Molecular and phenotypic characterization of diarrheagenic Escherichia coli isolated from groundwater in rural areas in southern Brazil


ABSTRACT

Water-borne diseases like diarrheagenic Escherichia coli (DEC)-induced gastroenteritis are major public health problems in developing countries. In this study, the microbiological quality of water from mines and shallow wells was analyzed for human consumption. Genotypic and phenotypic characterization of DEC strains was performed. A total of 210 water samples was analyzed, of which 153 (72.9%) contained total coliforms and 96 (45.7%) E. coli. Of the E. coli isolates, 27 (28.1%) contained DEC genes. The DEC isolates included 48.1% Shiga toxin-producing E. coli (STEC), 29.6% enteroaggregative E. coli (EAEC), 14.9% enteropathogenic E. coli (EPEC), 3.7% enterotoxigenic E. coli (ETEC), and 3.7% enteroinvasive E. coli (EIEC). All the STECs had cytotoxic effects on Vero cells and 14.8% of the DEC isolates were resistant to at least one of the antibiotics tested. All DEC formed biofilms and 92.6% adhered to HEp-2 cells with a prevalence of aggregative adhesion (74%). We identified 25 different serotypes. One EPEC isolate was serotype O44037:H7, reported for the first time in Brazil. Phylogenetically, 63% of the strains belonged to group B1. The analyzed waters were potential reservoirs for DEC and could act as a source for infection of humans. Preventive measures are needed to avoid such contamination.

Key words | DEC, Escherichia coli, groundwater, water for consumption

INTRODUCTION

Waterborne diseases are major public health problems in developing countries. It is estimated that contaminated water caused more than 15 million deaths, of which more than 80% were children under five years of age (WHO 2014). Although the total number of deaths attributable to diarrhea declined substantially between 1990 and 2012, a lack of access to safe drinking water, sanitation, and adequate hygiene still accounts for the death of more than a thousand children each day worldwide (Chakravarty et al. 2017). Despite reductions in mortality, the morbidity attributable to diarrhea remains unchanged at about 1.7 billion cases per year (Chakravarty et al. 2017).

Among the various contaminants in drinking water, microorganisms are considered one of the most serious in regards to public health hazards. The United States Environmental Protection Agency (USEPA) recommends the use of Escherichia coli as an indicator organism of fecal contamination by humans and other endothermic animals, as well as a potential indicator of the presence of pathogenic microorganisms (USEPA 2017).

Diarrheagenic E. coli (DEC) is one of the major groups of etiologic agents responsible for intestinal infections. These microorganisms account for up to 40% of acute diarrhea episodes in children in developing countries (Miliwebsky et al. 2016). This group of bacteria also plays a considerable causative role in diarrhea in Brazil, in both children and adults (Spano et al. 2017).

DEC strains are classified into eight pathotypes: enteropathogenic E. coli (EPEC), enterotoxigenic E. coli (ETEC), enteroinvasive E. coli (EIEC), Shiga toxin-producing E. coli (STEC), enteroaggregative E. coli (EAEC), diffusely adherent E. coli (DAEC), adherent invasive E. coli (AIEC), and Shiga toxin producing enteroaggregative E. coli (STEAEC). The STEC pathotype also contains the sub-category enterohemorrhagic E. coli (EHEC) (Clements et al. 2012).

DEC transmission typically involves a fecal–oral route involving contaminated sources of food or water and may be involved in outbreaks of waterborne diarrhea (Park et al. 2018).

Alternative groundwater extraction sources, such as mines and shallow wells, are more susceptible to contamination by domestic sanitary sewage because of their shallower depth, and are often used for water supply in rural areas. Therefore, the use of these sources for potable water is a health risk for this population (WHO 2011).

The objective of the current study was to evaluate the microorganisms of groundwater, non-treated water from mines and shallow wells that are used as sources of water for human consumption in southern Brazil and to characterize the genotypic and phenotypic properties of the DEC strains isolated.

METHODS

Water samples

A total of 210 in natura water samples for human consumption were collected from shallow wells and mines located in rural areas of the northern state of Paraná, southern Brazil, from May to August 2017. The properties were chosen by sanitary surveillance technicians and the samples were collected only once from each property, always with the inspection of sanitary surveillance technicians. The samples were collected in 500 mL sterile glass vials and transported in chilled isothermal boxes to the laboratory where they were stored at 4 °C until analyzed. The time span from collection to analysis did not exceed 6 hours.

