	Intermediate	Sensitive
Amikacin	28.5	19.2	52.3
Amoxicillin	71.4	19.0	9.6
Amoxicillin/ clavulanic acid	14.4	9.6	76.0
Ampicillin	71.4	4.8	23.8
Ceftriaxone	0.0	33.3	66.7
Cephalothin	85.7	14.3	0.0
Ciprofloxacin	19.2	28.5	52.3
Cotrimoxazole	38.2	28.5	33.3
Gentamicin	71.4	9.6	19.0
Nalidixic acid	23.8	38.1	38.1
Streptomycin	81.0	14.2	4.8
Tetracycline	38.2	28.5	33.3

demonstrated cross-infection between a dog and a child that lived in the same home in a city of the state of São Paulo, Brazil.

Hemolytic ***E. coli*** are a common occurrence both in healthy and in dogs with intestinal and extra-intestinal infections (BEUTIN, 1999). α-hemolytic activity was predominant (52.3%), among the STEC isolates examined; however, the significance of α-hemolysin production in strains causing enteric disease in dogs and its impact on potential virulence require further investigation.

Close contact between household pets and humans provides favorable conditions for the transmission of bacteria by direct contact (petting, licking, physical injuries, etc) or through the domestic environment (contamination of food, furnishings, etc). Children are at greater risk than adults, because of their closer physical contact with dogs as well as with household environment contaminated by pets. Bacteria resistant to antimicrobials selected for use in animal pets can reach a human host and exchange their resistance genes with bacteria residing in or on the host or vice versa (GUARDABASSI et al., 2004).

Members of most antimicrobials like tetracyclines, macrolides, lincosamides, aminoglycosides, penicillins and cephalosporins have for long periods been in use both in human and veterinary medicine; the same resistance genes have been identified in bacteria from humans and pet animals (PHILLIPS et al., 2004).

NORMAND et al. (2000) reported the results of analyses of ***E. coli*** isolates obtained from clinical cases in companion animals (dogs and cats) in the United Kingdom between 1989 and 1997. The percentages of antimicrobial resistance described agree with those reported in the present study, except for gentamicin (2.0%) and enrofloxacin (3.0%). Although the authorization of fluoroquinolones for use in small animal veterinary practice is quite recent in Europe (mid 1990s), resistance to this antimicrobial class is appearing in pet animal bacteria (GUARDABASSI et al., 2004). In the present study the percentage of resistance to ciprofloxacin was high (19.2%); the liberalization of this compound for veterinary use in Brazil is more recent than in Europe, and could indicate its misuse in veterinary practice in Brazil.

CARATTOLI et al. (2005) analyzed 298 ***E. coli*** isolates from specimens of 204 dogs submitted to routine diagnostic investigation in Italy between 2001 and 2003. The reported percentages of bacterial resistance were quite similar to that found in our study for tetracycline, nalidixic acid, cotrimoxazole and fluoroquinolones but very different regarding gentamicin (8.1%) and amikacin (0.7%).

To conclude, the present study showed the presence of STEC and AAEC strains in bacterial isolates from diarrheic dogs, as well as their high level of resistance to antimicrobial agents.

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