Prevalence of virulence factors in *Escherichia coli* isolated from healthy animals and water sources in Brazil

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ABSTRACT

The aim of this work was to verify the presence of seven virulence factors (ST, LT, *eae*, stx_1, stx_2, INV and EAEC) among *Escherichia coli* strains isolated from healthy humans, bovines, chickens, sheep, pigs and goats, from two sewage treatment plants and from the Tietê River. We have found a high prevalence of *eae*, stx_1 and stx_2 in ruminants. The EAEC gene was only found in humans and sewage. No strains presented ST, LT or INV. BOX-PCR fingerprints revealed a high diversity among the strains analysed and a non-clonal origin of strains that presented the same virulence factors. Therefore, we concluded that ruminants may constitute an important reservoir of most diarrheagenic *E. coli* in Brazil, except for EAEC strains. These results emphasize the importance of the identification of the animal source of fecal contamination for the correct water risk assessment.

Key words | BOX-PCR, *Escherichia coli*, fecal contamination, virulence factors

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INTRODUCTION

Fecal contamination of water systems is the major cause of waterborne enteric diseases in the world, responsible for killing about 2 million children a year (WHO 2007). This situation has worsened in recent years as a result of an increase in the contamination of drinking, recreational and irrigation water with emerging pathogens such as *Giardia*, *Cryptosporidium* and *Escherichia coli* O157: H7, among others.

Escherichia coli is a bacterium widely spread among warm-blooded animals. It is responsible for many intestinal and extra-intestinal diseases. According to Moreno *et al.* (2010), diarrheagenic *E. coli* is one of the most important enteric pathogens in Brazilian infants. The intestinal pathogenic strains belong to the pathotypes: enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC). These pathotypes present specific virulence factors and symptoms which can be used for *E. coli* strains identification (Kaper *et al.* 2004).

Several studies have demonstrated the importance of animals in the epidemiology of some *E. coli* pathotypes (reviewed in Nataro & Kaper 1998). Ruminants have been described as the main reservoir of STEC and EHEC (Beutin *et al.* 1993; Brett *et al.* 2003). However, some studies have indicated that healthy adult human carriers could be responsible for the dissemination of EAEC (Oundo *et al.* 2008). Therefore, the aim of this work was to verify the distribution of virulence factors (VFs) specific for the main *E. coli*

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pathotypes among *E. coli* strains from different sources in Brazil.

MATERIALS AND METHODS

Bacterial strains

Two hundred and twenty-two strains of *E. coli* were isolated from fecal samples, collected with sterile swabs, of a variety of non-diarrheic hosts including humans. Each strain was isolated from a single healthy animal. Furthermore, 47 strains were isolated from two sewage treatment plants (Barueri and Jesus Neto, São Paulo State, Brazil) and 15 strains were isolated from the Tietê River in Salesópolis City, São Paulo State, Brazil (Table 1).

The human, sewage and river strains were isolated as described in Orsi *et al.* (2008). Briefly, samples were subjected to the membrane filter technique with mTEC agar (Difco) with β-d-indoxyl glucoside (final concentration 100 mg l^{-1}) and incubated for 2 h at 35 ± 0.5°C and 22–24 h at 44.5 ± 0.2°C. Typical colonies (yellow) were transferred to EC broth and incubated at 44.5°C for 24 h. Positive cultures were streaked on EMB agar (Merck). Isolated colonies were tested for citrate utilization, lactose fermentation, oxidase, L-lysine decarboxylase, motility, glucose and sucrose fermentation, tryptophan deamination, indole production, urea hydrolysis and sulfide production. Isolates with an *E. coli* profile were inoculated into LB broth at 37°C overnight. One isolated colony from each EC positive sample was selected for further analyses. The animal strains were isolated using MacConkey and EMB plates and the typical *E. coli* colonies were tested as described above.

The study was approved by the Research Ethics Committee of the State University of Campinas School of Medical Sciences.

Table 1 | Source, number and sampling site of the 284 *E. coli* strains used in this study

Source	Number of strains	Sampling site (City/State)
Humans	74	São Paulo/São Paulo
Bovines	49	Jaboticabal/ São Paulo
Chickens	15	Ribeirão Preto/São Paulo
Pigs	39	Jaboticabal/ São Paulo
Goats	16	Jaboticabal/São Paulo
Sheep	29	Jaboticabal/ São Paulo
Sewage	47	São Paulo/ São Paulo
Tietê River	15	Salesópolis/ São Paulo

