

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

Camila Carlos, Fabiana Alexandrino, Monica A. M. Vieira, Nancy C. Stoppe, Maria Inês Z. Sato, Tânia A. T. Gomes and Laura M. M. Ottoboni

### ABSTRACT

The aim of this work was to verify the presence of seven virulence factors (ST, LT, *eae*, *stx*<sub>1</sub>, *stx*<sub>2</sub>, 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*<sub>1</sub> and *stx*<sub>2</sub> 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

Camila Carlos  
Fabiana Alexandrino  
Nancy C. Stoppe  
Laura M. M. Ottoboni (corresponding author)  
Centro de Biologia Molecular e  
Engenharia Genética,  
Universidade Estadual de Campinas - UNICAMP,  
C.P. 6010, 13083-875 Campinas,  
S.P., Brasil  
Tel: +55 19 3521 1136  
Fax: +55 19 3521 1089  
E-mail: ottoboni@unicamp.br

Monica A. M. Vieira  
Tânia A. T. Gomes  
Departamento de Microbiologia,  
Imunologia e Parasitologia,  
Universidade Federal de São Paulo - UNIFESP,  
04023-062 São Paulo,  
S.P., Brasil

Maria Inês Z. Sato  
Departamento de Análises Ambientais,  
Companhia Ambiental do Estado de  
São Paulo - CETESB,  
05459-900 São Paulo,  
S.P., Brasil

### 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*

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  $\beta$ -d-indoxyl glucoside (final concentration 100 mg l<sup>-1</sup>) 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.

### 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 [ $\alpha^{32}$ 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  $\mu$ l) consisted of 10 ng of DNA, 50 pmoles of the BOXA1R primer (5'-CTACGGCAAGGCGACGCTGAGG-3'; Versalovic *et al.* (1994)), 1  $\times$  Taq polymerase buffer, 0.1 mM of each dNTP, 2 mM MgCl<sub>2</sub> 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 $\times$  TBE buffer gels

**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

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

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

(Sambrook *et al.* 1989). The gels containing ethidium bromide (0.5 µg ml<sup>-1</sup>) were run at 5.0 Vcm<sup>-1</sup>. 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*, *stx1*, *stx2*, 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 *stx1* and *stx2* and only two strains from sheep presented *eae* and *stx2*.

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 *stx1* 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 *stx2* 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 *stx1* were from sheep, 25.7% from bovines and 22.9% from goats. The *stx2* 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 *stx1*).

**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\*

|             | <i>eae</i> | <i>stx1</i> | <i>stx2</i> | 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*<sub>1</sub>, *stx*<sub>2</sub> 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.

## ACKNOWLEDGEMENTS

This work was supported by a grant from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2007/55312-6). C.C. received a fellowship from FAPESP (FAPESP 2007/57025-4). L.M.M.O. received a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors thank Dr Wanderley Dias da Silveira and Dr Luiz A. Amaral for providing the *E. coli* strains from animal feces.

## REFERENCES

- Beutin, L., Stroehrer, U. H. & Manning, P. 1993 Isolation of enterohemolysin (Ehly2)-associated sequences encoded on temperate phages of *Escherichia coli*. *Gene* **132**, 95–99.
- Brett, K. N., Ramachandran, V., Hornitzky, M. A., Bettelheim, K. A., Walker, M. J. & Djordjevic, S. P. 2003 *stx1c* is the most common Shiga-toxin 1 subtype among Shiga toxin producing *Escherichia*