Analysis of total coliforms and E. coli

The technique used for the detection and quantification of total coliforms and E. coli was the defined substrate method Colilert (IDEXX Laboratories, Sovereign, USA), in accordance with methodology approved by the USEPA (2017), as described by Schuroff et al. (2014). After incubation of the Quanti-Tray cartons (WP2000), quantitative estimates of the most probable number (MPN) of total coliforms and E. coli were determined according to the manufacturer’s instructions.

For the isolation of the E. coli strains, the carton wells that displayed changes in the media color to yellow and acquired blue-fluorescent coloration against UV light were used to seed MacConkey agar plates (Difco®, USA) and incubated at 37 °C for 24 hours. After incubation, three to five colonies from each plate demonstrating presumptive characteristics of E. coli were submitted for biochemical identification using EPM, MILi, and Simmons Citrate media (PROBAC, BR). Isolates biochemically identified as E. coli were stored at −80 °C in heart and brain infusion broth (BHI; Difco®, USA) containing 20% (v/v) glycerol (Sigma, USA).

DEC genotypical characterization

DNA was extracted from the bacterial isolates by boiling, as previously described by Lascowski et al. (2013). The supernatants containing the DNA were then used for the identification of the DEC strains and their classification into phylogenetic groups. A total of 370 E. coli isolates, obtained from 96 water samples that were positive for E. coli, were analyzed for DEC identification. The stx1, stx2, and eae genes were used to identify STEC and EHEC strains; eae and bfp for identifying typical EPEC (tEPEC) and atypical EPEC (aEPEC) strains; ST-1a, ST-1b, and elt for identifying ETEC strains; ipaH for identifying EIEC strains; aatA, aggR, aaiA, and aaiC for identifying typical
EAEC (tEAEC) and atypical (aEAEC). The results are summarized in Table 1.

The strain identification was performed using polymerase chain reaction (PCR) amplification of the specific target genes on a GeneAmp® PCR System 9,700 thermocycler (Applied Biosystems, USA). Each of the bacterial DNA amplification reactions contained 2 μL of the bacterial-DNA lysate, 0.2 mM dNTPs, 2.0 mM MgCl₂, 20 pmol of each oligonucleotide primer, 1 U of Taq DNA polymerase (Invitrogen™), 1× reaction buffer, and sterile Milli-Q (Millipore) water at a final volume of 25 μL. Seven microliters of the amplified product was analyzed by 1.5% – 2% agarose gel electrophoresis (Invitrogen™) using Tris Borate EDTA (TBE) buffer. A 100 bp ladder (Invitrogen™) was used as a molecular size marker. The gels were stained with SYBR SAFE solution (Invitrogen™) and visualized under ultraviolet light transillumination (Vilbert Loumart, France). The positive controls included EHEC EDL933 for the stx1, stx2, and eae genes; EPEC E2348/69 for bfpA; EAEC 042 for aaiA, aaiC, aatA, and aggR; ETEC H10407 for ST-1a and elt; ETEC 4083 for ST-1b; and EIEC EDL1284 for ipaH.