Note: All strains were isolated from healthy animals

Search for virulence factor encoding genes of diarrheagenic *E. coli*

The screening for diarrheagenic *E. coli* virulence genes in the 284 *E. coli* strains was performed by colony hybridization using as probes, DNA fragments obtained by PCR amplification of prototype strains presenting the following virulence genes: *eae* (*E. coli* A/E gene encoding intimin), EAEC (EAEC plasmid), *ipaH* (*E. coli* invasiveness plasmid), LT-I (heat-labile enterotoxin type I), ST (comprising the genes encoding heat-stable enterotoxin type I of human and porcine origin), and Stx-I and II (Shiga toxin I and II genes). The characteristics of the amplicons used as probes, the prototype strains and references for the specific primers used are shown in Table 2. The amplicons were labelled with [α^{32}P]dCTP (GE Healthcare) using the kit Ready-To-Go DNA Labelling Beads (GE, United Kingdom). The colony hybridization assays were performed under stringent conditions (Sambrook *et al.* 1989).

BOX-PCR genotyping

All the 284 *E. coli* strains were analysed by BOX-PCR. For this, genomic DNA was isolated from the strains with the Wizard Genomic DNA Purification Kit (Promega), following the manufacturer's instructions. The BOX-PCR reactions were carried out as described by Versalovic *et al.* (1994) and Orsi *et al.* (2007) with modifications. The reaction mixtures (20 μl) consisted of 10 ng of DNA, 50 pmoles of the BOXA1R primer (5′-CTACGGCAAGGCGACGCTGAGG-3′; Versalovic *et al.* (1994)), 1 × Taq polymerase buffer, 0.1 mM of each dNTP, 2 mM $MgCl_2$ and 0.5 units of Taq polymerase (Fermentas). PCR reactions were performed in duplicate in a PT 100 thermocycler (MJ Research Inc.). The amplification conditions included an initial denaturation step at 95°C for 7 min, followed by 30 amplification cycles (94°C for 1 min, 53°C for 1 min, 56°C for 4 min) and a final extension at 65°C for 16 min. The amplification products were separated by electrophoresis on 2.0% agarose, 1× TBE buffer gels

Table 2 | Characteristics of the DNA amplicons used as probes for detection of diarrheagenic *E. coli* virulence factors

Probe	Virulence factor	*E. coli* pathotype	Amplicon size (pb)	Prototype strains	Reference
ST	Heat-stable enterotoxin	ETEC	190	3671–1/85	Stacy-Phipps *et al.* (1995)
LT	Heat -labile enterotoxin	ETEC	696	2871–5/85	Schultz *et al.* (1994)
eae	Intimin	EPEC	917	E2348/69	Gannon *et al.* (1993)
stx1	Shiga toxin 1	EHEC	348	EDL933	Cebula *et al.* (1995)
stx2	Shiga toxin 2	EHEC	548	EDL933	Cebula *et al.* (1995)
EAEC	EAEC plasmid	EAEC	630	17–2	Schmidt *et al.* (1995)
INV	*ipaH* sequence	EIEC	424	2401–1/85	Venkatesan *et al.* (1989)

(Sambrook *et al.* 1989). The gels containing ethidium bromide (0.5 μg ml^{-1}) were run at 5.0 Vcm^{-1}. The results were visualized and recorded with a gel documentation system (Kodak).

The BOX-PCR fingerprints were analysed with the software GelCompar II (Applied Maths). A dendrogram was constructed by using the Jaccard similarity coefficient (Hassan *et al.* 2007) and the UPGMA (unweighted pair group method with arithmetic mean) algorithm. Strains that did not produce fingerprints were excluded from the analysis.

RESULTS

The 284 *E. coli* strains from different sources were tested for the presence of seven VFs (ST, LT, *eae*, stx_1, stx_2, INV and EAEC) genes representing five diarrheagenic *E. coli* pathotypes. None of the strains presented the ST, LT and INV encoding genes. The strains isolated from the Tietê River presented no VF gene while 58 strains (20.4%) presented at least one VF gene (Table 3). Twenty-one of these strains (two from goats, eight from bovines and 11 from sheep) presented two VFs genes (Table 3). Most of them presented the genes stx_1 and stx_2 and only two strains from sheep presented *eae* and stx_2.

Seven strains (2.5%), one from humans, two from pigs and four from sheep, presented the *eae* gene. Thirty-five strains (12.3%) presented the stx_1 gene. Among these strains, one was isolated from pigs, eight from goats, nine from bovines and 17 from sheep. Twenty-eight strains (9.9%) presented the stx_2 gene. These strains were isolated from chickens (one strain), pigs (one strain), goats (four strains), sheep (nine strains) and bovines (13 strains). Nine strains (3.2%) isolated from humans (four strains) and sewage (five strains) presented the EAEC gene.