- coli* isolates from sheep but not among isolates from cattle. *J. Clin. Microbiol.* **41**, 926–936.
- Carlos, C., Pires, M. M., Stoppe, N. C., Hachich, E. M., Sato, M. I. Z., Gomes, T. A. T., Amaral, L. A. & Ottoboni, L. M. M. 2010 *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of fecal contamination. *BMC Microbiol.* **10**, 161.
- Cebula, T. A., Payne, W. L. & Feng, P. 1995 Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their Shiga-like toxin by mismatch amplification mutation assay-multiplex PCR. *J. Clin. Microbiol.* **33**, 248–250.
- Cerqueira, A. M., Guth, B. E., Joaquim, R. M. & Andrade, J. R. 1999 High occurrence of Shiga toxin-producing *Escherichia coli* (STEC) in healthy cattle in Rio de Janeiro State, Brazil. *Vet. Microbiol.* **70**, 111–121.
- Clermont, O., Bonacorsi, S. & Bingen, E. 2000 Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl. Environ. Microbiol.* **66**, 4555–4558.
- Escobar-Páramo, P., Blanc-Potard, A. B., Bui, H., Le Bouguenec, C. & Denamur, E. 2004 A specific genetic background is required for acquisition and expression of virulence factors in *Escherichia coli*. *Mol. Biol. Evol.* **2**, 1085–1094.
- Gannon, V. P. J., Rashed, M., King, K. R. & Golsteyn, T. E. J. 1993 Detection and characterization of the *eae* gene of shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. *J. Clin. Microbiol.*, **31**, 1268–1274.
- Hassan, W. M., Ellender, R. D. & Wang, S. Y. 2007 Fidelity of bacterial source tracking: *Escherichia coli* vs. *Enterococcus* spp and minimizing assignment of isolates from nonlibrary sources. *J. Appl. Microbiol.* **102**, 591–598.
- Herzer, P. J., Inouye, S., Inouye, M. & Whittan, T. S. 1990 Phylogenetic distribution of branched RNS-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. *J. Bacteriol.* **172**, 6175–6181.
- Kaper, J. B., Nataro, J. P. & Mobley, H. L. T. 2004 Pathogenic *Escherichia coli*. *Nature Rev.* **2**, 123–140.
- Moreno, A. C. R., Filho, A. F., Gomes, T. A. T., Ramos, S. T. S., Montemor, L. P. G., Tavares, V. C., Filho, L. S., Irino, K. & Martinez, M. B. 2010 Etiology of childhood diarrhea in the northeast of Brazil: significant emergent diarrheal pathogens. *Diagn. Microb. Infect. Dis.* **66**, 50–57.
- Nataro, J. P. & Kaper, J. B. 1998 Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* **11**, 142–201.
- Orsi, R. H., Stoppe, N. C., Sato, M. I., Gomes, T. A., Prado, P. I., Manfio, G. P. & Ottoboni, L. M. M. 2007 Genetic variability and pathogenicity potential of *Escherichia coli* isolated from recreational water reservoirs. *Res. Microbiol.* **158**, 420–427.
- Orsi, R. H., Stoppe, N. C., Sato, M. I. Z., Prado, P. I. & Ottoboni, L. M. M. 2008 Phylogenetic group distribution among *Escherichia coli* isolated from rivers in São Paulo State, Brazil. *World J. Microb. Biot.* **24**, 1573–1577.
- Oundo, J. O., Kariuki, S. M., Boga, H. I., Muli, F. W. & Iijima, Y. 2008 High incidence of enteroaggregative *Escherichia coli* among food handlers in three areas of Kenya: A possible transmission route of travelers' diarrhea. *J. Travel Med.* **1**, 31–38.
- Rocha, P. S., Luvizotto, G. L., Kosmehl, T., Böttcher, M., Storch, V., Braunbeck, T. & Hollert, H. 2009 Sediment genotoxicity in the Tietê River (São Paulo, Brazil): In vitro comet assay versus in situ micronucleus assay studies. *Ecotox. Environ. Safe.* **72**, 1842–1848.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, 2nd edn.
- Schmidt, H., Knop, C., Franke, S., Aleksie, S., Heeseman, J. & Karch, H. 1995 Development of PCR for screening of enteroaggregative *Escherichia coli*. *J. Clin. Microbiol.* **33**, 701–705.
- Schultz, C., Pool, G. J., van Ketel, R., de Wever, B., Speelman, P. & Dankert, J. 1994 Detection of enterotoxigenic *Escherichia coli* in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. *J. Clin. Microbiol.* **31**, 1268–1274.
- Selander, R. K., Caugant, D. A., Ochman, H., Musser, M., Gilmour, M. N. & Whittam, T. S. 1986 Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl. Environ. Microbiol.* **51**, 873–884.
- Stacy-Phipps, S., Mecca, J. J. & Weiss, J. B. 1995 Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* during course of infection. *J. Clin. Microbiol.* **33**, 1054–1059.
- Uber, A. P., Trabulsi, L. R., Irino, K., Beutin, L., Ghilardi, A. C., Gomes, T. A., Liberatore, A. M., de Castro, A. F. & Elias, W. P. 2006 Enteroaggregative *Escherichia coli* from humans and animals differ in major phenotypical traits and virulence genes. *FEMS Microbiol. Lett.* **256**, 251–257.
- Venkatesan, M. M., Buysse, J. M. & Kopecko, D. J. 1989 Use of *Shigella flexneri ipaC* and *ipaH* sequences for the general identification of *Shigella* spp. and enteroinvasive *Escherichia coli*. *J. Clin. Microbiol.* **27**, 2687–2691.
- Versalovic, J., Schneider, M., de Bruijn, F. J. & Lupski, L. R. 1994 Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Method. Mol. Cell Biol.* **5**, 25–40.
- Vettorato, M. P., de Castro, A. F. P., Cergole-Novella, M. C., Camargo, F. L. L., Irino, K. & Guth, B. E. C. 2009 Shiga toxin-producing *Escherichia coli* and atypical enteropathogenic *Escherichia coli* strains isolated from healthy sheep of different populations in São Paulo, Brazil. *Let. Appl. Microbiol.* **49**, 53–59.
- WHO (World Health Organization) 2007 *Water Supply, Sanitation and Hygiene Development*, [http://www.who.int/water\\_sanitation\\_health/hygiene/en/index.html](http://www.who.int/water_sanitation_health/hygiene/en/index.html), accessed 24 November 2010.