Determination of phylogenetic groups

The strains positive for the presence of DEC virulence genes were subsequently tested by PCR for the presence of the genes chuA, yjaA, arpA, and trpA and for the DNA fragment TSPE4.C2. The results were used for characterization of the strains relative to the phylogenetic groups A, B1, B2, C, D, E, and F according to Clermont et al. (2013).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'–3')</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>bfpA</td>
<td>(F) CAATGGTGCTTGCCTTGGT</td>
<td>326</td>
<td>Gunzburg et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>(R) GCCGCTTTATCCAAACCTGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eae</td>
<td>(F) GACCCGGCACAAGCATAGC</td>
<td>384</td>
<td>Paton &amp; Paton (1998)</td>
</tr>
<tr>
<td></td>
<td>(R) CCACCTGCAAGCAACAGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx1</td>
<td>(F) ATAAATCGCCATTCGTTGACTAC</td>
<td>180</td>
<td>Paton &amp; Paton (1998)</td>
</tr>
<tr>
<td></td>
<td>(R) AGAACGCCCACTGAGATCATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx2</td>
<td>(F) GGCACGTCTGAACATGTCCC</td>
<td>255</td>
<td>Paton &amp; Paton (1998)</td>
</tr>
<tr>
<td></td>
<td>(R) TCGCAGTTATCTGACATTCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST 1a</td>
<td>(F) TCTGTATTATTTCTCCCTTC</td>
<td>186</td>
<td>Schultsz et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>(R) ATAAACATCACGCAACAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST 1b</td>
<td>(F) CCCTCAGGATGCTAAACAG</td>
<td>166</td>
<td>Schultsz et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>(R) TTAATACGCACCGGTACAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>elt</td>
<td>(F) GGCACAGATTACGCTGCC</td>
<td>450</td>
<td>Aranda et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>(R) CGGTCTCTATATTCCTGGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aatA</td>
<td>(F) CTGGCGAAAGACTGTACATC</td>
<td>630</td>
<td>Schmidt et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>(R) AATGTATAGAAATCCGCTGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aggR</td>
<td>(F) GCAATCAGATTARACGCGATACA</td>
<td>426</td>
<td>Boisen et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>(R) CATCTTTATGTCAAGAGATCTGGG</td>
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<td></td>
</tr>
<tr>
<td>aaiA</td>
<td>(F) CCCACGACAGATAACG</td>
<td>476</td>
<td>Dudley et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>(R) GTTTTCAGGATTGCCATTAG</td>
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<td></td>
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<tr>
<td>aaiC</td>
<td>(F) ATTCCTTCAGCCATGTCAC</td>
<td>215</td>
<td>Lima et al. (2013)</td>
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<tr>
<td></td>
<td>(R) ACGCTCCCTGATAAAACA</td>
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</tr>
<tr>
<td>ipaH</td>
<td>(F) GTTCCTTGACGCCTTTCGCCATGTC</td>
<td>600</td>
<td>Aranda et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>(R) GCCGTCGACCACCTCTGAGATC</td>
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</tr>
</tbody>
</table>
Serotyping

The serotyping of the DEC isolates was performed by micro-agglutination (Navarro et al. 2016) using rabbit sera against somatic antigens (O1 to O187) and flagellar antigens (H1 to H53) from E. coli and against 46 different somatic antigens from Shigella sp.

Adherence assay

The DEC isolates were tested for adhesion in HEp-2 cells according to the technique described by Cravioto et al. (1999). The assay was performed allowing 6 hours of bacterial–cell interaction and then evaluated using light microscopy.

Biofilm formation test

Biofilm formation was evaluated using the method described by Wakimoto et al. (2004). Biofilm formation was considered positive when the optical density at 570 nm (OD 570) was greater than 0.2 OD units. EAEC strain 042 was used as a positive control and E. coli HB101 (E. coli K-12) was used as a negative control.

Antimicrobial susceptibility test

The bacterial strains were submitted to antimicrobial susceptibility testing using the disk diffusion technique as described by the Clinical Laboratory Standards Institute (CLSI 2016). The antimicrobial agents used were ampicillin (AMP) 10 μg, cefoxitin (CFO) 30 μg, cephalothin (CFL) 30 μg, ciprofloxacin (CIP) 5 μg, nalidixic acid (NAL) 30 μg, piperacillin-tazobactam (PPT) 100/10 μg, ampicillin-sulbactam (ASB) 10/10 μg, amicacin (AMI) 30 μg, gentamicin (GEN) 10 μg, and chloramphenicol (CLO) 30 μg.

STEC cytotoxicity in Vero cells

Production of Stx toxin by the STEC isolates was verified by Vero cell cytotoxicity assays as previously described by Beutin et al. (2002), with modifications. Supernatants from E. coli strains EDL933 and HB101 were used as positive and negative controls, respectively. The cytotoxicity of the isolates was quantified according to the metabolic activity of the cells by the MTT assay (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Sigma-Aldrich, USA) which evaluates the metabolism of tetrazolium salts by the mitochondria in viable cells (Murakami et al. 2000). The percentage of cytotoxicity was calculated using the following formula: (absorbance of the sample – absorbance of the negative control)/(absorbance of the positive control – absorbance of the negative control) × 100. The absorbance was measured by spectrophotometry at 570 nm in a Multiskan EX ELISA reader (Labsystems, Finland).

An isolate was considered cytotoxic when it induced 50% or greater cell death.

RESULTS

Presence of total coliforms and E. coli

Of the 210 water samples collected from mines and shallow wells, 72.9% (153/210) were contaminated with coliforms. The prevalence of E. coli was 45.7% (96/210). Of the 96 water samples positive for E. coli, 370 E. coli isolates were isolated and evaluated for the presence of DEC genes.