Fifty-seven per cent of the strains that presented the *eae* gene were from sheep, whereas 48.6% of the strains with the stx_1 were from sheep, 25.7% from bovines and 22.9% from goats. The stx_2 gene was found in 46.4% of the strains from bovines, 32.1% from sheep and 14.28% from goats.

The BOX-PCR fingerprints presented a high variability, with the similarities ranging from 5.57 to 100%. The dendrogram structure was complex, and 26 clusters were formed with a similarity higher than 20% (data not shown). Strains that presented the same virulence factors were not clustered together indicating that these potential pathogenic strains are highly diverse. No significant association between the genetic fingerprint/bands and the VFs genes was found. The highest similarity between two strains with the same VFs genes was 66.67% (two strains from sheep harbouring stx_1).

Table 3 | Distribution of genes encoding virulence factors among the *E. coli* strains from different sources and percentage of strains carrying at least one gene*

	eae	*stx1*	*stx2*	EAEC	% of strains with at least one gene
Sheep	4	17	9	0	66.5
Goats	0	8	4	0	62.5
Bovines	0	9	13	0	28.6
Sewage	0	0	0	5	10.6
Chickens	0	0	1	0	6.7
Humans	1	0	0	4	6.7
Pigs	2	1	1	0	10.2
Tietê River	0	0	0	0	0
Total	**7**	**35**	**28**	**9**	**20.4**

*None of the strains carried the virulence genes: ST, LT and INV

DISCUSSION

In this work we have investigated the presence of seven virulence factors in *E. coli* strains from non-diarrheic humans and animals, from sewage and from the Tietê River in order to identify the major reservoirs of enteric pathogenic *E. coli* strains and to assess the genetic variability of these strains. A high prevalence of stx_1, stx_2 and *eae* genes was found in sheep, bovines and goats. Recently, Vettorato *et al.* (2009) have found a high prevalence of these genes among *E. coli* strains isolated from sheep in the State of São Paulo, Brazil. Cerqueira *et al.* (1999) have found a high occurrence of *stx* genes in *E. coli* strains from healthy cattle in the State of Rio de Janeiro, Brazil. Our results have shown the importance of ruminants as reservoirs of potentially pathogenic *E. coli* strains.

Although none of the strains from animals presented the EAEC gene, this gene was found in four strains isolated from healthy humans and in five from the sewage of the Jesus Neto sewage treatment plant. The strains from sewage presented different BOX-PCR fingerprints, suggesting that they have a non-clonal origin. EAEC is an emerging enteric pathogen responsible for several outbreaks in humans around the world (Kaper *et al.* 2004). Apparently, it is rare to find EAEC strains in animals and they probably do not constitute an important reservoir of this pathotype (Uber *et al.* 2006). Therefore, we suggest that healthy adult human carriers may have a role in the epidemiology of the EAEC strains.

The Tietê River is one of the most polluted rivers in Brazil (Rocha *et al.* 2009). However, it rises in an environmentally protected area in the city of Salesópolis and, since the sampling site was in this preserved area, a low level of fecal contamination was expected. In fact, no VF genes were found in the *E. coli* strains isolated from this sampling site.

Escherichia coli strains can be separated into four main phylogenetic groups: A, B1, B2 and D (Selander *et al.* 1986; Herzer *et al.* 1990). Clermont *et al.* (2000) described a simple PCR-based method that uses a combination of the *chuA* and *yjaA* genes and the DNA fragment TSPE4.C2 to assign *E. coli* strains to the phylogenetic groups. The strains analysed in this work were classified into one of these four main phylogenetic groups (A, B1, B2 and D) in a previous work (Carlos *et al.* 2010). Most of the strains bearing virulence factors belonged to groups A and B1, 31.03 and 55.2%, respectively, 12.1% belonged to group D and 1.7% to group B2. Escobar-Páramo *et al.* (2004) have also found a remarkable association between diarrheagenic virulence genes and the phylogenetic groups A and B1. These authors concluded that there is a lack of compatibility between these genes and the genetic background of groups D and B2. The same authors have suggested that the acquisition of some VFs requires a particular genetic background, such as the VFs of enterohemorrhagic, enterotoxigenic and enteroinvasive *E. coli*, whereas other VFs can be found in different genetic backgrounds, such as those of enteroaggregative, enteropathogenic and diffusely adhering *E. coli*.

In the present study, we have found no association between a particular genetic fingerprint (as detected by BOX-PCR) and a specific VF gene. An interpretation of this result is that a broad range of genetic backgrounds can harbour the VFs tested and the potentially pathogenic strains found can have different origins.

Our results indicate that water contaminated with feces from sheep, bovines and goats might present a higher risk of enteric pathogenic *E. coli* than contamination with human feces. This result emphasizes the importance of the identification of the animal source of fecal contamination for the effective management and risk assessment of water systems.

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