DEC identification

DEC-related genes were present in 28.1% of the 96 E. coli positive samples, of which 48.1% included isolates with genotypic characteristics of STEC (eae – and stx+), 92.3% were positive for stx2, and 7.7% positive for stx1. The remaining DEC isolates were 11.1% tEAEC (aggR+), 18.6% aEAEC (aaiA+ and aggR–), 3.7% tEPEC (eae+ and bfp+), 11.1% EPECa (eae), 3.7% ETEC (ST-1a+), and 3.7% for EIEC (ipaH+) (Table 2).

Phylogenetic classification

Of the 27 isolates determined to be DEC strains based on genotypic analysis, 63% were classified to the phylogenetic group B1, 18.5% to the phylogenetic group C, 11.1% to the phylogenetic group B2, and 7.4% to the phylogenetic group E. Of the 13 STEC isolates, 61.5% belonged to group C, 15.4% to group E, and 7.7% to group B2. None
of the isolates belonged to phylogenetic groups A, D, or F (Table 2).

Serotyping

Among the 27 DEC isolates, 25 different serotypes were identified. Two tEAEC isolates shared the same O64474:H2 serotype, and two other strains from genotypes STEC and aEAEC shared the serotype O91:H21 (Table 2). In genotype aEPEC, we found the serotypes O44037:H7, O145:H34, and O109:H21 and in genotype tEPEC, serotype O132:H34. In the STEC isolates, serotypes O103:H7, O185:H16, O4:H12, O73:H12, O103:H16, O139:H7, O91:H10, O150:H8, O49:H49, O6:H49, O91:H21, O64474:H12, and O107:HNT were identified. In tEAEC DEC strains, serotypes O64474:H2 and O8:H48 were identified. For

Table 2  Genotypic and phenotypic characteristics of DEC isolated from water samples from mines and shallow wells of the northern region of Paraná, Brazil

<table>
<thead>
<tr>
<th>No. of isolate</th>
<th>Genotypic profile</th>
<th>Serotype</th>
<th>Stx(^a)</th>
<th>HEP-2(^b)</th>
<th>Biofilm formation</th>
<th>Resistance profile</th>
<th>Phylogenetic group</th>
<th>Pathotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>185</td>
<td>eae</td>
<td>O44037:H7</td>
<td>ND</td>
<td>CLA</td>
<td>+</td>
<td>PPT</td>
<td>B1</td>
<td>aEPEC</td>
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<tr>
<td>245</td>
<td>eae</td>
<td>O145:H34</td>
<td>ND</td>
<td>LAL</td>
<td>+</td>
<td>Susceptible</td>
<td>B2</td>
<td>aEPEC</td>
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<tr>
<td>349</td>
<td>eae</td>
<td>O109:H21</td>
<td>ND</td>
<td>LAL</td>
<td>+</td>
<td>Susceptible</td>
<td>B2</td>
<td>aEPEC</td>
</tr>
<tr>
<td>326</td>
<td>eae, bfp</td>
<td>O132:H34</td>
<td>ND</td>
<td>LA/AA</td>
<td>+</td>
<td>Susceptible</td>
<td>B1</td>
<td>tEPEC</td>
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<tr>
<td>20</td>
<td>stx2</td>
<td>O103:H7</td>
<td>+</td>
<td>AA</td>
<td>+</td>
<td>Susceptible</td>
<td>B1</td>
<td>STEC</td>
</tr>
<tr>
<td>8</td>
<td>stx2</td>
<td>O185:H16</td>
<td>+</td>
<td>AA</td>
<td>+</td>
<td>Susceptible</td>
<td>B1</td>
<td>STEC</td>
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<tr>
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<td>O4:H12</td>
<td>+</td>
<td>AA</td>
<td>+</td>
<td>PPT</td>
<td>C</td>
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<tr>
<td>222</td>
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<td>AA</td>
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<td>B1</td>
<td>STEC</td>
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<tr>
<td>15</td>
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<td>NA</td>
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<td>STEC</td>
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<tr>
<td>22</td>
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<td>STEC</td>
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<td>B1</td>
<td>STEC</td>
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<td>E</td>
<td>STEC</td>
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<td>E</td>
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<td>CFO(^c)</td>
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<td>AA</td>
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<td>B1</td>
<td>tEAEC</td>
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<td>AA</td>
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<tr>
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<td>AA</td>
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<td>CFO</td>
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<td>358</td>
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<td>AA</td>
<td>+</td>
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<td>B1</td>
<td>aEAEC</td>
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<td>AA</td>
<td>+</td>
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<td>5</td>
<td>stl-a</td>
<td>O150:H20</td>
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<td>AA</td>
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<td>ETEC</td>
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<td>ipaH</td>
<td>O93:H20</td>
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<td>AA</td>
<td>+</td>
<td>Susceptible</td>
<td>B1</td>
<td>EIEC</td>
</tr>
</tbody>
</table>

ND, not done; CLA, chain-like adherence; LAL, localized adherence-like; LA, localized adherence; AA, aggregative adherence; NA, non-adherence; UND, undefined pattern; DA, diffuse adherence; PPT, piperacillin-tazobactam; CFO, cefoxitin; H-, non-mobile; NTH, non-typing H antigen.

\(^a\)Cytotoxic effect on Vero cells.

\(^b\)Adherence patterns to HEp-2 cells.

\(^c\)Intermediate resistance.
genotype EAECa strains, serotypes O69:H –, O48:H21, O88:HNT, O91:H21, and O140:H21 were detected. In ETEC and EIEC isolates, we identified serotypes O150:H2O and O93:H2O, respectively.

Adherence in HEp-2 cells

Of the 27 isolates from the DEC, 92.6% were adherent. For the 13 STEC isolates, 69.2% demonstrated aggregative adherence (AA), 15.4% were non-adherent (NA), 7.7% demonstrated diffuse adherence (DA), and 7.7% were non-characterized. All EAEC, EIEC, and ETEC isolates demonstrated AA phenotypes. Of the three aEPEC isolates, 33.3% presented chain-like adhesion (CLA) and 66.7% showed localized-like adherence (LAL). The tEPEC strain had a mixed adhesion pattern of localized with aggregative adherence (LA/AA).

Biofilm

Biofilm formation (O.D. 570 > 0.2) was identified in all DEC isolates (Figure 1).

Susceptibility to antimicrobials

Susceptibility to ten antibiotics was examined in the 27 DEC isolates. Most (85.2%) did not demonstrate resistance to the antibiotics tested. Only 14.8% were resistant to one of the antibiotics. Two isolates (7.4%) were resistant to PPT, one (3.7%) to cefoxitin, and one (3.7%) presented intermediate resistance to cefoxitin.

Cytotoxic effect in Vero cells

All the supernatants of the STEC isolates induced cytotoxic effects in Vero cells. The level of cytotoxic activity ranged from 51.9% to 69.8% (Figure 2).

DISCUSSION

The identification of fecal microorganisms in drinking water is desirable in order to reduce the potential contact between humans and enteric pathogens. *E. coli* is the most commonly used microorganism as an indicator of recent fecal contamination (WHO, 2014). Our results showed that 72.9% of the water samples analyzed were unfit for human consumption, according to Ministry of Health Ordinance No. 2914/2011, which states that drinking water must not have total coliforms and *E. coli* (Brazil, 2011). In general, groundwater contamination is related to land use such as livestock and domestic septic systems. To avoid further contamination and to mitigate the risks to human health, appropriate management actions must be implemented (Gambero et al., 2017).

Epidemiological studies in Brazil involving DEC isolates in water intended for human consumption are scarce. In the
present study, five DEC pathotypes were found with STEC carriers of the stx2 gene being the most prevalent.

All strains of STEC isolated in this study were cytotoxic in Vero cells. Considering that epidemiologically the Stx2 toxin is strongly associated with serious diseases in humans causing serious damage to endothelial cells, which can lead to hemorrhagic (CH) and uremic hemolytic syndrome (HUS), STECs are a public health concern (Mohawk & O’Brien 2014).

Lascowski et al. (2015), studying STEC in water samples from the same region in the northern region of Paraná, Brazil, found that of the 12 STEC strains isolated, 10 had the stx2 gene (88.3%). Another study by Schuroff et al. (2017), also in this region, while searching for EPEC and STEC in decanter sludge and filter wash water samples from two water treatment plants, found 2.9% of the DEC strains to be EPEC and 4.7% to be STEC genotypes, thus showing that STEC is a pathotype often found in this region.

EPEC, EHEC, and EAEC have been associated with outbreaks of gastroenteritis in South Korea due to the consumption of contaminated groundwater (Park et al. 2018). In our study, aEAEC was the second most prevalent pathotype. Furthermore, a study conducted in the capital of Zimbabwe, South Africa, showed that 63% of drinking water samples were contaminated by E. coli, and of all the DEC strains evaluated, only EAEC carrying the aaiC gene was found (Navab-Daneshmand et al. 2018).

A study by Assis et al. (2014), also in the state of Paraná, Brazil, showed that of the DEC strains isolated from patients with diarrhea, aEPEC was the most commonly found pathotype. In our study, this pathotype was the third most prevalent, showing that contaminated water can be a source of infection by aEPEC in humans. Kambire et al. (2017) found that 68% of E. coli strains isolated from water belonged to the DEC group; 90% of which were ETEC. These findings differ from the results found in our study, where the prevalence of ETEC was dramatically less (1%). Huang et al. (2012) found DEC in 29.1% of samples from water treatment plants in Taiwan, but no ETEC isolates were found, similarly to our results, where in 210 water samples we isolated only one ETEC. The DEC pathotypes’ prevalence seems to change according to the geographical region, but further studies are required to better understand this. Overall, the detection of DEC genes in drinking water highlights the potential risk for environmental transmissibility of these pathogenic strains in various parts of the world.

The assignment of E. coli isolates to phylogenetic groups provides evidence that strains of various phylogenetic groups differ in their phenotypic and genotypic characteristics, ecological niche, ancestry, and the ability to cause disease. Therefore, analyses of phylogenetic groups, together with the detection of virulence factors genes, may be useful tools to predict potential health risks associated with E. coli strains found in the environment (Ishii et al. 2007).
In our study, the B1 phylogenetic group was the most prevalent (62.96%). Walk et al. (2007) demonstrated that most E. coli strains belonging to this phylogenetic group may persist in the environment. Duriez et al. (2001) suggest that strains of phylogenetic groups A, B1, and D predominate in the intestinal microbiota, and that these strains must acquire virulence factors in order to become pathogenic. In contrast, strains belonging to the phylogenetic group B2 are rare, but appear to be potentially virulent and are more often isolated in patients admitted to intensive care units (Mereghetti et al. 2002). In our study, the prevalence of B2 strains was at a much lower frequency than that of B1 strains, which is consistent with the previous studies.

Phylogroup C was the second most prevalent among our isolates, which typically is one of the less frequently detected groups. This phylogroup is proposed to contain strains closely related to, but distinct from, phylogroup B1 (Clermont et al. 2013). This, in part, points to the conclusion that DEC strains isolated from groundwater may constitute a genetically heterogeneous population.

We found 25 different serotypes, including serotypes O64474:H2, O64474:H12, and O44037:H7 that have the same O antigen as does Shigella boydii 16 (Navarro et al. 2010) and S. boydii 18 (Navarro et al. 2016). Serogroup O64474 was first described by Navarro et al. (2010) in ETEC isolates associated with diarrhea in children in Egypt, Bangladesh, and Mexico between 1980 and 2007 and distributed among strains containing flagellar H2, H10, H12, and H34 antigens. In Brazil, serotype O64474: H8 was recently found in E. coli isolated from cheese and contained ExPEC genes (de Campos et al. 2017). In our study, two isolates of tEAE presented the serotype O64474:H2, and one STEC of the serotype O64474:H12. Together, these studies show that serogroup O64474 may be found in both DEC and non-diarrheagenic E. coli with ExPEC genes.

Serogroup 44037 was also described by Navarro et al. (2016) in a study of 23 strains of E. coli isolated from feces of children and cattle used for the characterization of the somatic antigen and H antigen. Serotyping was performed in 187 O antigens and 53 H antigens of E. coli, and 46 O antigens of Shigella. The 23 strains show a positive reaction to only the O antigens of S. boydii 18. Thus, they suggest a new serogroup of E. coli, O44037, with at least five serotypes (H2, H3, H9, H16, and H48) that have some characteristics of ETEC. In our study, an isolate of aEPEC was identified as serotype 44037:H7, being the first of this serotype to be reported in Brazil. This relationship between Shigella antigens and E. coli antigens has been reported by several investigators including Liu et al. (2008) who found 21 O antigens of Shigella identical or closely related to those found in E. coli.

More than 380 O:H serotypes have already been described for STEC, but only a limited number are associated with disease in humans. Serogroup O103 is a classic serogroup (Karmali et al. 2010) and was found in two strains of STEC in this study. However, the H2 antigen is the most frequently found antigen associated with the O103 antigen (Park et al. 2018), but was not identified in the present study, confirming the heterogeneity within this pathotype.

We also found two strains of STEC with the serotypes O91:H21 and O91:H10. Based on the literature, these serotypes are most commonly associated with STEC strains that cause HUS; however, we also found the serotype O91:H21 in a strain of aEAE. The presence of the stx2 and eae genes has been a reliable predictor for the ability of strains to cause HUS, but some strains of E. coli, such as those of the O91: H21/H10 serotypes, have no eae gene yet have the capacity to cause this syndrome. In Germany, STEC serogroup O91 is the most prevalent type in adult patients, and the second most isolated type in food samples (Mellmann et al. 2009).

Among the EPEC strains, we found serotype O145:H34 (aEPEC) and O132:H34 (tEPEC), which have been reported to be related to strains of aEPEC and EHEC, strains capable of causing enteric infections in humans (Prager et al. 2009). Serogroup O109 is also related to aEPEC (Peeters et al. 1984) and EHEC (Akiyama et al. 2017), and was found in one of our aEPEC isolates. Thus, we note that the pathotypes of DEC have a great heterogeneity in relation to the serotypes. That is, the same serotype can be found in two or more different subgroups of DEC.

An important initial step in the colonization of the human gastrointestinal tract by bacteria is adhesion of the microorganism to the surface of the host. In this study, 92.6% of the isolated DEC were adherent. Of these, 74% demonstrated an AA pattern in cultures of HEP-2 cells,
Despite being characteristic of EAEC; the AA phenotype was also found in STEC strains, ETEC, EIEC, and EPEC, which did not present the EAEC genes screened.

Chain-like adhesion was first described by Gioppo et al. (2000) in tEAEC. In our study this phenotype was observed in an aEPEC strain.

In 2011, a strain of EAEC O104:H4 harboring the stx gene and exhibiting the AA phenotype caused a major outbreak in Germany, drawing attention to the aggregative phenotype as an important factor in pathogenesis (Bielaszewska et al. 2011).

Studies have attempted to identify genes coding for mixed adhesion phenotypes in DEC, such as the study by Scaletsky et al. (1999) with EPEC strains exhibiting LA and DA, and the work of Garcia et al. (2016) in which EPEC expressed LA and AA. In our study, we also found a tEPEC strain exhibiting the LA and AA adhesion patterns. Further studies are planned to better characterize this strain.

All the DEC strains studied have the ability to form biofilms, which facilitates the survival of microorganisms in adverse environments such as in drinking water systems where they represent a potential source of contamination (Wingender & Flemming 2011). In the host, biofilm formation protects the bacteria from exposure to innate immune defenses and facilitates the spread of resistance to antibiotics and other virulence factors, thus contributing to the persistence of infection (Kostakioti et al. 2013).

Few studies have been conducted to estimate the frequency of bacteria resistant to antibiotics in isolates from groundwater intended for consumption. Of the isolated DEC strains in our study, 7.4% demonstrated resistance to PPT and 7.4% to cefoxitin. In Ireland, high levels of resistance (93%) were found to aminoglycosides in isolates from groundwater from private wells. In California, among E. coli isolates from groundwater samples at a dairy farm, only one sample showed resistance to ceftriaxone, chloramphenicol, and tetracycline (Li et al. 2014). These data, in concordance with findings from the present study, point to a variable frequency of antibiotic resistance in E. coli isolated from groundwater.

The presence of antibiotic-resistant bacteria in the environment represents a serious public health problem because, potentially, it reduces the efficacy of antibiotics used to treat infections, contributing to a higher incidence of disease and higher mortality (Amaya et al. 2011). Although in our study we found a relatively high sensitivity to antimicrobial agents, we highlight the potential risk of contamination of groundwater with resistant bacteria. We believe there is a need for further research to establish the prevalence of antibiotic resistance in the hydrogeological environment, since groundwater is an important resource worldwide, especially in rural areas.

**CONCLUSIONS**

In conclusion, we infer from our findings that the natural groundwater that was analyzed in the present work was a potential source of DEC transmission. New studies are required and should contribute to an improved understanding of the epidemiology of these pathogens. We emphasize the need for changes in the policies and behavior of the population that uses these water sources. In addition, knowledge regarding the presence of these pathogens should serve to alert the regulatory agencies, health officials, and educators, the potential drivers for appropriate interventions and reassessment of current contamination and disease prevention strategies.

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